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The expression of extracellular matrix-degrading metalloproteinases is regulated by cell-extracellular matrix interactions

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The Expression of Extracellular Matrix-Degrading Metalloproteinases Is Regulated by Cell-Extracellular Matrix Interactions

by

Patrice Tremble

DISSERTATION

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Dedication

I would like to dedicate this thesis to my parents,

Carolyn and John Tremble, in recognition of their continued encouragement and support

Preface

I would first like to thank my thesis advisor, Dr. Zena Werb for allowing me to train in her laboratory. Her advice and cogent observations played a significant role in formulating the questions in the research described in this thesis; her enthusiasm for this research was inspirational during the dry spells that every scientist faces. I very much appreciate her encouragement and patience, the opportunities that she gave to me, the investment of her time and money, and yes, even the occasional well placed foot, that was required during my tenure in her laboratory. I would also like to thank Zena for providing a hospitable laboratory environment that was conducive to this research.

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I would like to thank the members of the Werb lab, past and present for interesting discussions and advice concerning just about everything.

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Finally, I would like to thank Dr. Rusty Williams for the training I received in his lab; without his help and support I would not have started a doctoral degree.

The published, in press, and in preparation papers included as part of this thesis each represent work that was substantially or wholly performed by Patrice Tremble. The Werb et al. (1989) paper represents the initial development of the model system described. Ms. Tremble performed 90% of the experiments and did 50% of the writing. The work presented in the Tremble et al. (1993) SPARC paper, the Tremble et al. (1993) tenascin paper and the Tremble et al. nuclear signaling manuscript (in preparation) represent 100% experimental effort, 80% of the conceptual effort and 100% writing of the first draft of these papers by Ms. Tremble. The co-authors prepared the specialized reagents used in the biological studies. Thus the work performed by Ms. Tremble represented by these papers is comparable in quality, quantity and writing to a standard Ph.D. dissertation.

Dr. Zena Werb

Ph.D. Thesis Advisor

Publications Generated in this Thesis

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Tremble, P., C.H. Damsky, and Z. Werb. Regulation of metalloproteinase gene expression by the fibronectin receptor involves a TPA responsive element and c *-fos* .

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Abstract

The Expression of Extracellular Matrix-Degrading Metalloproteinases Is Regulated by Cell-Extracellular Matrix Interactions. Patrice Tremble

The expression of interstitial collagenase (CL) in cultured rabbit synovial fibroblasts (RSF) increases in cells plated on the 120 kD chymotryptic fragment of fibronectin that contains the RGD site (120FN), or in cells plated on a function perturbing anti-$\alpha_5\beta_1$ fibronectin receptor antibody. The increase in collagenase mRNA and protein is evident within 2-4 h of treatment, while the expression of collagenase remains at basal levels in cells plated on intact fibronectin (FN), collagen, laminin or vitronectin. These results suggest that different ligands of the fibronectin receptor are functionally distinct, and that signals initiated by the interaction of the integrin fibronectin receptor with ligand can lead to changes in gene expression. In transient assays, the expression of reporter-constructs containing fusions of promoter sequences from the human collagenase gene and the gene encoding the bacterial gene for chloramphenicol acyl transferase was co-regulated with the endogenous collagenase gene. Sequences in the promoter between -139/-42 mediated the increase in collagenase in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibodies. A CAT construct containing only the AP1 and PEA3 sites in the collagenase gene (-90/-66) was upregulated in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibodies; substitutions in either the AP1 (-72/-67) or the PEA3 site (-90/-81) prevented this induction. The increase in collagenase is preceded by an increase in mRNA coding for cFos. In cells plated on 120FN, the level of nuclear cFos protein also increased, when compared to cells plated on intact FN, as determined by immunofluorescence and immunoblotting of nuclear extracts. In cells incubated with anti-sense fos oligonucleotides, the expression of collagenase-CAT constructs as well as the endogenous collagenase gene in cells plated on 120FN or treated with phorbol esters remains near the basal level seen in cells plated on intact fibronectin. Addition of control, sense-fos oligonucleotides did not diminish the upregulation of collagenase induced by 120FN or phorbol

esters. These results suggest that the interaction of integrin receptors with a subset of extracellular matrix molecules, in this case degradation products of fibronectin, modulates metalloproteinase expression, thereby affecting ECM structure and composition. SPARC and tenascin (TN) are ECM molecules that are expressed transiently, in developmentally regulated patterns during development, and in adults are reexpressed in actively growing, repairing or remodeling tissues. TN in the context of a fibronectin matrix, upregulated the expression of collagenase. Collagenase protein was upregulated within 4 h of treatment and was preceded by and increase in nuclear c-Fos. Anti-TN68, which recognizes an epitope in the FN type III repeats in the TN arm, blocked this effect of tenascin and an antibody recognizing an epitope in the EGF repeats had no effect. In RSF, SPARC upregulated the expression of collagenase in the context of interstitial collagens, vitronectin and laminin, but not in the context of collagen type IV or EHS matrix. Synthetic peptides representing sequences in the carboxyl-terminal EF hand or α-helical domains of SPARC also induced the expression of collagenase. The upregulation of collagenase by SPARC is indirect, mediated by an uncharacterized intermediate secreted by cells within 7 h of SPARC addition. In summary, perturbations in the interaction of rabbit synovial fibroblasts with the extracellular matrix, either through interaction of cells with degradation products of fibronectin, or with matrices supplemented with SPARC or Tenascin upregulate the expression of collagenase. These studies show that signals generated by the interaction of an integrin fibronectin receptor can alter the pattern of collagenase gene expression, that the upregulation of collagenase gene expression is preceded by an accumulation of cFos and requires an intact AP1 and PEA3 site in the collagenase promoter. These data support the idea that the cell and the extracellular matrix interact reciprocally, and suggest that extracellular matrix remodelling can be initiated or augmented as a consequence of the interaction of cells with specific matrices.

Chapter1

General Introduction

The interaction of cells with molecules in the extracellular matrix is an important feature in many biological processes. This interaction underlies many basic cellular decisions such as whether a stationary cell will decide to remain in place or, alternatively, whether migrating cells will stop moving and decide to adhere. Cell-cell and Cell-extracellular matrix (ECM) interactions participate in seemingly diverse processes such as the directed cell migrations and tissue patterning that occurs during development, in the tissue renewal that occurs after injury, or in the extravasation of leucocytes and tumor cells. One important corollary to the above statement is that cells must be able to modify both the composition of the extracellular matrix surrounding the cell as well as their interactions with the extracelluar matrix with which they come in contact.

The network of matrix that surrounds or underlies cells is assembled from a number of proteins and proteoglycans that are secreted by cells. One feature common to the macromolecules of the extracellular matrix is that they are large, multidomain molecules that are capable of both interacting with cells and interacting with other molecules in the matrix to form an intricate scaffold for cells (reviewed by Hay, 1991). Tissue specific matrices are assembled from some combination of the collagens, the laminins, fibronectin, vitronectin, fibrinogen and proteoglycans; molecules present to a lesser extent in the ECM include thrombospondin, SPARC, tenascin and entactin.

Molecules in the extracellular matrix

Collagen plays an important structural role in the ECM, and there are 18 collagen subtypes identified to date. Fibrillar collagen type I and the network-forming collagen type IV are the best characterized collagen molecules. A loose prototype for the structure of fibrillar collagen can be taken as that described for collagen type I- three separate protein α chains, twisted in a left-handed helix, that intertwine to form a larger triple helical species. The other collagen species have been loosely sorted into 6 subgroups: fibrillar (types I-VI), fibril-associated (IX,X11,XIV), network-forming (IV), filamentous (VI, XII), short chain (XIII) and long chain (X,VII) (reviewed

by Linsmeyer, 1989). Fibrillar collagens are not necessarily assembled from fibrils of one subtype, and this co-assembly of different fibrillar collagens may affect matrix properties. For example, heterogeneous mixtures of collagen have been observed in collagen isolated from the cornea (types I and V), cartilage (II and XI) and skin or tendon (I and III). It is interesting that matrix turnover is altered by heterotypic assembly of collagenous fibrils; not all collagens are cleaved by the same panel of proteinases. For example the co-distribution of Type I and V fibrils in the matrix of the cornea masks epitopes on collagen V that are susceptable to cleavage by the 92 kD gelatinase, an enzyme that degrades type V collagen. Another modification of collagenous matrices is caused by the fibril associated collagens. These collagens have been found associated with fibrillar collagens. The fibril associated collagens have multiple, distinct collagenous domains and non-collagenous domains which align with the periodical structure of fibrillar collagen, and thus may alter physical parameters of the collagen fibril. For example collagen IX is often found associated with collagen II and collagen XII has been associated with type I collagen. In both cases the non-collagenous domains of the fibril associated collagens are thought to modify matrix structure and turnover. The short chain collagens serve particular functions in the organization of cartilage and endothelial cell matrix, while the long chain collagen VII is found in the anchoring fibers that link squamous epithelial to stroma. Collagen IV, the first non-fibrillar collagen described is an important component of basement membranes (Hay, 1991 and references therein).

Yamada (1991) has reviewed the structural features common to several non-collagenous interactive glycoproteins in the ECM- fibronectin, laminin, vitronectin, and to some extent tenascin and thrombospondin. These glycoproteins interact with heparin, with other ECM glycoproteins, as well as with cell surface receptors. They oligomerize to form higher order polymers.

cDNA cloning has revealed common structural motifs in these interactive ECM proteins

such as homologies with EGF (EGF repeats) or fibronectin type III repeats. An excellent review of common structural motifs in ECM proteins is by Engel (1991). These structural building blocks can assemble to form matrix molecules that have very different effects on cellular behavior. Within one type of matrix molecule diversity in ECM composition and structure can also be generated by variations in RNA splicing. There are differences in fibronectin and tenascin molecules that are generated by alternative splicing to include or exclude specific domains in these molecules. It is perhaps significant that certain variants of these molecules are present transiently at key times and locations during development, but the expression of these variants is rapidly upregulated underneath wounded skin (ffrench-Constant, et al., 1989; Mackie, 1988; Prieto et al., 1991).

The extracellular matrix glycoprotein laminin is an important constituent of basement membranes. Laminin has been implicated or is involved in establishment of adhesion, cell polarity, differentiation, and neurite outgrowth. Laminin is a large trimeric glycoprotein. Structurally, the laminin cross is generated by disulfide linkage of three chains to yield one long arm, and three shorter arms of which one is slightly longer then the other two arms. The longest (A) chain runs the length of the molecule with the 2 B chains forming the side arms and continuing down the length of the A chain (reviewed by Kleinman and Weeks, 1989). The laminin molecule is differentially sensitive to proteolysis and fragments of the Ln trimer have been analyzed for their biological function (reviewed by Kleinman and Weeks, 1989; Beck et al., 1990). Laminin isolated from Engelbarth-Holm-Swarm (EHS) tumors consists of an A, B1, and B2 chains. Recently additional laminin isoforms, merosin, s-laminin (a B1 chain variant, present at synapses), kalinin and k-laminin have been identified (Hunter et al., 1989; Engvall, 1990; Marinkovich et al., 1992; Joseph and Baker, 1992). However most of the work to define functional epitopes on the laminin molecule have used laminin from EHS tumors and is composed of A, B1 and B2 chains. The development of laminin purification scheme from the

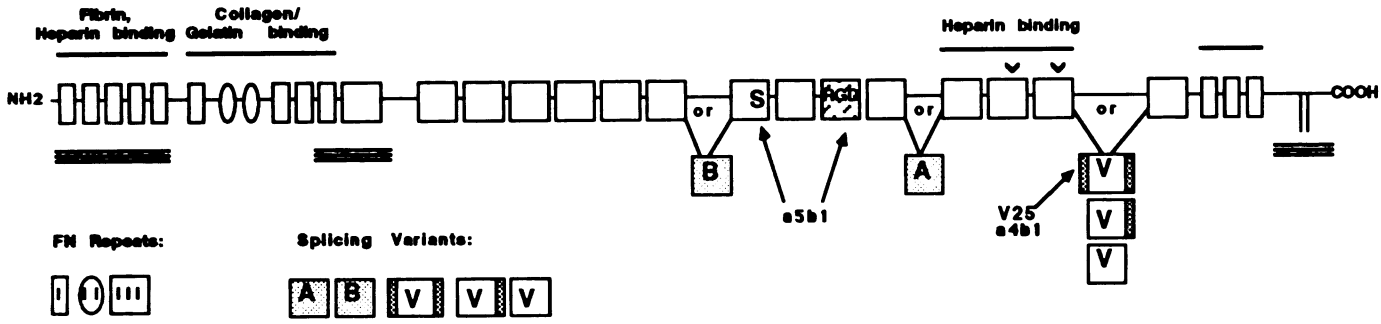
matrix formed by EHS tumors has allowed characterization of the molecule at the protein level; as the chains of laminin have been cloned, synthetic peptides spanning the sequences in proteolytic fragments of laminin having biological activity have been used to fine map the functional epitopes (Shubitz et al., 1991; Hall et al., 1990). Cellular interactions with laminin depend to a large extent on the complement of cell surface receptors expressed by the cell, but many epitopes on laminin that interact with cells have been mapped (Kleinman and Weeks, 1989; Beck and Engel, 1990; Shubitz et al., 1990; Hall et al., 1990))

Fibronectin is probably the best characterized ECM glycoprotein, due to the fact that the chains of the dimer are the product of one alternatively spliced gene, and that plasma fibronectin is easily purified from blood in quantities that are sufficient to do biochemistry. The gross molecular dissection of the canonical RGD cell adhesion site that is recognized by integrin receptors in the fibronectin molecule using classic techniques of protein biochemistry and epitope specific monoclonal antibodies set the standard for years to come (reviewed by Ruoslahti, 1989; Schwarzbauer, 1991).

Cloning and sequencing of fibronectin (FN) has shown that the fibronectin molecule is composed of three repeating units, type I, type II and type III; the type I and type II repeats contain disulfide linkages. These repeats are organized as shown in Fig.1. The type I units (I-1 to I-5) participate in fibronectin assembly, gelatin binding (I-6 to I9, in conjunction with the type II repeats), and interact with fibrinogen (I-10 to I-12). Generally heparin-binding regions contain type I repeats; type II repeats are structurally similar to kringles and are present in the collagen binding domain; type III repeats make up cell and heparin binding domains. Three regions of the molecule are alternatively spliced; this alternative splicing can generate 20 FN isoforms. One region, the V or IICS region is alternatively spliced to include or exclude all or part of a type III repeat. Two other type III repeats, EDA or EDB are included or excluded in cellular-FN (cFN). Generally it is thought that half of the chains in plasma FN , synthesized in the liver, contain

Figure 1. Domain structure of fibronectin. Fibronectin is assembled from three repeating subunits, type I, type II and type III repeats. Fibronectin is the product of a single gene, with isoforms generated by alternative splicing of three regions, designated EDA, EDB and the V region. EDA and EDB are represented by shaded squares marked A and B. The V region has a number of splice variants; in rat and mouse there are three (shown), while there are 5 splice variants in human fibronectin. The entire V region may be excluded, or smaller segments may be spliced in or out (stippled region). The type III repeat containing the Arg-Gly-Asp (RGD) site is indicated; an adjacent site that potentiates adhesion via $\alpha_5\beta_1$ (a5 b1) is also indicated (S). Sequences in the V25 region are recognized by $\alpha_4\beta_1$ (a4 b1) and epitopes in FNIII $_{13\text{-}14}$ are important for heparin binding (carots). The regions of fibronectin that are involved in matrix assembly are underlined. The approximate location of the 120 kD chymotryptic fragment of fibronectin is indicated.

Figure 1


Fibrin, Heparin binding
Collagen/ Gelatin binding
Heparin binding
NH2
COOH
or
S
RGD
or
or
B
A
V
V
V
α5β1
V25 α4β1
FN Repeats:
I
II
III
Splicing Variants:
A
B
V
V
V

some form of the V region (V+); EDA and EDB are not included in plasma FN (pFN). cFN is generally V+ and the inclusion of EDA or EDB is regulated in a tissue specific, developmentally regulated manner. Thus the most striking difference between cFN and pFN is the inclusion or exclusion of the V region. There are hints that the various isoforms may be functionally different. EDA and EDB are transiently expressed during development but the expression of FN isoforms that contain EDA and EDB is upregulated underneath the dermis in healing skin wounds and in smooth muscle cells that have assumed the "synthetic" phenotype (ffrench-Constant et al., 1989). In addition a segment in the V domain (IIICS) may be included or excluded in FN isoforms, this segment is recognized by the $\alpha_4\beta_1$ FN receptor(Mould et al., 1989). FN secreted by cells in which both chains of the FN dimer contain the V region is not as efficiently incorporated into fibrinogen clots, compared to pFN which contains only one copy of the V region per dimer (Wilson and Schwarzbauer, 1992).

FN is a secreted protein that is further assembled by cells into an insoluble matrix. A number of epitopes on FN that play a role in supporting cell adhesion and/or FN matrix assembly. The classical RGD sequence that is recognized by $\alpha_5\beta_1$ is located in the $FNIII_{10}$ repeat, and this site is within 15 -20 kD of a second site that potentiates adhesion to the RGD site (Pierschbacher and Ruoslahti, 1987; Obara et al., 1988; Nagai et al., 1991). The $\alpha_4\beta_1$ integrin recognizes a sequence in the V25 region (also termed the CS1 region) in the alternatively spliced V region of FN (Mold et al., 1990; Guan et al., 1990). There are heparin-binding sequences at the amino-terminal of FN and the $FNIII_{12\text{-}14}$ repeats have been shown to interact with heparin (Barkalow and Schwartzbauer, 1992). Fibronectin also interacts with molecules in the extracellular matrix. Fibrin-, heparin- and gelatin/collagen-binding domains have been identified (reviewed by Ruoslahti, 1988;Schwarzbauer, 1991). The epitopes in FN required for the assembly of an insoluble FN matrix are beginning to be characterized. This is a complex issue, because FN matrix assembly requires both that FN associate with cell surface receptors ($\alpha_5\beta_1$, and as of yet

unidentified non-integrin assembly receptor MacDonald, 1988; Blystone and Kaplan, 1992), and with other molecules of FN. An elegant study by Schwarzbauer (1989) clearly demonstrated that a dimeric FN molecule that contains the amino-terminal type I repeats 1-5 are required for matrix assembly. However there is also compelling data that suggest the $FNIII_1$ repeat is also involved in matrix assembly (Chernousov et al., 1991; Darribere et al., 1992; 1992; Morla et al., 1993) possibly mediating FN-FN interactions. Studies have demonstrated differences in the adhesion, spreading and focal contact formation in cells adhering to FN when compared to fragments of FN that contain only the cell binding domain. Cells will adhere and spread on fragments of FN, but can not form focal contacts; in some cell types, activation of protein kinase C will allow focal contact formation on fragments of FN (Woods and Couchman, 1989, 1992). Recently, a sequence in the $FNIII_{12-14}$ repeats has been identified that also can complement focal contact formation (Woods et al., 1993).

An emerging hypothesis in cell adhesion is that several molecules on the cell surface function as co-receptors for the extracellular matrix (reviewed by Damsky and Werb, 1992). The concerted interaction of these cell surface receptors with molecules in the extracellular matrix may be required for appropriate adhesion. Syndecan-like molecules are thought to be one type of cell surface receptor for the heparin-binding domains of FN (reviewed by Bernfield et al., 1992) as are receptors of the integrin family (reviewed by Hynes, 1992).

Receptors for Extracellular Matrix

The integrins and the syndecan (-like) molecules are the best characterized cell surface receptors for extracellular matrix (recently reviewed by Hynes, 1992; and by Bernfield et al., 1992). Syndecans are transmembrane proteoglycans that contain heparan sulfate and to a lesser extent chondroitin sulfate. Syndecans are associated with epithelial cells, although syndecan-like molecules have been identified on fibroblasts (reviewed by Bernfield et al., 1992). These molecules are composed of a membrane-spanning core protein that contains N-linked sugars and

glycoseaminogclycan chains. The cytoplasmic tail of the syndecan core proteins is very short and contains several tyrosine residues; another interesting feature of these molecules is that they contain a proteolytic cleavage site near the transmembrane domain (Saunders et al., 1989; David et al., 1990). Through the heparan sulfate chains, syndecan interacts with collagen types I, III and V (Koda and Bernfield, 1984), with fibronectin (Saunders and Bernfield, (1988) and with tenascin (Salmivirta et al., 1991). In adherent cells syndecan, like integrin receptors associates with the actin cytoskeleton (Rapraeger, 1986). The sequence of the cytoplasmic tail is conserved in all family members, suggesting that 1) the function of syndecan might be modified by phosphorylation and 2) that sequences in the carboxyl-terminal tail are required for syndecan localization (reviewed by Bernfield et al., 1992). Syndecans, therefore are good candidates for a co-receptor for various molecules in the matrix. Although not shown directly, syndecan may also be a co-receptor for heparin binding growth factors. Cell surface heparin sulfate proteoglycans are required for the interaction of basic fibroblast growth factors with the high affinity receptor for basic fibroblast growth factor (Klagsbrun and Baird, 1991), and cells deficient in cell surface heparan sulfate proteogyycans do not respond to basic fibroblast growth factor (Yayon et al., 1991; Rapraeger et al., 1991).

The integrin family of adhesion receptors are heterodimeric receptors consisting of an α chain selected from one of 14 α subunits and a β chain selected from one of 8 β subunits (reviewed by Albeda and Buck, 1991; Hynes, 1992). To some extent, these receptors are loosely grouped by the β chain usage, however the sorting of α chains with β chains is non entirely independent. To date 20 $\alpha\beta$ heterodimers have been characterized (reviewed by Hynes, 1992). Biochemical studies have defined the ligand specificities of the integrins and there is considerable overlap in the recognition of individual matrix molecules by different integrins. Some integrins recognize only one ligand, while others recognize several matrix molecules. Individual matrix molecules are often recognized by more that one $\alpha\beta$ heterodimer; for example

there are at least 8 heterodimers that interact with fibronectin (reviewed by Albeda and Buck, 1991; Hynes, 1992). The considerable overlap and duplication of ligand specificities of the integrins is puzzling, because cells often express more than one integrin receptor for specific matrix molecules, suggesting that the interaction of cells with the same ligand, via different integrins, may not be equivalent. Most of the α and β chains have been cloned and sequenced (reviewed by Hynes, 1992), and the composition of several chains is regulated by alternative splicing. Splicing patterns may be regulated during development, so it is entirely possible that the interaction of the same $\alpha\beta$ pair with the same ligand may not always have similar consequences.

Integrin receptors have been traditionally defined as adhesion receptors, serving as the membrane-spanning link between the extracellular cytoskeletal network (reviewed by Burridge, 1989; Turner and Burridge, 1990). In adhering cells these receptors play a central role nucleating and colocalizing with focal contacts and focal adhesions (Turner and Burridge, 1990), while on highly motile cells, integrins are diffusely distributed on the cell surface (Couchman and Reese, 1979; Duband et al., 1988). The cytoplasmic domain of the β chain is thought to be involved in targeting integrin receptors to focal adhesions (Solowaska et al., 1989; Marcantonio et al., 1990; Reszka et al., 1992).

Proteinases involved in extracellular matrix remodeling

Extracellular matrix deposition results from the summation of both synthesis and deposition of ECM molecules, as well as the regulated degradation and clearance of these molecules from the external milleau. Controlled proteolysis is a feature of ECM remodeling during morphogenesis and of cellular migration and invasion seen in implantation of embryos, while uncontrolled proteolysis is involved in the extravasation seen in metastasizing tumor cells (Brenner et al., 1989; Fisher et al., 1989; Liotta, 1991). The evolution of proteolytic cascades, a well characterized feature of the remodelling of wounded tissue associated with wound contraction

and healing, allows close control of degradative processes, that unchecked would be detrimental. There are several tiers of regulation of proteolytic activities including transcriptional regulation, spatial sequestration either by using specific receptors for proteinases and inhibitors or vectorial secretion, and finally regulation of the amount of active enzyme present in a given situation either by limiting the activation of the proenzyme or through specific inhibitors of these proteinases.

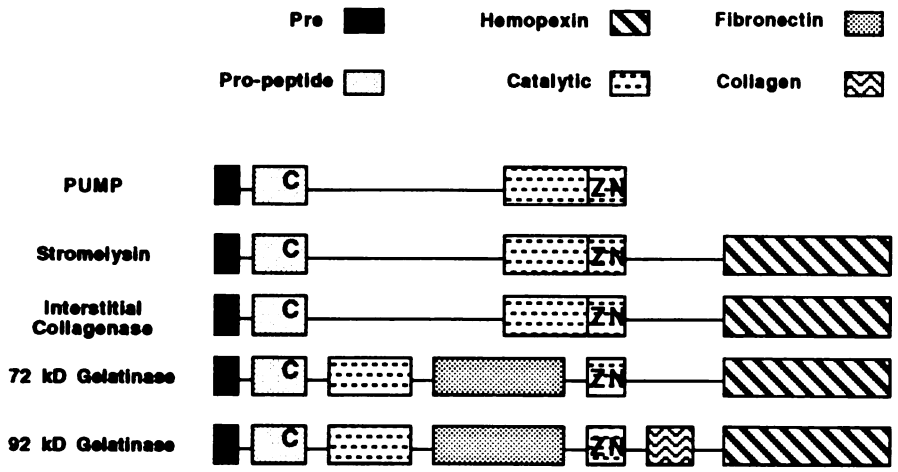
The secreted proteinases that effect much of the matrix degradation occurring in tissue remodelling have a neutral pH optima and are members of either the serine or metalloproteinase class of enzymes. Meshing of the activities of the serine and metalloproteinases classes in proteolytic cascades allows precise control of matrix degradation. Both families of proteins contain enzymes that have broad specificity (stromelysin, Pump, plasmin), as well as proteinases that have a limited substrate specificity (plasminogen activators(PA) and interstitial collagenase). The involvement of two classes of proteinases in matrix remodelling allows yet another level of control, with independent regulation and activation of individual proteinases from both groups in a matrix remodelling cascade (reviewed by Alexander and Werb, 1989).

Metalloproteinases and metalloproteinase inhibitors

The matrix metalloproteinases are grouped primarily by (i) coordination of a zinc molecule in catalysis, (ii) requiring calcium for enzyme activity, (iii) inhibition by tissue inhibitor of metalloproteinase (TIMP), and, more loosely, by (iv) degradation of extracellular matrix molecules as substrates. Structural motifs, determined by protein and cDNA sequencing include a zinc coordinating motif (VAAHEnGH) used in catalysis, and the cysteine switch sequence, PRCGnPDV, present N-terminal to the cleavage site that generates active metalloproteinases. Additional homology units have been inferred from information derived by cDNA cloning and sequencing. Specific metalloproteinases show a 71 -85% homology across species, while all family members share a 40 -60% homology. As can be seen in Figure 2 the homology between

Figure 2. Domain structure of metalloproteinases. Comparison of cDNA sequences has revealed structural similarities between metalloproteinases. The simplest metalloproteinase, Pump (matrilysin), contains a signal peptide (pre), a propeptide that is cleaved to allow activation of pro-metalloproteinases (pro-peptide). The location of the highly conserved cysteine switch is indicated by C. The catalytic domain contains three histidine residues that are believed to coordinated zinc in the active site (ZN). All other family members contain a hemopexin domain, and the 92 and 72- kD gelatinases contain sequences that resembles the collagen-binding domains of fibronectin; the 92 kD gelatinase contains homology to sequences to collagen type V. The hemopexin, fibronectin- and collagen-like domains may affect substrate specificity.

Figure 2


Pre
Hemopexin
Fibronectin
Pro-peptide
Catalytic
Collagen
PUMP
Stromelysin
Interstitial Collagenase
72 kD Gelatinase
92 kD Gelatinase
C
ZN

family members is concentrated in discrete domains, the zinc-chelating region, the proenzyme alignment site and the hemopexin domain (reviewed recently by Woessner, 1991; Matrisian, 1992; and by Birkedal-Hansen et al., 1993).

Family members include interstitial collagenase (MMP1), a 72 kD type IV collagenase (gelatinase, MMP2), stromelysin (transin, MMP3), Pump (MMP7), neutrophil collagenase, (MMP8), and a 92kD type IV collagenase (gelatinase, MMP9). (reviewed by Woessner, 1991, Matrisian, 1992).

MMP1, interstitial collagenase and MMP3, stromelysin are the best characterized metalloproteinases. Collagenase is secreted as an inactive proenzyme having a mass of 53 kD, with a glycosylated species having a mass of 57 kD. *In vitro*, collagenase can be activated chemically (as can all of the metalloproteinases) by incubation with organomercurial compounds, chaotropes, or by limited proteolysis. Limited proteolysis of the propeptide releases conformational constraints in the cysteine switch region and permits a collagenase catalysed autoactivating cleavage of a 10 kD propeptide (reviewed by Woessner, 1989). *In vivo* , the proteins that activate collagenase are not yet known, but stromelysin and plasmin are good candidates. Active collagenase has a mass of about 42 kD; although smaller cleavage products are detected by zymography. This metalloproteinase has a unique substrate specificity, cleaving a single site on the native triple helix of collagen type I, II and III (reviewed by Birkedal-Hansen, 1993). Further denaturation of this collagen allows its degradation and clearance by the metalloproteinases with a broader substrate specificity such as the gelatinases or stromelysin.

MMP3, or stromelysin was characterized as a secreted metalloproteinase that degrades fibronectin, proteoglycans, laminin, collagen type IV, and casein, but significantly, not collagen types I,II or III (Chin et al., 1985). This protein was subsequently cloned twice, as transin, a rat gene that was expressed at high levels in response to growth factors or oncogene expression (Matrisian et al., 1986); Fini et al. (1987) isolated a clone from rabbit by virtue of its ability to

activate collagenase that was homologous with stromelysin. Stromelysin is synthesized as a secreted proenzyme with a mass of 57 kD (unglycolsylated) and 61 kD (glycosylated). As with other metalloproteinases, stromelysin is activated by chaotropes, organomercurials, or limited proteolysis by a mechanism involving cleavage of a fragment using the cysteine switch as described above. Again, little is known about in vivo activation of this enzyme.

Apart from stromelysin and transin, cDNA cloning has identified other metalloproteinases with homology to stromelysin/transin. Interestingly, comparison of the promoter regions indicate that transin-1 and -2 are not likely to be co-regulated (Breathnach et al., 1987; Sirum and Brinckerhoff, 1989; Basset et al., 1990).

The other metalloproteinases are less well characterized. As can be seen in Figure 2 the Zn chelating and cysteine switch motifs are conserved; the addition of other domains may alter matrix binding capability (reviewed by Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993). MMP2, or the 72 kD gelatinase was identified by Collier et al. (1988). The inactive 72 kD proenzyme is constitutively secreted by many mesenchymal cells and is activated by chaotropes, organomercurials or limited proteolysis to a 66 kD active species that degrades gelatin, collagen IV,V VII and X, fibronectin, elastin and proteoglycans. cDNA sequence information reveals that the zinc chelating and cysteine switch structural motifs that characterize the MMP's are conserved, as is the hemopexin domain, Interestingly, a domain with a motif similar in sequence to the collagen/gelatin binding fibronectin type II repeats is located upstream from the Zn-chelating domain (Collier et al., 1992). One tempting hypothesis is that this domain is involved in the association of MMP2 with ECM substrates.

Tryggvasson's group have cloned both the 72 kD gelatinase(MMP2) and the 92 kD gelatinase (MMP9)(Reponen et al., 1992; Huhtala et al., 1991). The substrate specificity of the 92 kD gelatinase overlaps that of the 72 kD gelatinase; it includes gelatin and collagen types IV and V. Alignment of cDNAs shows that the propeptide, amino-terminal domains, the fibronectin

type II motif, the Zn chelating domain and the hemopexin domain are highly homologous. However, there is a unique 48 bp segment, similar in sequence to the helical domain of the type V collagen α2 chain, inserted in exon 9. The 92 kD gelatinase is activated by similar processes as other family members to generate an active species of about 84 kD (reviewed by Woessner, 1991, Matrisian, 1991; Birkedal-Hansen et al., 1993).

As a group, the matrix metalloproteinase family have an overlapping and complimentary range of matrix substrates. The synthesis and secretion of MMPs varies with the cell type and hormonal stimulation, and convergent or divergent regulation of MMP synthesis could yield different matrix remodelling cascades. Although in many cases several MMPs are co-regulated, examples of divergent regulation of these enzymes are being identified (MacNaul et al., 1990; Huhtala et al., 1991; Overall et al., 1991). The independent regulation of metalloproteinases is also suggested by the fact that the regulatory elements in the transin-1 and transin-2 promoters, and of the elements in the 92 kD gelatinase and the 72 kD gelatinase, two pair of enzymes with similar substrate specificities, are very different (Breathnach et al., 1987; Huhtala et al., 1991).

Metalloproteinases are regulated by controlling the rate of synthesis, the rate of activation, and importantly, the proteolytic activity of the active species. A family of proteins, the tissue inhibitors of metalloproteinases (TIMP) has been defined (Carmichael et al., 1986; Stetler-Stevenson et al., 1989). The family includes TIMP (28.5 kD) and TIMP2 (21 kD), which have been purified, cloned and sequenced. There are almost certainly other family members, because several proteins that inhibit metalloproteinase activity have been observed on inhibitor gels of proteins isolated from glial cells, transformed fibroblasts, and endothelial cells (Banda et al., 1988, Apodaca et al., 1990; Pavloff et al., 1992). TIMP1 and TIMP2 are the best characterized members of this inhibitor family. TIMP1 forms a stoichiometric (1:1), high affinity, reversible complex with collagenase and stromelysin; the region of TIMP1 involved in inhibition of enzyme activity has not yet been determined, nor has the enzyme binding site for TIMP1.

Another interesting feature of TIMPs was revealed during development of purification schemes for gelatinases and TIMPs from culture supernatants. TIMP2 stably associates intracellularly with the 92 kD and the 72 kD gelatinase using a site distinct from that involved in inhibition of proteolytic activity (Howard and Banda, 1991), suggesting a chaperone-like function for TIMP.

The pan-specific proteinase inhibitor α2-macroglobulin and a proteinase inhibitor from the placenta inhibits metalloproteinase activity, as does α1-macroglobulin and a protein found in avian eggs, ovostatin. Generally, the TIMPs are thought to be the important inhibitor for matrix metalloproteinases during matrix remodelling.

Plasmin/Plasminogen Activators

The serine proteinases plasminogen and plasminogen activators participate in localized proteolysis in tissue remodelling during embryogenesis, development and tissue repair in wound healing, as well as in the invasive processes occurring in metastasis. There is good evidence that the activity of plasminogen activators and plasminogen act in concert with the matrix remodelling metalloproteinases to degrade ECM (Werb et al., 1977, Mignatti 1985).

Plasmin is a serine proteinase active at neutral pH. Plasmin is very active and degrades a wide range of ECM substrates. However the activity of plasmin is tempered by the presence of an excess of α2 antiplasmin - active plasmin has a short half-life (Linjen,1987; Blasi, 1987). Thus the activation of plasminogen to plasmin plays a crucial role in determining the level of plasmin activity.

In thrombosis, plasmin is formed by the proteolytic cleavage of its zymogen, plasminogen through the action of one of two serine proteinases, tissue plasminogen activator (tPA, 70 kD) and urokinase plasminogen activator (uPA, 50 kD). The PAs have a more specific range of substrates- tPA specifically cleaves plasminogen, while uPA *in vitro* degrades FN as well. tPA and uPA are distinct proteins, the product of separate genes, although these proteins are 40% homologous. Both PAs are secreted as single chain proenzymes which are proteolytically

activated to an active two chain species. Single chain uPA is fully active only after cleavage. Structurally, the light chain (A,24 kD) contains kringle and EGF motifs, the heavy chain (B, 30 kD) contains the catalytic species, which is similar to other serine protease such as trypsin, thrombin and plasmin. Two chain uPA may be further cleaved to generate an active 33 kD species consisting of a 21 residue light chain and the catalytic chain tPA is thought to function in thrombosis and the clotting cascade, while uPA is thought to be active in extracellular matrix remodelling as well (reviewed by Vassalli et al., 1991).

Urokinase interacts with a cell surface receptor for urokinase. Receptor bound uPA is catalytically active and can interact with substrates and inhibitors. Receptor bound uPA is not immediately sequestered by receptor mediated endocytosis, but remains at the cell surface (t1/2 > 5 h); it appears that interaction with a plasminogen activator stimulates recycling of the bound protease-inhibitor complex (Estreicher,1990; Blasi et al., 1987). The uPA receptor is present on many cell types, fibroblasts and monocytes included. Both single chain and the cleaved two-chain species of uPA interact with the receptor; the EGF repeats in uPA are the domain in uPA recognized by the uPA receptor. Thus the smallest (33 kD form) of uPA does not associate with the receptor (Blasi et al. 1987).

Interaction of uPA with its receptor helps to localize uPA to pericellular regions. The uPA receptor has been immunolocalized to focal contacts (Pollanen, et al., 1989). Recently the uPA receptor was shown to accumulate on the leading edge of the plasma membrane in U937 (monocytic) cells migrating in a gradient of the chemotactic peptide f-Met-Leu-Phe (Estreicher, 1990). There is some evidence (Ossowski, 1991) that receptor bound uPA is the active species in matrix remodelling. Thus the interaction of uPA with its receptor is critical in the regulation of PA activity.

There are several well characterized inhibitors of plasminogen activators. Protease nexin and aprotinin, two inhibitors of serine proteases with broad specificity, inhibit plasmin activity.

There are also distinct inhibitors of plasminogen activators, the endothelial-type inhibitor, PAI1, and the placental-type inhibitor, PAI2. PAI1 is secreted by several cell types including endothelial cells, smooth muscle cells and is in plasma and platelets. Circulating PAI1 has a short half life (2 h) while matrix bound PAI1 is more stable; recently PAI1 has been shown to associate with vitronectin in the extracellular matrix (Kost et al., 1992; Mimuro et al., 1993). PAI2 does not circulate, it is found in the placenta and epidermis and is thought to be synthesized by macrophages. PAI2 complexes with uPA, tPA and plasmin.

AP1 and PEA3 sites in promoters of urokinase and several metalloproteinases mediate the inducible transcription of these genes

Collagenase, stromelysin, urokinase, and TIMP are proteinases or proteinase inhibitors that are sometimes co-regulated at critical times during development and tissue remodeling. *In vitro* these genes are upregulated in response to growth factors, cytokines and oncogenes. The activator protein-1 (AP1) and polyomavirus enhancer activator-3 (PEA3) sites in the promoters of collagenase, stromelysin, urokinase and TIMP play a role in this induction. These inducible elements have been studied using transient expression of promoter-reporter constructs (Angel et al., 1987; Gutman and Wasylyk, 1990; Wasylyk et al., 1991; Buttice and Kurkinen, 1991, 1993; Campbell et al., 1991; Auble and Brinckerhoff, 1992). Collagenase, stromelysin and TIMP all contain an AP1 and PEA3 site within 100 bp of the start of transcription. Collagenase can be considered the simplest of these promoters, with the AP1 and PEA3 sites playing the primary role in the regulation of collagenase by growth factors and cytokines, additional upstream sequences in the promoters of stromelysin and TIMP also respond to growth factors, cytokines and TPA play a significant role in the regulation of stromelysin and TIMP. In the collagenase promoter, disruption or deletion of the AP1 site in the endogenous promoter, or in a synthetic promoter containing 4 tandem copies of the PEA3/AP1 motif from the collagenase promoter, ablates the basal and induced expression of collagenase (Angel et al., 1987; Gutman and Wasylyk, 1990;

Auble and Brinckerhoff, 1992) ; disruption of the PEA3 site in a synthetic construct containing 4 tandem copies of the collagenase PEA3/AP1 motif compromises, but does not ablate transcription of the synthetic construct (Gutman and Wasylyk, 1990). This suggests that the requirement for AP1 is obligate for the expression of collagenase, and proteins interacting at the PEA3 site augment the expression of collagenase induced by TPA. Sequences flanking the PEA3 sites in the rabbit promoter are required for full induction of collagenase by TPA (Auble and Brinckerhoff, 1992).

Stromelysin, like collagenase, contains an AP1 and PEA3 site within 60 bp of the start of transcription, as well as two PEA3 sites about 200 bp upstream from the start site (Wasylyk et al., 1991; Sirum-Connoly and Brinckerhoff, 1991; Buttice and Kurkinen, 1992, 1993). The AP1 site is required for basal and induced transcription of stromelysin; the expression from reporter constructs in which the AP1 site is deleted or altered is below basal levels (Butticce and Kurkinen, 1992, 1993; Sirum-Connoly and Brinckerhoff, 1991). Reporter constructs containing only a functioning AP1 from stromelysin promoter are upregulated by IL-1, TPA and H-*ras*, although not to the same extent as constructs containing the longer segment of the promoter that includes the additional PEA3 sites (Wasylyk et al., 1991; Sirum-Connoly and Brinckerhoff, 1991). In human diploid fibroblasts, inclusion of the PEA3 site in constructs containing the promoter-proximal sequences augments the regulation of this short promoter fragment by TPA and IL-1 (Sirum-Connoly and Brinckerhoff, 1991). Reporter constructs containing another segment of the stromelysin promoter are also regulated by TPA. Constructs containing the two PEA3 sites (-209 to -191) respond independently to TPA, and are required for the full expression of stromelysin by TPA or oncogenes (Wasylyk et al., 1991; Butticce and Kurkinen, 1993). There is some suggestion that sequences between the upstream PEA3 element and the PEA3/AP1 site near the transcription start site also plays a role in the regulation of stromelysin by TPA or IL-1 (Sirum-Connoly and Brinckerhoff, 1991).

In the human urokinase gene the enhancer is located 1.7 kb upstream of the transcription start site. There is a single AP1 site 45 bp 3' to a combined PEA3/AP1 motif. Substitutions in the AP1 site or either site in the combined PEA3/AP1 motif significantly compromises induction of urokinase. In some cell types, intervening sequences between the AP1 and PEA3/AP1 elements, recognized by as of yet unidentified cellular factors, mediate a cooperative interaction between the two inducible elements (Nerlov et al., 1992).

The gene for TIMP1, an inhibitor of metalloproteinases, also contains AP1 and PEA3 elements that are required for its expression. TIMP1 differs from collagenase, stromelysin and urokinase in that its promoter does not contain TATAA sequences. However, like collagenase and stromelysin, there are AP1 and PEA3 sites within 100 bp of the transcription start site. Reporter constructs containing this segment of the promoter are weakly induced by serum. Promoter mapping has also shown that there is a serum response element that contains AP1 and PEA3 sites located at -800 in the TIMP promoter. While the AP1 and PEA3 sites near the start of transcription confer inducibility on a heterologous promoter, there is some suggestion that the AP1 and PEA3 sites adjacent to the transcription start site are acting to recruit RNA polymerase and/or transcription factors to the transcription start site. Indeed a number of genes that lack TATAA sequences contain AP1 and PEA3 sites immediately adjacent to the transcription start sites, and in the polyoma virus late genes both the AP1 and PEA3 sites are required for the transcription of late genes (Yoo et al., 1991 and references therein).

Glucocorticoids and retinoids repress the expression of collagenase and stromelysin (Frisch and Ruley, 1987; Lafyatis et al., 1990;, Jonat et al., 1990; Yang-Yen et al., 1990). Deletion analysis of the collagenase promoter has shown that the AP1 site, and not the upstream glucocorticoid response elements (GRE) mediates the repression of transcription caused by glucocorticoids and retenoids (Jonat et al., 1990, Yang-Yen et al., 1990; Schule et al., 1991). Summarizing the work from several groups (Jonat et al., 1990; Schule et al., 1991) repression of

collagenase by glucocorticoids is thought to involve a protein-protein interaction between AP-1 (Jun) and the glucocorticoid receptor; although the DNA-binding domain of the glucocorticoid receptor is necessary for this repression, the glucocorticoid receptor does not interact directly with the AP1 site, nor does it decrease the amount of AP1 bound to the AP1 site (Jonat et al., 1991; Yang-Yen et al., 1990). Interestingly, AP1 also diminishes glucocorticoid receptor-stimulated transcription from GREs. Although AP1 does not bind to GREs, the leucine zipper in Jun is required for the repression of transcription of GREs by Jun, again suggesting that protein-protein interactions are important (Yang-Yen et al., 1990). Additionally the glucocorticoid receptor has been shown to associate with AP1 by co-immunoprecipitation (Jonat et al., 1990; Yang-Yen et al., 1990).

TGFβ also represses the synthesis of collagenase and stromelysin (reviewed in Kerr et al., 1990). A deletion analysis of the rat stromelysin promoter has defined a sequence at -709 to -700 that is involved in the TGFβ regulated repression of stromelysin expression. This element, a TGFβ inhibitory element (TIE), is present in the promoters of other genes regulated by TGFβ - collagenase, uPA, elastase and proliferin. Surprisingly, in gel retardation assays using the consensus TIE as the probe, oligonucleotides encoding the TRE compete for proteins interacting with the TIE. Further investigation determined that Fos and Jun proteins did indeed interact with the TIE, and, significantly, in NIH 3T3 cells transfected with an anti-sense fos plasmid, TGFβ did not block the induction of stromelysin by EGF (Kerr et al., 1990). Thus the TRE and the proto-oncogenes Jun and Fos are significantly involved in both stimulating and repressing collagenase expression.

Does extracellular matrix play a role in orchestrating phenotype?

In the adult animal extracellular matrix may function primarily as a stable scaffold or as tissue infrastructure. However in developing animals, in tissues that are repairing, expanding or resorbing, or in tissues that are in a constant state of renewal, there must be a mechanism to

eliminate unnecessary extracellular matrix or to rapidly alter the composition of matrix presented to the cell. Over the years, a concept proposing a reciprocal interaction between cells and the surrounding extracellular matrix has evolved (Bernfield et al., 1984; Bissell et al., 1987). The cornerstones of this concept are: 1) that cells and ECM are closely associated, and in fact, link the cytoskeleton to molecules in the extracellular space; 2) extracellular matrix itself, or in conjunction with hormones and growth factors may orchestrate or coordinate the behavior of a number of cells, because a particular extracelluar matrix can span many cells; and 3) cells in turn can respond to the extracellular matrix that is presented to them, in some cases modifying this extracellular matrix.

The idea that the interaction with the appropriate extracellular matrix affects cellular behavior has progressed over the past 40 years from the observations of Grobstein (1954) on the tissue interactions occurring during mouse development to refined systems, where many of the matrix molecules and the cell surface receptors for these molecules are characterized. The awesome power of genetic analysis has described the consequences of perturbing specific cell-extracellular matrix interactions. In *Caenorhabditis elegans*, the cuticle is composed of a network of collagens, and mutations in collagen genes affect body shape (von Mende et al., 1988; Kramer et al., 1988). In Drosophila, a null mutant for laminin A is an embryonic lethal (Horsch and Goodman, 1991), and mutations in the Drosophila integrins, the PS molecules, have profound affects on wing and muscle development (Leptin et al., 1991; Volk et al., 1990). Adams and Watt (1993) have compiled a comprehensive review delineating what is known about the role that extracellular matrix plays in maintaining or inducing a differentiated phenotype *in vivo* and *in vitro*.

There are a number of cell culture systems in which the interaction of cells with molecules in the extracellular matrix modulates the maintenance or assumption of a differentiated phenotype (reviewed by Adams and Watt, 1993). Some of the most direct evidence documenting

the importance of cell-ECM interactions comes from studies characterizing keratinocyte differentiation (Adams and Watt, 1989, 1991), and from studies on the expression of tissue specific genes in mammary epithelial cells (Streuli et al., 1991). An element regulating the expression of milk proteins in mammary epithelial cells *in vitro* has been described in the promoter of casein; this element is regulated by the concerted action of lactogenic hormones only in the context of the appropriate extracellular matrix (Schmidhauser et al., 1991). The studies using mammary epithelial cells are strengthened by studies characterizing the expansion and involution of mammary tissue in mice (Talhouk et al., 1991).

The studies in this thesis describe another cell culture system that may be useful in dissecting cell-extracellular matrix interactions. As stated previously, closely controlled remodelling is a necessary component of normal processes that occur in tissue morphogenesis and in the repair of injured tissue. Factors that affect the balance of proteinase activity, in either direction, have detrimental consequences for the organism as a whole; for example unchecked proteolysis is one feature of tumor cell metastasis. Several lines of evidence suggest that the matrix itself may have a role in regulating ECM turnover. The proteolysis of ECM can release matrix-bound growth factors, to indirectly regulate the expression of proteinases or their inhibitors (Saksela and Rifkin, 1990). In synovial cells, the expression of metalloproteinases correlates with dissembly of the actin cytoskeleton, whether it is induced by growth factors phorbol esters or cytochalasins (Aggeler et al., 1984, McDonnell et al., 1991). The experiments in chapter 2, show that perturbations in the interaction of synovial fibroblasts with the extracellular matrix upregulates the expression of collagenase, stromelysin and the 92 kD gelatinase. This increase in the expression of was observed in RSF treated with RGD peptides or a function-perturbing anti-$\alpha_5\beta_1$ as well as in cells that were plated on RGD-containing fragments of fibronectin. These proteinases have overlapping and complementary substrate specificities, suggesting that in synovial fibroblasts, changes in the composition of extracellular matrix can

initiate or amplify a remodelling cascade, and thereby alter matrix composition or structure.

The upregulation of collagenase in synovial fibroblasts by degradation products of fibronectin suggest that this culture system could be useful in analyzing the consequences of other types of cell-extracellular matrix interactions. Another mechanism that can be used to rapidly alter the composition of extracellular matrix is the addition or deposition of matrix-associated molecules like SPARC or tenascin to an established matrix. SPARC and tenascin are two structurally dissimilar molecules that, when added to cells in culture, destabilize adhesion to the extracellular matrix. SPARC and tenascin are expressed transiently during devolopment in regions of tissue where extracellular matrix is likely to be remodeled. In adult animals the expression of these molecules again correlates with extracellular matrix remodeling; SPARC and tenascin are absent or are present at low levels in most tissues, but are re-expressed in response to injury. The studies in chapters 3 and 4 show that the expression of collagenase is upregulated in RSF plated on matrices supplemented with SPARC or tenascin.

Analysis of the expression of collagenase in synovial fibroblasts in cells plated on the function-perturbing anti- $\alpha_5\beta_1$ antibody is also useful for studying signaling through integrin receptors because it contains a ligand that targets a specific receptor, and uses a specific cellular response that can serve as an intracellular handle to follow signals generated at this receptor. The experiments in chapter 5 describe some of the elements in the nuclear signaling pathway that upregulate the expression of collagenase.

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Chapter 2

Signal Transduction through the Fibronectin Receptor Induces Collagenase and Stromelysin Gene Expression

This chapter approaches the question of whether ECM signals to cells through integrins and explores the nature of the ECM signaling event.

Signal Transduction through the Fibronectin Receptor Induces Collagenase and Stromelysin Gene Expression

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Abstract. We have investigated the effects of ligation of the fibronectin receptor (FnR) on gene expression in rabbit synovial fibroblasts. Monoclonal antibodies to the FnR that block initial adhesion of fibroblasts to fibronectin induced the expression of genes encoding the secreted extracellular matrix-degrading metalloproteinases collagenase and stromelysin. That induction was a direct consequence of interaction with the FnR was shown by the accumulation of mRNA for stromelysin and collagenase. Monoclonal antibodies to several other membrane glycoprotein receptors had no effect on metalloproteinase gene expression. Less than 2 h of treatment of the fibroblasts with anti-FnR in solution was sufficient to trigger the change in gene expression, and induction was blocked by dexamethasone. Unlike other inducers of metalloproteinase expression, including phorbol diesters and growth factors, addition of the anti-FnR in solution to cells adherent to serum-derived adhesion proteins or collagen produced no detectable change in cell shape or actin microfilament organization. Inductive effects were potentiated by cross-linking of the ligand. Fab fragments of anti-FnR were ineffective unless cross-linked or immobilized on the substrate. Adhesion of fibroblasts to native fibronectin did not induce metalloproteinases. However, adhesion to covalently immobilized peptides containing the arg-gly-asp sequence that were derived from fibronectin, varying in size from hexapeptides up to 120 kD, induced collagenase and stromelysin gene expression. This suggests that degradation products of fibronectin are the natural inductive ligands for the FnR. These data demonstrate that signals leading to changes in gene expression are transduced by the FnR, a member of the integrin family of extracellular matrix receptors. The signaling of changes in gene expression by the FnR is distinct from signaling involving cell shape and actin cytoarchitecture. At least two distinct signals are generated: the binding of fibronectin-derived fragments and adhesion-blocking antibodies to the FnR triggers events different from those triggered by binding of the native fibronectin ligand. Because the genes regulated by this integrin are for enzymes that degrade the extracellular matrix, these results suggest that information transduced by the binding of various ligands to integrins may orchestrate the expression of genes regulating cell behavior in the extracellular environment.

THE interactions of cells with components of the extracellular matrix (ECM)[1], such as fibronectin (Fn), laminin (Ln), tenascin, and collagens of more than 12 types, play an important role in morphogenesis, tissue repair and regeneration, and metastasis (Liotta et al., 1986; Chiquet-Ehrismann et al., 1986; Humphries et al., 1986; Gehlsen et al., 1988*a*,*b*). During development and remodeling, cells in tissues constantly alter their morphology, migration, and adhesion to ECM components. Temporal, spatial, and cell type-specific regulation of the expression of this large variety of ECM molecules and their receptors provides a powerful set of mechanisms for generating the diversity required for the proper orchestration of cell behavior during differentiation, morphogenesis, and tissue remodeling.

The integrin multigene family of transmembrane, heterodimeric adhesion receptors mediates cell attachment to a variety of ECM molecules, including Fn, Ln, collagen types I, IV, and VI, vitronectin (Vn), fibrinogen, and thrombospondin (reviewed by Ruoslahti and Pierschbacher, 1986; Buck and Horwitz, 1987). Antibody and peptide inhibition studies have implicated these receptors in processes as di-

Portions of this work have appeared previously in abstract form (Tremble, P. M., C. Damsky, and Z. Werb. 1988. *J. Cell Biol.* 107:150a).

1. *Abbreviations used in this paper*: CM, cell-conditioned medium; DME-LH, serum-free DME supplemented with 0.2% lactalbumin hydrolysate; ECM, extracellular matrix; Fn, fibronectin; FnR, fibronectin receptor; LDL-R, low density lipoprotein receptor; Ln, laminin; RSF, rabbit synovial fibroblasts; TIMP, tissue inhibitor of metalloproteinases; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Vn, vitronectin.

verse as neurite outgrowth (Bozyczko and Horwitz, 1986; Hall et al., 1987; Tomaselli et al., 1987), gastrulation (Boucaut et al., 1984), neural crest cell migration (Thiery et al., 1985; Bronner-Fraser, 1986), trophoblast outgrowth (Damsky et al., 1985*b*; Richa et al., 1985; Armant et al., 1986; Sutherland et al., 1988), platelet aggregation (Pytela et al., 1986; Phillips et al., 1988), muscle cell attachment to tendons (Bogaert et al., 1987), and tumor cell metastasis (Humphries et al., 1986; Gehlsen et al., 1988*a*).

In addition to establishing a particular set of contacts with the ECM, cells must be able to modify these contacts in a closely regulated fashion. Mechanisms by which this might be accomplished include regulation of the amounts of ligands and receptors present or alteration of ligand-receptor affinity. Proteolysis of ligands or receptors is likely to be involved in the processes of migration, invasion, and tissue repair (Liotta et al., 1986). Inhibition of proteinases inhibits invasion but not attachment of tumor cells (Mignatti et al., 1986; Schultz et al., 1988). Proteinases are present focally at adhesion sites in some cells, actively modulating their actin cytoskeleton and attachments to the substrate (Beckerle et al., 1987; Pöllänen et al., 1988). There is also a strong correlation between changes in the actin cytoskeleton and the induction of expression of collagenase and stromelysin (Aggeler et al., 1984*a*,*b*; Werb et al., 1986), two members of the metalloproteinase gene family (Whitham et al., 1986). Agents that promote cell spreading, such as Fn (Werb and Aggeler, 1978), and agents that inhibit differentiation of some cell types, such as transforming growth factor-β (Edwards et al., 1987), inhibit the phenotypic expression of collagenase. On the other hand, substances that promote cell rounding, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), some proteinases, and cytochalasin B, induce expression of collagenase (Harris et al., 1975; Aggeler et al., 1984*a*,*b*; Werb et al., 1986; Unemori and Werb, 1986, 1988). Taken together, these data suggest that proteinases play a prominent role in regulating adhesion of cells to ECM.

Given the necessity for closely regulating the establishment and modulation of cell-ECM interactions, we used an adhesion-blocking monoclonal antibody against the Fn receptor (FnR) and Fn-derived peptides to explore the possibility that interference with normal cell-Fn interactions can alter gene expression. We show that ligation of the FnR by these ligands, but not by native Fn, induces the expression of metalloproteinases. In contrast to other agents that stimulate expression of these genes, the anti-FnR-mediated induction can occur in the absence of a major change in cell shape or in the reorganization of the actin cytoskeleton. These data suggest that the status of the Fn-FnR interaction is an important signaling mechanism in regulating the expression of genes relevant to matrix remodeling during differentiation.

Materials and Methods

Cells and Cell Culture

Rabbit synovial fibroblasts (RSF) isolated as described previously (Aggeler et al., 1984*a*) and used between passages 1 and 6 were cultured in DME supplemented with 10% FBS. Cells ($1\text{-}2 \times 10^5$) were plated in 16-mm wells for 14 h in this medium before washing and replacement with serum-free DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH) for experiments. JAR human choriocarcinoma cells were cultured and maintained as described by Damsky et al. (1985*a*).

Preparation and Characterization of mAbs against Integrins

Whole JAR human choriocarcinoma cells were removed from the culture dishes with 5 mM EDTA, followed by washing with PBS containing Ca^{2+} and Mg^{2+}. A Lewis rat was immunized by a series of fortnightly injections of 10^7 JAR cells, twice intraperitoneally and twice intrasplenically. 4 d after the last injection the immune spleen cells were removed and fused with mouse Sp2/0 plasmacytoma cells and cultured according to the procedure of Kennett (1980) as modified by Wheelock et al. (1987). All wells with growing cells were screened by testing the ability of culture supernatants to inhibit the attachment of JAR cells to Fn or Ln in the attachment assay described by Giancotti et al. (1985) and Tomaselli et al. (1987). Two supernatants inhibited cell attachment to Fn only, whereas two others inhibited cell attachment to both Fn and Ln. After subcloning of the latter, subsequent characterization showed that the two mAbs also inhibited cell attachment to collagen types I and IV. None of the supernatants inhibited attachment to Vn. Detailed characterization of these mAbs will appear elsewhere (Hall, D. E., E. Crowley, and C. H. Damsky, manuscript submitted for publication). One mAb that inhibited cell attachment to Fn only (BIIG2) and one mAb that inhibited cell attachment to Fn, Ln, and the collagens (AIIB2) were selected for further study and designated anti-FnR and anti-β_1, respectively. An unrelated cell-binding rat mAb, BIVF2, of the same subtype was used as a control. The second rat anti-FnR mAb (BIE5) was used in certain experiments where indicated.

The mAbs were further characterized by immunoprecipitation to determine which polypeptides on RSF were recognized by anti-FnR and anti-β_1. Proteins in RSF ($\sim 5 \times 10^6$) were labeled by incubation for 24 h with 50 μCi/ml of [^{3}H]6-D-glucosamine (sp act 25 Ci/mmol; New England Nuclear, Boston, MA) in a low glucose (1 g/liter) formulation of DME. Cells were harvested in 2 mM EDTA, washed, and lysed in 10 mM Tris-acetate buffer, pH 8, containing 0.5% NP-40, 150 mM NaCl, and 2 mM PMSF. Precipitation of integrins by the anti-FnR and anti-β_1 mAbs was carried out according to the procedure described by Tomaselli et al. (1987). Immunoprecipitates were analyzed on 7% SDS-polyacrylamide gels under nonreducing conditions, followed by fluorography.

Preparation of Anti-FnR IgG and Monovalent Fab Fragments

IgG was purified from BIIG2 culture supernatant by anti-rat IgG affinity chromatography with goat anti-rat IgG immobilized on CNBr-activated agarose (Sigma Chemical Co., St. Louis, MO). The IgG was eluted with glycine, pH 2.6, neutralized immediately with Tris base, and dialyzed against PBS, pH 8.0. Affinity-purified BIIG2 in PBS, pH 8.0, was digested with papain (100 mg antibody per 1 mg papain) for 2 h at 37°C (Parham, 1986). The proteins were then alkylated with iodoacetamide (8 mM final concentration); the reaction mixture was dialyzed against 10 mM Tris-buffered saline, pH 8.0, or PBS, pH 8.0; and Fab fragments were separated from Fc fragments and undigested antibody by DEAE-cellulose or protein A-agarose chromatography. IgG and Fab concentrations are expressed in molar concentrations assuming molecular masses of 160 and 50 kD, respectively.

Preparation of ECM Ligand and Antibody Substrates

Culture dishes (Costar, Cambridge, MA; 24- or 48-well plates) were incubated with Fn or Vn at 10 μg/ml in PBS, pH 7.4, overnight at 4°C. Unoccupied sites were blocked with 0.2% bovine serum albumin at ambient temperature for 2 h before cell plating. To assess the extent of collagenase expression on various substrates, cells were plated on Fn, Vn, anti-FnR IgG, or Fab. In some cases these substrates were covalently linked to 12-mm glass coverslips as described below.

Covalent Protein Coating of Glass Coverslips for Specific Adhesion

For covalently linked Fn, type I collagen, peptides containing the arg-gly-asp (RGD) cell recognition sequence, or purified anti-FnR IgG or Fab, polypeptides were conjugated to glass coverslips as follows. Coverslips were washed sequentially with 20% concentrated H_2SO_4, water, 0.1 N NaOH, and water. Dried coverslips were exposed to γ-aminopropyltriethoxysilane (Sigma Chemical Co.) for 4 min at ambient temperature, followed by water and PBS rinses. The coverslips were incubated with 0.25% glutaraldehyde in PBS for 30 min at 22°C, washed several times with PBS, and covered

with ECM proteins or antibody solutions (20–700 μg/ml) for 1 h at ambient temperature, washed with PBS, and used immediately. All steps after the glutaraldehyde treatment were done with sterile reagents and utensils. Freshly trypsinized cells resuspended in DME-LH were plated on the glass coverslips containing immobilized proteins in 24-well plates.

Preparation of mAbs against Collagenase

Rabbit collagenase was purified from cultures of rabbit skin. Fragments of rabbit skin were cultured in DME-LH, and the cell-conditioned medium (CM) was collected every 2 d for up to 16–18 d. Medium from day 4 on was pooled and brought to 25% saturation with $(NH_4)_2SO_4$. The supernatant liquid was dialyzed extensively against 20 mM NH_4HCO_3, freeze-dried, reconstituted in water, and dialyzed against 10 mM Tris-HCl buffer, pH 8.4, with 0.05% Brij-35. Collagenase was purified by DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography, followed by zinc-chelate affinity chromatography (Cawston and Tyler, 1979; Chin et al., 1985). The collagenase, which was eluted with 50 mM sodium acetate, pH 4.7, was purified 460-fold.

BALB/c mice were immunized with 100 μg of purified collagenase as described by Oi and Herzenberg (1980), except that Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant was used for booster injections. The dispersed spleen cells of the mouse were fused with a subclone of the mouse myeloma line P3-X63-Ag8 that does not produce immunoglobulin. The fusion protocol was essentially the same as that described previously (Oi and Herzenberg, 1980), except that warm PBS instead of serum-free medium was added to the polyethylene glycol pellet, and the fused cells were centrifuged at 200 *g* for 8 min and resuspended in 12 ml of RPMI medium containing 15% FBS; 50 μl were placed in each well of a 24-well Costar plate for hybrid selection in medium containing 1×10^{-4} M hypoxanthine, 1×10^{-6} M aminopterin, and 1.6×10^{-5} M thymidine. As the hybrid cells grew, the medium was tested for production of specific antibody by a two-step, solid-phase ELISA, essentially as described by Maggio (1980), with alkaline phosphatase–conjugated anti-mouse IgG as secondary antibody. IgG secretors were subcloned and characterized by immunoblotting and immunoprecipitation of collagenase from TPA-treated RSF and by immunofluorescent staining of TPA-treated RSF. Relevant hybridoma clones were cultured in Ventrex HL-1 serum-free medium (Fisher Scientific Co., Pittsburgh, PA) or injected into BALB/c mice for ascites tumor production of antibody. mAb obtained from both sources was purified over a protein A–Sepharose column (Pharmacia Fine Chemicals). An "oligoclonal" mixture of five anticollagenase mAbs was used in this study.

Biosynthetic Labeling of Proteins Secreted by RSF

RSF proteins were biosynthetically labeled with 25–50 μCi/ml of [^{35}S]methionine (sp act 1,265 Ci/mmol) for 2–4 h in methionine-free DME at 37°C. Proteins were precipitated from the CM with quinine sulfate–SDS and resuspended in Laemmli sample buffer, as previously described (Unemori and Werb, 1986), and samples were analyzed on 10% gels with a 3% stacking gel under reducing conditions. Collagenase and stromelysin were immunoprecipitated from [^{35}S]methionine-labeled secreted proteins with 1–2 μg of monoclonal anti-rabbit collagenase IgG or 10 μg polyclonal sheep anti-stromelysin (Chin et al., 1985), followed by formalin-fixed *Staphylococcus aureus* (Zysorbin; Zymed Labs, Burlingame, CA). Nonimmune mouse IgG (2 μg) or sheep IgG (10 μg) replaced the immune IgG as controls. Total secreted proteins were separated on 7–15% or 10% SDS-polyacrylamide gels and then analyzed by fluorography as described previously (Unemori and Werb, 1986, 1988). All experiments were performed at least twice.

SDS–Substrate Gels for Analysis of Proteinases

CM was subjected to substrate gel electrophoresis in 10% polyacrylamide gels impregnated with 1 mg/ml gelatin or casein (Unemori and Werb, 1986; Herron et al., 1986). Unconcentrated CM was mixed with Laemmli sample buffer (lacking β-mercaptoethanol and modified to contain a final concentration of 2.5% SDS) and electrophoresed under nonreducing conditions. After electrophoresis, the gel was incubated in 2% Triton X-100 for 30 min at 37°C to remove SDS and then incubated for 18–24 h at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH 8, containing 5 mM $CaCl_2$). After staining with Coomassie blue R250, gelatin- or casein-degrading enzymes present in the CM were identified as clear zones in a blue background.

RNA Isolation, Blotting, and Hybridization

Total RNA was isolated from cultured RSF by the method of either Chirgwin et al. (1979) or Cathala et al. (1983). RNA was separated on agarose gels, transferred to nylon membranes, and probed with ^{32}P-labeled cDNA probes as previously described (Maniatis et al., 1982; Frisch et al., 1987). cDNA clones for rabbit collagenase (pCL1), stromelysin (pSL2; Frisch et al., 1987), human tissue inhibitor of metalloproteinases (TIMP) (SP65 TIMP/erythroid-potentiating activity; sequence identical to that described by Docherty et al., 1985; gift of M. Wrann, Sandoz Research Laboratories, Vienna, Austria), human 68-kD gelatinase/type IV collagenase (Collier et al., 1988; gift of G. Goldberg, Washington University, St. Louis, MO), and human γ-actin (Engel et al., 1981; gift of L. Kedes, Stanford University, Stanford, CA) were used to generate probes.

Colocalization of Collagenase and Actin Microfilament Bundles in RSF

Cells were plated in 24-well plates (Costar) in DME containing 10% FBS on 11-mm round glass coverslips for 8 h to permit spreading. Medium was then replaced with DME-LH. Anti-FnR IgG or control IgG was added at a concentration of 15 μg/ml. At various times, medium containing IgG was removed and replaced with DME-LH. At 24 h, coverslips were rinsed with DME-LH and fixed for 10 min in 3% paraformaldehyde in PBS containing 0.5 mM Ca^{2+} and then stained by a modification of the protocol of Damsky et al. (1985*a*). Briefly, after fixation, cells on coverslips were rinsed, made permeable with acetone at 4°C for 3 min, and incubated with PBS containing 0.2% bovine serum albumin. Cells were exposed for 1 h to a cocktail of mouse monoclonal antibodies against rabbit collagenase (Unemori and Werb, 1988). Cells were rinsed and exposed for 1 h to biotinylated goat anti-mouse IgG (Sigma Chemical Co.), rinsed again, and exposed for 30 min to a mixture of 1:100 dilution of FITC-labeled streptavidin (Amersham Corp., Arlington Heights, IL) and 1 μg/ml of rhodamine-phalloidin (Molecular Probes, Inc., Junction City, OR). The washed coverslips were then mounted in Gelvatol containing phenylenediamine to reduce quenching of the fluorescein signal (Platt and Michael, 1983) and examined with a phase epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with filters suitable for FITC and rhodamine and a 63× Planapo oil immersion objective. Cells were photographed with Eastman Kodak Co. (Rochester, NY) Tri-X film and developed with Accufine.

Other Antibodies and Reagents

A mouse anti-FnR mAb (gift of D. Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA) was analyzed in certain experiments where indicated. A mouse mAb binding to the rabbit low density lipoprotein receptor (LDL-R) (gift of T. Innerarity, Gladstone Foundation Laboratories, University of California, San Francisco, CA) and a mouse mAb to the transferrin receptor (AMAC Inc., Westport, ME) were used as controls for antibodies binding to other membrane receptors. Gly-arg-gly-asp-ser-pro (GRGDSP) and gly-arg-gly-glu-ser-pro (GRGESP) peptides were gifts of E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) or were purchased from Telios, Inc. (La Jolla, CA). Gly-D-arg-gly-asp-ser-pro-ala-ser-ser-lys (GdRGDSPASSK), gly-arg-gly-asp-asn-pro (GRGDNP), and the 120-kD and 60-kD cell-binding fragments of Fn were purchased from Telios, Inc. Vn was a gift of Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA); anti-Fn antibody, Ln, and Fn were purchased from Collaborative Research Inc., Waltham, MA. Type I collagen (Vitrogen) was purchased from Collagen Corp. (Palo Alto, CA).

Results

An mAb Recognizes the FnR in Rabbit Fibroblasts

We first tested an mAb to the FnR (BIIG2), one of the β_1 family of integrins, that was produced from a rat immunized with JAR human choriocarcinoma cells. Anti-FnR interfered with the initial attachment of many human cell types (data not shown), as well as RSF, to Fn (Fig. 1 *A*), but not to Ln, collagen types I and IV, or Vn (data not shown). Anti-FnR immunoprecipitated a dimer migrating at 110 and 150 kD under nonreducing conditions from NP-40 extracts of [^{3}H]glucosamine-labeled RSF (Fig. 1 *B*). We then used another mAb, anti-β_1, which recognizes the common β-chain of the β_1-integrins, to determine which other members of this subfamily are present in RSF. Anti-β_1 interfered with

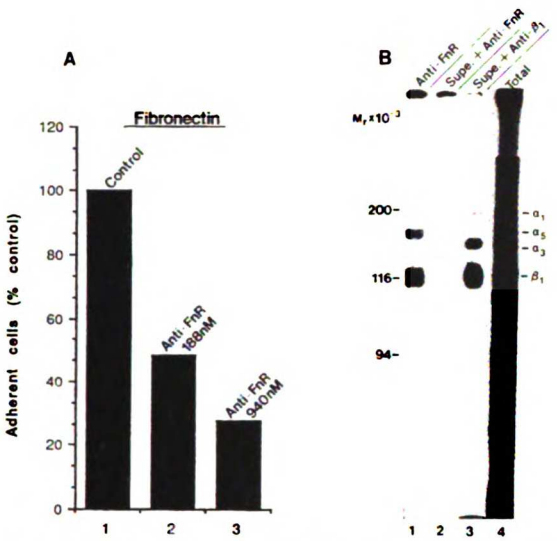


Figure 1. Characterization of an mAb that recognizes the FnR. (*A*) Inhibition of initial adhesion of RSF on culture wells coated with 10 μg/ml of Fn by anti-FnR (BIIG2) at 0.43 or 1.1 μM compared to the control mAb (BIVF2) at 0.63 μM. (*B*) Immunoprecipitation of integrins from NP-40 lysates of [^{3}H]glucosamine-labeled RSF. The immunoprecipitates were separated by SDS-PAGE under non-reducing conditions. The lysate was immunoprecipitated with anti-FnR (lane *1*), and the supernatant (*Supe*) was immunoprecipitated sequentially with anti-FnR (lane *2*) and anti-β_1 (lane *3*); total lysate is shown in lane *4*. The molecular weight standards ($\times 10^{-3}$) and the migration of the FnR α-chain (α_5), VLA_1 α-chain (α_1), VLA_3 α-chain (α_3), and β_1-chain are indicated.

attachment of RSF and several human cell types to Fn, Ln, and collagen types I and IV, but not to Vn, a member of the β_3-subfamily of integrins (data not shown). Anti-β_1 immunoprecipitated the 110-kD band of the β_1-chain and two α-chains at 140 and 190 kD that are distinct from the FnR α-chain (Fig. 1 *B*). Sequential depletion experiments (data not shown) indicated that these α-chains correspond to the integrins VLA_1 and VLA_3 (Takada et al., 1987). Thus, RSF express three members of the β_1-family of integrins, including the FnR (VLA_5).

Anti-FnR Induces Expression of Collagenase and Stromelysin

Alteration of cell adhesion and shape by proteinases such as trypsin (Werb and Aggeler, 1978), culture on surfaces of varying adhesivity (Aggeler et al., 1984*b*), and collagen gel tension (Unemori and Werb, 1986) results in a change in gene expression in fibroblasts characterized by synthesis and secretion of the metalloproteinases collagenase and stromelysin. It was therefore of interest to determine whether perturbation of the FnR would affect collagenase and stromelysin gene expression. Accordingly, we cultured RSF for 24 h in uncoated tissue culture wells in medium containing serum and then treated them with anti-FnR or an unrelated rat mAb as a control. After treatment of RSF with anti-FnR, a striking induction of several secreted proteinases was observed by zymography of the CM in SDS-substrate gels containing gelatin (Fig. 2 *A*) or casein (Fig. 2 *B*). In particular, bands corresponding to the proenzymes of the metalloproteinases collagenase (Fig. 2 *A*) and stromelysin (Fig. 2 *B*) were visible. The induction of collagenase and stromelysin activity by anti-FnR was selective, because the expression of several other metalloproteinases, including the 68-kD gelatinase/type IV collagenase, was unchanged by the treatment.

Analysis of newly synthesized secreted proteins showed that polypeptides migrating at 51, 53, and 57 kD were induced in a concentration-dependent fashion by anti-FnR but not by control mAb (Fig. 3 *A*). mAbs recognizing two other membrane glycoprotein receptors, the LDL-R and the transferrin receptor, did not induce these polypeptides at concentrations of up to 625 nM (data not shown). Treatment of RSF with <25 nM (4 μg/ml) anti-FnR was effective in inducing expression of these polypeptides. Immunoprecipitation with specific antibodies indicated that the 53- and 57-kD bands were procollagenase and the 51-kD band was prostromelysin (Fig. 3 *B*). After treatment with anti-FnR, the two proen-

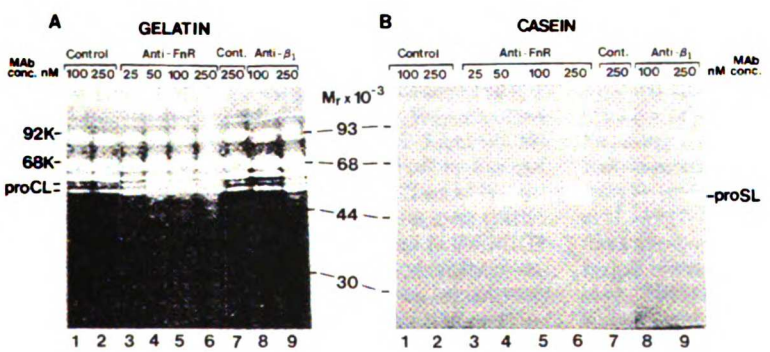


Figure 2. SDS-substrate gel zymography of secreted metalloproteinases induced by treating RSF with anti-FnR. RSF cultured in 48-well plates for 24 h in DME supplemented with 10% FBS were treated with various concentrations of control mAb (lanes *1*, *2*, and *7*), anti-FnR (lanes *3–6*), or anti-β_1 (lanes *8* and *9*) as culture supernatants in DME-LH for 16 h. The cultures were then washed and incubated in DME-LH for 24 h. Samples (10 μl) of the CM were then separated on SDS-substrate gels containing either gelatin (*A*) or casein (*B*), and the zymograms were developed. The migration of prestained molecular weight standards and the bands corresponding to procollagenase (*proCL*), prostromelysin (*proSL*), 68-kD gelatinase (*68K*), and 92-kD gelatinase (*92K*) (Unemori and Werb, 1988) are indicated.

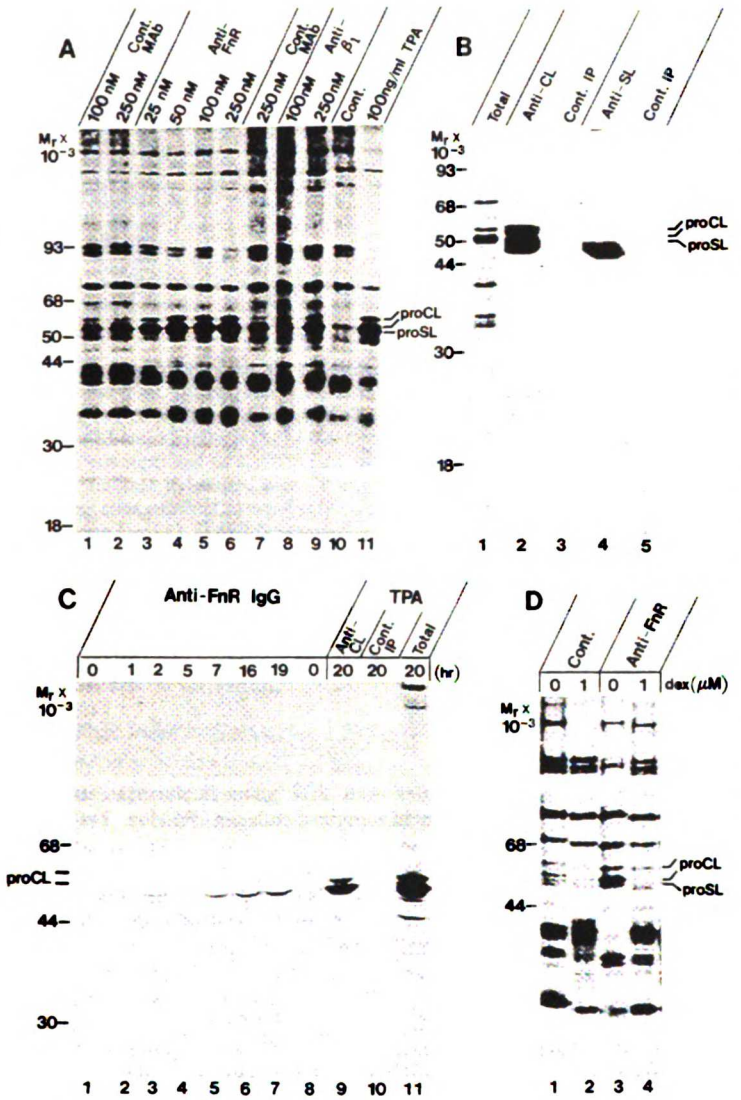


Figure 3. Expression of collagenase and stromelysin gene products by RSF treated with anti-FnR mAb. (*A*) RSF were treated with various concentrations of control mAb (lanes *1*, *2*, and *7*), anti-FnR (lanes *3–6*), or anti-β_1 (lanes *8* and *9*) as culture supernatants, or TPA (lanes *10* and *11*) as described in the legend to Fig. 2, then biosynthetically labeled with [^{35}S]methionine for 4 h. The secreted proteins were analyzed by SDS-PAGE followed by fluorography. (*B*) Collagenase and stromelysin bands were identified by immunoprecipitation of labeled secreted proteins from treated RSF. Total secreted proteins in 50 μl of CM are shown in lane *1*. Proteins were immunoprecipitated from 400 μl of CM with anticollagenase IgG (lane *2*), nonimmune mouse IgG (lane *3*), antistromelysin IgG (lane *4*), or nonimmune sheep IgG (lane *5*), and analyzed by SDS-PAGE followed by fluorography. (*C*) Time course of induction of collagenase secretion by anti-FnR. Confluent RSF were incubated with 310 nM anti-FnR in DME-LH for 1–19 h and then anti-FnR was removed and the cells were cultured in DME-LH for the remaining time up to a total time of 20 h (lanes *1–8*). The cells were then labeled with [^{35}S]methionine for 4 h. As a positive control, RSF were treated with TPA for 20 h (lane *9*) before labeling. The CM containing the biosynthetically labeled proteins was collected, and collagenase was immunoprecipitated with anticollagenase and separated by SDS-PAGE. Lane *10* shows immunoprecipitation with control mouse mAb, and lane *11* shows the total secreted proteins of TPA-treated cells. (*D*) Effect of dexamethasone on induction of collagenase by anti-FnR. RSF were untreated (lane *1*), treated with 1 μM dexamethasone (lane *2*), or treated with anti-FnR as described in *A*, with (lane *4*) or without (lane *3*) 1 μM dexamethasone before biosynthetic labeling with [^{35}S]methionine and separation of secreted proteins by SDS-PAGE. Molecular weight standards ($\times 10^{-3}$) and the migration of procollagenase (*proCL*) and prostromelysin (*proSL*) are indicated.

zymes accounted for as much as 5% of the total secreted proteins of RSF. Less than 2 h of exposure to anti-FnR was required for collagenase expression to be evident at the 24-h evaluation point (Fig. 3 *C*). Anti-β_1 also induced collagenase and stromelysin but was less effective than anti-FnR (Fig. 3 *A*). Two human fibroblast lines (MRC-5 and WI-38) also responded to treatment with the anti-FnR by induced expression of collagenase (data not shown). Two additional adhesion-blocking anti-FnR antibodies, the rat mAb (BIE5) and a mouse mAb, were qualitatively similar to the BIIG2 anti-FnR mAb in inducing collagenase and stromelysin expression in RSF (data not shown). In addition, dexamethasone suppressed the expression of the metalloproteinases induced by anti-FnR (Fig. 3 *D*). In the following sections we concentrate on collagenase expression, although similar induction was generally seen for stromelysin.

We next used RNA blotting analysis to identify collagenase and stromelysin transcripts in RNA extracted from RSF treated with anti-FnR. Untreated RSF contained little mRNA for either metalloproteinase. Treatment of RSF with anti-FnR induced expression of mRNA for collagenase and stromelysin coordinately (Fig. 4) as in treatment with TPA (Frisch et al., 1987; Unemori and Werb, 1988) but in lower amounts (data not shown). In contrast, the expression of mRNA transcripts of the 68-kD gelatinase/type IV collagenase (Collier et al., 1988) and actin was constitutive and was not affected by treatment with anti-FnR (Fig. 4). The mRNA for TIMP decreased very slightly in response to anti-FnR, whereas it

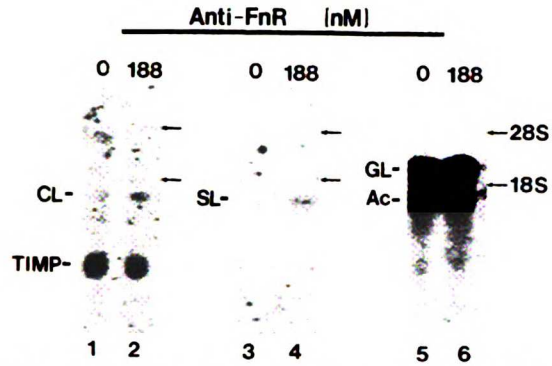


Figure 4. Regulation of metalloproteinase mRNA expression in RSF by anti-FnR. Blotting analysis of RNA isolated from untreated RSF (lanes *1*, *3*, and *5*) or RSF treated with 188 nM anti-FnR (lanes *2*, *4*, and *6*) for 14 h followed by 14 h in DME-LH alone. Total RNA (10 μg) was separated on agarose gels, transferred to nylon membranes, and then hybridized with ^{32}P-labeled inserts from cDNAs encoding collagenase (*CL*) and TIMP (lanes *1* and *2*), stromelysin (*SL*) (lanes *3* and *4*), or 68-kD gelatinase (*GL*) and actin (*Ac*) (lanes *5* and *6*). Migration of 28-S and 18-S rRNA are also indicated.

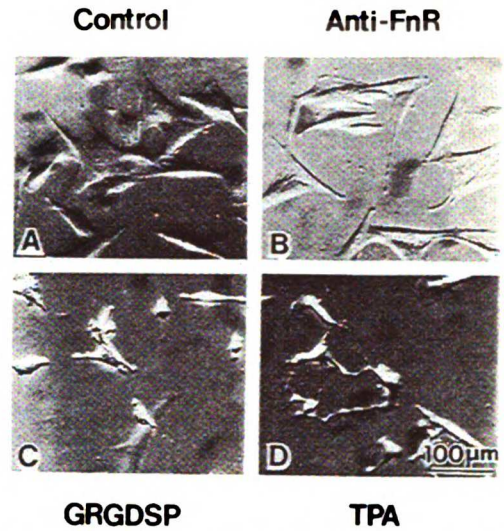


Figure 5. Low power morphological appearance of RSF treated with anti-FnR or a peptide containing RGD. RSF were treated with (*A*) control mAb (250 nM), (*B*) anti-FnR (250 nM), (*C*) GRGDSP (100 μg/ml), or (*D*) TPA (50 ng/ml) for 24 h. Phase-contrast microscopy. Untreated RSF and RSF treated with GRGESP (not shown) were indistinguishable from RSF treated with control mAb in *A*.

is induced by TPA (Murphy et al., 1985; Herron et al., 1986; Unemori and Werb, 1986).

Anti-FnR Induces Collagenase Expression in the Absence of Changes in Cell Shape

Previous experiments using TPA, cytochalasin D, calcium ionophore, collagen gel contraction, and poorly adhesive substrates indicated that induction of collagenase is strongly correlated with cell rounding and a substantial reorganization of the actin cytoskeleton, as determined by staining of actin filaments with rhodamine-phalloidin (Aggeler et al., 1984*a*,*b*; Unemori and Werb, 1986, 1988; Werb et al., 1986). When RSF were plated in the presence of serum that contained at least two adhesion proteins (Vn, Fn), they showed both a marked shape change (Fig. 5) and induction of collagenase expression (Fig. 6) after treatment with TPA. Under the same conditions, however, anti-FnR induced collagenase expression in the absence of apparent cytoskeletal reorganization: RSF maintained both a flattened morphology and elaborate arrays of rhodamine-phalloidin-staining actin microfilament bundles throughout the induction period (Fig. 5), and yet the cells went on to express collagenase (Fig. 6). These results are summarized in Table I. The adherent area of the anti-FnR mAb-treated cells differed from control RSF by <5% (data not shown).

Further evidence that shape change can be divorced from collagenase induction is indicated by the observations on cells plated and spread on immobilized anti-FnR as the substrate. Under these conditions, the cells displayed a flattened morphology but expressed collagenase (Table I; Fig. 7 *A*). Cells spread on type I collagen, Fn, or anti-LDL-R immobilized by the same procedure did not express collagenase. Therefore, immobilized anti-FnR acts as an inductive substrate, whereas several other substrates including Fn, the natural ligand of the FnR, do not.

To explore the relationship between cell shape and metalloproteinase gene expression further, anti-FnR mAb was added to RSF cultured on covalently immobilized purified ECM substrates. Anti-FnR induced collagenase expression by RSF cultured on type I collagen, Fn (Fig. 7 *A*), and Vn

Table I. Lack of Correlation between Induction of Collagenase and Stromelysin Gene Expression and Changes in Cell Shape

Inducing agent	Cell substrate	Cell shape	Collagenase/stromelysin induced*
None	Serum	Flat	No
None	Collagen	Flat	No
None	Fn	Flat	No
None	Anti-LDL-R	Flat	No
TPA	Serum	Rounded	Yes
Cytochalasin‡	Serum	Rounded	Yes
Collagen gel contraction‡	Serum	Rounded	Yes
Proteinases‡	Serum	Rounded	Yes
Soluble anti-FnR	Serum	Flat	Yes
Soluble anti-FnR	Fn	Rounded	Yes
Soluble anti-FnR	Collagen	Flat	Yes
Immobilized anti-FnR	Anti-FnR	Flat	Yes
GRGESP	Serum	Flat	No
GRGDSP	Serum	Rounded	Yes
Immobilized anti-FnR plus soluble GRGDSP	Anti-FnR	Rounded	Yes
GRGDSP	Collagen	Flat	No
Immobilized GRGDSP	GRGDSP	Flat	Yes
Immobilized Fn peptides	Fn peptides	Flat	Yes

* Induction of proteinases was determined by incorporation of [^{35}S]methionine into newly synthesized secreted proteins and/or by immunocytochemistry.
‡ Data are from Unemori and Werb (1986); Werb and Aggeler (1978); and Werb et al. (1986).

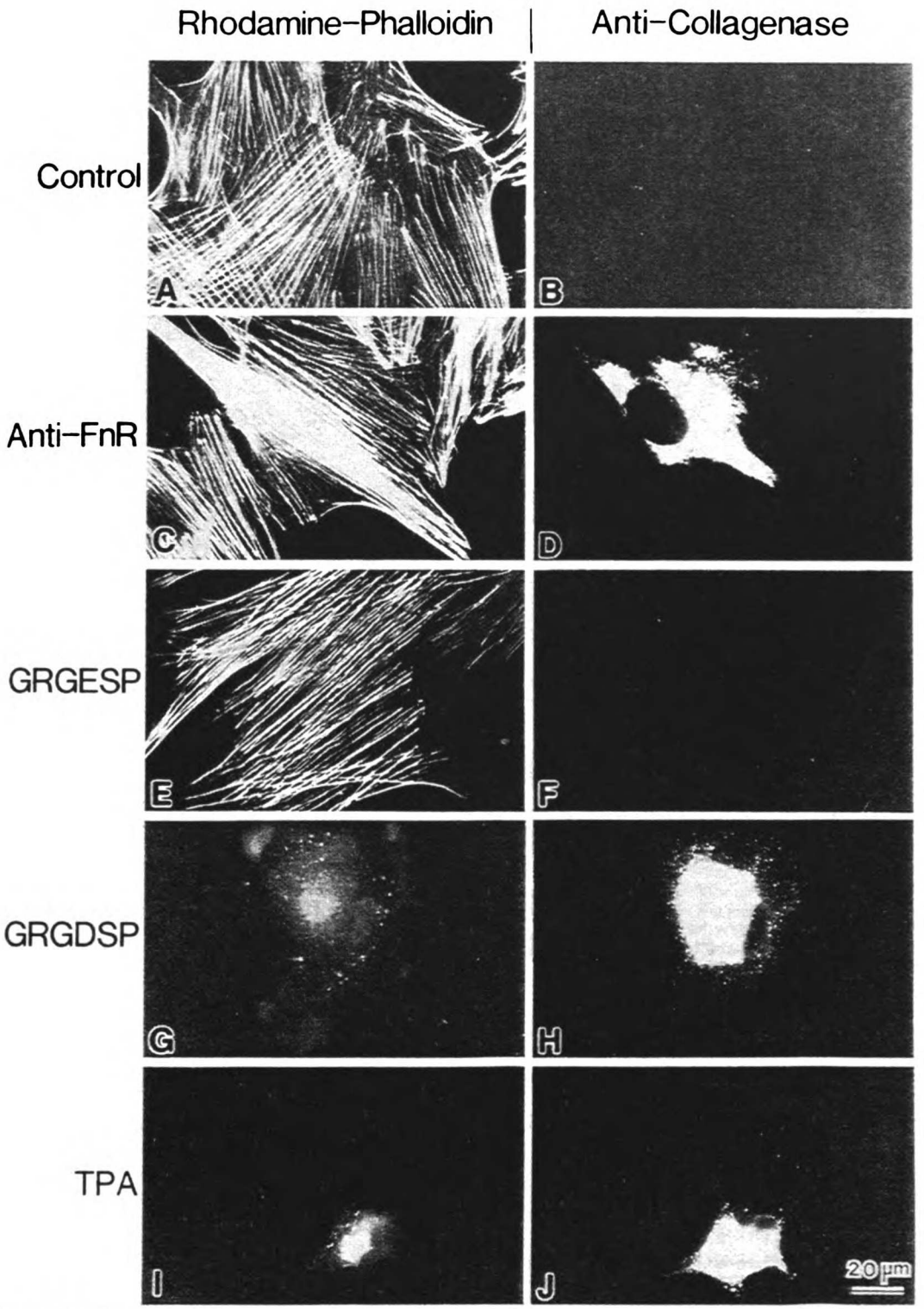


Figure 6. Localization of collagenase and actin microfilaments in RSF treated with anti-FnR or a peptide containing RGD. RSF plated on glass coverslips were left untreated (*A* and *B*) or treated with (*C* and *D*) anti-FnR (250 nM), (*E* and *F*) GRGESP (100 μg/ml), (*G* and *H*) GRGDSP (100 μg/ml), or (*I* and *J*) TPA (50 ng/ml), fixed, and double stained with (*left*) rhodamine-phalloidin to visualize actin microfilaments and (*right*) anticollagenase mAb followed by biotinylated anti-mouse IgG and fluorescein-streptavidin to visualize intracellular collagenase. Paired fluorescence micrographs are shown.

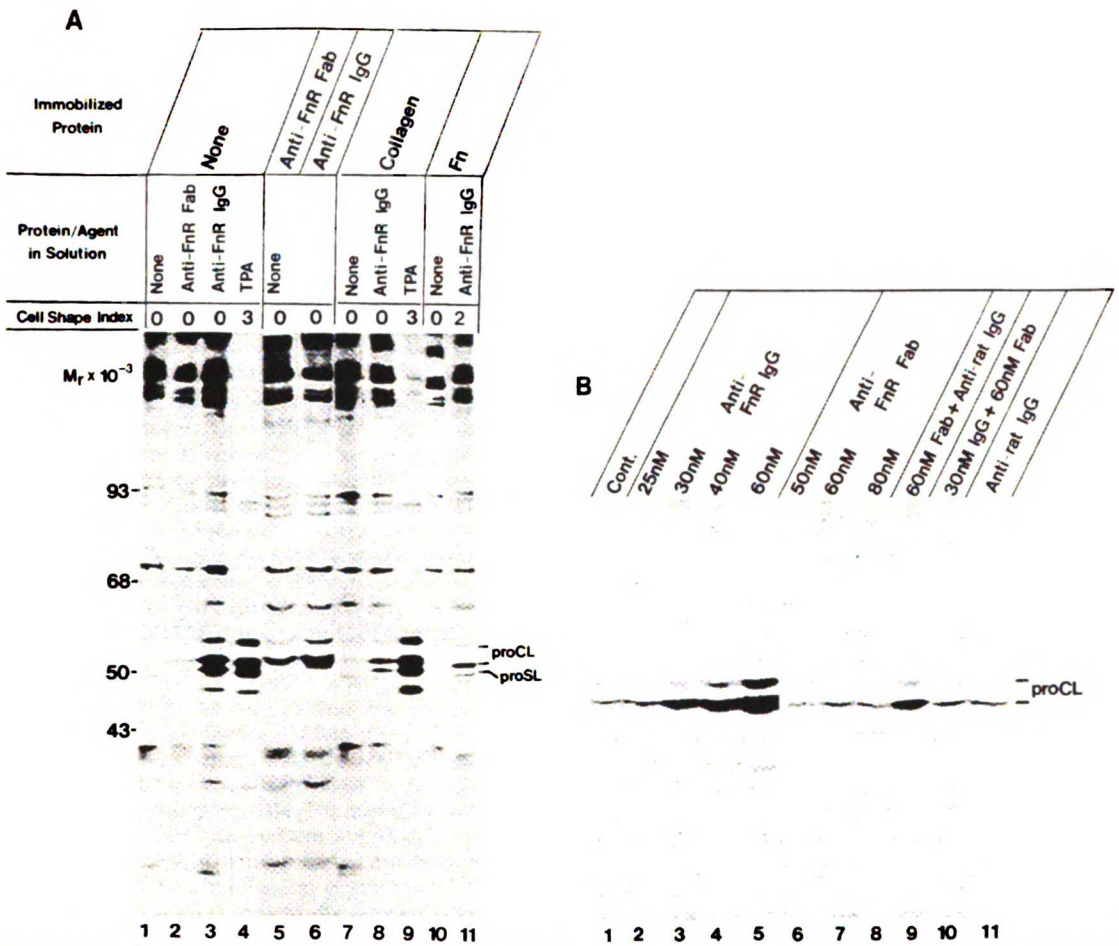


Figure 7. Induction of metalloproteinase expression by immobilized anti-FnR and requirement for cross-linked anti-FnR mAb. (*A*) RSF were plated on uncoated coverslips (lanes *1–4*) in DME supplemented with 10% FBS for 4 h, then placed in DME-LH, or on coverslips coated with covalently immobilized anti-FnR Fab (lane *5*), anti-FnR IgG (lane *6*), type I collagen (lanes *7–9*), or Fn (lanes *10* and *11*) in DME-LH. Soluble anti-FnR Fab at 400 nM (lane *2*), anti-FnR IgG at 115 nM (lanes *3*, *8*, and *11*), or TPA at 100 ng/ml (lanes *4* and *9*) was added. After 20 h the medium was removed and cultures were incubated with [^{35}S]methionine for 4 h to label proteins. The labeled secreted proteins were analyzed by SDS-PAGE followed by fluorography. The cell shape of the treated RSF, rated on a scale of 0–4, indicating flat to round but still attached (Aggeler et al., 1984*b*), is indicated across the top of the gel. The data are from two different experiments. (*B*) To examine the requirement for cross-linking, confluent RSF in 48-well plates were left untreated (lane *1*) or treated with 25–60 nM anti-FnR IgG (lanes *2–5*), 50–80 nM anti-FnR Fab (lanes *6–8*), 60 nM anti-FnR Fab plus 80 μg/ml rabbit anti-rat IgG (lane *9*), or 30 nM anti-FnR IgG plus 60 nM anti-FnR Fab (lane *10*). Control rabbit anti-rat IgG (80 μg/ml) alone is shown in lane *11*. The migration of procollagenase (*proCL*) is indicated.

(data not shown) but changed the cell shape only in cells cultured on Fn. Antibody to the Fn substrate itself also rounded up cells cultured on Fn and induced expression of collagenase in these cells (Table I).

Cross-linking Potentiates Induction of Collagenase Expression by Anti-FnR

The bivalent nature of antigen-antibody interactions suggests that cross-linking of the FnR in the plane of the membrane by anti-FnR may be important to the induction process. To evaluate this possibility, RSF were treated with monovalent anti-FnR Fab or bivalent anti-FnR IgG. Even at twice the equimolar concentration, monovalent anti-FnR Fab was much less effective than bivalent anti-FnR IgG in inducing expression of metalloproteinase, and competing anti-FnR Fab reduced the inducing effect of anti-FnR IgG (Fig. 7 *B*). Inducing activity of the anti-FnR Fab was increased when a secondary anti-rat IgG was added to cross-link the FnR artificially by interacting in a bivalent manner with the anti-FnR Fab.

A second line of evidence that cross-linking of the FnR by anti-FnR is important comes from the observation that immobilization of monovalent anti-FnR Fab on the substrate enhanced its collagenase-inducing activity (Fig. 7 *A*). These data suggest that aggregation of the FnR by anti-FnR, and not just occupancy by the anti-FnR mAb, is important in the gene induction events (Table I).

Fibronectin-derived Peptides Induce Collagenase and Stromelysin Expression

Although antibodies to receptors may behave as high affinity ligands, the anti-FnR mAb did not mimic the effects of native Fn. Therefore, we sought another physiological ligand with the same effector functions as the antibody. Hexapeptides containing RGD, which is present in Fn, Vn, and other ECM ligands that interact with integrins, interfere with cell adhesion (Pytela et al., 1986; Pierschbacher and Ruoslahti, 1987) and differentiation events (Menko and Boettiger, 1987). If the effects of anti-FnR on metalloproteinase gene expression are due to interference with the interaction of RSF with Fn, then RGD peptides should also induce metalloproteinases. We found that the GRGDSP peptide, but not the control GRGESP peptide, when added to RSF cultured in the presence of serum, induced a concentration-dependent increase in collagenase and stromelysin synthesis and secretion, as analyzed by zymography (Fig. 8 *A*) and by biosynthetic labeling of newly synthesized secreted proteins (Fig. 8 *B*). However, unlike the treatment with anti-FnR mAb, treatment with the GRGDSP peptide caused a marked shape change and reorganization of actin microfilament bundles along with the induction of collagenase expression (Figs. 5, 6, and 8, *A* and *B*). The GRGESP peptide produced no shape change, actin rearrangement, or collagenase induction.

Because the GRGDSP sequence is not recognized by the collagen receptor of the integrin class, binding to collagen is not reversed by this peptide (Dedhar et al., 1987). In contrast to cells plated in serum, when the GRGDSP peptide was added to RSF plated on a collagen substrate the cells did not round up, and metalloproteinase gene expression was not induced (Table I). These results were surprising because they suggested that the RGD peptide induces metalloproteinase gene expression by a mechanism dependent on a change in cytoarchitecture rather than the shape-independent mechanism induced by the anti-FnR. On the other hand, the GRGDSP peptide caused cell rounding in the RSF plated on immobilized anti-FnR (Table I), suggesting that anti-FnR recognizes a site on the FnR close to that bound by the RGD cell-recognition sequence on Fn.

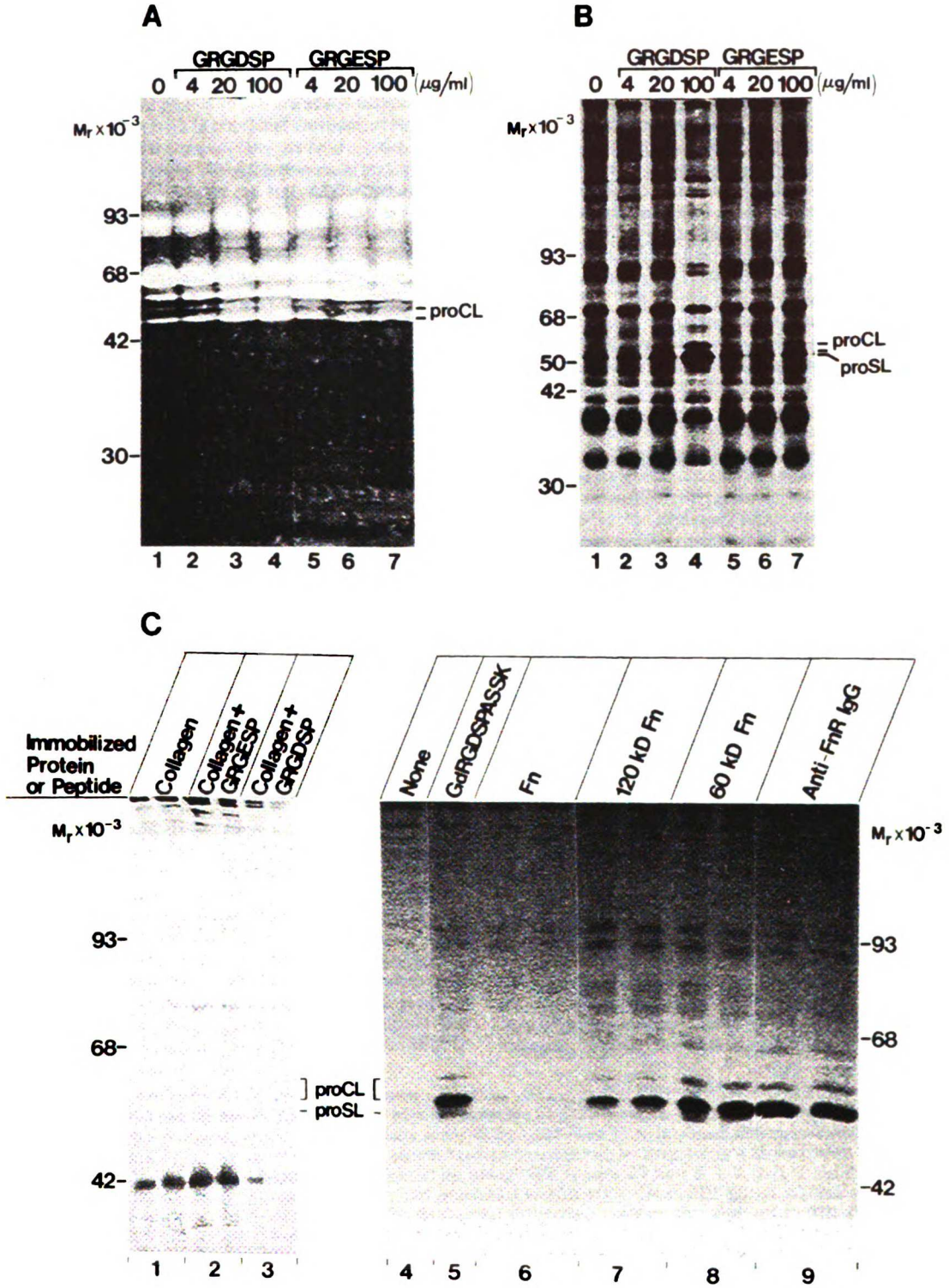
These disparate observations can be reconciled with the results with the anti-FnR mAb when we consider that the RGD peptide is a monovalent ligand. If the RGD peptide behaves as the natural inductive ligand for the FnR receptor only when it is present in a multimeric form, then immobilization of the peptide on the substrate should potentiate its inductive effect. RSF spread on immobilized GRGDSP, but not on GRGESP, rendering GRGESP inappropriate as a control. However, when GRGDSP or GRGESP was immobilized by cross-linking in the presence of collagen, RSF spread and assembled an organized actin cytoskeleton on both substrates. Collagenase expression was induced only in the cells spread on the immobilized GRGDSP, not on GRGESP or collagen alone (Fig. 8 *C*). Soluble, monovalent GRGDSP had no effect on RSF plated on collagen (Table I). Because GRGDSP is recognized not only by the FnR but also by the Vn receptor and other integrins, it has less selectivity than does the anti-FnR mAb. Therefore, we tested two other peptides, GdRGDSPASSK and GRGDNP, which have a higher specificity for the FnR (Pierschbacher and Ruoslahti, 1987); these peptides were also effective in inducing collagenase synthesis when immobilized.

We then tested whether larger fragments of Fn that contain the cell-binding domain and RGD sequence can mimic the inductive effects of the anti-FnR mAb. Collagenase and stromelysin expression was induced when RSF spread on immobilized 60- and 120-kD Fn fragments, in contrast to the lack of expression on immobilized native Fn (Fig. 8 *C*; Table I). The induction of metalloproteinases was dependent on the concentration of the immobilized Fn-derived peptides in the presence of collagen (data not shown). Although collagenase expression was also induced when the Fn fragments were added in solution (data not shown), we could not verify their monovalent status because of problems with aggregation of the fragments or their sticking to the surface of the culture dishes. Taken together, these data suggest that binding of Fn-derived peptides to the FnR triggers events different from those triggered by binding of native Fn.

Discussion

Our data demonstrate that perturbation of the interaction of Fn with its specific heterodimeric integrin receptor can alter gene expression. Treatment of attached and spread fibroblasts with an mAb against the specific integrin heterodimeric FnR, or with peptides containing the RGD cell-recognition sequence of Fn, but not with native Fn, induced expression of the genes for the metalloproteinases collagenase and stromelysin. Phorbol diesters, cytochalasins B and D, growth factors, and poorly adhesive substrates have also been shown to induce expression of these genes in fibroblasts (Aggeler et al., 1984*a*,*b*; Unemori and Werb, 1986, 1988; Edwards et al., 1987; Frisch and Ruley, 1987; Werb, 1989). The induction of proteinase expression by triggering the FnR and by these other treatments is similar in at least two respects. In all cases, there is a lag period before increased enzyme secretion is detectable. Once the inducing agent has been present for several hours, it can then be removed and the cells will go on to produce metalloproteinases over the next 24–48 h. Furthermore, proteinase induction by anti-FnR, as well as by the other inducers (Frisch and Ruley, 1987; Werb, 1989), is inhibitable by dexamethasone.

Despite these similarities, there is at least one important distinguishing feature between induction of proteinase expression mediated by the FnR and induction by previously reported treatments. Fibroblasts are induced to express metalloproteinases by anti-FnR even though they can remain flat and well spread throughout the induction period. Other treatments induce significant shape changes, which correlate with the extent of collagenase induction (Aggeler et al., 1984*b*; Werb et al., 1986). The divorcing of shape change from proteinase induction of collagenase and stromelysin gene expression by FnR has been documented in two ways. First, the organization of microfilament bundles, as detected by rhodamine-phalloidin, is not substantially altered during exposure to anti-FnR, whereas other agents, including the peptide containing RGD added in solution to cells spread in the presence of the adhesion proteins in serum, cause persistent and pronounced cell rounding and/or cytoskeletal reorganization. Although we cannot rule out the possibility that altered ligation of the FnR by the anti-FnR in solution induces more subtle or transient changes in cytoarchitecture, such as those seen in the first few minutes after the administration of growth factors such as epidermal growth factor


A
GRGDSP
GRGESP
0 4 20 100 4 20 100 (μg/ml)
Mr x10-3
93-
68-
42-
30-
proCL
1 2 3 4 5 6 7
B
GRGDSP GRGESP
0 4 20 100 4 20 100 (μg/ml)
Mr x10-3
93-
68-
50-
42-
30-
proCL
proSL
1 2 3 4 5 6 7
C
Immobilized Protein or Peptide
Collagen
Collagen + GRGESP
Collagen + GRGDSP
Mr x10-3
93-
68-
42-
proCL
proSL
1 2 3
None
GdRGDSPASSK
Fn
120 kD Fn
60 kD Fn
Anti-FnR IgG
Mr x10-3
93-
68-
42-
4 5 6 7 8 9

(Chinkers et al., 1979), in previous studies induction of proteinases was correlated with marked generalized alterations of the cytoskeleton of several hours' duration (Werb et al., 1986). In addition, studies on endothelial cells have shown that the FnR and Vn receptors are organized independently by their ligands, but both lead to local assembly of focal contacts and cytoskeletal proteins (Dejana et al., 1988). Thus, in cells exposed to serum, the cytoarchitecture may be maintained by the Vn receptor in the face of disruption of the FnR-Fn interaction. Second, proteinase expression by fibroblasts is stimulated even if they are cultured on substrates of anti-FnR, or of Fn-derived peptides containing RGD, that have been covalently linked to the culture dish. In this case, the fibroblasts spread on the mAb and the other immobilized inductive ligands as well as they do on noninductive collagen and Fn substrates and assume a highly flattened morphology, but are able to express proteinases. When Fn is conjugated to the dish, the cells spread but do not express proteinases. Thus, cells can distinguish whether they are attached to the substrate via their natural intact ligand or via Fn subfragments and the epitope recognized by the anti-FnR mAb, even though in all cases the cells have a similar morphology and degree of spreading. Because the selection screens for the anti-FnR mAb depended on inhibition of adhesion of cells to Fn, and peptides containing RGD also inhibit adhesion, it is likely that the anti-FnR mAb is a high affinity ligand for the subset of FnR configurations recognized by Fn subfragments. There are few data in the literature suggesting that Fn and Fn-derived peptides are recognized differently. However, monocytes, a cell type that expresses the FnR, show chemotaxis toward the cell-binding fragment of Fn but not native Fn in solution (Clark et al., 1988). Interestingly, these cells will respond to GRGDSP by activating their complement receptors only when bound to a surface (Wright and Meyer, 1985). Taken together, these observations suggest that the conformation of the binding site in Fn recognized by the FnR is likely to be altered when Fn is degraded by enzymes such as stromelysin (Chin et al., 1985).

There are other systems, such as adipocyte (Spiegelman and Ginty, 1983) and chondrocyte (Zanetti and Solursh, 1984) differentiation, in which the strong relationship between changes in cell shape and the induction of gene expression has also been observed. In these experiments, ECM molecules that reverse or prevent the shape change, such as Fn, prevent the induction of new gene expression. It is plausible that during induction of adipocyte-specific genes in preadipocytes and cartilage-specific genes in chondroblasts, as well as during induction of metalloproteinases in RSF by shape-altering reagents, the correlated reorganization of the actin cytoskeleton may act indirectly to alter adhesion of the cells via the FnR or other integrins. Therefore, these inductive signals may actually be transduced by the integrins.

Although RSF respond to anti-FnR whether it is presented in solution or substrate-bound, the two phenomena are fundamentally different in several respects and could be regulated by separate signaling mechanisms. In the first case, RSF are presented with soluble mAb after they have spread on the multiple adhesion proteins from serum and have formed a stable cytoskeletal framework. The effect of the soluble mAb would then be expected to be directly inductive for gene expression. In contrast, when freshly trypsinized, rounded RSF, with a disorganized cytoskeleton and diffuse FnR distribution, are plated on the immobilized anti-FnR, it is the interaction with mAb, not the native adhesion ligands, that induces the spreading of the cells. Because cell rounding and actin disorganization are correlated with metalloproteinase expression, it is possible that, upon exposure of cells to the native ligand, the inhibitory signal usually generated by interaction of Fn and other adhesion proteins with integrins stops the induction of metalloproteinases. However, when RSF are spreading on anti-FnR or Fn-derived peptides, the inhibitory signal does not go into effect, and gene expression is induced by a default pathway. These mechanisms remain to be explored.

Our data showing that anti-FnR and peptides containing RGD are inductive for metalloproteinase gene expression when multimeric or immobilized, but not when in solution in monovalent form, suggest that aggregation of the receptor is a prerequisite for transfer of information. The effects are specific to the FnR; cross-linking of other glycoprotein receptors such as the LDL-R and transferrin receptor did not induce the metalloproteinases. Although the distribution of the FnR on RSF was not determined directly in the present study, because this anti-FnR mAb does not stain well, there is a correlation between receptor aggregation and receptor function for two other integrins, Mac-1 of the β_2-subclass and GPIIb/IIIa of the β_3-subclass (Detmers et al., 1987; Isenberg et al., 1987). Therefore, cross-linking of the FnR may constitute part of the mechanism for transducing the signal for collagenase induction as it does for a variety of other receptors, including those for insulin (Kahn et al., 1978) and epidermal growth factor (Wakshull and Wharton, 1985). It is of interest in this regard that a fibrinogen decapeptide is able to induce aggregation of GPIIb/IIIa in platelets. However, aggregation alone clearly does not trigger the biological response, because the FnR is aggregated in focal contacts

Figure 8. Induction of metalloproteinase expression by peptides containing an RGD sequence. (*A* and *B*) Monolayers of RSF cultured to confluence in medium containing serum in 48-well plates were left untreated (lane *1*) or incubated with various concentrations of the GRGDSP peptide (lanes *2–4*), which contains the cell adhesion recognition sequence for integrins, or with the control GRGESP peptide (lanes *5–7*) in DME-LH for 24 h. The cell shape indices of the treated RSF in lanes *1–7* were 0, 1, 2, 3, 0, 0, and 0, respectively. (*A*) Samples (10 μl) of the CM were then analyzed for secreted proteinases by zymography on an SDS-gelatin substrate gel. (*B*) The cells were then biosynthetically labeled with [^{35}S]methionine for 4 h and the secreted proteins were analyzed by SDS-PAGE followed by fluorography. (*C*) RSF were plated on coverslips coated with immobilized proteins or peptides prepared by covalently cross-linking type I collagen at 1 mg/ml (lane *1*), collagen at 1 mg/ml plus GRGESP at 40 μg/ml (lane *2*), collagen at 1 mg/ml plus GRGDSP at 40 μg/ml (lane *3*), GdRGDSPASSK at 200 μg/ml (lane *5*), native Fn at 10 μg/ml (lane *6*), 120-kD fragment of Fn at 10 μg/ml (lane *7*), 60-kD fragment of Fn at 10 μg/ml (lane *8*), or anti-FnR mAb at 140 μg/ml (lane *9*). Lane *4*, RSF plated on plain glass coverslips. Some of the samples are shown in duplicate. After incubation for 30 h (lanes *1–3*) or 34 h (lanes *4–9*) in DME-LH, the cells were biosynthetically labeled with [^{35}S]methionine for 4 h and the secreted proteins were analyzed by SDS-PAGE followed by fluorography. Molecular weight standards ($\times 10^{-3}$) and the migration of procollagenase (*proCL*) and prostromelysin (*proSL*) are indicated.

when cells adhere to Fn, but no collagenase is induced under those conditions. Thus, it is likely that the inductive ligands produce a change in receptor conformation and/or interaction with the membrane along with oligomerization of the receptor and that this change contributes to signal transduction. The anti-FnR mAb and Fn-derived peptides induce a different subset of signals and responses than does the natural ligand Fn. In platelets, it is possible that different ligands induce different conformations of GPIIb/IIIa (Phillips et al., 1988). Taken together, all these results point to the conclusion that the nature of the interaction of the FnR with its ligand is an important factor in regulating gene expression. Thus, in addition to mediating cell attachment and spreading on Fn and formation of a membrane-actin cytoskeleton complex, the FnR complex can mediate signal transduction between the external environment and the cell interior. The nature of the signaling molecules is currently under investigation.

The integrins are good candidates to be involved in signal transduction. Integrins are transmembrane heterodimers that interact directly with their ECM ligands (Horwitz et al., 1985; Pytela et al., 1986; Tomaselli et al., 1988; Gehlsen et al., 1988*b*; Gailit and Ruoslahti, 1988) and with talin, a molecule associated with the cytoskeleton (Burridge, 1986; Buck et al., 1986). Tyrosine phosphorylation on the β_1-chain may regulate affinity for both Fn and talin (Burridge, 1986; Hirst et al., 1986; Buck and Horwitz, 1987). Our data suggest that integrins can act as a kind of homeostatic system for modulating ECM structure and organization in response to the needs of the cell. The high affinity interactions of mAbs with integrins mimic the interactions of integrins with fragments of ECM ligands, and thus make them useful for studying the pleiotropic ligands and functions of these receptors.

ECM remodeling is particularly important during embryonic development, in wound healing, in chronic inflammation, and in metastasis and embryo implantation (Fairbairn et al., 1985; Mignatti et al., 1986; Schultz et al., 1988; Sutherland et al., 1988). As a result of wounding, for example, there are likely to be significant changes in the environment of the cells at the wound site, including generation of fragments of ECM ligands, with an ordered series of events requiring ECM degradation, cell migration, ECM resynthesis, and remodeling. The pleiotropic responses of cells to interaction of their integrins with a changing population of ligands may therefore not only reflect the changes in the cellular environment but may actively mediate them through changes in expression of ECM and EMC-degrading molecules.

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Chapter 3

SPARC, a Secreted Protein Associated with Morphogenesis and Tissue Remodeling, Induces Expression of Metalloproteinases in Fibroblasts Through a Novel Extracellular Matrix-dependent Pathway

This chapter addresses the question of how an ECM molecule transiently present during repair and morphogenesis may trigger signalling events through unique non-integrin pathways involving interaction between SPARC and certain molecules in the ECM.

SPARC, a Secreted Protein Associated with Morphogenesis and Tissue Remodeling, Induces Expression of Metalloproteinases in Fibroblasts Through a Novel Extracellular Matrix-dependent Pathway

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Abstract. SPARC (osteonectin/BM40) is a secreted protein that modifies the interaction of cells with extracellular matrix (ECM). When we added SPARC to cultured rabbit synovial fibroblasts and analyzed the secreted proteins, we observed an increase in the expression of three metalloproteinases—collagenase, stromelysin, and the 92-kD gelatinase—that together can degrade both interstitial and basement membrane matrices. We further characterized the regulation of one of these metalloproteinases, collagenase, and showed that both collagenase mRNA and protein are upregulated in fibroblasts treated with SPARC. Experiments with synthetic SPARC peptides indicated that a region in the neutral α-helical domain III of the SPARC molecule, which previously had no described function, was involved in the regulation of collagenase expression by SPARC. A sequence in the carboxyl-terminal Ca^{2+}-binding domain IV exhibited similar activity, but to a lesser extent. SPARC induced collagenase expression in cells plated on collagen types I, II, III, and V, and on vitronectin, but not on collagen type IV. SPARC also increased collagenase expression in fibroblasts plated on ECM produced by smooth muscle cells, but not in fibroblasts plated on a basement membrane-like ECM from Engelbreth-Holm-Swarm sarcoma. Collagenase was induced within 4 h in cells treated with phorbol diesters or plated on fibronectin fragments, but was induced after 8 h in cells treated with SPARC. A number of proteins were transiently secreted by SPARC-treated cells within 6 h of treatment. Conditioned medium that was harvested from cultures 7 h after the addition of SPARC, and depleted of residual SPARC, induced collagenase expression in untreated fibroblasts; thus, part of the regulation of collagenase expression by SPARC appears to be indirect and proceeds through a secreted intermediate. Because the interactions of cells with ECM play an important role in regulation of cell behavior and tissue morphogenesis, these results suggest that molecules like SPARC are important in modulating tissue remodeling and cell-ECM interactions.

THE interactions of a cell with its surrounding extracellular matrix (ECM)[1] can play an important role in the regulation of cell behavior and tissue architecture. While most cells in adult tissues remain anchored in place through specific interactions with tissue matrices, subsets of differentiated cells are specialized to move through the ECM. Biological cues can also induce normally stationary, adherent cells to move over and through the ECM. In some cases, a specific interaction of a cell with certain matrices can help to stabilize or maintain a phenotype or particular tissue structure (Menko and Boettiger, 1987; Sorokin et al., 1990; Adams and Watt, 1990); in actively remodeling tissues, however, the cellular interaction with ECM is not static but is constantly changing (Mackie et al., 1988; ffrench-Constant et al., 1989; Gladson and Cheresh, 1991; Damsky et al., 1992).

Diversity in cell-ECM interactions is the consequence of several factors: the integration of the transcriptional regulation of ECM components and their receptors, the net accumulation of ECM constituents resulting from the balance of synthesis and degradation of ECM molecules, and the assembly of the ECM components into tissue-specific matrices (Cheresh et al., 1989; Dahl and Grabel, 1989; Dustin and Springer, 1991; for review see Hay, 1991; Damsky et al.,

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1. *Abbreviations used in this paper:* CM, conditioned medium; DESPARC, a 90% pure fraction of SPARC; ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1; LH, lactalbumin hydrolysate; RGD, Arg-Gly-Asp; RSF, rabbit synovial fibroblasts; RT, reverse transcription; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

1992). Cues provided by tissue-specific matrices are interpreted by cells in the decision to adhere, invade, migrate, secrete, or differentiate. There is strong correlative evidence that information transduced into the cell by adhesion of cells to specific matrices can lead to alterations in cell phenotype or to changes in gene expression (Menko and Boettiger, 1987; Werb et al., 1989; Sorokin et al., 1990; Adams and Watt, 1990; Talhouk et al., 1992; for review see Burridge et al., 1988; Hynes, 1992). Apart from establishing adhesive contacts with the ECM, a cell must somehow modify these contacts with the matrix, i.e., to de-adhere or break the interaction with the matrix. Controlled proteolysis might be one mechanism used by cells to diminish adhesive contacts with the ECM. Proteinases have been shown to be present at focal contacts in certain cells and can act to modulate the assembly of the actin cytoskeleton in these cells (Beckerle et al., 1987; Estreicher et al., 1990). ECM-degrading metalloproteinases are specifically enhanced in fibroblasts plated on fragments of fibronectin when compared with fibronectin or collagen type I (Werb et al., 1989), a response that could also diminish adhesion of cells to the ECM. Another way to effect changes in cell adhesion is to modify existing matrices by deposition or synthesis of additional matrix-associated molecules. Several ECM-associated molecules, including SPARC, thrombospondin, dermatan sulfate proteoglycans, and tenascin, have been shown to perturb the adhesion of cells to the matrix (Sage et al., 1989*c*; Lawler et al., 1988; Murphy-Ullrich and Hook, 1989; Chiquet-Ehrismann, 1991; Murphy-Ullrich et al., 1991). Thrombospondin and tenascin have both adhesive and anti-adhesive properties, and may interact with cells in a way that Arg-Gly-Asp (RGD) inhibits (Lawler et al., 1988; Bourdon and Ruoslahti, 1989), whereas SPARC acts in a manner insensitive to RGD (Sage et al., 1989*c*).

SPARC, tenascin, and thrombospondin are expressed transiently in tissues that are actively remodeling their matrix, where cells are dividing or migrating (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). Although SPARC is expressed transiently in a wide range of tissues during development, in adults SPARC is expressed chiefly in rapidly renewing populations of cells (Sage et al., 1989*a*,*b*). Metalloproteinase expression is also increased in regions of tissue where increased cell division, migration, or remodeling is observed (for review see Alexander and Werb, 1991). This is interesting because the expression of the ECM-degrading metalloproteinases collagenase and stromelysin correlates with perturbation of the actin cytoskeleton (Unemori and Werb, 1986), and tenascin, SPARC, and thrombospondin in culture have been shown to destabilize actin in focal contacts (Murphy-Ullrich et al., 1991).

The expression of collagenase and stromelysin is induced in cultured rabbit synovial fibroblasts (RSF) plated on fibronectin fragments or on anti-fibronectin receptor antibody but not on substrates of intact plasma fibronectin (Werb et al., 1989). This culture system might therefore be useful in the dissection of information transduced by cell-ECM interactions. Because SPARC diminishes adhesion of cultured cells by destabilizing focal contacts (Sage et al., 1989*c*; Murphy-Ullrich et al., 1991), we designed experiments to determine whether the addition of SPARC to cultured synovial fibroblasts alters the expression of metalloproteinases in these cells.

Materials and Methods

Cells and Cell Culture

RSF, isolated as described previously (Aggeler et al., 1984*a*,*b*) and used between passages 1 and 10, were cultured in DME (Cell Culture Facility, University of California, San Francisco), supplemented with 10% FBS (Hyclone Labs., Logan, UT). Cells (0.5–1 × 10^5) were plated in uncoated 24 or 48-well tissue culture dishes (Costar Corp., Cambridge, MA), and cultured in DME containing 10% FBS for 3–4 h, after which the monolayers were washed and incubated further in DME supplemented with 0.2% lactalbumin hydrolysate (LH) (GIBCO BRL, Gaithersburg, MD). Cells were plated on ECM-coated dishes at a density of 10^5 and were cultured in one of two serum-free media, DME-LH or Fibroblast Growth Medium (Clonetics Corp., San Diego, CA).

Preparation of ECM Ligand Substrates

Fibronectin was purchased from Collaborative Research (Bedford, MA) and Boehringer Mannheim Corp. (Indianapolis, IN); fibronectin fragments and vitronectin were purchased from Telios Pharmaceuticals (San Diego, CA). Bovine collagen type I (Vitrogen) was purchased in a solution of 0.1 N HCl from the Collagen Corp. (Palo Alto, CA); collagen types I, II, III, IV, V were purchased from Collaborative Research or from Eureka Laboratories (Sacramento, CA) and reconstituted as directed by the manufacturer. Culture dishes were coated with fibronectin or vitronectin at 10–20 μg/ml by incubating them overnight (9–15 h) in PBS, pH 7.4, at 4°C. Human collagen types I, II, III, IV, V, or bovine collagen type I were diluted from acidic solutions into distilled water to a concentration of 20 μg/ml and culture dishes were further incubated in this solution overnight (9–15 h) at 4°C. Unoccupied binding sites were blocked by incubation with 0.2% BSA (Sigma Chem. Co., St. Louis, MO) at ambient temperature for 2 h; wells were then washed three times with PBS and used immediately. Culture dishes coated with a basement membrane matrix from Engelbreth-Holm-Swarm (EHS) sarcoma (Alexander and Werb, 1992) or the smooth muscle matrix from R22 rat smooth muscle cells (Werb et al., 1980) were prepared as described previously.

Addition of SPARC or SPARC Peptides to Cell Cultures

SPARC was prepared from mouse parietal yolk sac (PYS-2) cells as described previously (Sage et al., 1989*c*). Purified SPARC was solubilized in PBS at a concentration of 250 μg/ml and used at a final concentration of 10–40 μg/ml; SPARC was used as a soluble ligand except where noted otherwise. In many experiments we used a slightly less pure (90%) fraction of SPARC (DESPARC), isolated by anion-exchange chromatography, which contains a carrier protein for SPARC; this protein was more stable and available in larger quantities than pure SPARC. The synthetic peptides used in this study were synthesized, characterized, and used as described previously (Lane and Sage, 1990).

Antibodies

The mouse anti-rabbit collagenase mAbs used for immunoprecipitations and analysis of secreted collagenase in this study were described by Werb et al. (1989). The mouse anti-human stromelysin antibody, SL188.2 (Wilhelm et al., 1992) used in the immunoblots was a generous gift of Dr. Scott Wilhelm (Miles Research, West Haven, CT). The horseradish peroxidase-conjugated and biotinylated secondary antibodies were purchased from Sigma Chem. Co. Texas red-streptavidin conjugate was purchased from Amersham Corp. (Arlington Heights, IL). Goat anti-SPARC antibody was prepared with purified SPARC as antigen, and its specificity characterized by immunoprecipitation and immunoblotting; the titer was measured by ELISA, and was maximal at 1:500 and half-maximal at 1:250.

To deplete SPARC from the medium, we incubated 500 μl of antiserum or normal goat serum with 100 μl packed protein G-Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 1 h and washed them extensively with PBS, and then incubated the beads with 200 μl conditioned medium (CM) for 1 h.

Biosynthetic Labeling of Secreted Proteins and Analysis of Metalloproteinases

Proteins were biosynthetically labeled by incubating cultures with 50–100

μCi/ml of [^{35}S]methionine (Expresslabel; New England Nuclear, Boston, MA) for 2–4 h in methionine-free DME at 37°C. Secreted proteins were precipitated from the CM with quinine sulfate and SDS as described (Unemori and Werb, 1986). Samples were then analyzed by SDS-PAGE using the Laemmli buffering system followed by fluorography (En^{3}Hance, New England Nuclear), as previously described (Unemori and Werb, 1986). Alternatively, collagenase in the radiolabeled CM was immunoprecipitated with a mixture of anti-collagenase mAbs (Werb et al., 1989). Zymography was used to analyze the gelatinases in the CM (Werb et al., 1989). The stromelysin and collagenase protein in the enriched culture supernatants was also identified by immunoblotting procedures (Harlow and Lane, 1988) with the use of anti-human stromelysin mAb (Wilhelm et al., 1992) and anti-rabbit collagenase mAbs (Werb et al., 1989). Briefly the CM was resolved by SDS-PAGE and the proteins were transferred to Immobilon P membranes (New England Nuclear). Unoccupied binding sites on the membrane were blocked by incubation with 3% BSA in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5), washed once with TBS, and incubated with the primary antibody for 1–2 h. The membrane was washed briefly with TBS, and then washed three times for 20 min with a solution of TBS containing 0.5% Tween-20 (Sigma Chem. Co.). After incubation with a horseradish peroxidase-conjugated secondary anti-mouse antibody, the membrane was washed as described above, and specific bands were visualized by enhanced chemiluminescence (Amersham Corp.) as described by the manufacturer. To quantify the amount of collagenase in the CM, we applied dilutions of the CM to nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) using a slot-blot filtration manifold (Schleicher and Schuell, Inc.). The filters were blocked and incubated with anti-collagenase antibodies as described above. The resulting films were scanned by laser densitometry and the amounts of collagenase in the CM were compared.

RNA Isolation, Polymerase Chain Reaction, Hybridization Conditions, and cDNA Probes

Total cellular RNA was isolated from cultured cells and 1-μg samples were analyzed by reverse transcription (RT) and amplification of specific sequences by the PCR (Rappolee et al., 1989). Synthetic primers used to amplify collagenase cDNA sequences were selected from regions of identity in the rabbit and human cDNA sequences. The collagenase primer pair (nucleotides 1154–1174 and 1433–1453 in the rabbit collagenase gene [Brenner et al., 1989]) amplified a 300-bp fragment of the transcribed cDNA. Synthetic primers used to amplify cDNA sequences coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) spanned the sequence 3308–3337 and 3649–3672 in the rat gene, and produced a 241-bp fragment (Rappolee et al., 1989). Amplifications with both primer pairs were performed with 4 mM $MgCl_2$ at an annealing temperature of 60°C.

PCR products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. For quantification of the amplified product, negatives of the gels were analyzed by scanning densitometry, and the area under the peak corresponding to specific bands was plotted against the dilution of the RT mixture used in that amplification. The amounts of GAPDH and collagenase mRNA in the RT mixture were compared in the linear portion of the curve. To analyze mRNA by blotting, we applied a series of linear dilutions of total RNA to nylon membranes using a slot-blot filtration manifold (Sambrook et al., 1989). The filter was probed with ^{32}P-labeled cDNA inserts from pCL1, a clone of rabbit collagenase (Frisch et al., 1987), and human γ-actin (Engel et al., 1981; a gift of L. Kedes, Stanford University, Palo Alto, CA) as described (Werb et al., 1989).

Results

The Expression of Metalloproteinases Is Upregulated in Synovial Fibroblasts Treated with SPARC

To determine if SPARC alters expression of proteins synthesized by RSF, we added purified SPARC or DESPARC to adherent RSF in culture. We compared the expression of proteins secreted by SPARC-treated cells with that of proteins secreted by untreated cells. We also incubated cells with the phorbol diester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which rapidly induces the expression of metalloproteinases in many cell types (Frisch and Werb, 1989). SPARC induced the synthesis of biosynthetically labeled secreted proteins migrating between 50 and 60 kD that are characteristic of the proenzyme forms of collagenase and stromelysin (Fig. 1). We detected no difference in the pattern of metalloproteinase expression in RSF induced by pure SPARC and DESPARC, and, unless otherwise stated, the two were used interchangeably in later experiments.

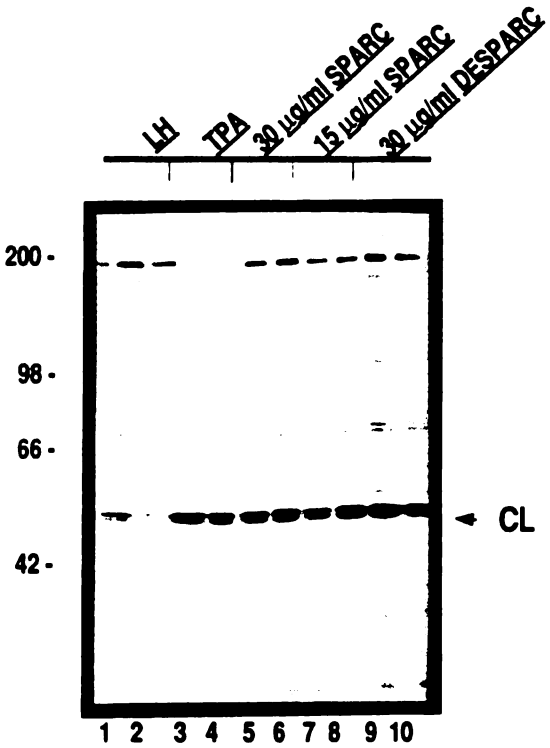

Figure 1. SPARC induces the synthesis and secretion of proteins in synovial fibroblasts. Freshly trypsinized RSF were cultured in DME-10% FBS in 48-well plates for 3 h. The cells were incubated further in DME-LH (*LH*, lanes *1* and *2*, in duplicate) or DME-LH supplemented with either 100 ng/ml TPA (lanes *3* and *4*, in duplicate), 30 μg/ml pure SPARC (lanes *5* and *6*, in duplicate), 15 μg/ml pure SPARC (lanes *7* and *8*, in duplicate), or 30 μg/ml DESPARC (lanes *9* and *10*, in duplicate) for 30 h. The CM was removed, and the proteins were biosynthetically labeled by incubation of cells with [^{35}S]methionine in methionine-free DME for 2 h. The radiolabeled secreted proteins were concentrated with quinine sulfate-SDS and analyzed by SDS-PAGE and autoradiography. Procollagenase (*CL*), migrating at 53 and 57 kD, is indicated on the right. Molecular weight (× 10^{-3}) markers are indicated on the left.

To identify and quantify the metalloproteinases in the CM harvested from SPARC-treated RSF, we used mAbs against collagenase and stromelysin. Immunoprecipitable collagenase represented >30% of the radiolabeled protein secreted by RSF after treatment with SPARC for 40 h (Fig. 2 *A*). Increases in total collagenase and stromelysin were also detected by immunoblotting CM harvested from SPARC-treated RSF (Fig. 2 *B*). Changes in proteolytic activity were evident on zymograms that used a gelatin substrate incorporated into the resolving gel. Increases in the gelatinolytic doublet of collagenase, migrating at 57 and 53 kD, and of the

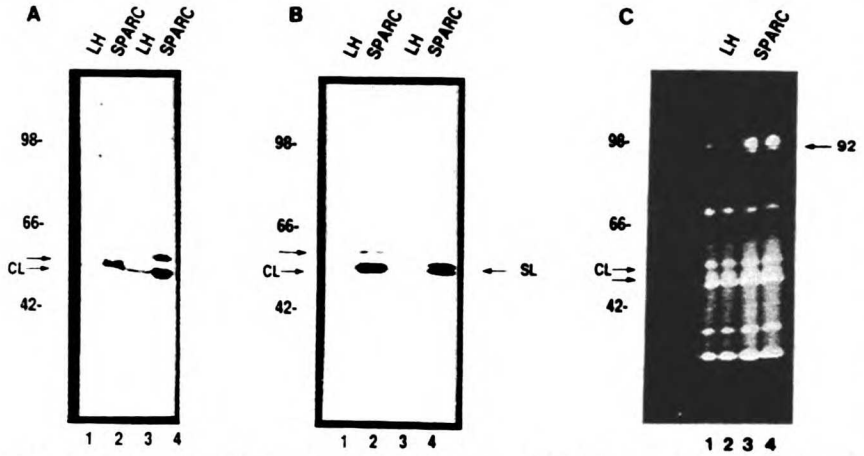

Figure 2. SPARC induces the expression of collagenase protein in synovial fibroblasts. (*A*) Freshly trypsinized RSF plated in 48-well plates were allowed to attach and spread in DME-10% FBS for 3 h. The medium was removed, the monolayers were washed and cells were incubated further in DME-LH or in DME-LH supplemented with 30 μg/ml DESPARC for 40 h. The CM was removed and saved for later analysis. The proteins were biosynthetically labeled for 3 h by incubation of cells with 50 μCi/ml of [^{35}S]methionine in methionine-free DME. Samples of CM containing radiolabeled secreted proteins from untreated RSF (*LH*, lanes *1* and *3*) or RSF treated with DESPARC (*SPARC*, lanes *2* and *4*) were concentrated with quinine sulfate-SDS (lanes *1* and *2*) to show total proteins secreted into the medium, or proteins secreted into the medium were immunoprecipitated with anti-collagenase mAbs (lanes *3* and *4*) and the precipitates were separated on a 10% SDS-polyacrylamide gel and analyzed by autoradiography. The doublet of procollagenase (*CL*) is indicated by arrows. (*B*) CM harvested from untreated RSF (*LH*, lanes *1* and *3*) or SPARC-treated RSF (*SPARC*, lanes *2* and *4*) was separated on a 10% SDS-polyacrylamide gel under denaturing conditions, and the proteins were transferred to membranes and analyzed by immunoblotting with anti-collagenase mAbs (lanes *1* and *2*) or an anti-stromelysin mAb (lanes *3* and *4*). Procollagenase (*CL*) and prostromelysin (*SL*) are indicated by arrows. (*C*) The proteinase content of the 40-h CM harvested from untreated RSF or from RSF treated with SPARC was analyzed by zymography. The proteins were separated on a 10% polyacrylamide gel that contained 0.1% gelatin under nondenaturing conditions. The zymograms were stained with Coomassie blue R250 after development overnight (14 h). Proteolytic degradation of gelatin appears as a clear band on a dark background. Note the induction of gelatinases migrating at 92, 57, and 53 kD corresponding to the proenzyme forms of the 92-kD gelatinase (*92*) and collagenase (*CL*). Molecular weight ($\times 10^{-3}$) markers are indicated on the left.

92-kD gelatinase show that these metalloproteinases are induced by SPARC (Fig. 2 *C*). In the rest of the experiments described in this report, we concentrated on the effects of SPARC on the regulation of collagenase gene expression.

Regardless of the basal level of collagenase secretion, SPARC reproducibly upregulated the expression of collagenase in RSF. The induction of collagenase in RSF treated with SPARC, measured as radiolabeled secreted collagenase protein, was quantified by scanning densitometry of autoradiographs (Table I). There was a mean 5.3-fold increase (± 2.7 SD) in the expression of collagenase in cultures treated with SPARC, compared with untreated cultures (n = 8 experiments, 2–3 replicates per treatment, $p < 0.01$; Student's t test). By comparison TPA induced an 8.6-fold increase (± 1.8 SD) in collagenase expression. Induction of collagenase expression in RSF was found using three preparations of SPARC and in RSF strains derived from six rabbits.

In the preceding experiments we examined the effect of SPARC on the amounts of collagenase protein secreted by RSF. We used RT-PCR to analyze the effect of SPARC on mRNA for collagenase. The accumulation of collagenase mRNA increased fivefold in RSF treated with SPARC (Table I), and when normalized for mRNA for the housekeeping gene GAPDH (Fig. 3), this increase correlated with the expression of collagenase protein by RSF treated with SPARC. The data analyzed in Fig. 3 *B* are an average of three experiments. In a separate blotting analysis of RNA from one experiment, a series of sequential dilutions of RNA was applied to nylon membranes with a filtration manifold, and the membrane was probed with ^{32}P-labeled cDNA probes for collagenase and γ-actin. The autoradiographs were scanned and, normalizing to the level of γ-actin expression, we saw a threefold increase in collagenase mRNA in cultures treated with SPARC compared with untreated control cultures; this increase was comparable to the levels obtained with RT-PCR (data not shown).

Previous studies have indicated that induction of collagenase and stromelysin gene expression strongly correlates with cell rounding, although treatment of RSF with a func-

Table I. Collagenase Expression in RSF Treated with SPARC and SPARC Peptides

Peptide*	Collagenase induction	G_0/S delay‡	Shape change§
	(*-fold*)		
SPARC	5.3 ± 2.7‖	Yes	Yes
1.1	1.2 ± 0.4	No	Yes
2.1	1.1 ± 0.	Yes	No
3.2	10.0 ± 1.7‖	No	No
3.4	1.1 ± 0.5‖	No	No
4.2	4.3 ± 0.5‖	No	Yes
TPA	8.6 ± 1.8‖	ND	Yes

To facilitate comparison of results from five experiments, we expressed data as "-fold induction" by normalizing data from treated to untreated cultures in individual experiments. For SPARC, eight different experiments were averaged. Data are shown as mean ± SD.

* See Fig. 6 A for location of peptides in the SPARC molecule. Peptides had the same structure as that described by Lane and Sage (1990) and were added to RSF at 0.8 mM for 30 h. SPARC was added at 30 μg/ml.

‡ From Funk and Sage (1991).

§ From Lane and Sage (1990).

‖ Significantly different from untreated controls ($p < 0.05$, Kruskall Wallis test).

ND, Not done.

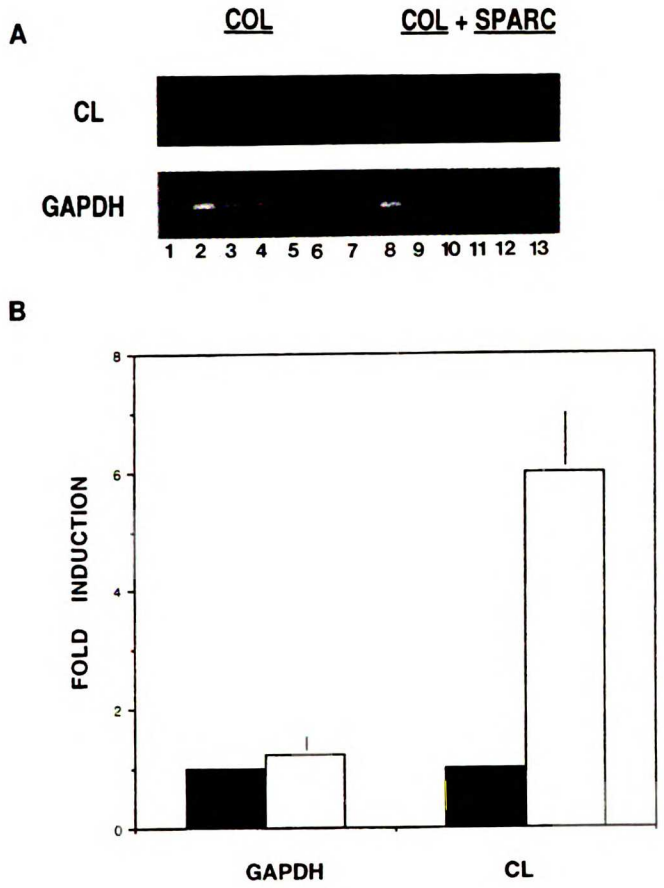

Figure 3. The expression of collagenase mRNA is up-regulated by SPARC. Freshly trypsinized RSF were cultured on type I collagen-coated wells in DME-LH or in DME-LH containing 30 μg/ml SPARC for 30 h. The total cellular RNA was isolated and analyzed by RT-PCR. (*A*) Sequences in cDNA were amplified with the use of specific primers for collagenase cDNA and GAPDH cDNA to yield 300- and 241-bp fragments, respectively. The products were separated on agarose gels and stained with ethidium bromide. For amplification of the collagenase (*CL*) mRNA, the cDNA derived by RT of 2 μg RNA from control (*COL*) and SPARC-treated RSF (*COL + SPARC*) was diluted to 10^{-2} (lanes *2* and *8*), 5×10^{-2} (lanes *3* and *9*), 10^{-3} (lanes *4* and *10*), 5×10^{-3} (lanes *5* and *11*), 10^{-4} (lanes *6* and *12*), or 5×10^{-4} (lanes *7* and *13*). For amplification of GAPDH mRNA, the cDNA derived by RT of 2 μg RNA from untreated RSF and RSF plated on collagen and treated with SPARC was diluted to 10^{-1} (lanes *2* and *8*), 5×10^{-1} (lanes *3* and *9*), 10^{-2} (lanes *4* and *10*), 5×10^{-2} (lanes *5* and *11*), 10^{-3} (lanes *6* and *12*), and 5×10^{-3} (lanes *7* and *13*); lane *1* contained no template. (*B*) The data from three independent experiments were quantified by densitometry and are expressed as -fold induction (the ratio of values from SPARC-treated to untreated RSF values). Shaded bars represent mean values from untreated cells plated on collagen, and open bars represent values from cells plated on collagen and treated with SPARC. Lines at the end of bars indicate SD.

tion-perturbing anti-fibronectin receptor antibody can induce collagenase without significant change in cell shape (Aggeler et al., 1984*b*; Werb et al., 1989). As in the regulation of collagenase in cells plated on fibronectin fragments or treated with anti-fibronectin receptor antibody, SPARC induced collagenase expression without a major morphological change (Fig. 4). This contrasts with the marked rounding of endothelial cells and nuchal fibroblasts treated with SPARC (Sage et al., 1989*c*).

The Induction of Collagenase by SPARC Is ECM-Specific

Because SPARC expression is tightly regulated during development and tissue remodeling, we wished to determine if there are particular ECM contexts in which SPARC initiates a remodeling cascade involving metalloproteinase expression. Accordingly, we plated fibroblasts in serum-free medium in wells that had been coated with collagen types I, II, III, IV, or V, with vitronectin, or with the more complex matrices synthesized by EHS tumor cells and R22 smooth muscle cells.

SPARC increased collagenase synthesis 2–3-fold in RSF that were plated on vitronectin or collagens of types I, II, III, and V, but not in cells plated on collagen type IV (Fig. 5). SPARC was an inductive ligand for cells plated on R22 ma-

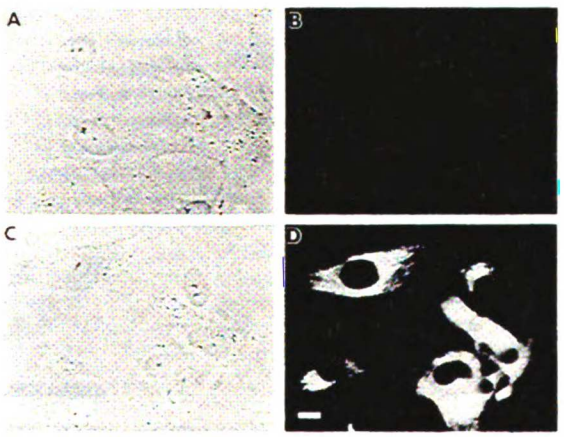

Figure 4. SPARC induces collagenase without inducing cell rounding in RSF. Freshly trypsinized RSF were cultured on glass coverslips in DME-10% FBS for 3 h. The cells were incubated in DME-LH (*A* and *B*) or in DME-LH supplemented with 30 μg/ml DESPARC (*C* and *D*) for 30 h, then fixed, permeabilized, and stained with anti-collagenase mAbs. Paired phase-contrast (*A* and *C*) and immunofluorescent micrographs (*B* and *D*) are shown. Bar, 10 μm.

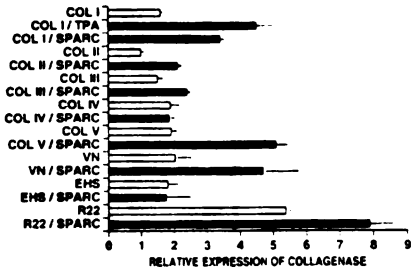

Figure 5. SPARC induces collagenase expression in matrix-specific fashion. RSF were plated in 48-well plates that were coated with ECM proteins—collagens (*COL*) of types I, II, III, IV, or V; vitronectin (*VN*), EHS matrix, R22 matrix—in Fibroblast Growth Medium alone or with 30 μg/ml DESPARC (*SPARC*) and incubated for 30 h. The proteins were biosynthetically labeled by incubating cells with [^{35}S]methionine, and the labeled secreted collagenase was quantified by scanning densitometry after SDS-PAGE and autoradiography. Open bars indicate collagenase synthesized by untreated RSF plated on the indicated matrix molecule. Shaded bars indicate total collagenase secreted into the culture medium from RSF plated on the indicated matrix molecule and treated with 30 μg/ml SPARC. Means of duplicate samples are shown; lines at end of bars indicate range.

trix (which consists predominantly of elastin and collagen types I and III), but not for cells plated on EHS matrix (predominantly laminin, collagen type IV, and heparan sulfate proteoglycan) (Fig. 5). Because the basal secretion of collagenase on EHS matrix was no lower than it was on other matrices in this experiment, and because cells plated on another basement membrane component (pure collagen type IV) did not regulate collagenase in response to SPARC, it is unlikely that TGF-β, which is known to be associated with some preparations of EHS matrix, abrogated the effect of SPARC in these experiments. These results indicate that cells treated with SPARC synthesize more collagenase than do untreated fibroblasts plated on the matrices.

SPARC Peptides Also Induce Collagenase Expression

Sequence analysis has indicated that there are four unique structural domains in the SPARC molecule (Mason et al., 1986; Engel et al., 1987): I, an acidic Ca^{2+}-binding domain; II, a cysteine-rich domain; III, a neutral α-helical sequence containing a serine proteinase-sensitive site; and IV, a high-affinity Ca^{2+}-binding EF handlike structure (Fig. 6). One approach that has been used successfully to define the structural motifs of the SPARC molecule that affect cell

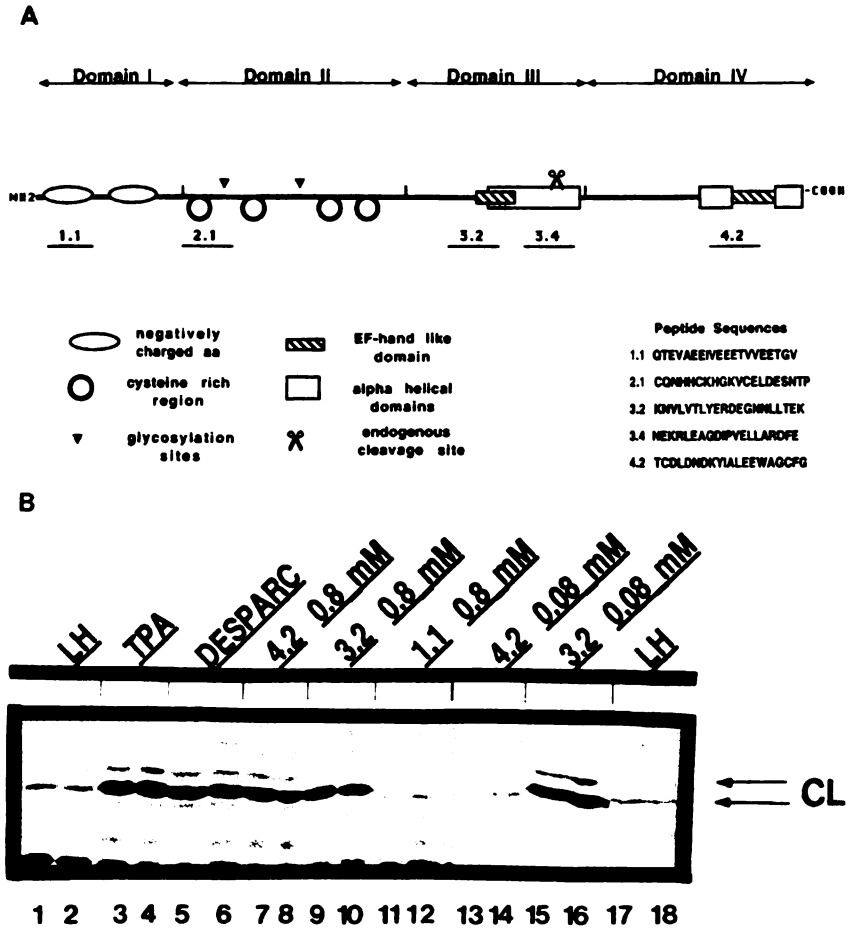

Figure 6. SPARC peptides from domains III and IV regulate collagenase expression. (*A*) Schematic diagram of the SPARC molecule showing the location of specific domains and the synthetic peptides (1.1, 2.1, 3.2, 3.4, and 4.2) used in this study. (*B*) The synthetic peptides (0.8 mM or 0.08 mM) or 30 μg/ml DESPARC were added to RSF plated in 48-well plates. Cultures were incubated for 24 h, and then radiolabeled with [^{35}S]methionine for 2 h. Biosynthetically labeled secreted proteins were analyzed by SDS-PAGE and autoradiography. This panel shows the induction of collagenase in RSF incubated with DME-LH alone (*LH*, lanes *1*, *2*, *17*, and *18*), with 100 ng/ml TPA (lanes *3* and *4*), with DESPARC (lanes *5* and *6*), or with synthetic peptide 4.2 (0.8 mM) (lanes *7* and *8*), peptide 3.2 (0.8 mM) (lanes *9* and *10*), peptide 1.1 (0.8 mM) (lanes *11* and *12*), peptide 4.2 (0.08 mM) (lanes *13* and *14*), or peptide 3.2 (0.08 mM) (lanes *15* and *16*).

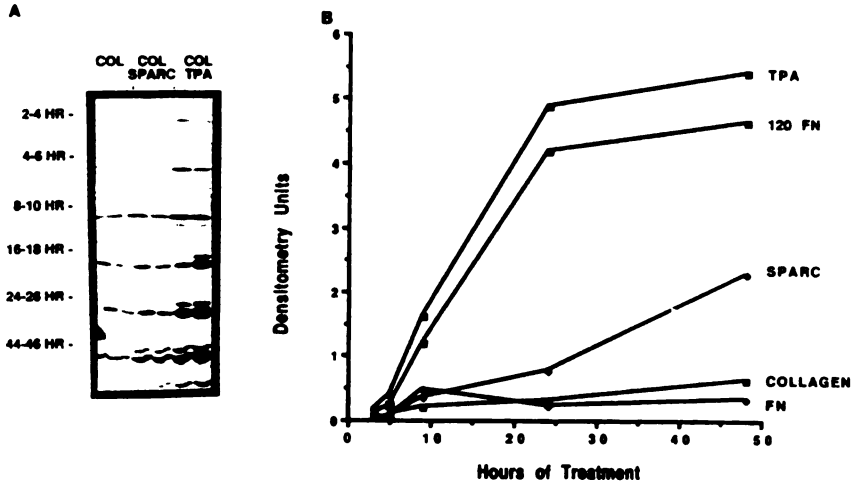

Figure 7. SPARC induces collagenase expression in RSF with slower kinetics than is seen for TPA or fibronectin fragments. Freshly trypsinized RSF were plated in wells coated with collagen type I in DME-LH alone (*COL*), or in DME-LH supplemented with 30 μg/ml SPARC (*COL SPARC*), or with 100 ng/ml TPA (*COL TPA*), or in wells coated with fibronectin (*FN*), or with the 120-kD chymotrypsin fragment of fibronectin (*120 FN*). The cultures were incubated for 2–44 h, and proteins were biosynthetically labeled by incubation with 100 μCi/ml of [^{35}S]methionine for 2 h. The radiolabeled secreted collagenase was analyzed by immunoprecipitation with anti-collagenase mAbs, and the precipitates were resolved by electrophoresis on a 10% SDS-polyacrylamide gel. (*A*) Autoradiogram of the immunoprecipitated radiolabeled collagenase protein. (*B*) Quantification of secreted collagenase by densitometry of autoradiographs.

adhesion (Lane and Sage, 1990) or the cell cycle (Funk and Sage, 1991) is the synthesis of peptides corresponding to regions in domains I–IV and the monitoring of their efficacy in mimicking SPARC function. We tested a panel of synthetic peptides spanning portions of each of the four domains of the SPARC molecule (Lane and Sage, 1990) to define which domains in the SPARC molecule confer regulation of metalloproteinases in RSF.

Peptides in domain I (1.1) and domain IV (4.2) destabilize adhesion of cells to the ECM and thus mimic the antiadhesive properties of SPARC. Addition of peptide 4.2, at 0.8 mM, to cultures of adherent RSF induced collagenase expression (Fig. 6, Table I). We detected no significant induction of collagenase in response to peptide 1.1 (Fig. 6). Peptide 2.1, like intact SPARC, delays the G_o/S transition when added to endothelial cells in culture (Funk and Sage, 1991); however, it did not induce collagenase expression in RSF (Fig. 6, Table I). Surprisingly, we saw an induction of collagenase in cells treated with peptide 3.2, which is in the neutral α-helical domain III; peptide 3.2 was inductive at 0.08 mM, which is a tenfold lower concentration than was inductive with peptide 4.2. Similar results were obtained with two different preparations of peptide 3.2. Peptides representing nearby sequences, both amino-terminal (peptide 2.1) and carboxyl-terminal (peptide 3.4), did not induce collagenase expression (Table I). These results suggest that regions in domain III and IV in the SPARC molecule induce metalloproteinase expression in fibroblasts.

The Regulation of Collagenase Expression by SPARC Is Temporally Distinct from Induction by TPA or Fibronectin Fragments

Previous experiments have shown that fibronectin fragments and TPA induce collagenase expression within 4 h (Werb et al., 1989). To characterize the timing of the induction of collagenase by SPARC, we compared the time course of collagenase induction by SPARC, TPA, and fragments of fibronectin. RSF were plated in serum-free medium in collagen-coated wells, some of which were supplemented with 30 μg/ml DESPARC or 100 ng/ml TPA, or in wells that had been coated with fibronectin or the 120-kD chymotrypsin fragment of fibronectin. At various times (2–44 h) after plating we incubated the cultures with [^{35}S]methionine for 2 h, immunoprecipitated the radiolabeled secreted collagenase from the CM, and analyzed the immunoprecipitates by SDS-PAGE and autoradiography. Collagenase synthesis was detected in SPARC-treated cultures after 8 h but was apparent within 4 h in cultures plated on fibronectin fragments or treated with TPA (Fig. 7). When we analyzed the total proteins secreted by RSF after a 2–6-h incubation with SPARC, we noted the transient induction of several proteins that were not secreted by RSF treated with TPA (Fig. 8).

SPARC Induces Collagenase Expression by an Indirect Mechanism

The data presented in Figs. 6, 7, and 8 suggest at least two potential mechanisms by which SPARC may regulate collagenase expression in fibroblasts. The slow induction of collagenase (>10 h) in RSF treated with SPARC is reminiscent of the time course of collagenase induction in RSF elicited by agents that act by shape-dependent mechanisms, such as cytochalasins or culture in retracted collagen gels (Unemori and Werb, 1986). In our experiments, however, SPARC did not appear to alter the morphology of RSF. Alternatively, the induction of collagenase in cells treated with SPARC may be mediated by one of the molecules induced in SPARC-treated cultures before collagenase expression is induced. Although the experiment with SPARC peptides also suggests that two domains in the SPARC molecule regulate the expression of collagenase, possibly by distinct mechanisms, for simplicity we decided to clarify the mechanism by which native, intact SPARC affects the expression of collagenase in fibroblasts.

To determine if the increase in collagenase expression was a direct response to treatment of cells with SPARC, or was

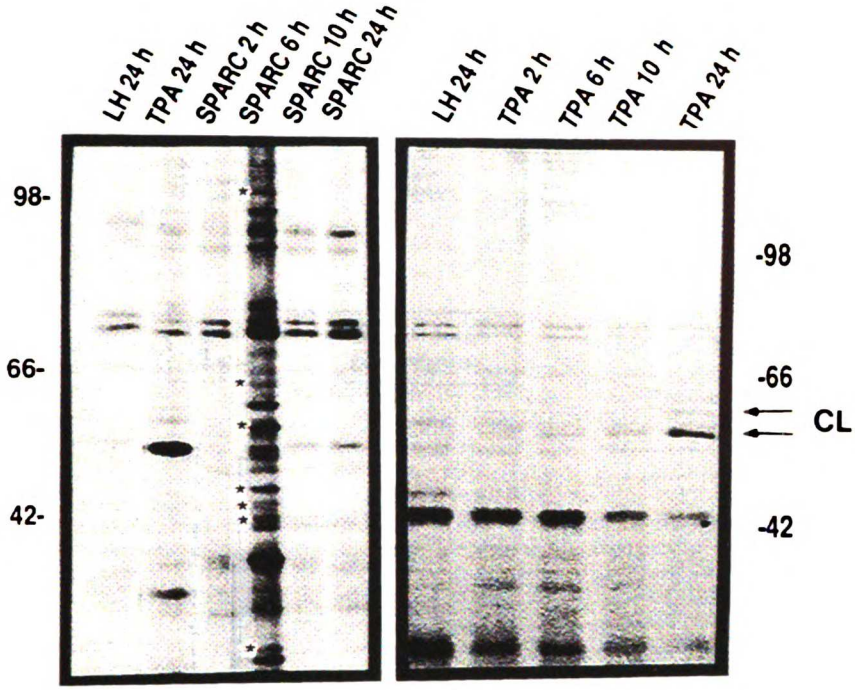

Figure 8. SPARC treatment transiently induces the expression of several novel secreted proteins. RSF were plated in 48-well plates in DME-10% FBS for 3 h. The medium was then replaced with DME-LH alone (*LH*) or with DME-LH supplemented with 100 ng/ml TPA (*TPA*), or 30 μg/ml DESPARC (*SPARC*). After incubation of cells for 2, 6, 10, or 24 h, the proteins were biosynthetically labeled by incubating cultures with [^{35}S]methionine, and the total secreted proteins were concentrated and analyzed by SDS-PAGE and autoradiography. The bands representing procollagenase are indicated at the right. The novel SPARC-induced proteins are indicated by asterisks. Molecular weight ($\times 10^{-3}$) markers are indicated. Data shown are from one experiment; however, the same results were obtained in a duplicate experiment.

secondary to the action of a SPARC-induced molecule, we plated three sets of parallel cultures of RSF in wells coated with collagen type I, with or without 30 μg/ml pure SPARC or DESPARC (Fig. 9 *A*). One set of cultures was incubated with pure SPARC or DESPARC for 24 h. Another set of cultures was incubated with or without pure SPARC or DESPARC for 7 h, the medium was removed and saved, and the cultures were incubated further in DME-LH for 17 h. From the medium that was removed and saved, we depleted the residual SPARC by incubation of the CM with goat anti-SPARC IgG bound to protein G beads; we incubated a third set of cultures, plated on collagen type I, with this SPARC-depleted CM for 15 h. To confirm that SPARC, and not a contaminating molecule in the preparation of DESPARC, induced the expression of collagenase, we removed SPARC from a duplicate sample of SPARC-supplemented culture medium by preadsorption with goat anti-SPARC IgG-loaded protein G beads before the addition of this medium to cultures. To ensure that SPARC was depleted from the samples incubated with anti-SPARC IgG beads, we subjected duplicate samples of SPARC, or CM harvested from SPARC-treated or control cultures, before and after incubation with anti-SPARC IgG, to analysis by SDS-PAGE under nondenaturing conditions. No residual SPARC was detected in the SPARC-depleted CM by immunoblotting (data not shown). To eliminate the possibility that serum-derived factors associated with the washed IgG-loaded protein G beads may induce collagenase expression, we also incubated cultures for 24 h with medium that had been preabsorbed with protein G beads loaded with IgG from normal goat serum. All cultures were incubated with [^{35}S]methionine 24 h after initial plating, and the biosynthetically labeled secreted collagenase was analyzed by immunoprecipitation. These experiments were performed with both DESPARC and pure SPARC. The data represent an average of three experiments.

We saw a 3.5-fold increase in collagenase expression in cultures incubated with SPARC for 24 h (Fig. 9 *B*). In cultures incubated with SPARC for only 7 h, collagenase expression remained at basal levels, similar to that of untreated controls. Interestingly, we saw a 2.5-fold increase in collagenase expression in cells incubated with the SPARC-depleted CM harvested from cultures treated with SPARC for 7 h, whereas cells cultured in SPARC-depleted CM harvested from untreated cells synthesized basal levels of collagenase. Cells that were incubated with medium from which SPARC was preadsorbed before incubation, or cells incubated with medium that was preincubated with protein G beads containing normal goat serum IgG, also synthesized basal levels of collagenase. These results indicate that the mechanism of regulation of collagenase by SPARC is different from that of TPA or fibronectin fragments. The increased expression of collagenase associated with SPARC may, therefore, be mediated in part through a secreted factor that is produced during the first 7 h of treatment with SPARC.

The upregulation of collagenase expression in response to peptide growth factors and cytokines is rapid and is initiated within 2–6 h of exposure to the inducing agent (Frisch and Werb, 1989). If the regulation of collagenase by SPARC is mediated by the production of a secreted intermediate such as a cytokine, one prediction would be that the induction of collagenase expression in cells incubated with SPARC-depleted CM harvested from cells treated with SPARC for 7 h would be faster than that seen in cultures incubated with SPARC. To analyze the kinetics of induction, we compared the number of cells staining positive by immunofluorescence for cell-associated collagenase from cultures treated with

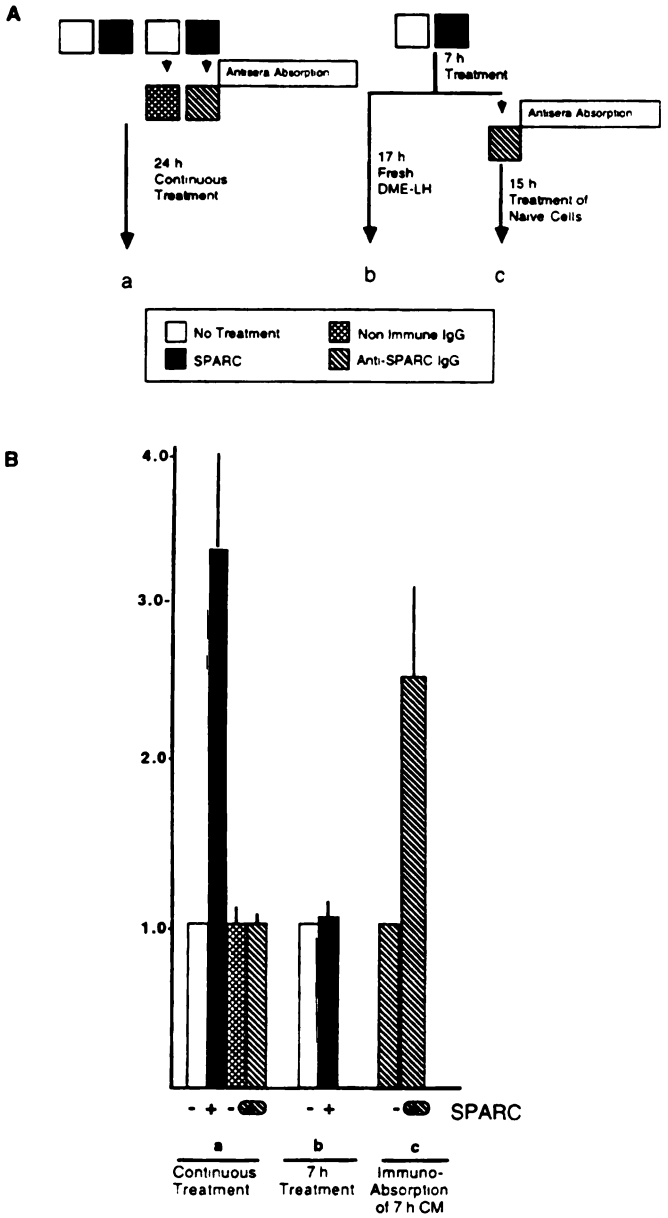

Figure 9. SPARC indirectly induces collagenase expression through a secreted intermediate. (*A*) RSF were plated on wells coated with collagen type I and incubated in Fibroblast Growth Medium, with or without 30 μg/ml DESPARC. One set of cultures (*a*) was incubated for 24 h. Experimental controls included RSF plated on type I collagen–coated wells in Fibroblast Growth Medium supplemented with DESPARC that had been preincubated with protein G beads loaded with goat anti-SPARC IgG, or in Fibroblast Growth Medium containing protein G beads loaded with IgG from normal goat serum. In a second set of cultures (*b*), the CM was removed 7 h after plating, and the cultures were incubated further in unsupplemented Fibroblast Growth Medium for 17 h. The CM that was harvested from cells incubated for 7 h in Fibroblast Growth Medium with or without SPARC was depleted of residual SPARC by immunoprecipitation with protein G beads loaded with goat anti-SPARC IgG; this SPARC-depleted medium was added to a third set of cultures (*c*) that had been plated on wells coated with collagen type I, and the cultures were incubated for an additional 15 h. At 24 h after the initiation of the experiment, proteins were biosynthetically labeled by incubation of cells with [^{35}S]methionine. The radiolabeled secreted collagenase was immunoprecipitated with anti-collagenase mAbs, and analyzed by SDS-PAGE followed by autoradiography. (*B*) The autoradiograms of the RSF treated as described in *A* from three independent experiments were quantified by densitometry. The data are expressed as -fold induction. Plus and minus signs indicate treatment with or without SPARC.

SPARC for 35 h, or with SPARC-depleted CM harvested from SPARC-treated cultures for 10 or 35 h (counting about 500 cells in 4–5 microscopic fields). We then studied RSF plated on collagen-coated coverslips and treated with SPARC-depleted CM from cultures incubated in serum-free medium with or without 30 μg/ml pure SPARC for 6.5 h at 10 and 35 h of treatment. RSF treated with SPARC for 10 h showed little induction of collagenase (see Fig. 7). However, at 10 h we saw a twofold increase in the total secreted collagenase and a 3.5-fold increase in the collagenase-positive cells in the cultures treated with CM from SPARC-treated cultures, whereas there was no change in secreted collagenase from cells treated with control CM. The increase in collagenase-positive cells compared with the increase in secreted collagenase reflects the short time elapsed to accumulate induced collagenase during the 10 h of culture time. By 35 h, the cells incubated with SPARC continuously showed a 4.3-fold increase in secreted collagenase and a 3.0-fold increase in collagenase-positive cells, which was similar to the fourfold increase in secreted collagenase and 2.7-fold increase in collagenase-positive cells seen in cultures treated with 6.5 h CM from SPARC-treated cells, compared with untreated controls or cells treated with CM from control cultures. The increase in collagenase-positive cells in cultures

incubated with SPARC for 35 h, or with SPARC-depleted, SPARC-conditioned medium for 10 and 35 h was significantly different from control cultures at all time points ($p < 0.05$, Student-Newman-Kuels test). The increase in the collagenase-positive cells in cultures treated for 10 h with SPARC-depleted CM from SPARC-treated cultures was not significantly different from cultures treated with pure SPARC for 35 h. Taken together, these data support the concept that a SPARC-induced secreted intermediate is at least a part of the cascade by which SPARC induces the expression of collagenase in RSF.

Discussion

The decision not to adhere to a substrate has developmental and biological consequences. This concept has been derived from studies on neurite outgrowth and patterning of the nervous system (Tomaselli et al., 1987; Dodd et al., 1988; Klambt et al., 1991; Bovolenta and Dodd, 1991), from studies of the complex adhesive interactions of cells in the immune system (Kieffer and Phillips, 1990; Butcher, 1991) and from characterization of the migration of embryonic cell populations (Riou et al., 1990; ffrench-Constant et al., 1991; Hynes and Lander, 1992). One proposed mechanism for diminishing the adhesion of cells to the ECM is through ECM-associated molecules with anti-adhesive properties (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). A number of structurally dissimilar molecules with anti-adhesive properties that modulate cell-ECM interactions have been described: SPARC, tenascin, thrombospondin, dermatan sulfate proteoglycans, and scatter factor have defined anti-adhesive effects on cells in culture (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). The expression of these proteins is tightly regulated to produce developmental patterns and to respond to tissue injury; moreover, they localize to regions of tissue where cell division, migration, and ECM remodeling are prevalent (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991).

In this report we have presented evidence that one of these anti-adhesive, ECM-associated molecules, SPARC, at a concentration of ~0.5 nM, which is within physiological levels (Maillard et al., 1992), initiates a cascade of events that result in the increased expression of the ECM-remodeling metalloproteinases. SPARC induced the expression of collagenase, stomelysin, and 92-kD gelatinase, metalloproteinases capable of degrading both basement membranes and interstitial connective tissue matrices. Collagenase specifically cleaves native, fibrillar collagens, whereas the 92-kD gelatinase degrades type IV collagen and denatured collagen. Stromelysin can degrade many molecules in the ECM, including proteoglycans and fibronectin (for review see Alexander and Werb, 1991). These results suggest that SPARC, in addition to its characterized effects on cell shape, may alter the nature of the ECM presented to the cell.

Cellular responses to growth factors have traditionally been divided by temporal parameters into immediate, intermediate, and delayed effects. Likewise, cells can respond to SPARC within minutes of treatment, but there are also downstream effects that can be measured hours to days after exposure. Immediate responses include actin disassembly and accumulation of mRNA coding for plasminogen activator inhibitor-1 in bovine aortic endothelial cells (Hasselaar et al., 1991). Intermediate responses include a delay in the G_0/S transition in cycling bovine aortic endothelial cells (Funk and Sage, 1991). The generation of a SPARC-induced secreted molecule that regulates collagenase expression in synovial fibroblasts is another intermediate response to SPARC, whereas a delayed response, the induction of metalloproteinases in cells treated with SPARC, occurs over a period of days.

When we further characterized the regulation of collagenase in RSF, we noted that the regulation of collagenase by SPARC was distinct from the regulation of collagenase conferred by TPA or ligands acting through the fibronectin receptor. Collagenase expression induced by SPARC proceeded with kinetics similar to that conferred by treatments that act by a shape-dependent mechanism, such as culture on polyhydroxyethyl methacrylate, in retracted collagen gels, or with cytochalasins. However, there are several reasons why it is unlikely that SPARC acts solely through a shape-dependent mechanism: (*a*) In contrast to the marked changes in the morphology of bovine nuchal fibroblasts or aortic endothelial cells mediated by SPARC (Sage et al., 1989*c*), RSF in culture remained well spread, although we may not have detected the subtle, transient alterations in the cytoskeleton that were described by Murphy-Ullrich et al. (1991); (*b*) we detected the synthesis of several novel SPARC-induced proteins that were secreted transiently before collagenase expression; (*c*) supernatants that were removed from SPARC-treated cultures and depleted of SPARC induced collagenase expression in untreated fibroblasts, whereas cultures from which SPARC was removed after 7 h of incubation did not, after further incubation, increase their expression of collagenase; furthermore, (*d*) the increase in cells staining for collagenase in cultures incubated with this SPARC-depleted, SPARC-conditioned medium for only 10 h was the same as that seen in cultures incubated continuously with pure SPARC for 35 h.

Thus, one important feature of this induction is the synthesis or secretion of an intermediary molecule. The nature of this secreted intermediate is not known at present. In preliminary experiments in which we used RT-PCR to measure cytokines expressed by RSF after incubation with SPARC for 7 h, we have observed increases in mRNA for basic FGF and tumor necrosis factor-α and a decrease in mRNA for TGF-β (unpublished observations). It is known that tumor necrosis factor-α and basic FGF upregulate the expression of collagenase in cultured fibroblasts (for review see Frisch and Werb, 1989). Because TGF-β both inhibits the expression of metalloproteinases and induces the expression of the tissue inhibitor of metalloproteinases, further studies to characterize this secreted intermediate will prove interesting.

There are other examples of autocrine regulation of collagenase expression in fibroblasts. The induction of collagenase by ultraviolet irradiation of fibroblasts also proceeds through an uncharacterized, secreted intermediate (for review see Herrlich et al., 1992). In synovial cells both serum amyloid alpha and β_2-microglobulin, which are induced in cells treated with TPA or interleukin-1 (IL-1), have been shown to induce the expression of collagenase in untreated fibroblasts (Brinckerhoff et al., 1989). In other culture systems, changes in adhesion correlate with the synthesis or secretion of growth factors (Hedin et al., 1989; Shaw

et al., 1990). Collagenase expression in fibroblasts is increased in response to a number of growth factors and cytokines that can be synthesized or stored in fibroblasts, including PDGF, basic FGF, tumor necrosis factor-α, and IL-1 (for review see Frisch and Werb, 1989). It is possible that any of these factors may be the secreted intermediate.

Collagenase expression is regulated in synovial fibroblasts by cytochalasins, by culture on a substrate with altered adhesive properties (polyhydroxyethyl methacrylate), or in retracted collagen gels through mechanisms that involve a change in the actin cytoskeleton (Aggeler et al., 1984*b*; Unemori and Werb, 1986), in which cell shape is altered from an extended, well-spread morphology to a rounded, cuboidal morphology. There may also be a cytoskeletal component to the induction of collagenase in RSF treated with SPARC. Although these cells remained spread and adherent, nuchal fibroblasts and aortic endothelial cells rapidly reorganize their actin cytoskeleton and lose their focal contacts after exposure to SPARC (Lane and Sage, 1990). Although a SPARC receptor has not yet been fully characterized, we cannot exclude the possibility that SPARC, acting through a specific cell surface receptor for SPARC, induces collagenase expression in fibroblasts in vivo.

That SPARC induced collagenase expression in cells plated on a smooth muscle matrix is consistent with the observations of Raines et al. (1992), who noted that SPARC and PDGF are increased in the neointima of atherosclerotic plaque. SPARC has been shown to delay the G_o/S transition of cycling cells (Funk and Sage, 1991), to play a role in calcifying tissue (Termine et al., 1981), and to have defined effects on adhesion of cells to ECM (Lane and Sage, 1990). SPARC binds to PDGF in such a way as to block the interaction of PDGF (AB and BB) with its receptor (Raines et al., 1992). Although both PDGF and IL-1 induce collagenase expression in synovial cells, the addition of PDGF to IL-1–stimulated cells diminishes the proliferation and metalloproteinase expression induced by IL-1 (Kumkumian et al., 1989). Therefore, the sequestration of PDGF by SPARC may have several distinct consequences, such as modifying the cellular response to specific cues and/or initiating a mitogenic or remodeling cascade in the atherosclerotic plaque.

We have used the induction of metalloproteinase expression in cultured cells as an assay to expand the structure-function analysis of the SPARC protein. Domain IV contains a high-affinity Ca^{2+}-binding site that diminishes the adhesion of cells in culture; this domain also mediates the interaction of SPARC with immobilized collagen type I. Our work supports the existing evidence that this carboxyl-terminal Ca^{2+} binding domain is important for SPARC function. We anticipated that peptide 4.2, derived from SPARC sequences representing the carboxyl-terminal Ca^{2+}-binding domain, would induce collagenase expression in RSF because this peptide causes cultured fibroblasts and aortic endothelial cells to assume a rounder morphology (Lane and Sage, 1990). In addition, antibodies raised against peptide 4.2 react against native SPARC. Peptide 4.2 binds to collagen type I and blocks the interaction of native SPARC with collagen type I (Lane and Sage, 1990). In contrast, peptide 1.1, which spans sequences in the amino-terminal Ca^{+}-binding domain I of the molecule, also induces a cell shape change; however, peptide 1.1 did not cause significant changes in collagenase expression in RSF. Peptide 1.1 does not inhibit SPARC binding to immobilized collagen I (Lane and Sage, 1990). Our data also suggest that the presumptive extended α-helical domain is important for some functions of SPARC. We did not anticipate that peptide 3.2, located in a stable helical structure of domain III in the SPARC molecule (Engel et al., 1987; Bolander et al., 1988; Lane and Sage, 1990), would regulate collagenase expression in RSF to a significant degree. It is possible that proteolytic cleavage changes the conformational constraints in the SPARC molecule and that these changes in turn alter the accessibility and/or function of the α-helical domain. Comparison of SPARC cDNA sequences from *Xenopus* and several mammalian species shows that there is a high degree of sequence homology in the carboxyl-terminal domain IV and in domain III, but not in the negatively charged domain I (Damjanovski et al., 1992). Taken together, these data indicate that domains III and IV are functionally important for the interaction of SPARC with ECM or cells and for functioning of SPARC in vivo.

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Chapter 4

The Extracellular Matrix Ligands Fibronectin and Tenascin Collaborate in Regulating Gene Expression in Fibroblasts

This chapter explores the mechanism by which the interaction between two ECM molecules can regulate gene expression, possibly by altering the interaction of integrins with molecules in the ECM.

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The Extracellular Matrix Ligands Fibronectin and Tenascin Collaborate in Regulating Gene Expression in Fibroblasts

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[1] Abbreviations used: BSA, bovine serum albumin; CM, conditioned medium; DME, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LH, lactalbumin hydrolysate; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RSF, rabbit synovial fibroblasts; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TN, tenascin; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; 120FN, 120 kD fragment of fibronectin.

Tenascin is a large oligomeric glycoprotein that is present transiently in the extracellular matrix of cells, and is involved in morphogenetic movements, tissue patterning, and tissue repair. It has multiple domains, both adhesive and anti-adhesive, that interact with cells and with fibronectin and other extracellular matrix macromolecules. We have studied the consequences of the interaction of tenascin with a fibronectin matrix on gene expression in rabbit synovial fibroblasts. Fibroblasts plated on a mixed substrate of fibronectin and tenascin, but not on fibronectin alone, upregulated synthesis of four genes: collagenase, stromelysin, the 92 kD gelatinase, and c-*fos*. Although the fibroblasts spread well on both fibronectin and fibronectin-tenascin substrates, nuclear c-Fos increased within 1 h in cells that were plated on fibronectin-tenascin, but not on fibronectin alone. Collagenase was upregulated within 4 h of plating on a fibronectin-tenascin substrate, but not on fibronectin alone, and exhibited kinetics similar to those for induction of collagenase gene expression by signaling through the integrin fibronectin receptor. Collagenase was not upregulated in cells plated on substrates of type I collagen or vitronectin and tenascin, or when soluble tenascin was added to cells adherent to serum proteins (predominantly vitronectin), suggesting that tenascin has an effect only in the context of fibronectin. The domain of tenascin responsible for the inductive effect of the fibronectin-tenascin substrate was mapped by using blocking monoclonal antibodies. Anti-TN68, a monoclonal antibody that recognizes an epitope in the carboxyl-terminal type III repeats involved in the interaction of tenascin with both fibronectin and cells, blocked the inductive effect, whereas anti-TNM1, a monoclonal antibody that recognizes an epitope in the amino-terminal anti-adhesive region of epidermal growth factor-like repeats, had no effect on the induction of collagenase by fibronectin-tenascin. These data suggest that transient alteration of the composition of extracellular matrix by transient addition of proteins like tenascin may regulate the expression of genes involved in cell migration, tissue remodeling, and tissue invasion, in regions of tissue undergoing phenotypic changes.

INTRODUCTION

Tenascin (TN)[1] is a large oligomeric glycoprotein in the extracellular matrix (ECM) that is thought to play a role in the morphogenetic movement of cells and the patterning of tissues during development. The structure of TN (also called GMEM, cytotactin, hexabrachion, or J1; reviewed by Erickson and Bourdon, 1989) is determined by a linear array of four motifs: a cysteine-rich, amino-terminal domain that plays a role in TN arm association; a number of epidermal growth factor (EGF)-like repeats; a series of fibronectin (FN) type III repeats, the number of which is determined by alternative splicing; and a carboxyl-terminal fibrinogen-like globular domain. The cDNA sequence of chicken, mouse, and human TN has shown that TN isoforms are a result of alternative splicing of FN type III repeats (Spring *et al.*, 1989; Nies *et al.*, 1991; Weller *et al.*, 1991; Siri *et al.*, 1991; Nishi *et al.*, 1991). Although the number of alternatively spliced type III repeats varies with species, the type III repeats in similar positions share considerable homology (Gulcher *et al.*, 1991).

In vitro, TN binds to immobilized FN (Chiquet-Ehrismann *et al.*, 1991) and to cell surface proteoglycans (Salmivirta *et al.*, 1991). There is some tissue specificity in the interaction of heparan sulfate proteoglycans and TN, because the integral membrane heparan sulfate proteoglycan syndecan, isolated from toothbud extracts, interacts with immobilized TN in cell-free binding assays, whereas preparations of syndecan isolated from mammary gland do not (Salmivirta *et al.*, 1991). The carbohydrate side chains of heparan sulfate and chondroitin sulfate proteoglycans play a role in the interaction with TN (Salmivirta *et al.*, 1991; Murphy-Ullrich *et al.*, 1991); however, the core protein of cytotactin-binding proteoglycan, which contains chondroitin sulfate proteoglycan, mediates the interaction with TN (Hoffman *et al.*, 1988).

Tenascin expression is seen in two types of tissues. The smallest isoform of TN is expressed in regions of dense connective tissue such as gizzard or myotendinous junctions, where tensile strength is important (Chiquet-Ehrismann *et al.*, 1991). In both adult and

embryonic tissues the expression of TN is also seen in regions of tissue in which ECM remodeling, cell division, and cell migrations take place. The temporal and spatial distribution of TN expression in the early embryo (Chiquet-Ehrismann *et al.*, 1986; Prieto *et al.*, 1990), coupled with the observations that TN diminishes cell adhesion and migration (Chiquet-Ehrismann *et al.*, 1986; Friedlander *et al.*, 1988; Lotz *et al.*, 1989; Halfter *et al.*, 1989; Riou *et al.*, 1990), suggest that TN is an important molecule used in directed migrations early in development. The transient expression of specific TN isoforms in the developing nervous system suggests that TN has important functions here as well (Steindler *et al.*, 1989; Prieto *et al.*, 1990). TN may also play a role in condensing tissue, because the expression of TN is upregulated during epithelial mesenchymal induction (Ekblom and Aufderheide, 1989; Salmivirta *et al.*, 1991); the distribution of TN in condensing tissues may also change, because TN localization shifts from a diffuse presence to a perichondral localization during cartilage condensation (Chiquet-Ehrismann *et al.*, 1986). In adults, the expression of TN is more limited; it is present in regions of continuous tissue renewal, such as in the intestinal crypts (Weller *et al.*, 1991), and is strikingly induced in the dermis beneath a wound epithelium after tissue injury (Mackie *et al.*, 1988; Whitby *et al.*, 1991). The transient expression of TN in discrete developmental patterns and its upregulation in regions of repairing tissues suggest an important role for TN in remodeling tissue.

The function of TN in remodeling tissue has not yet been clearly defined. However, a common response of fibroblasts, epithelial cells, neurons, and glia cultured in the presence of TN is a diminution of cell adhesion (for review see Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). This destabilization of cell adhesion to the ECM could enhance cell motility, as suggested by Tucker and McKay (1991). Alternatively, the addition of TN to an ECM could change the structure of the mesenchymal matrix, as suggested by Whitby *et al.* (1991) and Ekblom and Aufderheide (1989). Finally, the presence of TN in ECM could directly or indirectly signal cells to alter their expression of genes involved in ECM remodeling. This possibility is

supported by similar patterns of distribution of TN and ECM-degrading matrix metalloproteinases (MMPs) in repairing and remodeling tissue (reviewed by Alexander and Werb, 1991). The interaction of FN and TN molecules is clearly documented (Chiquet-Ehrismann *et al.*, 1991), and other studies demonstrate that TN inhibits or perturbs adhesion of cells to intact FN and FN fragments that contain the classical Arg-Gly-Asp (RGD) sequence (Chiquet-Ehrismann *et al.*, 1988; Lotz *et al.*, 1989). MMP expression is upregulated in cells in response to stimuli that subtly perturb adhesion or alter the actin cytoskeleton (Aggeler *et al.*, 1984; Unemori and Werb, 1986; Werb *et al.*, 1989). We therefore investigated whether perturbations in cell behavior generated by addition of TN to a substrate of FN might regulate cellular gene expression. As our model system, we chose rabbit fibroblasts, which respond to FN fragments or anti-FN receptor antibodies by induction of MMP expression, and we investigated the regulation of collagenase as a model gene.

MATERIALS AND METHODS

Cells and Cell Culture

Rabbit synovial fibroblasts (RSF), isolated as described previously (Aggeler *et al.*, 1984), were cultured in Dulbecco's modified Eagle's medium (DME) (Cell Culture Facility, University of California, San Francisco) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Denver, CO). RSF, used between passages 2 and 10, were subcultured 48 h before experimental procedures. Cells used in experiments were added to ECM-coated wells at a density of 1-2 x 10^5 in DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH); the plates were rotated to ensure even suspension of cells and incubated further at 37° C before analysis of MMP expression.

Antibodies

The anti-collagenase monoclonal antibodies (mAbs) were characterized and used as described (Werb *et al.*, 1989). The anti-stromelysin mAb (SL188.2) was a generous gift of Dr. Scott Wilhelm, Miles Research (West Haven, CT) (Wilhelm *et al.*, 1992). The anti-Fos antibody and matching peptide immunogen were purchased from CRB Biologics, Cambridge, England. Adsorption of the anti-Fos antibody with the peptide immunogen was performed as described in the technical information supplied with the antibody. The mAb anti-TNM1 was first described by Chiquet and Fambrough (1984), and the polyclonal anti-TN antibodies and mAb anti-TN68 were prepared and described by Spring *et al.* (1989). The biotinylated and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma (St. Louis, MO), and the Texas Red-labeled streptavidin was purchased from Amersham (Arlington Heights, IL).

Preparation of Extracellular Matrix Substrates

Human plasma FN was purchased from Collaborative Research (Waltham, MA) or Boehringer Mannheim Biochemical (Indianapolis, IN), reconstituted as directed by the manufacturer, and frozen at -70° C in single-use aliquots. The 120 kD chymotryptic fragment of human plasma FN (120FN) was purchased from Telios Pharmaceuticals (La Jolla, CA), reconstituted as directed, and stored in single-use aliquots at -70° C. Chicken TN was purified as described previously (Chiquet-Ehrismann *et al.*, 1991). The anti-TN mAbs TNM1 and TN68 and anti-TN polyclonal antibodies were used as described by Spring *et al.* (1989). For coating of wells, 24- or 48-well culture dishes (Costar, Cambridge, MA) were incubated with 0.1 or 0.2 ml of 25 µg/ml FN or 120FN in phosphate-buffered saline (PBS) overnight (9-15 h) at 4° C. They were then washed three times with PBS and incubated in 0.2% bovine serum albumin (BSA) in PBS for 30 min at ambient temperature to reduce nonspecific binding to the tissue culture dish. The wells were then washed three times with PBS and used immediately. Mixed substrates were prepared by sequential incubation with ECM proteins. Except where noted otherwise, wells were incubated

overnight (9-15 h) at 4° C with 25 μg/ml FN in PBS, washed with PBS, and incubated at ambient temperature for 2 h with 0.1-0.2 ml of a 5-10 μg/ml solution of TN in PBS. This solution was aspirated, the wells were washed with PBS, and the wells were incubated in 0.2% BSA in PBS to prevent nonspecific adhesion to the tissue culture dish. After washing three times with PBS, the plates were used immediately. In some experiments, an additional 45-min incubation with anti-TN monoclonal antibodies (50 μg/well) at ambient temperature was included before the incubation with 0.2% BSA in PBS. Type I collagen-coated wells were prepared by incubating wells in 48-well plates overnight at 4° C with a solution of 50 μg/ml pepsin-treated bovine skin collagen (Vitrogen; Collagen Corp., Palo Alto, CA) in distilled water, then washed with PBS, and uncoated sites were blocked with 0.2% BSA in PBS. The blocking solution was aspirated, the wells were washed three times with PBS, and 0.1-0.2 ml of 5-10 μg/ml TN in DME-LH was added to the wells before the addition of cells. Wells were coated with bovine vitronectin or mixtures of vitronectin and TN as described for type I collagen-coated wells, except that wells were incubated overnight with a solution of 10 μg/ml vitronectin (Telios Pharmaceuticals) in PBS.

Biosynthetic Labeling of Proteins Secreted by RSF

RSF were added to ECM-coated wells and cultured in DME-LH for up to 48 h, after which the conditioned medium (CM) was removed and saved for later analysis. Cultures of RSF were biosynthetically labeled by incubation with 50-70 μCi/ml [^{35}S]methionine (Express Label, New England Nuclear, Boston, MA) for 3-4 h in methionine-free DME (Gibco/BRL, Gaithersburg, MD). Radiolabeled secreted proteins were precipitated from the CM with quinine sulfate and sodium dodecyl sulfate (SDS) or by immunoprecipitation with specific antibodies as described previously (Werb *et al.*, 1989). Precipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography (En^{3}Hance; New England Nuclear). Radiolabeled collagenase secreted into the culture supernatants was quantified by scanning autoradiograms

with an LKB densitometer and GSXL software (LKB-Pharmacia, Piscataway, NJ).

Analysis of Specific Proteinases

Proteinases in the CM harvested from cell cultures were analyzed by using gelatin zymography as described previously (Werb *et al.*, 1989). CM was separated on nonreducing 10% polyacrylamide gels that contained 0.1% gelatin. After electrophoresis the gel was soaked in 2.5% Triton X-100 to remove SDS and incubated for 18 h in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$). The gels were stained with Coomassie Blue R 250 (BioRad, Richmond, CA); gelatinases appear as a clear zone on a blue background; in reverse prints they appear as a dark band on a clear background.

Alternatively, samples of CM were separated by denaturing SDS-PAGE, and the proteins were transferred to membranes (ImmobilonP; New England Nuclear) (Harlow and Lane, 1989). Nonspecific sites on the membranes were blocked with a solution of 3% BSA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), after which the membranes were incubated with anti-collagenase or anti-stromelysin mAb in TBS containing 0.5% Tween-20 (Sigma) for 1-2 h at ambient temperature. Before and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma), the membranes were washed three times for 20 min with 0.5% Tween-20 in TBS. Specific bands were detected by enhanced chemiluminescence (Amersham) as described by the manufacturer.

RNA Isolation and Analysis

Total cellular RNA was isolated from cultured RSF, and 1-μg aliquots were analyzed by RT followed by amplification of specific sequences in the polymerase chain reaction (PCR) as described by Rappolee *et al.* (1989). Synthetic primers used to amplify collagenase cDNA sequences were selected from regions of identity in the rabbit and human cDNA sequences as described previously (Brenner *et al.*, 1989). The collagenase primer pair represented the

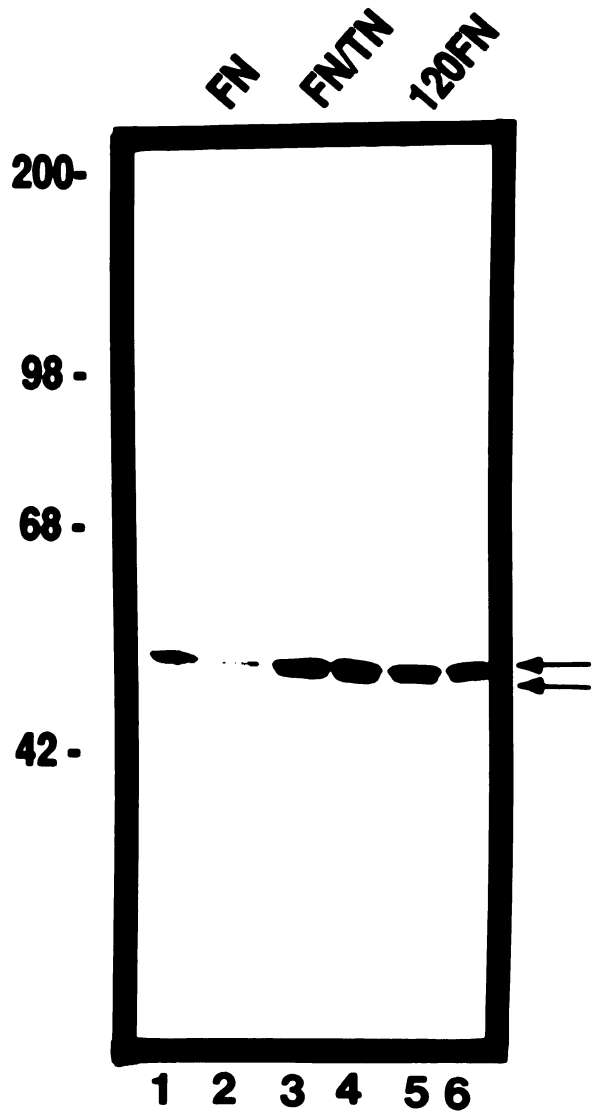
nucleotides 1154-1174 and 1433-1453 in the rabbit collagenase cDNA and amplified a 300-bp fragment of the transcribed cDNA. Amplifications with this primer were performed with 4.0 mM $MgCl_2$ at an annealing temperature of 60° C. Synthetic primers used to amplify cDNA sequences coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) span the sequence 3308-3337 and 3649-3672 in the rat cDNA and produce a 241-bp fragment, as described by Rappolee *et al.* (1992). Reactions with this primer were performed with 4.0 mM $MgCl_2$ at an annealing temperature of 60° C. For amplification of the collagenase mRNA, the cDNA derived from reverse transcription (RT) of 1 μg RNA from cells plated on FN, 120FN, or FN/TN was diluted to 10^{-3}, 10^{-4}, 10^{-5}, and 5 x 10^{-6}. For amplification of GAPDH mRNA, the cDNA derived by RT of 1 μg RNA from cells plated on FN, 120FN, and FN/TN was diluted to 5 x 10^{-2}, 10^{-2}, 10^{-3}, and 5 x 10^{-4}. Control samples contained no template. The products were separated on agarose gels and deleted by staining with ethidium bromide. We used scanning densitometry (LKB-Pharmacia) to quantify the level of collagenase and GAPDH products in amplifications from dilutions of RT mix from RNA harvested from cells plated on FN, 120FN, or FN/TN. The densitometer readings were plotted against the dilutions of the RT mix in the amplification; estimates of concentration were made on the linear portion of the graph.

Immunofluorescence

In experiments analyzing collagenase expression, cells were plated on acid-washed glass coverslips coated with ECM proteins as described above. At the indicated time after plating, coverslips were rinsed in PBS and cells were fixed for 5 min in 2% paraformaldehyde in PBS. After fixation, cultures were made permeable by incubation in 0.25% Triton X-100 in PBS at ambient temperature for 2 min. Coverslips were rinsed in PBS and blocked for several hours with a solution of 1% BSA in PBS before a 1 h incubation with a cocktail of five mAbs against rabbit collagenase (Werb *et al.*, 1989). Cells were washed, incubated for 1 h with biotinylated goat anti-mouse IgG, rinsed, and incubated for 1-h with Texas Red-streptavidin. The cells were

Figure 1. Analysis of proteins secreted by RSF plated on various matrices. Fibroblasts were plated in DME-LH in wells that were coated with FN (lanes *1,2*), 120FN (lanes *3,4*) or a mixed substrate of FN and TN (lanes *5,6*) and incubated for 24 h. The secreted proteins were then biosynthetically labeled by incubating cells with [^{35}S]methionine for 4 h. The radiolabeled secreted proteins were concentrated with quinine sulfate and SDS, and the precipitates were analyzed by SDS-PAGE and fluorography. Procollagenase (CL) migrating at 53 and 57 kD is indicated by *arrows*. Molecular weight (x 10^{-3}) markers are indicated at the left.

Figure 1



photographed on a Zeiss photomicroscope II with phase-contrast and epifluorescence and 25x or 63x water immersion lenses.

The coverslips that were stained with anti-Fos antibodies were fixed with ice-cold 4% paraformaldehyde in PBS and incubated on ice for 30 min, then made permeable at ambient temperature with 0.25% Triton X-100 in PBS for 5 min. Nonspecific sites were blocked by incubating coverslip cultures for at least 3 h with 0.2% BSA in PBS. Cells were then incubated with the anti-Fos antibody for 1 h at ambient temperature at temperature followed by 10 h at 4° C. Coverslips were washed and then incubated with biotinylated anti-rabbit IgG, washed again with PBS, then incubated with Texas Red-streptavidin. No staining was detected in cells that were incubated with a mixture of anti-Fos antibody and an excess of the peptide immunogen. The cells were photographed as described above.

RESULTS

Expression of Secreted Proteins Is Altered in Cells Plated on a Substrate of FN and TN

In previous studies we found that secreted proteins are sensitive indicators of altered phenotype in a variety of cell types, including fibroblasts (Aggeler *et al.*, 1984; Werb *et al.*, 1989; Tremble *et al.*, 1993). Accordingly, we plated RSF on a substrate of FN, 120FN, or a mixture of FN and TN (FN/TN) and cultured the cells for 24 h. The cultures were then incubated with [^{35}S]methionine for 4 h, and the radiolabeled secreted proteins were analyzed. Early-passage RSF plated on FN secrete little radiolabeled protein. We noted the induction of proteins migrating near 55 kD that are characteristic of the proenzyme forms of the MMPs collagenase and stromelysin and that are increased in fibroblasts plated on a substrate of 120FN, but not on intact FN (Werb *et al.*, 1989) (Fig. 1). Collagenase was specifically immunoprecipitated from the CM harvested from these cultures with an anti-collagenase mAb (Fig. 2A). We used scanning densitometry to quantify the amounts of collagenase synthesized by cells plated on FN/TN and by cells plated on FN. More

Figure 2. Collagenase and stromelysin are upregulated in cells plated on a mixed substrate of FN/TN. Freshly trypsinized RSF were plated in DME-LH in wells that had been coated with FN or FN/TN and incubated for 24 h. The CM was removed and saved for later analysis, and the cultures were incubated with [^{35}S]methionine for 2 h. (*A*) Samples of CM containing radiolabeled secreted proteins from cultures plated on FN (lanes *1,3*) or FN/TN (lanes *2,4*) were concentrated with quinine sulfate and SDS to show total proteins secreted into the medium (lanes *1,2*), or proteins secreted into the medium were immunoprecipitated with an anti-collagenase mAb (lanes *3,4*), and the precipitates were analyzed by SDS-PAGE and fluorography. The doublet of procollagenase (CL) is indicated. (*B*) The proteinase content of the 40-h CM harvested from cells plated in wells coated with FN or FN/TN was analyzed by gelatin zymography as described in Materials and Methods. Proteolytic degradation of gelatin, printed as a negative image, appears as dark bands. Note the induction of gelatinases migrating at 92, 57, and 53 kD, corresponding to the proenzyme forms of the 92 kD gelatinase and collagenase. (*C*) CM harvested from cells plated on FN (lanes *1,3*) or FN/TN (lanes *2,4*) was separated by SDS-PAGE under denaturing conditions, and the proteins were transferred to membranes and analyzed by immunoblotting with anti-collagenase (lanes *1,2*) and anti-stromelysin (SL) (lanes *3,4*) mAbs. Procollagenase (CL) and prostromelysin (SL) are indicated by *arrows*. Molecular weight ($x10^{-3}$) markers are indicated at the left.

Figure 2

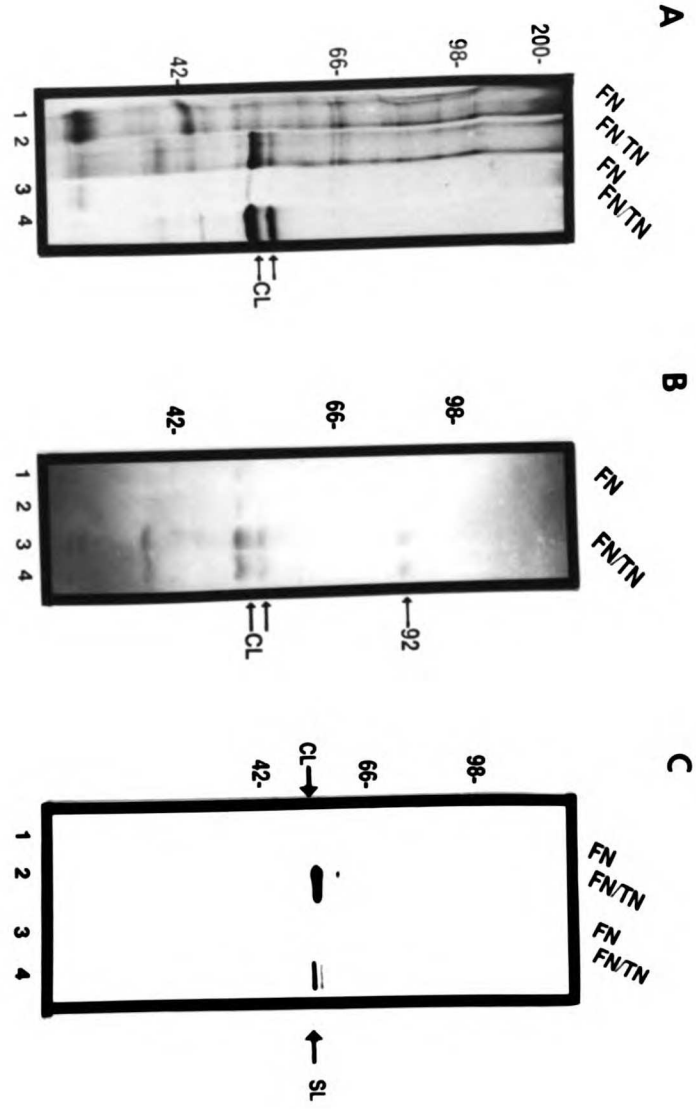
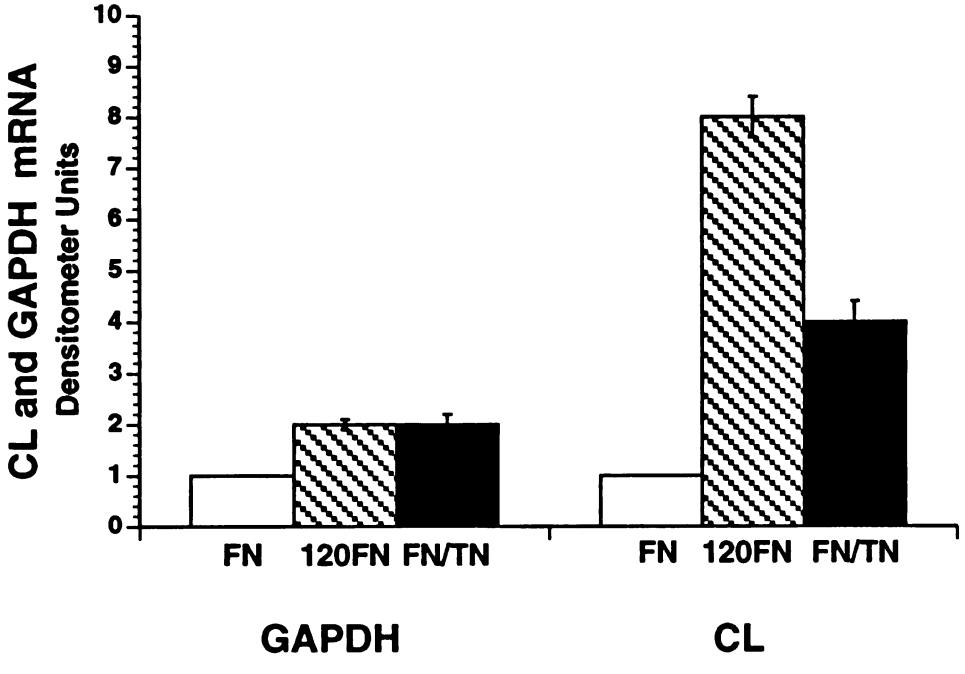

A
200-
98-
66-
42-
1 2 3 4
FN
FN/TN
FN
FN/TN
CL
B
98-
66-
42-
1 2 3 4
FN
FN/TN
CL
92
C
CL
98-
66-
42-
1 2 3 4
FN
FN/TN
FN
FN/TN
SL

Figure 3. The expression of collagenase mRNA is upregulated in cells plated on FN/TN. RSF were plated in DME-LH in wells coated with FN, 120FN, or FN/TN and cultured for 30 h. Total cellular RNA was isolated and analyzed by RT-PCR. Sequences in cDNA were amplified with specific primers for collagenase cDNA and GAPDH cDNA to yield 300-bp and 241-bp fragments, respectively. The products were separated on agarose gels and stained with ethidium bromide. The data were quantified by densitometry and expressed as -fold induction compared with cultures plated on FN, as described in Materials and Methods. The data are an average of two experiments.

Figure 3


CL and GAPDH mRNA
Densitometer Units
10
9
8
7
6
5
4
3
2
1
0
FN
120FN
FN/TN
FN
120FN
FN/TN
GAPDH
CL

than 50% of the total biosynthetically labeled protein secreted by the cells plated on FN/TN was procollagenase. Pooling the data from six experiments, in which two different preparations of TN and RSF cultures derived from several different rabbits were used, we saw a 2.2-fold increase (± 0.5 S.D.) (n=6, $p<0.005$, Student's t-test) in collagenase expression in cells plated on FN/TN when compared with cultures plated on FN alone.

To characterize the induced secreted proteins further, we analyzed the CM by zymography on gelatin substrate gels. Gelatin-degrading proteinases migrating at 92 kD and 55 kD were upregulated in cultures plated on FN/TN (Fig. 2B). These gelatinases are the proenzyme forms of the 92 kD gelatinase (also called type IV collagenase) and interstitial collagenase. Immunoblotting procedures confirmed that the expression of collagenase and stromelysin proteins increased in cells plated on FN/TN when compared with cells plated on FN (Fig. 2C). In subsequent experiments we used collagenase as a model gene to investigate the mechanisms that underlie the effects of a FN/TN substrate.

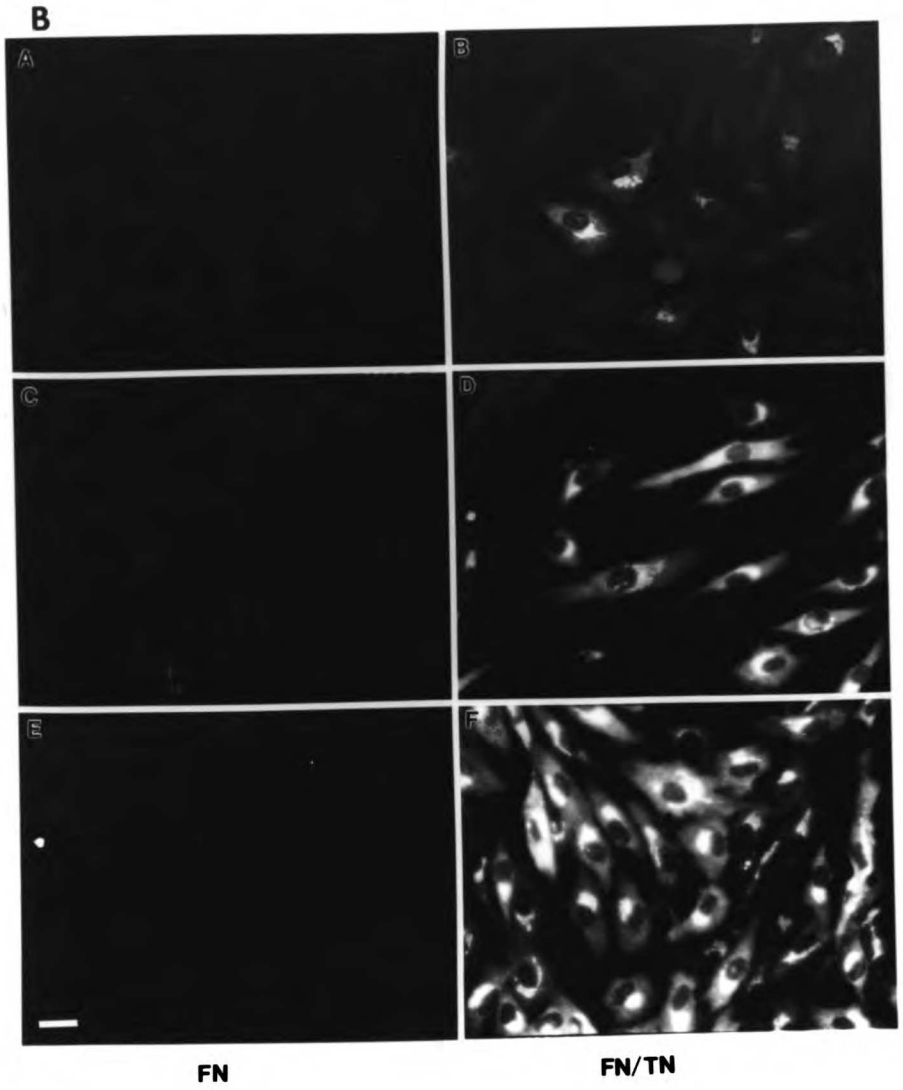
The increase in collagenase protein expression resulted from an increase in collagenase mRNA. Total RNA harvested from cells grown for 24 h on FN, 120FN, or FN/TN was analyzed for the presence of collagenase mRNA or the mRNA for the housekeeping gene GAPDH with the use of RT-PCR and sequence-specific primer pairs (Fig. 3). Collagenase mRNA increased 5-fold in cells plated on a FN/TN substrate and 8-fold in cells plated on 120FN, when compared with cells plated on intact FN. There was a 2-fold increase in GAPDH mRNA harvested from cells plated on 120FN or FN/TN. Comparing collagenase mRNA with GAPDH mRNA, we saw a net 2.5-fold increase in collagenase expression in cells plated on FN/TN and a 4-fold increase in cells plated on 120FN.

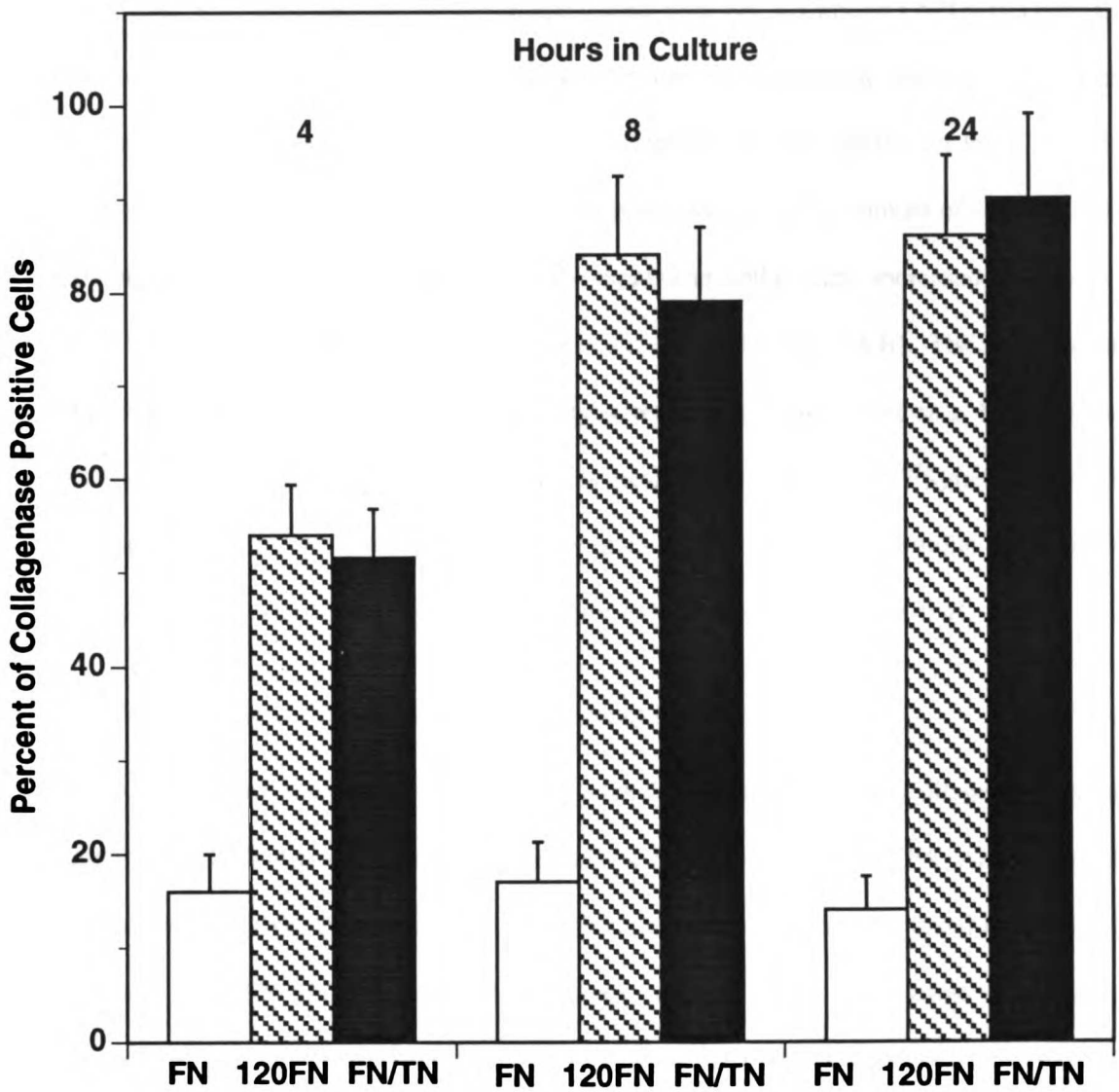
Collagenase Expression Is Upregulated within 4 h after Plating on a Substrate of FN/TN

The kinetics of the regulation of collagenase expression has proved to be an important first clue describing the mechanism underlying the regulation of gene expression by various ECM

Figure 4. The increases in collagenase expression in cultures plated on FN/TN are temporally similar to those seen in cells plated on 120FN. Freshly trypsinized RSF were plated in DME-LH on glass coverslips coated with FN, 120FN, or FN followed by TN, and cultured for 4, 8, or 24 h. The cultures were fixed and permeabilized, and the intracellular collagenase was detected with an anti-collagenase mAb. (*A*) The percentage of cells staining for intracellular collagenase was determined by counting at least 50 cells in five microscopic fields and recording the number of cells expressing collagenase. Bars indicate standard deviation. (*B*) Collagenase expression in cells plated on FN (*A,C,E*) or FN/TN (*B,D,F*) for 4 h (*A,B*), 8 h (*C,D*), or 24 h (*E,F*). Bar = 20 μm.

Figure 4




Hours in Culture
4
8
24
Percent of Collagenase Positive Cells
100
80
60
40
20
0
FN
120FN
FN/TN
FN
120FN
FN/TN
FN
120FN
FN/TN

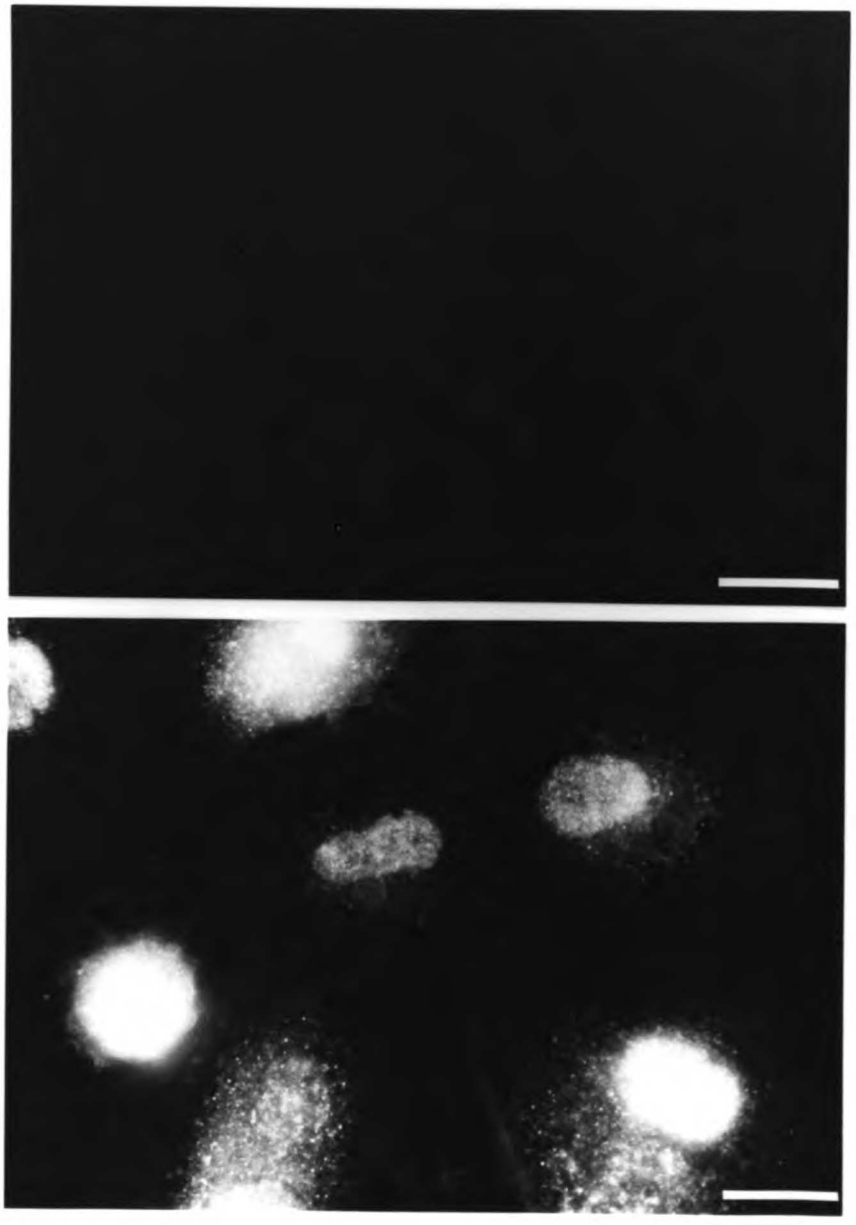
substrates. For example, the rapid induction of collagenase in cells plated on 120FN is similar to the induction of collagenase by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Werb *et al.*, 1989), whereas the induction of collagenase in cells cultured in retracted collagen gels or treated with SPARC or cytochalasins is much slower (Unemori and Werb, 1986; Tremble *et al.*, 1993). We used immunofluorescent techniques to determine whether the regulation of collagenase expression in cells plated on a substrate of FN/TN proceeds with rapid or slow kinetic parameters. RSF were cultured on coverslips coated with FN, 120FN, or FN/TN in DME-LH for 4, 8, or 24 h, and then fixed and processed for immunofluorescent staining of intracellular collagenase. We quantified the expression of collagenase in RSF plated on these matrices by counting at least 50 cells in five microscopic fields and recording the number of cells stained with anti-collagenase mAbs. The number of cells expressing collagenase increased by 4 h in cells plated on 120FN or FN/TN, and continued to increase for 20 h (Fig. 4A,B). The rapid increase in collagenase expression, detected by immunocytochemistry, in cells plated on a FN/TN substrate paralleled the induction of collagenase that was measured by analysis of [^{35}S]methionine-labeled collagenase secreted by cells plated on 120FN (data not shown) and was similar to the induction of collagenase in cells plated on anti-FN receptor mAb or with TPA (Werb *et al.*, 1989).

c-Fos Is Expressed in Cells Plated on a Substrate of FN/TN

The expression of recorder constructs that contain segments of the collagenase promoter, including the TPA response element, is upregulated in cells treated with TPA (Angel *et al.*, 1988; Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1991) and in cells plated on 120FN when compared with FN (P. Tremble, C.H. Damsky, and Z. Werb, manuscript in preparation). Induction of collagenase has been linked to binding of AP1 (which consists of heterodimers of the inducible nuclear proteins c-Fos and c-Jun or homodimers of Jun family members) to the TPA response element in the collagenase promoter. In cells treated with TPA, increases in both the expression and the activation of Fos and Jun family members precede the upregulation

Figure 5. Nuclear Fos is increased in cells plated on FN/TN. RSF were plated in DME-LH on coverslips coated with FN (*top*) or FN/TN (*bottom*) and cultured for 1 h. The coverslips were then fixed and stained with an anti-peptide antibody raised against a peptide sequence in c-Fos as described in Materials and Methods. As a control, cells were stained with antibody in the presence of an excess of peptide, and these showed no immunofluorescent signal (not shown). Bar = 20 μm.

Figure 5



of collagenase by phorbol esters (Angel *et al.*, 1988; reviewed by Angel and Karin, 1992). We used immunocytochemistry with an antibody raised against a c-Fos peptide sequence to show a nuclear localization of c-Fos protein in cells plated on a substrate of FN/TN (Fig. 5). Expression of c-Fos was scored by recording the number of cells that were stained for nuclear c-Fos in at least four microscopic fields in two experiments. Nuclear c-Fos expression was seen in 91.5% (± 9.8 S.D.) of cells within 1 h of plating on FN/TN, but only in 20% (± 16.3 S.D.) of cells plated on FN (Fig. 5).

Collagenase Upregulation in RSF Is Specific to a Mixed Substrate of FN/TN

Collagenase could be induced in cells plated on FN/TN through an alteration of FN, an alteration of TN, or a cooperative effect between both molecules. Determining if TN works alone or in the context of other matrix molecules may clarify this issue. Accordingly, we plated cells in DME-LH in wells that were coated with FN, vitronectin, or type I collagen in the presence or absence of TN. We also measured collagenase secreted by adherent RSF that were plated in DME containing 10% FBS and treated with soluble TN. We used SDS-PAGE, autoradiography, and densitometry to compare the biosynthetically labeled collagenase secreted into the CM in these experiments. TN did not induce the expression of collagenase in the context of a mixed substrate of either type I collagen or vitronectin and TN, or when added in solution to cells cultured on serum proteins (predominantly vitronectin) (Fig. 6). We were unable to analyze the expression of collagenase in cells plated on TN alone, because they adhered poorly and had a rounded morphology. Because a change in cell shape alone induces collagenase and stromelysin in RSF (Aggeler *et al.*, 1984; Unemori and Werb, 1986), it was not possible to determine if TN alone had a specific effect because of the nonspecific effect due to morphologic alteration.

When we tested a range of TN concentrations on a FN substrate, we found that not all combinations of FN/TN matrices were equally inductive as substrates (Fig. 7). Collagenase expression was seen at concentrations of up to 25 μg/ml TN added to wells coated with 25 μg/ml

Figure 6. Collagenase expression in cells plated on mixed substrates of collagen and TN or vitronectin and TN or treated with soluble TN does not differ from that in untreated cultures. Freshly trypsinized RSF were plated in DME-LH in wells coated with FN, FN/TN, collagen (COL), collagen and TN (COL/TN), vitronectin (VN), or vitronectin and TN (VN/TN). Adherent cells, plated on uncoated wells in DME-10% FBS for 6 h, were washed with DME-LH and further incubated in DME-LH (Serum) or in DME-LH supplemented with 80 μg/ml TN (Serum /sol.TN). The cultures were incubated with [^{35}S]methionine after 30 h in culture, and the radiolabeled secreted proteins were concentrated and analyzed by SDS-PAGE and autoradiography. Collagenase expression was measured by scanning densitometry of the autoradiograms, and the data were compared as -fold induction, which is taken as the amount of collagenase secreted by cultures incubated with TN, divided by the amount of collagenase secreted by cultures on the same matrix substrate in the absence of TN. Averages of the arbitrary densitometry units (±S.D) are shown. The data for FN, FN/TN, collagen, and collagen/TN summarize two separate experiments, and the data for VN, VN/TN, Serum, and Serum /sol.TN are an average of duplicates in one experiment.

Figure 6

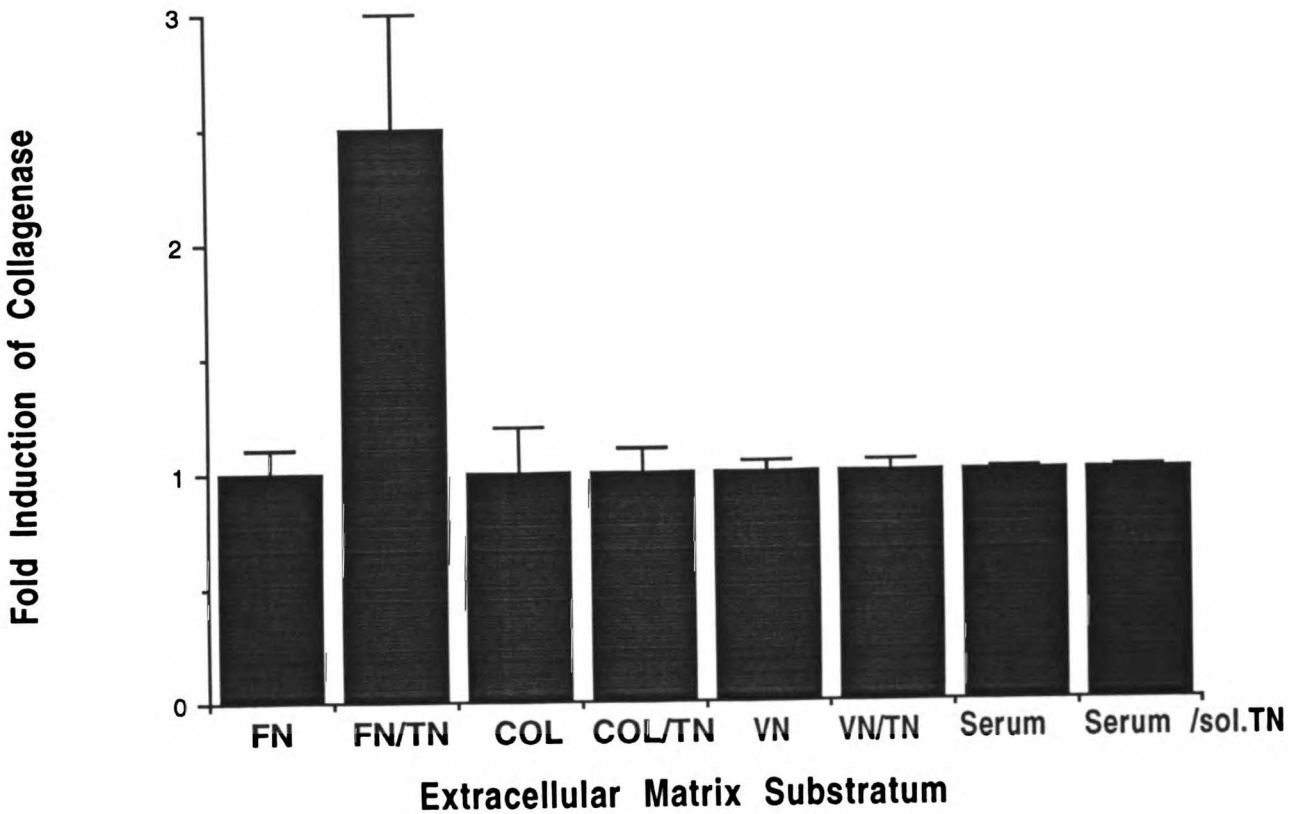
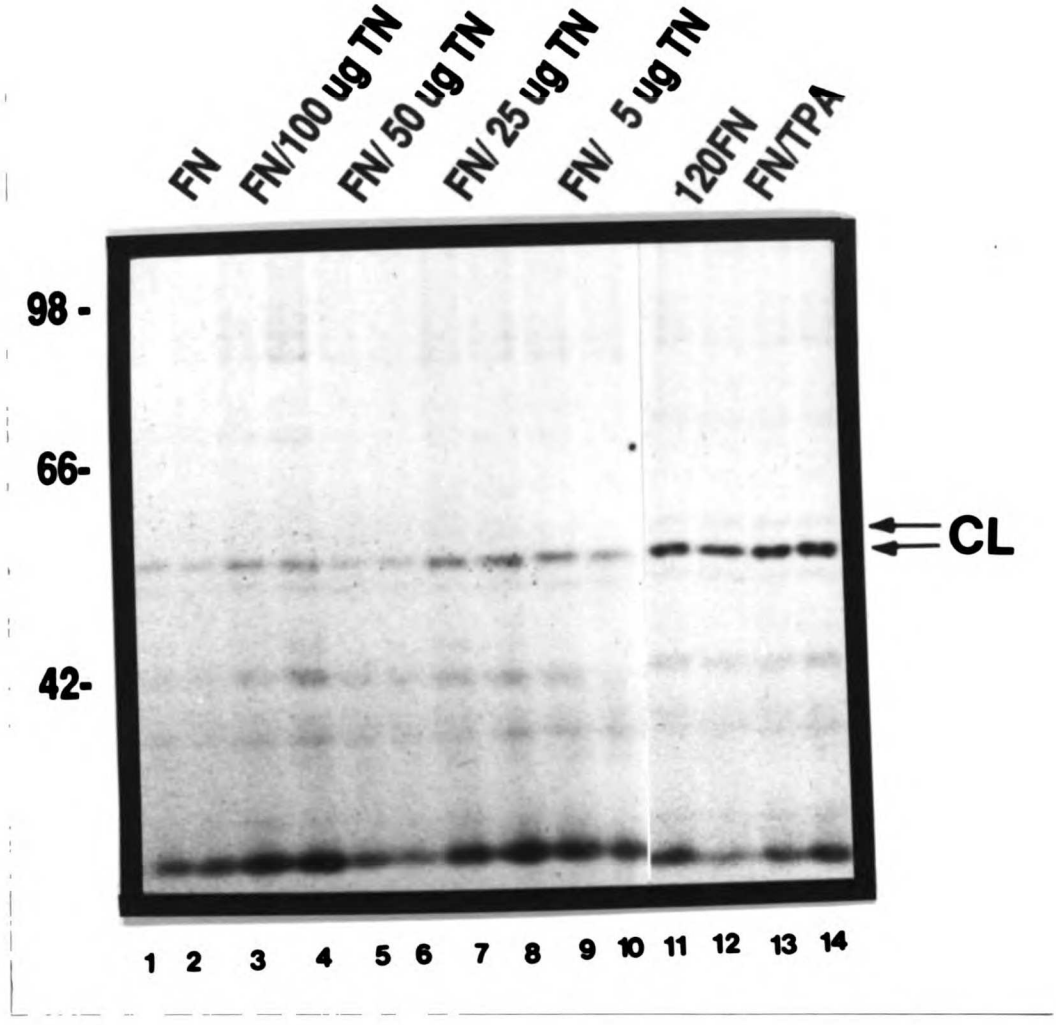

Fold Induction of Collagenase
3
2
1
0
FN
FN/TN
COL
COL/TN
VN
VN/TN
Serum
Serum /sol.TN
Extracellular Matrix Substratum

Figure 7. Not all FN/TN matrices are inductive substrates. Freshly trypsinized RSF were plated in DME-LH in wells coated with FN (lanes *1,2*), 120 FN (lanes *11,12*), FN in DME-LH supplemented with 100 μg/ml TPA (lanes *13,14*), or mixed substrates of FN and TN (lanes *3-10*) that were prepared by incubating FN-coated wells with 100 μg TN (lanes *3,4*), 50 μg TN (lanes *5,6*), 25 μg TN (lanes *7,8*), or 5 μg TN (lanes *9,10*), as described in Materials and Methods. The cultures were incubated for 24 h and then incubated with [^{35}S]methionine, and the secreted proteins were precipitated and analyzed by SDS-PAGE and autoradiography. Molecular weight ($x10^{-3}$) markers are indicated at the left.

Figure 7


FN
FN/100 ug TN
FN/ 50 ug TN
FN/ 25 ug TN
FN/ 5 ug TN
120FN
FN/TPA
98 -
66-
42-
CL
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FN. At 50 μg/ml TN, the response was reproducibly lower. At 100 μg/ml TN, adhesion was compromised and collagenase expression increased again, presumably because of the shape-dependent mechanism. This suggests that specific ratios of FN and TN combine to form inductive matrices. Early-passage fibroblasts in culture often synthesize and secrete TN into the culture medium. We analyzed the CM harvested from the RSF used in these experiments by immunoblotting and did not detect TN (data not shown).

The Distal Type III Repeats of TN Upregulate Collagenase Expression in RSF Plated on FN/TN

TN contains three defined protein structural domains—EGF-like repeats, FN type III repeats, and a fibrinogen globular domain—that have been well characterized in other proteins (Spring *et al.*, 1989; reviewed by Engel, 1991). Spring *et al.* (1989) defined both adhesive and anti-adhesive regions in the TN arm and showed that an epitope in the EGF-like repeats has an anti-adhesive effect on fibroblasts in culture, whereas the sites in the TN arm that interact with cells map to regions in the type III repeats (Spring *et al.*, 1989; Lochter *et al.*, 1991; Murphy-Ullrich *et al.*, 1991). The type III repeats of the TN arm not only are important for the interaction of TN with cells, but also modify the interaction of TN with FN (Chiquet *et al.*, 1991; Chiquet-Ehrismann *et al.*, 1991). To determine which of these regions confers regulation of collagenase expression in RSF plated on FN/TN, we used two anti-TN antibodies, anti-TNM1, which recognizes an epitope in the EGF-like repeats of the TN arm, and anti-TN68, which recognizes an epitope in the FN type III repeats (Spring *et al.*, 1989).

We plated cells on substrates of FN, FN/TN, or FN/TN that had been incubated with the mAbs anti-TNM1, anti-TN68, or a mixture of both anti-TNM1 and anti-TN68, and cultured them in DME-LH for 24 h. The cells were incubated with [^{35}S]methionine, and the secreted proteins were analyzed by SDS-PAGE and autoradiography. Collagenase expression was upregulated in cells plated on FN/TN or on FN/TN that had been incubated with anti-TNM1 (Fig. 8). However, when cells were plated on a substrate of FN/TN that had been incubated with anti-TN68,

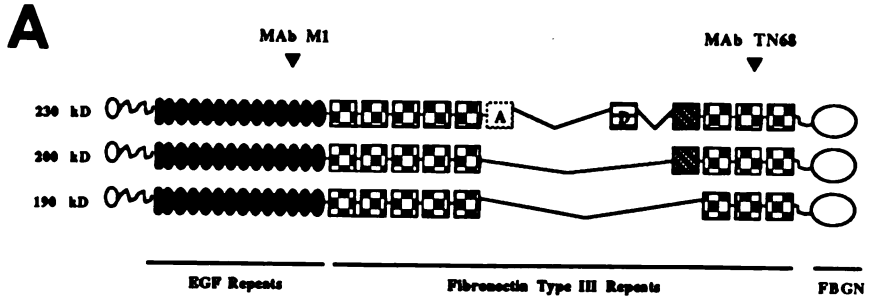
collagenase expression was reduced nearly to the levels seen in cells plated on FN alone. A combination of anti-TNM1 and anti-TN68 was no more effective than anti-TN68 alone in abrogating the regulation of collagenase expression in cells plated on a substrate of FN/TN. Thus, the distal part of the TN molecule containing the FN type III repeats is involved in mediating the changes in gene expression in cells plated on FN/TN matrices.

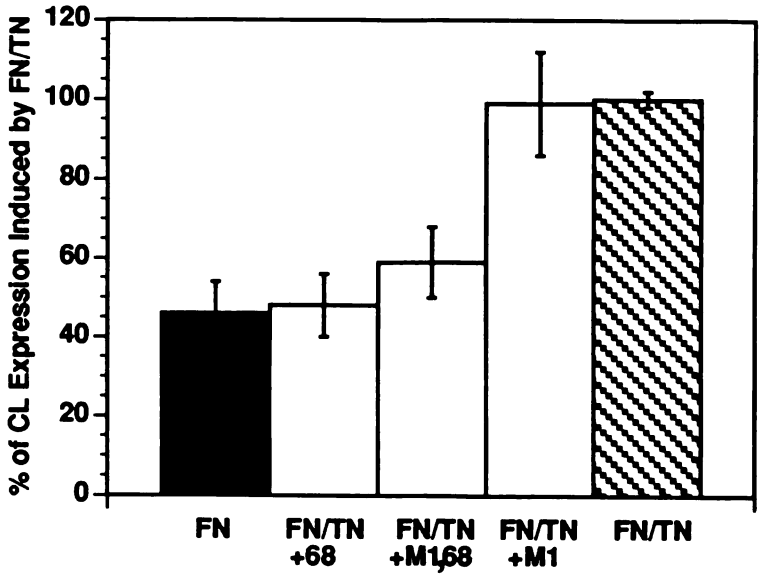
DISCUSSION

Our results suggest that the interaction of TN with FN alters cellular signaling and rapidly induces the expression of c-Fos. This is followed by an increase in metalloproteinase expression, an effect not seen in cell cultures incubated with TN or cultured on FN alone. Moreover, the carboxyl-terminal FN type III repeats in the TN arm function in this interaction. That the integrity of the TN arm near the carboxyl-terminal FN type III repeats, and not the anti-adhesive site mapped to the EGF-like repeats by Spring *et al.* (1989), is necessary for the regulation of collagenase was demonstrated by experiments in which anti-TN68, but not anti-TNM1, blocked the inductive effect of FN/TN matrices. mAb anti-TN68 defines an epitope in the tenth type III repeat that is included in all TN isoforms, is involved in cellular responses to both soluble and substrate-bound TN, and blocks the TN-induced destabilization of cellular adhesion to FN (Chiquet-Ehrismann *et al.*, 1986; Spring *et al.*, 1989; Halfter *et al.*, 1989; Riou *et al.*, 1990). Cells attach but do not spread on recombinant TN molecules or proteolytic fragments of TN that contain the distal type III repeats (Friedlander *et al.*, 1988; Spring *et al.*, 1989), showing that this portion of the TN arm interacts with molecules on the cell surface. The interaction of TN with cells and substrate is undoubtedly more complex, because adjacent regions in the alternatively spliced type III repeats alter the interaction of TN with FN substrates (Chiquet-Ehrismann *et al.*, 1991), promote neurite outgrowth (Wehrle and Chiquet, 1990; Lochter *et al.*, 1991), and destabilize the adhesion of adherent cultured endothelial cells (Murphy-Ullrich *et al.*, 1991). The nature of the interaction of TN with cells may also be specified by the type of cell surface

Figure 8. Induction of collagenase expression in fibroblasts plated on FN/TN is diminished by anti-TN68. (*A*) Structure of the TN isoforms from chicken. Each arm of the TN molecule consists of an amino-terminal globular domain, EGF-like repeats, FN type III repeats, and a carboxyl-terminal globular domain homologous to fibrinogen (FBGN). The epitopes recognized by anti-TNM1 and anti-TN68 are indicated. (*B*) Induction of collagenase expression in fibroblasts plated on FN/TN and anti-TN68. Fibroblasts were plated in serum-free medium in wells that were coated with FN, FN/TN, or a FN/TN mixture that had been incubated with 250 μg of anti-TN antibodies TNM1 or TN68, or with 250 μg of both antibodies. Cultures were incubated for 24 h; the secreted proteins were biosynthetically labeled by incubation of cells with [^{35}S]methionine, precipitated with quinine sulfate, and analyzed by SDS-PAGE, fluorography, and autoradiography. Collagenase protein secreted by fibroblasts was quantified with scanning densitometry, and the data, an average of two experiments, were expressed as -fold induction. Bars indicate standard deviation.

Figure 8


A
MAb M1
MAb TN68
230 kD
200 kD
190 kD
A
B
EGF Repeats
Fibronectin Type III Repeats
FBGN


B
% of CL Expression Induced by FN/TN
120
100
80
60
40
20
0
FN
FN/TN +68
FN/TN +M1,68
FN/TN +M1
FN/TN

molecules expressed by cells (Salmivirta *et al.*, 1991) and by the isoforms and glycosylation of TN expressed by cells (Hoffman and Edelman, 1987; Chiquet *et al.*, 1991; Prieto *et al.*, 1990).

Cells interacting with FN/TN matrices behaved like cells adhering to anti-FN receptor antibodies or to the cell-binding fragment of FN (Werb *et al.*, 1989). There are several possible explanations for how the addition of TN to the FN substrate produces an inductive matrix. One possibility is that TN sterically hinders the accessibility of ECM substrate to the cell surface (Lightner and Erickson, 1990). It is clear from our results that the addition of soluble TN to cultured fibroblasts, adhering largely to vitronectin derived from serum proteins, does not induce collagenase synthesis in these cells. Likewise, collagenase expression in cells plated on mixed substrates of collagen or vitronectin and TN did not differ from that in cultures plated on collagen alone. This is informative, because it suggests that TN alone is not the inductive molecule, but rather that the combination of FN and TN upregulates collagenase expression in RSF. If a strictly steric mechanism were operating, TN would also induce the expression of collagenase on collagen. Alternatively, the induction of collagenase in fibroblasts plated on a mixture of FN and TN could result from a specific interaction of a mixed substrate of FN/TN with the cell surface. TN disrupts adhesion to FN and FN fragments that have the cell-binding domain containing RGD (Chiquet-Ehrismann *et al.*, 1986) and decreases cellular adhesion to FN only in cells in which RGD also perturbs cellular adhesion to FN (Chiquet-Ehrismann *et al.*, 1988). This suggests that there may be similarities in the interaction of cells with FN/TN and the interaction of cells with the large cell-binding fragment of FN. For example, fibroblasts plated on intact FN express basal levels of MMP, whereas cells plated on 120FN or FN/TN upregulate the expression of MMP with similar kinetics. Similarly, the formation of focal adhesions in cells is diminished in cells plated on 120FN (Woods and Couchman, 1992) and in adherent cells treated with TN (Murphy-Ullrich *et al.*, 1991). The implication that the carboxyl-terminal type III repeats in the TN arm are a necessary component of an inductive matrix also supports this idea, because the

alternatively spliced and carboxyl-terminal type III repeats modulate the interaction of TN with FN (Chiquet *et al.*, 1991).

But how does FN/TN exert its inductive effect? FN, like TN, is a large, multidomain molecule in the ECM that interacts with other ECM molecules and is recognized by several classes of cell surface receptors (reviewed by Hynes, 1992). Intact FN in the matrix is recognized by integrin receptors, a matrix assembly receptor, and cell surface proteoglycans, whereas 120FN, which contains the cell-binding domain of FN, interacts primarily with integrin receptors (reviewed by Damsky and Werb, 1992). It is clear that cells plated on 120FN or treated with anti-FN receptor antibody upregulate the expression of collagenase (Werb *et al.*, 1989). Interestingly, like FN, both of these ligands interact with the integrin FN receptor, but unlike FN, these ligands do not contain domains that can interact with cell surface proteoglycans. It may be that the coordinate interaction of several classes of cell surface adhesion receptors (e.g., integrins and proteoglycans) with FN is necessary to restrict expression of MMP (reviewed by Damsky and Werb, 1992). This hypothesis is supported by data that show that focal contact formation is compromised in cells plated on 120FN (Woods and Couchman, 1992) and in cells treated with TN (Murphy-Ullrich *et al.*, 1991). Furthermore, focal contact formation in cells plated on 120FN or in the presence of TN is stabilized by treatments that appear to allow an interaction with cell surface proteoglycans. The addition of a catalytic amount of the amino-terminal heparin-binding domain of FN to cells plated on 120FN initiates or stabilizes focal contact formation (Woods and Couchman, 1992). Likewise, the addition of chondroitin sulfate proteoglycans blocks the destabilization of focal contacts in cultured cells caused by the addition of TN or recombinant TN molecules containing only the alternatively spliced FN type III repeats in the TN arm (Murphy-Ullrich *et al.*, 1991). Indeed, TN fragments that contain the carboxyl-terminal type III repeats (isolated by chromatography on anti-TN68) bind to a heparin affinity column (Chiquet *et al.*, 1991). Taken together, these observations support the idea that TN, in the context of a FN-

rich matrix, might interact with cell surface proteoglycans, thereby disrupting the adhesion of cells to FN. This could result in signaling information transduced either through the FN receptor, which may now sense intact FN to be like 120FN, or through a cooperative interaction of a TN-dependent receptor in concert with the integrin FN receptor.

In vitro, two examples have been described in which alterations in the FN/TN ratio are seen in conjunction with remodeling tissue or migrating cells. Gatchalian *et al.* (1989) have shown that TN accumulates around denervated synaptic sites and that a specific population of fibroblasts proliferate in perisynaptic spaces. They further have shown that the TN/FN ratio synthesized by fibroblasts enzymatically dissociated from denervated muscle end plate is increased when compared with fibroblasts from innervated muscle. In view of our observations that MMP expression is upregulated in cultured cells plated on a mixed substrate of FN and TN and that TN supports and fosters neurite outgrowth, it is interesting to speculate that concentrations of proteinases and inhibitors may also be altered in this region. Together with cytokines released locally by macrophages, perturbations in adhesion caused by the interaction of cells with an altered FN/TN substrate may also play a role in the remodeling of the perisynaptic site. In healing skin wounds TN accumulates in granulation tissue and in the dermis underneath the migrating epidermis (Mackie *et al.*, 1988; Whitby *et al.*, 1991). Studies comparing the closure and healing of fetal wounds, which heal with little or no scar formation, with that of adult wounds, which do scar, show that although the localization of TN immunostaining is similar in fetal and adult wounds, the temporal pattern of TN expression is distinct. In embryonic wounds TN expression is evident within 4 h of wounding, and back to normal levels within 72 h (Whitby *et al.*, 1991), whereas in adult tissues, TN expression increases at a much slower rate and is still present in the dermis 14 days after wounding (Whitby and Ferguson, 1991; Mackie *et al.*, 1988). FN isoforms expressed underneath the wounded tissue also revert to include isoforms expressed during development; ffrench-Constant *et al.* (1989)

have shown that the expression of FN isoforms containing alternatively spliced type III repeats, characteristic of embryonic matrices, is upregulated underneath the migrating wound epithelium. It is tempting to hypothesize that signals generated through alterations in cellular adhesion to the ECM by FN/TN regulate the ECM remodeling phenotype, including scar formation in response to injury. Alterations in the TN/FN ratio have also been described in cells in the intestinal crypts, another tissue that is continuously differentiating, renewing, and remodeling (Weller *et al.*, 1991), suggesting that cell-ECM interactions play an important role in the regulation of both the structure (remodeling) and function (differentiation) of renewing tissues.

In addition to serving as a scaffold for tissue structure, ECM plays important and diverse roles during development and morphogenesis, acting to coordinate the interaction of cell surface receptors for ECM with ligand, as well as by acting in concert with various growth factors or growth factor receptors (reviewed by Adams and Watt, 1993). As would be expected, there is considerable variation in the composition of tissue-specific matrices (reviewed by Hay, 1990; Adams and Watt, 1993). Diversity in the cell-ECM interactions can be generated by a change in the composition of ECM resulting from an alteration of synthesis or degradation of particular ECM molecules (reviewed by Alexander and Werb, 1991; Adams and Watt, 1993). Differences in the splicing patterns of ECM molecules (ffrench-Constant *et al.*, 1989; Prieto *et al.*, 1990; Weller *et al*, 1991) or post-translational modification of ECM molecules (Jones *et al.*, 1986) also alter the interaction of matrix molecules with cells or with other molecules in the ECM. We have previously shown that SPARC upregulates the expression of a number of genes (including ECM-degrading MMPs) in RSF, albeit by a mechanism distinct from that used for transducing information from FN/TN matrices (Tremble *et al.*, 1993). Taken together with the observations in this study, our results suggest that the addition of matrix-associated molecules such as SPARC and TN to an established ECM changes the nature of the matrix and, furthermore, that fibroblasts interacting with this functionally distinct matrix upregulate the expression of a number of genes,

some of which regulate the turnover of ECM itself.

The initiation of an ECM remodeling cascade in response to specific cues in the matrix that are determined by ECM composition and, conversely, the regulation of matrix and tissue structure by proteolysis are likely to be significant in wound healing and/or tissue morphogenesis. For example, MMPs and their inhibitors play a significant role in the expansion, maturation, and resorption of the mouse mammary gland (Talhouk *et al.*, 1992). Controlled proteolysis may also be necessary for the migration of cells seen in both normal developmental processes and the metastatic spread of tumor cells. Indeed, inhibition of MMPs slows or ablates the extravasation of metastatic melanoma cells (Schultz *et al.*, 1988). Likewise, the migration of cultured embryonic mesenchymal cells through basement membrane-like matrices requires a net increase in the proteolytic activity of these cells (Alexander and Werb, 1992). In addition to directly facilitating migration, upregulation of MMPs in migrating cells may also modify ECM presented to cells in directed cell migrations. We have shown that the augmentation of a FN matrix with TN induces the expression of MMPs in cultured cells. The three MMPs that are induced in cells plated on FN/TN can act in concert to degrade a wide range of tissue matrices: The 92 kD gelatinase degrades type IV collagen and denatured interstitial collagen; collagenase degrades native fibrillar collagens; and stromelysin degrades a wide range of matrix molecules, including types I and IV collagen, FN, and proteoglycans (reviewed by Alexander and Werb, 1991). Extrapolating, we suggest that the transient expression of TN may also trigger localized expression of MMP to remove and remodel matrices that are no longer functional, or to facilitate cell migration. In a previous study, we demonstrated that MMP expression is induced in synovial fibroblasts when plated on an agonistic antibody that interacts specifically with the $\alpha_5\beta_1$ FN receptor, or on 120FN, but not when plated on intact FN (Werb *et al.*, 1989). This suggests that the interaction of integrin-type FN receptor with a subset of ligands, in this case an anti-FN receptor antibody, or with FN fragments initiates a remodeling cascade. The same receptor may

be involved in detecting FN/TN ligands. The work reported here further extends the paradigm that ECM ligands, their receptors, proteinases, and their inhibitors, collaborate in the construction, turnover, and remodeling of ECM by cells.

Recently, targeted disruption of the TN gene by homologous recombination in embryonic stem cells has allowed the derivation of mutant mice that do not express TN (Saga *et al.*, 1992). Although these mice have no gross abnormalities, analysis of tissue structure at higher resolution may reveal differences between wild-type and TN null mice. It is also possible that alterations in phenotype will be observed in TN null mice under specific, challenging conditions, such as in repair of wounded tissue. These results, however, question the significance of TN during development and morphogenesis; it may be that developmental cues provided by other molecules in the ECM (which may or may not be TN analogs) can functionally replace the developmental cues normally provided by TN, and that manipulation of more than one molecule will be necessary to characterize a TN null mouse. Several molecules with structural similarities to TN have been described: undulin, which has similarities to FN and TN (Just *et al.*, 1991), and TN-MHC and restrictin, which more closely resemble TN (Matsumoto *et al.*, 1992; Norenberg *et al.*, 1992). A study of the expression of specific TN isoforms, or genetically altered TN isoforms in a TN-null background, may be informative as to the role of TN during developmental interactions, whereas a null phenotype is not informative. There also are species differences in TN structure and distribution that may be significant in generation of phenotype; for example, chicken TN (used in these experiments) and human TN have an RGD sequence in one of the type III repeats, whereas mouse TN does not contain this sequence. Likewise, the pattern of MMP expression differs between species; collagenase is the dominant MMP expressed in human and rabbit fibroblasts, whereas mouse fibroblasts secrete little collagenase. Information gleaned through characterization of TN structure-function relationships *in vitro*, identifying domains in the TN molecule that interact with cells or with the ECM (Friedlander *et al.*, 1988; Spring *et al.*, 1989;

Murphy-Ullrich *et al.*, 1991) and the consequences of these interactions (Wehrle and Chiquet, 1990; Lochter *et al.*, 1991; present study), will help to clarify the role of TN in developing and remodeling tissues and will facilitate analysis of the effects of TN *in vivo*.

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Chapter 5

Components of the Nuclear Signaling Cascade that Regulate Collagenase Gene Expression in Response to Integrin-Derived Signals

This chapter addresses the extracellular signaling events of the fibronectin-fibronectin receptor interaction and defines components in the nuclear signaling cascade.

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Components of the Nuclear Signaling Cascade that Regulate Collagenase Gene Expression in Response to Integrin-Derived Signals

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The nature of signals transduced by integrins is only beginning to be elucidated, but it is clear that these receptors can have at least two types of roles in signaling cascades — that is, integrin occupancy can modify or be modified by signals transduced by other ligand-receptor pairs or they may themselves initiate a signaling cascade. This implies a cooperative processing of information transduced by integrin (reviewed by Damsky and Werb, 1992). The affinity of β_1, β_2, or β_3 integrins for ligand can be upregulated in response to signals initiated in response to the activation of other cell surface receptors (reviewed by Hynes, 1992; Damsky and Werb, 1992). The ligand binding activity of β_1 integrins can also be down regulated by intracellular signals (Dahl and Grabel,1989; Grandori and Hynes, referenced in Hynes, 1992). Inactivation of β_1 integrin ligand binding function has been also observed in differentiating keratinocytes (Hotchin and Watt, 1992) and retinal tectal neurons (Neugebauer and Reichardt, 1991), although it is not yet known how these inactivations occur. Thus adhesiveness of integrins can be affected, in either direction, by signaling cascades initiated by other cell surface receptors. The opposite can also be true, because integrin occupancy can affect or is a prerequisite for several intracellular processes. In activated platelets, the phosphorylation of a number of intracellular proteins requires the occupancy of $\alpha IIb\beta_3$ by ligand (Lipfert et al., 1992). Clustering of $\alpha_5\beta_1$ with anti-fibronectin receptor antibody somehow regulates the Na^+/H^+ antiporter, alkalinizing the cytoplasm (Schwartz et al., 1990). It is also thought that clustering of $\alpha_5\beta_1$ or adhesion to FN augments the lipid metabolism induced by PDGF (McNamee et al., 1993).

Ultimately, the end point of signaling cascades is the regulation of cell phenotype or gene expression. Occupancy of integrin receptors by specific ligands demonstrate that integrins play a role in the regulation of myoblast terminal differentiation (Menko and Boettiger, 1987). The interaction of cells with FN or anti-integrin antibodies has been shown to inhibit the terminal differentiation of keratinocytes (Adams and Watt, 1989). Consequences of cell-ECM interactions have also been examined at the level of gene expression. Yamada et al. (1991) have

shown that in T lymphocytes under some conditions, occupancy of the fibronectin receptor induces the transcription factor AP-1, leading to upregulation of interleukin-2. An enhancer element in the casein gene, BCE-1, that upregulates casein expression in mammary epithelial cells in response to concurrent signals from matrix receptors and prolactin receptors has also been described (Schmidhauser et al., 1992). We have previously shown that rabbit synovial fibroblasts (RSF) plated on fibronectin fragments or anti-fibronectin receptor antibodies upregulate the expression of three extracellular matrix-degrading metalloproteinases (MMP) (collagenase, stromelysin and the 92 kD gelatinase) while RSF plated on plasma fibronectin express basal levels of these metalloproteinases (Werb et al., 1989). mRNA coding for collagenase and collagenase protein are upregulated in RSF plated on inductive ligands, with increases in collagenase protein detectable within 2-4 h of treatment (Werb et al., 1989; Tremble et al., 1993), suggesting that the induced expression of collagenase is a direct consequence of signals generated by integrins. In the present study we used several constructs containing fragments of the human collagenase promoter fused to a chloramphenicol acyl transferase (CAT) recorder gene to further describe the regulation of collagenase in RSF by integrin-derived signals.

Materials and Methods

Cells and Cell Culture

Rabbit synovial fibroblasts, isolated as described previously (Aggeler et al., 1984), were cultured in DME (Cell Culture Facility, University of California, San Francisco) supplemented with 10% FBS (Hyclone, Denver, CO) and used between passages 2 and 10. Except where noted otherwise, RSF were subcultured 48 h before experimental procedures. Cells used in experiments were added to ECM-coated wells at a density of 1-2 x 10^5 in DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH); the plates were rotated to ensure even

distribution of cells and incubated further at 37° C before analysis of MMP expression.

Antibodies

The anti-collagenase mAbs were characterized and used as described (Werb et al., 1989). The anti-stromelysin mAb (SL188.2) was a generous gift of Dr. Scott Wilhelm, Miles Research (West Haven, CT) (Wilhelm et al., 1992). The polyclonal anti-Fos antibody and matching peptide immunogen, purchased from CRB Biologics, Cambridge, England were used in immunofluorescent studies. Adsorption of the anti-Fos antibody with the peptide immunogen was performed as described in the technical information supplied with the antibody. Antibodies to cFos (Ab-1) and cJun (Ab-2) that were used in western immunoblotting were purchased from Oncogene Science, (Uniondale, NY). The monoclonal anti-fibronectin receptor antibody, BIIG2, was used as described in Werb et al.(1989). The biotinylated and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma (St. Louis, MO), and the Texas Red-labeled streptavidin was purchased from Amersham (Arlington Heights, IL).

Preparation of Extracellular Matrix Substrates

Human plasma FN was purchased from Collaborative Research (Waltham, MA) or Boehringer Mannheim Biochemical (Indianapolis, IN), reconstituted as directed by the manufacturer, and frozen at -70° C in single-use aliquots. The 120 kD chymotryptic fragment of human plasma FN (120FN) was purchased from Telios Pharmaceuticals (La Jolla, CA), reconstituted as directed, and stored in single-use aliquots at -70° C. For coating of wells, 24- or 48-well culture dishes (Costar, Cambridge, MA) were incubated with 0.2 or 0.1 ml of 25 µg/ml FN or 120FN in PBS overnight (9-15 h) at 4° C. They were then washed three times with PBS and incubated in 0.2% BSA in PBS for 30 min at ambient temperature to reduce nonspecific binding to the tissue culture dish. The wells were then washed three times with PBS and used immediately. For coating with anti-FNR antibody, wells were incubated with Protein-G purified anti-fibronectin receptor

antibody (50 μg/ml) in PBS overnight (9-15 h) at 4° C, and further processed as described above. Larger plates were coated with FN or 120FN at a concentration of 3μg/cm^2; larger dishes were coated with anti-FNR antibody at a concentration of 1.5μg/cm^2 .

Biosynthetic Labeling of Proteins Secreted by RSF

RSF were added to ECM-coated wells and cultured in DME-LH for up to 48 h, after which the conditioned medium (CM) was removed and saved for later analysis. Cultures of RSF were biosynthetically labeled by incubation with 50-70 μCi/ml [^{35}S]methionine (Express Label, New England Nuclear, Boston, MA) for 3-4 h in methionine-free DME (GIBCO, Grand Island, NY). Radiolabeled secreted proteins were precipitated from the CM with quinine sulfate and SDS or by immunoprecipitation with specific antibodies as described previously (Werb et al., 1989). Precipitates were analyzed by SDS-PAGE and fluorography (En^{3}Hance; New England Nuclear). Radiolabeled collagenase secreted into the culture supernatants was quantified by scanning autoradiograms with an LKB densitometer and GSXL software (LKB-Pharmacia, Piscataway, NJ).

Analysis of Specific Proteinases

Proteinases in the CM harvested from cell cultures were analyzed by using gelatin zymography as described previously (Werb et al., 1989). Proteins in the CM were separated on nonreducing 10% SDS-polyacrylamide gels that contained 0.1% gelatin. After electrophoresis the gel was soaked in 2.5% Triton X-100 to remove SDS and incubated for 18 h in substrate buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$). The gels were stained with Coomassie Blue R 250 (BioRad, Richmond, CA); zones containing gelatinases appear as a clear zone on a blue background.

To allow quantitation of collagenase protein in the CM harvested from test cultures by immunoblotting, we applied several dilutions of CM directly to nitrocellulose using a slot-blot filtration apparatus (Schleicher and Schuell, Keene, NH). Nonspecific sites on the membranes

were blocked with a solution of 3% BSA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), after which the membranes were incubated with anti-collagenase or anti-stromelysin mAb in TBS containing 0.5% Tween-20 (Sigma) for 1-2 h at ambient temperature. Before and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma), the membranes were washed three times for 20 min with 0.5% Tween-20 in TBS. Specific bands were detected by enhanced chemiluminescence (Amersham) as described by the manufacturer. After detection, the films were scanned using a densitometer and GelScan-XL software (LKB-Pharmacia, Piscataway, NJ).

RNA Isolation and Analysis

Total cellular RNA was isolated from cultured RSF by the method of Chirgwin (1979). Alternatively, poly-A RNA was isolated using a micro-Fast Track kit (Invitrogen, San Diego, CA). RNA was separated on agarose gels, transferred to nylon membranes, and probed with ^{32}P labeled cDNA probes as previously described (Maniatis et al., 1982). Plasmids containing cDNA coding for c-*fos* (kindly provided by Dr. Leslie Rall, San Francisco, CA), c-*jun* (Moye-Rowley et al., 1989) and glyceraldehyde-3-phosphate dehydrogenase (Fort et al., 1985) were used to generate probes.

Analysis of c-fos and c-jun

c-*fos* was analyzed using nucleic acid hybridization techniques described above. The cFos and cJun protein in nuclei harvested from cultures plated on matrix ligands was also analyzed using immunoblotting. Nuclear extracts were prepared by the method of Bos et al. (1988). The total protein content of these extracts was measured using the micro-BCA Assay (Pierce, Rockford, IL), and samples, normalized for protein content, were reduced and separated on a 10% SDS-polyacrylamide gel using Lamelli buffers (Harlow and Lane 1989) After electrophoresis, the proteins were transferred to ImmobilonP membranes (Millipore, Boston MA)(Harlow and Lane, 1989). Incubations with anti-cFos or anti-cJun antibodies were performed as suggested by the

manufacturer, followed by incubation with secondary antibodies and detection with enhanced chemiluminescence as described for the anti-collagenase antibody.

Immunofluorescence

In experiments analyzing the expression of immunoreactive collagenase, cells were plated on acid-washed glass coverslips coated with ECM proteins as described above. At the indicated time after plating, coverslips were rinsed in PBS and cells were fixed for 5 min in 2% paraformaldehyde in PBS. After fixation, cultures were made permeable by incubation in 0.25% Triton X-100 in PBS at ambient temperature for 2 min. Coverslips were rinsed in PBS, blocked for several hours with a solution of 1% BSA in PBS before a 60-min incubation with a cocktail of five mAbs against rabbit collagenase (Werb et al., 1989). Cells were washed, incubated for 1 h with biotinylated goat anti-mouse IgG, rinsed, and incubated for 1 h with Texas Red-streptavidin. The cells were photographed on a Zeiss photomicroscope II with phase-contrast and epifluorescence using 25x or 63x water immersion lenses.

The coverslips that were stained with anti-Fos antibodies were fixed with ice-cold 4% paraformaldehyde in PBS and incubated on ice for 30 min, then made permeable at ambient temperature with 0.25% Triton X-100 in PBS for 5 min. Nonspecific sites were blocked by incubating coverslip cultures for at least 3 h with 0.2% BSA in PBS. Cells were then incubated with the anti-Fos antibody for 1 h at ambient temperature at temperature followed by 10 h at 4° C. Coverslips were washed and then incubated with biotinylated anti-rabbit IgG, washed again with PBS, then incubated with Texas Red-streptavidin. No staining was detected in cells that were incubated with a mixture of anti-Fos antibody and an excess of the peptide immunogen. The cells were photographed as described above.

Reporter Plasmids

Several CAT reporter plasmids containing fragments of the human collagenase promoter regulating the expression of the enzyme chloramphenicol acyl transferase used in this study were

generously provided by Dr. Peter Herrlich and Dr. H. Rahmsdorf, Karlsruhe, Germany. These plasmids, -1200/-42 tkCAT, -139/-42 tkCAT, -66/-42 tkCAT, contained the indicated fragments of the human collagenase promoter inserted in front of a minimal thymidine kinase promoter in the plasmid pBLCAT2 first described in Angel et al., (1987). A reporter plasmids containing only the minimal SV40 AP1 site (Harshman et al., 1988), was generously provided by Dr. W.S. Moye-Rowley, (Univ. Iowa, Iowa City) The reporter construct $(AP1)_3$, containing three tandem repeats of the AP1 site in the proliferin promoter (Diamond et al., 1990) was provided by Dr. Susan Logan. The expression of β-galactosidase from co-transfected ΔGRE-βgal, which contains an RSV promoter, was used to normalize for transfection efficiency. The plasmids pCH110 (Pharmacia, Piscataway, NJ) and ΔGRE-βgal , provided by Dr. J.A. Miner, (Univ. of Cal., San Francisco), were used to normalize for transfection efficiency.

The plasmids mAPcol-tkCAT, mPEAcol-tkCAT and WTcol-tkCAT were constructed with synthetic oligonucleotides representing the AP1 and PEA3 sites in the human collagenase promoter (Angel et al., 1987a). WTcol-tkCAT contains the AP1 and PEA3 sites present in the human collagenase promoter. mAPcol-tkCAT contains a substitution of AT to TG in the AP1 site; in gel shift assays, oligonucleotides containing this substitution do not interact with AP1 (Gutman and Wasylyk, 1989). mPEAcol-tkCAT contains substitution of G to A in the PEA3 site of the collagenase promoter. Oligonucleotides containing this substitution do not interact with PEA3 in gel shift assays (Gutman and Wasylyk, 1989). The annealed oligonucleotides were subcloned into the *BamHI-HindIII* site in pBLCAT2. The insertion of the oligo was confirmed by hybridization analysis using end labeled oligos.

Synthesis of Oligonucleotides

Oligonucleotides were synthesized on a PCR-Mate, model #391 from Applied Biosystems (Foster City, CA), using columns and reagents from Applied Biosystems. Full length oligonucleotides were purified using OPC cartridges as supplied and described by Applied

Biosystems. The sequences of the WT, mAP1, and mPEA oligonucleotides are listed above, and were derived from Gutman and Wasylyk (1989). Antisense and sense fos oligonucleotides represented the 18 residues from and including the start codon for c-*fos*, ATG ATG TTC TCG GGC TTC AA (sense fos); TTG AAG CCC GAG AAC ATC AT (anti-sense fos) (Verma et al., 1986).

Transfections

Except in experiments where antisense and sense oligonucleotides were added to cells concurrently with the reporter construct, CAT plasmids were introduced into cells by either calcium phosphate coprecipitation of DNA (Sambrook et al., 1989) or by using the Lipofectin reagent (Gibco/BRL, Bethesda, MD). Typically 10-20 μg of CAT construct and 2 μg of β-galactosidase construct were coprecipitated with calcium phosphate (Sambrook et al., 1989); the precipitates were added to a 75 cm^2 flask of cells (subcultured 24 h prior to transfections) in DME containing 10% FBS. The cultures were incubated with the DNA precipitate for 5-6 h and allowed to recover overnight (9-15 h) in DME-LH, after which the cells were plated on ECM-coated wells as described in individual experiments. In transfection experiments using the Lipofectin reagent, a 100 μl aliquot containing 10 μg of CAT construct and 2 μg of β-galactosidase construct was mixed with 100 μl of a 50% solution of Lipofectin in DME. The DNA encapsulated in Lipofectin was added to a 75 cm^2 flask of cells (subcultured 24 h prior to transfection) in unsupplemented DME, and the cultures were incubated in this mixture overnight (9-15 h). These cells were then subcultured, and plated on ECM-coated wells as described in individual experiments. The cultures were terminated and cell extracts were prepared 50 h after plating on ECM coated wells.

In some experiments both oligonucleotides and plasmids were introduced into cells by electroporation. In these experiments, RSF in suspension were washed three times with DME that was supplemented with 20 mM Hepes, pH 7.5 and resuspended in Hepes buffered DME at a

concentration of 6.2 x 10^7 cells per ml. 0.8 ml aliquots of this cell suspension were added to disposable cuvettes (Gibco/BRL), along with 60 μg -139/-42 tkCAT and either sense c-*fos* (FOS-OLIGO) or anti-sense c-*fos* (SOF-OLIGO) oligonucleotides (25 μM final concentration). The cuvettes were placed on ice for 2 min, then cells were electroporated in a water filled electroporation chamber (Cellporator, Gibco/BRL) using settings of 280 V and 500 μF. Cells were allowed to recover for 3 min at ambient temperature prior to removal of the cells from the cuvette and plating of the cells DME-LH on ECM-coated wells in DME-LH supplemented with the appropriate oligonucleotide. The cultures were terminated and cell extracts were prepared after 50 h incubation.

CAT Assays

Assay of chloramphenicol acyl tranferase in cell extracts transfected with CAT plasmids was measured in assays that used either [^{3}H]-labeled chloramphenicol and thin layer chromatography to separate the acetylated chloramphenicol from unacetylated chloramphenicol (Gorman et al., 1982, Sambrook et al., 1989), or [^{14}C]-labeled coenzyme A and ethyl acetate to extract acetylated chloramphenicol products (Sleigh, 1986; Sambrook et al., 1989). Assay of β-galactosidase activity was performed as described in Sambrook et al. (1989).

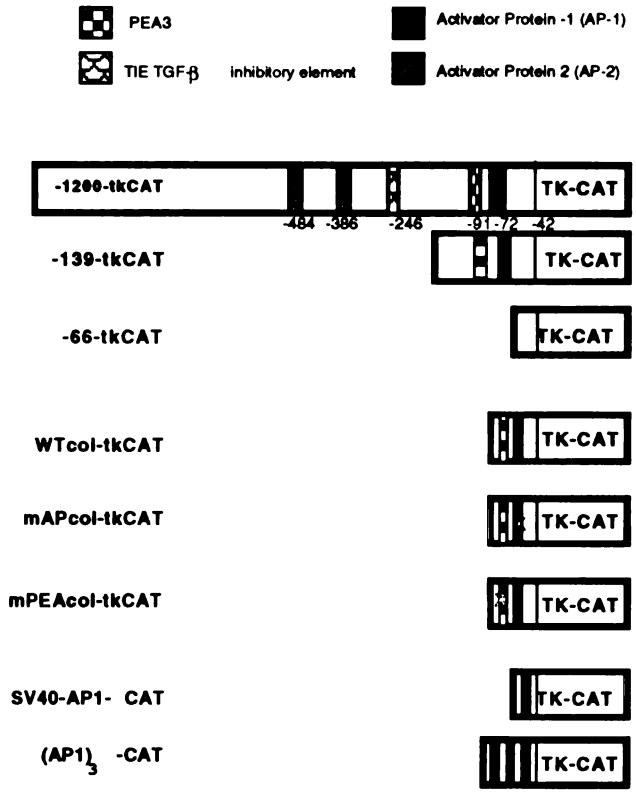
Results

Elements in the -139/-42 segment of the human collagenase promoter regulate transcription in cells adhering to anti-$\alpha_5\beta_1$ antibody and fibronectin fragments.

To define the elements in the collagenase promoter that are required for the regulation of collagenase by integrins or adhesion-derived signals, we first established that the collagenase-CAT promoter-reporter plasmids transiently introduced into cells were coregulated with the endogenous collagenase gene. We used constructs in which segments of the human collagenase gene between -1200 to -42, -139 to -42, or -66 to -42 were placed upstream of the minimal

Figure 1. Reporter plasmids used in this study. (A). The collagenase-reporter constructs used in this study contain segments of the human collagenase promoter (-1200/-42, -139/-42, and -66/-42) inserted upstream from the minimal thymidine kinase promoter and the gene coding for chloramphenicol acyl transferase in pBLCAT2 (Angel et al., 1987). The inducible elements that have been defined in the sequences in the human collagenase promoter are shown. They include an AP1 (activator protein 1) site that is also known as a TPA responsive element (TRE) at -72/-67, a PEA3 (polyoma enhancer activator 3) site at -90 to -82, a TGF-β inhibitory element at -246 to -237, and AP2 sites (activator protein 2) at -484/-464 and -234/-214 (Angel et al., 1987, Gutman and Wasylyk, 1990, Kerr et al., 1990). (B) Promoter constructs containing only the AP1 and PEA3 sequence from the human collagenase promoter, or derivatives of these sequences were made as described in the Materials and Methods. The construct WTcol-tkCAT contains the native AP1 and PEA3 sequences from the human collagenase gene (-90/-67) inserted into pBLCAT2. In mAPcol-tkCAT the AP1 site was disrupted by changing AT to TG, which prevents formation of complexes by this oligonucleotide and AP1 in gel shift assays (Gutman and Wasylyk, 1990). A G to A substitution in the PEA3 sequence generated the mPEA3col-tkCAT construct; oligonucleotides with this sequence do not form complexes with Ets-1 in gel shift assays (Gutman and Wasylyk, 1990). (C). Reporter constructs containing AP1 sequences from other genes were also tested. $(AP1)_3$ contains three tandem copies of the AP1 sequence from the proliferin gene. SV40AP1 contains a single copy of the SV40 TRE inserted into pUC19tkCAT (Moye-Rowley et al., 1988).

Figure 1

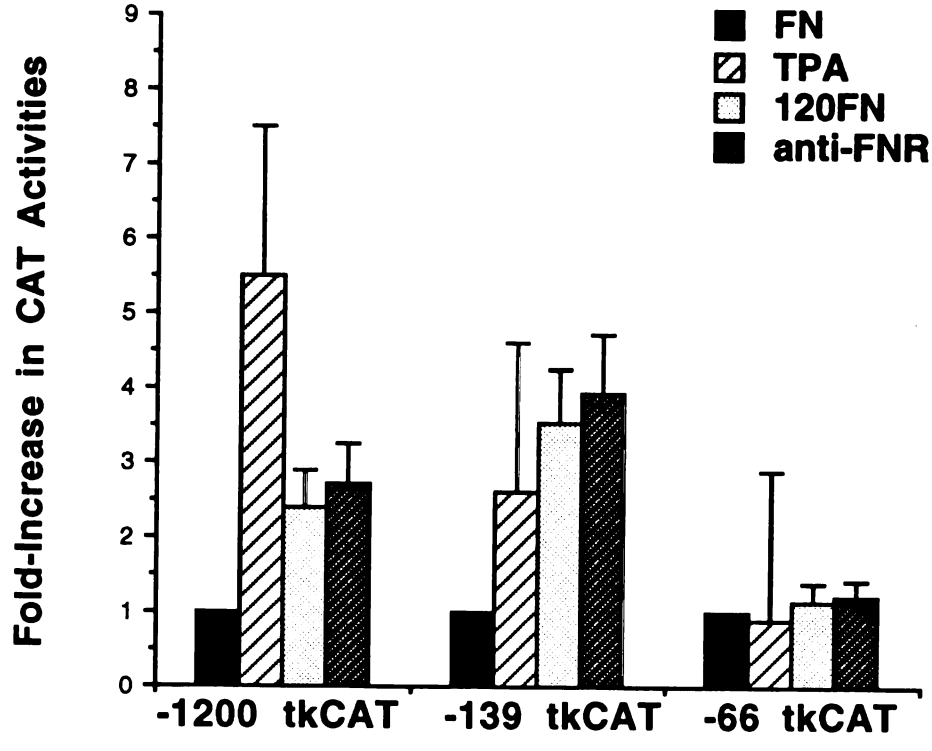

PEA3
TIE TGF-β
inhibitory element
Activator Protein -1 (AP-1)
Activator Protein 2 (AP-2)
-1200-tkCAT
TK-CAT
-484
-386
-246
-91
-72
-42
-139-tkCAT
TK-CAT
-66-tkCAT
TK-CAT
WTcol-tkCAT
TK-CAT
mAPcol-tkCAT
TK-CAT
mPEAcol-tkCAT
TK-CAT
SV40-AP1- CAT
TK-CAT
(AP1)3 -CAT
TK-CAT

pBLCAT2 (Angel et al., 1987). These plasmids (-1200/-42 tkCAT, -139/-42 tkCAT, or -66/-42 tkCAT; Fig. 1), along with an constitutve expression vector for β-galactosidase used to control for transfection efficiency, were introduced into RSF by calcium phosphate precipitation. The transfected cells were subcultured and plated in DME-LH on 35 mM dishes that were coated with FN, 120FN or anti-$\alpha_5\beta_1$ antibody. The upregulation of endogenous collagenase as well as promoter-reporter constructs driven by the collagenase promoter in response to phorbol esters has been well characterized (Angel et al., 1987; Auble et al., 1992). Therefore transfected cells, plated on FN in DME-LH that was supplemented with 200 ng/ml TPA were included as a positive control. After culture for 48-50 h, the conditioned medium was removed and saved to determine the induction of the endogenous collagenase gene, and cell extracts were prepared for analysis of CAT activity (Sambrook et al., 1989). The expression of CAT was compared by measuring enzyme activity. To normalize for transfection efficiency, CAT activity was analyzed from amounts of cell extract that gave equal β-galactosidase activity. Data were compared as the amount of CAT activity from promoter-reporter constructs over that seen from the parent pBLCAT2 construct alone.

Like the endogenous gene, the expression of the two reporter constructs containing sequences upstream from -66 increased in cells treated with TPA or plated on 120FN or anti-$\alpha_5\beta_1$ antibody (Fig. 2). To facilitate comparison between experiments, the data in this figure are presented as fold-induction, that is the expression of a particular reporter construct in cells plated on inductive ligands for the fibronectin receptor over that seen with this construct in cells plated on fibronectin. In cells plated on 120FN the expression of -1200tkCAT was 2.4 ±0.8-fold times that on fibronectin, and in RSF plated on anti-$\alpha_5\beta_1$ antibody CAT activity was 2.7 ±0.7-fold times the fibronectin controls. TPA increased the expression of -1200tkCAT 5.5±2.1-fold. In RSF transfected with -139tkCAT and plated on 120FN, CAT activity increased to 3.54± 1.5-fold times that seen on fibronectin. CAT activity from -139tkCAT in RSF plated on anti-$\alpha_5\beta_1$ antibody was

Figure 2. Promoter-reporter constructs containing sequences from the human collagenase promoter are induced in RSF plated on fibronectin fragments or anti-$\alpha_5\beta_1$ antibody (anti-FNR). RSF were transfected with 20 μg of the indicated collagenase-CAT construct and 2 μg of ΔGRE-β gal, a constitutively expressed vector for β-galactosidase, by incubation with calcium phosphate-DNA precipitates. RSF were incubated with the precipitates for 6 h. The precipitates were removed and the cultures were glycerol shocked for 2 min; cultures were allowed to recover overnight (9-14 h) in DME-LH. The transfected RSF were subcultured and plated in DME-LH on ECM-coated wells. As a positive control one set of RSF cultures was plated on fibronectin in DME-LH that was supplemented with 100 ng/ml TPA. Cell extracts were prepared from RSF that were cultured for 48-50 h and the expression of CAT was determined from enzyme activities. CAT activities were normalized to β-galactosidase activity and are presented as the ratio of the CAT activity in cell extracts from RSF transfected with one collagenase-tkCAT construct plated on fibronectin fragments, anti-$\alpha_5\beta_1$ antibody or treated with TPA, to the CAT activity in cell extracts prepared from RSF transfected with the same promoter-reporter construct that were plated on fibronectin. The results are the average of 9 experiments which used 6 different isolates of RSF. The expression are of -1200 tkCAT and -139 tkCAT constructs in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibody was upregulated to about the same extent when compared to cells plated on fibronectin; however the amount of -139 tkCAT expression was reproducibly higher than seen with -1200 tkCAT. For example, in two sets of experiments which used RSF of similar passage number that were isolated from the same rabbit, the CAT activity from -1200 tkCAT in RSF plated on fibronectin was 3.3± 0.1 times that of the parent pBLCAT2 vector; the expression from -139 tkCAT in RSF plated on FN was 15± 1.2 times that of the parent pBLCAT2 vector, and the expression from -66 tkCAT was 1.0± 0.2 times that of the parent pBLCAT2. In these experiments the baseline CAT activity from pBLCAT2 was 500±50 cpm.

Figure 2


Fold-Increase in CAT Activities
0
1
2
3
4
5
6
7
8
9
FN
TPA
120FN
anti-FNR
-1200 tkCAT
-139 tkCAT
-66 tkCAT

3.92± 1.7-fold times that seen in RSF plated on fibronectin; the expression of -139tkCAT increased 2.6±0.5-fold in RSF treated with TPA compared to control cultures. The expression of -66tkCAT was similar in cells plated on fibronectin, 120FN, anti-$\alpha_5\beta_1$ antibody and in cells treated with TPA (1-, 0.91±0.6-, 1.1±0.3-, 1.2± 0.2-fold, respectively). In the experiments plotted in Fig. 2 the CAT activities in cell extracts were normalized to equal amounts of β-galactosidase activity, however normalizing CAT activities to equal cellular protein gave the same results. In cells plated on 120FN or anti-$\alpha_5\beta_1$ antibody, the fold-induction of the reporter construct containing the longer -1200/-42 segment of the human collagenase promoter was similar to the reporter construct containing the -139/-42 segment of the promoter. We therefore assumed that the elements necessary for the induction of collagenase in cells plated on fibronectin fragments and anti-$\alpha_5\beta_1$ antibody are included in the -139/-42 segment of the promoter. In the collagenase promoter, two elements that confer inducibility by growth factors and TPA (Angel and Karin, 1992; Gutman and Wasylyk, 1992 and the references therein) have been described in this region— an activator protein 1 (AP1) site at -72/-67 and a polyomavirus enhancer activator 3 (PEA3) site at -90/-82. The region of the collagenase promoter from -139/-42 are nearly identical in the human and rabbit genes; in addition to the AP1 and PEA3 sites described in the human promoter, the sequences that have been described in this segment of the rabbit promoter that are involved in response to TPA are also present in the -139tkCAT construct (Angel et al., 1987; Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1992).

Both the AP1 and PEA3 sites are required to activate collagenase transcription in cells adhering to anti-$\alpha_5\beta_1$ antibody or fibronectin fragments.

To date, studies using promoter-reporter constructs to analyze the increases in gene expression regulated by the collagenase promoter show that a functional AP1 site is always required, but that the upstream PEA3 site or other adjacent sequences may also function in the regulation of collagenase by growth factors and TPA (Angel et al., 1987, Gutman and Wasylyk,

Figure 3. The AP1 and PEA3 sites are involved in the regulation of collagenase by ECM ligands or TPA. RSF were transfected with 20 μg of one of several CAT reporter plasmids containing segments of the human collagenase promoter, along with 2 μg of ΔGRE-βgal. Plasmids were introduced by incubation of RSF with calcium phosphate-DNA precipitates as described in the legend to Figure 2. After transfection, RSF were subcultured and plated in DME-LH on ECM coated wells; one set of cultures plated on fibronectin were cultured in DME-LH supplemented with 100 ng/ml TPA. Cell extracts were harvested 48-50 h after plating on ECM substrates and the expression of CAT was analyzed by comparing CAT enzyme activity in cell extracts. CAT activity was normalized to β-galactosidase activity, and the data are expressed as the ratio of CAT activity from collagenase promoter-reporter plasmids to that seen in cells transfected with the parent pBLCAT2 construct. *A)* The data in this panel, from one experiment show that the expression of both WTcol-tkCAT and -139 tkCAT is upregulated in cells treated with TPA or plated on 120FN or anti-$\alpha_5\beta_1$ antibody (anti-FNR), when compared to cultures plated on fibronectin. The CAT assays were done in duplicate and the duplicates varied by less than 10%. *B)* Panel B averages the results of 5 experiments in which the expression of WTcol-tkCAT, mPEAcol-tkCAT and mAPcol-tkCAT was analyzed. Experimental procedures were as described above. In cells transfected with WTcol-tkCAT, CAT expression in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibody (anti-FNR), or treated with TPA was significantly different ($P < 0.01$, 0.005, 0.005 respectively, Student's t-test) from that seen in cells plated on fibronectin; the mean ± SD are shown. CAT activity in extracts prepared from cells transfected with pBLCAT2 ranged from 200-500 counts per minute in the individual experiments.

Figure 3A

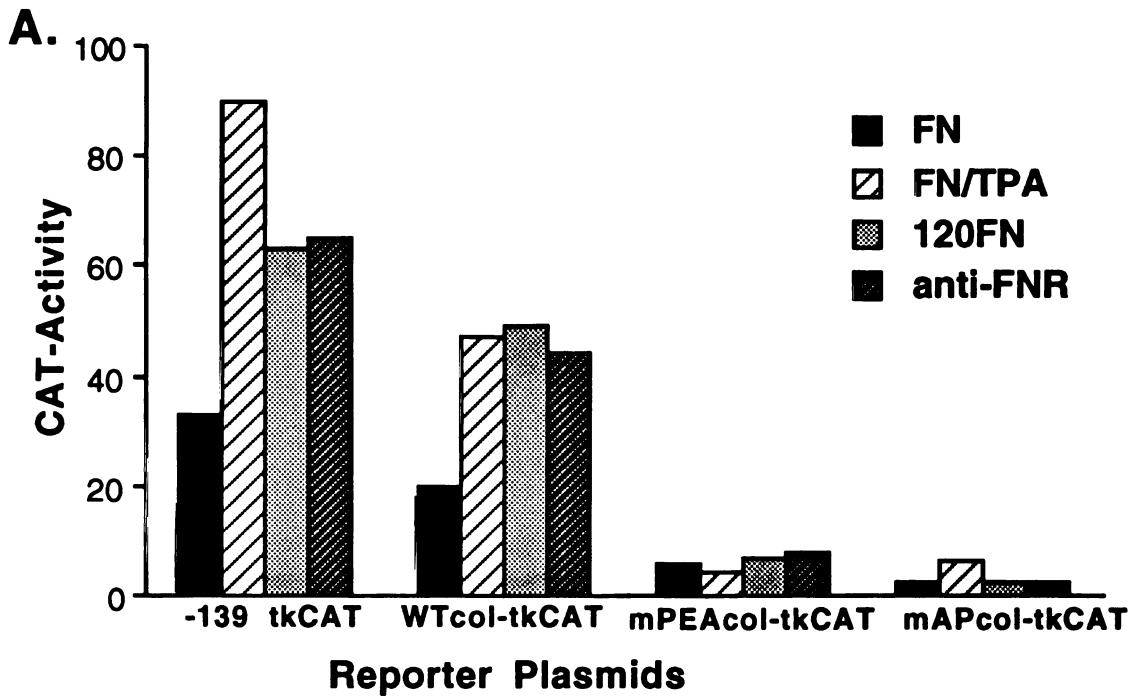
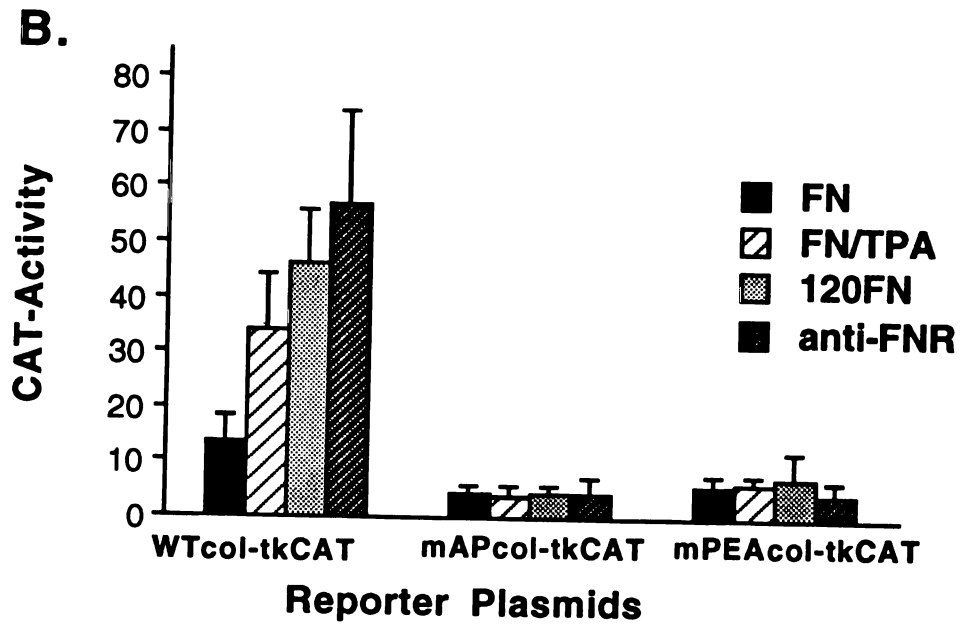

A.
100
80
60
40
20
0
CAT-Activity
FN
FN/TPA
120FN
anti-FNR
-139 tkCAT
WTcol-tkCAT
mPEAcol-tkCAT
mAPcol-tkCAT
Reporter Plasmids

Figure 3B


B.
CAT-Activity
80
70
60
50
40
30
20
10
0
FN
FN/TPA
120FN
anti-FNR
WTcol-tkCAT
mAPcol-tkCAT
mPEAcol-tkCAT
Reporter Plasmids

1990; Auble and Brinckerhoff, 1992). As an initial analysis, we compared the expression of a promoter-reporter construct containing only the PEA3 and AP1 sites, from the human collagenase promoter, WTcol-tkCAT, with -139 tkCAT in cells that were plated on fibronectin, 120FN and anti-$\alpha_5\beta_1$ antibody. We found that the expression of WTcol-tkCAT was upregulated in cells plated on 120FN and anti-$\alpha_5\beta_1$ antibody as well as in RSF that were treated with TPA (Fig. 3). (Fig. 3). While the overall amount of CAT activity in cell extracts from cells transfected with WTcol-tkCAT was lower than that in cell extracts prepared from RSF transfected with -139tkCAT, the increase in expression of these constructs in cells plated on inductive ligands compared to the expression seen in cells plated on fibronectin was similar. Thus, as has been seen in studies describing the regulation of collagenase in response to phorbol esters, growth factors and oncogenes, the AP1 and PEA3 sites in the context of the collagenase promoter are important for the upregulation of collagenase in response to signaling cascades initiated by integrins (Angel et al., 1987; Brenner et al., 1989; Gutman and Wasylyk, 1990; Kim et al., 1990; Lafayatis et al., 1990; Auble and Brinckerhoff, 1992) The expression of reporter constructs that contain only AP1 sites increased in cells plated on anti-$\alpha_5\beta_1$ antibodies or fibronectin fragments. We next compared the regulation of a CAT construct containing three tandem repeats of the AP1 site from the proliferin gene in a tkCAT reporter construct $(AP1)_3$ with WTcol-tkCAT and -139 tkCAT in RSF plated on different substrates. We found that $(AP1)_3$, WTcol-tkCAT and -139tkCAT were regulated similarly in cells plated on 120FN, anti-$\alpha_5\beta_1$ antibody or treated with TPA.

To address cooperation between AP1 and PEA3 sites in the collagenase promoter, we compared the regulation of a minimal promoter, WTcol-tkCAT with derivatives that contain substitutions in either the AP1 site (mAPcol-tkCAT) or PEA3 site (mPEAcol-tkCAT). The expression of mAPcol-tkCAT and mPEAcol-tkCAT was near background and the expression of these constructs was similar in RSF treated with TPA or plated on the various ECM substrates

(Fig. 3). In contrast, the expression of WTcol-tkCAT was upregulated in cultures treated with TPA or plated on agonistic ECM ligands. These data suggest that both AP1 and PEA3 sites are required for integrin/ECM regulation of the collagenase gene.

The accumulation of nuclear c-Fos precedes the induction of collagenase in cells plated on 120FN

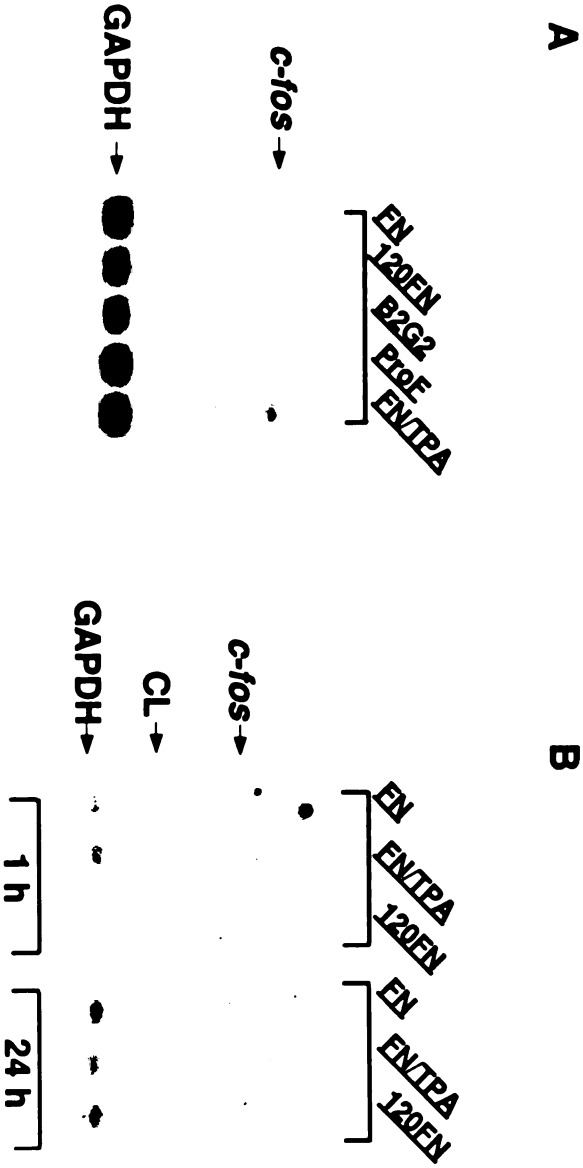
The results from the previous experiments, which show that the expression of collagenase-CAT constructs or CAT constructs containing minimal promoters with functional AP-1 sites is upregulated in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibody suggest that AP1 is necessary for the inducible transcription in response to integrin-derived signals. Proteins of the Jun family regulate transcription from AP1 sites, binding to AP1 sites with high affinity as heterodimeric complexes of Fos and Jun family members, or with lower affinity as homodimers of Jun family members (reviewed by Angel and Karin, 1992). In response to many stimuli, increases in the expression of collagenase often are preceded by a transient increase in the expression of c-*fos* and c-*jun*, implying that the increase in transcription of collagenase is mediated by heterodimeric complexes of Fos and Jun (reviewed by Angel and Karin, 1992). *In vitro*, Fos/Jun heterodimers and Jun homodimers transactivate constructs that contain a TPA-responsive element (TRE) (Lee et al., 1987). It is of note the the increase in the expression of collagenase in response to TNF-α results from an increase in the expression of only c-*jun* (Brenner et al., 1989).

Because the AP1 site in the collagenase promoter is essential for the upregulation of collagenase in response to integrin-derived signals, we next measured the accumulation of c*fos* mRNA and protein in cells that were plated on inductive and non-inductive ligands for the fibronectin receptor. We analyzed the expression of cFos protein by immunoblotting or immunofluorescent techniques. Analysis of c*fos* mRNA was by northern hybridization.

We first analyzed the mRNA coding for c*fos* and glyceraldehyde-3-phosphate dehydrogenase prepared from cells that were plated for 1 h on fibronectin, 120FN, anti-$\alpha_5\beta_1$

Figure 4. mRNA coding for c-*fos* increases in RSF plated on fibronectin fragments, anti-$\alpha_5\beta_1$ antibodies, or treated with TPA. (A). RSF were plated on wells coated with fibronectin (FN) 120FN, anti-$\alpha_5\beta_1$ antibody (BIIG2), or a poly-RGD compound (ProF), and cultured in DME-LH. In fibroblasts, TPA upregulates the expression of c-*fos* and metalloproteinases; as a positive control, one sample was treated with 100 ng/ml TPA (FN/TPA). After incubation for 1 h, the experiment was terminated and polyA$^+$ RNA was isolated from cell lysates. 5 μg aliquots of polyA$^+$ RNA were separated on agarose gels, the RNA was transferred to nylon membranes and probed with ^{32}P labeled cDNA inserts coding for c-*fos* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B). The increase in c-*fos* mRNA in RSF plated on 120FN or treated with TPA correlates with the expression of collagenase. Parallel cultures of RSF were plated in DME-LH on wells coated with fibronectin (FN), 120FN or plated on FN and treated with 100 ng/ml TPA (FN/TPA). Cultures were terminated after 1 h or 24 h of incubation and total RNA was prepared. 20 μg aliquots of RNA were separated by electrophoresis and RNA was transferred to nylon membranes which were hybridized with ^{32}P labeled cDNA inserts coding for c-*fos*, collagenase (CL), and GAPDH.

Figure 4


A
c-fos
GAPDH
EN
120EN
B2G2
ProF
EN/TPA
B
c-fos
CL
GAPDH
1 h
24 h
EN
EN/TPA
120EN
EN
EN/TPA
120EN

antibody or a poly-RGD compound. As a positive control we incubated fibroblasts in DME-LH supplemented with 100 ng/ml TPA (Fig. 4). We detected a modest increase in c*fos* mRNA in cells plated on 120FN or treated with TPA when compared to cultures that were plated in DME-LH on FN coated wells. The increase in the expression of mRNA coding for c-*fos* correlated with the upregulation of collagenase expression is shown in Fig. 4B. Total RNA was prepared from RSF that were cultured for 1h or 24h on wells coated with fibronectin or 120FN in the presence or absence of TPA. Hybridization analysis using [^{32}P]labeled probes for c-*fos*, collagenase and GAPDH again showed that c-*fos* mRNA increases in cells plated on 120FN or treated with TPA for 1h when compared to control cultures plated on FN. Analysis of the expression of collagenase in the parallel cultures that were incubated for 24 h showed an increase in mRNA encoding collagenase in RSF that were plated on 120FN or treated with TPA.

We used immunocytochemistry and an antibody generated against a Fos peptide to detect immunoreactive Fos protein in RSF that were treated with TPA or plated on different ECM substrates and in RSF that were treated with TPA. RSF were plated in DME-LH on coverslips coated with fibronectin or 120FN and cultured for 1 h or 24 h, after which the cultures were fixed and stained with an anti-peptide Fos antibody (1h) or anti-collagenase antibody (24h). To characterize the anti-Fos antibody specificity, one set of TPA-treated cultures was incubated with a mixture of the anti-peptide antibody and peptide immunogen.

As can be seen in Fig. 5, adherent, quiescent cells incubated in DME-LH had very low amounts of nuclear c-Fos, while staining of nuclear c-Fos was upregulated in RSF treated with TPA for 60 min. Cultures stained with anti-Fos antibody preparations that had been preincubated with immunogen showed no detectable nuclear staining for c-Fos (Fig 5.). We saw a striking increase in staining for nuclear c-Fos in RSF plated on 120FN for 1 h compared to control cultures of RSF that were plated on FN. The upregulation and nuclear accumulation of c-Fos compared well with the expression of collagenase in cultures plated on 120FN for 24 h. The

Figure 5. Nuclear Fos is higher in cells plated on 120FN than in cells plated on fibronectin. (A). We used a polyclonal antibody generated against a Fos peptide sequence (CRB Biologics) to analyze the immunoreactive cFos in RSF plated on fibronectin or 120FN. RSF were plated in DME-LH on coverslips that were coated with fibronectin (A,B,C), 120FN (D,E,F) and incubated for 1 h (A,B,D,E), or 24 h (C,F). The cultures were terminated by addition of paraformaldehyde and processed for immunocytochemistry to analyze the expression of Fos (A,B,D,E) or cell-associated collagenase (C,F). Phorbol esters rapidly upregulate the expression of the metalloproteinases as well as the nuclear proto-oncogenes c-*fos* and c-*jun* in fibroblasts. Therefore as a positive control to show that we could detect the presence of Fos protein by immunocytochemistry, RSF that had been plated in medium containing serum on glass coverslips and allowed to quiesce for 5 d were incubated in DME-LH supplemented with 100 ng/ml TPA for either 1 h, then fixed and processed for detection of Fos protein (G,H,I,J). In one set of TPA-treated cultures, the anti-Fos peptide antibody was preincubated with an excess of the peptide immunogen prior to addition to the fixed cultures (I,J). (K). We also compared the level of nuclear Fos protein that accumulates in the nuclei of cells freshly plated on FN or 120FN, or in RSF plated on FN and stimulated with TPA. Nuclear extracts were prepared using the method of Bos et al. (1987) from RSF (1×10^6) that were incubated for 1 h in DME-LH on 100 mM dishes coated with FN or 120FN. Extracts were also prepared from RSF plated on FN cells that were incubated for 1 h with 100 ng/ml TPA. Equal amounts of nuclear protein were separated on a 10% SDS-polyacrylamide gel under reducing conditions. The proteins were transferred to Immobilon membranes and the membranes were probed with an anti-Fos peptide monoclonal antibody (against a different domain of the Fos protein than the antibody used in the immunofluorescent studies) (Ab-1, Oncogene Science). The membrane was then incubated with an anti-mouse IgG antibody conjugatd with horseradish-peroxidase. Immunoreactive Fos was detected using the enhanced chemiluminescence reagent. The membrane was stripped and

Figure 5

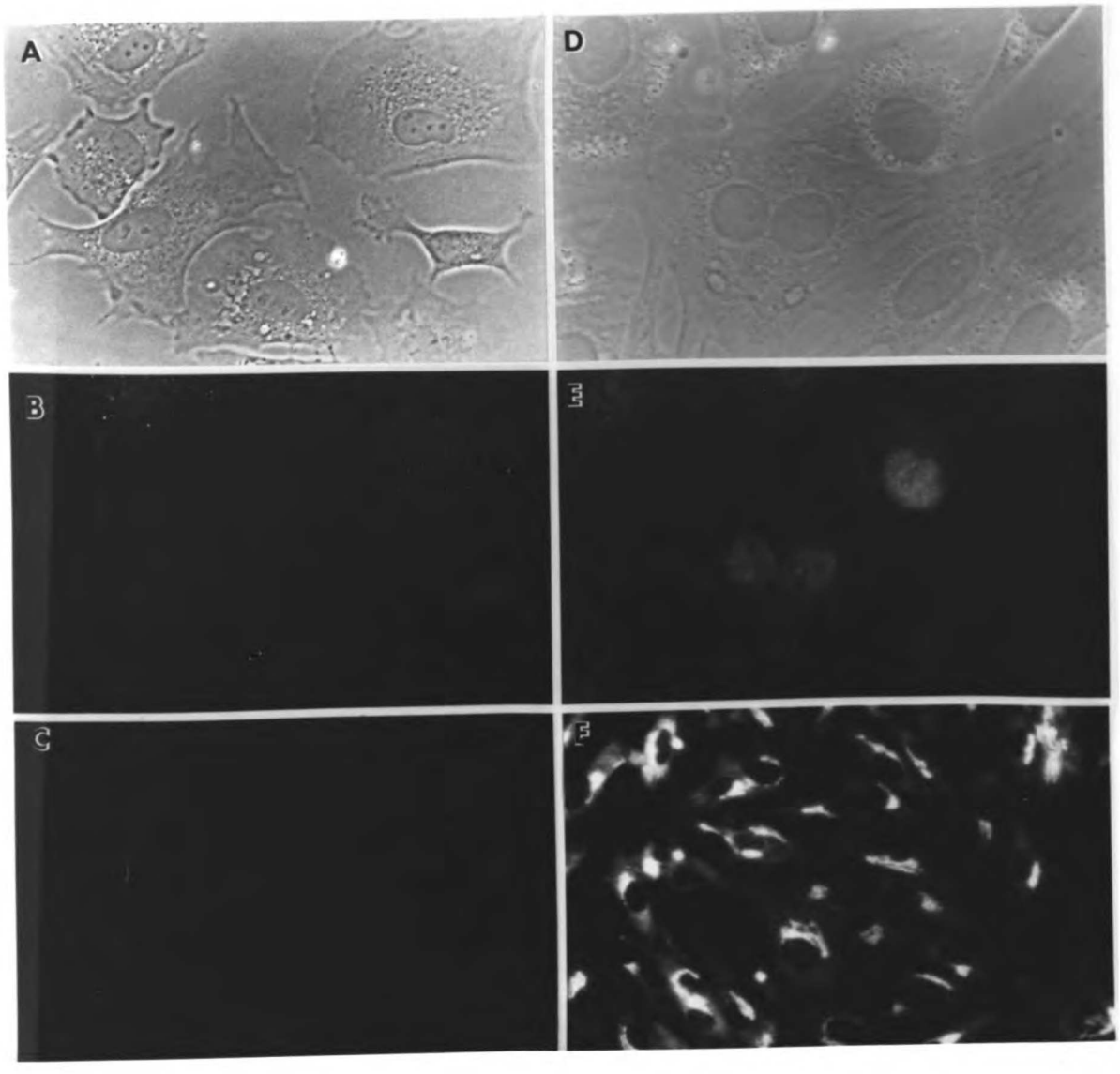


Figure 5

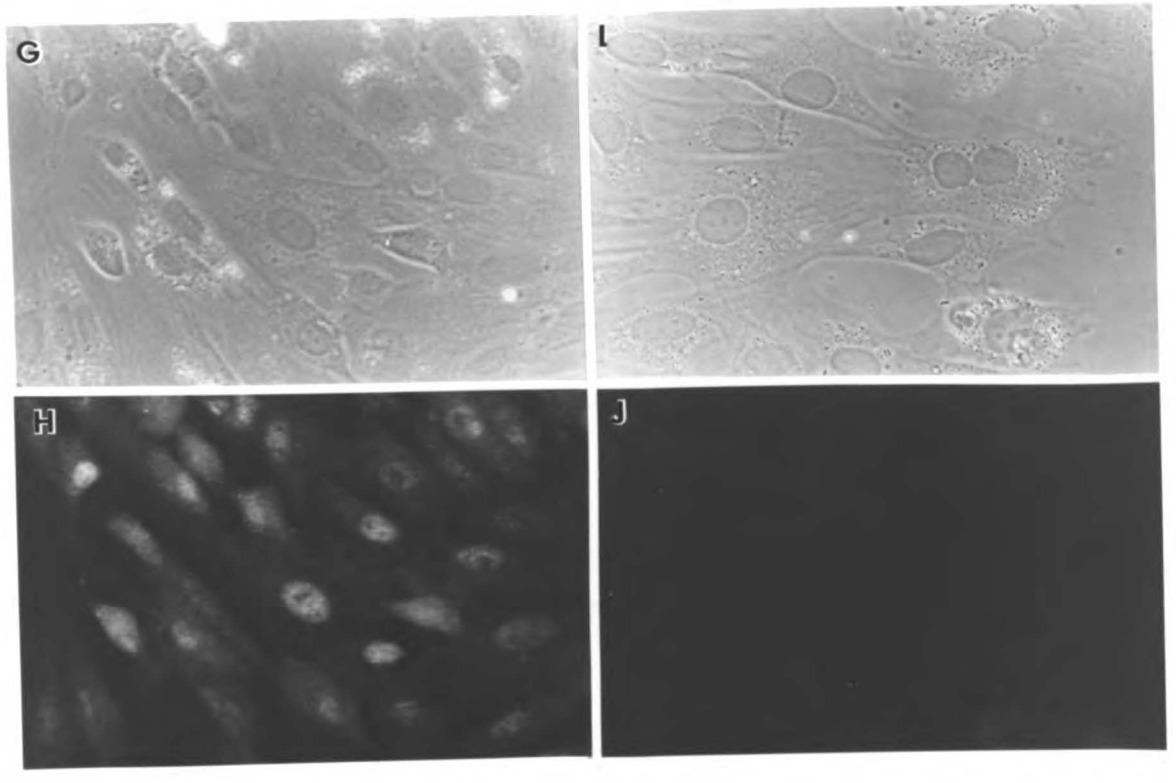
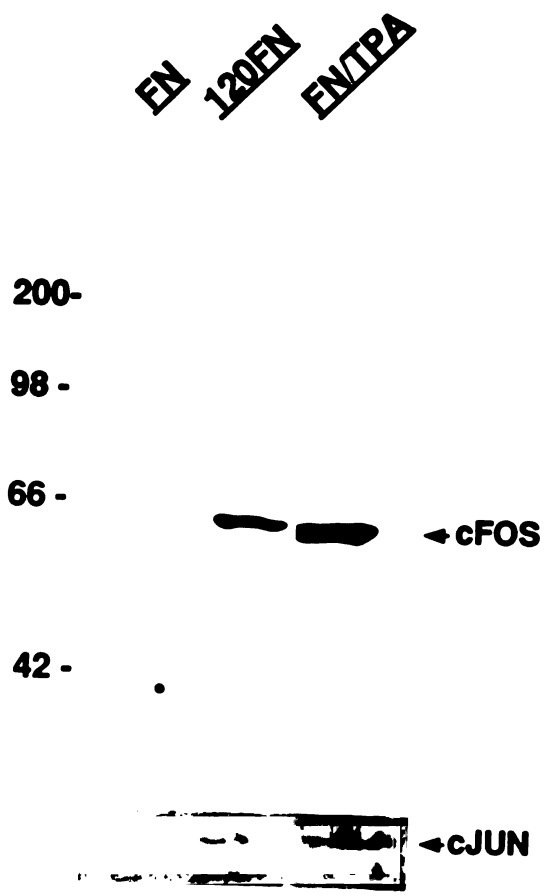


Figure 5



results from two experiments were compared by scoring cells having immunoreactive Fos or collagenase (5 randomly selected fields, 25-50 cells/field per treatment were counted). In cells plated on FN 16% stained for nuclear Fos at a low level, whereas immunoreactive Fos staining was readily detected in RSF plated on 120FN (80%) or treated with TPA (89%). The proportion of cells staining for Fos closely paralleled the percentage of cells with immunoreactive, cell-associated collagenase: 80% of RSF treated with TPA and 76% of RSF plated on 120FN were collagenase-positive, while only 10% of the cells plated on FN had immunoreactive collagenase.

We also biochemically analyzed nuclear Fos protein that accumulated in the nuclei of cells freshly plated on FN or 120FN, or in quiescent cells stimulated with TPA. Nuclear extracts were prepared from RSF (1×10^6) that were either treated for 1 h in DME-LH supplemented with 100 ng/ml TPA or were cultured for 1 h in DME-LH on 100 mm dishes coated with FN or 120FN using the method of Bos et al., 1987. Fos and Jun proteins in these nuclear extracts were analyzed by Western blotting using an anti-Fos monoclonal antibody (against a different domain of the fos protein than the antibody used in the immunofluorescent studies) and an anti-peptide Jun antibody as probes. Nuclear Fos was substantially higher in nuclei harvested from cells plated on FN fragments or from cells treated with TPA than in nuclei harvested from quiescent cultures or from cells plated on FN (Fig. 5). When we reprobed the blot with an antibody against cJun we also saw an increase in the level of cJun protein in nuclei harvested from cells plated on 120FN or treated with TPA compared to control cultures (quiescent RSF or RSF that were plated on FN).

Addition of antisense cfos *oligonucleotides to cells blocks the upregulation of collagenase gene expression in RSF plated on 120FN*

The previous experiments suggest that heterodimeric complexes containing both Fos and Jun nuclear proteins mediate at least part of the increased transcription through the AP-1 site in the promoter. If the upregulation of collagenase expression was dependent on the increased expression of c*fos*, preventing this increase in c*fos* expression should therefore diminish the

Figure 6 Antisense fos oligonucleotides compromise the induction of collagenase in cells treaed with TPA or plated on ECM ligands. *A)* Oligonucleotides that were complementary (antisense fos oligonucleotides, or SOF-OLIGO) or identical (sense-fos or FOS-OLIGO) to c-*fos* mRNA sequences were introduced into RSF by electroporation along with -139 tkCAT. The electroporated cells were plated in DME-LH on wells coated with fibronectin (FN) or 120FN, and cultures were incubated for 70 h. As a positive control, one set of cultures was incubated in DME-LH containing 100 ng/ml TPA. RSF were cultured in DME-LH supplemented with the indicated oligonucleotide at a concentration of 25 μM; oligonucleotides were replenished every 12 h. Cell extracts were prepared and CAT expression was measured by comparing enzyme activity from equal amounts of cell extracts. Acetylated chloramphenicol (arrows) was separated from chloramphenicol (arrowhead) by thin layer chromatography, and the chromatograms were analyzed by autoradiography. The construct -66 tkCAT which is just 3′ to the AP1 site was not upregulated by TPA or in cells plated on 120FN. *B)* The data shown in panel A were quantified by excising and counting the acetylated chloramphenicol and the unmodified chloramphenicol. The data are presented as pmoles acetylated CAT/mg cell extract. The data average the results of two experiments (means ± SD).

Figure 6A

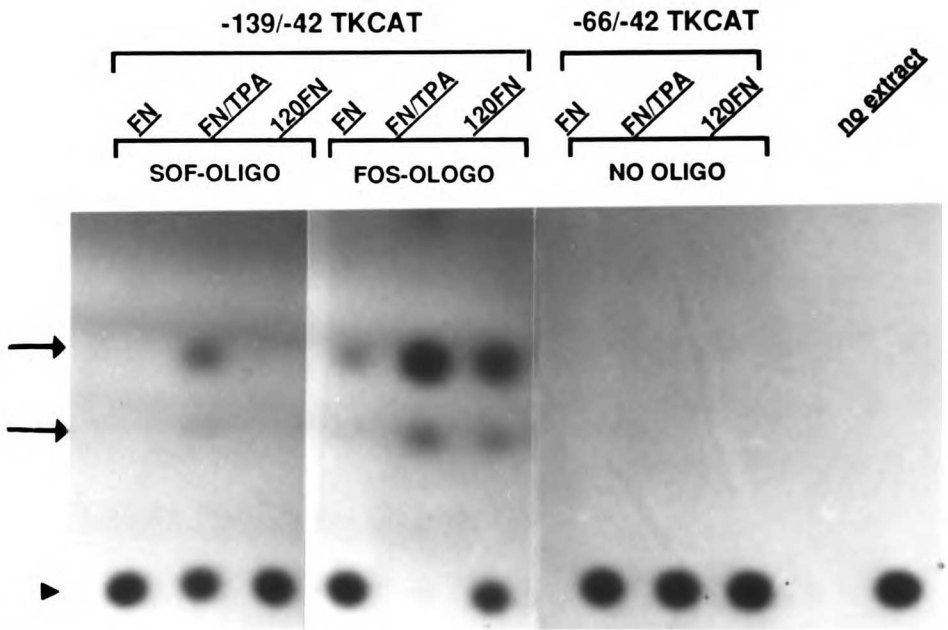
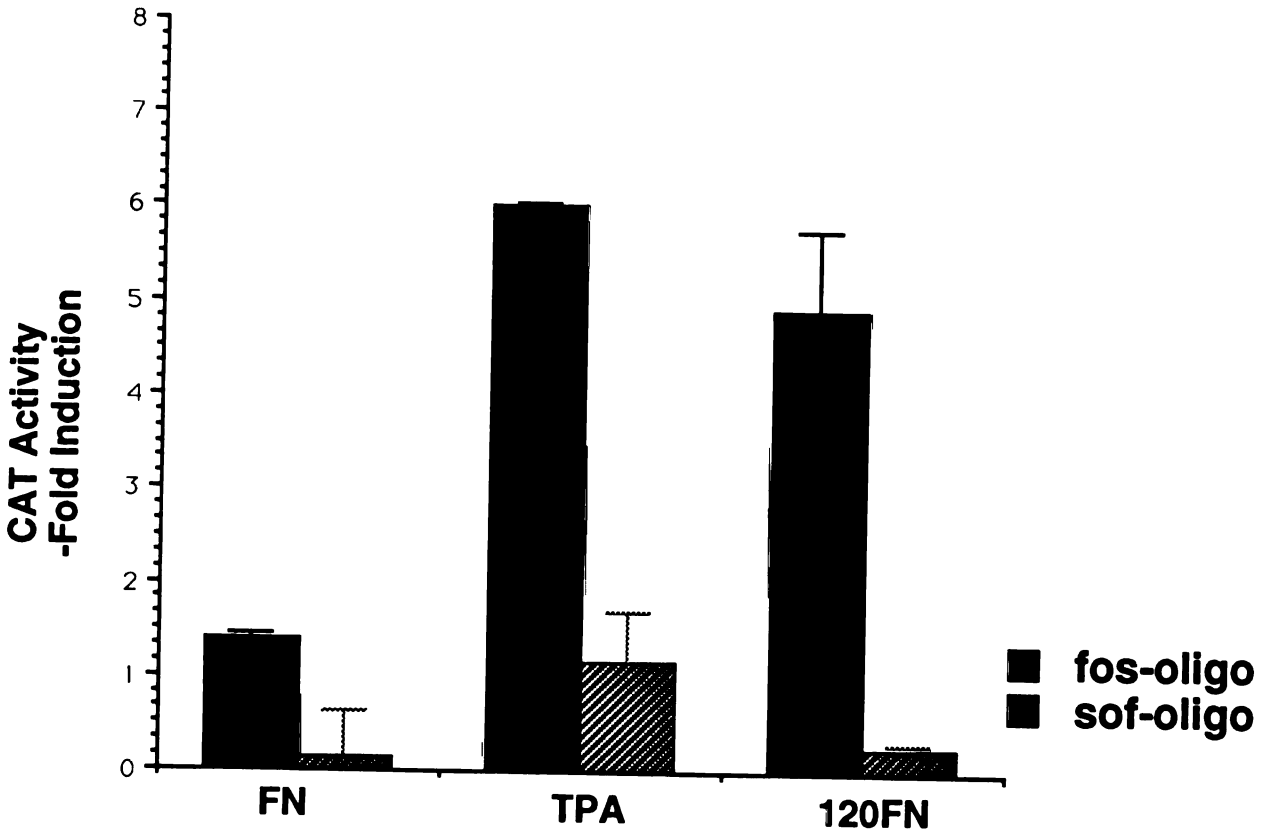

-139/-42 TKCAT
-66/-42 TKCAT
FN
FN/TPA
120FN
FN
FN/TPA
120FN
FN
FN/TPA
120FN
no extract
SOF-OLIGO
FOS-OLOGO
NO OLIGO

Figure 6B


CAT Activity
-Fold Induction
8
7
6
5
4
3
2
1
0
FN
TPA
120FN
fos-oligo
sof-oligo

expression of collagenase. This approach has been used successfully to analyze the role of c*fos* in regulating the synthesis of stromelysin by EGF (Kerr et al., 1988; McDonnell et al., 1990) or IL-1 (Quinones et al., 1988; Buttice and Kurkinen, 1991, 1993). In an attempt to perturb the expression of collagenase by altering the upregulation of cFos, we introduced synthetic DNA oligonucleotides that were complimentary to c*fos* mRNA sequences (antisense-fos oligonucleotides or SOF-OLIGO) or identical to the c*fos* mRNA (sense-fos oligonucleotides or FOS-OLIGO) along with collagenase-CAT constructs (-139/-42 tkCAT) into RSF by electroporation. As a positive control, RSF were also cultured on FN-coated wells in DME-LH supplemented with 100 ng/ml TPA. In these experiments, we analyzed the expression of both the endogenous collagenase gene and the regulation of -139/-42 tkCAT. The regulation of the endogenous collagenase gene was measured either as the accumulation of radiolabeled collagenase secreted by RSF incubated with [^{35}S] methionine, or by assay of the proteinase activity in the conditioned medium on zymograms that contained gelatin or casein as a substrate.

We first compared the expression of CAT from the collagenase promoter by analysis of CAT enzyme activity in equal amounts of cell extracts. In cells transfected with a mixture of -139/-42 tkCAT and FOS-OLIGO, the expression of CAT was upregulated in cells plated on 120FN and in cells treated with TPA, while CAT expression in cells transfected with the -66/-42 tkCAT construct was not detectable (Fig 6A). Compared to cultures treated with FOS-oligonucleotides, inclusion of SOF-OLIGO severely compromised both the basal expression of -139tkCAT in cells plated on fibronectin and the induced expression of -139/-42 tkCAT in cells plated on 120FN or treated with TPA (Fig. 6A,B). Overall, expression of CAT was lower in RSF incubated with oligonucleotides. CAT activity in cells treated with sense FOS-OLIGO that were plated on FN or 120FN was 70-80% that of cultures that were not supplemented with oligonucleotides, and the addition of control FOS-OLIGO oligonucleotides did not affect the upregulation of -139tkCAT in cells treated with TPA or plated on 120FN. In comparison, both

the basal and induced transcription of -139/-42 tkCAT was reduced in cells treated with SOF- OLIGO (less than 6% of that seen in RSF plated on FN that were transfected with -139/-42 tkCAT).

We then analyzed the expression of the endogenous collagenase gene in cells that were incubated with FOS- or SOF- OLIGOS, measuring either the radiolabeled proteins secreted by RSF or the proteinases in the conditioned medium using zymography. The radiolabeled proteins harvested from RSF plated on matrix coated wells in the presence or absence of oligonucleotides were concentrated and analyzed by SDS-PAGE, fluorography and autoradiography. The pattern of proteins synthesized by RSF incubated with FOS-OLIGO was the same as that of RSF cultured without oligonucleotides; in both cases the expression of collagenase was upregulated in RSF plated on 120FN or treated with TPA, while the synthesis of other proteins was unaffected (Fig. 7). In cultures incubated with the SOF-OLIGO, the amount of collagenase synthesized by RSF plated on 120FN or treated with TPA was lower than that seen in control cultures (untreated or treated with FOS-OLIGO), while the synthesis of other proteins was not affected.

Analysis of the proteinases in the CM from control cultures, or cultures treated with FOS- or SOF-OLIGO using zymography on a gelatin substrate gel showed again, that basal and induced expression of collagenase is lower in cultures treated with the SOF-OLIGO, compared to the activity seen in cultures that were treated with the FOS-OLIGO. We have previously noted that the expression of both stromelysin and the 92 kD gelatinase is upregulated in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibody compared to cells plated on FN (Werb et al., 1989). AP1 sites in the promoters of stromelysin and uPA are crucial for the upregulation of these proteinases in response to phorbol esters and growth factors (Buttice and Kurkinen, 1993; Nerlov et al., 1990), and the promoter region of the 92 kD gelatinase contains AP1 sequences (Huhtala et al., 1991). When we analyzed stromelysin, the 92 kD gelatinase and uPA in the CM harvested from these experiments, we found that introduction of SOF-OLIGO, but not FOS-OLIGO, also diminished

Figure 7 The regulation of endogenous collagenase, stromelysin, the 92 kD gelatinase and urokinase by TPA or in cells plated on120FN is also diminished by incubation with SOF-OLIGOs. Aliquots of cells transfected with -139 tkCAT and FOS or SOF oligonucleotides were plated in DME-LH on a 48 well plate that was coated with fibronectin (FN) or 120FN. As indicated, some RSF were incubated in the presence of 100 ng/ml TPA and/or oligonucleotides (25 μM). RSF were cultured for 70 h after which the culture supernatants were removed and saved for analysis by zymography. *A)* The cultures were biosynthetically labeled by incubation with [^{35}S] methionine. The radiolabeled proteins in the medium were concentrated and analyzed by SDS-PAGE and autoradiography. The migration of collagenase (CL) is indicated at the left, and the migration of the molecular weight standards is indicated at the right. *(B,C)* The proteases in the conditioned medium harvested prior to radiolabeling the cultures were analyzed by zymography, incorporating gelatin (panel B), casein or casein + plasminogen (panel C) into the resolving gel. Proteinases appear as a clear band on a dark background. *B)* In the presence of sense fos oligonucleotides (FOS-OLIGO), the expression of gelatinases migrating at 55 kD (CL) and 92 kD are upregulated in cells treated with TPA or plated on 120FN (FOS-OLIGO). The expression of these gelatinases is diminished in cells incubated with the anti-sense fos oligonucleotides (SOF-OLIGO). The migration of the gelatinases migrating at 55 kD (CL), and 92 kD (92) are indicated at the right; the migration of the molecular weight standards is indicated at the right. *C)* In the presence of sense fos oligonucleotides (FOS-OLIGO), the expression of caseinases migrating at 92 kD (92) and 53 kD (SL) was upregulated in cultures treated with TPA or cultured on 120FN; these caseinases were expressed at lower levels in cultures treated with antisense fos oligonucleotides (SOF-OLIGO). In a parallel gel, casein + plasminogen was incorporated into the resolving gel. The expression of a plasminogen activator migrating at 40 kD (uPA) is upregulated in culture supernatants harvested from cells treated with TPA or plated on 120FN and incubated in the presence of sense fos oligonucleotides (FOS-OLIGO), while this

activity is not detected in culture supernatants harvested from RSF incubated with antisense fos oligonucleotides (SOF-OLIGO). The migration of the caseinolytic stromelysin migrating at 53 kD (SL) and the 92 kD gelatinase (92) and of the urokinase-type plasminogen activator (uPA) are indicated at the right; the molecular weight standards are indicated at the left. The same samples were applied to the casein and casein + plasminogen zymograms, but to allow resolution of the plasminogen activator, the zymogram containing casein + plasminogen was developed for a shorter time than the zymogram containing only casein.

Figure 7A

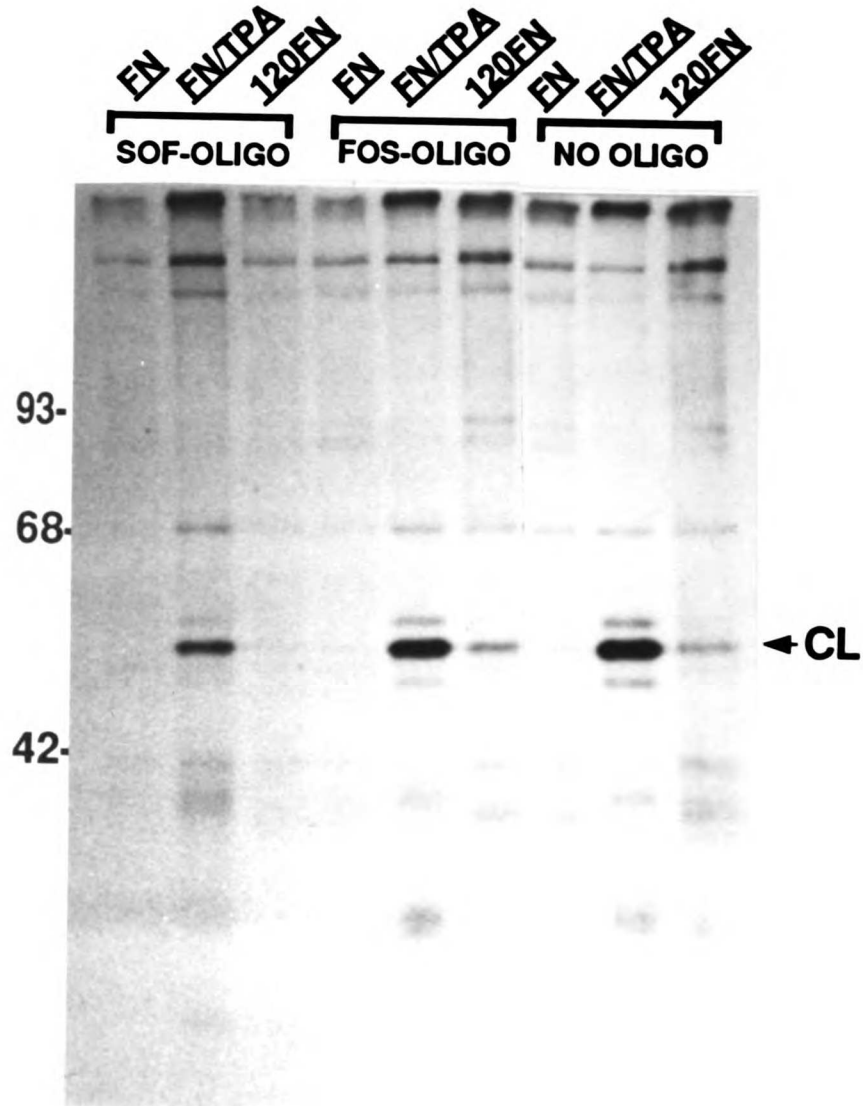

EN
EN/TPA
120EN
EN
EN/TPA
120EN
EN
EN/TPA
120EN
SOF-OLIGO
FOS-OLIGO
NO OLIGO
93-
68-
42-
CL

Figure 7B

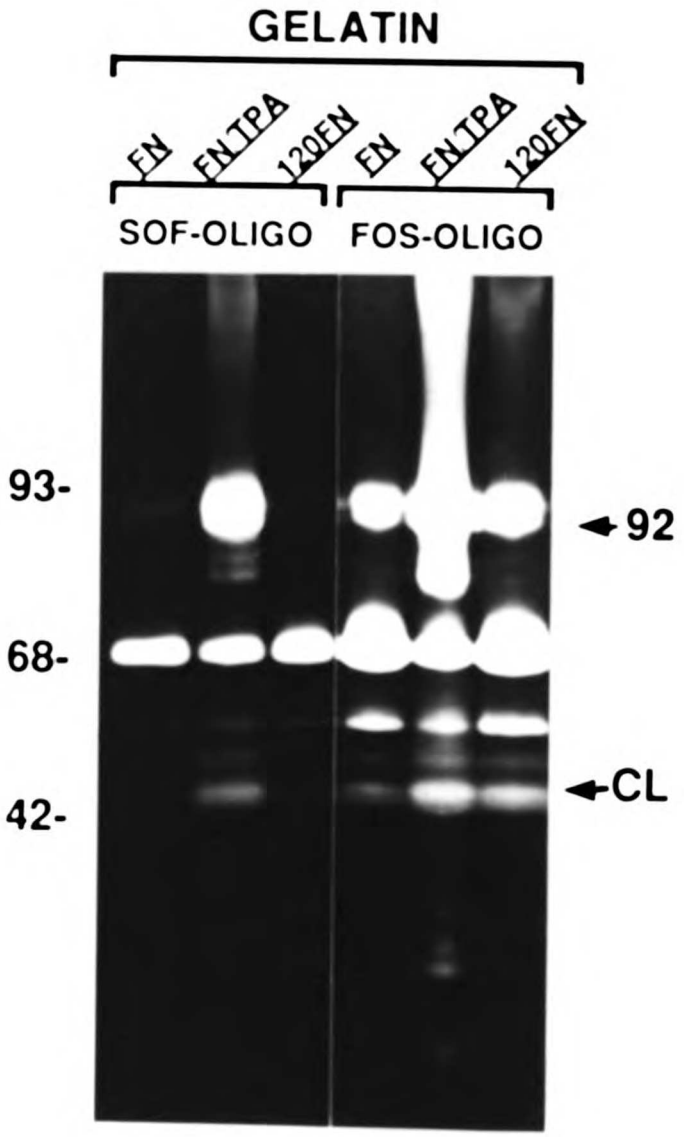
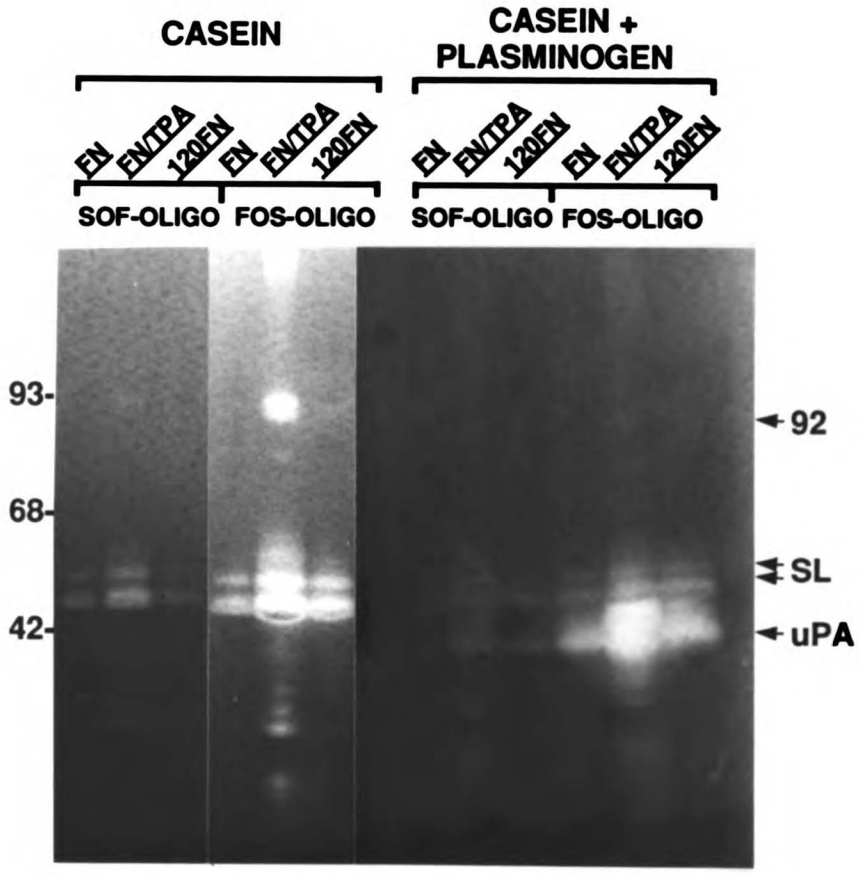

GELATIN
FN
FN/TPA
120FN
FN
FN/TPA
120FN
SOF-OLIGO
FOS-OLIGO
93-
68-
42-
92
CL

Figure 7C


CASEIN
CASEIN + PLASMINOGEN
EN
EN/TPA
120EN
EN
EN/TPA
120EN
EN
EN/TPA
120EN
EN
EN/TPA
120EN
SOF-OLIGO
FOS-OLIGO
SOF-OLIGO
FOS-OLIGO
93-
68-
42-
92
SL
uPA

the induction of these proteinases in cells plated on inductive ligands for the fibronectin receptor.

Discussion

This study describes a nuclear signalling pathway, triggered by the interaction of RSF with the 120 kD cell binding fragment of fibronectin or a function perturbing anti-$\alpha_5\beta_1$ antibody, which upregulates the expression of an ECM-degrading metalloproteinase, collagenase. More importantly, these experiments show that cells perceive and respond to changes in the ECM, and indicate a mechanism by which signalling pathways initiated by integrins converge with those responding to signals initiated by other cell surface receptors. We have characterized, at the molecular level, the increase in collagenase gene expression in RSF adhering to agonistic ligands for the fibronectin receptor (FNR). We show that sequences in the -139 to -42 segment of the human collagenase promoter regulate this increase in collagenase expression. This region of the promoter contains two defined inducible elements, an AP1 site (TRE, -72/-67) and a PEA3 site (-90/-82) (Angel et al., 1987; Gutman and Wasylyk, 1990) and is similar to sequences found in the promoter of rabbit collagenase that also respond to TPA (Auble and Brinckerhoff, 1992). The $\alpha_5\beta_1$ FNR is specifically implicated in this pathway, because the expression of endogenous collagenase and collagenase promoter constructs is increased in cells plated on anti-$\alpha_5\beta_1$ antibody. Our results show that the AP1 site from -72 to -67 is crucial for the upregulation of collagenase in RSF adhering to agonistic ligands for the fibronectin receptor. Likewise, the PEA3 site from -90 to -82 also plays a role in this induction. Several lines of evidence show that a functional AP1 site is needed in the collagenase promoter to upregulate the expression of collagenase by ligation of the FNR. Constructs containing promoter sequences 3′ to the AP1 site are not upregulated, nor are constructs containing an altered AP1 site. The activity of AP1 is regulated by the signaling pathway initiated at the FNR because the expression of reporter plasmids containing a minimal promoter and three AP1 sites in series also increases in RSF plated on fibronectin fragments or anti-$\alpha_5\beta_1$ antibody. We showed that an increase in the

expression of one component of AP1, c*fos*, and an increase in the nuclear localization of cFos correlate with the upregulation of collagenase in cells plated on 120FN. This correlation was strengthened by our experiments showing that incubation of cells with SOF-OLIGO, but not FOS-OLIGO specifically decreases the expression of collagenase. However, comparison of the regulation of reporter constructs that contain substitutions in the AP1 and PEA3 sites, WTcol-tkCAT , mAPcol-tkCAT and mPEAcol-tkCAT, indicate that the PEA3 site is also necessary for the regulation of collagenase in cells plated on agonistic ligands of the FNR.

Our results compare well with other studies analyzing the regulation of reporter constructs containing segments of the human collagenase promoter by tumor promoters (Angel et al., 1987; Kim et al., 1990) and growth factors (Brenner et al., 1989; Lafyatis et al., 1990). Curiously, the basal transcription and the fold-induction of -139/-42 tkCAT in cells plated on agonistic ligands of the FNR was higher than from -1200/-42 tkCAT construct. In contrast to the regulation of -139/-142 tkCAT by ECM ligands, TPA upregulated the expression of -1200/-42 tkCAT to a greater extent than -139/-42 tkCAT, suggesting that additional sequences upstream from -139 respond to TPA. Our observations that the overall level of transcription from the -139/-42 tkCAT construct was higher than that from the -1200/-42 tkCAT construct, but that the fold induction of -1200/42 tkCAT in response to TPA is higher, agree with the results of Angel et al. (1987), who analyzed the regulation of these constructs by TPA in HeLa tk^- cells and L tk^- cells.

Our results using the human collagenase promoter differ slightly from an analysis of the regulation of the promoter from the rabbit collagenase gene in synovial fibroblasts treated with TPA (Auble and Brinckerhoff, 1991). We agree that inclusion of sequences 70 to 100 bp 5′ to the AP1 site in the collagenase promoter increased the induction of the reporter construct by TPA, but in the study by Auble and Brinckerhoff, both the basal and the induced transcription from a -182 to +60 fragment of the rabbit promoter was lower than transcription from a longer (-1176 to +60) segment of the promoter. There are a number of possible reasons that may explain

the differences between the studies: in the -139/-42 tkCAT construct, the region of the of the human collagenase gene (-42-105) that contains the AP1, PEA3 and a nearby TTCA sequence that was identified in the rabbit promoter is identical to the sequence of the rabbit gene; however there is no homology in the sequence from -106/-139. The reporter constructs in our experiments are chimeric promoters that contain sequences from the human collagenase gene and the thymidine kinase promoter, again there may be differences between the regulation of chimeric and native promoters. It is of note that our results are similar to other studies that compared the regulation of these human collagenase promoter constructs by tumor promoters (Angel et al., 1987; Kim et al., 1990) and growth factors (Lafyatis et al., 1990) in both normal and transformed cell lines.

The induction of collagenase expression in response to growth factors, cytokines, transforming oncogenes or a change in cell shape reflects an increase in the level of gene transcription, because in most cell types, collagenase is not stored prior to secretion. The promoter of collagenase has been particularly well characterized (Angel et al., 1987; Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1992), and has been used to define inducible elements, notably the TPA responsive element (TRE or AP1 site) and the PEA3 or ETS site, that may play a role in the regulation of gene expression during cell growth differentiation or transformation (reviewed by Gutman and Wasylyk, 1991; Angel and Karin, 1992)

The TRE was first defined in the collagenase promoter (Angel et al., 1987), and has also been found in the promoters of many other cellular and viral genes (reviewed by Jones et al., 1988; Gutman and Wasylyk, 1990; Angel and Karin, 1992). The TRE is recognized by AP1, a dimeric transcription factor that is composed of nuclear proteins in the Jun and Fos families (reviewed by Angel and Karin, 1992). Homodimeric complexes of Jun family members activate transcription from AP1 sites, albeit with a lower efficiency than heterodimeric complexes of cFos and cJun. AP1 has been shown to transactivate promoter-reporter constructs that contain TRE

(Lee et al., 1987). With three known members of the Jun family and 4 characterized members of the Fos family, there are at least 18 variants of AP1, and the fact that there are AP1 sites in the promoters of Fos and Jun family members suggests that the regulation of transcription by AP1 is not simple.

Both an increased synthesis of Jun and Fos family members as well as post-translational modifications of these proteins play a role in regulating AP1-mediated transcription (reviewed by Morgan and Curran, 1991). Post-translational modification of Fos or Jun proteins initiated by extracellular events is one mechanism for regulation of AP1 activity. Critical amino acids on Fos or Jun proteins can be modified by phosphorylation or by a redox reaction to either enhance or diminish AP1 activity (Barber and Verma, 1987; Guis et al., 1990; Offir et al., 1990; Abate et al., 1990a,b; Boyle et al., 1991; Xanthoukakas and Curran, 1992). A dominant inhibitor of AP1, IP1, present in the cytoplasm has also been described (Auwerx and Sassone-Corsi, 1991). The results from our experiments using anti-$\alpha_5\beta_1$ antibody or fibronectin fragments as a substrate show that ligation of $\alpha_5\beta_1$ increases the AP1-dependent transcription of reporter constructs (-139/-42 tkCAT, AP_3-CAT) when compared to cells plated on fibronectin. This increase in transcription of reporter constructs that contain TREs correlates with an increase in the amount and nuclear localization of cFos in cells plated on 120FN. In our experiments, post-translational modification of AP1 activity probably plays a significant role in the regulation of collagenase, because while the increase in *cfos* message was modest, there was a striking upregulation of nuclear Fos protein.

Changes in the amount or activity of components of AP1 correlate with adhesion to specific matrices in other systems as well. In T-lymphocytes, the expression of *cfos* and the AP1-driven transcription of IL-1 is upregulated when CD3 and the fibronectin receptor are occupied with anti-receptor antibodies or ligand (Yamada et al., 1991). The expression of *cfos* is also upregulated in monocytes adhering to fibronectin (Shaw et al., 1990). While the exact signal

generated through the interaction of monocytes, T-lymphocytes and RSF with fibronectin is unclear, changes in the nature of a cells interaction with the substrate correlates with an increase in the expression of c*fos* or c*jun*. Destabilization of actin or tubulin supported cytoarchitecture has been shown to lead to an increase in c*fos* mRNA in some cell types (Zambetti et al., 1991), or an accumulation of c*jun* mRNA in others (Botteri et al., 1990).

PEA3 was first characterized as an activity in studies on the polyomavirus promoter (Martin et al., 1988; reviewed by Jones, 1988). It was noted that the activity characterized as PEA3 was also induced in response to serum, TPA and several oncogenes (Wasylyk et al, 1989). PEA3 sites have been identified in the enhancers or promoters of c*fos*, stromelysin, urokinase, collagenase, TIMP, polyoma virus late genes, BK papova virus late genes, the mouse DNA polymerase B gene, and the mouse ERA-1 gene (a gene induced rapidly after retinoic acid treatment of F9 embryonal carcinoma cells) (see Yoo et al., 1991 and references therein). In gel shift assays, members of the *ets* family, Ets1 and/or Ets2, have been shown to interact specifically with oligos representing the PEA3 sites in the promoters of the collagenase, stromelysin, urokinase, and TIMP genes (Gutman and Wasylyk, 1990; Nerlov et al., 1990;Wasylyk et al., 1991; Campbell et al., 1991; Buttice and Kurkinen, 1993). Recombinant Ets1 or Ets2 proteins transactivate reporter constructs containing the promoter from the polyomavirus early genes, c*fos*, collagenase, and stromelysin genes, suggesting that genes containing PEA3 sites are regulated by *ets* family members. The Ets superfamily (*ets, PEA3, erg, elk, PU.1, E74, GABP-α, Spi-1 and fli-1)* are grouped through homology of cDNA sequence in the DNA-binding domain of these proteins, while the other domains in the family members are unique (reviewed by Gutman and Wasylyk, 1990). The expression of several *ets* family members are developmentally regulated (reviewed in Gutman and Wasylyk, 1990). *c-ets1* mRNA is abundant in T and B lymphocytes, and is also present transiently in several mesodermal tissues during organogenesis in the chick embryo, notably endothelial cells undergoing vasculogenesis or angiogenesis

(Vandenbunder et al., 1989). In humans, *ets-1* is present in endothelial cells of developing vessels but is absent from fully developed vessels; however if angiogenesis is reinitiated, the expression of *ets* increases (Werner et al., 1992).

Collagenase, stromelysin, urokinase, and TIMP are proteinases or proteinase inhibitors that are sometimes co-regulated at critical times during development and tissue remodeling. *In vitro* these genes are upregulated in response to growth factors, cytokines and oncogenes. The AP1 and PEA3 sites in the promoters of collagenase , stromelysin, urokinase and TIMP play a role in this induction. These inducible elements have been studied using transient expression of promoter-reporter constructs (Angel et al., 1987; Gutman and Wasylyk, 1990; Wasylyk et al., 1991; Buttice and Kurkinen, 1991, 1993; Campbell et al., 1991; Auble and Brinckerhoff, 1992). Collagenase, stromelysin and TIMP all contain an AP1 and PEA3 site within 100 bp of the start of transcription. Collagenase can be considered the simplest of these promoters, with the AP1 and PEA3 sites playing the primary role in the regulation of collagenase by growth factors and cytokines, additional upstream sequences in the promoters of stromelysin and TIMP also respond to growth factors, cytokines and TPA play a significant role in the regulation of stromelysin and TIMP. In the collagenase promoter, disruption or deletion of the AP1 site in the endogenous promoter, or in a synthetic promoter containing 4 tandem copies of the PEA3/AP1 motif from the collagenase promoter, ablates the basal and induced expression of collagenase (Angel et al., 1987; Gutman and Wasylyk, 1990; Auble and Brinckerhoff, 1992) ; disruption of the PEA3 site in a synthetic construct containing 4 tandem copies of the collagenase PEA3/AP1 motif compromises, but does not ablate transcription of the synthetic construct (Gutman and Wasylyk, 1990). This suggests that the requirement for AP1 is obligate for the expression of collagenase, and proteins interacting at the PEA3 site augment the expression of collagenase induced by TPA. Sequences flanking the PEA3 sites in the rabbit promoter are required for full induction of collagenase by TPA (Auble and Brinckerhoff, 1992). Our results, which show that a functional

AP1 site is necessary for the basal and induced transcription of a reporter construct containing the PEA3 and AP1 sequences from the collagenase promoter and that substitutions in the PEA3 site substantially decrease the expression of these constructs, are in agreement with the previous studies which show that both the PEA3 and AP1 sites in the collagenase promoter are required for expression of collagenase.(Gutman and Wasylyk, 1991; Auble and Brinckerhoff, 1992)

Stromelysin, like collagenase, contains an AP1 and PEA3 site within 60 bp of the start of transcription, as well as 2 PEA3 sites about 200 bp upstream from the start site (Wasylyk et al., 1991; Sirum-Connoly and Brinckerhoff, 1991; Buttice and Kurkinen, 1992, 1993). The AP1 site is required for basal and induced transcription of stromelysin; the expression from reporter constructs in which the AP1 site is deleted or altered is below basal levels (Butticce and Kurkinen, 1992, 1993; Sirum-Connoly and Brinckerhoff, 1991). Reporter constructs containing only a functioning AP1 from stromelysin promoter are upregulated by IL-1, TPA and H-*ras*, although not to the same extent as constructs containing the longer segment of the promoter that includes the additional PEA3 sites (Wasylyk et al., 1991; Sirum-Connoly and Brinckerhoff, 1991). In human diploid fibroblasts, inclusion of the PEA3 site in constructs containing the promoter-proximal sequences augments the regulation of this short promoter fragment by TPA and IL-1 (Sirum-Connoly and Brinckerhoff, 1991). Reporter constructs containing another segment of the stromelysin promoter are also regulated by TPA. Constructs containing the two PEA3 sites (-209 to -191) respond independently to TPA, and are required for the full expression of stromelysin by TPA or oncogenes (Wasylyk et al., 1991; Butticce and Kurkinen, 1993). There is some suggestion that sequences between the upstream PEA3 element and the PEA3/AP1 site near the transcription start site also plays a role in the regulation of stromelysin by TPA or IL-1 (Sirum-Connoly and Brinckerhoff, 1991).

In the human urokinase gene the enhancer is located 1.7 kb upstream of the transcription start site. There is a single AP1 site 45 bp 3′ to a combined PEA3/AP1 motif. Substitutions in

the AP1 site or either site in the combined PEA3/AP1 motif significantly compromises induction of urokinase. In some cell types, intervening sequences between the AP1 and PEA3/AP1 elements, recognized by as of yet unidentified cellular factors, mediate a cooperative interaction between the two inducible elements (Nerlov et al., 1992).

TIMP1, an inhibitor of metalloproteinases, also contains AP1 and PEA3 elements that are required for expression of TIMP. TIMP differs from collagenase, stromelysin and urokinase in that TIMP1 does not contain TATAA sequences. However, like collagenase and stromelysin, there are AP1 and PEA3 sites within 100 bp of the transcription start site. Reporter constructs containing this segment of the promoter are weakly induced by serum. Promoter mapping has also shown that there is a serum response element that contains AP1 and PEA3 sites located at -800 in the TIMP promoter. While the AP1 and PEA3 sites near the start of transcription confer inducibility on a heterologous promoter, there is some suggestion that the AP1 and PEA3 sites adjacent to the transcription start site are acting to recruit RNA polymerase and/or transcription factors to the transcription start site. Indeed a number of genes that lack TATAA sequences contain AP1 and PEA3 sites immediately adjacent to the transcription start sites, and in the polyoma virus late genes both the AP1 and PEA3 sites are required for the transcription of late genes (Yoo et al., 1991 and references therein).

The significant inducible elements in the collagenase, stromelysin, urokinase, and TIMP1 promoters are AP1 and PEA3, but there are differences in the regulation of these promoters by growth factors, oncogenes and TPA. The regulation of these genes is influenced by the transcription factors present in the cell. It is well known that AP1 is lacking in F9 teratocarcinoma cells, however it is less obvious that in HeLa cells, the transcription of collagenase and stromelysin reporter constructs is dominated by AP1, while PEA3 and AP1 appear to have equal roles in HepG2 cells (Angel et al., 1987b; Buttice and Kurkinen, 1992,1993; Nerlov et al., 1992) There may also be some specificity or selectivity of protein complexes for

specific AP1 sites because in gel shift assays, a subset of proteins that interact with the AP1 site at -59 in the TIMP promoter do not interact with the consensus AP1 site in the collagenase promoter (Edwards et al., 1993). Signaling cascades initiated at the fibronectin receptor regulates AP1-mediated expression of collagenase. In all probability, this increase in AP1 augments the regulation of the stromelysin and urokinase genes as well. These observations describe, at a molecular level, how changes in cell adhesion translate into changes in gene expression, emphasizing that cell-ECM interactions have important consequences.

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Chapter 6

Overall Conclusions and Future Directions

It is well known that the interaction of cells with specific components in the extracellular matrix can have widely ranging effects on cell behavior and phenotype. Numerous observations, *in vivo* and *in vitro*, show that particular cell-ECM interactions, or changes in these interactions correlate with the stabilization or selection of cell phenotype (reviewed by Adams and Watt, 1993). The studies in this thesis show that, in rabbit synovial fibroblasts, the expression extracellular matrix-degrading metalloproteinases is upregulated in cells plated on degradation products of fibronectin, anti-$\alpha_5\beta_1$ antibodies, on fibronectin matrices supplemented with tenascin, and on certain ECM substrates in the presence of SPARC. These observations are informative, because they support the concepts that 1) the extracellular matrix contains information that is interpreted by cells, and 2) that this information can be translated into changes in gene expression. These studies also support the concept that the interaction of a cell and its ECM is reciprocal. While cellular responses to cues provided in cell-ECM interactions can initiate or stabilize cellular behavior, the composition and structure of the ECM itself is not static. The fact that, in RSF, the proteinases that are upregulated in cells plated on inductive matrices are the enzymes that are primarily responsible for ECM turnover suggest that cell-ECM interactions can initiate or amplify ECM remodeling cascades *in vivo*. Taken together, the studies in this thesis suggest that the regulation of collagenase gene expression in cultured synovial fibroblasts is a model system that can be used to study each of the steps in the regulation of gene expression initiated by cell-ECM interactions: the nature of the signaling ECM ligand, the identity and function of the ECM receptor(s), the cytoplasmic signaling cascade and the regulation of the nuclear signaling cascade.

The initial characterization of this system (Chapter 2) shows that the expression of collagenase increases in cells treated with anti-$\alpha_5\beta_1$ antibody and in cells plated on fibronectin fragments, but remains at basal levels in cells plated in serum proteins, or on intact fibronectin, vitronectin, collagen or laminin. This induction of collagenase protein occurs within 4 h of

treatment and results from an increase in mRNA coding for collagenase. In the limited case of cells treated with or plated on the function-perturbing anti-$\alpha_5\beta_1$ antibody, I demonstrated that the regulation of gene expression induced by the interaction of a specific integrin with ligand. The situation is likely to be more complex in cells plated on fibronectin fragments, because several integrin receptors that are present on RSF mediate adhesion to the cell binding domain of fibronectin. The experiments in Chapters 3 and 4 show that there are other types of matrices that can regulate the expression of collagenase.

The useful features of this system include both the ability to follow the consequences of the interaction of a specific integrin with ligand on the regulation of the expression of a specific gene and the ability to easily analyze the expression of collagenase at the level of mRNA, protein, and the regulatory elements in the promoter that control gene expression. Chapter 5 defines components of the nuclear signaling pathway that regulate the expression of collagenase in cells plated on inductive ligands of the fibronectin receptor. The promoters of collagenase and other metalloproteinases are well characterized, and many inducible elements in these promoters have been identified using promoter-reporter fusion constructs. In the human collagenase promoter, the sequences between -139/-42 mediate the upregulation of collagenase in RSF plated on anti-$\alpha_5\beta_1$ antibodies or fibronectin fragments. Both the AP1 (-72/-67) and PEA3 (-92/-81) sites are required for this increase in expression. Transcription from AP1 sites is mediated by homodimers of the Jun family of nuclear proto-oncogenes, and more efficiently by heterodimers of Jun and Fos family members (reviewed by Angel and Karin, 1992). The amount of mRNA coding for cFos increases in cells plated on anti-$\alpha_5\beta_1$ antibody or on fragments of fibronectin that contain the RGD sequence, as does Fos protein in the nuclei of cells plated on fibronectin fragments. Incubation of cultures with short oligonucleotides complementary to sequences near the c-*fos* start site of transcription significantly compromised the upregulation of collagenase in cells plated on fibronectin fragments. This strengthened the conclusion that the transcription from the

AP1 site in the collagenase promoter is a necessary component of the regulation of collagenase by cell-ECM interactions. The results of this study suggest another mechanism by which signaling cascades initiated by cell-ECM interactions converge with signaling cascades initiated by other cell surface receptors, because to date, the AP1 site in the collagenase promoter is required for the expression of collagenase induced by growth factors or drugs (Auble and Brinckerhoff, 1992; Angel et al., 1987).

Other groups have noted that signaling pathways regulated by adhesion/integrins converge with pathways used by other receptors. The regulation of transcription of specific genes in response to cell-ECM interactions is beginning to be characterized. In monocytes, the expression of a number of genes can be upregulated or downregulated in response to cell adhesion to the substratum (monocyte adhesion or MAD genes) (reviewed by Juliano and Haskill, 1993). Adhesion per se induces the expression of several MAD genes, because they are upregulated in monocytes adhering to plastic, but the composition of the matrix can alter the subset of MAD genes induced in adhering monocytes (Eirmen et al., 1989; Sporn et al., 1990). Crosslinking of β_1 integrins on monocytes with anti-β_1 $[Fab]_2'$ fragments upregulates the expression of MAD genes, implying that β_1 play a role in the regulation of MAD genes (reviewed by Juliano and Haskill, 1993). Comparison of the sequences in the promoters of several MAD genes show that in monocytes, genes that are upregulated by adhesion contain sites for the transcription factor NF-κB, and to a lesser extent sites for AP1 and the cyclic AMP response element, suggesting again that signaling pathways initiated by adhesion converge with other signaling cascades (reviewed by Juliano and Haskill, 1993). The expression of the casein gene in mammary epithelial cells is upregulated by prolactin only in the context of the appropriate matrix. BCE-1, an element in the casein promoter is regulated by concurrent signals generated by cell-ECM interactions, and by the interaction of prolactin with its cell surface receptor. Molecular analysis of BCE-1 has not yet separated the sequences that respond to matrix

from those responding to prolactin, suggesting that signals from both pathways are required for transcription from this element (Schmidhauser et al., 1992).

The interaction of cells with ECM plays a role in establishing and stabilizing normal cytoarchitecture. In adhering cells, integrin receptors are recruited into focal contacts and focal adhesions, and they nucleate the assembly of cytoskeletal proteins into stable structures (reviewed by Burridge et al., 1988. However, the studies in this thesis suggest that under come conditions, the interaction of cells with specific combinations of molecules in the ECM initiates a signaling cascade that can alter the pattern of collagenase gene expression in cells. Although we have established that the $\alpha_5\beta_1$ fibronectin receptor plays a role in this signaling cascade, and partially defined components of the nuclear cascade that are involved in this cascade, the nature of this dynamic interaction of cells with ECM is not yet fully characterized. The resolution of several fundamental issues is necessary before we can describe the attributes of the signaling receptor(s) involved in this cascade. The data from Chapter 2 and Chapter 4 can be interpreted to suggest that the concurrent occupancy of integrin and non-integrin receptors for ECM is required to maintain basal (uninduced) expression of collagenase. 120FN, a monomer, lacks both the heparin-binding and matrix-assembly domains found in intact, dimeric fibronectin; these domains interact with non-integrin receptors for fibronectin (reviewed by Schwarzbauer,1991a). Tenascin also interacts eith cell surface heparin-sulfate proteoglycans (Salmivirta et al., 1991). In a tenascin supplemented fibronectin matrix tenascin may compete with fibronectin for cell surgace proteoglycans, thereby perturbing the interaction of cells with fibronectin. Together, these observations imply that the spatial arrangement or localization of receptors in the cell membrane play a role in the signaling cascade; it may be that the signaling receptor is diffusely present on the cell surface.

An alternative hypothesis, which also agrees with the studies in chapter 2 and chapter 4, is that a ligand -induced conformational change in the $\alpha_5\beta_1$ fibronectin receptor, or a change in the

interaction of ligand with the receptor, initiates the cascade of events that regulates the expression of collagenase. In this case, 120FN and a tenascin-supplemented fibronectin matrix are perceived differently from intact, dimeric, fibronectin. Experiments are underway to discriminate between these ideas, although it is equally probable that facets of each hypothesis may be true.

The nature of the cytoplasmic signal has not yet been determined. It may be that the concentration and/or spatial arrangement of integrin receptors in focal contacts somehow regulates the expression of collagenase in cells plated on fibronectin fragments or fibronectin/tenascin substrates. While we do not know if the activated integrin receptor is localized to focal contacts, or diffusely present on the cell surface, crosslinking of at least two fibronectin receptors is required, because bivalent anti-fibronectin receptor antibody, but not monovalent anti-fibronectin receptor fab, upregulates the expression of collagenase. An alternative hypothesis is that traditional signaling pathways are utilized to transduce signals through integrin receptors. The cytoplasmic tails of integrin receptors are very short. Analysis of cDNA sequence has determined that these receptors lack obvious homolgy with other signaling receptors (reviewed by Hynes, 1992). However, studies defining the post-receptor events that immediately follow the interaction of GPIIbIIIa with ligand in activated platelets, show that a number of proteins, including FAK, a tyrosine kinase that is associated with focal adhesions, are phosphorylated on tyrosine (Lipfert et al., 1992; Huang et al., 1993). This suggests that, like the T-cell receptor for antigen, integrin receptors may associate a signaling machine that is likely to include protein kinases and phosphatases. Experiments are underway to characterize the pattern of phosphorylated proteins in cells plated on fibronectin, 120FN and anti-fibronectin receptor antibody.

Future Directions

Several approaches can be taken to expand this work. Unanswered questions abound, but they

can be sorted into several broad experimentally tractable categories.

-What is the nature of the "inductive" ligand?

Fibronectin is a dimeric molecule that interacts with several cell surface receptors (reviewed by Schwarzbauer, 1993, Chapter1); the monomeric 120kD chymotryptic fragment of fibronectin lacks both heparin binding domains, but contains the RGD-containing cell binding domain and synergy site recognized by $\alpha_5\beta_1$. 120FN may be recognized differently from fibronectin because it is monomeric or because it lacks specific domains that are present in intact fibronectin. Alternatively, the conformational constraints on the 120FN molecule could differ from those of intact fibronectin, and affect the interaction of ligand with receptor (e.g. interactions that stabilize the interaction of fibronectin with the receptor are missing in 120FN). One approach to answer these questions is to use recombinant fibronectin molecules of known composition to define the "inductive" fibronectin molecule; in addition, these molecules can be tested as both monomers and dimers (Obara et al., 1989; Guan et al., 1990; Schwarzbauer, 1992).

There is also precedent for the argument that the conformation of the RGD site affects the recognition of this sequence by integrin receptors (Pierschbacher and Ruoslahti, 1989) because studies using synthetic peptides having different sequences surrounding the RGD site show that these peptides have different affinities for different $\alpha\beta$ heterodimers. Sequences surrounding the RGD site also affect the affinity of the ligand for integrin receptors- peptides and fragments of fibronectin or vitronectin have a lower affinity interaction with the receptor (Orlando and Cheresh, 1991).

That a substrate of anti-$\alpha_5\beta_1$ antibody is an inductive ligand can be interpreted to support either idea— anti-$\alpha_5\beta_1$ antibodies do not contain the additional epitopes in fibronectin that interact with other cell surface receptors, or that anti-$\alpha_5\beta_1$ antibodies induce or stabilize a conformation of the receptor that signals.

-Why is a mixed substrate of fibronectin and tenascin perceived as an inductive substrate?

The upregulation of collagenase in RSF plated on mixed substrates of fibronectin and tenascin is reminiscent of that seen in cells plated on 120FN or anti-$\alpha_5\beta_1$ antibodies. In both cases, the expression of collagenase protein is upregulated within 4 h of treatment and is preceded by an increase in nuclear cFos. An issue that has not yet been resolved is which cell surface receptor recognizes the mixed substrate of fibronectin and tenascin. We also do not know what makes the mixture of fibronectin and tenascin an inductive substrate. The inductive substratum could result from a conformational change in either fibronectin or tenascin resulting from the interaction of the two molecules. It could also be possible that the interaction of tenascin and fibronectin masks an epitope on fibronectin, making a fibronectin/tenascin substrate resemble a degradation product of fibronectin. Another possibility is that tenascin interacts with cell-surface heparin sulfate proteoglycans and prevents the coordinated interaction of cell surface receptors with fibronectin.

-What is the SPARC-induced secreted intermediate that upregulates the expression of collagenase in RSF plated on interstitial matrices?

Like tenascin, SPARC is another matrix molecule that in some cases diminishes the interaction of cells with the ECM (Sage and Bornstein, 1992). We've shown that in the context of primarily intersitial matrix molecules, SPARC upregulates the expression of collagenase. This induction is indirect, caused at least in part by a SPARC-induced secreted intermediate. The nature of this factor has not yet been determined and would be a fruitful area of investigation. The experiments that showed synthetic peptides from the C-terminal EF-hand domain and the α-helical domains in the SPARC molecule play a role in the regulation of collagenase by SPARC. There are two cleavage sites in the α-helical domain of SPARC, and there is some suggestion that under some conditions, the fragment of SPARC that contains the amino-terminal may interact with collagen-containing matrices with a higher affinity (Tyree et al., 1989). The precise cleavage site is not known, but it would be intersting to know if the amino-terminal fragment of SPARC contains the

residues in peptide 3.2. A third issue in the analysis of the effect of SPARC is the nature of the receptor(s) that recognize SPARC/ECM complexes. The ECM specificity of the SPARC effect closely resembles the ECM-binding specificities of the Syndecan family of heparin sulfate proteoglycans. Reagents for perturbing interactions with syndecan are now becoming available, and these experiments may soon be feasible.

-What is the function of other anti-adhesive molecules?

Taken together, the experiments showing that addition of SPARC and tenascin to matrices can regulate the expression of collagenase suggest that the addition of molecules of molecules like decorin and thrombospondin, which also perturb the interaction of cells with fibronectin matrices may also regulate the expression of collagenase.

-Do other cell surface receptors for ECM play a role in the regulation of collagenase in RSF plated on 120FN?

There is growing evidence that in some cell types, the concurrent interaction of more than one class of cell surface receptor for fibronectin with ligand is required before focal contact formation is initiated (Woods and Couchman, 1991, and references therein). It is perhaps significant that the inductive ligands for the fibronectin receptor, 120FN and anti-$\alpha_5\beta_1$ antibody, lack domains that interact with heparan containing molecules on the cell surface. If occupancy of a co-receptor were required to maintain the basal expression of collagenase, removal of cell surface heparin by treating RSF with heparinase or heparitinase, or by perturbing the addition of sulfated glycoseaminioglycan side chains on heparin sulfate proteoglycans should make fibronectin an inductive molecule.

$\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin receptors interact at some level to mediate some cellular functions. Both $\alpha_v\beta_3$ and $\alpha_v\beta_1$ have been shown to interact with fibronectin (Charo et al., 1990; Zhang et al., 1993). These receptors can play a role in the attachment and spreading of cells on fibronectin,

but they can not fill the role of $\alpha_5\beta_1$ in matrix assembly or cell migration on fibronectin (Zhang et al., 1993). There is some suggestion of cooperation between $\alpha_5\beta_1$ and $\alpha_v\beta_3$, because in CHO cells that lack the $\alpha_5\beta_1$ fibronectin receptor, the $\alpha_v\beta_3$ mediated migration on vitronectin is diminished (Bauer et al., 1992), and in a melanoma cell line, both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ mediate attachment to a fibronectin substrate (Charo et al., 1990).

Although the vitronectin receptor is a signaling receptor (Leavesley et al., 1993), if this receptor is a component of this signaling cascade, it is very likely to be an interplay between the receptors that regulates the expression of collagnease, because function perturbing antibodies to the $\alpha_v\beta_3$ receptor do not affect the regulation of collagenase. One possible hypothesis is that because $\alpha_v\beta_3$ interacts with fibronectin and fibronectin fragments with a lower affinity than $\alpha_5\beta_1$ (Charo et al., 1990), some cellular processes are affected. This is supported by the observation that the initial attachment strength (this is affected by the affinity of the receptor, but is not equivalent to the the affinity of the receptor) closely correlates with the migrating phenotype in smooth muscle cells and that migrating cells are not tightly adherent (DiMilla et al., 1993).

Another approach to identify co-receptors on the cell surface would be to mark cell surface proteins (e.g. biotinylation or iodination) and allow these cells to interact with matrix ligands immobilized on magnetic or polyacrylamide beads. After lysing the cells with detergent, with luck the cell surface receptors that associate with the matrix macromolecules and intracellular proteins that associate with the receptors can be concentrated by isolating the beads. The isolated proteins, separated on gels could be visualized by autoradiography or by incubation with streptavidin conjugates.

-Which domains of the receptor are used in the signaling cascade?

The role that cytoplasmic domains of integrin α and β chains play in cellular processes is beginning to be elucidated. Recombinant receptor proteins that contain substitutions or truncations of the cytoplasmic tails, or chimeric receptors containing the extracellular domain of

one α or β chain and the transmembrane domain of another family member (e.g. β_1/β_3, α_2/α_4, α_2/α_5, α_{IIb}/α_5) have been introduced into cells and the function of these receptors analyzed (Ref). Together, these studies suggests that the information contained in the cytoplasmic domain of the β_1 and β_3 chains is necessary and sufficient for recruitment into focal adhesions (Ylanne et al., 1993; Reszka et al., 1993; LaFlamme et al., 1992; O'Toole et al., 1991; Hayashi et al., 1990; Marcantonio et al., 1990). but that there may be a slight bias against the recruitment of unoccupied receptors into focal contacts (LaFlamme et al., 1992; Ylanne et al., 1993). Most studies suggest that removal of 5-15 amino acids of the C-terminal of β_1 chains diminishes the localization of this chain to focal contacts (Ylanne et al., 1993; Solowaska et al., 1991; Hayashi et al., 1990), but there is a suggestion that sequences in β_1 adjacent to the membrane may also affect receptor localization to focal contacts (Marcantonio et al., 1991). Reszka et al. (1993) have identified specific residues in the cytoplasmic domain of β_1 that play a role in targeting receptors to focal adhesions. These studies also hint that phosphorylation of serine or tyrosine residues on β_1 chains may diminish recruitment of integrin to focal adhesions.

β chains can pair with one of a number of α chains. Summarizing the results from a number of studies analyzing the function of recombinant α chains suggest that the cytoplasmic domain of α chains restricts recruitment of αβ heterodimers to focal contacts to those initiated by adhesion of these receptors to their specific matrix ligand. α chains also affect both the assembly of matrix molecules into extracellular matrix, as well as the stabilization of the cytoskeleton assembly nucleated by integrins (Ylanne et al., 1993; Bauer et al., 1993; Zhang et al., 1992). The analysis of chimeric receptors suggests that cytoplasmic domains of α chains may have more specific affects. α_{IIb} truncations or α_{IIb}/α_5 receptor chimeras suggest that the cytoplasmic sequences in α_{IIb} may regulate the affinity of $\alpha_{IIb}\beta_3$ for ligand (O'Toole et al., 1991). The role of α_5, α_4, and α_2 cytoplasmic domains play in cellular functions has been studied in chimeric receptors that contain the extracellular domain of α_2. The native α_2 receptor and the chimeric

receptors recognized collagen and laminin equally well; cells containing wild type receptors or the α_2/α_5 chimera were capable of collagen gel contraction, while cells containing the α_2/α_4 construct migrated on a collagen substrate (Chan et al., 1992).

It has been demonstrated that the β_1 chain is required for phosphorylation of a number of substrates in adhering cells (Guan et al., 1991), and truncations in α_5 do not appear to alter the pattern of phosphoproteins in CHO cells (Bauer et al., 1993). Analysis of these constructs in RSF may prove interesting because the cells in which the recombinant receptors were analyzed in all probability do not regulate the expression of collagenase in response to cell-ECM interactions.

-What is the initial cytoplasmic signal?

We have had some success in dissecting parts of the nuclear signaling pathway that regulate the expression of collagenase in cells plated on anti-$\alpha_5\beta_1$ antibodies or on 120FN. Identifying receptor proximal and intermediary components of this pathway is the next logical series of experiments. To date, ras and raf kinase are intermediates in the transduction pathway leading to the upregulation of collagenase in response to many growth factors, drugs, or the overexpression of non-nuclear oncogenes (Kolch et al., 1993; Kolch et al., 1993b; Kyriakis et al., 1993; Bruder et al., 1992; Schonthal et al., 1988). There are expression vectors that direct the synthesis of recombinant raf proteins that have a dominant-negative effect on endogenous raf kinase function (Bruder et al., 1992). These constructs can be used to determine if raf kinase is involved in the upregulation of collagenase induced by ECM-derived signals.

It is becoming clear that integrins may also participate directly in signaling pathways. The intracellular domains of integrins are noticeably short, however proteins that associate with the cytoplasmic domain of integrins are being identified. Integrin associated protein (IAP) is a 50 kD protein found on many cell types that is physically and functionally associated with β_3 integrins (Brown et al., 1990) and copurifies with $\alpha_v\beta_3$. Analysis of the cDNA sequence reveals a strong

homology of IAP with the transmembrane sequences found in ion channels (Lindberg and Brown, 1992, Molecular Biology of the Cell, supplement,v3 p 95a #547). Hendley et al. (1992) have shown that the migration of neutrophils on vitronectin requires transient intracellular calcium spikes. Coupled with the observations that a rise in intracellular calcium, presumably through voltage gated channels, occurs in cells adhering to vitronectin or to anti- $\alpha_v\beta_3$ or anti-$\alpha_v\beta_1$ antibodies and that $\alpha_v\beta_3$ dependent migration towards vitronectin is inhibited by the removal of extracellular calcium (Leavesley et al., 1993) suggest that some combination of $\alpha_v\beta_3$ and IAP may be a functional signaling apparatus. Pullman and Bodmer (1992) have cloned another protein, called cell adhesion regulator (CAR) that affects integrin function. Expression of CAR in cells that do not adhere well to collagen type I caused a 2.6 fold increase in adhesion to collagen type I, collagen type IV and laminin. This adhesion was blocked by anti-β_1, anti-α_2, or anti-α_3, antibodies, and was not due to an increased expression of integrins at the cell surface. CAR is a myristolated cytoplasmic protein that has a consensus site for tyrosine phosphorylation; substitution of other amino acids for this tyrosine ablates the enhancement of cell adhesion by CAR. This work strongly suggests that tyrosine kinases may modify cell adhesion.

The interplay of tyrosine kinases and integrin receptors is becoming clearer in studies of a focal adhesion associated kinase, pp125^{FAK}, first identified as a substrate of the src tyrosine kinase (Shaller et al., 1991). pp125^{FAK} localizes to focal adhesions; however it lacks SH_2 or SH_3 domains, and apart from the catalytic kinase motif, is not homologous to other tyrosine kinases (Shaller et al., 1992). This kinase is phosphorylated in cells that are transformed by v-src or have an overexpressed constitutively active c-src or in response to polypeptide growth factors (Zachary et al., 1992; Guan and Shalloway, 1992). Interestingly, this kinase is not phosphorylated when anchorage dependent fibroblasts are not adherent but is rapidly phosphorylated upon adhesion of cells to fibronectin, laminin, collagen or anti-integrin antibodies (Guan et al., 1991; Turner et al., 1991; Burridge et al., 1991; Komberg et al., 1992)

Increased phosphorylation of pp125^{FAK}, induced by adhesion to the ECM or by clustering of integrins with anti-integrin antibodies correlates with an increased tyrosine kinase activity of pp125^{FAK} (Lipfert et al., 1992; Guan and Shalloway, 1992). In platelets, increased phosphorylation of pp125^{FAK} is only seen in aggregated platelets in which $\alpha IIb\beta_3$ interacts with fibrinogen (Lipfert et al., 1992), implying that pp125^{FAK} may well be one point of integration of signaling by different receptor-effector pathways (reviewed by Zachary and Rozengurt, 1992). Ligation of gpIIbIIIa with antibodies that recognize or stabilize the active conformation of gpIIbIIIa induces the phosphorylation of several proteins on tyrosine in the absence of other agonists. The phosphorylation of these proteins precedes the phosphorylation of FAK and does not occur in platelets treated with cytochalasins, suggesting that the nucleation of cytoskeleton assembly caused by integrin-ligand interactions may juxtapose kinases and substrates, and that kinases other than FAK may also be assembled into this complex (Huang et al., 1993).

An obvious set of experiments is the analysis of the pattern of tyrosine phosphorylation of proteins in cells plated on inductive and non-inductive ligands for the fibronectin receptor, and where possible identifying these proteins. Secondly, it would be interesting to characterize proteins and phosphoproteins that may associate with $\alpha_5\beta_1$, and to determine if the distribution of these proteins is altered in the focal adhesions assembled on fibronectin or 120FN.

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