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Transmembrane transforming growth factor-α tethers to the PDZ domain-containing, Golgi membrane-associated protein p59/GRASP55

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Transforming growth factor-α (TGF-α) and related proteins represent a family of transmembrane growth factors with representatives in flies and worms. Little is known about the transport of TGF-α and other transmembrane growth factors to the cell surface and its regulation. p59 was purified as a cytoplasmic protein, which at endogenous levels associates with transmembrane TGF-α. cDNA cloning of p59 revealed a 452 amino acid sequence with two PDZ domains. p59 is myristoylated and palmitoylated, and associates with the Golgi system, where it co-localizes with TGF-α. Its first PDZ domain interacts with the C-terminus of transmembrane TGF-α and select transmembrane proteins. p59 is the human homolog of GRASP55, which is structurally related to GRASP65. GRASP55 and GRASP65 have been shown to play a role in stacking of the Golgi cisternae *in vitro*. C-terminal mutations of transmembrane TGF-α, which decrease or abolish the interaction with p59, also strongly impair cell surface expression of TGF-α. Our observations suggest a role for membrane tethering of p59/GRASP55 to select transmembrane proteins, including TGF-α, in maturation and transport to the cell surface.

Keywords: GRASP55/membrane tethering/p59/
PDZ domain/transforming growth factor-α

Introduction

Cell behavior depends on communication between adjacent cells, which is often mediated by interactions between transmembrane proteins. One protein frequently serves as a signaling receptor and the other one as ligand, which activates receptor signaling. Several transmembrane ligands belong to the transforming growth factor-α (TGF-α) family of growth and differentiation factors, which interact with and activate transmembrane tyrosine kinase receptors (Derynck, 1992; Massagué and Pandiella, 1993; Lee *et al*., 1995). The signaling pathways that are induced by receptor activation have been well studied (Schlessinger and Ullrich, 1992; Hackel *et al*., 1999). In contrast, little is known about how the presentation of the ligand to the receptor is regulated. Genetic analyses in *Drosophila* and *Caenorhabditis elegans* have demonstrated the need for regulation and the existence of regulatory proteins for ligand presentation (Perrimon and Perkins, 1997; Mohgal and Sternberg, 1999), but the biochemical and cell biological characterization of this regulation have remained poorly explored. Using TGF-α as a model, we have been studying mechanisms and proteins that regulate the presentation of transmembrane TGF-α to its receptor.

TGF-α is expressed primarily by ectodermal and epithelial cells, and is the prototype of a growth factor family that includes Spitz and Gurken in *Drosophila* and Lin-3 in *C.elegans* (Derynck, 1992; Massagué and Pandiella, 1993; Lee *et al*., 1995). With few exceptions, all TGF-α family members are transmembrane proteins, which undergo a regulated cleavage of their extracellular domain to release the soluble growth factor (Massagué and Pandiella, 1993). Both the transmembrane and soluble forms of TGF-α are functional ligands, which activate the epidermal growth factor receptor (EGFR) (Brachmann *et al*., 1989; Wong *et al*., 1989), but their modes of interaction with the receptor may differ substantially. Transmembrane TGF-α is the predominant form of TGF-α in normal cells and tissues (Derynck, 1992; Lee *et al*., 1995), and its ectodomain cleavage can be induced by growth factor stimulation and activation of MAP kinase signaling cascades (Baselga *et al*., 1996; Fan and Derynck, 1999; Merlos-Suarez and Arribas, 1999).

TGF-α exerts, through its ability to activate the EGFR, a diversity of activities, although its primary ones are associated with its ability to induce cell proliferation and differentiation in epithelial and neuronal cells (Derynck *et al*., 1992; Lee *et al*., 1995). Increased TGF-α/EGFR signaling also contributes to malignant transformation and tumor development, especially of carcinomas (Khazaie *et al*., 1993). Considering its multiple activities in normal development and tumorigenesis, it is important to understand how the presentation of TGF-α is regulated, especially since it may also provide information on the regulation of other transmembrane growth and differentiation factors.

To study how the presentation of transmembrane TGF-α is regulated, we identified proteins that interact with transmembrane TGF-α. The functional importance of the 38 amino acid cytoplasmic domain of TGF-α is suggested by its stringent sequence conservation among species and the presence of many cysteines, which could be involved in cytoplasmic protein–protein interactions (Derynck *et al*., 1984; Lee *et al*., 1985). Our approach was to detect and identify endogenous proteins that associate with TGF-α and then to design methods for their large-scale purification. Sufficient quantities of TGF-α-associated proteins would then allow peptide microsequencing

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and cDNA cloning. This approach allows for identification of proteins that normally interact at endogenous levels with TGF-α. In contrast, other methods identify proteins with the ability to interact with TGF-α, and these proteins have to be verified for their interaction with TGF-α at endogenous levels.

Using this approach, we reported the association of a transmembrane protein (p106) and a cytoplasmic protein (p86) with transmembrane TGF-α (Shum *et al*., 1994). We now report the identification, purification and characterization of the predominant cytoplasmic protein to associate with transmembrane TGF-α. This protein, p59, has two PDZ domains and associates through its first PDZ domain with the C-terminus of the TGF-α cytoplasmic domain. Remarkably, p59 is the human homolog of the recently identified GRASP55, which has been proposed to play a role in stacking of the Golgi apparatus (Shorter *et al*., 1999). p59/GRASP55 is closely related to GRASP65, which similarly has been implicated in stacking of the Golgi membranes (Barr *et al*., 1997). GRASP65 interacts with GM130, another Golgi-associated protein (Barr *et al*., 1998), and GM130 interacts in turn with p115 (Nakamura *et al*., 1997). p115 and GM130 have both been implicated in vesicle transport within the Golgi system (Seemann *et al*., 2000). C-terminal deletions of transmembrane TGF-α that decrease or abolish the interaction of transmembrane TGF-α with p59/GRASP55 result in strongly decreased TGF-α levels at the cell surface. Our findings suggest that GRASP55 and GRASP65, and their associated proteins, may regulate the transport and presentation of a defined set of transmembrane proteins, such as transmembrane TGF-α.

Results

Detection of p59, a transmembrane TGF-α-associated protein

We previously identified p106 and p86 as proteins that interact specifically with transmembrane TGF-α (Shum *et al*., 1994) and therefore may play a role in its biological presentation or activity. Because of the low levels of endogenous TGF-α, their detection required the generation of stably transfected Chinese hamster ovary (CHO) cells, which express high levels of transmembrane TGF-α, yet lack endogenous EGFR. In addition, the interaction of these proteins with transmembrane TGF-α needed to be stabilized using the cell-permeable, reversible chemical cross-linker dithiobis-succinimidyl-proprionate (DSP) prior to immunoprecipitation using a monoclonal antibody against the ectodomain of TGF-α. The associated proteins were then de-cross-linked from transmembrane TGF-α, separated by SDS–PAGE and visualized by autoradiography of the ^{35}S-labeled proteins. p106 is a tyrosine-phosphorylated, transmembrane protein, whereas p86 is a cytoplasmic protein that interacts with the cytoplasmic domain of transmembrane TGF-α (Shum *et al*., 1994).

Since the antibody heavy and light chains precluded the identification of associated proteins with a similar mobility on SDS–PAGE gels, we coupled the α1 anti-TGF-α antibody to protein A–Sepharose. This modification allowed us to detect p59, which previously was not well visualized because of the presence of IgG heavy chains on the gel. As in the case of p106 and p86 (Shum *et al*., 1994),

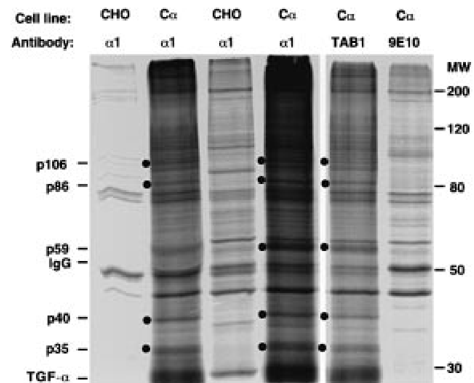


Fig. 1. Co-immunoprecipitation of associated proteins with transmembrane TGF-α. TGF-α-expressing CHO cells (Cα) or control CHO cells were ^{35}S-labeled, treated with the chemical cross-linker DSP and lysed. Samples were then immunoprecipitated (lanes 1 and 2) or immunoaffinity purified (lanes 3–6) using the monoclonal antibodies α1 or TAB1 against TGF-α, or 9E10 against a myc epitope as control, as indicated. Proteins were then resolved on SDS–PAGE. The positions of the TGF-α-associated proteins are indicated to the left of the panel and with bullets to the left of individual lanes. Note the detection of p59 in lanes 4 and 5, which relied on immunoaffinity purification, and is much less visible in the immunoprecipitation in lane 2. Only the largest, fully glycosylated TGF-α band is apparent on the autoradiogram.

p59 co-immunoprecipitated with transmembrane TGF-α when the TAB1 or the α1 anti-TGF-α monoclonal antibody was used. p59 was not detected in this way using CHO cells that did not express TGF-α, or when the 9E10 anti-myc antibody was used instead of an anti-TGF-α antibody. Besides p106, p86 and p59, two other proteins, p40 and p35, were also identified as endogenous proteins that associated specifically with transmembrane TGF-α. Among these, p59 was the predominant protein to co-precipitate with transmembrane TGF-α (Figure 1).

We further enriched p59 from contaminating proteins, which did not interact with transmembrane TGF-α, using Cibacron blue–Sepharose chromatography. p59 did not bind to Cibacron blue–Sepharose, while most contaminating proteins, as well as p106 and p86, did (Figure 2A). In addition, two-dimensional electrophoresis (isoelectric focusing followed by SDS–PAGE) of the anti-TGF-α-immunopurified proteins also resulted in substantial further purification of p59, as well as of p106 (Figure 2B).

Purification and cDNA characterization of p59

Two large-scale purifications allowed us to obtain p59 peptide sequences. For the first purification, TGF-α complexes were immunopurified from 8.8 m^2 of TGF-α-expressing CHO cells (2.5×10^9 cells), which had been treated with the cross-linker DSP. After de-cross-linking, the sample was purified further using Cibacron blue chromatography, followed by preparative SDS–PAGE (Figure 2C). The proteins were then transferred onto PVDF, stained with amido black, and the p59 band was excised, digested with trypsin and subjected to Edman degradation. Four peptide sequences were obtained. The second purification started from 15 m^2 of TGF-α-expressing CHO cells (4.3×10^9 cells). Following immunoaffinity purification, proteins were resolved by two-dimensional electrophoresis and visualized by copper staining (Figure 2D). The p59 protein was excised and eluted, and trypsin digestion followed by Edman degrad-

ation revealed two peptide sequences, one of which corresponded to a sequence from the first purification. All sequences from both purifications were later found in the full-size p59 protein sequence, deduced from the coding cDNA sequence (Figure 3A).

The peptide sequences allowed us to isolate and characterize cDNAs for the full-size p59 coding sequence. Screening of human expressed sequence tag (EST) databases with these sequences allowed the isolation of partial p59 cDNAs, and nested PCR-based cDNA cloning from a human cDNA library extended the sequence to the full-size coding cDNA sequence (Figure 3A). Partial and overlapping cDNA sequences were then ligated to obtain a contiguous coding sequence for human p59, which could be expressed in mammalian cells. Transfection of COS-1 cells with an expression plasmid for Flag-tagged p59 resulted in expression of a 59 kDa protein that migrated somewhat more slowly than endogenous p59, as predicted by the tag addition to the full-size p59 (see below). Therefore, even though no in-frame stop codon precedes the start codon, the proposed amino acid sequence corresponds to the full-size sequence.

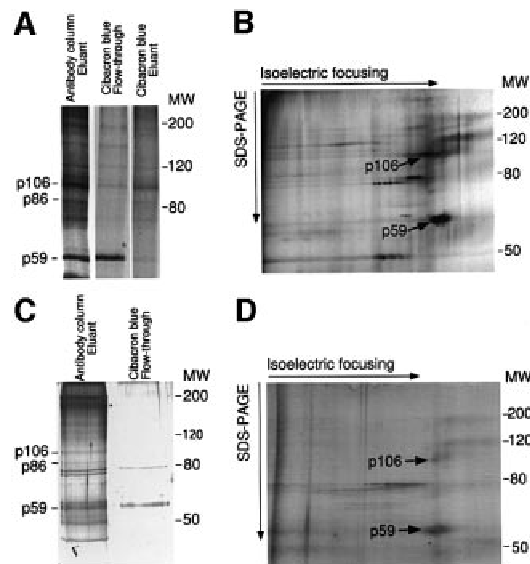


Fig. 2. Purification of p59. (**A**) Analytical Cibacron blue purification of p59. The left lane shows a ^{35}S-labeled Cα cell lysate sample following anti-TGF-α immunoaffinity purification (TGF-α ran off the gel) and prior to Cibacron blue–Sepharose. The next two lanes show p59 in the flow-through fraction but not in the eluant. (**B**) Analytical two-dimensional gel electrophoresis of p59. A similar immunoaffinity-purified sample to that in (A) was subjected to two-dimensional SDS–PAGE. As in (A), proteins are visualized by autoradiography. (**C**) Preparative Cibacron blue purification of p59. Unlabeled cell lysate starting from 18 m^2 of confluent Cα cells was purified by anti-TGF-α immunoaffinity chromatography and subjected to Cibacron blue–Sepharose. One percent samples of the eluant and flow-through fractions were analyzed by SDS–PAGE, and proteins were visualized by silver staining. (**D**) Preparative two-dimensional gel electrophoresis of p59. Cell lysate starting from 25 m^2 of confluent Cα cells was purified by anti-TGF-α immunoaffinity chromatography and then subjected to preparative two-dimensional gel electrophoresis. A 1% sample was analyzed and proteins are visualized by silver staining. The positions of p59, p86 and p106 are marked.

Predicted polypeptide sequence of p59

The predicted human p59 protein sequence is 452 amino acids long and contains all peptide sequences obtained from microsequencing (Figure 3A). The lack of a signal sequence or a hydrophobic sequence of significant length predicts it to be a cytosolic protein. The N-terminal 210 residues of p59 contain two domains with considerable sequence similarity and identity to each other (Figure 3B). These domains share significant homology in their N-termini with members of the htrA-like class of PDZ domains (Ponting, 1997), which are found primarily in prokaryotes (Figure 3C). The sequence following the PDZ domains of p59 has no apparent sequence motifs.

p59 has sequence similarity to GRASP65 (Figure 3D), a Golgi membrane-associated protein, which also has two PDZ domains in its N-terminal half (Barr *et al.*, 1997). GRASP65 is required for stacking of the cisternae in a cell-free system (Barr *et al.*, 1997) and is able to associate with the Golgi protein GM130 (Barr *et al.*, 1998). The sequence similarity between p59 and GRASP65 is predominant in the two PDZ domains, but sparse in the C-terminal half of the two proteins (Figure 3D). Recently, a GRASP65-related mouse protein, GRASP55, was identified and proposed also to be required for cisternal stacking of the Golgi apparatus *in vitro* (Shorter *et al.*, 1999). While GRASP65 localizes to the *cis*-Golgi (Barr *et al.*, 1997) and interacts with GM130 (Barr *et al.*, 1998), GRASP55 was present in the medial-Golgi and did not interact detectably with GM130 (Shorter *et al.*, 1999). The high degree of sequence identity with GRASP55 (Figure 3D) suggests that p59 is the homolog of mouse GRASP55.

p59/GRASP55 is myristoylated and palmitoylated

To start characterizing the p59 protein, we expressed p59 with a C-terminal Flag epitope tag. This Flag-tagged version migrated slightly more slowly than endogenous p59, detected in association with transmembrane TGF-α, which is consistent with the 2 kDa size of the Flag epitope sequence (Figure 4A).

GRASP65 has been shown previously to be myristoylated, and this type of fatty acylation is linked to a glycine at N-terminal position 2 (Barr *et al.*, 1997). Since this residue was also present in p59, we assessed whether p59 is myristoylated. As shown in Figure 4B, [^{3}H]myristic acid was incorporated during biosynthesis of p59. We also assessed whether p59 is modified by palmitoylation. This type of fatty acylation occurs at cysteines and contributes, similarly to myristoylation, to membrane anchoring of cytosolic proteins (Resh, 1999). As shown in Figure 4C, *in vivo* expressed p59 was palmitoylated.

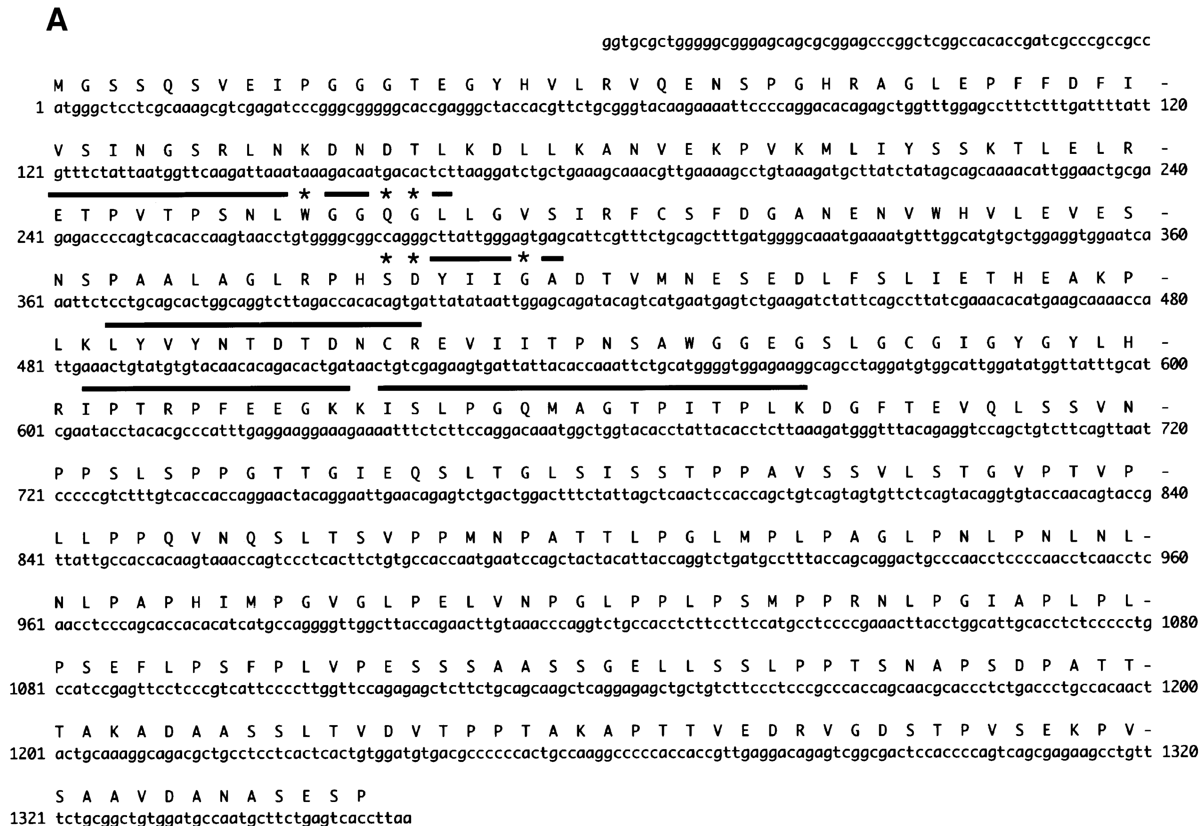
p59/GRASP55 co-localizes with TGF-α in the Golgi apparatus

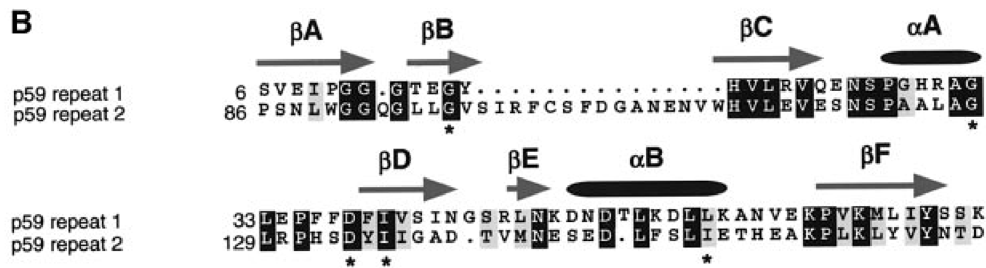
To assess the subcellular localization of p59 relative to TGF-α, we performed anti-Flag immunofluorescence in transfected HeLa cells, which expressed Flag-tagged p59 and TGF-α. As shown in Figure 5A, p59 and TGF-α co-localized with an intense perinuclear staining. Similar patterns of staining were also observed in MDCK and 3T3-F442A cells (data not shown). The immunolocalization of p59 coincided largely with the immunofluorescence staining of the Golgi-associated GM130 protein (Nakamura *et al.*, 1997), as shown in Figure 5B, and wheat germ agglutinin, another marker for the Golgi apparatus (Tartakoff and Vassalli, 1983; data not shown). We

therefore conclude that p59 is associated primarily with the Golgi apparatus, where it co-localizes with TGF-α. Finally, the p59 immunofluorescence pattern was different from that of BiP/GRP78, a marker for the endoplasmic reticulum (Takemoto *et al*., 1992; Figure 5C), further suggesting a distinct localization of p59 in the Golgi and not in the endoplasmic reticulum.

p59/GRASP55 associates with transmembrane TGF-α and preferentially with a processing intermediate

Previous results established that transmembrane TGF-α is expressed as multiple forms due to differential glycosylation and processing (Bringman *et al*., 1987). Three predominant forms were immunopurified using the anti-TGF-α antibody resin (Figure 6A). The middle form corresponds to the initial precursor, which contains endoglycosidase H (endo H)-sensitive N-glycosylation, and the largest form is derived from the middle one following modification of its glycosylation and is endo H resistant (Figure 6A; Bringman *et al*., 1987). Treatment of cells with deoxymannojirimycin prior to immunoprecipitation did not affect the mobility of the middle band (Figure 6A), confirming that it corresponds to a precursor with immature glycosylation. Deoxymannojirimycin blocks the action of mannosidase in the *cis*-Golgi and prevents maturation of N-linked carbohydrates (Bischoff *et al*., 1986). The smallest form is generated after removal





C

p59 repeat 1	16	EGY HVLRVQENSPGHRAGLEPFFDFIVSIN	
p59 repeat 2	96	LGVSIRF (8) NVWHVLEVESNSPAALAGLRPHSDYIIGAD	
Pseudomonas aeruginosa	272	LGVVIQE(14) SGALVAQLVEDGPAAKGGLQVG DVILSLN	U32853
Bartonella henselae	302	LGVQIQP(14) KGALITDPLK GPAAKAGIKAG DVIISVN	L20127
E. coli	398	EGAEMSN(4) QGVVVNNVKTGTPAAQIGLKKG DVIIGAN	P09376
E. coli	382	EGATLSD(7) KGIKIDEVVKGSPAAQAGLQKD DVIIGVN	P39099
E. coli	259	IGIGGRE(14) QGIVVNEVSPDGPAANAGIQVN DLIISVD	P31137

of the glycosylated prosequence and derives from the larger and middle forms (Bringman *et al*., 1987).

Since p59 was identified through its association with transmembrane TGF-α, we assessed whether recombinant p59 associated with transmembrane TGF-α. As shown in Figure 6B, immunoprecipitation of transmembrane TGF-α from transfected cell lysates co-precipitated p59. Conversely, immunoprecipitation of p59 resulted in co-precipitation of the three TGF-α forms, but the middle TGF-α band was strongly enriched, indicating a preferential association of p59 with this TGF-α form with immature glycosylation (Figure 6B). In contrast to endogenous p59, the association of transfected, Flag-tagged p59 with transmembrane TGF-α could be detected without chemical cross-linking (data not shown).

Consistent with the predicted intracellular localization of p59, the extracellular domain of transmembrane TGF-α was not required for interaction with p59. p59 co-precipitated with chimeric proteins in which either a myc epitope tag or the EGFR extracellular domain replaced the ectodomain of transmembrane TGF-α (Figure 6C).

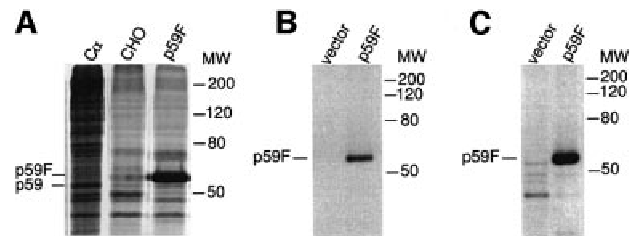


Fig. 4. p59 is myristoylated and palmitoylated. (**A**) Immunoprecipitation of Flag-tagged p59 (p59F) from transfected cells in comparison with control transfected cells and TGF-α-expressing CHO cells (Cα) in which endogenous p59 was co-immunoprecipitated with TGF-α using an anti-TGF-α antibody (lane 1; as in Figures 1 and 2A). (**B**) *In vitro* translation of p59 in the presence of [^{3}H]myristic acid and immunoprecipitation shows that p59 is ^{3}H-myristoylated. (**C**) *In vivo* labeling of transfected cells using [^{3}H]palmitic acid, followed by immunoprecipitation, shows that p59 is ^{3}H-palmitoylated.

D

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p59        1  MGSSQSVEIPGGGTEGYHVLRVQENSPGHRAGLEPFFDFIVSINGSRLNKDNDTLKDLLK
GRASP55    1  MGSSQSVEIPGGGTEGYHVLRVQENSPGHRAGLEPFFDFIVSISGSRLNKDNDTLKDLLK
GRASP65    1  MGLGASSEQPAGG.EGFHLHGVQENSPAQQAGLEPYFDFIITIGHSRLNKENDTLKALLK

p59       61  ANVEKPVKMLIYSSKTLELRETPVTPSNLWGGQGLLGVSIRFCSFDGANENVWHVLEVES
GRASP55   61  ANVEKPVKMLIYSSKTLELREASVTPSNLWGGQGLLGVSIRFCSFDGANENVWHVLEVES
GRASP65   60  ANVEKPVKLEVFNMKTMRVREVEVVPSNMWGGQGLLGASVRFCSFRRASEHVWHVLDVEP

p59      121  NSPAALAGLRPHSDYIIGADTVMNESEDLFSLIETHEAKPLKLYVYNTDTDNCREVIITP
GRASP55  121  NSPAALAGLRPHSDYIIGADTVMNESEDLFSLIETHEAKPLKLYVYNTDTDNCREVIITP
GRASP65  120  SSPAALAGLRPYTDYIVGSDQILQESEDFFTLIESHEGKPLKLMVYNSESDSCREVTVTP

p59      181  NSAWGGEGSLGCGIGYGYLHRIPTRPFEEGKKISLPGQMAGTPITPLKDGFTEVQLSSVN
GRASP55  181  NSAWGGEGSLGCGIGYGYLHRIPTRPFEEGKKISLPGQMTGTPITPLKDGFTQVQLSSVS
GRASP65  180  NAAWGGEGSLGCGIGYGYLHRIPTQPSSQYKK...P.PSASSPGTPAKTPQPNAFPLGAP

p59      241  PPSLSPPGTTGIE.QSLTGLSISSTPPAVSSVLSTGVPTVPLLP....PQVNQSLTSVP.
GRASP55  241  PPSLSPPGTAGVE.QSLSGLSISSAPPAVSNVLSTGVPTVPLLP....PQVNQSLASVP.
GRASP65  236  PPWPIPQDSSGPELGSRQSDYMEALPQVPGGFMEEQLPG.PGSPGHGTADYGGCLHSMEI

p59      295  PMNPATTLPGLMPLPAGLPNLPNL....NLNLP.APHIMPGVGLPELVNPGLPP..LPSM
GRASP55  295  PMNPAATLPSLMPLSAGLPNLPNLPSLSNFNLP.APHIMPGVGLPELGKPGLPP..LPSL
GRASP65  295  PLQPPPPVQRVM..DPGFLDVSGMSLLDSNNTSVCPSLSSSSLLTPTAVSALGPEDIGSS

p59      348  PPRNLPGIAPLPLPSEFLPSFPLVPESSSAASSGELLSSLPPTSNAPSDPATTTAKADAA
GRASP55  352  PPRNVPGIAPLPMPSDFLPSFPLVPEGSSAASAGEPLSSL.PAMGPPSDPVMTTAKAD.T
GRASP65  353  TSSHERGGEATWSGSEFEISFPDSP.GSQAQVDHLPRLTLPDGLTSAASP....EQGLSA

p59      408  SSLTVDVTPPTAKAPTTVEDRVGDSTPVSEKP.VSAAVDANASESP
GRASP55  410  SSLTVDVMSPASKVPTTVEDRVSDCTPAMEKP.VSAVTDANASGAS
GRASP65  408  ELLEAQTEEPHTRSACIAWHKLRGHPANSRLPHIQSLGCVKAPGDIWCSLAVYLSSCSLY

GRASP65  468  RGMGFATVHMYSWIERNRTLEQCPASIEAGDGSNVSVKHWHLPGRERLQARHNVHMKMGW

GRASP65  528  GTRGCVHKRPHWYRGAPRIPMPFLILILTLDERSSILGHLISRMEDSGPFRGTCLC
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Fig. 3. Polypeptide sequence of human p59. (**A**) cDNA sequence and corresponding amino acid sequence of human p59. The two PDZ domains are underlined. Peptide sequences obtained from microsequencing are overlined, with asterisks above ambiguous residues. (**B**) Sequence alignment of the two PDZ domains. Identical (black boxes) and similar (shaded boxes) residues are marked. The asterisks mark highly conserved residues among PDZ domains. The predicted α-helices and β-sheets, characteristic for PDZ domains, are shown by overlining the corresponding amino acids. (**C**) Alignment of the N-terminal two-thirds of the two PDZ sequences of p59 with the corresponding sequences in other htrA-like PDZ domains. The DDBJ/EMBL/GenBank accession numbers of these sequences are shown to the right. (**D**) Comparison of the human p59 amino acid sequence with the mouse GRASP55 and GRASP65 sequences. Black boxes show identical residues, while similar residues are shaded.

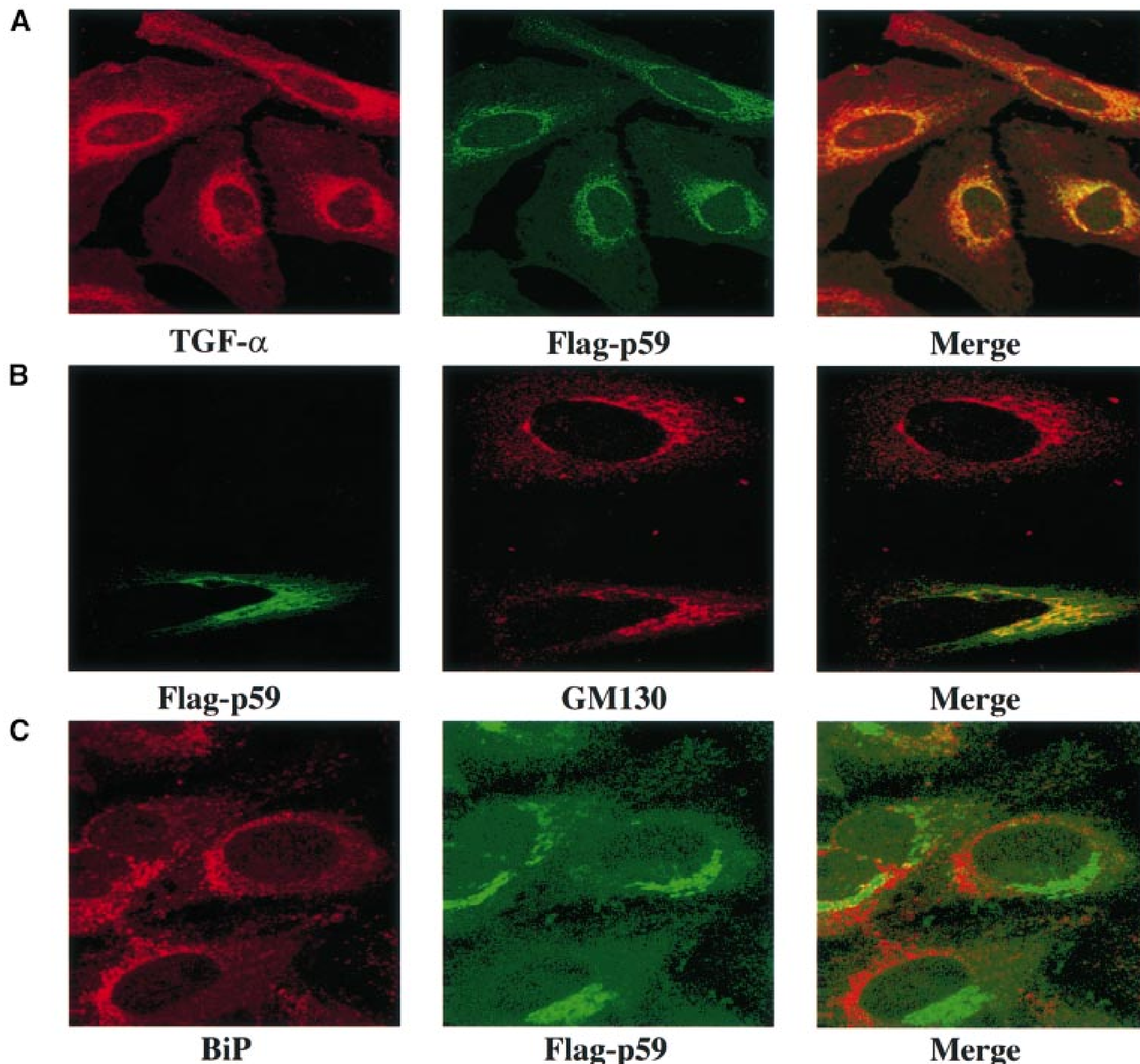

Fig. 5. Subcellular localization of p59, as assessed by immunofluorescence, in transfected HeLa cells. (**A**) Immunofluorescent localization of TGF-α or Flag-tagged p59 in permeabilized cells, showing that p59 and TGF-α co-localize. (**B**) Immunofluorescent detection of Flag-tagged p59 or the endogenous Golgi marker GM130. Note that only one of the two cells shown was transfected to express Flag-p59. These results strongly suggest that p59 and GM130 co-localize in the Golgi. (**C**) Immunofluorescent detection of Flag-tagged p59 or endogenous BiP/GRP78, a resident protein of the endoplasmic reticulum. The data show that p59 does not localize in the endoplasmic reticulum.

p59/GRASP55 associates through its first PDZ domain with the hydrophobic C-terminus of transmembrane TGF-α

We next defined the sequence of transmembrane TGF-α with which p59 interacts. Deletion of the cytoplasmic domain in the TGF-αΔ122 mutant (Shum *et al.*, 1994, 1996) abolished the interaction, which is consistent with the intracellular localization of p59 (Figure 7A). The cytoplasmic domain of transmembrane TGF-α contains a cysteine pair at positions 153 and 154, which is palmitoylated and followed by six amino acids ending in Thr-Val-Val. Deletion of these six amino acids in the Δ152 mutant (Shum *et al.*, 1996) also abolished its ability to interact with p59 (Figure 7A).

We also determined the sequence in p59 that interacts with transmembrane TGF-α. Among the deletion mutants, a p59 derivative that lacks the two PDZ domains was unable to associate with TGF-α (data not shown). In addition, deletion of the first PDZ domain in p59ΔPDZ1 also abolished the interaction with transmembrane TGF-α (Figure 7B). Since the two PDZ domains do not overlap and are therefore structurally independent of each other, this result suggests that the N-terminal PDZ domain mediates the interaction with the C-terminus of TGF-α. Accordingly, the first PDZ domain of p59, when expressed as a separate polypeptide, interacted with transmembrane TGF-α (Figure 7B). We were unable to express the second PDZ domain by itself.

Specificity of association of p59/GRASP55 with transmembrane proteins

To characterize further the interaction of p59 with transmembrane TGF-α, we assessed the ability of another PDZ domain protein to interact with transmembrane TGF-α. NHERF/EBP50 (Weinman *et al.*, 1995; Reczek *et al.*, 1997) has two PDZ domains in its N-terminal half

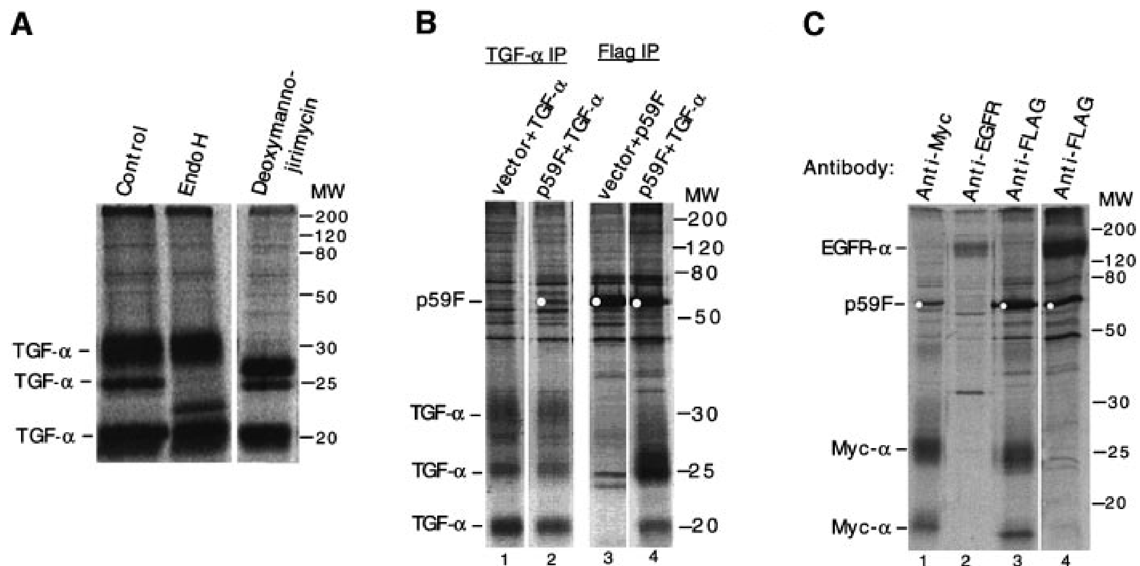

Fig. 6. p59 associates preferentially with an immaturely glycosylated form of transmembrane TGF-α. (**A**) TGF-α-expressing CHO cells (Cα) were ^{35}S-labeled and transmembrane TGF-α was immunoprecipitated. The sample in the second lane was treated with endo H after immunoprecipitation, while the sample in the third lane was obtained from cells grown in the presence of deoxymannojirimycin. (**B**) Co-immunoprecipitation of p59 and transmembrane TGF-α from transfected, ^{35}S-labeled 293 cells. Lanes 1 and 2: anti-TGF-α co-precipitates p59 (lane 2), but p59 is not detected, when cells are not transfected for Flag-tagged p59 expression (lane 1). Note the ratios of the three TGF-α forms. Lanes 3 and 4: immunoprecipitation of Flag-tagged p59 co-precipitates TGF-α. In comparison with the anti-TGF-α immunoprecipitations in lanes 1 and 2, the second TGF-α band, which is a processing intermediate, is highly enriched. No TGF-α was detected in lane 3, in which the cells were not transfected with a TGF-α expression plasmid. (**C**) Replacement of the ectodomain of transmembrane TGF-α with another ectodomain does not affect p59 association. 293 cells were co-transfected with cDNAs for Flag-tagged p59 and TGF-αΔE, a chimera in which the ectodomain of TGF-α has been replaced by a myc epitope tag (lanes 1–3), or EGFR-α, a chimera in which the ectodomain of TGF-α has been replaced by the ectodomain of EGFR (lane 4). Cells were ^{35}S-labeled, lysed, and samples were immunopurified using the Sepharose-conjugated antibodies, as indicated.

and a similar molecular weight to that of p59, and has been shown to interact with the cytoplasmic terminus of the β2-adrenergic receptor (Hall *et al*., 1998; Cao *et al*., 1999). As shown in Figure 8A, EBP50 was unable to interact with transmembrane TGF-α. Finally, we also assessed the ability of p59 to interact with other transmembrane proteins. As shown in Figure 8B, p59 associated with two other single transmembrane proteins, membrane-type matrix metalloproteinase 1 (Sato *et al*., 1994) and kit ligand (Flanagan *et al*., 1991), which, similarly to transmembrane TGF-α, have a C-terminal valine. p59 did not interact with L-selectin, which has a C-terminal tyrosine (Siegelman and Weisman, 1989).

Impaired interaction of p59/GRASP55 with transmembrane TGF-α mutants results in strongly decreased TGF-α levels at the cell surface

The interaction of p59 with the hydrophobic C-terminus of transmembrane TGF-α in the Golgi suggested a possible role for p59 in transport of TGF-α to the cell surface. We therefore evaluated whether transmembrane TGF-α mutants with impaired interaction with p59 showed differences in cell surface levels of transmembrane TGF-α in transfected CHO cells. Three versions of transmembrane TGF-α were evaluated (Figure 9). Consistent with Figure 7A, p59 interacted with wild-type transmembrane TGF-α, but not or only barely detectably with the Δ152 mutant of TGF-α, which lacks the C-terminal eight amino acids (Figure 9A). Deletion of the C-terminal two amino acids of transmembrane TGF-α in the Δ158 mutant showed a much decreased interaction of p59 when compared with wild-type transmembrane TGF-α (Figure 9A). Cell surface biotinylation followed by anti-TGF-α immunoprecipitation of transfected CHO cells allowed us to assess the transmembrane TGF-α levels at the cell surface. While wild-type TGF-α was easily detectable at the cell surface, no TGF-αΔ152 was detected and only a very low level of TGF-αΔ158 was apparent at the cell surface (Figure 9B), even though the mutations did not drastically affect the total levels of transmembrane TGF-α in the cell lysates (Figure 9C). We therefore conclude that the cell surface levels of transmembrane TGF-α correlate with its ability to interact with p59.

Discussion

To gain insight into the mechanisms that regulate the presentation and processing of transmembrane TGF-α, we identified and purified proteins which, at the endogenous level, associate with transmembrane TGF-α. Since the endogenous TGF-α levels of epithelial cells are too low to allow such an approach, we generated CHO cells, which overexpress TGF-α and have high levels of transmembrane TGF-α at the cell surface. CHO cells lack EGFR expression (Livneh *et al*., 1986) and, therefore, TGF-α cannot interact with its receptor. While the purification of endogenously interacting proteins is extremely labor intensive, this approach has the advantage that we know that the associated proteins naturally interact with transmembrane TGF-α. In contrast, yeast two-hybrid screening or glutathione *S*-transferase (GST)-based adsorption chromatography identify only candidate proteins, and it still remains to be shown whether or not they associate naturally at the endogenous level. In addition, the seven cysteines in the 38 amino acid short cytoplasmic domain (Derynck *et al*., 1984) and the

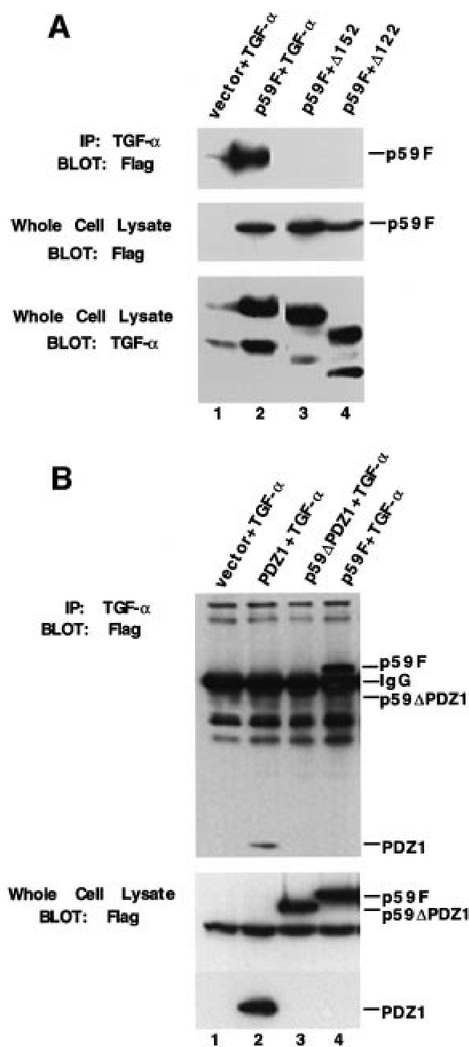

Fig. 7. p59 associates through its first PDZ domain with the C-terminal sequence of transmembrane TGF-α. (**A**) The C-terminal sequence of transmembrane TGF-α interacts with p59. CHO cells were transfected with Flag-tagged p59 (lanes 2–4) and full-size TGF-α (lanes 1 and 2), TGF-αΔ152 (lane 3) or TGF-αΔ122 (lane 4). Immunoprecipitation with anti-TGF-α, followed by anti-p59 western blotting (top panel), showed that only full-size TGF-α, but not TGF-αΔ152 or TGF-αΔ122, associated with p59. The middle and lower panels show expression of Flag-tagged p59 and transmembrane TGF-α, as assessed by western blotting. (**B**) p59 interacts with transmembrane TGF-α through its first PDZ domain. CHO cells were transfected to express transmembrane TGF-α and PDZ1, a segment of p59 containing the first PDZ domain (lane 2), p59ΔPDZ1, a p59 mutant that lacks the first PDZ domain (lane 3), or full-size p59 (lane 4). Anti-TGF-α immunoprecipitation followed by western blotting for Flag-tagged p59 revealed the association of full-size p59 and PDZ1, but not of p59ΔPDZ1. The lower panel shows the expression levels of p59 and its mutants, as assessed by anti-Flag western blotting.

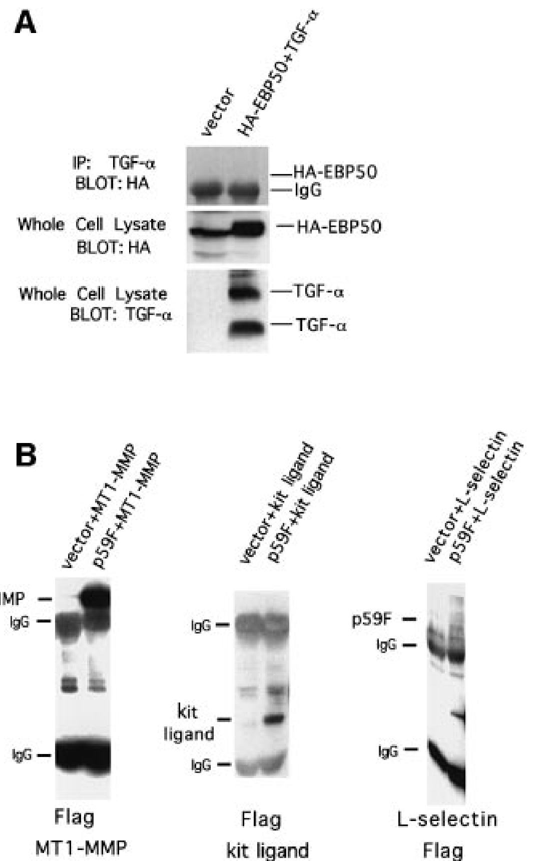

Fig. 8. Specificity of the interaction of p59 with transmembrane TGF-α. (**A**) Cells were transfected with expression plasmids for TGF-α and HA-tagged EBP50. Immunoprecipitation with anti-TGF-α followed by an anti-HA western blot (top panel) did not reveal TGF-α-associated EBP50, even though EBP50 and TGF-α were expressed, as assessed by western blots (middle and lower panels). (**B**) p59 interacts with matrix metalloproteinase 1 (MT1-MMP) and kit ligand, but not L-selectin. 293 cells were transfected with the indicated expression plasmids. In the left and middle panels, anti-Flag immunoprecipitation for p59, followed by western blotting for MT1-MMP or kit ligand, respectively, indicated the association of either transmembrane protein with p59. In the right panel, immunoprecipitation for L-selectin, followed by anti-Flag western blotting for p59, did not detect associated p59.

palmitoylation of two of them (Shum *et al*., 1996) make GST adsorption chromatography and yeast two-hybrid screening less reliable.

We identified p59 as the major endogenous protein associated with the cytoplasmic domain of TGF-α. p59 contains two PDZ domains with structural similarity to each other, followed by ~250 amino acids without structural motifs. PDZ domains frequently interact with hydrophobic C-termini with the consensus sequence S/T/Y–X–V/I-COO$^-$ (Ranganathan and Ross, 1997; Songyang *et al*., 1997). The two PDZ domains of p59 have considerable sequence similarity over their entire length, and resemble bacterial htrA repeats. htrA-like PDZ domains are distinguishable from other PDZ domains primarily through sequence similarity in their N-terminal halves (Ponting, 1997). In addition, the PDZ domains of p59 are also closely related to two PDZ domains of unknown function, one in *Saccharomyces cerevisiae* and another in *Schizosaccharomyces pombe* (data not shown).

Transmembrane TGF-α interacts through its C-terminus with the first PDZ domain of p59. The cytoplasmic domain of TGF-α ends with Ser-Glu-Thr-Val-Val (Derynck *et al*., 1984; Lee *et al*., 1985), which conforms to the hydrophobic sequence requirements for binding to a PDZ domain (Ranganathan and Ross, 1997; Songyang *et al*., 1997). Accordingly, deletion of the C-terminal eight amino acids of transmembrane TGF-α abolishes interaction with p59, and deletion of the last two amino acids strongly decreases the interaction. Conversely, p59 without its first PDZ domain is unable to interact with

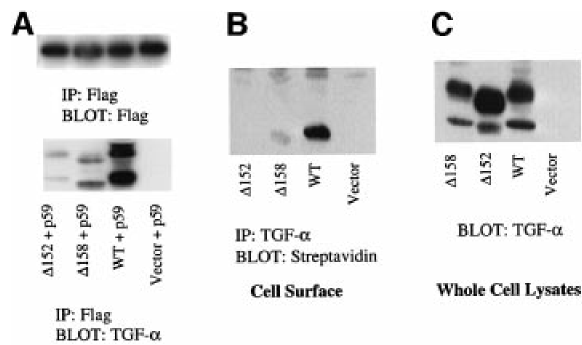

Fig. 9. The ability of transmembrane TGF-α mutants to associate with p59 correlates with the levels of transmembrane TGF-α at the cell surface. (**A**) Wild-type (WT) transmembrane TGF-α, and the TGF-αΔ158 and Δ152 mutants, which lack the C-terminal two and eight amino acids, respectively, were co-expressed with Flag-tagged p59 in CHO cells and their ability to interact was assessed by co-immunoprecipitation analysis. The top panel shows the expression levels of p59 and the lower panel shows the TGF-α that co-precipitates with p59. The control for the total levels of transmembrane TGF-α is essentially as in (C). (**B**) Cell surface levels of transmembrane TGF-α in transfected CHO cells. Cell surface biotinylation, followed by cell lysis and anti-TGF-α immunoprecipitation, allowed detection of TGF-α at the cell surface, but only of the lower TGF-α band. While wild-type TGF-α is well expressed at the cell surface, the Δ158 mutation resulted in strongly decreased cell surface expression and the Δ152 mutation abolished detectable cell surface expression of TGF-α. (**C**) Total levels of transmembrane TGF-α in the cell lysates corresponding to (B), as assessed using anti-TGF-α western blotting. This control very closely resembles the total TGF-α levels for (A) (data not shown).

transmembrane TGF-α, and the first PDZ domain by itself interacts with transmembrane TGF-α. The interaction between p59 and transmembrane TGF-α is presumably facilitated by fatty acylation of p59. Indeed, p59 is both myristoylated and palmitoylated, strongly suggesting that p59 is membrane anchored, while the short cytoplasmic domain of TGF-α is also palmitoylated (Shum *et al*., 1996). Fatty acylation has previously been shown to be important for targeting of PDZ domain proteins. Thus, PSD-95 is palmitoylated and this palmitoylation is required for clustering at the cell membrane and interaction with its binding partner Kv 1.4 (Craven *et al*., 1999). In addition, GRASP65, which is structurally related to p59, requires myristoylation for membrane anchoring (Barr *et al*., 1998).

Our results also illustrate specificity in the interaction of p59 with transmembrane TGF-α. Transmembrane TGF-α did not associate with EBP50, which, similarly to p59, has two PDZ domains in its N-terminal half (Weinman *et al*., 1995; Reczek *et al*., 1997). In addition, the p59 mutant without its first PDZ domain, yet containing its second PDZ domain, was also unable to interact. This interaction specificity may result from differences in affinity and specificity of the PDZ domains and subcellular localization. Conversely, p59 interacts not only with transmembrane TGF-α, but also with matrix metalloproteinase 1 and kit ligand, both transmembrane proteins with a C-terminal valine (Flanagan *et al*., 1991), but not with L-selectin with its C-terminal tyrosine (Siegelman and Weisman, 1989) and the truncated forms of transmembrane TGF-α. This raises the possibility that p59 may interact with a defined class of transmembrane proteins, although their structural requirements for interaction with p59 at endogenous levels need to be defined further.

Syntenin, another PDZ domain protein, has been identified recently as a protein that interacts with the C-terminal sequence of transmembrane TGF-α (Fernandez-Larrea *et al*., 1999). Syntenin is a 32–35 kDa protein with two PDZ domains in its C-terminal half and originally was isolated through its interaction with the C-terminal Phe-Tyr-Ala sequence of syndecan (Grootjans *et al*., 1997). The interaction of syntenin with syndecan and the Thr-Val-Val sequence of TGF-α suggests a relaxed specificity for recognition by the PDZ domains of syntenin. Syntenin was localized in the endoplasmic reticulum and *cis*-Golgi (Fernandez-Larrea *et al*., 1999). Whereas syntenin interacted with transmembrane TGF-α in HeLa cells (Fernandez-Larrea *et al*., 1999), we did not detect syntenin in association with transmembrane TGF-α in our preparations. This may reflect differences in the experimental system and cell source, although p59 and syntenin may also interact differentially with transmembrane TGF-α, depending on the subcellular location.

The interaction of p59 with transmembrane TGF-α in the Golgi is in contrast to the interaction of most PDZ domain proteins at the plasma membrane. The localization of p59 largely coincided with that of GM130, a Golgi membrane-associated protein with predominant staining in the *cis* compartment (Nakamura *et al*., 1997), and of wheat germ agglutinin, which also stains the Golgi apparatus (Tartakoff and Vassalli, 1983; data not shown). The p59 immunofluorescence did not localize to the endoplasmic reticulum. Besides their co-localization by immunofluorescence, the interaction of p59 with transmembrane TGF-α in the Golgi was also apparent from the biochemical nature of the interacting TGF-α. TGF-α is usually detected as three forms, a result of differential glycosylation and processing. The larger form derives from the middle, immature form as a result of extensive N-glycosylation of the prosequence, whereas the smaller form arises after removal of the prosequence (Bringman *et al*., 1987). p59 interacted primarily with the TGF-α form of intermediate size, which is a glycosylation intermediate. The insensitivity of this TGF-α form to deoxymannojirimycin further supports its localization in the Golgi. In contrast, the largest form is endo H resistant, indicative of its mature glycosylation status after transit through the Golgi. We therefore conclude that p59 interacts with transmembrane TGF-α in the Golgi, presumably prior to transit in the *trans*-Golgi, where endo H-resistant carbohydrates are added.

The sequence similarity of p59 to the recently published mouse sequence of GRASP55 (Shorter *et al*., 1999) suggests that p59 is the human homolog of GRASP55. GRASP55 was localized to the intermediate Golgi compartment and is thought to play a role in assembly and membrane stacking of the Golgi apparatus (Shorter *et al*., 1999). Accordingly, recombinant GRASP55 or antibodies against GRASP55 block stacking of Golgi cisternae in a cell-free system (Shorter *et al*., 1999). This activity is the basis for its name: 'Golgi reassembly stacking protein of 55 kDa'. GRASP65, which is structurally related to p59/GRASP55 and similarly has two PDZ domains in its N-terminal half, also plays an important role in stacking of Golgi cisternae *in vitro* (Barr *et al*., 1997), and localizes primarily to the *cis*-Golgi, where it associates with the membranes (Barr *et al*., 1997, 1998). The sequence

similarity of GRASP65 and p59/GRASP65 and the association of p59 with transmembrane TGF-α suggest that GRASP65 may also interact through its first PDZ domain with transmembrane proteins. Accordingly, similarly to p59/GRASP55, GRASP65 can interact with transmembrane TGF-α (data not shown), although endogenous GRASP65 was not detected in association with transmembrane TGF-α. The dimeric or trimeric nature of GRASP65 (Barr *et al*., 1998), and therefore presumably of GRASP55 as well, raises the possibility that transmembrane TGF-α may occur as a dimer.

GRASP65 interacts with GM130 (Barr *et al*., 1998), another membrane-associated protein in the *cis*-Golgi (Nakamura *et al*., 1997), and this interaction is thought to tether GM130 to the Golgi membrane. The sequence in GRASP65 required for interaction with GM130 is conserved in p59/GRASP55 (Figure 3B), even though no interaction of p59/GRASP55 with GM130 was detected *in vivo* (Shorter *et al*., 1999). This raises the possibility that GM130 or a related protein may participate in the complex of transmembrane TGF-α with p59/GRASP55. GM130 also interacts with p115 (Nakamura *et al*., 1997), which is required for intra-Golgi transport and transcytotic membrane traffic, and is also implicated in docking of coat protomer I (COPI) vesicles to Golgi membranes (Nelson *et al*., 1998; Alvarez *et al*., 1999). In addition, p115 also plays a role in cisternal stacking of the Golgi complex *in vitro* (Shorter and Warren, 1999), similarly to GRASP65 (and GRASP55). Collectively, these observations suggest that the tethering complex of p115 and GM130 may play a role in vesicle transport (Seemann *et al*., 2000). We therefore tested whether C-terminal deletions of transmembrane TGF-α, which impair the interaction with p59/GRASP55, would affect cell surface transport of transmembrane TGF-α. Deletion of the C-terminal two amino acids strongly decreased, while deletion of the C-terminal eight amino acids abolished interaction with p59. The interaction of transmembrane TGF-α with p59 correlated remarkably well with the cell surface levels of TGF-α. While wild-type TGF-α was expressed efficiently at the cell surface, deletion of the C-terminal two amino acids strongly decreased, and deletion of the C-terminal two amino acids totally abolished expression of cell surface TGF-α. This strong decrease in cell surface TGF-α levels is consistent with the observation that deletion of the C-terminal valine of TGF-α enhances intracellular retention (Briley *et al*., 1997; Urena *et al*., 1999).

The finding that p59/GRASP55 interacts with transmembrane TGF-α provides some new insights. First, our results indicate that p59/GRASP55, and presumably GRASP65 (and by extension the associated GM130–p115 complex), interacts with Golgi membranes not merely through myristoylation, but also through direct association with the cytoplasmic domains of select transmembrane proteins. Secondly, the correlation of the cell surface levels of transmembrane TGF-α with the ability to interact with p59 suggests that p59/GRASP55 and possibly GRASP65, together with the associated GM130–p115 complex, may participate in the secretory pathway of select transmembrane proteins. In this way, p59/GRASP55 may play an important role in transport and maturation of TGF-α through the Golgi apparatus. Thus, besides a role in Golgi assembly, a major role for GRASP55 and GRASP65 may reside in their ability to regulate transport of select cargo proteins through the Golgi apparatus. Further studies will be required to characterize the function of p59/GRASP55 in the maturation and intracellular transport of transmembrane TGF-α and other, select transmembrane proteins.

Materials and methods

Cell culture

CHO cells and Cα cells, a CHO derivative cell line that overexpresses TGF-α (Shum *et al*., 1994), were grown in F-12 Ham's nutrient mix (Gibco-BRL, Gaithersburg, MD). The media for Cα cells were supplemented with geneticin (Gibco-BRL) at 400 μg/ml. MDCK cells, obtained from R.Coffey (Vanderbilt University), were grown in Dulbecco's modified Eagle's medium (DMEM)-H21 with 4.5 g/l glucose (Gibco-BRL) supplemented with non-essential amino acids. COS-1, 3T3-F442A and 293 cells were grown in DMEM-H16, 3 g/l glucose (Gibco-BRL). All media were supplemented with 10% fetal calf serum (Hyclone Labs, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin.

Antibodies

The monoclonal antibody α1mAb has been described (Bringman *et al*., 1987). The TAB1 monoclonal antibody was a gift from Berlex Corporation of Richmond, CA. Goat affinity-purified anti-TGF-α polyclonal antibody was from Santa Cruz Biotechnology. Rabbit anti-mouse immunoglobulin was from Jackson ImmunoResearch Labs (West Grove, PA). The anti-myc monoclonal 9E10 (Evan *et al*., 1985) was either a gift of J.M.Bishop (University of California, San Francisco, CA) or purchased from Babco (Richmond, CA). The M2 anti-Flag antibody and M2 immunoaffinity resin were purchased from Kodak Eastman (New Haven, CT), while anti-HA monoclonal antibody was from Boehringer Mannheim. 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), and monoclonal antibody CA21 (Kahn *et al*., 1994) against L-selectin was obtained from T.Kishimoto (Boehringer Ingelheim, Inc.). MT1-MMP was detected using monoclonal antibody Ab-4 (Calbiochem, La Jolla, CA) and c-kit ligand was detected using a goat anti-human SCF antiserum (R&D Systems). Anti-GRP78 (BiP) antiserum was from Alexis Biochemicals (San Diego, CA). Rabbit polyclonal anti-GRASP65 was kindly provided by Dr F.Barr (University of Glasgow, Scotland, UK).

Immunoaffinity resins

The α1, TAB and anti-myc 9E10 monoclonal antibodies were covalently coupled to protein A–Sepharose (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 1 h at room temperature. A 3 mg aliquot of antibody was incubated per milliliter of hydrated beads. The beads were then washed twice with 10 vols of 3 M NaCl, 50 mM sodium borate pH 9.0 to remove unbound antibody. Antibodies were then coupled to the beads by incubation at room temperature for 30 min with a 20 mM solution of the chemical cross-linker dimethylpimelimidate in 3 M NaCl, 0.1 M sodium borate pH 9.0. After removal of the cross-linking solution, the reactions were quenched for 2 h in 0.2 M ethanolamine pH 8.0, at room temperature. The beads were then washed with and resuspended in phosphate-buffered saline (PBS).

Metabolic labeling

Cells 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. Labeling was typically performed 24 h after transfection (Shum *et al*., 1994).

Chemical cross-linking

Cells were washed twice with cold PBS, and then incubated at 4°C for 30 min with 2 mM DSP (Pierce, Rockford, IL) in PBS (Shum *et al*., 1994). Cells were subsequently washed twice with cold Ca/Mg-free PBS with 0.04% (w/v) EDTA before further processing.

Immunoprecipitations and immunoaffinity purification

For immunoprecipitations, ^{35}S-labeled cells were lysed in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM

phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin (buffer F). The lysate was cleared for 30 min by centrifugation at 16 000 *g* at 4°C, and subsequently incubated for 1 h at 4°C with antibody resin or α1 antibody and protein A–Sepharose (Pharmacia, Piscataway, NJ) pre-loaded 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 Tris–HCl pH 7.5, and once with 50 mM Tris pH 7.5. The beads were resuspended in reducing sample buffer, heated for 5 min at 100°C, and proteins were resolved by SDS–PAGE (Shum *et al*., 1994).

Immunoaffinity purification

For analytical purification, Cα and CHO cells were ^{35}S-labeled, treated with DSP, lysed with buffer F and clarified by centrifugation as described above. Cleared lysates were incubated with immunoaffinity resin for 1 h at 4°C. Lysate from each 10 cm diameter dish was incubated with 15 μl of an immunoaffinity resin described above. Samples to be analyzed at this point were washed three times with buffer F, once with 500 mM NaCl, 50 mM Tris pH 7.5, and once with 50 mM Tris pH 7.5. For samples to be purified further, the resin was packed into a column, washed with 10 column volumes of buffer F, 5 vols of 500 mM NaCl, 50 mM Tris pH 7.5, and 5 vols of 50 mM Tris pH 7.5. TGF-α complexes were eluted with five column volumes of 100 mM glycine pH 2.5 and the pH was then neutralized with 1 M Tris pH 10. Cross-linking was reversed by treatment with 100 mM dithiothreitol (DTT) for 30 min at 37°C. The sample was then desalted and concentrated using a 30 kDa molecular weight cut-off Microcon concentrator (Amicon, Beverly, MA).

Two large-scale purifications were performed, starting from 8.8 m^2 (2.5×10^9) and 15 m^2 (4.3×10^9) of confluent, DSP-treated Cα cells. Confluent cells were cross-linked with DSP, lysed with buffer F and clarified by centrifugation; 1–2 ml of lysis buffer were used per 100 cm^2 of cells. For each purification, lysates were incubated with 30 ml of α1mAb immunoaffinity resin for 12 h at 4°C with agitation. The slurries were packed into a column and the flow-through fraction was collected. The column was then washed with 10 vols of buffer F, 5 vols of 0.5 M NaCl, 50 mM Tris pH 7.5, and 5 vols of 50 mM Tris pH 7.5. Bound proteins were eluted with five column volumes of 100 mM glycine pH 2.5, 0.1% Triton X-100. Fractions (1 ml) of this elution were collected and neutralized with 1 M Tris pH 10. Fractions, which contained protein as determined by anti-TGF-α western blotting or India ink staining, were pooled, treated with 100 mM DTT for 30 min at 37°C to reverse cross-linking, concentrated, and desalted using NAP-50 columns (Pharmacia, Piscataway, NJ) and lyophilization. The flow-through fraction was then re-incubated with the antibody resin, and residual TGF-α complexes were purified as above.

Cibacron blue chromatography

Blue Sepharose High Performance, an agarose resin coupled to the Cibacron blue 3G-A, was obtained from Pharmacia. This resin was equilibrated in buffer F and then incubated for 1 h at 4°C with immunoaffinity-purified transmembrane TGF-α complexes, which were prepared as described above. For analytical experiments, 50 μl of resin were incubated with cell lysate from a 10 cm dish. Following pelleting of the beads by centrifugation, the supernatants were removed and then desalted and concentrated using a Microcon. The supernatants contained proteins, which did not bind to the resin, and were designated as flow-through fractions. After three washes with buffer F, the bound proteins were eluted in 0.5 ml of 50 mM Tris pH 7.5, 1 M NaCl, 10 mM ATP. The eluted material was then desalted and concentrated with a Microcon. Flow-through and bound fractions were resolved by SDS–PAGE and visualized by autoradiography.

For preparative chromatography, immunoaffinity-purified material from the first large-scale purification was incubated with 30 ml of Blue Sepharose High Performance resin for 1 h 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 Tris pH 7.5, concentrated by lyophilization, and resolved using SDS–PAGE. Samples were electrotransferred onto polyvinylidine difluoride (PVDF) membrane, and proteins were visualized with amido black staining. The p59 band was excised and processed for peptide microsequencing.

Two-dimensional electrophoresis

Material eluted from the α1mAb immunoaffinity resin was equilibrated with isoelectric focusing sample buffer consisting of 9 M urea, 4% NP-40, 2% β-mercaptoethanol and 2% pH 3–10 ampholytes (Bio-Rad) at 37°C for 30 min. Samples were then loaded onto a tube gel and subjected to equilibrium isoelectric focusing in a Biorad Mini-PROTEAN II 2-D cell for 5 h (analytical gels) or 8 h (preparative gel) at 500 V. Tubes were extruded, equilibrated in Laemmli sample buffer, loaded onto a 1.5 mm Laemmli slab gel, and proteins were separated electrophoretically. Proteins were visualized by autoradiography and silver staining. For preparative purposes, proteins were visualized by copper staining, and the p59 spot was excised and processed for peptide microsequencing.

Trypsin digestion, HPLC separation and peptide microsequencing

Purified proteins, separated by SDS–PAGE or on a two-dimensional gel, were electrotransferred to a PVDF membrane. Individual proteins were excised and submitted to *in situ* digestion with trypsin. The resulting peptide mixture was separated by microbore HPLC using a Zorbax C18 1.0 × 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions from the chromatogram were chosen based on differential UV absorbance at 205, 277 and 292 nm, peak symmetry and resolution. Peaks were screened further for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-MS) and selected fractions were subjected to automated Edman degradation on an Applied Biosystems 494A or 477A (Foster City, CA) protein sequencer. Details of strategies for the selection of peptide fractions and their microsequencing have been described (Lane *et al*., 1991). Alternatively, tryptic peptide sequences were determined by microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (San Jose, CA) as described (Hunt *et al*., 1992).

cDNA cloning and p59 expression plasmids

Hbc 551 (DDBJ/EMBL/GenBank accession No. T10844), a cDNA that codes for amino acids 143–452 of p59, was obtained from G.Bell (University of Chicago). The myc epitope tag sequence was added in-frame to the 3′ end of the coding sequence by ligating an *Xho*I–*Eco*RI fragment of hbc551 into the *Xho*I–*Eco*RI sites of pRK5myc (Feng *et al*., 1995), thereby generating a C-terminally tagged version. The missing 5′ coding sequence was obtained with two rounds of nested PCR from a human placental cDNA library. In the initial round, the λgt10 reverse primer (5′ GGTGGCTTATGAGTATTTCTTCC 3′) was used together with a p59 antisense primer (rev1: 5′ TGGATTCATTGGTGGCAC-AGAAGT 3′) in a PCR. The reaction products were then used as template for a second PCR, using the same λgt10 primer and an antisense primer closer to the 5′ end of the coding stand (rev2: 5′ CGACAGTTATCA-GTGTCTGTGTTG 3′). This second PCR generated an incomplete, 5′-extended cDNA segment for p59 and allowed the design of an additional antisense primer (rev3: 5′ CCCCACAGGTTACTTGG 3′) closer to the 5′ end than rev2. A second nested PCR was performed using the λgt10 primer and, sequentially, rev2 and rev3. The product of these reactions, which coded for an additional 142 amino acids, was ligated in-frame to the 5′ end of the original, partial p59 cDNA. PCRs were used to introduce a *Sal*I site at the 3′ end of the coding region of this extended cDNA, and the *Sal*I–*Eco*RI fragment containing the full-size coding sequence was ligated into the *Sal*I–*Eco*RI sites of pRK5-Flag (Feng *et al*., 1995) to generate pRK5-p59F. pRK5-p59F allowed expression of a full-size, C-terminally Flag-tagged version of p59. pRK5hygro-p59F corresponds to pRK5-p59F with a hygromycin resistance expression unit incorporated into the plasmid (Shi *et al*., 2000).

To express the first PDZ domain of p59, we generated plasmid pRK5-p59PDZ1. This plasmid is identical to plasmid pRK5-p59F, except that the full-size coding sequence was replaced by that for the p59 segment from amino acid 1 to 107, flanked by *Sal*I and *Eco*RI sites. This segment with its flanking sequences was generated by PCR-based amplification. A p59 deletion mutant lacking the first PDZ domain was generated similarly by PCR to contain the sequence from amino acid 84 to 453. The corresponding plasmid was named pRK5-ΔPDZ1. Both deletion mutants have a C-terminal Flag sequence.

Expression plasmids for other proteins

pRK7-α (Shum *et al*., 1994) and pRK7-αΔE (Shi *et al*., 2000) have been described. pRK7-EGFRα was constructed by fusing a PCR-generated fragment corresponding to the extracellular domain of the human EGFR (amino acids 1–645; Lin *et al*., 1984) in-frame with a DNA segment coding for the transmembrane and intracellular domains (amino acids 99–160) of TGF-α. The resulting chimera was subcloned into the mammalian expression plasmid pRK7 (Graycar *et al*., 1989). The L-selectin expression plasmid Leu8-14 (Migaki *et al*., 1996) was provided by T.Kishimoto (Boehringer Ingelheim, Inc.). The expression plasmid pKLM1, encoding full-size kit ligand (Flanagan *et al*., 1991), was from J.Flanagan (Harvard Medical School, Boston, MA). An expression plasmid for matrix metalloproteinase I (MT1-MMP) was

generated by subcloning the *Eco*RI–*Hin*dIII fragment from plasmid pCR3.1-Uni (Pei and Weiss, 1996; obtained from S.J.Weiss, University of Michigan) into the *Eco*RI–*Hin*dIII sites of pRK7 (Graycar *et al*., 1989). To express the PDZ domain protein EBP-50, we subcloned the coding sequence for EBP-50 with a C-terminal hemagglutinin tag from a corresponding cDNA3.0 vector (Cao *et al*., 1999; obtained from M.Van Zastrow, UCSF) into the *Hin*dIII–*Xho*I sites of pRK5 (Graycar *et al*., 1989). The GRASP65 expression plasmid was a gift from Dr F.Barr (University of Glasgow, Scotland, UK).

Transfection, immunoprecipitations and western blotting

COS-1, 293 and CHO cells were transfected using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions, while 3T3-F442A cells (Green and Kehinde, 1976) were transfected with Lipofectamine with the PLUS reagent (Gibco-BRL). MDCK cells were transfected with pRK5hygro-p59F using the calcium phosphate method. Following transfection, cells were grown to confluence and then split into medium containing hygromycin (Boehringer Mannheim, Indianapolis, IN) at 400 μg/ml. Single MDCK cell clones were isolated and expanded.

p59 and transmembrane TGF-α were detected 48 h after transfection by western blotting using the M2 anti-Flag antibody or an anti-TGF-α antibody. For the TGF-α–p59 co-immunoprecipitation experiments (Figure 6B and C), cells were lysed in buffer F, and the lysates were cleared by centrifugation and subsequently incubated for 1 h at 4°C with M2 anti-Flag, α1mAb or anti-EGFR (R-1) immunoaffinity resins, coupled to protein A–Sepharose (Pharmacia, Piscataway, NJ) pre-loaded 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 Tris pH 7.5, and once with 50 mM Tris pH 7.5. The beads were resuspended in protein sample buffer, heated for 5 min at 100°C, and proteins were resolved with SDS–PAGE. The experiments in Figure 6A and B were carried out similarly except that the samples were first incubated with free α1 antibody, followed by incubation with protein A–Sepharose.

To determine cell surface levels of TGF-α, transfected cells were washed twice with 0.5 mM Mg^{2+}-containing, Ca^{2+}-free PBS and incubated with 0.5 mg/ml EZ-link sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 45 min. Excess biotin was quenched in 50 mM glycine twice for 10 min, and cells were lysed for immunoprecipitation of TGF-α. Following gel electrophoresis and transfer onto PVDF membrane, biotinylated proteins were detected using horseradish peroxidase-conjugated streptavidin (Pierce).

For immunoprecipitation of EBP-50, kit ligand or L-selectin, we used the same conditions and the following antibodies: anti-HA antibody for EBP-50, CA21 for L-selectin and anti-human SCF for kit ligand.

To detect MT1-MMP, transfected cells were washed twice in ice-cold PBS, and lysed in 1.5% Triton X-114 (Sigma) containing proteinase inhibitor mix (Calbiochem). After 15 min, the cell lysate was collected by scraping and cleared at 10 000 *g* for 5 min. The cleared supernatant was incubated for 2 min at 37°C and again cleared at 10 000 *g* for 5 min. The upper water phase was removed carefully and the lower Triton fraction was diluted 4-fold with water prior to immunoprecipitation or western blotting. Full-length MT1-MMP was detected using monoclonal antibody Ab-4 (Calbiochem).

Myristoylation and palmitoylation

To detect myristoylation of p59, *in vitro* transcription–translation reactions were carried out using the Promega TNT kit. A 0.5 μg aliquot of plasmid pRK5-p59F was incubated with SP6 polymerase for 2 h at 30°C with complete amino acid mix plus 1 μl of [^{3}H]myristic acid (1 mCi/ml; Amersham). After addition of buffer F, Flag-tagged p59 was immunoprecipitated using M2 Flag antibody and then processed and exposed to film to detect ^{3}H-labeled proteins (Barr *et al*., 1997).

To detect palmitoylation, 293 cells, transfected with pRK5-p59F, were labeled overnight with 1 mCi/ml [^{3}H]palmitic acid (Amersham) in medium containing 10% dialyzed fetal calf serum, 10% tryptose phosphate, 5 mM pyruvate, 1% dimethylsulfoxide. Following two washes with PBS, cells were lysed with buffer F. p59 was immunoprecipitated using M2 Flag antibody, analyzed by SDS–PAGE under non-reducing conditions, enhanced, and exposed to film to detect ^{3}H-labeled proteins (Shum *et al*., 1996).

Immunofluorescence

Cells were grown to 70% confluence on tissue culture chamber slides and transfected. At 24 h post-transfection cells were fixed with formaldehyde. Cells were permeabilized with 0.5% Triton X-100, blocked for 1 h in 5% bovine serum albumin (BSA) and incubated with primary antibody, and then Texas red- or fluorescein-labeled secondary antibodies (Jackson ImmunoResearch). In some cases, Alexa 488 goat anti-rabbit and Alexa 594 goat anti-rat IgG (H+L) conjugates (Molecular Probes, Eugene, OR) were used for better results. Oregon green-labeled α1 monoclonal antibody was used for direct immunofluorescence of TGF-α. For anti-Flag staining of MDCK cells, an additional amplification step was added. Samples were incubated sequentially with M2 antibody, unlabeled rabbit anti-mouse antisera, and finally a fluorescein-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). Immunofluorescence was visualized using a Bio-Rad MRC-1024 confocal microscope equipped with a krypton–argon laser and an Optiphot II Nikon microscope with a Plan Apo 60× 1.4 NA objective.

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