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An analysis of the localization and function of the c-Src kinase during cell adhesion

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An Analysis of the Localization and Function of the

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by

Kenneth B. Kaplan

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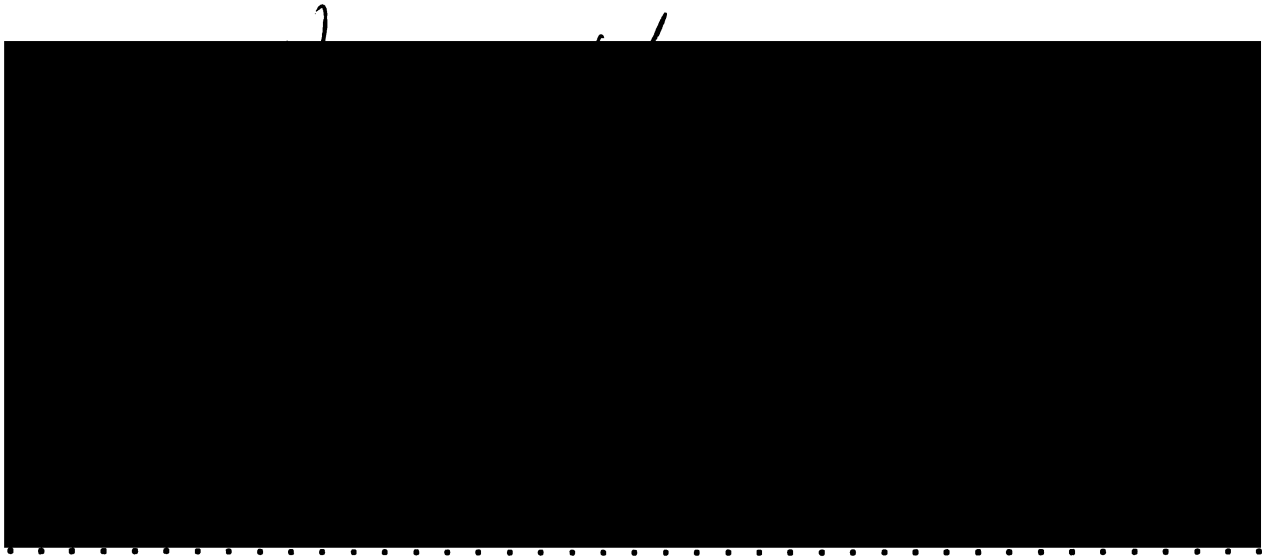

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Analysis of the Localization and Function of the c-Src Kinase during Cellular Adhesion

Kenneth B. Kaplan

Abstract

The cellular role of the cytoplasmic tyrosine kinase c-Src was examined by characterizing the regulation of its subcellular distribution in mammalian fibroblasts. Under normal growth conditions, both immunofluorescence studies and biochemical fractionation revealed that the majority of c-Src is associated with endosomal membranes. However, mutational analyses of c-Src revealed that its subcellular distribution can be altered by changes in the negative regulatory tyrosine at position 527 (Y527). Either amino acid substitution or the absence of phosphorylation at Y527 results in a dramatic redistribution of c-Src from endosomal membranes to focal adhesions, independent of kinase activity and cellular transformation. Additional mutations revealed that the association of c-Src with focal adhesions is mediated by the first 251 amino acids and requires the SH3 domain as well as protein myristylation. Significantly, the presence of the amino-terminal half of c-Src in focal adhesions alters both their structural and biochemical properties, suggesting that c-Src may regulate cellular adhesion. Consistent with this possibility, *src*$^{-/-}$ fibroblasts were shown to be defective in cell spreading and could be complemented by expression of the amino terminal half of c-Src, confirming that interactions between c-Src and focal adhesions directly affect cellular adhesion.. Although the kinase domain is not required to complement the adhesion defect in *src*$^{-/-}$ fibroblasts, mutations in the amino terminus of c-Src revealed that both the SH2 and SH3 domains are involved in stimulating cell spreading. Consistent with a physiological role of c-Src in cellular adhesion , we demonstrated that the kinase activity and

subcellular distribution of c-Src are regulated during the adhesion of fibroblasts on fibronectin. In conclusion, these results strongly implicate c-Src in the regulation of focal adhesions and the spreading of fibroblasts on fibronectin.

INVOLVEMENT OF COAUTHORS: ADVISOR'S STATEMENT

Kenneth Kaplan's dissertation work resulted in the publication of two articles and a third that is intended for submission at a later date. Some of the work involved coauthors whose citation and contributions are described below:

Chapter 2:

Kaplan, K. B., Swedlow, J. R., Varmus, H. E. and Morgan, D. O. 1992. Association of p60c-src with endosomal membranes in mammalian fibroblasts. *J Cell Biol.* 118:321-33

Kenneth Kaplan carried out the majority of the work in this publication. Jason R. Swedlow contributed to the optical sectioning analysis of immunofluorescence experiments. Both myself and Dr. Harold E. Varmus supervised the work.

Chapter 3:

Kaplan, K.B., Bibbins, K.B., Swedlow, J.R., Arnaud, M., Morgan, D.O. and Varmus, H.E. Association of the amino terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. in press The EMBO J. 1994

The majority of the work presented in this chapter was performed by Kenneth Kaplan. Kristen Bibbins aided in the generation of cell lines expressing mutant constructs, Jason Swedlow contributed to the optical sectioning analysis of immunofluorescence experiments, Martha Arnaud was a rotation student who helped to characterize the detergent fractionation protocol. This work was supervised by both myself and Dr. Harold E. Varmus.

Chapter 4:

Kaplan, K.B., Swedlow, J.R., Morgan, D.O. and Varmus, H.E. C-Src enhances the spreading of *src-/-* fibroblasts on fibronectin by a kinase-independent mechanism. Manuscript in preparation. 1994

Kenneth Kaplan performed the vast majority of the work included in this chapter. Jason Swedlow collaborated in analyzing immunofluorescence experiments by optical sectioning microscopy. This work was supervised by both myself and Dr. Varmus.

David O. Morgan

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Introduction

The proto-oncogene c-src

Oncogenes are mutations in normal genes, whose gene products are involved in the regulation of cell growth, and which ultimately lead to the development of cancer. The study of oncogenes has lead to the identification of genes that are involved in both stimulating (dominant oncogenes) and repressing (tumor suppressor genes) the growth of cells. The goal of cancer research has been to identify the precise biochemical and molecular mechanisms by which oncogene protein products induce the transformation of a normal cell into a cancer cell. The study of cellular transformation in culture has provided a system to address these questions and has identified a large number of oncogenic proteins. The precise alterations in the cell that lead to transformation are less clear, due largely to the many changes that occur during transformation and the complexity of the cellular processes affected. Nonetheless, advances in the biology of the cancer cell have been significant, providing a detailed description of the normal cellular processes that become subverted by oncogene products and that ultimately give rise to a cancer cell.

One approach to further resolving the changes associated with transformation is to examine the normal function of cellular homologues of oncogenes (proto-oncogenes). This approach has the advantage of addressing basic problems in cell biology that are directly linked to transformation through the deregulation of the proto-oncogene product. In addition, the study of the basic biology of the cell also benefits from the availability of dominant mutations that arise in many oncogenic proteins. Therefore, the study of the normal function of proto-oncogene products and their involvement in cellular transformation can simultaneously advance our understanding of cancer and the biology of the cell.

One of the most intensely studied oncogene products is the cytoplasmic tyrosine kinase c-Src, the product of the first discovered proto-oncogene (*c-src*). A great deal has been learned concerning the regulation of the kinase and the general mechanism by which mutations in *c-src* lead to cellular transformation. In addition, the use of Rous sarcoma virus to transform cells in culture has generated a detailed description of the cellular changes associated with transformation. Despite these prodigious efforts, it has been difficult to identify the exact cellular changes that lead to cellular transformation. Similarly, the normal cellular role of c-Src remains obscured by the large number of changes that occur during cellular transformation. It will be the effort of this work to understand the normal cellular function of the c-Src kinase and ultimately to relate this function to the changes that lead to cellular transformation.

The src-family of cytoplasmic tyrosine kinases

The fact that *c-src* is expressed in a variety of cells and is a member of large family of cytoplasmic kinases (Eiseman and Bolen 1990) suggests that it is involved in an essential and well conserved cellular process. The src family of cytoplasmic tyrosine kinases consists of nine known members: src, yes, fyn, fgr, lyn, lck, hck, blk, and yrk (Bolen 1993). Four of the members (src, yes, lyn, and fyn) are expressed in a wide variety of tissues, although the levels of expression vary in different cell types . Notably, c-Src comprises 0.2-0.4% of total cell protein in platelets and is also highly expressed in neurons and osteoclasts. Four of the other family members (lck, fgr, hck, and blk) are expressed primarily in cells of hematopoietic origin. It should be noted that there is often expression of multiple src-family members in the same cell, although some src-family members are only expressed in a limited number of cell types. The overlapping expression of src-family members is consistent with

their serving a potentially redundant function to ensure the integrity of important cellular processes.

Consistent with a shared cellular function, members of the src-family possess common structural features that have been divided into several distinct domains (Figure 1-1) (Cooper 1989; Jove and Hanafusa 1987; Parsons and Weber 1989). These domains were originally defined based on sequence comparisons among the src-family members and were designated src-homology (SH) regions. At the amino terminus is a non-conserved domain (unique domain=amino acids 18-84 in c-Src) that mediates the attachment of src-family members to cellular membranes and contains sequences modified by the addition of fatty-acid side chains as well as phosphorylations (see *Src regulation*). Membrane attachment is a hallmark of src-family members and distinguishes them from other cytoplasmic tyrosine kinases. The modification of c-Src involves the addition of a myristic acid to the glycine at position 2 (Kamps, et al. 1986) and requires the presence of the first 7 amino acids (Kaplan, et al. 1990). However, myristylation is not sufficient for the association of c-Src with cellular membranes, which requires the first 14 amino acids of c-Src as well as unidentified cellular proteins (Kaplan, et al. 1990; Resh 1989). While the exact mechanism of membrane attachment is not known, it is critical for the transformation of cells by the viral Src protein (v-Src) (Buss, et al. 1986; Kamps, et al. 1986) and is likely to be important for the normal function of c-Src as well.

The src-homology domain 3 (SH3; amino acids 84-149) and src-homology domain 2 (SH2; 149-260) comprise the remaining sequences of the amino terminal half of c-Src. These sequences were originally defined through mutants of v-Src defective in cellular transformation and led to the proposal that these domains mediate protein interactions involved in regulating the kinase activity or substrate recognition of v-Src (Cooper 1989). In neurons, an

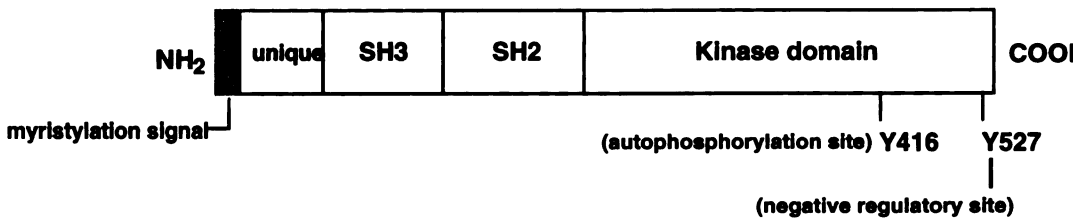
NH2
unique
SH3
SH2
Kinase domain
COOH
myristylation signal
(autophosphorylation site) Y416
Y527
(negative regulatory site)

Figure 1-1

insertion of up to 17 amino acids occurs within the SH3 domain as a result of alternatively spliced exons (Pyper and Bolen 1989). These inserted amino acids result in the high specific kinase activity of neuronal c-Src (Levy, et al. 1987) and may alter the binding of cellular proteins to this domain in neurons (Meyerson, et al. 1992). It has been proposed that the SH2 and SH3 domains serve a general biochemical function in the cell based on their presence in a number of signal transduction related proteins, including non-catalytic "adapter" proteins (Cantley, et al. 1991).

Biochemical analysis of isolated SH2 and SH3 domains and the solution of their three dimensional structures (Pawson and Gish 1992; Schlessinger 1994) has confirmed their unique biochemical characteristics. The SH3 domain is a compact beta-barrel made of five anti-parallel beta-strands with conserved aromatic amino acids located close to each other on one side of the molecule. (Musacchio, et al. 1992). Identification of ligands for the SH3 domain indicate that proline-rich peptides bind to a hydrophobic pocket on the surface of the protein (Ren, et al. 1993; Yu, et al. 1992). There are substantial differences in the identities of the amino acids that make up the receptor sites in SH3 domains from different proteins; this may account for their ability to bind different ligands (Koyama, et al. 1993), although the issue of specificity remains to be resolved.

In contrast, the specificity of SH2-binding to tyrosine phosphorylated proteins has been characterized in great detail. Studies of signaling proteins containing the SH2 domain as well as peptide binding experiments demonstrate the phosphotyrosine dependent binding of SH2 domains (Margolis, et al. 1990; Mayer, et al. 1991; Moran, et al. 1990). A series of structural studies indicate that the SH2 domain consists of an anti-parallel beta-sheet flanked by two alpha-helices, with peptide binding mediated by the sheet, intervening loops and one of the helices. The recognition of phosphotyrosine

peptides by the Src-SH2 domain involves primary interactions of the phosphotyrosine and a nearby isoleucine residue on the consensus peptide (PQ(pY)EEI). These residues are tightly bound by two well-defined pockets on the protein surface of the SH2 domain, resulting in a complex that resembles a "two-pronged plug engaging a two-holed socket" (Overduin, et al. 1992; Waksman, et al. 1992; Waksman, et al. 1993). Mutations in the conserved pockets that coordinate the binding of phosphotyrosine residues dramatically impair the ability of the SH2 domain to bind target proteins (Bibbins, et al. 1993; Koch, et al. 1992). In general, the SH3 and SH2 domains mediate protein-protein interactions and may serve to bring together substrate and kinase, or to target c-Src to specific regions of the cell. On the other hand, these domains may also mediate the intramolecular interaction with regulatory phosphorylation sites in the carboxy terminus (discussed below).

The carboxy terminal half of c-Src includes the catalytic domain of the enzyme and contains many of the conserved hallmarks of tyrosine-specific protein kinases (Parsons and Weber 1989). The ATP binding site and the autophosphorylation site (Y416) are conserved among many tyrosine kinases and are required for the full catalytic activity of c-Src. Mutations in the nucleotide binding site abolish catalytic activity, while substitution of Y416 with a phenylalanine reduces the enzymatic activity of c-Src approximately five-fold (Bryant and Parsons 1984; Kmiecik, et al. 1988). However, the major site of src-family kinase regulation is in the well conserved carboxy terminal regulatory domain. Deletion of the carboxy terminus (as in v-Src) and mutations in c-Src have implicated the carboxy terminal 19 amino acids in the regulation of the kinase. These residues contain a tyrosine at position 527 (Y527) that is the major site of *in vivo* tyrosine phosphorylation in c-Src. Phosphorylation of Y527 (pY527) is required to maintain the normally repressed kinase activity of c-Src.

Replacement of Y527 with a phenylalanine results in a highly active kinase and cellular transformation (Cooper, et al. 1986; Courtneidge 1985; Laudano and Buchanan 1986). The absolute conservation of this negative regulatory site in src-family members suggests a common regulatory mechanism for controlling the kinase activity of src-family members.

Regulation of c-Src

The regulation of the kinase activity of c-Src has been proposed to involve the intramolecular interaction of the amino and carboxy termini (Cooper 1989). The model predicts that pY527 interacts intramolecularly with the SH2 domain, resulting in a protein in a "closed" conformation and repressed kinase activity. Alternatively, disruption of the intramolecular interaction, either by dephosphorylation or mutation of Y527, gives rise to a protein in an "open" conformation possessing high levels of kinase activity (Figure 1-2) (Courtneidge 1985; Kmiecik and Shalloway 1987). As predicted, mutations in the SH2 domain that eliminate phosphotyrosine binding also derepress the kinase activity of c-Src (Hirai and Varmus 1990; O'Brien, et al. 1990; Seidel-Dugan, et al. 1992). Studies of protein conformation of wild type and mutant c-Src proteins reveal changes in both the protease sensitivity and the ability of synthetic phosphotyrosine peptides to bind the SH2 domain in the activated form of the protein (Y527F) (MacAuley and Cooper 1989; Roussel, et al. 1991). In addition, binding studies with purified SH2 domains confirm the ability of peptides representing the carboxy terminus of c-Src (i.e., Y527) to bind the SH2 domain in solution (Bibbins, et al. 1993).

The mutations in the SH3 domain of c-Src also deregulate the c-Src kinase, although the exact role of the SH3 domain in kinase regulation is unclear (Kato, et al. 1986; Seidel-Dugan, et al. 1992). Analysis of amino

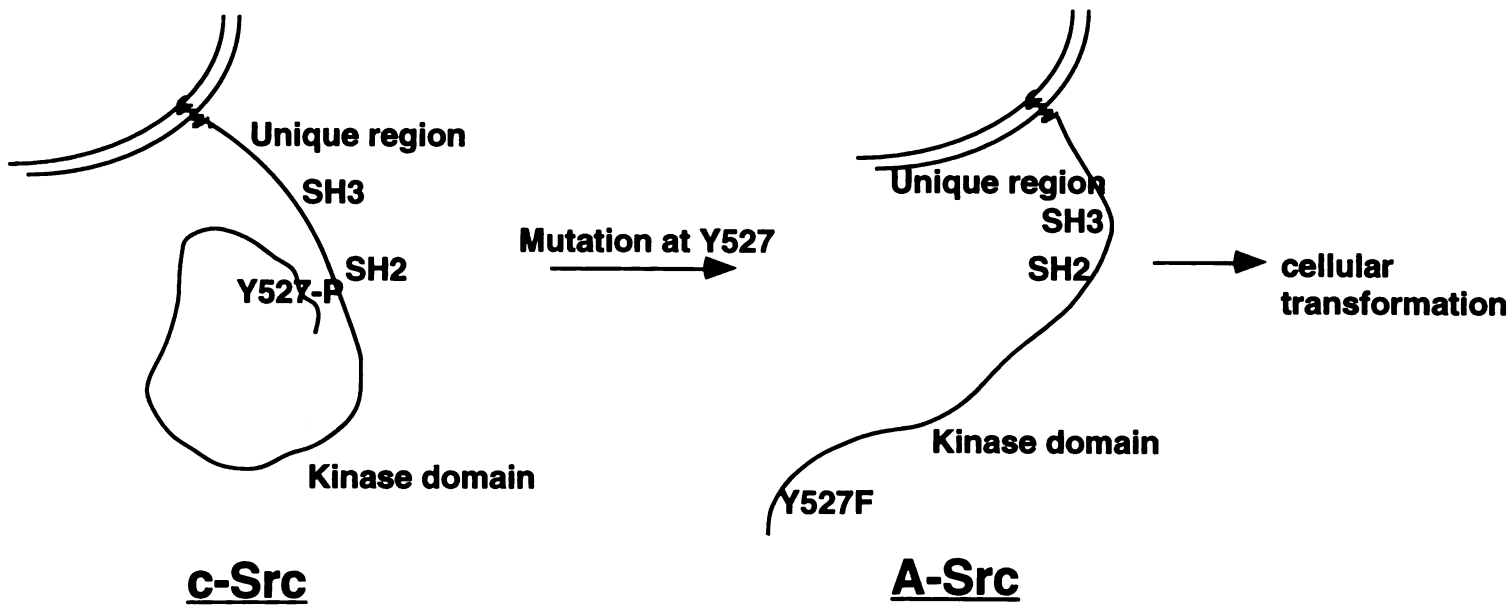
Unique region
SH3
SH2
Y527-P
Kinase domain
c-Src
Mutation at Y527
Unique region
SH3
SH2
Kinase domain
Y527F
A-Src
cellular
transformation

Figure 1-2

terminal mutants of c-Src in yeast suggest that the SH3 domain may also be involved in the intra-molecular interaction between the amino and carboxy termini. In addition to requiring the integrity of the SH2 domain, suppression of the kinase activity of c-Src in yeast also requires an intact SH3 domain, even though pY527 can still interact with the SH2 domain in these mutants (Murphy, et al. 1993; Okada, et al. 1993; Superti-Furga, et al. 1993). Recent structural studies of co-crystals containing both the amino terminus of lck and the carboxy terminal regulatory peptide demonstrates the binding of the tail peptide at the intermolecular SH3/SH2 contact, consistent with the ability of the SH3 domain to influence binding of the carboxy and amino termini (Eck, et al. 1994). It is also possible that the presence of SH3 stabilizes the SH2/Y527 interaction. In either case, it is clear that the amino terminal domains of c-Src regulate the activity of the kinase through a complex set of interactions with the carboxy terminus.

A basic prediction of the regulatory model is that activation of the c-Src kinase should be accompanied by the dephosphorylation of Y527. The complete dephosphorylation of Y527 has been observed only in polyoma virus infected cells and leads to transformation (Courtneidge 1985). The exact mechanism involved in the activation of c-Src by middle T antigen remains unclear, but may involve a change in c-Src conformation following middle T antigen binding that allows a tyrosine phosphatase access to pY527. A more physiologically relevant example of Y527 regulation is associated with the augmented kinase activity of c-Src during mitosis (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989; Shenoy, et al. 1989). In this case, pY527 undergoes only a partial dephosphorylation (50-70%) that correlates with a modest increase in kinase activity (2-4 fold) (Bagrodia, et al. 1991). A lower but significant level of pY527 dephosphorylation (10-15%) also accompanies the

increase in kinase activity of c-Src after stimulation of platelets by thrombin (Clark and Brugge 1993). Finally, the interaction of the SH2 domain of c-Src with the PDGF-receptor following PDGF treatment of cells implies that the SH2/pY527 interaction has been disrupted, although dephosphorylation of Y527 has not been demonstrated (Gould and Hunter 1988; Kypta, et al. 1990; Twamley-Stein, et al. 1993).

Dephosphorylation of Y527 and activation of c-Src is likely to occur in at least two distinct steps: (i) the disruption of the intramolecular interaction between pY527 and SH2, and (ii) the action of a tyrosine phosphatase to dephosphorylate pY527. While the sequence of these steps is not clear, it is reasonable to assume pY527 must be exposed before it can be dephosphorylated. This suggests that the SH2/pY527 interaction is able to block the dephosphorylation of pY527 by a tyrosine phosphatase. Consistent with this suggestion, the SH2 domain of PLCγ is able to protect tyrosine phosphorylated residues on the EGF receptor from the action of tyrosine phosphatases, while the SH2 domain of lck has been observed to similarly protect the negative regulatory tyrosine (Y505) (Gervais, et al. 1993; Rotin, et al. 1992). These results support a two step mechanism for the activation of the c-Src kinase; pY527 is first exposed and then dephosphorylated by a tyrosine phosphatase (Taylor and Shalloway 1993). The exposure of pY527 may result from the competition of other tyrosine phosphorylated proteins for the SH2 binding site, or a change in c-Src conformation that reduces the affinity of the SH2 domain for pY527. Consistent with the former mechanism, binding of peptides to the SH2 domain of c-Src results in an increase in kinase activity, possibly through the displacement of pY527 from the SH2 domain (Liu, et al. 1993a). Alternatively, phosphorylation of amino terminal residues have been observed during the activation of c-Src by PDGF and during mitosis and may

result in a change in protein conformation that exposes pY527 (Taylor and Shalloway 1993). However, the correlation between the phosphorylation of the amino terminus and the dephosphorylation of pY527 is not absolute. For example, mutating the mitotic phosphorylation sites only partially (50%) reduces dephosphorylation of Y527 and activation of c-Src (Shenoy, et al. 1992). These results may indicate that a combination of events, including amino terminal modifications and regulation of phosphatases or kinases specific for Y527, leads to the activation of the c-Src kinase.

Recently, advances have been made concerning the identity and potential regulation of the kinases and phosphatases specific for Y527. Studies in yeast suggest that Y527 is likely to be phosphorylated by a tyrosine kinase other than Src (Cooper and Runge 1987; Kornbluth, et al. 1987). The recent purification and cloning of a novel tyrosine kinase (the *c-src* kinase; CSK) and the findings that it can phosphorylate Y527 *in vitro* and *in vivo*, confirmed the existence of a distinct Y527 kinase. CSK has been reported to phosphorylate several src-family members and recent genetic experiments in mice have confirmed that CSK is the major source of Y527 phosphorylation (Imamoto and Soriano 1993; Nada, et al. 1993; Okada, et al. 1991). Interestingly, CSK is highly homologous to src-family members containing a carboxy terminal kinase domain and amino terminal SH2 and SH3 domains. However, CSK lacks sequences for membrane attachment, the autophosphorylation site and the regulatory carboxy terminal tyrosine (Sabe, et al. 1992). The absence of an obvious negative regulatory site suggests that CSK activity may be regulated by other means inside the cell. The recent finding that CSK associates with focal adhesions raises the intriguing possibility that negative regulation of c-Src is confined to a particular subcellular compartment (Sabe, et al. 1994).

In addition to the possible regulation of CSK, the subsequent dephosphorylation of pY527 may also be regulated. The inhibition of tyrosine phosphatases with sodium vanadate prevents the activation of the c-Src kinase during mitosis and confirms the involvement of tyrosine phosphatases in c-Src regulation (Bagrodia, et al. 1993). The identity of the Y527 phosphatase is unknown, although overexpression of the receptor-like protein tyrosine phosphatase, PTP alpha, in cells results in persistent activation of the c-Src kinase and cellular transformation (Zheng, et al. 1992). However, the high levels of PTP alpha expression required to observe c-Src activation and the dephosphorylation of Y527 raises questions concerning the specificity of the phosphatase under these conditions.

Signals that regulate c-Src

Increases in tyrosine phosphorylation during cell signaling have implicated tyrosine kinases, including c-Src, in transmitting these signals from the cell surface to the cytoplasm and nucleus. While many growth factor receptors are themselves tyrosine kinases, it has been proposed that src-family members may participate by amplifying signals transmitted by receptor tyrosine kinases. In particular, studies of the activation of growth factor receptors have directly implicated src-family members. These receptors transmit signals by nucleating the association of a complex of signaling proteins, many of which bind to tyrosine phosphorylated sites on the receptor following ligand activation (Cantley, et al. 1991). For example, the treatment of cells with platelet derived growth factor (PDGF) stimulates the phosphorylation of tyrosines on the cytoplasmic portion of the receptor, resulting in the association of a number of proteins including src-family members (Kypta, et al. 1990). Activation of the PDGF receptor also results in the phosphorylation of amino terminal sites on c-

Src and is accompanied by a modest increase in the kinase activity (Gould and Hunter 1988; Ralston and Bishop 1985). More recent studies have implicated the SH2 domain of c-Src in binding to tyrosine phosphorylated residues at the juxta-membrane region of the receptor (Mori, et al. 1993; Twamley, et al. 1992). Both the increase in kinase activity and the SH2-dependent association of c-Src with the PDGF-receptor suggest that the phosphorylation of Y527 is regulated after PDGF stimulation. The mechanism for Y527 regulation may involve a combination of amino terminal phosphorylations as well as the actions of a tyrosine phosphatase. Interestingly, the cytoplasmic tyrosine phosphatase, Syp, has also been characterized to associate with the PDGF receptor via its SH2 domain (Kazlauskas, et al. 1993). Although its relationship to c-Src is unclear, it is a prime candidate to regulate the phosphorylation state of Y527.

The activity of c-Src may also be regulated through stimulation of non-tyrosine kinase receptors. For example, thrombin treatment of platelets results in the activation of the c-Src kinase and alteration in its subcellular distribution. The change in subcellular distribution of c-Src is dependent on the presence of the integrin receptor, GPIIb-IIIa (Clark and Brugge 1993; Horvath, et al. 1992; Pumiglia and Feinstein 1993), suggesting that c-Src may also respond to signals transmitted through integrins. The relationship between c-Src and integrin receptors is further supported by the phosphorylation of integrins in v-Src-transformed cells (Tapley, et al. 1989). While the relationship between c-Src and integrins is intriguing, a direct association between c-Src and integrins has not been established. Other cells expressing high levels of *c-src*, including neurons and osteoclasts, provide potentially useful systems to further examine the relationship between c-Src and integrins.

The ability of multiple receptors to regulate c-Src may reflect the need for c-Src function in response to a broad set of stimuli. Other signals that regulate

c-Src remain to be explored and may be as varied as the cell types in which *c-src* is expressed.

Transformation by Src

Transformation is the acquisition, by cells *in vitro*, of the characteristics associated with cancer cells *in vivo*. The changes in transformed cells resemble many of the changes that occur as normal cells progress through the cell cycle; transformed cells have altered cell morphologies accompanied by the loss of adhesion and disassembly of the cytoskeleton. Unlike normal cells, transformed cells do not become pause in their cell cycles in the absence of growth factors. These changes lead to the properties that we associate with cancer, namely the loss of contact inhibition, the ability of cells to grow in suspension and the deregulation of cellular adhesion. While similar changes are observed in most transformed cells, there are characteristics of transformation that may reflect the specific activities and normal functions of each proto-oncogene.

Transformation of cells by v-Src is accompanied by high levels of tyrosine phosphorylated proteins, resulting from mutations in the regulatory domain of Src that deregulate the kinase. One of the most dramatic changes in v-Src transformed cells is the drastic alteration in cell morphology (Parsons and Weber 1989). The earliest change seen in cells expressing temperature sensitive alleles of v-src, after shift to the permissive temperature, is an increase in membrane ruffling. Membrane ruffling is followed by the rounding of cells and a disorganization of the microfilament network. In addition, a number of cytoskeleton related proteins are phosphorylated (discussed below), actin cables disappear and focal adhesions are rearranged into plaques or podosomes (Boschek, et al. 1981; Carley, et al. 1981; Shriver and

Rohrschneider 1981). While changes in cell shape result from transformation by many oncogenes, the drastic morphological changes in v-Src transformed cells and the association of v-Src with the cytoskeleton (discussed below) suggests that at least a portion of these changes may be specific to Src. Furthermore, mutations in the amino terminus of v-src give rise to cells with a fusiform morphology, indicating that cell rounding can be separated from other aspects of transformation by v-Src (i.e., anchorage independent growth). These mutants may affect the ability of the amino terminus of c-Src to interact with proteins involved in controlling cell morphology, independent of transformation (Parsons and Weber 1989).

Changes in cell morphology are accompanied by equally dramatic changes in the growth properties of v-src transformed cells. A variety of functional studies have suggested that v-Src activates the ras signal transduction pathway. This appears to be the case in the nerve growth factor induced differentiation of PC12 cells into neuron-like cells. Expression of v-src in undifferentiated PC12 cells results in NGF-independent neuronal differentiation (Alema, et al. 1985; Sassone-Corsi, et al. 1989) that depends on the activity of ras (Kremer, et al. 1991). These results and the increasing number of ras regulators phosphorylated by v-Src (discussed below), implies that v-Src can influence the regulation of a number of cell processes through the ras signaling pathway. However, the involvement of c-Src in the ras-signaling pathway has been more difficult to establish, perhaps because of the distinct activities of c-Src and v-Src.

In general, the changes in transformed cells are difficult to interpret with respect to the normal function of c-Src. There are several reasons to be critical of efforts to divine the cellular function of c-Src through changes that occur in v-Src transformed cells. It is significant that complete dephosphorylation of Y527

and maximal activation of the c-Src kinase has not been observed under normal physiological conditions. In fact, mutation or complete dephosphorylation of Y527 is only associated with abnormally high levels of kinase activity and cellular transformation, suggesting that cells normally do not contain highly active c-Src. This raises the possibility that a hallmark of Src-transformed cells, large numbers of tyrosine phosphorylated proteins, may not be directly related to the normal function of c-Src. Therefore, it has been assumed that a only a proportion of the changes associated with Src-transformation may be informative about the cellular function of c-Src.

Src substrates and associated proteins

The attempts to identify proteins relevant for transformation by v-Src have focused on finding substrates of the v-Src kinase and proteins that directly associate with v-Src. The holy grail in studying transformation by v-Src has been to discover the identity of the phosphotyrosine proteins that lead to transformation. However, identification of these substrates has been hampered by the promiscuous nature of tyrosine phosphorylation in cells transformed by v-Src. A number of approaches have revealed many candidate substrates, although it has been difficult to confirm their direct phosphorylation by v-Src. For the convenience of discussion, these putative substrates can be divided into three categories: (i) factors associated with signal transduction via receptor tyrosine kinases, (ii) factors associated with the cytoskeleton, and (iii) a group of phosphorylated factors whose significance is unclear.

One of the most productive strategies for studying putative substrates of v-Src has been the identification of proteins that are also tyrosine phosphorylated following growth factor stimulation of receptor tyrosine kinases. These proteins are believed to become tyrosine phosphorylated following their

association with the cytoplasmic domain of activated growth factor receptors. The fact that many of these proteins are also phosphorylated in cells transformed by v-Src is consistent with their phenotype of unregulated cell growth. Interestingly, many of the tyrosine phosphorylated proteins have been implicated in the regulation of the Ras signaling pathway. For example, Grb2, Shc, and SOS are non-catalytic "adapter" proteins that contain multiple SH2 and SH3 domains and have been proposed to link growth factor receptor tyrosine kinases to Ras signal transduction (Medema and Bos 1993). The tyrosine phosphorylation of these proteins may be involved in regulating their association following receptor tyrosine kinase activation, or in transformed cells (McGlade, et al. 1992; Pelicci, et al. 1992; Segatto, et al. 1993). In addition to these "adapter' proteins, effectors of Ras activity, including the 120Kd GTPase activating protein (GAP), may also be regulated in v-Src transformed cells (Bouton, et al. 1991). The phosphorylation of GAP may be of particular interest, as GAP has been reported to form an SH2-mediated complex with v-Src (Brott, et al. 1991; Ellis, et al. 1990; Fantl, et al. 1992; Koch, et al. 1992). These findings provide circumstantial evidence that v-Src can affect the ras signaling pathway, although the exact consequences of these tyrosine phosphorylations remains to be explored.

In addition to ras-related targets, v-Src has been reported to phosphorylate several other proteins associated with growth factor receptors. These include PLCγ, the p85 subunit of the PI3 kinase, and the MAP kinases, all proteins thought to be important in the growth factor induced signal transduction (Cantley, et al. 1991). The association of p85 with the SH3 domain of v-Src underscores the potential relevance of this phosphorylation event (Liu, et al. 1993b; Otsu, et al. 1991). It is unclear whether this association results in the direct phosphorylation of p85 by v-Src, or if there are other kinases involved. In

general, the fact that these substrates are shared with receptor tyrosine kinases suggests that v-Src may function similarly to activated receptor tyrosine kinases, effectively signaling in the absence of receptor-ligand engagement. Since receptor tyrosine kinases are equally capable of phosphorylating these substrates, it is difficult to know what purpose their phosphorylation by c-Src would serve. Rather, it seems likely that these proteins represent fortuitous phosphorylations caused by the promiscuous activities of v-Src, independent of the normal role of c-Src.

The second major category of potential Src substrates includes proteins associated with cytoskeletal formation or regulation. Some of these substrates have been identified due to their relatively high levels of phosphorylation in v-Src transformed cells (Parsons and Weber 1989), while others have been identified through the generation of monoclononal antibodies to prominent v-Src substrates (Kanner, et al. 1990). One of the first described phosphorylations that links v-Src with cytoskeletal regulation is phosphorylation of the fibronectin receptor in chicken embryo fibroblasts (Hirst, et al. 1986). Significantly, the distribution of fibronectin receptors and their ability to interact with other cytoskeletal proteins are compromised in transformed cells (Tapley, et al. 1989). However, the stoichiometry of tyrosine phosphorylation is low and it has been difficult to show that the functional changes observed in transformed cells are directly due to the phosphorylation of integrins. The identification of other cytoskeletal targets in v-Src transformed cells suggests that disruption of the cytoskeleton during transformation may result from the accumulated modifications of multiple proteins. For example, the focal adhesion-associated proteins vinculin, talin and ezrin are also tyrosine phosphorylated in transformed cells (Parsons and Weber 1989). Again, the low levels of phosphorylation in transformed cells and the absence of phosphorylation in

normal cells raises questions concerning the relevance of these modifications for both transformation and c-Src function. More recently, the focal adhesion protein, paxillin, has been shown to be phosphorylated in v-Src transformed cells (Glenney and Zokas 1989). In contrast to the other cytoskeletal proteins, paxillin is also tyrosine phosphorylated following growth factor stimulation, suggesting that this modification may be physiologically relevant (Rankin and Rozengurt 1993). In general however, the consequences of these phosphorylations for the physiology of cell adhesion and morphology remains unknown. A more detailed understanding of how these proteins function to link integrins to the cytoskeleton will undoubtedly shed light on the mechanisms underlying v-Src disruption of the cytoskeleton and ability of c-Src to regulate these structures in normal cells.

The identification of less abundant cytoskeletal protein substrates have been advanced by the generation of monoclonal antibodies that recognize tyrosine phosphorylated proteins in v-Src transformed cells (Kanner, et al. 1990). The subsequent cDNA cloning of several of these potential substrates has revealed their connection with the cytoskeleton. For example, the 85kD protein, cortactin, colocalizes with and is able to bind to F-actin in peripheral extensions of normal cells and is found in rosettes (podosomes) of Src-transformed cells (Wu, et al. 1991). Cortactin contains an SH3 domain and has also been reported to be phosphorylated in response to growth factors (Wu and Parsons 1993). These phosphorylations have been shown to be dependent on the levels of c-Src in the cell, providing indirect evidence that cortactin may also be a substrate of c-Src (Maa, et al. 1992). Another protein identified in v-Src transformed cells, AFAP-110, shows no relation to any known proteins but associates with v-Src via the SH3 and SH2 domains (Flynn, et al. 1993; Kanner, et al. 1991). AFAP-110 also binds to F-actin and can be found in

rosettes in Src-transformed cells in a very similar pattern to cortactin, suggesting that these two proteins may be phosphorylated in the same subcellular compartment. The characterization of other candidate substrates recognized by monoclonal antibodies (p130, p120, p210, p118) may provide additional insight into the relationship between the cytoskeleton and Src proteins.

The identification of a novel cytoplasmic tyrosine kinase (FAK) that associates with focal adhesions as a putative substrate of v-Src supports a long held suspicion that v-Src regulates other tyrosine kinases in transformed cells (Schaller, et al. 1992). The tyrosine kinase, FAK, is a 125kD protein that contains a consensus autophosphorylation site and no src-homology domains. While little is known about the regulation of FAK, it appears that it is highly phosphorylated in transformed cells and during cell adhesion on fibronectin (Guan and Shalloway 1992; Kornberg, et al. 1992). The phosphorylation of FAK and its association with focal adhesions naturally implicates this kinase in the regulation of cell adhesion and may be related to the loss of adhesion observed in cells transformed by v-Src. This connection is further supported by the direct interaction of FAK with Src-family members in chicken embryo fibroblasts and transformed cells (Cobb, et al. 1994). Recent binding studies have shown that this association is mediated through the interaction of the SH2 domain of Src and the autophosphorylation site of FAK (Schaller, et al. 1994). Further analysis of the regulation of FAK and the effect of tyrosine phosphorylations are required to fully understand the role it plays in transformation by v-Src. Importantly, FAK represents one of the few targets of v-Src that may also interact with c-Src, strongly implying that c-Src may also be involved in regulating FAK.

Finally, the third category of potential v-Src substrates includes a number of proteins whose significance for transformation is less clear. These putative

substrates include the EGF receptor, glycolytic enzymes (enolase, phosphoglycerate mutase and lactate dehydrogenase), clathrin heavy chain, calpactin (p36), and more recently caveolin. The questionable significance of these phosphorylations arises due to their poor correlation with transformation, the low stoichiometry of phosphorylation, or simply the lack of any clear consequences of these phosphorylations. For example, the consequences of tyrosine phosphorylation for calpactin remain unknown and do not correlate with cellular transformation (Parsons and Weber 1989). It is possible that calpactin is phosphorylated due to the abundance of both calpactin and v-Src in the same cell. Nonetheless, the role of calpactin in regulating membrane fusion with the cytoskeleton provides an intriguing, although tenuous, connection between a v-Src substrate and the increased membrane ruffling in transformed cells.

Perhaps more significant is the recent finding that v-Src can phosphorylate caveolin, a structural protein that forms complex structures on the plasma membrane known as caveolae (Rothberg, et al. 1992). Reports that caveolae are involved in signal transduction events at the plasma membrane may implicate c-Src in the potential regulation of these structures following receptor activation (Sargiacomo, et al. 1993). However, the ability of c-Src to phosphorylate caveolin and the consequences of these potential modifications for the function of caveolae are unknown.

In general, the plethora of potential Src substrates makes the determination of the relevance of these phosphorylations daunting. One approach to resolving this dilemma is to focus the analysis on the proper subcellular compartment. For example, the reported presence of Src at the plasma membrane makes caveolin an attractive candidate for a relevant Src substrate. On the other hand, the association of v-Src with adhesion plaques

(discussed below) similarly highlights the potential relevance of the cytoskeletal substrates described previously. Thus, it is important to possess a detailed description of the sub-cellular distribution of Src proteins in order to evaluate the relevance of potential substrates and their implications for c-Src function and transformation by v-Src.

Sub-cellular distribution of Src proteins

In most cases, Src proteins have been found to associate with cellular membranes. Initially, immunofluorescence studies of v-Src transformed cells revealed a concentration of staining at regions of cell/cell contact, in podosomes (adhesion plaques) at the bottom of the cell and concentrated in a peri-nuclear position (Nigg, et al. 1982; Rohrschneider 1980; Rohrschneider 1979). On the basis of immuno-electron microscopy and crude membrane fractionation, the great proportion of v-Src was reported to associate specifically with plasma membranes (Courtneidge, et al. 1980; Willingham, et al. 1979). The presence of v-Src at the plasma membrane is consistent with its proposed role in relaying signals from outside the cell and, as mentioned, would place v-Src in a prime position for modifying proteins associated with growth factor receptors. However, these experiments fail to distinguish between the three distinct populations of Src observed in immunofluorescence studies (cell/cell contacts, podosomes, and peri-nuclear), therefore leaving open the possibility that only a portion of Src associates with plasma membranes.

In addition to plasma membranes, v-Src clearly associates with podosomes (adhesion plaques) in transformed cells. These structures are similar to the focal adhesions found in normal cells, although they differ slightly in structure and composition (Nakamura, et al. 1993; Nigg, et al. 1986). The presence of v-Src in adhesion plaques underscores the potential relevance of

the phosphorylation of focal adhesion components in transformed cells. It is also possible that v-Src associates with cytoskeletal structures other than adhesion plaques. Detergent fractionation of cells has described a detergent-insoluble population of v-Src that may associate with various components of the cytoskeleton (Hamaguchi and Hanafusa 1987). Deletion mutants of v-Src have implicated sequences in the amino terminus in the interaction with the detergent-insoluble fraction of the cell (Fukui, et al. 1991). Whether this population of v-Src is associated with adhesion plaques or some other part of the cytoskeleton remains to be determined. In contrast, c-Src does not associate with the detergent-insoluble fraction of cells, emphasizing the different distribution of c-Src. The fact that alleles of v-Src contain multiple mutations and there is a major rearrangement of the cellular architecture in transformed cells may help to explain the differences in localization between v-Src and c-Src.

While v-Src has been reported to associate with adhesion plaques, the detergent-insoluble fraction of cells and the plasma membrane, the majority of c-Src is detected in a punctate staining pattern, concentrated at the peri-nuclear region of the cell, that is characteristic of membrane vesicles (Courtneidge, et al. 1980; Resh and Erikson 1985; Rohrschneider 1980). Consistent with this possibility, c-Src has been reported to associate with vesicles in other cell types. For example, c-Src is enriched in neuronal growth cones and associates with specialized secretory vesicles in differentiated PC12 cells and neuroendocrine cells (Grandori and Hanafusa 1988; Linstedt, et al. 1992; Sobue and Kanda 1988). Similarly, osteoclasts contain high levels of c-Src on intra-cellular membranes and vacuoles near the ruffled membrane (Horne, et al. 1992; Tanaka, et al. 1992). The only exception to the presence of c-Src on intra-cellular membranes may be platelets. Detailed studies of platelets have

indicated that c-Src is associated with the both the plasma membrane and membranes of the surface-connected canalicular system (Ferrell, et al. 1990). However, the complex organization of platelets makes it difficult to compare these structures to compartments in other cell types. Taken together, these experiments demonstrate the overlapping, yet distinct, distribution of the normal and transforming Src proteins. These differences suggest that the normal function of c-Src may comprise only a subset of the cellular events that occur during transformation.

The physiological role of c-Src

The expression of c-Src in a wide variety of cell types suggests that it is involved in one or more essential cellular functions. However, genetic experiments have shown that mice homozygous for a disruption of the *c-src* gene develop osteopetrosis after birth (Soriano, et al. 1991). Osteopetrosis in *src*$^{-/-}$ mice develops from a cell-autonomous defect in osteoclasts (Lowe, et al. 1993), specialized secretory cells involved in degrading bone matrix in order to form properly shaped bones. When cultured in vitro, *src*$^{-/-}$ osteoclasts fail to form pores in a model substrate, implicating c-Src in a basic cellular function required for the proper function of osteoclasts (Boyce, et al. 1992). The presence of c-Src on intracellular membranes and at the ruffled border of osteoclasts (Boyce, et al. 1993; Horne, et al. 1992; Tanaka, et al. 1992) is consistent with the possibility that c-Src may regulate membrane trafficking responsible for transporting lysosomal proteases to the osteoclast lumen that makes contact with the bone matrix. Alternatively, the involvement of v-Src in cellular adhesion could implicate c-Src in the regulation of osteoclast adhesion on bone matrix, an essential event for proper function of osteoclasts (Teti, et al. 1991). In either case, osteoclasts are extremely dependent on c-Src and are

unable to compensate for the loss of c-Src in spite of the presence of multiple src-family members (Horne, et al. 1992).

The otherwise normal development of mice lacking c-Src suggests that other src-family members can functionally compensate for the loss of c-Src in cells other than osteoclasts. The finding that src kinases can compensate biochemically for each other is consistent with a degree of functional overlap among src-family members. For example, an increase in the activity of the lyn kinase has been observed in macrophages lacking hck, presumably in order to compensate for the absence of the normally highly expressed hck (Lowell, et al. 1994). Functional redundancy between src-family members is also supported by the increased severity of phenotypes associated with mice lacking multiple src-family members (Lowell, et al. 1994). Although compensation by src-family members may allow the relatively normal development of mice lacking one or more src genes, it is possible that closer examination of specific cell types may reveal compromised cellular processes. These kinds of detailed analyses in cells lacking c-Src have only recently been attempted. Neurons lacking c-Src have recently been shown to be impaired for neurite outgrowth on the cell adhesion molecule L1 (Ignelzi, et al. 1994). These results hold the promise that cells derived from src-deficient mice can be used to directly examine the role of c-Src in individual cellular processes.

While it is tempting to speculate that the physiological function of c-Src is intimately associated with cellular transformation, it has been difficult to identify the characteristics of cellular transformation relevant for c-Src. The disparate subcellular distributions of v-Src and c-Src support the contention that the mutant and wild type proteins are involved in distinct cellular processes. One way to focus on the cellular processes specific for c-Src function is to determine the precise subcellular compartments where it acts. A detailed understanding

of these subcellular compartments will allow a directed biochemical analysis of c-Src substrates and associated proteins. Furthermore, the identification of relevant subcellular compartments will allow the examination of the associated cellular processes in src-deficient cells. The general cellular processes regulated by c-Src are likely to be shared by the many cells that express c-Src, although the details of these processes may reflect the specific requirements of each cellular environment. Therefore, findings related to c-Src function in one cell type will probably be applicable to c-Src function in other cell types. Finally, by understanding the cellular function of c-Src, the exact cellular mechanisms by which a normal cell is transformed into a cancer cell can also begin to be addressed.

These studies examine the subcellular distribution of c-Src in mammalian fibroblasts, the regulation of the c-Src distribution and the implications of these findings for the cellular function of c-Src. We find that under normal conditions c-Src is predominantly associated with endosomal membranes. The association of c-Src with endosomal membranes may implicate c-Src in the regulation of endosomal membranes and protein trafficking, although there is no evidence in support of this possibility. However, the subcellular distribution of c-Src is regulated by dephosphorylation of Y527 which leads to the association of c-Src with focal adhesions via sequences in the amino terminal half of the protein. Association with focal adhesions occurs independently of the kinase domain and leads to alterations in the structural and biochemical properties of focal adhesions. Finally, the distribution of c-Src is regulated during adhesion of fibroblasts on fibronectin. Dephosphorylation of Y527 is accompanied by a transient increase in the kinase activity of c-Src and the subsequent redistribution of c-Src to newly formed focal adhesions. *Src*$^{-/-}$ fibroblasts are defective in cell spreading and can be complemented by the

amino terminal half of c-Src. These results suggest that c-Src is regulated during cellular adhesion and may function to positively influence cell spreading in mammalian fibroblasts.

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Chapter Two:

Association of c-Src with Endosomal Membranes in Mammalian Fibroblasts

Abstract

We have examined the sub-cellular localization of c-Src in mammalian fibroblasts. Analysis of indirect immunofluorescence by three dimensional optical sectioning microscopy revealed a granular cytoplasmic staining that co-localized with the microtubule organizing center. Immunofluorescence experiments with antibodies against a number of membrane markers demonstrated a striking co-localization between c-Src and the cation-independent mannose-6-phosphate receptor (CI-MPR), a marker that identifies endosomes. Both c-Src and the CI-MPR were found to cluster at the spindle poles throughout mitosis. In addition, treatment of interphase and mitotic cells with Brefeldin A resulted in a clustering of c-Src and CI-MPR at a peri-centriolar position. Biochemical fractionation of cellular membranes showed that a major proportion of c-Src co-enriched with endocytic membranes. Treatment of membranes containing horseradish peroxidase to alter their apparent density also altered the density of c-Src-containing membranes. Similar density shift experiments with total cellular membranes revealed that the majority of membrane-associated c-Src in the cell is associated with endosomes, while very little is associated with plasma membranes. These results support a role for c-Src in the regulation of endosomal membranes and protein trafficking.

Introduction

C-Src is a member of a family of cytoplasmic tyrosine kinases that are associated with cellular membranes and are thought to be involved in signal transduction events underlying growth control (for review see (Cooper 1989). The normal function of c-Src is not clear, although studies of mutant forms of the

protein (e.g.. the viral transforming protein v-Src) have implicated c-Src in the control of cell growth and proliferation. On the other hand, c-Src is expressed at high levels in terminally differentiated cells such as platelets and neurons (Cotton and Brugge 1983; Golden, et al. 1986). In addition, c-Src is activated during mitosis in fibroblasts and thus may mediate certain mitotic events (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989; Shenoy, et al. 1989). Finally, a homozygous disruption of the *c-src* gene in mice is not lethal, but instead leads to defects in bone remodeling (probably as a result of defective osteoclast function) (Soriano, et al. 1991).

Clues about c-Src function have been obtained from studies of the sub-cellular location of src proteins. Immunofluorescence and biochemical fractionation studies have suggested that both c-Src and v-Src are localized to peri-nuclear and plasma membranes, while transforming proteins have also been found in association with adhesion plaques (Courtneidge, et al. 1980; Resh and Erikson 1985; Rohrschneider 1980). Immunofluorescence studies of c-Src have also revealed a punctate staining pattern that is dependent on membrane attachment domains in the amino-terminal region of the protein (Kaplan, et al. 1990). This punctate pattern is characteristic of membrane vesicles, suggesting that c-Src is associated with cellular membranes distinct from the plasma membrane. Although these membranes remain uncharacterized, analysis of c-Src over-expressed in 3T3 cells indicates a possible connection with endosomal membranes (David-Pfeuty and Nouvian-Dooghe 1990). In addition, recent efforts to localize c-Src in differentiated PC12 cells and neuroendocrine cells have shown that a significant proportion of c-Src is associated with an endosomally derived population of vesicles (Linstedt et al, personal communication)(Grandori and Hanafusa 1988).

To gain insight into the normal role of c-Src, we have used high resolution immunofluorescence analysis and biochemical fractionation to characterize the nature of c-Src-containing membranes. Our results indicate that c-Src is mainly associated with endosomal membranes and is particularly enriched in a population of late endosomes.

Results

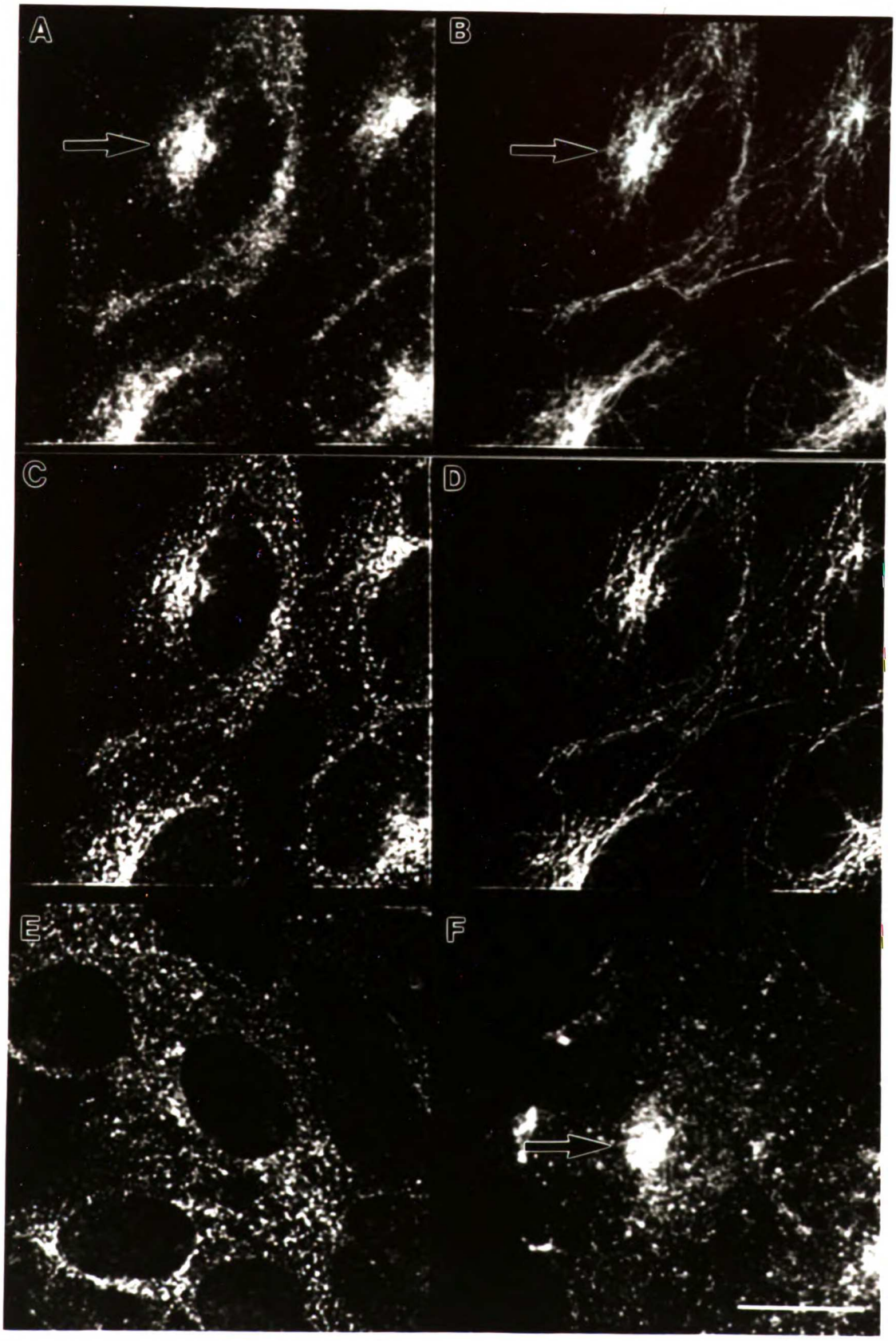
Co-staining of c-Src with late endosomes at the MTOC

Rat-1 fibroblasts over-expressing chicken c-Src (RTCS cell line) were analyzed by indirect immunofluorescence with a monoclonal antibody against c-Src (MAb 327). To enhance resolution, immunofluorescence was recorded with an optical sectioning microscope and both individual optical sections and three dimensional reconstructions of the images were analyzed. Two general populations of c-Src were apparent: a granular staining throughout the cytoplasm and a concentrated staining adjacent to the nucleus (Figure. 2-1A). Staining with a different monoclonal antibody against c-Src (MAb 2-17) demonstrated a similar staining pattern, and signals with either antibody were blocked by addition of purified chicken c-Src produced with the baculovirus expression system (Morgan, et al. 1991)(data not shown).

Staining with antibodies against tubulin revealed that c-Src co-localizes with the microtubule organizing center (MTOC), the region of the cell where many membranous organelles are located (Figure. 2-1B). The concentration of c-Src at the MTOC was not simply due to the increased cell thickness in this region of the cell, as analysis of optical sections showed a distinct region of staining along the vertical axis of the cell where c-Src is concentrated (Figure. 2-1C). In addition, c-Src concentrated at the MTOC co-stains with individual

Figure 2-1

Co-staining of c-Src and the MTOC. Projections comprising multiple optical sections of RTCS fibroblasts (see Methods) were obtained with MAb 327 against c-Src (A) and MAb tub 2.1 against tubulin (B). Individual optical sections that represent information from the middle of the cells stained with MAb 327 against c-Src (C) and MAb tub2.1 against tubulin (D) are also presented. A single optical section is presented from RTCS cells treated for 30 minutes with 0.5ug/ml nocodazole to depolymerize microtubules, and stained with MAb 327 (E). A projection of Rat-1 fibroblasts expressing only endogenous *c-src* was obtained with MAb 327 against c-Src (F). Endogenous c-Src signal was enhanced with a rabbit anti-mouse antiserum (sandwich antibody) between the primary monoclonal antibody and fluorophore-conjugated secondary antibody. Arrows indicate the MTOC (Scale bar=10μm).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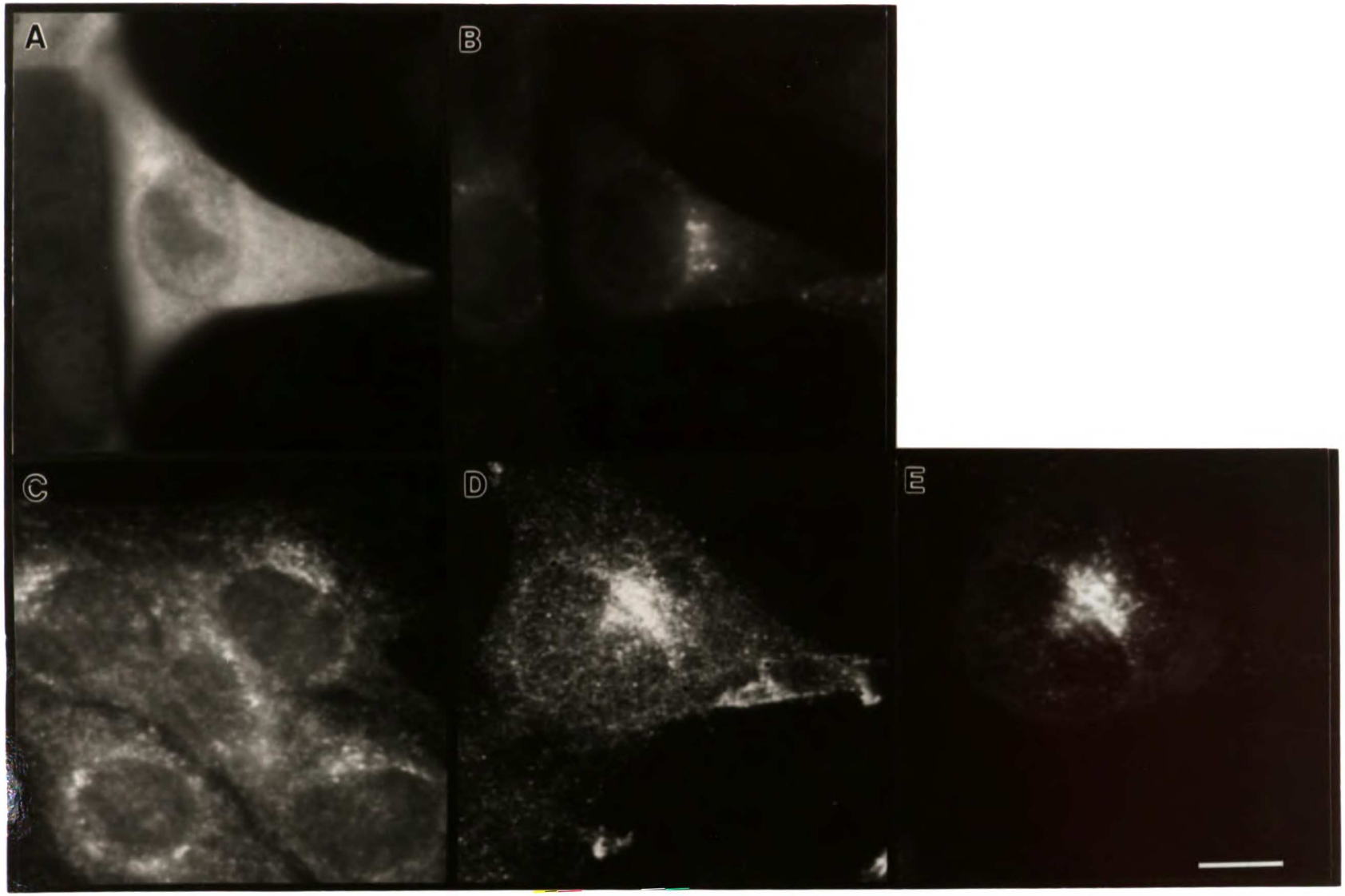
cellular microtubules when optical sections from cells stained with antibodies against tubulin (Figure. 2-1D) are compared to the identical sections stained with antibodies against c-Src (Figure. 2-1C). This staining pattern is also not due to the over-expression of chicken c-Src in these cells, as endogenous c-Src in Rat-1 fibroblasts exhibited an identical pattern (Figure. 2-1F). In addition, the depolymerization of cellular microtubules with the drug nocodazole dispersed c-Src (Figure. 2-1E) and suggested a direct association with the MTOC. C-Src rapidly returned to the peri-nuclear region of the cell upon removal of nocodazole and the repolymerization of cellular microtubules (data not shown).

C-Src is myristylated at its NH_2-terminus (Buss and Sefton 1985), and the myristylation signal is necessary but not sufficient for association with cellular membranes (Kaplan, et al. 1990). To address the issue of whether membrane association of c-Src is required for the localization of c-Src at the MTOC, we characterized a 3T3 fibroblast cell line expressing c-Src in which the second amino acid glycine has been replaced with alanine. In v-Src, this amino acid change results in a protein that cannot be myristylated and therefore does not associate with cellular membranes (Buss, et al. 1986; Kamps, et al. 1986). Immunofluorescence analysis of cells expressing c-Src containing this substitution showed a diffuse cytoplasmic staining, indicating that membrane attachment is required for co-localization at the MTOC, as previously suggested (Kaplan, et al. 1990)(Figure. 2-2A,B). We also failed to observe the granular cytoplasmic c-Src staining pattern, indicating that membrane association is required for this localization as well. To determine whether membrane association alone is sufficient for localization of c-Src at the MTOC, we analyzed a membrane-associated fusion protein comprising the first 14 amino acids of c-Src fused to pyruvate kinase (Kaplan, et al. 1990). When expressed in Rat-1

Figure 2-2:

Myristylation of c-Src is required for localization at the MTOC and for co-staining with CI-MPR. 3T3 fibroblasts expressing a non-myristylated mutant form of chicken c-Src stained with MAb 327 (A) and anti-sera against CI-MPR (B) are presented as single unprocessed images. RTCS fibroblasts were stained with MAb 10E6 against cis/medial golgi and a single unprocessed image is presented (C). RTCS fibroblasts stained with MAb 327 (D) and anti-sera against CI-MPR (E) are presented as projections (Scale bar=10μm).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cells this protein exhibited a punctate staining pattern and did not co-localize with the MTOC (data not shown). We therefore conclude that myristylation is necessary but not sufficient to allow MTOC localization of c-Src, supporting the notion that there are other domains of c-Src that contain information important for localization (Kaplan, et al. 1990).

Membrane organelles involved in various secretory and endocytic processes have been localized to the MTOC. To identify candidate membranes that contain c-Src, co-localization studies were performed with a number of antibodies against markers of specific membrane compartments. The golgi compartment was identified with several markers, including fluorescein-conjugated wheat germ agglutinin and two monoclonal antibodies, 10E6 and 8B3, specific for cis/medial golgi elements (Wood, et al. 1991). These markers stained a peri-nuclear region of the cell that was distinct from c-Src, suggesting that c-Src is not found in cis/medial golgi membranes (Figure. 2-2C). Similarly, staining of the endoplasmic reticulum with antibodies against BiP (Bole, et al. 1989) and staining of lysosomes with antibodies against lgp120 (Lewis, et al. 1985) revealed that these two membrane compartments are clearly distinct from c-Src at the MTOC (data not shown).

In contrast, staining with antibodies to the cation-independent mannose 6-phosphate receptor (CI-MPR), a marker of endosomes and trans golgi network (TGN), revealed a striking co-localization with c-Src at the MTOC (Figure 2-2D,E). While all endosomal membranes are believed to contain the CI-MPR, the CI-MPR-positive endosomes located at the MTOC represent vesicles at a late stage in the endocytic pathway (late endosomes or pre-lysosomes)(Messner, et al. 1989). CI-MPR-positive vesicles also require intact microtubules for their localization, unlike early endosomes (KBK, unpublished observations) (Matteoni and Kreis 1987). Thus, our results suggest that c-Src is

associated with membranes at the MTOC that represent a late stage in the endocytic pathway. While no direct attempts were made to distinguish CI-MPR-positive endosomes from CI-MPR-positive TGN, we observed that c-Src did not exhibit the tubular staining pattern characteristic of the TGN (data not shown).

C-Src and CI-MPR co-localize after treatment with BFA

To further analyze c-Src-containing membranes and to distinguish these membranes from cis/medial golgi, we treated cells with brefeldin A (BFA). BFA disrupts the normal distribution of cis/medial golgi membranes, causing retrograde transport to the endoplasmic reticulum. This results in a reticular staining pattern when the golgi apparatus is visualized by immunofluorescence (Lippincott-Schwartz, et al. 1990; Lippincott-Schwartz, et al. 1989). In contrast , BFA treatment does not result in the transport of CI-MPR-containing membranes into the endoplasmic reticulum (Chege and Pfeffer 1990; Lippincott-Schwartz, et al. 1991; Wood, et al. 1991). If c-Src is associated with CI-MPR-containing late endosomes, then the behavior of c-Src and CI-MPR after BFA treatment should be similar, and yet distinct from cis/medial golgi membranes.

Consistent with previous findings, we found that after BFA treatment the golgi marker 10E6 exhibited a punctate/reticular staining pattern characteristic of endoplasmic reticulum staining in these cells (Figure. 2-3A). We also observed that CI-MPR-positive membranes do not enter the endoplasmic reticulum after BFA treatment. After BFA treatment, both c-Src and CI-MPR (Figure.2-3B,C) formed a tight cluster at the centrosome in a staining pattern that is distinct from cis/medial golgi membranes. This clustering of membranes containing c-Src and CI-MPR at the centrosome is less diffuse than the staining pattern at the MTOC in untreated cells. The clustering occurs after 30 minutes

Figure 2-3

Brefeldin A treatment causes c-Src and CI-MPR membranes to cluster at the centrosome. RTCS fibroblasts were treated with 1μg/ml brefeldin A for 30 minutes at 37°C and then processed for immunofluorescence. A single unprocessed image of staining with MAb 10E6 against cis/medial golgi is presented (A), while projections of multiple optical sections are presented for staining with MAb 327 against c-Src (B) and anti-sera against CI-MPR (C). Arrows indicate clustered c-Src and CI-MPR staining at the centrosome (Scale bar=10μm).

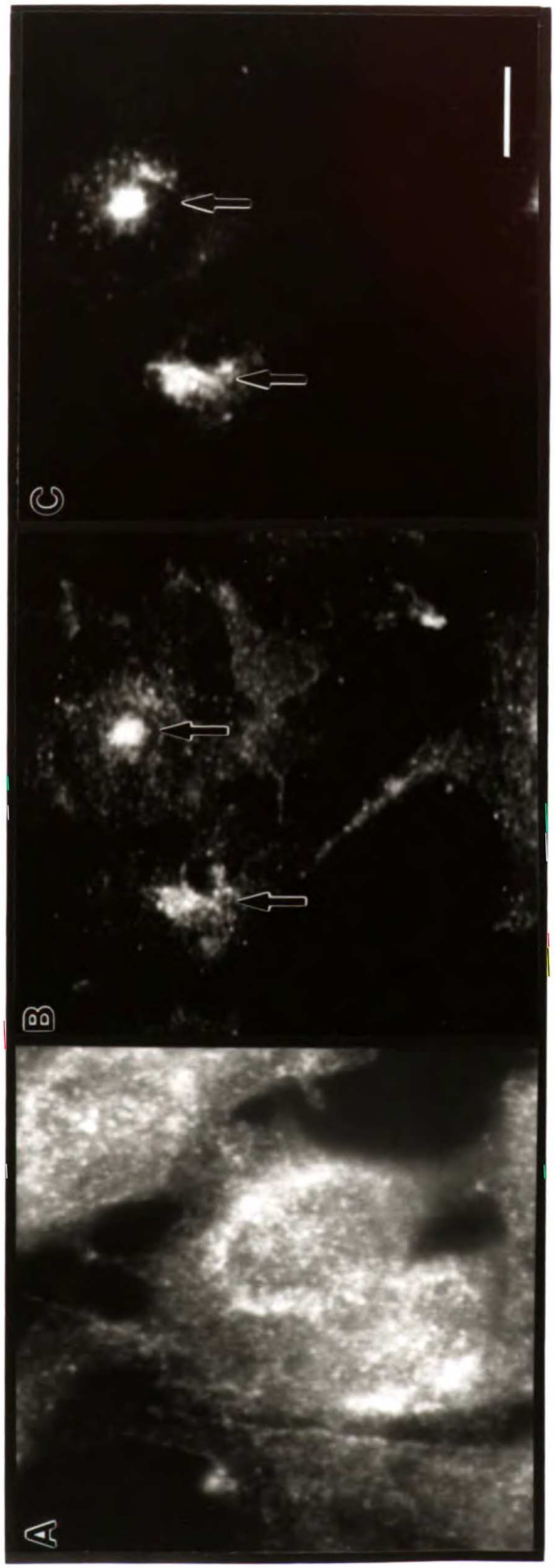

C
B
A

Figure 2-4

Co-staining of c-Src and CI-MPR at the spindle poles of mitotic cells. Panel A-C represent projections of the same RTCS fibroblast prophase cell stained with MAb 327 against c-Src (A), anti-sera against CI-MPR (B) and Hoechst stain (C) to reveal the chromatin. Projections were obtained of Rat-1 fibroblasts stained with MAb 327 against c-Src plus a sandwich antibody (see figure 1 legend; D) and with Hoechst stain to reveal the chromatin (E). Arrows indicate peri-centriolar staining in mitotic cells (Scale bar=10μm).

A
B
C
D
E

of BFA treatment, is reversible, and is dependent on intact microtubules, since pre-treatment with nocodazole abolishes the effect (data not shown). In untreated populations of fibroblasts, some cells (5-10%) exhibit a similar clustering of c-Src and CI-MPR staining at the centrosome, suggesting that the clustering of endosomes in this region can occur in the absence of BFA. These results further support the notion that c-Src and the CI-MPR are in a similar cellular compartment that is distinct from the cis/medial golgi apparatus.

Co-localization of c-Src and CI-MPR at the spindle poles during mitosis

The activation of c-Src during mitosis suggests that it may play a role in mitosis. Therefore we also examined the localization of c-Src in mitotic cells (identified by the state of their chromatin after Hoechst staining; see Methods). During mitosis, c-Src was observed to cluster at the dividing centrosomes (Figure 2-4A; arrows). Co-staining with anti-CI-MPR clearly demonstrated that the CI-MPR is clustered at the same peri-centriolar position as c-Src (Figure 2-4B; arrows). Reconstructive analysis of three dimensional optical sections confirmed that c-Src staining corresponded to the position of migrating centrioles as determined by tubulin staining (data not shown). Analysis of additional stages of mitosis showed that the peri-centriolar populations of c-Src and CI-MPR were present throughout mitosis and appear positioned to form membrane organelles at the MTOC following telophase.

Over-expression of c-Src did not affect staining, as similar patterns were apparent when endogenous levels of c-Src were examined in Rat-1 cells (Figure 2-4D,E). The mitotic localization of both CI-MPR and c-Src is dependent on a properly formed spindle, since cells treated with nocodazole no longer show peri-centriolar staining of c-Src or CI-MPR (data not shown). In addition,

staining of cells with antibodies specific for the golgi apparatus revealed that golgi membranes became vesicularized and were scattered throughout the cell during mitosis as previously reported for HeLa cells (Lucocq and Warren 1987; Moskalewski and Thyberg 1990). This contrast between golgi staining and c-Src /CI-MPR staining during mitosis provides additional evidence that c-Src associates with endosomal membranes and not with cis/medial golgi elements.

Biochemical fractionation reveals that c-Src co-enriches with endosomal membranes

We next examined the association of c-Src with endosomal membranes by standard cell fractionation techniques. RTCS cells were allowed to internalize the fluid phase marker horseradish peroxidase (HRP) to label the endocytic compartment. Cellular membranes isolated after a 100,000xg spin (P100 pellet) were placed at the bottom of a sucrose step gradient consisting of 45%, 32%, and 18% steps. Membranes were allowed to float to equilibrium during centrifugation. We determined that the endocytic marker HRP and thus endocytic membranes from these cells were enriched at the 18%/32% interphase of the gradient (data not shown). We analyzed the fractions from the gradient by immunoblotting to identify c-Src and observed a major peak at the 18%/32% interface (Figure 2-5A). Based on immunoblots with ^{125}I-labeled antibody, we estimate that 65% of the total c-Src on the gradient was at the 18%/32% interface (Figure 2-5B). A similar pattern of enrichment was observed with cells expressing endogenous levels of c-Src (data not shown). The density at the 18%/32% interface is characteristic of endosomal membranes and is therefore consistent with the enrichment of c-Src with endocytic vesicles. Analysis of markers for membrane compartments demonstrated that fractions enriched for c-Src are distinct from lysosomal (data not shown), golgi apparatus,

and plasma membrane markers (Figure 2-5C-E) , although a small amount of plasma membrane marker was found at the 18%/32% interface (see methods).

C-Src was sometimes observed in the 45% and 32% fractions. To address whether this material was membrane-associated, each fraction from the sucrose step gradient was diluted to a final concentration of 8.5% sucrose, pelleted at 100,000xg and the pellet analyzed for c-Src by immunoblotting. Approximately 90% of the c-Src in these fractions remained in the supernatant and was therefore presumed to be soluble. In addition, treatment of cellular membranes with a water soluble, membrane impermeable cross-linking reagent (DTSSP) prevented the appearance of c-Src in the 45% or 32% fractions, and resulted in a corresponding increase in the amount of c-Src at the 18%/32% interface. We therefore suspect that a under normal homogenization conditions a minor fraction of c-Src dissociates from cellular membranes and sediments near the bottom of the sucrose gradient.

We were particularly interested in separating plasma membranes and endosomal membranes because of the reported association of c-Src with plasma membranes (Courtneidge, et al. 1980). To further separate membranes, material from the 18%/32% interface of the sucrose gradient was run on a Percoll gradient and fractions were analyzed for the presence of c-Src. In multiple experiments, c-Src migrated as a single peak in the low density fractions and paralleled the endocytic marker (HRP) profile (e.g. Figure 2-6A,B) . In similar but separate experiments with total cellular membranes, c-Src was also detected in low density fractions (data not shown) while golgi markers, iodinated plasma membrane proteins (Figure 2-6C) and lysosomal markers (data not shown) were not enriched in c-Src-containing fractions. To rule out

Figure 2-5

Enrichment of c-Src and endosomal membranes on sucrose step gradients. RTCS fibroblasts were incubated with HRP for 30 minutes at 37°C and cell membranes were isolated and loaded on the bottom of a sucrose step gradient (see Methods). After centrifugation, fractions were analyzed by immunoblot with MAb 327 detected by ^{125}I labeled goat ant-mouse antibodies (A). Bands were cut from the blot and counted (B). Fractions were also analyzed for HRP activity (endosomal marker) (C), ADPE I activity (plasma membrane marker) (D), and galactosyl transferase activity (cis/medial golgi marker) (E).

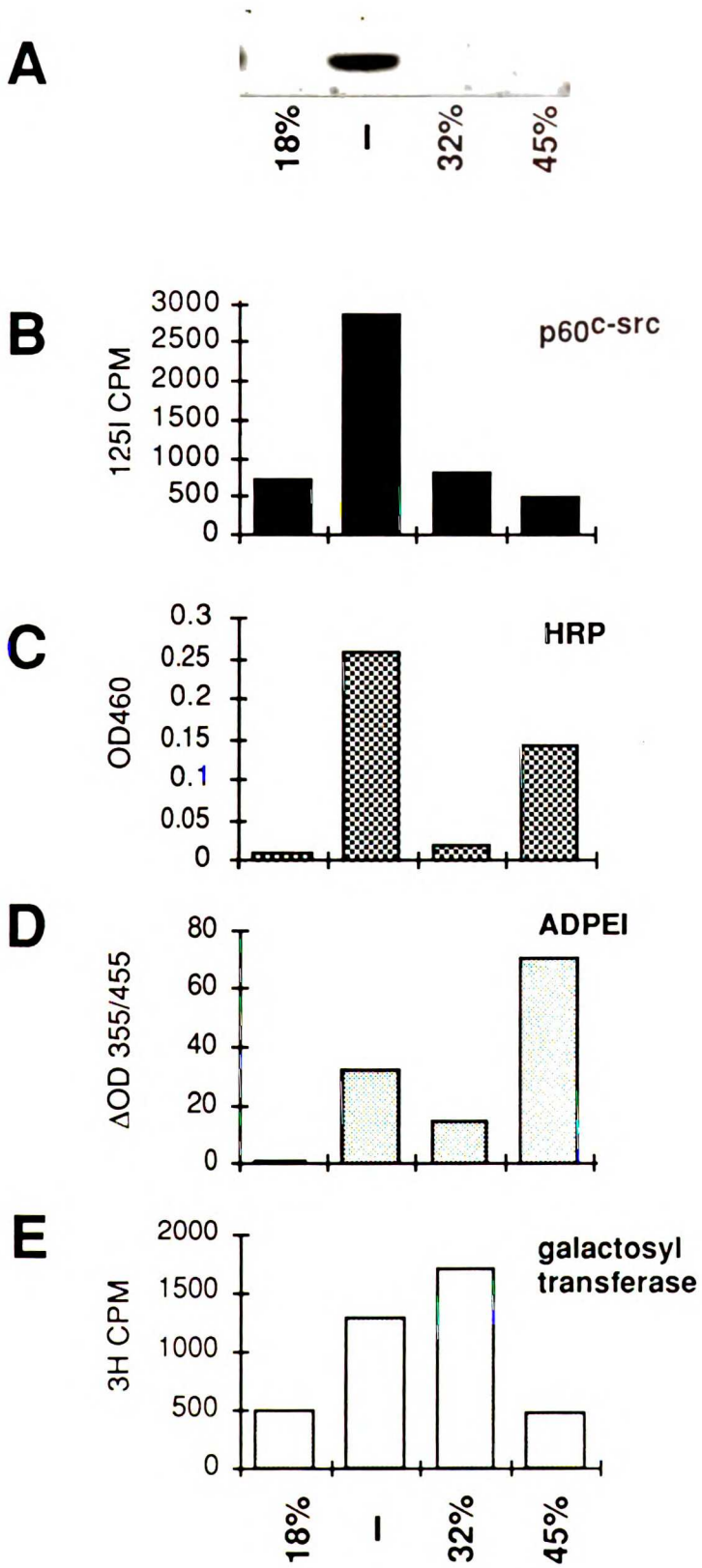
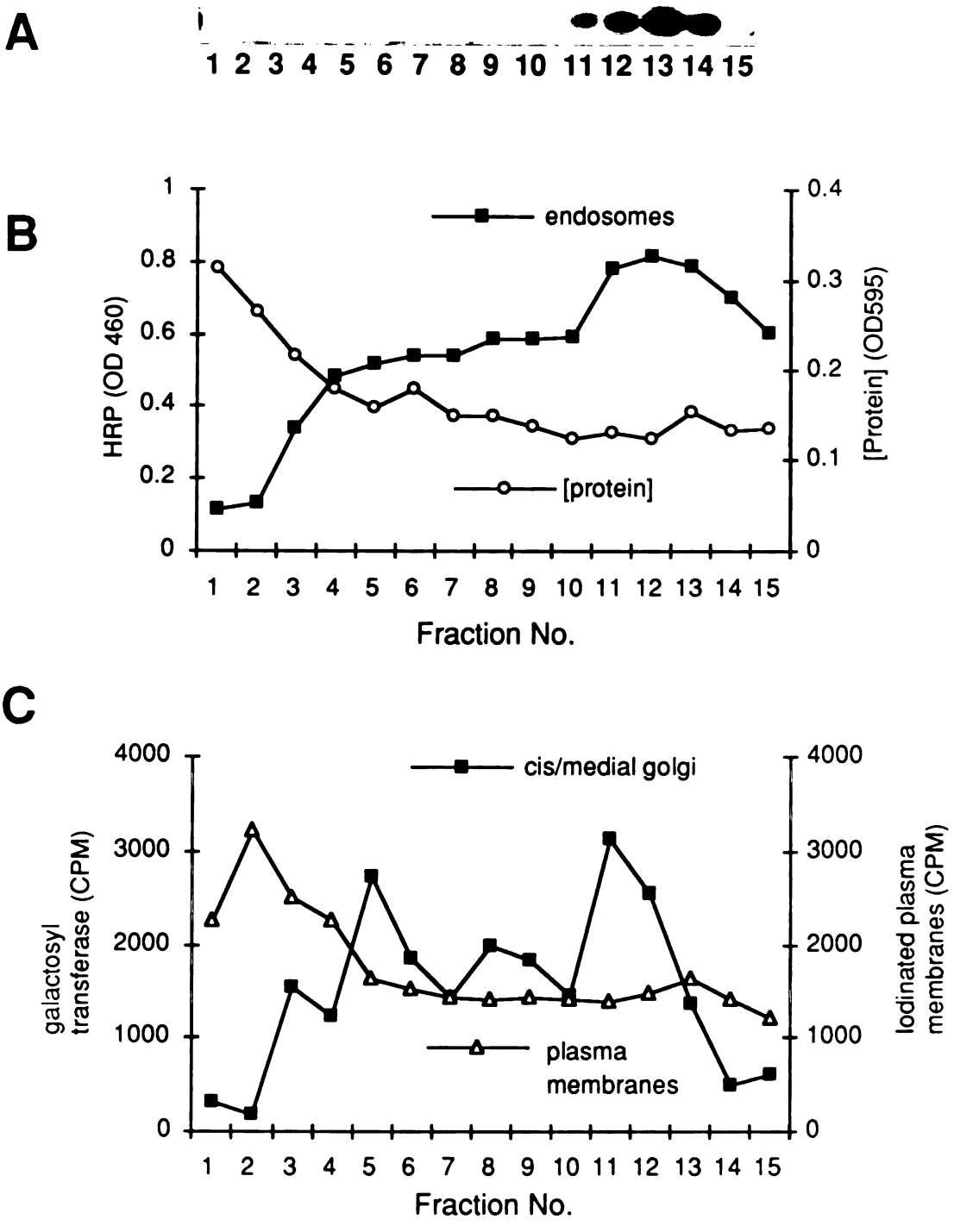

A
18%
I
32%
45%
B
125I CPM
3000
2500
2000
1500
1000
500
0
p60c-src
C
OD460
0.3
0.25
0.2
0.15
0.1
0.05
0
HRP
D
ΔOD 355/455
80
60
40
20
0
ADPEI
E
3H CPM
2000
1500
1000
500
0
galactosyl
transferase
18%
I
32%
45%

Figure 2-6

Separation of c-Src from plasma membranes on Percoll gradients. RTCS fibroblasts were incubated with HRP and cell membranes from the 18%/32% interface of a sucrose step gradient were isolated and loaded on a Percoll gradient (in the absence of $CaCl_2$; see Methods). Fractions were collected from the bottom of the gradient (1=highest density and 15=lowest density) and analyzed by immunoblot with MAb 327 against c-Src (A), for protein concentration (B, o——o) and HRP activity (B, ■——■). Whole cellular membranes from a P100 fraction were isolated in homogenization buffer (+2mM $CaCl_2$; see Methods), run on parallel Percoll gradients and analyzed for the golgi marker galactosyl transferase (C, ■——■) and a gamma counter was used to detect the presence of iodinated plasma membrane proteins (C, ▲——▲). The addition of $CaCl_2$ to the homogenization buffer in this experiment (see Methods; Figure 2-6c) further separated iodinated plasma membrane proteins from endosomal membranes by altering the apparent density of plasma membranes on the Percoll gradient


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
HRP (OD 460)
[Protein] (OD595)
endosomes
[protein]
Fraction No.
C
galactosyl transferase (CPM)
Iodinated plasma membranes (CPM)
cis/medial golgi
plasma membranes
Fraction No.

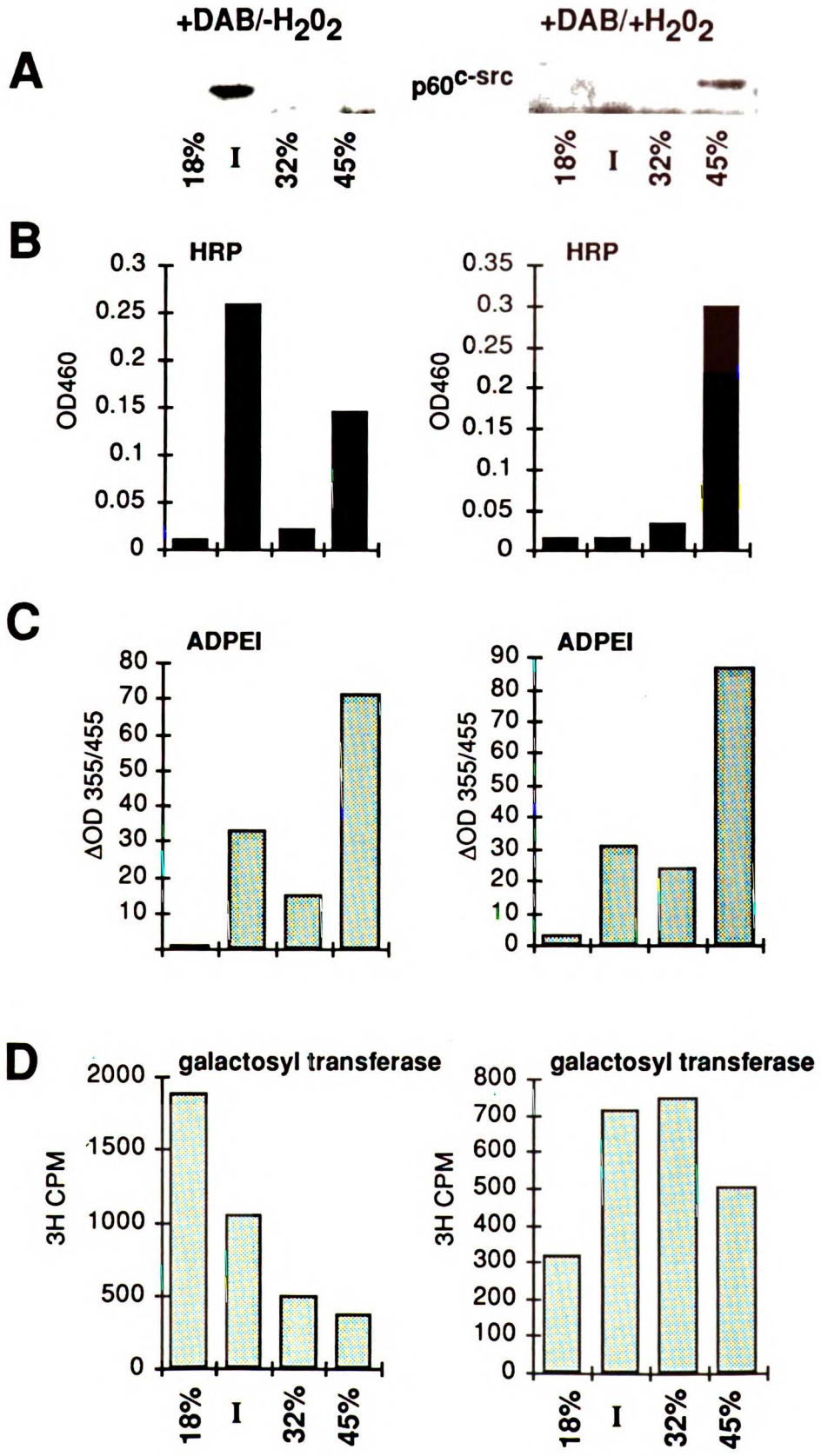
the possibility that soluble c-Src present in cell homogenates was simply associating with endosomal membranes during cell lysis, we mixed c-Src found in S100 fractions with membranes from cells not expressing c-Src (due to a homozygous mutation at the src gene). C-Src did not associate with endosomal membranes under these conditions, indicating that soluble c-Src does not preferentially associate with endosomal membranes after cell lysis (data not shown).

Shift in density of endocytic membranes alters membrane bound c-Src density

To determine if c-Src is directly associated with endocytic vesicles inside the cell we specifically altered the density of endocytic membranes as described previously (Courtoy, et al. 1984; Stoorvogel, et al. 1991). Cells were allowed to internalize horseradish peroxidase to saturate the endocytic compartment (see methods). Cellular membranes were isolated and sedimented on a sucrose step gradient as above. The 18%/32% interface was isolated and split equally. Half of the interface membranes were treated with diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2), a substrate and catalyst for HRP. The modification of DAB by HRP in endocytic vesicles results in an increase in vesicle density. Each set of membranes was analyzed on separate sucrose step gradients for the presence of c-Src and HRP activity. As expected, DAB and H_2O_2 treatment resulted in a shift of the endocytic marker HRP to the 45% fraction (Figure 2-7B). Treatment of membranes with DAB and H_2O_2 also resulted in a shift of membranes containing c-Src (Figure 2-7A) and CI-MPR (data not shown) to the 45% fraction. Other membrane markers present in the 18%/32% interface did not undergo a shift in density to the 45% fraction after treatment with DAB (Figure 2-7C and 2-7D). The variation in the

Figure 2-7

DAB-induced density shift of c-Src in HRP-containing endosomes. Material from the 18%/32% interface of a sucrose step gradient was treated with DAB in the presence (right) or absence (left) of H_2O_2. C-Src was detected by immunoprecipitation and immunoblotting with MAb 327 (A). Fractions were also analyzed for HRP activity (B), ADPE I activity (plasma membrane marker) (C) and galactosyl transferase activity (cis/medial golgi marker) (D) from gradients treated or untreated with H_2O_2.


A
+DAB/-H202
+DAB/+H202
p60c-src
18%
I
32%
45%
B
HRP
OD460
0.3
0.25
0.2
0.15
0.1
0.05
0
0.35
C
ADPEI
ΔOD 355/455
80
70
60
50
40
30
20
10
90
D
galactosyl transferase
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700
600
400
300
200
100

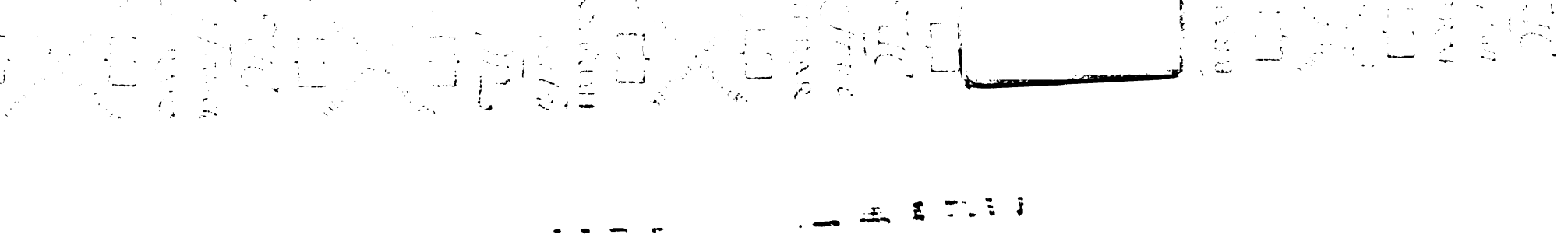
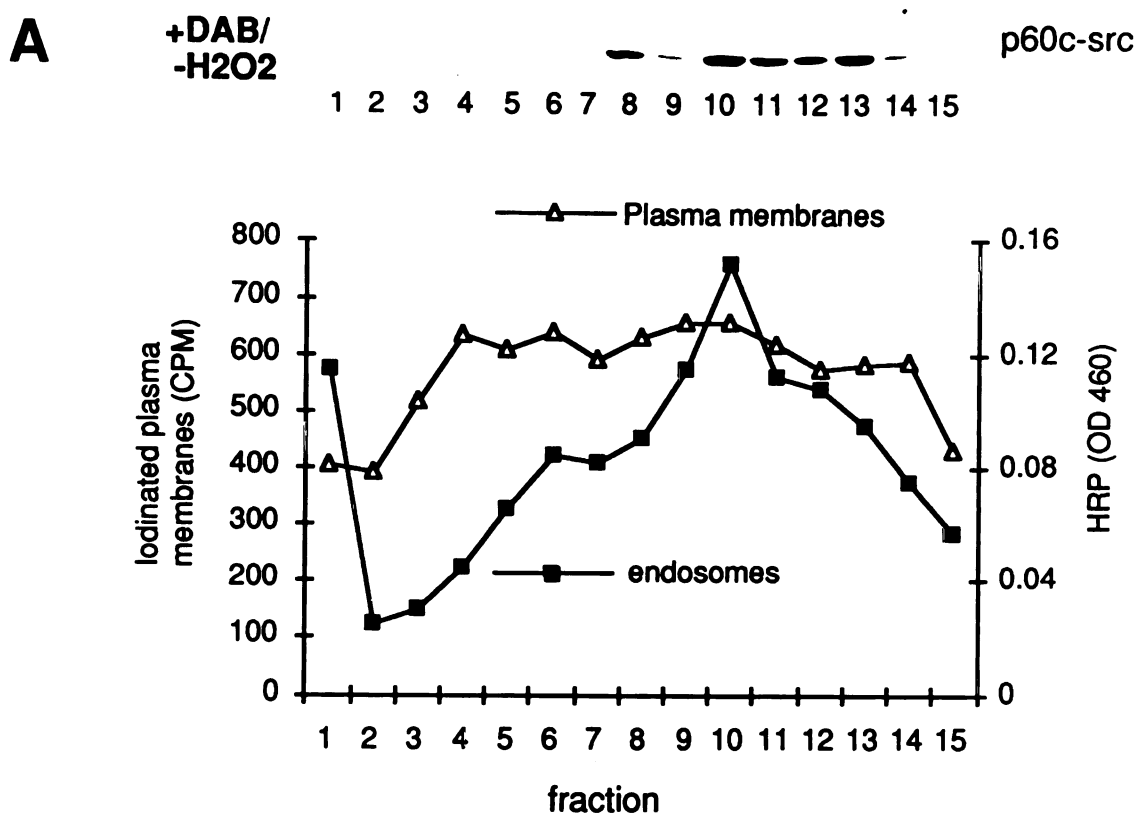
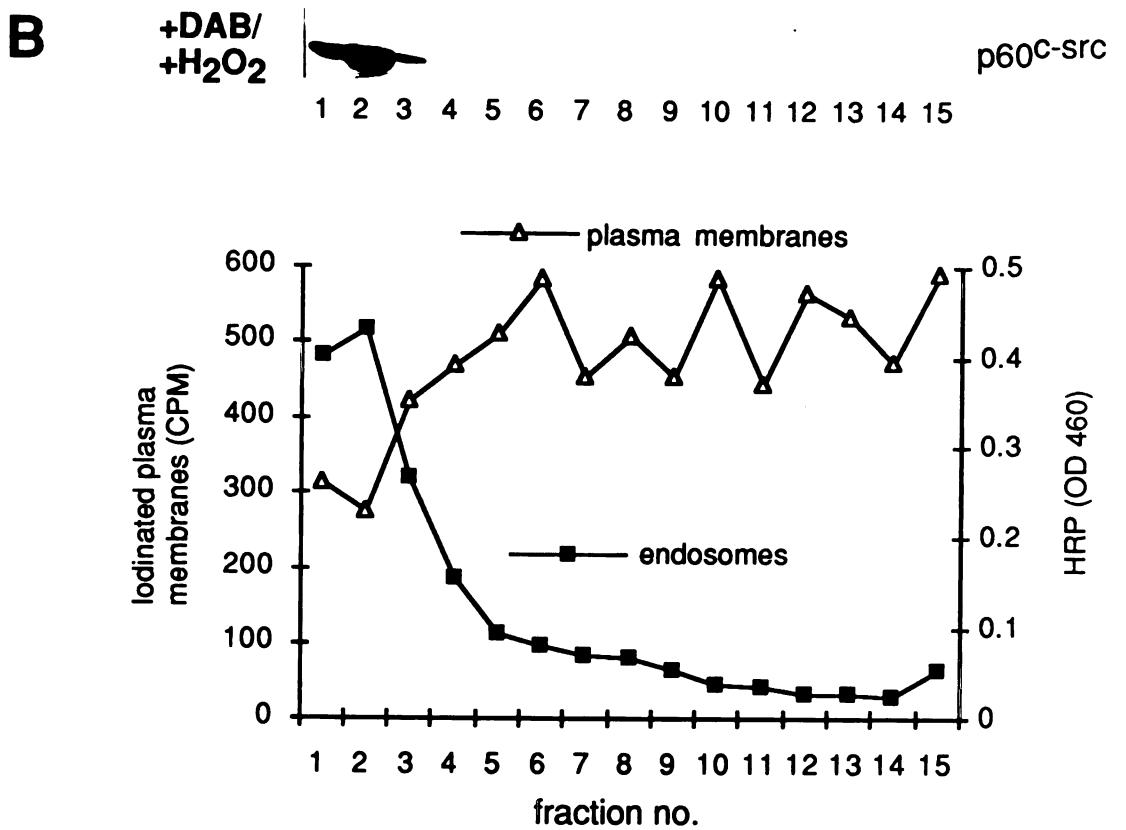


Figure 2-8

DAB-induced density shift of c-Src from total cellular membranes. RTCS fibroblasts were incubated with HRP and total cellular membranes were isolated. Membranes were treated with DAB alone (A), or DAB/H_2O_2 (B) and then run on Percoll gradients. Fractions were collected as in figure 6. C-Src was detected by immunoprecipitation and immunoblotting with MAb 327. Fractions were also analyzed for HRP activity to detect endosomal membranes (■——■) and a gamma counter was used to detect the presence of iodinated plasma membrane proteins (▵——▵). Note that plasma membranes do not behave as in figure 6 due to the absence of $CaCl_2$ (see figure 6 legend).


A
+DAB/
-H2O2
p60c-src
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Plasma membranes
endosomes
Iodinated plasma membranes (CPM)
HRP (OD 460)
800
700
600
500
400
300
200
100
0
0.16
0.12
0.08
0.04
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
fraction


B
+DAB/
+H2O2
p60c-src
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
plasma membranes
endosomes
Iodinated plasma membranes (CPM)
HRP (OD 460)
600
500
400
300
200
100
0
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
fraction no.

distribution of the golgi marker galactosyl transferase (Figure 2-7D) was found to be dependent on homogenization conditions rather than DAB treatment, suggesting that the DAB-induced density shift was specific for endosomal membranes. To demonstrate dependence of the density shift on the HRP reaction, samples treated only with DAB (or only with H_2O_2, data not shown) exhibited no shift in the apparent density of c-Src-associated membranes (Figure 2-7A, left).

Association of the majority of c-Src with endosomal membranes

All of the c-Src isolated from the 18%/32% fraction shifted density in these experiments, suggesting that all of the c-Src in this fraction is associated with membranes accessible to the endocytic marker HRP. To determine the amount of c-Src in the entire cell that is associated with endosomal membranes, we performed density shift experiments on the entire population of cellular membranes isolated from a 100,000xg pellet. The proportion of c-Src in this pellet that undergoes a density shift should represent the proportion of membrane-bound c-Src associated with endocytic membranes. After treatment with DAB and H_2O_2, total cellular membranes were separated on a Percoll gradient and fractions were analyzed to identify c-Src and relevant membrane markers. C-Src from untreated membranes paralleled the peak of endosomal membranes (HRP) in low density fractions (Figure 2-8A). Treatment of membranes with DAB and H_2O_2 shifted the density of all detectable c-Src and the endocytic marker HRP to higher density fractions (Figure 2-8B, C).

The shift in density of all detectable c-Src suggested that the majority of c-Src is associated with endosomal membranes and little or no c-Src is associated with plasma membranes. However, it was possible that HRP bound to the plasma membrane was incorporated into vesicles, resulting in a shift in

density of plasma membranes after DAB/H_2O_2 treatment. To address this possibility, attempts were made to determine the fate of plasma membranes after DAB/H_2O_2 treatment. Identification of plasma membranes by the measurement of enzymatic markers were impeded by low enzymatic activities in Percoll. Instead, HRP-containing cells were surface iodinated at 0^oC to identify plasma membranes. Measurement of ^{125}I after density shift revealed that there was no detectable shift in labeled plasma membrane proteins, while the c-Src-associated membranes underwent a shift in density as expected (Figure 2-8). To further address the specificity of the density shift, HRP was bound to the surface of cells at 0^oC to prevent endocytosis and cell membranes were harvested and treated with DAB and H_2O_2. No detectable density shift of c-Src was observed (data not shown) despite high levels of HRP activity in the membrane pellet. Thus, only HRP internalized into the endocytic pathway is able to shift the density of c-Src-containing membranes.

It was also possible that there was a preferential solubilization of plasma membrane associated c-Src during preparation of cellular membranes. The ~10-20% of total c-Src that we detected in the soluble S100 fraction may therefore represent a plasma membrane-associated fraction of c-Src. To address this possibility, cell extracts were treated with DTSSP (as described above) to reduce the amount of c-Src in the S100 fraction. C-Src was fractionated on Percoll gradients and did not co-sediment with plasma membranes (data not shown). It is therefore unlikely that the population of soluble c-Src came from the plasma membrane.

Discussion

We have found that the majority of membrane-associated c-Src in mammalian fibroblasts is localized to a compartment that probably represents a population of endosomes. Our immunofluorescence experiments demonstrate that a significant proportion of c-Src is found at the MTOC and co-localizes with CI-MPR, a marker of late endosomes. Biochemical fractionation and density shift experiments indicate that the majority of c-Src in the cell associates with endosomal membranes accessible to the endocytic marker HRP. These results raise the possibility that c-Src plays a role in endosomal function.

Previous immunofluorescence and electron microscopic studies of v-Src tend to support localization of the viral src protein at the plasma membrane (Rohrschneider 1979; Willingham, et al. 1979). Some src family members are thought to interact with cell surface proteins, and a putative plasma membrane receptor for has been identified in cross-linking experiments (Resh and Ling 1990). In contrast, our results suggest that very little c-Src is associated with plasma membranes. Although we sometimes observed c-Src staining at cell/cell contacts, we suspect that this does not represent plasma membrane association but rather is due to enrichment of membranes at bundles of microtubules (see Figure 2-1). Dependence on intact microtubules for this localization further supports the notion that c-Src is microtubule-associated at cell/cell contacts. The discrepancy between previous localization studies and our results may arise in part from differences between the transforming protein v-Src and the non-transforming protein c-Src.

Our results also differ from previous studies that have used biochemical fractionation to localize src proteins. These studies demonstrated an enrichment of both v-Src and c-Src in plasma membranes (Courtneidge, et al. 1980; Resh and Erikson 1985). Fractionation techniques employed in these studies did not differentiate between plasma membranes and endosomal

membranes, which have similar densities and are likely to co-sediment. We have used variable conditions during homogenization to alter the sedimentation of plasma membranes, allowing them to be separated from endosomes (see Methods). In addition, the specific shift in density of HRP-containing membranes convincingly separated endosomes from plasma membranes. Based on results from these fractionation experiments we have concluded that no detectable c-Src is associated with plasma membranes. It remains possible that a very small proportion of c-Src is associated with plasma membranes but is undetectable under our conditions.

Our results suggest that c-Src is associated with the microtubule cytoskeleton via endocytic vesicles, in contrast to previous localization and biochemical studies suggesting an association of v-Src with the actin cytoskeleton (Hamaguchi and Hanafusa 1987; Henderson and Rohrschneider 1987; Resh and Erikson 1985; Rohrschneider 1980; Rohrschneider 1979). Analysis of optical sections near the bottom of cells over-expressing chicken c-Src showed only punctate, microtubule-associated staining and no localization to adhesion plaques. We found that c-Src co-localizes with a number of microtubule-related structures, including microtubule bundles at points of cell/cell contact, the microtubule organizing center, and a region associated with the spindle pole during mitosis (Figure 2-1, 2-4). In all cases this staining is dependent on intact microtubules. Ample evidence has demonstrated that endocytic membranes can be transported along microtubules (DeBrabander, et al. 1988; Goergi, et al. 1990; Matteoni and Kreis 1987). It is therefore likely that co-localization of c-Src with various microtubule structures reflects the transport of endosomal membranes along cellular microtubules. It is even conceivable that c-Src may regulate microtubules or microtubule-associated motors involved in endosomal membrane transport.

The microtubule-dependent alteration of c-Src staining induced by BFA treatment provides additional evidence for c-Src transport along microtubules. BFA is known to cause microtubule-dependent retrograde transport of golgi elements into the endoplasmic reticulum (Lippincott-Schwartz, et al. 1990; Lippincott-Schwartz, et al. 1989). We have shown that treatment of cells with BFA also affects the transport of endosomal membranes containing c-Src. BFA treatment results in the clustering of c-Src-containing membranes at the centrosome, which is distinct from the normally diffuse staining of c-Src at the MTOC (Figure 2-3). Similar findings have been reported for the effect of BFA on a wide range of endocytic membranes in both rat and canine cells (Lippincott-Schwartz, et al. 1991; Wood, et al. 1991). While the mechanism of action of BFA remains unclear, the behavior of c-Src and CI-MPR after BFA treatment emphasizes the similar nature of the membrane compartment occupied by these proteins.

Studies of cells in early stages of mitosis show that c-Src and CI-MPR are found at the dividing centrosomes (Figure 2- 4). The presence of both CI-MPR and c-Src at the spindle poles during mitosis further suggests that both of these proteins reside in a similar membrane compartment that is distinct from cis/medial golgi membranes, which are scattered throughout the cell during mitosis (KBK unpublished observations) (Lucocq and Warren 1987; Moskalewski and Thyberg 1990). Since c-Src is known to be activated during mitosis and phosphorylated by $p34^{cdc2}$ (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989), it is possible that c-Src is mediating mitotic effects of $p34^{cdc2}$ at the peri-centriolar region of the cell. Several processes associated with endosomal membranes are known to be regulated during mitosis. For example, it has been shown in vitro that early endosomal fusion events are

inhibited by addition of active Xenopus $p34^{cdc2}$ (Tuomikoski, et al. 1989; Woodman, et al. 1992).

Work with secretory cells suggests that c-Src may be involved in regulating the function of specialized secretory vesicles. In addition to being enriched in secretory granules of chromaffin cells, c-Src is associated with a 38-kD protein that may be important in the function of these secretory organelles (Grandori and Hanafusa 1988). These compartments may be analogous to the endosomal compartment in fibroblasts. The association of c-Src with endosomally-derived synaptic vesicles in PC-12 cells is consistent with this proposal (Johnston, et al. 1989)(Linstedt, personal communication) .

Endosomal membranes are dynamic structures involved in the trafficking of proteins throughout the cell and are known to be regulated at the level of both transport and fusion. In this regard it may be relevant to consider the phenotype of mice that are genetically null for the *c-src* gene through homologous recombination (Soriano, et al. 1991). The defect in bone modeling (osteopetrosis) exhibited by these mice arises ultimately from an inability to dissolve bone tissue during development. The osteoclasts responsible for dissolving bone tissue are known to be highly specialized secretory cells that secrete lysosomal enzymes. In the absence of c-Src these cells may be defective in regulating the trafficking of lysosomal proteins.

Chapter Three:

Association of the amino terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527

Abstract

We have characterized the mechanism by which the sub-cellular distribution of c-Src is controlled by the phosphorylation of tyrosine 527. Mutation of this tyrosine dramatically redistributes c-Src from endosomal membranes to focal adhesions. Redistribution to focal adhesions occurs independently of kinase activity and cellular transformation. In cells lacking the regulatory kinase (CSK) that phosphorylates tyrosine 527, c-Src is also found predominantly in focal adhesions, confirming that phosphorylation of tyrosine 527 affects the location of c-Src inside the cell. The first 251 amino acids of c-Src are sufficient to allow association with focal adhesions, indicating that at least one signal for positioning c-Src in focal adhesions resides in the amino-terminal half. Point mutations and deletions in the first 251 amino acids of c-Src reveal that association with focal adhesions requires the myristylation site needed for membrane attachment, as well as the SH3 domain. Expression of the amino-terminal region alters both the structural and biochemical properties of focal adhesions. Focal adhesions containing this non-catalytic portion of c-Src are larger and exhibit increased levels of phosphotyrosine staining. Our results suggest that c-Src may regulate focal adhesions and cellular adhesion by a kinase-independent mechanism.

Introduction

The tyrosine kinase c-Src belongs to a family of cytoplasmic tyrosine kinases that associate with cellular membranes and are thought to be involved in signal transduction events underlying growth control (for review see (Cooper 1989). The normal function of c-Src is not clear. Studies of mutant forms of the

protein (e.g., the viral transforming protein v-Src) have implicated c-Src in the regulation of growth control, although c-Src is also expressed at high levels in terminally differentiated cells such as platelets and neurons (Cotton and Brugge 1983; Golden, et al. 1986). In addition, the kinase activity of c-Src increases during mitosis in fibroblasts and thus may mediate certain mitotic events (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989; Shenoy, et al. 1989). Recent genetic studies with mice containing a homozygous disruption of the *c-src* gene suggest a specific role for c-Src in the normal physiological function of osteoclasts (Boyce, et al. 1992; Soriano, et al. 1991), but have not led to any clear understanding of the intra-cellular activities of c-Src required for function.

The conserved SH2 and SH3 domains in the amino terminus of c-Src are shared with a number of proteins involved in signal transduction (see Figure 3- 2- 1). The SH2 domain interacts with phosphotyrosine-containing sequences (Mayer, et al. 1991; Pawson and Gish 1992), while the SH3 domain is thought to mediate interactions with proline-rich regions in target proteins (Ren, et al. 1993). The amino terminus of c-Src also contains the "unique" domain (amino acids 2-90), which shares little homology with other Src-family members and is involved in membrane attachment through a myristic acid attachment site (Buss and Sefton 1985; Kaplan, et al. 1990).

While the amino terminus of c-Src mediates protein/protein interactions, carboxy terminal phosphorylations are involved in regulating the kinase activity of c-Src. Autophosphorylation at tyrosine 416 (Y416) is required for full activity of the kinase (Kmiecik, et al. 1988; Piwnica-Worms, et al. 1987) and phosphorylation at tyrosine 527 (Y527) negatively regulates c-Src activity (Courtneidge 1985; Kmiecik and Shalloway 1987). Normally, Y527 is thought to be extensively phosphorylated in the cell by the c-Src kinase (CSK) (Imamoto

and Soriano 1993), resulting in an intramolecular interaction between phosphorylated Y527 and the SH2 domain (MacAuley and Cooper 1989). Deleting Y527 (as in v-Src) or mutating it to a phenylalanine (Y527F; SrcA) disrupts this interaction and results in a highly active kinase, exposing amino terminal domains (the "open" conformation) (Liu, et al. 1993a; Roussel, et al. 1991) that now may interact with target proteins.

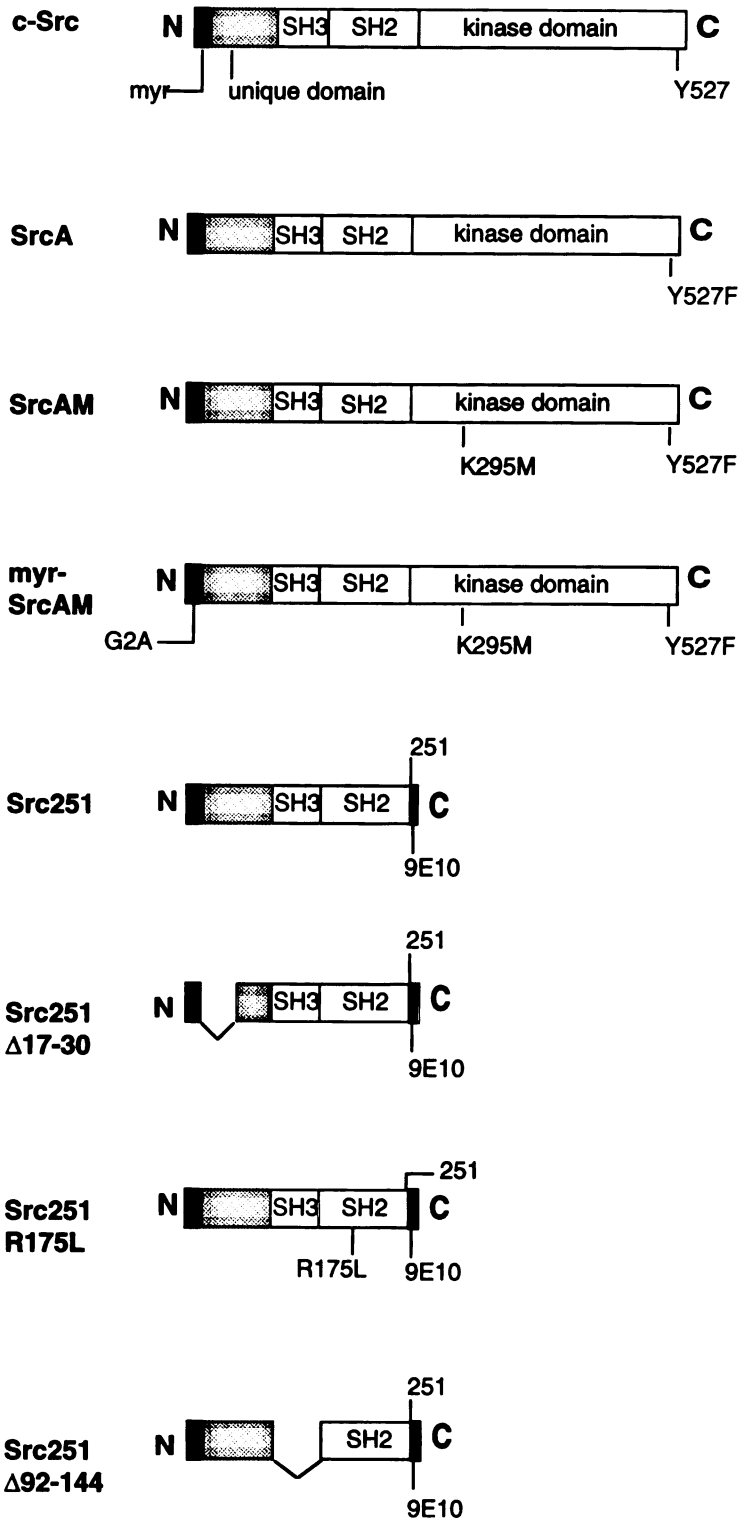
Interactions between amino terminal domains of c-Src and target proteins may also be responsible for determining the sub-cellular location of Src proteins and thus may provide clues about the function of c-Src. Recent studies have shown that c-Src co-localizes primarily with markers of endosomal membranes (David-Pfeuty and Nouvian-Dooghe 1990; Kaplan, et al. 1992), and biochemical fractionation suggests that the majority of c-Src directly associates with endosomal membranes in fibroblasts (Kaplan, et al. 1992). C-Src also associates with specialized secretory vesicles in a number of cell types, suggesting a possible role for c-Src in regulating some aspect of directed secretion (Grandori and Hanafusa 1988; Linstedt, et al. 1992). In contrast to c-Src, v-Src is abundant in both endosomal membranes (KBK, unpublished observations; (Redmond, et al. 1992)) and rosette-like structures (also called adhesion plaques) that contain proteins normally found in focal adhesions (David-Pfeuty and Singer 1980; Nigg, et al. 1982; Rohrschneider 1980). The distribution of v-Src in adhesion plaques correlates with the detergent-insolubility of v-Src and contrasts with the detergent-solubility of c-Src (Burr, et al. 1980; Hamaguchi and Hanafusa 1987).

The different locations of v-Src and c-Src may be due to differences in their protein sequence (multiple scattered amino acid changes and multiple differences at the carboxy terminus) or may be due to changes in the cellular environment that accompany cellular transformation. To distinguish between

these possibilities and to address the relevance of the sub-cellular distribution of v-Src to the normal function of c-Src, we have investigated the mechanisms that control the sub-cellular distribution of c-Src. Through high-resolution immunofluorescence analysis and biochemical fractionation of fibroblasts expressing various mutants of c-Src, we show that phosphorylation of Y527 regulates the distribution of c-Src inside the cell. Normally, Y527 is phosphorylated and c-Src associates predominantly with endosomal membranes. Under conditions where Y527 is not phosphorylated, c-Src associates with focal adhesions. The first 251 amino acids of c-Src are sufficient for distribution to focal adhesions and require membrane attachment and the SH3 domain. Interestingly, the association of the non-catalytic amino terminal half of c-Src in focal adhesions results in the alteration of the structure and an increase in the level of phosphotyrosine staining of focal adhesions. These results suggest that the amino terminal half of c-Src may regulate focal adhesions and influence cell adhesion in the absence of its kinase domain.

Figure 3-1

Predicted products of chicken *c-src* alleles used in this report. Abbreviations are as follows: Myr, myristylation site; 9E10, myc epitope tag (see Materials and Methods), N, amino terminus; C, carboxy terminus. At the left are the names of the proteins, as used in the text.


c-Src
N
SH3
SH2
kinase domain
C
myr
unique domain
Y527
SrcA
N
SH3
SH2
kinase domain
C
Y527F
SrcAM
N
SH3
SH2
kinase domain
C
K295M
Y527F
myr-
SrcAM
N
SH3
SH2
kinase domain
C
G2A
K295M
Y527F
Src251
251
N
SH3
SH2
C
9E10
Src251
Δ17-30
251
N
SH3
SH2
C
9E10
Src251
R175L
251
N
SH3
SH2
C
R175L
9E10
Src251
Δ92-144
251
N
SH2
C
9E10

Results

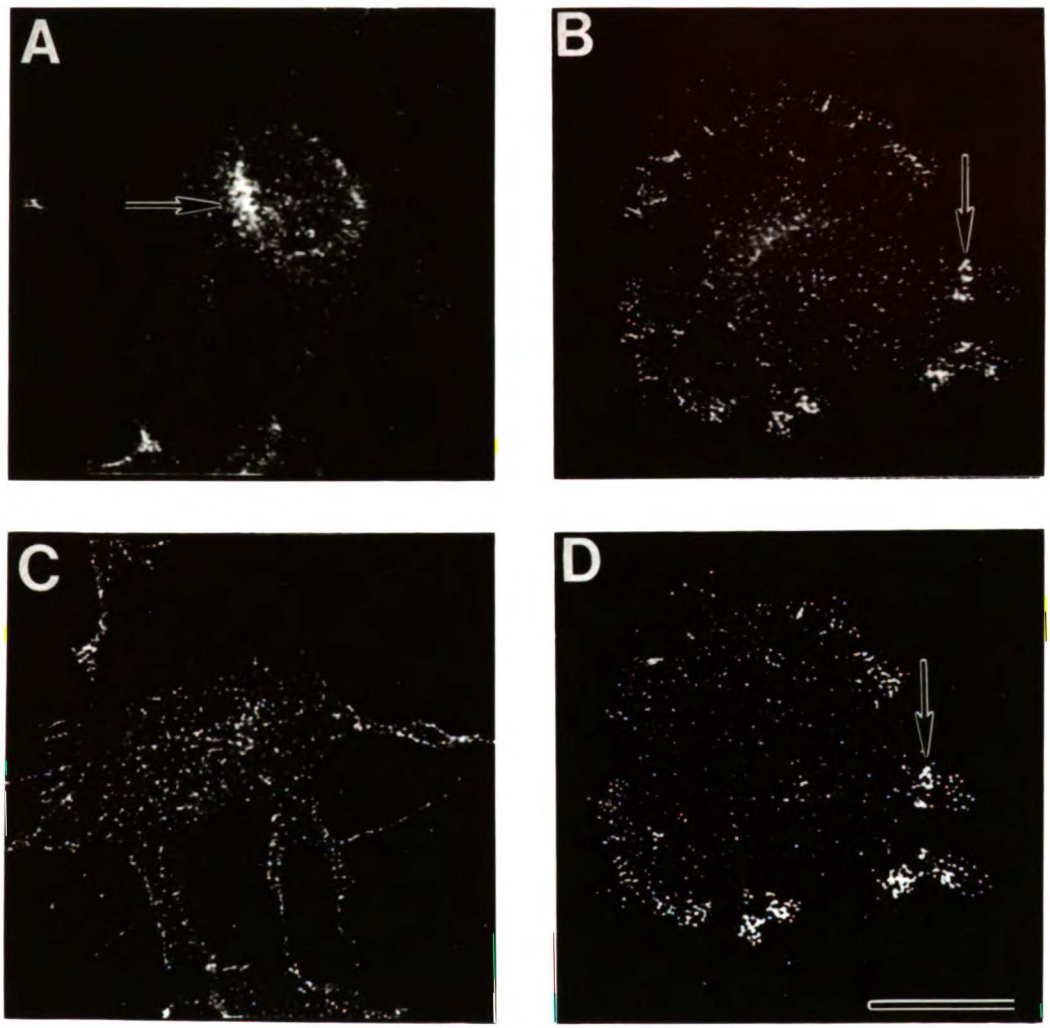
Requirements for Src translocation to focal adhesions

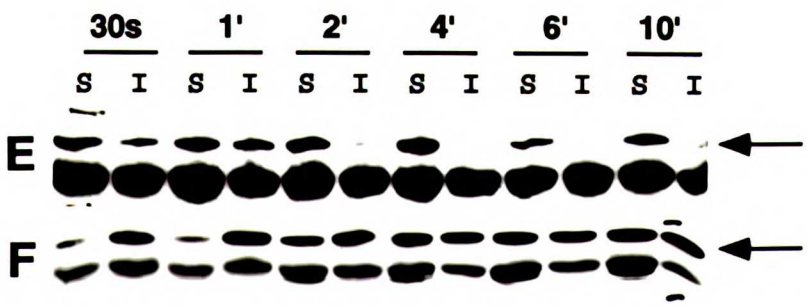
To examine the properties of v-Src required for association with adhesion plaques, we analyzed the sub-cellular distribution of the mutant forms of c-Src shown in Figure 3-1. We expressed the mutant c-Src proteins in *src*$^{-/-}$ fibroblasts to avoid any confusion with endogenous c-Src. Consistent with previous studies (David-Pfeuty and Nouvian-Dooghe 1990; Kaplan, et al. 1992), wild type c-Src in *src*$^{-/-}$ fibroblasts associated primarily with endosomal membranes, as determined by standard immunofluorescence experiments analyzed by optical sectioning microscopy. Optical sections in the middle of the cell reveal a concentration of c-Src staining in a peri-nuclear location (Figure 3-2a) that co-localizes with markers of endosomal membranes (data not shown) (Kaplan, et al. 1992). Optical sections at the bottom of the cell show a diffuse punctate staining pattern with no detectable staining in focal adhesion structures (Figure 3-2c).

Previous studies of the localization of Src have involved v-Src, a protein whose sequence differs from that of c-Src at several positions. To address specifically the role of Y527 in regulating the distribution of c-Src, we examined cells expressing a protein with a single amino acid substitution, Y527F (SrcA). *Src*$^{-/-}$ fibroblasts expressing SrcA were morphologically transformed as expected, and immunofluorescence showed that the single amino acid change caused SrcA to associate with focal adhesions (Figure 3-2 b, d). Projections of all optical sections (Figure 3-2b) or optical sections on the bottom of the cell (Figure 3-2d) exhibited intense Src-staining in structures that co-localize with markers of focal adhesions (data not shown). In cells expressing higher levels of SrcA, a significant amount of staining was also observed in the peri-nuclear

Figure 3-2

C-Src associates with endosomes and SrcA with focal adhesions. *Src*$^{-/-}$ fibroblasts expressing either c-Src (A, C) or SrcA (B, D) were stained with MAb 327. Indirect immunofluorescence was analyzed by optical sectioning microscopy (see Methods) and projections comprising multiple optical sections (A, B) and individual optical sections near the bottom of the same cell (C, D) are presented. Structures corresponding to endosomes or focal adhesions are indicated in cells expressing c-Src (arrow; A) or SrcA (arrow; B, D), respectively (Scale bar=10μM). Cells expressing either c-Src (E) or SrcA (F) were fractionated in Triton X-100-containing buffer for indicated times and detergent-soluble (S) and detergent-insoluble (I) fractions were immunoprecipitated and immunoblotted with MAb 327. Arrows indicate the Src band; the band below Src represents the immunoglobulin heavy chain, recognized by the anti-mouse secondary antibody.


A
B
C
D


30s
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4'
6'
10'
S I S I S I S I S I S I
E
F

region of the cell, costaining with endosomal membrane markers (data not shown). The association of SrcA with focal adhesions correlated with a reduction in its detergent (Triton X-100) solubility when compared to wild type c-Src (Figure 3-2e,f). The detergent-insoluble SrcA protein was found predominantly in focal adhesions in cells extracted with detergent

Since cellular transformation did not direct c-Src to focal adhesions, we examined whether the kinase activity of SrcA and consequent cellular transformation are necessary for association of c-Src with focal adhesions. We expressed a kinase-deficient version of c-Src containing an amino acid change in the ATP binding site (K295M), as well as the change at Y527 (c-SrcAM). Optical-sectioning microscopy revealed that c-SrcAM was present in focal adhesions (Figure 3-4a-b). Optical sections at the bottom of the cell revealed Src-containing structures clustered at the cell periphery (arrows in Figure 3-4b), co-localizing with several focal adhesion markers, including phosphotyrosine, vinculin and talin (data not shown; see Figure 3-5). Optical sections from the middle of the cell reveal a fraction of c-SrcAM in the peri-nuclear region (Figure 3-4a), indicating that c-SrcAM associates with endosomal membranes as well as focal adhesions. The presence of c-SrcAM in focal adhesions correlated with an increase in detergent-insolubility (Figure 3-4c). The level of detergent-insoluble Src was observed to be slightly greater in transformed cells (Figure 3-2e), perhaps reflecting the increased levels of c-SrcAM expression and consequently, higher levels of Src associated with endosomal membranes (Figure 3-2b, 4a). Nonetheless, the changes in solubility between c-Src and c-SrcAM clearly suggest that the enzymatic and transforming activities of SrcA are not required for association with focal adhesions.

To determine if the presence of c-SrcAM in focal adhesions requires membrane attachment, we examined cells expressing a mutant c-SrcAM

Figure 3-3

Transformation does not alter c-Src localization. *Src*$^{-/-}$ cells expressing chicken c-Src were transformed by deletion mutant of SrcA (Δ92-144) and stained with MAb327 to specifically visualize c-Src. A projection comprising multiple optical sections shows a strong peri-nuclear staining pattern (arrow; A), while sections on the bottom of the cell show that c-Src is not in focal adhesions (B) which are apparent in the same section when co-stained with anti-phosphotyrosine antibodies (arrows; C). (Same scale as Figure 3-2)

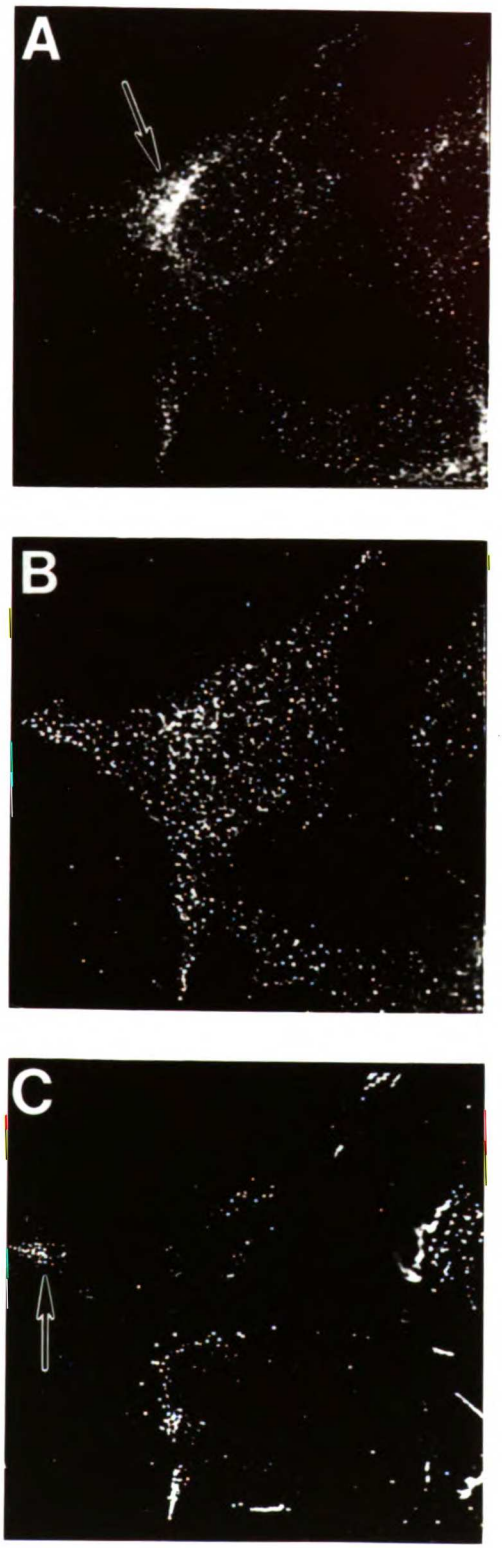
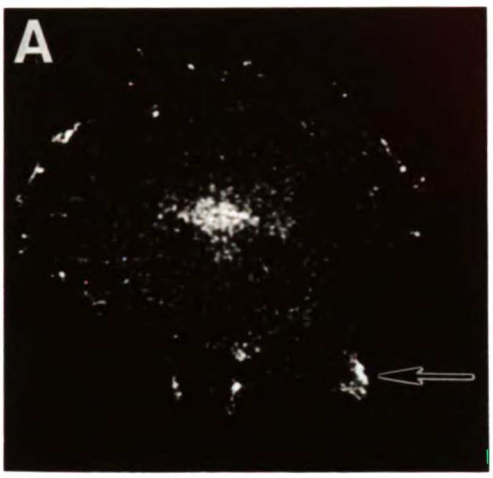
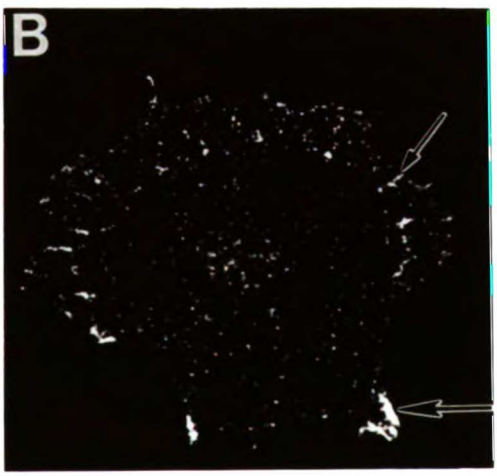

A
B
C

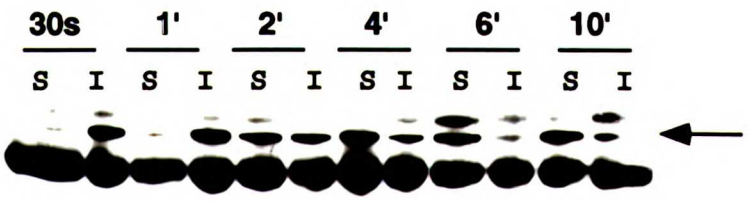
Figure 3-4

Kinase activity is not required for localization of c-Src to focal adhesions. Projections of *src*$^{-/-}$ fibroblasts expressing a kinase inactive SrcA protein (SrcAM) stained with MAb 327 reveals both endosomal membrane (arrow; A) and focal adhesion staining in optical sections on the bottom of the cell (arrows; B), (same scale as Figure 3-2). Detergent fractionation of cells expressing the SrcAM protein was carried out as in Figure 3-2 (C). Arrows indicate the Src band; the band below Src represents the immunoglobulin heavy chain.





C



protein that contains an additional amino acid change in the amino terminus (G2A), which eliminates the consensus myristylation signal and prevents association with cellular membranes (data not shown). This protein has a diffuse cytoplasmic distribution inside the cell, does not associate with focal adhesions or cellular membranes by immunofluorescence analysis (Figure 3-5, a-c), and is completely soluble by detergent fractionation (Figure 3-6a). This is also consistent with myristylation defective alleles of v-Src which are not transforming and no longer associate with the cytoskeleton of cells (Hamaguchi and Hanafusa 1987; Kamps, et al. 1985). Therefore, membrane association plays an important role in c-Src distribution, either in the trafficking of c-Src to focal adhesions or by anchoring c-Src in membranes associated with focal adhesions.

Although c-Src association with focal adhesions requires myristylation, it is not generally sufficient to localize proteins to focal adhesions. We therefore attempted to identify other domains of c-Src involved in focal adhesion association. In addition to activating the kinase, mutation of Y527 is thought to alter the folding of c-Src to expose amino terminal domains (Liu, et al. 1993a; Roussel, et al. 1991). To determine whether a signal(s) within the amino terminus directs c-Src to focal adhesions, a truncated version of c-Src containing only the amino terminal 251 amino acids (Src251) was expressed in *src*$^{-/-}$ fibroblasts. The truncated protein distributed to focal adhesions (Figure 3-5d-f), with very little staining associated with peri-nuclear membranes. The staining pattern of Src251 co-localized with that of a number of focal adhesion markers, including phosphotyrosine staining observed in optical sections at the bottom of the cell (Figure 3-5f). Src251 protein is found in the detergent-insoluble fraction, consistent with a correlation between detergent-insolubility and association with focal adhesions (Figure 3-6b). These results demonstrate

Figure 3-5

Myristylation and the SH3 domain are required for localization of c-Src to focal adhesions. The column on the left represents cells stained with MAb 327 after expressing the indicated mutant proteins. Results are displayed as a projection of optical sections (arrows indicate Src staining in focal adhesions where applicable; a, d, g, j, m). The middle column represents an optical section on the bottom of the same cell stained with MAb327 (b, e, h, k, n). The column on the right depicts the same optical section on the bottom of the cell co-stained with anti-phosphotyrosine antibodies to reveal the focal adhesions (arrows indicate staining of focal adhesions; c, f, i, l, o). (Scale bar=10μM)

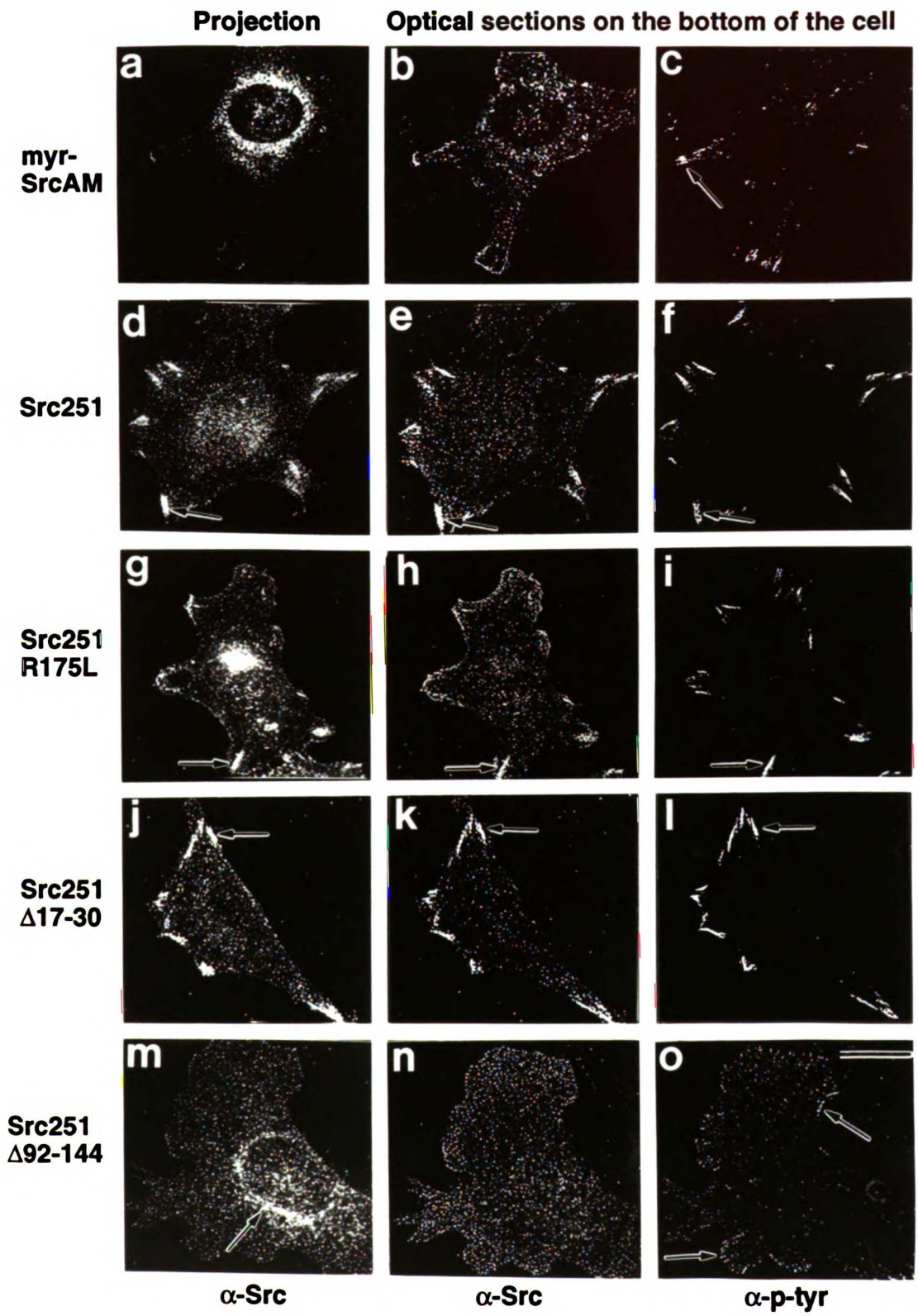
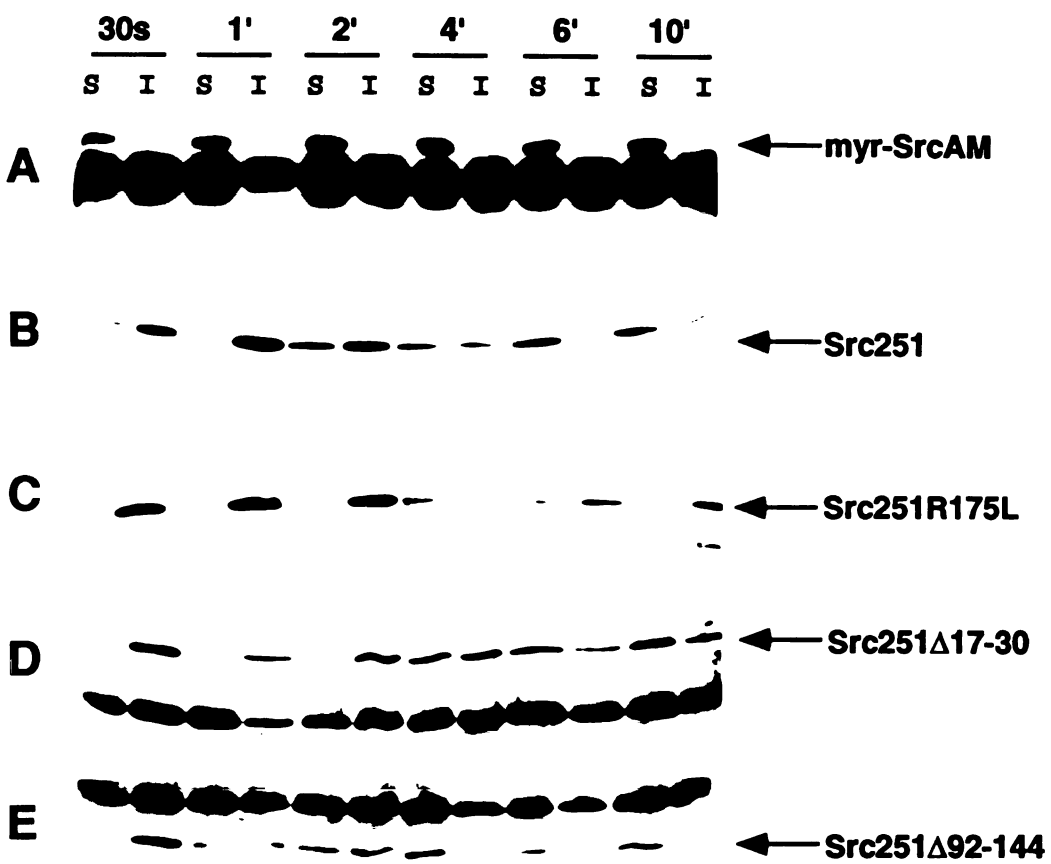

Projection
Optical sections on the bottom of the cell
a
b
c
myr-
SrcAM
d
e
f
Src251
g
h
i
Src251
R175L
j
k
l
Src251
Δ17-30
m
n
o
Src251
Δ92-144
α-Src
α-Src
α-p-tyr

Figure 3-6

Insolubility of c-Src in Triton X-100 requires the SH3 domain and myristylation. Cells expressing mutant Src-proteins were fractionated in Triton X-100 buffer as in Figure 3-2. Soluble (S) and insoluble (I) fractions were immunoprecipitated and immunoblotted to reveal the Src protein with MAb 327 (A, B, C, D) or MAb 9E10 (E). Arrows indicate the Src protein in each experiment; the additional bands are either immunoglobulin heavy chain (A) or light chain (C, E).


30s
1'
2'
4'
6'
10'
S I S I S I S I S I S I
A
myr-SrcAM
B
Src251
C
Src251R175L
D
Src251Δ17-30
E
Src251Δ92-144

that the amino terminal 251 amino acids of c-Src are sufficient for association with focal adhesions.

NH_2-terminal sequences that direct c-Src to focal adhesions

The presence of Src251 in focal adhesions indicates that sequences in the first 251 amino acids are directly involved in the association with focal adhesions. The requirement for myristylation implies that membrane attachment is an important factor for accumulating c-Src in focal adhesions. The contribution of other amino terminal domains, including the unique domain, the SH3 domain, and the SH2 domain, was evaluated by examining a series of deletions and point mutations in the context of the truncated c-Src protein, Src251. The same mutants were also made in the context of the full length c-SrcAM protein and similar results were obtained (see discussion).

Contribution of the SH2 domain was investigated by analyzing a version of Src251 containing a mutation (R175L) in the SH2 domain (Src251R175L). This mutation eliminates the ability of the SH2 domain to bind phosphotyrosine ligands *in vitro* (Bibbins, et al. 1993; Mayer, et al. 1992). When expressed in *src*$^{-/-}$ fibroblasts, Src251R175L was found in focal adhesions (Figure 3-5g-i). Optical sections at the bottom of the cell clearly reveal large structures at the cell periphery that co-stain with antibodies against Src and phosphotyrosine (Figure 3-5h-i). While impairment of SH2 function does not prevent the appearance of the amino terminal half of Src in focal adhesions, there is a slight change in the staining pattern. Cells expressing the Src251R175L protein had a higher level of staining in peri-nuclear membranes, possibly due to the higher levels of protein expression compared to wild type Src251 (~5-10X). Therefore, disruption of phosphotyrosine binding by the SH2 domain can subtly affect the relative distribution of Src251 between endosomes and focal adhesions,

without disrupting the ability of Src251 to associate with focal adhesions. Disruption of phosphotyrosine binding did not alter the detergent insolubility of this protein, when compared to the Src251 protein (Figure 3-6b,d).

To address the role of the unique domain (aa 17-90) in targeting c-Src to focal adhesions, we examined the distribution of two deletion mutants. In the first, amino acids 17-30 were deleted from the Src251 protein (Src251Δ17-30). Analysis of immunofluorescence staining revealed that deletion of these amino acids had no effect on the ability of Src251 to associate with focal adhesions (Figure 3-5 j-l). A second unique domain deletion, removing amino acids 30-92, partially reduced the amount of Src251 in focal adhesions but cell lines expressing detectable levels of the protein proved difficult to isolate, hampering further analysis (data not shown). On the basis of the reduced staining in focal adhesions and the disruption of an SH3-associated antibody epitope, we suspected that the Δ30-92 deletion may affect an SH3-related localization signal.

To test this possibility, we expressed a deletion of the SH3 domain (92-144) in the context of Src251 (Src251Δ92-144; see Figure 3-1). Deletion of the SH3 domain completely abolished the association of Src251 with focal adhesions (Figure 3-5m-o). Src staining was not observed in focal adhesions at the bottom of the cell (Figure 3-5n). A projection of all optical sections revealed that the majority of Src251Δ92-144 distributes very tightly around the nucleus (Figure 3-5m), in contrast to Src251, which shows very little staining in this region (compare to Src251; Figure 3-5b). In addition, focal adhesions in cells expressing Src251Δ92-144 are less pronounced than in cells expressing Src251 (arrows, Figure 3-5f, o). Src251Δ92-144 is also more detergent-soluble than Src251(Figure 3-6d). Some protein was retained in the detergent-insoluble fraction (52% insoluble), but only at the earliest time point after lysis

(30s) and it became mostly soluble by the two minute time point (60% soluble). The drastic change in subcellular distribution of Src251 lacking amino acids 92-144 strongly implicates the SH3 domain in the association of the amino terminal half of c-Src with focal adhesions, but does not rule out some contribution from the SH2 domain.

Structural alteration of focal adhesions by Src-251

The ability of the amino terminal half of c-Src to associate with focal adhesions suggests that c-Src may have a specific role at this intracellular site. Consistent with this possibility, we observed that expression of the NH_2-terminal portion of Src (Src-251) in *src*$^{-/-}$ fibroblasts altered the appearance of the focal adhesions. Focal adhesions containing Src251 (see arrows, Figure 3-7a) show a dramatic increase in the level of phosphotyrosine staining, as well as an alteration of the overall structure of focal adhesions (see arrows, Figure 3-7b). Calculations made from digitized data of optical sections at the bottom of the cell indicate that the mean pixel intensity of antiphosphotyrosine staining (see Methods) in focal adhesions in cells expressing Src251 increases by an average of 3.9 fold (n=5) over non-expressing cells. The alteration of phosphotyrosine staining in focal adhesions is not simply due to the detection of phosphotyrosine in the Src251 protein, as the kinase domain has been deleted and anti-phosphotyrosine antibodies do not recognize the Src251 protein by immunoblotting (data not shown). Both the relative thickness and length of focal adhesions increase, resulting in bundled fibres of dense phosphotyrosine staining (see arrows Figure 3-7b). In contrast, non-expressing cells in the same field (top right; Figure 3-7c; overexposed image) have quantitatively smaller focal adhesions as measured by comparing the relative areas of focal adhesion staining (5.6X larger in Src251 cells; n=5). The altered appearance of focal

Figure 3-7

The non-catalytic amino terminal half of Src increases phosphotyrosine and alters the structure of focal adhesions. Pools of *src*$^{-/-}$ fibroblasts expressing Src251 were co-stained with MAb 327 (A) and anti-phosphotyrosine antibodies to reveal focal adhesions (B) and are presented as projections of multiple optical sections. An alternative scaling of the same data in panel B is provided to reveal the non-expressing cells more clearly (C). Alternatively, cells were co-stained with a polyclonal antibody against Src, N-16 (D) and a MAb against paxillin (E). Cells expressing Src251 are indicated with arrows while the position of non-expressing cells is indicated with arrowheads (A, D). Nuclear staining with polyclonal antibody N-16 was determined to be non-specific, as it also stains the nucleus of non-expressing cells (D). (Scale bar=20μM)

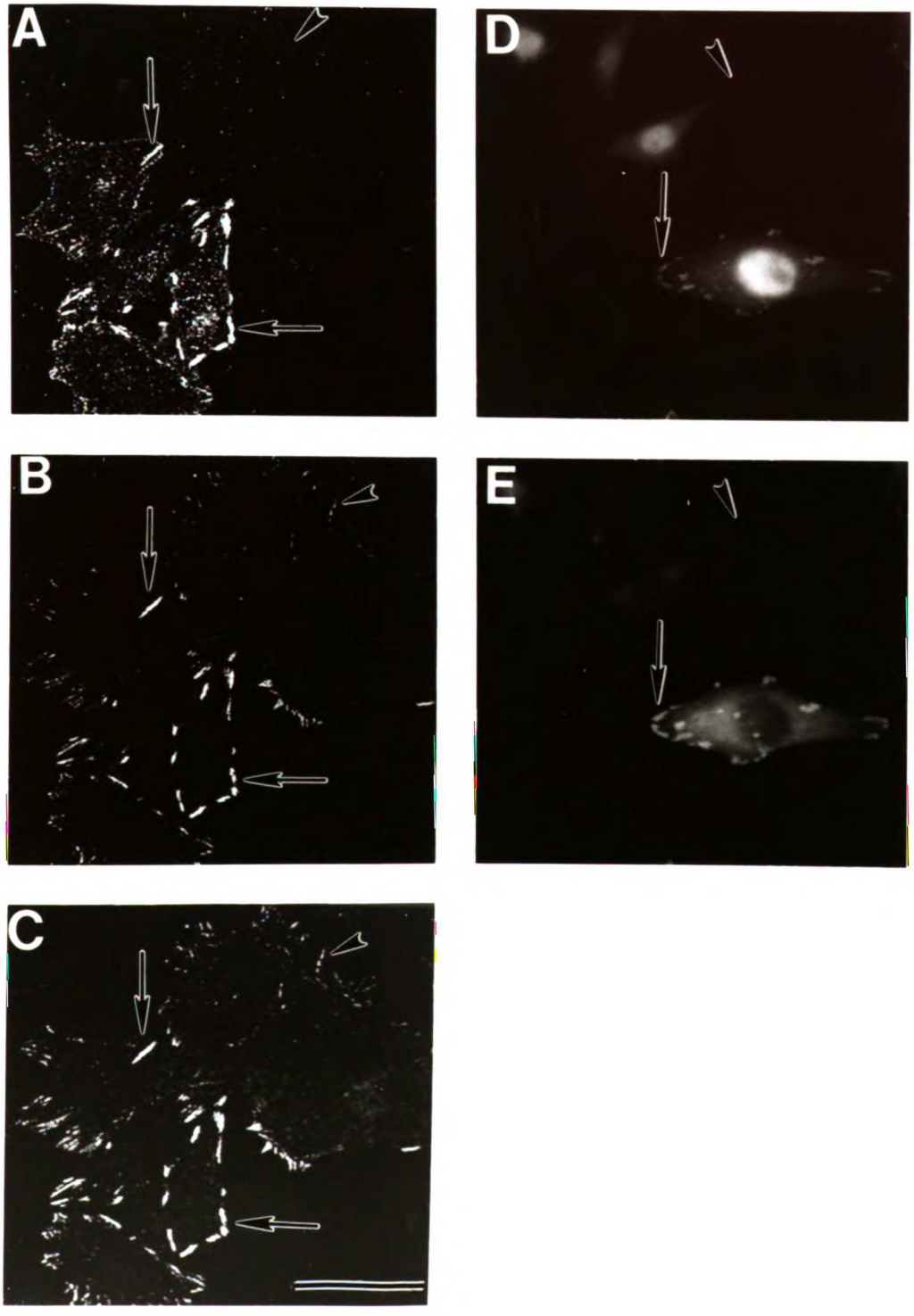
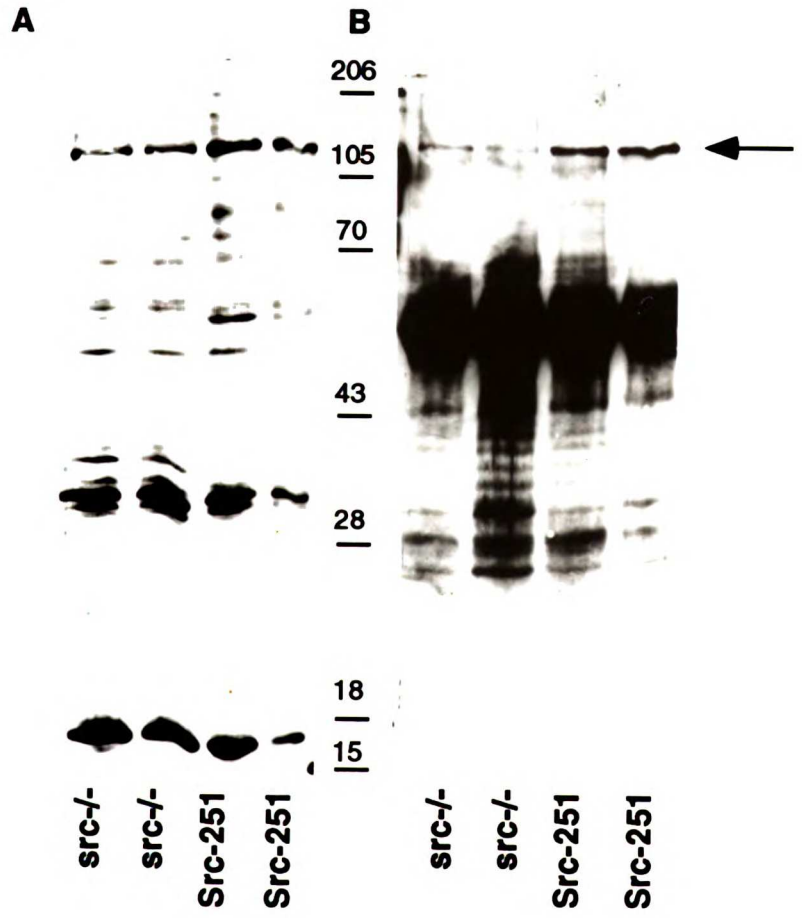

A
D
B
E
C

Figure 3-8

Biochemical changes in the phosphotyrosine content of proteins in cells expressing the amino terminal half of Src. Two *src*$^{-/-}$ fibroblast cell lines expressing Src251 and the two parental *src*$^{-/-}$ lines were lysed in Triton X-100 buffer for 1 minute and the detergent-insoluble fractions were analyzed by immunoblotting with the anti-phosphotyrosine antibody MAb 4G10 (A). Similar Triton X-100-insoluble fractions were immunoprecipitated with antibodies against p125FAK (MAb 2A7) and analyzed as above (B) The arrow indicates the p125FAK band immunoprecipitated from lysates. Molecular weight markers are given in kilodaltons between panels (A) and(B).


A
B
206
105
70
43
28
18
15
src-/-
src-/-
Src-251
Src-251
src-/-
src-/-
Src-251
Src-251

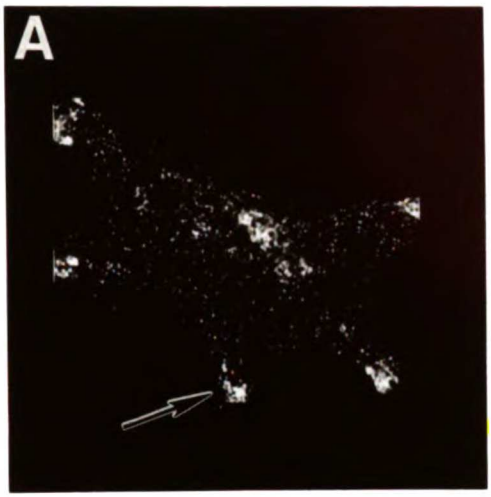
adhesions is also observed by standard immunofluorescence staining with antibodies against paxillin, another marker of focal adhesions (Turner, et al. 1990) (arrow; Figure 3-7d,e).

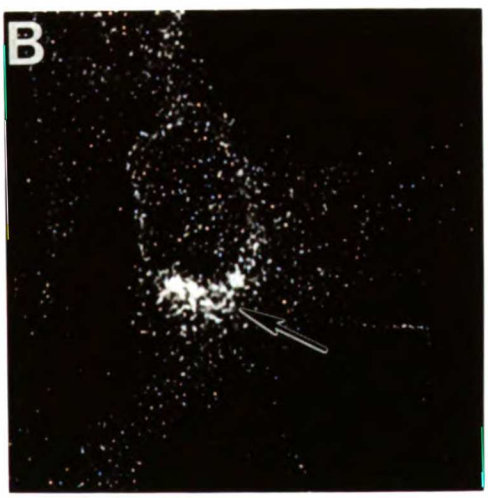
The Src-induced changes in focal adhesions observed by immunofluorescence were further characterized by biochemical fractionation. We fractionated *src*$^{-/-}$ fibroblasts and cells expressing Src-251 in Triton X-100 buffer and both detergent-soluble and insoluble fractions were analyzed for phosphotyrosine-containing proteins. Several proteins in the detergent-insoluble fraction exhibited increased levels of phosphotyrosine in cells expressing Src251 (Figure 3-8a). Protein(s) with an apparent molecular weight of ~120-125Kd underwent the most dramatic change in the levels of phosphotyrosine in several cell lines expressing Src251. Quantitation of the increase in phosphotyrosine signal of the p120-125 complex based on ^{125}I-labeled immunoblots showed an average increase of 2.3 fold (n=4) in cells expressing Src251 compared to the parental cells.

On the basis of the molecular weight of the complex and the reported interaction of the v-Src protein with p125-FAK (focal adhesion kinase) (Cobb, et al. 1994), we tested whether phosphorylation of p125-FAK on tyrosine increased in cells expressing Src-251. P125-FAK was immunoprecipitated from the detergent-insoluble fraction and exhibited 2-3X higher levels of phosphotyrosine compared to p125FAK from the parental *src*$^{-/-}$ fibroblasts (Figure 3-8b). The amount of FAK protein immunoprecipitated from the detergent-insoluble fraction was equal in the parental cell line and the Src-251 expressing cells, indicating that the elevated signal was due to an increase in tyrosine phosphorylation of p125-FAK and not a change in protein distribution (data not shown).

Figure 3-9

Presence of c-SrcM in focal adhesions in cells lacking the c-Src kinase (CSK). c-SrcM was expressed in either *csk*$^{-/-}$ (A) or *csk*$^{-/+}$ cells (B) and cells were stained with MAb 327. Projections of optical sections depict focal adhesion staining (arrows; A) and staining of peri-nuclear membranes (arrow; B). (Scale is the same as Figure 3-2). Triton X-100 fractionation of c-SrcM in *csk*$^{-/-}$ cells (C) or in *csk*$^{-/+}$ cells (D) was conducted as in Figure 3-2. Arrows indicate the Src band; the band below Src represents the immunoglobulin heavy chain.


A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10'
S I S I S I S I S I S I
C
D

Regulation of c-Src association with focal adhesions

Our data suggest that Y527 regulates the sub-cellular distribution of c-Src by controlling the accessibility of amino terminal sequences that mediate association with focal adhesions. A prediction of this model is that a wild-type c-Src protein that associates predominantly with endosomal membranes would redistribute to focal adhesions under conditions where Y527 is not phosphorylated (Figure 3-10). To test this model, we expressed full-length kinase-inactive c-Src (c-SrcM) in cells lacking the Y527.kinase (CSK) due to gene disruption by homologous recombination (Imamoto and Soriano 1993) (*csk*$^{-/-}$).

As predicted, Y527 was not appreciably phosphorylated in *csk*$^{-/-}$ cells, whereas phosphorylation of Y527 was readily detected in *csk*$^{-/+}$ cells derived from heterozygous littermates (Imamoto and Soriano 1993; KBK, unpublished observations). The c-SrcM protein expressed in *csk*$^{-/-}$ cells was present in focal adhesions (Figure 3-9a) and was highly insoluble in detergent (Figure 3-9c). In contrast, c-SrcM expressed in cells containing CSK (*csk*$^{-/+}$) associated primarily with endosomal membranes, not with focal adhesions, and appeared to be detergent-soluble (Figure 3-9b,c). As a control, we also expressed a protein lacking the regulatory Y527 (c-SrcAM) in *csk*$^{-/+}$ cells; it was able to associate with focal adhesions (data not shown). These results support the model that the dephosphorylation of Y527 re-distributes c-Src to focal adhesions and suggests that c-Src may translocate to focal adhesions in response to appropriate cellular signals.

Figure 3-10

Model of the regulation of c-Src localization. When phosphorylated at tyrosine 527, c-Src is in a "closed" conformation and associates primarily with endosomal membranes. Removal of the phosphate at tyrosine 527 results in an "open" conformation that exposes amino terminal domains involved in focal adhesion localization. This process can be reversed through phosphorylation of tyrosine 527 by CSK, followed by an enrichment of c-Src in endosomal membranes.

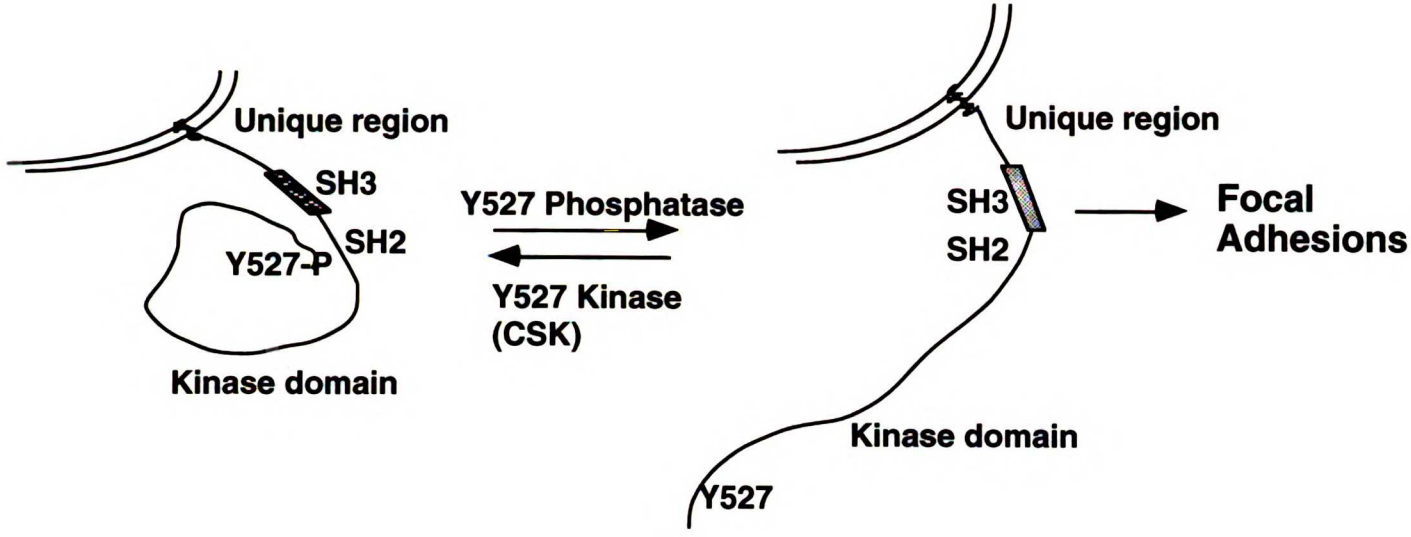

Unique region
SH3
SH2
Y527-P
Kinase domain
Y527 Phosphatase
Y527 Kinase
(CSK)
Unique region
SH3
SH2
Focal
Adhesions
Kinase domain
Y527

Figure 3-10

Discussion

We have shown that the subcellular distribution of c-Src is controlled by the phosphorylation of tyrosine 527 (Y527), the same tyrosine that serves as a negative regulator of c-Src kinase activity. When Y527 is mutated, association of c-Src with focal adhesions occurs independently of kinase activity and is dependent on membrane attachment. Sequences in the amino terminal half of c-Src are sufficient for association with focal adhesions, and mutational analyses demonstrate the involvement of the SH3 domain and myristylation in focal adhesion association. The presence of the amino terminal 251 amino acids of Src in focal adhesions can dramatically alter their structural and biochemical properties, implicating c-Src in the regulation of focal adhesions and therefore in cellular adhesion.

Mechanism for regulating c-Src distribution

While the v-Src protein associates with adhesion plaques in transformed cells, c-Src protein associates with endosomal membranes and is absent from adhesion plaques. The ability of Y527 to regulate the distribution of c-Src between these two sub-cellular compartments is probably related to the ability of phosphorylated Y527 to bind intra-molecularly to the SH2 domain. Disruption of the intra-molecular interaction by mutation of Y527 correlates with a change in protein conformation (MacAuley and Cooper 1989). In addition to exposing the SH2 domain (Liu, et al. 1993a; Roussel, et al. 1991), we propose that this change in conformation also exposes SH3 related signals that are sufficient to allow c-Src to distribute to focal adhesions (see model). The phosphorylation of other proteins by c-Src appears to be irrelevant for c-Src distribution, as kinase-inactive c-Src associates with both endosomes and focal

adhesions. The fact that the same site is used to regulate both the location and the kinase activity of c-Src may reflect the importance of coordinating the location and the activity of the kinase inside the cell, perhaps as means for ensuring that normal c-Src does not disrupt cellular adhesion.

Focal adhesion localization signals

Clearly, multiple signals in the amino terminus are involved in the association of c-Src with focal adhesions. Myristylation and therefore membrane association is required to place c-Src in focal adhesions. Consistent with our findings, myristylation has been shown to be required for maintaining the detergent-insolubility of v-Src (Hamaguchi and Hanafusa 1987). Membrane association may be required either for c-Src attachment to the plasma membrane within focal adhesions or for proper trafficking to focal adhesions. It is unclear whether the c-Src that is associated with intra-cellular membranes represents protein trafficking to focal adhesions, or cycling out of them. In either case, understanding how Src transits from endosomal membranes to focal adhesions will be important in determining the function of c-Src in these two compartments.

Chimeric proteins that fuse the first 14 amino acids of v-Src to pyruvate kinase do not enter focal adhesions even though they are myristylated and membrane-associated (Kaplan, et al. 1990), KBK; unpublished observations), consistent with the requirement of both SH3 sequences and membrane attachment for focal adhesion association. Presumably, SH3 interacts with other proteins, either those in the focal adhesions or those involved in membrane trafficking, to induce c-Src to associate with focal adhesions. While the precise mechanism remains unclear, recent findings that the SH3 domain of c-Src interacts with dynamin and PI3 kinase (proteins involved in regulating

endocytosis) as well as paxillin (a component of focal adhesions) provide evidence for a connection between c-Src, endosomal membranes and focal adhesions (Gout, et al. 1993; Liu, et al. 1993b; Weng, et al. 1993). In this regard, it is interesting that endosomal membranes containing Src251Δ92-144 have an altered staining pattern compared to those containing c-Src (Figure 3-2a) or Src251 (Figure 3-5d-f). The Src251Δ92-144 pattern of staining in the middle of the cell is tightly clustered around the nucleus and may represent an intermediate membrane compartment that requires the SH3 domain of c-Src for proper sorting. The residual amount of detergent insolubility of Src251Δ92-144 may represent protein complexes associated with this intermediate membrane compartment, possibly associated with caveolar structures on these membranes (Anderson 1993). A clearer understanding of these putative complexes may reveal the nature of the proteins that interact with the amino terminus of c-Src both in focal adhesions and endosomal membranes.

The SH2 domain mediates protein/protein interactions primarily through interactions with phosphorylated tyrosine residues. However, unlike SH3, the phosphotyrosine binding ability of SH2 is not absolutely required for Src251 to associate with focal adhesions. Studies of deletion mutants in v-Src indicate that the amino terminal half of SH2 (149-169) is required for the association of v-Src with the detergent insoluble fraction of the cell (Fukui, et al. 1991). However, the function of these sequences (149-169) is unclear, as a deletion of the carboxy terminal half of SH2 (169-204) does not affect the association of v-Src with the detergent-insoluble fraction but is likely to abolish the ability of SH2 to bind phosphotyrosine. It is possible that the deletion of amino acids 149-169 decreases the stability of the neighboring SH3 domain resulting in detergent solubility. In our experiments, the analyses of SH2 deletion mutants were hampered by gross changes in protein stability that made simple interpretation

impossible (KBK; unpublished observations). However, the single amino acid change at position 175 (R175L) in c-Src has been shown previously to abolish the binding of phosphotyrosine ligands to SH2 *in vitro* (Bibbins, et al. 1993). Consistent with disruption of phosphotyrosine binding, we have demonstrated that p125FAK (focal adhesion kinase) co-immunoprecipitates with Src251, but not with R175LSrc251 from cell extracts (KBK; unpublished observations). Despite the inability of this mutant protein to mediate phosphotyrosine interactions, it associates with focal adhesions. The absence of SH2 function results in a subtle redistribution of this mutant protein and may be explained, in part, by higher levels of protein expression. Alternatively, there may be a role for SH2 in regulating the overall efficiency of Src-traffic to focal adhesions.

The role of the carboxy terminus of c-Src

In general, our observations indicate that the carboxy terminus of c-Src does not interact with focal adhesions, but instead serves to regulate the accessibility of amino terminal signals. Phosphorylation of Y527 by CSK enables the carboxy terminus to associate with the amino terminus, blocking the interaction of amino terminal domains with other proteins. Conversely, dephosphorylation of Y527 prevents the intra-molecular interaction and allows other proteins access to amino terminal domains. Deletion of the carboxy terminus, as in Src251, permanently exposes the amino terminus that contains the necessary information for association with focal adhesions.

The carboxy terminus may also play some role in focal adhesions by influencing amino terminal signals. By comparing deletions made in the truncated Src251 protein with the same deletions made in the full length c-SrcAM protein, the carboxy terminus was observed to influence deletions involving sequences in the region of the SH3 domain. Deletions of amino acids

17-30 and the SH2 domain were unaffected by the carboxy terminus. In contrast, the carboxy terminus altered the accessibility of antibody epitopes and slightly enhanced the focal adhesion distribution of mutants with deletions encompassing amino acids 30-144 (including Δ30-92 and Δ92-144). These results suggest that the carboxy terminus of c-Src affects the conformation of the SH3 domain and thus the presentation of the signal for association with focal adhesions. Support for the putative interaction of the SH3 domain and the carboxy terminus comes from recent studies of c-Src regulation in yeast, which demonstrated that efficient regulation by Y527 required the SH3 domain (Murphy, et al. 1993; Superti-Furga, et al. 1993). Taken together, these observations suggest that the carboxy terminus does not direct c-Src to focal adhesions, but can influence the presentation of amino terminal signals. Dissecting the exact nature of the SH3-related signal will require extensive mutagenesis and biochemical characterization of proteins that interact with this region in order to identify the exact sequences involved in associating with focal adhesions.

Regulation of focal adhesions by src

The striking structural and biochemical alteration of focal adhesions in cells expressing the amino terminal half of Src provides compelling evidence that Src may normally regulate these structures. Alteration of focal adhesion structure is dependent on the SH3 domain, which may be needed primarily for trafficking or may interact directly with focal adhesion structures. The fact that only low levels of Src251 protein expression were ever achieved in *src*$^{-/-}$ fibroblasts, suggests that Src may influence the catalytic activity of other kinases or phosphatases in focal adhesions. The tyrosine kinase p125FAK is one potential target for Src regulation in focal adhesions. On the other hand,

mutants that no longer associate with p125FAK are still able to alter the structure of focal adhesions, suggesting that Src may have other relevant interactions in focal adhesions (KBK; unpublished observations). The detection of other Src family members in the adherens junctions of polarized cells (Tsukita, et al. 1991) and associated with focal adhesion proteins in chicken embryo fibroblasts (Cobb, et al. 1994) suggests that Src-family members may generally be involved in transducing signals at sites of adhesion.

Upstream signals for regulation of c-Src redistribution

Our results imply that the amino terminal region of Src is capable of regulating focal adhesions in the absence of kinase activity. It may be that this is the primary mode of regulation used by c-Src in focal adhesions and that the kinase activity of Src is required in other compartments. It is also possible that kinase activity will provide an additional level of regulation in focal adhesions by phosphorylating specific substrates located in focal adhesions. In either case, our results predict that upstream signals that influence the phosphorylation of Y527 will influence both the kinase activity and the sub-cellular distribution of c-Src, possibly resulting in changes in focal adhesions and therefore in cell adhesion. These signals could originate from various cell surface receptors or from internal regulators of cellular events. Examples of c-Src regulation during the cell cycle (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989; Shenoy, et al. 1989) and in response to growth factors (Kypta, et al. 1990; Ralston and Bishop 1985) may provide a link between cell growth and cellular adhesion. It will be important to identify the signals that influence the sub-cellular distribution of c-Src, which presumably act through the Y527 kinase (CSK) or a Y527 phosphatase. By understanding the cellular

signals that lead to the redistribution of Src to focal adhesions, we will gain insight into the possible role of c-Src in cellular adhesion events.

Chapter Four:

A role for c-Src in fibronectin-mediated adhesion of fibroblasts

Abstract

Analysis of the tyrosine kinase c-Src during cellular adhesion showed a transient increase in specific kinase activity that was associated with a decrease in phosphorylation of the negative regulatory tyrosine (Y527). Activation of c-Src was followed by a redistribution of c-Src to newly formed focal adhesions. The role of c-Src in cellular adhesion was addressed by examining fibroblasts lacking c-Src (*src*$^{-/-}$). *Src*$^{-/-}$ fibroblasts exhibited a defect in cell adhesion on fibronectin that could be complemented by expression of chicken c-Src. Expression of c-Src mutants revealed that the SH2 and SH3 domains, but not the kinase domain, are required to rescue the adhesion defect in *src*$^{-/-}$ fibroblasts. These results suggest that the enzymatic activity and subcellular distribution of c-Src are coordinately regulated during cellular adhesion and that c-Src can affect adhesion by a kinase-independent mechanism.

Introduction:

The tyrosine kinase c-Src belongs to a family of cytoplasmic tyrosine kinases that associate with cellular membranes and whose normal function is not clear (for review see (Cooper 1989). Studies of mutant forms of the protein (e.g., the viral transforming protein v-Src) have implicated c-Src in the regulation of growth control. In addition, the kinase activity of c-Src increases during mitosis in fibroblasts and thus may mediate certain mitotic events (Chackalaparampil and Shalloway 1988; Morgan, et al. 1989; Shenoy, et al. 1989). However, high levels of c-Src expression in terminally differentiated cells such as platelets and neurons suggest a specific cellular function for c-Src unrelated to growth control (Cotton and Brugge 1983; Golden, et al. 1986).

Recent genetic studies with mice containing a homozygous disruption of the c-src gene implicates c-Src in the normal physiological function of osteoclasts (Boyce, et al. 1993; Boyce, et al. 1992; Soriano, et al. 1991), but have not led to any clear understanding of the cellular function of c-Src.

Several common sequence motifs are found in c-Src and related src-family kinases. Most notable are the conserved SH2 and SH3 domains in the amino terminal half of c-Src that are shared with a number of proteins involved in signal transduction. The SH2 domain interacts with phosphotyrosine-containing sequences (Mayer, et al. 1991; Pawson and Gish 1992), while the SH3 domain is thought to mediate interactions with proline-rich regions (Ren, et al. 1993). The amino terminus of c-Src also contains the "unique" domain (amino acids 2-90), which shares little homology with other Src-family members and is involved in membrane attachment (Kaplan, et al. 1990).

While the amino terminal half of c-Src mediates protein/protein interactions, the carboxy terminal half contains the kinase domain and two tyrosine phosphorylation sites that regulate the kinase activity. Autophosphorylation at tyrosine 416 (Y416) is required for full kinase activity (Kmiecik, et al. 1988; Piwnica-Worms, et al. 1987) and phosphorylation at tyrosine 527 (pY527) inhibits c-Src activity (Courtneidge 1985; Kmiecik and Shalloway 1987). Normally, Y527 is extensively phosphorylated in the cell by the c-Src kinase (CSK) (Imamoto and Soriano 1993), resulting in an intramolecular interaction between phospho-Y527 and the SH2 domain (MacAuley and Cooper 1989). Deleting Y527 (as in v-Src) or mutating it to a phenylalanine (Y527F) disrupts this interaction and results in a highly active kinase with exposed amino terminal domains (the "open" conformation) (Liu, et al. 1993a; Roussel, et al. 1991) that now may interact with target proteins.

Recently we have shown that exposure of the amino terminal domains of c-Src can also influence its sub-cellular location. While c-Src normally associates with endosomal membranes (David-Pfeuty and Nouvian-Dooghe 1990; Kaplan, et al. 1992), decreased phosphorylation at Y527, either by amino acid substitution or expression of c-Src in cells lacking CSK, results in the association of c-Src with focal adhesions (Kaplan et al, submitted for publication). The amino terminal half of c-Src mediates association with focal adhesions and, significantly, can alter the structure and biochemical properties of focal adhesions even in the absence of c-Src-kinase activity (Kaplan et al, submitted for publication). Our observation that Y527 phosphorylation regulates c-Src localization and focal adhesion structure raises the possibility that c-Src plays a role in cellular adhesion events.

The phosphorylation of integrins in v-Src transformed cells, as well as alterations in the adhesive properties of src-transformants have long implicated c-Src in adhesion-related events (Aneskievich, et al. 1991; Hirst, et al. 1986; Nigg, et al. 1986; Pasquale, et al. 1986). The translocation of c-Src to the cytoskeleton during platelet activation is dependent on the integrin alpha IIb/beta III, arguing for a functional connection between integrins and c-Src (Clark and Brugge 1993; Horvath, et al. 1992). In addition, the presence of c-Src in neuronal growth cones and the reduction in the rate of axon growth in src-deficient neurons implicates c-Src in adhesion events involved in neuronal development (Ignelzi, et al. 1994). Finally, the SH2-dependent association of c-Src with the focal adhesion kinase (FAK) provides a direct biochemical link between c-Src and a tyrosine kinase thought to be involved in cellular adhesion (Cobb, et al. 1994; Guan and Shalloway 1992; Kornberg, et al. 1992; Schaller, et al. 1994; Turner, et al. 1993).

We have examined the role of c-Src in cellular adhesion. We find that when fibroblasts are plated on fibronectin, there is a transient increase in the kinase activity of c-Src, followed by the redistribution of c-Src to newly formed focal adhesions. In addition, c-src-deficient (*src*$^{-/-}$) fibroblasts, derived from mice homozygous for a c-src gene disruption (Soriano, et al. 1991), exhibit a defect in adhesion on fibronectin that is rescued by expression of c-Src. Complementation of the adhesion defect by c-Src requires the SH2 and SH3 domains but not the kinase domain. These results provide a direct link between the biochemical association of c-Src with focal adhesions and events required for efficient cellular adhesion.

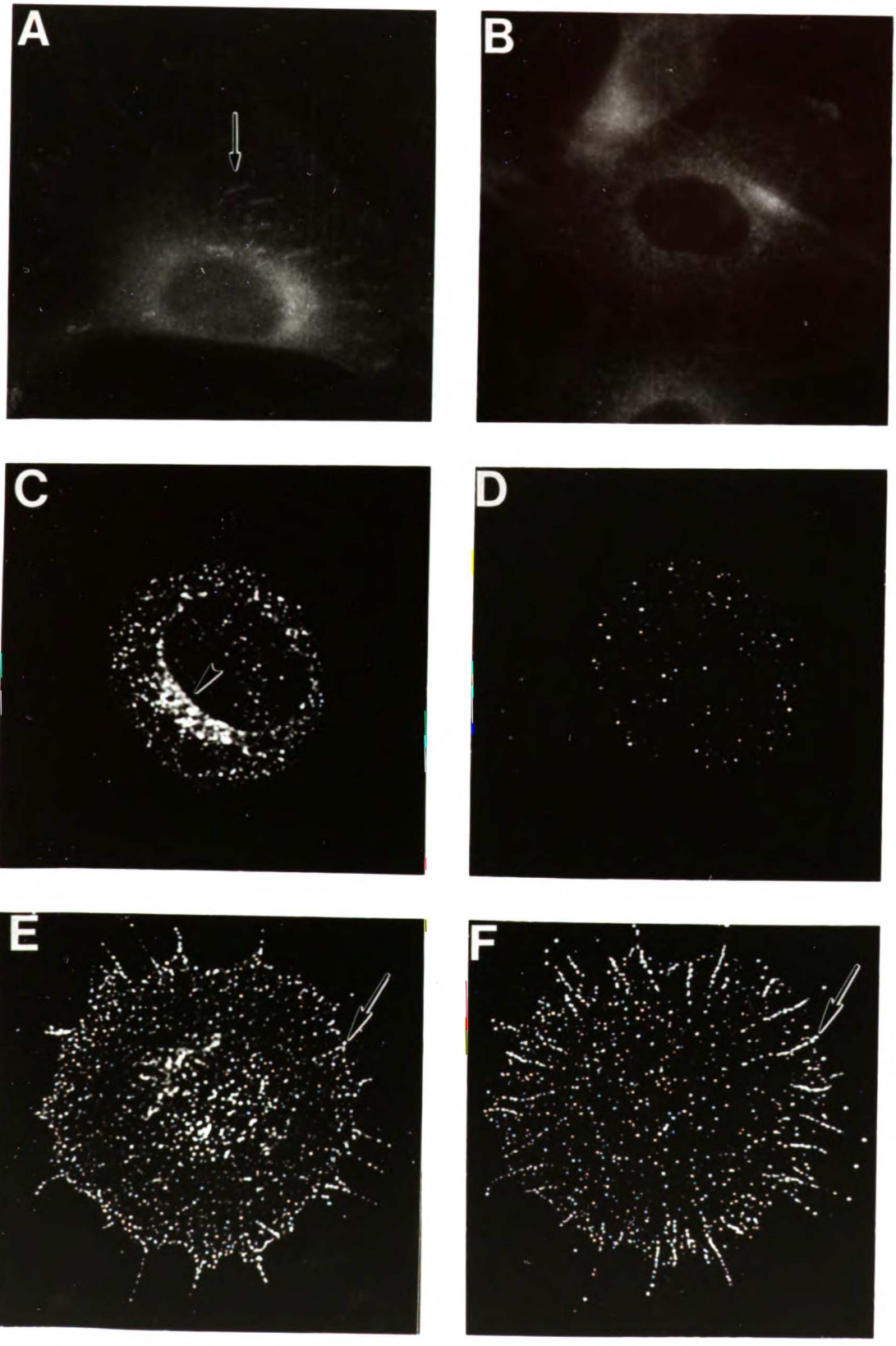
Results:

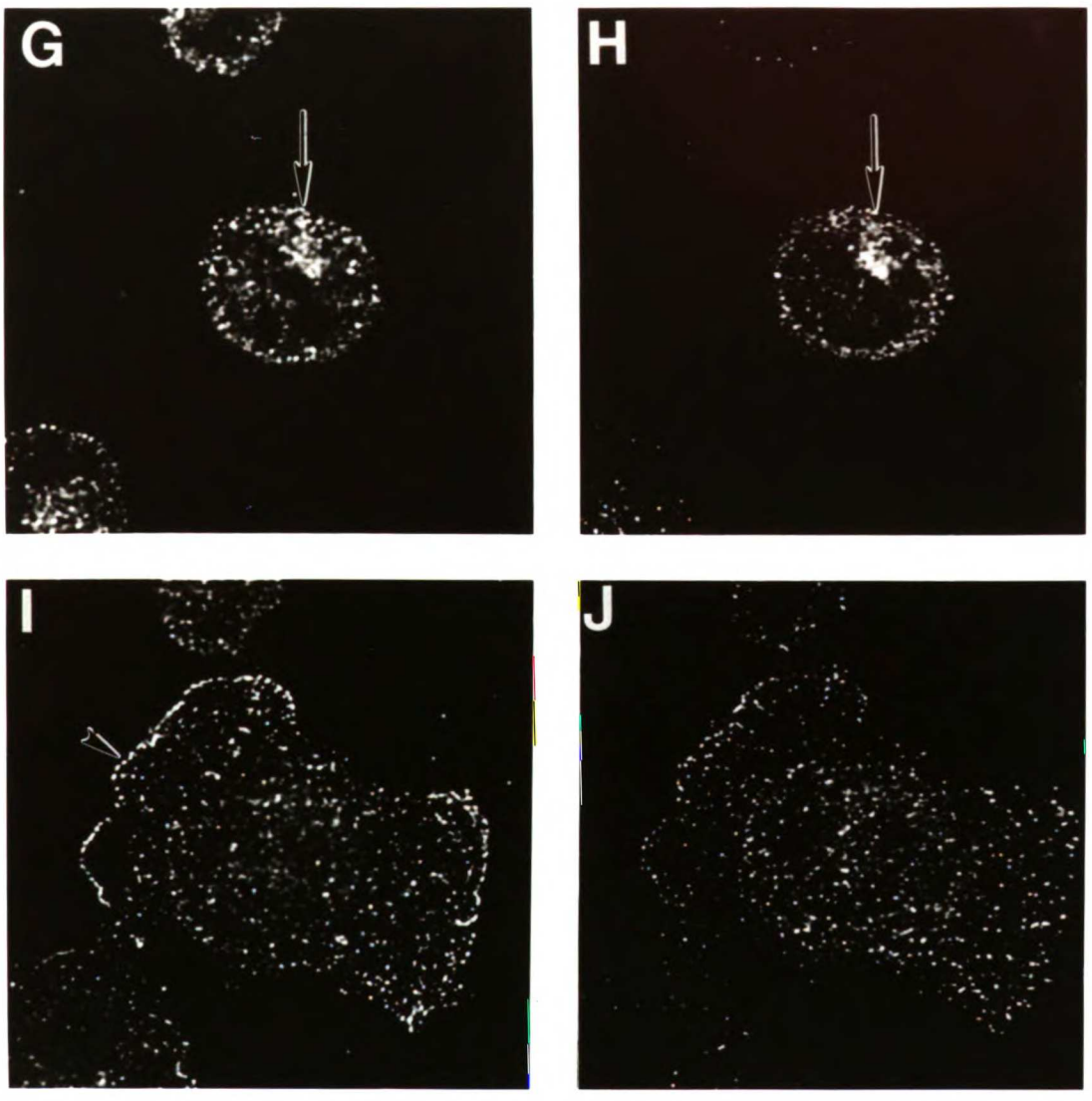
Redistribution of c-Src during adhesion on fibronectin

Our recent demonstration that the phosphorylation of Y527 regulates association of c-Src with focal adhesions (Kaplan et al, in press) led us to examine the subcellular distribution of c-Src expressed in *src*$^{-/-}$ fibroblasts during cellular adhesion. Standard immunofluorescence revealed that a significant proportion of c-Src associates with focal adhesions after cells were plated on fibronectin-coated coverslips, when compared to cells plated on uncoated-coverslips (arrows; Figure 4-1 a, b). Analysis of immunofluorescence by optical-sectioning microscopy of cells plated for short periods of time (20 min) on fibronectin showed the association of c-Src with newly formed focal adhesions early in the process of cellular adhesion. Optical sections from the bottom of the cell revealed c-Src in a symmetrical pattern of fibre-like structures at the periphery of the adhering cell (Figure 4-1e). Co-staining of the same cell with anti-phosphotyrosine antibodies revealed similar structures that were also recognized by antibodies against vinculin, another marker of focal adhesions

Figure 4-1:

C-Src associates with focal adhesions in cells plated on fibronectin. *Src*$^{-/-}$ fibroblasts expressing c-Src were plated on fibronectin-coated (A) or uncoated (B) coverslips for 12 hours and stained with MAb 327 (arrow indicates focal adhesion structures). Cells plated for 20 min. (C, D, E, F), or 10 min. (G, H, I, J) on fibronectin-coated coverslips were stained with MAb 327 (C, E, G, I) and anti-phosphotyrosine antibodies (D, F, H, J). Indirect immunofluorescence was analyzed by optical sectioning microscopy (see Methods) and sections at the middle of the cell (C, D), at the top of the cell (G,H) and at the bottom of the cell (E, F, I, J) are presented. (Scale bar=10μM).


A
B
C
D
E
F


G
H
I
J
α-Src
α-p-tyr

(Figure 4-1f and data not shown). A significant proportion of c-Src was also observed at the microtubule organizing center in optical sections from the middle of the cell (Figure 4-1c) and co-staining experiments with endosomal markers suggest that this staining represents an endosomal compartment (data not shown; (Kaplan, et al. 1992)). Significantly, this population of c-Src does not co-stain with anti-phosphotyrosine antibodies, further distinguishing c-Src in the middle and at the bottom of the adhering cell (Figure 4-1d, f).

We also examined the redistribution of c-Src by Triton X-100 detergent-fractionation. Increased detergent-insolubility of c-Src is associated with the presence of c-Src in focal adhesions (Kaplan et al, submitted for publication). The solubility of c-Src was examined in cells adherent on uncoated, BSA-coated and fibronectin-coated plates. In cells plated either on uncoated or BSA-coated wells, c-Src was predominantly soluble, even at early time points of lysis (30s; Figure 4-2a,b). In contrast, c-Src in cells plated on fibronectin-coated wells was predominantly insoluble at the earliest points of lysis (30s, 1 min; Figure 4-2c), and a significant portion remained insoluble even at the later time points (4 min; Figure 4-2c). These results suggest fibronectin-mediated adhesion causes the redistribution of c-Src to the detergent-insoluble fraction.

In contrast to the focal adhesion distribution of c-Src after cell spreading, a strikingly different distribution was observed early in the process of cellular adhesion. Fibroblasts expressing c-Src were allowed to attach to fibronectin-coated coverslips for brief periods of time (10 min); under these conditions very few cells (<1%) had spread. Optical sections at the bottom of the cell did not reveal extensive phosphotyrosine-containing structures, consistent with the lack of focal adhesion formation at this early stage of cellular adhesion (Figure 4-1j). Some cells contained a small amount of c-Src at the bottom of the cell, at the leading edge of the spreading cell membrane (arrowhead; Figure 4-1i). In

contrast to spread cells (Figure 1c), the majority of c-Src was located in optical sections at the top of newly attached cells (arrows; Figure 4-1g), in a perinuclear location that costains with endosomal-membrane markers (data not shown). Interestingly, endosomal membrane-associated c-Src (Figure 4-1g) co-stained with phosphotyrosine-containing proteins in the same optical section of the cell (arrow; Figure 4-1h). The costaining of c-Src with phosphotyrosine proteins at this position in the cell was only observed at this very early stage of cell attachment to fibronectin. This unusual staining pattern at the top of the cell suggests that biochemical properties of this population of c-Src may be altered during the early stages of cellular adhesion.

Activation of c-Src during cellular adhesion

To address the biochemical properties of c-Src during binding of cells to fibronectin, we examined the possibility that re-distribution of c-Src during cellular adhesion is accompanied by a change in kinase activity. *Src*$^{-/-}$ cells expressing chicken c-Src were incubated on fibronectin-coated dishes for various times at 37°C in order to examine the behavior of c-Src at different stages of adhesion (see Methods). Adhesion of fibroblasts to fibronectin-coated dishes was divided into several distinct stages (Figure 4-3): (i) cell attachment to fibronectin-coated plates, presumably via cell surface integrins (<5 min), (ii) cell spreading, including the appearance of membrane projections (10-15 min), and (iii) the complete flattening of cells into a normal fibroblast (45 min). Cells isolated from each of these stages of adhesion were fractionated into detergent-soluble and detergent-insoluble fractions and the kinase activity of c-Src was measured in Src immunoprecipitates.

Greater than 95% of cells had attached to fibronectin-coated plates after five minutes at 37°C (Figure 4-3a) and detergent fractionation revealed that the

Figure 4-2:

Cells plated on fibronectin induce c-Src redistribution to the Triton X-100 insoluble fraction. Cells expressing c-Src plated on uncoated-wells (A), BSA-coated wells (B) and fibronectin-coated wells (C) were fractionated in Triton X-100-containing buffer for indicated times and detergent-soluble (S) and detergent-insoluble (I) fractions were immunoprecipitated and immunoblotted with MAb 327. The upper band in each panel represents the 60kD c-Src protein and the lower band represents the immunoglobulin heavy chain, recognized by the anti-mouse secondary antibody.

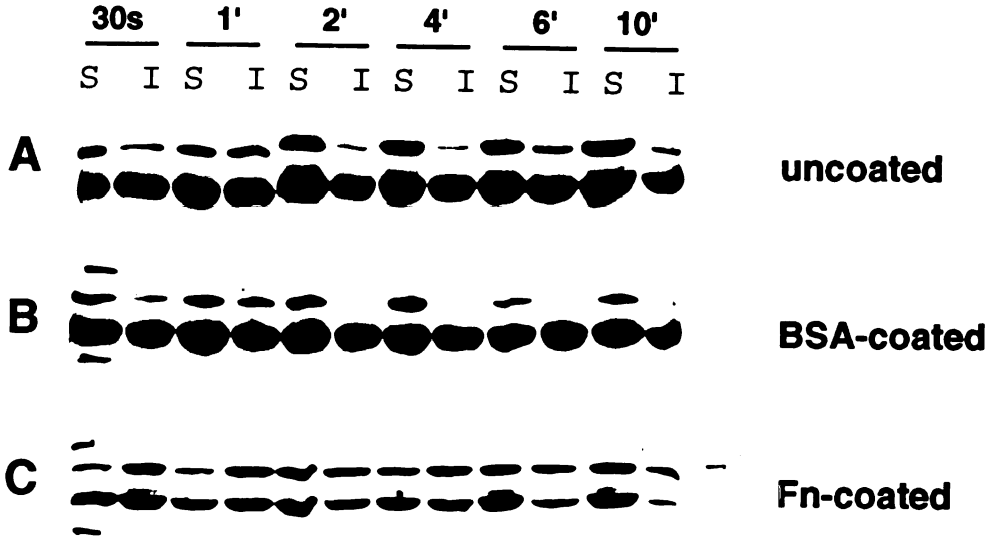
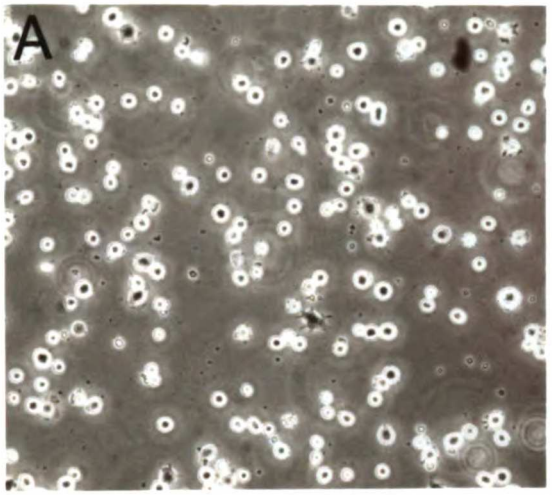
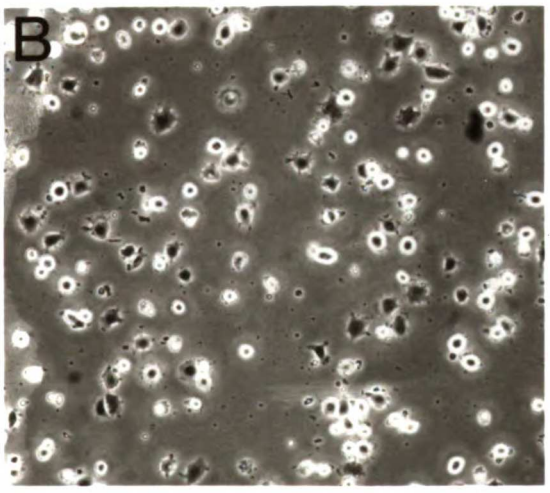

30s
1'
2'
4'
6'
10'
S I S I S I S I S I S I
A
uncoated
B
BSA-coated
C
Fn-coated

Figure 4-3:

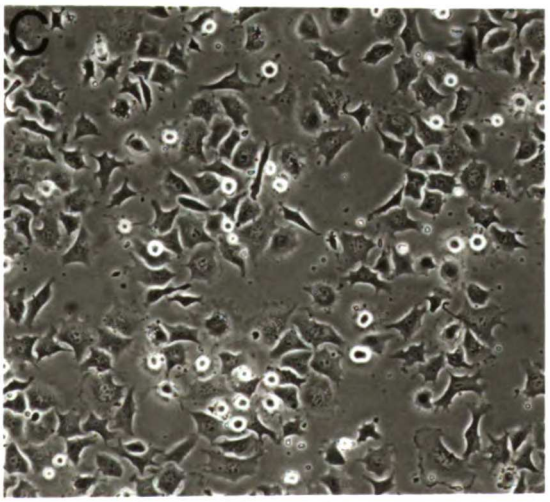
Cellular adhesion of fibroblasts expressing c-Src occurs in three major stages. *Src*$^{-/-}$ fibroblasts expressing c-Src were plated on fibronectin-coated dishes for 5 min, 15 min, and 45 min at 37°C and were photographed by phase-contrast microscopy. These cells were subsequently prepared for analysis in Figure 4-4. (Scale bar=15μM)



5' on Fn



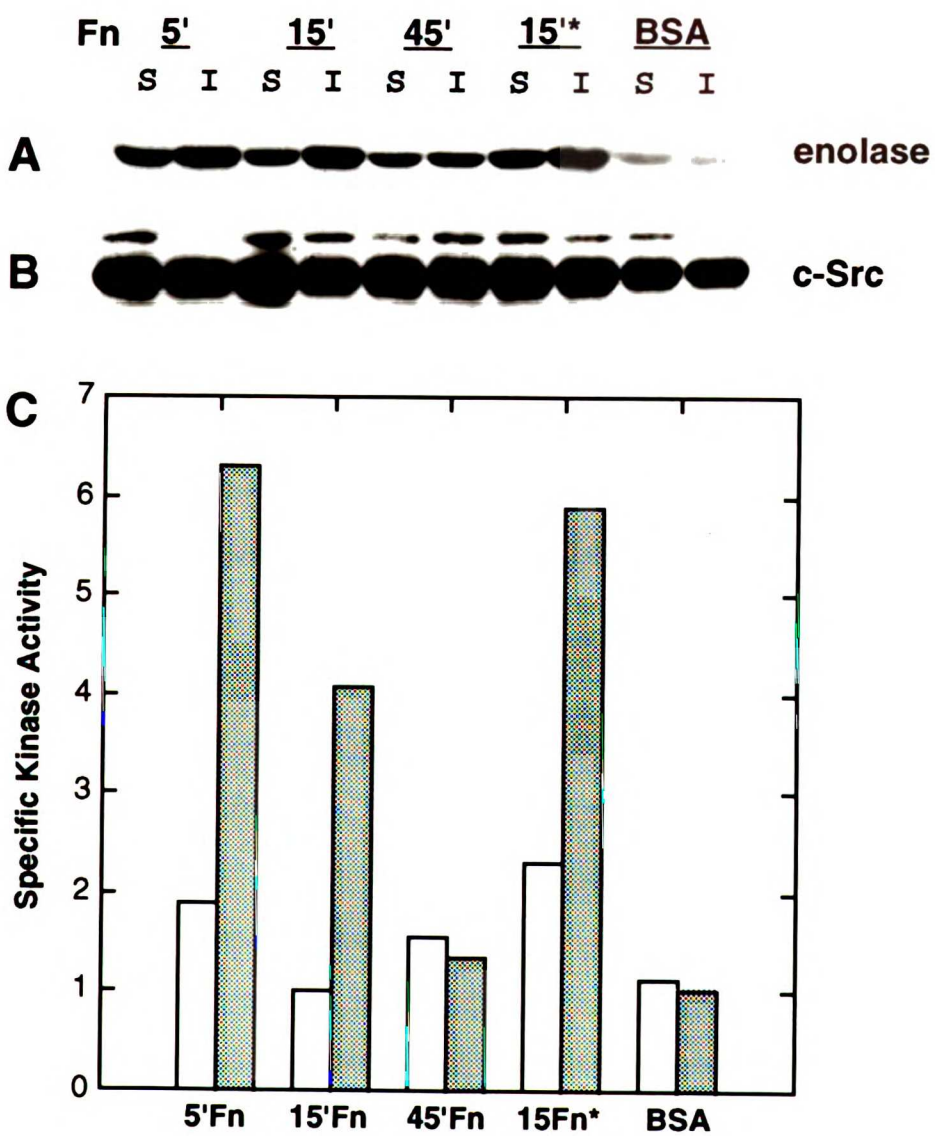
15' on Fn



45' on Fn

Figure 4-4:

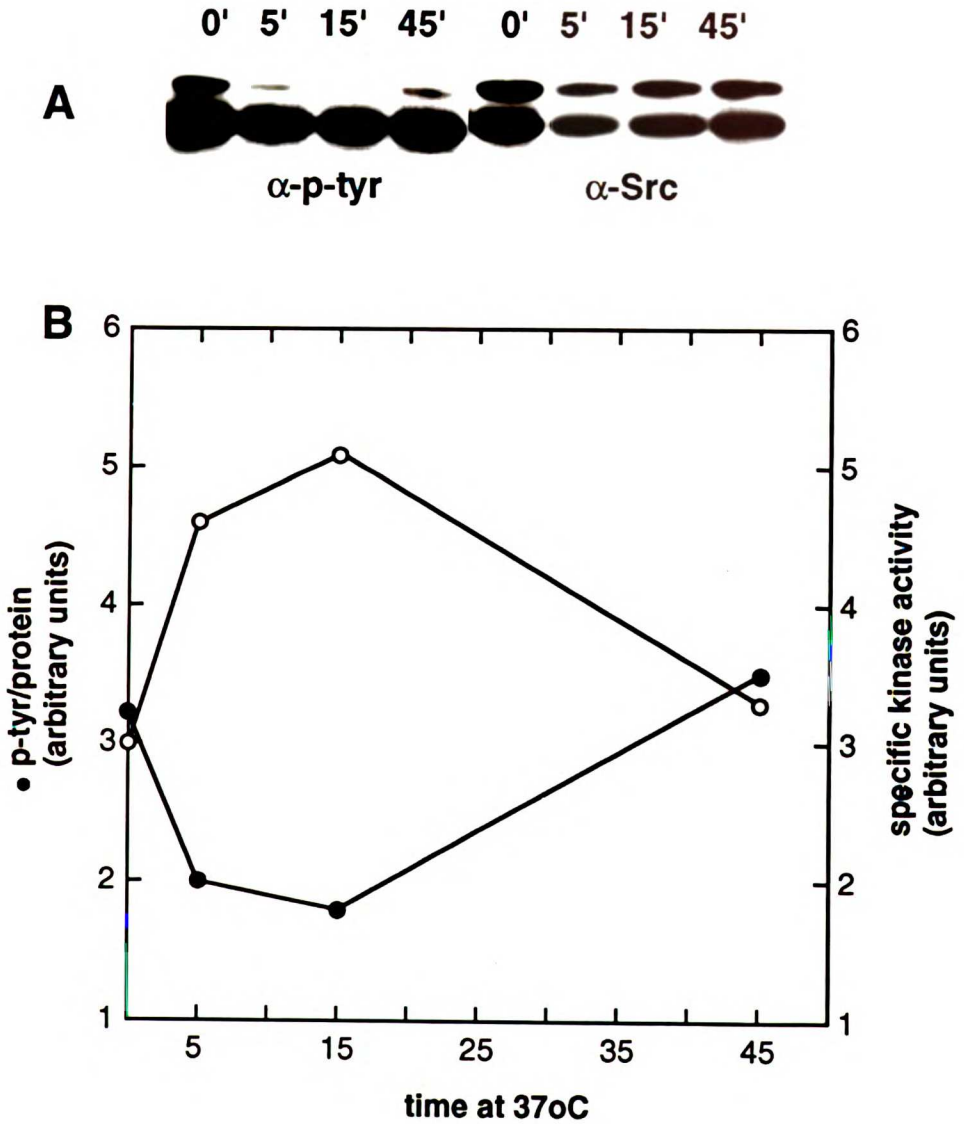
The kinase activity of c-Src is transiently increased after plating cells on fibronectin. *Src*$^{-/-}$ fibroblasts expressing c-Src plated on fibronectin-coated plates for the indicated times (see Figure 3) were fractionated in Triton X-100-containing buffer for 1' and detergent-soluble (S, ☐) and detergent-insoluble (I, ▩) fractions were immunoprecipitated with MAb 327. Cells in the 15'* lane were first plated on BSA-coated wells for 45', replated for 15' on fibronectin-coated wells and then similarly analyzed. Immunoprecipitates were analyzed by measuring the incorporation of ^{32}P into the exogenous substrate, enolase (A) and immunoblotting with MAb 327 (B); the upper band is c-Src and the lower band represents the immunoglobulin heavy chain, recognized by the ^{125}I-conjugated anti-mouse secondary antibody. The specific kinase activity was calculated by dividing the area of the enolase bands by the area of the c-Src bands as determined by PhosphorImager analysis. Cells plated on BSA for 45' and then replated on fibronectin for 15' were treated as described above.


Fn 5' 15' 45' 15'* BSA
S I S I S I S I S I
A
enolase
B
c-Src
C
Specific Kinase Activity
0
1
2
3
4
5
6
7
5'Fn
15'Fn
45'Fn
15Fn*
BSA

majority of c-Src remained in the soluble fraction (Figure 4-4a). The amount of c-Src protein was used to calculate the specific kinase activity of c-Src immunoprecipitated from each fraction. We observed a 3.4 fold increase in total kinase activity (i.e., from soluble and insoluble fractions), and a 5.7 fold increase in the detergent-insoluble fraction when compared to c-Src isolated from cells plated on BSA-coated wells (Figure 4-4b, c). The activity of the kinase decreased as cells continued to spread on fibronectin. In fifteen minutes, approximately 30% of cells had spread (Figure 4-3b) and the specific kinase activity of c-Src was reduced by 38%, although the kinase activity remained high compared to the BSA control (2.1X; Figure 4-4b, c). The large portion of c-Src (40%) observed in the detergent-insoluble fraction correlated with a decrease in specific kinase activity in the detergent-insoluble fraction (Figure 4-4b, c). This trend continued as the specific kinase activity of c-Src dropped to control levels after cell spreading had occurred (Figure 4-3c) and the majority of c-Src (60%) was found in the detergent-insoluble fraction (Figure 4-4a, b, c). Prolonged incubation of cells had no permanent effect on the regulatory mechanisms, as c-Src could be activated when cells were replated on fibronectin-coated wells after first attaching to BSA-coated wells for forty five minutes (Figure 4-4c, 15'*). In addition, c-Src isolated from trypsinized cells exhibited a low level of kinase activity, suggesting c-Src is primarily regulated during adhesion and not during cell rounding (data not shown). The activation of the c-Src kinase during cell attachment to fibronectin is consistent with high levels of phosphotyrosine staining at the top of attached cells (Figure 1h). The phosphotyrosine staining may represent either c-Src itself, which appears to be highly autophosphorylated (KBK; unpublished observations), or other protein substrates of the activated c-Src kinase (Figure 4-2j). Finally, these results are

Figure 4-5:

Dephosphorylation of Y527 is inversely related to the increase in kinase activity of c-Src after plating cells on fibronectin. *Src*$^{-/-}$ cells expressing c-SrcY416F plated on fibronectin-coated plates for the indicated times were lysed and immunoprecipitated with MAb 327. Immunoprecipitates were analyzed by measuring the incorporation of ^{32}P into the exogenous substrate, enolase, immunoblotting with MAb 327 (A; α-Src) and anti-phosphotyrosine antibodies (A; α-p-tyr); the upper band is c-Src and the lower band represents the immunoglobulin heavy chain, recognized by the ^{125}I-conjugated anti-mouse secondary antibody. The specific phosphotyrosine/Src ratio was calculated by dividing the areas calculated for the phosphotyrosine bands by the area of the c-Src bands and is presented graphically(—o—). The specific kinase activity was calculated as in Figure 4 and is presented graphically (—●—).


A
0' 5' 15' 45' 0' 5' 15' 45'
α-p-tyr
α-Src
B
• p-tyr/protein (arbitrary units)
specific kinase activity (arbitrary units)
time at 37oC
1
2
3
4
5
6
5
15
25
35
45

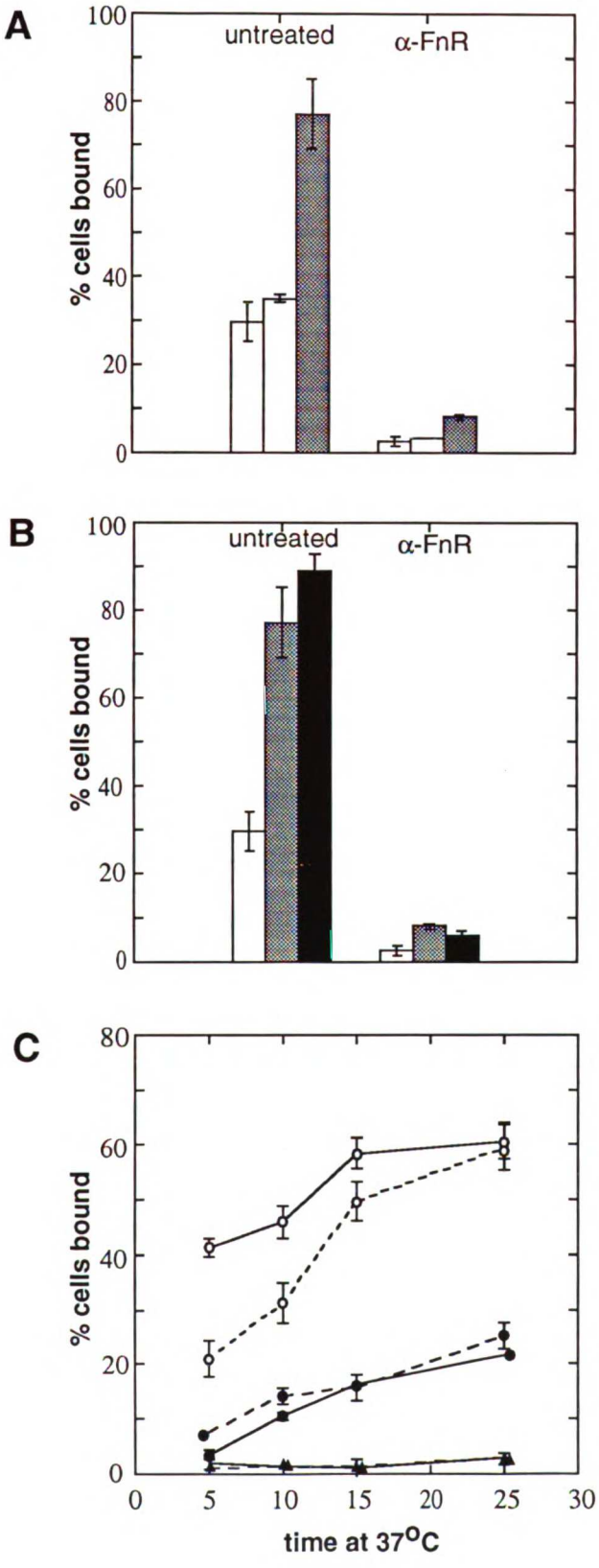
consistent with the regulation of c-Src distribution through dephosphorylation of the negative regulatory site at Y527.

Activation of the c-Src kinase is accompanied by the dephosphorylation of tyrosine 527 during cellular adhesion

The role of Y527 during cellular adhesion was addressed by the indirect analysis c-Src tyrosine phosphorylation *in vivo*. We examined the changes in phosphotyrosine of a mutant c-Src protein containing a single amino acid substitution at the auto-phosphorylation site (c-SrcY416F). Immunoblotting of wild type c-Src with anti-phosphotyrosine antibodies revealed that Y416 and Y527 are the major sites of tyrosine phosphorylation (data not shown). Thus, the recognition of c-SrcY416F by anti-phosphotyrosine antibodies depends on the level of phosphorylation of Y527, allowing us to monitor phosphorylation of this site by immunoblotting. *Src*$^{-/-}$ fibroblasts expressing c-SrcY416F were plated on fibronectin coated wells for 5', 15', and 45' at 37°C (see above), cells lysed and c-SrcY416F isolated by immunoprecipitation for analysis by in vitro kinase assay and anti-phosphotyrosine immunoblotting. C-SrcY416F from cells incubated at 37°C for five to fifteen minutes showed a 37-45% decrease in relative levels of phosphotyrosine staining, which then returned to the levels observed in unplated cells (Figure 4-5b). Consistent with its proposed negative regulatory role, the relative decrease in phosphotyrosine levels was inversely related to the activation of the kinase during cellular adhesion. These data support a role for Y527 in regulating c-Src kinase activity during cellular adhesion.

Figure 4-6:

Src$^{-/-}$ fibroblasts exhibit a defect in adhesion on fibronectin. The percentage of cells bound after two independently derived *src*$^{-/-}$ cell lines (□) and a *src*$^{-/+}$ cell line (▩) were plated on fibronectin for 5 min. at 37°C with (α-FnR; 1/50 dilution) or without (untreated) anti-fibronectin receptor antibody are compared graphically (A). A *src*$^{-/-}$ (□), *src*$^{-/+}$ (▩) and the same *src*$^{-/-}$ cell line expressing chicken c-Src (■) were similarly compared after plating on fibronectin for 5 min (B), *src*$^{-/-}$ cell line (- - - -) and the same cell line expressing chicken c-Src (——) plated on fibronectin-coated plates (o), collagen-coated plates (●), or BSA-coated plates (▲) for the indicated times were analyzed as above, and the percentage of cells that remained bound are displayed as a function time at 37°C (C).


A
100
80
60
40
20
0
% cells bound
untreated
α-FnR
B
100
80
60
40
20
0
% cells bound
untreated
α-FnR
C
80
60
40
20
0
% cells bound
0
5
10
15
20
25
30
time at 37°C

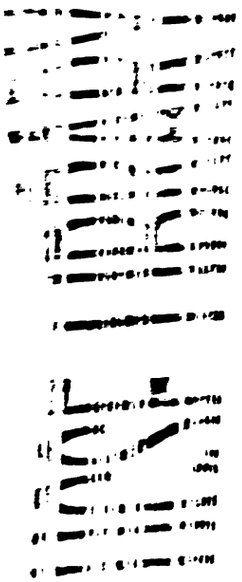
Src$^{-/-}$ fibroblasts are defective in cellular adhesion events

The regulation of c-Src during adhesion of cells on fibronectin led us to examine the ability of *src$^{-/-}$* fibroblasts to adhere to fibronectin-coated dishes. Fibroblasts lacking c-src (*src$^{-/-}$*) were compared to fibroblasts derived from mice heterozygous for the disruption of the c-src gene (*src$^{-/+}$*). Cells were allowed to attach to fibronectin-coated wells at 37°C and loosely attached cells were removed by washing. The percentage of cells that remained bound were calculated based on the level of an internal enzymatic marker (β-hexosamindase (Hall, et al. 1990). Multiple *src$^{-/-}$* fibroblast cell lines consistently adhered less well than *src$^{-/+}$* fibroblasts to fibronectin-coated plates (Figure 4-6a; 3.1X and 2.9X less adherent). Antibodies to the fibronectin receptor efficiently blocked adhesion of all cells in this assay, demonstrating the specificity of the assay for integrin-mediated adhesion (Figure 4-6a). The difference in cell adhesion required incubation at 37°C, as cells incubated at 4°C demonstrated no difference in binding to substrate (data not shown).

The ability of low levels of c-Src (~3-4X over endogenous levels in *src$^{-/+}$* cells; data not shown) to restore wild-type adhesion to *src$^{-/-}$* fibroblasts confirmed the involvement of c-Src in adhesion (Figure 4-6b). The increase in adhesion was not due to clonal variation between cell lines, as similar results were obtained with multiple c-Src expressing sub-clones derived from independently isolated *src$^{-/-}$* cell lines (mean=2.3X, n=12; data not shown). The reduced adhesion of *src$^{-/-}$* fibroblasts was most apparent at early times after plating. Initial binding of cells (1-2 min. at 37°C) to substrate was equal for all cells; greater than 95% of cells attached to the substrate, but were easily removed during washing (data not shown). Differences in adhesion were most apparent after 5-10 min. at 37°C. Incubation at 37°C for longer than 25 minutes equalized the level of adhesion between *src$^{-/-}$* and c-src expressing cells

Figure 4-7:

C-Src enhances the rate of cell spreading of *src*$^{-/-}$ fibroblasts by a kinase independent mechanism that requires SH2 and SH3. *Src*$^{-/-}$ fibroblasts (A) and *src*$^{-/-}$ fibroblasts expressing chicken c-Src (B), K295Lc-Src (C), Src251 (D), Src251ΔSH3 (E) and Src251R175L (F) were plated on fibronectin-coated plates for 15' at 37°C and individual fields from multiple experiments were photographed by phase contrast microscopy. A portion of the entire microscope field is displayed and selected fields are quantitated in Figure 8. (Scale bar=15μM)



C
F
B
E
A
D

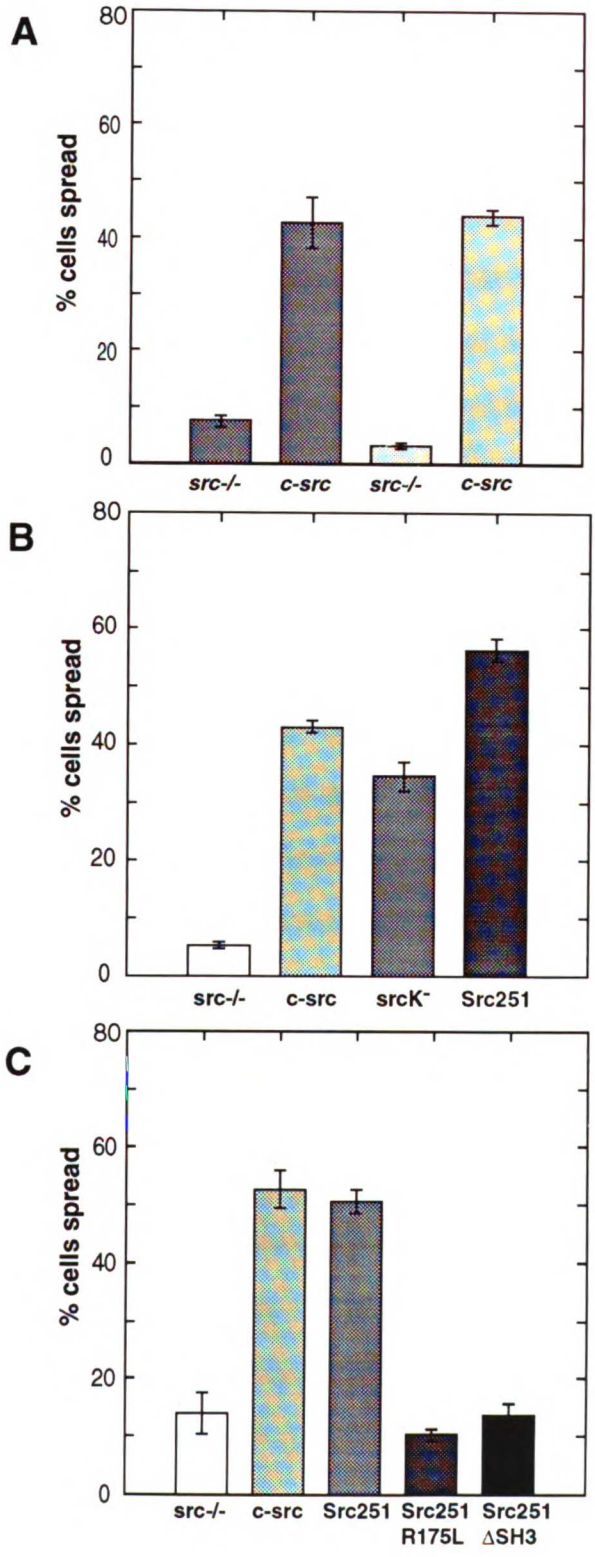
(Figure 4-6c), suggesting that *src*$^{-/-}$ cells are deficient during an early stage of cellular adhesion. Adhesion on plates blocked with BSA was negligible, ruling out non-specific binding of cells in the assay. In addition, differences in cellular adhesion were not observed in cells plated on collagen-coated wells over the same time course, suggesting that c-Src may function specifically during fibronectin-mediated cellular adhesion (Figure 4-6c).

C-Src enhances the ability of cells to spread on fibronectin by a kinase independent-mechanism.

The early regulation of c-Src during cellular adhesion on fibronectin led us to more closely examine *src*$^{-/-}$ fibroblasts during the early stages of adhesion. A direct comparison of *src*$^{-/-}$ fibroblasts with cells expressing c-Src revealed a dramatic delay in the ability of *src*$^{-/-}$ fibroblasts to spread on fibronectin. Little cell spreading was observed after five minutes incubation of cells at 37°C, although all cells attached to fibronectin-coated plates equally well (data not shown). Cells expressing c-Src exhibited a significant amount of cell spreading after fifteen minutes at 37°C, while little change in cell morphology was observed in *src*$^{-/-}$ fibroblasts (Figure 4-7a, b). The amount of cell spreading was quantitated by photographing multiple microscope fields and counting the number of spread cells as a percentage of cells in the entire field (see Methods). Results obtained using different cell lines in multiple experiments (Figure 4-8a) consistently showed a dramatic increase in cell spreading in c-Src expressing fibroblasts, compared to the parental *src*$^{-/-}$ cell line (mean=8.1, n=11). *Src*$^{-/-}$ fibroblasts maintained at 37°C for longer time periods (~45') eventually spread and became as flat as c-Src expressing cells, indicating that c-Src expression accelerates the rate of cell spreading, but is not absolutely required. These results support a role for c-Src during the spreading

Figure 4-8:

Quantitation of fibronectin-mediated cell spreading of *src*$^{-/-}$ fibroblasts expressing wild type and mutant c-Src proteins. The percentages of cells that had spread were calculated based on the total number of cells in a single microscope field (5 fields/bar, >500 cells/field; error bars indicate the variation among the 5 fields of cells counted). Independent experiments were conducted to analyze the percentage of cell spreading in two *src*$^{-/-}$ cell lines and subclones expressing chicken c-Src (A, B, C), K295Lc-Src (B), Src251 (B, C), Src251R175L (C) and Src251ΔSH3 (C) after plating on fibronectin as described in Figure 7.


A
% cells spread
80
60
40
20
0
src-/-
c-src
src-/-
c-src
B
% cells spread
80
60
40
20
0
src-/-
c-src
srcK⁻
Src251
C
% cells spread
80
60
40
20
0
src-/-
c-src
Src251
Src251 R175L
Src251 ΔSH3

of fibroblasts on fibronectin and correlate with the altered adhesion properties observed in *src*$^{-/-}$ fibroblasts.

The enhancement of cell spreading by c-Src is independent of kinase activity and requires the SH2 and SH3 domains

The ability of c-Src to enhance cell spreading may result from the transient increase in kinase activity, the redistribution of c-Src to focal adhesions, or some combination of these events. In order to distinguish among these possibilities, we analyzed a kinase deficient c-Src protein containing a single amino acid change in the catalytic region of the kinase (K295L). Surprisingly, the kinase activity of c-Src is not required to complement the cell spreading defect in *src*$^{-/-}$ fibroblasts (Figure 4-7c and Figure 4-8b). This protein was able to redistribute normally during adhesion, suggesting that the association of c-Src with focal adhesions is sufficient to stimulate cell spreading in the absence of an increase in enzymatic activity (data not shown).

The relationship between focal adhesion association and cell spreading was further addressed by examining mutant c-Src proteins that associate primarily with focal adhesions. A mutant protein lacking the entire carboxy terminal half of c-Src (Src251) associates with focal adhesions (Kaplan et al, submitted for publication) and was able to induce a dramatic increase (mean=10.6X, n=8) in the rate of cell spreading of *src*$^{-/-}$ fibroblasts (Figure 4-7d and Figure 4-8c). In general, these cells spread faster (1.4X) than cells expressing wild type c-Src (Figure 4-7b,c and Figure 4-8b), perhaps reflecting the increased levels of Src251 associated with focal adhesions. These results further support the kinase-independent influence of c-Src on cell spreading and links focal adhesion association of c-Src with enhanced cell spreading.

We also analyzed a series of mutants to identify the amino terminal domains required to enhance cell spreading. A deletion of the SH3 domain (Src251ΔSH3) has been shown previously to abolish Src251 association with focal adhesions (KBK; submitted for publication). Cells expressing Src251ΔSH3 do not exhibit enhanced cell spreading (Figure 4-7e and Figure 4-8c), further supporting the importance of focal adhesion association for enhanced cell spreading.

The importance of the SH2 domain was tested with a protein containing a single amino acid change (R175L) in the conserved region of the SH2 domain, abolishing phosphotyrosine binding *in vitro.* Despite the ability of Src251R175L to associate with focal adhesions (KBK; submitted for publication), cells expressing significant levels of Src251R175L showed no enhancement of cell spreading on fibronectin (Figure 4-7f and Figure 4-8c). In fact, these cells showed a consistently reduced ability to spread when compared to the parental *src*$^{-/-}$ fibroblast cell line (Figure 4-7a, f). This result implies that focal adhesion association is required, but not sufficient for the kinase-independent enhancement of cell spreading by c-Src and that both the SH2 and SH3 domains are involved in regulating cellular adhesion.

Discussion

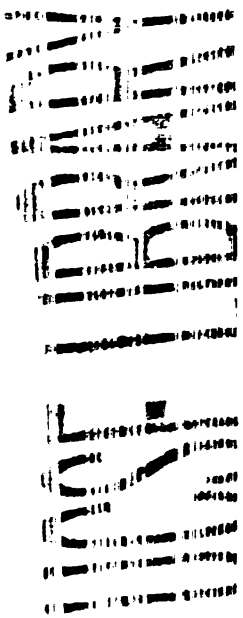
We have shown that c-Src is regulated and functions in a kinase-independent manner during the early steps of fibronectin-mediated cellular adhesion of fibroblasts. During cell attachment to fibronectin, a reduction in Y527 phosphorylation accompanies an increase in the kinase activity of c-Src that is concomitant with the redistribution of c-Src to newly formed focal adhesions in spread cells. The cellular adhesion defect exhibited by *src*$^{-/-}$ fibroblasts occurs during cell spreading and is consistent with the regulatory

changes in c-Src kinase activity and subcellular distribution. Surprisingly, c-Src is able to enhance the spreading of *src*$^{-/-}$ fibroblasts on fibronectin by a kinase-independent mechanism that involves the SH2 and SH3 domains of c-Src. The regulation of c-Src and the phenotype of *src*$^{-/-}$ fibroblasts during adhesion on fibronectin strongly argues that c-Src is involved in regulating the early stages of cellular adhesion.

Redistribution of c-Src to focal adhesions

Our previous work showed that a single amino acid substitution, Y527F, or expression of c-Src in cells lacking CSK (*csk*$^{-/-}$) was sufficient to allow the association of c-Src with focal adhesions (Kaplan et al, submitted for publication). We propose that redistribution of c-Src during cellular adhesion represents a biologically relevant regulation of the sub-cellular distribution of c-Src through Y527 dephosphorylation. These results also represent a novel regulatory scheme for coordinating the activity of a kinase and its subcellular distribution. Our results suggest that c-Src becomes activated while associated with endosomal membranes through dephosphorylation of Y527. Exposed amino terminal domains mediate the association of c-Src with focal adhesions where it becomes deactivated.

The mechanism by which c-Src traffics to focal adhesions following Y527 dephosphorylation is not clear. Adhesion could trigger a shift in the equilibrium of c-Src between endosomes and focal adhesions via a cytoplasmic intermediate, or an activation of a vesicular trafficking pathway that is repressed in adherent cells. Staining of fibronectin-attached cells with endosomal membrane markers revealed qualitative changes in membranes at the top of the cell that are consistent with a general change in membrane trafficking (KBK; unpublished observations). A more detailed biochemical analysis of the

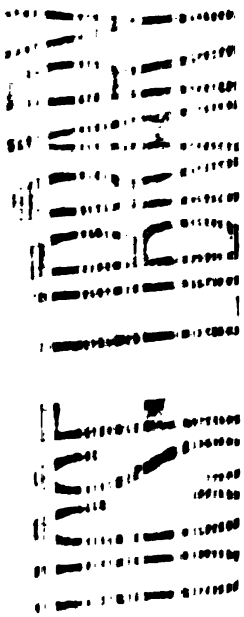


behavior of endosomal membranes and c-Src during adhesion will be needed to address the exact nature of c-Src trafficking to focal adhesions.

Regulation of c-Src during cellular adhesion on fibronectin

Regulation of the enzymatic activity of c-Src is largely ascribed to the phosphorylation state of the negative regulatory site, Y527. Mutation of this site results in a highly active kinase (Yaciuk, et al. 1988) and a decrease in Y527 phosphorylation has been observed to correlate with kinase activation during mitosis and platelet activation (Bagrodia, et al. 1991; Clark and Brugge 1993). During adhesion, we also observed similar levels of dephosphorylation of Y527 as reported for c-Src during mitosis, consistent with the proposed models for kinase activation. The proportion of c-Src that remains phosphorylated at Y527 may reflect a sub-population of c-Src that is not activated during cellular adhesion, or the rapid rephosphorylation of this site following activation, presumably by CSK (Okada, et al. 1991). Recent reports that CSK associates with focal adhesions is consistent with the latter possibility (Sabe, et al. 1994).

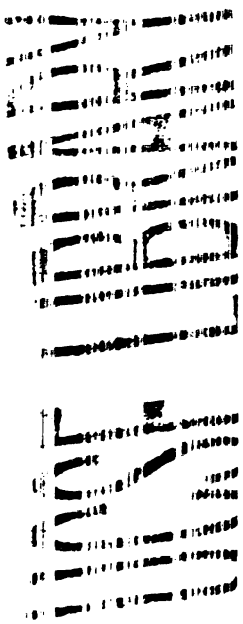
It is also possible that dephosphorylation of Y527 is not the sole regulatory event. Amino terminal modifications such as those seen during mitosis, platelet activation and PDGF stimulation may also be involved in regulating the activity of the c-Src kinase in concert with Y527 dephosphorylation (Chackalaparampil and Shalloway 1988; Clark and Brugge 1993; Gould and Hunter 1988; Morgan, et al. 1989; Ralston and Bishop 1985; Shenoy, et al. 1989). It should be noted that the increase in kinase activity of c-SrcY416F was 50-60% of wild type c-Src levels, supporting the notion that modifications of amino acids other than Y527 will be important in the regulation of the c-Src kinase activity.



The signals that regulate c-Src activity during adhesion are uncharacterized but may potentially consist of both integrin-related and -independent signals. Our experiments were performed in low serum (0.2%) and so the contribution of serum factors are likely to be negligible. In addition, experiments using antibodies to cross-link fibronectin receptors resulted in a modest activation of c-Src in adherent cells, but failed to activate c-Src from cells in suspension (KBK; unpublished observations). This may indicate that a combination of signals act together to regulate c-Src (i.e., integrin cross-linking and adhesion), or may reflect inefficient receptor cross-linking with antibodies. Regulation of c-Src in platelets depends on multiple signals, including thrombin and the integrin GPIIb-IIIa and may represent an example of how multiple signals can activate c-Src (Clark and Brugge 1993). In either case, experiments addressing the signals that regulate c-Src activity during adhesion will provide insights into c-Src regulation and the events required for cellular adhesion.

Kinase-independent regulation of cellular adhesion

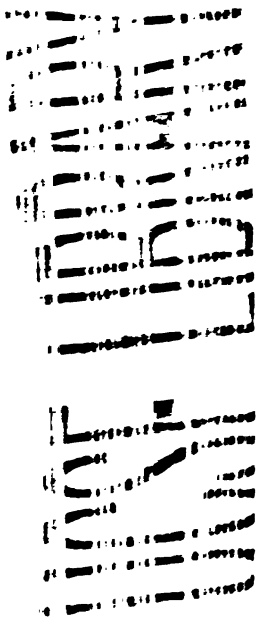
Although the increase in kinase activity of c-Src is transient during cellular adhesion, c-Src remains associated with the detergent-insoluble fraction after the kinase activity has returned to basal levels. The low kinase activity and the kinetics of phosphorylation of Y527 suggests that the kinase activity of c-Src associated with focal adhesions is low. Furthermore, the catalytic activity of c-Src is not required to complement the adhesion defect in *src*$^{-/-}$ cells suggesting that the kinase is irrelevant in the enhancement of cell spreading. This idea is consistent with our previous findings that the amino terminal half of c-Src (Src251) associates with and modulates the structure of focal adhesions by a kinase-independent mechanism (Kaplan et al, submitted for publication). The function of the kinase domain in cellular adhesion is



unclear, but may serve to phosphorylate proteins prior to cell spreading to further amplify integrin-mediated signals. Alternatively, the kinase domain of c-Src may serve to negatively regulate the association of c-Src with focal adhesions, by preventing association of other proteins through steric hindrance or phosphorylation.

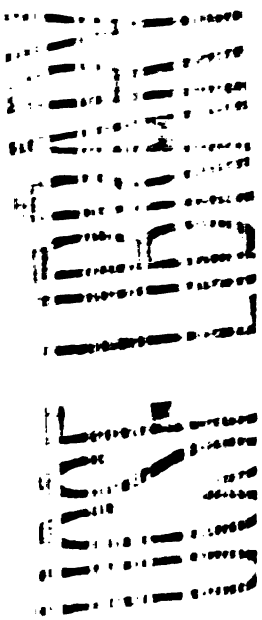
The amino terminus of c-Src is sufficient to enhance cell spreading and the association of c-Src with focal adhesions. Thus, deletion of the SH3 domain abrogates the enhancement of cell spreading, probably by preventing Src251 from associating with focal adhesions (Kaplan et al, submitted for publication). While the SH2 domain is not required for c-Src association with focal adhesions (Kaplan et al, submitted for publication), it is clearly required for the Src-induced enhancement of cell spreading. This may be due to the capacity of the SH2 domain to catalyze the formation of large complexes of proteins important in coordinating signal transduction during cellular adhesion. The interaction of the SH2 domain and the focal adhesion kinase (FAK) lends credence to the formation of a signaling complex involved in regulating cellular adhesion (Cobb, et al. 1994; Schaller, et al. 1994). Furthermore, the phosphorylation of integrins in v-src transformed cells as well as in response to a variety of stimuli may serve to recruit proteins, such as c-Src and FAK, to sites of integrin clustering (Dahl and Grabel 1989; Danilov and Juliano 1989; Falcioni, et al. 1989; Freed, et al. 1989). Further analyses of these complexes will identify additional components and reveal the biochemical consequences of complex formation important for understanding the exact function of these proteins in regulating cellular adhesion.

Mechanism of c-Src regulation of cellular adhesion



The seemingly paradoxical roles of the oncogenic and the proto-oncogenic forms of c-Src in adhesion, raises questions concerning the mechanism by which c-Src regulates cellular adhesion. Although c-Src and v-Src are capable of associating with focal adhesions, the consequences for the cell appear to be quite different. Our results indicate that c-Src positively influences cellular adhesion, whereas v-Src results in morphological transformation and an apparent reduction in cellular adhesion. The different levels of kinase activity of the two proteins in focal adhesions may partially explain this paradox. While very few changes in phosphotyrosine levels were observed during adhesion of cells expressing c-Src (KBK; unpublished observations), v-Src transformed cells exhibit a dramatic increase in phosphotyrosine-containing proteins, especially proteins normally associated with focal adhesions (Hirst, et al. 1986; Nigg, et al. 1986; Pasquale, et al. 1986). The cellular consequences of these modifications remains obscure, although it is likely that they substantially alter focal adhesion structure and contribute to the decrease in cell adhesion (Kellie, et al. 1986; Kellie, et al. 1986; Nakamura, et al. 1993; Nermut, et al. 1991).

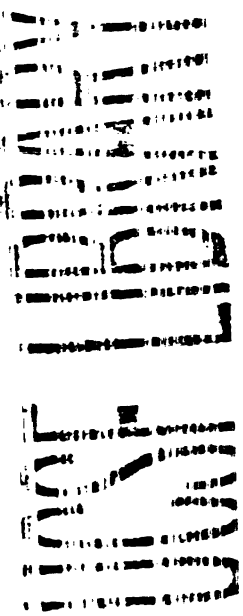
The potential involvement of c-Src in adhesion related functions has intriguing implications for situations where c-Src is known to be regulated. For example, the regulation of c-Src during mitosis when cell adhesion is tightly controlled, supports a role for c-Src in regulating cell spreading following mitosis. In this regard, it is interesting to note the presence of c-Src in both endosomal membranes and at the contractile ring in telophase cells, reminiscent of the distribution of c-Src between endosomes and focal adhesions during cellular adhesion on fibronectin (KBK; unpublished observations). While the importance of c-Src in osteoclasts has been shown from genetic studies, the exact role of c-Src in osteoclasts remains unclear. The



fact that osteoclasts form a highly adherent interaction with the bone matrix to facilitate proper bone absorption emphasizes the potential involvement of c-Src in regulating osteoclast adhesion (Boyce, et al. 1992; Hall, et al. 1994; Lowe, et al. 1993). In addition, the regulation of c-Src after growth factor stimulation may attest to the link between the cell cycle and adhesion in fibroblasts. Further experiments in specialized cells like osteoclasts, may provide a more detailed picture of the importance of c-Src in cellular adhesion, as will the additional characterization of the complexes that regulate adhesion in both normal and transformed cells.

Chapter Five

Future directions



Summary

Our characterization of the subcellular distribution of c-Src indicates that c-Src may function in cellular processes associated with endosomal membranes and focal adhesions. To test the relevance of these findings for the function of c-Src, we have characterized the behavior of fibroblasts lacking c-Src during cellular adhesion. The results of these experiments implicate c-Src in the formation of focal adhesions and the spreading of fibroblasts on fibronectin. Furthermore, our analyses raise questions concerning the precise mechanisms by which c-Src influences cellular adhesion and provides a foundation for further experimentation. In the following chapter, I will discuss the major findings of our work and their implications for future analyses.

On the basis of our results, it is apparent that c-Src is found in two, distinct subcellular compartments: endosomal membranes and focal adhesions. Under normal growth conditions, the majority of c-Src associates with endosomal membranes while none is detected in focal adhesions. However, during the adhesion of cells on fibronectin, a significant proportion of c-Src redistributes to focal adhesions. Biochemical analysis of c-Src demonstrates that redistribution to focal adhesions is controlled through phosphorylation of Y527; dephosphorylation of Y527 leads to the redistribution of c-Src to focal adhesions as well as a transient increase in kinase activity. Mutational analysis of c-Src revealed that association with focal adhesions is mediated by signals in the amino-terminal half of the protein. Significantly, the amino terminal half of c-Src can also induce alterations in the structural and biochemical properties of focal adhesions, suggesting that c-Src can directly regulate cellular adhesion. Consistent with this possibility, *src*$^{-/-}$ fibroblasts were shown to be defective in cell spreading and could be complemented by

expression of the amino terminal half of c-Src, confirming that interactions between c-Src and focal adhesions directly affect cell spreading.

The subcellular distribution of c-Src

The presence of c-Src in two distinct subcellular compartments suggests that there may be a dynamic relationship between endosomal membranes and focal adhesions. We propose that c-Src is maintained in an equilibrium between endosomes and focal adhesions that is regulated by the phosphorylation of Y527. Under normal growth conditions, all detectable c-Src associates with endosomal membranes and is heavily phosphorylated at Y527. The dephosphorylation of Y527 results in the disruption of the intramolecular interaction between Y527 and the SH2 domain of c-Src. The resulting change in c-Src conformation leads to an increase in kinase activity and the exposure of amino terminal domains that mediate the association of c-Src with focal adhesions.

Although the trafficking of c-Src between endosomes and focal adhesions can be inferred from our data, the details of Src-trafficking in the cell remain to be explored. One possibility is that c-Src associates with focal adhesions immediately following synthesis and is then endocytosed after phosphorylation of Y527. Alternatively, it is possible that Y527 is phosphorylated co-translationally, resulting in the immediate association of c-Src with endosomal membranes. Examination of the kinetics of Y527 phosphorylation following c-Src synthesis will be important in establishing the direction of c-Src trafficking between focal adhesions and endosomes.

Regardless of the course of c-Src traffic in the cell, it is reasonable to propose that small fluctuations in Y527 phosphorylation, or Src-protein conformation, result in the cycling of c-Src between these two compartments.

This suggests that some proportion of c-Src is always present in focal adhesions. Thus, our inability to detect c-Src in focal adhesions in normally growing cells may simply reflect the small proportion of c-Src associated with focal adhesions under these conditions. Only the engagement of large numbers of fibronectin receptors (i.e., fibronectin plated cells) allows a sufficient increase in the proportion of c-Src in focal adhesions to be detected by immunofluorescence. Development of more sensitive assays for monitoring the association of c-Src with focal adhesions, perhaps by assessing the association of c-Src with specific focal adhesion proteins, will allow for a more detailed assessment of c-Src trafficking.

The presence of c-Src in multiple subcellular compartments potentially implicates it in a number of cellular processes. As discussed in Chapter 2, it is possible that c-Src has a role in regulating endosomal membranes in addition to its role in cellular adhesion. However, the characterization of endosomal membranes in *src*$^{-/-}$ fibroblasts did not reveal any alterations in endosome behavior in the absence of c-Src (KBK; unpublished observations). These results may indicate that the proper parameters of endosome-function were not assayed or that c-Src does not directly regulate the behavior of these membranes. Nonetheless, it remains an intriguing possibility that there is a functional connection between endosomal membranes and focal adhesions. Although there is little evidence linking endocytosis with cellular adhesion, it is possible that uptake of focal adhesion components can alter the dynamics of focal adhesions. For example, by regulating the uptake of a focal adhesion component that is limiting for assembly, c-Src may indirectly modulate the stability of focal adhesion complexes. Considered more generally, it may be useful for the cell to link endocytosis and cellular adhesion as a means for controlling the insertion of new membranes at sites of cell spreading or at the

leading edge of the motile cell. A detailed analysis of endocytosis within focal adhesions will be a first step in addressing the possible relationship between endocytosis and cellular adhesion.

A second possible role for c-Src in membrane regulation is in the modulation of secretory events associated with endosomal membranes at sites of cell/substrate contact. The relationship between endosomes and specialized secretory vesicles has been established in the case of synaptic vesicles isolated from neurons. Synaptic vesicles are specialized secretory vesicles derived from endosomal membranes in neurons that fuse with plasma membranes at the synaptic junction (Linstedt and Kelly 1991; Sudhof and Jahn 1991). Interestingly, sites of synaptic vesicle fusion contain proteins also present in focal adhesions, suggesting that these two structures share common biological functions. Although an analogous set of membranes has not been characterized in fibroblasts, c-Src associates with endosomally-derived synaptic vesicles in PC12 cells (Linstedt, et al. 1992), implying that c-Src may associate with similar membranes in fibroblasts. Further characterization of c-Src-containing vesicles in fibroblasts will be important in fully understanding the role of c-Src on membrane vesicles as well as in cellular adhesion.

Signals that regulate c-Src distribution

The involvement of tyrosine phosphorylation (i.e., Y527) in controlling the distribution of c-Src naturally implicates other kinases and phosphatases in the regulation of c-Src distribution. For example, we demonstrate that the absence of CSK (the kinase that phosphorylates Y527) results in the dramatic redistribution of c-Src to focal adhesions. It is therefore possible that regulation of the kinase activity of CSK will also alter the subcellular distribution of c-Src. Although little is known concerning regulation of the enzymatic activity of CSK,

recent reports have found that CSK and c-Src simultaneously associate with focal adhesions, implying that Y527 phosphorylation is confined to a specific subcellular compartment (Howell and Cooper 1994). The spatial confinement of CSK may ensure that there are low levels of c-Src kinase activity in focal adhesions and may stimulate the cycling of c-Src from focal adhesions to endosomes. Additional mechanisms that regulate CSK activity remain to be addressed and will be important in determining how c-Src distribution is controlled in the cell.

In addition to kinases, we imagine that the cell also employs a tyrosine phosphatase to control the rapid redistribution of c-Src during cellular adhesion. The high levels of tyrosine phosphatase activity reported in unattached cells may be involved in regulating a number of events during cellular adhesion, including the redistribution of c-Src (KBK; unpublished observations, (Maher 1993)). Consistent with this possibility, we have observed that inhibitors of tyrosine phosphatases completely block cellular adhesion in mammalian fibroblasts (KBK; unpublished observations). However, the pleotrophic effects associated with phosphatase-inhibitors makes it difficult to ascertain the precise role of tyrosine phosphatases during cellular adhesion. Identification and characterization of the specific phosphatase involved in Y527 dephosphorylation during adhesion will provide insights into the regulation of c-Src and cellular adhesion.

Upstream signals that control Y527 phosphorylation are likely to be initiated, at least in part, by the engagement of integrin receptors. Preliminary results suggest that cross-linking integrins with antibodies can modulate the kinase activity of c-Src in adherent cells; in contrast, cross-linking of integrins had no effect on the kinase activity of c-Src in non-adherent cells (KBK; unpublished observations). These results may reflect the requirement of a

specific level of integrin engagement to achieve activation of the c-Src kinase or that additional, non-integrin related signals, are required for the regulation of c-Src. A more detailed characterization of the signals generated by integrin cross-linking will address the precise requirements and mechanisms involved in regulating c-Src. In addition, it will be important to determine whether these signals directly modulate the phosphorylation state of Y527 or result in amino terminal modifications of c-Src; similar modifications have been implicated in the regulation of c-Src in a variety of situations (e.g., mitosis, PDGF stimulation, and platelet activation; see Introduction). Therefore, the identification of amino-terminal modifications of c-Src during cellular adhesion may help to define the exact nature of adhesion-generated signals.

Regulation of focal adhesions by c-Src

Based on our results, c-Src is able alter focal adhesions by both kinase-dependent and -independent mechanisms; each results in a distinct set of changes in the properties of focal adhesions. On the one hand, the presence of active c-Src in focal adhesions appears to fragment, and in some cases, completely disperse focal adhesions proteins. This is most readily observed in *csk*$^{-/-}$ cells expressing wild c-Src. Focal adhesions in these cells are confined to the cell periphery and appear fragmented compared to the tightly organized structures seen in non-expressor cells (KBK; unpublished observations). These results are consistent with the ability of transforming mutants of c-Src to disrupt focal adhesions and interfere with cellular adhesion. On the other hand, the presence of non-catalytic mutants of c-Src in focal adhesions results in large, well-formed focal adhesions, implying that c-Src can induce opposite states of focal adhesion stability, depending on the levels of kinase activity. Therefore, it is tempting to speculate that c-Src controls both the assembly and disassembly

of focal adhesions, contributing directly to their dynamic behavior during cellular adhesion.

A likely sequence of events would involve the dephosphorylation of Y527, resulting in an active c-Src kinase that disassembles focal adhesions, possibly to facilitate reassembly at new sites of cell/substrate contact. The ensuing inactivation of the c-Src kinase would result in the stimulation of focal adhesion assembly, similar to the changes induced by the amino terminal half of c-Src. The continual cycling of focal adhesion stability would result in the dynamic properties required for complex cellular processes, including cell motility and adhesion. In such a scenario, it is obviously important for the cell to tightly coordinate both the subcellular distribution and the kinase activity of c-Src. A prediction of this model is that cellular processes dependent on dynamic focal adhesions will be compromised in cells expressing mutants of c-Src. In this regard, it is worth noting that expression of the amino terminal half of c-Src is toxic to cells, suggesting that stabilization of focal adhesions may interfere with cell growth (KBK; unpublished observations). A more detailed examination of these cells may reveal relevant phenotypes that arise from the improper regulation of focal adhesion assembly (e.g., decreased cell motility).

Although our data suggest that the kinase domain of c-Src is not required to enhance cell spreading, it is possible that the kinase is involved at an earlier step in cellular adhesion. The high levels of c-Src kinase activity prior to focal adhesion formation suggests that c-Src may phosphorylate substrates in other subcellular compartments. Whether these putative substrates influence adhesion directly or serve to coordinate other cellular processes during adhesion remains to be examined. Identification of adhesion-related c-Src substrates will provide further insights into the exact contribution of the kinase domain during cellular adhesion. However, the apparently small changes in

tyrosine phosphorylation that accompany the adhesion of cells on fibronectin may make identification of such substrates difficult. Analysis of proteins that associate with the amino terminal half of c-Src may provide an alternative strategy to identify candidate substrates.

Regardless of the role of the kinase domain, we have established that the amino terminal half of c-Src alters the structure of focal adhesions, increasing the size and amount of phosphotyrosine in focal adhesions, in the absence of kinase activity. The ability of the amino terminal half of c-Src to alter focal adhesion structure depends primarily on the SH3 domain (see Chapter 3). Although the exact mechanisms by which SH3 influences the structure of focal adhesions remains to be resolved, it is clear that the SH3 domain is at least required for the association of the amino terminal half of c-Src with focal adhesions. It has not been possible to separate the targeting function of SH3 from its regulatory role in focal adhesions. Additional mutational studies may address this issue by identifying mutations which allow c-Src to associate with focal adhesions but not alter their structure. Alternatively, it may be that these two events are not separable; thus, the SH3-mediated interactions required for focal adhesion association may be the same interactions involved in altering focal adhesion structure.

Resolution of this question is also central to understanding how SH3 mediates the targeting of c-Src to focal adhesions. The SH3 domain may target c-Src to focal adhesions by directly associating with resident focal adhesion proteins or by regulating the trafficking of c-Src to focal adhesions. Consistent with the latter possibility, deletion mutants lacking the SH3 domain appear to associate with a distinct population of membrane vesicles when compared to c-Src positive vesicles (KBK; unpublished observations). In the absence of SH3, c-Src may become trapped in an intermediate membrane-compartment and

therefore is unable to reach focal adhesions. In this light, it is interesting to note that both dynamin and the PI3 kinase have been identified as SH3-ligands; dynamin and the PI3 kinase have both been implicated in the regulation of membrane trafficking (Gout, et al. 1993; Herskovits, et al. 1993; Liu, et al. 1993b). Further characterization of these interactions and their effects on endosomal membranes may shed light on the role of the SH3 domain in targeting c-Src to focal adhesions. In general, the characterization of proteins that bind specifically to the SH3 domain of c-Src *in vivo* will also address the mechanisms underlying the targeting of c-Src to focal adhesions.

While only the SH3 domain is required to alter focal adhesion structure, both the SH2 and SH3 domains are required for the enhancement of cell spreading. As discussed above, the role of the SH3 domain appears to be primarily involved in targeting c-Src to focal adhesions. In contrast, the SH2 domain is primarily involved in regulating cell spreading on fibronectin, possibly by governing the activities of other kinases and phosphatases associated with cellular adhesion. One candidate is the focal adhesion kinase (FAK), which has been shown to directly associate with the amino terminal half of c-Src via the SH2 domain (Schaller, et al. 1994). In addition, FAK exhibits increased phosphotyrosine levels in cells expressing the amino terminal half of c-Src. Mutants in the SH2 domain that disrupt FAK association with Src also fail to complement the adhesion defect in *src*$^{-/-}$ fibroblasts, implying that FAK is involved in regulating cell spreading through c-Src. Interestingly, association of the amino terminal half of c-Src with FAK correlates with the reduction of FAK kinase activity as well as the enhancement of cell spreading (KBK; unpublished observations). In contrast, high levels of FAK kinase activity have been reported in Src-transformed cells (Guan and Shalloway 1992), suggesting that the kinase activity of FAK is inversely related to the state of cell adhesion. Further

characterization of FAK regulation and other SH2-associated proteins in focal adhesions will provide insights into the precise mechanism by which c-Src regulates cellular adhesion.

In conclusion, these studies provide convincing evidence that c-Src is involved in regulating cellular adhesion in mammalian fibroblasts. This finding is consistent with certain characteristics of Src-transformed cells; these include the disruption of focal adhesions and a loss of cell adhesion in Src-transformed cells (i.e., cell rounding). In addition to linking the normal function of c-Src to transformation, these results may be relevant for the function of the osteoclast cell type in *src*$^{-/-}$ mice. Many studies have demonstrated the importance of osteoclast adhesion in the degradation of bone matrix. An adhesion defect in osteoclasts would inhibit the reabsorbtion of bone and result in the characteristic osteopetrosis observed in *src*$^{-/-}$ mice. Our results provide a basic understanding of the biochemical mechanisms that govern c-Src during cellular adhesion and the basis for further exploration of the role of c-Src in cellular adhesion.

Appendix: Materials and methods

Cell lines

The RTCS cell line was derived from a Rat-1 cell line infected with a retroviral construct expressing chicken c-Src (Morgan, et al. 1989) and expresses approximately 7.5-fold more c-Src than the parental Rat-1 cell line (determined by western blot; KBK, unpublished observations). In some experiments, we used cell lines derived from the spontaneous immortalization of mouse embryo fibroblasts isolated from mice heterozygous or homozygous for a disruption of the *c-src* gene were used (cells kindly provided by P. Soriano). Cells were cultured in DME H-16 with 10% Fetal Calf Serum at 37°C in 5% CO_2.

The *src*$^{-/-}$ cell lines were derived from the spontaneous immortalization of mouse embryo fibroblasts homozygous for a disruption in the *c-src* gene (cells kindly provided by P. Soriano). Cells were infected with retroviruses containing a selectable marker, hygromycin B, and the indicated *c-src* constructs. Infected cells were selected and maintained in 125 ug/ml hygromycin B in DME H-21 with 10% fetal calf serum at 37°C in 5% CO_2. Colonies or pools were isolated and analyzed for expression of constructs by immunoblotting. Several different *src*$^{-/-}$ cell lines derived from *src*$^{-/-}$ littermates were used to confirm all results.

Constructs

Mutant *c-src* cDNAs were constructed by standard site-directed mutagenesis (mutants outlined in Figure 4-1). Constructs containing only the first 251 amino acids of Src were derived from plasmids containing deletions and mutations in the *c-src* cDNA by polymerase chain reaction (PCR) with Vent polymerase. PCR primers were designed with restriction sites to facilitate subsequent cloning steps. The 3' PCR primer codes for in frame sequence that adds the 9E10-myc epitope tag to the carboxy terminus of all PCR-generated

constructs (i.e., those composed of the first 251 amino acids). The following amino acids were added after amino acid 251 of Src to constructs containing the 9E10-myc epitope tag: VDMEQKLIAEEDLN. Two separate PCR reactions were carried out for each construct and similar results were obtained. All mutant constructs constructed by PCR were also sequenced to confirm predicted DNA sequence of clones.

All Src proteins were expressed with the retroviral vector, HyTCX (kindly provided by J. Murphy), derived from the LNCX vector. The neomycin selectable marker was replaced with a fusion of hygromycin B and thymidine kinase (Lupton, et al. 1991) to allow positive or negative selection of the provirus. Chicken *c-src* cDNA constructs were cloned into the polylinker downstream of the CMV promoter and the DNA was then transfected into PE501 packaging cells and selected for hygromycin B resistance. Viruses were harvested from pools of resistant cells and used to infect *src*$^{-/-}$ fibroblasts.

Antibodies

Monoclonal antibodies (MAb's) against c-Src were kindly provided by Joan Brugge (MAb327), Sara Courtneidge (MAb2-17) (Lipsich, et al. 1983) and S. Parsons (MAb EC10). Polyclonal rabbit sera against the CI-MPR were kindly provided by Peter Lobel (Rutgers University) and Bill Brown (Cornell University). MAb's against cis/medial golgi markers (10E6 and 8B3) were also kindly provided by Bill Brown (Cornell University). Antibodies against BiP binding protein were obtained from David Bole (University of Michigan). MAb against ß-tubulin (tub2.1) was purchased from Sigma Chemical Co. Texas Red and fluorescein-labeled secondary anti-rabbit and anti-mouse antibodies were purchased from the Accurate Chemical Co. and used at recommended dilutions.

The hybridoma cell line for monoclonal antibody 9E10 was kindly provided by G. Ramsey and ascites fluid was prepared by Babco Inc. Polyclonal rabbit sera against phosphotyrosine and human Src was purchased from Upstate Biotechnology Inc. (UBI). The monoclonal anti-phosphotyrosine antibody 4G10 was prepared from hybridoma supernatants. Monoclonal antibodies against paxillin was purchased from Transduction Laboratories. Antibodies against FAK were kindly provided by T. Parsons and include the polyclonal sera BC3, as well as a mouse monoclonal antibody, 2A7. The blocking antibodies against the fibronectin receptor (GP140) and antibodies to the alpha and beta chains of the fibronectin receptor were kindly provided by C. Damsky.

Immunofluorescence

Cells were plated in 6 well plates on glass coverslips 18-36 h. before analysis. Following specified treatments, cells were washed twice with phosphate buffered saline (PBS) and then fixed for 20 minutes at 20°C in freshly made 3.7% paraformaldehyde/PBS (Polysciences Inc.). Fixed cells were washed twice with PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were rinsed and then blocked in PBS+0.2% gelatin+0.02% NaN_3. Primary antibodies were used at the following dilutions for 20 minutes at 20°C in a humidified chamber: MAb 327 (1mg/ml): 1/100; tub2.1: 1/200; CI-MPR: 1/200; MAb 2-17: 1/200; MAb 10E6/8B3: undiluted cell culture supernatants. Coverslips were then treated with secondary antibodies at the appropriate dilutions. Endogenous levels of c-Src in Rat-1 fibroblasts were detected with an unlabeled rabbit anti-mouse antibody (used at 1μg/ml) followed by goat anti-rabbit antibody labeled with Texas red. Controls performed with only secondary

and tertiary antibodies resulted in negligible signals. Coverslips were washed in PBS+0.2% gelatin+0.02% NaN_3, followed by PBS, and finally incubated in Hoechst stain (No. 33258, Sigma Chemical Co.) for 5 minutes at 20°C before mounting in a solution of 3% n-propyl gallate in glycerol. The specificity of the c-Src signal was demonstrated by adding excess amounts of purified baculovirus-produced chicken c-Src (Morgan, et al. 1991).

In co-staining experiments with MAb tub2.1 and MAb 327, MAb tub 2.1 was directly labeled with Texas Red (Harlow and Lane 1988). The labeled MAb tub2.1 was separated from unincorporated Texas Red by gel filtration on a G-50 Sephadex column. Staining with MAb 327 was carried out as above and followed by an additional fixation step before incubation with MAb tub 2.1-Texas red. This process fixed the anti-mouse antibody and ensured no cross-reactivity between the fluorescein-conjugated anti-mouse antibody and the Mab tub 2.1. No tubulin staining was seen in the fluorescein channel, indicating that there was no interaction between fluorescein-labeled anti-mouse antibodies and MAb tub 2.1.

Additional controls revealed no cross-staining of the polyclonal anti-phosphotyrosine antibody with anti-mouse secondary antibody.

Three-Dimensional Optical Sectioning Microscopy and Image Processing

Three-dimensional images of immunostained cells were recorded using a Peltier-cooled charge-coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ) with a 1,340 x 1,037 pixel CCD chip (Kodak-Videk; Eastman Kodak Co., Rochester, NY). The camera was mounted on a fluorescence microscope workstation controlled by a SGI 4D-35 computer (Silicon Graphics Corp., Mountain View, CA) and equipped with bandpass excitation and

emission filters mounted on motorized wheels and a multi-wavelength dichroic mirror (Chroma, Inc., Brattleboro, VT). Spatial fluctuations in illumination intensity caused by wandering of the arc were eliminated by passing the illumination light through a fiber-optic scrambler (Kam, et al. 1993). To correct for temporal fluctuations in illumination intensity due to power instabilities in the Hg-arc lamp, the intensity of the lamp was directly measured using a avalanche photodiode-based photon counting module (EG & G, Vaudreuil, Quebec). Optical sections (512 x 512 pixels; effective pixel size = 0.1117 μm; except in Figure 4-7 where images have been modified to increase the field size; effective pixel size=0.234μm) were recorded with an Olympus Plan ApoChromat 60x/NA1.4 lens at 0.2 μm intervals by changing the microscope focus with a computer-controlled Nanomover motor (Melles Griot, Inc., Rochester, NY). Multiple wavelength three-dimensional images were recorded in a single focal series by alternating the appropriate bandpass excitation and emission filters for fluorescein isothiocyanate and Texas Red. Out-of-focus information in the images was then removed using iterative, constrained, three-dimensional deconvolution (Agard, et al. 1989; Hiraoka, et al. 1991). This computational technique deblurs an image by moving out-of-focus intensity back to its originating point based on an empirical measure of the "point-spread" function, the blurring of an image caused by the limited resolution of the objective lens . Data are presented both as projections of the entire set of optical sections (Agard, et al. 1989), or as individual optical sections where noted. Some data are presented as unprocessed images recorded by the CCD camera.

The c-Src content in individual focal adhesions of different cells was measured by manually tracing a border around individual clusters of phosphotyrosine staining at the interface of the cell and substrate (i. e., focal adhesions). The c-Src intensity in all pixels that contained anti-

phosphotyrosine staining was summed and divided by the total number of anti-phosphotyrosine staining pixels. In addition, the mean pixel area for each focal adhesion was also calculated. The values reported in the text are means of five focal adhesions in separate cells.

Membrane Isolation and Fractionation

RTCS cells were plated 18-36 h. before harvesting and allowed to reach a final confluency of 75-85%. Cells were loaded with horseradish peroxidase (HRP) (Sigma Chemical Co.) (7.5-10 mg/ml in DME H-16, 10% FCS) at 37°C for 30 minutes (sufficient to saturate the endocytic pathway in these cells). Cells were chilled on ice, washed 4 times in ice-cold PBS and then scraped into homogenization buffer (20 mM Hepes pH7.4, 150 mM KCl, 2 mM $MgCl_2$, 10 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1μg/ml leupeptin, 10μg/ml pepstatin, 0.1mg/ml soybean trypsin inhibitor, 1unit/ml aprotinin, 0.25M sucrose). Cells were homogenized in a Wheaton 2ml dounce with an A pestle for 30-40 strokes. After homogenization, nuclear membranes were pelleted for 10 minutes at 2000xg in an SS-34 rotor at 4°C. The post-nuclear supernatant (S1) was collected and loaded over a 62% sucrose cushion and spun at 100,000xg in an SW50.1 rotor for 35 minutes at 4°C. Membranes at the interface were resuspended in the sucrose cushion (adjusted to 45% sucrose) and placed at the bottom of a sucrose step gradient consisting of 45%, 32%, and 18% steps. The gradient was centrifuged at 35,000 rpm in an SW50.1 rotor for 60 minutes at 4°C. The 18%/32% interface was collected with a 23 gauge needle and a 2.5 ml syringe. The remaining fractions were collected from the top of the gradient with a pasteur pipet and analyzed for c-Src and membrane markers.

Under these homogenization conditions (30-40 strokes) 30-40% of HRP activity was left in the nuclear pellet and plasma membranes were mainly observed in the 45% sucrose fraction. Harsher homogenization conditions (80 strokes) left less than 1% of the total HRP activity in the nuclear pellet and the majority of plasma membranes were observed in the 32% and interface (18%/32%) fractions on sucrose gradients.

To analyze the 18%/32% sucrose interface on Percoll gradients, the fraction was mixed into a 27% Percoll solution (8.5% sucrose) and centrifuged over a 62% sucrose cushion in a 50Ti rotor at 16K for 60 minutes at 4^{o}C. Fractions were collected from the bottom of the tube and assayed for membrane markers and c-Src by immunoblotting.

Membrane markers were assayed as follows. The plasma membrane marker alkaline diphoshoesterase I (ADPE I) was measured with thymidine-5'-monophosphate-p-nitrophenyl ester (Sigma Chemical Co.) and assayed using a Beckman fluorometer at excitation 355nM and emission 455nM (Poole, et al. 1983). The lysosomal marker ß-hexosaminidase was measured with the fluorometric substrate 4-methyl umbelliferyl-N-acetyl-ß-D-glucosaminide (Sigma Chemical Co.) and samples were read on a Beckman fluorometer at excitation 350nM and emission 450nM. The golgi marker galactosyl transferase was measured by incorporation of UDP-[3H]-galactose (Amersham) into BSA (Aoki, et al. 1990). Horse radish peroxidase activity was measured using o-dianisidine (Sigma Chemical Co.) prepared at 0.11µg/ml in PBS pH 5.0, 0.1% Triton X-100. Activity was measured at OD_{460} at several time points (or at several volumes of extract) to ensure linearity of the assay.

Density Shift and DAB Cytochemistry

HRP-containing membranes from the 18%/32% interface or from the P100 fraction (isolated under relatively harsh homogenization conditions; see above) were treated with diaminobenzidine (DAB) in homogenization buffer to a final concentration of 6 μg/ml. 10 μl of 30% H_2O_2 was added and the mixture incubated in the dark at 0°C for 10 minutes. After treatment, membranes were loaded on another sucrose gradient or a Percoll gradient as described above, centrifuged and analyzed for c-Src and membrane markers. In control experiments no density shift was observed if DAB or H_2O_2 were added separately.

To inhibit HRP entry in some experiments, cells were chilled on ice and cold media containing HRP was incubated with the cells at 0°C for 20 minutes. Half of the cells were placed at 37°C for 40 minutes. Both sets of plates were then washed and membranes harvested as above. DAB cytochemistry was carried out and fractions from Percoll gradients were analyzed for c-Src and membrane markers.

Monitoring of plasma membranes by surface iodination was performed with cells loaded for 30 minutes at 37°C with HRP. Cells were chilled on ice, washed 4 times with ice cold PBS and then dislodged from the plate using PBS + 1mM EDTA. Cells were extensively washed in ice cold PBS and then labeled using sulfo-SHPP (Pierce) labeled with iodobeads (Thompson, et al. 1987). Free ^{125}I counts were washed out of the cells using ice cold PBS and membranes were isolated, DAB cytochemistry performed and analyzed as above.

Triton X-100 fractionation

Cell lines expressing specific Src-proteins were plated on 6-well tissue culture plates (~500,000 cells/well). The next day cells were chilled on ice for

15-20' and then washed 2X with ice cold PBS. Cells were lysed for indicated times (30s, 1', 2', 4', 6', 10') in 200µl of Triton X-100 lysis buffer (0.3M sucrose, 0.5% triton X-100, 10mM PIPES (pH 6.8), 100mM KCl, 1mM CaCl2, 2.5mM MgCl2, 1mM $Na_3(VO)_4$, and the following protease inhibitors: 1mM phenyl methyl sulfonyl fluoride (PMSF), 1 ug/ml leupeptin, 0.023 units aprotinin). Cells were then rinsed briefly (<5s) in 200µl Triton X-100 lysis buffer and combined with the lysate, resulting in a final volume of 400µl; this fraction is referred to as the detergent-soluble fraction. The detergent-insoluble fraction was prepared from the remaining cells which were lysed in 400µl RIPA buffer (50mM Hepes (pH 7.4), 1% deoxycholate acid, 1% triton X-100, 0.1% SDS, 150mM NaCl, 1mM EDTA, 1mM $Na_3(VO)_4$ and protease inhibitors as described above) for 5 minutes on ice, after which cells were scraped off the dish. Lysates were centrifuged at 16,000xg at 4°C for 15 minutes to clear cell debris and were transferred to fresh tubes before being immunoprecipitated as described below. Cell debris pellets were monitored and not found to contain greater than 5% of the total Src-protein.

Immunoprecipitation and Immunoblotting

For immunoprecipitating proteins from fractionated lysates, each fraction was diluted two times with RIPA buffer. Antibodies were added as indicated (0.5ul of 1mg/ml MAb 327, 1ul of MAb EC10 ascites, 1ul MAb 9E10 ascites, 2ul Mab2A7 ascites) and incubated at 4°C for 1-4hr. Protein A sepharose conjugated to a rabbit anti-mouse antibody was added (40µl/fraction of 50% slurry) and incubated at 4°C for 1-2hr. Beads were washed 2-3X in 1 ml of ice cold RIPA buffer, boiled in loading buffer and loaded onto 10% PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose in a BioRad electroblot apparatus, and blots were blocked in TBST (10 mM Tris pH 8.0,150

mM NaCl, 0.2% tween-20) plus 2% BSA (bovine serum albumin; Sigma Chemical Co; fraction IV) for 1hr at 20°C. Blots were incubated first in primary antibody (MAb327; 0.25 μg/ml, BC3 polyclonal; 1:2000, MAb 4G10 hybridoma supernatant; 1:4) diluted in TBST 2% BSA at 20°C for 1-2hr, then in secondary anti-mouse HRP-coupled (horse radish peroxidase) antibody (Boeringher Mannheim; diluted 1/20,000 in TBST 2% BSA) for 1 hour at 20°C. Proteins were detected with the chemi-luminescence procedure as described for the Amersham ECL detection system. To detect p125-FAK antibodies, HRP-linked goat anti-rabbit antibodies (Amersham) were used in the chemi-luminescence protocol. In some cases, Src and phosphotyrosine western blots were incubated with ^{125}I-labeled goat anti-mouse IgG (Amersham; 30mCi/mg) for 1-4hr at 4°C, after which blots were washed and the bands quantitated with a Molecular Dynamics PhosphorImager.

In vitro kinase assay

Cells were fractionated for 1 minute in Triton X-100 lysis buffer and the remaining cells were lysed in RIPA buffer as described above. Immunoprecipitations were carried out as described above. After washing beads in RIPA buffer, beads were washed an additional 3X in kinase buffer (20mM Tris-HCl, pH7.2, 5mM $MnCl_2$). Beads were split equally to two tubes; one sample was analyzed by immunoblot (described above) and the other was analyzed in a kinase assay. Approximately 30μg of rabbit muscle enolase (Sigma Chem. Co.) was denatured in 0.025M sodium acetate, pH 3.3 at 30°C for 10'. The denatured enolase was resuspended in 400μl kinase buffer, neutralized with 35μl Tris-HCl, pH8.8 and adjusted to 10μM ATP. Each kinase reaction received 30μl of enolase mix and 10μCi of Amersham Redivu-γ-ATP (3000Ci/mM) and was incubated at room temperature for 5' with constant

mixing. The reaction was terminated by adding an equal volume of loading buffer, boiling and loading onto 10% PAGE gels. Gels were prepared for standard autoradiography and quantitated using a Molecular Dynamics PhosphorImager. Specific kinase activity was calculated by dividing the area of the enolase band by the area of the c-Src band determined by immunoblotting.

Cell Adhesion and Spreading

Cells grown for 24-36 hours were trypsinized in 1ml 0.05% trypsin, EDTA and immediately resuspended in SBTI-media (DME H-21, 10mM Hepes, pH 7.2, 1 mg/ml soybean trypsin inhibitor). Cells were adjusted to a final concentration of 300,000 cells/ml and 50μl of each cell line (15,000cells/well) were plated in 96-well tissue culture plates, coated with the indicated substrate (5μg/ml human fibronectin, 5μg/ml collagen IV, 1μg/ml BSA). Cells were added to duplicate wells (6 wells/data point) containing 50μl of adhesion media (DME H-21, 0.2% FCS, 10mM HEPES, pH7.2) and spun gently to the bottom of each well (1000rpm, 30s in a clinical centrifuge). The plates were incubated at 37°C for the indicated period of time and then chilled on ice for 15'. Loosely bound cells were removed by washing 5X in 150μl adhesion media. The number of cells left in each well was determined by measuring an internal enzymatic marker, β-hexosamindase. A flouremetric substrate was added in 60μl of buffer (0.25% Triton X-100, 5mM p-nitrophenyl N-acetyl-B-D glucosaminide (Sigma Chem. Co.), 40mM citric acid/80mM Na_2HPO_4, pH4.75), incubated at 37°C for 3-4 hours and the reaction was stopped by adding 90μl stop buffer (50mM glycine/250mM Na_2CO_3, pH10.0, 0.5mM EDTA). The OD_{410}nm was determined using a Molecular Devices Microplate Reader. A standard curve for each cell line was used to calculate the number of cells left in each well as a

percentage of the total cells plated. The error bars represent the variation in the same experiment as recorded over six identically treated wells.

To measure cell spreading, cells were resuspended in SBTI-media (see above) and plated in 4ml adhesion media on 10cm tissue culture dishes coated with fibronectin (5µg/ml overnight at 4°C). Plates were incubated at 37°C for 15-20' and then chilled on ice for 15'. Five microscope fields from each plate were photographed (magnification; 150X, each field contained between 400-1000 cells) and both rounded and spread cells were counted. A spread cell was defined as containing less than 10% refractility and the final number of spread cells was calculated as a percentage of the total cells in each field. The error bars indicate the range over five fields in a given dish and therefore reflect the variation in cell spreading over the whole dish. The longer incubation at 37°C (i.e., 15-20') was required because cells were not spun down onto the plate, as was done in the adhesion assay (see above).

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Literature cited

Agard, D. A., Hiraoka, P., Shaw, P. and Sedat, J. W. (1989) Methods Cell Biol. 30, 353-377

Alema, S., Casalbore, P., Agostini, E. and Tato, F. (1985) Nature. 316, 557-9

Anderson, R. G. (1993) Proc Natl Acad Sci U S A. 90, 10909-13

Aneskievich, B. J., Haimovich, B. and Boettiger, D. (1991) Oncogene. 6, 1381-90

Aoki, D., Apperti, H. E., Johnson, D., Wong, S. S. and Fukada, M. N. (1990) European Molecular Biology Organization. 9, 3171-3178

Bagrodia, S., Chackalaparampil, I., Kmiecik, T. E. and Shalloway, D. (1991) Nature. 349, 172-5

Bagrodia, S., Taylor, S. J. and Shalloway, D. (1993) Mol Cell Biol. 13, 1464-70

Bibbins, K. B., Boeuf, H. and Varmus, H. E. (1993) Molecular and Cellular Biology. 13, 7278-7287

Bole, D. G., Hendershot, L. M. and Kearney, J. F. (1989) Journal of Cell Biology. 102, 1558-1566

Bolen, J. B. (1993) Oncogene. 8, 2025-31

Boschek, C. B., Jockusch, B. M., Friis, R. R., Back, R., Grundmann, E. and Bauer, H. (1981) Cell. 24, 175-84

Bouton, A. H., Kanner, S. B., Vines, R. R., Wang, H. C., Gibbs, J. B. and Parsons, J. T. (1991) Mol Cell Biol. 11, 945-53

Boyce, B. F., Chen, H., Soriano, P. and Mundy, G. R. (1993) Bone. 14, 335-40

Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P. and Mundy, G. R. (1992) J Clin Invest. 90, 1622-7

Brott, B. K., Decker, S., Shafer, J., Gibbs, J. B. and Jove, R. (1991) Proc Natl Acad Sci U S A. 88, 755-9

Bryant, D. L. and Parsons, J. T. (1984) Mol Cell Biol. 4, 862-6

Burr, J. G., Dreyfuss, G., Penman, S. and Buchanan, J. M. (1980) Proc Natl Acad Sci U S A. 77, 3484-8

Buss, J. E., Kamps, M. P., Gould, K. and Sefton, B. M. (1986) Journal of Virology. 58, 468-474

Buss, J. E. and Sefton, B. M. (1985) Journal of Virology. 53, 7-12

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell. 64, 281-302

Carley, W. W., Barak, L. S. and Webb, W. W. (1981) J Cell Biol. 90, 797-802

Chackalaparampil, I. and Shalloway, D. (1988) Cell. 52, 801-810

Chege, W. N. and Pfeffer, S. R. (1990) Journal of Cell Biology. 111, 893-899

Clark, E. A. and Brugge, J. S. (1993) Mol Cell Biol. 13, 1863-71

Cobb, B. S., Schaller, M. D., Leu, T. H. and Parsons, J. T. (1994) Mol Cell Biol. 14, 147-55

Cooper, J. A., Gould, K. L., Cartwright, C. A. and Hunter, T. (1986) Science. 231, 1431-1434

Cooper, J. A. and Runge, K. (1987) Oncogene Res. 1, 297-310

Cotton, P. C. and Brugge, J. S. (1983) Molecular and Cell Biology. 3, 1157

Courtneidge, S. A. (1985) Embo J. 4, 1471-7

Courtneidge, S. A., Levinson, A. D. and Bishop, J. M. (1980) Proceedings National Academy of Science USA. 77, 3783-3787

Courtoy, P. J., Quintart, J. and Baudhuin, P. (1984) Journal of Cell Biology. 98, 870-876

Dahl, S. C. and Grabel, L. B. (1989) J Cell Biol. 108, 183-90

Danilov, Y. N. and Juliano, R. L. (1989) J Cell Biol. 108, 1925-33

David-Pfeuty, T. and Nouvian-Dooghe, Y. (1990) Journal of Cell Biology. 111, 3097-3116

David-Pfeuty, T. and Singer, S. J. (1980) Proceedings of the National Academy of Science. 77, 6687-6691

DeBrabander, M., Nuydens, R., Geerts, H. and Hopkins, C. R. (1988) Cell Motility and Cytoskeleton. 9, 30-47

Eck, M. J., Atwell, S. K., Shoelson, S. E. and Harrison, S. C. (1994) Nature. 368, 764-9

Eiseman, E. and Bolen, J. B. (1990) Cancer Cells. 2, 303-10

Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990) Nature. 343, 377-81

Falcioni, R., Perrotti, N., Piaggio, G., Kennel, S. K. and Sacchi, A. (1989) Mol Carcinog. 2, 361-8

Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F. and Williams, L. T. (1992) Cell. 69, 413-23

Ferrell, J., Jr., Noble, J. A., Martin, G. S., Jacques, Y. V. and Bainton, D. F. (1990) Oncogene. 5, 1033-6

Flynn, D. C., Leu, T. H., Reynolds, A. B. and Parsons, J. T. (1993) Mol Cell Biol. 13, 7892-900

Freed, E., Gailit, J., van der Geer, P., Ruoslahti, E. and Hunter, T. (1989) Embo J. 8, 2955-65

Fukui, Y., O'Brien, M. C. and Hanafusa, H. (1991) Mol Cell Biol. 11, 1207-13

Gervais, F. G., Chow, L. M., Lee, J. M., Branton, P. E. and Veillette, A. (1993) Mol Cell Biol. 13, 7112-21

Glenney, J., Jr. and Zokas, L. (1989) J Cell Biol. 108, 2401-8

Goergi, A., Mottola-Harthshorn, C., Warner, A., Fields, B. and Chen, L. B. (1990) Proceedings National Academy of Science USA. 87, 6579-6583

Golden, A., Nemeth, S. P. and Brugge, J. S. (1986) Proceedings National Academy of Science USA. 83, 852-856

Gould, K. L. and Hunter, T. (1988) Molecular and Cell Biology. 8, 345

Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W. and et, a. (1993) Cell. 75, 25-36

Grandori, C. and Hanafusa, H. (1988) Journal of Cell Biology. 107, 2125-2135

Guan, J. L. and Shalloway, D. (1992) Nature. 358, 690-2

Hall, D. E., Reichardt, L. F., Crowley, E., Holley, B., Moezzi, H., Sonnenberg, A. and Damsky, C. H. (1990) J Cell Biol. 110, 2175-84

Hall, T. J., Schaeublin, M. and Missbach, M. (1994) Biochem Biophys Res Commun. 199, 1237-44

Hamaguchi, M. and Hanafusa, H. (1987) Proceedings National Academy of Science USA. 84, 2312-2316

Henderson, D. and Rohrschneider, L. (1987) Experimental Cell Research. 168, 411-421

Herskovits, J. S., Shpetner, H. S., Burgess, C. C. and Vallee, R. B. (1993) Proc Natl Acad Sci U S A. 90, 11468-72

Hirai, H. and Varmus, H. E. (1990) Mol Cell Biol. 10, 1307-18

Hiraoka, Y., Swedlow, J. R., Paddy, M. R., Agard, D. A. and Sedat, J. W. (1991) Sem in Cell Biol. 2, 153-165

Hirst, R., Horwitz, A., Buck, C. and Rohrschneider, L. (1986) Proc Natl Acad Sci U S A. 83, 6470-4

Horne, W. C., Neff, L., Chatterjee, D., Lomri, A., Levy, J. B. and Baron, R. (1992) J Cell Biol. 119, 1003-13

Horvath, A. R., Muszbek, L. and Kellie, S. (1992) Embo J. 11, 855-61

Howell, B. W. and Cooper, J., A. (1994) Molecular and Cellular Biology. 14, 5402-5411

Ignelzi, M., Jr., Miller, D. R., Soriano, P. and Maness, P. F. (1994) Neuron. 12, 873-84

Imamoto, A. and Soriano, P. (1993) Cell. 73, 1117-24

Johnston, P. A., Cameron, P. L., Stukenbrok, H., Jahn, R., De Camilli, P. and Sudhof, T. C. (1989) European Molecular Biology Organization. 8, 2863-2872

Kam, Z., Jones, M. O., Chen, H., Agard, D. A. and Sedat, J. W. (1993) Bioimaging. 1, 71-81

Kamps, M. P., Buss, J. E. and Sefton, B. M. (1985) Proc Natl Acad Sci U S A. 82, 4625-8

Kamps, M. P., Buss, J. E. and Sefton, B. M. (1986) Cell. 45, 105-112

Kanner, S. B., Reynolds, A. B., Vines, R. R. and Parsons, J. T. (1990) Proc Natl Acad Sci U S A. 87, 3328-32

Kanner, S. B., Reynolds, A. B., Wang, H. C., Vines, R. R. and Parsons, J. T. (1991) Embo J. 10, 1689-98

Kaplan, J. M., Varmus, H. E. and Bishop, J. M. (1990) Molecular and Cell Biology. 10, 1000-1009

Kaplan, K. B., Swedlow, J. R., Varmus, H. E. and Morgan, D. O. (1992) J Cell Biol. 118, 321-33

Kato, J. Y., Takeya, T., Grandori, C., Iba, H., Levy, J. B. and Hanafusa, H. (1986) Mol Cell Biol. 6, 4155-60

Kazlauskas, A., Feng, G. S., Pawson, T. and Valius, M. (1993) Proc Natl Acad Sci U S A. 90, 6939-43

Kellie, S., Patel, B., Mitchell, A., Critchley, D. R., Wigglesworth, N. M. and Wyke, J. A. (1986) J Cell Sci. 82, 129-42

Kellie, S., Patel, B., Wigglesworth, N. M., Critchley, D. R. and Wyke, J. A. (1986) Exp Cell Res. 165, 216-28

Kmiecik, T. E., Johnson, P. J. and Shalloway, D. (1988) Mol Cell Biol. 8, 4541-6

Kmiecik, T. E. and Shalloway, D. (1987) Cell. 49, 65-73

Koch, C. A., Moran, M. F., Anderson, D., Liu, X. Q., Mbamalu, G. and Pawson, T. (1992) Mol Cell Biol. 12, 1366-74

Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M. and Juliano, R. L. (1992) J Biol Chem. 267, 23439-42

Kornbluth, S., Jove, R. and Hanafusa, H. (1987) Proc Natl Acad Sci U S A. 84, 4455-9

Koyama, S., Yu, H., Dalgarno, D. C., Shin, T. B., Zydowsky, L. D. and Schreiber, S. L. (1993) Cell. 72, 945-52

Kremer, N. E., D'Arcangelo, G., Thomas, S. M., DeMarco, M., Brugge, J. S. and Halegoua, S. (1991) J Cell Biol. 115, 809-19

Kypta, R. M., Goldberg, Y., Ulug, E. T. and Courtneidge, S. A. (1990) Cell. 62, 481-492

Laudano, A. P. and Buchanan, J. M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 892

Levy, J. B., Dorai, T., Wang, L. H. and Brugge, J. S. (1987) Mol Cell Biol. 7, 4142-5

Lewis, V., Green, S. A., Marsh, M., Vihko, P., Helenius, A. and Mellman, I. (1985) Journal of Cell Biology. 100, 1839-1847

Linstedt, A. D. and Kelly, R. B. (1991) Neuron. 7, 1-9

Linstedt, A. D., Vetter, M. L., Bishop, J. M. and Kelly, R. B. (1992) J Cell Biol. 117, 1077-84

Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P., Yuan, L. C. and Klausner, R. D. (1990) Cell. 60, 821-836

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L. and Klausner, R. D. (1991) Cell. 67, 601-616

Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. and Klausner, R. D. (1989) Cell. 56, 801-813

Lipsich, L. A., Lewis, A. J. and Brugge, J. S. (1983) Journal of Virology. 48, 352-360

Liu, X., Brodeur, S. R., Gish, G., Zhou, S., Cantley, L. C., Laudano, A. P. and Pawson, T. (1993a) Oncogene. 8, 1119-26

Liu, X., Marengere, L. E., Koch, C. A. and Pawson, T. (1993b) Mol Cell Biol. 13, 5225-32

Lowe, C., Yoneda, T., Boyce, B. F., Chen, H., Mundy, G. R. and Soriano, P. (1993) Proc Natl Acad Sci U S A. 90, 4485-9

Lowell, C. A., Soriano, P. and Varmus, H. E. (1994) Genes Dev. 8, 387-98

Lucocq, J. M. and Warren, G. (1987) European Molecular Biology Organization. 6, 3239-3246

Lupton, S. D., Brunton, L. L., Kalberg, V. A. and Overell, R. W. (1991) Mol Cell Biol. 11, 3374-8

Maa, M. C., Wilson, L. K., Moyers, J. S., Vines, R. R., Parsons, J. T. and Parsons, S. J. (1992) Oncogene. 7, 2429-38

MacAuley, A. and Cooper, J. A. (1989) Molecular and Cellular Biology. 9, 2648-2656

Maher, P. A. (1993) Proc Natl Acad Sci U S A. 90, 11177-81

Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T. and Schlessinger, J. (1990) Embo J. 9, 4375-80

Matteoni, R. and Kreis, T. E. (1987) Journal of Cell Biology. 105, 1253-1265

Mayer, B. J., Jackson, P. K. and Baltimore, D. (1991) Proc Natl Acad Sci U S A. 88, 627-31

Mayer, B. J., Jackson, P. K., Van Etten, R. A. and Baltimore, D. (1992) Mol Cell Biol. 12, 609-18

McGlade, J., Cheng, A., Pelicci, G., Pelicci, P. G. and Pawson, T. (1992) Proc Natl Acad Sci U S A. 89, 8869-73

Medema, R. H. and Bos, J. L. (1993) Crit Rev Oncog. 4, 615-61

Messner, D. J., Griffiths, G. and Kornfeld, S. (1989) Journal of Cell Biology. 108, 2149-2162

Meyerson, G., Pfenninger, K. H. and Pahlman, S. (1992) J Cell Sci. 103, 233-43

Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S. and Pawson, T. (1990) Proc Natl Acad Sci U S A. 87, 8622-6

Morgan, D. O., Kaplan, J. M., Bishop, J. M. and Varmus, H. E. (1989) Cell. 57, 775-786

Morgan, D. O., Kaplan, J. M., Bishop, J. M. and Varmus, H. E. (1991) Methods in Enzymology. 200, [53]

Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtneidge, S. A., Claesson-Welsh, L. and Heldin, C. H. (1993) Embo J. 12, 2257-64

Moskalewski, S. and Thyberg, J. (1990) J. Submicrosc. Cytol. and Pathol. 22, 159-171

Murphy, S. M., Bergman, M. and Morgan, D. O. (1993) Mol Cell Biol. 13, 5290-300

Musacchio, A., Noble, M., Pauptit, R., Wierenga, R. and Saraste, M. (1992) Nature. 359, 851-5

Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M. and Aizawa, S. (1993) Cell. 73, 1125-35

Nakamura, N., Tanaka, J. and Sobue, K. (1993) J Cell Sci. 106, 1057-69

Nermut, M. V., Eason, P., Hirst, E. M. and Kellie, S. (1991) Exp Cell Res. 193, 382-97

Nigg, E. A., Sefton, B. M., Hunter, T., Walter, G. and Singer, S. J. (1982) Proc Natl Acad Sci U S A. 79, 5322-6

Nigg, E. A., Sefton, B. M., Singer, S. J. and Vogt, P. K. (1986) Virology. 151, 50-65

O'Brien, M. C., Fukui, Y. and Hanafusa, H. (1990) Mol Cell Biol. 10, 2855-62

Okada, M., Howell, B. W., Broome, M. A. and Cooper, J. A. (1993) J Biol Chem. 268, 18070-5

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T. and Nakagawa, H. (1991) J Biol Chem. 266, 24249-52

Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N. and et, a. (1991) Cell. 65, 91-104

Overduin, M., Rios, C. B., Mayer, B. J., Baltimore, D. and Cowburn, D. (1992) Cell. 70, 697-704

Pasquale, E. B., Maher, P. A. and Singer, S. J. (1986) Proc Natl Acad Sci U S A. 83, 5507-11

Pawson, T. and Gish, G. D. (1992) Cell. 71, 359-62

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P. G. (1992) Cell. 70, 93-104

Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E. and Cheng, S. H. (1987) Cell. 49, 75-82

Poole, R. R. J., Maurey, K. M. and Storrie, B. (1983) Cell Biol Int. Reports. 7, 361

Pumiglia, K. M. and Feinstein, M. B. (1993) Biochem J. 294, 253-60

Pyper, J. M. and Bolen, J. B. (1989) J Neurosci Res. 24, 89-96

Ralston, R. and Bishop, J. M. (1985) Proceedings National Academy of Science USA. 82, 7845

Rankin, S. and Rozengurt, E. (1993) J Biol Chem. 268, 704-10

Redmond, T., Brott, B. K., Jove, R. and Welsh, M. J. (1992) Cell Growth Differ. 3, 567-76

Ren, R., Mayer, B. J., Cicchetti, P. and Baltimore, D. (1993) Science. 259, 1157-61

Resh, M. D. (1989) Cell. 1989, 58

Resh, M. D. and Erikson, R. L. (1985) Journal of Cell Biology. 100, 409-417

Resh, M. D. and Ling, H. (1990) Nature. 346, 84-86

Rohrschneider, L. R. (1980) Proceedings National Academy of Science USA. 77, 3514-3518

Rohrschneider, L. R. (1979) Cell. 16, 11-24

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. and Anderson, R. G. (1992) Cell. 68, 673-82

Rotin, D., Margolis, B., Mohammad, M., Daly, R. J., Daum, G., Li, N., Fischer, E. H., Burgess, W. H., Ullrich, A. and Schlessinger, J. (1992) EMBO J. 11, 559-567

Roussel, R. R., Brodeur, S. R., Shalloway, D. and Laudano, A. P. (1991) Proc Natl Acad Sci U S A. 88, 10696-700

Sabe, H., Hata, A., Okada, M., Nakagawa, H. and Hanafusa, H. (1994) Proc Natl Acad Sci U S A. 91, 3984-8

Sabe, H., Knudsen, B., Okada, M., Nada, S., Nakagawa, H. and Hanafusa, H. (1992) Proc Natl Acad Sci U S A. 89, 2190-4

Sargiacomo, M., Sudol, M., Tang, Z. and Lisanti, M. P. (1993) J Cell Biol. 122, 789-807

Sassone-Corsi, P., Der, C. J. and Verma, I. M. (1989) Mol Cell Biol. 9, 3174-83

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) Proc Natl Acad Sci U S A. 89, 5192-6

Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994) Mol Cell Biol. 14, 1680-8

Schlessinger, J. (1994) Curr Opin Genet Dev. 4, 25-30

Segatto, O., Pelicci, G., Giuli, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Pawson, T. and Pelicci, P. G. (1993) Oncogene. 8, 2105-12

Seidel-Dugan, C., Meyer, B. E., Thomas, S. M. and Brugge, J. S. (1992) Mol Cell Biol. 12, 1835-45

Shenoy, S., Chackalaparampil, I., Bagrodia, S., Lin, P. H. and Shalloway, D. (1992) Proc Natl Acad Sci U S A. 89, 7237-41

Shenoy, S., Choi, J. K., Bagrodia, S., Copeland, T. D., Maller, J. L. and Shalloway, D. (1989) Cell. 57, 761-772

Shriver, K. and Rohrschneider, L. (1981) J Cell Biol. 89, 525-35

Sobue, K. and Kanda, K. (1988) Biochem Biophys Res Commun. 157, 1383-9

Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Cell. 64, 693-702

Stoorvogel, W., J., S. G., Geuze, H. J., Oorschot, V. and Schwartz, A. L. (1991) Cell. 65, 417-427

Sudhof, T. C. and Jahn, R. (1991) Neuron. 6, 665-677

Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A. and Draetta, G. (1993) Embo J. 12, 2625-34

Tanaka, S., Takahashi, N., Udagawa, N., Sasaki, T., Fukui, Y., Kurokawa, T. and Suda, T. (1992) Febs Lett. 313, 85-9

Tapley, P., Horwitz, A., Buck, C., Duggan, K. and Rohrschneider, L. (1989) Oncogene. 4, 325-33

Taylor, S. J. and Shalloway, D. (1993) Current Opinion in Genetics and Development. 3, 26-34

Teti, A., Marchisio, P. C. and Zallone, A. Z. (1991) Am J Physiol. 261, C1-7

Thompson, J. A., Lau, A. L. and Cunningham, D. D. (1987) Biochemistry. 26, 743-750

Tsukita, S., Kumiko, O., Akiyama, T., Yu, Y. and Yamamoto, T. (1991) Journal of Cell Biology. 113, 867-879

Tuomikoski, T., Felix, M.-A., Doree, M. and Gruenberg, J. (1989) Nature. 342, 942-945

Turner, C. E., Glenney, J., Jr. and Burridge, K. (1990) J Cell Biol. 111, 1059-68

Turner, C. E., Schaller, M. D. and Parsons, J. T. (1993) J Cell Sci. 105, 637-45

Twamley, G. M., Kypta, R. M., Hall, B. and Courtneidge, S. A. (1992) Oncogene. 7, 1893-901

Twamley-Stein, G. M., Pepperkok, R., Ansorge, W. and Courtneidge, S. A. (1993) Proc Natl Acad Sci U S A. 90, 7696-700

Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J. and Overduin, M. (1992) Nature. 358, 646-53

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. and Kuriyan, J. (1993) Cell. 72, 779-90

Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S. and Seidel-Dugan, C. (1993) J Biol Chem. 268, 14956-63

Willingham, M. C., Jay, G. and Pastan, I. (1979) Cell. 16, 125-3l

Wood, S. A., Park, J. E. and Brown, W. J. (1991) Cell. 67, 591-600

Woodman, P. G., Mundy, D. I., Cohen, P. and Warren, G. (1992) Journal of Cell Biology. 116, 331-338

Wu, H. and Parsons, J. T. (1993) J Cell Biol. 120, 1417-26

Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R. and Parsons, J. T. (1991) Mol Cell Biol. 11, 5113-24

Yaciuk, P., Cannella, M. T. and Shalloway, D. (1988) Oncogene Res. 3, 207-12

Yu, H., Rosen, M. K., Shin, T. B., Seidel-Dugan, C., Brugge, J. S. and Schreiber, S. L. (1992) Science. 258, 1665-8

Zheng, X. M., Wang, Y. and Pallen, C. J. (1992) Nature. 359, 336-9