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The Carboxyl-Terminal Valine Residues of ProTGFα Are Required for Its Efficient Maturation and Intracellular Routing

Glenn P. Briley,*† M. Andrew Hissong,†‡ Marie L. Chiu,* and David C. Lee*‡§

*University of North Carolina Lineberger Comprehensive Cancer Center, and ‡Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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Soluble forms of transforming growth factor-α (TGFα) are derived by proteolytic processing of an integral membrane glycoprotein precursor (proTGFα). Previous studies indicated that phorbol ester-induced cleavage of proTGFα in CHO cells is dependent on the presence of a valine residue located at the carboxyl terminus of the precursor's cytoplasmic domain. We reassessed this requirement with epitope-tagged constructs introduced into transformed rat liver epithelial cells that normally express and process TGFα. We found that proTGFα mutants lacking the terminal valine residues showed greatly reduced maturation to the fully glycosylated form. Additionally, they were present at substantially reduced levels on the cell surface and, instead, accumulated in the endoplasmic reticulum. Consistent with these results, enzyme-linked immunosorbant assay (ELISA) and Western blot analyses revealed little or no soluble TGFα in medium conditioned by cells expressing the mutant constructs. Finally, a truncated proTGFα mutant lacking most of the cytoplasmic domain but retaining a carboxyl-terminal valine was processed and cleaved in a near-normal manner. These results, some of which were reproduced in CHO cells, indicate that the predominant effect of the carboxyl-terminal valines is to ensure normal maturation and routing of the precursor.

INTRODUCTION

Polypeptide growth factors are divided into two general categories that are distinguished by whether or not the initial translation product is a soluble protein. Thus, some are expressed as integral membrane growth factors that contain receptor-binding sequences in their extracellular domains. Examples of this latter category include mammalian, worm, and fly members of the epidermal growth factor (EGF)/neuregulin superfamily (reviewed in Lee *et al.*, 1995), colony-stimulating factor-1 (Kawasaki *et al.*, 1985), the c-kit ligand (KL; reviewed in Massagué and Pandiella, 1993), ligands for the eph receptor family (Davis *et al.*, 1994), and *Drosophila* bride-of-sevenless (Boss; Hart *et al.*, 1990). Additionally, tumor necrosis factor-α (TNFα) is a type II membrane glycoprotein with its amino terminus in the cytoplasm (Kriegler *et al.*, 1988). Most tested members of this class are biologically active as integral membrane proteins (Brachmann *et al.*, 1989; Wong *et al.*, 1989; Perez *et al.*, 1990; Dobashi and Stearn, 1991; Davis *et al.*, 1994), but many are nevertheless cleaved to release soluble receptor ligands (reviewed by Ehlers and Riordan, 1991; also Massagué and Pandiella, 1993). These facts suggest an appealing model in which the activities of membrane-tethered growth factors are spatially regulated via production or activation of specific proteases. Although there is limited evidence to validate this model, it is consistent with studies of the spontaneous

† These two authors contributed equally to this work.
§ Corresponding author: Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295.

mouse mutant Sl^d, which show that exclusive production of a soluble bioactive KL (with complete loss of its integral membrane precursor) yields a spectrum of mouse phenotypes that are similar to those resulting from loss of the KL receptor c-*kit* (Brannan *et al.*, 1991).

Besides providing a mechanism for spatial regulation, membrane-anchored growth factors could have other roles as well. Because internalization of a receptor complex with membrane-anchored growth factor could be substantially slower than that of a complex with soluble ligand, signaling consequences could be different. For example, activation of mitogen-activated protein kinase might be prolonged, thereby promoting cellular differentiation instead of cellular proliferation (Traverse *et al.*, 1992). Integral membrane growth factors might also possess activities that are distinct from those of their soluble derivatives. For example, binding to receptor could simultaneously enhance cell–cell adhesion and induce the receptor's tyrosine kinase activity, as suggested by some model studies (Anklesaria *et al.*, 1990; Flanagan *et al.*, 1991; Kaneko *et al.*, 1991). An especially interesting possibility is that integral membrane growth factors could participate in bidirectional signaling and, thus, function as both receptor and ligand. This hypothesis, first put forth by Pfeffer and Ullrich (1985), is consistent with the recent demonstration of receptor-binding–induced tyrosine phosphorylation of eph-receptor ligands (Holland *et al.*, 1996), and possibly with the identification of a multiprotein proTGFα complex (Shum *et al.*, 1994). Critically important to the future evaluation of these various possibilities is greater knowledge of the processing proteases and their regulation. Although candidate activities have been described (Cappelluti *et al.*, 1993; Harano and Mizuno, 1994), none of the relevant proteases have yet been cloned or characterized.

The integral membrane glycoprotein precursor to soluble TGFα, proTGFα has emerged as a principal model for studies of membrane-anchored growth factors and their proteolytic processing. proTGFα contains 159 or 160 amino acids, depending on the use of alternative splice acceptor sites (Derynck *et al.*, 1984; Lee *et al.*, 1985; Blasband *et al.*, 1990). Its extracellular domain contains the mature 50-amino acid TGFα sequence preceded by a short sequence containing the *N*-glycosylation site (*O*-glycosylation also occurs but the sites have not been mapped), together with a signal peptide. In addition to the transmembrane domain, it also includes a short cytoplasmic region of approximately 40 amino acids that is rich in cysteines and modified by palmitoylation (Bringman *et al.*, 1987; Shum *et al.*, 1996). Its overall structure is distinct from that of the much larger prepro EGF, which contains multiple EGF-like sequences in its extracellular domain and also has a more extensive cytoplasmic domain (Gray *et al.*, 1983; Scott *et al.*, 1983). Although proTGFα generally resembles precursors to the other EGF family members, amphiregulin, heparin-binding EGF, betacellulin, and epiregulin, these precursors share no significant homology outside of the EGF-like motif, and there is little similarity in the apparent proteolytic cleavage sites. Findings that cleavage-resistant forms of proTGFα activate EGF receptor in coculture models (Brachmann *et al.*, 1989; Wong *et al.*, 1989) have been extended by subsequent observations that intact precursors to other EGF family members are also bioactive (Mroczkowski *et al.*, 1989; Dobashi and Stearn, 1991). This raises unanswered questions as to the relative importance of membrane-tethered versus soluble EGF family growth factors in various in vivo contexts.

Release of the fully processed 50-amino acid TGFα peptide (Marquardt *et al.*, 1983) from proTGFα depends on cleavage of alanine–valine bonds at the amino and carboxyl termini, with both cleavage sites flanked by additional small apolar amino acids (alanine, valine, or leucine). The similarity of these proTGFα processing sites to those typically cleaved by elastase enzymes has been previously noted, and exogenous elastases will cleave larger proTGFα derivatives to a species that comigrates with the 50-amino acid protein (Ignotz *et al.*, 1986; Luetteke *et al.*, 1988; Mueller *et al.*, 1990). However, studies comparing the effects of various protease inhibitors on proTGFα processing suggest that elastase-like enzymes may not be responsible for these events in situ (Pandiella *et al.*, 1992). Moreover, despite the apparent similarity of the two cleavage sites, distinct enzymes could be involved. Hence, while the amino-terminal cleavage occurs at a much faster rate in transfected CHO cells (Teixidó *et al.*, 1990), biological fluids and medium conditioned by tumor cell lines contain larger soluble TGFα proteins that are apparently derived by cleavage of the carboxyl-terminal cleavage site only (Ignotz *et al.*, 1986; Bringman *et al.*, 1987; Luetteke *et al.*, 1988).

More recently, attention has focused on the activation of TGFα secretion by diverse treatments including exposure to serum factors, calcium ionophores, and particularly phorbol esters (phorbol 12-myristate 13-acetate [PMA]; Pandiella and Massagué, 1991a,b; Pandiella *et al.*, 1992). Release in response to these agents occurs rapidly (i.e., within minutes) and is attributable to proteolysis of cell surface proTGFα (Bosenberg *et al.*, 1993). The phenomenon of activated cleavage is not restricted to proTGFα, and the ectodomains of other cell surface proteins, including KL, are also rapidly shed (Stein and Rettenmier, 1991; Huang *et al.*, 1992). Nevertheless, activated processing of proTGFα and KL demonstrate different, albeit overlapping, sensitivities to different protease inhibitors (Pandiella *et al.*, 1992). Most surprisingly, PMA-induced cleavage was found to be dependent on the presence of a valine residue located at the carboxyl terminus of the cytoplasmic domain of proTGFα (Bosenberg *et al.*, 1992).

Additionally, a chimeric precursor containing the ectodomain of proTGFα and the cytoplasmic region of KL (which also includes a carboxyl-terminal valine) also undergoes activated cleavage (Bosenberg *et al.*, 1992). This indicated that the cytoplasmic sequences of proTGFα could be replaced by the equivalent domain from another integral membrane growth factor that similarly undergoes activated cleavage. These findings were interpreted to suggest a novel proteolytic cleavage activity that acts on proTGFα sequences in the extracellular environment but is regulated by intracellular signals involving the proTGFα carboxyl-terminal valine. As a result, attention has focused on the PMA-induced proteolytic activity, even though the relationship of this activity to constitutive proTGFα processing has not been firmly established.

In this report, we reexamine the role of cytoplasmic determinants and, particularly, the carboxyl-terminal valine residues in proTGFα maturation and processing. In so doing, we have used *ras*-transformed rat liver epithelial cells as a model system because these cells actively express and process proTGFα encoded by the endogenous gene. Our results indicate that the carboxyl-terminal valines are predominantly required for precursor routing and maturation and that the cytoplasmic domain is otherwise not required for constitutive secretion.

MATERIALS AND METHODS

Cell Lines and Materials

R1 cells (kindly provided by S. Strom, University of Pittsburgh) were grown in Eagle's minimal essential medium containing 10% fetal bovine serum and 50 μg/ml gentamicin. Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 50 μg/ml gentamicin. All media and supplements were from Life Technologies (Grand Island, NY) unless otherwise noted. Mouse monoclonal antibodies against the hemagglutinin (HA) epitope were from Berkeley Antibody (Richmond, CA; clone 16B12) or from Boehringer Mannheim (Indianapolis, IN; clone 12CA5). Antibody directed against the FLAG epitope (M2) was obtained from Eastman Kodak (New Haven, CT). Rabbit anti-GRP78 (BiP) polyclonal antiserum was obtained from Affinity Bioreagents (Neshanic Station, NJ). Rhodamine-conjugated donkey anti-mouse and fluorescein-conjugated donkey anti-rabbit IgGs were obtained from Chemicon International (Temecula, CA), and peroxidase-conjugated goat anti-mouse IgG1 and IgG2b were from Boehringer Mannheim. The vectors pCEP4 and pcDNA3 were obtained from Invitrogen (San Diego, CA). Oligonucleotide primers were synthesized by the University of North Carolina Nucleic Acids Core Facility.

Mutant Construction and Cell Transfection

PCR-based site-directed mutagenesis was used to insert the HA (YPYDVPDYA) and FLAG (DYKDDDDK) epitopes and to create the ΔV mutant. The oligonucleotide primer prHA was used to introduce the HA epitope in proTGFα, primers prFLAGΔV and prΔV were used to produce FLAG-tagged wild-type (Wt) and ΔV variants, and primer prΔV was used to generate ΔV^{HA}. The sequences of these primers were as follows: prHA, 5′-CTGGGCACTTGTTGAAAGCGTAGTCTGGGACGTCGTATGGGTAGTGAGACACCACTGCAGCC-3′; prFLAG, 5′-GGATCTTCAGACCACCTTGTCATCGTCGTCCTTGTAGTCTGTCTCAGAGTGGCAGC-3′; prFLAGΔV, 5′-CCTCTGGGATCTTCACTTGTCATCGTCGTCCTTGTAGTCTGTCTCAGAGTGGCAGC-3′; prΔV, 5′-CCCTCGAGTCATGTCTCAGAGTGGCAGCAAGCAGTCC-3′.

These primers were used in mutagenic reactions with Vent DNA polymerase (New England Biolabs, Beverly, MA) and a 540-bp TGFα cDNA in pBluescript (Stratagene, La Jolla, CA). The first PCR used vector T7 sequencing primer and mutagenic primer; purified product was then used as the forward primer in the second PCR with the reverse vector T3 primer. Resulting products were purified, digested with *Eco*RI, and subcloned into modified pCEP4 (R1) or pcDNA3 (CHO) vectors. The authenticity of cloned sequences was confirmed by the University of North Carolina Automated DNA Sequencing Facility.

The two cytoplasmic truncation mutants (T129C/V) were produced by using the Muta-Gene Phagemid In Vitro Mutagenesis Version 2 Protocol (Bio-Rad, Richmond, CA). Briefly, epitope-tagged proTGFα construct was introduced into *Escherichia coli* strain CJ236 for production of single-stranded uracil-containing template. The single-stranded DNA was annealed to two phosphorylated oligonucleotide primers that were designed to produce constructs encoding truncated proTGFα proteins terminating with either a natural cysteine at position 129 (prT129C) or, alternatively, a valine (prT129V; Figure 1B). The sequences of the two primers were as follows: prT129C, 5′-GACGAGGGCACGGCACCATCAACAGTGTTTGCGGAGCTG-3′; prT129V, 5′-GACGAGGGCACGGCACCATCAGACGTGTTTGCGGAGCTGACA-3′.

Annealing reactions also included a primer designed to eliminate a novel diagnostic *Bst*BI restriction endonuclease site present in parental vector sequence. Synthesis reactions were then performed, and the resulting products were transfected into BMH cells for inactivation of the uracil-containing DNA. Plasmid DNAs isolated from the BMH cells were digested with *Bst*BI to eliminate DNA lacking the desired mutation, and the digestion products were transfected into bacterial strain DH5α for the final amplification. The resulting truncated proTGFα coding sequences were confirmed as described above and subcloned into the eukaryotic expression vector pCEP4. This vector, which carries a hygromycin B-selectable marker, was modified by removal of the Epstein–Barr virus origin of replication and nuclear antigen. For CHO cell transfections, constructs were also cloned into pcDNA3.

Expression vectors were introduced into R1 and CHO cells by calcium phosphate transfection or with Lipofectamine (GIBCO/BRL). Multiple geneticin-(CHO) or hygromycin B-(R1) resistant clones were isolated, and those expressing appropriate levels of vector-encoded transcript were identified by Northern blot analysis of total RNA (Berkowitz *et al.*, 1996).

Cell Lysate Analyses

For Western blot analysis, near-confluent monolayers were dissolved in lysis buffer [1% Triton X-100, 25 mM Tris, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 18 μg/ml aprotinin, 10 μg/ml leupeptin] and placed on ice for 30 min. Clarified lysates were incubated with anti-HA or anti-FLAG (3 μg/500 μg of lysate protein) for a minimum of 4 h at 4°C, and tagged TGFα-antibody complexes were incubated for 1 h after the addition of protein G-agarose (Life Technologies, Grand Island, NY) that had been washed once in ice-cold lysis buffer. Pelleted complexes were washed four times with ice-cold lysis buffer, resuspended in sample buffer [62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol (β-ME), 0.25% phenol red], and boiled for 5 min before loading on 15% polyacrylamide gels. After electrophoresis, gels were soaked for 10 min in 1× Tris-glycine (25 mM Tris base, 192 mM glycine, pH 8.3), and proteins were transferred to prewetted Immobilon-P membrane (Millipore, Bedford, MA). Blots were blocked in TBS-T (20 mM Tris base, 140 mM NaCl,

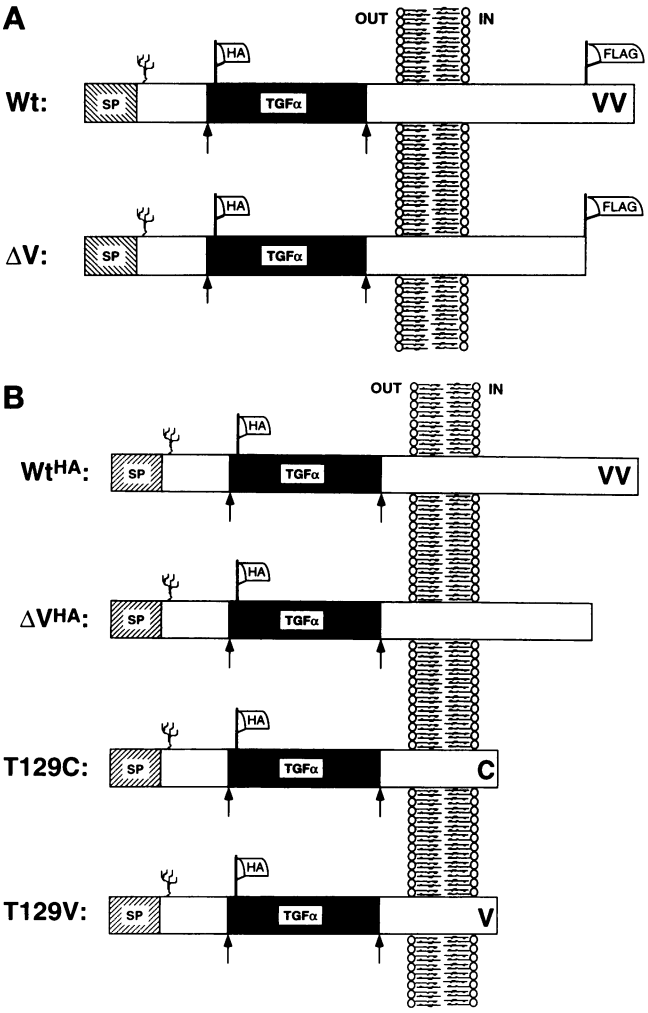

Figure 1. (A) Diagram of dual-tagged proTGFα proteins. Wt denotes the Wt proTGFα sequence, and ΔV is a mutant lacking the two carboxyl-terminal valine residues. The positions of the inserted HA and FLAG epitopes are indicated. The solid box marks the fully processed 50-amino acid TGFα sequence, the shaded box denotes the signal peptide, and the adjacent single site of *N*-linked glycosylation is indicated by the branching symbol. Arrows mark the positions where proteolytic cleavage must occur to release the mature 50-amino acid TGFα. (B) As in A, except that the indicated proTGFα proteins are tagged only with the HA epitope. Wt^{HA}, Wt proTGFα; ΔV^{HA}, a mutant lacking the two carboxyl-terminal valines; T129C, a truncation mutant lacking most of the cytoplasmic domain and terminating with a natural cysteine residue; T129V, an alternate truncation mutant terminating with a valine residue in place of the cysteine.

pH 7.6, 0.05% Tween 20) containing 5% nonfat dry milk for ≥2 h, rinsed in 1× TBS-T for 10 min, and incubated with primary antibody for 60 min. Blots were washed for four 10-min periods in TBS-T, and then incubated with secondary antibody for an additional 60 min. Peroxidase-conjugated goat anti-mouse IgG1 and IgG2b secondary antibodies (diluted to 1:10,000) were used to detect anti-FLAG and anti-HA, respectively. Blots were incubated in SuperSignal Substrate (Pierce, Rockford, IL) for 5 min before exposing them to x-ray film to detect chemiluminescence.

For pulse-chase analyses, cells were grown to approximately 90% confluence in complete medium, washed for two 20-min periods with cysteine-free Eagle's minimal essential medium at 37°C, and incubated in 0.6 ml of cysteine-free medium containing 200 μCi/ml [^{35}S]cysteine (Amersham International, Arlington Heights, IL) for 20 min at 37°C. The medium was removed, and the cells were washed in phosphate-buffered saline (PBS) and incubated in complete medium for the indicated chase periods. They were then scraped in lysis buffer and incubated on ice for 30 min. After clarification by centrifugation, TGFα proteins were immunoprecipitated (with anti-FLAG or anti-HA) and resolved on 15% polyacrylamide gels. Gels were fixed in 25% isopropanol and 10% acetic acid for 30 min, soaked in Amplify for 30 min (Amersham International), dried, and exposed to x-ray film at −70°C.

Immunofluorescence

Cells were grown to approximately 70% confluence on coverslips, rinsed three times with solution A (PBS, 0.5% bovine serum albumin) at 4°C, and then incubated for 60 min at 4°C in a 1:40 dilution of anti-HA in solution A. Cells were rinsed as before, fixed for 10 min in 3.7% paraformaldehyde in PBS, rinsed again, and incubated for 60 min at room temperature (RT) in a 1:40 dilution of rhodamine-conjugated donkey anti-mouse antibody in solution A. Coverslips were then rinsed three times in solution A, once in H_20, and mounted. For cytoplasmic staining, cells were rinsed, fixed as above, permeabilized in 0.5% Triton X-100 for 10 min, and incubated for 60 min at RT in solution A containing a 1:40 dilution of anti-FLAG. Coverslips were rinsed and the rhodamine-conjugated secondary antibody applied as above. The procedure for anti-BiP staining was identical except that cells were incubated with primary antibody for 2 h, and the primary antibody was visualized with a 1:40 dilution of fluorescein isothiocyanate-conjugated donkey anti-rabbit in solution A.

Glycosidase Treatments

After immunoprecipitation of protein–antibody complexes with protein G-agarose, pellets were resuspended in 40 μl of endoglycosidase H buffer (3.6 mM Na_2HPO_4/NaH_2PO_4 [pH 5.5], 0.05% SDS, 4 μg/ml PMSF, 70 mM β-ME) and boiled for 5 min. After centrifugation at 12,000 rpm for 20 s, supernatant was collected and incubated with 4 mU of endoglycosidase H for 4 h at 37°C. After the addition of 44 μl of 2× sample buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% β-ME), samples were boiled for 5 min and resolved by SDS-PAGE. For neuraminidase treatment, immunoprecipitates were resuspended in 40 μl of 50 mM sodium acetate (pH 5.2), incubated with 40 mU of neuraminidase (sialidase, Boehringer Mannheim) for 30 min at 37°C, and processed as described above.

Medium Analyses

For medium conditioning, cells were grown to approximately 75% confluence in 150-mm dishes, rinsed in serum-free medium for two 20-min periods at 37°C, and placed in serum-free medium for the indicated time periods. Conditioned medium was clarified and treated with 1 mM PMSF, and medium proteins were concentrated by using Sep-Pak Plus C18 cartridges (Waters, Milford, MA) as previously described (Sandgren *et al.*, 1990). Resulting samples were normalized relative to the amount of lysate protein prior to analysis.

For ELISAs, HA peptide or Sep-Pak-enriched samples were incubated in 96-well radioimmunoassay plates overnight at RT (Costar, Cambridge, MA). Each well was rinsed three times with wash solution (100 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.05% Tween 20), and blocking solution (0.25% bovine albumin, 100 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, pH 7.5, 0.05% Tween 20) was added for 60 min at RT. Cells were rinsed three times with wash buffer and incubated with a 1:1000 dilution of mouse anti-HA antibody in blocking buffer for 60 min at RT. Cells were washed four times and

incubated with a 1:1000 dilution of alkaline phosphatase-conjugated secondary antibody in blocking buffer for 60 min at RT. Wells were washed four times, and *p*-nitrophenyl phosphate (Sigma 104 phosphate substrate) was added. After 60 min at RT, plates were read on a DuPont V_{max} kinetic microplate reader. Sep-Pak-enriched medium proteins were also analyzed by Western blot as described above.

RESULTS

Construction of Epitope-tagged TGFα Precursors

Typically, studies of proTGFα processing have been performed with transfected cells that do not express the endogenous TGFα gene (e.g., CHO cells). We wished instead to examine processing of Wt and mutant proTGFα proteins in cells that normally produce and secrete this growth factor. We chose a *ras*-transformed rat liver epithelial cell (RLEC) line, R1, because it contains relatively high levels of TGFα mRNA (Berkowitz *et al.*, 1996) and actively secretes TGFα into the medium. Additionally, EGF receptor (EGFR) is undetectable in this cell line. The latter point has practical importance because we wished to monitor TGFα secretion partly by measuring its accumulation in the medium. An unpublished comparison of related RLEC clones that express equivalent levels of TGFα mRNA revealed an inverse correlation between the amount of EGFR protein detected by Western blot and the level of TGFα in conditioned medium. This suggested that TGFα is rapidly internalized when EGFR is present at significant levels.

To distinguish TGFα species expressed from exogenous vectors (versus those produced from the endogenous gene), we tagged the transfected proTGFα with two distinct epitopes (Figure 1A). The influenza HA epitope was incorporated between amino acids 42 and 43, which correspond to positions 4 (His) and 5 (Phe) of the fully processed 50-amino acid TGFα. This region of the mature growth factor is relatively unimportant for biological activity (Defeo-Jones *et al.*, 1988), and it was recently tagged by others (Arribas and Massagué, 1995). The FLAG epitope, on the other hand, was incorporated between amino acids 157 and 158 (of 159-amino acid proTGFα), immediately preceding the two terminal valine residues of the Wt proTGFα cytoplasmic domain. We chose to retain the two terminal valine residues in their normal position because a prior study indicated that they were essential for activated cleavage of proTGFα (Bosenberg *et al.*, 1992). However, to test this thesis with R1 cells, we also created a variant of the dual-tagged TGFα precursor (ΔV) that lacked the two terminal valines and hence contained the FLAG sequence at its carboxyl terminus (Figure 1A). The two proTGFα variants were cloned into the mammalian expression vector pCEP4 under the control of the cytomegalovirus promoter, and the resulting vectors were transfected into R1 cells. Stable transformants expressing comparable levels of the two novel TGFα transcripts (Wt^{low} and ΔV) were then selected (Figure 2A). For comparison, we also isolated a Wt clone (Wt^{high}) that expressed the

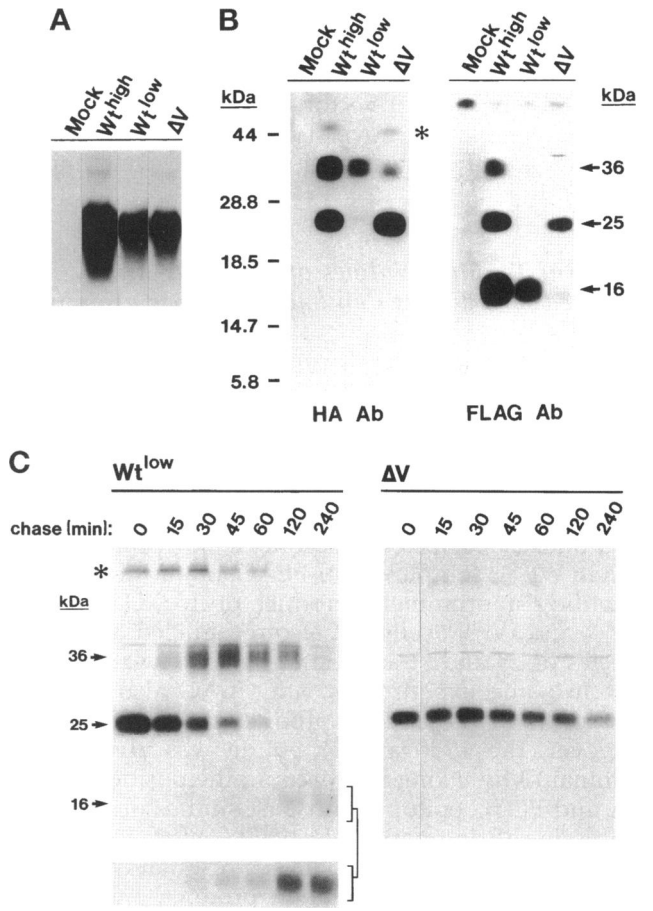

Figure 2. Expression and localization of dual-tagged proTGFα constructs. (A) Northern blot analysis. R1 cells were stably transfected with either parental pCEP4 vector (Mock) or pCEP4 containing epitope-tagged Wt or mutant (ΔV) proTGFα sequences. Total RNA was prepared from selected clones and examined by Northern blot analysis for expression of vector-encoded transcripts. The probe was the coding portion of mouse TGFα cDNA. Wt^{high} cells expressed severalfold higher levels of the appropriately sized transcript that Wt^{low} cells. (B) Western blot analysis. Total cell lysates were immunoprecipitated with anti-FLAG, and the proteins resolved by SDS-PAGE, transferred to blots, and probed with anti-HA (left) or anti-FLAG (right). The positions of molecular weight markers are shown on the left, and the apparent sizes of tagged proTGFα species are shown on the right. An asterisk denotes an apparently specific but variably detected band of approximately 44 kDa. (C) Pulse–chase experiment. Cell clones expressing the indicated proTGFα proteins were incubated in cysteine-free medium supplemented with [^{35}S]cysteine for 20 min at 37°C and then transferred to complete medium without labeled cysteine for the indicated times. Cells were lysed, proteins were immunoprecipitated with anti-FLAG, and recovered proteins were analyzed by SDS-PAGE/autoradiography. The apparent molecular weights of prominent bands are shown on the left, and an asterisk marks a minor variably observed band. Below, a section of an overexposed autoradiogram more clearly reveals the presence of the 16-kDa tail in Wt^{low} samples.

transfected vector at levels severalfold higher than those of Wt^{low} (Figure 2A), and we established a line (Mock) that was stably transfected with the parental (non-TGFα-encoding) vector. All clones used in this study expressed the novel TGFα transcripts at levels that were significantly higher than those of the endogenous mRNA.

Carboxyl-Terminal Valines of ProTGFα Are Required for Efficient Cell Surface Localization in R1 Cells

To examine expression of Wt proTGFα, we immunoprecipitated cell lysates with the FLAG antibody and then performed Western blot analyses using either anti-HA (Figure 2B, left) or anti-FLAG (Figure 2B, right). Anti-HA identified a prominent protein of approximately 36 kDa present in both Wt^{low} and Wt^{high} cells but also detected a second major protein of 25 kDa in Wt^{high} samples. Anti-FLAG, on the other hand, identified a prominent product of 16 kDa in both Wt^{low} and Wt^{high} cells but also recognized additional species of 25 and 36 kDa in the Wt^{high} lysates. (Note that in some experiments, anti-FLAG also detected low levels of the 36-kDa product in Wt^{low} samples; however, the 16-kDa polypeptide was always predominant.) By taking into account the positions of the HA and FLAG epitopes and past studies of proTGFα processing (Bringman *et al.*, 1987; Gentry *et al.*, 1987; Teixidó *et al.*, 1990), these data suggest that the 25-kDa protein is an initial translation product, and the 36-kDa protein corresponds to a more extensively glycosylated form. Because the 16-kDa protein includes the carboxyl-terminal FLAG epitope but not the HA tag, it presumably corresponds to a proteolytic "tail" product that contains only the juxtamembrane, transmembrane, and cytoplasmic sequences. These conclusions were corroborated by pulse–chase experiments in which ^{35}S-labeled lysate proteins were immunoprecipitated with anti-FLAG. Analysis of Wt^{low} cells clearly established a temporal sequence in which the initial 25-kDa protein was converted to the 36-kDa product, and the latter then to the 16-kDa tail (Figure 2C, left; note that an overexposure of the Wt^{low} tail is shown below). This temporal sequence, which was confirmed with Wt^{high} cells, is consistent with previous descriptions of proTGFα processing. The relative accumulation of the 25-kDa protein in Wt^{high} versus Wt^{low} cells indicates that maturation to the 36-kDa product was rate-limiting when proTGFα was expressed at particularly high levels.

We similarly examined proTGFα expression in the ΔV clone of R1 cells by anti-FLAG immunoprecipitation followed by anti-HA or anti-FLAG Western blot analysis. Because this clone expressed vector-derived RNA at a level that was comparable to that of Wt^{low} cells, we expected a similar profile of epitope-tagged proteins. In fact, the pattern was quite different. Thus, anti-HA identified a prominent 25-kDa product, with only minor amounts of the 36-kDa protein detected (Figure 2B, left). Anti-FLAG also predominantly identified the 25-kDa protein, with only minor amounts of the 16-kDa tail detected after long exposures (Figure 2B, right). These results suggested that the 25-kDa product was accumulating in ΔV cells, with greatly reduced maturation to the 36-kDa species. This interpretation was supported by pulse–chase analyses, which clearly showed that the initial 25-kDa protein turned over with little or no conversion to the 36- or 16-kDa products (Figure 2C, right).

To confirm impaired surface localization of the ΔV mutant, we performed immunofluorescent staining of intact cells. The use of anti-HA yielded marked surface staining of Wt^{low} cells (Figure 3c). In contrast, the surface staining of ΔV (Figure 3b) cells was only barely increased relative to that of the mock-transfected control cells. Anti-FLAG staining of fixed Wt^{low} cells yielded a conspicuous punctate staining pattern in the cytoplasm (Figure 3d), consistent with vesicular transport of proTGFα to the cell surface. In contrast, anti-FLAG staining of permeabilized ΔV cells was largely restricted to a perinuclear structure (Figure 3e). An antibody directed against an established endoplasmic reticulum (ER) marker protein, BiP/GRP78 (Collier *et al.*, 1993; Green *et al.*, 1995), produced similar staining of permeabilized ΔV cells (Figure 3f), whereas staining with a Golgi-specific marker yielded a distinct pattern. These results corroborated the preceding findings and indicated that the ΔV protein (i.e., the predominant 25-kDa form) accumulated in the rough endoplasmic reticulum (RER).

We also examined the effects of specific deglycosidases on TGFα species present in cell lysates. Endoglycosidase H (Figure 4, lanes E), which is not active against the complex carbohydrate of mature glycoproteins, did not affect the mobility of the 36-kDa protein from Wt^{low} cells, but it reduced the apparent size of the 25-kDa product in the same lysate by approximately 5 kDa. In contrast, neuraminidase, which removes the terminal sialic acid residues of mature glycoproteins, reduced the size of the 36-kDa protein by approximately 3 kDa without affecting the 25-kDa product (Figure 4, lanes N). Similar results were obtained with ΔV samples (Figure 4, right), except that only minor amounts of the 36-kDa form were present as expected. Collectively, these various data confirm that the 25-kDa protein is an ER form, whereas the 36-kDa protein has exited this compartment and likely accounts for the surface immunostaining.

Thus, these various results reveal a correlation among extensive glycosylation of proTGFα, its movement from the RER to the cell surface, and its apparent proteolytic processing. They are supported and extended by studies of TGFα secretion described later.

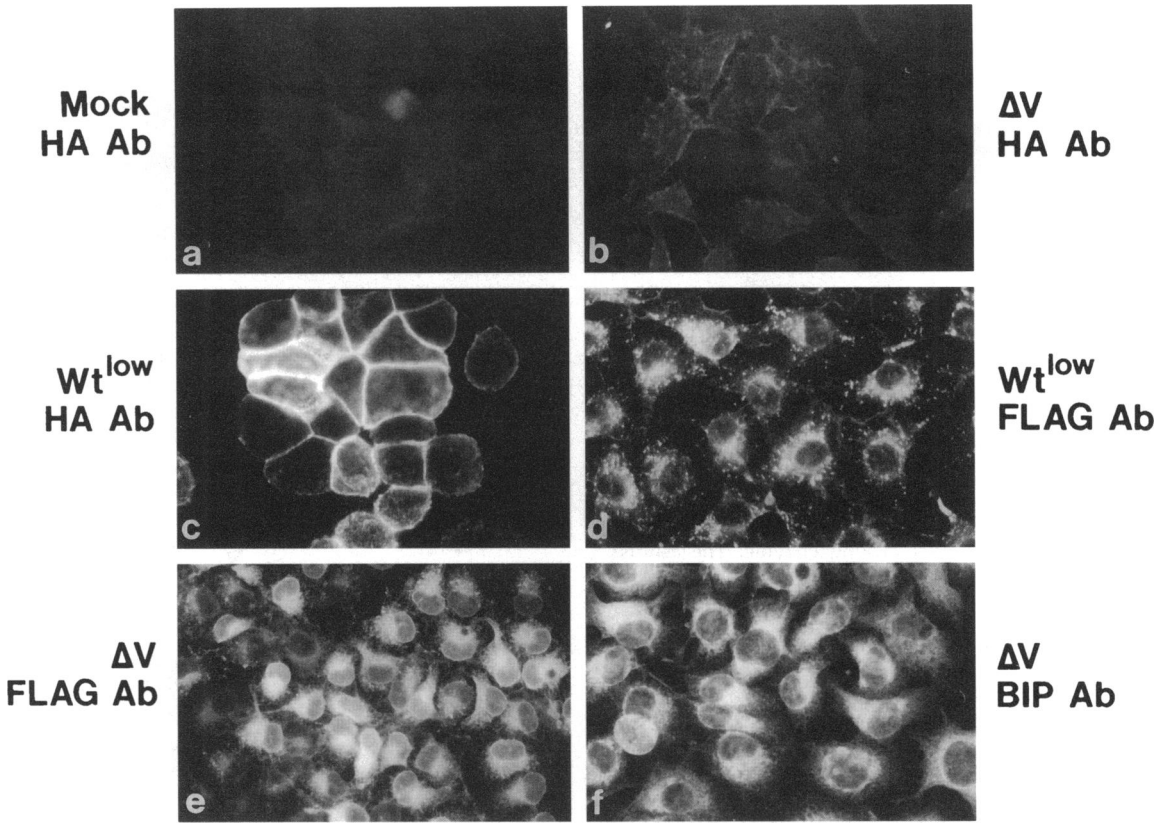

Figure 3. (a–c) Cell surface staining of tagged proTGFα proteins. Intact cells from the indicated clones were incubated with anti-HA for 60 min at 4°C, fixed for 10 min in paraformaldehyde, and incubated with rhodamine-conjugated anti-mouse IgG for 1 h at RT. (d–f) Cytoplasmic staining of tagged proTGFα proteins. The indicated cells were fixed as above, permeabilized, and incubated with either anti-FLAG or anti-BiP for 1 or 2 h, respectively. Primary antibody was then visualized with fluorescein isothiocyanate-conjugated anti-rabbit.

Similar Results Are Obtained with CHO Cell Clones and with Non-FLAG-tagged ProTGFα

Since a previous study found that (nontagged) ΔV-like proTGFα mutants were normally expressed on the surface of CHO cells (Bosenberg *et al.*, 1992), we performed the following experiments. First, we confirmed that routing of the ΔV mutant was also impaired in CHO cells. CHO cell clones stably transfected with epitope-tagged Wt and ΔV constructs were selected, and the expression of TGFα species in these cells was then compared. Pulse–chase analysis of a CHO Wt clone revealed a temporal pattern of proTGFα maturation and processing that was similar to the R1 Wt pattern, except that minor products of 22 and 23 kDa were also detected (Figure 5A, top). In marked contrast, examination of a CHO ΔV clone showed that the initial 25-kDa species largely turned over with little evidence of subsequent maturation or processing (Figure 5A, bottom). These results were confirmed by Western blot analysis. Anti-HA (Figure 5B, left) and anti-FLAG (Figure 5B, right) detected predominant 36-kDa surface and 16-kDa tail forms, respectively, in the CHO Wt clone, but the 25-kDa ER-localized protein was the major form recognized by both antibodies in CHO ΔV samples. Moreover, anti-HA immunostaining of intact CHO clones revealed a marked decrease in surface staining of CHO cells expressing the ΔV mutant compared with those expressing the Wt protein. Thus, removal of the carboxyl-terminal valines of proTGFα had similar consequences in R1 and CHO cells.

Second, to exclude the possibility that our results were influenced by placement of the FLAG epitope immediately preceding the carboxyl-terminal valines, we made new Wt and ΔV constructs (Wt^{HA} and ΔV^{HA}) that were tagged only with the HA epitope (Figure 1B). Pulse–chase analysis of an R1 clone expressing Wt^{HA} showed the expected conversion of a 24-kDa species to a modified protein of 35 kDa (these products are slightly smaller than those of the double-tagged precursor due to the absence of the FLAG epitope; Figure 6A, top). Because these constructs were tagged only with the HA epitope, we could not monitor the appearance of the proTGFα tail. Nevertheless, pulse–chase analysis of an R1 ΔV^{HA} clone clearly revealed that in the absence of the carboxyl-

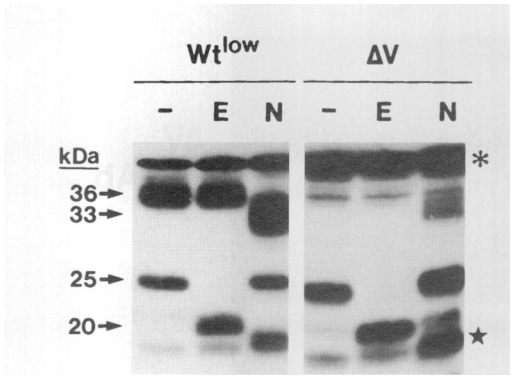

Figure 4. Effect of glycosidases on tagged proTGFα proteins. Anti-FLAG immunoprecipitates from the indicated lysates were resuspended in the appropriate buffers and incubated either with endoglycosidase H (E) or neuraminidase (N) at 37°C for 4 h or 30 min, respectively. Samples were then analyzed by SDS-PAGE. The apparent molecular weights of prominent species are shown on the left. The star marks a band also generated when samples were incubated in neuraminidase buffer lacking enzyme. The asterisk denotes a nonspecific band.

terminal valines, the initial 24-kDa protein turned over with greatly reduced conversion to the 35-kDa surface form (Figure 6A, bottom). Western blots of anti-HA-immunoprecipitated cell lysates confirmed that although the 35-kDa surface form was the predominant species detected by anti-HA in samples from Wt^{HA} cells (Figure 6B, left), the 24-kDa product was the primary form recognized in ΔV^{HA} samples (Figure 6B, right). (Note that as was the case with the dual-tagged ΔV mutant, minor amounts of the larger surface form were detected when samples were overloaded, suggesting that although maturation of the ΔV mutants was dramatically impaired, it was not completely blocked.) Finally, anti-HA surface staining of ΔV^{HA} cells was consistently reduced compared with that of Wt^{HA} cells. These various results, which are consistent with studies of TGFα secretion described below, corroborate those obtained with the dual-tagged proTGFα proteins and confirm that carboxyl-terminal valine residues of proTGFα have a critical role in precursor maturation and routing.

TGFα Is Constitutively Secreted by Clones Expressing Wt but Not ΔV Forms of ProTGFα

To measure constitutive secretion of TGFα species by Wt and ΔV clones, we used an HA-specific ELISA. Serum-free medium conditioned for 24 h by Wt^{low} or Wt^{HA} cells contained significant amounts of ELISA activity relative to the subtracted background activity of medium from control (Mock) cells. Comparison with an HA peptide standard indicated that soluble TGFα levels ranged from 5 to 10 ng/ml in medium conditioned by cells expressing Wt precursor, consistent with active secretion of HA-tagged protein (Figure 7). In contrast, medium conditioned by cells expressing the ΔV or ΔV^{HA} constructs contained markedly reduced activity (<1 ng/ml TGFα) that was only minimally increased relative to background.

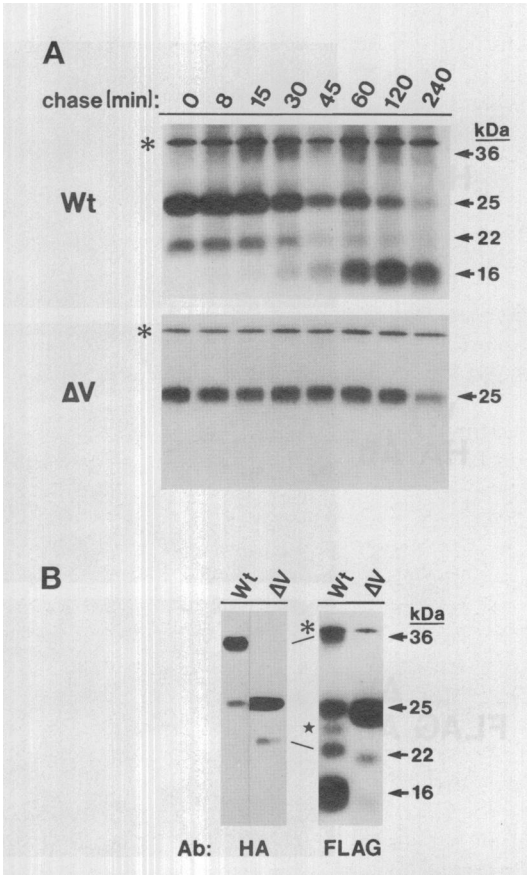

Figure 5. Expression of Wt and mutant (ΔV) proTGFα proteins expressed in CHO cells. (A) Pulse–chase analysis. (B) Western blot analysis. Experiments were performed as described in Figure 2. Asterisks denote nonspecific bands.

Consistent with the ELISA results, Western blot analysis using anti-HA revealed that a prominent HA-tagged species of approximately 26 kDa was present in medium conditioned by Wt^{low} and Wt^{HA} clones but not by ΔV or ΔV^{HA} cells (Figure 8A). Further characterization of the secreted 26-kDa protein revealed that it was not recognized by anti-FLAG and was thus distinguished from the 25-kDa lysate protein that contains the FLAG epitope and cytoplasmic sequences (see Figure 2B). Additionally, it was quantitatively converted to a much smaller HA-tagged protein by pancreatic elastase, and it contained *N*-linked carbohydrate since its apparent molecular weight was reduced by several kilodaltons after treatment with *N*-glycanase (Figure 8B). These data indicate that the 26-kDa protein corresponds to ectodomain sequences of proTGFα and includes the 50-amino acid TGFα polypeptide together with amino-terminal precursor sequences containing the single *N*-glycosylation site. It

is presumably generated by selective proteolytic cleavage of the precursor's carboxyl-terminal Ala-Val (or adjacent) bond. Thus, the ELISA and Western blot assays extend and corroborate the protein and immunofluorescence analyses described above.

Addition of a Carboxyl-Terminal Valine Enhances Maturation of a Truncated ProTGFα Lacking Most of the Cytoplasmic Domain

We constructed two variants of a truncated proTGFα that lacked most of the cytoplasmic domain: T129C terminated with a natural cysteine residue at position 129, and T129V replaced Cys^{129} with a carboxyl-terminal valine (Figure 1B). Both variants were tagged with the HA epitope as described for full-length proTGFα and introduced into R1 cells. Lysates from T129C and T129V clones were immunoprecipitated with anti-HA and then examined by Western blot analysis using the same antibody. Figure 9A shows that the predominant protein identified in both T129V and T129C samples was a 19-kDa species that was predicted, on the basis of size, to correspond to the initial translation product. However, both samples also contained an additional anti-HA-recognized species of approximately 28 kDa; this latter protein presumably corresponds to the fully glycosylated truncated proTGFα. The observation that this 28-kDa protein was present at significantly higher levels in T129V versus T129 cells suggests that the addition of a carboxyl-terminal valine also enhanced the maturation of the proTGFα truncation mutant. This interpretation is consistent with the finding that T129V cells showed enhanced anti-HA surface immunostaining compared with T129C cells (Figure 9B) and also secreted higher levels of TGFα into the medium. Thus, the TGFα-specific ELISA activity of T129V medium was roughly threefold higher than that of T129C cells and in fact approached that of cells expressing the Wt proTGFα counterpart (Wt^{HA}) (Figure 7). In agreement, Western blot analyses showed that the secreted 26-kDa protein was present at significantly higher levels in T129V medium compared with T129C samples (Figure 8A).

Collectively, the results of the above described Western blot, surface immunofluorescence, and medium analyses indicate that the valine requirement was recapitulated even when the cytoplasmic sequence of proTGFα had been largely deleted. This, in turn, suggests that the role of the carboxyl-terminal valine in full-length proTGFα maturation is unrelated to correct folding of the cytoplasmic domain of the precursor.

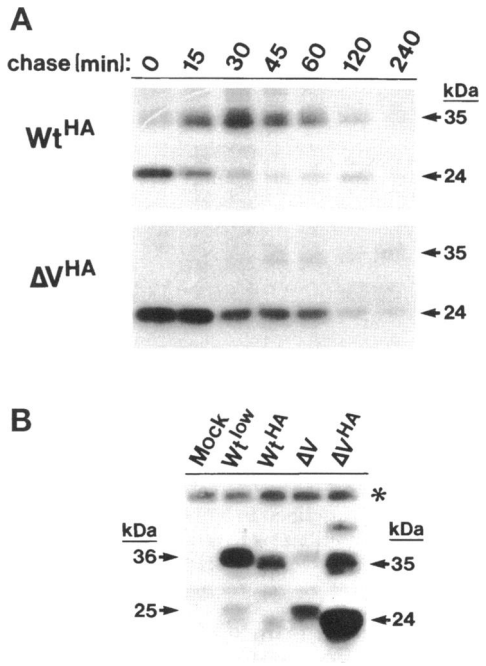

Figure 6. Expression of Wt and mutant (ΔV) proTGFα proteins tagged only with the HA epitope. (A) Pulse–chase analysis. (B) Western blot analysis. Experiments were performed as described in Figure 2, except that immunoprecipitations were performed with anti-HA. Note that lanes 2 and 4 of the Western blot include dual-tagged Wt^{low} and ΔV samples for comparison. The apparent molecular weights of the dual-tagged proteins are shown on the left, and those of the HA-tagged proteins are shown on the right. The asterisk marks a nonspecific band.

DISCUSSION

Our results show that carboxyl-terminal valine residues of proTGFα are required for its normal glycosylation and routing to the cell surface. Specifically, proTGFα mutants lacking the terminal valines were substantially reduced in size, consistent with incomplete glycosylation, and they remained sensitive to endoglycosidase H. This enzyme removes asparagine-linked carbohydrate chains from polypeptides in the RER but has no effect on the processed oligosaccharides of proteins that have been transferred to the Golgi (Kornfeld and Kornfeld, 1985). The resulting suggestion that these proTGFα mutants accumulate in the RER was corroborated by localization studies, which showed them to be present at substantially reduced levels on the cell surface and instead to colocalize with an RER marker. Finally, consistent with these findings and evidence that cleavage of proTGFα occurs at the cell surface (Bosenberg *et al.*, 1993), cells expressing these mutants secreted dramatically lower levels of soluble TGFα species. The finding that the ΔV form of proTGFα is present at markedly reduced levels on the cell surface contradicts a previous report that proTGFα mutants lacking the carboxyl-terminal valines were localized normally to the surface of CHO cells but not subject to phorbol ester-induced proteolytic processing (Bosenberg *et al.*, 1992). However, we

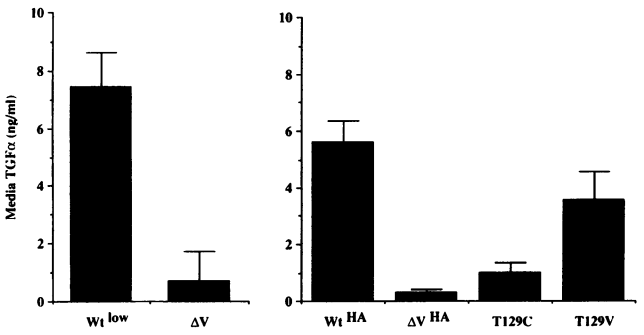

Figure 7. ELISA measurements of TGFα secretion. Cells from the indicated clones were grown to approximately 75% confluency, switched to serum-free medium, and incubated at 37°C for an additional 24 h. The resulting conditioned medium was clarified and concentrated by chromatography on Sep-Pak Plus C_{18} cartridges. Equivalent protein samples were then analyzed by using an HA-specific ELISA as described in MATERIALS AND METHODS. Medium TGFα concentrations were estimated by comparisons with a standard curve generated with an HA peptide standard.

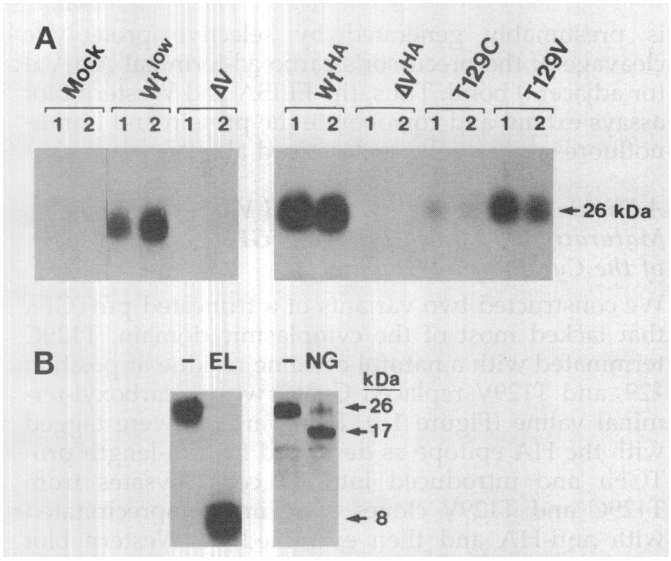

Figure 8. Western blot analyses of soluble TGFα species. (A) Conditioned medium from the indicated cell clones was collected and concentrated. Equal amounts of Sep-Pak-concentrated protein from each sample was then resolved by PAGE, transferred to blots, and probed with anti-HA. A resulting predominant product of 26 kDa is marked. Lanes 1 and 2 are duplicate samples from separate cultures. (B) Indicated samples of Wt^{low} conditioned medium were treated with 3 μg/ml pancreatic elastase (EL; Elastin Products, Owensville, MO) for 1 h at RT or 12.5 U/ml *N*-glycanase (NG; Genzyme, Cambridge, MA) for 2 h at 37°C prior to blotting with anti-HA. The apparent molecular weights of resulting species are shown on the right.

reproduced our results in CHO cells and with both full-length and truncated proTGFα molecules that lacked the cytoplasmic FLAG tag. In light of these results, it seems possible that the reported reconstitution of phorbol ester-induced processing of a proTGFα/KL chimera whose cytoplasmic domain (including a carboxyl-terminal valine) corresponded to that of KL (Bosenberg *et al.*, 1992) could instead be accounted for by reconstituted transport of the chimeric protein from the RER to the cell surface.

The aforementioned findings are consistent with literature reports of mutations affecting the intracellular trafficking (i.e., maturation) of a variety of secreted or integral membrane proteins. For example, human α-1-proteinase inhibitor (A1Pi) deficiency disease results from frameshift mutations that yield abnormal carboxyl-terminal sequence and premature terminations. Studies of similar deliberately produced mutations in transfected cells revealed that a nearly full-length protein containing at least 391 of 394 amino acids was required for movement of newly synthesized A1Pi from the RER to the Golgi (Brodbeck and Brown, 1992). Comparison of nested A1Pi mutants revealed an especially critical role for a proline residue located four amino acids upstream from the carboxyl terminus of the full-length protein. Similarly, variable spontaneous deletions of the carboxyl-terminal 22 amino acids of thyroxine-binding globulin caused that nascent protein to be retained in the RER with resultant lack of secretion (Miura *et al.*, 1994). Illustrating that even relatively subtle mutations can have comparable effect, P-glycoprotein mutants bearing individual substitutions of several glycine residues in the cytoplasmic domain had reduced mass (presumably due to incomplete glycosylation), were endoglycosidase H sensitive, and failed to confer normal multidrug resistance when introduced into cells. Collectively, these results suggest that the P-glycoprotein point mutants were retained in the RER (Loo and Clarke, 1994). Likewise, a mutant c-kit (W^n) containing a single point mutation in the tyrosine kinase domain was reduced in size and absent from the cell surface. Studies of cells transfected with W^n cDNA revealed that the mutant protein was inefficiently glycosylated, endoglycosidase-H-sensitive, and retained in the RER (Koshimizu *et al.*, 1994). Though the precise mechanisms by which mutations like these affect protein trafficking is unknown, incorrect folding of the nascent polypeptide is a favored hypothesis (Lehrman *et al.*, 1985; Pfeffer and Rothman, 1987; Lodish, 1988). Alternatively, mutant proteins may not properly interact with specific transport receptors. In support of the former hypothesis, mutations of the cytoplasmic domain of influenza virus HA (Doyle *et al.*, 1986) and single point substitutions distant from the oligosaccharide attachment site of hepatic α_1-antitrypsin (Loebermann *et al.*, 1984) that affect transport from the RER to the Golgi also cause protein denaturation or misfolding. On the other hand, the fact that the valine requirement was recapitulated with a proTGFα mutant that lacked virtually all of the cytoplasmic domain suggests that the effect of removing the carboxyl-terminal valine is unrelated

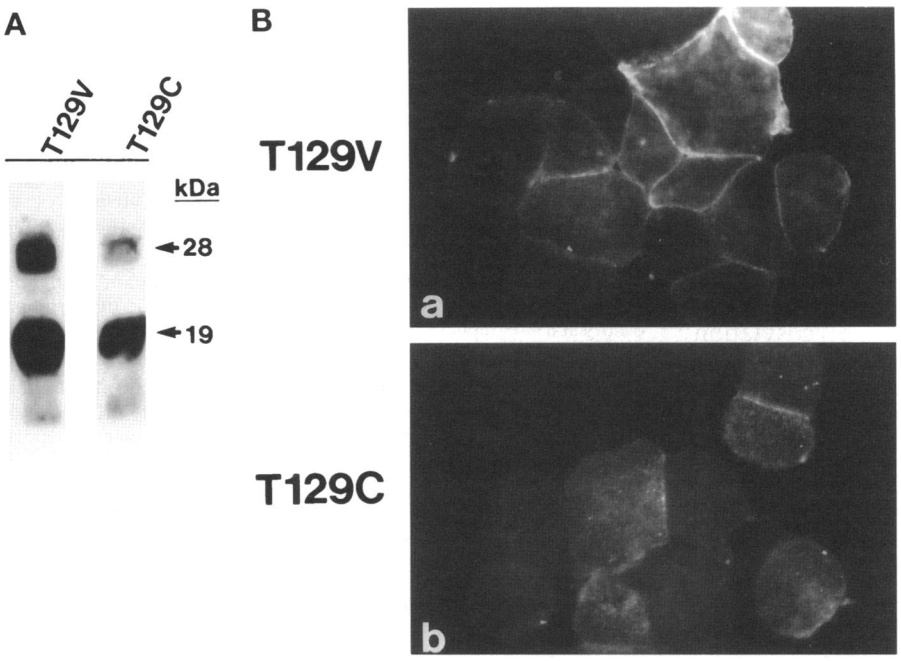

Figure 9. Expression and localization of proTGFα truncation mutants. (A) Western blot analysis. Cells expressing the indicated proTGFα mutants were lysed and immunoprecipitated with anti-HA. Proteins were resolved by PAGE, transferred to blots, and probed with anti-HA. (B) Surface immunofluorescence. Intact cells were probed with anti-HA as described in Figure 3, a–d.

to cytoplasmic domain foldings. It is interesting to note in this regard that the carboxyl-terminal valine of KL was not required for routing of that protein to the cell surface (Cheng and Flanagan, 1994), even though its removal inhibited activated processing of a chimeric protein containing the proTGFα ectodomain and KL cytoplasmic sequences (Bosenberg *et al.*, 1992). These latter results suggest that the valine requirement may in part be determined by proTGFα extracellular sequences.

Our finding that the truncation mutant T129V gave rise to near Wt levels of soluble TGFα suggests that the cytoplasmic domain of proTGFα is not essential for constitutive proteolytic processing. This is reminiscent of the finding that cleavage of KL is also not dependent on the cytoplasmic domain of that precursor. Of course, we cannot exclude possible quantitative differences in the proteolytic processing of full-length and truncated proteins. Additionally, we have not examined whether truncation of the cytoplasmic domain affects phorbol ester-induced cleavage or determined whether secretion of the truncated protein occurs through the same pathway. Importantly, our data also do not exclude possible roles for the cytoplasmic domains of proTGFα and KL in other proposed activities such as bidirectional signaling. In this regard, the KL mutant, Sl^{17H}, is of particular interest. The Sl^{17H} protein contains Wt extracellular and transmembrane domains, but its cytoplasmic region is replaced with a foreign sequence of similar length (Brannan *et al.*, 1992). Interestingly, this mutant is associated with severe effects on male germ cell development, although other cell lineages are also impacted. Although this phenotype could be indicative of a signaling role for the KL cytoplasmic domain, there are other possible explanations. For example, although the SL^{17H} protein is found on the cell surface (Cheng and Flanagan, 1994), it may be present at reduced levels compared with Wt KL, perhaps as a result of inefficient intracellular routing and maturation. If so, the impairment to RER–Golgi transport could be exaggerated in male germ cells, or this particular cell type might be more sensitive to the levels of surface or soluble KL. Finally, a recent study (Shum *et al.*, 1996) also concluded that proTGFα cytoplasmic sequences were not required for processing, but surface localization and secretion were not directly measured.

Finally, our results also indicate biased recognition of the juxtamembrane cleavage site of proTGFα. Thus, medium conditioned by the R1 clones contained a prominent HA-tagged TGFα of 26 kDa, with little or no evidence of the fully processed 6-kDa form. Because this larger species included the mature TGFα peptide and amino-terminal sequences containing the *N*-linked glycosylation site, we presume that proteolytic cleavage was restricted to the carboxyl-terminal Ala-Val bond in R1 cells (see Figure 1). This contrasts with reports of rapid cleavage at the amino-terminal site of proTGFα in transfected CHO cells (Teixidó *et al.*, 1990) but is consistent with the presence of relatively large TGFα species in a variety of cell cultures and biological fluids. For example, we previously characterized a 21-kDa TGFα protein that is the prominent TGFα species secreted by the chemically induced

rat hepatocellular carcinoma line JM1 (Luetteke *et al.*, 1988). Moreover, soluble TGFα species ranging from 18 to 42 kDa in size have been detected in medium conditioned by retrovirus-transformed rat cells (Linsley *et al.*, 1985; Teixidó *et al.*, 1988), untransformed bovine anterior pituitary cells (Kobrin *et al.*, 1986), and even CHO cells (Bringman *et al.*, 1987). The complete lack of the fully processed 6-kDa form in the present samples is surprising: perhaps, cleavage at the amino-terminal site is dampened by the nearby placement of the HA epitope. Alternatively, processing at this site could be inhibited by the more extensive glycosylation of proTGFα that occurs in R1 versus CHO cells. Finally, cleavage at the amino and carboxyl sites may be mediated by different proteases (Teixidó *et al.*, 1990), in which case R1 cells could be deficient in the respective protease. Future experiments will be partly aimed at distinguishing between these various possibilities.

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