Potential Role of Two Novel Elastase-like Enzymes in Processing Pro-Transforming Growth Factor-α[†]

Erika Cappelluti,[‡] Stephen C. Strom,[§] and Robert B. Harris*,[‡]

Department of Biochemistry and Molecular Biophysics and Department of Pathology, Virginia Commonwealth University, Richmond, Virginia 23298

Received July 7, 1992; Revised Manuscript Received October 30, 1992

ABSTRACT: Transforming growth factor-α (TGF-α) is a mitogenic peptide produced by tumor cells and by virally and chemically transformed cells in culture. TGF-α is almost certainly derived from its precursor protein (pro-TGF-α) by limited proteolysis, but the physiologically relevant processing enzyme(s) is(are) unknown. We now report that oncogenically transformed rat liver epithelial cells (known to secrete TGF-α) and Schwann cells in culture transfected with SV40 T-antigen (which are now reported to express mRNA encoding pro-TGF-α) contain membrane associated, neutral pH, serine proteinases which are elastase-like in their substrate specificity, but elastase is not known to be associated with these cell types. In both cell types, the enzyme is associated with a subcellular fraction enriched for microsomes and plasma membranes. Furthermore, the enzyme appears to be specifically induced 4-fold in the transformed epithelial cells as compared with the level of enzyme present in the nontransformed parental cells. The enzymes have been purified $\approx$20 000-fold to near homogeneity (50–60 units/mg) and are virtually identical with regard to their molecular weights (38 000) and other physiochemical properties. Results obtained with numerous synthetic peptide substrates show the enzymes prefer nonpolar residues such as Ala and Val in the P_1 and P_2 positions, but promiscuity of cleavage specificity observed with long-chain peptide substrates is attributed to the absence of structure in these peptides. Thus, although these enzymes may be involved in processing pro-TGF-α at the plasma membrane of the cell, it is just as likely that these enzymes play other physiological roles in the parental and/or transformed cells and that there is no specific endoproteolytic processing enzyme of pro-TGF-α.

Transforming growth factor-α (TGF-α)[1] is a mitogenic peptide produced by tumor cells and by virally and chemically transformed cells in culture (Derynck, 1986, 1988). TGF-α is almost certainly derived from its precursor protein (pro-TGF-α) by limited proteolysis (Lee et al., 1985; Ignotz et al., 1986). cDNA sequence analysis shows that the sequence of TGF-α within the rat and human precursor proteins is not framed by the usual processing site sequence present in most other mammalian pro-hormones [see Harris (1989)]; the putative processing sites in pro-TGF-α more closely resemble elastase or thermolysin-like cleavage sequences (Figure 1). Thus, both pancreatic and polymorphonuclear leukocytic elastase are capable of processing pro-TGF-α to TGF-α (Ignotz et al., 1986; Leutteke et al., 1988; Mueller et al., 1990). No endoproteolysis of the pro-hormone appears to take place until the pro-hormone reaches the plasma membrane (Teixido et al., 1990), and cleavage of membrane-bound pro-TGF-α appears to be an important regulatory step in generating soluble TGF-α. However, the physiologically relevant processing enzyme(s) of pro-TGF-α is(are) unknown. Nonetheless, sequence similarities at the likely N- and C-terminal processing sites within pro-TGF-α suggest that the same enzyme catalyzes the hydrolysis of both the Ala^{39}–Val^{40} and Ala^{89}–Val^{90} bonds to liberate TGF-α (Figure 1).

It was our intention to determine whether transformed cells which express pro-TGF-α also contain an enzyme that would specifically cleave the appropriate bonds and thereby function as a processing enzyme of pro-TGF-α. We now report that oncogenically transformed RLEC (known to secrete TGF-α; Faust et al., 1988; Strom et al., 1991) and Schwann cells in culture transfected with SV40 T-antigen (which are now reported to express mRNA encoding pro-TGF-α) contain membrane associated, neutral pH, serine proteinases which exclusively cleave the Ala–MCA bond in the synthetic substrates Succ-Ala-MCA, Succ-AA-MCA, and Succ-AAA-MCA (or 2NA). The enzymes are inhibited with α_1-antiproteinase and MeO-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone, two well-characterized inhibitors of pancreatic and leukocyte elastases (Powers, 1977; Travis et al., 1983), and thus, the enzymes are elastase-like. However, elastase is not known to be associated with these cell types. The enzymes have been purified to near homogeneity from confluent RLEC and TSC, and in both cell types, the enzymes are associated with a subcellular fraction enriched for microsomes and/or

[†] This work was supported by grants from the USPHS, NIH, NS 24933 (R.B.H.), from Virginia Commonwealth University, No. 87062 (R.B.H.), and from the American Cancer Society, No. CN43B (S.C.S.). The work was done during the tenure of an Established Investigatorship Award to R.B.H. from the American Heart Association. This work comprises part of the Ph.D. Thesis of E.C. to the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, June 1992.

* Address correspondence to this author at the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Box 614, Richmond, VA 23298-0614.

[‡] Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University.

[§] Department of Pathology, Virginia Commonwealth University, Box 662, Richmond, VA 23298-0662.

[1] Abbreviations: Ac, acetyl; BOC, *tert*-butyloxycarbonyl; Bz, benzoyl, FPLC, fast protein liquid chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MCA, methylcourmarinamide; 2NA, 2-naphthylamide; RLEC, rat liver epithelial cells; R10, transformed rat liver epithelial cells; TSC, transfected Schwann cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Succ, succinyl; TGF-α, transforming growth factor-α; Tris, tris(hydroxymethyl)aminomethane. The nomenclature of Schecter and Berger (1967) is used to denote the position of amino acids in the various substrates used.

0006-2960/93/0432-551$04.00/0

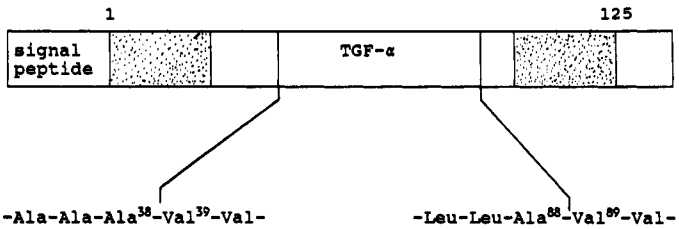

FIGURE 1: Schematic diagram of the sequence of rat pre-pro-TGFα. The sequences of mature rat and human TGF-α are identical at 46 of 50 residues, and both peptides show strong sequence homology with human and rat epidermal growth factor (EGF). cDNA analysis reveals the presence of hydrophobic domains, suggesting that pro-TGF-α is a transmembrane protein (Ignotz et al., 1986; Teixido et al., 1987; Teixido & Massague, 1988; Wong et al., 1989; Brachmann et al., 1989; Teixido et al., 1990; Anklesaria et al., 1990); the C-terminal domain spans the plasma membrane. The putative N- and C-terminal processing sites within the precursor protein are indicated. Endoproteolysis takes place at Ala-Val residues that flank the sequence of mature TGF-α in many different cell lines.

plasma membranes. Furthermore, the enzyme appears to be specifically induced 4-fold in the transformed RLEC as compared with the level of enzyme present in the nontransformed parental cells.

EXPERIMENTAL PROCEDURES

Cell Culture. RLEC and RLEC transformed with pSV2-neo and mutated Ha-*ras* oncogene (designated R10 cells) were prepared, cultured, and frozen in liquid nitrogen as described previously (Faust et al., 1988; Houck et al., 1989; Strom et al., 1991). To initiate cultures of nontransformed and transformed RLEC, two bullets of each cell type (≈10^7 cells/bullet) were removed from the liquid nitrogen, thawed rapidly at 37 °C, and introduced into one 162 cm^2 canted culture flask (Corning) containing 30 mL of modified Eagle's medium (MEM) with nonessential amino acids supplemented with 5% fetal bovine serum (Gibco). The cells were adherent and grown to near confluency at 37 °C (5% CO_2); they were harvested by trypsinization (0.05% (w/v) trypsin–0.53 mM EDTA solution, Gibco) and reseeded into six 162 cm^2 canted culture flasks (30 mL of media/flask). The passed cells were grown to confluence, and 24 h prior to harvesting, the serum-containing media was removed and replaced with serum-free MEM. After 24 h, the cells were harvested by trypsinization (the reaction was stopped by addition of 3 mg of ovalbumin/mL as an alternate substrate for trypsin), collected by centrifugation (200*g*, 30 min), resuspended in 50 mM Hepes–250 mM sucrose buffer, pH 7.5, and washed 2–3 times with the same buffer. Approximately 1.5 × 10^8 RLEC and transformed RLEC were obtained.

Neonatal rat Schwann cells transformed with the SV40 large T antigen were a generous gift of Dr. George H. DeVries, Virginia Commonwealth University, Department of Biochemistry and Molecular Biophysics. The cells were cultured as described (Tennekoon et al., 1987); 24 h prior to harvesting, serum-free medium was added to the cells at confluence (≈1.5 × 10^8 cells). The next day, the cells were harvested by trypsinization, pelleted by centrifugation, and successively washed as described for the RLEC.

Northern Hybridization Analysis. To determine the presence of mRNA encoding pro-TGF-α in either transformed Schwann or transformed RLEC, Northern hybridization analyses were carried out according to Strom et al. (1991).

Preparation of Peptide Substrates. Synthetic peptides whose sequences are based on the amino acid sequence of pro-TGF-α at the putative processing sites of TGF-α were prepared by solid-phase peptide synthesis (Milligen/Biosearch Model 9600 synthesizer) using the appropriate BOC-amino acid substituted Merrifield resin (all from Advanced ChemTech; substitution levels ranged from 0.3 to 0.62 mmol/g of resin). After synthesis, the peptides were acylated directly on the resin using either acetic anhydride or succinic anhydride in dimethylformamide. The peptides were cleaved from the resin using anhydrous HF and were extracted and recovered by lyophilization from water, essentially as described (You et al., 1991). The peptides were purified to homogeneity by preparative reverse-phase HPLC, and where indicated, Ala-Ala-2-NA (or Ala-MCA) was coupled in solution phase (Baxter et al., 1986). The purity of each peptide was confirmed by amino acid compositional analysis, UV spectral analysis, and by reverse-phase HPLC. Succ-Ala-Ala-Ala-MCA was purchased from Bachem Bioscience.

Fluorometric Enzyme Assay. Enzyme-catalyzed release of 2NA or MCA from the fluorogenic substrates was monitored spectrofluorometrically (Soler & Harris, 1988). Briefly, enzyme and substrate were incubated in the cuvette (at 37 °C) in 50 mM Hepes buffer, pH 7.5 (total reaction volume 150 μL). The increase in fluorescence due to released 2NA or MCA was continuously recorded (emission 420 nm, excitation 310 nm). There was no increase in fluorescence in the absence of added enzyme. The slope was converted to nanomoles of 2NA or MCA produced per minute using a calibration curve of relative fluorescence versus known concentrations of 2NA or MCA. As little as 10 pmol of either fluorophore can be detected.

Enzyme Kinetics and Inhibitor Studies. Values for K_m and V_{max} were determined from double-reciprocal plots of v^{-1} versus S^{-1} where less than 10% of the initial substrate was hydrolyzed. k_{cat} was calculated according to $k_{cat} = V_{max}/[E_t]$; $[E_t]$ is the total concentration of enzyme in the preparation.

When greater than 10% of the substrate was hydrolyzed (e.g., to accumulate enough product for quantitation by reverse-phase HPLC), $[S]_{av}$ [average substrate concentration) was used to determine kinetic parameters according to the equation

$$[S]_{av} = ([S]_f + [S]_i)/2$$

where $[S]_i$ and $[S]_f$ are the initial and final substrate concentrations, respectively. Even at 50% hydrolysis, substitution of $[S]_{av}$ for [S] results in <5% error in determined kinetic parameters (Lee & Wilson, 1971).

One milliunit of enzyme catalyzes the formation of 1 nmoL of product/min at 37 °C at the indicated pH.

Enzyme Assay by Reverse-Phase HPLC. C18 reverse-phase binary gradient HPLC (Shimadzu LC 6A) was used to separate and identify the hydrolytic products obtained with the various peptide substrates. Generally, peptides were resolved in linear gradients of 0.1% (v/v) trifluoroacetic acid to 70–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The elution profile was monitored at 215 nm. The identity and concentration of each separated hydrolytic product was

determined by (i) amino acid analysis and/or (ii) by amino-terminal sequence analysis (automated Edman degradation, Applied Biosystems Model 470A/120A sequencer).

Protein Determination. Protein concentration was measured by the Bradford microassay done according to the manufacturer's instructions (Bio-Rad). Bovine serum albumin was the standard.

Sucrose Densitry Gradient Ultracentrifugation. The sedimentation coefficient, $S_{20,w}$, of the elastase-like enzyme was determined in 5–20% sucrose gradients in an SW 50.1 rotor (108000g_{max}, 16 h, 4 °C), essentially as described previously (Harris & Wilson, 1984). The data were analyzed by the method of Martin and Ames (1961), and the $S_{20,w}$ and stokes radius values (determined by calibrated gel permeation chromatography) were used to calculate the apparent molecular weight of the enzyme [see Harris & Wilson (1984)].

SDS–PAGE and Native Gel Electrophoresis. Samples were lyophilized and subjected to SDS–PAGE analysis using the Tris–glycine system of Laemmli (1970).

The molecular weight of the pro-TGF-α processing enzyme from each cell type was also determined by native gel electrophoresis using a modification of the Tris–imidazole system of Hedrick and Smith (1968).

Enzyme Preparation and Subcellular Fractionation. The elastase-like enzyme was purified from the two cell types. Following the last wash procedure already described, the cells were suspended in 2 mL of 10 mM Hepes–0.1%(v/v)Triton X-100–50 mM KCl buffer, pH 7.5, and vigorously sonicated (100 × 2 s bursts, power setting 2, Heat Systems Ultrasonics Probe Sonciator) to disrupt the cells and release soluble and membrane associated cellular components. Cellular debris was removed by centrifugation, and the supernatant (about 2 mL) was mixed for 15 min in a batch procedure with DE-52 anion-exchange resin (Whatman) at a ratio of 10 mg of protein/mL of settled resin. After mixing, the resin was settled by low-speed centrifugation and washed with an equal volume of equilibrating buffer. The enzyme binds quantitatively and elution is accomplised using 2 mL of Hepes buffer containing 500 mM NaCl, pH 7.5 (for Schwann cells), or 1 M NaCl, pH 7.5 (for RLEC). During all steps of purification, enzyme activity was measured using Succ-Ala-Ala-Ala-MCA as substrate.

The DE-52 enzyme preparation was next subjected to successive chromatography procedures. In the first step, the enzyme preparation is applied to phenyl-Sepharose equilibrated in the high ionic strength buffer used to desorb the enzyme from the DE-52. The enzyme does not adsorb to this matrix and washes through with equilibrating buffer. However, >85% of the total protein in the preparation does adsorb to the phenyl-Sepharose under these conditions and so this is a good purification step. The enzyme is then applied to a gel filtration FPLC Superose-12 gel filtration column equilibrated in 30 mM Hepes–30 mM NaCl buffer, pH 7.5. Finally, the fractions with activity are pooled and applied to an FLPC Mono Q anion-exchange column equilibrated in 30 mM Hepes–30 mM NaCl buffer, pH 7.5. Elution is accomplished with a linear gradient of 30–350 mM NaCl. The appropriate fraction (1 mL) from this chromatography are then pooled and were used for all subsequent experiments.

To determine the subcellular distribution of the enzyme activity, a modification of the method of Walter and Blobel (1983) was routinely employed and the data were analyzed by the method of relative specific activities (DeDuve, 1975). Fractions enriched for nuclei, mitochondria, lysosomes, microsomes, and plasma membranes were obtained and examined for the presence of enzyme activity.

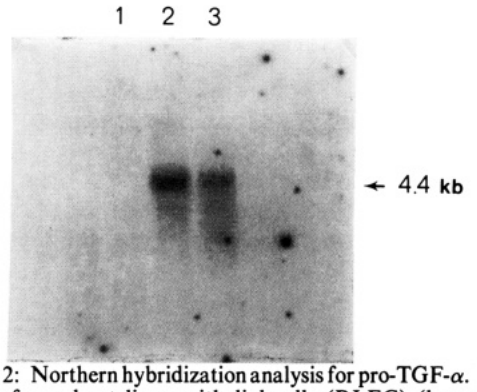

FIGURE 2: Northern hybridization analysis for pro-TGF-α. Parental, nontransformed rat liver epithelial cells (RLEC) (lane 1), SV40 T-antigen transformed Schwann cells (lane 2), or H-*ras*-transformed rat liver epithelial cells (R10) (lane 3) were grown to confluence and solubilized in 4 M guanidine isothiocyanate–0.05 M sodium citrate–0.1 M 2-mercaptoethanol. Cellular RNA was prepared by centrifugation through a 5.7 M CsCl cushion (Beckman SW 41 rotor, 16–20 h, 134000g_{max}); mRNA was collected by a single selection over oligo(dT)–cellulose (Collaborative Research). Samples of mRNA (10 μg) were subjected to electrophoresis, transblotted to Nytran membranes, probed with a ^{32}P-random prime-labeled cDNA for rat TGF-α (Ala^{39}–Ala^{89}) (Lee et al., 1985), and washed under stringent conditions, essentially as described (Strom et al., 1991).

RESULTS

Selection of Cell Lines. Oncogenically transformed RLEC are known to synthesize and process pro-TGF-α and to secrete bioactive TGF-α (Faust et al., 1988; Strom et al., 1991) at about 10 ng/mL of conditioned medium (Strom, unpublished observation). Hence, it seemed reasonable to use these cells as a potential source of an enzyme(s) which might function as a processing enzyme of pro-TGF-α. TGF-α is also secreted from anterior pituitary cells in culture (Kobrin et al., 1986) and from several areas of the adult brain (Wilcox & Derynck, 1988; Samsoondar et al., 1988), and because TSC (which envelope both myelinated and unmyelinated nerves) respond to numerous peptide and proteinacious mitogens (Yoshino et al., 1984; DeVries et al., 1982; DeCoster & DeVries, 1989), it seemd worthwile to determine whether pro-TGF-α was synthesized in (and TGF-α was secreted from) these cells.

Northern blot analysis clearly shows the presence of a 4.4-kb mRNA from the transfected Schwann cell and the transformed RLEC (Figure 2). No hybridization signal was detected in the lane containing RNA from the parental RLEC line not transformed with the *ras* oncogene. Hence, at least for rat liver epithelial cells, TGF-α expression is limited to the transformed cells.

Identification of an Elastase-like Enzyme. Having identified two cell types which express pro-TGF-α, we next determined whether they contain an enzyme(s) that might be capable of catalyzing the formation of TGF-α from the precursor protein.

Approximately 5 × 10^7 RLEC, transformed RLEC, and TSC were vigorously sonicated in buffer containing detergent, and the preparations were clarified by high-speed centrifugation. An enzyme was present in each of these sonicates that rapidly liberates the fluorophores, MCA or 2-NA, from the appropriate substrate. Hydrolysis is linear with time and proprotional to enzyme and substrate concentration. The identity of the hydrolytic products (Succ-Ala-Ala-Ala, 2NA, and MCA) was confirmed by reverse-phase HPLC and compositional analysis. Hydrolysis occurred exclusively at

Table I: Release of the R10 and TSC Enzyme

sonication conditions[a]	R10 cells: activity released[b] (milliunits/(mg of protein·10^6 cells))	R10 cells: % total	TSC: activity released[b] (milliunits/(mg of protein·10^6 cells))	TSC: % total
(1) 10 mM Hepes, buffer, pH 7.5, 50 × 2 s bursts	1.39×10^{-3}	4	1.10×10^{-2}	13
(2) as (1), with 50 mM KCl	1.12×10^{-2}	31	4.50×10^{-2}	53
(3) as (1), with 0.1% Triton X-100	1.11×10^{-2}	31	3.01×10^{-2}	35
(4) as (2), with 0.1% Triton X-100	3.60×10^{-2}	100	8.50×10^{-2}	100

[a] About 5×10^7 of each cell type were individually subjected to various procedures to effect release of the enzyme from the cells. After each treatment, the sonicate was subjected to centrifugation, and an aliquot of the supernatant was assayed for enzyme activity using 0.67 mM Succ-Ala-Ala-Ala-MCA as substrate. [b] Total activity released in two sonication treatments. A third sonication treatment of each pellet released no additional enzyme activity.

the Ala–MCA(or 2NA) bond; there was no cleavage of the Ala–Ala bonds. This was shown in experiments where aminopeptidase M was included in the assay buffer. If Ala-Ala-MCA (or 2NA) or Ala-MCA (or 2-NA) were formed, inclusion of aminopeptidase M would cause an increase in the hydrolysis rate as the Ala residues weer successively removed by this enzyme. However, aminopeptidase M did not affect the measured rate of hydrolysis. Furthermore, Ala-MCA is not a substrate of the purified enzyme (see Table V).

This enzyme activity is associated with the cells and is not a component of the culture medium as indicated by the following experiments: (1) each of the cell pellets was intentionally spiked with [4,5-^{3}H]Leu and after two washes of the cell pellets in serum-free buffer greater than 99.9% of the radiolabeled Leu was recovered in the washes, consistent with the idea that Leu does not fortuitously associate with the cells. The cells were then washed a third time prior to sonication; (2) the cells were grown in serum-free medium prior to collection; and (3) 100 mL of serum containing medium was concentrated 20-fold by ultrafiltration. There was no measurable enzyme activity in the ultra-retentate or the ultra-filtrate. Not surprisingly, 20-fold concentrated serum-free medium also did not possess measurable enzyme activity. An aliquot of conditioned medium, however, contained marginal activity, suggesting that some enzyme may be lost to the medium from the cells during growth.

To further confirm the cellular association of the enzyme and to establish whether the enzymes are membrane bound, washed R10 or TS cells were sonicated in Hepes buffer alone, buffer containing 50 mM KCl, buffer containing 0.1%(v/v) Triton X-100, or buffer containing 50 mM KCl and 0.1%-(v/v) Triton X-100. The activity released on two sonication treatments represents 4%, 31%, 31%, and 100% of the total activity for the R10 cells, respectively, and 13%, 53%, 35%, and 100% for the Schwann cells (Table I). The second sonication treatment of each pellet released little additional enzyme activity, and the third sonication treatment of the resulting pellets released no additional enzyme activity. These results suggest that the enzyme in each cell type is associated with a membrane component of the cells and that sonication in the presence of detergent and salt is required to liberate all the assayable activity.

Mechanical scrapping, trypsin (0.05% w/v), or collagenase (2 units/mL of media) treatments were used to harvest R10 or TS cells. The specific activity of the enzyme activity from either cell type was the same regardless of the particular method of collection, but the greatest number of cells and the greatest number of milliunits were obtained by harvesting either cell type according to the trypsin. Hence, trypsin was routinely employed for all subsequent experiments.

There was a good correlation of increase in cell number with total protein and enzyme activity from either cell source. Cells reached confluence by day 5 (R10) or day 3 (TSC) in culture, and at this point, the specific activity of the enzyme remained constant [(≈4 milliunits/mg)·10^6 cell equivalents]. Thus, the maximum level of enzyme activity and total protein was observed in confluent cells.

Subcellular Distribution of Enzyme Activity. Because the enzyme is associated with a cellular membrane and does not appear to be a cytosolic component, we determined the distribution and enrichment of the enzyme in the various subcellular fractions prepared from each cell type. The patterns of the histograms obtained for the experimental enzymes from the transformed R10 (Figure 3) or Schwann cells (Figure 4) are consistent with an enrichment of this enzyme into the microsomal subfraction. On the basis of the distribution of the marker enzymes, we know that this subfraction contains microsomes and plasma membranes.

Induction of Enzyme Activity in Transformed RLEC. The enzyme appears to be specifically induced in *ras*-transformed RLEC as compared to the parental, nontransformed RLEC. While the total protein and the activity levels of the various marker enzymes were essentially constant between the two cell types (Table II), the activity level of the experimental enzyme shows an apparent 4-fold increase in the transformed RLEC when corrected for milligrams of protein and cell number.

Purification of the Candidate Processing Enzyme. The enzyme has been purified to near electrophoretic homogeneity from transformed RLEC and from TSC. Typically, the enzyme is purified from 1.2×10^8 cells which have been harvested, washed, and pelleted as described. The cells are disrupted by sonication in buffer containing detergent and KCl. The sonicate from either cell type contains about 8 mg of protein and 30 milliunits of activity (Table III). DE-52 anion-exchange chromatography and successive chromatographies on phenyl-Sepharose, FPLC Superose-12, and FPLC Mono Q yielded highly purified enzyme with a specific activity range of 50–65 units/mg of protein. The enzyme is obtained in 10% overall yield from both cell types (approximately 10 ng of enzyme/1.2×10^8 cells) and has been purified about 20 000-fold relative to the cell sonicate. With increasing purity, there is a notable decrease in enzyme stability. To minimize loss of activity, the enzyme is stored in 33% (v/v) glycerol at 4 °C. The highly purified enzyme preparations also lose activity during the course of assay. To overcome this problem, the enzyme is routinely assayed in buffer containing 1 mg/mL bovine serum albumin which seems to stabilize the enzyme toward loss of activity. In this respect, this enzyme behaves in a similar fashion to atrial granule serine proteinase, a processing enzyme of pro-atrial natriuretic factor (Wypij & Harris, 1992).

Physicochemical Properties of the Candidate Enzyme. The pH optimum of the enzyme purified from either transformed

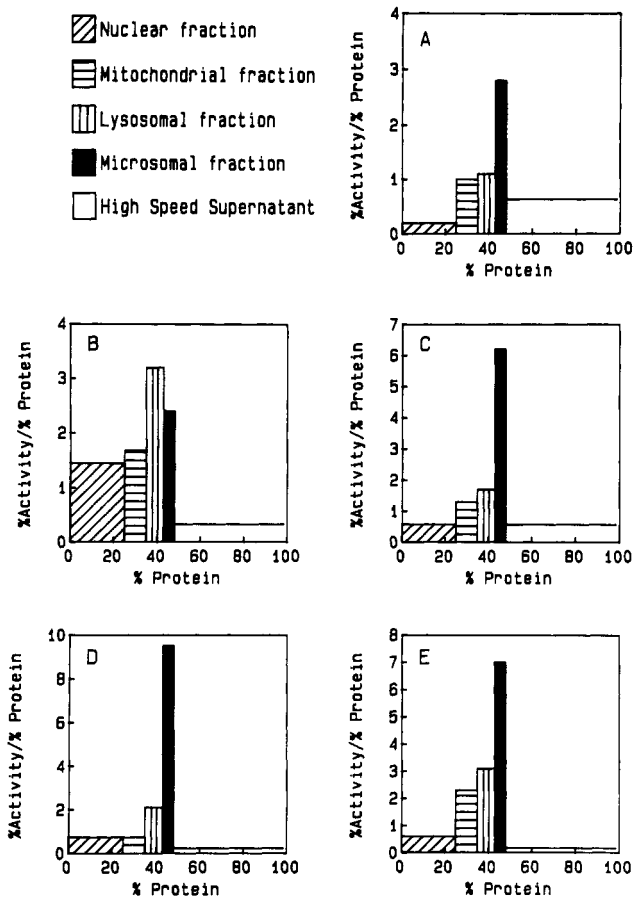

FIGURE 3: Enrichment of enzyme activities in subcellular fractions prepared from R10 cells as analyzed by the method of relative specific activities. About 3×10^7 cells obtained following the last wash procedure were resuspended in 50 mM potassium acetate–250 mM sucrose–50 mM triethanolamine–6 mM magnesium acetate–1 mM EDTA–1 mM dithiothreitol solution, pH 7.5, and sonicated (15 × 2 s bursts, power setting 2) to disrupt the cells. The preparation was subjected to differential and sucrose cushion centrifugations to yield subcellular fractions enriched for nuclei (5 min, 1000*g*), mitochondria (5 min, 2000*g*), lysosomes (10 min, 10000*g*), and microsomes and plasma membranes (2.5 h, 140000*g*). Each of the subfractions was vigorously sonicated in 10 mM Hepes–0.15% (v/v) Triton X-100–50 mM KCl buffer and centrifuged at 75000*g* to remove organellar debris. Histograms representing the distribution of (A) the elastase-like enzyme, (B) *N*-acetylglucoseaminidase as a marker enzyme for lysosomes (Barrett, 1972), (C) 2′–3′-cyclic nucleotide 3′-phosphodiesterase (Yoshino et al., 1985) and (E) 5′-nucleotidase (Kaulen et al., 1970) as marker enzymes for the plasma membrane, and (D) glucose-6-phosphatase as a marker enzyme for the endoplasmic recticulum (Appelmans et al., 1955) are shown. By the method of relative specific activities, the total protein content of all subfractions is taken as 100% and the relative specific activity of each of the enzyme activities is plotted as a function of the percent of total protein present in each subcellular fraction.

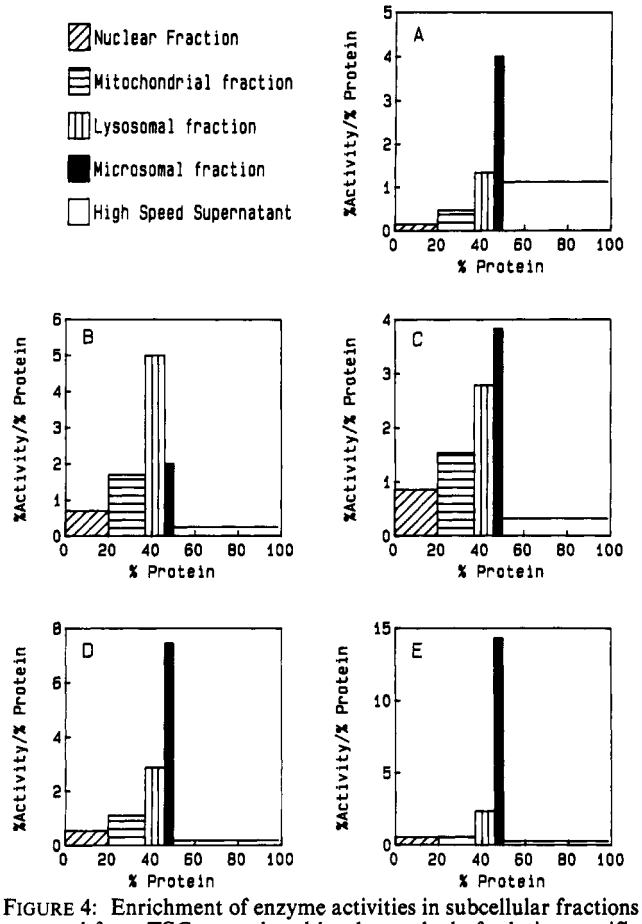

FIGURE 4: Enrichment of enzyme activities in subcellular fractions prepared from TSC as analyzed by the method of relative specific activities. See Figure 3.

Table II: Induction of the Transformed RLEC Enzyme[a]

	parental cells	transformed cells	*x*-fold increase
protein (mg/10^6 cells)	3.6×10^{-2}	3.5×10^{-2}	1.0 ± 0.1
glucose-6-phosphatase (milliunits/(mg of protein·10^6 cells))	21.4	24.5	1.2 ● 0.1
N-acetylglucoseaminidase (milliunits/(mg of protein·10^6 cells))	1.0×10^{-2}	1.6×10^{-2}	1.3 ± 0.1
5′-nucleotidase (millunits/(mg of protein·10^6 cells))	1.2×10^{-2}	1.5×10^{-2}	1.1 ± 0.4
experimental enzyme (milliunits/(mg of protein·10^6 cells))	7.0×10^{-2}	27.3×10^{-2}	3.5 ± 0.3

[a] A total of (3–5) × 10^7 RLEC or transformed RLEC (R10 cells) were sonicated, and the preparation was clarified by centrifugation. The supernatants were analyzed for total protein content and each enzyme as shown. Each activity was corrected for total protein and total cell number. The *x*-fold increase represents the mean ±1 SD from three experiments.

cell type for hydrolysis of Succ-Ala-Ala-Ala-2NA (or MCA) is 7.5; at pH values less than 5.5 or greater than 8.5, the enzyme is essentially inactive. Activity is constant and optimal at 37–41 °C; at 25 or 45 °C the enzyme activity is 40% of the control levels.

The enzyme from each cell type elutes as a single peak on HPLC or FPLC Superose-12 gel permeation chromatography corresponding to a Stoke's radius of approximately 2.7 nm, M_r 37 000. The $S_{20,w}$ of the two enzyme preparations as determined by sucrose density gradient ultracentrifugation is 3.52, corresponding to a molecular weight of 34 500 by comparison with marker proteins.

The purified enzyme preparation from each cell type was subjected to SDS–AGE analysis under reducing or nonreducing conditions. Under nonreducing conditions, two protein bands (M_r 38 000 and M_r 50 000) were detected after silver-staining (Figure 5). Under reducing conditions, the same two protein bands were visualized. On native gel electrophoresis (Figure 6), the molecular weight of the enzyme from both cell types was calculated to be 37 600. Thus, the M_r 38 000 protein band in the SDS–PAGE can be attributed to the experimental enzyme.

Inhibition Studies. The inhibition profiles of the purified enzymes were determined using numerous well-characterized reagents (Table IV). The enzymes from both cell types are

Table III: Purification of the R10 and TSC Enzymes[a]

enzyme preparation	protein (mgs)	activity (milliunits)	sp act. (milliunits/mg)	*x*-fold purified
R10 cells				
whole cell sonicate	8.5	26.2	3.1	1.0
DE-52 enzyme pool	4.3	24.8	5.8	1.9
phenyl-Sepharose	0.62	20.8	33.5	10.8
FPLC Superose-12	0.12	6.5	53	17.1
FPLC Mono Q enzyme	3.8×10^{-5}	2.4	64 000	≈21 000
TS cells				
whole cell sonicate	8.6	21.0	2.4	1.0
DE-52 enzyme pool	5.4	14.0	2.6	1.1
phenyl-Sepharose	0.69	10.4	15.1	6.2
FPLC Superose-12	0.24	6.4	26.6	11.1
FPLC Mono Q enzyme	3.8×10^{-5}	1.9	50 800	≈21 000

[a] The results shown are for a typical experiment. Five such fractions using about 1.5×10^8 of each cell type were done. Total protein of the FPLC Mono Q enzyme preparation is estimated from SDS–PAGE analyses.

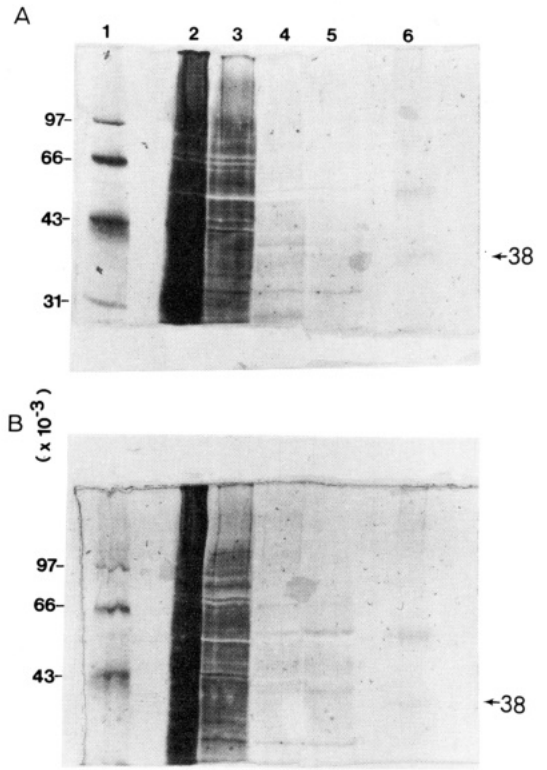

FIGURE 5: SDS–PAGE analysis of the purification of the candidate processing enzyme of pro-TGF-α from R10 and TSC. Samples and molecular weight markers (Bio-Rad) were reconstituted in loading buffer with or without 2% 2-mercaptoethanol, boiled for 5 min, and subjected to electrophoresis (30 mA constant current). After electrophoresis, proteins were visualized using silver strain (Harlow & Lane, 1988), and apparent molecular weights were determined from a calibration curve of log molecular weight versus relative mobility of each marker protein. (A) R10 cells: Lane 1, molecular weight markers; lane 2, whole cell sonicate; lane 3, DE-52 enzyme preparation; lane 4, phenyl-Sepharose enzyme preparation; lane 5, FPLC Superose-12 enzyme preparation; lane 6, FPLC Mono Q enzyme preparation. (B) TS cells: lane 1, molecular weight markers; lane 2, whole cell sonicate; lane 3, DE-52 enzyme preparation; lane 4, phenyl-Sepharose enzyme preparation; lane 5, FPLC Superose-12 enzyme preparation; lane 6, FPLC Mono Q enzyme preparation.

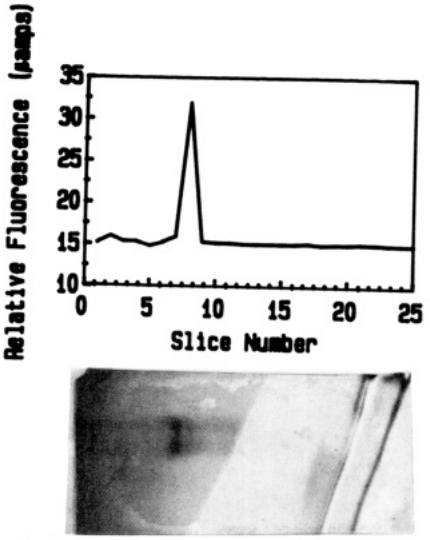

FIGURE 6: Native gel electrophoresis of the candidate processing enzyme from R10 cells. An aliquot of the R10 Mono Q enzyme preparation (or TSC preparation, not shown) was dialyzed against 0.06 M imidazole hydrochlorides 33% (v/v) glycerol buffer, pH 6.7, overnight at 4 °C. The enzyme preparation and marker proteins were then subjected to electrophoresis under nondenaturing conditions (4 °C) at 10 mA for 45 min, then 5 mA for 75 min. Following electrophoresis, half of the gel was stained for protein using silver stain; individual lanes of the second half of the gel which contained identical samples were cut into 2-mm sections and submersed individually in 0.67 mM Succ-Ala-Ala-Ala-MCA substrate solution in 1 mg/mL BSA, 50 mM Hepes buffer, pH 7.5, to detect the presence of the elastase-like enzyme activity. Diffusion was allowed to proceed at 37 °C for 16 h and the release of fluorophore into the solution was measured. The figure shows a portion of the silver-stained 12% acrylamide gel and enzyme activity (in terms of relative fluorescence units) present in each gel slice. The relative mobility of the marker proteins in 7.5, 10, and 12% polyacrylamide gels were used to construct a calibration curve of molecular weights (Hedrick & Smith, 1968). The apparent molecular weight (≈38 000) of the elastase-like enzyme was then determined from the calibration curve.

inhibited by general serine proteinase inhibitors including DFP and TPCK. The enzyme prepared from either R10 or TSC is also inhibited by known elastase inhibitors including α_1-antiproteinase and MeO-Succ-Ala-Ala-Pro-Val-chloromethyl ketone, a potent irreversible inhibitor of pancreatic and leukocyte elastases. This latter compound was also an irreversible inhibitor of the R10 enzyme (k_i 1×10^3 min^{-1} M^{-1}) and of the TSC enzyme (k_i 1.4×10^3 min^{-1} M^{-1}).

In contrast, chelating agents (EDTA, EGTA) and thiol reagents (iodoacetic acid, *N*-ethylmaleimide) had little or no effect on activity. The enzymes are not inhibited by phosphoramidon, captopril, or leupeptin and thus cannot be considered as endopeptidase EC 3.4.24.11 (enkephalinase), angiotensin I converting enzyme (EC 3.4.15.1), or cathepsin B (EC 3.4.22.1). The candidate processing enzymes can therefore be categorized as neutral pH, serine proteinases which are also inhibited by known elastase inhibitors. However, elastase is not known to be associated with either cell type.

Substrate Specificity of the Elastase-like Enzyme. Several different synthetic substrates were used to assess the specificity of the elastase-like enzyme purified from both cell types. Succ-Ala-Ala-Ala-MCA served as the reference substrate (Table

Table IV: Inhibition Profile of the R10 and TSC Enzymes[a]

inhibitor[a]	concentration	R10 enzyme % inhibition	TSC enzyme % inhibition
DFP	1.0 mM	75	50
	1.5	90	80
PMSF	1.5	36	
	3.2		42
TPCK	0.1	100	100
α_1-antiproteinase	140 μg/mL	54	50
	250	81	
MeO-Succ-AAPV-CH_2-Cl	10 μM	18	
	100	50	59
	250	80	77
EDTA	4.5 mM	0	0
EGTA	5.0 mM	0	0
phosphoramidon	1.5 μM	3	24
captopril	33.3 μM	8	5
iodoacetic acid	1.0 mM	0	0
pepstatin	1.0 μM	67	20
leupeptin	1.0 mM	38	33

[a] Enzyme and inhibitor were preincubated (30 min, 25 °C) prior to the addition of substrate (0.67 mM Succ-Ala-Ala-Ala-MCA). % inhibition is calculated relative to a control enzyme sample incubated identically in the absence of inhibitor. Abbreviations: DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; TPCK, tosyllysyline chloromethyl ketone.

V). The enzyme readily hydrolyzes the Ala–MCA bond of the reference substrate. Cleavage of the corresponding bond in pro-TGF-α (Ala^{38}–Val^{39} and Ala^{88}–Val^{89}) would yield a recognized bioactive form of TGF-α. Thus, the same enzyme could possibly catalyze cleavage at both the N- and C-terminal sites.

Ala-MCA was not a substrate for either the R10 or TSC enzyme preparation (which also shows both preparations do not contain aminopeptidase M) (Table V). Succ-Ala-MCA was a better substrate than its nonsuccinylated counterpart, but it was a much poorer substrate than the reference substrate; substrate inhibition was observed with Succ-Ala-MCA making the calculation of kinetic constants arbitrary. Succ-Ala-Ala-MCA was also a substrate for the enzymes and was cleaved exclusively at the Ala–MCA bond. The K_m values are essentially the same for Succ-Ala-Ala-MCA and Succ-Ala-Ala-Ala-MCA in both R10 and TSC. k_{cat} values for Succ-Ala-Ala-MCA and Succ-Ala-Ala-Ala-MCA are basically the same within each cell type though the values obtained using R10 enzyme are 2-fold higher than those obtained using TSC enzyme. Succ-Ala-MCA shows only limited solubility in the assay buffer, and for this reason, 10% methanol had to be included in the reaction mixtures. However, methanol suppresses hydrolysis of the reference peptide; even in 2% methanol, k_{cat} is considerably lower than that obtained in the absence of methanol. Examination of k_{cat}/K_m for the substrates shows that the tripeptide is about equivalent to the dipeptide and both are clearly better than the monoamino acid substrate.

Additional Substrates Specificity Studies of Pro-TGF-α Processing Enzyme. Additional substrates were prepared to assess the importance of residues at or near the cleavage site on hydrolysis. Peptides (i–v) (Figure 7) encompass extended sequences at the N-terminal processing site and peptide vi encompasses the sequence at the C-terminal site. Hydrolytic products were separated by reverse-phase HPLC and identified by compositional and/or sequence analysis (Table VI).

Succ-G-A-A-A (i) was designed so that Ala would occupy positions P_3 through P_1 and the unique Gly at P_4 would be helpful when calculating the compositional analyses of the hydrolytic products. We did not expect to observe any products derived from i. However, i was a substrate for both enzymes and hydrolysis took place between the second and third Ala residues. This finding suggests that the pro-TGF-α processing enzymes require Ala in both the P_2 and P_1 positions but can accommodate residues other than Ala in the P_3 position. By comparison, this peptide was not a substrate for porcine pancreatic elastase.

The remaining substrates encompassing the putative N-terminal processing site in the pro-hormone were good substrates for pancreatic elastase; hydrolysis generally yielded multiple products (Table VI). In contrast, the R10 and TSC enzymes hydrolyzed each of the peptides at one unique bond. Surprisingly, hydrolysis did not take place at Ala–Val bonds as expected, and the cleavage point shifted depending upon the nature of the substrate. For example, both enzymes hydrolyzed Succ-G-A-A-A-V-V-S (ii) between the second and third Ala residues, rather than at the Ala–Val bond. Succ-G-A-A-A-V-V-S-H-F-NH_2 (iii) and its nonsuccinylated counterpart (iv) were hydrolyzed at the Val–Ser bond while Succ-A-A-A-V-V-S-H-F-N-K (v) was hydrolyzed exclusively at the Ser-His bond. The substrate encompassing the putative C-terminal processing site in the pro-hormone (vi) was also not hydrolyzed at the Ala–Val bond but was instead cleaved exclusively between the Ala-Ala doublet.

There is no clear difference in catalytic rates obtained using the N-terminal substrates or the C-terminal substrate with each enzyme. Furthermore, the individual kinetic constants for each of the long-chain substrates are not very different from each other (Table VII). These enzymes are thus somewhat promiscuous with regard to substrate specificity. They appear to prefer nonpolar amino acids in the P_1 position of their substrates but can easily accommodate other amino acids. Despite their permissiveness, however, oxidized insulin B chain (a nonrelated peptide) was not a substrate for either enzyme. In contrast, pancreatic elastase hydrolyzd the oxidized insulin B chain at multiple residues giving rise to at least six products in less than 6 h of incubation.

DISCUSSION

TGF-α is synthesized as part of a higher molecular weight precursor protein, pro-TGF-α, which undergoes limited endoproteolysis to yield mature TGF-α. However, specific processing endoproteinases of pro-TGF-α are unknown. Two elastase-like enzymes were discovered and purified from *ras*-transformed rat liver epithelial cells (R10) (which express mRNA encoding pro-TGF-α and secrete TGF-α) and from transfected Schwann cells (TSC) which are shown here for the first time to also express mRNA encoding pro-TGF-α. We do not yet know the molecular nature of the secreted form(s) of TGF-α from TSC and do not know the possible function of this growth factor in the peripheral nervous system.

Expression and secretion of TGF-α and other EGF-like growth factors occur at a very low, essentially non-existent, level in nontransformed cells. Transformation of RLEC with *ras* induces the expression and secretion of TGF-α (Faust et al., 1988), and in the present studies, we also found that the enzyme levels were specifically induced 4-fold. The mechanism of intracellular induction, however, is not clear. It may be that the transformed cells are generating more processing enzyme in response to the increased levels of pro-TGF-α. However, it is also possible that the turnover rate of individual enzyme molecules increases or that induction of this enzyme comes about as a result of the introduction of the *ras* oncogene into these cells. Alternatively, transformation may increase the level of other regulators (activators) of the enzyme activity.

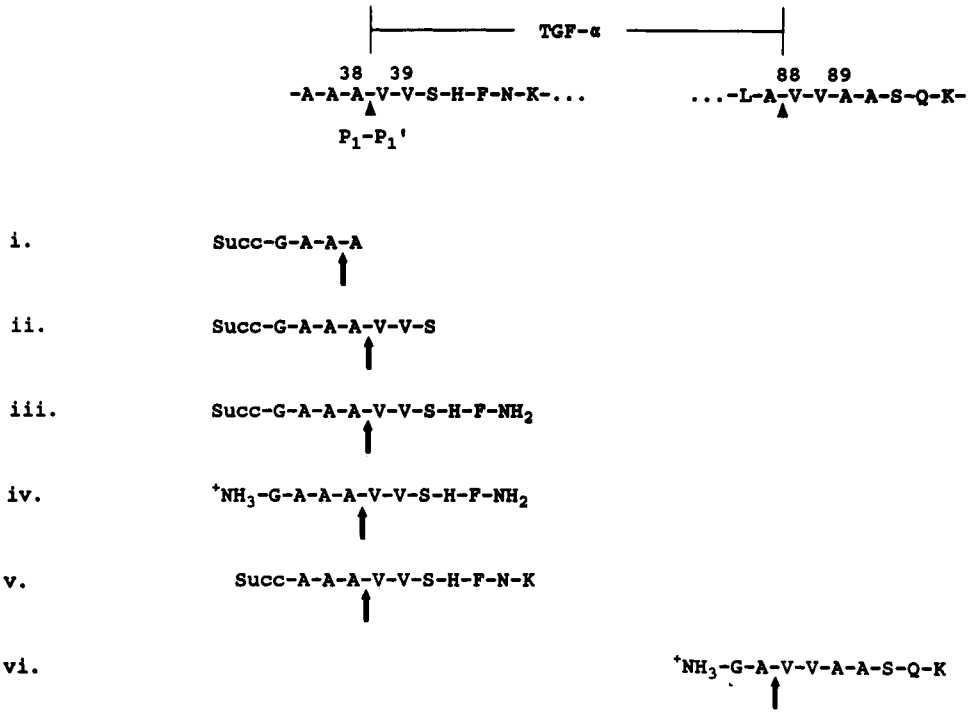

FIGURE 7: Design of additional synthetic peptides based on the sequence of pro-TGF-α at the processing sites. The amino acid sequence in pro-TGF-α is shown. Synthetic peptides incorporating potential processing sites (indicated by arrows) in pro-TGF-α were synthesized, purified, and characterized. Their sequences are aligned with that of pro-TGF-α.

Table V: Hydrolysis of Synthetic Peptide Substrates by the R10 and TSC Enzymes[a]

	R10 enzyme			TSC enzyme		
substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot mM^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot mM^{-1}$)
10% methanol						
Ala-MCA	*b*			*b*		
Succ-Ala-MCA	*c*			*c*		
Succ-Ala-Ala-MCA	0.21	1.8×10^4	8.4×10^4	0.10	3.8×10^3	3.8×10^4
Succ-Ala-Ala-Ala-MCA	0.19	1.3×10^4	6.8×10^4	0.10	3.7×10^3	3.7×10^4
2% methanol						
Succ-Ala-Ala-MCA	0.25	1.2×10^4	4.8×10^4	0.25	7.6×10^3	3.0×10^4
Succ-Ala-Ala-Ala-MCA	0.36	1.4×10^4	3.8×10^4	0.28	8.6×10^3	3.1×10^4
0% methanol						
Succ-Ala-Ala-Ala-MCA	0.44	2.2×10^4	4.9×10^4	0.50	23.8×10^3	4.8×10^4

[a] Enzyme and substrate were incubated at 37 °C in buffer containing 10% (v/v), 2%, or 0% methanol to facilitate substrate solubility. Release of MCA was quantitated fluorometrically. [b] Not a substrate. [c] Substrate inhibition.

Table VI: Hydrolytic Products Derived from Synthetic Peptide Substrates Using Porcine Pancreatic Elastase or the R10 or TSC Enzymes[a]

peptide substrate	PPE	R10 enzyme	TSC enzyme
(i) Succ-GAAA	no products	Succ-GAA	Succ-GAA
(ii) Succ-GAAAVVS	no products	Succ-GAA, AVVS	Succ-GAA, AVVS
(iii) Succ-GAAAVVSHF-NH_2	Succ-GAAAVV, SHF-NH_2	Succ-GAAAVV, SHF-NH_2	Succ-GAAAVV, SHF-NH_2
(iv) GAAAVVSHF-NH_2	GAAAVV, SHF-NH_2	GAAAVV, SHF-NH_2	GAAAVV, SHF-NH_2
(v) Succ-AAAVVSHFNK	many products	Succ-AAAVVS, HFNK	Succ-AAAVVS, HFNK
(vi) GAVVAASQK	many products	GAVVA, ASQK	GAVVA, ASQK

[a] Porcine pancreatic elastase (PPE), R10 cell enzyme, or TSC enzyme (0.03 milliunit each) was allowed to react with each peptide (0.5–0.67 mM final substrate concentration) in 50 mM Hepes buffer containing 1 mg/mL BSA, pH 7.5, for 6 h at 37 °C. An aliquot of the acid-quenched reaction mixture was subjected to reverse-phase HPLC. The identity of the separated peaks was identified by compositional and/or N-terminal sequence analysis.

In any case, transfection with *ras* causes RLEC to become tumorigenic and exhibit other morphological changes (Strom et al., 1991) which ultimately apparently induce an increase in the level of the experimental enzyme.

The enzymes from the two unrelated cell types share many common features. Both enzymes are membrane associated with the plasma membrane of the cells and are not components of the growth medium. Both enzymes are single polypeptide chains and were purified about 20 000-fold to near electrophoretic homogeneity. Both enzymes are $\approx$38 000 M_r, and both enzymes are inhibited by serine proteinase and elastase inhibitors. These results show that both enzymes are elastase-like, but elastase is not known to be associated with other cell type.

Initially, we expected to isolate a specific candidate processing enzyme of pro-TGF-α. However, as the cleavage specificity of the enzymes were examined and their permissiveness revealed, it became increasingly clear that (1) the synthetic peptide substrates we used may not truly represent the processing sites in pro-TGF-α (including potential con-

Table VII: Hydrolysis of Additional Synthetic Peptide Substrates by R10 and TSC Enzymes[a]

substrate	R10 enzyme			TSC enzyme		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot mM^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot mM^{1}$)
NH_3^+-A-MCA	[b]			[b]		
Succ-AAA-MCA	0.440	2.2×10^4	4.9×10^4	0.440	2.2×10^4	4.9×10^4
Succ-GAAAVVS-OH	0.282	6.8×10^3	2.4×10^4	0.282	6.8×10^3	2.4×10^4
Succ-GAAAVVSHF-NH_2	0.530	6.1×10^3	0.6×10^4	0.530	6.1×10^3	0.6×10^4
Succ-AAAVVSHFNK-OH	0.224	1.1×10^4	4.9×10^4	0.224	1.1×10^4	4.9×10^4
NH_3^+-GAVVAASQK-OH	0.393	4.5×10^3	6.3×10^4	0.393	4.5×10^3	6.3×10^4
ox. insulin B chain	[b]			[b]		

[a] Enzyme and substrate were incubated at 37 °C for 6 h. Release of MCA was quantitated fluorometrically. Reaction mixtures containing nonfluorogenic substrates were analyzed by reverse-phase HPLC, and the separated products were identified by compositional analysis. [b] Not a substrate.

formational elements encompassing these sites (Harris, 1989; Rangaraju & Harris, 1991) or (2) the secreted forms of TGF-α have heterogeneous N-termini or (3) these enzymes also serve as general cellular proteases capable of cleaving other constituents of the cell proteinaceous substrates presented to the cell. For instance, the putative processing sites in pro-tumor necrosis factor also contain scissile Ala–Val bonds that flank the mature peptide hormone (Kriegler et al., 1988; Perez et al. 1990). The only consensus of specificity discerned is that the enzymes prefer a nonpolar amino acid in he P_1 position. With the synthetic peptides tested, other residues are readily accommodated at other P and P′ positions. Preference for nonpolar amino acids in P_1 closely parallels the specificity of pancreatic and leukocyte elastases.

Even with the long-chain peptide substrates tested, hydrolysis did not take place at the expected bonds. It is possible that because these substrates fail to assume the appropriate conformation, hydrolysis becomes permissive and is not restricted to cleavage at Ala–Val bond. In any case, the processing enzymes are more restrictive than pancreatic elastase and only cleave at a single bond. With Succ-A-A-A-V-V-S-H-F-N-K (which mimics the sequence Ala^{36}–Lys^{45} in pro-TGF-α) hydrolysis occurred exclusively at the Ser–His bond, which may not, at first, appear to be an elastase-like cleavage. However, pancreatic elastase does cleave a Ser–His bond in oxidized insulin B chain (Sampath Narayanan & Anwar, 1969; also repeated for these studies) and so His is likely considered a nonpolar residue from the enzyme's standpoint.

There was no dramatic difference between the catalytic constants obtained using the substrates based on the N- or C-terminal processing site sequences, suggesting that these enzymes are capable of acting with equal facility at either processing site within pro-TGF-α. These results contrast with those obtained by Pandiella and Massague (1991), who suggested that two different enzymes may be responsible for hydrolysis at either terminus. These investigators found that cleavage at the N-terminus occurred considerably faster than cleavage at the C-terminus, resulting in the accumulation of pro-TGF-α at the cell surface capable of affecting adjacent cells in a mechanism defined as juxtacrine stimulation. It is just as likely, however, that differential processing comes about as a result of structural constraints present in the intact prohormone resident in the plasma membrane which are relaxed after endoproteolysis occurs at the N-terminal site. These conformational constraints may help regulate endoproteolysis (Rangaraju & Harris, 1991; Benjannet et al., 1992; You et al., 1992).

It would appear, then, that a specific processing endoproteinase of pro-TGF-α does not exist, at least in these cell lines. Rather, these enzymes appear to be normal cell constituents. If they are physiologically involved in processing pro-TGF-α, then processing must take place at the plasma membrane where the enzyme and its substrate reside. The regulatory mechanism of this event is unknown, but processing may be initiated under some cellular signal, such as membrane perturbation. In any case, the mechanism of processing pro-TGF-α clearly occurs in marked contrast with processing of other mammalian precursor proteins in endocrine and neuroendocrine tissues.

ACKNOWLEDGMENT

We thank Dr. James C. Powers, Georgia Institute Technology, for the generous supply of elastase inhibitors.

REFERENCES

Anklesaria, P., Teixido, J., Laiho, M., Pierce, J. H., Greenberger, J. S., & Massague, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3289–3293.

Appelmans, F., Wattiaux, R., & deDuve, C. (1955) *Biochem. J. 59*, 438–445.

Barrett, A. J. (1972) in *Lysosomes—a laboratory handbook* (Dingle, J. T., Ed.) pp 46–135.

Baxter, J. H., Wilson, I. B., & Harris, R. B. (1986) *Peptides 7*, 407–411.

Benjannet, S., Reudelhuber, T., Mercures, C., Rondeau, N., Chretien, M., & Seidah, N. G. (1992) *J. Biol. Chem. 267*, 11417–11423.

Brachmann, R., Lindquist, P. B., Nagashima, M., Kohr, W., Lipari, T., Napier, M., & Derynck, R. (1989) *Cell 56*, 691–700.

Bringman, T. S., Lindquist, P. B., & Derynck, R. (1987) *Cell 48*, 429–440.

DeCoster, M. A., & DeVries, G. H. (1989) *J. Neurosci. Res. 22*, 283–288.

deDuve, C. (1975) *Science 189*, 186–194.

Derynck, R. J. (1986) *J. Cell. Biochem. 32*, 1–12.

Derynck, R. J. (1988) *Cell 54*, 593–595.

DeVries, G. H., Salzer, J. L., & Bunge, R. P. (1982) *Dev. Brain Res. 3*, 259–299.

Faust, J. B., Rogers, K. T., Lee, D. C., & Strom, S. (1988) *Proc. Am. Assoc. Cancer Res. 29*, 212 (Abstract).

Harlow, E., & Lane, D. (1988) in *Antibodies: A Laboratory Manual*, pp 651–652, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Harris, R. B. (1989) *Arch. Biochem. Biophys. 275*, 313–333.

Harris, R. B., & Wilson, I. B. (1984) *Arch. Biochem. Biophys. 233*; 667–675.

Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.

Houck, K. A., Michalopoulos, G. K., & Strom, S. C. (1989) *Oncogene 4*, 19–25.

Ignotz, R. A.; Kelly, B., Davis, R. J., & Massague, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6307–6311.

Kaulen, H. D., Henning, R., & Stoffel, W. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 1555.

Kobrin, M. S., Samsoondar, J., & Kudlow, J. E. (1986) *J. Biol. Chem. 261*, 14414–14419.

Kriegler, M., Perez, C., DeFay, K., Albert, I., & Lu, S. D. (1988) *Cell 53*, 45–53.

Laemmli, U. K. (1970) *Nature 227*, 680–688.

Lee, D. C., Rose, T. M., Webb, N. R., & Todaro, G. J. (1985) *Nature 313*, 489–491.

Lee, H. J., & Wilson, I. B. (1971) *Biochim. Biophys. Acta 242*, 519–522.

Luetteke, N. C., Michalopoulos, G. K., Teixido, J., Gilmore, R., Massague, J., & Lee, D. C. (1988) *Biochemistry 27*, 6487–6494.

Marquardt, H., & Todaro, G. J. (1982) *J. Biol. Chem. 257*, 5220–5225.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.

Mueller, S. G., Paterson, A. J., & Kudlow, J. E. (1990) *Mol. Cell. Biol. 10*, 4596–4602.

Pandiella, A., & Massague, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1726–1730.

Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., & Kriegler, M. (1990) *Cell 63*, 251–258.

Powers, J. C. (1977) in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins* (Weinstein, B., Ed.) Vol. 4, pp 65–178, Marcel Dekker, New York.

Rangaraju, N. S., & Harris, R. B. (1991) *Arch. Biochem. Biophys. 290*, 418–426.

Samsoondar, J., Kobrin, M. S., & Kudlow, J. E. (1986) *J. Biol. Chem. 261*, 14408–14413.

Schecter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Soler, D. F., & Harris, R. B. (1988) *Int. J. Pept. Protein Res. 32*, 35–40.

Strom, S. C., Faust, J. B., Cappelluti, E., Harris, R. B., & Lalwani, N. D. (1991) *Dig. Dis. Sci. 36*, 642–652.

Teixido, J., & Massague, J. (1988) *J. Biol. Chem. 263*, 3924–3929.

Teixido, J., Gilmore, R., Lee, D. C., & Massague, J. (1987) *Nature 326*, 883–885.

Teixido, J., Wong, S. T., Lee, D. C., & Massague, J. (1990) *J. Biol. Chem. 265*, 6410–6415.

Tennekoon, G. O., Yoshino, J. E., Peden, K. W. C., Bigbee, J., Rutkowski, J. L., Kishimoto, Y., DeVries, G. H., & McKhann, G. M. (1987) *J. Cell Biol. 105*, 2315–2325.

Travis, J., & Salvesen, G. (1983) *Annu. Rev. Biochem. 52*, 655–709.

Walter, P., & Blobel, G. (1983) *Methods Enzymol. 96*, 84–93.

Wilcox & Derynck, R. (1988) *J. Neurosci. 8*, 1901–1904.

Wong, S. T., Winchell, L. F., McCune, B. K., Earp, H. S., Teixido, J., Massague, J., Herman, B., & Lee, D. C. (1989) *Cell 56*, 495–506.

Wypij, D. M., & Harris, R. B. (1992) *Life Sci. 50*, 523–531.

Yoshino, J. E., Dinnneen, M. P., Lewis, B. L., Meador-Woodruff, J. H., & DeVries, G. H. (1984) *J. Cell Biol. 99*, 2309–2313.

Yoshino, J. E., Dinneen, M. P., Sprinkle, T. J., & DeVries, G. H. (1985) *Brain Res. 325*, 199–203.

You, J. L., Scarsdale, J. N., & Harris, R. B. (1991) *J. Protein Chem. 10*, 301–311.

You, J. L., Milton, S. C. F., Milton, R., deLisle, Rangaraju, N. S., & Harris, R. B. (1992) *J. Protein Chem.* (in press).