Identification and Analysis of Discrete Functional Domains in the Pro Region of Pre-Pro-Transforming Growth Factor Beta 1

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Abstract. A series of site-specific insertion and deletion mutants was prepared in the pro domain of transforming growth factor β1 (TGFβ1) encoded by simian TGFβ1 cDNA. These mutants were transiently expressed in COS-1 cells and the ability of each to be properly processed, folded correctly, and secreted was determined by immunoblot analysis of cells and culture supernatants. Insertions in regions corresponding to amino acid residues 50, 154, and 170 blocked secretion; culture supernatants from COS-1 cells showed no immunologically reactive proteins, whereas intact cells contained high levels of the mutant polypeptides. Insertions in the middle portion of the pro domain at residues 81, 85, and 144 affected disulfide maturation of the mature TGFβ1. An insertion at residue 110, on the other hand, appeared to destabilize the mature TGFβ1 polypeptide, resulting in degraded growth factor. Relatively small (10 amino acids) to large (125 amino acids) deletion mutations in the pro domain of TGFβ1, when expressed as the full-length pre-pro-TGFβ1, appeared to block secretion. By contrast, if the pro domain (designated β1-latency-associated peptide [β1-LAP]) was expressed independently, deletion mutants in the region 40–110 were readily secreted by the COS-1 cells, whereas deletions in residues 110–210 either destabilized the structure of the protein or blocked its intracellular transport. Cross-linking assays employing radioiodinated TGFβ1 and biological assays indicate that residues 50–85 of β1-LAP are required for association with mature TGFβ1.

Transforming growth factor-beta-1 (TGFβ1) is a potent regulator of cell growth and differentiation (Roberts and Sporn, 1988, 1990; Barnard et al., 1990). Following the initial purification and characterization of TGFβ1 as a homodimeric, 24-kD polypeptide (Assoian et al., 1983; Frolik et al., 1983; Roberts et al., 1983), several distinct but related TGFβ family members have been cloned and analyzed by cDNA sequencing (Derynck et al., 1985, 1988; deMartin et al., 1987; ten-Dijke et al., 1988; Hanks et al., 1988; Jakowlew et al., 1988a,b; Madisen et al., 1988), indicating that they share similar structural properties. These novel TGFβs are encoded as large precursors and each is processed from the carboxyl-terminus of its precursor by proteolytic cleavage. The mature TGFβs all show considerable sequence similarity (~80%) and contain nine cysteine residues which can be perfectly aligned in sequence. The pro domains of each precursor contain three aligned cysteine residues as well as several sites for N-linked glycosylation. Since the pro portions of each precursor show less sequence similarity (~50%), each form of the growth factor may be secreted and activated differently.

Studies on posttranslational modification and processing of the TGFβ1 precursor have been derived from sequence analysis of cDNA clones (Derynck et al., 1985, 1986; Sharples et al., 1987) and from the high-level expression of these proteins in CHO cells (Gentry et al., 1987, 1988, 1989); these studies have indicated that a signal sequence of 29 amino acids is removed from the amino-terminus between Gly-29/Leu-30 (Gentry et al., 1988). Glycosylation occurs at all three predicted N-linked sites within the pro region followed by phosphorylation at mannose residues of the attached oligosaccharides (Purchio et al., 1988; Brunner et al., 1988). At some stage during synthesis or transit, proteolytic cleavage at dibasic residues and disulfide bond formation occur and the mature growth factor is released (Gentry et al., 1988). The precise order of the processing, and the pathway of the intracellular routine of TGFβ1 precursor have not been characterized.

The independently expressed pro domain of TGFβ1, when cotransfected with the mature TGFβ1, aids in the folding of the growth factor within the cell resulting in a small percentage of secreted, mature TGFβ1 (Gray and Mason, 1990). Furthermore, the pro portion of TGFβ1 represents a functional binding protein for mature TGFβ1 (Gentry et al., 1987; Miyazomo et al., 1988; Wakefield et al., 1989; Gentry and Nash, 1990). The dimer of this pro domain, termed β1-latency-associated peptide (β1-LAP), appears to be important for its biological function (Brunner et al., 1989; Gentry and Nash, 1990). The resulting latent form may then be acti-
vated by extreme pH values, by heat, or by treatment with selected chaotropic agents or proteases (Lawrence et al., 1985; Pircher et al., 1986; Lyons et al., 1988, 1990; Gentry and Nash, 1990). These results suggest that the TGFβ1 pro domain serves an important role in disulfide folding of mature TGFβ1 and latent complex formation.

As an initial step towards understanding the mechanisms by which cells control TGFβ1 activities, we undertook a systematic dissection of the pro domain of TGFβ1 by site-specific insertion and deletion mutagenesis. Our approach was to place a unique 12-mer oligonucleotide at specific restriction sites in the pro domain of TGFβ1, resulting in the in-frame placement of four amino acids. Such insertions should disrupt the local protein structure and allow our assignment of functional regions. The mutant TGFβ1 molecules were transfected in COS-1 cells and the proteins were identified by immunoblotting and then analyzed for proteolytic processing, secretion, and disulfide maturation. We found by these approaches that the structural integrity of the pro region, when expressed as pre-pro-TGFβ1, is essential for secretory exit of mature and precursor forms of TGFβ1 from cells. Several insertions resulted in mutants of TGFβ1 precursors which were not secreted, while insertions at other sites resulted in high levels of disulfide-linked TGFβ1 aggregates, indicating that mature TGFβ1 was not properly folded at disulfide bonds. Independently expressed deletion mutants of β-LAP reveal that the amino-terminal portion of the pro region is involved in noncovalent association of the mature TGFβ1 to form a latent complex.

Materials and Methods

Cell Culture Condition

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 DMEM supplemented with FBS (10% vol/vol), penicillin (100 U/ml), and streptomycin (100 μg/ml). These cells were passaged by treatment with 0.5% (wt/vol) trypsin (Gibco Laboratories, Grand Island, NY) at a 1:5 splitting ratio.

Mutagenesis

The basic strategy used to construct a series of in-phase linker insertion mutations in the pro region of TGFβ1 peptide was based on the method of Stone et al. (1984). The pUC19-TGFβ1 vector DNA was linearized at NodI, filled in with Klenow DNA polymerase, and then digested with HindIII. The HindIII to EcoRI (blunt) fragment of pre-pro-TGFβ1 mutant was ligated into the pCDM8 vector.

The placement of unique restriction sites within the precursor of TGFβ1 cDNA allowed for the creation of in-frame deletion mutants by using pairs of insertion mutants. Pairs with BglII linkers inserted in the same reading frame were directly recombined by ligating the appropriate fragments isolated after digestion of plasmid with both BglII and HindIII. In-frame deletions between BglII sites that were not in the proper reading frame required either digestion with single stranded-specific mung bean nuclease or were filled in with the Klenow DNA polymerase and ligated. In all cases, ligation of ends produced by BglII digestion and treatment with mung bean nuclease generates unique EcoRI sites which could be used for screening mini-preparation DNA samples. The predominant plasmids bearing deletions at different sites were identified, mapped by restriction enzyme analysis, and/or sequenced. The deletion mutants expressing pre-pro-TGFβ1 were identified by Δ followed by the positions of the two insertion mutants used for their construction (see Table 2).

To prepare deletion mutants which express the pro region independently of pre-pro-TGFβ1, the plasmid PBS Stop278 was utilized (Gentry and Nash, 1990). Each deletion mutant cDNA was excised from its respective pCDM8 expression vector with HindIII and BstXI and used to replace the wild-type sequence of the pro domain of TGFβ1 in the plasmid vector pCDM8-Stop278. These deletion mutants expressing the pro domain independently were named as β-LAP deletion mutants, and designated L, followed by the same definition as previously described for the pre-pro-TGFβ1 deletion mutants (see Table 2).

Transient Expression

The pCDM8 constructs containing the mutant genes were transfected into COS-1 cells by a modified DEAE-dextran/chloroquine method (Seed and Aruffo, 1987). Each transfection was repeated at least three times. COS-1 cells were plated onto 10-cm plates and transfected at 30%-60% confluence. After a brief incubation with DME containing 10% Nu-serum (Collaborative Biomedical Products, Lexington, MA), 1 ml of DME containing 5% (L-15) or 12% SDS-polyacrylamide gels (Laemmli, 1970). The cells were lysed with an equal volume of 2x sample preparation buffer and heated at 95°C for 4 min before analysis. After further incubation of the cells for 4 h in DME supplemented with 10% FBS, the medium was changed to serum-free DME (5 ml/10-cm dish) and collected after 48 h. Each experiment included control plates containing cells transfected by pCDM8 and pCDM8-TGFβ1 or pCDM8-Stop278. The transfected cells were washed three times with cold PBS, scraped from the culture plate in cold PBS, and collected by low-speed centrifugation.

Immunoblot Analysis

Secreted and cellular forms of the precursor for TGFβ1 were analyzed by immunoblotting (Gentry et al., 1987). Serum-free conditioned medium was collected at the end of transfection. PMSF was added to the medium at a final concentration of 1 mM, which was then clarified by low-speed centrifugation. The medium was then dialyzed against 0.2 M acetic acid and 1-M aliquots were lyophilized. Each lyophilized sample was resuspended in 20 μl of sample preparation buffer and fractionated on 7.5-17.5% gradient SDS-polyacrylamide gels (Laemmli, 1970). The cells were lysed with equal volume of 2X sample preparation buffer and heated at 95°C for 4 min before analysis. After electrophoresis, proteins were electrochemically transferred to nitrocellulose and stained for immunoreactive mature and precursor forms of the TGFβ1 proteins as described previously (Gentry et al., 1987). The antisera used were directed against residues 225-236 of the mature portion of TGFβ1 or residues 369-381 of the mature growth factor (see Fig. 3). In some experiments, affinity-purified antipeptide antibodies were utilized for immunostaining.

Affinity-purified antipeptide antibodies were prepared using the procedures described by Smith and Fiser (1984). Briefly, concentrated culture supernatants from CHO cells expressing high levels of rTGFβ1 precursor (Gentry et al., 1987) were fractionated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The protein bands corresponding to the mature and pro region of TGFβ1 were visualized after the membrane was stained with 0.5% ponceau S, excised, and incubated overnight with the antipeptide antisera, respectively. The antibodies were eluted with 0.1 M glycine-HCl, 0.1 M NaCl (pH 2.5), and immediately neutralized with 1 M Tris-HCl, pH 8.0. Alkaline phosphatase-conjugated-Protein A or
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Fig. 2 shows an immunoblot of cells transfected by pre-pro-TGFβ1 and its insertion mutants. Proteins were electrophoresed under reducing conditions and the blot was incubated with serum against mature TGFβ1 followed by staining with alkaline phosphatase–conjugated Protein A. The pCDM8 control transfection showed no immunoreactive TGFβ1 precursors. In all other cases, however, the precursor of TGFβ1 and its insertion mutants were readily expressed in COS-1 cells and produced proteins possessing molecular masses of ∼44 kD (Fig. 2). These results indicate that wild-type and insertion mutants of the TGFβ1 precursors were expressed by COS-1 cells and that steady-state levels of the intracellular mutant pre-pro-TGFβ1s were comparable to that of wild-type. The intracellular form of the TGFβ1 precursor probably represents the partially glycosylated pro-TGFβ1, as observed previously in CHO cells expressing high levels of rTGFβ1 precursor (Sha et al., 1989). Use of alkaline phosphatase–conjugated goat anti-rabbit IgG for staining resulted in greater sensitivity, and consistently showed the existence of mature TGFβ1 in the intact cells transfected with wild-type, pre-pro-TGFβ1 (see Fig. 4 B).

An immunoblot of serum-free media from each of the transfectants is shown in Fig. 3 B. Under nonreducing condition of electrophoresis, peptide-specific antisera against mature TGFβ1 identified a 100–120-kD protein, and a 24-kD protein in medium from cells transfected with wild-type, pre-pro-TGFβ1 (pCDM8-TGFβ1). These immunoreactive forms comigrated with rTGFβ1 precursors produced by CHO cells, suggesting that the high molecular weight band probably represented a complex of the pro-TGFβ1 (form a), the pro region (form b) and the mature TGFβ1 (form c) linked by intersubunit disulfide bonds. The low molecular weight band corresponds to mature TGFβ1 dimer (Gentry et al., 1987, 1988). These forms were not present in the supernatants from cells transfected with pCDM8 vector. Mutants In50, In110, In154, and In170 showed no immunostainable levels of mature or larger molecular mass forms of TGFβ1.

also prepared (Table II); it covers the whole sequence of the pro region in blocks from 10 to 125 residues. Some of the deletions were designed to encompass one, two, or all three potential glycosylation signals in the pro region.

pCDM8 vectors containing insertion mutants in the precursor of TGFβ1 were transiently expressed in COS-1 cells. As controls, the pCDM8 vector itself without an insert and pCDM8 containing a wild-type, pre-pro-TGFβ1 cDNA (pCDM8-TGFβ1) were used as controls. After transfection, the cells and the serum-free supernatants from the transfected cells were analyzed by Western blot analysis using either mature TGFβ1 specific or pro region–specific antibodies (Gentry et al., 1987). Immunoblotting was necessary since these antibodies do not recognize the native protein and can not be used for immunoprecipitation analysis.

Effects of the Insertion Mutations in the Pro Region of TGFβ1 on Synthesis, Proteolytic Processing, Disulfide Maturation, and Secretion of Mature TGFβ1 Polypeptides

Table I. TGFβ1 Insertion Mutants

| Insertion mutants | Restriction sites mutated | Codons inserted* | Secreted TGFβ1 monomer (% of wild-type)† | Secreted TGFβ1 dimer (% of wild-type)† | Biologically active TGFβ1 (% of wild-type)§ | Disulfide folding percentage† |
|-------------------|--------------------------|-----------------|----------------------------------------|----------------------------------------|----------------------------------------|-----------------------------|
| CDM8              |                          |                 | 2                                      | 7                                      | 8.0 ± 2.0                              | ND                          |
| CDM8-TGFβ1        |                          |                 | 100                                    | 100                                    | 100 ± 11.3                             | 20                          |
| In40              | Alu                      | E(GRSS)L        | 59                                     | 88                                     | 46.8 ± 3.5                             | 30                          |
| In50              | BstUI                    | R(EDLP)G        | 3                                      | 6                                      | 10.0 ± 2.9                             | ND                          |
| In81              | RsaI                     | L(WKFH)N        | 33                                     | 13                                     | 9.0 ± 1.8                              | 5                           |
| In85              | BstUI                    | R(EDLP)E        | 31                                     | 14                                     | 9.7 ± 2.0                              | 6                           |
| In110             | BstUI                    | R(EDLP)V        | 7                                      | 4                                      | 8.5 ± 2.3                              | ND                          |
| In144             | RsaI                     | V(EDLP)P        | 38                                     | 15                                     | 10.3 ± 2.9                             | 4                           |
| In154             | Alu                      | E(GRSS)L        | 2                                      | 7                                      | 6.9 ± 2.0                              | ND                          |
| In170             | Alu                      | E(GRSS)L        | 3                                      | 7                                      | 8.7 ± 1.5                              | ND                          |
| In171             | RsaI                     | L(WKFH)Q        | 13                                     | 23                                     | 25.4 ± 8.8                             | 29                          |
| In210             | BstUI                    | R(EDLP)G        | 31                                     | 32                                     | 29.8 ± 3.3                             | 20                          |
| In273             | Alu                      | Q(REDLPS)S      | 48                                     | 53                                     | 40.5 ± 8.4                             | 23                          |

* In parentheses are the inserted amino acids, whereas original codons are shown outside. Amino acids appearing below the plane are deleted due to insertional interruption.
† The immunoblot run under reducing conditions (shown in Fig. 3 C) was quantitated by densitometry as described in Materials and Methods. The amounts of 12-kD monomeric TGFβ1 (form c) in the supernatants are expressed as a percentage of wild-type TGFβ1 (CDM8-TGFβ1).
‡ The amounts of mature TGFβ1 dimer were quantitated by densitometry scanning of the nonreducing immunoblot shown in Fig. 3 B (form C) and expressed as a percentage of wild-type TGFβ1 (CDM8-TGFβ1).
§ The amounts of bioactive TGFβ1 secreted by COS-1 cell transfectants were determined as described in Materials and Methods. Wild-type TGFβ1 produced ∼70 ng/ml active TGFβ1. The results were expressed as a percentage of wild-type activity ± standard deviation (CDM8-TGFβ1). Results are based on six independent determinations.
∥ The immunoreactive disulfide complex (form A) and the mature TGFβ1 dimer (form C) shown in Fig. 3 B were quantitated by densitometry as described in Materials and Methods. The disulfide folding percentage shown is based on the following equation, [C/(A+C)] x 100, using the results obtained from densitometer scanning. A disulfide folding percentage of 20 for wild-type, pre-pro-TGFβ1 is similar to results obtained from CHO cells expressing large amounts of rTGFβ1 and its precursors (unpublished observations). For more information see Results section.

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* TGFSI deletion mutant was expressed as pre-pro-TGFβ1 by transfected COS-1 cells, and designated as Δ, followed by the numbers of the paired insertion mutants used for its construction.

† +, the immunoreactive TGFβ1 in the supernatant from cells expressing TGFβ1 deletion mutant was detected by immunoblot as shown in Fig. 4 A. -, not detected.

§ The cross-linking of the pro region dimer of TGFβ1 in the supernatants from cells transfected by Stop278 or S1-LAP deletion mutants with radioiodinated TGFSI is described in Materials and Methods. After SDS-PAGE, the protein bands were excised and the amount of cross-linking was assessed by a γ counter (Fig. 6 B). The results are represented as a percentage of the wild-type. Nonspecific binding was <20%. ND, not determined.

precursors. All other insertional mutants produced lower levels of rTGFSI polypeptides. These mutants, except In210, migrated at similar positions to wild-type, TGFβ1 precursors on SDS-gels; however, In210 migrated as a larger molecular mass. Since we did restriction analysis as well as DNA sequencing, and did not find additional insertions beside the introduced linker sequence, the retarded mobility of the high molecular form of In210 may be due to differences in the disulfide folding pattern of this molecule. Note that for In210, pro-TGFβ1 and its pro domain migrate similar to other mutants on the reducing gels shown in Fig. 3 (C and D).

Under reducing conditions of sample preparation (Fig. 3 C), the immunoblot probed with the same antibodies revealed the existence of two forms of recombinant TGFSI precursors in the supernatant from the wild-type transfectant, a 44–56-kD band representing uncleaved pro-TGFβ1 (form a), and a 12-kD band corresponding to mature TGFβ1 monomer (form c) (Gentry et al., 1987; 1988). Immunoreactive mature or precursor forms of TGFβ1 were not detected in the supernatants from cells transfected with insertion mutants In50, In154, and M170. In110 still made a small amount of mature monomer. The other mutants appeared to secrete lower levels of immunostained monomer than CDM8-TGFSI (Fig. 3 C, band c). To determine the degree of proteolytic processing, immunoblots (Fig. 3 C) were scanned by densitometry. The percentage of mature TGFβ1 (band c) relative to precursor (form a) generated by wild-type or its various insertion mutants was similar (~40%). Therefore, the amount of mature 12-kD species is representative of the level of secreted TGFβ1; these values are listed in Table I using 100 for wild-type, mature TGFβ1.

The amount of mature, monomeric TGFβ1 (Fig. 3 C) detected by immunoblotting appeared greater than that of mature, dimeric TGFβ1 detected under nonreducing conditions, especially for mutants In81, In85, and In144 (Fig. 3 B). Such an inconsistency may be due to a greater percentage of mature, monomeric TGFβ1 forming disulfide cross-links through Cys-33 of their precursors (Fig. 3 B) (Gentry et al., 1988). To test this possibility, immunoblots showing the mature dimers (form C) and larger disulfide aggregates of TGFβ1 (form A) were scanned by densitometry (Fig. 3 B). After subtracting the appropriate background (i.e., pCDM8),
Figure 3. Immunoblots of culture supernatants from transfected COS-1 cells expressing pre-pro-TGFβ1 insertion mutants. Serum-free media from transfected COS-1 cells were collected and processed as described in Materials and Methods. Conditioned, concentrated medium from CHO cells (2 μl) which express high levels of rTGFβ1 precursor (Gentry et al., 1987) was used in these experiments as a standard; this is indicated above the immunoblots as TGFβ1. Molecular size standards in kilodaltons are shown on the left of each panel. Results are representative of three independent transfection experiments. (A) The diagram shows a schematic representation of the structure of pre-pro-TGFβ1 polypeptide and the sites of insertion mutations. Pre-TGFβ1, the pro region of TGFβ1, and mature TGFβ1 are indicated by a, b, and c, respectively. The asterisks denote N-linked glycosylation signals. Positions of the insertion mutants are designated by overlying circles. The shaded circles indicate reduced amounts of TGFβ1 precursor in the supernatant, and the open circles represent no detectable amount of the precursors in the supernatant. (B) Medium from cells (1 ml) expressing the mutants was fractionated on a 12% SDS polyacrylamide gel under nonreducing condition and stained for immunoreactive TGFβ1 precursor using anti-TGFβ1(369-381). The identity of each mutant is located above each lane. The letter A represents the disulfide aggregates expressed by the transfected COS-1 cells, and C denotes the mature TGFβ1 dimer secreted. (C) Immunoblot of supernatants fractionated on 7.5-20% gradient SDS-polyacrylamide gels under reducing condition. Blots were probed with some antibodies as described above. (D) Immunoblot of supernatants fractionated on 7.5-20% gradient SDS-polyacrylamide gels. Blots were probed with anti-TGFβ1(225-236) antibodies.

the relative amount of mature, dimeric TGFβ1 was then calculated using the formula \([C/(A + C)] \times 100\). This result is listed in Table I as disulfide折叠 percentage.

The arbitrary value of disulfide folding percentage refers to the relative amount of mature, dimeric TGFβ1 present in the culture medium. A value of 100% would mean that all of pre-pro-TGFβ1 is processed properly and that the mature growth factor contains no disulfide formation with Cys-33 of the pro domain. A value of 0%, on the other hand, would mean that mature TGFβ1 was linked to Cys-33 of the larger disulfide form. Wild-type, pre-pro-TGFβ1 transfectants produced mature TGFβ1, which represented ~20% of the total immunoreactive TGFβ1 (Fig. 3 B), a value in close agreement with results from CHO cells expressing large amounts of this growth factor (unpublished observations). Mutants In81, In85, and In144 produced lower amounts of dimer ranging from 4-6%. Mutants in the amino- and carboxyl-portion of the TGFβ1 pro domain (In40, In71, In210, and In273) had virtually no effect on this Disulfide Folding Percentage (Table I), displaying values ranging from 20-30%.

These results indicate that regions surrounding residues 81, 85, and 144 of the TGFβ1 pro domain may play an important role in the disulfide modeling of mature TGFβ1.

The corresponding biological activities of wild-type, mature TGFβ1 and mutants were determined in a standard bioassay, using growth inhibition of mink lung epithelial cells (Table I). Wild-type, mature TGFβ1 (pCDM8-TGFβ1) produced ~70 ng/ml of TGFβ1, a value similar to a previous report (Brunner et al., 1989). This value has been normalized to 100 for wild-type TGFβ (Table I). Mutants In50, In154, and In170, which do not show immunoreactive TGFβ1 monomers and dimers in their supernatants, had biological activities similar to that produced by pCDM8 vector alone. The amounts of biologically active TGFβ correlated well with the amount of mature TGFβ1 dimers detected by immunological methods (see above; Fig. 3 B; Table I).

Processed pro domains in the culture supernatants from various transfected COS-1 cells were demonstrated by immunoblot analysis with antibodies against the pro region of TGFβ1 (Fig. 3 D). Wild-type, precursor forms of TGFβ1 were apparent as two forms, both of which comigrated with the rTGFβ1 precursor. A 44-56-kD band represents the un-
Deletion Mutations in the TGFβ1 Pro Region Abolished Secretion of Mature Growth Factor

To examine the role of the pro domain in secretion, a large deletion of the TGFβ1 precursor was made using the two naturally occurring SsrI restriction sites (Fig. 1). The deletion removes 162 amino acid residues in the pro domain including all three glycosylation signals. When this deletion construct was expressed in COS-1 cells, no secreted product was detected. When cells were examined, an immunoreactive protein corresponding to the size of the modified protein was readily detected and was present at higher levels than normal pro-TGFβ1 (data not shown), suggesting that either glycosylation or the pro domain was important in secretion (Sha et al., 1989).

To examine the pro domain in more detail, deletion mutants of pre-pro-TGFβ1 were transfected into COS-1 cells and the secreted and cell-associated mutant proteins were detected by immunoblotting using affinity-purified antipeptide antiserum as shown in Fig. 4. The results are summarized in Table II. Immunoblots of supernatants probed with mature (Fig. 4A) or pro region–specific antibodies (data not shown) from cells transfected with wild-type, pre-pro-TGFβ1 confirmed that TGFβ1 was expressed and secreted normally. However, when any of the deletion mutants were expressed in COS-1 cells, immunoblots of supernatants did not show any corresponding immunoreactive TGFβ1 precursor proteins (Fig. 4A). These mutants of TGFβ1 were readily expressed in COS-1 cells, as demonstrated by immunoblotting of cell extracts (Fig. 4B). Under reducing conditions, these affinity-purified antibodies against mature TGFβ1 identified the partially glycosylated TGFβ1 precursor (Sha et al., 1989) and the mature form of wild-type TGFβ1 (see pCDM8-TGFβ1 in the left two immunoblots), confirming that proteolytic processing of the TGFβ1 precursor occurred intracellularly. In cells transfected with the deletion mutants, mature forms were not detected, while all mutant precursor forms were expressed and appeared to accumulate to different extents. Previous work has demonstrated that proteolytic processing of rTGFβ1 precursor by CHO cells occurred in acidic intracellular organelles (Sha et al., 1989), where endoproteolytic cleavages at paired basic residues commonly occur within acidic, clathrin-coated vesicles (Orci et al., 1987). The results from our studies indicate that deletion mutants within the TGFβ1 pro domain might either affect routing of TGFβ1 precursor to the proper intracellular compartment for proteolytic processing, or alternatively affect protein stability.

The mutant precursor forms migrated at shifted molecular weights relative to wild-type, pre-pro-TGFβ1. Mutant proteins would probably not migrate according to size since carbohydrate addition would not be uniform; Δ50–85, Δ81–171, Δ85–210, Δ110–144, Δ144–210, and Δ171–210 contain deletions in N-linked carbohydrate addition sites.

Expression of β1-LAP Deletion Mutants Independent of Mature TGFβ1

The pro region of TGFβ1, when expressed independent of the mature growth factor in recombinant systems, was able to fold properly and be secreted from the transfected cells (Gentry and Nash, 1990). The insertion and deletion studies indicated that disruption of the pro domain of pre-pro-TGFβ1 affected folding and secretion of mature TGFβ1. These results imply that information for protein conformation and/or secretory transit of pre-pro-TGFβ1 are probably stored in the pro domain, and that mature TGFβ1 must somehow associate with this domain using covalent and/or noncovalent bonds for proper folding and secretion from the cells.

Figure 4. Determination of secreted and cell-associated precursor forms of TGFβ1 deletion mutants. (A) Immunoblot of supernatants from COS-1 cells transfected by deletion mutants of pre-pro-TGFβ1. The Δ in each case represents the pre-pro-TGFβ1 deletion mutant; numbers following the Δ represent insertion mutants used for its construction. Supernatant from the various transfecteds were fractionated on 7.5–20% gradient SDS-polyacrylamide gels under reducing conditions. The secreted protein was probed with affinity-purified anti-TGFβ1369-381 antibodies. The results have been repeated three times and appear to be consistent. Marker proteins are shown to the left. (B) Immunoblots of transfected cells expressing pre-pro-TGFβ1 deletion mutants. Cells were collected as described in Materials and Methods. For each mutant, 10^6 cells were analyzed by immunoblotting under reducing conditions. Affinity-purified anti-TGFβ1369-381 antibodies were used for these immunoblots. Alkaline phosphatase–conjugated anti-rabbit IgG was used for the immunoblots in the left two panels. Alkaline phosphatase–conjugated Protein A was used for the right panel. Markers are shown to the left.
Deletion mutants were prepared in β1-LAP and placed into the same expression vector (Table II). The β1-LAP deletion mutants were transfected into COS-1 cells, and tested for their secreted pro region by immunoblotting with affinity-purified antiserum against the pro domain (Fig. 5). Under reducing conditions (Fig. 5 A), cells transfected with β1-LAP (Stop278) produced a 30-42-kD immunoreactive protein representing the wild-type, pro domain (Gentry and Nash, 1990). Interestingly, the pro regions in the supernatants from cells transfected with L40-50, L50-81, L50-85, and L85-110 were also detected, suggesting that deletions in the amino-terminal residues 40-110 of the pro region did not abolish secretion of β1-LAP. However, mutants containing deletions in residues 110-210 were not detected in the supernatants from COS-1 cells. In cells expressing these mutants, the levels of the mutant proteins were slightly higher than that produced by wild-type β1-LAP (data not shown), suggesting that these β1-LAP mutants may have protein secretion defects. Mutant L85-210 reproducibly showed a small amount of mutant protein secreted, while smaller deletion mutants covering the same region gave no detectable, secreted pro region.

To demonstrate the different molecular sizes of wild-type β1-LAP (Stop278) and its deletion mutants, the culture supernatants were treated with N-glycanase, and then further analyzed by immunoblotting (Fig. 5 B). Under reducing condition, digestion with N-glycanase caused wild-type β1-LAP (Stop278) to migrate as a sharp band of 26-28 kD, which corresponds to the calculated molecular size of unglycosylated pro peptide (Brunner et al., 1988; Sha et al., 1989). Similarly, the protein bands of β1-LAP deletion mutants were shifted and had gel mobilities corresponding to their sizes following deletion mutations.

Deletions in the Amino-terminal Portion of β1-LAP Severely Reduce Binding to Mature TGFβ1

Mutants that were able to secrete the β1-LAP pro regions were examined for their abilities to generate functional dimer forms by immunoblotting of culture supernatants under nonreducing conditions (Fig. 6 A). Consistent with a previous report (Gentry and Nash, 1990), β1-LAP (Stop278) produced a major protein band with a molecular mass around 80 kD, and a minor band of 40-42 kD. The two protein species represent the dimer and monomer forms of β1-LAP, respectively. The β1-LAP deletion mutants also appeared to form dimers.

Previous studies reported that the independently expressed β1-LAP could associate with biologically active, mature TGFβ1 to form a latent complex in vitro (Gentry and Nash, 1990). Removal of the amino-terminal sequence of the pro region by protease treatment resulted in dissociation of the latent complex (Lyonset al., 1990). We examined the association of the β1-LAP deletion mutants with radioiodinated mature TGFβ1 by cross-linking the two proteins (Gentry and Nash, 1990). Equivalent amounts of supernatants from cells transfected with Stop278 or L40-50, L50-81, L50-85, and L85-110 were incubated with radioiodinated TGFβ1 and subsequently treated with cross-linker (Fig. 6 B). The cross-linked proteins were fractionated by nonreducing SDS-PAGE followed by autoradiography (see Materials and Methods). Incorporation of radioiodinated TGFβ1 into wild-type and mutant β1-LAPs was then determined and expressed as percentage of the wild-type (Table II). When β1-LAP (Stop278) supernatant without cross-linker was included as a control, only the 24-kD radioiodinated, mature TGFβ1 was detected. After addition of cross-linker, a large aggregate of
Figure 6. Detection of dimeric forms of β1-LAP deletion mutants and subsequent cross-linking with radioiodinated, mature TGFβ1. (A) Supernatants from the β1-LAP deletion mutants were analyzed by SDS-PAGE on a 12% SDS polyacrylamide gel under nonreducing conditions; immunoreactive protein was identified by affinity-purified anti-TGFβ1225-236. Markers are shown to the left. (B) Culture medium from the control transfectants or from cells expressing β1-LAP deletion mutants was first normalized and then incubated with radioiodinated, mature TGFβ1 and cross-linked with bis(sulfosuccinimidyl) suberate as described in Materials and Methods. Cross-linked TGFβ1 complexes were fractionated on 7.5% SDS-polyacrylamide gel followed by autoradiography. The + and − at the bottom of the figure indicate whether or not cross-linker was added. γ-Spectroscopy counts of each mutant are shown in Table II.

cross-linked TGFβ1 was observed at the top of the gel (>200 kD). A limited amount of cross-linked TGFβ1 complex was observed in the lane marked Stop278. This lane contains wild-type β1-LAP protein which efficiently binds to mature TGFβ1 and prevents excessive aggregation. Cross-linking β1-LAP (Stop278) generated a 100–110-kD species which represented 47% of the input TGFβ1 radioactivity. No such protein was detected in the pCDM8 medium. This 100–110-kD species represents the cross-linked complex containing one dimer of the pro domain (80 kD) and one mature TGFβ1 dimer (24 kD) (Gentry and Nash, 1990).

Deletion mutations in the amino-terminal part of the pro region affected formation of the cross-linked complex (Fig. 6 B). Mutant L40–50 and L85–110 reduced the amount of the cross-linked complex formed giving 10 and 12% of wild-type β1-LAP (Stop278), respectively. Mutants L50–81 and L50–85 did not form cross-linked complexes. The complex of L85–110 had a lower molecular size compared with wild-type β1-LAP (Stop278), probably from its 25 residue deletion. The mobility shift of L40–50 was not observed under these conditions due to its smaller deletion. A minor cross-linked species (45 kD) was observed in all mutants and pCDM8 supernatants.

β1-LAP (Stop278) and the four β1-LAP deletion mutants were also tested by bioassay for their abilities to regulate the growth inhibition activity of the mature TGFβ1. Consistent with a previous report (Gentry and Nash, 1990), β1-LAP (Stop278) medium inhibited the TGFβ1 bioactivity, whereas L40–50 and L85–110 had 2–4% the activity of Stop278, and L50–81 and L50–85 were not inhibitory (data not shown). These results indicate the importance of the amino-terminal region (residue 50–85) of β1-LAP in latent complex formation. Although this region contains the first glycosylation signal, one of the deletion mutants of β1-LAP (L50–85) that lacks this site was readily secreted, suggesting that this N-linked oligosaccharide addition site is not essential for secretory transport of β1-LAP. The carboxyl-terminal half of β1-LAP, on the other hand, appears to contain sequences that are essential for secretory exit of β1-LAP and pre-pro-TGFβ1.

Discussion

TGFβ1 possesses multiple functional properties that affect cell growth and differentiation (Robert and Sporn, 1990). TGFβ1 is synthesized as a precursor, which subsequently undergoes extensive posttranslational processing events for mature TGFβ1, which is secreted (Derynck et al., 1985; Gentry et al., 1988; Brunner et al., 1988). The secreted TGFβ1 exists as a biologically inactive or latent form that is not able to bind to its cell surface receptors (Lawrence et al., 1985). Latency results from the binding of mature growth factor to a dimer of its own pro region, termed β1-LAP (Gentry et al., 1987; Miyazono et al., 1988; Wakefield et al., 1988; Gentry and Nash, 1990). Since the presence of TGFβ1 receptors is universal (Wakefield et al., 1987), latent complex formation and its activation through an unknown...
Mechanism must be an important part of the control of TGFβ1 function. The pro domain also aids in proper folding of the mature growth factor intracellularly (Gray and Mason, 1990). Despite these known functional roles, very little is known about structure/function relationships of the pro region. Using mutants generated by linker insertion, we have demonstrated that the structural integrity of the pro region is required for export of mature TGFβ1. In addition, we have defined several regions in the pro domain that are important in the folding, stability, secretion, and latent complex formation of TGFβ1.

An illustration demonstrating the results from our experiments and how these results relate to the sequence homology of human TGFβ1, β2, or β3 is shown in Fig. 7. Linker insertion mutagenesis at three sites in the pro domain of simian TGFβ1 (amino acids 50, 154, and 170) abolished detection of all three forms of mutant TGFβ1 precursor in culture supernatants, whereas insertion in other regions resulted in diminished levels of the secreted precursor to TGFβ1 (Fig. 3). Deletion mutations have provided similar results. All deletion mutants, even the mutant lacking 10 amino acids, were not secreted (Fig. 4). This does not appear to be the result of decreased protein expression, since mutant proteins were expressed intracellularly at levels comparable to wild-type, pre-pro-TGFβ1 (Fig. 2). Previous work has indicated that the independently expressed mature TGFβ1 was not properly folded and/or secreted from transformed cells unless coexpressed with its pro domain (Gray and Mason, 1990). Together with our results, we speculate that either the primary sequence of the pro domain and/or its resultant conformation is essential in secretory export of mature TGFβ1.

Does the amino acid sequence of the inserted linker play a specific role in the degradation of the mutant TGFβ1 precursor? Table I lists the four different linker sequences which were placed into the precursor of TGFβ1. In40 and In273 resulted in the insertion of an Arg residue, an amino acid sensitive to hydrolysis by plasmin and many cellular proteases. In154 and In170 also contain insertions that result in the placement of specific Arg residues. Why then are In40 and In273 stable to this insertion, and In154 and In170 unstable? Perhaps insertions at the ends of the molecule (In40 and In273) will not affect the biological activity of β1-LAP.

Placement of this linker sequence toward the middle of the molecule (In154 and In170) somehow disrupts the normal functional properties of β1-LAP, either through the inclusion of a positive charge or through the addition of an amino acid that is recognized by cellular proteases. SDS-gel electrophoresis of these mutant TGFβ1 molecules did not reveal the presence of any degraded protein precursor either in the cell or in the culture medium (Figs. 2 and 3). Insertions after an Arg residue for In50 may have resulted in degraded protein by providing a hydrolyzable "kink" in the precursor polypeptide chain, resulting in degradation. However, this seems unlikely, since In85, In110, and In210 also contain insertions after Arg residues and produce mutants that were readily detected in the culture medium (Fig. 3). Moreover, In50 produced intracellular levels of precursor similar or greater to wild-type TGFβ1 with no detectable degradation products (Fig. 2). We have yet to test the effects of two different four amino acid insertions at the same position in the TGFβ1 precursor.

Independently expressed β1-LAPs lacking amino-terminal sequences 40–110 were readily secreted from cells and these mutants appeared to exist as dimeric structures. By contrast, β1-LAPs with mutations between residues 110 and 210 were apparently not secreted (Fig. 5). Cross-linking studies and bioassays indicated that the secreted β1-LAP deletion mutants interacted marginally with mature TGFβ1 (L40–50 and L85–110) or not at all (L50–81 and L50–85), suggesting an importance of these deleted sequences in latent complex formation (Fig. 6). These results indicate that amino acid residues 50–85 of β1-LAP are probably involved in maintaining the structure of the latent complex. Residues 40–50 and 85–110 may only be superficially involved in binding mature TGFβ1.

A similar suggestion has recently been made looking at the latent form of rTGFβ1 precursor after plasmin digestion. In those studies, activation of the latent complex occurred after removal of the amino-terminal portion of the pro domain after plasmin digestion; these results suggest that this region of β1-LAP is important for stabilizing the latent complex (Lyons et al., 1990). Analysis of the TGFβ1 precursor sequence indicates that residues 40–60 are the largest conserved continuous sequence of the pro domain in all three TGFβs, and appear to be rich in basic residues. It is likely that residues 40–60 may be involved in the binding of TGFβ1 through electrostatic interactions. This interaction could stabilize the entire protein complex, which is essential to form the latent complex. Residues 110–210 of β1-LAP may also be involved in the protein interaction; no such re-
region mutants were secreted from COS-1 cells (Fig. 5). Based on these results, it appears that latent complex formation is required for proper secretory exit of the mature growth factor or its stability. In deletions that eliminate residues 40–110, the β1-LAPs were still folded into a transportable structure. However, when expressed as pre-pro-TGFβ1, these mutants were not detected as secreted forms. We presume that the mature growth factor was unable to associate with the mutant pro domain and resulted in an altered conformation that could affect the mature TGFβ1 stability or its export. Insertion mutant In50, which did not show secreted TGFβ1, might also fail to form the latent complex, resulting in a defect in protein secretion (Fig. 3).

The region corresponding to residues 110–210 of pre-pro-TGFβ1 appears to be essential in export of the mature growth factor (Figs. 4 and 5). Mutants In54 and In170 had secretion or stability defects when expressed both as pre-pro-TGFβ1 (Fig. 3) and β1-LAPs (data not shown). Regions containing amino acids 154 and 170 appear to lie in strictly conserved regions among the sequenced human TGFβ1s (Fig. 7). TGFβ1 precursor polypeptides could not be detected in COS-1 supernatants when the linker sequence GRSS was inserted in these regions. Presumably this region of the protein could form secondary structures which may be essential for proper folding of the pro domain and may be used for secretory exit.

One of the best known examples concerning domains involved in protein transport has come from studies of intracellular transport of vesicular stomatitis virus (VSV) G protein (Rose and Bergmann, 1983; Dams et al., 1987, 1988). Using in vitro mutagenesis of the VSV G cDNA, these researchers found that deletion of the carboxyl-domain, a domain lying in the cellular cytoplasm, had profound effects on secretion of the VSV G protein without altering the whole protein conformation, indicating that this domain must fulfill structural requirements for intracellular routing independent of other protein domains. In addition, assembly of the VSV G protein into a trimer was required for normal transport. Experiments to determine the cell-associated mutant TGFβ1 precursors by immunoblotting under nonreducing conditions showed that dimers of In50, In54, and In170, which were not secreted, were readily identified intracellularly along with wild-type, pre-pro-TGFβ1 (data not shown) indicating that formation of a dimer was not a prerequisite for transport of TGFβ1.

The pro domain of TGFβ1 may function analogously to the pro domain of somatostatin, a domain that is important for secretion of the mature somatostatin. This neuropeptide has a structure analogous to pre-pro-TGFβ1 consisting of a signal peptide followed by a pro peptide and the mature hormone (Hobart et al., 1980). The pre-pro sequence, when fused to α-globin mediated the cytoplasmic protein to be secreted in a regulatory pathway. In contrast, if α-globin was fused to a signal peptide alone, it was translocated to ER, but in the absence of the pro domain it was rapidly degraded, suggesting that the pro peptide protected nascent precursor from protease attack, probably by enhancing its folding and facilitating efficient export (Stoller and Shields, 1989). It is likely that mutation in the pro region of TGFβ1 resulted in misfolding of the newly synthesized precursor, which was recognized and degraded by intracellular enzymes. To confirm this, additional experiments using antibodies that immunoprecipitate wild-type and mutant TGFβ1 precursors to determine the kinetics of intracellular routing of these TGFβ1 forms will be necessary to provide important information about their eventual fate.

N-linked oligosaccharides are often crucial in promoting export of protein. However, this requirement for carbohydrate in transport of membrane or secretory proteins is not universal and is highly protein specific as well as cell specific (reviewed by Olden et al., 1982). Using glycosylation inhibitors, we have determined that glycosylation and its maturation state affected secretion of rTGFβ1 precursors from CHO cells (Sha et al., 1989). The effects of oligosaccharides on protein secretion most likely results from incorrect protein folding and protein instability as reported by many investigators (Grafl et al., 1987; Slicker et al., 1986; Dams et al., 1988). Initially, when we designed the deletion mutants, we hoped to see some correlation with carbohydrate sites and secretion. Deletion mutants lacking each of the three glycosylation signals in the pro portion of pre-pro-TGFβ1 were prepared to assess the role of the individual oligosaccharides on protein secretion (Fig. 1). However, all deletion mutants of pre-pro-TGFβ1 were not secreted (Fig. 4 A). Deletion mutant Δ81–171 which lacks the first and second glycosylation signals was partially degraded in the COS-1 cells (Fig. 4 B), indicating that these carbohydrates may be important for protein stability. The first carbohydrate moiety does not appear to play a role in the secretion of β1-LAP, since such mutants that delete this N-linked addition site were readily observed in the culture medium (Fig. 6). Selective removal of glycosylation sites without disrupting sequences on either side of this signal using site-directed mutagenesis are in progress and should allow for proper evaluation of the role of individual oligosaccharides in proper folding and secretion of mature TGFβ1 and β1-LAP.

Insertion mutations which reduced transport did not apparently affect proteolytic processing of mature TGFβ1, suggesting that the dibasic cleavage site before the aminoterminus of mature TGFβ1 is readily exposed on the protein even with altered folding. The intracellular forms of the deletion mutants which had defects in secretion were not processed properly. We suggest that the misfolded mutant proteins were probably not transferred to the Golgi apparatus where the proteolytic processing of protein precursors begins (Steiner et al., 1984). Dibasic cleavage sites releasing the mature portion of TGFβ1 are usually not accessible to protease attack in wild-type, pre-pro-TGFβ1. Insertion mutation in region 110 secreted the pro piece of TGFβ1 but very little mature TGFβ1. Antibodies against mature TGFβ1 detected the existence of a mutant precursor intracellularly, suggesting that the pro domain affects proper folding of the mature TGFβ1. The misfolded mutant was probably degraded during secretion.

Unlike most tissue culture cells which secrete mature TGFβ1 and the pro form together as a latent complex, the transfected CHO and COS-1 cells were unable to completely process TGFβ1 precursor (pro-TGFβ1), and secreted all three forms of rTGFβ1. Furthermore, these transformed cells released a disulfide aggregate of rTGFβ1 precursor, where the pro domain is disulfide linked to mature TGFβ1 through Cys-33 and an unknown Cys residue in the mature region (Gentry et al., 1987, 1988; Brunner et al., 1989; Lyons et al., 1990). Latent TGFβ1 is secreted from tissue culture cells as a tri-peptide complex containing a cysteine-rich "binding
protein”, the pro domain of TGFβ1, and mature TGFβ1 (Miyazono et al., 1988; Wakefield et al., 1988; Kanazaki et al., 1990; Tsuji et al., 1990). Thus, it appears that secretion of this larger disulfide-linked form may represent an expression artifact. From our insertion mutation studies, we found that mutations in amino acid 81, 85, and 144 of the pro domain resulted in a large amount of disulfide aggregate, further indicating that the pro domain plays an important role in the proper folding of mature TGFβ1.

In summary, site-specific insertion and deletion mutagenesis of the pro domain of pre-pro-TGFβ1 indicate that the structural integrity of the pro region is required in secretory transport of TGFβ1 precursors. Additionally, our results highlight the amino-terminal region of the pro domain as being important for interaction with the mature TGFβ1 and indicated that this interaction must be maintained for latent complex formation and protease secretion. Finally, a comparison of the amino acid sequences of the pro domains from TGFβ1, β2, and β3 indicate that, even though these domains share <50% amino acid identity, functionally important regions identified by insertional mutagenesis are strictly conserved among them. The sequence homology in these regions presumably reflects their biological importance.

We gratefully acknowledge Dave Dignam, Manohar Ratnam, Melvin Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Weggoner for the typescript. We would like to thank Ron Manger for supplying the colorimetric protocol for measuring cell growth; Mary Harte and Grainne Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Waggoner for the typescript. We would like to thank Ron Manger for supplying the colorimetric protocol for measuring cell growth; Mary Harte and Grainne Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Waggoner for the typescript. We would like to thank Ron Manger for supplying the colorimetric protocol for measuring cell growth; Mary Harte and Grainne Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Waggoner for the typescript. We would like to thank Ron Manger for supplying the colorimetric protocol for measuring cell growth; Mary Harte and Grainne Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Waggoner for the typescript. We would like to thank Ron Manger for supplying the colorimetric protocol for measuring cell growth; Mary Harte and Grainne Soloff, and James Trempe for critical review of the manuscript; other members of the Department for helpful comments; and Kay Waggoner for the typescript.
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