Association of the Transmembrane TGF-α Precursor with a Protein Kinase Complex

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Abstract. A variety of growth factors including transforming growth factor-α (TGF-α) are synthesized as transmembrane precursors. The short cytoplasmic domain of the transmembrane TGF-α precursor lacks any apparent motif associated with signal transduction. However, the sequence conservation of this cytoplasmic domain and its abundance of cysteine residues, reminiscent of the cytoplasmic domains of CD4 and CD8, suggest a biological function. In this study, we showed that transmembrane TGF-α was rapidly internalized after interaction with a specific antibody and that this internalization was greatly decreased when the COOH-terminal 31 amino acids were removed. Chemical cross-linking experiments revealed two associated proteins of 86 and 106 kD which coimmunoprecipitated with the TGF-α precursor. The association of p86 was dependent on the presence of the COOH-terminal cytoplasmic 31 amino acids of the TGF-α precursor, whereas p106 still remained associated when this segment was deleted. In addition, p106 was tyrosine-phosphorylated and exposed on the cell surface. The protein complex associated with transmembrane TGF-α displayed kinase activities towards tyrosine, serine, and threonine residues. These activities were not associated with transmembrane TGF-α when the COOH-terminal segment was truncated. The association of a protein kinase complex with transmembrane TGF-α may provide the basic elements for a "reverse" mode of signaling through the cytoplasmic domain of this growth factor, which may lead to two-directional communication during ligand-receptor interaction.

TGF-α is synthesized as a transmembrane precursor (Derynck et al., 1984; Lee et al., 1985) and specific proteolytic cleavages in the extracellular domain produce soluble TGF-α (Bringman et al., 1987; Gentry et al., 1987). However, the uncleaved transmembrane TGF-α precursor often represents the predominant species in TGF-α producing cells (Derynck, 1992). TGF-α is synthesized by many normal cells and tissues, predominantly various epithelia including skin, brain (Coffey et al., 1987; Wilcox and Derynck, 1988*a*,*b*; Derynck, 1992), and tumor cells (for review see Derynck, 1992). TGF-α binds to the same receptor as EGF as a result of structural similarity (Massagué, 1983; Marquardt et al., 1984). Other members of the EGF/TGF-α family also exist as cleavable transmembrane growth factors. Besides EGF and TGF-α, this family includes amphiregulin (Shoyab et al., 1989; Plowman et al., 1990), HB-EGF (Higashiyama et al., 1991, 1992), betacellulin (Shing et al., 1993), and vaccinia virus growth factor (Stroobant et al., 1985), all of which bind to the EGF/TGF-α receptor. In addition, the differentially spliced forms of neuregulins (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993), lin 3 in Caenorhabditis elegans (Hill and Sternberg, 1992) and spitz (Rutledge et al., 1992), and gurken (Neumann-Silberberg and Schüpbach, 1993) gene products in Drosophila also belong to this family.

The cytoplasmic domain of the TGF-α precursor is only 39 amino acids long, contains 7 cysteines, and is highly conserved among species (Derynck et al., 1984; Lee et al., 1985; Zurfluh et al., 1990; Chiang et al., 1991), thus suggesting a defined biological function. However, examination of this sequence does not reveal any known functional motifs. Recently, it has been demonstrated that the COOH-terminal valines in the TGF-α precursor are required for efficient cleavage of the ectodomain of transmembrane TGF-α and subsequent release of soluble TGF-α (Bosenberg et al., 1992). Since the short cytoplasmic domain of TGF-α is unlikely to have enzymatic activity, its potential function may be mediated through proteins that interact with the cytoplasmic domain which may associate noncovalently through the cysteine-rich regions. This is modeled after the T cell membrane glycoproteins CD4 and CD8, which have short cytoplasmic domains that interact with the associated lck kinase

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via cysteine–cysteine interactions (Turner et al., 1990; Shaw et al., 1989, 1990).

It is possible that the biological significance of the cytoplasmic domain of TGF-α resides in its role in cell–cell communication. Direct cell–cell communication is critical in various biological processes and frequently involves physical interactions between cell surface molecules on adjacent cells (Singer, 1992; Greenwald and Rubin, 1992). In several cases, heterotypic interactions between two different membrane-associated polypeptides mediate cell–cell communication. Such interactions occur between Notch and Delta or Serrate, which mediate cell adhesion and may play a role in neural patterning in Drosophila (Fehon et al., 1990; Heitzler and Simpson, 1991; Rebay et al., 1991), and bride of sevenless and sevenless which determine the cell differentiation of the R7 cell in the Drosophila eye (Kramer et al., 1991). A possible role of transmembrane TGF-α in direct cell communication is supported by the interaction of transmembrane TGF-α with its receptor on a neighboring cell resulting in signal transduction events similar to those elicited by its soluble counterpart (Brachmann et al., 1989; Wong et al., 1989). In addition, normal TGF-α synthesis usually coincides with coexpression of the EGF/TGF-α receptor in the same cells or adjacent cells, and TGF-α overexpression in tumor cells is often accompanied by a high level expression of its receptor (DiMarco et al., 1989, 1990). Various other growth and differentiation factors are also synthesized as transmembrane polypeptides (for review see Ehlers and Riordan, 1991; Massagué and Pandiella, 1993). In addition to the TGF-α precursor, the transmembrane forms of CSF-1 (Stein et al., 1990), TNF-α (Perez et al., 1990), and stem cell factor (Flanagan et al., 1991; Brannan et al., 1992) are also known to be biologically active, thus raising the possibility that they may also participate in direct cell–cell communication when interacting with their respective receptors on neighboring cells. Thus, studies on the biological role of transmembrane TGF-α can significantly contribute to our understanding of other transmembrane growth factors and similar cell–cell communication systems.

To initiate our studies of the biological role of the cytoplasmic domain of the TGF-α precursor, we examined the association of cellular proteins with transmembrane TGF-α. Using chemical cross-linking and immunoprecipitations, we detected two proteins, p86 and a tyrosine-phosphorylated p106, which associate with transmembrane TGF-α. This protein complex had protein kinase activity which was dependent on the presence of the cytoplasmic domain of transmembrane TGF-α. The existence of a protein kinase complex in association with transmembrane TGF-α suggests that this enzymatic activity might be regulated by ligand-receptor interaction. If this is the case, the interaction between transmembrane TGF-α and its receptor could provide the necessary elements for two-directional communication in the context of direct cell–cell contact.

Materials and Methods

Plasmid Constructions

pRK7-α was constructed by excising the cDNA encoding the wild-type full length TGF-α as an EcoRI–HindIII fragment from pMTE4E (Rosenthal et al., 1986) and subcloning into pRK7 in which transcription was driven by the CMV promotor. pRK7αΔC was constructed by polymerase chain reaction mutagenesis such that the sequence coding for the COOH-terminal 31 amino acids of the TGF-α precursor was deleted and the codons for the two cytoplasmic cysteines proximal to the transmembrane region (positions 123 and 124, Derynck et al., 1984) were replaced by serine codons. The mutations were verified by double stranded DNA sequencing by Sequenase Ver 2.0 (USB).

Stable Transfections

CHO cells were maintained in F12 Ham's Nutrient Mix (GIBCO BRL, Gaithersburg, MD) with 10% FCS (Hyclone Labs., Logan, CT), 100 U/ml penicillin, and 100 μg/ml streptomycin. The day before transfection, CHO cells were split 1:5 and allowed to adhere and grow overnight. On the following day, cells were washed twice with 10 mM Tris (pH 7.4), 150 mM sodium chloride and transfected overnight using the calcium phosphate method (Sambrook, 1989), after which the cells were washed and grown to confluence in complete medium. The neomycin gene was cotransfected with the full length or the truncated form of TGF-α to confer neomycin resistance. Transfected cells were selected with 400 μg/ml Geneticin (GIBCO BRL) and single clones were isolated and expanded. The expression levels of the proteins were analyzed by Northern analysis, immunofluorescence, and immunoprecipitation. CHO cells transfected with pRK7-α and pRK7-αΔc were designated as Cα and CαΔc cells, respectively.

Antibodies

The monoclonal antibody αlmAb was described before (Bringman et al., 1987). The TABmAb monoclonal antibody, raised against the 50 amino acid human TGF-α, was a generous gift from Dr. R. Harkins (Berlex Inc.). Both antibodies, purified to homogeneity, were used for immunostaining and immunoprecipitation.

Immunofluorescence Analysis

Cells were grown on tissue culture chamber slides (Nunc) for immunostaining. The primary antibody, αlmAb, as well as the secondary antibody, rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs., West Grove, PA) were used at 5 μg/ml. Total cell staining was achieved by fixing cells in methanol for 10 min and incubating with primary and secondary antibodies sequentially each for 1 h. Surface staining was performed by incubating live cells with primary antibody for 1 h on ice, fixing with 4% paraformaldehyde in PBS for 15 min, and incubating with secondary antibody. Internalization of TGF-α was allowed by incubating the cultures at 37°C for the designated period of time. Cells were permeabilized by incubating with 0.2% Triton X-100 in PBS for 10 min after fixation. The slides were rinsed in PBS twice after each incubation and finally mounted with Fluoromount G (Fisher America, Inc., Waukesha, WI) for observation. A Zeiss Axioplan Microscope was used for photomicrography.

Internalization Assay

Cells were grown to confluence in 24-well plates. Primary and secondary antibodies were αlmAb used at 3 μg/ml and horseradish peroxidase-conjugated goat anti-mouse IgG (GIBCO BRL) used at 2 μg/ml, respectively. Conditions for incubations, fixation, and permeabilization were performed similar to immunofluorescence analysis as described above. The final colorimetric detection was carried out by incubating for 15 min with 0.8 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Sigma Chem. Co., St. Louis, MO) at 1 mg/ml and 0.015% hydrogen peroxide in 100 mM citrate-phosphate buffer at pH 4. The absorbence of the product was read at 405 nm with a spectrophotometer (Shimadzu Corp., Tokyo, Japan). The percent internalization was calculated from the difference in absorbance between paired samples with and without permeabilization, and was expressed as a fraction of the total initial absorbance at time 0. These values were obtained after subtraction of background absorbance from parental CHO cells and normalization of non-specific diffusion.

Pulse-Chase ^{35}S-labeling and Immunoprecipitation of Cell Surface TGF-α

Equal numbers of Cα and CαΔC cells were plated in 6-cm diam cell culture dishes and cultured to 80% confluence. To minimize cleavage and release of cell surface TGF-α precursor, cells were serum-starved overnight. On the following day, cells were preincubated for 1 h in F-12 Ham's nutrient mix lacking cysteine, methionine, and serum. Cells were then pulsed for 20 min with 1 ml of fresh medium containing 250 μCi of [^{35}S]cysteine/methionine

protein labeling mix (New England Nuclear, Boston, MA). After two washes with cold PBS, cells were incubated (chased) with 1 ml of F-12 medium containing 0.1 mg/ml of each cysteine and methionine for 0, 30, 60, or 90 min, and cell surface TGF-α was immunoprecipitated.

To prevent internalization of the cell surface TGF-α all manipulations up to cell lysis were strictly performed at 0°C. Cells were kept on ice for 5 min, washed twice with ice-cold PBS, and then incubated with 7 μg αlmAb antibody for 1 h. The cells were then extensively washed three times with ice-cold PBS and lysed as described below. To further ensure that equivalent numbers of cells were compared, the amount of radioactivity in each sample was determined by scintillation counting, and immune complexes from equal amounts of cell lysate were collected and processed as described below.

Metabolic Labeling, Cross-linking, Immunoprecipitation, and SDS-PAGE

Cells were grown to 80% confluence and labeled with 160 μCi/ml [^{35}S]-cysteine-methionine protein labeling mix (NEN) overnight in cysteine/methionine-free F12 Ham's Nutrient Mix with 10% dialyzed FCS. Cells were washed twice with cold PBS and incubated with 2 mM dithiobis-succinimidyl-propionate (DSP)[1] (Pierce, Rockford, IL), a homobifunctional hydrophobic reversible cross-linker, for 30 min at 4°C. Subsequently, cells were washed twice with cold calcium, magnesium-free PBS after which the cells were lysed in buffer containing 50 mM Tris (pH 7.4), 100 mM sodium chloride, 2 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. The lysate was sheared twice through a 25-G needle and centrifuged at 14,000 rpm for 20 min. The supernatant was collected and precleared with protein A-Sepharose (Pharmacia) preloaded with rabbit anti-mouse IgG (Jackson ImmunoResearch Labs.) for 1 h at 4°C. The precleared supernatant was used for immunoprecipitation by adding TABmAb or α1 mAb to a final concentration of 5 μg/ml and protein A-Sepharose preloaded with rabbit anti-mouse IgG and allowed to incubate at 4°C for 2 h. Then, the immunoprecipitate was washed three times with lysis buffer, once with 50 mM Tris (pH 7.4) and 500 mM sodium chloride and finally once with 50 mM Tris (pH 7.4). The beads were resuspended in protein sample buffer, heated for 5 min at 100°C and proteins were separated by SDS-PAGE according to Laemmli (1970) using the Minigel Apparatus (Biorad Labs., Hercules, CA). A hydrophilic cross-linker dithiobis-sulfo-succinimidyl-propionate (DTSSP) (Pierce) was used similarly in control experiments.

Cell Surface Biotinylation

After metabolic labeling, the cells were washed twice with ice-cold PBS*, i.e., PBS containing 0.5 mM $MgCl_2$ pH 7.8, and incubated for 45 min at 4°C in a 1 mg/ml solution of sulfosuccinimdyl 6-(biotinamido) hexanoate (NHS-LC-biotin, Pierce Chem. Co.) in PBS*. Cells were then washed twice with ice-cold PBS*, cross-linked with DSP, incubated with PBS/50 mM Tris pH 7.4 for 15 min at 4°C and immunoprecipitated as described above. Immunoprecipitates were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Biotinylated proteins were detected with the Enhanced Chemiluminescence system (Amersham Corp., Arlington Heights, IL) using streptavidin-conjugated horseradish peroxidase as probe. The blot was subsequently incubated for 1 h in a 0.02% NaN_3 solution, dried at room temperature overnight, and exposed for autoradiography to visualize ^{35}S-labeled proteins.

Anti-Phosphotyrosine Western Blotting

The immunoprecipitate was resolved on denaturing protein gel and electrotransferred onto nitrocellulose using a Mini Transblot Apparatus (Bio-Rad Labs.). The immunoblot was first blocked with 3% gelatin (BioRad Labs.) in NTBS (10 mM Tris pH 8, 150 mM sodium chloride, 0.05% NP-40) for 1 h at room temperature. Subsequently, the blot was incubated with anti-phosphotyrosine monoclonal antibody, PY20 (Zymed Labs., S. San Francisco, CA) at 1 μg/ml overnight at room temperature, washed in NTBS twice, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI) at 0.2 μg/ml for 1 h. The blots was washed again and developed with NBT/BCIP substrate (Promega Corp.) until the desired intensity. The reaction was terminated with distilled water.

1. *Abbreviations used in this paper*: DSP, dithiobis-succinimidyl-propionate; DTSSP, dithiobis-sulfo-succinimidyl-propionate.

Kinase Assay and Tyrosine Kinase Inhibitors

The immunoprecipitate was prepared as described above. In addition, it was washed once in kinase buffer containing 25 mM Hepes (pH 7.4) and 10 mM $MgCl_2$. Substrates such as acid-denatured enolase (Sigma Chem. Co.), histone 2B (Boeringer Mannheim Corp., Indianapolis, IN) or casein (Sigma Chem. Co.) were used at 100 ng/ml. The kinase reaction was performed by adding substrate to the immunoprecipitate in kinase buffer and allowed to proceed with the addition of 1 μCi ^{32}P-γ-ATP. The reaction was incubated for 30 min at room temperature and terminated by adding gel electrophoresis protein sample buffer. The product was analyzed by SDS-PAGE. Various tyrosine kinase inhibitors (GIBCO BRL) were used: methyl-hydroxy-cinnamate at 300 μM, lavendustin A at 2 μM, genistein at 180 μM, tyrphostin at 240 μM, hydroxydihydroxylbenzyl-aminobenzoic acid at 0.3 μM, herbimycin A at 50 μg/ml, staurosporine at 1 μM, H7 at 500 μM, and HA1004 at 500 μM.

Phosphoaminoacid Analysis

Phosphoamino acid analysis was performed according to Kamps and Sefton (1989). Briefly, the kinase product used for phosphoamino acid analysis was first electrotransferred onto PVDF membrane. The transferred band on the membrane was excised and hydrolyzed in 200 μl of 6 N boiling hydrochloric acid (Pierce) for 1 h at 110°C. Then the slice of membrane was removed and the hydrolyzed material was vacuum dried. The product was analyzed by two-dimensional electrophoresis on a TLC plate. The first dimension was performed using pH 1.9 buffer containing 15% acetic acid and 4% formic acid, and the second dimension using pH 3.5 buffer containing 5% acetic acid and 0.5% pyridine. 2 μg of amino acid standards containing tyrosine, serine, and threonine were used in parallel and the positions of migration of these standards were revealed by spraying the TLC plate after electrophoresis with ninhydrin (Sigma) and drying it at 80°C.

Results

Expression of Full-Length and Truncated TGFα Precursors in CHO Cells

A cDNA encoding the full length TGF-α precursor of 160 amino acids long (Derynck et al., 1984) was subcloned into a plasmid that expressed transmembrane TGF-α and its secreted derivatives under the control of the cytomegalovirus promoter. This plasmid, pRK7-α, was cotransfected with a plasmid encoding neomycin-resistance into CHO cells. These cells were chosen since they lack EGF/TGF-α receptors (Livneh et al., 1986; Clark et al., 1988) thus avoiding any interaction of transfected TGF-α with its receptor. CHO cells expressing the transfected TGF-α precursor were obtained following selection in G418 and were named Cα cells. Immunofluorescence using monoclonal antibody αlmAb directed against the 50–amino acid extracellular domain of TGF-α (Bringman et al., 1987), revealed that Cα cells expressed a high level of cell surface associated TGF-α precursor. Staining of permeabilized cells also detected TGF-α in the perinuclear area presumably in association with the endoplasmic reticulum and Golgi apparatus (Fig. 1). There was no detectable immunofluorescence in untransfected CHO cells (Fig. 1) or in control experiments when the primary TGF-α antibody was omitted (data not shown). Immunoprecipitation using another antibody TABmAb confirmed the synthesis of TGF-α in Cα cells and its absence in untransfected CHO cells (Fig. 2). As previously described (Bringman et al., 1987), TGF-α ranging from 20 to 30 kD and corresponding to different glycosylated and processed forms of transmembrane TGF-α were detected by immunoprecipitation of the ^{35}S-labeled cell lysate (Fig. 2). The predominant TGF-α form in the medium was the 6-kD fully processed 50 amino acids TGF-α; however, larger glycosylated secreted

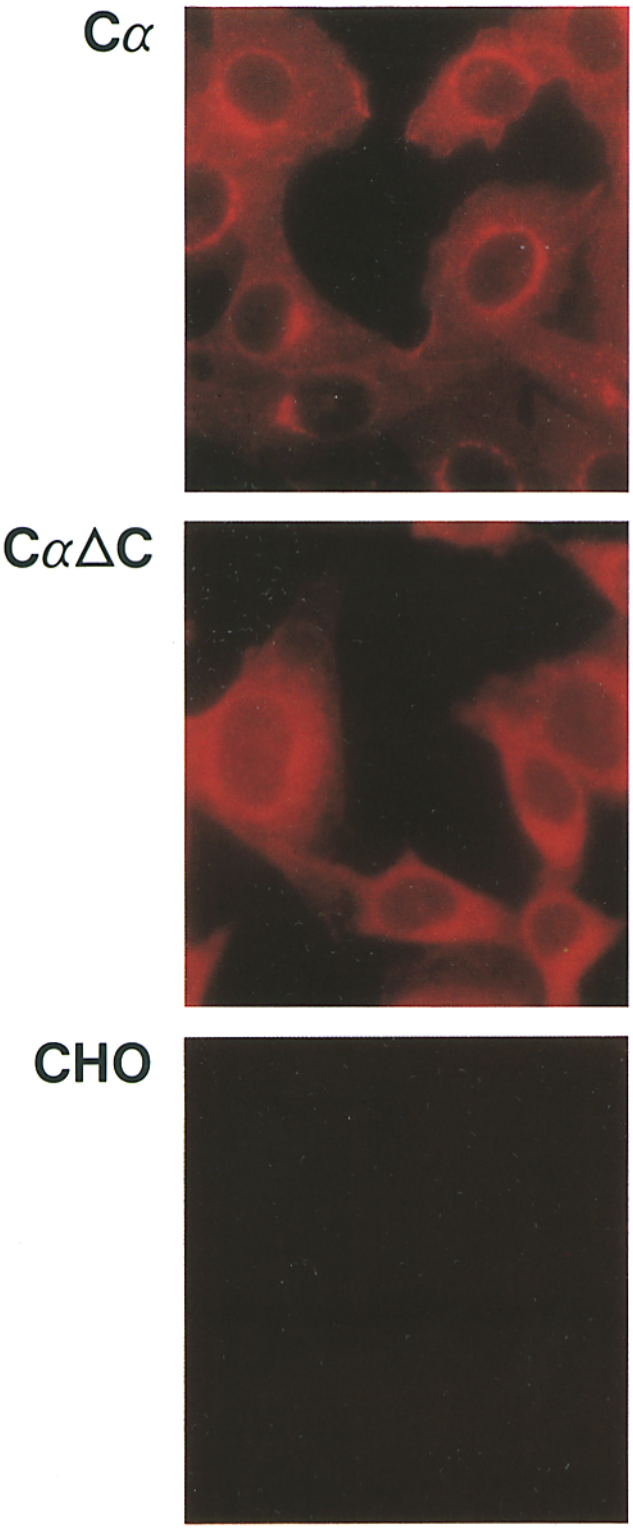

Figure 1. Immunofluorescence visualization of TGF-α in Cα cells expressing the full size TGF-α precursor, and CαΔC cells expressing the truncated TGF-α precursor. Cells were fixed, permeabilized, and immunostained with α1mAb and a rhodamine-conjugated secondary antibody. TGF-α was predominantly detectable at the cell surface and in the perinuclear region, presumably in the Golgi apparatus and the endoplasmic reticulum. Parental CHO cells were negative.

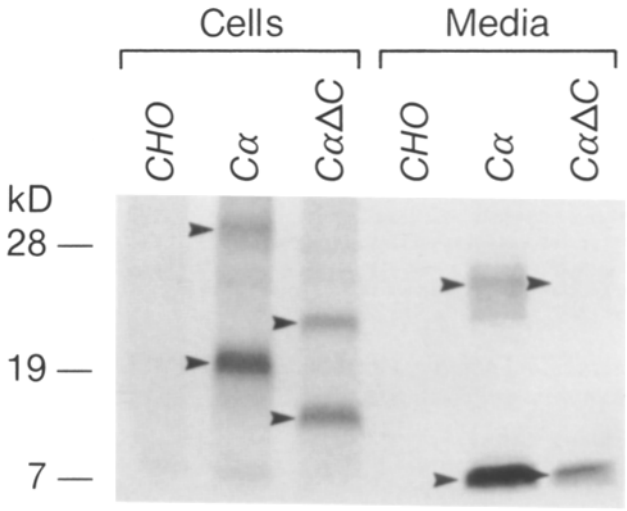

Figure 2. Immunoprecipitation of TGF-α from the media and lysates of transfected CHO cells. Electrophoretic analysis of immunoprecipitates from ^{35}S-labeled Cα cells using the α1mAb revealed immunoreactive species between 20–30 kD (lane *2*), which, based on previous analyses, presumably correspond to the different glycosylated forms of transmembrane TGF-α precursor. The smaller TGF-α forms of 12–25 kD in the CαΔC cell lysates (lane *3*) are consistent with the length of the cytoplasmic deletion of transmembrane TGF-α in these cells. In the media, the 6-kD species corresponded to the 50 amino acids, fully processed form of TGF-α, whereas the larger species corresponds to the secreted glycosylated form (lane *5*). The lower expression of the soluble forms in CαΔC cells compared with the Cα cells is consistent with the previously reported inefficient processing of cytoplasmic truncated form of TGF-α (lane *6*). Parental CHO cells were negative (lanes *1* and *4*). The positions of molecular weight markers are shown on the left.

TGF-α forms were detectable as well (Fig. 2). Consistent with our previous analysis of the processing of transmembrane TGF-α (Bringman et al., 1987) we conclude that the Cα cells synthesize transmembrane TGF-α, some of which is cleaved to release soluble TGF-α.

To study the function of the cytoplasmic domain of TGF-α, a similar expression plasmid, pRK7-αΔC, was constructed to drive the expression of a truncated form of TGF-α in which the COOH-terminal 31 amino acids corresponding to most of the cytoplasmic domain were deleted. In addition, the two cytoplasmic cysteines (residues 123 and 124, Derynck et al., 1984) proximal to the transmembrane region were replaced by serines in pRK7-αΔC. Transfected CHO cells expressing the truncated transmembrane TGF-α were named CαΔc cells. Similar to the expression of the full length TGF-α precursor in Cα cells, the CαΔC cells expressed the transmembrane TGF-α at the cell surface, as assessed by immunofluorescence (Fig. 1). Immunoprecipitation analysis of the cell lysate demonstrated several forms of the truncated TGF-α precursor ranging in sizes from 12 to 25 kD (Fig. 2), presumably corresponding to different glycosylated forms (Bringman et al., 1987). The smaller sizes of the TGF-α species in CαΔC cells as compared with those in Cα cells were consistent with the length of the deleted segment. The conditioned medium of CαΔC cells showed the 6-kD form of TGF-α and the glycosylated TGF-α species, albeit at a much lower level than in Cα cells (Fig.

2). The lower degree of proteolytic processing of the truncated transmembrane TGF-α compared with the full size transmembrane TGF-α may reflect the requirement for the cytoplasmic domain for efficient proteolysis (Bosenberg et al., 1992). The immunoprecipitations and cell surface staining indicated that Cα and CαΔC cells expressed similar levels of transmembrane TGF-α.

To further compare the cell surface expression of TGF-α in the two cell lines, pulse chase analysis followed by immunoprecipitation of the ^{35}S-labeled cell surface TGF-α forms was performed. By comparison with our previous findings (Bringman et al., 1987, unpublished data) and consistent with our results in Fig. 2, several cell surface forms of TGF-α were detected (Fig. 3). Cα cells expressing the full size precursor express two major, relatively stable, cell surface forms presumably corresponding to fully glycosylated transmembrane TGF-α (30 kD) and transmembrane TGF-α without the proregion (20 kD), whereas the unglycosylated form with the proregion can only be found as a major species of 26 kD at the earliest time point and is considerably less stable (Fig. 3, lanes *1–4*). Also, the truncated form of transmembrane TGF-α can be found as two species presumably corresponding to the glycosylated form (25 kD) and the transmembrane form without the proregion (12 kD), whereas again the unglycosylated form (21 kD) with the proregion is highly unstable and can be detected only at the earliest time point (Figs. 2 and 3, lanes *5–8*). As for the steady state immunoprecipitations (Fig. 2), the intensity of the ^{35}S-labeled TGF-α bands was greater for Cα than for CαΔC cells (Fig. 3). However, the truncated protein lacks seven of the fourteen cysteines present in the full length protein. Thus, an equivalent amount of the truncated TGF-α is expected to have a level of [^{35}S]cysteine incorporation of only half of the full length transmembrane TGF-α. We therefore conclude that the expression of the truncated TGF-α form is within the same range as the full size transmembrane TGF-α.

Internalization of the TGF-α Precursor

Most cell surface receptors are internalized after exposure to their ligands or monoclonal antibodies that mimic the ligand. Required for internalization is a structural motif in the cytoplasmic domain that results in a tight reverse β turn and usually contains a tyrosine (for review see Trowbridge, 1991). Internalization can also occur when a membrane-associated protein lacks an internalization sequence yet physically interacts with a cytoplasmic protein containing the necessary internalization signal (Amigorena et al., 1992). To examine whether the TGF-α precursor which lacks a tyrosine residue in its cytoplasmic domain can be internalized, we evaluated the ability of the monoclonal antibody αlmAb to induce internalization of transmembrane TGF-α.

Immunofluorescence staining of live cells at 0°C using the TGF-α monoclonal antibody revealed a homogeneous staining pattern at the cell surface of both the Cα and CαΔC cells, which expressed the full length and truncated TGF-α precursor, respectively (Fig. 4 *A*). When we exposed the transfected cells to αlmAb at 0°C, and subsequently incubated them at 37°C for 30 min, it was apparent that there was internalization of transmembrane TGF-α as detected using the secondary rhodamine-conjugated antibody after cell fixation. Comparison of the immunofluorescent staining between permeabilized and non-permeabilized cells revealed different patterns. Non-permeabilized cells showed surface

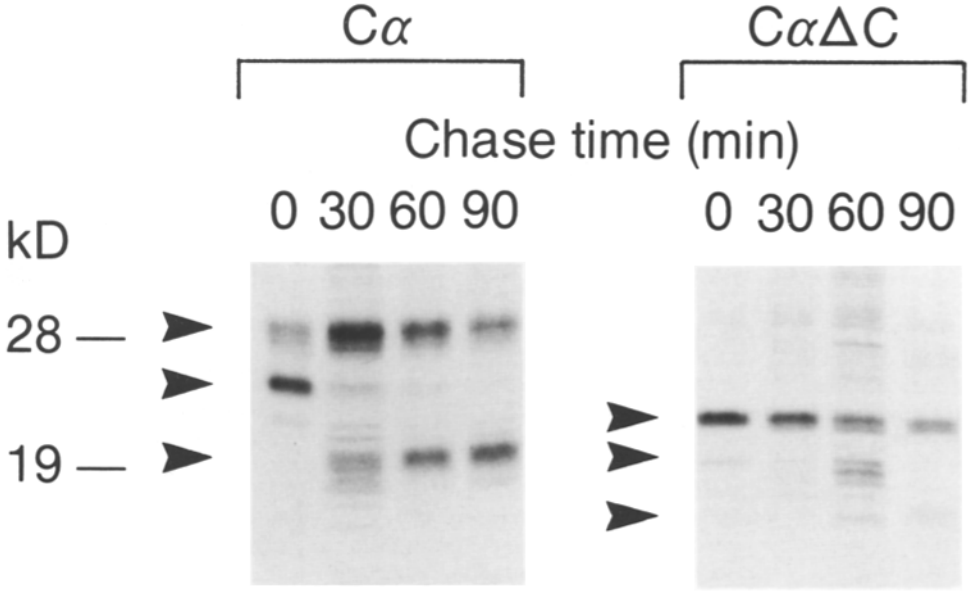

Figure 3. Pulse-chase analysis of full length and truncated cell surface TGF-α. Equal numbers of Cα and CαΔC cells were pulse-labeled for 20 min with [^{35}S]cysteine/methionine, and then chased with excess unlabeled cysteine and methionine for the indicated times. Intact cells were incubated with αlmAb antibody for 1 h at 0°C and the cell surface TGF-α forms were immunoprecipitated. Note that the full length TGF-α species made by Cα cells have twice as many cysteines and will incorporate twice the amount of radiolabel as the truncated TGF-α forms from CαΔC cells. Arrow heads indicate the TGF-α species. A high level of non-specific background bands, due to insufficient washing of the immunoprecipitate, is seen in lane *7* (60 min, (60 min, CαΔC). The positions of molecular weight markers are shown on the left.

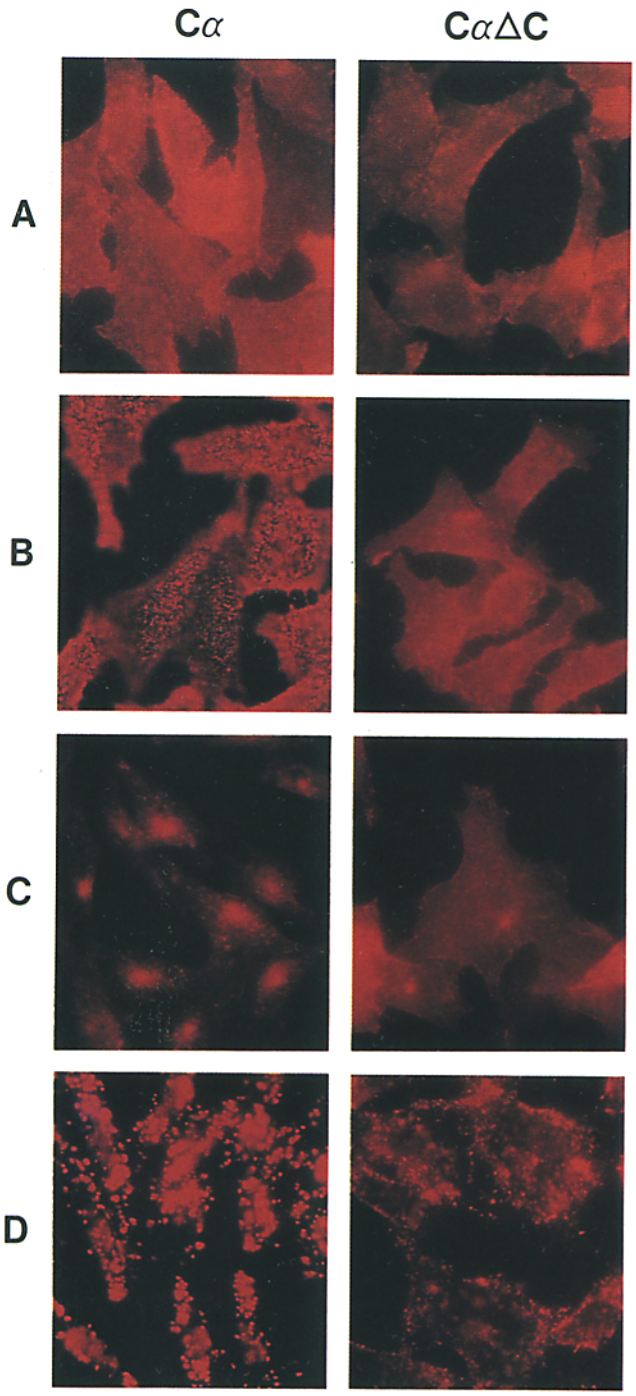

Figure 4. Internalization analysis of full length and truncated TGF-α precursor by immunofluorescence using α1mAb antibody. (*A*) Live cells were exposed to α1mAb at 0°C, incubated without antibodies for 30 min at 0°C, fixed, permeabilized, and stained with secondary antibody. A homogenous surface staining could be observed in both Cα and CαΔC cells. (*B*) Live cells were exposed to α1mAb at 0°C, and then incubated for 30 min at 37°C, fixed without permeabilization and stained with secondary antibody. A homogenous staining pattern was observed in both Cα and CαΔC cells. In addition, a slightly punctated pattern was apparent in the Cα cells only, possibly representing clusters of transmembrane TGF-α on the cell surface. (*C*) Cells were treated identically as in *B* except that the cells were permeabilized after fixation. In addition to surface staining, large clusters of TGF-α can be seen consistently in all Cα cells. These intracellular clusters could only be derived from surface TGF-α after internalization since only the surface population of TGF-α molecule was allowed to interact with anti-TGF-α antibody. The intracellular clusters were considerably less predominant and less consistently detected in CαΔC cells. (*D*) Live cells were allowed to interact with both α1mAb and secondary antibody sequentially at 0°C, followed by incubation at 37°C for 30 min. Clusters of TGF-α could be observed in Cα cells which were less pronounced in CαΔC cells.

staining which was homogeneously distributed but in a punctated pattern indicative of internalization of clustered surface receptors (Fig. 4 *B*). This punctated pattern was less apparent in cells expressing the truncated TGF-α precursor. In the permeabilized cells, there was, in addition, clustered immunofluorescent TGF-α, which by comparison with the pattern of non-permeabilized cells, has to be attributed to intracellular TGF-α (Fig. 4 *C*). In Cα cells, these clusters were consistently seen in all cells, whereas in CαΔC cells, the clusters were much smaller, less consistent and detectable in only a fraction of the cells. Since the staining detected only the complexes of transmembrane TGF-α with αlmAb that were initially formed at the cell surface of live cells, we conclude that the intracellular clusters of immunofluorescent complexes can only be derived from cell surface TGF-α that was subsequently internalized.

Sequential exposure of live cells to the anti-TGF-α primary antibody and the rhodamine-conjugated secondary antibody on ice, followed by incubation at 37°C, resulted in the appearance of TGF-α clusters resembling internalized clusters of cell surface polypeptides (Fig. 4 *D*). The immunofluorescent patterns of the internalized TGF-α after exposure of Cα cells to only the primary antibody or to both antibodies were different. In the former case, most cells showed one major inclusion and many smaller clusters, whereas, in the latter case, the punctated pattern of large clusters was observed scattered in each cell. The clusters were smaller and the internalization pattern seemed to be less pronounced in the CαΔC cells expressing cytoplasmic truncated TGF-α when compared with that in Cα cells.

Thus, the immunofluorescence analyses of antibody-treated cells suggested an efficient internalization of the transmembrane TGF-α, which was attenuated when most of the cytoplasmic domain was deleted. To quantitate the efficiency of internalization, we employed an antibody-based colorimetric internalization assay (Ishii et al., 1993). Confluent cells were exposed to the anti-TGF-α primary antibody at 0°C, and then incubated at 37°C for different periods of time between 0 to 90 min. Subsequently, cells were fixed with or without permeabilization and a horseradish peroxidase-conjugated secondary antibody was allowed to bind. Incubation of the peroxidase-conjugated antibody complexes with a peroxidase substrate allowed a relative quantitation of the cell surface bound TGF-α, based on the colorimetric determination of the substrate.

After subtraction of background absorption using the parental CHO cells and normalization for a low level of nonspecific decrease of total absorption by cells, the kinetics of internalization of transmembrane TGF-α induced by the monoclonal antibody were quantitated. In a period of 90 min, as much as 38.5% of the full length TGF-α precursor was internalized compared to only 11.9% of the truncated

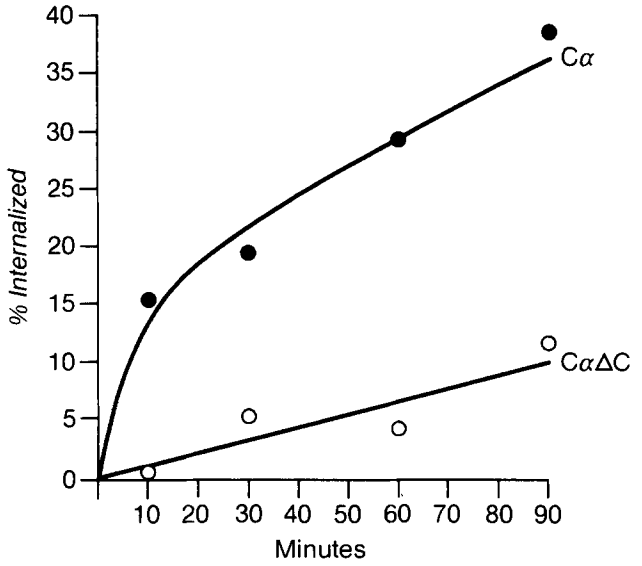

Figure 5. Internalization analysis of full length and truncated TGF-α precursor by colorimetric assay using αlmAb antibody. To quantitate the internalization efficiency, we performed an antibody-based colorimetric assay using conditions described in Fig. 4. The percentage of internalized, antibody-coupled cell surface TGF-α was expressed as a function of time for both Cα and CαΔC cells. The percent internalization was calculated as described in Materials and Methods. In a period of 90 min, 38.5% of the full length TGF-α precursor was internalized compared with only 11.9% of the truncated precursor. The rate of internalization of the full length molecule was rapid in the initial 10 min, since as much as 15% of the surface population was internalized during this period.

form (Fig. 5). In Cα cells, the internalization rate was rapid in the initial 10 min during which 15% of the surface TGF-α precursor was internalized. In contrast to the divalent αlmAb, monovalent Fab fragments of αlmAb bound with a much lower efficiency to cell surface TGF-α. Whereas no internalization was detectable at 37°C, the low level of binding did not allow us to conclude that the internalization depended on the divalent nature of the antibody (data not shown). The low level of apparent internalization of the truncated transmembrane TGF-α in CαΔC cells may result from normal membrane turnover.

Thus, our data indicate that the anti-TGF-α monoclonal antibody induces internalization of transmembrane TGF-α and that truncation of the cytoplasmic domain strongly decreases the efficiency of internalization. This suggests that either the cytoplasmic domain contains an internalization signal that lacks a tyrosine residue or that the internalization is due to association of at least one protein with a cytoplasmic internalization signal.

Association of p106 and p86 with the Transmembrane TGF-α Precursor

To detect proteins associated with transmembrane TGF-α, we performed low stringency immunoprecipitations using a variety of lysis and wash conditions. These analyses did not allow the unambiguous detection of proteins associated with transmembrane TGF-α, because the lowest stringency conditions resulted in a highly complex pattern of non-specific bands following gel electrophoresis (data not shown). We therefore used the chemical cross-linker dithiobis-succinimidyl-propionate (DSP) to stabilize protein–protein associations in the cells before immunoprecipitation. DSP is a hydrophobic, homobifunctional cross-linker with a 12 Å spacer arm. Cross-linking with DSP can be reversed with reducing agents, thus allowing gel electrophoretic analysis and resolution of the cross-linked components (Lomant and Fairbanks, 1976). DSP has been used successfully in identifying proteins associated with several cell surface receptors (de Gunzburg et al., 1989; Cochet et al., 1991; Shakelford and Trowbridge, 1991; Mittler et al., 1991; Takeda et al., 1992; Yoshimura and Lodish, 1992). In contrast to DSP which is cell permeable, its hydrophilic analog dithiobis-sulfo-succinimidyl-propionate (DTSSP) is membrane impermeable. Thus, a comparison of the results of DSP and DTSSP allows us to determine whether protein–protein interactions that are stabilized by cross-linker occur inside or outside the cell (Staros, 1982). The use of these cross-linking agents before immunoprecipitation allows high stringency washes of the immune complexes while maintaining the protein–protein associations.

We treated the transfected or parental CHO cells with DSP and coimmunoprecipitated the transmembrane TGF-α polypeptides and any cross-linked associated proteins using TGF-α specific monoclonal antibodies. The cross-linked immune complexes were then washed under high stringency conditions and reduced with dithiothreitol or β-mercaptoethanol before denaturing gel electrophoresis. Comparison of the immunoprecipitates from the Cα and parental CHO cell lysates showed, besides the TGF-α forms, the presence of two specific components with apparent molecular weights of 106 and 86 kD in the TGF-α expressing cells (Fig. 6 *A*). These bands were consistently seen in a large number (>20) of analyses, irrespective of whether TAB mAb or αlmAb was used as anti-TGF-α antibody, and were not observed when control antibodies were used. An additional 160-kD protein was seen in about half of the analyses (data not shown). The detection of the 86- and 106-kD protein was seen in about half of the analyses (data not shown). The detection of the 86- and 106-kD proteins (to which we refer as p86 and p106) in Cα cells was totally dependent on the use of DSP and these proteins were thus not detected under identical reaction conditions in which DSP was omitted (Fig. 6 *B*). When DTSSP was used instead of DSP as the cross-linking agent, the two specific bands were not detected (Fig. 6 *B*), suggesting that cell permeation of the cross-linker was required and thus, that the interactions of these proteins with transmembrane TGF-α that were stabilized by DSP did not occur extracellularly. In another variation of the experiment, total cell lysates instead of intact cells were treated with DSP followed by immunoprecipitation with the anti-TGF-α antibody. In these experiments, we did not detect either of these two proteins, suggesting that an intact cell membrane that retained the transmembrane TGF-α and the natural proximity of the proteins were essential for successful cross-linking (Fig. 6 *B*). Finally, binding of the monoclonal antibody to transmembrane TGF-α on intact cells, followed by cross-linking with DSP and subsequent immunoprecipitation of the cell lysate gave identical results as when cross-linking preceded the exposure to antibody (data not shown). Thus, these associated proteins interacted with transmembrane

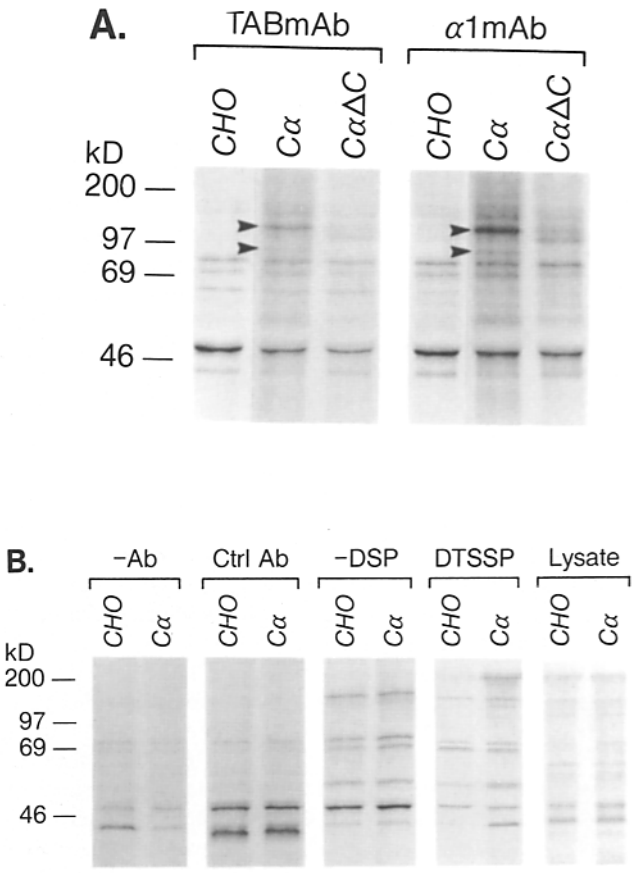

Figure 6. Immunoprecipitation of TGF-α associated proteins p106 and p86 after chemical cross-linking. (*A*) ^{35}S-Labeled Cα and CαΔC cells were exposed to the cross-linker DSP, after which they were immunoprecipitated for TGF-α under high stringency. Two specific bands (*arrows*) could be observed in immunoprecipitates from Cα cells (lanes *2* and *5*), which had electrophoretic mobilities of 106 and 86 kD. The 106-kD protein could also be immunoprecipitated from CαΔC cells but at lower abundance (lanes *3* and *6*). Both proteins were absent in parental CHO cells (lanes *1* and *4*). The association of these proteins was consistently detected using two different anti-TGF-α antibodies in more than twenty experiments. The TGF-α proteins were allowed to run off the gel. The position of molecular weight markers are shown on the left. (*B*) Control experiments using various conditions demonstrating the specificity of the detection of p106 and p86 in cross-linked anti-TGF-α immunoprecipitations. These proteins were not detected in negative controls including no primary antibody (lanes *1* and *2*), control monoclonal antibody (lanes *3* and *4*), and no cross-linker (lanes *5* and *6*). In addition, the use of DTSSP, a hydrophilic membrane impermeable analogue of DSP, did not allow the detection of p106 and p86 (lanes *7* and *8*). Lastly, cell lysates instead of intact cells were exposed to DSP after which TGF-α was immunoprecipitated (lanes *9* and *10*). In all these cases, electrophoretic patterns of immunoprecipitates from CHO cells and CαΔC cells were identical, and p106 and p86 were not detectable. The position of molecular weight markers are shown on the left.

TGF-α precursor at the cell surface, since only this population had been exposed to the immunoprecipitating antibody.

To characterize further the interaction of these two proteins with transmembrane TGF-α, we performed similar cross-linked immunoprecipitations using CαΔC cells. In these experiments, p106 was seen in immunoprecipitates of CαΔC cells, albeit it at a lower intensity than in Cα cells (Fig. 6 *A*), even though equal amounts of transmembrane TGF-α were immunoprecipitated (data not shown). In contrast, p86 was never detected in the CαΔC cells expressing the truncated transmembrane TGF-α (Fig. 6 *A*), suggesting that the cytoplasmic domain of TGF-α was required for association of this protein. A protein band just below the p106 band was frequently but not always observed in immunoprecipitates of CαΔC cells (Fig. 6 *A*) and was also often detectable at low intensity in Cα cells. Its low and variable intensity and inconsistent presence did not allow us to draw any conclusions about its relevance.

Characterization of p106 and p86

The p106 and p86 proteins did not react in Western blotting analyses using an antibody against TGF-α (data not shown). Because of the similarity in apparent molecular weights with the p85 and p110 subunits of phosphatidyl inositol-3 kinase, we also tested the recognition of p86 and p106 by several different phosphatidyl inositol-3 kinase antibodies in Western analysis, but no reactivity was detectable (data not shown).

Immunoblotting of the cross-linked TGF-α immunoprecipitate from Cα cells using an anti-phosphotyrosine antibody, PY20, demonstrated that the 106-kD, but not the 86-kD protein, was tyrosine phosphorylated. This 106-kD band that reacted with the anti-phosphotyrosine antibody was not detected in immunoprecipitates of CHO cells (Fig. 7 *A*). When the immunoblotting was performed using immunoprecipitates from ^{35}S-labeled cells, and the immunoblot subsequently exposed for autoradiography, the images of the tyrosine-phosphorylated 106-kD band and the specific coimmunoprecipitated ^{35}S-labeled p106 band were superimposable (data not shown). Thus, the coimmunoprecipitated p106 that interacted with transmembrane TGF-α was tyrosine-phosphorylated.

Since p106 and p86 interacted with transmembrane TGF-α, we evaluated further whether any of these associated proteins were exposed at the cell surface. Cell surface proteins of ^{35}S-metabolically labeled CHO and Cα cells were biotinylated with NHS-LC biotin, which is membrane impermeable and thus only reacts with extracellularly exposed proteins, and cross-linked immunoprecipitations were performed. A biotinylated protein with a molecular weight of 106 kD was immunoprecipitated from Cα cells, but not from CHO cells (Fig. 7 *B*). This protein was identical in molecular weight to p106 from previous analyses and the image of the biotinylated band was in fact superimposable on that of the coimmunoprecipitated ^{35}S-labeled p106. This band was not detected when the NHS-LC-biotin was omitted from the experiment (data not shown). In contrast to p106, p86 was not biotinylated.

Association of Kinase Activity with the Cytoplasmic Domain of the TGF-α Precursor

To characterize further the proteins associated with the TGF-α precursor, we performed in vitro kinase assays using exogenous substrates such as acid-denatured enolase, histone 2B, and casein. Live cells were exposed to the cross-linker DSP and immunoprecipitated using monoclonal TGF-α antibodies, as outlined above. After extensive washing of the cross-linked immune complexes, in vitro kinase reactions were

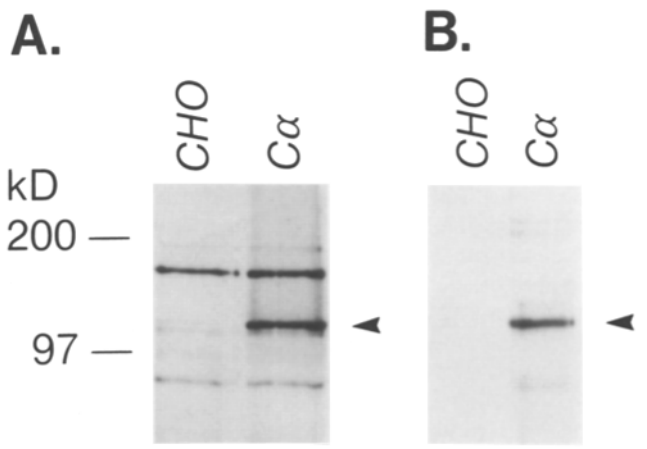

Figure 7. Characterization of p106 and p86. (*A*) ^{35}S-labeled cells were exposed to the cross-linker DSP and immunoprecipitated with anti-TGF-α TABmAb. The immunoprecipitates were subjected to Western analysis using an anti-phosphotyrosine antibody (PY20) and alkaline phosphatase-conjugated secondary antibody. A band corresponding to 106 kD (*arrow*) was seen in immunoprecipitates from Cα cells (lane *2*) but not in parental CHO cells (lane *1*) and was superimposable on the p106 in the ^{35}S-labeled pattern (data not shown). p86 did not react with the anti-phosphotyrosine antibody and was present in the ^{35}S-image (date not shown). The TGF-α proteins were allowed to run off the gel. The position of molecular weight markers are shown on the left. (*B*) ^{35}S-labeled cells were surface biotinylated, and then exposed to DSP and immunoprecipitated for TGF-α. Biotinylated proteins were visualized using streptavidin-conjugated horseradish peroxidase as probe. A band corresponding to 106-kD (*arrow*) was seen in immunoprecipitates from Cα cells (lane *2*) but not parental CHO cells (lane *1*). The image of this biotinylated 106-kD protein was superimposable to that of the ^{35}S-labeled associated p106. p86 was present in the ^{35}S image but not labeled by surface biotin (data not shown). The TGF-α proteins were allowed to run off the gel.

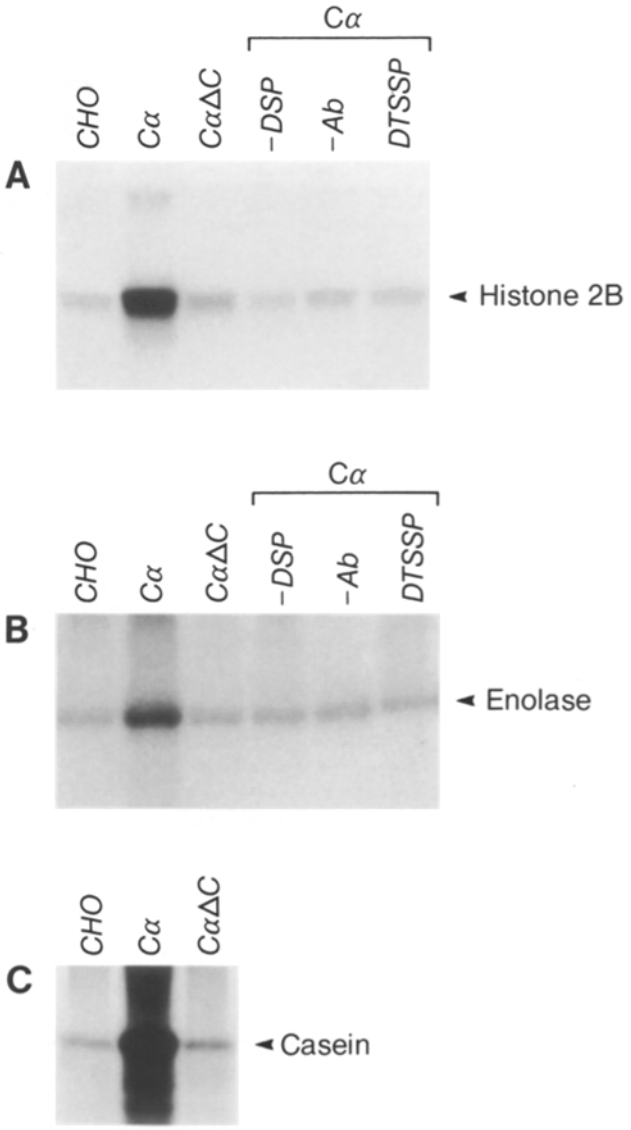

Figure 8. In vitro kinase assay of TGF-α associated protein kinase complex. Cells were exposed to the cross-linker DSP and immunoprecipitated for TGF-α with TABmAb. These immunoprecipitates were subjected to in vitro kinase assay using the exogenous substrates histone 2B (*A*, lanes *1–3*), acid-denatured enolase (*B*, lanes *1–3*), and casein (*C*, lanes *1–3*) at 100 ng/ml, with 1 μCi or γ^{32}P-ATP and allowed to incubate for 30 min at room temperature. Only immune complexes from Cα cells phosphorylated the three substrates whereas those from parental CHO cells and CαΔC cells expressing the truncated form of TGF-α were negative. Control experiments on Cα cells but using no cross-linker, no antibody, or DTSSP instead of DSP were also negative using either histone 2B (*A*, lanes *4–6*) or enolase (*B*, lanes *4–6*) as substrate.

carried out using the exogenous substrates in the presence of γ^{32}P-ATP. Dissociation of the cross-linked TGF-α precursor and associated protein complexes using DTT before the assay resulted in unfavorable kinase reaction conditions. We therefore performed kinase reactions using the cross-linked immunoprecipitated complex coupled to protein A-Sepharose. Protein complexes immunoprecipitated with the TGF-α specific antibody from Cα cells phosphorylated all three substrates (Fig. 8, *A*, *B*, and *C*). The rate and extent of this activity was comparable to that of documented kinases, such as the type II TGF-β receptor kinase, which was used as a positive control (data not shown). Under these reaction conditions, no other ^{32}P-phosphorylated proteins were detectable, either in the presence or absence of the exogenous substrates, suggesting that under these conditions autophosphorylation was not detectable. In contrast to Cα cells, immunoprecipitates from CαΔC cells were negative as were control immunoprecipitates using no primary antibody, or DTSSP as cross-linker or no cross-linker. Also, cross-linked immunoprecipitates from untransfected CHO cells were similarly negative in the kinase assays. In all these cases, a low background level of radioactivity was associated with the substrate protein (Fig. 8, *A*, *B*, and *C*).

Although these kinase substrates have documented preferences as to the type of kinase action, that is, tyrosine-specific or serine/threonine-specific, we performed phosphoamino acid analysis on the products of these kinase reactions to confirm the nature of the phosphorylated residues. The ^{32}P-phosphorylated enolase and histone 2B were hydrolyzed and the radiolabeled amino acids were separated by two-dimensional electrophoretic analysis. The ^{32}P-labeled enolase contained phosphorylated tyrosine and no detectable phosphoserine or phosphothreonine (Fig. 9 *A*). The low background level of ^{32}P associated with enolase in control ex-

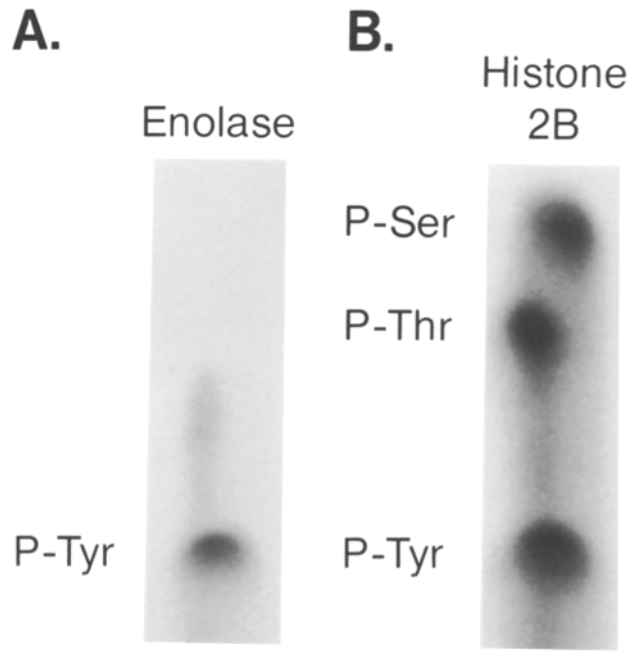

Figure 9. Phosphoamino acid analysis of substrates phosphorylated by the protein kinase complex associated with transmembrane TGF-α. Cα cells were exposed to DSP, immunoprecipitated for TGF-α, and in vitro kinase assays were performed on enolase or histone 2B as exogenous substrate. The ^{32}P-phosphorylated substrates were electrophoretically separated and electrotransferred, onto PVDF membrane, from which the radioactive bands were excised, hydrolyzed, and examined by two-dimensional electrophoresis. Acid-denatured enolase was phosphorylated on tyrosine residue exclusively (*A*), whereas histone 2B was phosphorylated on serine, threonine, and tyrosine (*B*).

periments was due to a minimal level of phosphotyrosine and unincorporated phosphate. In the case of histone 2B, the phosphorylated substrate had high levels of phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 9 *B*). The low background level of ^{32}P on histone in the control reactions was due to phosphorylated serine and threonine, but not tyrosine, and free phosphate (data not shown). Thus, the kinase activities associated with the cross-linked immunoprecipitate of the TGF-α precursor phosphorylated tyrosine in both enolase and histone 2B, and serine and threonine in histone 2B.

To examine further the kinase activity associated with transmembrane TGF-α, we introduced a panel of tyrosine-specific kinase inhibitors into the kinase assays. Methyl-hydroxycinnamate, lavendustin A, hydroxydihydroxybenzyl-amino-benzoic acid, and tyrphostin completely abolished the kinase activity, whereas genistein reduced the activity (Fig. 10 *A*). Herbimycin A had no effect on the activity (Fig. 10 *A*). This pattern of inhibition was identical when either enolase or histone 2B was used as substrate (Fig. 10 *B*). In addition, we also used three other protein kinase inhibitors which have a preference for serine/threonine kinase, including staurosporine, H7 and HA1004, in the presence of histone 2B as exogenous substrate. Staurosporine inhibited the kinase activity entirely whereas H7 and HA1004 reduced the activity at relatively high concentrations (Fig. 10 *B*).

Discussion

A variety of growth factors, especially the members of the EGF/TGF-α family are synthesized as transmembrane precursor molecules (Massagué and Pandiella, 1993). This has been well substantiated in the case of TGF-α (Bringman et al., 1987; Gentry et al., 1987), which under certain conditions is cleaved to release the soluble growth factor by a regulated process (Pandiella and Massagué, 1991*a*,*b*). On the other hand, growth factor receptors themselves, which are normally transmembrane proteins, can also exist as soluble forms of the extracellular domain, as in the case of the EGF/TGF-α receptor (Ullrich et al., 1984; Weber et al., 1984) and several other receptors (Rubin et al., 1985; Leung et al., 1987; Mosley et al., 1989; Zupan et al., 1989; Loetscher et al., 1990; Schall et al., 1990). Thus, the concept of ligand-receptor relationship in which a soluble ligand binds to a transmembrane receptor should be expanded. Instead, ligands and receptors could sometimes be considered as components of direct cell–cell communication systems

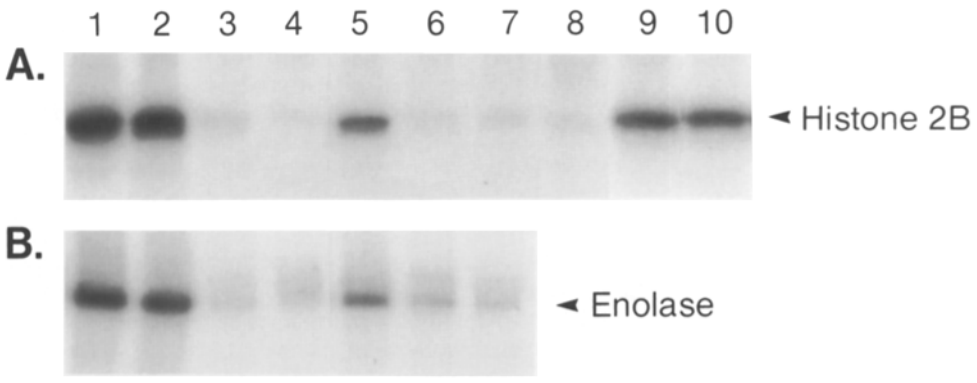

Figure 10. Effect of protein kinase inhibitors on the kinase activities of transmembrane TGF-α associated protein kinase complex. The in vitro kinase activity of the TGF-α associated protein kinase complex immunoprecipitated from Cα cells was tested as in Fig. 8 in the presence of kinase inhibitors: 50 μg/ml herbimycin A (*2*), 0.3 μM hydroxy-dihydroxy-benzyl-aminobenzoic acid (*3*), 240 μM tyrphostin (*4*), 180 μM genistein (*5*), 2 μM lavendustin A (*6*), and 300 μM methyl-hydroxycinnamate (*7*). Except for herbimycin A and genistein, these inhibitors abolished the kinase activities of the complex. Herbimycin A had no effect and genistein inhibited partially the kinase activity. This pattern of inhibition was observed using both histone 2B (*A*) or enolase (*B*) as substrate. In addition, staurosporine at 1 μM also abolished the kinase activities (*8*), whereas H7 (*9*) and HA1004 (*10*) only reduced the activities even at concentrations as high as 500 μM, when histone was used as substrate.

between transmembrane molecules. That transmembrane protein–protein interactions can result in signal transduction has been established in the case of bride of sevenless and sevenless (Kramer et al., 1991), c-kit and steel factor (Flanagan et al., 1991; Brannan et al., 1992), and the TGF-α precursor and EGF/TGF-α receptor (Brachmann et al., 1989; Wong et al., 1989). Whereas in these cases only unidirectional signaling through the receptor has been documented, these interactions raise the possibility of two-directional communication which involves signals associated with the cytoplasmic domain of the transmembrane ligand. It has been postulated before that the EGF precursor may be a receptor (Pfeffer and Ullrich, 1985), but, so far, no experimental evidence has been presented.

Based on these observations and the extreme sequence conservation of the cytoplasmic domain of the TGF-α precursor, we began examining the function of this domain. Since the cytoplasmic region is very short and lacks any known motifs involved in signal transduction, a possible function would require an interaction with a protein which mediates cytoplasmic signals. In this context, cytoplasmic kinases are often involved in control of growth and differentiation. As an example, CD4 and CD8 have short cysteine-rich cytoplasmic domains, and are associated with the $p56^{lck}$ kinase (Turner et al., 1990; Shaw et al., 1989, 1990). Similarly, the ζ chain of the T cell receptor is devoid of intrinsic enzymatic capacity but may mediate its action through ZAP-70 (Chan et al., 1991). We thus evaluated the association of proteins with transmembrane TGF-α, using a CHO cell line which lacks EGF/TGF-α receptors (Livneh et al., 1986; Clark et al., 1988) that could interfere with our analyses.

The presence of associated proteins was first suggested by the induction of internalization of transmembrane TGF-α by a specific monoclonal antibody. The kinetics of internalization resembled ligand-induced internalization of cell surface receptors, since the rate was rapid during the initial 10 min and progressed more slowly thereafter (Wileman et al., 1985). In addition, efficient internalization was dependent on the presence of the cytoplasmic domain. Ligand- or single antibody-induced internalization of transmembrane proteins such as receptors requires an internalization motif in the cytoplasmic domain, which usually contains a critical tyrosine (Trowbridge, 1991). Such is the case in the transferrin receptor (Collawn et al., 1990; McGraw and Maxfield, 1990), LDL receptor (Chen et al., 1990; Bansal and Gierasch, 1991), mannose-6-phosphate receptor (Jadot et al., 1992), insulin receptor (Reynet et al., 1990; Rajagopalan et al., 1991), EGF receptor (Sorkin et al., 1992), and lysosomal acid phosphatase (Eberle et al., 1991; Lehmann et al., 1992). However, the cytoplasmic domain of the TGF-α precursor (Derynck et al., 1984) does not contain a tyrosine residue which could be responsible for its internalization. This raises the possibility that the internalization is due to a cytoplasmic internalization motif in an associated protein, as in the case of the FcγRIII receptor which has a short cytoplasmic domain without internalization sequence, yet has an associated polypeptide responsible for internalization (Amigorena et al., 1992). Alternatively, it is possible that the cytoplasmic domain of TGF-α contains an internalization sequence that lacks tyrosine yet results in the required tight β turn (Letourneur and Klausner, 1992; Chang et al., 1993).

Since the internalization studies only suggested association of proteins with the cytoplasmic domain of transmembrane TGF-α, we performed immunoprecipitation analyses. Low stringency immunoprecipitations resulted in such high background that, even though suggestive, the association of specific proteins could not be convincingly demonstrated. We thus used the membrane permeable and reversible cross-linker DSP to covalently stabilize protein–protein interactions in combination with high stringency coimmunoprecipitation. DSP has been used successfully to detect the tyrosine kinase jak2 associated with the erythropoietin receptor (Yoshimura and Lodish, 1992; Witthuhn et al., 1993) and several tyrosine phosphorylated proteins associated with the EGF/TGF-α receptor (Cochet et al., 1991) and the interleukin-2 receptor (Shackelford and Trowbridge, 1991). Furthermore, the association of a protein p60 with $p21^{ras}$ (de Gunzburg et al., 1989) and the presence of CD45 in the CD4 or CD8 complex (Mittler et al., 1991; Takeda et al., 1992) were also detected by this method. Two proteins, p106 and p86, were specifically coimmunoprecipitated with the TGF-α precursor using TGF-α specific antibodies. The inability to detect associated proteins using the membrane-impermeable analog DTSSP suggests intracellular interactions which also require intact cell membranes based on control experiments using cell lysates. Furthermore, these proteins are associated with transmembrane TGF-α at the cell surface since exposure of intact cells to anti-TGF-α antibody allowed coimmunoprecipitation of p106 and p86 with the surface population of transmembrane TGF-α. Whether both p106 and p86 interact directly with TGF-α, or only one of them is in direct contact with transmembrane TGF-α and the second interacts with the first one is unknown. It is also possible that additional proteins are associated with the transmembrane TGF-α protein complex in these cells. As an example, an associated protein of ~160 kD was detected in about half of the cross-linked immunoprecipitation experiments. Indeed, the use of this cross-linker with a defined spacer length allows for other as yet undetected proteins.

p106 is tyrosine-phosphorylated and associated with the full-length and truncated transmembrane TGF-α. Its association with the truncated version was less than with the full-length polypeptide, even though the immunoprecipitated levels of both TGF-α precursors were very similar. Furthermore, the association was only detected with DSP and not with DTSSP. Thus, this interaction may involve physical contact with the transmembrane region of TGF-α, but the COOH-terminal 31 amino acids may contribute to reinforce this interaction as well. Cell surface biotinylation indicated that p106 may have an extracellular domain. The association of the transmembrane p106 with the TGF-α precursor may bear some similarity with the association of DRAP-27, a 27-kD transmembrane protein (Mitamura et al., 1992), with the diphtheria toxin receptor, which corresponds to HB-EGF (Naglich et al., 1992).

In contrast to p106, the association of p86 was totally dependent on the COOH-terminal 31 amino acids of transmembrane TGF-α. Similarly, the kinase activity was not detected in immunoprecipitates of truncated transmembrane TGF-α. Thus, p86 is a likely candidate for this kinase activity associated with the cytoplasmic domain, although the enzymatic activity may be due to another as yet undetectable protein which, similar to p86, associates with the cytoplasmic domain. Phosphoamino acid analyses revealed that the kinase phosphorylates in vitro not only on tyrosine but also on serine and threonine. The kinase activity can be abolished

by inhibitors with specificity for tyrosine kinases, and by staurosporine, which inhibits many serine/threonine kinases. The identical patterns of differential sensitivity to these inhibitors using either enolase and histone 2B as substrates suggest that the dual specificity may reside in a single enzyme, but does not rule out the possibility of two different kinases.

The biological relevance of our findings is as yet unclear and awaits further characterization of the associated proteins and their interactions with transmembrane TGF-α in cell lines that naturally express TGF-α. However, the endogenous coexpression of the EGF/TGF-α receptor with its intrinsic kinase activity and its many associated proteins precludes such analyses in the absence of specific antibodies for p86 and p106. The association of kinase activity and the tyrosine phosphorylation of p106 in the cross-linked immunoprecipitates suggests that p106 may represent a target for the kinase, since these proteins are likely to be in direct contact. Thus, the protein complex associated with transmembrane TGF-α in the transfected CHO cells contains at least two associated proteins: p86 is associated with the cytoplasmic domain of TGF-α and may represent the kinase, whereas p106 may be a transmembrane protein that is phosphorylated by this kinase. Alternatively, p106 and p86 may represent two subunits of a kinase that are both required for enzymatic activity. How the associated proteins interact with the cytoplasmic domain of TGF-α is currently unknown. However, the many cysteines in the short cytoplasmic sequence may be involved in protein–protein interactions, similar to the interactions between the lck kinase and the cytoplasmic domains of CD4 and CD8 (Turner et al., 1990; Shaw et al., 1989, 1990).

Phosphorylation, especially tyrosine phosphorylation, is a pivotal event in signal transduction related to cell growth and differentiation (Hanks, 1991). The association of cell surface TGF-α with a protein complex with kinase activity suggests that it may be involved in signal transduction. This makes it now possible that interaction of the EGF/TGF-α receptor or its secreted extracellular domain (Soderquist et al., 1988; Petch et al., 1990) with transmembrane TGF-α may induce or modulate signals through transmembrane TGF-α. The biological consequences of any signaling associated with transmembrane TGF-α and specifically its cytoplasmic domain remains to be determined.

One possibility is that the associated protein complex may be involved in the cleavage process of transmembrane TGF-α, since processing of TGF-α at the cell surface may be due to a membrane-bound proteolytic system which can be activated (Pandiella and Masagué, 1991*a*,*b*; Bosenberg et al., 1993). In this scenario, p86 and p106 may be regulatory or effector components of the proteolytic system leading to the cleavage and subsequent release of the soluble TGF-α.

An intriguing possibility is that signal transduction associated with the cytoplasmic domain of transmembrane TGF-α allows for two-directional communication between adjacent cells after physical interaction of transmembrane TGF-α with its receptor. It is well documented that activation of the EGF/TGF-α receptor of one cell by cell surface TGF-α of an adjacent cell induces receptor-mediated signaling in the receptor expressing cell (Brachmann et al., 1989; Wong et al., 1989). This interaction may now be accompanied by an induction of signal transduction the transmembrane TGF-α associated complex, which may lead to biological changes in the TGF-α expressing cell. If so, these changes may, by analogy with other developmentally regulated cell–cell communication systems (Singer, 1992; Greenwald and Rubin, 1992), be related to cell differentiation and proliferation. Thus, expression of transmembrane TGF-α with its associated complex may result in biological consequences that are qualitatively different from soluble TGF-α. Our current findings serve as a basis to characterize the TGF-α associated protein complex and to explore its regulation by occupation by the EGF/TGF-α receptor and the consequences for the TGF-α producing cells.

We thank Dr. David Morgan for helpful discussions.

This work was supported by a fellowship from the Tobacco-Related Disease Research Program to L. Shum, by grants from the American Cancer Society and the National Cancer Institute to R. Derynck, and by the Markey Charitable Trust to the Programs in Biological Sciences.

Received for publication 3 January 1994 and in revised form 10 March 1994.

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