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Identification and characterization of proteins that associate with transmembrane transforming growth factor alpha

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Identification and Characterization of Proteins that Associate with Transmembrane Transforming Growth Factor Alpha

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DISSERTATION

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Abstract

Identification and Characterization of Proteins that Associate with Transmembrane Transforming Growth Factor Alpha.

Alfred Chung Kuo

Intercellular communication plays an essential role in processes such as tissue organization, proliferation, and differentiation. In many cases, cells send messages in the form of growth factors, which are polypeptides that convey a signal by binding to and modulating the activity of specific cellular receptors. Transforming growth factor alpha (TGFα) is one of the best-characterized growth factors. This protein plays roles in normal tissue growth and development, and has also been implicated in cancer and other disease processes. TGFα is synthesized as a transmembrane molecule that can be converted into soluble forms by proteolysis of its extracellular domain. The cytoplasmic domain of transmembrane TGFα is highly conserved, suggesting that it is functionally important. Since there are no known motifs in the cytoplasmic domain, the proteins which bind to transmembrane TGFα may mediate these functions. Protein purification and yeast two-hybrid screening were used to identify polypeptides that interact with transmembrane TGFα. Several candidates were obtained, including filamin, p86/Mem3, and p59, a novel Golgi protein that contains two atypical PDZ domains. These proteins may play roles in a variety of processes, including the post-translational modification, intracellular trafficking, and cytoskeletal attachment of transmembrane TGFα. Therefore, the study of the associated proteins may shed light on both transmembrane TGFα and these basic cellular processes.

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List of Abbreviations

B	bound
BSA	bovine serum albumin
C	centigrade
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD9	cluster of differentiation 9
cdk2	cyclin-dependent kinase 2
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
COPII	coat protein complex II
COS-1	CV-1 origin, SV40 line 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSP	dithiobissuccinimidyl-proprionate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Elk-L	eph-like-kinase ligand
endo H	endoglycosidase H
Eph	erythropoietin-producing human hepatocellular carcinoma cell-line gene
ER	endoplasmic reticulum
ERGIC-53	endoplasmic reticulum Golgi intermediate compartment 53

List of Abbreviations, continued

EST	expressed sequence tag
FT	flow-through
g	gravity
g	gram
GM130	Golgi matrix protein 130
GRASP65	Golgi reassembly stacking protein 65
HB-EGF	heparin-binding epidermal growth factor
HPLC	high performance liquid chromatography
Htk-L	hepatoma transmembrane-kinase ligand
htrA	high termperature requirement A
IgG	immunoglobulin
kb	kilobase
kD	kilodalton
l	liter
M	molar
mAb	monoclonal antibody
mCi	millicurie
MDCK	Madin-Darby canine kidney
Mem3	maternal embryonic 3
Mer5	murine erythroleukemia 5
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid

List of Abbreviations, continued

MW	molecular weight
nCi	nanocurie
NIH	National Institutes of Health
PBS	phosphate buffered saline
PC12	pheochromocytoma 12
PCR	polymerase chain reaction
PDZ	PSD95, Dlg, ZO-1
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PKD	polycystic kidney disease
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. pombe	*Schizosaccharomyces pombe*
SSC	saline sodium citrate
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TM	transmembrane
TrisCl	tris[hydroxymethyl]aminomethane hydrochloride
U	unit
μCi	microcurie
μg	microgram
μl	microliter
UV	ultraviolet

List of Abbreviations, continued

V	volt
VPS35	vacuolar protein sorting 35
W T	wild-type
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter 1: Biology of Transforming Growth Factor α

Introduction

Multicellular organisms rely on intercellular communication to drive essential processes such as tissue proliferation, differentiation, and organization. In many cases, cells send messages in the form of growth factors, which are polypeptides that convey a signal by binding to and modulating the activity of specific cellular receptors. Growth factors can be grouped into two categories based on their precursor structure: those that are synthesized as soluble molecules and those that are produced as membrane-anchored molecules, which may then be subsequently solubilized.

Transforming growth factor α (TGFα) is one of the best-characterized membrane-bound growth factors. TGFα is synthesized as a transmembrane molecule that can be released from the cell by regulated proteolysis of its extracellular domain (Pandiella and Massague, 1991). It was originally identified in the medium of cells transformed by certain sarcoma viruses (De Larco and Todaro, 1978). When grown in this conditioned medium, uninfected cells undergo reversible phenotypic transformation, hence the name of the growth factor. Biochemical purification of this activity showed that it was a mixture of two proteins, TGFα and TGFβ, a struncturally unrelated molecule (Anzano, et al., 1983).

TGFα is related to several other growth factors, including epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), amphiregulin (also known as Schwannoma-derived growth factor), epiregulin, and betacellulin. All of these molecules contain an EGF domain, an extracellular motif with six cysteines, which form three characteristic disulfide bonds. In addition, these molecules are all synthesized as transmembrane proteins.

The effects of TGFα are mediated by its interaction with and activation of the epidermal growth factor receptor (EGFR) (Massague, 1983, Todaro, et al., 1980). The EGFR is a transmembrane polypeptide that is expressed in many epithelial and mesenchymal cells. It contains an intracellular domain with tyrosine kinase activity as well as an extracellular portion, which binds EGF domains. Occupation of the EGFR with ligand leads to dimerization of the receptor, autophosphorylation of its cytoplasmic domain, and subsequent binding and phosphorylation of associated target-proteins. This in turn initiates signaling cascades, which ultimately trigger alterations in gene expression. These transcriptional changes lead to DNA synthesis and cell division in most cell types. In other cases, such as TGFα treatment of rat PC12 pheochromocytoma cells, EGFR activation leads to differentiation (Zhang, et al., 1990). Since EGF stimulates these same cells to proliferate, the final outcome of receptor stimulation depends on ligand as well as cell type.

TGFα is expressed in a wide range of adult and embryonic tissues and is likely to have roles in normal tissue physiology and development. As its name and history imply, transfection and expression of TGFα in a number of cell lines is sufficient to induce phenotypic transformation, suggesting that TGFα has a role in oncogenesis. Consistent with this, TGFα is expressed in many mammalian cancers. In addition, the growth factor has been implicated in several other disease processes.

TGFα in Normal Physiology

The widespread expression of TGFα in both adult and fetal tissues suggests that it has a role in normal development and tissue physiology. In the adult animal, TGFα is expressed in most if not all epithelia, including skin (Coffey, et al., 1987), mammary epithelia and gut (Beauchamp, et al.,

1989), where its mitogenic activity likely contributes to normal tissue proliferation and/or maintenance. For example, after hepatic injury, TGFα production increases in the liver, hinting that the growth factor has a role in regeneration of this organ (Mead and Fausto, 1989, Russell, et al., 1993). Several types of hematopoietic cells also express TGFα, including eosinophils (Walz, et al., 1993, Wong, et al., 1990), erythroid precursor cells (Walz, et al., 1995), neutrophils (Calafat, et al., 1997), monocytes (Calafat, et al., 1997), and activated macrophages (Madtes, et al., 1988). Additionally, studies have shown that TGFα is capable of inducing angiogenesis (Schreiber, et al., 1986) and bone remodeling (Ibbotson, et al., 1986, Stern, et al., 1985), presumbably by stimulating EGFRs on endothelial cells and osteoclasts respectively. Finally, TGFα is expressed in many areas of the brain (Wilcox and Derynck, 1988), where it may play a neurotrophic role, although its exact function in this tissue is unknown.

TGFα is also expressed during mammalian embryogenesis, peaking in mice at days 9-11 *post coitum*. In the fetus, high levels of expression are found in ectodermal structures including the branchial arches, the oral and naropharyngeal epithelia, the otic vesicle, and the mesonephric tubules of the kidney (Wilcox and Derynck, 1988). Just as in the adult, the principal role of TGFα during development is likely to drive cellular proliferation.

While these *in vitro* studies and expression patterns suggest possible roles for TGFα in select cell types and tissues, direct experimental evidence is generally not available for the contribution of TGFα to each of these processes *in vivo*. Mice lacking TGFα, either generated by natural mutation or by targeted removal, are viable and reproduce normally. In fact, the phenotype only consists of wavy hair and curly whiskers (Luetteke, et al., 1993, Mann, et al., 1993). This is perhaps not surprising due to the coexpression of TGFα-

related growth factors in the same cells that produce TGFα. These family members can activate EGFRs and presumably compensate for the absence of TGFα.

Mice in which the EGFR has been removed by targeted disruption give a better indication of the importance of the TGFα family as a whole (Miettinen, et al., 1995, Sibilia and Wagner, 1995). These mice die before maturity, which, depending on the background strain, can be during mid-gestation, perinatally, or at 20 weeks. The viable mice display epithelial defects resulting in problems of the lungs, kidneys, digestive tract, and nervous system, and eventually die of failure of one or more of these organs. However, the individual contribution of a given ligand to the physiology of each organ remains unknown.

TGFα and cancer

The enhanced expression of TGFα in many tumors suggests that it has a role in these cancers. TGFα is expressed in many sarcomas and most carcinomas, especially renal, squamous, mammary, and neuroectodermal carcinomas (Derynck, et al., 1987). Although it may be expressed in adjacent tissues, the level of expression of TGFα is often many fold greater in the tumor tissue. The underlying molecular basis for TGFα's overexpression in tumors is unknown. No promoter or gene mutations resulting in enhanced TGFα transcription have yet been discovered. However, genetic changes at other loci can increase the levels of TGFα mRNA and protein. For example, immortalized fibroblasts can be induced to express TGFα by introduction of many but not all oncogenes; stimulators of tyrosine phosphorylation are able to do so, whereas nuclear oncogenes are not (Ciardiello, et al., 1990).

TGFα is thought to act in oncogenesis as an autocrine stimulator of mitogenesis (Sporn and Roberts, 1980). Cancer cells that expresss TGFα often also express the EGFR. Therefore, TGFα produced by these cells could activate receptors on the same cells, driving proliferation in an autocrine fashion. In addition, stimulation of the tyrosine kinase activity of the EGFR induces transcription of TGFα (Coffey, et al., 1987), thereby amplifying this autocrine loop. Consistent with this, overexpression of the growth factor is sufficient to drive malignant transformation in some cell lines and tissues. Expression of TGFα in non-transformed Rat-1 fibroblasts leads to anchorage-independent growth and tumor formation in nude mice (Rosenthal, et al., 1986). Immortalized mouse mammary epithelial cells behave similarly (McGeady, et al., 1989, Shankar, et al., 1989). Transgenic mice overexpressing TGFα develop epithelial hyperplasia in several organs, and depending on the background, pancreatic metaplasia and carcinoma of the breast, coagulation gland, and/or liver (Jhappan, et al., 1990, Sandgren, et al., 1990).

Conversely, reduction of TGFα activity can decrease the growth of some tumors and of transformed cells that express the growth factor. For example, expression of a TGFα antisense gene in a transformed rat epithelial cell-line slows the growth of tumors formed by these cells (Laird, et al., 1994). Likewise, antibodies that neutralize TGFα can induce apoptosis in hepatocellular carcinomas that express the growth factor (Seki, et al., 1997) and slow the growth of mesothelioma cells that express the growth factor and its receptor (Walker, et al., 1995).

However, overexpression of TGFα alone does not induce malignant transformation in all circumstances. In many cases, other factors act in concert with TGFα. For instance, overexpression of TGFα in NIH 3T3 cells is insufficient to drive their transformation (Finzi, et al., 1987). In the original

identification of transforming growth factor, both TGFα and TGFβ were required to efficiently drive transformation. Expression of the hepatitis B virus surface antigen along with TGFα in transgenic mice leads to faster appearance of hepatocellular carcinomas than in mice singly transgenic for either one of these proteins (Jakubczak, et al., 1997). Nuclear oncogenes may also potentiate the effects of TGFα in cancer. For instance, in epithelial cell lines derived from chemically-treated liver, the increased expression of both c-myc and TGFα correlates with increased tumorigenicity (Lee, et al., 1991). In transgenic mice overexpressing TGFα and/or c-myc, the doubly-transgenic animals develop breast and salivary gland tumors with increased frequency relative to singly transgenic animals (Amundadottir, et al., 1995).

Besides driving the proliferation of tumor cells, TGFα may contribute in other ways to the pathology of cancer. For instance, TGFα-stimulated angiogenesis may contribute to the vascularization of a tumor (Schreiber, et al., 1986). Hypercalcemia seen in many cancer patients may result in part from TGFα-stimulated bone resorption (Ibbotson, et al., 1986).

TGFα in other Disease Processes

Besides a possible role in oncogenesis, TGFα has been implicated in other disease processes. Psoriasis, an idiopathic condition involving hyperproliferation of keratinocytes and consequent thickening of the epidermis, also is characterized by increased TGFα production by these cells (Elder, et al., 1989). Transgenic mice overexpressing TGFα develop hypochloridria and thickening of the gastric mucosa, hallmarks of Ménétrier's disease in humans. Accordingly, patients with this disease also have elevated gastric mucosal levels of TGFα (Dempsey, et al., 1992, Takagi, et al., 1992).

TGFα may also play a role in polycystic kidney disease (PKD). Transgenic overexpression of TGFα in mice leads to renal enlargement, glomerular mesangial expansion, and renal epithelial cyst formation (Lowden, et al., 1994). In addition, TGFα expression is increased in renal cysts induced by glucocorticoids in mice (Ogborn and Sareen, 1996), and TGFα overexpression accelerates the growth of cysts in a mouse model of autosomal dominant PKD (Gattone, et al., 1996).

Finally, some forms of cleft palate have been associated with particular alleles of TGFα. During palatogenesis, TGFα, EGF and EGFR expression are localized to the medial edge epithelium, the region where closure occurs (Dixon, et al., 1991). Their levels are elevated at the time of palatal fusion. An association between restriction fragment length polymorphisms in the TGFα gene with non-syndromic cleft lip with or without cleft palate has been established (Ardinger, et al., 1989), although other genetic and environmental factors play probably more significant roles in the development of these syndromes (Hibbert and Field, 1996, Hwang, et al., 1995).

Chapter 2: Transmembrane TGFα

Structure and Processing of Transmembrane TGFα

TGFα is synthesized as a 160 amino acid type I transmembrane molecule that is designated as pre-proTGFα. Starting from its amino terminus, pre-proTGFα consists of a 22 residue signal peptide that is rapidly removed, a 17 residue pro-region, a 50 residue EGF domain, a transmembrane domain, and a 39 residue cytoplasmic domain. Fully processed, secreted TGFα consists of the 50 residue EGF domain and is generated by proteolysis of the extracellular portion of the transmembrane molecule. This cleavage occurs at the plasma membrane (Bosenberg, et al., 1993, Teixido, et al., 1990).

The maturation of pre-proTGFα involves the transport of the molecule to the cell surface and a series of post-translational modifications. Besides removal of its signal peptide, which generates a molecule designated as proTGFα, it undergoes cleavages at both the amino- and carboxy-termini of the 50-residue EGF motif. Both of these sites are cleaved between alanine and valine in the sequence Ala-Val-Val. Cleavage at the amino-terminal site removes the pro-region and cleavage at the carboxy-terminal site releases soluble TGFα from the cell. Since either proteolytic event can occur first, two soluble forms of TGFα can be released. One form includes the pro-region and the other form lacks it (Bringman, et al., 1987). The protease(s) responsible for the two cleavages have not yet been identified, although several candidates have been partially purified (Cappelluti, et al., 1993, Harano and Mizuno, 1994). ProTGFα also undergoes N- and O-linked glycosylation of its pro-region (Bringman, et al., 1987, Teixido and Massague, 1988, Teixido, et al., 1990) as well as palmitoylation of its cytoplasmic domain (Bringman, et al., 1987).

The post-translational modifications result in the generation of multiple transmembrane forms of TGFα, including three predominant species in Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells (Fig. 1) (Bringman, et al., 1987, Dempsey and Coffey, 1994, Shum, et al., 1994). Of these three, an intermediate-sized form of 25 kD is the initial precursor (Dempsey and Coffey, 1994). This species probably contains an N-linked high-mannose oligosaccharide in the pro-region (Briley, et al., 1997). The 30 kD, largest form contains additional N- and O-linked glycosylation in the pro-region (Brachman, et al., 1989, Bringman, et al., 1987, Teixido and Massague, 1988). Finally, the smallest transmembrane-form of 20 kD results from the proteolytic removal of the pro-region (Brachman, et al., 1989). Soluble forms of the growth factor are generated from both the 30 kD and 20 kD transmembrane forms, yielding respectively, 1) a protein that contains the complex N-glycosylated pro-region and the EGF domain and 2) the mature, soluble 50 residue growth factor (Bringman, et al., 1987).

I analyzed the processing of transmembrane TGFα in transfected human embryonic kidney 293 cells. My results were consistent with the published ones and they extended them further. The α1 anti-TGFα monoclonal antibody (Bringman, et al., 1987) specifically immunoprecipitated proteins of 30, 25, and 20 kD from these cells (Fig. 2). These bands were not seen in immunoprecipitations from mock-transfected cells. Pulse-chase analysis showed that the 25 kD species is the precursor that is converted sequentially into the 30 kD species and finally into the 20 kD form (Fig. 2a). A 22 kD band was also seen during early time points. This species is probably a minor, transient intermediate since it was not seen in steady-state immunoprecipitations.

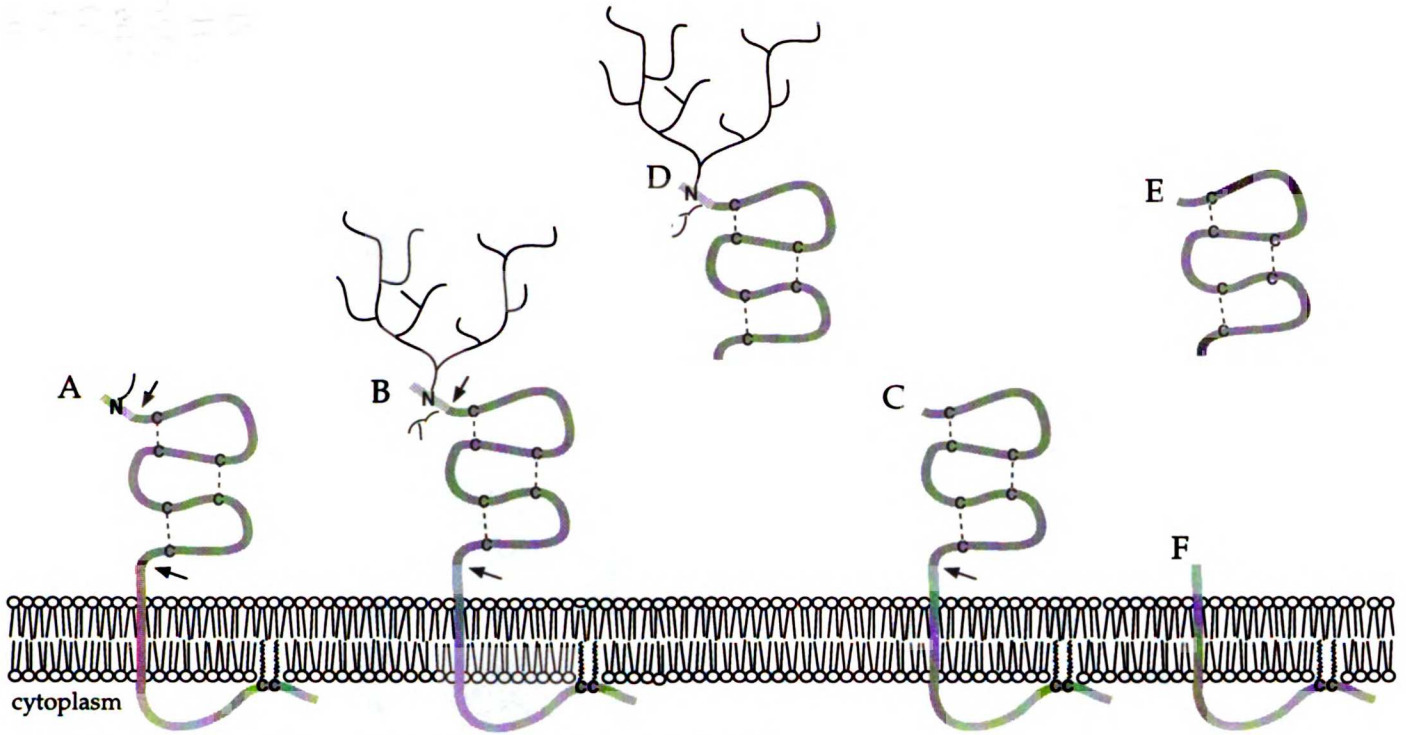

Figure 1. The Transmembrane and Soluble forms of TGFα

Schematic diagram of the five major TGFα species, with the pro-region (lightest gray), EGF repeat (dark gray), cysteine palmitoylation sites, and N-glycosylation site illustrated. Potential cleavage sites are indicated with arrows. (A). The 25 kD endo H-sensitive transmembrane precursor. Additional glycosylation of this species forms a 30 kD molecule (B). The pro-regions of both (A) and (B) can be proteolytically clipped, leading to a 20 kD transmembrane molecule (C). Soluble TGFα can be generated by proteolysis of either the 30 kD molecule, leading to form (D), or of the 20 kD species, leading to the mature 50 residue growth factor (E). In both cases, a short transmembrane tail remains behind (F).

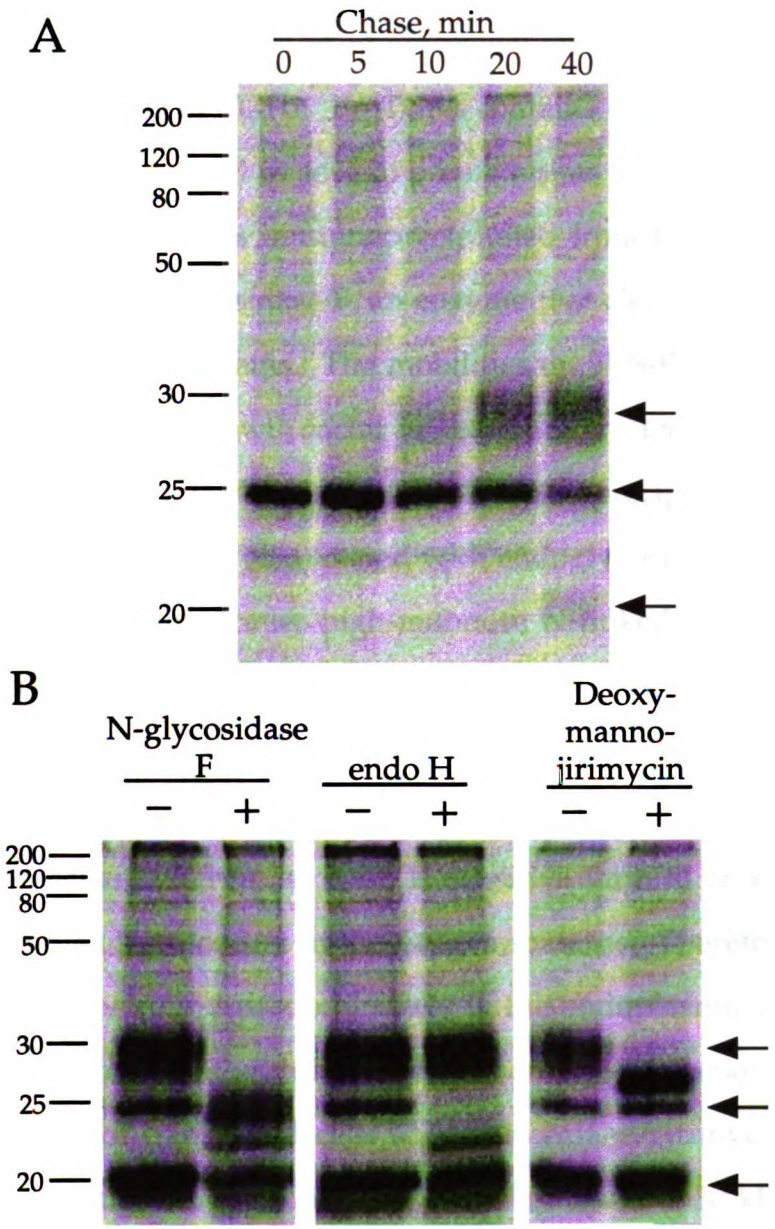

Figure 2. Processing of transmembrane TGFα in 293 cells

A. Pulse-chase analysis. 293 cells were transfected with an expression plasmid for TGFα, labeled for five minutes with [^{35}S]-cysteine and methionine, incubated for the indicated times in unlabeled media, lysed, and immunoprecipitated for transmembrane TGFα.

B. Glycosylation analysis. 293 cells were transfected as in (A), [^{35}S]-labeled, and immunoprecipitated for TGFα. Deoxymannojirimycin was added to culture medium and glycosidase digestions were performed on immunoprecipitates. Arrows indicate positions of the three major transmembrane TGFα species in the absence of glycosidase or inhibitor treatment.

Next, I analyzed the N-linked glycosylation of the different transmembrane forms of TGFα. ProTGFα contains a single N-glycosylation site in its pro-region as well as several possible sites for O-glycosylation. Transmembrane TGFα was immunoprecipitated from transfected 293 cells and digested with N-glycosidase F, an enzyme that cleaves all N-linked oligosaccharides from proteins. The mobilities on SDS-PAGE of the 25 kD and 30 kD forms of the growth factor increased after digestion, indicating that these species are N-glycosylated (Fig. 2b). Immunoprecipitated transmembrane TGFα was also treated with endoglycosidase H (endo H), an enzyme that selectively cleaves high-mannose N-linked oligosaccharides from proteins. Only the 25 kD form was susceptible to cleavage by endo H, indicating that it carries a high-mannose oligosaccharide and that the 30 kD form carries a complex N-linked carbohydrate (Fig. 2b). The conversion of N-linked oligosaccharides from an endo H-sensitive form to an endo H resistant, complex form occurs in the Golgi apparatus. Therefore, the 25 kD form is likely to be found predominantly in the endoplasmic reticulum and the early Golgi, consistent with its identification as a precursor species.

Treatment of transfected cells with deoxymannojirimycin prior to the immunoprecipitation of proTGFα also indicated that the 30 kD form contains a complex-type N-linked carbohydrate. This chemical prevents the conversion of high-mannose N-linked oligosaccharides into complex ones. In this case, the mobility of the intermediate-sized form of transmembrane TGFα was unchanged while the largest form of TGFα displayed an increased mobility. Thus it normally contains complex-type N-linked oligosaccharides.

Two additional conclusions can be drawn from these results. First, the smallest species of transmembrane TGFα is not N-glycosylated since its

mobility was not changed by any of these treatments. Second, the 30 kD species contains post-translational modifications besides N-glycosylation. If the conversion of the 25 kD form into the 30 kD form involved only the modification of the N-linked oligosaccharide, then the two forms would have identical mobilities after N-glycosidase digestion or deoxymannojirimycin treatment. This was not the case, indicating that the conversion of the precursor to the largest form involves processing of the N-linked oligosaccharide as well as additional modifications (Fig. 2b). This additional processing probably includes O-glycosylation of the pro-region (Teixido and Massague, 1988).

Activity of Transmembrane TGFα

Unlike classical prohormones, which are inactive prior to proteolytic cleavage, proTGFα is a biologically active molecule. Transmembrane TGFα is the predominant form of the growth factor in many normal and neoplastic tissues (Hoffman, et al., 1997, Russell, et al., 1993) and cell lines (Brachman, et al., 1989, Derynck, et al., 1987, Teixido, et al., 1990, Wong, et al., 1989). Like soluble TGFα, transmembrane TGFα can bind to and activate EGFRs on adjacent cells (Brachman, et al., 1989, Wong, et al., 1989). However, despite sharing this biochemical activity, the two forms differ in several aspects and may well have different biological effects. This is suggested, for instance, by the differential accumulation of soluble TGFα in regenerating rat liver versus normal liver (Russell, et al., 1993).

The two forms of the growth factor differ in their spatial distributions, which may in turn allow them to serve different functions. Since soluble TGFα can diffuse widely, it can stimulate not only neighboring cells, but also distant ones. In contrast, proTGFα cannot diffuse and instead has much more

localized effects. Obviously, proTGFα on a cell can only activate EGFRs on direct neighbors; the binding of receptor to ligand is in effect a form of cell-cell interaction, and could conceivably contribute to cell-cell adhesion, something the soluble growth factor cannot do. Furthermore, since the plasma membrane can be compartmentalized, proTGFα can be selectively expressed on a limited portion of the membrane, thereby activating only those cells that contact that area. For instance, in polarized Madin-Darby canine kidney epithelial cells, TGFα is sorted to the basolateral membrane (Dempsey and Coffey, 1994). A similar process also occurs in the case of the *Drosophila* TGFα-relative Gurken, which localizes to the dorsal face of the oocyte and thereby determines dorsal-ventral patterning (Neuman-Silberberg and Schupbach, 1993).

The two forms of TGFα may also differ in their ability to induce ligand-mediated endocytosis of the EGFR. After occupation of the receptor with soluble ligand, such internalization is responsible for much of the downregulation of EGFR activity (Chen, et al., 1989). This process is more difficult to imagine in the case of the transmembrane ligand; internalization would involve engulfment of not only the ligand, but also a portion of the membrane of the cell expressing it. Surprisingly, such an event happens when the *Drosophila* transmembrane ligand Boss interacts with its receptor Sevenless (Cagan, et al., 1992), so it could also occur with proTGFα. Alternatively, proTGFα bound to its receptor could be proteolytically cleaved, allowing subsequent conventional internalization of this newly solubilized molecule. In either case, endocytosis would be more energetically expensive and would probably occur with slower kinetics than internalization of soluble TGFα, potentially leading to prolonged activation of the receptor.

The cytoplasmic domain

A major structural difference between transmembrane and soluble TGFα is the presence of the cytoplasmic domain in proTGFα. This 39 residue domain contains seven cysteines. Two of these cysteines are palmitoylated and may mediate the association of other proteins with the cytoplasmic domain (Shum, et al., 1996). The cytoplasmic domain is also highly conserved. Among rat, mouse, pig, and man, there are only two conservative substitutions in this region (Fig. 3).

While there are no known motifs, this structural conservation as well as mutational analysis indicate that sequences in this domain are functionally significant. For example, the two carboxy-terminal valines of the cytoplasmic domain are important for the post-translational processing of TGFα. In CHO cells, mutant forms of the growth factor that lack these valines are defective in several aspects of maturation. In contrast to wild-type TGFα, these mutants accumulate in an endo H-sensitive form (Briley, et al., 1997). Cells expressing these mutants also fail to release appreciable amounts of soluble TGFα (Bosenberg, et al., 1992). Two explanations for the latter observation have been advanced. First, the valines might be required only for the efficient proteolysis of transmembrane TGFα at the plasma membrane, with the truncated growth factor moving normally to the cell surface (Bosenberg, et al., 1992). This would not explain the accumulation of endo H-sensitive forms of the growth factor. Alternatively, the valines might be required for efficient transport of proTGFα to the cell surface; reduced amounts of growth factor at the plasma membrane would then lead to reduced secretion of TGFα (Briley, et al., 1997). Inefficient intracellular transport of the mutants would also explain the accumulation of endo H-sensitive forms, since the

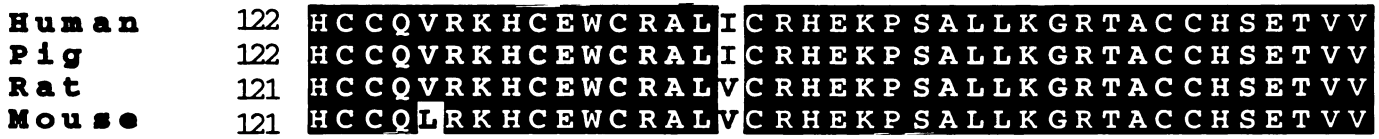

Figure 3. Conservation of the cytoplasmic domain of TGFα

Identical residues are in black boxes and conservative replacements are in gray boxes. Residue numbers are indicated to the left of the sequences.

conversion to endo H resistance requires transport from the endoplasmic reticulum to the Golgi apparatus.

To distinguish between these two possibilities, I compared the processing of wild-type TGFα and TGFα-Δ158, a mutant lacking the carboxy-terminal divaline, in 293 cells. I found defects in the precursor-processing, cell-surface transport, and secretion of the mutant growth factor. In contrast to wild-type transmembrane TGFα, where the 25 kD form is only a minor species, immunoprecipitation of transmembrane forms of the Δ158 mutant showed a striking accumulation of the precursor (Fig. 4a). To measure the accumulation of growth factor at the cell surface, I compared the amounts of full-length and mutant transmembrane TGFα immunoprecipitated from intact cells to the total amounts precipitated from lysed cells. The former is a measure of the amount of growth factor at the cell surface and the latter is a measure of the total amount of growth factor, so this ratio reflects cell surface accumulation. A smaller proportion of mutant transmembrane TGFα was found at the cell surface compared to wild-type transmembrane TGFα (Fig. 4b). This could be due to decreased transport to the cell surface or a decreased half-life of the molecules at the plasma membrane. Since secretion is the major mechanism by which cell-surface TGFα is removed, I compared the amounts of soluble mutant and wild-type growth factor that could be immunoprecipitated from the media of cells expressing equivalent amounts of transmembrane TGFα. Less soluble TGFα was immunoprecipitated from cells expressing the Δ158 mutant (Fig. 4c), so the decreased accumulation of transmembrane forms of the mutant growth factor at the cell surface compared to wild-type proTGFα is not due to increased secretion and instead is probably due to decreased transport to the plasma membrane.

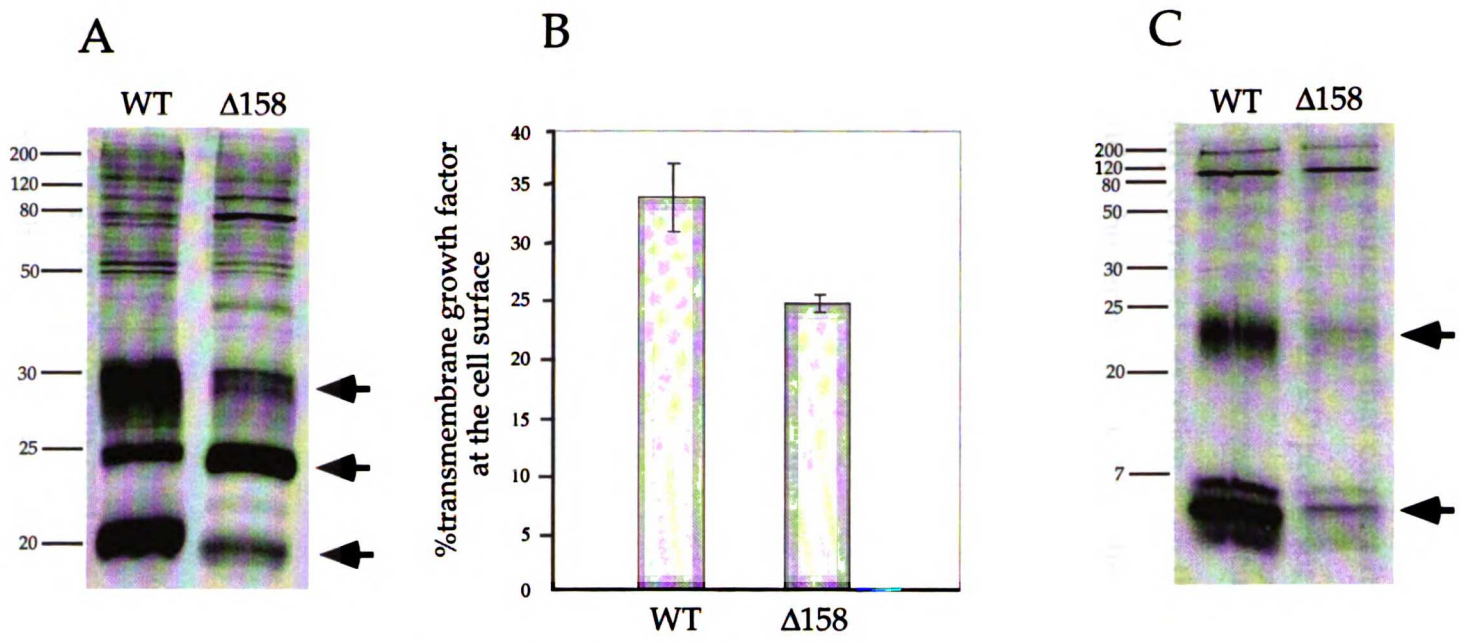

Figure 4. Comparison of wild-type TGFα and the Δ158 mutant

293 cells were transfected with expression plasmids for either wild-type TGFα or Δ158, [^{35}S]-labeled, and immunoprecipitated for total transmembrane growth factor (A), cell-surface and intracellular transmembrane TGFα (B), or soluble growth factor (C). Arrows in (A) and (C) indicate positions of TGFα.

A. Comparison of transmembrane forms of wild-type TGFα and the Δ158 mutant.

B. Percentages of immunoprecipitable transmembrane growth factors which were found at the plasma membrane. Results are from three independent transfections.

C. Comparison of amounts of soluble growth factor immunoprecipitated from the media of cells expressing similar amounts of transmembrane TGFα.

The simplest explanation for the mutant phenotype is a single defect in the transport of the molecule from the endoplasmic reticulum through the Golgi. This would account for the accumulation of the 25 kD precursor form because the modifications that convert it to the other forms occur in the Golgi. Retention of the 25 kD precursor in the endoplasmic reticulum or early Golgi would lead to decreased accumulation at the cell surface and consequently decreased secretion from the cell.

Besides the terminal divaline, the cytoplasmic domain also contains a leucine pair, a motif that can serve as an endocytosis signal for transmembrane molecules (Letourneur and Klausner, 1992). TGFα's dileucine motif may play a similar role. Treatment of cells with anti-TGFα antibody induces efficient endocytosis of wild-type cell-surface TGFα. In contrast, mutant molecules lacking the 31 carboxy-terminal residues, including the two leucines, fail to internalize under similar conditions (Shum, et al., 1994).

Cytoplasmic residues may also be important for the vectoral transport of proTGFα. As mentioned, when TGFα is expressed in polarized MDCK cells, the growth factor is efficiently sorted to the basolateral membrane. As with other polarized molecules, cytoplasmic determinants of proTGFα are probably important for this targeting (Dempsey and Coffey, 1994).

Associated proteins

Besides the dileucine and the carboxy-terminal divaline mentioned above, the cytoplasmic domain contains no known motif or biochemical activity. Since this region is comprised of only 39 residues, the proteins that bind to it may mediate many functions of this domain, including its proper processing and sorting. The seven cysteines in this domain may mediate

some of these protein-protein interactions. For example, CD8 and CD4, two other transmembrane proteins with short cysteine-containing cytoplasmic domains, interact with the kinase Lck via cysteine-cysteine interactions (Turner, et al., 1990). In addition, the cysteine-rich domain of Raf-1 has been shown to bind a number of proteins, including Ras, c-Jun, and 14-3-3 proteins (Clark, et al., 1997, Radziwill, et al., 1995, Warne, et al., 1993). While no associated proteins have yet been characterized, three proteins, a kinase, and proteins of MW 106 kD and 86 kD, can be specifically chemically crosslinked to and coimmunoprecipitated with transmembrane TGFα (Shum, et al., 1994). The presence of the two most distal cysteines in the cytoplasmic domain of TGFα are required for the interaction with p86 (Shum, et al., 1996).

The functions of these coimmunoprecipitating proteins are unknown and they could play a role in any process involving transmembrane TGFα. For instance, they could participate in the previously mentioned maturation of proTGFα. The defect in the processing of the Δ158 mutant could arise from its inability to bind to a protein that recognizes the carboxy-terminal valines of the wild-type molecule. Such a protein could act in the post-translational modification of TGFα or in the trafficking of the growth factor. Other associated proteins could link transmembrane TGFα to the cytoskeleton. They could potentiate proTGFα's stimulation of the EGFR, something that the integral membrane protein CD9 may do for transmembrane HB-EGF (Higashiyama, et al., 1995). When grown in the presence of HB-EGF-expressing cells that had been fixed and therefore could not secrete the growth factor, cells dependent on EGFR stimulation were stimulated to proliferate. Coexpression of CD9 in the HB-EGF expressing cells increased this stimulation. The kinase could be involved in the regulation of any of these events or, more speculatively, in propagating a signal into the growth-factor

expressing cell upon receptor binding. Just like the EGFR, proTGFα is a transmembrane molecule with an associated cytoplasmic kinase. Such bidirectional cell-cell communication occurs in the ephrins, an unrelated family of transmembrane ligands for receptor tyrosine kinases. Not only do ephrins activate the Eph class of receptor tyrosine kinases, but binding of receptor ectodomains to the transmembrane ephrins Elk-L and Htk-L induces tyrosine phosphorylation of their intracellular domains (Holland, et al., 1996).

Materials and Methods

Reagents

Unless mentioned otherwise, all chemicals were purchased from Sigma, St. Louis, MO. The α1 anti-TGFα monoclonal antibody was previously described (Bringman, et al., 1987). The Endoglycosidase H Deglycosylation kit, N-glycosidase F, and deoxymannojirimycin were purchased from Boehringer Mannheim (Indianapolis, IN). pRK7α, a mammalian expression plasmid for wild-type TGFα, and pRK7α-Δ158, an expression plasmid for the mutant growth-factor lacking the carboxy-terminal divaline, were described previously (Shum, et al., 1994, Shum, et al., 1996).

Cell Culture and Transfection

Human embryonic kidney 293 cells were grown in DMEM-H16, 3 g/l glucose (GIBCO BRL, Gaithersburg, MD) with 10% fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transiently transfected using Lipofectamine (GIBCO BRL) according to the manufacturer's instructions.

Metabolic labeling

Transfected cells were labeled overnight with 160 μCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN, Boston, MA) in cysteine- and methionine-free media with 10% dialyzed fetal calf serum. Labeling was performed 24 hours after cells were transfected.

Immunoprecipitations

For whole-cell immunoprecipitations, [^{35}S]-labeled cells were lysed in buffer containing 50 mM TrisCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin (Buffer F). The lysate was cleared by centrifugation for 30 minutes at 16,000*g* at 4°C, and subsequently incubated for one hour at 4°C with α1 mAb and protein A-sepharose (Pharmacia, Piscataway, NJ) preloaded with rabbit anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). Beads were washed three times with Buffer F, once with 0.5 M NaCl, 50 mM TrisCl, pH 7.5, and once with 50 mM TrisCl, pH 7.5. The beads were resuspended in reducing protein sample buffer, heated for five minutes at 100°C, and proteins were resolved with SDS-PAGE.

For cell surface immunoprecipitations, intact [^{35}S]-labeled cells were washed three times for five minutes each with ice-cold PBS containing 2% BSA. Cells were then incubated for one hour on ice with α1 mAB diluted in PBS containing 2% BSA. Excess antibody was removed by three ten-minute washes with ice-cold PBS containing 2% BSA. Cells were lysed as above and immune complexes were collected by incubation for one hour at 4°C with protein A-sepharose, preloaded with anti-mouse IgG rabbit antiserum. Samples were then processed in the same fashion as for whole cell immunoprecipitations.

For immunoprecipitation of soluble growth factor, media from [^{35}S]-labeled cells was collected, cleared by centrifugation, and then incubated for one hour at 4°C with α1mAB and protein A-sepharose preloaded with anti-mouse IgG rabbit antiserum. Immunoprecipitates were then washed and resolved as described above.

[^{35}S] Pulse-Chase labeling and analysis

Transfected 293 cells were grown to 80% confluence, incubated for 30 minutes in cysteine- and methionine-free media, pulsed for five minutes with 250 μCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN) in cysteine/methionine-free media with 10% dialyzed fetal calf serum, and then chased with unlabeled media for 0, 5, 10, 20, or 40 minutes. Cells were lysed in buffer F and transmembrane TGFα was then immunoprecipitated as described above.

Glycosylation analysis

[^{35}S]-cysteine-methionine labeled transmembrane-TGFα was immunoprecipitated from transfected 293 cells as described above, and then digested using the Endoglycosidase H Deglycosylation Kit (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer's instructions. For N-glycosidase F digestions, immunoprecipitated samples were denatured by heating for five minutes at 100°C in the presence of 1% SDS, and digestions were performed overnight at 37°C in 20 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 1% octylglucoside, 0.2% SDS with 2 units N-glycosidase F. Deoxymannojirimycin was added at 20 μg/ml to culture media during the metabolic labeling period.

Chapter 3: Proteins Associated with Transmembrane TGFα

Introduction

To better understand the function of transmembrane TGFα, it is important to study the proteins with which it interacts. The structural and functional characterization of associated polypeptides should provide insight not only into the physiology of transmembrane TGFα, but is also likely to shed light on fundamental cellular processes such as the intracellular trafficking of proteins. Thus, the results from these studies should be applicable not only to transmembrane TGFα, but also to the TGFα family as a whole and to other transmembrane proteins. Therefore, I used two methods, protein purification and yeast two-hybrid screening, to identify proteins that interact with transmembrane TGFα. The biochemical purification and characterization of proteins that associate with transmembrane TGFα are discussed in this chapter, Chapter 4, and Chapter 5. The results from a yeast two-hybrid screen to identify proteins that intact with the cytoplasmic domain of transmembrane TGFα are discussed in Chapter 6.

Since we previously identified proteins that coimmunoprecipitate with transmembrane TGFα (Shum, et al., 1994), I chose to use the coimmunopurification of these proteins with transmembrane TGFα as the basis for their preparative purification. Microsequencing of the purified material would then yield peptide sequence, which would serve as a basis to isolate cDNAs encoding the polypeptides. Proteins of MW 86 kD and 106 kD as well as a protein-kinase activity coimmunoprecipitate with transmembrane TGFα from Cα cells, a CHO derivative that stably overexpresses transmembrane TGFα (Shum, et al., 1994). Coimmunoprecipitation of these proteins with proTGFα is dependent on the

prior treatment of cells with the chemical-crosslinker dithiobissuccinimidyl-proprionate (DSP). DSP crosslinking can be reversed by treatment with reducing agents, allowing subsequent separation of the associated proteins from transmembrane TGFα.

Immunoprecipitation using free antibody is impractical for preparative purifications, since it would consume large amounts of antibody and since the immunoglobulins would contaminate the purified fractions of associated proteins. Therefore, I generated a column in which an anti-TGFα monoclonal antibody was covalently linked to a support resin. Such a resin can be reused in several purifications and also will not contaminate purified proteins with antibody chains. Using this resin in analytical immunoaffinity purifications experiments, p86 and p106 were shown to copurifiy with transmembrane TGFα. Furthermore, I identified three additional proteins of MW 59 kD, 40 kD, and 35 kD that also copurify with proTGFα. In control experiments using cell lysates of CHO cells that do not express transmembrane TGFα, these proteins were not retained on the anti-TGFα antibody column.

Materials and Methods

Chemicals

Unless mentioned otherwise, all chemicals were purchased from Sigma, St. Louis, MO.

Cell culture

CHO and Cα cells, a CHO derivative that stably overexpresses TGFα, were grown in F-12 Ham's Nutrient Mix (GIBCO BRL, Gaithersburg, MD) with 10%

fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. The media for Cα cells was supplemented with Geneticin (GIBCO BRL) at 400 μg/ml.

Antibodies

The monoclonal antibody α1 mAB was previously described (Bringman, et al., 1987). The TAB1 monoclonal antibody was a gift from Berlex Corporation of Richmond, CA. Rabbit anti-mouse immunoglobulin was from Jackson ImmunoResearch Labs (West Grove, PA). The anti-myc monoclonal antibody 9E10 was a gift of Dr. J.M. Bishop (University of California, San Francisco).

Immunoaffinity resins

The α1 mAb, the TAB mAb, and the anti-myc mAb 9E10 were coupled to protein A-Sepharose as previously described (Harlow and Lane, 1988). Briefly, antibodies were bound to protein A-Sepharose beads by incubation in 3 M NaCl, 50 mM sodium borate, pH 9.0, for one hour at room temperature. The beads were then washed twice with ten volumes of 3 M NaCl, 50 mM sodium borate, pH 9.0 to remove unbound antibody. Antibodies were then covalently coupled to the beads by incubation at room temperature for 30 minutes with a 20 mM solution of the chemical-crosslinker dimethylpimelimidate in 3 M NaCl, 0.1 M sodium borate, pH 9.0. After removal of the crosslinking solution, the reactions were quenched by incubating the beads for two hours at room temperature in 0.2 M ethanolamine, pH 8.0. The beads were then washed with and resuspended in PBS.

Metabolic labeling

Cells were grown to 80% confluence and labeled overnight with 160 μCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN, Boston, MA) in cysteine/methionine-free media with 10% dialyzed fetal calf serum.

Crosslinking

Cells were washed twice with cold PBS, and then incubated at 4°C for 30 minutes with 2 mM dithiobissuccinimidyl-proprionate (DSP, Pierce, Rockford, IL) in PBS. Cells were subsequently washed twice with cold calcium/magnesium-free PBS with 0.04% (w/v) EDTA before further processing.

Immunoprecipitations

[^{35}S]-labeled cells were lysed in buffer containing 50 mM TrisCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin (Buffer F). The lysate was cleared by centrifugation at 16000*g* for 30 minutes at 4°C and subsequently incubated for one hour at 4°C with α1 mAb and protein A-Sepharose (Pharmacia, Piscataway, NJ) preloaded with rabbit anti-mouse IgG (Jackson ImmunoResearch Labs). Beads were washed three times with Buffer F, once with 0.5 M NaCl, 50 mM TrisCl, pH 7.5, and once with 50 mM TrisCl, pH 7.5. The beads were resuspended in reducing protein sample buffer, heated for 5 minutes at 100°C, and proteins were resolved with SDS-PAGE.

Immunoaffinity purification

Cα and CHO cells were [^{35}S]-labeled, crosslinked with DSP, lysed with Buffer F, and clarified by centrifugation as described above. Cleared lysates were

incubated with immunoaffinity resin for one hour at 4°C. Samples to be visualized at this point were washed three times with Buffer F, once with 500 mM NaCl, 50 mM TrisCl, pH 7.5, and once with 50 mM TrisCl, pH 7.5. For samples to be purified further, the resin was packed into a column, washed with ten column-volumes of buffer F, five volumes of 500 mM NaCl, 50 mM TrisCl, pH 7.5, and five volumes of 50 mM TrisCl, pH 7.5. TGFα complexes were eluted with 100 mM glycine, pH 2.5 and the pH was then neutralized with 1 M TrisCl, pH 10. Crosslinking was reversed by treatment with 100 mM DTT for 30 minutes at 37°C. The sample was then desalted and concentrated using a 30 kD molecular-weight cut off Microcon concentrator (Amicon, Beverly, MA).

Chromatography

Q Sepharose, an anion-exchange resin, S Sephasore, a cation-exchange resin, Blue Sepharose High Performance, an agarose resin coupled to the dye Cibacron Blue 3G-A, Butyl Sepharose, a hydrophobic interaction resin, and Heparin Sepharose were purchased from Pharmacia. Resins were equilibrated in Buffer F and then incubated for one hour at 4°C with immunoaffinity-purified transmembrane-TGFα complexes that were prepared as described above. Following centrifugation of the beads, the supernatants were removed and then desalted and concentrated using a Microcon concentrator. The supernatants contained proteins that did not bind to the resins; these were designated as the flow-through fractions. After three washes with Buffer F, the proteins that bound to the resins were eluted. Polypeptides that bound to Butyl Sepharose were eluted with 0.1% Triton X-100 in 50 mM TrisCl, pH 7.5. Proteins were eluted from all other resins with 1 M NaCl, 50 mM TrisCl, pH 7.5, supplemented in the case of Blue Sepharose

with 10 mM ATP. The eluted material was then desalted and concentrated with a Microcon concentrator. Flow-through and bound fractions were resolved by SDS-PAGE and visualized with autoradiography.

Two-dimensional electrophoresis (Isoelectric focusing vs SDS-PAGE)

Material eluted from the α1mAb immunoaffinity resin was equilibrated with isoelectric-focusing sample-buffer consisting of 9 M urea, 4% Nonidet P-40, 2% β-mercaptoethanol, and 2% pH 3-10 ampholytes (Bio-Rad, Hercules, CA) at 37°C for 30 minutes. The sample was then loaded onto a tube gel and isoelectric-focused to equilibrium in a Bio-Rad Mini-PROTEAN II 2-D cell for five hours at 500 V. Tubes were extruded, equilibrated in Laemli sample buffer, loaded onto a 1.5 mm Laemli slab gel, and proteins were electrophoretically separated. Samples were visualized by autoradiography and silver staining.

Results and Discussion

Immunoaffinity detection of TGFα-associated proteins

Previously, we identified three proteins, p106, p86, and a kinase activity, that associate with transmembrane TGFα (Shum, et al., 1994). In these experiments, the proteins were reversibly crosslinked to transmembrane TGFα, coimmunoprecipitated using an anti-TGFα antibody, and then resolved by reducing SDS-PAGE. Therefore, the region around 50 kD in the gels was obscured by the presence of the heavy chains of the antibodies used in the immunoprecipitations. The heavy chains could also contaminate samples of the associated proteins destined for peptide

microsequencing. To examine this region and to make subsequent large-scale purifications easier, I prepared an immunoaffinity resin in which the α1 anti-TGFα monoclonal antibody was covalently linked to protein A-Sepharose beads.

Like the free anti-TGFα antibody, the anti-TGFα antibody bound to Sepharose allowed immunopurification of transmembrane TGFα from Cα cells (Fig. 5b). Both immunoaffinity purification (using antibody covalently coupled to beads) and immunoprecipitation (using free antibody combined with protein A-beads) resulted in the isolation of three predominant proteins of 30 kD, 25 kD, and 20 kD from Cα cells but not from parental CHO cells. These are the expected sizes of the three forms of transmembrane TGFα. In addition, p86 and p106 also copurified with transmembrane TGFα when the antibody resin was used to purify growth factor from DSP-treated Cα cells, but not from CHO cells (Fig. 5a). Consistent with prior results and with the low efficiency of chemical crosslinking, only small amounts of the associated proteins coprecipitated with TGFα.

Besides p106 and p86, three additional proteins of MW 59 kD, 40 kD, and 35 kD also coimmunopurified with transmembrane TGFα from DSP-treated Cα cells (Fig. 5). p40 and p35 were detected in both immunoprecipitations and immunoaffinity purifications of proTGFα. p59 was only clearly detected when the antibody resin was used, presumably because it was obscured in the gel by immunoglobulin heavy chain when free antibody was used (Fig 5). These proteins were not precipitated when immunopurifications were performed on lysates from parental CHO cells that do not express transmembrane TGFα (Fig. 5a). While trace amounts of the associated proteins coprecipitated with transmembrane TGFα in the

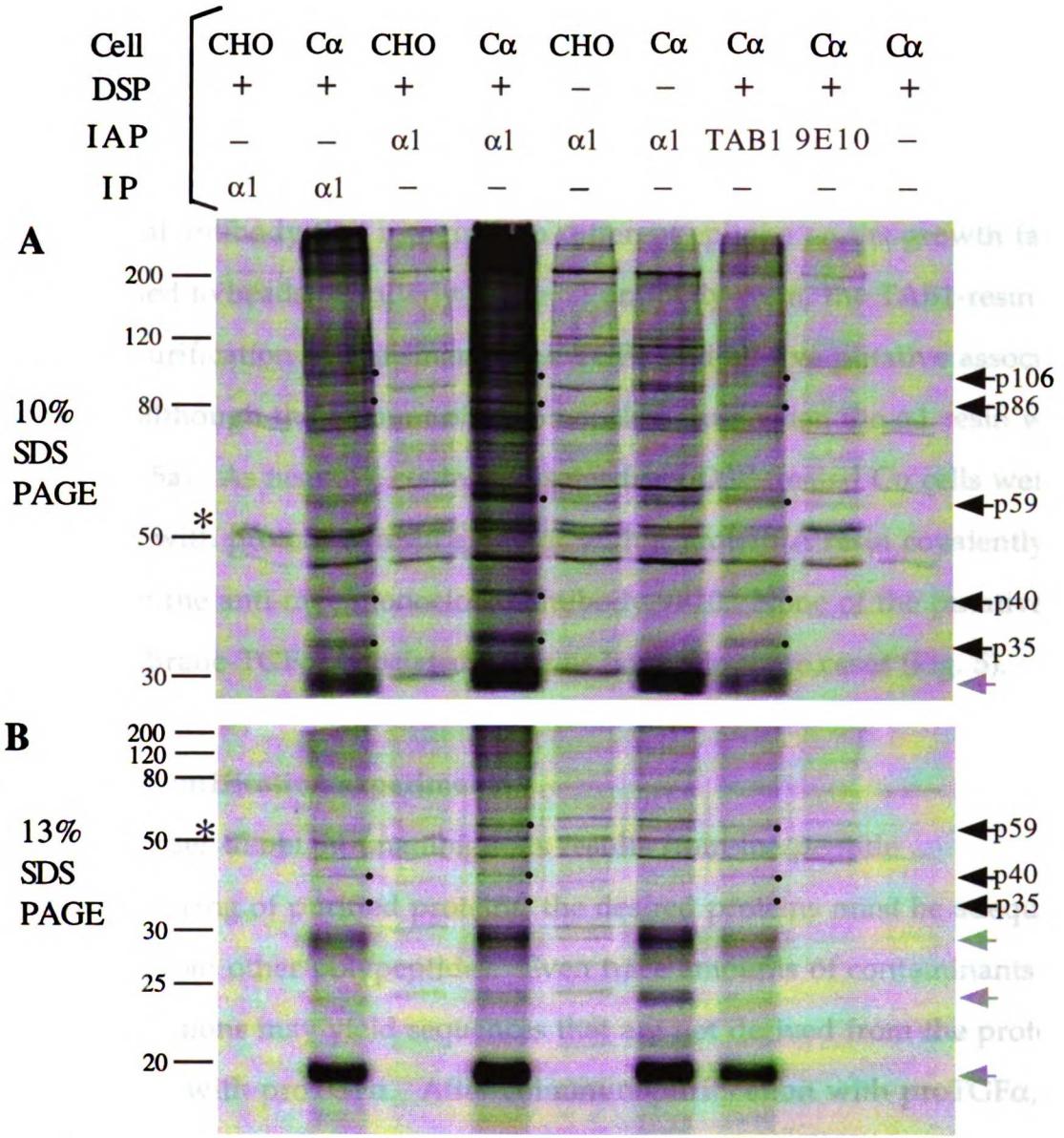

Figure 5. Coimmunopurification of proteins with transmembrane TGFα

The indicated cells were [^{35}S]-labeled, treated with DSP as indicated, and lysed. Samples were then either immunoprecipitated (IP) or immunoaffinity purified (IAP) using the indicated antibodies. To inspect different molecular weight ranges, samples were resolved on both 10% (A) and 13% (B) polyacrylamide gels. Gray arrows show transmembrane TGFα, and asterisks show positions of IgG heavy chain. The positions of associated proteins are indicated to the right of the panels with black arrows and next to the lanes with dots. α1 and TAB1 are anti-TGFα monoclonal antibodies and the 9E10 monoclonal antibody binds a myc-epitope. The samples in the far right lane were incubated with protein A beads alone.

absence of crosslinking, DSP-treatment of cells greatly increased these quantities (Fig. 5a).

To confirm that the proteins interacted with TGFα and were not directly recognized by the α1 monoclonal antibody, TAB1, another anti-TGFα monoclonal antibody that recognizes a different epitope on the growth factor, was coupled to beads. Similarly to the α1 antibody-resin, the TAB1-resin allowed purification of transmembrane TGFα and all five putative associated proteins, although the amount of p106 was less than when the α1 resin was used (Fig. 5a). As negative controls, lysates from DSP-treated Cα cells were incubated with protein A resin alone or with a protein A resin covalently coupled to the anti-myc monoclonal antibody 9E10. None of the putative transmembrane-TGFα-associated proteins bound in these cases (Fig. 5).

Analytical purification experiments

In order to obtain unambiguous results from the peptide microsequencing of purified proteins, the desired proteins must be adequately separated from other polypeptides. Even trace amounts of contaminants in purified fractions may yield sequences that are not derived from the proteins that interact with proTGFα. After coimmunopurification with proTGFα, p86, p40, and p35 migrated on SDS-PAGE as discrete bands that were well separated from other proteins. Therefore, anti-TGFα immunoaffinity chromatography followed by SDS-PAGE may adequately purify these three proteins. In contrast, p106 and p59 were not sufficiently purified by these two steps; they migrated close to or comigrated with background bands on SDS-PAGE. I therefore evaluated potential additional purification steps for the associated proteins. Since p106, p86, and p59 invariably coprecipitated with

proTGFα while p40 and p35 were not seen in all cases, I focused on the purification of the three larger proteins.

Using immunopurified, de-crosslinked proTGFα-complexes as starting material, I tested several chromatographic resins for their ability to further purify p106, p86, and p59. These resins were Q Sepharose (anion exchange), S Sepharose (cation exchange), butyl Sepharose (hydrophobic interaction), heparin Sepharose, and Cibacron blue Sepharose (Fig. 6). In each case, the proteins were incubated with the resin, unbound material was removed, and then a single stringent wash condition was used to elute the proteins that associated with the resin. Unbound material was designated as the flow-through fraction ("FT" lanes in Fig. 6), while the eluted material was designated as the bound fraction ("B" lanes in Fig 6). With the exception of Cibacron blue, all proteins behaved similarly with respect to a given resin. For instance, all proteins bound Q Sepharose, and consistent with this, did not bind S Sepharose. The resins that retained TGFα-associated proteins could be useful in further purification steps, since elution using a salt gradient might separate individual polypeptides.

In contrast to the other matrices, Cibacron blue Sepharose acted selectively. While most proteins, including contaminants, bound to the resin or partitioned between the resin and supernatant, p59 flowed through. Therefore, anti-TGFα immunochromatography, followed by Cibacron blue chromatography and SDS-PAGE should yield purified p59. Unfortunately, p106 and p86 were only poorly eluted from the resin and also poorly separated from contaminants.

Finally, two-dimensional isoelectric focusing vs. SDS-PAGE of immunopurified material was also tested for its ability to further purify the associated proteins (Fig. 7a). p106 and p59 were satisfactorily resolved by this

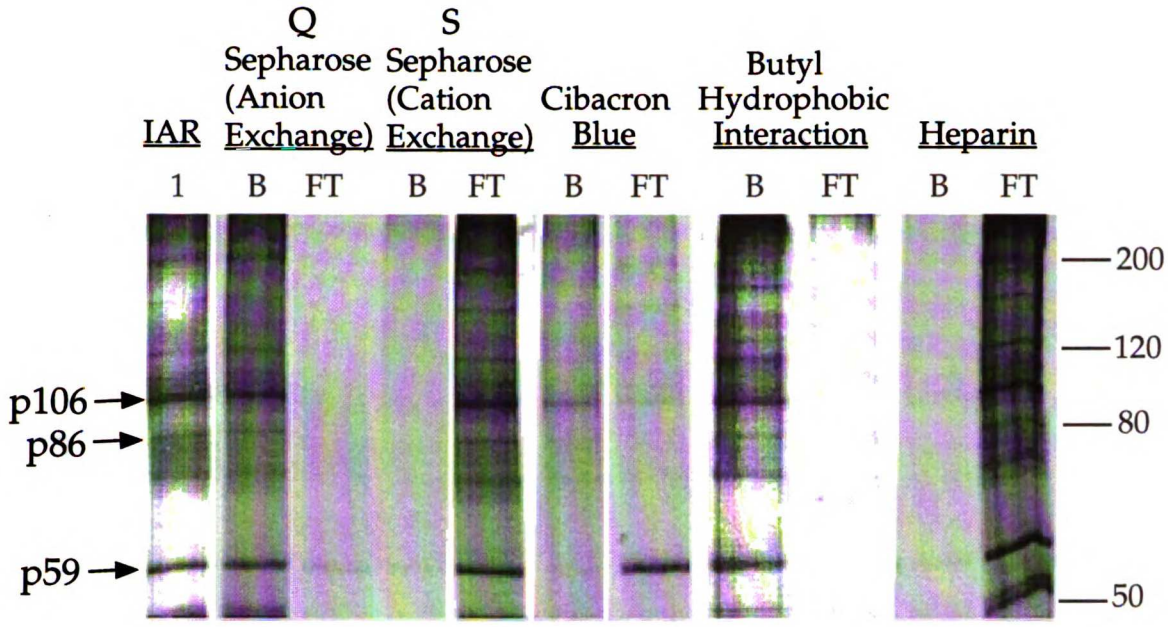

Figure 6. Chromatographic purification of transmembrane-TGFα-associated proteins

Material purified from [^{35}S]-labeled, DSP-treated Cα cells with the anti-TGFα immunoaffinity resin (IAR, lane 1) was treated with reducing agent to reverse crosslinking and subjected to further chromatographic steps as indicated. Lanes marked B are samples which bound the resin, while those marked FT are samples which did not bind and instead flowed through. The positions of p106, p86, and p59 are indicated with arrows.

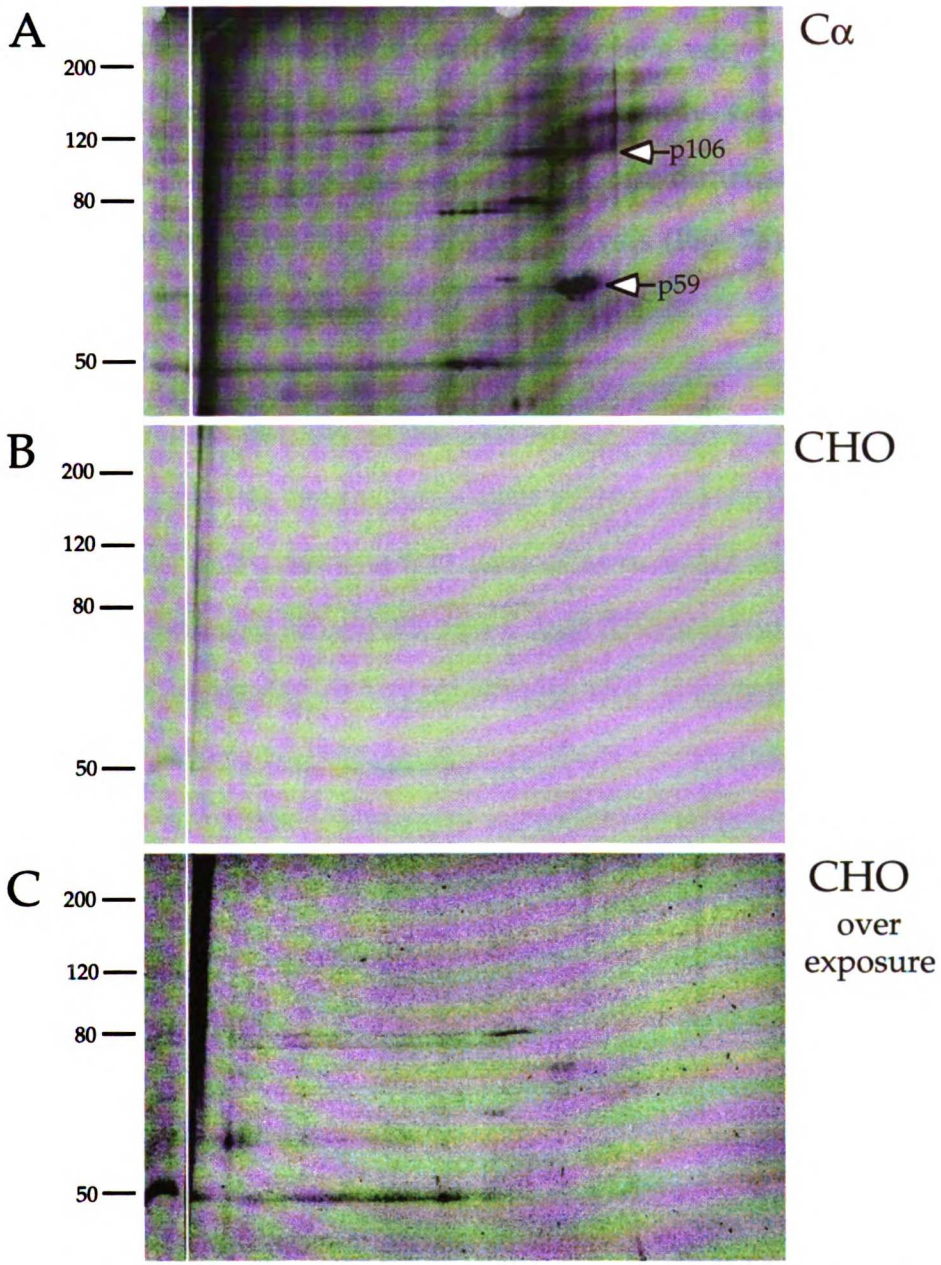

Figure 7. Two-dimensional electrophoresis of TGFα-associated proteins

Lysates from equal numbers of [^{35}S]-labeled, DSP-treated Cα (A) and CHO (B) cells, were immunopurified for TGFα, reduced to reverse crosslinking, and then resolved with two-dimensional isoelectric focusing (horizontal dimension) vs SDS-PAGE (vertical dimension). The positions of associated proteins were determined based on their mobilities and their absence from the CHO samples. Arrows indicate p106 and p59; p86 was not resolved by this technique. A portion of each sample was resolved by SDS-PAGE alone (left most lanes). Panel (C) is an overexposure of panel (B). Transmembrane TGFα is not shown.

technique, but the yield of p106 was low and p86 was not resolved. p86 may be insoluble under the conditions used, or may be a very basic protein that cannot enter the isoelectric-focusing gel. Interestingly, several additional Cα-specific proteins, which were not apparent on one-dimensional SDS-PAGE, were resolved by two-dimensional electrophoresis (Fig. 7a).

Chapter 4: Purification and Sequence Characterization of Transmembrane-TGFα-associated p59 and p86

Introduction

Using the protocols described in the previous chapter, I performed three preparative purifications of the proteins that coimmunopurify with transmembrane TGFα. This allowed the isolation of sufficient amounts of p86 and p59 to obtain peptide sequences through microsequencing. This information was used to obtain full-length cDNAs for the two proteins. Lower amounts of p106, p49 and p35 were obtained and their structural characterization remains to be performed. p59 is related to a Golgi protein, while p86 shows similarity to a yeast protein that is involved in the intracellular trafficking of proteins.

Materials and Methods

Chemicals

Unless mentioned otherwise, all chemicals were purchased from Sigma, St. Louis, MO.

Cell culture and transfection

CHO and Cα cells were grown in F12 Ham's Nutrient Mix (GIBCO BRL, Gaithersburg, MD), and COS-1 and 293 cells were grown in DMEM-H16, 3 g/l glucose (GIBCO BRL), in all cases supplemented with 10% fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cα cells were selected with 400 µg/ml Geneticin (G418; GIBCO BRL). To adapt adherent Cα cells to suspension culture, they were trypsinized and seeded at a

density of $2X10^5$ cells/ml in spinner flasks containing D-MEM/F-12 media (GIBCO BRL) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 400 µg/ml Geneticin. Following a month-long adaptation period, cells grew well and typically doubled every two to three days. Cells in long-term culture were selected with 400 µg/ml G418; those to be harvested were not grown under selection. COS-1 and 293 cells were transiently transfected using Lipofectamine (GIBCO BRL) according to the manufacturer's instructions

Analytical immunoaffinity purification and immunoprecipitation

Adherent cells grown to 80% confluence were labeled overnight with 160 µCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN, Boston, MA) in cysteine/methionine-free media with 10% dialyzed fetal calf serum. Suspension cells were labeled with the same media at a density of $1X10^6$ cells/ml. After 3 rinses with PBS, cells were crosslinked with 2 mM DSP in PBS for one hour at 4°C. Cells were washed three times with calcium/magnesium free PBS with 0.04% EDTA, and then lysed with buffer F. Lysates were incubated with α1mAb immunoaffinity resin for one hour at 4°C with shaking. Beads were washed three times with buffer F, once with 0.5 M NaCl, 50 mM TrisCl, pH 7.5, and once with 50 mM TrisCl, pH 7.5. Reducing protein sample buffer was added, the beads were boiled for five minutes, and proteins were resolved with SDS-PAGE and visualized with autoradiography. [^{35}S]-labeled COS-1 and 293 cells transfected with expression plasmids for epitope-tagged p59 proteins were immunopurified for myc using the 9E10 monoclonal antibody (a gift of Dr. J. M. Bishop, University of California, San Francisco) and for FLAG using the M2 anti-FLAG

immunoaffinity resin (Kodak Eastman, New Haven, CT). Precipitations were performed in the same manner as for the α1 monoclonal antibody.

Large-scale immunoaffinity purification

Adherent and suspension Cα cells were grown to confluence or a density of $1X10^6$ cells/ml respectively, crosslinked with DSP, lysed with Buffer F, and clarified by centrifugation. Lysates were then incubated with α1mAb immunoaffinity resin for 12 hours at 4°C with agitation. Slurries were packed into a column and washed with 10 column volumes of buffer F, five column volumes of 0.5 M NaCl, 50 mM TrisCl, pH 7.5, and five column volumes of 50 mM TrisCl, pH 7.5. The proteins that remained bound to the column were eluted with 100 mM glycine, pH 2.5, 0.1% Triton X-100. One milliliter fractions of this elution were collected and neutralized with 1 M TrisCl, pH 10. Fractions that contained protein were determined by anti-TGFα Western blotting or India ink staining, and these were pooled, treated with DTT to reverse crosslinking, concentrated, and desalted by NAP-50 columns (Pharmacia, Piscataway, NJ) and lyophilization. The sample from the suspension cells was resolved on SDS-PAGE and proteins were visualized with silver staining and Coomassie Brilliant Blue staining.

Cibacron Blue Chromatography

Immunoaffinity purified material was incubated with Blue Sepharose High Performance resin for one hour at 4°C. The resin was subsequently poured into a column and the flow-through was collected. This material was dialyzed against 0.01% Triton X-100, 10 mM TrisCl, pH 7.5, concentrated by lyophilization, and resolved using SDS-PAGE. Samples were

electrotransferred to PVDF, and proteins were visualized with amido-black staining. The band corresponding to p59 was excised and processed for peptide microsequencing.

Preparative Two-dimensional electrophoresis (Isoelectric focusing vs SDS-PAGE)

Material eluted from the α1mAb immunoaffinity column was equilibrated with isoelectric focusing sample buffer consisting of 9 M urea, 4% Nonidet P-40, 2% β-mercaptoethanol, and 2% pH 3-10 ampholytes (Bio-Rad, Hercules, CA) at 37°C for 30 minutes. The sample was then loaded onto a tube gel and equilibrium isoelectric focused for eight hours at 500 V on a Bio-Rad Mini-PROTEAN II 2-D cell. Tubes were extruded, equilibrated in Laemli sample buffer, loaded onto a 1.5 mm Laemli slab gel, and proteins were electrophoretically separated. Samples were visualized by silver staining or copper staining. In the latter case, the spot corresponding to p59 was excised and processed for peptide microsequencing.

Peptide microsequencing

Peptide microsequencing was performed by the Harvard University Microchemistry Facility (Cambridge, MA) under the direction of Dr. William Lane. The protein samples, either within a polyacrylamide gel slice or transferred to PVDF, were digested *in situ* with trypsin. Tryptic peptides were separated by microcapillary reverse-phase HPLC, and sequenced by either Edman degradation or tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

cDNA cloning of p59

Hbc 551 (Genbank accession T10844), a cDNA that codes for most of p59, was a gift of Dr. G. Bell of the University of Chicago. Sequence coding for a myc epitope-tag was added in-frame to this cDNA by ligating a *Xho* I/*Eco* RI fragment of hbc 551 into the corresponding sites of the mammalian expression vector pRK5myc (Feng, et al., 1995). The 5' sequence missing from hbc 551 was obtained with two rounds of nested PCR from a human placental cDNA library. In the initial round, the lambda gt10 reverse primer (5' GGT GGC TTA TGA GTA TTT CTT CC 3'), corresponding to vector sequence, was used along with an anti-sense p59 primer (rev1: 5' TGG ATT CAT TGG TGG CAC AGA AGT 3') in a PCR. The products of this reaction were then used as template for a second PCR, using the lambda primer and a second anti-sense primer closer to the 5' end of the coding stand (rev2: 5' CGA CAG TTA TCA GTG TCT GTG TTG 3'). This second reaction gave a product that gave an incomplete 5' extension of p59. Based on the extra sequence obtained, an additional anti-sense primer closer to the 5' end than rev2 was designed (rev3: 5' CCC CAC AGG TTA CTT GG 3'). A second nested PCR was performed using the lambda primer and sequentially, rev2 and rev3. The product of these reactions, which coded for an additional 142 residues, was ligated in frame to the original p59 cDNA. PCR was used to introduce a *Sal* I site at the 3' end of the coding region of this extended cDNA, and a *Sal* I/*Eco* RI fragment from this new construct was ligated into the corresponding sites of the expression vector pRK5FLAG (Feng, et al., 1995) to generate pRK5-p59F.

Results and Discussion

Purification of p59

Since p59 can be purified with anti-TGFα immunoaffinity chromatography followed by either Cibacron-blue chromatography or two-dimensional isoelectric focusing vs SDS-PAGE, each method was used in a separate preparative purification. For the first purification, TGFα complexes from 8.8 square meters of DSP-treated adherent Cα cells (2.5 $X10^9$ cells) were purified on an anti-TGFα antibody column. Following reversal of crosslinking, the sample was further purified on a Cibacron-blue column, and finally resolved using SDS-PAGE (Fig. 8a,b). Consistent with the analytical results, p59, but not p106 or p86, was adequately preparatively purified. Proteins were transferred to PVDF, stained with amido black, and the p59 band was excised and processed for peptide microsequencing. Sequences from four peptides were obtained (Fig. 9).

To confirm that the peptides corresponded to p59, an additional purification utilizing two-dimensional isoelectric focusing vs SDS-PAGE was undertaken. Fifteen square meters of Cα cells (4.3 $X10^9$ cells) were grown, crosslinked and harvested, and TGFα-containing complexes were again purifed on an anti-TGFα antibody column. One percent of the material from this column was then resolved on an analytical two-dimensional gel and proteins were visualized with silver staining (Fig. 8c). Since both p106 and p59 were adequately resolved, the remainder of the sample was then resolved on a preparative two-dimensional gel. Proteins were visualized with copper staining and the spots corresponding to p59 and p106 were excised, washed with water and processed for peptide microsequencing. Sequences from two p59 peptides were obtained, one of which corresponded to sequence obtained

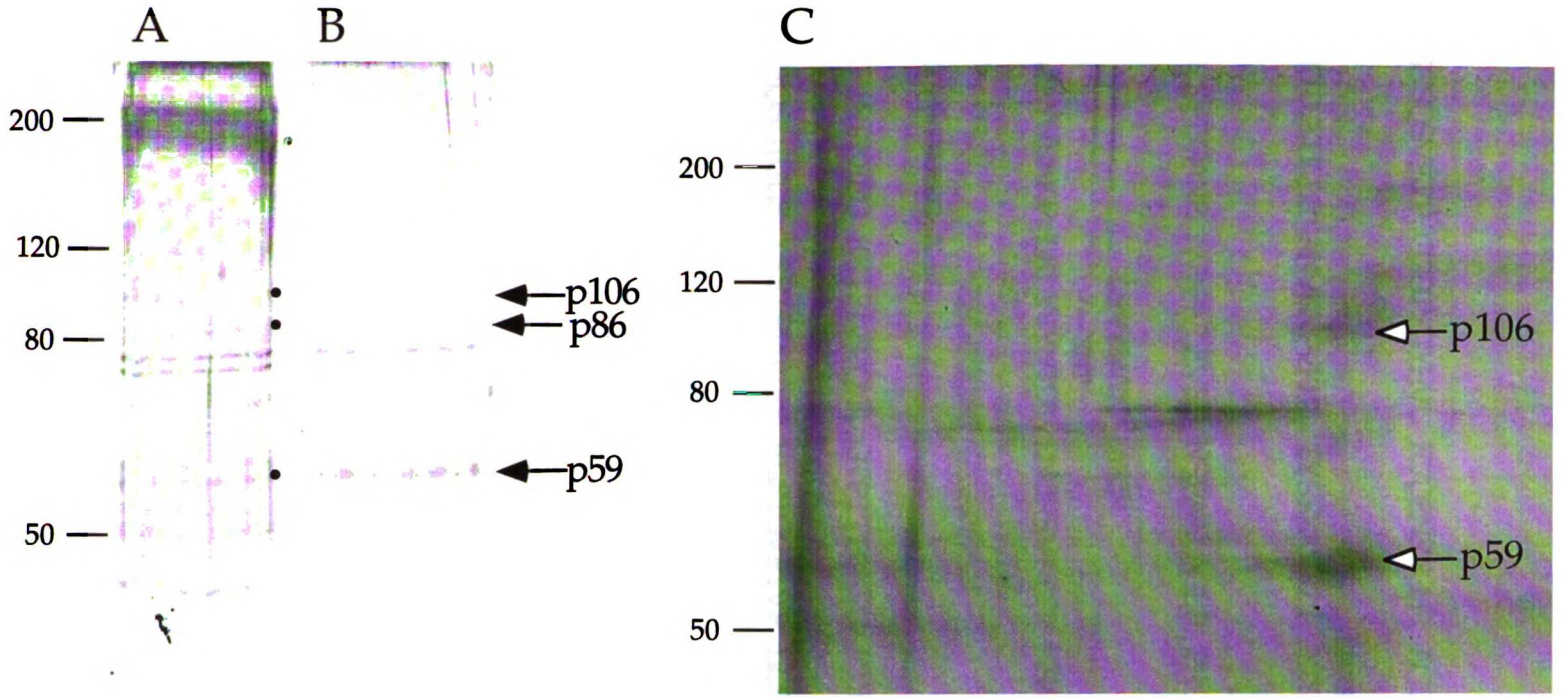

Figure 8. Preparative Purification of p59

Transmembrane-TGFα complexes from DSP-treated Cα cells were purified on an anti-TGFα immunoaffinity column (panel A), reduced, and then further purified using either Cibacron Blue Chromatography (panel B) or two-dimensional electrophoresis (panel C). Panels A and B were from the same purification, while panel C was from a separate preparation. Silver-stained gels of 1% of the material from each case are shown. Positions of associated proteins are indicated with arrows and dots. Transmembrane TGFα is not shown.

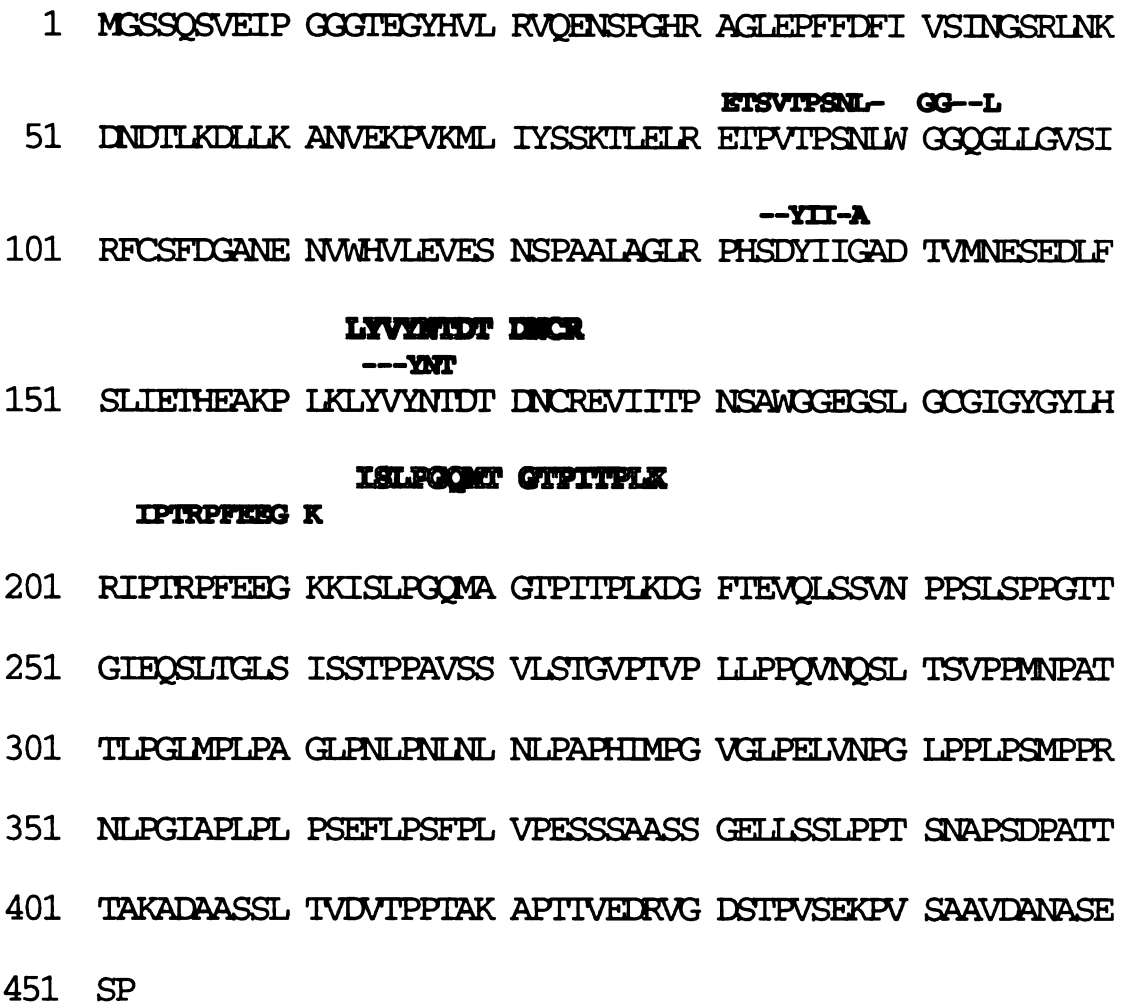

Figure 9. Sequence of Human p59

Peptide sequences from p59 purified from Cα cells are aligned above the protein sequence which was derived from the cDNA sequence. Peptides derived from anti-TGFα immunoaffinity chromatography followed by Cibacron Blue chromatography (first purification) are in bold, while those purified by immunoaffinity chromatography and two-dimensional electrophoresis (second purification) are outlined.

from the previous purification (Fig. 9). Inadequate amounts of p106 were present for sequencing.

cDNA cloning of p59

The peptide sequences were searched against the expressed sequence tag (EST) database and matched several overlapping human ESTs. The largest cDNA in this group was obtained and sequenced, but coded for a protein product with a predicted MW of only 35 kD. A protein's molecular mass determined from SDS-PAGE may not match that predicted from its sequence. Therefore, a myc-epitope tagged version of the protein encoded by this cDNA was expressed in and immunoprecipitated from COS-1 cells. The immunoprecipitated protein was 10 kD smaller than native p59 and therefore was not derived from a full-length coding sequence (Fig. 10a). This sequence had a stop codon and a poly-adenine tract in its 3' untranslated region, and therefore probably lacked upstream coding-sequence. To obtain this missing sequence, nested polymerase chain reaction (PCR) was performed using a human placental library as template and primers based on p59 and vector sequences as anchors. A PCR product was obtained and sequenced.

The PCR-generated DNA sequence included an ATG that could be the initiator codon, since it is in a strong Kozak consensus-sequence for translational initiation (Kozak, 1987). However, no in-frame stop-codon was found upstream of this ATG. This sequence, which coded for 142 residues, was ligated in-frame with the cDNA obtained previously, resulting in a sequence that coded for all of the peptide sequences obtained by microsequencing (Fig. 9). 293 cells were transfected with an expression plasmid for p59F, a FLAG-epitope tagged version of the protein encoded by this combined sequence, and immunoprecipitated for FLAG, yielding a

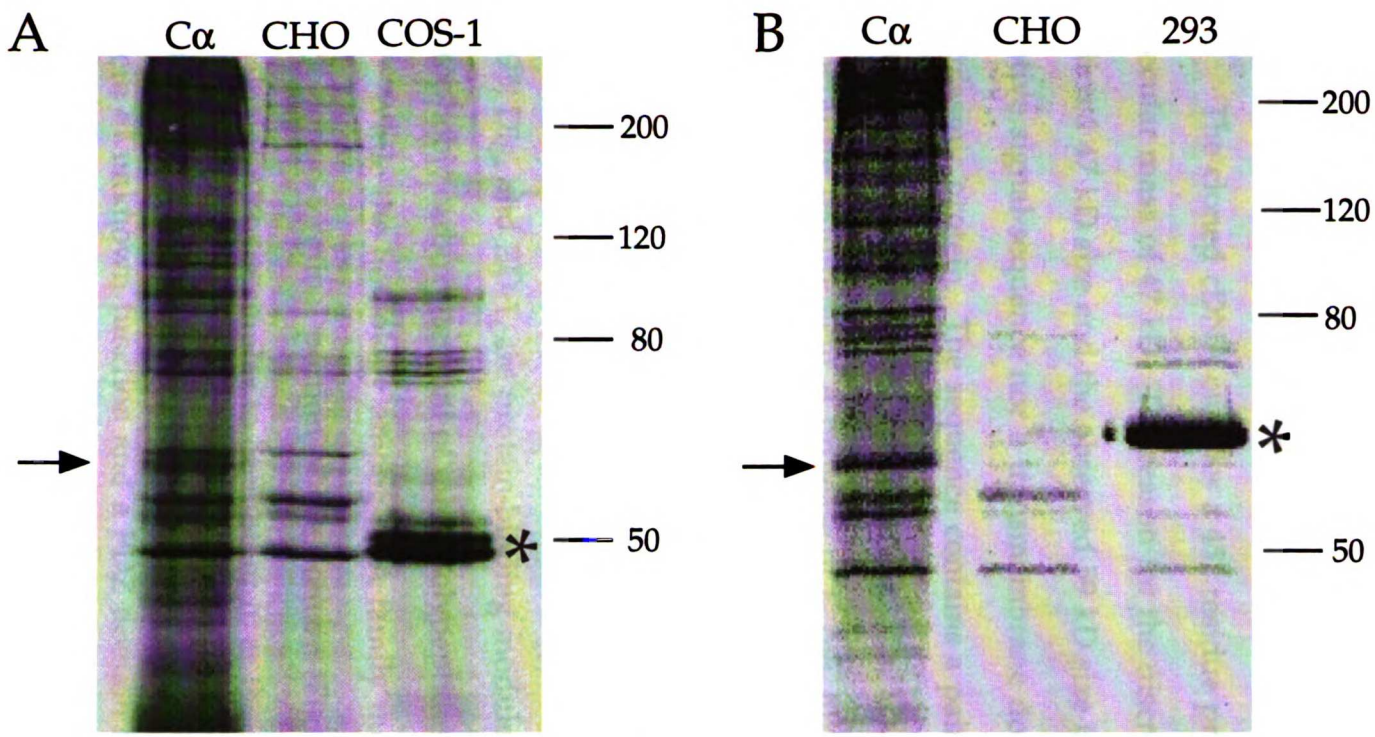

Figure 10. Comparison of recombinant, epitope-tagged p59 and endogenous p59

A. Anti-myc immunoprecipitation from COS-1 cells transfected with an expression plasmid for the myc-tagged version of the partial p59 which was first isolated (lane 3).
B. Anti-FLAG immunoprecipitation from 293 cells transfected with an expression plasmid for p59F, the FLAG-tagged full-length p59 (lane 3).
Both panels show anti-TGFα immunoaffinity precipitates from crosslinked Cα and CHO cells in the first two lanes (transmembrane TGFα is not shown). Arrows indicate endogenous p59, asterisks indicate recombinant p59. Endogenous p59 was identified based on its mobility, its position relative to other bands in the pattern, and its absence from precipitates from CHO cells. Samples are from [^{35}S]-labeled cells.

protein that migrated on SDS-PAGE at a position consistent with the size of endogenous p59 plus the extra two kD contributed by the epitope tag (Fig. 10b). The cDNA was therefore judged to be full-length.

p59 is a conserved protein that contains two htrA-like PDZ domains

The cloned cDNA encodes a 452 residue protein with a predicted molecular mass of 47 kD. The reason for the discrepancy between the predicted 47 kD size and the observed 59 kD size is unknown. It has neither a putative signal-sequence nor a transmembrane domain and thus is likely a cytoplasmic protein. The sequence contains a pair of htrA-like domains within its first two hundred residues (Fig. 11a, 12). Named after the high temperature requirement A protease of *E. coli*, htrA repeats are found predominantly in bacterial proteins. Just as in p59, many of these prokaryotic polypeptides have two tandemly repeated htrA domains. Sequence analysis and secondary structure prediction indicate that htrA repeats are a subclass of PDZ domain (Ponting, 1997). PDZ domains, named after the family members PSD95, Dlg, and ZO-1, are protein-binding modules that often bind to the hydrophobic carboxy-termini of other polypeptides (Songyang, et al., 1997).

While the homology among htrA family members as a whole lies only in the amino-terminal portion of the domain (Fig. 11a), the two htrA domains of p59 are similar to each other over their entire lengths (Fig. 11b). They are also closely related to a PDZ domain found in a budding-yeast protein of unknown function and a PDZ domain found in a fission-yeast protein of unknown function (Fig. 11b).

p59 also shares similarity with GRASP65 (Fig. 12), a rat Golgi peripheral-membrane protein that acts in the stacking of Golgi cisternae (Barr, et al., 1997). The amino-termini of these two proteins are closely

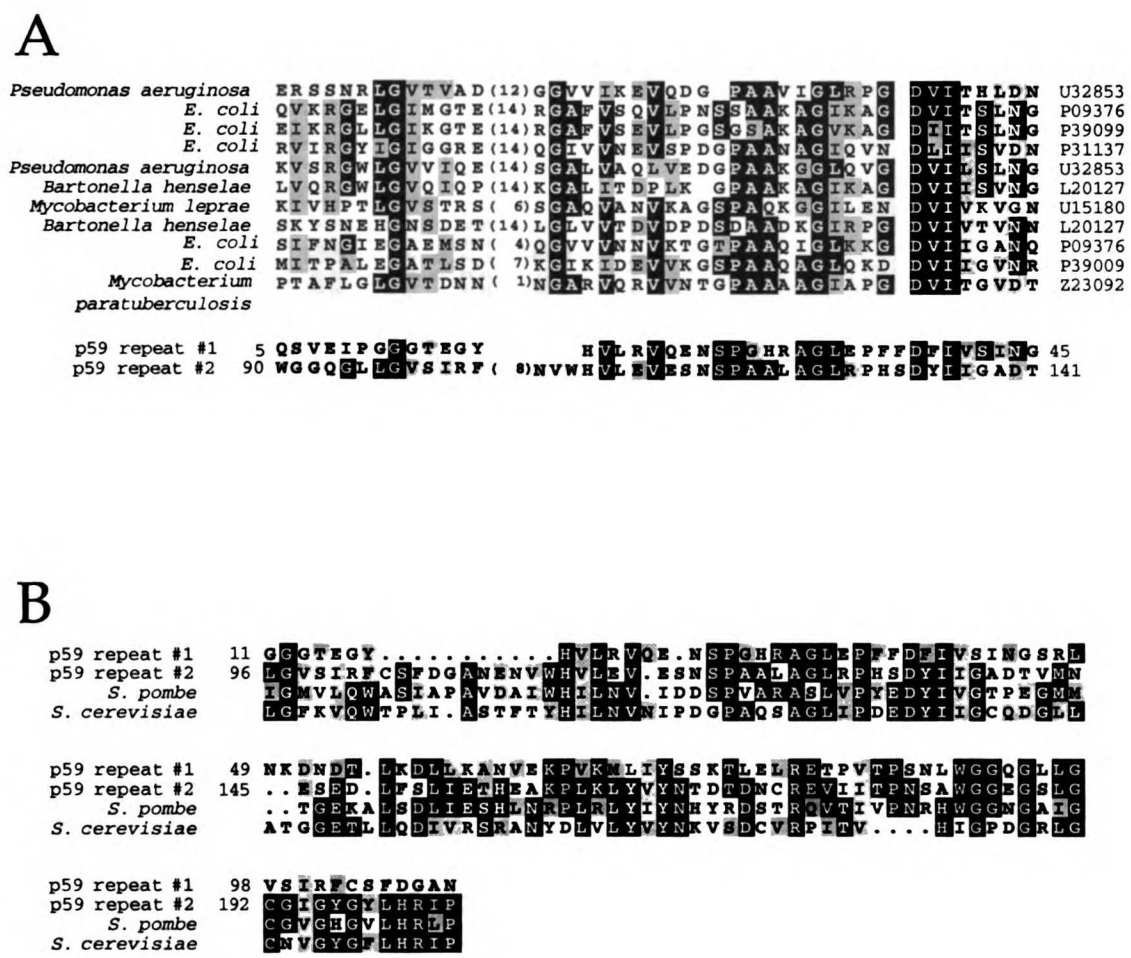

Figure 11. p59 contains two conserved htrA-like PDZ domains

A. Multiple alignment of the conserved amino-terminal portions of bacterial htrA-like domains from the indicated species with two repeats from p59. Data base accession codes for the bacterial sequences and the residue numbers for p59 are shown. Numbers in parentheses represent residues omitted from sequences. Patterned after Fig. 1 from Ponting, 1997.
B. Alignment of the PDZ domains from human p59 with domains from fission yeast (accession code Z69239) and budding yeast (accession code U33057) proteins. Residue numbers for p59 are shown. Identical residues are shown in black boxes and conservative replacements are in gray boxes.

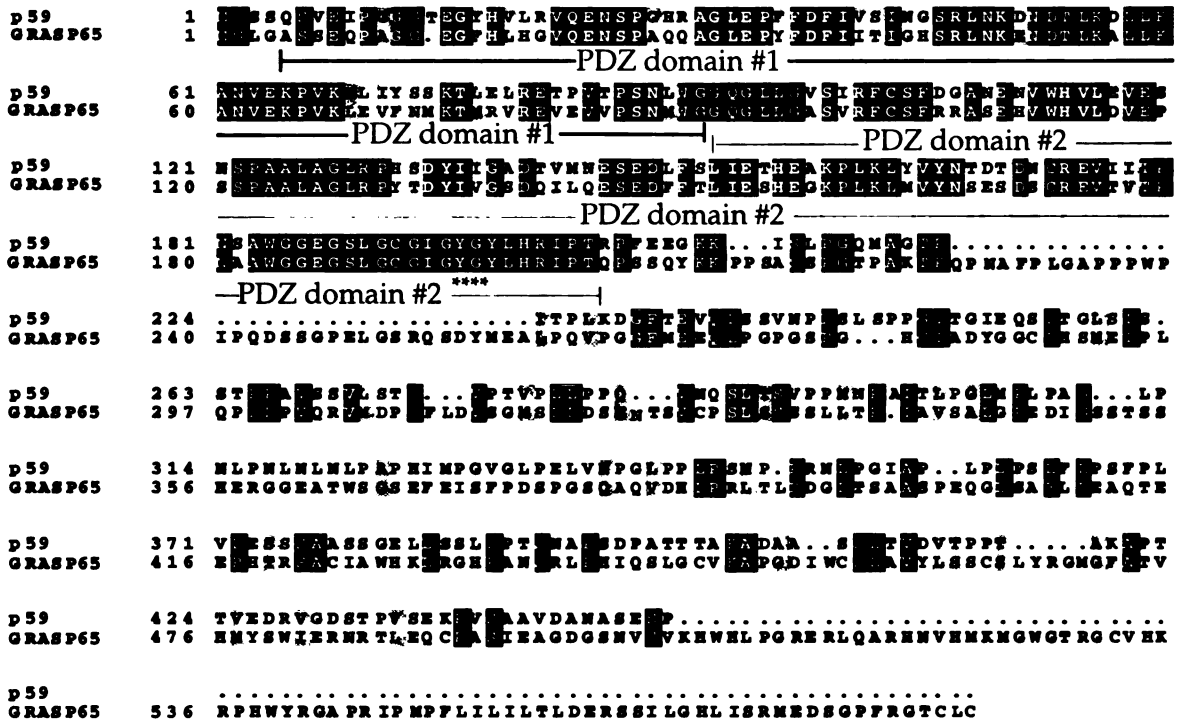

Figure 12. Alignment of p59 and GRASP65

The two PDZ domains are underlined. Note that their exact boundaries are unknown. Asterisks denote the sequence GYGY, which is found at the carboxy-terminus of PDZ domain #2 and is therefore unlikely to correspond to the GLGF of classical PDZ domains. Identical residues are shown in black boxes and conservative replacements are in gray boxes.

related; this region corresponds to the two htrA domains of p59. The conserved region mediates GRASP65's Golgi localization (Barr, et al., 1998), so p59 may also be a Golgi protein. In addition, GRASP65 and p59 both have glycines at position two. Myristoylation of this residue in GRASP65 mediates its attachment to intracellular membranes (Barr, et al., 1997), so p59 may also be myristolyated and linked to membranes. The second htrA domain of GRASP65 has been proposed to be a PDZ domain since it contains the sequence GYGY, which is reminiscent of the GLGF motif found in the carboxylate-binding loop of classical PDZ domains (Barr, et al., 1998, Cho, et al., 1992, Doyle, et al., 1996). However, the GYGY of GRASP65 probably does not serve such a binding function, since it is found at the carboxy-terminal portion of the domain while GLGF sequences are invariably found near the amino-termini of classical PDZ domains (Doyle, et al., 1996).

The sequences of GRASP65 and p59 diverge greatly downstream of their two PDZ domains and the portion of GRASP65 implicated in Golgi stacking lies in this non-conserved region. Thus, p59 and GRASP65 are structural relatives but are unlikely to be homologs and probably perform different functions. Neither TGFα nor a protein associated with it has any obvious connection to Golgi stacking.

Purification of p86

The initial two purifications did not yield sufficient p86 or p106 for peptide microsequencing, so a third large-scale purification was undertaken. In order to grow larger quantities of cells and purify larger amounts of protein, the adherent Cα cells were adapted to suspension culture. I then performed analytical immunoprecipitation experiments to determine if these suspension cells could be used as a source for the preparative purification of p106 and p86. First, I immunoprecipitated transmembrane TGFα from the

suspension cells. The α1 monoclonal precipitated three bands from these cells that were similar in size to the three transmembrane-TGFα species from adherent cells (Fig. 13a). However, the largest band from the suspension cells migrated slightly faster than the corresponding band from adherent cells. The reason for this difference is unknown. Despite this difference, p106 and p86 still coimmunoprecipitated with the growth factor from suspension cells that had been treated with DSP (Fig. 13b).

One hundred and ten liters ($6.3X10^{10}$ cells) of suspension cells were treated with chemical crosslinker and lysed, and TGFα-containing complexes from these cells were purified using immunoaffinity chromatography. One percent of the purified material was then resolved on SDS-PAGE and, based on silver staining, p86 was judged to be sufficiently pure and abundant for peptide microsequencing (Fig. 14). However, the amount of p106 was judged to be inadequate. The remainder of the material was resolved on SDS-PAGE and stained with Coomassie brilliant blue. The band corresponding to p86 was excised and processed for peptide microsequencing. Sequences from two peptides were obtained (Fig. 15).

p86 is a conserved protein

A search of the database revealed that these two peptides are derived from the same protein (Fig. 15), the likely homolog of mouse Mem3. Mem (maternal-embryonic) 3 has already been cloned and codes for a putative cytoplasmic protein with a predicted molecular mass of 86 kD (Hwang, et al., 1996). Mem3 has no known motifs and the biochemical role of the protein is unknown. Transcripts of Mem3 are supplied to the embryo by both maternal stores and transcription of the zygotic genome. In addition, it is expressed in many adult tissues (Hwang, et al., 1996).

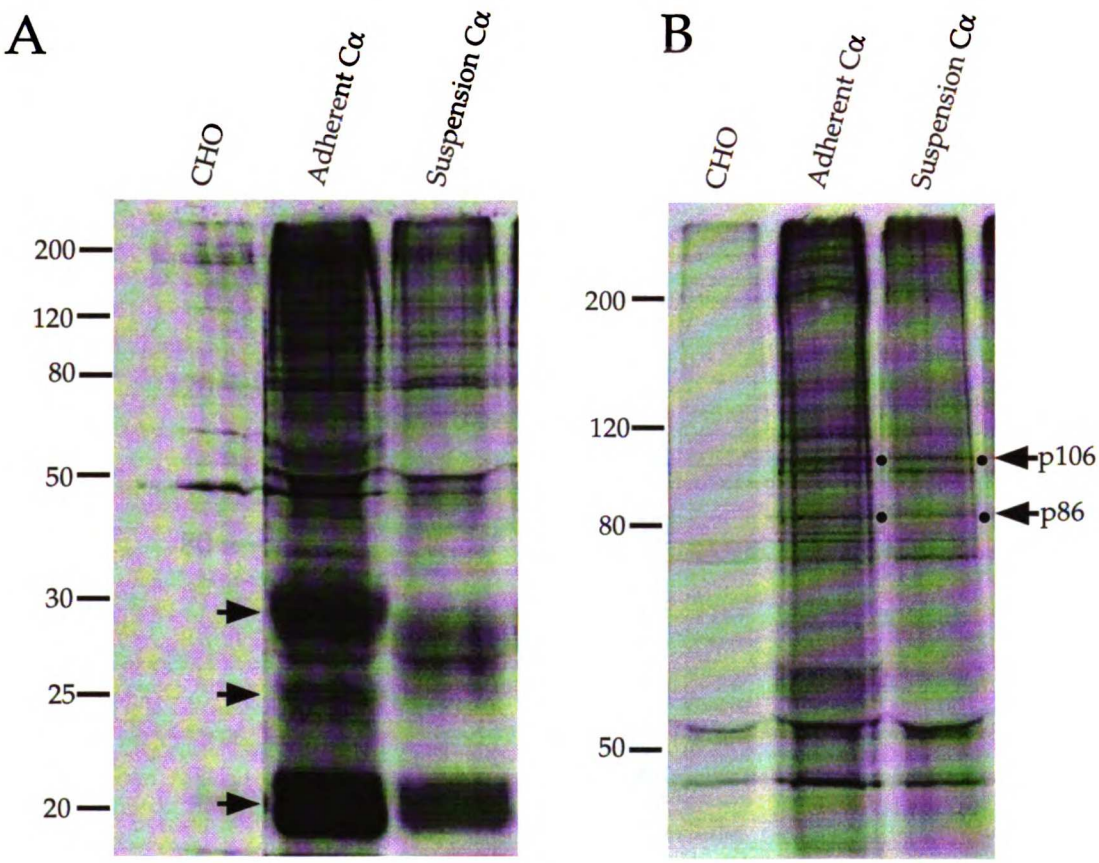

Figure 13. p106 and p86 coimmunoprecipita te with transmembrane TGFα from suspension Cα cells

CHO, adherent Cα, and suspension Cα cells were [^{35}S]-labeled, treated with DSP, lysed, and immunoprecipitated for transmembrane TGFα.
A. Immunoprecipita tion of transmembrane TGFα from suspension and adherent Cα cells. Arrows indicate the positions of the three transmembrane forms of TGFα which were purified from adherent Cα cells.
B. Coimmunoprecipitaion of p106 and p86 with transmembrane TGFα from suspension and adherent Cα cells. Arrows and dots indicate the positions of the two associated proteins. p106 and p86 were identified based on their mobilities, their positions within the pattern of bands, and their absence in the immuno-precipitates from CHO cells.

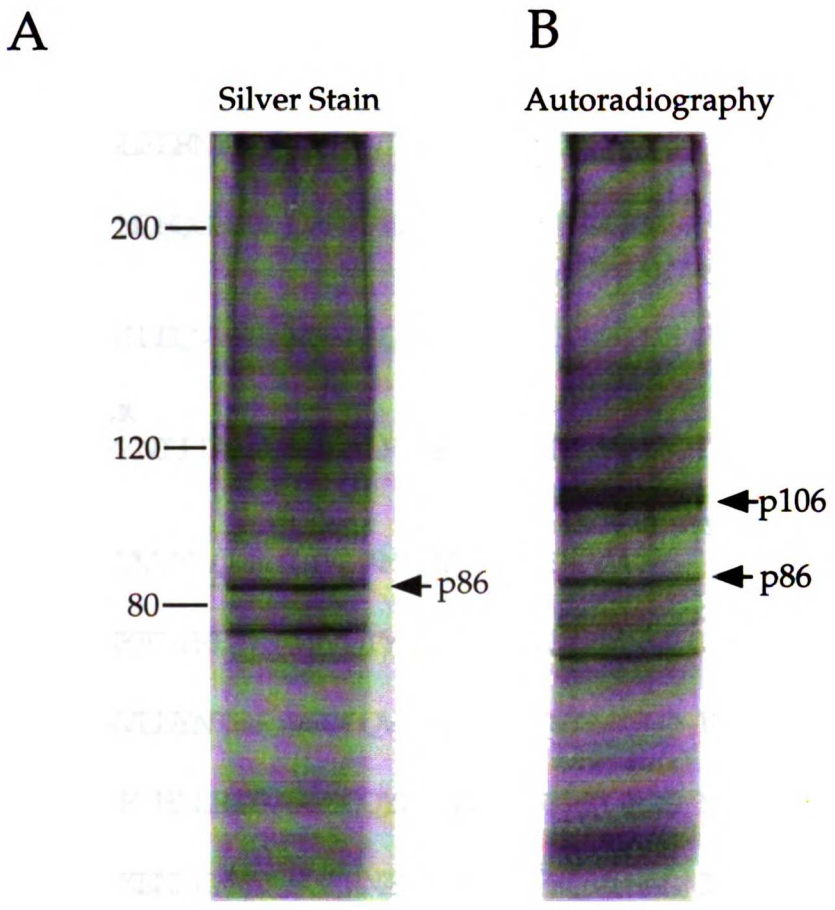

Figure 14. Preparative purification of p86

One hundred and ten liters of suspension Cα cells were treated with DSP, lysed, and then immunopurified for transmembrane TGFα and associated proteins. One percent of this sample was mixed with smaller amounts of [^{35}S]-labeled anti-TGFα immunoprecipitates and resolved with SDS-PAGE. The sample was visualized both with silver staining (A) and autoradiography (B). (A) and (B) represent the same lane. While both p106 and p86 were apparent on the autoradiogram, only p86 was detected by silver staining. p86 and p106 were identified based on their mobility and the pattern of bands. Transmembrane TGFα is not shown.

```
  1  MPPICLESSG PLCCHQRVIM NFIWLFLMNC TIWKVYLIDE FAKGERLADL
 51  YELVQYSGNI IPRLYLLITV GVVYVKSFPQ SRKDILKDLV EMCRGVQHPL
101  RGLFLRNYLL QCTRNILPDE GEPTDEETTG DISDSMDFVL LNFAEMNKLW
151  VRMQHQGHSR DREKRERERQ ELRILVGINL VALTLVSWRC KCGTLQQIVL
201  TGILEQVVNC RDALAQEISM ECIIQVFPDE FHLQTLNPFL RACAELHQNV
251  NVKNIIIALI DRLALFAHRE MEPGIPAELK LFDIFSQQVA TVIQSRRDMP
301  SEDVVSLQVS LINLAMKCYP DRVDYVDKVL ETTVEIFNKL NLEHIATSSA
351  VSKELTRLLK IPVDTYNNIL TVLKLKHFHP LFEYFDYESS PGKSMSCYVL
401  SNVLDYNTEI VSQDQVDSIM NLVSTLIQDQ PDQPVEDPDP EDFADEQSLV
451  GRFTHLLRSD DPDQQYLILN TARKHFGAGG NQRIRFTLPP LVFAAYQLAF
501  RYKENSKWMT SGKRNARRYF HLPHQTTSAL IKAELAELPL RLFLQGALAA
551  GEIGFENHET VAYEFMSQAF SLYEDEISDS KAQLAAITLI IGTFERMKCF
601  SEENHEPLRT ECALAASKLL KKPDQAEREH MCTSLWSGRN TDKNGEELHG
651  GKRVMECLKK ALKIANQCMD PSLQVQLFIE ILNRYTYFYE KENDAVTIQV
701  LNQLIQKIRE DLPNLESSEE TEQINKHFHN TLEHLRTRRE SPESEGPIYE
751  GLIL
```

Figure 15. Sequence of p86/Mem3

Sequences of peptides purified from Cα cells are in bold and are aligned over the murine Mem3 sequence.

p86/Mem3 is related to VPS (vacuolar protein sorting) 35, a *Saccharomyces cerevisiae* gene product involved in the sorting of vacuolar proteins (Fig. 16). Yeast mutant for this gene secrete some proteins that are normally targeted to the vacuole (Paravincini, et al., 1992). The gene product is a putative cytoplasmic protein that associates with intracellular membranes but whose exact biochemical activity is unknown.

Since VPS35 plays a role in sorting proteins to the vacuole, p86/Mem3 may act in the intracellular trafficking of transmembrane TGFα. More specifically, it may target proteins to the lysosome, the mammalian equivalent of the yeast vacuole. As such, it could play a role in the endocytosis and transport to the lysosome of cell-surface proTGFα. A house-keeping role for p86 would explain its widespread distribution and its accumulation in the oocyte. Mutational analysis of TGFα is consistent with a role for p86 in the endocytosis of the growth factor. Deletion of the carboxy-terminal 31 amino acids of the cytoplasmic domain dramatically slows antibody-induced internalization of TGFα and abolishes its binding to p86 (Shum, et al., 1994).

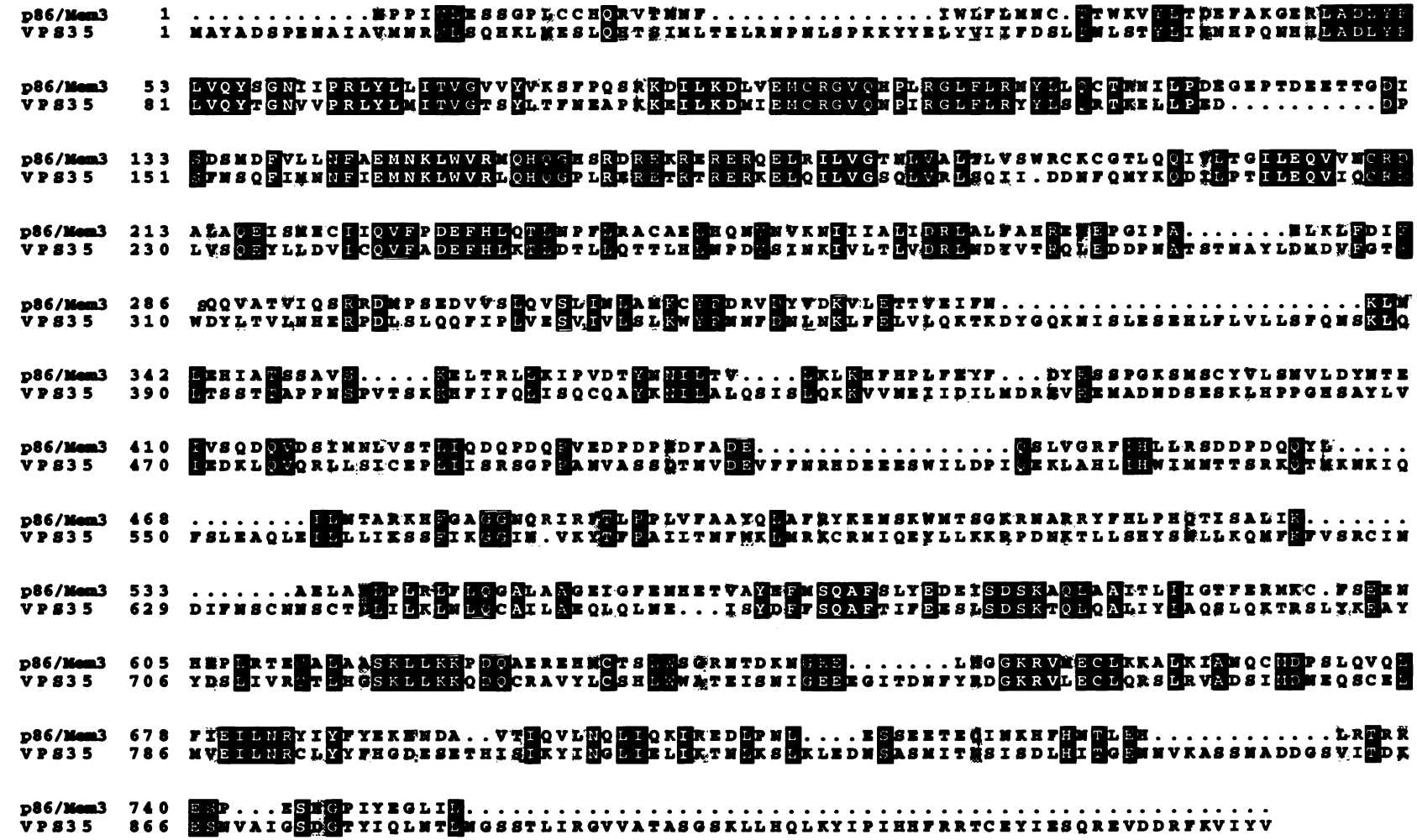

Figure 16. Comparison of p86/Mem3 and VPS35
Identical residues are shown in black boxes and conservative replacements are in gray boxes.

Chapter 5: Structural and Functional Characterization of p59

Introduction

In any biochemical purification, there is a risk that a contaminant may have been isolated instead of the desired polypeptide. This was initially a special concern in the case of the candidate p59 clone, since it is structurally related to GRASP65, a protein that acts in the stacking of Golgi cisternae. This process has no obvious connection to TGFα. Recombinant p59, which was expressed from a cDNA expression plasmid, migrated on gel with the same mobility as the endogenous p59 that associates with transmembrane TGFα. In addition, the recombinant protein also associated with transmembrane TGFα *in vivo* . These results demonstrate that it is authentic p59 rather than a contaminant. Overexpression of p59 altered the processing of wild-type transmembrane TGFα and partially rescued the phenotype of a mutant form of TGFα that is improperly processed. This suggests that p59 may play a role in the maturation of the growth factor.

Materials and Methods

Chemicals

Unless mentioned otherwise, all chemicals were purchased from Sigma, St. Louis, MO.

Cell culture

CHO and Cα cells were grown in F12 Ham's Nutrient Mix (GIBCO BRL, Gaithersburg, MD), in the latter case supplemented with Geneticin (GIBCO BRL) at 400 μg/ml. COS-1 and 293 cells were grown in DMEM-H16, 3 g/l

glucose (GIBCO BRL). MDCK cells were obtained from Dr. Robert Coffey (Vanderbilt University). These cells were grown in DMEM-H21 with 4.5 g/l glucose (GIBCO BRL) supplemented with non-essential amino acids. All media included 10% fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmids

pRK7α, a mammalian expression plasmid for wild-type TGFα, and pRK7α-Δ158, a mammalian expression plasmid for a mutant form of transmembrane TGFα that lacks the carboxy-terminal divaline, were described previously (Shum, et al., 1994, Shum, et al., 1996). pRK5-p59F, an expression plasmid for FLAG-tagged p59 was described in Chapter 4. pRK5-p59FΔN142 was constructed by replacing the *Eco* RI-*Bgl* II fragment of pRK5-p59F with the corresponding fragment from hbc 551. pRK-p59FΔN68 was generated by replacing the *Eco* RI-*Bgl* II fragment of pRK5-p59F with the corresponding fragment of the initial nested-PCR product described in Chapter 4. pRK5-p59FΔ149 was constructed by removing the *Bgl* II-*Sal* I fragment of pRK5-p59F, filling in the 5' single-stranded DNA-overhangs with the Klenow fragment of DNA Polymerase I (GIBCO BRL), and ligating the resultant blunt DNA ends together. pRK5-p59FΔ102-377 was constructed by digesting pRK5-p59F with *Pst* I, isolating the largest fragment generated, removing the 3' DNA overhangs of this fragment with Vent DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions, and ligating the resultant blunt DNA-ends together. pRK7-MycΔEα was provided by Kuh-Wei Tzung in our laboratory. pRK7-EGFRα was constructed by fusing a PCR-generated fragment corresponding to the extracellular domain of the human epidermal growth factor receptor in frame with a DNA segment

coding for the transmembrane and intracellular domains of TGFα. The resulting chimera was subcloned into the mammalian expression plasmid pRK7.

Transfections

COS-1 and 293 cells were transiently transfected using Lipofectamine (GIBCO BRL) according to the manufacturer's instructions, while F442A 3T3 cells were transfected with Lipofectamine with the PLUS reagent (GIBCO BRL). MDCK cells were transfected with pRK5hygro-p59F, a derivative of pRK5-p59F containing a hygromycin resistance cassette, using the calcium phosphate method (Sambrook, 1989). Following transfection, cells were grown to confluence and then split into medium containing hygromycin (Boehringer Mannheim, Indianapolis, IN) at 400 μg/ml. Single clones were isolated and expanded. Expression of p59F was assessed by immunofluorescence and immunoprecipitation.

Antibodies

The α1 monoclonal antibody was previously described (Bringman, et al., 1987). α1 mAb was coupled to Oregon Green purchased from Affinity Bioreagents (Eugene, OR) according to the manufacturer's instructions. The M2 anti-FLAG antibody and M2 immunoaffinity resin were purchased from Kodak Eastman (New Haven, CT). The 9E10 anti-myc monoclonal antibody was a gift of Dr. J. M. Bishop (University of California, San Francisco). The anti-EGFR immunoaffinity resin R-1 was from Santa Cruz Biotech (Santa Cruz, CA). The anti GM130 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY).

Metabolic labeling

For protein metabolic-labeling, cells were grown to 80% confluence and labeled overnight with 160 μCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN, Boston, MA) in cysteine/methionine-free media with 10% dialyzed fetal calf serum.

For palmitate labeling, cells were grown to 80% confluence and then labeled overnight with 1.1 mCi/ml [9,10-^{3}H] palmitic acid (NEN) in medium supplemented with 5 mM pyruvate, 1% DMSO, 10% tryptose, and 10% dialyzed fetal calf serum.

***In vitro* palmitoylation**

50 nCi of [palmitoyl-1-^{14}C] palmitoyl Coenzyme A (NEN) was added to a 25 μl TNT® reticulocyte coupled transcription/translation reaction (Promega, Madison, WI), using SP6 RNA polymerase and either pRK5 or pRK5-p59F as template. Samples were resolved with SDS-PAGE and visualized with phosphorimaging.

Crosslinking

Cells were washed twice with cold PBS, and then incubated at 4°C for 30 minutes with 2 mM DSP (Pierce, Rockford, IL) in PBS. Cells were subsequently washed twice with cold calcium/magnesium-free PBS with 0.04% EDTA before further processing.

Immunoprecipitation

Cells were lysed in buffer F, and the lysate was cleared by centrifugation and subsequently incubated for one hour at 4°C with primary antibody and protein A-sepharose (Pharmacia, Piscataway, NJ) preloaded with rabbit anti-

mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). In some cases, M2 anti-FLAG affinity resin, α1mAb immunoaffinity resin, or anti-EGFR (R-1) immunoaffinity resin was used instead of free antibody. Beads were washed three times with Buffer F, once with 0.5 M NaCl, 50 mM TrisCl, pH 7.5, and once with 50 mM TrisCl, pH 7.5. The beads were resuspended in protein sample buffer, heated for 5 minutes at 100°C, and proteins were resolved with SDS-PAGE.

Immunofluorescence

Cells were grown on tissue-culture chamber-slides to 70% confluence and then fixed with formaldehyde. Cells were incubated with primary antibody, and then Texas-red or Fluorescein-labeled secondary antibodies (Jackson Immunoresearch) were used for detection. In some cases, the Oregon Green-labled α1 mAb was used for direct immunofluorescence of TGFα. For anti-FLAG staining of MDCK cells, an additional amplification step was added. Samples were incubated sequentially with M2 mAb, unlabeled rabbit anti-mouse antisera, and finally a fluor-labeled anti-rabbit antibody. Alternatively, the signal was enhanced using Tyramide signal amplification (NEN) according to the manufacturer's instructions. Fluorescein-conjugated wheat germ agglutinin was purchased from Vector Labs (Burlingame, CA).

Northern blotting

Placental RNA was a gift of Dr. Susan Fisher (University of California, San Francisco). COS-1, CCL64, F442A preadipocyte, and F442A adipocyte RNA were provided by Dr. Lisa Choy in our laboratory. RNA from CHO, 293, and MDCK cells was prepared using Ultraspec (Biotecx Labs, Houston, TX) according to the manufacturer's instructions. RNA was electrophoresed in a

1% agarose gel containing 12% formaldehyde in MOPS buffer (4 mg/ml MOPS, 5 mM NaOAc, 1 mM EDTA, pH 7.0). RNA was subsequently tranferred to a Biotrans membrane (ICN, San Diego, CA) by capillary action in 10X SSC, UV crosslinked, and baked in an 80°C oven for 15 minutes. The blot was prehybridized for one hour at 65°C in prehybridization buffer (50 mM PIPES, pH 6.5, 100 mM NaCl, 50 mM $NaHPO_4$, 1 mM EDTA, 5% SDS, 60 µg/ml denatured single-stranded DNA). The blot was hybridized for 16 hours at 65°C in fresh prehybridization buffer to which a randomly-primed [^{32}P]-dCTP (NEN) labeled, *Bgl* II-*Sal* I restriction-fragment of pRK5-p59F was added as probe. The blot was washed once at room temperature and twice at 65°C for 20 minutes with 5% SDS in 0.5X SSC, before exposure to film.

Results

Expression of p59 in cell lines

An initial survey Northern blot was performed to evaluate the expression pattern of p59. RNA from five epithelial cell lines as well as placenta, pre-adipocytes and adipocytes were probed with a *Bgl* II-*Sal* I fragment of p59. A band of 2.6 kb was seen in all cell types examined, except for F442A preadipocytes and adipocytes (Fig. 17). These results indicate that p59 is not an essential cellular gene.

Subcellular localization of p59

Since the subcellular localization of p59 could give an indication of its function, anti-FLAG immunostaining was performed in a variety of cells transfected with p59F, which contains a FLAG epitope-tag (Fig. 18, 19). The

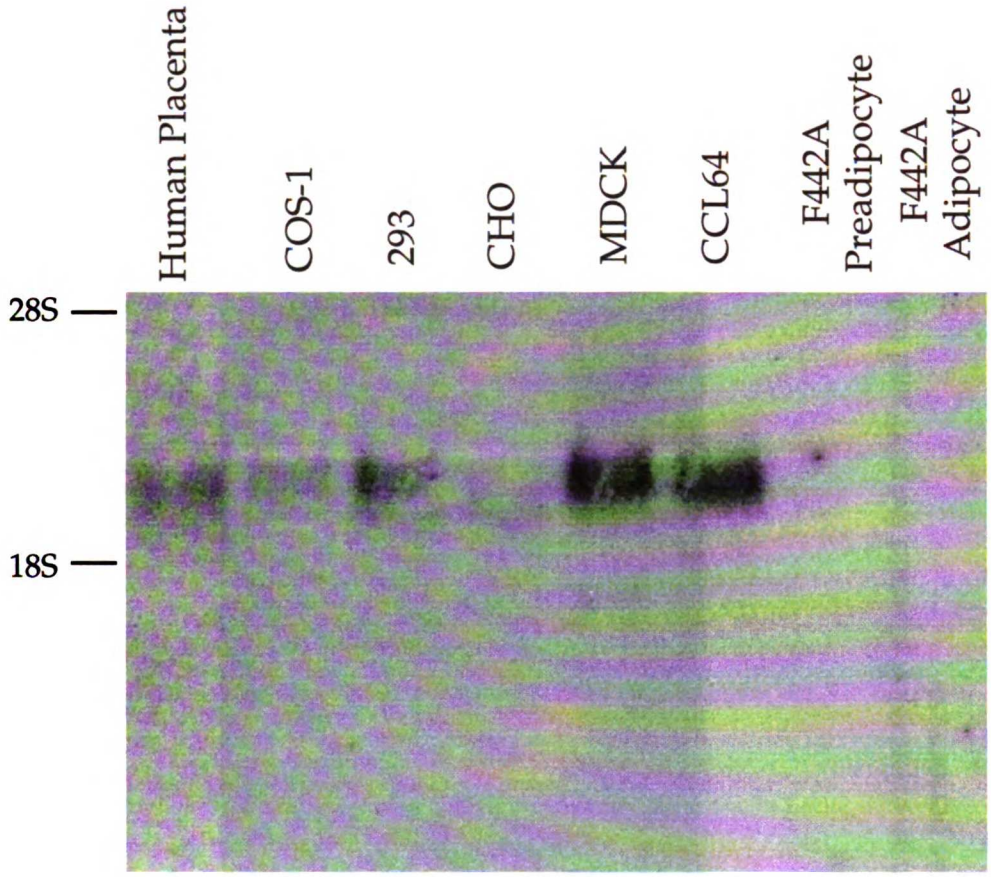

Figure 17. Northern hybridization for p59 mRNA

RNAs from the listed sources were hybridized with a p59 probe. The positions of 28S and 18S rRNAs are indicated.

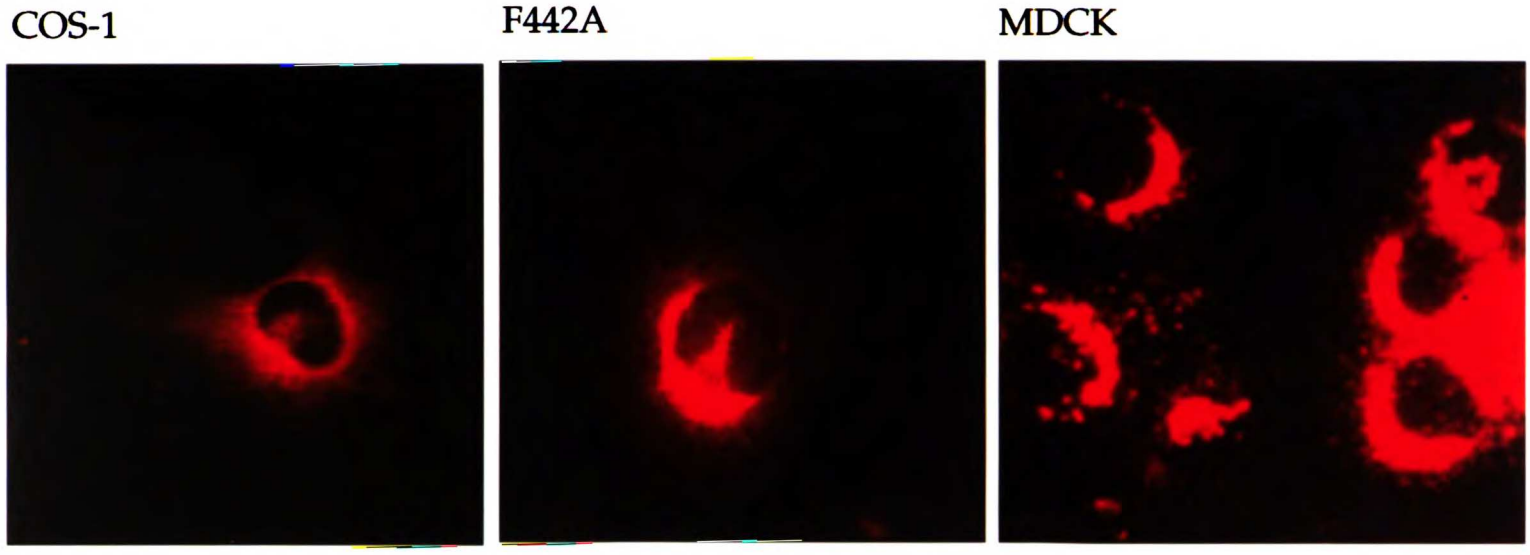

Figure 18. Subcellular localization of p59

The indicated cells were transfected with an expression plasmid for p59F and following permeabilization, anti-FLAG indirect immunofluorence was performed.

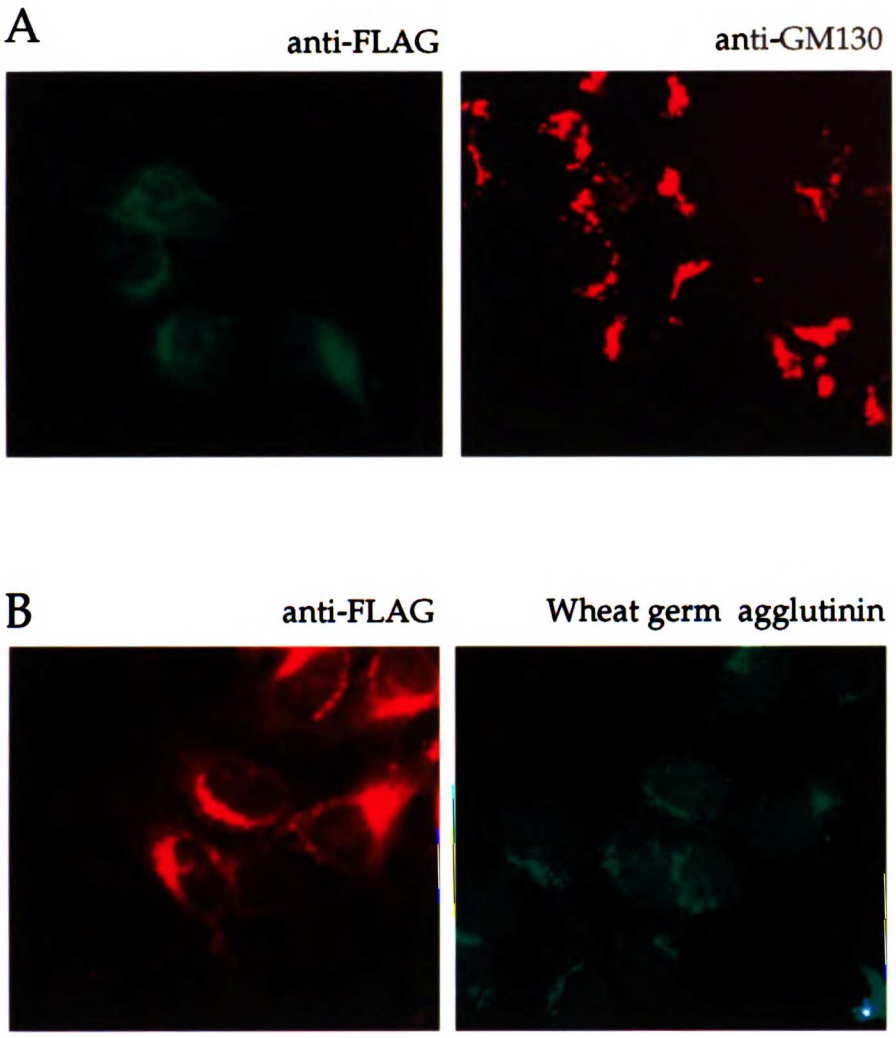

Figure 19. p59 colocalizes with Golgi markers

293 cells were transfected with an expression plasmid for p59F, permeabilized, and stained as indicated. Different cells are shown in (A) and (B). Identical fields are shown in the left and right panels in each case.

PDZ domains of p59 are very similar to the region of GRASP65 that directs the rat protein to the Golgi apparatus (Barr, et al., 1998), so a similar localization for p59 was expected. MDCK, 293, and F442A cells expressing p59F all exhibited very focal, intense perinuclear staining, consistent with Golgi staining. In addition, those cells expressing extremely high amounts of protein also had a network-like staining throughout the cytoplasm. Transfected COS-1 cells displayed both the perinuclear and reticular pattern of staining.

To determine if the perinuclear staining might represent Golgi localization, 293 cells that expressed p59F were costained for FLAG and for the Golgi marker GM130 (Fig. 19a). The signals in this case overlapped, suggesting that the perinuclear staining represented Golgi localization. Note that all cells expressed endogenous GM130 and were stained for this molecule, whereas only a subset of cells were transfected with and expressed p59F. 293 cells that expressed p59F were also costained with the anti-FLAG monoclonal and wheat germ agglutinin, which labels the trans-Golgi. Once again, the signals overlapped (Fig. 19b). When 293 cells were transfected with expression plasmids for both TGFα and p59F and stained for TGFα and FLAG, overlapping staining in the perinuclear region was also seen (Fig. 20).

p59 is palmitoylated

p59 lacks a putative signal-sequence and transmembrane regions, so its perinuclear localization is unlikely to be mediated by a direct protein anchorage into the Golgi membrane. Proteins that lack membrane-spanning domains can be anchored to membranes by acylation, such as myristoylation and palmitoylation. For instance, GRASP65, the structural relative of p59, is myristoylated and binds to Golgi membranes via this

anti-FLAG

anti-TGFα

Figure 20. Coimmunofluorescence of p59 and TGFα

293 cells were cotransfected with expression plasmids for p59F and TGFα, permeabilized, and stained as indicated.

modification (Barr, et al., 1997). p59 has a similar myristoylation site, which is the glycine at position two, and may also be myristoylated.

Since p59 also has several cysteines that may be palmitoylated, its palmitoylation status was determined. Cells were transfected with expression plasmids for p59F and TGFα, *in vivo* labeled with tritiated palmitate, and immunoaffinity purified for TGFα and FLAG. After SDS-PAGE, tritiated proteins were visualized using phosphorimaging. Transmembrane TGFα is a palmitoylated protein (Bringman, et al., 1987) and thus served as a positive control. As expected, the anti-TGFα immunoaffinity resin precipitated three labeled bands that were consistent in size with the three transmembrane forms of TGFα (Fig. 21a). Several larger bands were also seen in this sample, including one that migrated in the position expected for p59F. The anti-FLAG immunoaffinity resin precipitated a tritiated protein with a size consistent with that of p59F, suggesting that p59 incorporated the label (Fig. 21a).

During the time required for *in vivo* labeling, cells may convert palmitate to myristate by beta oxidation. To determine if the label incorporated during the *in vivo* labeling included palmitate, a coupled *in vitro* transcription and translation of p59F was performed in the presence of radiolabeled palmitoyl CoA, the activated form of the fatty acid. Under these conditions, no conversion to myristate should occur and any incorporation of label should represent palmitoylation. A protein corresponding to the size of p59F was labeled when the p59F cDNA was present (Fig. 21b). This protein was not seen when a control vector plasmid was used in the reaction, indicating that p59 can be palmitoylated.

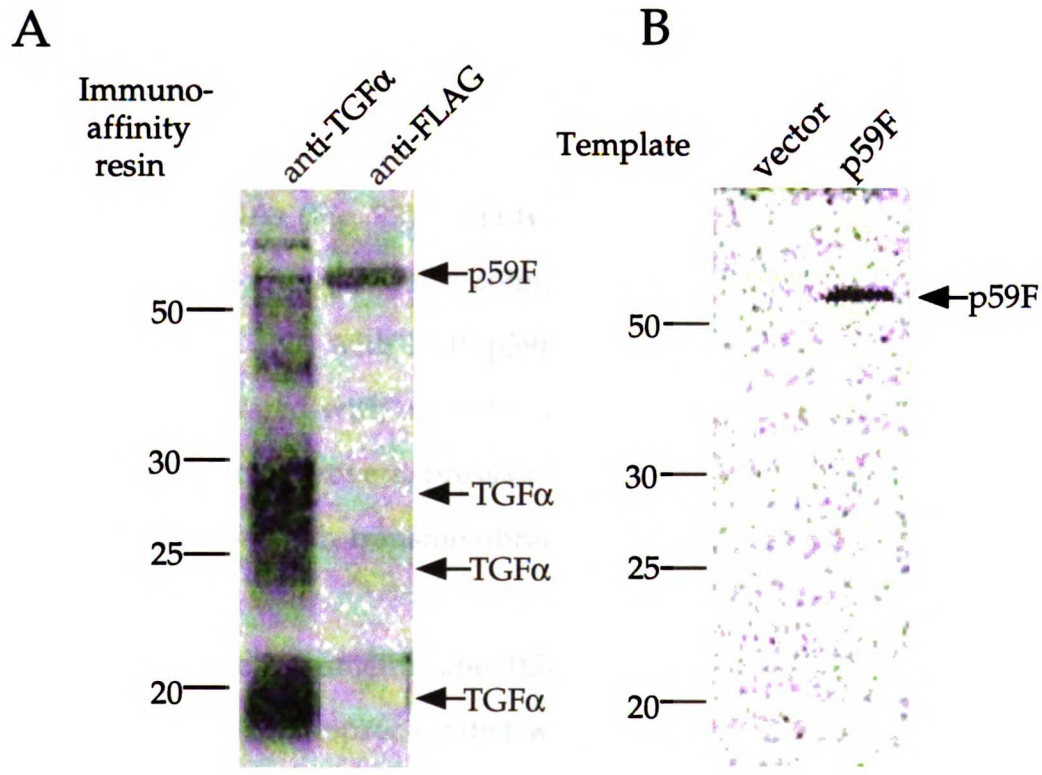

Figure 21. p59 is palmitoylated

A. *In vivo* palmitoylation. 293 cells were transfected with expression plasmids for TGFα and p59F, metabolically labeled with tritiated palmitate and immunoaffinity purified for TGFα (left lane) or FLAG (right lane). p59F contains a FLAG epitope and was precipitated by the anti-FLAG resin.
B. *In vitro* palmitoylation. Coupled transcription/translation reactions were performed in the presence of [^{14}C]-palmitoyl CoA using either control vector (left lane) or p59F (right lane) as template. Proteins migrate as indicated.

TGFα and p59 associate *in vivo*

The perinuclear colocalization of p59 and TGFα that was seen by immunofluorescence does not prove that they physically interact. To address this, coimmunopurification studies were performed in transfected 293 cells. Immunoaffinity resins specific for TGFα or FLAG each precipitated both transmembrane TGFα and p59F from lysates of cells that were cotransfected with the two cDNAs (Fig. 22). The two proteins therefore interact *in vivo*. In contrast to results from Cα cells, coimmunoprecipitation did not depend on prior treatment of cells with DSP; p59F and transmembrane TGFα coimmunoprecipitated with or without crosslinking (Fig. 23). This may be due to mass action; the overexpression of p59 apparently leads to an increased level of association with transmembrane TGFα that can be detected without crosslinking.

Of the three transmembrane TGFα species, the 25 kD form was preferentially coimmunoprecipitated with p59F (Fig. 22). As demonstrated in Chapter 2, this species is the initial endo H-sensitive precursor molecule. Since the conversion to endo H resistance occurs in the Golgi apparatus, this form is probably found predominantly in the endoplasmic reticulum and the Golgi apparatus. Thus, p59 may interact with transmembrane TGFα in these compartments, consistent with the perinuclear localization of significant amounts of both molecules (Fig. 20). If so, p59 should interact with proTGFα soon after the synthesis of the growth factor. To determine if the kinetics of the interaction between p59 and TGFα were consistent with such a rapid association, pulse-chase experiments were performed (Fig. 24). 293 cells were transfected with expression plasmids for p59F and TGFα, pulsed for five minutes with [^{35}S]-methionine and cysteine, chased for various times, and

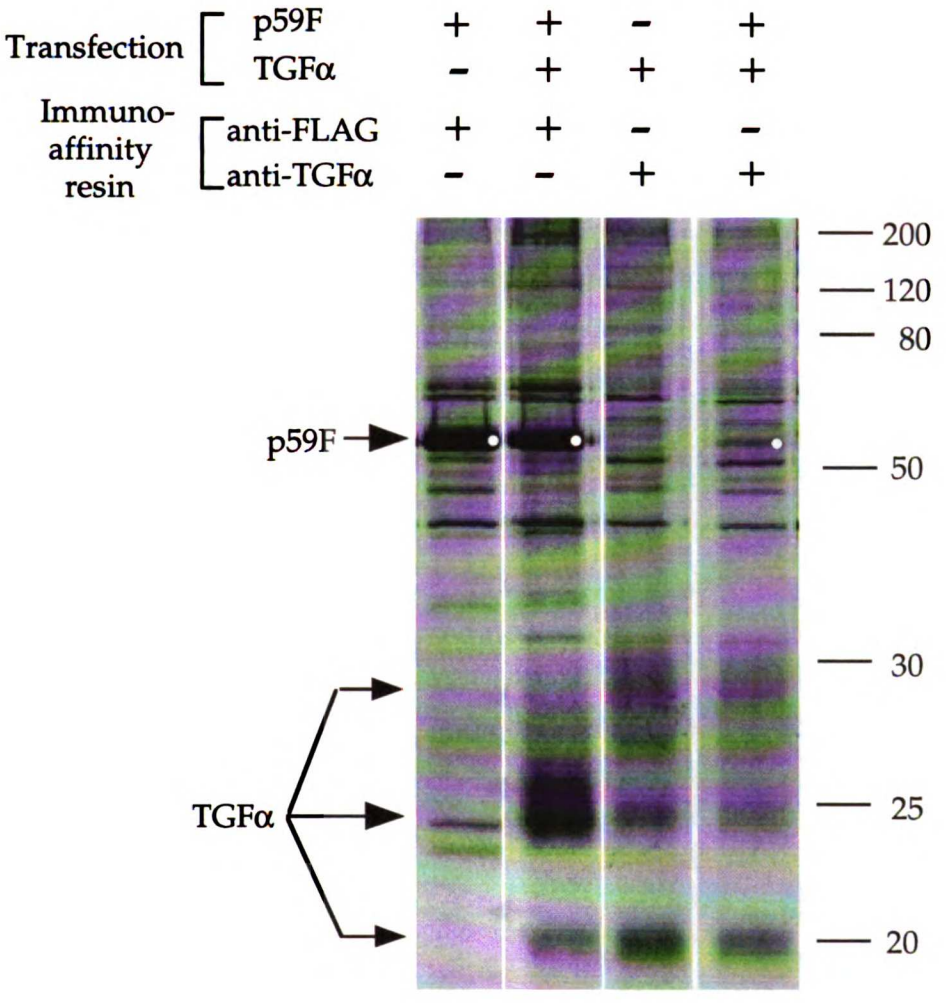

Figure 22. p59 interacts with transmembrane TGFα *in vivo*

293 cells were transfected with expression plasmids for the indicated proteins, [^{35}S]-labeled, DSP-treated, lysed, and immunoaffinity purified using the indicated antibody resins. p59F contains a FLAG epitope-tag and was precipitated by the anti-FLAG resin. Arrows indicate the positions of proteins. White dots within lanes indicate the positions of p59F. In cells co-transfected with expression plasmids for both p59F and TGFα, both the anti-FLAG and anti-TGFα resins precipitated both proteins. p59 preferentially coprecipitated the 25 kD precursor species of transmembrane TGFα.

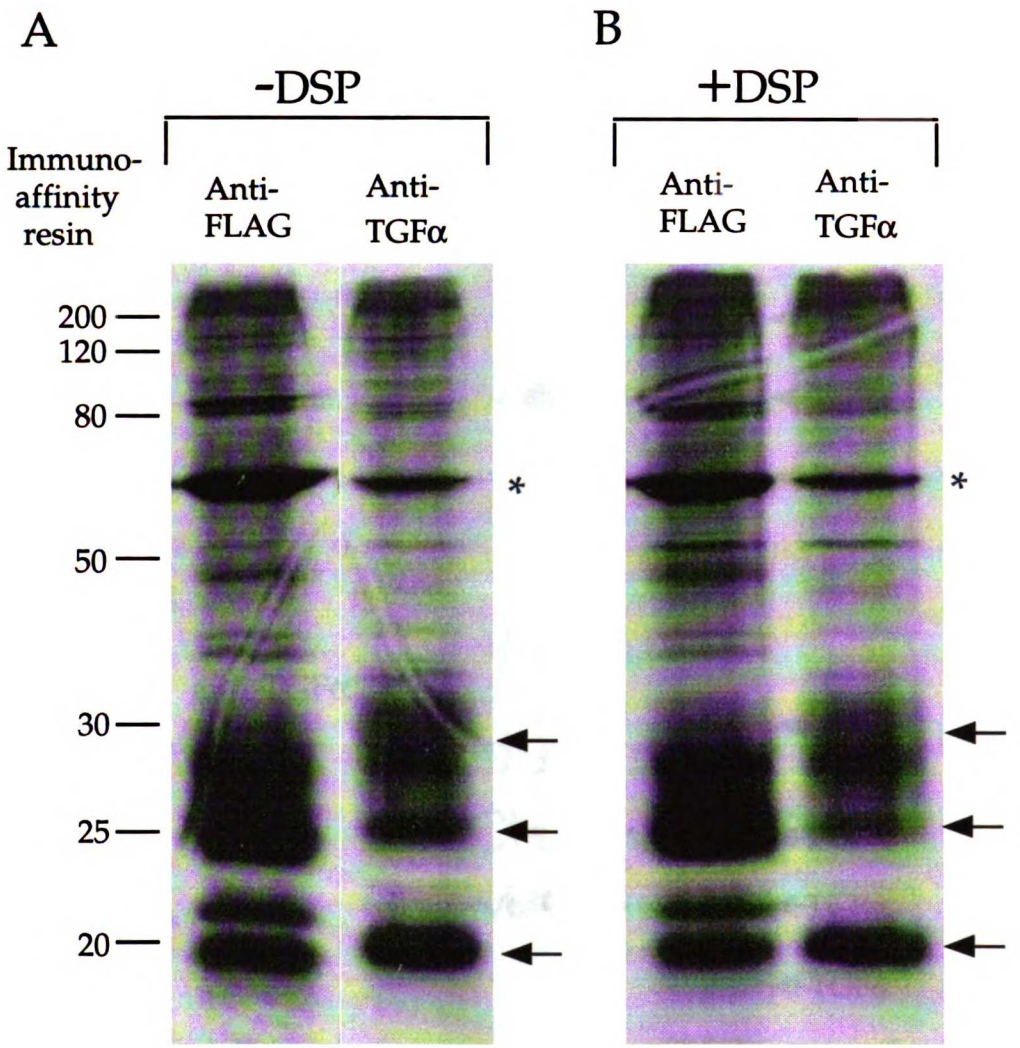

Figure 23. Overexpressed p59 and transmembrane TGFα coimmunopurify without crosslinking

293 cells were cotransfected with expression plasmids for TGFα and p59F, [^{35}S]-labeled, treated with DSP (panel B only), and lysed. Proteins were then purified using the indicated immunoaffinity resins. p59F contains a FLAG epitope-tag and was precipitated by the anti-FLAG resin. Asterisks indicates the positions of p59F and arrows indicate the positions of transmembrane TGFα. p59F and transmembrane TGFα coprecipitated with (panel B) or without (panel A) prior crosslinking of cells.

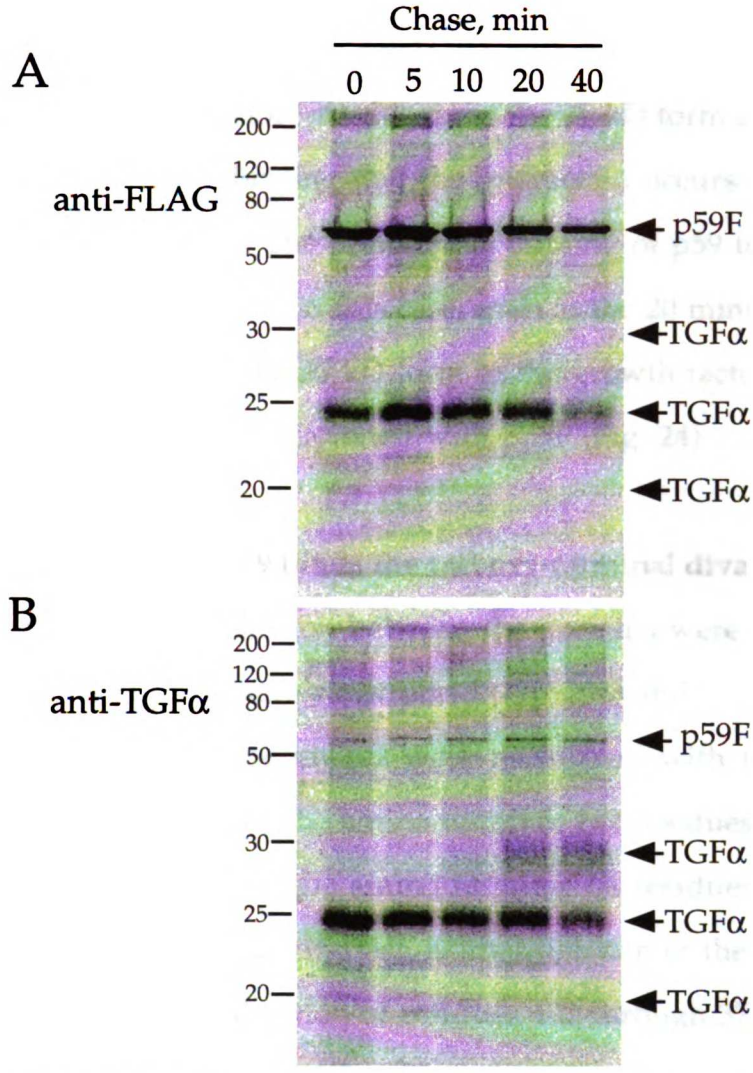

Figure 24. Pulse-chase analysis of the interaction between p59 and transmembrane TGFα

293 cells were cotransfected with expression plasmids for p59F and TGFα, labeled for five minutes with [^{35}S]-cysteine and methionine, and then incubated for the indicated chase times with non-radioactive media. Cells were then lysed and immunoaffinity purifications were performed using an anti-TGFα antibody resin (A) and an anti-FLAG antibody resin (B). The positions of p59F and the three major transmembrane TGFα species are indicated with arrows.

immunoprecipitated for FLAG and TGFα. [^{35}S]-labeled TGFα coimmunoprecipitated with p59F immediately after the five-minute pulse, indicating that p59 interacts with proTGFα within five minutes of the synthesis of the growth factor. At this time, the 25 kD form of proTGFα was the only form present, indicating that the interaction occurs in an intracellular compartment. The preferential binding of p59 to the 25 kD species of TGFα was absolute in this case; even at the 20 minute and 40 minute chase times, when the 30 kD form of the growth factor appeared, only the 25 kD form coimmunoprecipitated with p59F (Fig. 24).

The first PDZ domain of p59 binds the carboxy-terminal divaline of TGFα

The regions of interaction between the proteins were then mapped. Deletion mutants of p59F were constructed (Fig. 25a) and coimmunoprecipitation experiments were performed with transmembrane TGFα (Fig. 25b). Deletion of the amino terminal 142 residues abolished interaction, while deletion of the amino terminal 68 residues severely reduced binding to TGFα. In contrast, neither deletion of the carboxy terminal 149 residues nor deletion of residues 102 through 377 affected coimmunoprecipitation with TGFα. Therefore, the residues that are critical for coimmunoprecipitation with TGFα must lie in the amino terminal 102 residues of p59. This region corresponds to the first PDZ domain of p59.

Mutants of TGFα were also tested for their ability to coimmunoprecipitate with p59F. Since p59 is predicted to be a cytoplasmic protein, the extracellular domain of TGFα should not be important for the interaction of the two molecules. To test this, coimmunoprecipitation experiments were performed using 293 cells transfected with p59F and either

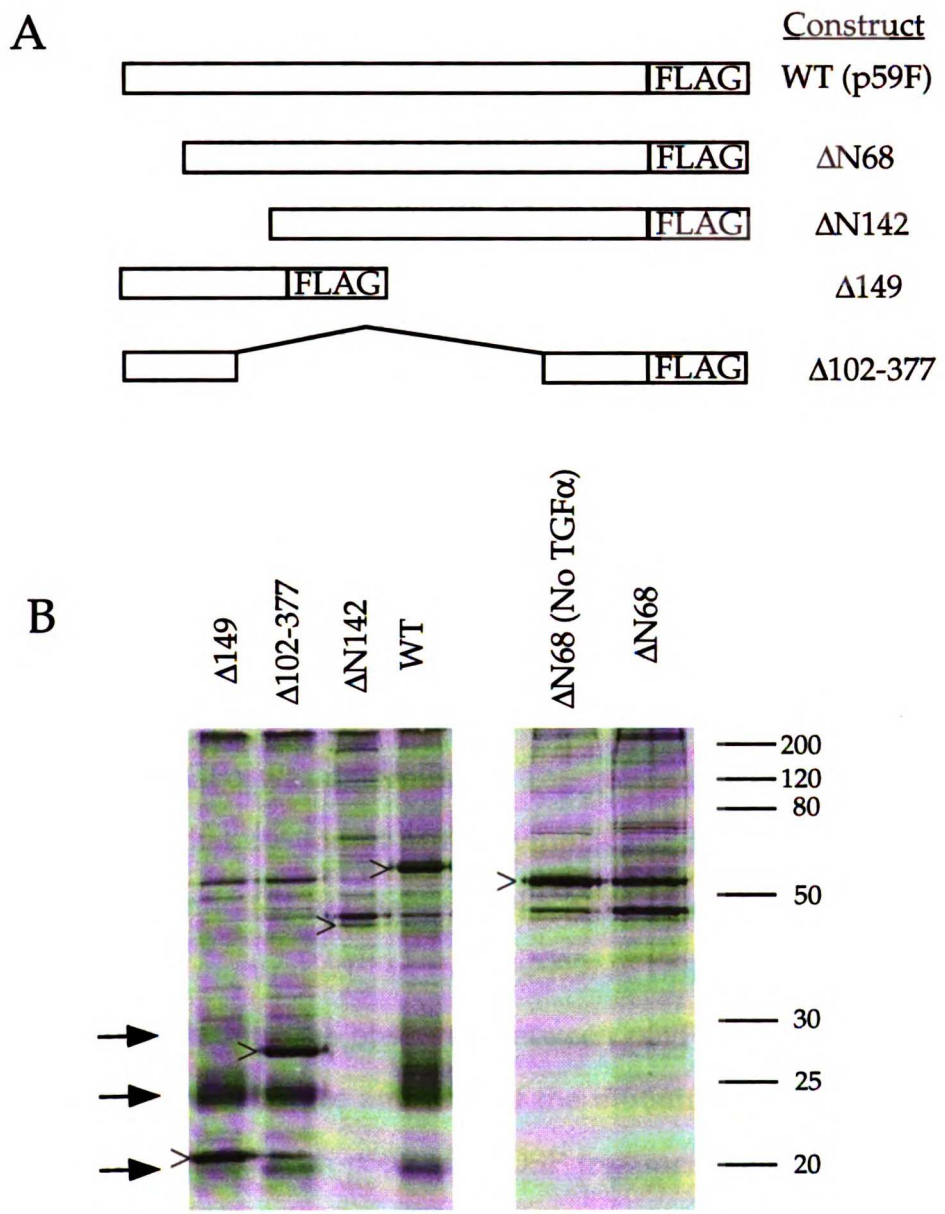

Figure 25. Interaction of transmembrane TGFα with p59 mutants
A. Schematic representation of p59 and its mutants. WT refers to the wild-type molecule. ΔN68 and ΔN142 are deletions of respectively the amino terminal 68 and 142 residues. Δ149 is a deletion which lacks the carboxy-terminal 303 residues. Δ102-377 lacks those residues. All constructs contain a carboxy-terminal FLAG epitope-tag.
B. Coimmunoprecipitation of transmembrane TGFα with p59 mutants. 293 cells were transfected with expression plasmids for TGFα and the indicated p59 proteins. Cells were then treated with DSP and immuno-affinity purified using an anti-FLAG antibody resin. The positions of the three transmembrane TGFα species are indicated with arrows. Arrow heads indicate the positions of p59 proteins.

of two chimeric molecules that contained the transmembrane and cytoplasmic domains of TGFα and heterologous extracellular domains. These were MycΔEα, in which the EGF repeat of TGFα was replaced by a myc epitope-tag, and EGFRα, in which the extracellular domain of the growth factor was replaced by the extracellular domain of the EGFR (Fig. 26a). Both transmembrane proteins coimmunoprecipitated with p59F, indicating that TGFα's extracellular domain is dispensible for interaction with p59 (Fig. 26b). Two forms of MycΔEα were seen that probably corresponded to a form that contained the pro-region and one that lacked the pro-region (Fig. 26a).

Since p59's amino terminal PDZ domain is important for interaction with TGFα and since PDZ domains often recognize hydrophobic carboxy-terminal determinants, the ability of p59 to coimmunoprecipitate with the Δ158 mutant of TGFα was determined. This mutant lacks the two carboxy-terminal valines that are found in the cytoplasmic domain of the wild-type molecule. As mentioned in Chapter 2, the Δ158 mutant exhibits defects in its post-translational maturation. In the absence of DSP treatment, no detectable coimmunoprecipitation was observed between p59F and the Δ158 mutant (Fig. 27a), and at most, trace amounts of the mutant transmembrane TGFα coimmunoprecipitated with p59F after DSP treatment (Fig. 27b). Therefore, p59 interacts much more weakly with the Δ158 mutant than with wild-type TGFα and may not bind the mutant at all. This lack of coprecipitation was not due to different subcellular localizations of p59F and the Δ158 mutant. The mutant growth factor is synthesized and reaches the cell surface, although with a decreased efficiency (Fig. 4), and must therefore pass through the endoplasmic reticulum and the Golgi apparatus. In addition, coimmunofluorescence of the two molecules in transfected 293 cells

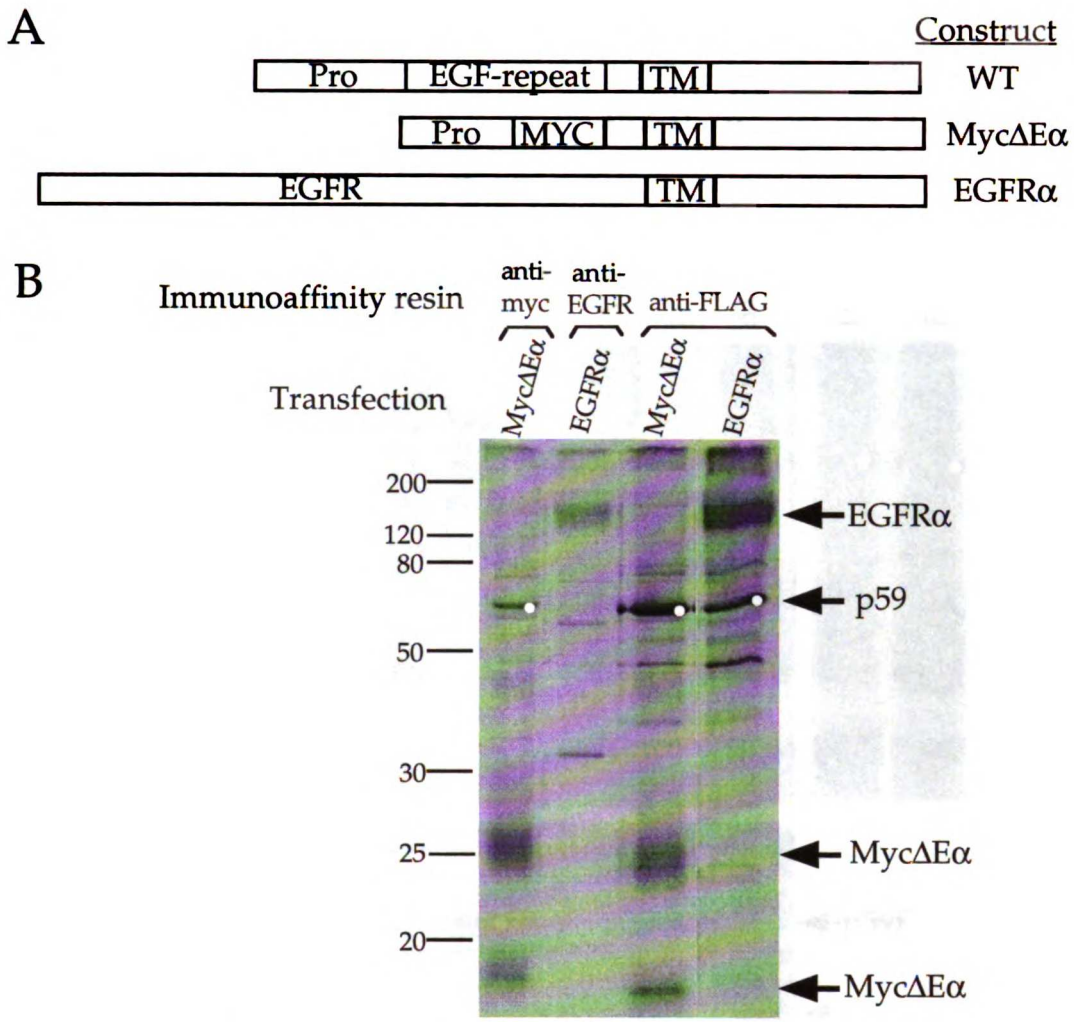

Figure 26. TGFα's extracellular domain is dispensible for association with p59

A. Schematic representation of mutants of TGFα's extracellular domain. MycΔEα is a cDNA in which the EGF repeat of TGFα has been replaced by a myc epitope-tag; the pro-region is retained. EGFRα is a cDNA in which TGFα's extracellular domain has been replaced by the extracellular domain of the epidermal growth factor receptor. TM refers to the transmembrane domain; WT refers to the wild-type growth factor. Diagrams are not to scale.

B. Coimmunopurification of p59F with TGFα mutants. 293 cells were cotransfected with expression plasmids for p59F and the indicated TGFα mutants. Cells were [^{35}S]-labeled, treated with DSP, and immunoaffinity purified as indicated. p59F contains a FLAG epitope-tag and was precipitated by the anti-FLAG resin. The larger form of MycΔEα likely contains the pro-region and the smaller form probably lacks the pro-region. Arrows indicate the positions of proteins. Dots within lanes indicate the positions of p59F.

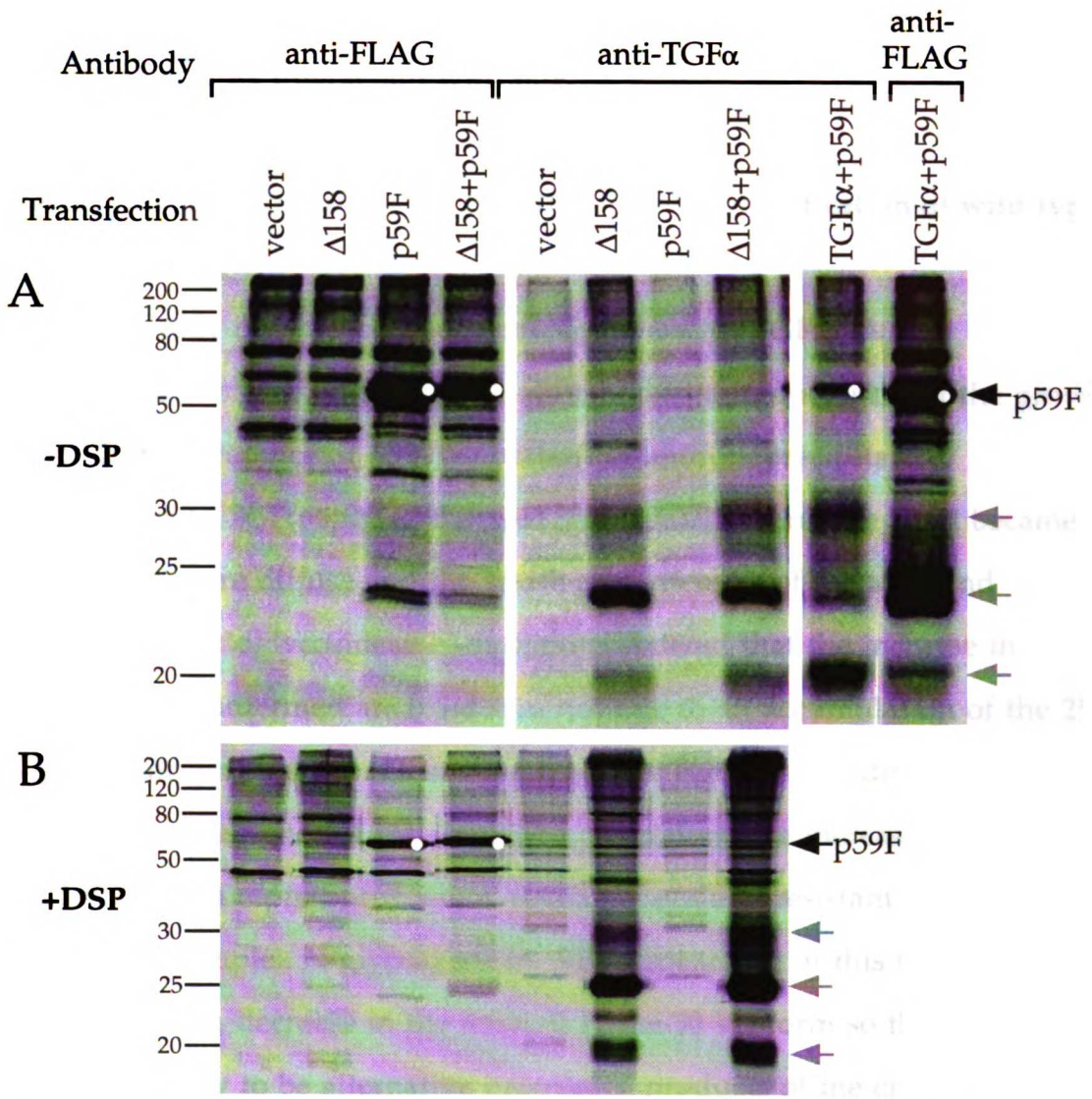

Figure 27. Interaction of p59 with wild-type transmembrane TGFα and the Δ158 mutant

293 cells were transfected with the the indicated expression plasmids and [^{35}S]-labeled. After treatment with DSP (panel B only), cells were lysed and then immunopurified using the indicated antibody resins. p59F contains a FLAG epitope-tag. Black arrows and white dots indicate the positions of p59F. Gray arrows indicate the positions of the mutant and wild-type transmembrane TGFα species. At most only a trace amount of the mutant growth factor copurified with p59F (B). In contrast, large amounts of wild-type transmembrane TGFα and p59F coimmunopurified (panel A, right two lanes).

demonstrated overlapping staining in the perinuclear region (Fig. 28). The carboxy-terminal valines of TGFα are therefore important for both its efficient maturation and its interaction with p59.

Overexpression of p59 alters the post-translational modification of wild-type and mutant TGFα

Not only did p59 coimmunoprecipitate with TGFα, but overexpression of p59 also led to an alteration in the processing of the growth factor. In the presence of p59, the pattern of immunoprecipitated transmembrane TGFα species changed (Fig. 29a). The middle band became broader and more diffuse and the width and intensity of the top band diminished. Endoglycosidase H digestions showed that the increase in intensity of the intermediate band was not due to an accumulation of the 25 kD precursor, since about the same amount of TGFα was endo H sensitive in the presence or absence of p59 (Fig. 29b). Therefore, the increased intensity in the 25 kD region represented a new species of endo H-resistant growth-factor with altered complex N-glycosylation. The appearance of this new form coincided with a decrease in the amount of the 30 kD form so these two species are likely to be alternative processing products of the endo H sensitive precursor. The ability of p59 overexpression to alter the processing of the precursor suggests that it may play a role in this pathway.

Although it did not coimmunoprecipitate with the Δ158 mutant, coexpression of p59 also had a functional consequence on the processing of the truncated growth factor. As demonstrated earlier, the Δ158 mutant accumulates in the initial 25 kD form (Fig. 4). When 293 cells were transiently transfected with either the Δ158 mutant alone or the Δ158 mutant and p59, the total amount of immunoprecipitated growth factor was similar,

anti-FLAG

anti-TGFα

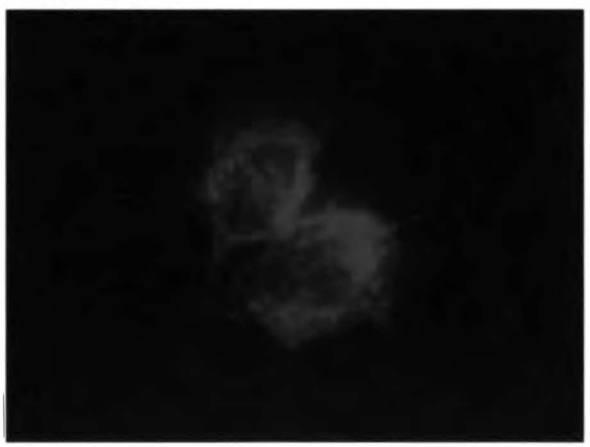

Figure 28. Coimmunofluorescence of p59 and the TGFα-Δ158 mutant

293 cells were cotransfected with expression plasmids for p59F and the Δ158 mutant, permeabilized, and stained as indicated.

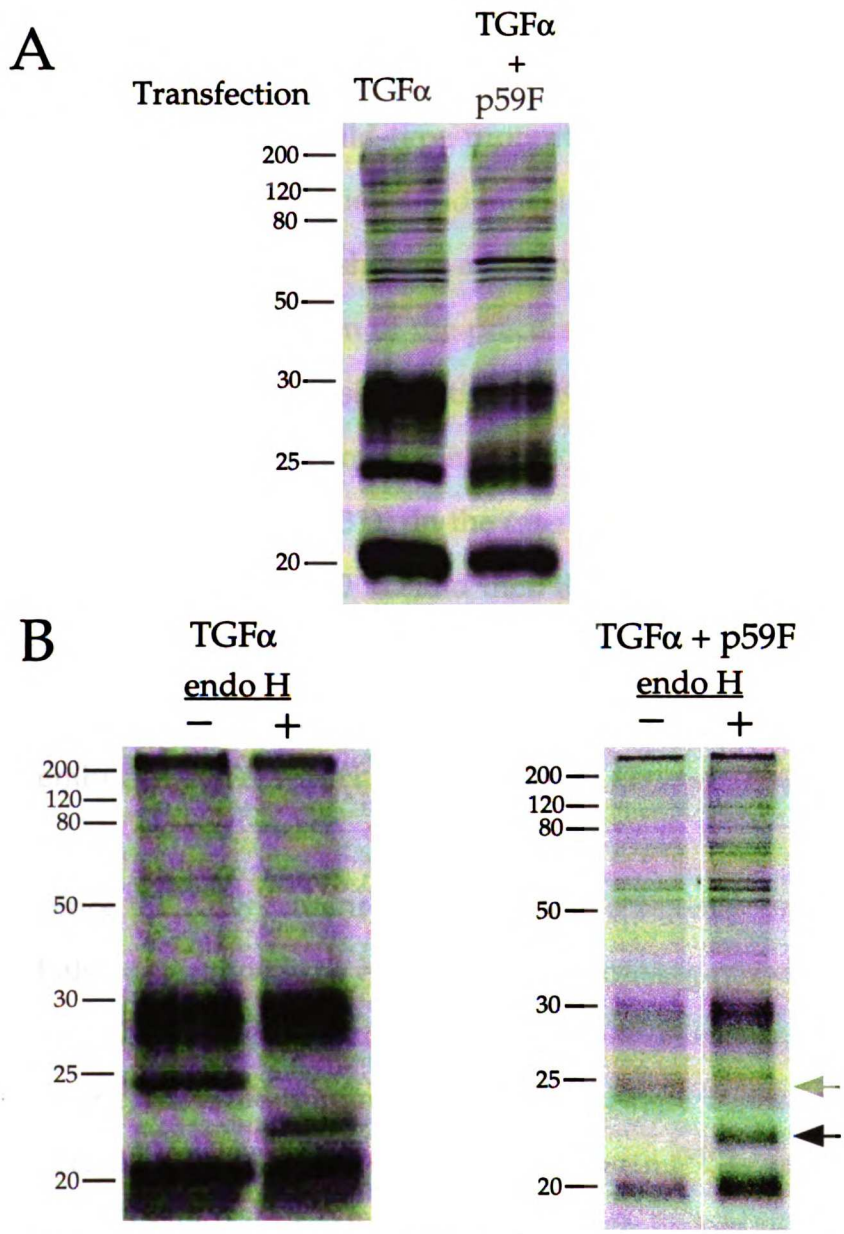

Figure 29. p59 alters the glycosylation of transmembrane TGFα

A. 293 cells were transfected with expression plasmids for the indicated proteins, [^{35}S]-labeled, lysed, and immunoprecipitated for transmembrane TGFα. The presence of p59 led to a decreased intensity of the top TGFα band and an increased intensity and broadening of the middle band.

B. 293 cells were transfected with TGFα alone (left panel) or TGFα and p59F (right panel), [^{35}S]-labeled, lysed, and immunoprecipitated for transmembrane TGFα. Immunoprecipitates were incubated with endo H as indicated. In the absence of p59, the 25 kD band was endo H sensitive. In the presence of p59, the middle band consisted of a mixture of endo H-sensitive (black arrow) and endo H-resistant forms (gray arrow). The presence of this new endo H-resistant form explains the broadening of the band seen in panel A.

yet the proportion of the 25 kD form in the latter case decreased (Fig. 30a). This decrease was statistically significant but did not return the ratio of transmembrane forms to wild-type proportions. An additional six independent transfections yielded identical results. Analysis of these data as a whole using a paired t-test showed a highly statistically significant (P=0.006) decrease in the accumulation of the precursor in the presence of p59. Pulse chase analysis of the processing of the Δ158 mutant indicates that this decrease was due to an enhanced conversion of the precursor to the other transmembrane forms (Fig. 31). In the case of wild-type, the 25 kD species was efficiently chased into the 30 kD and then the 20 kD forms. In contrast, the 25 kD form of the Δ158 mutant underwent little conversion into the other forms. However, in the presence of p59, more of the Δ158 mutant transmembrane TGFα was chased into the 30 kD and 20 kD forms at each chase point.

Since the Δ158 mutant is secreted less efficiently than wild-type growth factor, I also determined the effect of overexpressing p59 on this aspect of processing. 293 cells transfected with expression plasmids for the Δ158 mutant alone, the Δ158 mutant and p59F, wild-type TGFα and p59F, or wild-type TGFα alone were metabollically labeled with [^{35}S]-cysteine and methionine. Soluble and cell-associated growth factors were immunoprecipitated respectively from the culture medium and detergent lysates of the cells. The ratio of radioactive growth factor immunoprecipitated from the medium to that immunoprecipitated from cell-lysates was then calculated. This is a measure of secretion that normalizes for the amount of growth factor produced; simply measuring the amount of soluble TGFα released would not take into account differences in transfection efficiency or biosynthesis of the proteins. As expected, the ratio

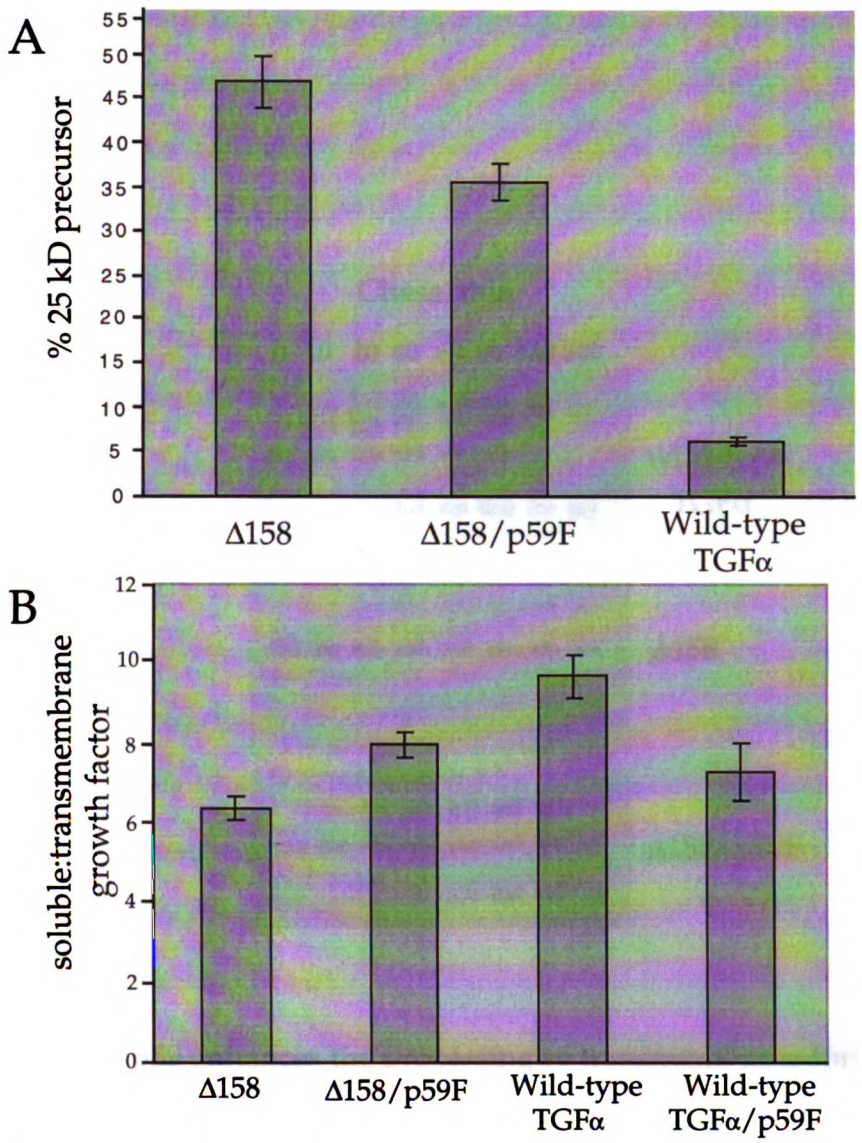

Figure 30. p59 partially suppresses the defect of the Δ158 mutant

293 cells were transfected with expression plasmids for the indicated proteins, [^{35}S]-labeled, and lysed. Transmembrane (A and B) and soluble TGFα (B) were immunoprecipitated, resolved by SDS-PAGE, and the radioactivity in the indicated species was quantitated by phosphorimaging. Results were calculated from at least three independent transfections.

A. Percentage of transmembrane TGFα in the 25 kD precursor form. This value was not calculated for wild-type TGFα coexpressed with p59F since an endo H-resistant form of the growth factor comigrated with the 25 kD, endo H-sensitive precursor under these conditions (Fig. 29).

B. Ratio of immunoprecipitated soluble to transmembrane TGFα.

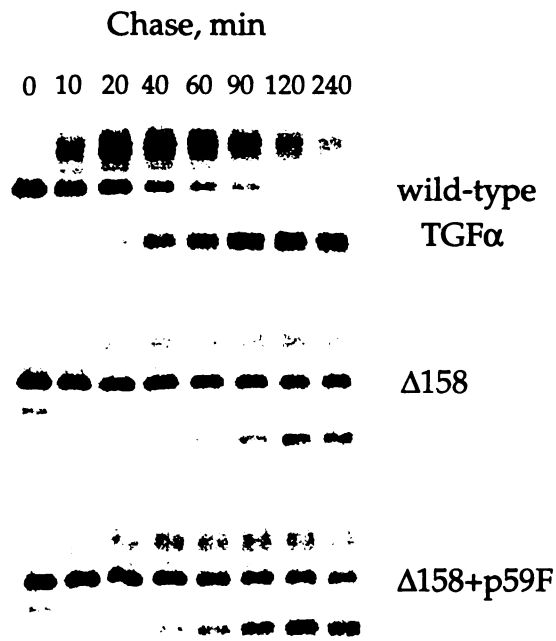

Figure 31. p59 enhances the processing of transmembrane forms of TGFα-Δ158

293 cells were transfected with expression plasmids for the indicated proteins, [^{35}S]-labeled for ten minutes, chased for the indicated times with unlabeled media, and then immunoprecipitated for transmembrane TGFα. At each chase point, a greater percentage of the mutant transmembrane TGFα was converted into the 30 kD and 20 kD forms in the presence of p59.

was higher for wild-type TGFα than for the mutant. In the presence of p59, this value decreased for wild-type TGFα, again indicating an perturbation of processing. In contrast, p59 increased the ratio of soluble to transmembrane growth factor for the Δ158 mutant, indicating an enhancement of secretion, although not to wild-type levels (Fig. 30b). This increased secretion likely arises as a downstream result of the enhanced maturation of the 25 kD precursor rather than a direct effect on proteolysis; p59 preferentially binds to the precursor and would be expected to have effects on this form rather than the transmembrane TGFα polypeptides at the cell surface.

Discussion

Several lines of evidence suggest that p59 plays a role in the maturation of the 25 kD TGFα precursor. For one, p59 preferentially associates with this form of the growth factor. In addition, the subcellular localizations of both proteins are consistent with such a role. Immunofluorescence showed significant amounts of both proteins in a perinuclear compartment that was consistent with Golgi localization; this organelle is a major site of protein sorting and modification.

More strikingly, overexpression of p59 altered the processing of the 25 kD forms of both wild-type TGFα and the Δ158 mutant. Processing was perturbed in the former case; glycosylation was altered and secretion was decreased. The reason for these effects and their significance are currently unclear. One possible explanation is that overexpression of p59 led to inappropriate binding to and retention of the growth factor within the cell.

In contrast, overexpression of p59 enhanced the maturation of the 25 kD form of the Δ158 mutant. This partial rescue of the mutant phenotype suggests that p59 acts in the same pathway that is perturbed in the mutant.

Since the carboxyl terminal valines of TGFα are required both for its efficient maturation and its coimmunoprecipitation with p59, the primary defect of Δ158 may be its inability to bind well to p59. If so, the overexpression of p59 may augment the maturation of the mutant growth factor by increasing the association of the two molecules. PDZ domains specifically recognize up to nine residues in their substrates (Songyang, et al., 1997), so the deletion of the two valines may not completely abolish interaction between p59 and TGFα. The effect of p59 overexpression on the processing of mutant forms of TGFα that absolutely cannot bind to p59 should therefore be examined.

The maturation of the endo H sensitive precursor involves carbohydrate modifications as well as transport from the endoplasmic reticulum through the Golgi apparatus. The p59-mediated enhancement of the secretion of the mutant TGFα precursor suggests that it acts in the latter process. Inhibition of N-glycosylation and the processing of N-linked glycoproteins have been reported to have little or no effect on TGFα secretion (Bringman, et al., 1987, Teixido and Massague, 1988), nor is O-glycosylation required for this process (Teixido, et al., 1990). Therefore, enhancement of glycosylation alone would not explain all effects of p59. In contrast, relief of a block in intracellular trafficking would lead to both increased processing of the precursor and increased generation of soluble growth factor. More protein would pass through the Golgi and be properly glycosylated and more would reach the plasma membrane and be proteolytically released.

The similarity between p59 and GRASP65 also suggests that p59 may play a role in trafficking. The second PDZ domain of p59 is almost identical to a domain in GRASP65 that has been shown to bind to the cis-Golgi matrix protein GM130 (Barr, et al., 1998). GM130 in turn binds to p115 (Nakamura, et al., 1997), a peripheral Golgi protein that acts in intercisternal transport at the

stage of vesicle docking (Barroso, et al., 1995, Waters, et al., 1992). p59, GM130, and p115 may therefore be components of a multiprotein trafficking complex. If so, they may selectively transport those cargo molecules, such as transmembrane TGFα, that are able to bind to the first PDZ domain of p59.

Transport of cargo proteins to the cell surface has been proposed to be a non-selective process whereby proteins are passively moved with the bulk-flow of lipids (Wieland, et al., 1987). However, recent evidence suggests otherwise; instead, it appears that transported proteins are specifically recognized by the trafficking machinery. Furthermore, some trafficking proteins apparently act only on a subset of cargo molecules. For instance, in budding yeast, components of the COPII coat of vesicles that mediate ER to Golgi transport selectively bind cargo proteins (Kuehn, et al., 1998). Combined deficiency of coagulation factors V and VIII arises from mutations in the intermediate compartment protein ERGIC-53 (Nichols, et al., 1998). In this syndrome, the plasma levels of these glycosylated factors are severely reduced without a decrease in the levels of all plasma proteins. Since ERGIC-53 has a lectin-like domain (Fiedler and Simons, 1994), it may recognize and sort proteins that have specific carbohydrate moieties. Similarly, p59 may utilize its initial PDZ domain to sort proteins that contain specific hydrophobic carboxy-terminal sequences.

Chapter 6: The Yeast Two-Hybrid Screen

Introduction

While coimmunoprecipitation yielded several candidate TGFα-interacting proteins, this technique probably failed to detect many others since it is a stringent procedure. For the purification of p59 and p86, chemical crosslinking was required to allow coprecipitation with TGFα. Therefore, associated molecules that lack crosslinkable residues in the proper geometry would not be identified by this procedure.

An alternative to biochemical purifications is the yeast two-hybrid screen. This method has been extensively used to detect proteins that associate with one another and relies on the reconstitution of transcriptional activation activity when a "bait" protein fused to a DNA-binding domain interacts with another hybrid consisting of a transcriptional activation domain fused to a "prey" protein. The resultant complex activates transcription of reporter genes, which is then scored.

This procedure has several advantages over biochemical purification. For one, the cDNA sequences of the candidate interacting clones can immediately be determined by DNA sequencing. In contrast, the cDNAs for interacting proteins that are detected and obtained by biochemical purification must still be isolated, based on directly determined peptide sequences, and then sequenced. Secondly, these analyses are performed under conditions that are more natural than *in vitro* assays of binding. Finally, the detection of protein-protein interactions is more sensitive than coimmunoprecipitations, and can be quantitated using enzymatic assays for β-galactosidase activity.

The two-hybrid screen also has several drawbacks. The screen usually yields many false-positive clones. Thus, all candidates must be evaluated for

their ability to interact using other methods. A major disadvantage is that the screen will not detect all interactions. One reason for this, and a factor that is particularly relevant to transmembrane TGFα, is that the screen detects associations between hybrid proteins rather than native ones, and these hybrids must be capable of entering the nucleus. Therefore, interactions with the transmembrane and extracellular domains of the growth factor are unlikely to be scored. The former would be insoluble and the latter would not be able to form disulfide bonds, which are essential for its structure. Thus, only the cytoplasmic domain can be used as bait, and only those proteins that associate with it can be detected. Even this domain may not work in the two-hybrid screen, since it is a very short, cysteine-rich sequence that may not fold properly when expressed as a fusion protein. It probably will not be palmitoylated either, a modification that may be important for its function. Finally, the large DNA-binding domain used in the fusion may occlude or decrease the accessibility of the short cytoplasmic sequence of transmembrane TGFα, thereby preventing potential interacting proteins from binding it.

I performed a two-hybrid screen to identify proteins that interact with the cytoplasmic domain of TGFα. Among the many proteins encoded by the cDNA clones obtained, filamin and Mer5 are likely to be physiologically relevant TGFα-binding proteins based on their known biological roles and their ability to coimmunoprecipitate with the growth factor.

Materials and Methods

Chemicals

Unless mentioned otherwise, all chemicals were purchased from Sigma, St. Louis, MO.

Plasmids

pASα was constructed by fusing a PCR product encoding the 39 amino acids comprising the cytoplasmic domain of transmembrane TGFα to the sequence coding for the GAL4 DNA-binding domain in the vector pAS1-CYH (a gift from Dr. Stephen Elledge, Baylor College). pAS-αΔC, a plasmid encoding a fusion between the GAL 4 DNA-binding domain and a truncated cytoplasmic domain of transmembrane TGFα lacking the carboxy-terminal 8 residues, was also constructed using PCR-based methods. Plasmids encoding GAL4 DNA-binding domain fusions to SNF, laminin, cdk2, and p53 were obtained from Dr. Stephen Elledge of Baylor College. The HeLa cell cDNA library was obtained from Clontech (Palo Alto, CA) and was amplified prior to use. Myc-tagged two-hybrid constructs were constructed by ligating the clone-specific inserts into the mammalian expression vector pRK5-Myc (Feng, et al., 1995).

Antibodies

The monoclonal antibody α1 mAb was previously described (Bringman, et al., 1987). The 9E10 anti-Myc antibody was a gift of Dr. J. M. Bishop (University of California, San Francisco) and was used for immunoprecipitations and Western blotting. The TGFα 1 monoclonal antibody was purchased from Oncogene Science (Cambridge, MA) and was used for Western blotting according to the manufacturer's protocol.

Yeast strains

Y190 was used in the initial two-hybrid screen. This strain is MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 + URA3::GAL-->lacZ, LYS2::GAL-->HIS3 cyh^r.

Y187 was used in secondary screens to eliminate clones that interacted with bait proteins unrelated to the cytoplasmic domain of TGFα. This strain is MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-->lacZ.

β-galactosidase assay

Cells were transferred to filter paper, flash-frozen in liquid nitrogen, soaked in assay buffer consisting of 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM β-mercaptoethanol, 0.01% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 120 mM sodium phosphate buffer, pH 7.0, and incubated at 30°C for 15 to 30 hours. At this point the yeast were scored for blue color, since β-galactosidase converts X-gal into blue products.

DNA hybridization

Clone plasmids were spotted onto nitrocellulose, prehybridized, and then hybridized with probe derived from random-prime labeling of unique insert sequence. After hybridization for 30 hours at 68°C, the blot was washed twice for fifteen minutes at room temperature with 2X SSC, 0.1% SDS and once for 30 minutes at 60°C with 0.1% SSC, 0.1% SDS. This was repeated a total of five times with separate probes.

Mammalian cell culture

COS-1 and 293 cells were grown in DMEM-H16, 3 g/l glucose (GIBCO BRL, Gaithersburg, MD), 10% fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with Lipofectamine (GIBCO-BRL) according to the manufacturer's instructions.

Metabolic labeling

Cells were grown to 80% confluence and labeled overnight with 160 μCi/ml [^{35}S]-cysteine-methionine protein labeling-mix (NEN, Boston, MA) in cysteine/methionine-free media with 10% dialyzed fetal calf serum.

Immunoprecipitation

Cells were lysed in buffer containing 50 mM TrisCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin (Buffer F). The lysate was cleared by centrifugation and subsequently incubated for one hour at 4°C with primary antibody and protein A-sepharose (Pharmacia, Piscataway, NJ) preloaded with rabbit anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). Beads were washed three times with Buffer F, resuspended in protein sample buffer, heated for 5 minutes at 100°C, and proteins were resolved with SDS-PAGE.

Results

The Two-hybrid screen

A bait construct, pASα was designed in which the DNA-binding domain of the yeast transcription factor GAL4 was fused to a sequence identical to the 39 residues that form the cytoplasmic domain of TGFα. This construct was introduced into *Saccharomyces cerevisiae* strain Y190, a histidine auxotroph that lacks GAL4 and that has the *lacZ* and His3 genes under GAL4 transcriptional control. The resultant strain required histidine for growth and did not express β-galactosidase, indicating that pASα was unable to activate transcription of the reporter genes by itself.

Since TGFα is highly expressed in HeLa cells (Derynck, et al., 1987), a library of HeLa cDNAs fused to the GAL4 activation domain was transformed into the reporter strain. GAL4 transcriptional activity would be reconstituted in cases where the TGFα and library fusion-proteins bound one another, leading to transcription of His3 and *lacZ*. In contrast to the parental strain, such yeast would be able to grow in the absence of histidine and would produce β-galactosidase. Eight hundred and sixty thousand transformants were obtained and selected for histidine prototrophy. The clones that grew in the absence of histidine were then screened for β-galactosidase production by incubation with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). In all, 91 of the colonies that grew without histidine also turned blue in the presence of X-gal, which indicated β-galactosidase production.

Secondary Screens

Several classes of false positives arise in the two-hybrid screen. One class is represented by clones that are able to activate transcription by themselves; these could be proteins that are able to independently bind to the GAL4 DNA-binding sites upstream of the reporter genes. Therefore, strains containing the library plasmids but lacking the TGFα plasmid were generated and assayed for β-galactosidase activity. One clone expressed *lacZ* and was discarded.

Another class of false-positives are those which require the presence of a DNA-binding domain hybrid, but which bind non-specifically to them. These "sticky" proteins would be expected to also bind other bait constructs unrelated to TGFα. To eliminate these, strains containing the library plasmids alone were mated to Y187 strains expressing fusions of the GAL4 DNA-binding domain and SNF, laminin, cdk2, and p53, respectively. None of these proteins share sequence homology with TGFα. The resultant diploids were assayed for β-galactosidase activity, and the 24 clones that expressed *lacZ* were discarded. The clones were also mated with a strain expressing the TGFα construct to reverify that the two proteins could interact. Sixteen of these did not express β-galactosidase and these were not pursued further.

To determine whether there was redundancy in the remaining clones, the library plasmids were recovered from the yeast. Restriction digests were performed on these plasmids, which were then grouped according to their restriction patterns. Representative plasmids from each category were spotted onto nitrocellulose and DNA hybridizations were performed using five of the inserts as probe. Based on the patterns of hybridization, the 50 remaining clones were reduced to 33.

Sequence of the clones

These 33 inserts were then sequenced and subjected to homology searches. Four of these inserts were represented more than once, leaving 29 unique clones. Eleven of these clones are novel, while the rest have already been cloned or are homologous to known genes (Table 1). Based on their functions, several of the known genes are unlikely to interact physiologically with the cytoplasmic domain of transmembrane TGFα. These include epithelin, which is an extracellular growth factor, RNA polymerase, NADH dehydrogenase, a cytochrome oxidase assembly factor, and cytochrome B-245 light chain. Such false positives are common in the two-hybrid screen.

Besides several unique polypeptides, the remaining characterized clones contain two families of proteins. The first, the thiol-specific antioxidant family includes clones 7, 12, and 13. In total this family made up 15 of the 91 clones isolated. The second family is composed of two members, clone 52 and clone 18, which are both similar to mannosyltransferases. Such polypeptides are involved in the biosynthesis of the carbohydrates that are used to glycosylate proteins. This process does not have an obvious connection to the cytoplasmic domain of TGFα, so these clones may also be false positives.

The last eight amino acids of TGFα's cytoplasmic domain are required for interaction with p86 and a kinase activity (Shum, et al., 1996). To determine if any of the two-hybrid clones specifically interacted with these residues, pAS-αΔC was constructed and transformed into strain Y187. This plasmid encodes a GAL4 DNA-binding domain/TGFα cytoplasmic domain fusion in which the last eight residues of transmembrane TGFα have been deleted. These yeast were then mated with cells containing the 29 positive

Table 1. Yeast Two-Hybrid Clones

clone	#times	homology
2	3	acetolactate synthase
4	1	epithelin: growth factor
5	2	cytochrome B-245 light chain
6	1	alpha tubulin
7	13	Mer 5
11	2	very low density lipoprotein receptor
12	2	Mer 5 homologue
14	1	novel
17	1	NAK1: orphan nuclear receptor
18	1	mannosyltransferase
19	2	tastin: cell adhesion molecule
20	1	novel
21	1	RNA polymerase II subunit
23	2	novel
28	1	filamin: actin binding protein
33	2	novel
35	1	novel
42	1	novel
45	1	TEGT: testis enhanced gene
46	1	OXA1L: cytochrome oxidase assembly
49	1	novel
52	1	Not56: mannosyltransferase
53	1	novel
54	1	novel
57	1	MCM: DNA replication factor
67	1	NADH dehydrogenase subunit
70	1	novel
73	1	novel
79	1	60s ribosomal protein

library-plasmids. Only clone 12 failed to activate transcription of the reporter genes, indicating that its interaction with the cytoplasmic domain of transmembrane TGFα was dependent on the carboxy-terminal eight residues.

Coimmunoprecipitations

Due to the high number of false positives, interactions found in the two-hybrid screen must be verified in mammalian cells. The best yet most stringent method is coprecipitation of the proteins from cells expressing both endogenous molecules or from transfected cells expressing both proteins. This, however, requires the isolation of cDNAs that contain full-length coding sequences. To bypass this labor-intensive procedure, I ligated the partial coding sequences for each of the candidate clones in-frame into the mammalian expression vector pRK5-Myc. This allows expression of the imcomplete polypeptide preceded with a start codon and an amino-terminal myc tag. Transfection of these cDNAs in COS-1 or 293 cells led to the expression of proteins that could be immunoprecipitated using the 9E10 anti-myc antibody (Fig. 32). Only twelve cDNA plasmids were chosen for subcloning, based on their derived polypeptide sequences.

These cDNA expression plasmids were subsequently cotransfected into 293 cells with or without an expression plasmid for TGFα and coimmunoprecipitation experiments were performed. Among the twelve clones, only the proteins derived from clone 7 (Mer5) and clone 28 (filamin) coimmunoprecipitated with transmembrane TGFα (Fig. 33). In both cases, the coprecipitation of the associated protein with transmembrane TGFα only worked when using the anti-myc antibody, but not the anti-TGFα antibody. Additionally, the forms of transmembrane TGFα that coimmunoprecipitated

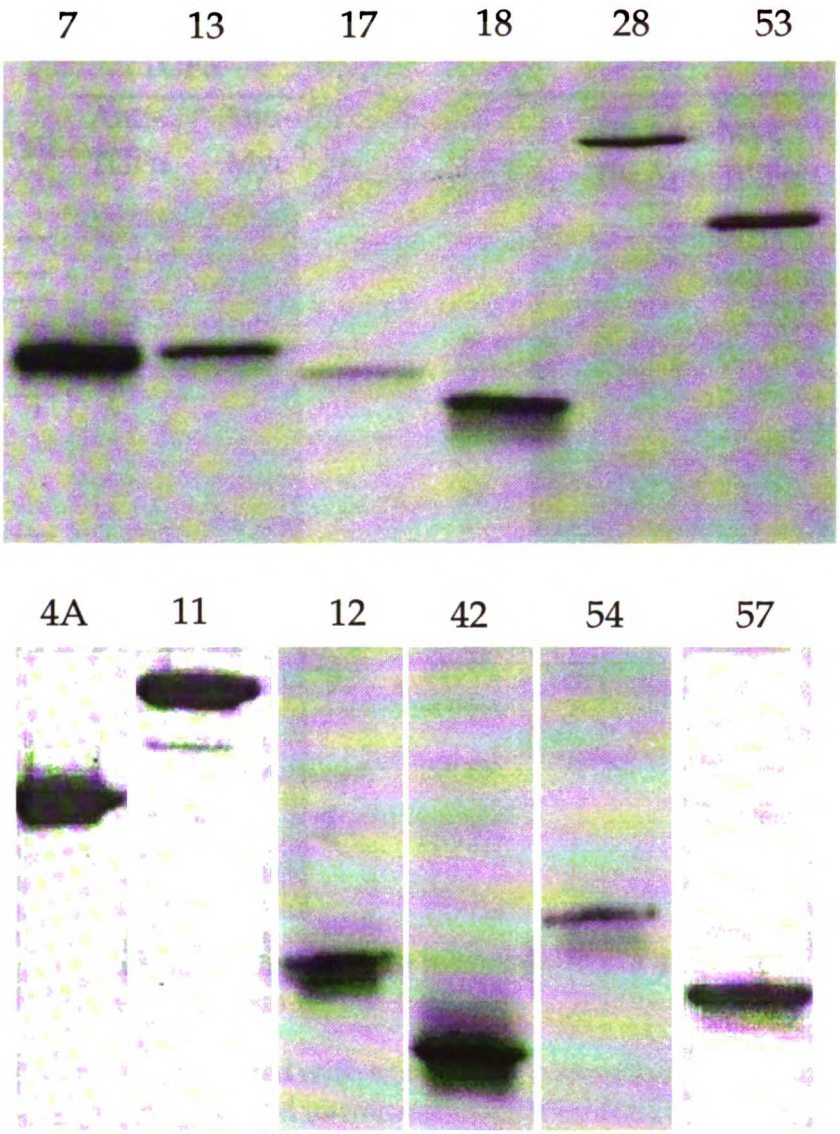

Figure 32. Expression of myc-tagged derivatives of the proteins encoded by yeast two-hybrid clones

The inserts from the indicated clones were ligated in-frame with the sequence encoding a myc-epitope tag in the mammalian expression plasmid pRK5myc. 293 cells were transiently transfected with these expression plasmids, [^{35}S]-labeled, and lysed. The recombinant proteins were then immunoprecipitated using the 9E10 anti-myc monoclonal antibody.

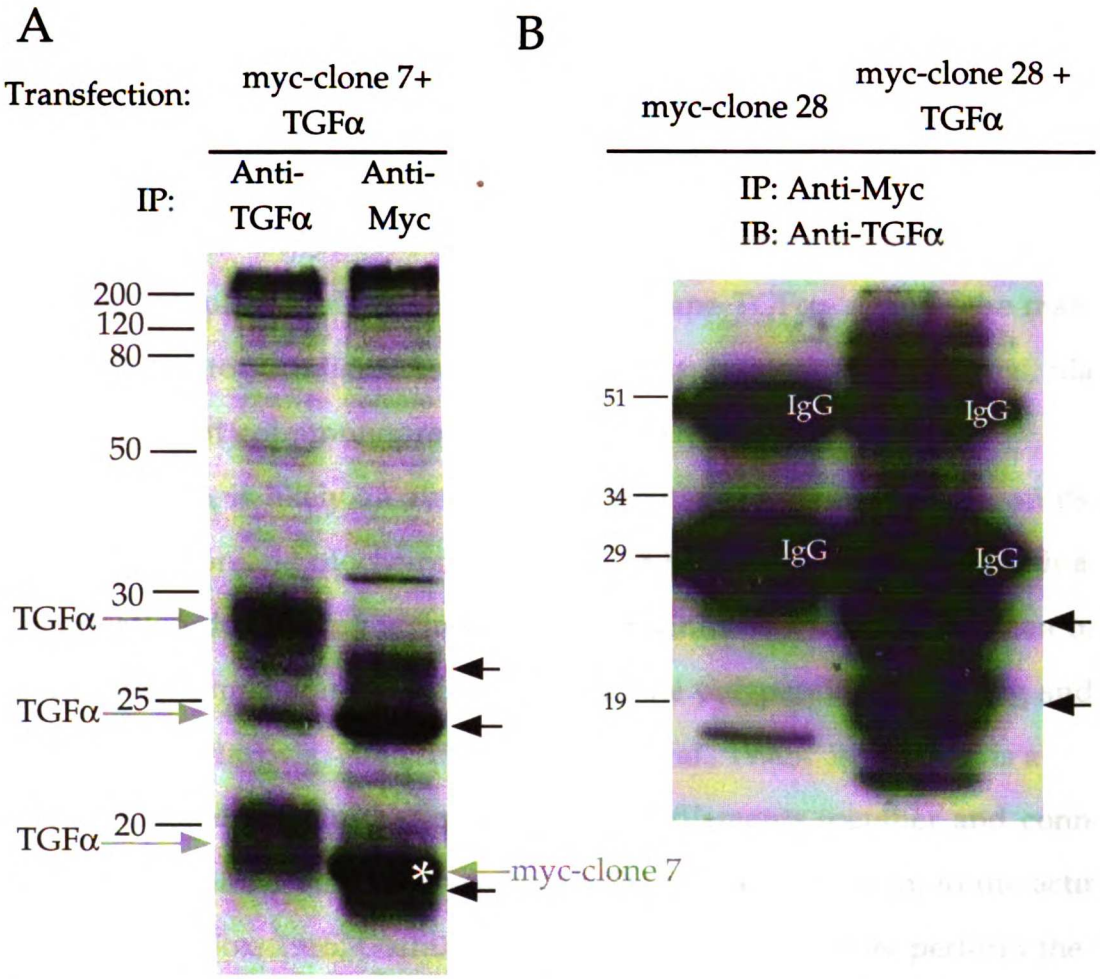

Figure 33. Coimmunoprecipitation of transmembrane TGFα with yeast two-hybrid clones

A. 293 cells were cotransfected with expression plasmids for TGFα and myc-clone7, [^{35}S]-labeled, and immunoprecipitated for TGFα or myc as indicated. The anti-myc antibody coprecipitated three proteins (arrows) which were similar in size to the three transmembrane forms of TGFα. Since these three bands were not present in myc-clone7 single transfections (Fig. 32), they likely represent TGFα–specific bands. The position of myc-clone7 is indicated with an asterisk.

B. 293 cells were transfected with an expression plasmid for myc-clone28 alone or with expression plasmids for myc-clone28 and TGFα as indicated, immunoprecipitated for myc, and then immunoblotted for TGFα.

Two proteins which migrated at the anticipated positions of the 25 kD and 20 kD forms of transmembrane TGF (arrows) were seen in anti-myc immunoprecipitations only when TGFα was coexpressed with myc-clone28. IgG light-chain obscured the region of the gel which would contain the 30 kD TGFα species.

with Mer5 in an anti-myc-immunoprecipitation migrated faster than those precipitated by the α1 monoclonal (Fig. 33a).

Discussion

I performed a yeast two-hybrid screen to identify proteins that interact with the cytoplasmic domain of transmembrane TGFα. Among the many cDNA clones for candidate associated proteins, two encoded proteins, filamin and Mer5, that coimmunoprecipitated with transmembrane TGFα.

Filamin, is likely an authentic interacting protein based both on its known function and biochemistry. It is a 280 kD modular protein, with a dimerization domain, an actin-binding domain, and a domain that can bind to the cytoplasmic domains of transmembrane polypeptides (Hartwig and Stossel, 1981, Hartwig, et al., 1980, Jammey, et al., 1990). As such, it is a multifunctional protein that crosslinks actin filaments together and connects membrane proteins such as glycoprotein Ib, a platelet integrin, to the actin cytoskeleton (Fox, 1985, Okita, et al., 1985). It could therefore perform the same role with transmembrane TGFα, especially since the portion of filamin encoded by the clone cDNA coimmunoprecipitates with proTGFα and corresponds to the domain that binds to transmembrane proteins (Gorlin, et al., 1990).

The second noteworthy candidate, Mer5, is a 27 kD protein that strongly interacts with TGFα in both the two-hybrid screen and in immunoprecipitation experiments. Mer5 and several close relatives, including clone 12, which interacts with proTGFα's carboxy-terminal eight amino acids, accounted for a large number of the positive clones obtained.

Mer5 is homologous to thiol-specific antioxidant, a protein that protects cells from reactive sulfur species (Chae, et al., 1994, Yim, et al., 1994).

They both contain two conserved cysteines that may form a disulfide bond and that are catalytically important. Since the TGFα cytoplasmic-domain bait has eight cysteines, the possibility exists that the binding between the two proteins is mediated by cysteine-cysteine interactions. This probable enzymatic function of Mer5 may also explain the increased electrophoretic mobility of the transmembrane TGFα that coimmunoprecipitates with it; Mer5 may change the oxidation state of some of the cysteines of the cytoplasmic domain of transmembrane TGFα thereby altering its migration.

The interaction of transmembrane TGFα and Mer5 is biologically plausible. Mer5 was originally identified as a house keeping gene whose expression in murine erythroleukemia cells increased when these cells were induced to differentiate (Yamamoto, et al., 1989). Like Mer5, TGFα is expressed in erythroid cells, raising the possibility that these two proteins may contribute to the differentiation of these cells. In addition, the expression of a Mer5-related gene, pag, is increased in serum-stimulated cells and may be induced during cellular proliferation (Prosperi, et al., 1993). Of course, TGFα stimulates proliferation, and its transcription can also be activated during this process.

Chapter 7: Future Experiments

In the course of its lifetime, a protein interacts with many other polypeptides. From its biosynthesis to its eventual degradation, protein-protein interactions play a central role in a polypeptide's lifespan and function, as it serves either as a substrate for protein machines or as a component of them. Transmembrane TGFα is no exception. Mutational analysis has shown that the growth factor's cytoplasmic domain is important for its maturation, internalization, and basolateral sorting (Briley, et al., 1997, Dempsey and Coffey, 1994, Shum, et al., 1994), so associated proteins are probably important for these functions. I have isolated two proteins, p59 and p86, that likely participate in the first two of these processes. In addition, using the yeast two-hybrid system, I have identified additional candidate TGFα interacting proteins that could have a number of different roles. This work is a first-step, as numerous questions and experiments remain. For instance, cDNAs for other coimmunoprecipitating proteins such as p106, p40, p35 and the kinase must still be isolated. The roles of the proteins as well as the functional significance of their interaction with transmembrane TGFα must be further characterized.

p59

While the overexpression of p59 can partially rescue the processing defect of the Δ158 mutant, the role of endogenous p59 in the normal processing of wild-type transmembrane TGFα has not been examined. To show that p59 plays a role in the maturation of transmembrane TGFα, processing of the growth factor should be examined in the absence of p59. Ideally, this would be accomplished by neutralizing p59 function in a cell line

that normally expresses it. Possibilities for this include antisense oligonucleotide abrogation of p59 mRNA expression, disruption of p59 protein function with dominant negative proteins, or microinjection of neutralizing antibodies. These approaches require the development of additional reagents. Alternatively, trafficking and modification of TGFα and the Δ158 mutant could be examined in a cell line that does not express p59, such as the F442A preadipocyte line. However, this approach is not ideal since other factors may perform p59's role in these cells. In either case, I predict TGFα's processing will be disrupted and more specifically, that the growth factor will accumulate in its 25 kD precursor form, leading to decreased cell-surface accumulation and decreased secretion.

p86

While Mem3 is a biologically plausible interacting protein, it must be shown to be identical to p86. As with any biochemical purification, the isolated protein may be a contaminant rather than the desired polypeptide. Therefore, expression studies and coimmunoprecipitation experiments must be performed to prove that it is a TGFα-associated protein. Coimmunoprecipitation experiments with a TGFα mutant that lacks the carboxy-terminal eight residues of the cytoplasmic domain will be especially informative, since this mutant does not coprecipitate with authentic p86 (Shum, et al., 1996).

If the cloned cDNA does encode genuine p86, additional experiments must be performed to determine its function. Mem3's yeast relative VPS35 acts in trafficking cargo to the vacuole so the mammalian protein may play a role in endocytosis, the homologous process. However, the yeast protein's exact biochemical role is unknown. If Mem3 truly binds to transmembrane

TGFα, this would suggest that it acts in the direct association and perhaps selection of cargo molecules. Subcellular localization studies might also give a clue to the role of p86. A protein involved in endocytosis would be expected to accumulate in areas such as coated pits, coated vesicles, endosomes, and lysosomes. In addition, experiments in which p86 function is disrupted by dominant negative interference or using antibody or antisense techniques would show whether it is important for trafficking or another process involving transmembrane TGFα.

Two-hybrid clones

While portions of filamin and Mer5 coimmunoprecipitate with TGFα, these interactions must be verified using full-length clones. In addition, the significance of their binding must be determined. Does filamin truly anchor TGFα to the actin cytoskeleton? Does Mer5 cooperate with TGFα during the differentiation of cells? Again, tissue expression patterns and subcellular localizations should be determined for these proteins and neutralization and overexpresssion experiments should be performed.

The remaining two-hybrid clones should also be characterized further. Even though they did not coimmunoprecipitate with transmembrane TGFα, such a requirement is extremely stringent and they remain excellent candidate interacting-proteins. Full-length clones should be obtained and used for additional *in vitro* and *in vivo* binding experiments. For the clones that do bind, experiments such as those outlined above should be performed to determine their biological roles as well as the significance of their interaction with TGFα.

The net result of this work will be a fuller understanding of transmembrane TGFα's role in the cell. Such knowledge will hopefully provide insights into the growth factor's contributions to normal cell physiology and to disease and development. In addition, these results might be applicable to other transmembrane growth factors and perhaps transmembrane molecules in general. Many of the processes, in which associated molecules likely act, such as trafficking and cytoskeletal anchorage, are clearly not limited to TGFα. Therefore, the study of these molecules in the context of transmembrane TGFα may shed light not only on the physiology of the growth factor, but also on these fundamental cellular processes.

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