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Recombinant Type 1 Transforming Growth Factor β Precursor Produced in Chinese Hamster Ovary Cells Is Glycosylated and Phosphorylated

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Analyses of cDNA clones coding for simian type 1 transforming growth factor β (TGF-β1) suggest that there are three potential sites for N-linked glycosylation located in the amino terminus of the precursor region. Analysis of [^{3}H]glucosamine-labeled serum-free supernatants from a line of Chinese hamster ovary cells which secrete high levels of recombinant TGF-β1 indicate that the TGF-β1 precursor, but not the mature form, is glycosylated. Digestion with neuraminidase resulted in a shift in migration of the two TGF-β1 precursor bands, which suggests that they contain sialic acid residues. Endoglycosidase H had no noticeable effect. Treatment with *N*-glycanase produced two faster-migrating sharp bands, the largest of which had a molecular weight of 39 kilodaltons. TGF-β1-specific transcripts produced by SP6 polymerase programmed the synthesis of a 42-kilodalton polypeptide which, we suggest, is the unmodified protein backbone of the precursor. Labeling with $^{32}P_i$ showed that the TGF-β1 precursor was phosphorylated in the amino portion of the molecule.

DNA sequence analyses of clones coding for human (2), murine (3), and simian (11) type 1 transforming growth factor β (TGF-β1) indicate that this protein is synthesized as a larger, 390-amino-acid precursor polypeptide, the carboxy terminus of which is proteolytically cleaved to yield the mature 112-amino-acid monomer. Analyses of these sequences further predict the existence of three potential sites of N-linked glycosylation which, in the case of simian TGF-β1, are located at amino acid residues 82, 136, and 176 (11).

We have recently succeeded in expressing high levels of biologically active, recombinant simian TGF-β1 in Chinese hamster ovary (CHO) cells (4). Using site-specific antipeptide antibodies directed against various regions of the TGF-β1 precursor, we demonstrated that these cells secrete both mature TGF-β1 and TGF-β1 precursor polypeptides. The structural features of the TGF-β1 precursor which are relevant to this study are illustrated in Fig. 1A. The hydrophobic leader cleaved is from the protein at amino acid residue 29 (L. E. Gentry et al., manuscript in preparation), producing a 361-amino-acid polypeptide (Fig. 1A, a). Subsequent modification and cleavage would result in a mature TGF-β1 monomer (Fig. 1A, c) and a 249-amino-acid protein consisting entirely of amino terminal precursor residues (Fig. 1A, b).

The cell line used in the experiments described below is designated TGFβ3-2000. The TGF-β1-related proteins secreted by these cells were analyzed by immunoblotting (Fig. 1B). As described previously (4), supernatants derived from TGFβ3-2000 cells contain a large (90- to 110-kilodalton [kDa]) form of TGF-β1 as well as the mature 24-kDa protein dimer, as determined by analysis with sodium dodecyl sulfate (SDS)-polyacrylamide gels under nonreducing conditions. When analyzed under reducing conditions, these supernatants are found to contain a 44- to 55-kDa band (Fig. 1B, lane 2, a), a 30- to 42-kDa band (Fig. 1B, lane 2, b), and a 12-kDa band (Fig. 1B, lane 2, c), which is the mature TGF-β1 monomer. Evidence that bands a, b, and c in Fig. 1B contain the regions of the TGF-β1 precursor shown in Fig. 1A has been presented previously (4). These bands can be easily visualized when [^{35}S]methionine- and [^{35}S]cysteine-labeled supernatants from TGFβ3-2000 cells are analyzed directly by SDS-polyacrylamide gel electrophoresis followed by fluorography (Fig. 1C, lane 1, and Fig. 1D, lane 1); these bands are not detected in supernatants from nontransfected CHO cells (4).

The diffuse nature of bands a and b in Fig. 1 suggested that they might be glycosylated. To investigate this possibility, TGFβ3-2000 cells were labeled with [^{3}H]glucosamine, and cell-free supernatants were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The 90- to 110-kDa form was labeled; no label was detected in the mature 24-kDa protein dimer (Fig. 1C, lane 2). When analyzed under reducing conditions (Fig. 1D, lane 2), bands a and b were labeled. No label was found in band c.

The nature of this glycosylation was further investigated by treating supernatants from TGFβ3-2000 cells with various glycolytic enzymes and then fractionating the digestion products on SDS-polyacrylamide gels. Figure 2A shows an immunoblot of these digestion products. Neuraminidase treatment caused bands a and b to migrate as faster but still diffuse bands (Fig. 2A, lane 3), indicating the presence of sialic acid residues. Endoglycosidase H, which predominantly cleaves high-mannose oligosaccharide chains, had no noticeable effect (Fig. 2A, lane 4). Digestion with *N*-glycanase, which removes N-linked carbohydrates, caused bands a and b to migrate as two sharp bands, the largest of which had a molecular weight of approximately 39 kDa (Fig. 2A, lane 2). As expected, no change in the migration of the mature 12-kDa monomer was noted. The same results were obtained with [^{35}S]methionine- and [^{35}S]cysteine-labeled supernatants from TGFβ3-2000 cells (Fig. 2B).

To determine the size of the unmodified TGF-β1 precursor, a 1,350-base-pair *Pst*I-*Eco*RI fragment containing the entire coding region of TGF-β1 (11) was subcloned into pSP64 and transcribed with SP6 polymerase (6). Analysis of

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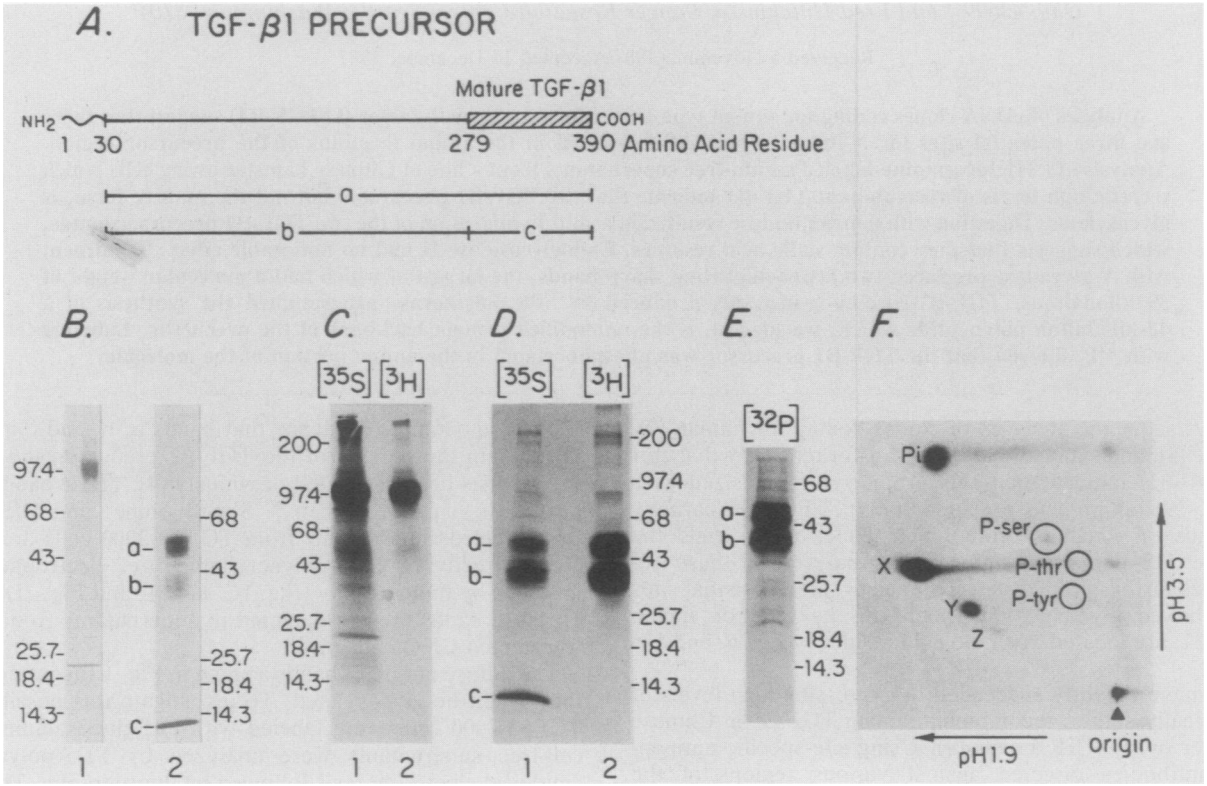

FIG. 1. TGF-β1-related proteins secreted by TGFβ3-2000 cells. (A) Line diagram of TGF-β1 precursor. See text for discussion. (B) TGFβ3-2000 cells were grown to confluency in 100-mm dishes as described previously (4). Serum-free supernatant (5 ml) was collected and dialyzed against 0.2 M acetic acid, and 0.5 ml samples were lyophilized and immunoblotted as described elsewhere (4) with a pool of antipeptide antibodies against amino acid residues 369 to 381 (antiTGFβ$_{369-381}$) and 81 to 94 (antiTGFβ$_{81-94}$). Lane 1, Nonreduced sample; lane 2, reduced sample. The numbers to the left of lane 1 and to the right of lane 2 indicate the positions of molecular weight standards in kilodaltons. (C) TGFβ3-2000 cells were grown to confluency in 60-mm tissue culture dishes and pulsed for 15 min in serum-free medium (3 ml) containing 100 to 200 μCi of [^{35}S]cysteine and [^{35}S]methionine per ml. Cells were then chased for 4 h in serum-free medium containing unlabeled methionine and cysteine. A 10-μl sample was analyzed on a 7.5 to 15% polyacrylamide–SDS gradient gel under nonreducing conditions as previously described (7) (lane 1). A second dish of cells was labeled for 24 h in serum-free medium containing 200 μCi of [^{3}H]glucosamine per ml, and the cell-free supernatant was dialyzed for 48 h against 0.2 M acetic acid. Of this material, 200 μl was lyophilized and analyzed on a 7.5 to 15% polyacrylamide–SDS gradient gel under nonreducing conditions. The gel was fluorographed and exposed for autoradiography with Cronex-4 X-ray film (Du Pont Co., Wilmington, Del.). (D) Same as panel C, except that samples were run under reducing conditions. (E) TGFβ3-2000 cells were labeled with 1 mCi of $^{32}P_i$ per ml in serum- and phosphate-free medium. Cell-free supernatants were treated as described above and fractionated on a 15% polyacrylamide–SDS gel under reducing conditions, and the gel was autoradiographed. (F) ^{32}P-labeled precursor was purified by two cycles of polyacrylamide-SDS gel electrophoresis and hydrolyzed for 1 h at 95°C in 6 M HCl (1). The digestion products were separated by electrophoresis at pH 1.9 and pH 3.5 and detected by autoradiography. Internal standards (P-Ser, P-Thr, P-Tyr) were detected with ninhydrin.

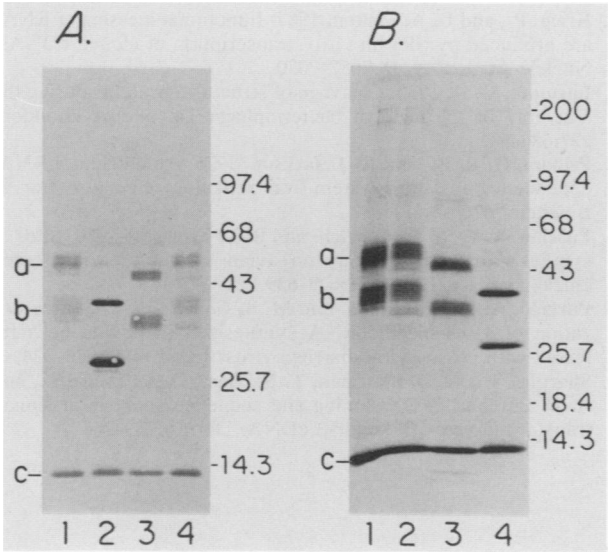

FIG. 2. Digestion of serum-free supernatants from TGFβ3-2000 cells with various glycolytic enzymes. (A) TGFβ3-2000 cells were grown to confluence and incubated for 24 h in serum-free medium. The medium was dialyzed against 0.2 M acetic acid and lyophilized. Samples were treated with neuraminidase (0.25 U/ml, lane 3), *N*-glycanase (20 U/ml, lane 2), or endoglycosidase H (0.2 U/ml, lane 4). The digestion products were fractionated by SDS-polyacrylamide gel electrophoresis under reducing conditions and analyzed by immunoblotting as described in the legend to Fig. 1. Lane 1 contains an untreated sample. The numbers to the right of panels A and B indicate the positions of molecular weight standards in kilodaltons. (B) Serum-free supernatants from TGFβ3-2000 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine as described in the legend to Fig. 1 and digested as described above with endoglycosidase H (lane 2), neuraminidase (lane 3), or *N*-glycanase (lane 4). Lane 1 contains an untreated sample. Digestion products were fractionated by SDS-polyacrylamide gel electrophoresis under reducing conditions, and the gels were autoradiographed. *N*-glycanase was purchased from Genzyme, Boston, Mass., and endoglycosidase H and neuraminidase were from Calbiochem-Behring, La Jolla, Calif. Buffer conditions were those recommended by the manufacturer.

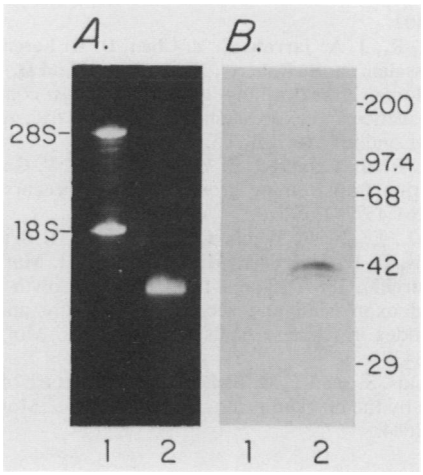

FIG. 3. Cell-free translation of TGF-β1-specific transcripts. (A) A 1,350-base-pair *Pst*I-*Eco*RI fragment containing the entire coding region of TGF-β1 (11) was subcloned into pSP64 (Pharmacia, Inc., Piscataway, N.J.), and 5 μg of linearized plasmid was transcribed with SP6 polymerase (6; Bethesda Research Laboratories, Inc., Baltimore, Md.) under ionic conditions described previously (5). The reaction product was digested with DNase, extracted twice with phenol-chloroform-isoamylalcohol (24:24:1), and ethanol precipitated. The RNA was dissolved in 50 μl of H_2O, and 10 μl of the RNA was fractionated in a 1% agarose–urea gel as previously described (10) (lane 2). Lane 1 contains reticulocyte rRNA markers. The gel was stained with ethidium bromide, illuminated with UV, and photographed. (B) The RNA described above was treated for 10 min at 22°C with 10 mM methyl mercury and adjusted to 20 mM 2-mercaptoethanol. A 1-μg portion of the RNA was translated in a message-dependent reticulocyte cell-free translation system (8) in a total volume of 50 μl with [^{35}S]methionine under ionic conditions described previously (9). The reactions were fractionated on a 10% polyacrylamide–SDS gel (7); the gel was fluorographed and exposed to Cronex-4 X-ray film (Du Pont). Lane 1, No added RNA; lane 2, 1 μg of TGF-β1 RNA.

these transcripts on agarose-urea gels indicated that a single RNA species was produced (Fig. 3A). This species programmed the synthesis of a 42-kDa polypeptide in a message-dependent reticulocyte cell-free translation system (Fig. 3B). Longer exposure of this gel revealed the presence of a minor 40-kDa product. The size (42 kDa) of the major cell-free translation product is in agreement with that expected for a 390-amino-acid protein and most likely corresponds to the unmodified TGF-β1 polypeptide backbone. The 39-kDa bands shown in Fig. 2A, lane 2, and Fig. 2B, lane 4, would then represent the deglycosylated protein core of TGF-β1 minus the hydrophobic leader sequence (Fig. 1A, a [Gentry et al., manuscript in preparation]). The band below this corresponds to the deglycosylated band b in Fig. 1A.

Incubation of TGFβ3-2000 cells in the presence of $^{32}P_i$ and subsequent fractionation of cell-free supernatants on SDS-polyacrylamide gels indicated that the TGF-β1 precursor, but not the mature 12-kDa monomer, was phosphorylated (Fig. 1E). Thin-layer electrophoresis of acid hydrolysates showed that most of the phosphate was not attached to serine, threonine, or tyrosine (Fig. 1F, X, Y, and Z). Preliminary experiments suggest that these phosphate residues are attached to the carbohydrate moiety of the TGF-β1 precursor (data not shown). Minor amounts of phosphate were found as phosphoserine (Fig. 1F).

The amino portions of the precursor region of TGF-β1 from human, rodent, and simian sources show a high degree of homology (2, 3, 11), suggesting that an important biological function is associated with this part of the molecule. The data shown in this report demonstrating that this portion of the TGF-β1 precursor is glycosylated and phosphorylated support this contention, since one might assume that a cell would not go through the expense of performing these secondary modifications if they were not for a specific function. These modifications may be important for dimerization of the precursor or for directing its movement out of the cell; perhaps this phosphoglycoprotein is free to perform other intra- or extracellular functions. Antibodies that recognize the native TGF-β1 precursor should be useful in localizing this protein and in determining its function.

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