The precursor for transforming growth factor-\(\alpha\), proTGF-\(\alpha\), is synthesized as an integral membrane glycoprotein with the mature TGF-\(\alpha\) sequence located in the extracellular domain. Retrovirally transformed rat embryo fibroblasts (FeSV-Fre cells) expressing the endogenous proTGF-\(\alpha\) gene release and accumulate in the medium mature TGF-\(\alpha\), as well as a heterogeneous (17–19 kDa) group of soluble, bioactive TGF-\(\alpha\) precursor forms. These precursors correspond to the heterogeneous glycosylated extracellular domain of proTGF-\(\alpha\) which is released from the membrane by proteolytic cleavage. They are designated mesoTGF-\(\alpha\) to denote their intermediate position in the proTGF-\(\alpha\) processing pathway. The nature of the carbohydrate linked to mesoTGF-\(\alpha\) has been examined by treatment with glycosidases and the use of metabolic inhibitors of glycosylation. The results indicate that the TGF-\(\alpha\) precursors from FeSV-Fre cells contain O-linked carbohydrate as well as sialylated N-linked carbohydrate. Heterogeneous N-linked glycosylation of an 11-kDa core polypeptide accounts for the heterogeneous nature of mesoTGF-\(\alpha\). MesoTGF-\(\alpha\) released by cells treated with inhibitors of N-linked carbohydrate processing appears as a 17-kDa species. Treatment with these inhibitors does not alter significantly the production of mesoTGF-\(\alpha\) or mature TGF-\(\alpha\) by the cells. However, treatment of cells with an inhibitor of co-translational N-linked glycosylation, tunicamycin, results in the accumulation of mesoTGF-\(\alpha\) in the medium and blocks the production of mature TGF-\(\alpha\) under conditions in which overall protein synthesis is only minimally affected. These findings suggest that the proTGF-\(\alpha\) processing activity is limiting in FeSV-Fre cells and other transformed cells that accumulate mesoTGF-\(\alpha\) in the medium and that proTGF-\(\alpha\) processing depends on a component whose function may require N-linked glycosylation.

Transforming growth factor-\(\alpha\) (TGF-\(\alpha\))\(^1\) is a 50-amino acid polypeptide hormone that is structurally related to epidermal growth factor (EGF) (1–5) and exerts its biological actions by binding to the EGF receptor (1, 6, 7). Binding of TGF-\(\alpha\) results in activation of the receptor-associated tyrosine kinase (8, 9) and induction of a mitogenic response in various cell types. Both TGF-\(\alpha\) and EGF exogenously added to fibroblasts enable them to form colonies in semisolid medium if other growth factors are also present (2, 10–12). Although TGF-\(\alpha\) is expressed in certain normal tissues (13, 14), oncogenic transformation frequently results in enhanced expression of TGF-\(\alpha\) (15). In contrast, oncogenic transformation has not yet been found to be associated with expression of EGF.

Analysis of rat and human cDNAs has indicated that TGF-\(\alpha\) is synthesized as part of a large precursor, proTGF-\(\alpha\), of 159 or 160 amino acids (4, 5). About 93% identity exists between the amino acid sequences of human and rat proTGF-\(\alpha\). The predicted structure of proTGF-\(\alpha\) includes a NH\(_2\)-terminal signal sequence of 23 uncharged amino acids followed by a domain of about 72 amino acids that includes the mature TGF-\(\alpha\) sequence. This domain is followed by a highly hydrophobic sequence of 25 amino acids, and then a cysteine-rich COOH-terminal domain of 39 amino acids (4, 5). proTGF-\(\alpha\) behaves as a membrane protein in cells transfected with proTGF-\(\alpha\) cDNA expression vectors (16, 17). Translation of rat proTGF-\(\alpha\) mRNA in vitro in the presence of microsomal membrane vesicles has shown that the NH\(_2\)-terminal domain of proTGF-\(\alpha\) is translocated across the endoplasmic reticulum membrane and co-translationally N-glycosylated (18). Translocation of proTGF-\(\alpha\) becomes arrested at the second hydrophobic sequence. This sequence acts as a membrane-spanning domain that leaves proTGF-\(\alpha\) as an integral membrane protein (18). The mature TGF-\(\alpha\) sequence is located in the extracellular domain of proTGF-\(\alpha\) flanked by valine- and alanine-rich sequences. Mature TGF-\(\alpha\) can be released from the precursor by the action of elastase-like enzymes (19).

Transformed cells that express TGF-\(\alpha\) accumulate in the medium substantial amounts of bioactive TGF-\(\alpha\) forms larger (>20 kDa) than the mature 6-kDa TGF-\(\alpha\) (2, 15, 16, 20–23). These larger TGF-\(\alpha\) forms are of interest because they constitute the most abundant forms of the factor found in the medium of various human tumor-derived cells (15). The larger TGF-\(\alpha\) forms have the properties of soluble precursors for TGF-\(\alpha\) and are similar to mature TGF-\(\alpha\) in their potency to bind to EGF/TGF-\(\alpha\) receptors and activate the receptor-associated tyrosine kinase in intact cells (19). Like mature TGF-\(\alpha\), the soluble TGF-\(\alpha\) precursors are mitogenically active and can contribute to the induction of soft agar colony formation by NRK-49F rat fibroblasts in the presence of TGF-\(\beta\). In the current studies, we have examined the basis for the heterogeneity of TGF-\(\alpha\) precursors released by retrovirally transformed rat fibroblasts and the relationship of these precursor forms to proTGF-\(\alpha\). The results indicate that these TGF-\(\alpha\) precursors are heterogeneously glycosylated forms of the extracellular domain of proTGF-\(\alpha\). We find that the generation of these forms and their conversion into mature
TGF-α in transformed rat fibroblasts are prevented by treatment with tunicamycin, an inhibitor of co-translational N-linked glycosylation.

**EXPERIMENTAL PROCEDURES**

**Source of TGF-α Precursors**—Serum-free medium conditioned by Snyder-Theilen feline sarcoma virus-transformed Fischer rat embryo (FeSV-Fre) fibroblasts was collected and dialyzed against 0.1 M acetic acid, lyophilized and fractionated over Bio-Gel P-60 molecular filtration columns as previously described (2). Some incubations were performed in the presence of tunicamycin (Behring Diagnostics), castanospermine (Boehringer Mannheim), desoxynojirimycin (Boehringer Mannheim) or swainsonine (Behring Diagnostics) as indicated. According to the supplier’s data sheet, the tunicamycin preparation used in these studies had the following isomer composition: 10% Aα, 20% Bβ, 25% Cτ, 20% Cτ, 10% Dα, and minor proportions of 12 other isomers.

**Electrophoresis and Immunoblot Assays**—Electrophoresis on decyl sulfate-polyacrylamide gels was performed as previously described (24). After electrophoresis, material in the gels was electro-transferred onto nitrocellulose sheets and subjected to immunoblot analysis as previously described (19). Anti-TGF-α antibodies were generated against a synthetic peptide that corresponds to the sequence between residues 33 and 50 of mature rat TGF-α (residues 71–88 in proTGF-α). These antibodies recognize TGF-α but not EGF (19). Anti-proTGF-α antibodies were generated against a mixture of two synthetic peptides that correspond to residues 138–151 and 145–161 in the COOH terminus of rat proTGF-α. The details for synthesis and purification of the peptide immunogens and generation of the antibodies have been described (19). Under the specific immunoblot conditions used (19) the amount of meso-TGF-α present in 50 ml of conditioned medium yielded a detectable signal after a 24-h autoradiographic exposure of the immunoblots to Kodak film with a Cronex Lightning Plus (Du Pont) screen at −70 °C.

**High Pressure Liquid Chromatography**—Fractions from the Bio-Gel P-60 step containing 17- and 19-kDa TGF-α precursors were pooled, lyophilized, and redissolved with 0.05% trifluoroacetic acid in water. Chromatograms were chromatographed at room temperature on a 4.6 × 250 mm Vydac C18 column (The Separations Group, Hesperia, CA) equilibrated with 0.05% trifluoroacetic acid, and eluted with a 0–20% gradient of acetonitrile (4%/min) followed by a slower (0.4%/min) gradient between 20 and 35% acetonitrile in 0.05% trifluoroacetic acid.

**Deglycosylations**—"Endoglycosidase F" digestions were performed using a protease-free preparation containing endoglycosidase F (endo-β-N-acetylglucosaminidase F) and peptide-N-glycosidase F (Boehringer Mannheim). According to the supplier, both enzymatic activities in this preparation are expressed at pH 7.0 (21). Incubations were carried out for 2 h at 37 °C after adding 50 milliunits of the enzyme to samples of lyophilized TGF-α precursor resuspended in 20–40 μl of a solution containing 60 mM sodium phosphate, pH 7.0, 20 mM EDTA, 10 μg/ml 2-mercaptoethanol, 10 μg/ml Nonidet P-40, and 1 mg/ml sodium dodecyl sulfate. Digestions with endoglycosidase H (endo-β-N-acetylglucosaminidase H, Boehringer Mannheim) were performed at 37 °C for 18–20 h in 40 μl of a solution containing 25 mM sodium citrate, pH 5.5, 10 μg/ml 2-mercaptoethanol, 1 mg/ml sodium dodecyl sulfate, and 2 milliunits of the enzyme.

**Electrophoresis and Immunoblot Assays**—Polypeptide digestion products were analyzed by 10% SDS-polyacrylamide gel electrophoresis (24) and heating at 100 °C for 2 min. For chemical deglycosylation, samples of TGF-α precursors were resuspended and dialyzed against 1% sodium dodecyl sulfate, and lyophilized. Treatment of the samples with trifluoromethanesulfonic acid (Sigma) was performed as previously described (25).

**RESULTS**

**Lack of COOH-terminal proTGF-α Sequences in the Secreted TGF-α Precursors**—FeSV-Fre cells, one of the highest producers of TGF-α by expression of the endogenous proTGF-α gene (1, 2) were used in the present studies. We tested the hypothesis (19) that the soluble TGF-α precursor forms released by FeSV-Fre cells into the medium lack the cytosolic COOH-terminal domain of proTGF-α. The estimated molecular mass of the 17–19-kDa TGF-α precursors released by FeSV-Fre cells is similar to the molecular mass predicted for the 159-amino acid rat proTGF-α. Indeed, the primary translation product of rat proTGF-α mRNA migrates as a 17-kDa polypeptide in sodium dodecyl sulfate-polyacrylamide gels (Fig. 1 and Ref. 18). Like the secreted TGF-α precursors, the product of in vitro translation of rat proTGF-α mRNA was recognized by antibodies raised against the synthetic peptide that corresponds to part of the sequence of mature TGF-α (Fig. 1). By immunizing rabbits with synthetic peptides that correspond to sequences in the COOH-terminal domain of rat proTGF-α, we raised a second set of antibodies that would discriminate between full length proTGF-α and fragments lacking the COOH-terminal domain (18). These antibodies recognized specifically the product of translation of rat proTGF-α mRNA, but not the 17–19-kDa TGF-α precursors released by FeSV-Fre cells (Fig. 1), suggesting that they lack the proTGF-α COOH-terminal domain.

The two most prominent TGF-α precursor forms released into the medium were separated from each other by reverse-phase HPLC for further structural analysis. These two species, 17 and 19 kDa, behaved as relatively hydrophilic poly-

![Fig. 1. The soluble 17–19-kDa TGF-α precursors are not recognized by antibodies against the COOH-terminal domain of proTGF-α.](image-url)
peptides eluting from C₄₆ columns at 25.5 and 24.5% acetonitrile, respectively (Fig. 2). These results suggest that the 17- and 19-kDa TGF-α precursors released by the cells are intermediate forms of the processing of proTGF-α that lack not only the COOH-terminal cytoplasmic domain but also the highly hydrophobic transmembrane domain of proTGF-α. These soluble TGF-α precursors will henceforth be termed mesoTGF-α to indicate their intermediate position in the proTGF-α processing pathway.

**Presence of N-Linked and O-Linked Carbohydrate in the TGF-α Precursors**—The sequence predicted for proTGF-α exhibits a site for N-linked glycosylation located at asparagine 25 in the extracellular domain of the molecule (4, 5). Endoglycosidase-sensitive carbohydrate does become incorporated into proTGF-α translated in vitro in the presence of microsomal membrane vesicles (18). We examined the presence of endoglycosidase-sensitive carbohydrate in the 17- and 19-kDa forms of mesoTGF-α. Samples of both polypeptides were treated with a preparation containing endoglycosidase F and peptide:N-glycosidase F that removes high mannose, hybrid, and complex N-linked carbohydrate from glycoproteins (26). Both polypeptides were converted to a 14-kDa product by this treatment (Fig. 3). Thus, the 17- and 19-kDa mesoTGF-α forms are glycoproteins that contain N-linked carbohydrate. Furthermore, these results indicate that heterogeneous N-linked glycosylation of a common polypeptide chain is the basis for the heterogeneity of mesoTGF-α found in the medium of FeSV-Fre cells.

Since the apparent molecular weight of endoglycosidase F-treated mesoTGF-α was still larger than that expected for the polypeptide backbone of the free extracellular domain of proTGF-α (residues 1-88 or 1-95, see Fig. 7), we examined the possibility that additional molecular mass might be contributed to the TGF-α precursors by O-linked carbohydrate chains. Treatment with O-glycanses, an enzyme that removes unsubstituted core disaccharide gal-galNAc linked to serine or threonine (27), converted the 19- and 17-kDa mesoTGF-α forms to 17- and 15-kDa products, respectively (Fig. 4). This action of O-glycanses was not dependent on pretreatment of mesoTGF-α with neuraminidase which is usually required to

![Fig. 3. Presence of N-linked carbohydrate in the mesoTGF-α.](image)

![Fig. 4. Presence of O-linked carbohydrate in the soluble TGF-α precursors.](image)
render O-linked oligosaccharides sensitive to O-glycanase if they are sialylated. However, treatment with neuraminidase did decrease the apparent molecular mass of mesoTGF-α by about 2 kDa (Fig. 4B). These results indicate that O-linked carbohydrate and sialylated N-linked carbohydrate are present in mesoTGF-α from FeSV-Fre cells. In confirmation of these observations, treatment with trifluoromethanesulfonic acid which removes both N- and O-linked carbohydrate from glycoproteins (25) converted both, the 17- and the 19-kDa mesoTGF-α forms into a 11-kDa product (Fig. 4B).

Tunicamycin Inhibits Normal Processing of TGF-α Precursors—N-Linked glycosylation is a requirement for the cell surface expression of some membrane glycoproteins (28, 29) but not others (30). N-Linked glycosylation is also important for normal release of many secretory proteins (31). To determine whether N-linked glycosylation is necessary for the processing and release of mesoTGF-α and TGF-α, we treated FeSV-Fre cells with tunicamycin, an agent that inhibits lipid carrier-dependent protein N-glycosylation (32). Exposure of FeSV-Fre cells to tunicamycin (20–100 ng/ml) inhibited in a concentration-dependent manner the accumulation of 17–19-kDa mesoTGF-α in the medium and concomitantly induced the appearance of lower amounts of a 14-kDa mesoTGF-α form (Fig. 5A). This form of mesoTGF-α has the molecular weight expected for the version of the polypeptide lacking N-linked carbohydrate but retaining O-linked carbohydrate, according to the results of in vitro deglycosylation experiments (see Fig. 3). At high concentration (1 μg/ml), tunicamycin inhibited completely the accumulation of mesoTGF-α in the medium of FeSV-Fre cells (Fig. 6A).

The most striking effect of tunicamycin on the TGF-α biosynthetic pathway in these cells was the inhibition of mature TGF-α production. Essentially complete inhibition of this process was observed with as little as 60 ng/ml tunicamycin and occurred under conditions in which the cells were still expressing and releasing a small amount of 14-kDa mesoTGF-α (Fig. 5A). These results suggest that treatment with tunicamycin may interfere with conversion of TGF-α precursors into mature TGF-α. Although tunicamycin at higher concentrations can inhibit protein synthesis (31, 32), the inhibition of TGF-α production occurred at concentrations of tunicamycin which decreased only marginally (~10%) overall protein synthesis but quantitatively blocked [3H]mannose incorporation into cell macromolecules (Fig. 5B). The potency of tunicamycin at inhibiting N-linked glycosylation of TGF-α precursors and overall protein synthesis was that expected from the isomer composition of the tunicamycin preparation used in these experiments, according to Duksin and Mahoney (31).

Processing of N-Linked Carbohydrate Is Not Essential for TGF-α Biosynthesis—Next we examined whether complete processing of the N-linked carbohydrate chains was necessary for expression of normal levels of mesoTGF-α and mature TGF-α by the cells. Inhibitors of various steps of the post-translational N-linked glycoprotein-processing pathway were

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**Fig. 5. Effect of tunicamycin on TGF-α production and total protein synthesis.** Cultures of FeSV-Fre cells were incubated for 16 h in the presence of serum-free medium containing the indicated concentrations of tunicamycin. The conditioned medium was collected and subjected to immunoblot analysis using anti-TGF-α antibodies and 125I-protein A. The autoradiogram from one representative experiment (n = 3) is shown in panel A. Other cultures of FeSV-Fre cells were incubated with tunicamycin as described above, but in cysteine- and serum-free medium containing 20 μCi/ml of [35S]cysteine (Du Pont-New England Nuclear) or in medium containing 20 μCi/ml of N-[2-3H]mannose (Du Pont-New England Nuclear). After incubation for 16 h, the cell cultures were rinsed twice with Krebs-Ringer Hepes buffer, solubilized with 1% sodium dodecyl sulfate, and macromolecules were precipitated with ice-cold 10% trichloroacetic acid, and collected on nitrocellulose filter discs. The radioactivity associated with the filters was determined and is plotted in panel B (△, [35S]cysteine; ▲, [3H]mannose, average of duplicate determinations). The radioactivity associated with the 17–19-kDa mesoTGF-α (○) and TGF-α ( ●) bands in the immunoblots was determined by densitometry of the autoradiogram shown in panel A and is also plotted in panel B. Data are expressed as percent of controls incubated without tunicamycin.

**Fig. 6. Effect of inhibitors of N-linked carbohydrate processing on the production of mesoTGF-α and TGF-α.** A, FeSV-Fre cells were incubated for 16 h in serum-free medium alone or with 1 μg/ml tunicamycin, 20 μg/ml castanospermine, 20 μg/ml desoxymannojirinycin, or 1 μg/ml swainsonine. The medium was then subjected to immunoblot analysis using anti-TGF-α antiserum and 125I-protein A. A resulting autoradiogram is shown. B, mesoTGF-α from the medium of control or castanospermine-treated cells was incubated with buffer alone, endoglycosidase H, or endoglycosidase F. Samples were then displayed by immunoblotting with anti-TGF-α and 125I-protein A, followed by autoradiography.
used. They included castanospermine which inhibits glucosidases I and II responsible for the removal of the outermost glucose residues of the gliomucoglycan (33); 1-deoxymannojirimycin which inhibits removal of L-1,2-linked mannoses by mannosidase I (34, 35); and swainsonine which inhibits removal of α-1,3-linked and α-1,6-linked mannosae by mannosidase II in the pathway of asparagine-linked complex-type oligosaccharide formation (36). In the presence of any of these agents, FeSV-Fre cells failed to accumulate 19-kDa mesoTGF-α but produced higher levels of 17-kDa mesoTGF-α, the amount of mesoTGF-α produced being similar to the overall amount produced by control cells (Fig. 6A). In contrast to mesoTGF-α from control cells, mesoTGF-α produced by cells treated with castanospermine was sensitive to digestion with endoglycosidase H, an enzyme that cleaves high mannosae and hybrid N-linked but not complex type oligosaccharide chains. These results suggest that heterogeneous N-linked glycosylation of TGF-α precursors is due to the variable processing of complex-type carbohydrate linked to asparagine 25. None of the inhibitors tested altered significantly the level of mature TGF-α accumulated in the medium of FeSV-Fre cells (Fig. 6A).

**DISCUSSION**

The results of this study combined with previous information (16–19) suggest a pathway for proTGF-α processing in FeSV-Fre rat fibroblasts whose features are illustrated and summarized in Fig. 7. Two soluble products of this pathway accumulate in the medium, the glycosylated partially processed mesoTGF-α and the mature TGF-α polypeptide. These results confirm the hypothesis that the heterogeneous 17–19-kDa TGF-α precursor forms produced by FeSV-Fre cells correspond to the extracellular domain of proTGF-α released into the medium by proteolytic cleavage. Several lines of evidence indicate that mesoTGF-α comprises residues 1–88 or 1–85 of the rat proTGF-α sequence. The results of proTGF-α mRNA translation experiments have suggested that contrary to most signal sequences, the hydrophobic signal sequence between amino acids 1 and 23 of proTGF-α is not removed by signal peptidase in the microsomal lumen (18). The molecular mass (11 kDa after complete deglycosylation, Fig. 4) and hydrophilic character of mesoTGF-α, as well as the transmembrane topology of proTGF-α virtually exclude the possibility that the hydrophobic sequence extends into or beyond the hydrophobic transmembrane domain. Whether proTGF-α is cleaved at the Ala<sup>Val</sup> or at the Lys<sup>M</sup>-Lys<sup>Val</sup> site to release mesoTGF-α is unknown at present. Cleavage at Ala<sup>Val</sup> must eventually occur to generate mature TGF-α, but on the basis of the available evidence we cannot exclude the possibility that cleavage at Lys<sup>M</sup>-Lys<sup>Val</sup> may occur first and serve as an initial step to make mesoTGF-α available for further processing by the elastase-like TGF-α converting activity.

The results also show that the TGF-α precursors in FeSV-Fre cells contain both O-linked and sialylated N-linked oligosaccharides. N-Linked glycosylation of proTGF-α takes place co-translationally (18) presumably at asparagine 25, the only canonical site for this type of glycosylation present in the extracellular domain of proTGF-α (4, 5). N-Linked glycosylation has also been noted in human proTGF-α (16). It has been suggested that the heterogeneity of intermediate TGF-α precursors released by cells could be due to the incomplete removal of the sequence between residues 89 and 96 (16), but the present results clearly show that the size heterogeneity of mesoTGF-α derives from processing of the N-linked oligosaccharide in this glycoprotein.

Inhibition of processing of the N-linked carbohydrate eliminates the size heterogeneity of mesoTGF-α but does not affect the overall level of mesoTGF-α or mature TGF-α released into the medium even when inhibition of oligosaccharide processing occurs early in the pathway, i.e. when glucosidase I is inhibited by castanospermine. Thus, incomplete processing of N-linked carbohydrate does not affect the expression or proteolytic processing of TGF-α precursors. In marked contrast to this phenomenon, inhibition of the initial steps of N-linked glycosylation by tunicamycin blocks the release of mature TGF-α by FeSV-Fre cells. Although tunicamycin at high concentrations can inhibit protein synthesis, its effect on TGF-α expression can be observed under conditions in which overall protein synthesis is quantitatively close (>90%) to normal. Inhibition of N-linked glycosylation could affect the transport of proTGF-α and its products to the cell surface by interfering directly with exocytotic sorting and traffic. The results are also compatible with the possibility that tunicamycin may inhibit the proTGF-α-processing enzymes in FeSV-Fre cells.

The tunicamycin sensitivity of the TGF-α biosynthetic pathway in FeSV-Fre fibroblasts, a transformed cell line expressing the endogenous proTGF-α gene, is in contrast to the relative resistance of TGF-α production to tunicamycin in Chinese hamster ovary cells transfected with a proTGF-α expression plasmid (16). This difference might be due to differences in the proTGF-α-processing enzyme(s) or in the secretory pathways in both cell types.

The results also suggest that the activity that converts proTGF-α into mature TGF-α is limiting in FeSV-Fre cells. This phenomenon and its consequence, the accumulation of mesoTGF-α in the medium, may not be unique to this cell type. TGF-α-related polypeptides with molecular weight similar to mesoTGF-α are found almost invariably in other
retrovirally transformed cells (20–23), and they are the predominant form of TGF-α released by several human tumor-derived cells (15, 23, 37). This result raises the interesting possibility that the proTGF-α-converting enzyme(s) might constitute a regulatory rate limiting step in the processing of this polypeptide.

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