Mechanism of Activation of Latent Recombinant Transforming Growth Factor $\beta 1$ by Plasmin

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Abstract. Medium conditioned by Chinese hamster ovary (CHO) cells transfected with the simian pre-pro-TGF β 1 cDNA contains high levels of latent TGF β 1. The amino-terminal region of the TGF β 1 precursor is secreted and can be detected in the conditioned medium by immunoblotting using peptide antibodies specific for amino-terminal peptides. Chemical crosslinking of CHO-conditioned medium using bis-(sulfosuccinimidyl)-suberate (BS³) followed by immunoblot analyses indicates that latent recombinant TGF β 1 contains both the cleaved amino-terminal glycopeptide and mature TGF β 1 polypeptide in a noncovalent association and that this association confers latency. The data presented here do not support the involvement of a

TRANSFORMING growth factor β (TGF β)¹ is an important regulator of cell growth and differentiation. $TGF\beta$ has been shown to be a potent inhibitor of proliferation of many cell types, both normal and transformed (Tucker et al., 1984b; Moses et al., 1985). In addition, TGF β may stimulate growth under specific conditions in certain cell types. The majority of cultured cells studied to date have been shown to secrete TGF β 1 in an inactive form (Lawrence et al., 1985; Pircher et al., 1986) that neither interacts with specific TGF β cell surface receptors nor elicits any of the known TGF β -induced biological responses. The presence of specific TGF β receptors on most cell types (Tucker et al., 1984a) and the ubiquity of the TGF β molecule itself (Roberts et al., 1983; Moses et al., 1985) suggest that activation of latent TGF β must be an integral component in the sequence of events leading to growth regulation by TGF β .

The active 25-kD TGF β molecule consists of two identical disulfide-linked 12.5-kD polypeptide chains (Assoian et al., 1983). The 12.5-kD monomer contains 112 amino acids and is synthesized as a larger pre-pro-TGF β 1 molecule of 390 amino acid residues as determined by cDNA sequencing (Derynck et al., 1985). The carboxy-terminal 12.5-kD mature peptide is proteolytically cleaved from the aminoterminal glycopeptide at arginine 278 (Derynck et al., 1985;

unique TGF β binding protein(s) in latent recombinant TGF β 1. Plasmin treatment of CHO-conditioned medium resulted in the appearance of TGF β competing activity. In addition, immunoblot analysis of plasmintreated CHO-conditioned medium indicates that the amino-terminal glycopeptide is partially degraded and that mature TGF β 1 is released. Thus, activation of latent TGF β 1 may occur by proteolytic nicking within the amino-terminal glycopeptide thereby causing a disruption of tertiary structure and noncovalent bonds, which results in the release of active, mature TGF β 1. Acid activation of latent TGF β , in comparison, appears to be due to dissociation of the amino-terminal glycopeptide from the mature polypeptide.

Gentry et al., 1987). In addition, a signal sequence of 29 amino acids is cleaved by a signal peptidase during transit of the precursor through the rough endoplasmic reticulum (Gentry et al., 1988). Other posttranslational modifications of TGF β that take place before secretion include glycosylation, mannose-6-phosphorylation of the glycoprotein and disulfide isomerization (Gentry et al., 1988; Purchio et al., 1988; Brunner et al., 1988). These events have been designated "processing" steps, and, while their order has not been well characterized, they are distinct from "activation" events that are required for the generation of biologically active TGF β from the latent molecule. Activation may be achieved by extremes of pH or by treatment with selected chaotrophic agents, including SDS and urea (Lawrence et al., 1985; Pircher et al., 1986). More recently, we have reported protease, specifically plasmin, activation of latent TGF β secreted by fibroblasts (Lyons et al., 1988). Recent findings of Sato and Rifkin (1989) indicate that activation of latent TGF β occurring during co-culture of bovine endothelial cells and pericytes is due to plasmin. This suggests that plasmin may be a physiological activator of latent TGFB.

Chinese hamster ovary (CHO) cells transfected with simian TGF β l cDNA secrete 200–1,000-fold more latent TGF β l than nontransfected cells providing an excellent source of material for use in activation studies (Gentry et al., 1987). The overexpression of latent recombinant TGF β l enabled the detection of the amino-terminal glycopeptide and mature

^{1.} Abbreviations used in this paper: BS³, bis(sulfosuccinimidyl)suberate; TGF β , transforming growth factor β .

polypeptide by immunoblotting with synthetic peptide antibodies (Gentry et al., 1987; Brunner et al., 1988). The present study indicates that a noncovalent association of the amino-terminal glycopeptide with the mature TGF β 1 polypeptide is responsible for latency of the 25-kD molecule and further suggests that proteolytic degradation of the TGF β 1 amino-terminal glycopeptide may provide a means of release of the active 25-kD TGF β molecule. The results of these experiments suggest that plasmin may degrade the aminoterminal glycopeptide and thereby release active TGF β 1.

Materials and Methods

Cell Culture and Collection of Conditioned Medium

CHO cells were transfected with simian pre-pro-TGF β l and adapted to grow in increasing concentrations of methotrexate (Gentry et al., 1987). For these studies, a cloned CHO cell line termed clone 17, was used and propagated as described (Brunner et al., 1988). Serum-free DME containing methotrexate (20 μ M) was added to confluent cultures and cell-conditioned medium was collected after 24 h. Crude-conditioned medium was centrifuged to remove cellular debris and stored at 4°C before use to prevent spontaneous activation. Acid activation of conditioned medium was performed by the addition of HCl to reach pH 1.5 for 1 h. The medium was then reneutralized using NaOH and used for biological and biochemical analyses.

AKR-2B (clone 84A) cells were used as indicator cells in radioreceptor assays. Cultures were maintained in McCoy's 5a medium supplemented with 5% FCS.

Radioreceptor Assay

TGF β radioreceptor assays were performed as previously described (Tucker et al., 1984*a*). Porcine TGF β (R & D Systems, Inc., Minneapolis, MN) was iodinated using a modified chloramine-T method (Frolik et al., 1984) and had a specific activity of ~50 Ci/g. Binding was performed in 1 ml/well of binding buffer (PBS, pH 7.4) containing [^{125}I]TGF β (0.25 ng; ~30,000 cpm). Nonspecific binding was determined in the presence of 30 ng porcine TGF β 1 and was <25%.

Peptide Antibodies

The synthetic peptide antibodies used in these studies have been characterized previously (Gentry et al., 1987). The antibodies are denoted as follows: No. 1125, amino-terminal peptide sequence 81–96; No. 7251, aminoterminal peptide sequence 225–236; and No. 978, mature sequence 369–383.

Chemical Cross-linking of CHO-conditioned Medium

CHO-conditioned medium (100 μ l) was incubated with bis(sulfosuccinimidyl)suberate (BS³) (Pierce Chemical Co., Rockford, IL) at the concentrations indicated for 45 min at 22°C. Medium was then centrifuged for 15 min at 12,000 g to remove precipitated material. Equivalent amounts of total protein from each treatment relative to that of 100 μ l of untreated conditioned medium was determined using a dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Samples were dried and prepared for PAGE followed by immunoblot analysis.

Plasmin Treatment of Latent TGF β 1

CHO-conditioned medium or latent TGF β 1 from human platelets (a gift of C.-H. Heldin) was incubated with plasmin as supplied (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) at the indicated concentrations for 2 h at 37°C. After incubation, PMSF and aprotinin were added to reach a final concentration of 2 mM and 0.55 TIU, respectively. As a control, serum-free MCDB 402 medium which had not been exposed to cells was also incubated with plasmin at 37°C for 2 h at which time protease inhibitors were added as described. Plasmin-treated conditioned medium (30 μ l), latent TGF β 1 from platelets (30 ng) or control medium was then examined for TGF β competing activity in a radioreceptor assay. Duplicate samples were also dried and prepared for PAGE followed by immunoblot analysis.

PAGE

After treatments described elsewhere, CHO-conditioned medium was subjected to PAGE using a standard protocol (Laemmli, 1970). Proteins were solubilized in a small volume ($20-50 \ \mu$ l) of Laemmli sample buffer ($62.5 \ m$ M Tris, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) in the presence of absence of 50 mM DTT. The samples were then boiled for 5 min and loaded on a 7.5–15% linear gradient polyacrylamide gel with a 4% stacking gel. Proteins were electrophoresed at 15 mA/0.75 mm gel for 4–5 h. Rainbow markers (Amersham Corp., Arlington Heights, IL) were used to determine molecular mass.

Immunoblot Analysis

After electrophoresis, proteins were transferred from the polyacrylamide gel to nitrocellulose (Schleicher & Schuell, Keene, NH) as described by Towbin et al. (Towbin et la., 1979). Primary antibodies were used at a 1:100 dilution. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used in combination with an avidin-biotin-alkaline phosphatase complex (Vector Laboratories). 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were used as substrates for color development of immune complexes.

Results

Effect of Acid Treatment on Latent Recombinant TGF^β1

Medium-conditioned by CHO cells transfected with simian pre-pro-TGF β 1 cDNA that was acidified as described in Materials and Methods contained $\sim 1 \mu g/ml$ of TGF β competing activity as measured in a TGF β radioreceptor assay (data not shown). This is in agreement with determinations based on the inhibition of DNA synthesis in mink lung cells (Gentry et al., 1987; 1988). Conditioned medium that had been acidtreated was also analyzed by immunoblotting with either amino-terminal (No. 1125) or mature (No. 978) peptide antibodies (Fig. 1) and compared with CHO-conditioned medium that was untreated (Fig. 2). Under nonreducing conditions, both amino-terminal and mature TGF β 1 peptide antibodies detect a 110-kD protein (Fig. 2 A). Only a small amount of 25-kD mature TGF β 1 can be detected as compared to the intensity of the 12.5-kD band visualized when reducing conditions were used (Fig. 2 B). Additional bands visualized under reducing conditions include two polypeptides with apparent molecular masses of 55 and 44 kD when an amino-terminal peptide antibody was used (Fig. 2 B). These polypeptides represent the uncleaved pro-TGF β l polypeptide (55 kD) and the amino-terminal glycopeptide (44 kD) as demonstrated by Gentry et al. (1987; 1988). The ma-

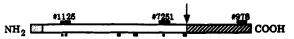


Figure 1. Diagrammatic representation of pre-pro-TGF β 1. Important sites and their relative positions along the 390-amino acid sequence of monomeric TGF β 1 are shown. The signal sequence (residues 1-29, stippled box), the amino-terminal (residues 30-278, open box), and mature (residues 279-390, diagonally hatched box) regions are indicated. The dibasic cleavage site at residue 278 is indicated by an arrow. Solid lines above the molecule represent peptide sequences used for antibody production and the antibody number is indicated. Potential plasmin cleavage sites are indicated by dots (single Arg residues) or lines (monobasic cleavage sequences; Benoit et al., 1987) below the molecule.

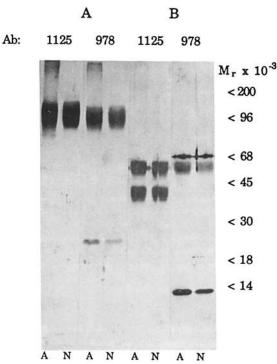


Figure 2. Immunoblot analysis of CHO cell-conditioned medium. Equal volumes (20 μ l) of acid-treated (A) or untreated (N) CHOconditioned medium were subjected to SDS-PAGE on 7.5–15% linear gradient gels. Separated proteins were then transferred to nitrocellulose and probed with peptide antibodies that are specific for amino-terminal (No. 1125) or mature (No. 978) sequences. The migration of rainbow molecular mass markers is shown. A, Samples electrophoresed under nonreducing conditions; B, Samples electrophoresed under reducing conditions in the presence of 50 mM DTT.

A

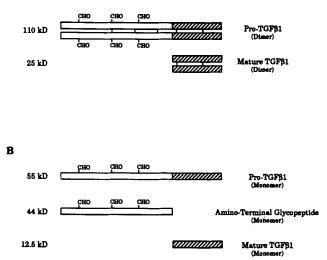


Figure 3. Interpretation of immunoblot analyses. A, Polypeptides observed under nonreducing conditions by immunoblot analysis and their apparent molecular masses are shown. Carbohydrate moieties (CHO) are indicated. Interchain disulfide linkages are shown as vertical lines, but do not indicate position or number. B, Polypeptides detected under reducing conditions and their apparent molecular masses are depicted.

ture sequence peptide antibody recognizes both a 12.5- and a 55-kD band, which further supports the view that the 55-kD band represents pro-TGF β 1. The presence of this molecular form may be the result of incomplete processing because of overexpression.

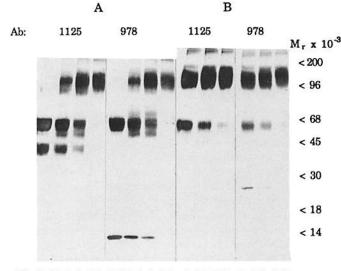
A diagrammatic representation of the polypeptides identified by immunoblot analysis with and without reduction is shown in Fig. 3. The immunoreactive banding pattern of the cell-conditioned medium was the same with or without acid treatment. As mature TGF β 1 polypeptides can be detected with or without prior acid treatment of the conditioned medium, it appears that the denaturing gel conditions (SDS) have the same effect as acid treatment. This is not an unanticipated result, as Lawrence et al. (1985) had shown that many dissociating conditions may lead to activation of latent TGFß. Thus, the nature of latent TGF β 1 cannot be determined from this analysis. The presence of SDS, a dissociating agent, during PAGE leads to the detection of mature TGF β 1 polypeptides in untreated cell-conditioned medium. Furthermore, under reducing conditions, a band of 85-90 kD detected by the amino-terminal antibody is predicted. However, a discrete band of such molecular mass was not observed. This may be because of the inability of this gel system to adequately separate broad bands of high molecular mass.

The intensity of the mature TGF β 1 polypeptides detected with antibody No. 978 is comparable to that obtained when a similar amount (100 ng) of pure, biologically active TGF β is analyzed under identical conditions (data not shown). The mature peptide antibody, No. 978, has greater affinity for the reduced, 12.5-kD polypeptide (L. E. Gentry, unpublished data) which accounts for the apparent inconsistency in the relative intensities of the 25- and 12.5-kD bands. Because of the glycosylation of the precursor sequence as demonstrated by Brunner et al. (1988) several bands (110, 55, and 44 kD) appear as broad, heavily stained regions. In contrast, the mature TGF β polypeptide that does not contain glycosylation sites, is detected as a sharp, discrete band.

Cross-linking of CHO-Conditioned Medium

To determine what proteins may be involved in the latent form of TGF β 1 secreted by the transfected CHO cells, chemical cross-linking of cell-conditioned medium was carried out before electrophoresis (Fig. 4). With increasing concentrations of the cross-linking agent BS³, there is a shift in the molecular mass of polypeptides observed with amino-terminal and mature sequence peptide antibodies. Under reducing conditions (Fig. 4 A), the 55- and 44-kD bands detected with an amino-terminal peptide antibody become less intense in conjunction with the appearance of a high molecular mass $(\sim 110 \text{-kD})$ immunoreactive band. This high molecular mass band was also detected with the mature peptide antibody and coincides with the loss of the 55- and 12.5-kD bands at increasing concentrations of BS3 (Fig. 4 A). In addition, transiently acid-treated conditioned medium was exposed to increasing concentrations of BS³ after reneutralization of the medium (data not shown). Under these conditions, the mature peptide antibody detected a 25-kD peptide at all BS³ concentrations. A high molecular mass band (~110 kD) also appeared.

These results can be compared with cross-linked conditioned medium analyzed under nonreducing conditions (Fig.



BS³, mM: 0 0.5 1.0 2.5 0 0.5 1.0 2.5 0 0.5 1.0 0 0.5 1.0

4 B). In the absence of BS³, the appearance of the 55-kD band suggests that spontaneous reduction has taken place. This has been observed previously and is believed to be the result of storage of conditioned medium (Gentry et al., 1987). The addition of BS³ to CHO-conditioned medium, however, yielded similar results to those described above. That is, a band of ~110 kD was recognized by both aminoterminal and mature peptide antibodies. This high molecular mass band represents a complex containing either two pro-TGF β 1 polypeptides (55 kD), two amino-terminal glycopeptides (44 kD) plus two mature polypeptides (12.5 kD), or a combination of these three polypeptides. These data provide strong evidence that the latent form involves the association of the amino-terminal glycopeptide and mature TGF β l polypeptide and that there are no additional proteins involved in this form of latent TGF β 1. Furthermore, these data indicate that once dissociated, the latent complex cannot be fully reconstituted as detectable quantities of mature TGF β 1 were observed.

Plasmin Activation of Latent TGF β 1

We have previously reported that plasmin, a broad spectrum, serine protease, activated 50-60% of the cell-secreted latent TGF β (Lyons et al., 1988). A similar study was conducted using either CHO-conditioned medium or latent TGF β 1purified from human platelets (Fig. 5). In each case, a smaller proportion of the latent TGF β 1 could be activated by plasmin than by acid treatment. Previous studies have shown that CHO-conditioned medium contained $\sim 1 \ \mu g/ml$ of TGF β 1 after acidification as measured by the inhibition of mink lung epithelial cells (Gentry et al., 1987; 1988) or using a specific TGF β radioreceptor assay (data not shown). Based on the knowledge that plasmin is not as effective as acidification in the activation of latent TGFA, a volume of CHO-conditioned medium was chosen (30 μ l) for plasmin treatment that would allow detection of any resulting biological activity by radioreceptor assay. Incubation of CHOconditioned medium with increasing concentrations of plasmin resulted in an increase in TGF β competing activity. The inhibition of [125]]TGF β binding increased to a maximum of Figure 4. Chemical cross-linking of CHO cell-conditioned medium. Conditioned medium was treated with the indicated concentrations of BS^3 for 45 min and subjected to immunoblot analysis using the indicated peptide antibodies. Rainbow molecular mass markers are indicated. A, Reducing conditions; B, nonreducing conditions.

75% at a plasmin concentration of 2 U/ml. While quantitation of active TGF β l after plasmin treatment is not possible from these experiments, it is clear that plasmin activates a smaller proportion of the latent recombinant TGF β l secreted by transfected CHO cells than does acidification.

Similarly, incubation of latent TGF β l purified from platelets with increasing concentrations of plasmin produced an increase in TGF β competing activity. For reasons cited above, the amount of latent platelet TGF β l used (30 ng) exceeded that which is needed to achieve 100% inhibition when acid treated. As seen with CHO-conditioned medium, plasmin treatment of latent platelet TGF β l produced biologically active TGF β l. However, the maximum inhibition of binding achieved in these experiments was 60%, indicating that only a portion of the acid activatable latent material was activated by plasmin. The ability of plasmin to activate only a portion of latent TGF β l from human platelets is in agreement with results obtained using CHO-conditioned medium.

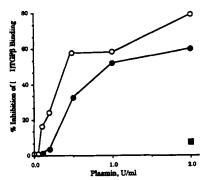


Figure 5. Radioreceptor assay of plasmin treated latent TGF β 1. Conditioned medium (O) or latent TGF β 1 from human platelets (•) was incubated with increasing concentrations of plasmin for 2 h at 37°C. Protease inhibitors were then added to a final concentration of 2 mM PMSF and 0.55 TIU aprotinin. Serum-free MCDB 402 that had not been exposed to cells was treated with plasmin (2 U/ml) as a control (•). Assays were performed in triplicate and the data have been corrected by subtraction of nonspecific binding. In this assay, 50% inhibition of binding can be achieved with 1 ng/ ml of pure TGF β 1.

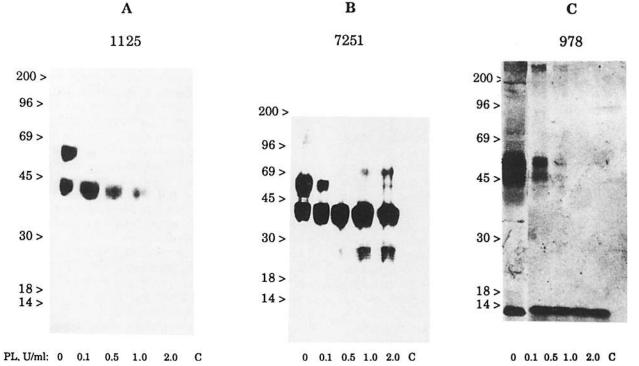


Figure 6. Immunoblot analysis of plasmin-treated CHO cell-conditioned medium. Conditioned medium was treated with the indicated concentrations of plasmin in a manner identical to that described in Fig. 4. Proteins were then electrophoresed under reducing conditions and probed with different peptide antibodies. Plasmin alone (2 U/ml) was examined for its ability to cross react with these peptide antibodies as a control (C). The migration of rainbow molecular mass markers are indicated. A, Antibody No. 1125; B, Antibody No. 7251; C, Antibody No. 978.

Effect of Plasmin on the TGF β 1 Amino-terminal Glycopeptide

In an attempt to address the question of how plasmin was able to activate latent recombinant TGF β 1, plasmin-treated CHO-conditioned medium was analyzed using immunoblotting techniques (Fig. 6). Two amino-terminal peptide antibodies were used to detect changes in immunoreactive polypeptides after plasmin treatment (Fig. 1). Both antibodies show that at the lowest concentration of plasmin used, the 55-kD band is lost and the 44-kD band becomes more intense. This concentration of plasmin, however, produced only minimal TGF β competing activity when examined by radioreceptor assay (Fig. 5). As noted earlier, the 55-kD band represents pro-TGF β 1. Thus, it appears that low concentrations of plasmin are able to process pro-TGF β 1 (55 kD) by proteolytic cleavage at the dibasic cleavage site. This, however, does not produce biologically active TGF β 1. At higher concentrations of plasmin, the intensity of the 44-kD band decreased and was eventually undetectable with antibody No. 1125 (Fig. 6 A). An identical immunoblot probed with a different amino-terminal specific antibody, No. 7251, shows that at higher concentrations of plasmin the 44-kD band is not lost and additional bands of lower molecular weight appear (Fig. 6 B). Finally, when a similar immunoblot was probed with the mature peptide antibody, No. 978, there was an increase in intensity of the 12.5-kD band at the lowest concentration of plasmin used (Fig. 6 C). With increasing concentrations of plasmin, there was a loss of the 55-kD band while the intensity of the 12.5-kD band remained constant. These results suggest that, at concentrations of plasmin which produce biological activity, there is both release of mature TGF β 1 and some degradation of the TGF β 1 amino-terminal glycopeptide.

The ability of the amino-terminal peptide antibodies, No. 1125 and No. 7251, to recognize precursor polypeptides after plasmin treatment of latent $TGF\beta 1$ differs significantly. Antibody No. 1125 does not detect a 44-kD band at plasmin doses which result in biological activity (Fig. 6 A), while antibody No. 7251 recognizes a 44-kD band at all plasmin doses used in this experiment (Fig. 6 B). This suggests that the regions at or near the peptide sequence of the TGF β 1 amino-terminal glycopeptide used to produce antibody No. 1125 are susceptible to proteolytic cleavage and that such protease action results in a loss of the epitope required for recognition by antibody No. 1125. The difference in susceptibility to protease activity may reflect the conformational availability of this region for proteolytic attack and/or represent an important functional domain of the TGF β 1 aminoterminal glycopeptide.

Discussion

All cellular responses to TGF β are the consequence of an interaction of the 25-kD TGF β homodimer with its specific cell surface receptor. However, platelets as well as a variety of cells in culture, release TGF β in an inactive form. Most cells have been shown to have TGF β receptors (Tucker et al., 1984b); however, latent TGF β is not able to compete for TGF β binding sites. Activation of latent TGF β must precede any interaction of the growth factor with its receptor and thus is an important point of control of TGF β function. Understanding the structure and composition of latent TGF β will provide necessary information for determination of the in vivo mechanism of TGF β activation. Thus, the emphasis of this study was to determine the molecular nature of latent TGF β and to address potential physiologically relevant methods of activation.

Previous studies by several investigators (Gentry et al., 1987, 1988; Purchio et al., 1988; Brunner et al., 1988) have shown that TGF β is synthesized as a larger molecule, contains a dibasic cleavage site (Fig. 1), and is glycosylated at sites within the amino-terminal region. The present report expands upon these findings to determine the molecular nature of the latent TGF β 1 molecule. Specifically, acid-activated CHO-conditioned medium was compared directly with untreated cell-conditioned medium using immunoblotting techniques to determine whether additional material would appear in the 25- or 12.5-kD bands upon activation. There was little difference between acid-treated and untreated cell-conditioned medium in this analysis, indicating that denaturing gel conditions (SDS) perform the same function as prior acid treatment. Chemical cross-linking experiments were therefore performed to ascertain the involvement of other proteins in the latent molecule. These studies demonstrate that the latent TGF β 1 secreted by transfected CHO cells contains both the amino-terminal glycopeptide and mature TGF β 1 polypeptide and that no other proteins are required for latency. In addition, when conditioned medium was exposed to acid conditions prior to cross-linking, mature $TGF\beta 1$ remained free from the cross-linked complex. This supports the hypothesis that mature TGF β 1 is noncovalently associated with the amino-terminal glycopeptide and that following activation by acid treatment, the latent complex can no longer be completely reconstituted.

Platelets are a rich source of TGF β and other investigators have shown that platelets release TGF β in an inactive form (Lawrence et al., 1984; Wakefield et al., 1988). The latent TGF β released by platelets and characterized by Miyazono et al. (1988), contains both the amino-terminal and mature TGF β polypeptides. The involvement of precursor sequences in latent TGF β from human platelets was further established by Wakefield et al. (1988) through the use of chemical crosslinking techniques. The carbohydrate moieties contained in the precursor peptide have recently been shown to be important in rendering platelet TGF^β inactive (Miyazono and Heldin, 1989), suggesting the involvement of carbohydrate structures in the stabilization of the association of amino-terminal and mature TGF β peptides in the latent molecule. Unlike cell-secreted latent TGF β , however, latent TGF β released by platelets contains an additional protein that is unrelated to the TGF β polypeptides. This binding protein appears to be covalently associated through disulfide linkage(s) to the amino-terminal glycopeptide of the latent complex. Although a binding protein appears to be an integral part of latent TGF β released by platelets, acid activation of this material can be achieved, suggesting that dissociation of mature, biologically active TGF β from this complex is possible. Thus, the role of the binding protein in this complex remains unclear. The present study demonstrates that such a binding protein is not necessary for latency of CHO cell-secreted TGF\$1.

Proteolytic activation of latent TGF β in cell-conditioned medium has been investigated in an earlier report (Lyons et al., 1988). Plasmin was shown to activate 50-60% of the acid activatable pool of latent TGF β secreted by fibroblasts. Conditioned medium from TGF^{β1}-transfected CHO cells was treated with plasmin in this study to determine if the recombinant latent TGF β 1 could be similarly activated. While quantitation was not possible, activation of recombinant latent TGF β 1 was achieved by plasmin treatment. In addition, latent TGF β 1 from a different source, human platelets, was also activated by the same concentrations of plasmin. Although latent platelet TGF β 1 contains an additional binding protein (Miyazono et al., 1988), activation of these two different forms of latent TGF β 1 by either acidification or plasmin treatment indicates that the platelet latent $TGF\beta I$ binding protein has little or no influence on the activation processes thus far examined.

Furthermore, at concentrations of plasmin that did not result in biological activity, there was a significant loss in detection of the 55-kD band (pro-TGF\$1 monomer) and an increase in the intensity of the 12.5-kD peptide (mature TGF β 1 monomer). This suggests that the dibasic cleavage site within the pro-TGF β 1 molecule, which separates the amino-terminal glycopeptide and mature peptide, may be a preferential site for plasmin action. However, proteolytic cleavage at this site is not sufficient to produce biological activity, indicating that the cleaved mature peptide remains in association with the amino-terminal glycopeptide at low plasmin concentrations. At higher plasmin concentrations, measurable biological activity was observed and additional bands were detected using an amino-terminal glycopeptide antibody for immunoblot analysis. This suggests that the appearance of plasmin-generated TGF β activity is accompanied by proteolytic cleavage within the amino-terminal glycopeptide.

The data presented in this study support the hypothesis of proteolytic degradation of the TGF β amino-terminal glycopeptide as a physiological mechanism of latent TGF β activation. A model of plasmin activation is presented (Fig. 7) and compared with acid activation. It should be noted that the latent molecule (110-kD) is shown to have been cleaved at the dibasic cleavage site between the amino-terminal glycopeptide and the mature region. This is believed to occur before secretion of the latent complex; however, overexpression of pre-pro-TGF β 1 by CHO cells may result in a significant amount of incompletely processed protein. As improper processing is considered to be an artefact of overexpression, only molecules that have been cleaved at the dibasic site are included in this model of activation of latent TGF β 1. Dissociation of mature TGF β from the amino-terminal glycopeptide is an essential feature for activation by either acid or plasmin. However, plasmin acts by cleaving the amino-terminal glycopeptide that is postulated to result in a conformational change in the amino-terminal glycopeptide such that mature TGF β is released. In contrast, acid acts by disrupting hydrogen and other noncovalent bonds through changes in ionic strength without degradation of the amino-terminal glycopeptide.

The importance of the amino-terminal glycopeptide in stabilizing the latent form of TGF β 1 has been further substantiated using mutant proteins (Brunner et al., 1989). The substitution of cysteine residues within the amino-terminal glycopeptide resulted in the secretion of biologically active TGF β 1. In addition, only monomeric amino-terminal glyco-

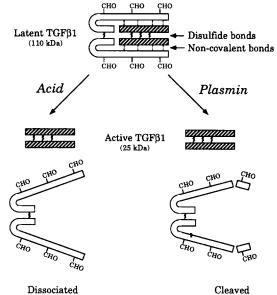




Figure 7. Model of acid and plasmin activation of cell-secreted latent TGF β 1. Open boxes represent amino-terminal glycopeptides and diagonally hatched boxes represent mature polypeptides. Processing has occurred by proteolytic cleavage at the dibasic site (Fig. 2) and the amino-terminal glycopeptides and mature polypeptides remain associated through noncovalent interactions (broken lines). Disulfide linkages are represented by \$, but do not indicate number or position; carbohydrate moieties (CHO) are indicated. The molecular mass of latent and mature TGFB1 is indicated. The predicted molecular mass of the dissociated amino-terminal glycopeptide is 85-90 kD; however, this molecular form was not separated from the 110 kD latent TGF β 1. The cleaved amino-terminal glycopeptide was not examined under nonreducing conditions. Under reducing conditions, however, several bands of \sim 25–27 kD were observed; thus, the predicted molecular mass of the plasmin-cleaved amino-terminal glycopeptide is ~50-55 kD.

peptides were observed. This study indicates that latent TGF β 1 is stabilized by dimerization of the amino-terminal glycopeptide and further supports the hypothesis that disruption of the amino-terminal glycopeptide will release mature, biologically active TGF β 1.

Plasmin has been shown to activate latent TGF β from several sources. In addition, the activation of latent TGF β during the co-culture of bovine endothelial cells and pericytes can be diminished by the addition of a specific plasmin inhibitor to the co-culture system (Sato and Rifkin, 1989). Finally, the ability of TGF β to increase message and activity levels of plasminogen activator inhibitor as well as decrease plasminogen activator activity (Laiho et al., 1986*a*, *b*; Keski-Oja et al., 1988), provides an excellent system for the negative feedback regulation of TGF β action formerly proposed (Lyons et al., 1988). These data make plasmin an attractive candidate for a physiological activator of latent TGF β .

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