Latency-associated Peptide of Transforming Growth Factor-β1 Is Not Subject to Physiological Mannose Phosphorylation*

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**Background:** Previous reports indicate latent TGF-β1 activation is mediated by mannose phosphorylation of its N-glycans.

**Results:** Minimal mannose phosphorylation was detected on secreted TGF-β1, and its activation in corneal fibroblasts is not Man-6-P-dependent.

**Conclusion:** Latent TGF-β1 is not subject to physiological mannose phosphorylation.

**Significance:** Because free Man-6-P reduces scarring by inhibiting TGF-β1 activation, further investigation into molecular mechanisms explaining this phenomenon is warranted.

Latent TGF-β1 was one of the first non-lysosomal glycoproteins reported to bear mannose 6-phosphate (Man-6-P) residues on its N-glycans. Prior studies have suggested that this sugar modification regulates the activation of latent TGF-β1 by allowing it to bind cell surface-localized Man-6-P receptors. Man-6-P has also been proposed as an anti-scarring therapy based on its ability to directly block the activation of latent TGF-β1. A complete understanding of the physiological relevance of latent TGF-β1 mannose phosphorylation, however, is still lacking. Here we investigate the degree of mannose phosphorylation on secreted latent TGF-β1 and examine its Man-6-P-dependent activation in primary human corneal stromal fibroblasts. Contrary to earlier reports, minimal to no Man-6-P modification was found on secreted and cell-associated latent TGF-β1 produced from multiple primary and transformed cell types. Results showed that the inability to detect Man-6-P residues was not due to masking by the latent TGF-β1-binding protein (LTBP). Moreover, the efficient processing of glycans on latent TGF-β1 to complex type structures was consistent with the lack of mannose phosphorylation during biosynthesis. We further demonstrated that the conversion of corneal stromal fibroblast to myofibroblasts, a well known TGF-β1-dependent process, was not altered by Man-6-P addition when latent forms of this growth factor were present. Collectively, these findings indicate that Man-6-P-dependent effects on latent TGF-β1 activation are not mediated by direct modification of its latency-associated peptide.

The delivery of soluble acid hydrolases to the lysosome is primarily mediated by the mannose 6-phosphate (Man-6-P) targeting system in vertebrates. The biosynthesis of Man-6-P residues on newly synthesized hydrolases is initiated by the heterohexameric enzyme UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) in the early Golgi. This enzyme selectively transfers GlcNAc 1-phosphate to lysosomal enzymes to form phosphodiester intermediates. The subsequent removal of the GlcNAc residues by the enzyme GlcNAc-1-phosphodiester α-N-acetylglucosaminidase, or “uncovering enzyme,” in the trans-Golgi network produces Man-6-P monoesters. These monoesters serve as recognition markers for the sorting of newly synthesized lysosomal proteins by the cation-dependent and the cation-independent mannose 6-phosphate receptors (CD-MPR and CI-MPR, respectively) (3, 4).

In addition to acid hydrolases, several non-lysosomal proteins, including latent transforming growth factor-β1 (TGF-β1) (5), leukemia-inhibitory factor (6, 7), prolierin (8), renin precursor (9), and T-cell activation antigen CD26 (10), have been identified as Man-6-P-modified glycoproteins. An understanding of the mechanisms by which mannose phosphorylation governs the properties of these proteins, however, remains limited. In light of the fact that many Man-6-P-modified non-lysosomal proteins are secreted glycoproteins, mannose phosphorylation is thought to facilitate their uptake from the extracellular space by cell surface Man-6-P receptors. Recent work has also demonstrated that the addition of phosphomannosyl residues on leukemia-inhibitory factor can limit its extracellular levels by another mechanism: direct sorting and disposal within lysosomes (11).

The functional relevance of mannose phosphorylation on non-lysosomal proteins, however, is not restricted to the regulation of extracellular half-life because past evidence suggests...
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that this modification may regulate activation of latent TGF-β1 (12–17). Following secretion, latent TGF-β1 must be activated and released from its latency-associated peptide (LAP) before it can bind to its receptor and exert its biological activity (18–21). Activation can occur by various mechanisms in vivo, including those governed by integrins and thrombospondin (22–27). Mannose phosphorylation has also been implicated in this activation process (12–14, 28). Stockinger and colleagues (29) have proposed a model in which cell surface-localized CI-MPR is not only responsible for the binding of Man-6-P-modified latent TGF-β1 but also serves as a scaffold for the proteolytic cleavage of plasminogen to plasmin. Along these lines, Dennis and Rifkin (14) demonstrated that the presence of free Man-6-P blocked the plasmin-dependent activation of TGF-β1 in co-cultures of bovine endothelial and smooth muscle cells, supporting a role for the CI-MPR in this process. Similar results were obtained by Ghabary et al. (12), who showed that the addition of Man-6-P to the culture medium or the use of CI-MPR-deficient MS-9 cells results in loss of activation of latent TGF-β1 in keratinocyte/fibroblast co-culture assays.

Supporting these studies, earlier reports provided biochemical evidence for the presence of Man-6-P on the N-glycans of overexpressed, latent TGF-β1 (5, 14, 29, 30). These experiments did not yield any information, however, regarding the percentage of TGF-β1 molecules that were modified. Many of the subsequent studies addressing the Man-6-P-dependent activation of TGF-β1 were based on indirect effects mediated by the addition of free Man-6-P to culture medium. Therefore, the possibility that other Man-6-P-sensitive glycoproteins contributed to the altered activation of latent TGF-β1 could not be ruled out. In light of these considerations, we felt that a quantitative determination of the extent of mannose phosphorylation on latent TGF-β1 and further investigation into its Man-6-P-dependent activation was needed in order to elucidate the glycosylation-dependent mechanisms that govern TGF-β1 activation.

In this report, we undertook a biochemical evaluation of latent TGF-β1 mannose phosphorylation (using both CI-MPR affinity chromatography and analysis of [3H]mannose-labeled glycans) and explored its Man-6-P-dependent activation in human corneal stromal fibroblasts. Contrary to earlier studies, we observed no mannose phosphorylation on latent TGF-β1 secreted from several lines, with the exception of CHO- and HeLa-expressed protein. Our results further showed that the inability to detect high levels of mannose phosphorylation was not due to masking by the latent TGF-β1 binding protein (LTBP). Consistent with a lack of mannose phosphorylation, we were unable to detect Man-6-P-dependent effects on activation in corneal stromal fibroblasts, suggesting that the impact of exogenous Man-6-P addition on TGF-β1 activation may arise due to indirect effects not associated with the mannose phosphorylation of latent TGF-β1 itself.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Reagents—CHO, HeLa, and human erythroleukemia (HEL and K562) cells were obtained from ATCC. CHO, HEL, and K526 cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and streptomycin. Primary HUVEC cells, maintained in EBM-2 basal medium (Lonza, Walkersville, MD) containing 2% FCS, were used no later than passage 7. The CI-MPR lectin affinity column was a generous gift from Dr. Peter Lobel (Robert Wood Johnson Medical School). LAP goat polyclonal and LTBP mouse monoclonal antibodies, purified LAP (from sf21 cells), and latent TGF-β1 (derived from CHO cells) were obtained from R&D Systems. The anti-cathepsin D rabbit polyclonal antibody was a generous gift from Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Mannose 6-phosphate and glucose 6-phosphate (both as disodium salt hydrate) as well as phorbol 12-myristate 13-acetate (PMA) and plasmin from human plasma were purchased from Sigma. Endoglycosidase H₄ (Endo H₄) and peptide: N-glycosidase F (PNGase F) were obtained from New England Biolabs (Ipswich, MA). The transfection reagents Lipofectamine PLUS and Opti-MEM were obtained from Invitrogen. Swainsonine was purchased from Sigma, and [2-3H]mannose was from PerkinElmer Life Sciences. Human donor corneas were obtained from the Wisconsin Lion’s Eye Bank (Madison, WI) under the approval of the Medical College of Wisconsin Institutional Review Board.

PMA Treatment of Human Erythroleukemia and Chronic Myelogenous Leukemia Cells—HEL and K562 cells were cultured in a roller bottle system and maintained at a cell density under 1.0 × 10⁶ cells/ml. PMA treatment was performed as described previously (21, 31). Upon differentiation, cells were cultured in serum-free medium for 48 h and collected for analysis.

CI-MPR Lectin Chromatography and Western Blot Analysis—HeLa and CHO cells were transfected as described previously (11). Cell lysates were prepared in detergent-containing buffer, and total protein concentration was determined using a BCA protein assay kit (Pierce). Medium from transfected CHO and HeLa cells and PMA-treated erythroleukemia cells was collected, concentrated using Centricon 10 spin columns (Amicon), and fractionated using an immobilized CI-MPR affinity column as described previously (11). Column fractions were precipitated with ethanol and resolved by SDS-PAGE. Immunoblot detection was performed with either a mouse monoclonal antibody to LTBP or a goat polyclonal antibody to LAP. Densitometry analysis of individual CI-MPR column fractions was performed by plotting the optical density versus distance followed by measurement of the area under the curve using ImageJ software (32). In some cases, aliquots of concentrated media were incubated with either 50 units of Endo H₄ or 15 units of PNGase F for 2 h prior to SDS-PAGE and Western blot analysis.

Plasmin Treatment of Medium Aliquots from Human Erythroleukemia Cells—Medium aliquots from HEL cells were concentrated (from 1 ml to 200 μl) and treated with and without 1 unit of plasmin buffered to pH 8.0 and supplemented with 3 mM MgCl₂, CaCl₂ for 2 h at 37°C. Medium aliquots were then boiled and brought to a volume of 1 ml followed by application to the CI-MPR chromatography column.
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Latent TGF-β1 Plasmid Construction and Transfections—The human latent TGF-β1 plasmid used in this study was synthesized by GeneArt (Invitrogen) and ligated into a pcDNA3.1 expression vector using flanking BamHI and XbaI restriction sites. Cell transfections were performed as described (11). In some cases, swainsonine (an inhibitor of Golgi-mannosidase II) was added (10 μM) to the cultures after transient transfection to limit N-glycan processing and serve as an Endo H1 control.

[2-3H]Mannose Labeling and Analysis of Mannose Phosphorylation on Endo H1-released Glycans—The determination of the percentage of latent TGF-β1 glycans that are Man-6-P-modified was performed as described earlier but without ammonium chloride in the culture medium (33–35). The percentage phosphorylation was calculated as the radioactivity (cpm) recovered in Endo H1-released glycans with one or two phosphates/total cpm in Endo H1-released glycans + 2 Endo H1-resistant complex glycans. The values for the complex glycans were multiplied by 2 to correct for the fact that they contain 3 mannose residues versus an average of 6 mannose residues per high mannose glycan.

Assessment of Myofibroblast Differentiation in Human Corneal Stromal Fibroblasts—Human corneal stromal cells were obtained by digesting corneas with collagenase. Stromal fibroblasts were maintained in DMEM high glucose, MITO+, and 5% FBS. To differentiate to myofibroblasts, stromal cells were grown on collagen-coated plates in serum-free medium (DMEM high glucose containing 1% RPMI vitamin mix, 100 μM non-essential amino acids, 1 mM pyruvate, and 100 μg/ml ascorbic acid) containing 10 ng/ml TGF-β1 ligand (0.35 nM) for 7 days. An equimolar amount of LAP and latent TGF-β1 (based on their respective molecular sizes) was used. Myofibroblast transformation was assessed morphologically by immunofluorescence using a monoclonal antibody to α-smooth muscle actin (α-SMA). Fixed cells were imaged using a Nikon Diaphot 300 equipped with a DVC camera and C-View software. Cells containing assembled α-SMA fibers were counted, and the various treatment groups were compared using a two-way analysis of variance and the Bonferroni post-test (GraphPad Software, Inc.).

RESULTS

Latent TGF-β1 Secreted from Differentiated Human Erythroleukemia Cells Is Not Mannose-phosphorylated—The identification of phosphomannosyl residues on latent TGF-β1 was initially done via the analysis of secreted protein overexpressed in transfected or transduced cultured cells (5). To estimate the extent of mannose phosphorylation on endogenously expressed latent TGF-β1, we took advantage of the fact that human erythroleukemia cell lines (HEL and K562) express and secrete latent TGF-β1 in a dose-dependent manner following stimulation with PMA, a potent inducer of megakaryocyte differentiation in these cells (21, 31). Because treatment of both HEL and K562 with 16 nM PMA was found to result in the highest production of latent TGF-β1 (data not shown), this concentration was used for subsequent experiments.

Following PMA stimulation, medium samples were resolved by SDS-PAGE, and Western blot analysis was performed using antisera against the LAP portion of TGF-β1. The various molecular sizes of latent TGF-β1 detected in these cells (as well as the other cell lines used in this study) under reducing and non-reducing conditions are illustrated in Fig. 1. The mature TGF-β1 ligand (not shown) does not alter the apparent molecular weight of these forms because it dissociates from LAP in the presence of SDS (36). Under non-reducing conditions, latent TGF-β1 was primarily secreted from HEL and K562 cells as a 270-kDa protein, corresponding to the large latent complex (LLC), containing the small latent complex (SLC) disulfide-linked to LTBP. In addition, a 100 kDa band corresponding to either the LAP or precursor dimer was observed in the non-reducing samples. Upon reduction with DTT, monomeric LAP migrated at a molecular mass of 40 kDa in both cell lines (Fig. 2A).

To determine whether latent TGF-β1 was mannose-phosphorylated, medium samples from PMA-stimulated erythroleukemia cells were subjected to CI-MPR affinity chromatography and Western blot analysis as described under “Materials and Methods.” The results of this analysis clearly showed that none of the secreted forms of latent TGF-β1 from HEL (Fig. 2, B–D) or K562 (data not shown) cultures bound to the column. As a control, PMA-stimulated HEL cell lysates were subjected to CI-MPR affinity chromatography as before, followed by Western blot analysis using antisera against cathepsin D, a highly Man-6-P-modified lysosomal protease containing a comparable amount of N-glycans as latent TGF-β1 (Fig. 2E). The high level of cathepsin D binding to the affinity column indicated that PMA stimulation did not alter global mannose phosphorylation in these cells. This analysis was extended to two other cultured cells, NCI-H23 adenocarcinoma and hFOB.
1.19 osteoblasts, both of which produce large quantities of latent TGF-β1, but no detectable mannose phosphorylation on this protein was observed in either cell line.

**Failure of Latent TGF-β1 to Bind Immobilized CI-MPR Is Not Due to Masking of N-Glycans by LTBP**—The presence of Man-6-P on LAP could be masked by its interaction with LTBP and thus prevent its binding to the CI-MPR. Indeed, previous reports have suggested that the proteolytic liberation of LLC from the ECM releases a hinge region within LTBP that can mask the glycans present on LAP (15). To explore this possibility, the interaction of LTBP with LAP was disrupted by reduction of disulfide bonds with DTT in HEL medium aliquots prior to Man-6-P binding analysis using the immobilized CI-MPR. The results of this experiment demonstrated that separation of LTBP from latent TGF-β1 does not increase binding of the Man-6-P receptor affinity column (Fig. 3A). To determine whether serine protease cleavage of LTBP might lead to a conformational change in LLC and increased accessibility of the N-glycans on latent TGF-β1, HEL medium samples were incubated in the presence or absence of plasmin prior to analysis. Again, no increase in column binding was detected (Fig. 3B, left). Proteolysis of LTBP was demonstrated in Fig. 3B (right) as a control for efficiency of plasmin digestion. These data suggest that LTBP masking of the N-glycan residues on latent TGF-β1 was not responsible for the lack of detectable binding to the Man-6-P receptor affinity column.

Prior experiments using co-cultured systems of endothelial and smooth muscle cell lines indicated that latent TGF-β1 can be activated through its interaction with Man-6-P receptors and specifically blocked by the addition of free Man-6-P (14). A human endothelial cell line, HUVEC, was used to assess TGF-β1 mannose phosphorylation levels in these cells and the possibility of glycan masking by LTBP. Western blot analysis confirmed that latent TGF-β1 is not mannose-phosphorylated in fractionated cell lysates or medium samples (Fig. 3, C and D, top). In addition, latent TGF-β1 reduced from LTBP does not bind to the CI-MPR column Fig. 3, C and D, bottom). Collectively, these data confirm that the latent TGF-β1 is poorly modified in human endothelial cells and LTBP does not mask CI-MPR binding.

**Latent TGF-β1 Expressed in CHO and HeLa Cells Contains Low Levels of Mannose Phosphorylation**—In light of the fact that phosphomannosyl residues were first identified on latent TGF-β1 overexpressed in CHO cells, we chose to analyze mannose phosphorylation levels in transfected CHO and HeLa cultures to gauge whether the extent of this modification is cell type-specific. Medium from cultures transfected with a latent TGF-β1 expression construct was collected and analyzed by Western blot under reducing conditions to determine the molecular forms of the molecule secreted by these cell lines. Equivalent medium samples were also subjected to mannose phosphorylation analysis as described above. The majority of latent TGF-β1 in transfected HeLa cells (cell-associated and secreted) was detected as the LAP monomer in HeLa cells (Fig. 4A), indicating that the majority of secreted TGF-β1 was processed from the precursor (49 kDa) by the trans-Golgi network-localized furin convertase (37). As shown in Fig. 4B (top), TGF-β1 precursor dimer (~90–100 kDa (31)) and the LAP dimer (82 kDa) from HeLa cell lysates do not bind the Man-6-P receptor column, but a small amount of bound LAP dimer from HeLa (percentage bound = 2.37 ± 0.02; Fig. 4B, bottom) as well as precursor and processed latent TGF-β1 from CHO (percentage bound = 3.33 ± 0.01; Fig. 4C) medium samples was detected. These data demonstrate that mannose phosphorylation on latent TGF-β1 overexpressed in CHO cells is present but only at low levels.

As a direct measure of mannose phosphorylation, an analysis of [3H]mannose-labeled N-glycans released from latent TGF-β in transfected HeLa and CHO cells was undertaken (Table I). This analysis allows for an accurate determination of the percentage of Endo H-released N-glycans that are Man-6-P-modified and provides an estimate of the extent of N-glycan pro-
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As a control, cells transfected with a cathepsin D expression construct were also analyzed. The results demonstrate that only 1.8/0.9% of the latent TGF-β1 oligosaccharides in HeLa cells and 2.7/1.0% in CHO cells bear Man-6-P, a finding consistent with the low level of Man-6-P receptor column binding. The respective values for cathepsin D were 9–12-fold higher. These results also demonstrate that the large majority (>86%) of N-glycans present on latent TGF-β are complex type.

Secreted Latent TGF-β1 Predominantly Bears Complex Type N-Glycans—The lack of phosphomannosyl residues on latent TGF-β1 suggests that no mannose phosphorylation occurred during biosynthesis (an outcome that would result in processing of the N-glycans to complex type). It is equally possible,
TABLE 1
Percentage mannose phosphorylation on overexpressed cathepsin D and latent TGF-β1
CHO and HeLa cells were transfected with either cathepsin D or latent TGF-β1 plasmids. Cultures were labeled with [2-3H]mannose, followed by immunoprecipitation of the secreted protein from the culture medium. Released N-glycans were subjected to mannose phosphorylation analysis by QAE-Sepharose affinity chromatography, and the average amount of Endo H-sensitive and -insensitive N-glycans on these molecules was determined.

|                  | CHO                     | HeLa                    |
|------------------|-------------------------|-------------------------|
|                  | Cathepsin D (n = 3)     | TGF-β1 (n = 4)          | Cathepsin D (n = 5) | TGF-β1 (n = 5) |
| Man-6-P-modified N-glycans (%) | 33.5 ± 5.9              | 2.7 ± 1.0               | 16.4 ± 1.1          | 1.8 ± 0.9    |
| EndoH-sensitive (%) | 64.2                   | 92.8                    | 63.4                | 86.3         |
| EndoH-sensitive (%) | 35.8                   | 7.20                    | 36.6                | 13.7         |

however, that these residues are effectively removed by phosphatases upon secretion into the culture medium (an outcome that would result in the preservation of the N-glycans as high mannose type). To address these two possibilities, medium samples were treated with either Endo H, which removes high mannose N-glycans, or N-glycanase, which removes all N-glycans, followed by immunoblot analysis. In HEI medium, the results show that fully processed latent TGF-β1 glycoforms are highly resistant to Endo H, consistent with the processing of N-glycans to complex type (Fig. 5A). A minor shift in the molecular weight of precursor and processed latent TGF-β1 from HeLa and CHO cell medium (Fig. 5, B and C) was observed following Endo H; however, these glycoforms were mostly complex type, consistent with the glycan analysis shown in Table 1.

Latent TGF-β1 Is Not Able to Stimulate Myofibroblast Conversion of Corneal Stromal Fibroblasts in a Man-6-P-dependent Fashion—Human corneal stromal fibroblasts are thought to exhibit Man-6-P-dependent effects on TGF-β1 activation. These effects are based on observations that the addition of Man-6-P to cultured keratocytes suppresses TGF-β1 myofibroblast transformation (39). Moreover, the presence of Man-6-P was shown to reduce corneal haze following refractive surgery in rabbits (40). Although these observations are believed to arise due to direct effects on the binding of latent TGF-β1 to cell surface CI-MPR, this mechanism is inconsistent with the present observations that latent TGF-β1 is only marginally modified with Man-6-P residues. To address whether latent forms of TGF-β1 were capable of stimulating myofibroblast differentiation and whether these molecules could be activated in a Man-6-P-dependent manner, cultured human corneal fibroblasts were incubated with various forms of active and latent TGF-β1 in the presence or absence of free Man-6-P. Expression of the myofibroblast-specific protein, α-SMA, was used to gauge the extent of myofibroblast transformation. As shown in Fig. 6A, the active TGF-β1 ligand was the only form capable of robustly increasing α-SMA levels and stimulating assembly of α-SMA fibers characteristic of myofibroblast transformation in corneal fibroblasts. The percentage of fibroblasts transformed to myofibroblasts under various conditions was quantified (Fig. 6B). These data clearly demonstrate minimal effects of the different forms of latent TGF-β1 on this process in the presence or absence of Man-6-P. We confirmed that the sources of latent TGF-β1 and LAP were poorly modified with Man-6-P residues (Fig. 6C; see also Fig. 4C). The lack of myofibroblast stimulation with LAP expressed in sf21 insect cells was not surprising because this form does not contain mature ligand.

DISCUSSION

Latent TGF-β1 was one of the first secreted glycoproteins reported to contain Man-6-P residues (5, 30). Subsequent studies have been aimed at understanding how mannose phosphorylation of latent TGF-β1 may regulate its physiological function. Much of this past work has focused on the role of latent TGF-β1 mannose phosphorylation in its activation at the cell surface, where the binding of modified latent TGF-β1 to the CI-MPR is thought to mediate its proteolytic activation (12, 14–17). In these studies, activation of latent TGF-β1 (gauged using reporter cell-based or growth inhibition assays that respond to liberated TGF-β1 ligand) was reduced by the addition of free Man-6-P or antiserum against the CI-MPR to the culture medium. In nearly all cases, however, the extent of mannose phosphorylation on the latent TGF-β1 molecules employed in these experiments was never determined, leaving open the possibility that Man-6-P-dependent effects on activation were not mediated by direct perturbation of latent TGF-β1.

We demonstrated here that latent TGF-β1 was poorly modified with Man-6-P residues, ranging from undetectable in differentiated erythroleukemia and HUVEC cells to 1–3% in transfected CHO and HeLa cells. The highest level of Man-6-P-modified latent TGF-β1 could be detected in transfected CHO cells, a noteworthy observation because these cells were also employed in the initial studies (5). Utilizing metabolic labeling experiments with [32P]orthophosphate and subsequent carbohydrate analysis, these investigators accurately identified Man-6-P on the N-glycans of latent TGF-β1 but also estimated in another study that only 5% of the [125I]-labeled TGF-β1 precursor bound to CI-MPR-coated plates (30). This minimal binding is consistent with our findings that less than 3% of CHO-derived protein binds to the affinity column. Due to the sensitivity of metabolic labeling, very low levels of Man-6-P might be detected on liberated glycans. Thus, one might reason that the original determination of Man-6-P-modified glycans on latent TGF-β1 was simply based on high sensitivity detection of a low abundance modification. This likelihood is supported by the fact that many serum glycoproteins can be identified as Man-6-P-modified using mass spectrometry-based enrichment and detection strategies (41). We believe these examples serve to reinforce the necessity of quantifying the extent of mannose phosphorylation on non-lysosomal glycoproteins prior to the analyses of its functional relevance.

The very low level of mannose phosphorylation on latent TGF-β1 suggests that this modification is a cell type-specific
artifact or a function of its overexpression. It is possible, however, that the Man-6-P-modified fraction of the molecule is relevant to its activation or regulation in specific physiological contexts. By virtue of its ability to associate with the plasma membrane following secretion, Man-6-P-modified latent TGF-β1 may be more readily activated in cell types where cell surface activation mechanisms, such as interaction with integrins, are present.

Over the past 2 decades, multiple reports have described the Man-6-P-dependent activation of latent TGF-β1. Ghahary et al. (12) showed, using a keratinocyte/fibroblast co-culture system, that ECM expression was mediated by Man-6-P-dependent activation of latent TGF-β1. Along these lines, Dennis and Rifkin (14) demonstrated similar effects on TGF-β1 activation in a bovine aortic endothelial and smooth muscle cell co-culture system when free Man-6-P was present. However, these
studies left an open question as to what other co-factors might be necessary for the activation mechanism because only the combined co-culture medium stimulated an effect. For instance, Godár et al. (29) later proposed an in vitro model for Man-6-P-dependent activation of TGF-β1 that involved the organization of a proteolytic cascade, including plasminogen and urokinase-type plasminogen activator receptor (uPAR), at the cell surface by the CI-MPR. Indeed, work by MacDonald and colleagues (42) has determined that uPAR itself is mannos phosphorylated. Therefore, it is possible that the addition of free Man-6-P abrogates the interaction of uPAR with cell surface CI-MPR. Failure to recruit the uPAR-uPA-plasminogen complex would potentially decrease TGF-β1 activation. Similar results have been determined through inhibition of plasm, suggesting that the conversion of plasminogen to plasmin is an important part of the activation mechanism (28, 43).

More recently, effects of free Man-6-P and its analogues on both TGF-β1 activation and downstream indicators of this activation have been documented in models of flexor tendon injury and renal fibrosis (44–46). How can the prior work demonstrate in models of flexor tendon injury and renal fibrosis (44–46). How can the prior work demonstrate in vivo TGF-β1 activation be justified in light of the findings presented here? Because proteases are capable of latent TGF-β1 activation, one possibility is that the addition of free Man-6-P is displacing Man-6-P-modified enzymes from cell surface-localized CI-MPR or interfering with their uptake in some manner that is indirectly influencing the activation. Moreover, free Man-6-P has been reported to decrease TGF-β1 receptor expression at the cell surface (46, 47), providing another mechanism to reduce TGF-β1 signaling. The results in human corneal stromal fibroblasts clearly demonstrated that latent forms of TGF-β1 were not capable of stimulating myofibroblast transformation.

Mannose 6-phosphate and its analogues have been proposed as candidate molecules to prevent hazy following refractive surgery on the cornea (39, 40). The proposed mechanism of action is thought to occur via decreased activation of latent TGF-β1, which would in turn limit the production of ECM and reduce pathology. Although the level of mannose phosphorylation on endogenous latent TGF-β1 has not been determined from these tissues, careful interpretation of such results and investigation into the possibility that other Man-6-P-modified proteins mediate the effects of activation is warranted.

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