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INTERACTIONS OF RECOMBINANT AND PLATELET TRANSFORMING GROWTH FACTOR-β1 PRECURSOR WITH THE INSULIN-LIKE GROWTH FACTOR II/MANNOSE 6-PHOSPHATE RECEPTOR

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SUMMARY: Recombinant transforming growth factor (TGF)-β1 precursor was recently found to contain mannose 6-phosphate (Purchio et al., 1988, J. Biol. Chem. 263, 14211-14215). In the present study, recombinant TGF-β1 precursor was shown to bind to the insulin-like growth factor (IGF)-II/mannose 6-phosphate (man6P) receptor on the plasma membrane of cells since: 1) Insulin, which induces an increase in cell surface IGF-II/man6P receptors on adipocytes, caused a 2.7-fold increase in TGF-β1 precursor binding to adipocytes; 2) Chinese hamster ovary cells selected for overexpression of the IGF-II/man6P receptor exhibited an increased binding of TGF-β1 precursor in comparison to the parental cells; and 3) the binding of ^{125}I-TGF-β1 precursor to these transfected cells and adipocytes was largely inhibited by man6P. After 15 minutes at 37°C, 75% of the recombinant TGF-β1 precursor was found to be internalized in the transfected cells. Additional studies with latent TGF-β1 isolated from platelets indicated that this material could also bind to the isolated IGF-II/man6P receptor.

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The abbreviations used are: TGF, transforming growth factor; man6P, mannose 6-phosphate; IGF, insulin-like growth factor; CHO, Chinese hamster ovary.

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These results demonstrate that the TGF-β1 precursor binds to the IGF-II/man6P receptor on cells and suggest that this interaction could be physiologically important.

Transforming growth factor (TGF) -β1 is a member of a growing family of structurally related peptides which includes TGF-β2 and β3, the inhibins, Mullerian-inhibiting substance, the product of the decapentaplegic complex of Drosophila, and two of the bone morphogenetic proteins (1-4). TGF-β1 was first identified by its ability to stimulate growth of rat fibroblasts (5). However, subsequent studies have shown that TGF-β1 can also inhibit growth of many cell types including epithelial cells, endothelial cells, and lymphocytes (1,3,6). TGF-β1 also can have effects on differentiation (both stimulatory and inhibitory) as well as modulate various functions of numerous cell types (1,3). Although many cells can synthesize TGF-β1, a major storage site of TGF-β is the alpha granule of platelets (1,3), consistent with the proposed role of TGF-β1 in wound healing (7,8).

The isolation, sequencing, and expression of cDNA clones for TGF-β1 have shown that this protein is synthesized as a pre-prohormone which is subsequently cleaved to yield the mature TGF-β1 (9,10). However, cleavage alone is not sufficient to generate active TGF-β1. The TGF-β1 released from platelets as well as other cell types is in a biologically inactive state (11,12). This material can be activated in vitro by transient acidification, proteolysis or via the use of various chaotropic agents (13,14). Recent studies of the latent TGF-β1 released from platelets have shown that the mature TGF-β remains non-covalently associated with the remainder of the TGF-β precursor after cleavage (15,16). In addition, an unidentified component (Mr ~ 125,000) was found to be linked via a disulfide bond to the cleaved TGF-β precursor remainder (15,16).

Studies of TGF-β1 released from CHO cells selected for overexpression of the cDNA encoding this protein have shown that this material is also in a latent state (10). Unlike the TGF-β1 released from platelets, the recombinant TGF-β1 does not contain the additional 125 kDa component. These results suggest that this component is not required for the latent state.

An understanding of the in vivo mechanism for activation of the latent TGF-β complex may be of importance in determining how the actions of TGF-β are targeted to specific cells. In addition, it may be important in understanding why certain tumor cells are

resistant to the growth inhibitory effects of TGF-β (3,17). A recent clue to a potential mechanism for activation of TGF-β came from the finding that the recombinant TGF-β1 precursor contained man6P (18). At least two specific receptors for man6P are present in cells (19,20), one of which (the cation-independent form) has recently been shown to be identical to the receptor for IGF-II (21-24). This receptor has been implicated in the targeting of lysosomal enzymes to the lysosomes (19,20). In addition, a fraction of these receptors is present on the plasma membrane of cells (19, 20). Moreover, insulin can induce a translocation of this IGF-II/man6P receptor from inside the cell to the plasma membrane (25,26). The present studies were therefore designed to analyze the interaction of this receptor with the TGF-β1 precursor.

MATERIALS AND METHODS

Materials: Recombinant IGF-II was the gift of Dr. M. Smith (Eli Lilly Co.) and was iodinated (160 Ci/g) as described previously (27). Recombinant TGF-β1 precursor was prepared and iodinated (190 Ci/g) as described (18). Purified latent TGF-β1 was isolated from platelets (15) and desalted on a Superose 6 column. The IGF-II/man6P receptor and polyclonal antibodies to this protein were as previously described (28). CHO cells overexpressing the human IGF-II/man6P receptor were produced as described previously for the human IGF-I receptor (27). Monoclonal antibodies to the IGF-II/man6P receptor (29) were a gift of Dr. A. Hasilik (Universität Munster). A polyclonal anti-peptide antibody (to residues 81-94) to the precursor sequence of TGF-β1 was prepared as described (10).

Binding and Internalization Studies: The epididymal fat pads from male Sprague-Dawley rats (180-220 g) were excised, minced, and collagenase-digested as described (30). The isolated adipocytes were pre-incubated with or without 1 μM insulin for 10 min at 37°C in 0.7 ml Krebs-Ringer buffer (107 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 7 mM $NaHCO_3$, 10 mM glucose, and 20 mM Hepes, pH 7.8) with 3% bovine serum albumin. The indicated labeled ligand was added and the incubations continued for 45 min at 37°C. The cells were separated from the media by centrifugation through silicon oil and the radioactivity in the cell layer counted.

Binding and internalization studies with transfected and control CHO cells were performed in 24-well culture plates. Confluent wells of cells were washed and incubated for 5 h at 4°C with the labeled ligand in 0.3 ml of binding buffer (100 mM Hepes, pH 7.6, 120 mM NaCl, 1.2 mM $MgSO_4$, 15 mM sodium acetate, 5 mM glucose). Cells were then washed 2 times, lysed with 0.1% sodium dodecyl sulfate and counted. For the internalization studies, the labeled ligands were added to the cells in serum-free Ham's F-12 medium containing 0.1% bovine serum albumin and 20 mM Hepes, pH 7.6. After the indicated periods of time at 37°C, the cells were put on ice and washed with pre-cooled Hepes buffered saline containing 0.3 mM $CaCl_2$. To determine the acid resistant fraction (i.e. the internalized ligand), cells were incubated for 5 min at 4°C with 0.2 M CH_3COOH, 0.5 M NaCl, pH 3.0 (31). The cells were then washed, solubilized, and counted as above.

Binding studies were also performed with isolated IGF-II/man6P receptor (18). For these studies, the purified receptor was adsorbed to microtiter wells coated with 100 nM of a monoclonal antibody (2C2) specific for the human IGF-II/man6P receptor (29). The labeled recombinant TGF-β1 precursor was then incubated with the receptor in the presence of the indicated concentrations of competing ligand for 3 h at 4°C. The wells were then washed, cut out, and counted.

Analysis by Western Blotting: Recombinant and platelet purified TGF-β1 precursor were electrophoresed on reduced, sodium dodecyl sulfate, polyacrylamide (12.5%) gels. The samples were transferred to a nitrocellulose filter (Schleicher and Schuell) and the filters were incubated in a blocking solution (3% bovine serum albumin and 0.1% Triton X-100 in Tris-buffered saline, pH 7.5) for 30 min at 24°C. The filters were then incubated overnight at 4°C with either: 1) a rabbit anti-peptide antibody to the precursor sequence of TGF-β1 (diluted 1:100) (10); or 2) purified IGF-II/man6P receptor, followed by a polyclonal antibody to this receptor (28). After washing four times, the filters were incubated with anti-rabbit IgG conjugated to alkaline phosphatase (1:5000, Promega) for 1 h at 4°C, washed, and then developed with the phosphatase substrate (Promega).

RESULTS

Binding and Internalization Studies: Binding of ^{125}I-IGF-II and recombinant TGF-β1 precursor to primary rat adipocytes was measured with and without insulin pretreatment. As previously reported (25,26), insulin caused a 2- to 3-fold increase (av.=2.4±0.3, n=4) in specific binding of IGF-II to the adipocytes (Fig. 1A). Insulin caused a similar (2.7±0.3, n=4) increase in the binding of recombinant TGF-β1 precursor to the adipocytes (Fig. 1B). The binding of the TGF-β1 precursor to the control and insulin-treated adipocytes was inhibited 65% and 81%, respectively, by 3 mM man6P (Fig. 1B). These results indicate that the recombinant TGF-β1 precursor was binding to the IGF-II/man6P receptor on adipocytes.

Additional binding studies were then performed with a line of CHO cells which were transfected with a cDNA coding for the IGF-II/man6P receptor (21) and selected for overexpression of the receptor protein. These transfected cells specifically bound ~10X more IGF-II than the parental CHO cells (Fig. 2A). These transfected cells also bound ~15X more recombinant TGF-β1 precursor than the parental CHO cells and this binding was inhibited by man6P (Fig. 2B). The inhibition by man6P was dose-dependent and half-maximal inhibition was observed at 10 μM (Fig. 3A). Mannose 1-phosphate was at least 1,000 times less effective at inhibiting binding of the TGF-β1 precursor (Fig. 3A). The binding of the precursor to the transfected cells was also inhibited by unlabeled

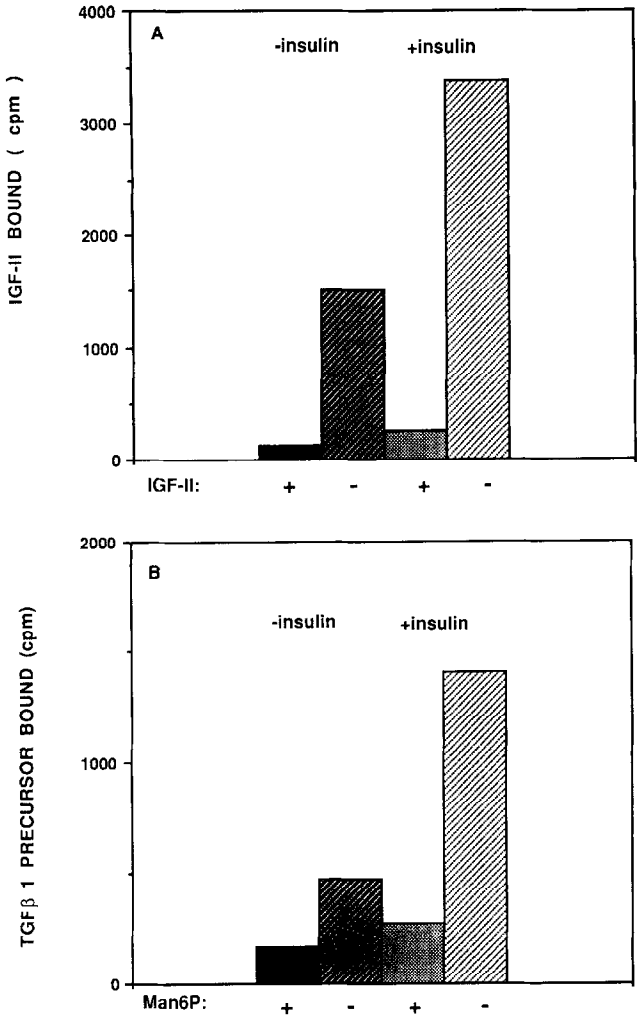


FIGURE 1: Insulin stimulated increase in IGF-II and recombinant TGF-β1 precursor binding to rat adipocytes. Rat adipocytes pretreated or not with insulin were incubated with either recombinant ^{125}I-TGF-β1 precursor (4.9 x 10^4 cpm) or ^{125}I-IGF-II (4.2 x 10^4 cpm). To assess non-specific binding, 100 nM IGF-II or 3 mM man6P were included as indicated. Results are averages of triplicate determinations.

TGF-β1 precursor but not by the mature TGF-β1 (Fig. 3B). One hundred nM IGF-II inhibited the binding of precursor TGF-β1 by 34%.

The bound TGF-β1 precursor was also tested for its ability to be internalized in the transfected cells. After various periods of time at 37°C, the cell surface TGF-β1 precursor was removed by an acid wash of the cells and the acid-resistant (i.e. internalized) (31) TGF-β1 precursor measured. After 15 min, >75% of the specifically bound TGF-β1 precursor was found to be in an acid-resistant compartment (Fig. 4). A further increase in total

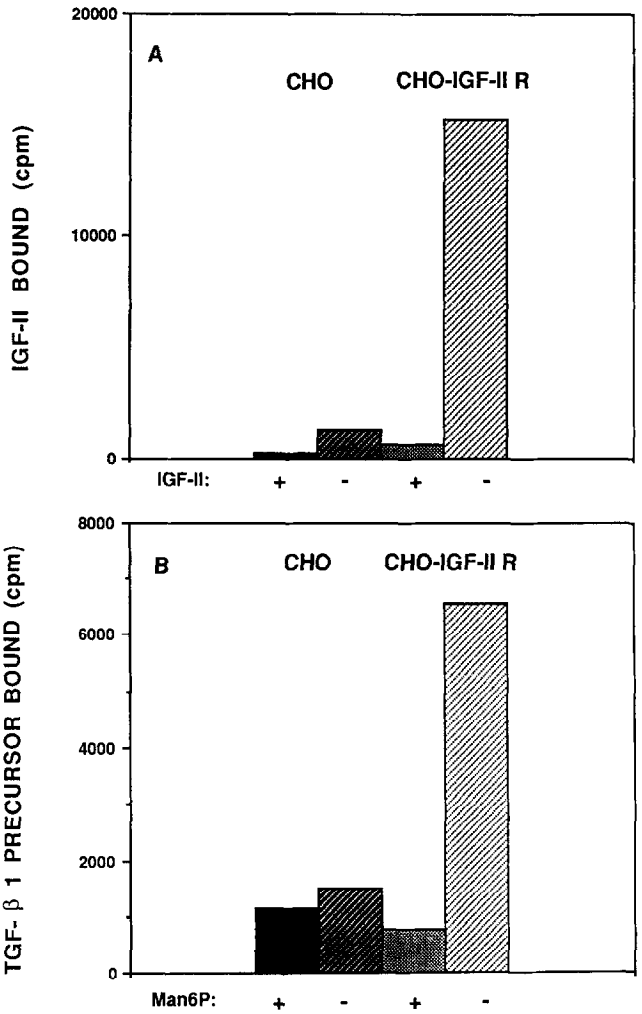
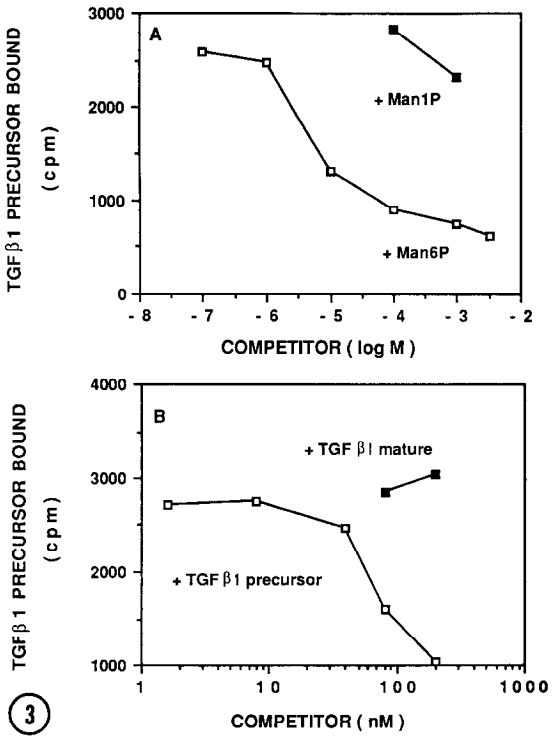


FIGURE 2: Binding of IGF-II and recombinant TGF-β1 precursor to CHO cells and CHO cells overexpressing the human IGF-II/man6P receptor (CHO-IGF-IIR). Cells were incubated with either ^{125}I-IGF-II (8.0 x 10^4 cpm) or recombinant ^{125}I-TGF-β1 precursor (1.3 x 10^5 cpm) and where indicated, either 5 mM man6P or 100 nM IGF-II was included to assess non-specific binding. Results are averages of triplicate determinations.

and internalized counts observed after 20 min appeared to be via a non-specific route since it was not blocked with man6P (Fig. 4).

Presence of man6P on latent TGF-β isolated from platelets: To determine whether latent TGF-β isolated from platelets also contained man6P, highly purified platelet TGF-β precursor (15) was tested for its ability to inhibit the binding of labeled recombinant TGF-β precursor to the isolated IGF-II/man6P receptor. The latent platelet TGF-β1 complex was found to be a potent inhibitor of this binding, with half-maximal inhibition at approximately 0.2 nM (Fig. 5).



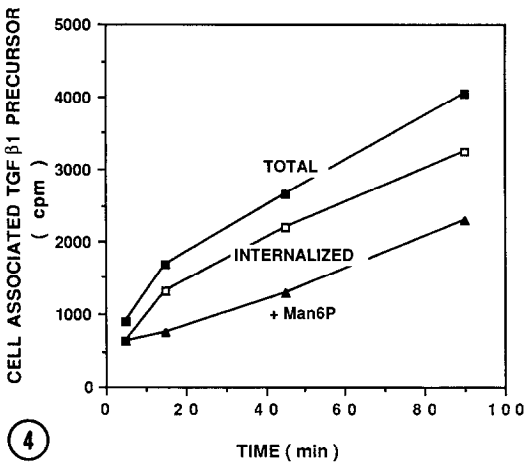
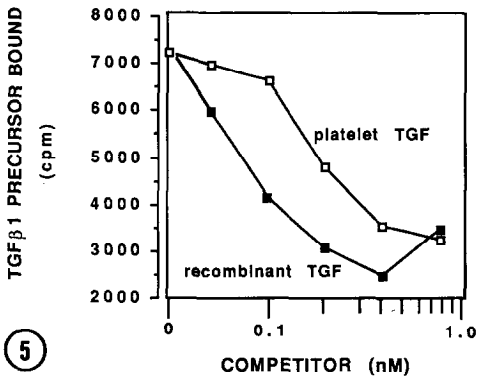


FIGURE 3: Specificity of recombinant TGF-β1 precursor binding to CHO cells overexpressing the human IGF-II/man6P receptor. Cells were incubated with recombinant ^{125}I-TGF-β1 precursor (6.7 x 10^4 cpm) and the indicated concentration of competing unlabeled ligand. Results are averages of triplicate determinations.

FIGURE 4: Internalization of recombinant TGF-β1 precursor. CHO cells overexpressing the IGF-II/man6P receptor were incubated at 37°C with recombinant ^{125}I-TGF-β1 precursor (5 x 10^4 cpm) in the presence or absence of 5 mM man6P. At the indicated times, either the total or the acid-resistant (i.e., internalized) cell associated counts were determined. Results are averages of triplicate determinations.

To further examine the platelet TGF-β1 complex for the presence of man6P, this material was electrophoresed on a reduced polyacrylamide SDS gel, transferred to a nitrocellulose membrane, and sequentially incubated with purified IGF-II/man6P receptor, rabbit antibodies to the receptor, alkaline phosphate-conjugated anti-rabbit Ig and a histochemical stain for alkaline phosphatase. Although this technique could detect the man6P on the uncleaved recombinant TGF-β1 precursor (Fig. 6, band a) and precursor remnant (Fig. 6, band b), no band of the appropriate molecular weight was detected with the platelet TGF-β1. In control experiments, an antibody specific for the sequence of the TGF-β1 precursor could detect the platelet TGF-β1 precursor remnant (Fig. 6, band c).



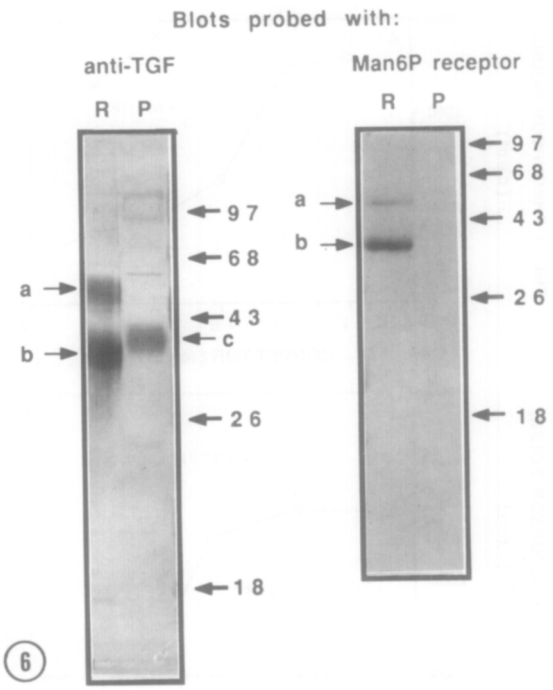


FIGURE 5: Binding of the platelet TGF-β1 precursor to the isolated IGF-II/man6P receptor. Purified IGF-II/man6P receptor adsorbed to microtiter wells was incubated with ^{125}I-labeled recombinant TGF-β1 precursor in the presence of the indicated concentrations of either purified latent TGF-β1 from platelets (□)or the recombinant TGF-β1 precursor (■). After 3 h at 4°C, the amount of receptor-bound radioactivity was determined.

FIGURE 6: Binding of the IGF-II/man6P receptor to recombinant TGF-β1 precursor but not the platelet TGF-β1 by Western blotting. Recombinant (R) (0.5 μg) and platelet (P) TGF-β1 precursor (2.5 μg) were electrophoresed on a reduced, SDS polyacrylamide gel, transferred to a nitrocellulose filter and reacted with either anti-peptide antibodies specific for a sequence in the precursor (left) or with the IGF-II/man6P receptor and antibodies to the receptor (right). The presence of bound rabbit antibodies was detected with alkaline phosphatase conjugated to anti-rabbit Ig and a histochemical stain for alkaline phosphatase. The positions of molecular weight markers (in kilodaltons) are indicated by arrows. The positions of the recombinant uncleaved precursor (a), recombinant precursor remnant (b), and platelet precursor remnant (c) are also indicated.

DISCUSSION

Prior studies have demonstrated that recombinant TGF-β1 precursor contains man6P and binds to the isolated IGF-II/man6P receptor (18). From the present studies, we can conclude that recombinant TGF-β1 precursor can bind to the IGF-II/man6P receptor on the plasma membrane of cells since: 1) The binding of recombinant TGF-β1 precursor to rat adipocytes was increased by the insulin-induced translocation of IGF-II/man6P receptor to the

plasma membrane (Fig. 1); 2) The binding of recombinant TGF-β1 precursor to CHO cells overexpressing the IGF-II/man6P receptor was also increased in comparison to the parental CHO cells (Fig. 2); and 3) The binding of TGF-β1 to both rat adipocytes and CHO cells was largely inhibited by man6P (Figs. 1 and 2). In the case of the transfected CHO cells, man6P half-maximally inhibited binding at a concentration of 10 μM (Fig. 3), a value close to that previously found (8 μM) to half-maximally inhibit binding to the isolated receptor (18). In addition, mannose 1-phosphate was a much less effective inhibitor than man6P (Fig. 3), consistent with the specificity of the IGF-II/man6P receptor for these two sugars (32).

After binding at 37°C to the IGF-II/man6P receptor on the transfected CHO cells, the recombinant TGF-β1 precursor rapidly became resistant to removal by an acid wash of these cells. After 10 min at 37°C, >75% of the specifically bound counts were resistant to an acid wash (Fig. 4). These results suggest that the TGF-β1 precursor has been internalized (31). After longer incubations at 37°C, a further increase in the uptake of label was observed. Since this additional increase was not blocked by man6P, it is likely due to the uptake of breakdown products of the degraded TGF-β1.

To determine whether latent TGF-β isolated from platelets could also bind to the IGF-II/man6P receptor, highly purified platelet TGF-β precursor (15) was tested for its ability to block the binding of recombinant TGF-β1 precursor to the isolated receptor. The platelet TGF-β precursor was found to be a potent inhibitor of this binding (IC_{50} = 0.2 nM) (Fig. 5). These results would suggest that the latent platelet TGF-β1 complex also contains man6P. Attempts to detect the binding of the IGF-II/man6P receptor to the platelet TGF-β1 precursor remnant after electrophoresis and transfer to nitrocellulose filters were, however, unsuccessful (Fig. 6). These negative results could be due to a lower amount of man6P being present on the platelet TGF-β1 precursor than on the recombinant TGF-β1 precursor. This would be consistent with the 3-fold weaker potency of the platelet TGF-β1 precursor than the recombinant precursor at displacing binding of the recombinant precursor to the isolated receptor (Fig. 5). It is possible that during the lengthy purification of the platelet TGF-β1 precursor, part of the phosphate is removed by phosphatases.

Recent studies of the platelet TGF-β1 precursor indicate that the carbohydrate structure may be important in maintaining this

complex in the inactive state (33). Digestion by endoglycosidase or sialidase was found to activate the latent platelet TGF-β1 complex. Moreover, incubation of the complex with man6P and sialic acid but not other sugars was also found to activate the latent TGF-β1. These results support a role for the carbohydrate in maintaining the platelet TGF-β1 precursor in its latent state. The present results would suggest that man6P on the TGF-β1 precursor can direct this molecule to cells via its interaction with the IGF-II/man6P receptor. This interaction could also lead to either its activation or degradation via its internalization and delivery to an endocytic compartment.

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