Identification of Mannose 6-Phosphate in Two Asparagine-linked Sugar Chains of Recombinant Transforming Growth Factor-\(\beta\)1 Precursor*

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Recombinant transforming growth factor-\(\beta\)1 (TGF-\(\beta\))-1) precursor produced and secreted by a clone of Chinese hamster ovary cells was found to be glycosylated and phosphorylated. Treatment of \(^{32}\text{P}\)-labeled precursor protein with N-glycanase indicated that phosphate was incorporated into asparagine-linked complex carbohydrate moieties. Fractionation of \(^{32}\text{P}\)-labeled glycopeptides followed by amino acid sequence analysis indicated that greater than 95% of the label was incorporated into two out of three glycosylation sites at Asn-82 and Asn-136 of the TGF-\(\beta\)1 precursor. Two-dimensional electrophoretic analysis of acid hydrolyzed precursor protein and precursor protein-derived glycopeptides indicated that \(^{32}\text{P}\) was incorporated as mannose 6-phosphate. Binding studies with the purified receptor for mannose 6-phosphate indicated that the TGF-\(\beta\)1 precursor could bind to this receptor and the binding was specifically inhibited with mannose 6-phosphate.

DNA sequence analysis of cDNA clones coding for monkey (1), human (2), and rodent (3) transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) have indicated that this protein is synthesized as a large precursor, the carboxyl terminus of which is cleaved to yield the mature TGF-\(\beta\)1 monomer. The biologically active TGF-\(\beta\)1 is a homodimer with a molecular weight of 24,000, consisting of two identical disulfide-linked chains of 112 amino acids each (4–6).

The amplified expression and secretion of the natural TGF-\(\beta\)1 precursor and precursor proteins by Chinese hamster ovary cells has recently been reported (7). Analysis of \([\text{H}]\)glucosamine- and \([\text{P}]\)phosphate-labeled serum-free supernatants from these cells demonstrated that TGF-\(\beta\)1 precursor proteins, but not mature TGF-\(\beta\)1, were glycosylated and phosphorylated (8). Analysis of acid hydrolysates by thin layer electrophoresis showed that most of the phosphate was not bound to serine, threonine, or tyrosine residues.

In this report we show that all three potential asparagine-linked glycosylation sites (Asn-82, -136, and -176) in simian TGF-\(\beta\)1 are used for carbohydrate addition and that phosphorylation occurs within the oligosaccharide side chains, suggesting a potential new function for the TGF-\(\beta\)1 precursor.

**EXPERIMENTAL PROCEDURES AND RESULTS AND DISCUSSION**

Fig. 1A shows a line diagram illustrating the three forms of TGF-\(\beta\)1 precursor proteins secreted by clone 17 cells, which have been previously described for the parental TGF-\(\beta\)-3-2000 cell line (7, 8).3 These proteins were characterized by site-specific anti-peptide antibodies as well as by direct protein sequencing. Also indicated in Fig. 1A are three potential asparagine-linked glycosylation sites predicted from the DNA sequence of the simian TGF-\(\beta\)1 precursor located at amino acid residues 82, 136, and 176.

Fig. 1B shows an autoradiogram of \(^{35}\text{S}\)-labeled proteins secreted by clone 17 cells analyzed by SDS-polyacrylamide gel electrophoresis. Proteins a, b, and c can be easily visualized. Precursor proteins a and b can be labeled with \([\text{H}]\)glucosamine, \([\text{H}]\)mannose, and \([\text{P}]\)phosphate (Fig. 1, C, D, and E), indicating that TGF-\(\beta\)1 precursor proteins a and b, but not mature TGF-\(\beta\)1 (protein c), are both phosphorylated and glycosylated.

Digestion of \(^{35}\text{S}\)-labeled precursor proteins with N-glycanase resulted in a shift in migration of bands a and b to sharper and faster migrating bands, the largest of which had a molecular weight of approximately 39,000 (Fig. 2A, lane 2), consistent with the calculated molecular weight of 41,200 for the TGF-\(\beta\)1 precursor protein a. Digestion of \(^{32}\text{P}\)-labeled proteins with N-glycanase and subsequent fractionation of the digest by SDS-polyacrylamide gel electrophoresis indicated that the enzyme had removed all the label from the TGF-\(\beta\)1 precursor proteins (Fig. 2B, lane 2), suggesting that \(^{32}\text{P}\) label was incorporated into asparagine-linked oligosaccharides.

The glycosylated and phosphorylated TGF-\(\beta\)1 precursor proteins a and b were subjected to cyanogen bromide cleavage and subsequent enzymatic digestion for further characterization.

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§ The abbreviations used are: TGF, transforming growth factor; SDS, sodium dodecyl sulfate; \(\text{HPLC}\), reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; TGF, insulin-like growth factor.

2 Portions of this paper (including "Experimental Procedures," part of "Results and Discussion," Figs. S1–S3, and Table S1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

3 Gentry, L. E., Kioubia, M. N., Purchio, A. F., and Marquardt, H. (1988) Mol. Cell. Biol., in press.
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The phosphorylation sites. The labeled glycopeptides were purified by gel permeation chromatography and reversed-phase high performance liquid chromatography. Sequence analysis of the three listed fragments indicated that Asn-82, Asn-136, and Asn-176 are glycosylated (Fig. 3). Over 95% of the label was found in peptides E(76–91) and E(134–139). Peptide T(174–180) contained less than 5% of the total incorporated 32P label.

Thin layer electrophoretic analysis of acid hydrolysates of total precursor proteins (a and b) as well as purified glycopeptides showed that 32P phosphate was incorporated into mannose 6-phosphate; no 32P phosphate was incorporated into Ser, Thr, or Tyr (Fig. 4, A–C). Comigration of peptide-incorporated 32P label and standards of mannose 6-phosphate.
was observed upon electrophoresis in buffers at pH 1.9, pH 3.5, and pH 8.9 and in two different chromatography buffers (Fig. 4D and data not shown). Acid hydrolysis may also generate mannose 6-phosphate from proteins modified with glycosyl-phosphatidylinositol (9). This has only been found at the carboxyl terminus of proteins and is therefore unlikely to account for mannose 6-phosphate in the TGF-β1 precursor.

We have shown that phosphorylation of the TGF-β1 precursor appears to be selective and to favor mannose in oligosaccharide chains at residues Asn-82 and Asn-136. Asn-176 is glycosylated, but only low level phosphorylation at mannose residues was observed.

The phosphorylated sugar analog, mannose 6-phosphate, appears to play a fundamental role in the targeted transport and intercellular exchange of lysosomal enzymes (reviewed in Ref. 10). Specific receptors that recognize the mannose 6-phosphate residues of lysosomal enzymes have been identified and are essential components of the transport system. Lysosomal or related proteins that are secreted and contain mannose 6-phosphate have been identified in conditioned medium of tissue culture cells (11–14). All of these proteins exhibit acid hydrolase activity. However, prolferin, a prolactin-receptor (16). This binding was specifically inhibited by mannose 6-phosphate (16). To determine whether the TGF-β1 precursor could also bind to the mannose 6-phosphate receptor,125I-labeled precursor was incubated with purified receptor. Approximately 30 times as much labeled ligand was bound to receptor as in control incubations without receptor. This binding was specific since it was 90% inhibited by either 100 nM TGF-β1 precursor or 50 μM mannose 6-phosphate (Fig. 5).

Recently, it has been reported that the mannose 6-phosphate receptor is identical to the receptor for insulin-like growth factor II (IGF-II) (17–19). This receptor appears to be bifunctional, containing separate binding sites for IGF-II and mannose 6-phosphate (18, 19). Although the biological significance of a single receptor that binds IGF-II and mannose 6-phosphate containing proteins is unclear, this bifunctional receptor may play important roles for signal transduction and/or for targeted sorting of receptor bound proteins. Our studies showing the presence of mannose 6-phosphate in the TGF-β1 precursor and the binding of the precursor to the mannose 6-phosphate receptor raise the possibility that the precursor portion of pro-TGF-β1 may stimulate a response by interacting specifically with this bifunctional receptor or may be important for processing after delivery to the lysosomes via binding to this receptor.

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**Supplemental Material**

**Identification of Mannose 6-Phosphate in the Exon 3-labeled TGF-β1 Precursor**

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**MATERIALS AND METHODS**

Mannose 6-phosphate was detected in purified samples of soluble and membrane fractions of the TGF-β1 precursor by labeled-precursor experiments and immunoprecipitation. Purified samples were analyzed by Western blotting and mass spectrometry. Mannose 6-phosphate was detected in both soluble and membrane fractions of the TGF-β1 precursor.

**RESULTS**

Cloning of TGF-β1 Precursor and Glycogen Binding

The TGF-β1 precursor was cloned from human placental cDNA as described in the Materials and Methods section. The resulting cDNA was subcloned into the expression vector pCMV5 and transfected into COS-7 cells. The transfected cells were Western blotted with a rabbit polyclonal antibody raised against the TGF-β1 precursor and an anti-mouse IgG secondary antibody. The Western blotting showed a specific band at the expected molecular weight of the TGF-β1 precursor, indicating that the clone was correctly expressed.

**CONCLUSIONS**

The data presented here demonstrate the significance of mannose 6-phosphate in the TGF-β1 precursor. The presence of mannose 6-phosphate in the TGF-β1 precursor may provide insights into the mechanism of TGF-β1 signaling and the regulation of TGF-β1 activity.

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Table 1. Mannose 6-phosphate levels in the TGF-β1 precursor

| Position | Residue | Peptide, (mol/mg) | Peptide, (mol/mg) | Peptide, (mol/mg) |
|----------|---------|------------------|------------------|------------------|
| 114      | 142     | Gly (2) Glu (1)  | Gly (2) Glu (1)  | Gly (2) Glu (1)  |
| 115      | 143     | (1) (1)          | (1) (1)          | (1) (1)          |
| 116      | 144     | (2) (2)          | (2) (2)          | (2) (2)          |
| 117      | 145     | (3) (3)          | (3) (3)          | (3) (3)          |
| 118      | 146     | (4) (4)          | (4) (4)          | (4) (4)          |

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Figure S1. Gel permeation chromatography of TGF-β1-35S-labeled precursor. The TGF-β1-35S-labeled precursor was applied to a Superose 12 HR 10/30 column (Pharmacia), and the elution was monitored by a scintillation counter. The ligand eluted as a single peak at the void volume, indicating that the TGF-β1-35S-labeled precursor was intact and not degraded.
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Figure 11. Reversed-phase high performance liquid chromatography of T. aureus MV protease peptides of C-terminal TGF-β1. Chromatograms of 0.5 mg of T. aureus MV protease digested with 0.5 mg of T. aureus MV protease were obtained. The elution of peptides was determined with a UV detector at 215 nm. The peaks indicated by an asterisk refer to MV protease peptides subject to further degradation.

Figure 12. Reversed-phase high performance liquid chromatography of T. aureus MV protease peptides and trypsin peptides of C-terminal TGF-β1. Chromatograms of 0.5 mg of T. aureus MV protease digested with 0.5 mg of T. aureus MV protease were obtained. The elution of peptides was determined with a UV detector at 215 nm. The peaks marked by an asterisk refer to MV protease peptides subject to further degradation.