The Pro Domain of Pre-Pro-Transforming Growth Factor β1 When Independently Expressed Is a Functional Binding Protein for the Mature Growth Factor†

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ABSTRACT: Transforming growth factor β1 (TGF-β1) is proteolytically derived from the carboxyl terminus of a 390 amino acid precursor molecule termed pre-pro-TGF-β1. Prevous studies have suggested that the pro piece of pre-pro-TGF-β1 may play an important role in the formation of an inactive, latent complex. These latent forms are thought to be important in the regulation of TGF-β1 activity. To understand this latent complex in more detail, we have expressed the pro domain of pre-pro-TGF-β1 in tissue culture cells independent of the mature growth factor. A stop codon was genetically engineered into the cDNA of pre-pro-TGF-β1 by changing the Arg-278 codon from *CGA* to the STOP codon *TGA*. The resulting protein is truncated just prior to the amino-terminal Ala residue of the mature growth factor. Transient expression studies and immunoblotting indicate that this pro piece is readily made and secreted by the COS-1 cells; the major form of the expressed pro piece, when analyzed by SDS–polyacrylamide gel electrophoresis, behaves as a disulfide-linked dimer (M_r 80000). Bioassays, using mink lung indicator cells, reveal that the pro domain forms an inactive complex with exogenously added mature TGF-β1. Treatment of this complex with heat or acid results in the release of active TGF-β1, indicating an in vitro structure similar to natural, latent TGF-β1 complexes. The pro piece from TGF-β1 was also found to form latent structures with two closely related family members, TGF-β1.2 and TGF-β2. Cross-linking studies using radioiodinated TGF-β1 suggest a one to one association between the dimer of the pro domain and mature TGF-β1.

Transforming growth factor β (TGF-β)[1] is a family of small polypeptide factors with potent activities which regulate cell growth and differentiation (Sporn et al., 1986; Keski-Oja et al., 1987; Roberts & Sporn, 1988). Several distinct but related TGF-β family members have been cloned and analyzed by cDNA sequencing (Derynck et al., 1985, 1988; de-Martin et al., 1987; ten-Dijke et al., 1988; Hanks et al., 1988; Jakowlew et al., 1988a,b; Madisen et al., 1988). In general, these modulatory factors are rich in conserved cysteines and appear to be dimeric, a form which is apparently necessary for biological activity. Most of our current knowledge about these polypeptide factors, however, stems from work with TGF-β1, the first member of this family discovered (Sporn et al., 1986; Keski-Oja et al., 1987; Roberts & Sporn, 1988). Consequently, its biological properties and structure have been intensely studied.

The molecular structure and processing of TGF-β1 have been deduced from sequence analysis of cDNA clones from several species (Derynck et al., 1985, 1986; Sharples et al., 1987; van Obberghen-Schilling et al., 1987) and from the high-level expression of these proteins in tissue culture cells (Gentry et al., 1987, 1988, 1989). These studies have indicated that this growth factor molecule is synthesized as a larger precursor (pre-pro-TGF-β1) containing a hydrophobic signal sequence, three N-linked glycosylational sites, and a multibasic proteolytic cleavage site. The first step in processing appears to involve signal peptide cleavage at the Gly-29–Leu-30 peptide bond followed by glycosylation at the three predicted N-linked sites within the pro domain (Gentry et al., 1988, 1989; Purchio et al., 1988). The glycosylated protein is sequentially processed during transit through the Golgi to yield complex-type sialated oligosaccharides (Brunner et al., 1988; Gentry et al., 1989; Sha et al., 1989). In addition, the carbohydrate side chains are phosphorylated at mannose residues to produce mannose 6-phosphate, a lysosomal targeting sugar (Purchio et al., 1988).

Recently, we have addressed several questions about the processing of TGF-β1 and also about the potential functional role of the pro region of this growth factor. Utilizing glycosylational inhibitors which affect different stages of carbohydrate remodeling, we were able to demonstrate that proper glycosylation is necessary for secretion of TGF-β1, implying one functional role for the pro region (Sha et al., 1989). Additionally, we showed that the TGF-β1 precursor is cleaved at the multibasic site to release the mature growth factor by a protease having an acidic pH optimum (Sha et al., 1989).

Besides a role in secretion, the pro region, due to its large size, serves other functions as well. One striking feature of TGF-β1 isolated from tissues or tissue culture cells is its existence in a biologically, inactive form, a form which can be activated by chaotropic agents or proteases (Pircher et al., 1984; Lawrence et al., 1985; Lyons et al., 1988). One postulated function for this latent form is a role in regulating the potent, biological activity of the TGF-β1 molecule. Early studies on the amplified expression of TGF-β1 in CHO cells revealed the release of latent TGF-β1 even though no other genes were cotransfected (Gentry et al., 1987). This immediately suggested that the pro piece of TGF-β1 may be involved in this latent form and possibly interact directly with the mature growth factor. Later, several studies indicated that the pro piece was found associated with the latent complex isolated from platelets (Miyazono et al., 1988; Wakefield et

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[1] Abbreviations: SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor β; CDM8, transient expression vector with no insert; CDM8-TGF-β1, expression vector containing the complete coding region of the precursor for TGF-β1; Stop278, expression vector containing the mutant TGF-β1 cDNA which has a stop codon at amino acid position 278; nTGF-β1, natural TGF-β1; rTGF-β1, recombinant TGF-β1.

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al., 1988) and with a partially purified latent form isolated from recombinant TGF-β1 expressing cell lines (Wakefield et al., 1989). Although these studies point to a functional role of the pro region in latency, no work has definitively indicated that the pro domain is the functional binding protein for the mature factor.

In this report, we have expressed the pro portion of TGF-β1 in tissue culture cells independent of the mature growth factor and have shown that the expressed pro piece binds specifically to mature TGF-β1 forming an inactive complex. This confirms the hypothesis that the pro domain serves as a functional binding protein to regulate the biological activity of TGF-β1. In addition, we have demonstrated that this in vitro complex behaves similarly to the latent forms of TGF-β1 isolated previously (Lawrence et al., 1985) whereby the mature factor may be released in an active form following treatment with acid or heat. Thus, through recombinant techniques, we have been able to artificially prepare latent complexes of TGF-β1. This should allow for comprehensive studies to evaluate activation mechanisms or to probe potential therapeutic uses of such latent forms.

Experimental Procedures

Cell Culture Conditions. COS-1 cells (ATCC CRL 1650), a simian fibroblast cell line transformed by an origin-defective mutant of SV40, and mink lung epithelial cells (ATCC CCL 64) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10% v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL). These cells were passaged by treatment with 0.5% (w/v) trypsin in normal saline containing 0.02% (w/v) ethylenediaminetetraacetic acid at a 1:5 splitting ratio.

DNA Manipulations, Site-Directed Mutagenesis, and Plasmid Constructions. Standard DNA manipulations were done as outlined previously (Maniatis et al., 1986). The *Pst*I–*Eco*RI fragment of the simain TGF-β1 cDNA (Sharples et al., 1987) was subcloned into M13mp19 for site-directed mutagenesis and to place a *Hin*dIII restriction site upstream of the *Pst*I site. Phosphorothioate-based site-directed mutagenesis was performed with a kit from Amersham, Inc., Arlington Heights, IL (Nakamaye & Eckstein, 1986; Sayers et al., 1988). The single-stranded M13 template containing the TGF-β1 cDNA was used along with the mutant, primer oligonucleotide 5′-TGTCCAGGGCTCAGCGGTGCC. This placed a stop codon at amino acid position 278 of the TGF-β1 protein. Following mutagenesis, single-lane dideoxy DNA sequence analysis was performed on isolated phage to identify mutants; greater than 50% of the isolated phage contained the desired mutation. Dideoxy sequencing was utilized to confirm the integrity of the mutant cDNA (Sanger & Coulson, 1975).

RF DNA from the M13 phage containing the TGF-β1 mutant was digested with *Bam*HI, and the overhang was filled in with Klenow polymerase. This blunt-end DNA was then digested with *Hin*dIII and the 1.0-kb fragment isolated from an agarose gel. The expression vector CDM8 (Seed, 1987) was cut with *Not*I, filled in with Klenow DNA polymerase, and then digested with *Hin*dIII. The TGF-β1 mutant [*Hin*dIII–*Bam*HI(blunt)] was then directionally ligated into the CDM8 vector. As a control, the TGF-β1 cDNA was also placed into the CDM8 expression vector. In this case, the cDNA in RF M13 was cut by *Eco*RI, filled in with Klenow, excised with *Hin*dIII, and ligated into *Hin*dIII,*Not*I(blunt) CDM8.

Transient Expression of CDM8 Constructs in COS-1 Cells. COS-1 cells were plated onto 15-cm round tissue culture dishes and allowed to reach a density between 30 and 60% confluent. The plasmids were transfected into the COS cells by a modified DEAE-dextran/chloroquine method (Seed & Aruffo, 1987). After transfection and further indubation of the cells for 48 h in complete medium, the medium was changed to serum-free DMEM and the supernatant collected 48 h later. The serum-free supernatant was concentrated 10-fold by ultrafiltration (YM10 membrane, 10000 molecular weight cutoff; Amicon Corp., Danvers, MA).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting. Proteins were fractionated by SDS–polyacrylamide gel electrophoresis on 7.5–20% gradient polyacrylamide gels (Laemmli, 1970). Following electrophoresis, proteins were transferred to nitrocellulose and stained fro immunoreactive TGF-β1 proteins as described previously (Gentry et al., 1987). The antiserum used was derived against residues 225–236 of the pro portion of the TGF-β1 protein.

Growth Inhibition Assays. Mv 1 Lu mink lung epithelial cells were utilized for growth inhibition assays essentially as described previously (Ikeda et al., 1987) using DNA synthesis as a measure of growth. Incorporation of the thymidine analogue 5′-[^{125}I]iododeoxyuridine into cellular DNA was assessed by a γ counter. The use of standard amounts of TGF-β1 indicates that 2–4 pM will inhibit the growth of these cells by 50% over the assay period. For assays measuring the effects of the pro region on biological activity, the pro region and TGF-β1 were combined and incubated for 2 h prior to addition to the cells.

Radioiodination of TGF-β1. TGF-β1 was purchased in a lyophilized, carrier-free form from R&D Systems, Minneapolis, MN. The growth factor was redissolved directly in 0.3 M potassium phosphate buffer, pH 7.2, and labeled with ^{125}I in the same vessel using Enzymobeads as described previously (Massague & Like, 1985). The radioiodinated protein was separated from free radioiodine by gel filtration on a column of Sephadex G-50 equilibrated in 0.5 M acetic acid/0.2% bovine serum albumin. The radioiodinated TGF-β1 was tested for biological activity and was shown to possess a specific activity of 31 μCi/μg of TGF-β1.

Cross-Linking of Radioiodinated TGF-β1 with Its Pro Region. Cross-linking experiments were performed in 0.4 M phosphate buffer, pH 7.2, containing medium expressing the pro region of TGF-β1 or control medium and ^{125}I-labeled TGF-β1. In a 20-μL reaction volume, 8 μL of concentrated expression medium and radioiodinated TGF-β1 (15000 dpm) were incubated for 2 h at 22 °C. For cross-linking, 5 μL of a fresh 5 mM solution of bis(sulfosuccinimidyl) suberate (Pierce Labs, Rockford, IL), dissolved in phosphate-buffered saline, was added and cross-linking allowed to proceed for 30 min. SDS–sample buffer (2×) without 2-mercaptoethanol was then added and the sample fractionated on SDS–polyacrylamide gels.

Results

Site-Directed Mutagenesis and Expression of the Pro Domain of TGF-β1. Due to spurious disulfide bond formation in the conditioned medium from cells expressing high levels of the full-length gene for TGF-β1 (Gentry et al., 1987, 1988, 1989), it was too difficult to isolate purified recombinant latent complexes for analysis. Consequently, we used site-directed mutagenesis followed by expression of the mutant protein in tissue culture cells. We altered residue 278 by changing the usual Arg-278 codon (CGA) to a STOP codon (TGA), resulting in a truncated form of the TGF-β1 gene product (Figure 1); the mutant was confirmed by dideoxy sequencing. Assuming proper signal peptide cleavage, the predicted protein would represent residues 30–277 of the TGF-β1 cDNA (the

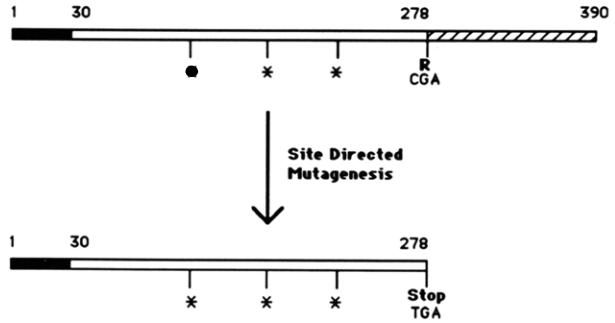

FIGURE 1: Scheme used to construct and express the TGF-β1 mutant (Stop278). The simian TGF-β1 cDNA (Sharples et al., 1987) was subcloned into M13mp19 and the single-stranded template used for site-directed mutagenesis as described under Experimental Procedures. The resulting mutant was subcloned into the expression plasmid CDM8 and used to transiently transfect COS-1 cells. For details, see Experimental Procedures.

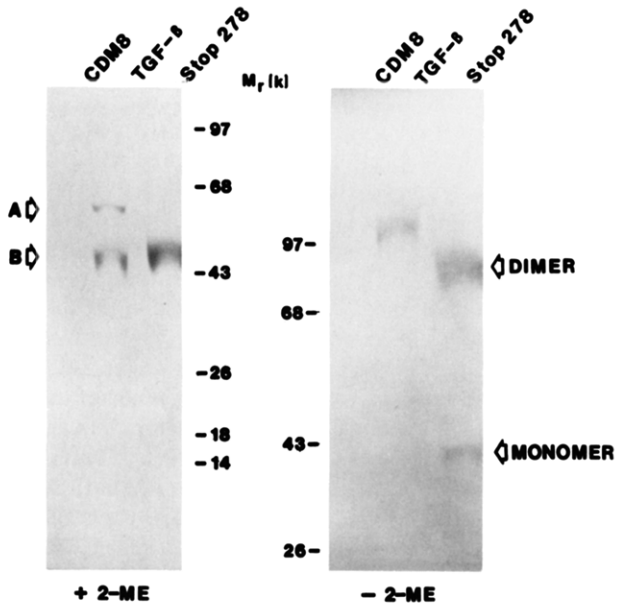

FIGURE 2: Immunoblot of culture supernatants from COS-1 cells expressing the pro piece of the TGF-β1 molecule (Stop278) and the complete TGF-β1 precursor. Supernatants from transfected cells were collected and processed as described in the text, and 5 μL was fractionated on 7.5–20% gradient SDS–polyacrylamide gels under reducing conditions (left panel) or on 7.5% SDS–polyacrylamide gels under nonreducing conditions (right panel). Proteins were stained for immunoreactive TGF-β1 by using antibodies specific for the pro portion of the molecule and techniques described previously (Gentry et al., 1987). Molecular weight standards are located in the center.

pro piece of TGF-β1 minus Arg-278) and would be expressed independent of the mature growth factor. Herein, this mutant will be referred to as Stop278.

For expression studies, the Stop278 TGF-β1 cDNA was placed into the expression vector CDM8 and transiently expressed in COS-1 cells. This vector contains the cytomegalovirus promoter sequences 5′ and simian virus 40 sequences 3′ which result in high-level expression and correct processing of transcribed cDNA sequences, respectively (Seed, 1987). As controls, the CDM8 vector itself without an insert and CDM8 containing a full TGF-β1 precursor cDNA (CDM8-TGF-β1) were utilized. After transient expression, cell-free tissue culture supernatants were collected and analyzed for expressed TGF-β1 proteins by immunoblotting using antibodies specific for the pro piece of TGF-β1 (Gentry et al., 1987).

Figure 2 shows the results which were obtained. Under reducing conditions (left panel), no immunoreactive material was observed in the culture supernatants from cells transfected

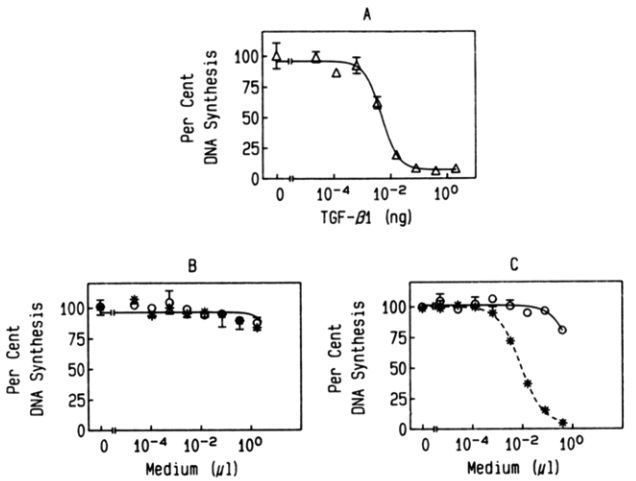

FIGURE 3: Biological assays indicate that the pro piece of TGF-β1 disrupts the growth inhibitory activity of mature TGF-β1. Culture supernatants were incubated in the absence or presence of TGF-β1 and assayed in triplicate for their ability to inhibit DNA synthesis of mink lung epithelial cells using 5′-[^{125}I]iododeoxyuridine to label newly synthesized DNA. (A) Assay using 800 pM TGF-β1 serially diluted 1:5 in complete medium to illustrate the sensitivity of the method. (B) Assay using 2 μL of control transfection medium [(*) CDM8] or 2 μL of medium from cells transfected with the pro piece of TGF-β1 [(○) Stop278] and serially diluted 1:5. (C) Same as (B) except that a final concentration of 800 pM TGF-β1 was added and incubated with the medium for 2 h prior to serial dilution and plating for the bioassay. Results are expressed as percent DNA synthesis based on controls receiving no TGF-β1. See Experimental Procedures for details.

with the CDM8 vector control (no insert present). On the other hand, culture medium from cells expressing the full-length coding region of TGF-β1 (CDM8-TGF-β1) contained two immunoreactive proteins. The sizes of these proteins were consistent with the sizes of the TGF-β1 precursors reported in our previous studies (Gentry et al., 1987, 1988, 1989); band A represents pro-TGF-β1 (residues 30–390), and band B corresponds to the cleaved pro portion (residues 30–278). In contrast, Stop278 transfectants produced only one protein band immunoreactive with these antibodies which comigrated with the pro portion detected in CDM8-TGF-β1. Under nonreducing conditions (right panel), a different pattern emerged; CDM8-TGF-β1 transfectants produced a large disulfide-linked complex of M_r 100 000–120 000 which had been observed previously (Gentry et al., 1987, 1988) where as Stop278 produced immunoreactive proteins at M_r ~80 000 and a smaller form of M_r ~40 000; by immunostaining, it appeared that the M_r 80 000 protein was the major form secreted by the mutant-transfected cells. Since under reducing conditions the Stop278 medium contained only one immunoreactive band, these proteins detected under nonreducing conditions probably represent the dimer and monomer forms of the pro domain, respectively.

Expressed Pro Domain Inactivates the Biological Activity of Mature TGF-β1. To demonstrate that the pro piece can regulate the bioactivity of TGF-β1, concentrated serum-free culture supernatants from CDM8 control or Stop278 transfectants were combined with TGF-β1, incubated for 2 h, and then analyzed by using a sensitive bioassay. The assay chosen was based on the potent growth inhibitory properties of mink lung epithelial cells following treatment with mature TGF-β1; growth inhibition was assessed by measuring DNA synthesis using the thymidine analogue 5′-[^{125}I]iododeoxyuridine (Ikeda et al., 1987).

The sensitivity of this microtiter plate assay and results are shown in Figure 3. Panel A demonstrates a typical dose

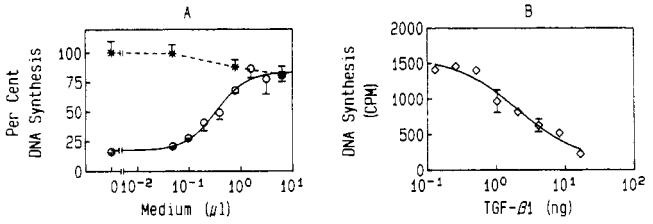

FIGURE 4: Dose response effect of medium from COS-1 cells expressing the pro piece of TGF-β1. (A) Medium collected and processed from cells expressing the pro piece (Stop278) was serially diluted in assay medium lacking TGF-β1. The diluted assay medium was used as is (*) or in combination with mature TGF-β1 [160 pM (○)], and triplicate bioassays were performed to measure DNA synthesis. (B) Medium from Stop278 (10 μL) was treated with different amounts of mature TGF-β1 and assayed, in triplicate, for inhibition of DNA synthesis.

response curve for mature TGF-β1 with half-maximal inhibition of DNA synthesis occurring following addition of 2–4 pM TGF-β1 (5–10 pg/well) (Ikeda et al., 1987; Gentry et al., 1988). Concentrated culture medium from CDM8 control and Stop278 transfectants had very little effect on cell growth except when using relatively large amounts of the medium (panel B and data not shown). If a large excess of TGF-β1 (~800 pM) was added to a small aliquot of the control medium and incubated prior to addition to the indicator cells (panel C), the medium, when serially diluted 1:5, showed a very similar dose response curve to the control shown in panel A, indicating no adverse effect on the added TGF-β1. However, when the same large excess of TGF-β1 (~800 pM) was incubated with the medium from the Stop278 transfectant and tested by bioassay (panel C), a dose response curve that was nearly identical with the control medium containing no TGF-β1 was obtained (compare to panel B). These results demonstrate that medium from the Stop278 transfectant was able to inactivate (i.e., regulate) the bioactivity of exogenously added TGF-β1.

A dose response effect of the inactivation of TGF-β1 bioactivity by Stop278 medium is shown in Figure 4. In panel A, a constant amount of TGF-β1 (160 pM) is titrated with the Stop278 medium to show a potent inactivation of TGF-β1 activity. Normally 160 pM TGF-β1 without any added Stop278 would completely inhibit (to background levels) the DNA synthesis of the mink lung indicator cells (panel A, 0 μL). However, in the presence of Stop278 medium, the TGF-β1 was inactive when incubated with relatively small amounts of the Stop278 medium prior to the assay. From the dose response curve, half-maximal inactivation of the added TGF-β1 was observed at approximately 0.5 μL of Stop278 medium; half-maximal inhibition of these cells by TGF-β1 occurs at 2–4 pM (5–10 pg/well) (Ikeda et al., 1987; Gentry et al., 1988), indicating that a large amount of TGF-β1 (156–158 pM) is regulated by the Stop278 medium. Panel B shows a similar biological dose response titration except that the Stop278 medium is held constant and the amount of mature TGF-β1 is varied. From these bioassays, it is clear that the Stop278 medium can potently regulate (inactivate) the biological activity of TGF-β1 whereas control medium was ineffective (data not shown; Figure 3, panel C).

Pro Domain Associates with Mature TGF-β1 To Form a Complex. The above results suggest that the pro piece associates with mature TGF-β1 to form an inactive complex. However, these results could be alternatively explained by high levels of protease activity within the Stop278 supernatant thereby degrading the active TGF-β1. To eliminate this alternative, TGF-β1 was radioiodinated using lactoperoxidase and glucose oxidase (Massague & Like, 1985). The radioiodinated growth factor was then incubated with Stop278 medium and the integrity of the TGF-β1 tested by nonreducing or reducing SDS–polyacrylamide gel electrophoresis. Incubation with Stop278 medium had no observable effect on the radioiodinated TGF-β1, indicating the absence of protease activity (data not shown and see below).

To confirm a direct interaction of TGF-β1 with its pro piece, the radioiodinated growth factor was incubated with Stop278 medium, and attempts were made at separating the free form with the form bound to its pro domain. The usual techniques for separating such forms (such as gel filtration, charcoal binding, nondenaturing polyacrylamide gel electrophoresis, etc.) were unsuccessful due in large part to the nonspecific absorption of TGF-β1 to many types of surfaces or to similar charges. To circumvent this problem, an approach using the water-soluble homobifunctional cross-linker bis(sulfosuccinimidyl) suberate was used to trap the growth factor bound to its pro piece.

Supernatants collected from control transfections or from Stop278 transfections were incubated with radioiodinated TGF-β1 for 2 h and subsequently treated with cross-linker. The cross-linked species were then resolved by nonreducing SDS–polyacrylamide gel electrophoresis (Figure 5A). As part of the controls, samples were included which did not receive any cross-linker. In these lanes, the radioiodinated TGF-β1 appeared intact and, by comparison with other gels, was at a concentration similar to the starting amount regardless of which medium was used for the incubation. In the CDM8 supernatants, the radioiodinated TGF-β1 was absent from the resolving gel following the addition of cross-linker. A small portion of the radioactivity was present at the top of the resolving gel whereas the remainder was present in the stacking gel (data not shown). The exact reason for the disappearance of TGF-β1 following cross-linking is not known, but may be due to formation of cross-linked TGF-β1 aggregates. Cross-linking of the radioiodinated species after incubation with Stop278 medium produced a major radioactive species ranging from M_r ~100 000 to M_r 110 000; quantitation indicated that this represented 45% of the input TGF-β1 radioactivity. Although SDS–polyacrylamide gels are sometimes unreliable for molecular mass determinations, the migration of this species appears to correspond to one mature TGF-β1 dimer (M_r ~25 000) cross-linked to the dimer of the pro domain (M_r ~80 000). No smaller cross-linked protein species were observed on the SDS–polyacrylamide gels, even though the Stop278 medium contained the monomeric form of the pro piece (see Figure 2). These results indicate that the dimer of the pro piece is the active form for binding mature TGF-β1 and suggest a one to one association of TGF-β1 to its pro domain.

Figure 5B shows that the cross-linking of radioiodinated TGF-β1 to its pro domain is competitively displaced by the addition of cold TGF-β1 to the incubation mixture. Greater than 10 ng of TGF-β1 was needed to prevent the radioiodinated TGF-β1 from binding to the pro domain in the Stop278 medium. This result is consistent with the high capacity of this medium to inactivate TGF-β1 in the bioassays discussed earlier. Also included in Figure 5 is a control where the Stop278 medium was denatured at 95 °C for 5 min prior to the addition of radioiodinated TGF-β1. Following addition of cross-linker, no M_r ~100 000–110 000 species (TGF-β1 cross-linked to its pro piece) was observed. This provides additional support that the cross-linking of the pro piece with TGF-β1 is due to a specific interaction.

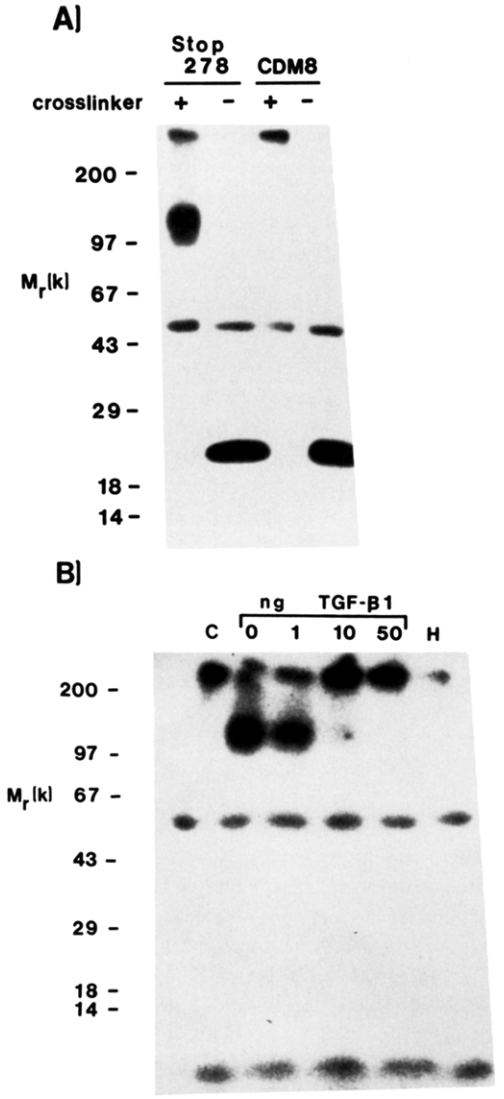

FIGURE 5: Mature TGF-β1 associates with the dimeric form of the pro piece to form a complex of $M_r \sim 100\,000$–$110\,000$. (A) Supernatants from control transfectants or from cells expressing the pro portion of TGF-β1 (Stop278) were incubated with ^{125}I-labeled, mature TGF-β1 and cross-linked with bis(sulfosuccinimidyl) suberate as described under Experimental Procedures. Cross-linked complexes were resolved on 7.5–20% gradient SDS–polyacrylamide gels and detected by autoradiography. (B) Unlabeled, mature TGF-β1 at the indicated amounts was added to the radioiodinated TGF-β1 and then incubated under similar conditions as described above. C represents control CDM8 medium. H shows results from Stop278 medium which received prior heating for 3 min at 95 °C before the addition of the ^{125}I-labeled, mature TGF-β1 and cross-linking. Complexes were separated on a 7.5–20% gradient SDS–polyacrylamide gel. Molecular weight markers are shown to the left of each panel.

In Vitro Complex between Mature TGF-β1 and Its Pro Piece Is Latent. The results from cross-linking shown above suggest that the association of mature TGF-β1 with is pro domain is also latent. Therefore, bioassays were setup to demonstrate that the in vitro complex can be activated by methods similar to the activation of naturally isolated latent complexes. Since the pro piece in the Stop278 medium may be in vast excess over the amount of mature TGF-β1, the half-maximal inhibitory point for the Stop278 medium (Figure 4A) was used for the assays. At this dilution of transfection medium, 156–158 pM TGF-β1 was complexed with the pro portion, and 2–4 pM was active. This dilution was used for both the control CDM8 medium and the Stop278 medium.

Following incubation of the transfectant medium with TGF-β1 for 2 h, the mixture was heated at 95 °C or transiently acidified (see Experimental Procedures) and then assayed for growth inhibition. The results of the bioassay are shown in Table I. Clearly, heat denatures the pro piece and completely destroys its ability to form this latent complex. Furthermore, heat treatment releases bioactive TGF-β1 from a preformed complex. The transient acidification results also showed a similar trend.

Table I: In Vitro Complex of TGF-β1 and Stop278 Is Latent

	DNA synthesis in CCL64 cells (cpm)[a]		
	control medium	CDM8 medium	Stop278 medium
no treatment, TGF-β1	176 ± 13	143 ± 8	894 ± 75
acid, then TGF-β1[b]	128 ± 3	118 ± 17	304 ± 47
TGF-β1, then acid[b]	129 ± 18	120 ± 13	377 ± 51
95 °C, then TGF-β1[c]	130 ● 7	128 ± 13	139 ● 13
TGF-β1, then 95 °C[c]	224 ● 31	149 ± 16	124 ● 11

[a] All assays included 400 pg of TGF-β1 and 0.8 μL of medium. Samples were incubated for 2 h prior to the assay. The data represent cpm of [^{125}I]iododeoxyuridine incorporated into the DNA of the mink lung indicator cells. Culture wells of mink lung cells without exogenously added TGF-β1 incorporated 1928 ± 65 cpm of [^{125}I]iododeoxyuridine into DNA. [b] Transient acidification was accomplished by treatment with 1 M HCl for 30 min at 22 °C followed by neutralization with 1 M NaOH. [c] Heated for 5 min.

Table II: Recombinant TGF-β1 Pro Region (Stop278) Interacts with Two Other TGF-β's

	DNA synthesis in CCL64 cells (cpm)[a]		
	control medium	CDM8 medium	Stop278 medium
TGF-β1	120 ± 11	121 ± 25	1681 ± 29
TGF-β2	183 ± 25	160 ± 13	854 ± 88
TGF-β1.2	150 ± 6	136 ± 5	1678 ● 80
no TGF-β	2115 ± 195	1875 ± 56	1933 ± 260

[a] All assays included 400 pg of the TGF-β's and 2 μL of the various media. The data represent cpm of [^{125}I]iododeoxyuridine incorporated into the DNA of the mink lung indicator cells.

Pro Domain Can Also Form Complexes with Mature TGF-β1.2 and TGF-β2. The TGF-β family of mature polypeptides are about 70% homologous to one another. Because of their close similarity in structure, two readily available members of this family, TGF-β1.2 and TGF-β2, were tested for their ability to interact with the pro piece of TGF-β1 (Table II). By use of a growth inhibition assay and mink lung indicator cells, the pro piece was shown to inactivate the biological activities of TGF-β1.2 and TGF-β2, suggesting a likely association. Close examination of these data indicates that TGF-β2 was not as efficiently regulated by the pro piece of TGF-β1. However, at the concentrations of TGF-βs used, greater than 95% of these growth factors are bound and inactivated by the pro domain. Thus, this difference in bioactivity observed for TGF-β2 is only slight in comparison to the sensitivity of the assay. This may suggest that TGF-β2 associates with the TGF-β1 pro piece with only a slightly lower affinity.

Discussion

Mature TGF-β1 is a biologically potent molecule exerting its effects on a wide variety of cells (Sporn et al., 1986; Keski-Oja et al., 1987; Roberts & Sporn, 1988). Since almost every cell type in the human body possesses receptors for this cellular modulator (Frolik et al., 1984; Tucker et al., 1984; Massague & Like, 1985; Ruff & Rizzino, 1986; Cheifetz et al., 1988; Segarini & Seyedin, 1988; Segarini et al., 1989), its activity must be efficiently controlled to prevent adverse effects on surrounding cells or tissues. In this report, we

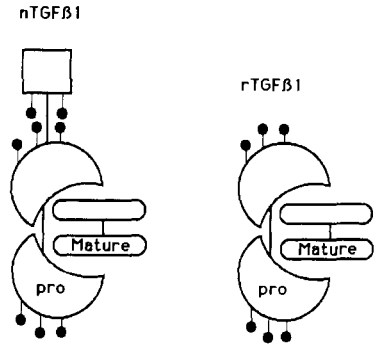

FIGURE 6: Proposed model for the structure of the latent complex of mature TGF-β1 with its pro portion from natural and recombinant sources. The dimer of the pro portion is responsible for binding the mature growth factor. Glycosylation groups are denoted by (●).

demonstrate that the regulatory protein for mature TGF-β1, the protein directly responsible for masking biological activity, is the dimer of its own pro region. Since the mature and pro portions are synthesized together, TGF-β1 is always released as an inactive complex awaiting subsequent activation mechanisms. Figure 6 illustrates the proposed model for the latent complex of recombinant and natural TGF-β1 (termed rTGF-β1 and nTGF-β1, respectively). From this and earlier reports, it is now clear that the latent complex is due to the binding of the mature growth factor to its own pro region (Gentry et al., 1987; Miyazono et al., 1988; Wakefield et al., 1989) and that the pro domain serves as the functional regulating protein. The difference between rTGF-β1 and nTGF-β1 is the existence of a third polypeptide cross-linked by disulfides to the pro region of nTGF-β1 (Miyazono et al., 1988; Wakefield et al., 1988). No such protein has been identified in recombinant TGF-β1 expressing cell lines (Wakefield et al., 1989) (unpublished observation). The function of this polypeptide is not known; however, since the COS-1 cell expressed pro domain is functional and binds to mature growth factor, it is not necessary for the formation of the latent complex. This polypeptide may serve other important roles such as directing the TGF-β1 latent complex to a site for specific activation.

The functional binding of the pro region of TGF-β1 to its mature growth factor is similar to the binding of neurophysin, the pro portion of pre-pro-oxytocin, to oxytocin. In this case, neurophysin shows a weak but specific association for oxytocin [for a comprehensive review of neurophysin function, see Walter (1975)]. The binding of mature TGF-β1 to its pro domain appears to represent a similar event; however, the affinity of this association is apparently greater than that of neurophysin/oxytocin (unpublished observations). Furthermore, neurophysin has not been demonstrated to play a role in regulation of oxytocin activity whereas the TGF-β1 latent complex, a 1:1 complex of mature TGF-β1 with its pro region dimer, appears important to its regulation. Other examples highlighting the functional importance of pro domains involve the proteolytic zymogens (Neurath, 1984). Here, the pro pieces play an indirect role in activation of enzyme activity. However, these pro domains are much smaller than that of TGF-β1 and, when proteolytically excised, allow for conformational changes associated with an active enzyme; they do not usually associate tightly with the enzyme following cleavage (Neurath, 1984).

On the basis of the cross-linking studies presented in this report, it is clear that the dimeric form of the pro region is the functional binding protein. These results are consistent with some recent work using site-directed mutagenesis (Brunner et al., 1989). In those studies, the cysteines of the pro region were sequentially changed into serines. When the TGF-β1 precursor lost its ability to form a dimer, the expressed, mature TGF-β1 was fully active, requiring no further activation. Here, we also demonstrate that monomeric forms of the pro region, present in our Stop278 expression medium (Figure 2), did not bind mature, radioiodinated TGF-β1 as judged by cross-linking studies followed by SDS–polyacrylamide gel electrophoresis (Figure 5).

A previous study has suggested that the pro domain, through its attached carbohydrate, is important for secretory exit of TGF-β1 (Sha et al., 1989). It is interesting to point out that the pro piece of TGF-β1 can be expressed and secreted independently of the mature growth factor; however, mature TGF-β1 is not released from transfected cells expressing deletion mutants of the TGF-β1 cDNA lacking small or large portions of the pro domain (Sha and Gentry, unpublished observations). The pro domain also does not appear to serve as a carrier for mature, biologically active TGF-β1 since the mature growth factor may be expressed and secreted without a functional binding pro region (Brunner et al., 1989). The above observations would tend to implicate the primary protein structure of the TGF-β1 pro domain as playing an important role for direct secretory transit.

TGF-β1 is one member of an increasingly growing family of polypeptide factors which show a high degree of homology within the mature TGF-β regions; however, their pro domains show limited homology (Derynck et al., 1985, 1988; de-Martin et al., 1987; ten-Dijke et al., 1988; Hanks et al., 1988; Jakowlew et al., 1988a,b; Madisen et al., 1988). We demonstrate in this report that two other TGF-β family members, TGF-β1.2 and TGF-β2, interact with the pro domain from TGF-β1, suggesting that other TGF-β members may also bind to this pro piece. The ability of TGF-β2 to interact with this region is consistent with expression work of TGF-β2 chimeras in which the pro region of TGF-β1 was fused to the mature TGF-β2 molecule (Madisen et al., 1989). In those studies, the mature TGF-β2 secreted by cells expressing the chimera was found as a latent complex. Other TGF-βs may also be released as latent forms provided that their pro regions, despite differences in primary structure, have similar functions and comparable binding sites to the TGF-β1 pro region.

The biological mechanism for activation of latent TGF-βs to release mature growth factor is not well understood. Earlier work has indicated that activation of TGF-β1 can occur by proteolysis (Lyons et al., 1988) or by removal of carbohydrate (Miyazono & Heldin, 1989). Acidic pH values such as those which surround necrotic tissue (Lawrence et al., 1985; Jullien et al., 1989) or which occur at the face of remodeling bone may also cause a release of active TGF-β1. Since this family of potent cellular modulators affects a wide variety of tissues and cells and since each TGF-β has a different structure for the latent complex, activation may represent a complex and highly regulated mechanism. The major differences in primary protein structure between the pro regions of the TGF-β family members may provide different regulatory modes for their specific activation. The ability to prepare latent complexes of TGF-β1 in vitro should provide material necessary to study the regulation of TGF-β1 in further detail.

ADDED IN PROOF

Recently, it was decided that the pro portion of each of the TGF-βs be called latency associated peptides (LAPs) to standardize the TGF-β literature. Thus, the TGF-β1 pro region described in this paper will be subsequently termed β1-LAP for clarity.

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