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# Characterization of Latent Recombinant TGF-β2 Produced by Chinese Hamster Ovary Cells

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**Abstract** Latent recombinant transforming growth factor-β2 (LrTGF-β2) complex has been purified from serum-free media conditioned by Chinese hamster ovary cells transfected with a plasmid encoding the TGF-β2 (414) *precursor. Under neutral* conditions, LrTGF-β2 had an apparent molecular weight of 130 kDa. The complex contained both mature and pro-region sequences. Acidification of LrTGF-β2 resulted in the release of mature 24 kDa TGF-β2 from the high molecular weight pro-region-containing complex, suggesting that TGF-β2 was non-covalently associated with this complex. These results were confirmed by crosslinking experiments performed on partially purified LrTGF-β2. Protein sequence analysis of the purified TGF-β2 pro-region indicated that signal peptide cleavage occurred between ser(20) and leu(21). The pro-region, which previously was found to contain mannose-6-phosphate, bound to the mannose-6-phosphate receptor. Proteolytic cleavage of mature TGF-β2 from pro-TGF-β2 was inhibited by monensin and chloroquin suggesting that binding to this receptor and subsequent transport to acidic vesicles may be involved in the processing of rTGF-β2 precursor.

**Key words:** recombinant TGF-β2, latency, activation

Transforming growth factor-β2 (TGF-β2) is a member of a family of related molecules which regulate the growth and differentiation of various cell types [1–5]. This family includes TGF-β1 [2,6], TGF-β2 [7–10], TGF-β3 [11–13], TGF-β4 [14], TGF-β5 [15], Mullerian inhibitory substance [16], and the inhibins [17].

Sequence analysis of cDNAs encoding TGF-βs 1 through 5 [6,11–15,18–21] indicate that these proteins are synthesized as larger precursor molecules, the carboxy terminus of which is cleaved to yield the 112-amino-acid monomer (114 amino acids in the case of TGF-β4 [14]). Further analyses of TGF-β2-specific cDNA clones predicted the existence of two TGF-β2 precursor proteins of 442 amino acids (TGF-β2 (442)) and 414 amino acids (TGF-β2 (414)) due to a 28-amino-acid insertion within the pro-region of TGF-β2 (442) [22].

TGF-β1 has been expressed to high levels in Chinese hamster ovary (CHO) cells [23] and a *human renal carcinoma* cell line [24]. Analysis of recombinant TGF-β1 (rTGF-β1) secreted by these cells indicated that it was secreted in a latent form, non-covalently complexed to high-molecular weight, di-sulfide-bonded pro-region-containing proteins [23–26]. Acidification of this complex was necessary to detect optimal biological activity. Natural TGF-β1 is also secreted by cells or released from platelets in a latent form [4] and can be activated by acid [27] or certain proteolytic enzymes [28]. Further characterization of latent platelet-derived TGF-β1 indicated that the mature TGF-β1 was non-covalently associated with a 125–160 kDa binding protein and pro-region molecules [29,30]. This high-molecular-weight binding protein has not been detected in the latent recombinant TGF-β1 (LrTGF-β1) complex and may not be necessary for latency [23,24,26].

We have recently expressed TGF-β2 to high levels in CHO cells and showed that recombinant TGF-β2 (rTGF-β2) was secreted in a latent form [31]. Here we further characterize the LrTGF-β2 complex and demonstrate that mature TGF-β2 is non-covalently associated with a high-molecular-weight pro-region-containing complex which can be dissociated by acid treatment. We also identify the site of signal peptide cleavage and demonstrate that the purified

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TGF-β2 pro-region-containing high-molecular-weight complex binds to the mannose-6-phosphate receptor.

## MATERIALS AND METHODS

### Cell Culture

Chinese hamster ovary (CHO) cell clones producing LrTGF-β1 (β1cl.17) and LrTGF-β2 (β2(414)cl.35) were propagated as described [23,31,32]. Secreted proteins were purified from serum-free media conditioned by the above clones.

### Growth-Inhibitory Assay

Samples were assayed for growth inhibition of mink lung cells (CCL-64) in 10% FBS as previously described [8,23]. In this assay, TGF-β1 and TGF-β2 have similar specific activity.

### Purification

Serum-free conditioned medium was clarified by centrifugation at 3,000*g* before solid ammonium sulphate was added to 80% saturation. Precipitation proceeded for 2 h at 4°C with constant stirring. The precipitate was recovered by centrifugation at 30,000*g* and dissolved in PBS pH 7.5. The samples were again centrifuged at 30,000*g* before any further processing.

**Gel filtration.** Samples in PBS were purified on a BioSil TSK-250 gel filtration column (Bio Rad 600 × 21.5 mm) equilibrated in PBS pH 7.5 and eluted in the same buffer at a flow rate of 2 ml/min. Fractions were collected at 2 min intervals. Gel filtration under acid conditions was performed on a BioSil TSK-250 column (Bio Rad 600 × 7.5 mm) equilibrated and eluted with 40% $CH_3CN$, 0.1% TFA, in $H_2O$ at a flow rate of 1 ml/min [8]. Fractions were collected at 1 min intervals.

**Reverse-phase chromatography.** Gel filtration-purified samples (fractions 30–32; Fig. 2D) were pooled, acidified with TFA (to 0.1%), and applied to a Bio Gel Phenyl RP+ column (Bio Rad 75 × 4.6 mm) equilibrated in 0.05% TFA. The column was eluted with a linear gradient of $CH_3CN$ containing 0.035% TFA at a flow rate of 1 ml/min. Fractions were collected at 1 min intervals.

### Polyacrylamide Gel Electrophoresis

All electrophoretic analyses were carried out on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) under reducing or non-reducing conditions [33]. All determinations were performed on 7.5–17.5% gradient gels. Total protein was visualized by staining with Coomassie brilliant blue R250 (Serva). Gels to be analyzed by autoradiography were dried under vacuum before exposure to x-ray film (DuPont Cronex-4).

### Western Blot Analysis

Samples analyzed by SDS-PAGE were transferred onto nitrocellulose filters and immunoblotted as previously described [23,34]. The antisera used were raised against specific sequences unique to the pro or mature regions of the proteins and they have been previously characterized. Antiserum to the mature region of TGF-β1 is termed anti-TGF-$\beta1_{369-381}$ [23]; antiserum to the mature region of TGF-β2 is termed anti-TGF-β2 $(414)_{367-379}$ [31,35]. Antiserum to the pro-region of TGF-β1 is termed anti-TGF-$\beta1_{81-94}$ [23]; antiserum to the pro-region of TGF-β2 is termed anti-TGF-$\beta2(414)_{51-66}$ [31].

### Chemical Crosslinking

Proteins were chemically crosslinked in PBS containing 0.01% BSA to reduce non-specific crosslinking. Samples to be crosslinked were incubated with 250 μM disuccinimidyl suberate (DSS) at 24°C for 30 min. The reaction was stopped by adding an equal volume of electrophoresis sample buffer and heating at 95°C for 5 min. Samples were analyzed by SDS-PAGE under non-reducing conditions and immunoblotting with the appropriate antibodies.

### Iodination of Proteins

The high-molecular-weight TGF-β2 pro-region-containing complex, obtained after chromatography on the Phenyl RP+ column, was labeled with [$^{125}$I]Na to a specific activity of 100–150 μCi/μg by the chloramine T method [36]. The iodinated protein was purified on a PD-10 column (Pharmacia) equilibrated with 4 mM HCl, 75 mM NaCl, and 0.1% BSA.

### Binding to Mannose-6-Phosphate Receptor (M-6-PR)

[$^{125}$I]-labeled TGF-β2 pro-region (Fig. 5C) was used in a solid-phase assay to measure binding to the M-6-PR/IGF-II receptor as described [32,37,38]. Binding studies employing the M-6-PR as a primary antibody in a Western blot were performed as described [37].

### Protein Sequencing

Proteins were recovered from SDS-polyacrylamide gels by electroblotting onto Immobilon membrane (Millipore Corp., Bedford, MA) by using a Mini-Transblot Electrophoretic Transfer Cell (BioRad Laboratories, Richmond, CA), as described [39]. The membrane was stained with Coomassie brilliant blue and destained, and the stained monomeric pro-region band ($M_r$ = 39,000) was excised with a razor blade for subsequent amino-terminal sequence analysis.

Samples were sequenced in a pulsed liquid protein sequencer (model 475A, Applied Biosystems, Inc.) with an on-line model 120A PTH analyzer. Data reduction and quantitation were performed by using a Nelson 760 interface, a Hewlett Packard 9816 computer, and model 900A/model 475A chromatogram analysis software.

## RESULTS

### Chromatographic Analysis of LrTGF-β2

Figure 1 shows the major TGF-β2-related proteins secreted by β2(414)cl.35 cells as detected by immunoblotting using a mixture of site-specific anti-peptide antibodies directed against the mature- and pro-region of TGF-β2(414). When immunoblotting is performed after proteins are fractionated by SDS-PAGE under reducing conditions, these cells are found to secrete a 30–42 kDa species (band b in Fig. 1C) containing pro-region sequences, a 12 kDa band corresponding to the TGF-β2 monomer (band c in Fig. 1C), and a small amount of uncleaved pro-TGF-β2 (band a, Fig. 1C). A line diagram of these proteins is shown in Figure 1A; they have been previously characterized and shown to represent a major portion of the total proteins secreted by β2(414)cl.35 cells [31].

Analysis of β2(414)cl.35-cell-secreted proteins by immunoblotting after fractionation by SDS-PAGE under non-reducing conditions (Fig. 1B) reveals major proteins of 85 kDa and 105 kDa as well as the mature TGF-β2 dimer (arrow, Fig. 1B). The 85 kDa protein contains only pro-region sequences while the 105 kDa protein contains both mature- and pro-region sequences [31]. Variable amounts of higher-molecular-weight (130 kDa) proteins, containing mature- and pro-region sequences, can also be seen [31] (Fig. 2D).

Serum- and cell-free medium conditioned by β2(414)cl.35 cells was size fractionated under neutral conditions and TGF-β2 purification was monitored by immunoblotting and bioactivity after acidification. LrTGF-β2 chromatographed as a 130 kDa complex as shown in Figure 2C. Immunoblotting indicated that most of the TGF-β2 was associated with a high-molecular-weight pro-region-containing complex (Fig. 2D). Acidification of LrTGF-β2 was required to detect optimal bioactivity (Table I). Similar results were obtained with LrTGF-β1 as shown in Figure 2A,B. These data are consistent with previous results reported for LrTGF-β1 [24,26].

When the LrTGF-β2 complex was treated with acid and size fractionated under acidic conditions, peak bioactivity was now detected in the 14 kDa region of the column (Fig. 3C). Immuno-

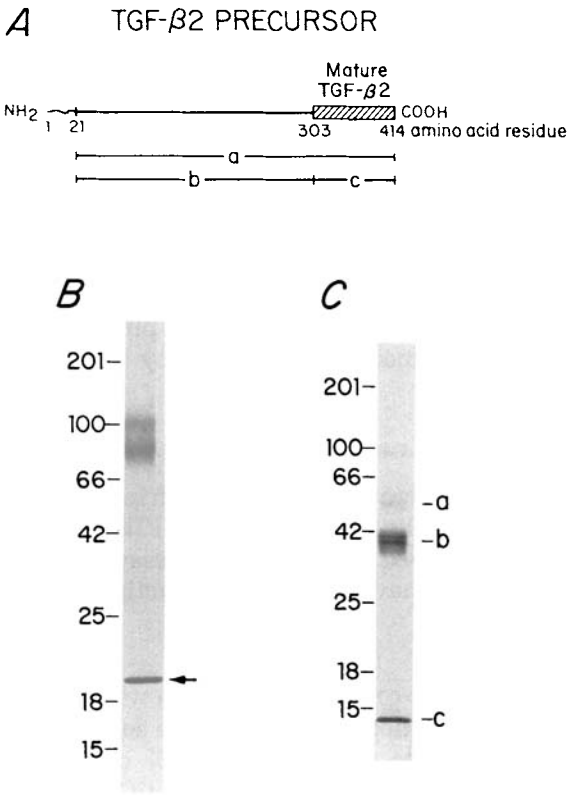


**Fig. 1.** **A:** A line diagram of the TGF-β2(414) precursor. Line a represents pro-TGF-β2; line b represents the pro-region of the TGF-β2 precursor; and line c represents the TGF-β2 manomer. These lowercase designations will be used throughout the text. The TGF-β2(442) precursor is identical to the TGF-β(414) precursor with the exception of a 28-amino-acid insertion within the pro-region of TGF-β(414) at position 116 [20,22]. **B:** Serum- and cell-free medium conditioned by β2(414)cl.35 cells was analyzed by immunoblotting with a mixture of anti-TGF-$\beta2(414)_{367-379}$ and anti-TGF-$\beta2(414)_{51-66}$ after SDS-PAGE under non-reducing conditions. **C:** Same as in B except SDS-PAGE was performed under reducing conditions. The arrow in B points to mature TGF-β2. Numbers on the left indicate the position of migration of molecular weight standards in kilodaltons.

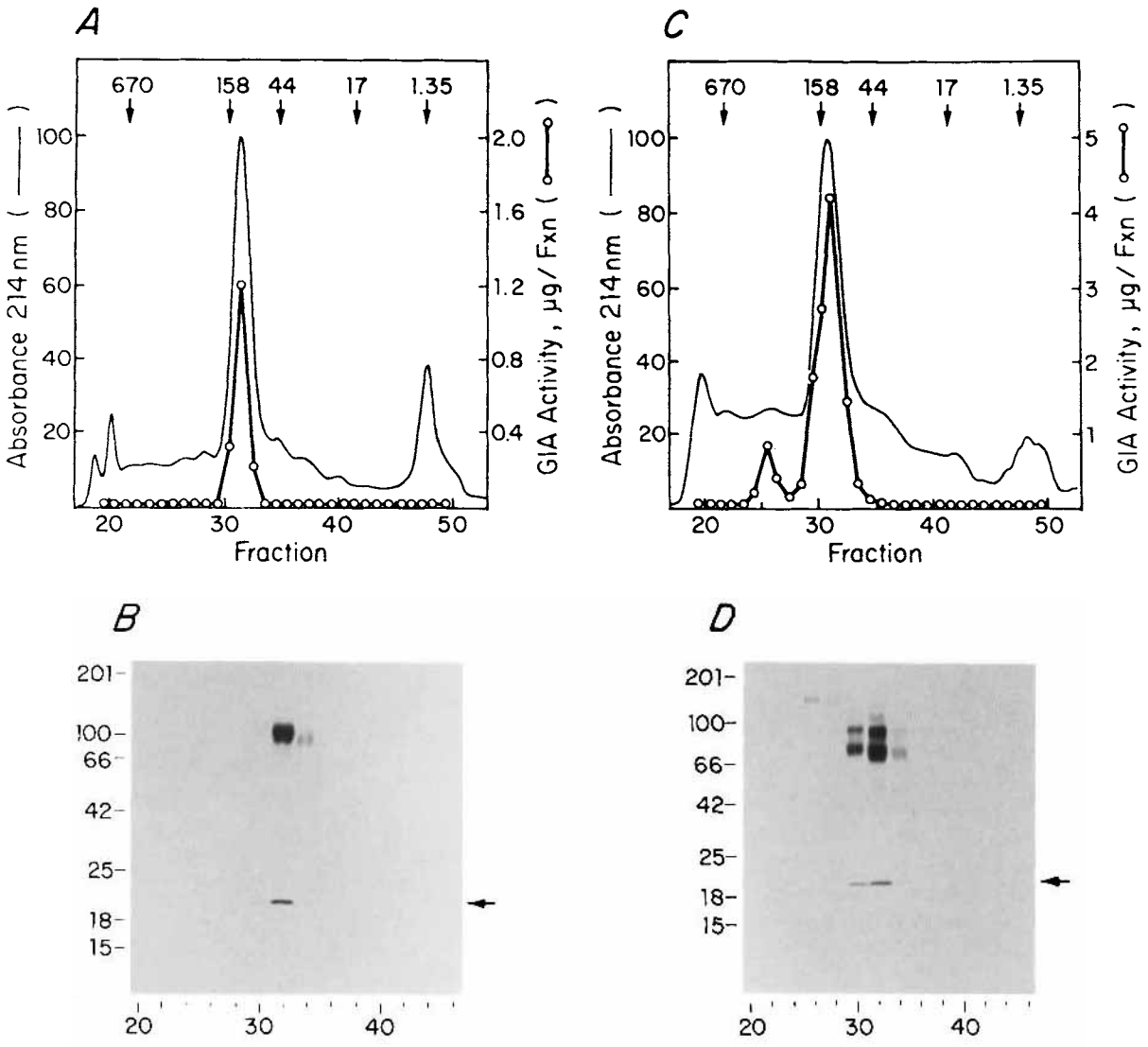


**Fig. 2.** Chromatograph analysis of LrTGF-β2 under neutral conditions. Serum-free medium, conditioned by β1cl.17 cells **(A,B)** or β2(414)cl.35 cells **(C,D)**, was fractionated on a TSK-250 gel filtration column under neutral conditions. The chromatographic runs were monitored at 214 nm (——) and aliquots of the fractions were tested for their ability to inhibit the growth of CCL64 cells (O—O, A,C) after acidification. Fractions were also subjected to Western blot analysis (B,D) using a mixture of antibodies directed against the pro- and mature-regions of the polypeptides. A and B represent TGF-β1, and C and D represent TGF-β2. Note that the mature TGF-βs (24 kDa indicated with an arrow) elute in the same fractions as the pro-regions at about 130 kDa. Numbers across the top of A and C indicate the position of elution of molecular weight standards in kilodaltons. Numbers on the left of panels B and D indicate the position of migration of molecular weight standards in kilodaltons.

blotting of the column fractions revealed that the 24 kDa dimer (fraction 18, Fig. 3D) was now well separated from the high-molecular-weight pro-region complex (fraction 12, Fig. 3D). The major components of this complex were the 85 kDa and 105 kDa proteins. Similar results were obtained with LrTGF-β1 (Fig. 3A,B). All the detectable bioactivity was associated with the 24 kDa dimer. The lower apparent molecular weight of TGF-β2 (ca. 14,000) seen by gel permeation chromatography may be due to non-specific adsorption or to the tightly folded structure of the dimeric growth factor and has been noted previously [8,26].

### Crosslinking of TGF-β2

The chromatographic analysis of LrTGF-β2 suggests that mature TGF-β2 is non-covalently associated with a high-molecular-weight pro-region-containing complex. To further character-

**TABLE I. Acid Activation of LrTGF-β2**

| | Neutral (U/μl)[a] | Acid (U/μl)[a] | Activation (fold) |
|---|---|---|---|
| TGF-β1 | 28 | 1,250 | 45 |
| TGF-β2 | 57 | 3,750 | 65 |

[a]One unit is defined as the amount of TGF-β causing inhibition of IUDR (iododeoxyuridine) uptake of CCL-64 cells by 50% of control. The same samples after gel filtration (Fig. 2) were assayed for growth-inhibitory activity before (neutral conditions) and after (acid conditions) acidification. It is evident that acidification of the sample is needed for maximal activity, providing a 45- and 65-fold increase of activation for TGF-β1 and TGF-β2, respectively.

ize this complex, crosslinking experiments were performed on peak fractions from the TSK column shown in Figure 2C. LrTGF-β2 (fraction 32, Fig. 2C) was fractionated by SDS-PAGE under non-reducing conditions and stained with Coomassie blue (Fig. 4A); note that the 105 kDa, 85 kDa, and 24 kDa mature dimer (arrow in Fig. 4A) comprise the major proteins in this sample. These proteins were crosslinked with DSS, fractionated by SDS-PAGE under non-reducing conditions, and analyzed by immunoblotting using anti-peptide antibodies directed against either mature sequences (Fig. 4B, lanes 1,2), pro-region-containing sequences (Fig. 4B, lanes 3,4), and a mixture of the two antisera (Fig. 4B, lanes 5,6). The amount of mature 24 kDa TGF-β2 is greatly reduced after crosslinking and appears as 105–130 kDa species (Fig. 4B, lanes 2,4,6). Similar results were obtained with LrTGF-β1 (data not shown). Crosslinking caused a shift in the molecular weight of the 85 kDa protein to 105 kDa, suggesting it had been crosslinked to mature TGF-β2 (Fig. 4B, lanes 4,6).

### Identification of Signal Peptide Cleavage

The TGF-β2 high-molecular-weight pro-region-containing complex was purified from β2(414)cl.35 conditioned medium by gel filtration and, after acidification, by reverse-phase chromatography. Figure 5 shows the analysis of this material by SDS-PAGE under reducing and non-reducing conditions (lanes 1,2, respectively) followed by Coomassie blue staining (Fig. 5A) or by immunoblotting with anti-TGF-β2$(414)_{51-66}$ (Fig. 5B). Note that the 105 kDa material resolves into a closely spaced doublet upon reverse-phase chromatography, possibly due to purification away from minor glycosylated forms. Autoradiography of $^{125}$I-labeled material is shown in Figure 5C. The proteins shown in Figure 5A lane 1 were transferred to Immobilon and the TGF-β2 pro-region (band b) was sequenced as described in Materials and Methods. The results are shown in Table II and indicate that signal peptide cleavage took place between serine (residue 20) and leucine (residue 21) in pre-pro-TGF-β2(414).

### Binding to the M-6-PR

Previous reports had indicated that the rTGF-β1 precursor contained M-6-P at two glycosylation sites located within the pro-region, and that this protein could specifically bind to the M-6-PR [32,37]. Since TGF-β2(414) also contains M-6-P residues in the pro-region [31], the purified high-molecular-weight pro-region-containing complex was iodinated with [$^{125}$I] (Fig. 5C) and tested for binding to this receptor. Figure 6 shows that this complex was capable of binding to the M-6-PR when the receptor was immobilized on microtiter wells (Fig. 6C,D). This binding was specific since it was inhibited in a dose-dependent manner by unlabeled complex ($IC_{50}$ = 3 mM) and M-6-P ($IC_{50}$ = 0.5 mM).

To confirm that the M-6-P receptor was binding to the TGF-β2 pro-region, the complex was fractionated by SDS-PAGE under reducing or non-reducing conditions, transferred to nitrocellulose, and tested for its ability to bind the M-6-P receptor. Bands of the appropriate molecular weight were detected (85 kDa and 105 kDa, Fig. 6A; 50 kDa and 38 kDa, Fig. 6B).

### Inhibition of Proteolytic Cleavage

Binding to the M-6-PR is thought to be involved in the transport of proteins from the Golgi to acidic vesicles where proteolytic cleavage can occur [for review, see 40]. Processing of rTGF-β1 has been shown to be inhibited by acid protease inhibitors such as ammonium chloride, methylamine, chloroquin, and monensin [41,42], agents which inhibit acid proteases. Figure 7 shows that cleavage of the 112-amino-acid TGF-β2 monomer from the TGF-β2 precursor was inhibited by both chloroquin and monensin; cleavage was more resistant to ammonium chloride treatment (Fig. 7, lane 4) and methylamine (data not shown).

## DISCUSSION

Analysis of conditioned media from β2-(414)cl.35 cells indicated that rTGF-β2 is se-

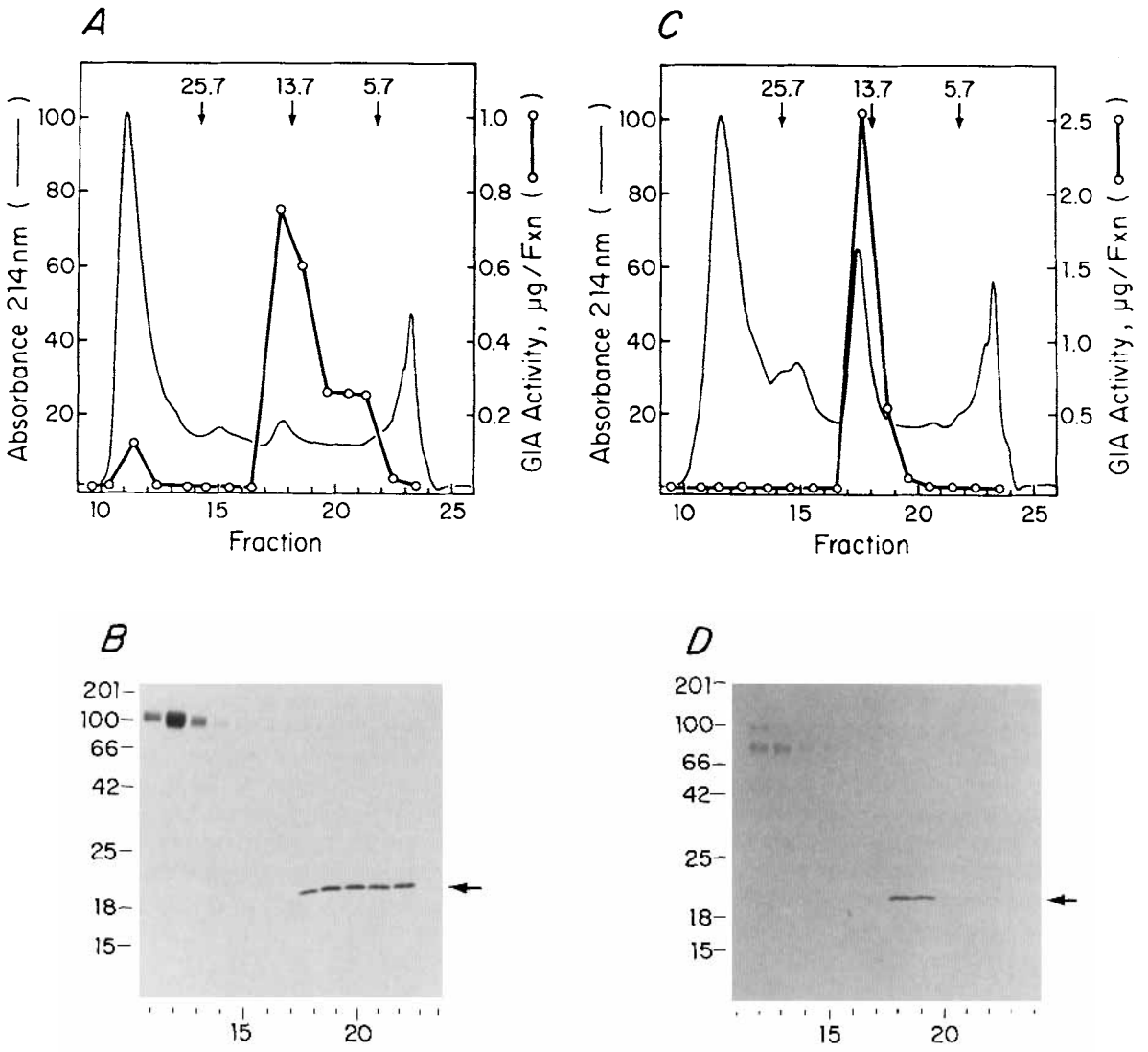


**Fig. 3.** Chromatographic analysis of LrTGF-β2 under acidic conditions. Selected fractions of the neutral TSK-250 column (Fig. 2) containing the LrTGF-β (130 kDa) were acidified and rechromatographed on the same column under acid conditions. The chromatography was monitored at 214 nm (——) and aliquots of the fractions were assayed for growth inhibitory activity on CCL64 cells (○—○) **(A,C).** Fractions were also subjected to Western blot analysis **(B,D)** using a mixture of mature- and pro-region-specific antibodies as in Figure 2B,D. A and B represent TGF-β1 and C and D represent TGF-β2. Note that under acid conditions, the pro-region-containing high-molecular-weight complex (fractions 12 and 13) is separated from the mature TGF-βs, designated by an arrow. Numbers on the left of B and D indicate the position of migration of molecular-weight standards in kilodaltons. Numbers across the top of A and C indicate the position of elution of molecular weight standards in kilodaltons.

creted as part of a large 130 kDa complex consisting of mature TGF-β2, an 85 kDa protein composed of pro-region dimers, and a 105 kDa protein containing mature- and pro-region sequences [31] (Figs. 1B, 2C,D). The LrTGF-β2 complex required acidification to detect bioactivity (Table I). Acidification of LrTGF-β2 releases the mature 24 kDa homodimer from a high-molecular-weight complex consisting mainly of the 85 kDa and 105 kDa proteins (Fig. 3C,D). Similar results are seen with the LrTGF-β1 complex (Figs. 2A,B and 3A,B) [23,24,26]. These data present the first clear demonstration of a non-covalent association between mature rTGF-β2 and a high-molecular-weight pro-region-containing complex. We propose that latency results from the formation of non-covalent bonds between rTGF-β2 and pro-region-containing proteins, and that disruption of these bonds (for example, by acidification) will lead to

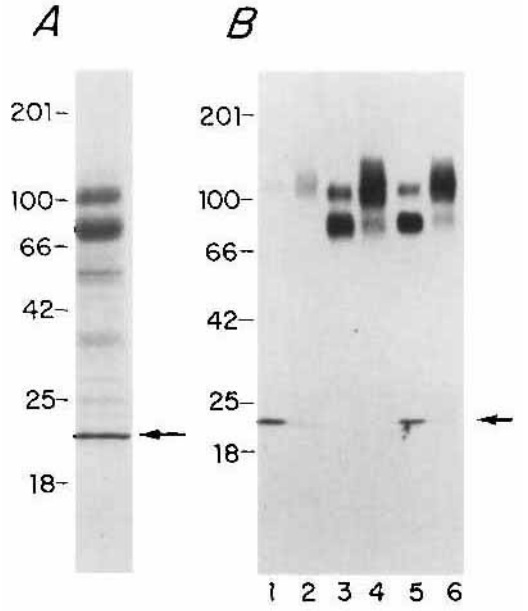


Fig. 4. Crosslinking of LrTGF-β2. Selected fractions of the neutral TSK-250 column were analyzed on a 7.5–17.5 gradient gel under non-reducing conditions and stained with Coomassie brilliant blue (**A**). The same samples were analyzed by immunoblotting before (**B**, odd-numbered lanes) and after (B, even-numbered lanes) crosslinking with DSS. **Lanes 1, 2** were visualized by using only mature region antibodies; **lanes 3, 4** were visualized by using only pro-region antibodies; and **lanes 5, 6** were visualized by using a mixture of pro- and mature-region antibodies. Note that when the samples are crosslinked, there is a decrease in the amount of the 24 kDa mature peptide (arrow) which appears as a larger molecular-weight complex containing pro-region.

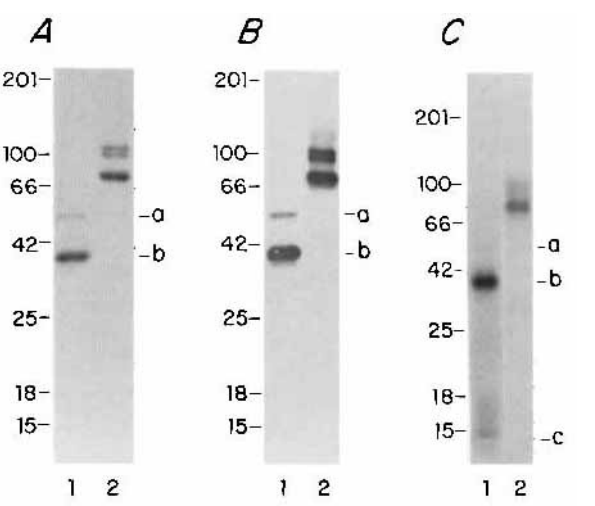


Fig. 5. Analysis of purified TGF-β2 high-molecular-weight pro-region-containing complex. LrTGF-β2 (fractions 30–32; Fig. 2D) were acidified and the TGF-β2 high-molecular-weight pro-region-containing complex was purified by reverse-phase chromatography as described in Materials and Methods. **A:** The purified protein was analyzed by SDS-PAGE under reducing (**lane 1**) or non-reducing (**lane 2**) conditions. Protein was visualized by staining Coomassie blue. **B:** Specific pro-region proteins were visualized by immunoblotting with pro-region-specific antibodies. **C:** Autoradiogram of the TGF-β2 pro-region-containing high-molecular weight complex labeled with $^{125}$Iodine to be used for M-6-P receptor binding.

activation. In addition, dimerization of pro-region-containing proteins may be required for latency.

This proposal is supported by crosslinking experiments shown in Figure 4. Crosslinking of LrTGF-β2 with DSS resulted in almost complete transfer of the 24 kDa dimer to a complex migrating at 105 kDa which most likely represents chemical linkage between mature TGF-β2 and the 85 kDa pro-region dimer. Previous reports on the crosslinking of LrTGF-β1 resulted in a similar transfer of mature dimer to high-molecular-weight forms [26].

In studies by Brunner et al. [25], mutant cDNAs were constructed which encoded TGF-β1 precursor proteins that were unable to dimerize due to mutations in Cys residues located within the pro-region. Transfection of these mutant DNAs into COS cells resulted in the secretion of TGF-β1, a significant portion of which was active without prior acidification, suggesting that dimerization of the pro-region-containing molecules was, in part, required to confer latency. Experiments aimed at identifying those Cys residues involved in interchain disulfide bonding within the pro-region of TGF-β2 precursor are in progress.

The TGF-β2 pro-region contains M-6-P [31] and is able to bind to the M-6-PR (Fig. 6). Cleavage of TGF-β2 from its precursor was in-

**TABLE II. Amino Acid Sequence Data for rpro-TGF-β2**

| Position (residue) | Yield (pmol) |
|---|---|
| 21 (Leu) | 19.4 |
| 22 (Ser) | 9.3 |
| 23 (Thr) | 15.4 |
| 24 (Cys) | ND[a] |
| 25 (Ser) | 6.7 |
| 26 (Thr) | 10.5 |
| 27 (Leu) | 9.3 |
| 28 (Asp) | 4.8 |
| 29 (Met) | 7.3 |
| 30 (Asp) | 4.5 |
| 31 (Gln) | 9.2 |
| 32 (Phe) | 9.9 |
| 33 (Met) | 4.9 |

[a]ND, not determined.

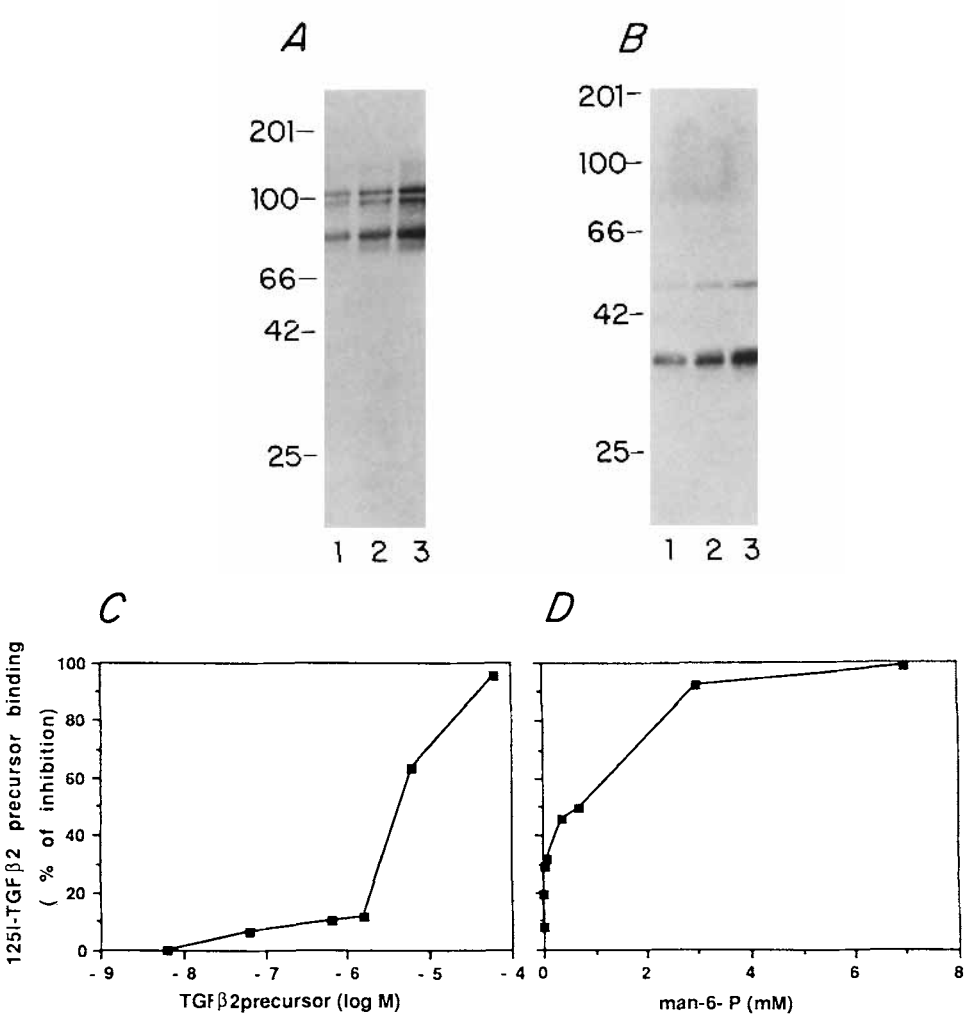


**Fig. 6.** Binding to M-6-PR. Purified TGF-β2 high-molecular-weight pro-region-containing complex was analyzed by SDS-PAGE under non-reducing (**A**) or reducing (**B**) conditions and transferred onto nitrocellulose, and purified M-6-P receptor was allowed to bind. Bound receptor was detected via the use of rabbit anti-receptor antibody and an alkaline-phosphatase-conjugated anti-rabbit immunoglobulin. The intensity of the bands increased with increasing amounts of complex (**lanes 1–3** contained 250, 500, and 1,000 ng, respectively) indicating specificity of binding. Iodinated complex was incubated with immunoimmobilized M-6-P receptor in the presence of either unlabeled complex (**C**) or M-6-P (**D**). Wells were washed, cut out, and counted. In the absence of competitor, 5,995 cpm were bound out of a total of 34,400 cpm added. Of these bound cpm, 1,645 cpm were bound to wells coated with the anti-receptor antibody in the absence of receptor (i.e., non-specific binding). Thus 4,350 cpm were specifically bound by receptor.

hibited by chloroquin and monensin, but not by methylamine and ammonium chloride (Fig. 7). Cleavage of natural TGF-β2 in BSC-40 cells (a monkey kidney cell line) also showed the same differential inhibition (Lioubin et al., manuscript in preparation) suggesting that these effects are not particular to CHO cells. All four of the above reagents were able to inhibit proteolytic processing of the TGF-β1 precursor [41,42]. Binding to the M-6-PR and subsequent transport to acidic vesicles for proteolytic cleavage may be part of the pathway involved in the processing of the TGF-β precursors. The observed differences in protease susceptibility suggest that different proteases may be involved in cleavage of mature TGF-β1 and TGF-β2 from their respective precursors; regulation of these proteases could give cells another level of control over when and where these molecules are activated.

Chloroquin, monensin, ammonium chloride, and methylamine are thought to act by raising the pH of acidic vesicles (for example, lysosomes) and thereby inhibit the action of acid proteases.

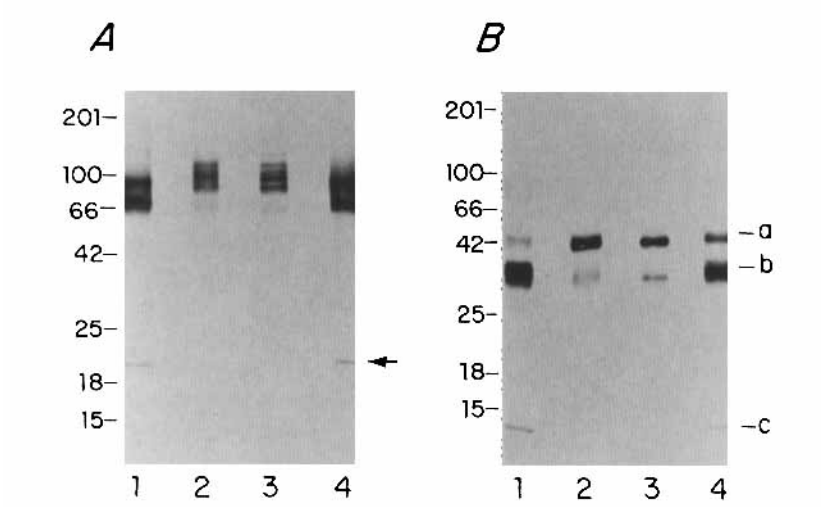


**Fig. 7.** Inhibition of proteolytic cleavage. β2(414)cl.35 cells were treated for 4 h with chloroquin **(lane 2)**, monensin **(lane 3)**, or ammonium chloride **(lane 4)**, and serum- and cell-free conditioned media were analyzed by immunoblotting under non-reducing **(A)** or reducing conditions **(B)** by using a mixture of anti-TGF-β2(414)$_{367-379}$ and antiTGF-β2(414)$_{51-66}$. **Lane 1** contains conditioned medium from untreated cells. Note that cleavage of the mature peptide (indicated by an arrow in A) is prevented by treatment with chloroquin and monensin (lanes 2,3). **B** shows the increased amount of uncleaved band a v. band b as a result of inhibition of proteolysis.

If this were the only mechanism of action, one would expect that ammonium chloride and methylamine would inhibit TGF-β2 processing. Differential effects between chloroquin, monensin, and methylamine with respect to overcoming multiple drug resistance (MDR) in tumor cells have been reported. Chloroquin and monensin can overcome MDR [43,44] whereas methylamine cannot [45]. Therefore, one should consider the possibility that these compounds may exert their effects at other intracellular targets involved in protein processing (for example, the Golgi). Experiments are in progress to purify the TGF-β1 and TGF-β2 precursors which should serve as in vitro substrates for their respective proteases and aid in the identification of these enzymes.

Natural TGF-β1 and -β2 are secreted by most cell lines as latent complexes [46–48]. Latent TGF-β1 has been purified from platelets as part of a 210 kDa complex consisting of pro-region-containing proteins and a 125–160 kDa binding protein [29,30]. This binding protein has not been found in LrTGF-β2 (this report) or LrTGF-β1 [24,26]. Preliminary data from our laboratory indicate that natural latent TGF-β2 secreted from BSC-1 cells and LrTGF-β2 secreted from CHO cells have similar structures and are structurally different from platelet-derived latent TGF-β1. Since disruption of the non-covalent bond between mature TGF-β and the high-molecular-weight complexes results in activation, the interaction of mature TGF-β1 or -β2 with these pro-region-containing proteins and other binding proteins may confer latency. Therefore multiple mechanisms of activation of structurally different latent TGF-β complexes can be thought of; this would give cells different levels of control over when and where TGF-βs become active.

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