Molecular Events in the Processing of Recombinant Type 1 Pre-Pro-Transforming Growth Factor Beta to the Mature Polypeptide

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Recently, the simian type 1 transforming growth factor beta (TGF-β1) cDNA was expressed at high levels in Chinese hamster ovary (CHO) cells by dihydrofolate reductase-induced gene amplification (L. E. Gentry, N. R. Webb, G. J. Lim, A. M. Brunner, J. E. Ranchalis, D. R. Twardzik, M. N. Lioubin, H. Marquardt, and A. F. Purchio, Mol. Cell. Biol. 7:3418–3427, 1987). We have now purified and characterized the recombinant proteins released by these cells. Analyses of the precursor proteins by amino acid sequencing identified potentially important proteolytic processing sites. Signal peptide cleavage occurs at the Gly-29–Leu-30 peptide bond of pre-pro-TGF-β1, yielding pro-TGF-β1 (30 to 390). In addition, proteolytic processing of the precursor to yield mature TGF-β1 occurs at the dibasic cleavage site immediately preceding Ala-279, indicating that CHO cells possess the appropriate processing enzyme. Greater than 95% of the biological activity detected in the conditioned medium of the CHO transfectant was due to mature, properly processed growth factor. Highly purified recombinant TGF-β1 had the same specific biological activity as natural TGF-β1. The concentration of TGF-β1 required for half-maximal inhibition of MvlLu mink lung epithelial cell growth was approximately 1 to 2 pM. Purified precursor inhibited mink lung cell proliferation at 50 to 60 pM concentrations. The purified precursor preparation was shown to consist of pro-TGF-β1 (30 to 390), the pro region of the precursor (30 to 278), and mature TGF-β1 (279 to 390) interlinked by at least one disulfide bond with the pro portion of the precursor. These recombinant forms of TGF-β1 should prove useful for further structural and functional studies.

Type 1 transforming growth factor beta (TGF-β1) belongs to a closely related family of polypeptides with potent cellular modulating activities (for reviews, see references 12 and 19). This growth factor molecule appears to be intimately associated with cell growth and differentiation and may play pivotal roles in the autocrine or paracrine regulation of these processes. Although numerous studies have addressed important cellular and physiological properties of TGF-β1, very little information is available concerning structural features and posttranslational processing events necessary for its expression and function.

The molecular cloning of TGF-β1 cDNA from several species (4, 5, 18) and analysis of the predicted protein sequences has provided some insight into the structure and processing of this polypeptide growth factor. Sequence analysis has predicted that the mature 112-amino-acid chain of TGF-β1 is derived from the C terminus of a 390-amino-acid pre-pro-TGF-β1 by proteolytic cleavage. Proteolytic cleavage is predicted to occur following a dibasic Arg-Arg sequence and immediately preceding the N-terminal Ala residue of the mature growth factor. Structural studies of mature TGF-β1 have revealed that TGF-β1 is homodimeric and interlinked by disulfide bonds (1, 6, 16). The precursor for TGF-β1 contains a typical hydrophobic signal peptide and three potential N-linked glycosylation sites (4, 5, 18).

We recently reported the high-level expression of the simian TGF-β1 cDNA (7) in Chinese hamster ovary (CHO) cells using dihydrofolate reductase gene amplification (9, 11). These CHO cell transfectants expressed biologically active growth factor and TGF-β1 precursor (7). Subsequent studies have revealed that the expressed TGF-β1 precursor is glycosylated and phosphorylated (3).

In this report, we present data on the purification and extensive characterization of the recombinant forms of the TGF-β1 precursor and mature growth factor (rTGF-β1). Our studies showed that TGF-β1 is synthesized in CHO cells as pre-pro-TGF-β1, which is processed at the carboxy-terminal side of Gly-29 and Arg-278. Most importantly, our results showed that mature rTGF-β1 is properly processed and possesses the same specific biological activity as natural TGF-β1 (nTGF-β1). Since the mature simian and human TGF-β1 molecules are identical in primary sequence, simian rTGF-β1 may thus be useful for therapeutic applications in humans.

MATERIALS AND METHODS

Cell culture conditions. CHO cell transfectants expressing TGF-β1 (TGF-β3-2000 cells) were propagated and passaged as described previously (7). Roller bottles (850 cm²) containing 50 ml of Dulbecco modified Eagle medium supplemented with fetal bovine serum (10%, vol/vol), penicillin (100 U/ml), streptomycin (100 μg/ml), L-proline (150 μg/ml), and methotrexate (20 μM) were seeded with the contents of one confluent 150-cm² round tissue culture dish of TGF-β3-2000 cells and grown at 37°C. After the cells attached and reached confluency (ca. 2 days), they were rinsed twice with 50 ml of serum-free medium supplemented as above and then incubated for 24 h in 50 ml of serum-free medium supplemented additionally with ascorbate and reduced glutathione at 100 and 20 μg/ml, respectively.

Processing of conditioned medium. Serum-free supernatants collected from roller bottles were centrifuged at 200 ×

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g to remove cellular debris and immediately adjusted to 10 mg of phenylmethylsulfonyl fluoride per liter, 50 trypsin inhibitory units of aprotinin (Sigma Chemical Co., St. Louis, Mo.) per liter, and 0.2 M acetic acid. Supernatants were then concentrated 40-fold by ultrafiltration (YM10 membrane, 10,000-molecular-weight cutoff; Amicon Corp., Danvers, Mass.), and the resulting concentrate was dialyzed extensively against 0.2 M acetic acid. The dialyzed material was lyophilized and stored at $-20°C$ prior to purification.

HPLC. Gel permeation chromatography was performed on a Bio-Sil TSK-250 column (21.5 by 600 mm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 0.1% trifluoroacetic acid (TFA) containing 40% acetonitrile by high-performance liquid chromatography (HPLC) as described previously (10). Reversed-phase HPLC was performed on a μBondapak C_{18} column (3.9 by 300 mm, 10-μm particle size; Waters Associates, Inc., Milford, Mass.) with a linear gradient composed of 0.05% TFA in water as the starting buffer and 0.045% TFA in acetonitrile as the limiting buffer (10). To minimize losses from nonspecific adsorption, fractions from the HPLC runs were collected in polypropylene tubes.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (13) using either a 7.5 to 15% acrylamide gradient or 15% acrylamide gels. Proteins were stained with Coomassie brilliant blue R-250.

Cleavage with cyanogen bromide. For CNBr cleavage at methionyl residues, 800 pmol of TGF-β1 precursor was dissolved in 30 μl of 70% formic acid and 16 μl of a solution containing 15 mg of CNBr in 100 μl of 70% formic acid was added (8). The reaction proceeded under nitrogen for 4 h at 30°C in the dark. The digest was chromatographed on a Bio-Sil TSK-250 gel permeation chromatography column.

Amino acid sequence analysis. Automated sequence analysis was performed on an amino acid sequencer (model 475A; Applied Biosystems, Inc., Foster City, Calif.). Phenylthiohydantoin-amino acid derivatives were separated by reversed-phase HPLC, on-line, on a PTH analyzer (model 120A; Applied Biosystems), as described previously (14).

Concanavalin A chromatography. TSK-250-purified precursor was dialyzed against 50 mM NH_4HCO_3 (pH 7.0) and then centrifuged at 15,000 × *g* prior to chromatography. A column containing 1 ml of concanavalin A covalently bound to Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.) was extensively washed with phosphate-buffered saline and equilibrated with 50 mM NH_4HCO_3 (pH 7.0). Samples to be absorbed were loaded and recirculated four times through the column before being washed with 10 column volumes of phosphate-buffered saline. Specifically bound material was eluted with 100 mM methyl-α-D-mannopyranoside in phosphate-buffered saline.

Growth inhibition assays. Growth inhibition assays were performed with Mv1Lu mink lung epithelial cells (CCL-64; American Type Culture Collection, Rockville, Md.) essentially as described previously (10). One unit of activity was defined as the amount of TGF-β1 required to give 50% maximal response in the assay.

RESULTS

Amino-terminal sequence of rTGF-β1 polypeptides. Conditioned medium from transfected CHO cells expressing high levels of simian rTGF-β1 was used for characterization. These cells have been shown to secrete three molecular forms of rTGF-β1 proteins (7). Figure 1 shows a Coomassie blue-stained SDS-polyacrylamide gel of serum-free medium
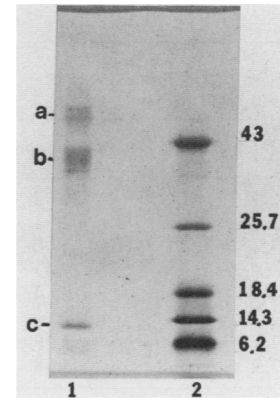

FIG. 1. Coomassie blue staining pattern of proteins from the conditioned medium of amplified CHO cells expressing TGF-β1. Conditioned medium was dialyzed, fractionated on 15% SDS-polyacrylamide gels under reducing conditions, and stained with Coomassie brilliant blue R-250. For reference, the letters a, b, and c are noted at the left of the figure to indicate rTGF-β1 molecules. Lane 1, 0.25 ml of conditioned medium; lane 2, marker proteins with the indicated molecular size in kilodaltons.

fractionated under reducing conditions. The largest form, a broadly migrating species ranging in size from 44 to 56 kilodaltons (kDa) (a, Fig. 1), has previously been shown (7) to possess immunological epitopes derived from the precursor and mature growth factor and most likely represents unprocessed TGF-β1 precursor. The 30- to 42-kDa polypeptide (b, Fig. 1) contains only epitopes derived from the TGF-β1 precursor, indicating that this species has undergone proteolytic cleavage to release the mature growth factor (7). The 14-kDa species (c, Fig. 1) represents the mature, fully processed TGF-β1 monomer.

The recombinant proteins (a and b, Fig. 1) were electroeluted from acrylamide slices and characterized by amino-terminal sequence analysis. The results are shown in Table 1. Sequence analysis revealed that the two larger precursor forms had identical amino-terminal sequences. Comparison of this sequence with that predicted from the simian TGF-β1 cDNA (18) indicated that both larger proteins underwent specific proteolytic cleavage at Gly-29–Leu-30 removing the first 29 amino acids of the intact pre-pro-TGF-β1 molecule. Cleavage of this hydrophobic 29-amino-acid leader sequence is most likely the result of a signal peptidase. The Gly-29–Leu-30 peptide bond is the predicted signal peptide cleavage site (20). Based on these results, the 44- to 56-kDa TGF-β1 polypeptide (a, Fig. 1) represents pro-TGF-β1 (30 to 390), whereas the 30- to 42-kDa species (b, Fig. 1) corresponds to the pro form of the precursor (30 to 278) lacking the signal peptide and mature TGF-β1 sequences. Sequence analysis of the 14-kDa polypeptide (data not shown) revealed an intact amino terminus beginning at Ala-279 of the mature growth factor, indicating that the CHO cells properly process the simian rTGF-β1 at the dibasic cleavage site.

Purification of biologically active rTGF-β1. The recombinant proteins released by transfected CHO cells were biologically active (7). Conditioned medium was first fractionated by gel permeation chromatography. A representative elution profile is shown in Fig. 2. Greater than 95% of the biological activity which was applied eluted at a molecular weight of approximately 15,000, based on marker proteins. The same elution pattern was of observed with nTGF-β1 with the same column conditions. The low apparent molecular weight of TGF-β1 as determined by gel permeation

TABLE 1. Amino-terminal sequence analysis of rTGF-β1 polypeptides

Cycle	Yield (pmol)		Position (Residue)	HPLC-purified mature rTGF-β1[b]			
				Amino terminal		Carboxy terminal	
	Polypeptide a[a]	Polypeptide b[a]		Yield (pmol)	Position (residue)	Yield (pmol)	Position (residue)
1	42.9	93.8	30 (Leu)	40.1	279 (Ala)	27.4	383 (Ile)
2	17.0	29.1	31 (Ser)	36.1	280 (Leu)	27.3	384 (Val)
3	13.2	27.0	32 (Thr)	26.8	281 (Asp)	13.5	385 (Arg)
4	ND[c]	ND	33 (Cys)	10.9	282 (Thr)	7.3	386 (Ser)
5	34.9	49.4	34 (Lys)	15.2	283 (Asn)	ND	387 (Cys)
6	12.5	20.0	35 (Thr)	14.8	284 (Tyr)	8.1	388 (Lys)
7	21.1	32.5	36 (Ile)	ND	285 (Cys)	ND	389 (Cys)
8	17.9	38.8	37 (Asp)	14.6	286 (Phe)	4.7	390 (Ser)

[a] Polypeptides a and b were electroeluted from a Coomassie blue-stained gel similar to that shown in Fig. 1 prior to amino-terminal sequence analysis.

[b] Sequence of purified mature rTGF-β1. The rTGF-β1 was cleaved with CNBr, and results indicate simultaneous sequences of amino and carboxy ends of the growth factor.

[c] ND, Not determined.

chromatography may be due to the tightly folded structure of the dimeric growth factor molecule or to nonspecific adsorption. TGF-β1 activity was observed in the void volume of the column and accounted for less than 5% of the total applied activity. SDS-acrylamide gel electrophoresis of pools A and B under nonreducing conditions revealed that the majority of the biological activity eluted as a 24-kDa polypeptide species, whereas the minor activity eluted as a large 95- to 110-kDa, component (data not shown).

To confirm that the 24-kDa component represented the properly processed rTGF-β1, we purified this species using reversed-phase HPLC for subsequent characterization. Pool B was fractionated on a μBondapak C₁₈ support (Fig. 3). The

biologically active component eluted on a shallow acetonitrile gradient as a homogeneous peak with the same retention time as nTGF-β1. Analysis on SDS-polyacrylamide gels under nonreducing and reducing conditions demonstrated that this active component comigrated with nTGF-β1 isolated from bovine spleen (Fig. 4). rTGF-β1 was further characterized by protein sequence analysis (Table 1). The purified polypeptide was chemically cleaved with cyanogen bromide prior to sequencing. Since mature TGF-β1 contains only one methionine at residue 382, two sequences were obtained simultaneously: one corresponding to the amino-terminal sequence of the growth factor (beginning at Ala-279) and one representing the carboxy-terminal 8 amino acids (beginning at Ile-383). Our results (Table 1) demon-
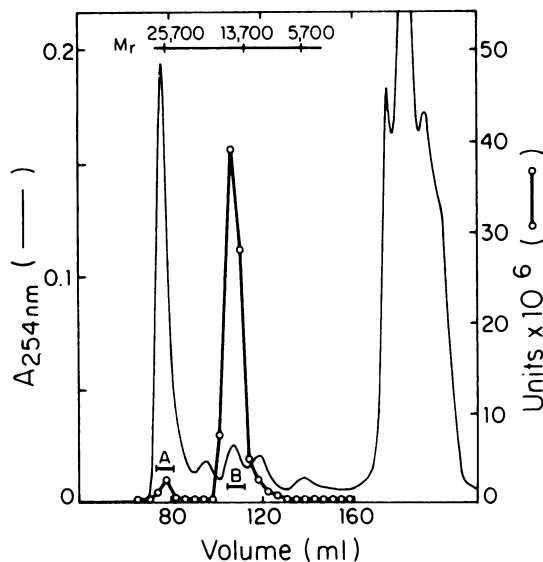

FIG. 2. Gel permeation chromatography on a Bio-Sil TSK-250 column (21.5 by 600 mm) of 20 mg of protein after ammonium sulfate precipitation of 500 ml of serum-free supernatant from TGF-β3-2000 cells. The column was equilibrated with 0.1% TFA in water containing 40% (vol/vol) acetonitrile at 2 ml/min at 22°C; 4-ml fractions were collected. Samples of the indicated fractions were assayed for growth inhibitory activity on mink lung epithelial cells (O). The following proteins were used as markers: α-chymotrypsinogen (M_r 25,700), bovine pancreatic RNase A (M_r 13,700), and insulin (M_r 5,700).
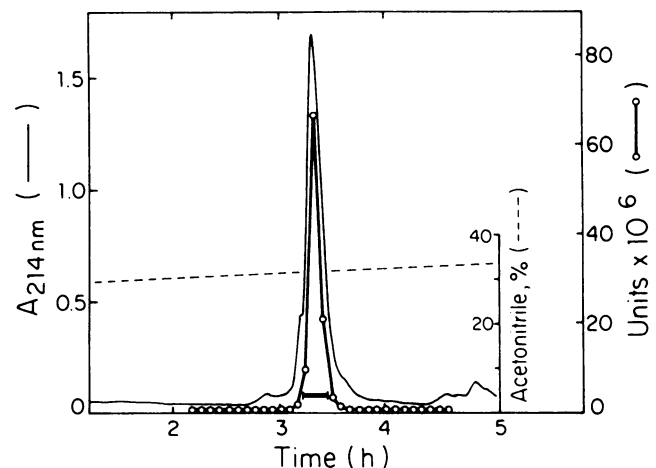
FIG. 3. Purification of rTGF-β1 by reversed-phase HPLC. Shown is the elution pattern of 0.65 mg of protein from gel permeation chromatography-purified rTGF-β1 (Fig. 2, pool B) on a μBondapak C₁₈ column (10-μm particle size, 3.9 by 300 mm). Elution was achieved with a linear 10-min gradient of 0.05% TFA in water to 30% acetonitrile in 0.045% TFA, followed by a linear 6-h gradient of 30 to 36% acetonitrile in 0.045% TFA and a 10-min gradient of 36 to 60% acetonitrile in 0.045% TFA. The column was operated at a flow rate of 0.2 ml/min at 22°C. Samples of the indicated fractions were assayed for growth inhibitory activity on mink lung epithelial cells (O). The horizontal bar indicates pooled rTGF-β1. UV-absorbing material was continuously monitored at 214 nm (——); – – –, concentration of acetonitrile.

FIG. 4. SDS-polyacrylamide gel analysis of purified rTGF-β1 proteins. Precursor and mature forms of TGF-β1 were fractionated by SDS-PAGE under nonreducing (A) or reducing (B) conditions and stained with Coomassie brilliant blue R-250. nTGF-β1 was isolated from bovine spleen and used for comparison. Marker proteins are indicated (kilodaltons) at the left and right of the figure.

strated that biologically active rTGF-β1 is properly processed.

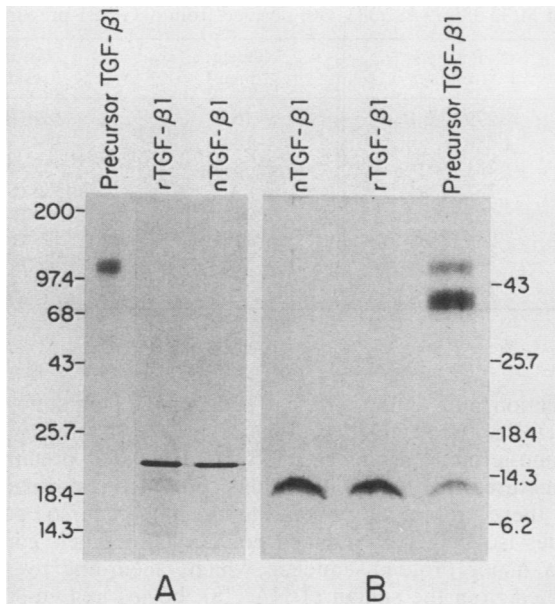A summary of the purification steps of rTGF-β1 is shown in Table 2. The growth factor was purified greater than 30-fold in two purification steps. In this particular experiment, the overall yield was 54% and resulted in 0.65 mg of rTGF-β1 per liter of conditioned culture medium. Processing of other preparations resulted in greater than 85% recovery of biologically active recombinant protein, a yield of more than 1 mg/liter.

Purification of rTGF-β1 precursor. The TGF-β1 precursor was purified by taking advantage of its glycosylated nature (3). Precursor eluting in the void volume of a TSK-250 column (pool A) was dialyzed against neutral buffer and fractionated on a concanavalin A lectin column. The precursor bound to concanavalin A was eluted specifically with α-methyl mannoside (data not shown). An SDS-polyacrylamide gel profile of the purified precursor stained with Coomassie blue is shown in Fig. 4. The eluted protein migrated at a molecular weight between 95,000 and 120,000. This large form was reactive with antibodies directed toward the precursor sequences and mature growth factor (data not

TABLE 2. Purification of mature rTGF-β1 from conditioned medium

Fraction	Protein (mg)[a]	10^7 Units[b]	Sp act (10^6 units/mg)	% Yield
Conditioned medium[c]	39.0	14	3.6	100
TSK (fraction B)	1.37	8.3	61	59
HPLC-C$_{18}$	0.65[d]	7.5	119	54

[a] Protein was determined assuming 1 absorbance unit at 280 nm = 1 mg of protein per ml.
[b] One unit of activity is defined in Materials and Methods.
[c] Started with 1 liter of conditioned medium.
[d] Calculated from amino acid analysis.

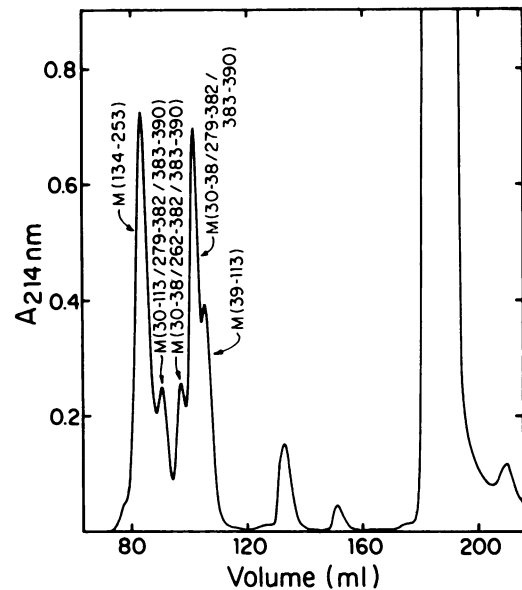

FIG. 5. Gel permeation chromatography on a Bio-Sil TSK-250 column (7.5 by 600 mm) of CNBr peptides of rTGF-β1-precursor. Shown is the elution pattern of 800 pmol of rTGF-β1-precursor cleaved with CNBr. The column was equilibrated with 0.1% TFA in water containing 40% acetonitrile at 0.25 ml/min at 22°C. UV-absorbing material was monitored at 214 nm. Peaks designated M refer to CNBr peptides subjected to Edman degradation; numbers refer to the position of that particular fragment or fragments connected by disulfide bonds in the complete sequence (18).

shown). No contaminating mature rTGF-β1 was detected in this preparation even on overloaded SDS-polyacrylamide gels.

Although the mature dimeric growth factor was absent from the preparation, the purified precursor when analyzed by reducing SDS-PAGE revealed a 14-kDa species which comigrated with monomeric rTGF-β1 (Fig. 4). Also apparent on the gels were the two precursor species, pro-TGF-β1 (30 to 390) and the 30- to 42-kDa pro region of the precursor (30 to 278; see also Fig. 1). Further attempts to fractionate this larger complex into separate components were unsuccessful. Amino-terminal sequence analysis of the concanavalin A-purified material revealed two amino-terminal sequences, one beginning at Leu-30 and the other at Ala-279.

Secreted rTGF-β1 precursor is a disulfide-linked complex. The results shown above strongly suggest that the 95- to 120-kDa rTGF-β1 precursor purified from the conditioned medium of the CHO cells represents a mixture of pro-TGF-β1 (30 to 390), the pro region of the precursor (30 to 278), and the mature chain of rTGF-β1 (279 to 390) linked by at least one disulfide bond to the pro portion. To confirm this observation, we digested the precursor with CNBr and purified the CNBr peptides to establish the chemical nature of this complex. A TSK-250 elution profile of CNBr-cleaved rTGF-β1 precursor is shown in Fig. 5. The various peaks were purified by homogeneity by reversed-phase HPLC (data not shown) and identified by amino acid sequencing. A major CNBr peptide fragment containing a disulfide bridge between precursor cysteine residue 33 and one cysteine residue of the mature growth factor is M(30-38/279-382/383-390), and its amino-terminal sequence analysis is shown in Table 3. This particular peptide fragment involves Cys-33 of the precursor. The amino-terminal CNBr peptide, M(30-38/262-382/383-390), represents a disulfide-linked peptide

TABLE 3. Amino-terminal sequence of disulfide cross-linked CNBr fragment M(30-38/279-382/383-390) derived from rTGF-β1 precursor

Cycle	Yield (pmol)	Position (residue)	Yield (pmol)	Position (residue)	Yield (pmol)	Position (residue)
1	22.6	30 (Leu)	25.5	279 (Ala)	16.0	383 (Ile)
2	10.6	31 (Ser)	28.3	280 (Leu)	18.5	384 (Val)
3	11.3	32 (Thr)	21.2	281 (Asp)	8.5	385 (Arg)
4	ND[a]	33 (Cys)	14.9	282 (Thr)	9.3	386 (Ser)
5	14.8	34 (Lys)	18.9	283 (Asn)	ND	387 (Cys)
6	10.9	35 (Thr)	21.3	284 (Tyr)	14.7	388 (Lys)
7	13.3	36 (Ile)	ND	285 (Cys)	ND	389 (Cys)
8	12.3	37 (Asp)	22.1	286 (Phe)	5.6	390 (Ser)

[a] ND, Not determined.

involving pro-TGF β1; no processing at the dibasic sequence preceding Ala-279 occurred in this TGF-β1 precursor species.

Quantitation of biological activity. The purified mature and precursor forms of rTGF-β1 were tested for biological activity on mink lung epithelial cells. Purified proteins were quantitated by amino acid analysis by using the predicted amino acid compositions. For the purified precursor preparation, an amino acid composition for pro-TGF-β1 was used. Figure 6 shows the biological activity profiles. Mature rTGF-β1 was a potent inhibitor of mink lung cell proliferation, displaying an activity curve superimposable with that of nTGF-β1, indicating that rTGF-β1 and nTGF-β1 possess identical specific activities. The precursor, on the other hand, was much less effective in inhibiting epithelial cells. Moreover, the inhibition curve of the precursor appeared to have a slightly altered slope when compared with that of mature growth factor.

DISCUSSION

Molecular cloning of the cDNA for TGF-β1 has suggested that this growth factor molecule undergoes a variety of posttranslational processing events before secretory exit (4, 5, 18). Attempts to define these processing events in tissue culture cells have been precluded owing to the low level of secretion of TGF-β1 proteins. To define some of these processing events, we have begun to examine the TGF-β1 proteins produced and released by transfected cells. CHO cells which express high levels of biologically active simian rTGF-β1 were used (7). In this study, we utilized protein

FIG. 6. Growth inhibition curves of purified TGF-β1 proteins obtained with mink lung indicator cells. Growth inhibition was assessed as described in the text. Concentration of TGF-β1 polypeptides was determined by amino acid analysis. Symbols: △, recombinant precursor protein; ●, nTGF-β1 from bovine spleen; ○, rTGF-β1.

purification and amino-terminal sequencing techniques to characterize the rTGF-β1 proteins.

Amino-terminal sequence analysis of isolated precursor and mature TGF-β1 polypeptides provided information about these proteolytic processing events. Precursor polypeptides isolated from reduced polyacrylamide gels generated a major protein sequence which, according to that predicted from the simian cDNA (18), begins at Leu-30 of the TGF-β1 precursor. No heterogeneity in the amino-terminal sequence was observed, indicating a specificity in proteolytic processing. This result identifies the Gly-29–Leu-30 peptide bond as the signal cleavage site and is consistent with that predicted by the signal peptide prediction method of von Heijne (20). Moreover, very recent protein sequence studies of isolated TGF-β1 precursor from the platelet latent complex have also implicated this site for signal peptide cleavage (15). In addition to signal peptide cleavage, purification and examination of the mature TGF-β1 revealed that rTGF-β1 is proteolytically processed at the predicted dibasic protease site (18), resulting in a mature polypeptide. Furthermore, protein sequence analysis of the carboxy-terminal CNBr fragment of mature TGF-β1 suggests an intact molecule. Thus, CHO cells possess the appropriate proteases necessary for correctly processing pre-pro-TGF-β1.

The major biological activity secreted by transfected CHO cells is the mature dimeric growth factor. Our results indicate that greater than 95% of the activity present in CHO conditioned medium copurifies with mature rTGF-β1, whereas less than 5% copurifies with the larger rTGF-β1 precursor. rTGF-β1 behaved identically to nTGF-β1 and possessed an identical specific biological activity. The rTGF-β1 precursor, on the other hand, was 50-fold less active than the mature growth factor. In addition, a comparison of mink lung cell inhibition profiles showed a slightly altered dose-response curve, suggesting a different receptor affinity for the precursor as compared with the mature TGF-β1.

Although these results suggest that the rTGF-β1 precursor is biologically active, an in-depth structural analysis revealed some intriguing anomalies complicating any definitive interpretations. Protein sequence analysis and SDS-PAGE revealed that the isolated precursor consists of pro-TGF-β1, mature TGF-β1, and the pro region of the precursor interlinked by disulfide bonds. Chemical cleavage of this mixture with CNBr and separation of CNBr peptides clearly showed that the Cys-33 of the precursor forms a disulfide bond with one cysteine residue of mature TGF-β1 (Fig. 7A). The existence of the mature TGF-β1 interconnected with the pro region severely limits any conclusions which can be made concerning the biological activity of the unprocessed precursor. The formation of this disulfide-linked complex in CHO
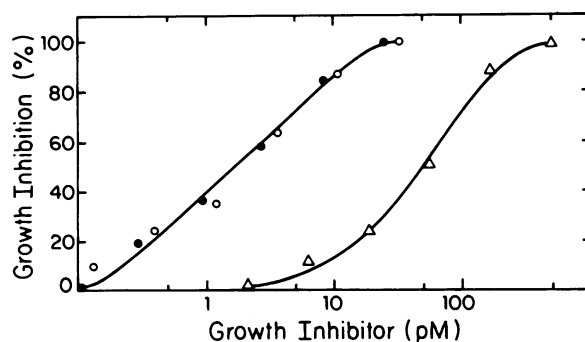
FIG. 7. (A) Proposed structure of TGF-β1 precursor highlighting its disulfide cross-linked nature. See text for details. (B) Summary of processing events of pre-pro-TGF-β1 in transfected CHO cells. Proteolytic processing sites are indicated. For details, please refer to the text. ♀, Glycosylation site; ▬, signal peptide; ☐, pro region; ▨, mature TGF-β1.

cells raises a question about its significance in tissues and cells which secrete TGF-β1 naturally. The very high level of secretion of rTGF-β1 by CHO cells may lead to an unnatural cross-linking owing to improperly folded rTGF-β1, resulting in an expression artifact. Alternatively, the disulfide-linked precursor complex may represent an important intermediate in TGF-β1 processing. It is interesting to note that disulfide-linked precursor complexes have been observed in latent isolated forms of TGF-β1 (15, 21).

On the basis of results presented in this report, we summarized the processing of pre-pro-TGF-β1 in the transfected CHO cells (Fig. 7B). The proposed processing scheme is by no means complete; however, it emphasizes several of the steps which have been at least partially defined. The order of the various processing steps is not well characterized, and for convenience we have placed these steps in succession. The first step involves signal peptide cleavage at the Gly-29–Leu-30 peptide bond. This cleavage event most likely occurs cotranslationally during transit of the precursor through the rough endoplasmic reticulum membrane (2, 22). Following cleavage of the signal peptide, core glycosylation units (17) are added to pro-TGF-β1 at each of the predicted N-glycosylation sites located at Asn-82, Asn-136, and Asn-177 (A. F. Purchio, J. Cooper, A. Brunner, M. N. Lioubin, L. E. Gentry, K. S. Kovacina, R. A. Roth, and H. Marquardt, J. Biol. Chem., in press). The core glycosylated pro-TGF-β1 is then sequentially processed during transit through the Golgi complex to yield a phosphorylated glycoprotein containing complex sialated oligosaccharides (3). At some stage during synthesis or transit, proteolytic cleavage at the dibasic residue and disulfide bond formation occur, releasing mature TGF-β1. Two important questions concerning processing are readily apparent from the proposed scheme. At what stage and in what compartment does proteolytic cleavage at the dibasic residues occur? In addition, what is the function of carbohydrate addition and processing? We are currently examining these intriguing questions.

The results presented in this report were useful for defining the proteolytic events which occur during TGF-β1 processing and for characterizing the recombinant precursor. More importantly, however, our results indicated that the mature rTGF-β1 secreted by transfected CHO cells behaves identically to nTGF-β1, indicating that CHO cells expressing rTGF-β1 may represent an excellent source for comprehensive structural studies of TGF-β1. Moreover, since the mature simian TGF-β1 is identical to human TGF-β1, this source of recombinant growth factor may eventually be useful for detailed therapeutic and physiological studies.

ACKNOWLEDGMENTS

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