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Cellular activation of latent transforming growth factor β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor

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ABSTRACT **The activation of latent transforming growth factor β (LTGF-β) normally seen in cocultures of bovine aortic endothelial and bovine smooth muscle cells can be inhibited by coculturing the cells with either mannose 6-phosphate (Man-6-*P*) or antibodies directed against the cation-independent Man-6-*P*/insulin-like growth factor type II receptor (anti-Man-6-*P*R). This result was established by measuring the ability of coculture conditioned medium (formed with or without Man-6-*P* or anti-Man-6-*P*R) to suppress bovine aortic endothelial cell migration and protease production, activities previously shown to be related to transforming growth factor β activity. The inhibition by Man-6-*P* is dose dependent, with maximal inhibition seen at 100 μM, and is specific because mannose 1-phosphate and glucose 6-phosphate do not interfere with activation of LTGF-β. The inhibitory effect of anti-Man-6-*P*R is also specific and dose dependent; maximal inhibition of activation occurs at 400 μg/ml. Control experiments indicate that Man-6-*P* and anti-Man-6-*P*R do not interfere with the basal level of migration of bovine aortic endothelial cells, the migration observed when exogenous transforming growth factor β is added, the activation of transforming growth factor β by plasmin or transient acidification, and the release of LTGF-β. Thus, binding to the cation-independent Man-6-*P*/insulin-like growth factor type II receptor appears to be a requirement for activation of LTGF-β.**

The transforming growth factor β (TGF-β) family is composed of a group of structurally related proteins including activins, inhibins, Müllerian inhibitory substance, and bone morphogenetic proteins (1). The best characterized member of this family is the 25-kDa homodimer TGF-β1. This protein can be isolated from a variety of cells *in vitro* and is found in high concentration in platelets, bone, and placenta (2–4). It is a multipotential protein that inhibits the growth of most epithelial cells, endothelial cells, and lymphocytes (5–7). TGF-β1 is also an inhibitor of endothelial cell migration, as well as an immunosuppressive agent and a potent inducer of matrix biosynthesis (8).

TGF-β1 is synthesized as a preproprotein of 390 amino acids that is converted to the mature form by cleavage between residues 278 and 279 (9). However, TGF-β isolated *in vitro* and *in vivo* is found predominantly as a high-molecular-weight latent complex (LTGF-β) in which the pro region is still associated with the mature molecule, even though the bond between residues 278 and 279 has been cleaved (2, 10). LTGF-β is composed of one mature TGF-β molecule noncovalently bound to the pro-region dimer, which is referred to as the latency-associated peptide (LAP). LTGF-β isolated from platelets contains an additional protein of 130 kDa that is disulfide bonded to the dimeric propeptide (10, 11).

LTGF-β can be activated *in vitro* by transient acidification, proteolysis, and the use of chaotropic agents (12–14). Although receptors for TGF-β1 are found in almost all cell types (15), these receptors do not recognize LTGF-β.

Miyazono and Heldin (16) have postulated that the carbohydrates of the LTGF-β molecules may be important in maintaining latency because enzymatic removal of the carbohydrate structures produces active TGF-β from the LTGF-β complex. Furthermore, Purchio *et al.* (17) analyzed the carbohydrate chains of the TGF-β precursor and determined that two of the three carbohydrate chains contain mannose 6-phosphate (Man-6-*P*). Mature TGF-β does not contain Man-6-*P* residues. Recombinant LTGF-β and LTGF-β isolated from platelets bind to the plasma membrane form of the cation-independent Man-6-*P*/insulin-like growth factor II (IGF-II) receptor (17, 18).

Recently, cocultures of endothelial cells and either smooth muscle cells or pericytes have been shown to convert LTGF-β to active TGF-β (19, 20). Thus, medium conditioned by homotypic cultures contains only LTGF-β, whereas conditioned medium (CM) from heterotypic cultures contains active TGF-β. The requirements for activation in these coculture systems are cell–cell contact (or the close apposition of the two different cell types) and plasmin and urokinase; inhibitors of plasmin and urokinase block activation of LTGF-β.

Because of the previously reported binding of LTGF-β to the cation-independent Man-6-*P*/IGF-II receptor, we have tested whether this interaction is part of the activation process of LTGF-β. We report Man-6-*P* and anti-Man-6-*P*R receptor (anti-Man-6-*P*R) specifically inhibit the activation of LTGF-β by bovine aortic endothelial (BAE)/bovine smooth muscle (BSM) cocultures, suggesting that binding to the cation-independent Man-6-*P*/IGF-II receptor is required for LTGF-β activation.

MATERIALS AND METHODS

Materials

Mannose 1-phosphate, glucose 6-phosphate, Man-6-*P*, aprotinin, and plasmin were purchased from Sigma. Anti-Man-6-*P*R was a gift from C. Gabel (Pfizer Pharmaceuticals,

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Abbreviations: BAE, bovine aortic endothelial; BSM, bovine smooth muscle; CM, conditioned medium; PA, plasminogen activator; αMEM, α modification of Eagle's minimal essential medium; IGF-II, insulin-like growth factor II; TGF-β, transforming growth factor β; LTGF-β, latent TGF β; LAP, latency-associated peptide; Man-6-*P*, mannose 6-phosphate; anti-Man-6-*P*R, anti-cation-independent mannose 6-phosphate receptor IgG; anti-TGF-β, anti-TGF-β IgG.

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Groton, CT) and does not recognize the cation-dependent Man-6-*P* receptor (21). TGF-β and anti-TGF-β IgG (anti-TGF-β) was purchased from R & D Systems (Minneapolis, MN). Anti-TGF-β has been shown to neutralize porcine, human, and bovine TGF-β1 (19, 22).

Methods

Cell Culture. BAE cells were isolated and grown in α minimal essential medium (αMEM)/10% calf serum, as described (23). BSM cells were explanted from bovine aortae and grown in Dulbecco's modified minimal essential medium/10% calf serum (19).

Wound Assays for BAE-Cell Migration. Wound assays were done as described (24). Briefly, confluent monolayers of BAE cells in 35-mm dishes were washed with phosphate-buffered saline (PBS), wounded with a razor blade, and washed again with PBS. Cells were incubated in serum-free αMEM or 25% CM as indicated for 20 hr at 37°C, and the cells were fixed with absolute methanol and stained with Giemsa. Cells that had migrated from the edge of the wound were counted in seven successive 125-μm increments at ×100 by using a light microscope with an ocular grid. The cell numbers represent the means from at least six different fields. The dose responsiveness of this assay to TGF-β has been described (25).

Preparation of CM. *Homotypic cultures.* BAE and BSM cells were seeded separately at either 1×10^6 cells per 60-mm dish or 4×10^5 cells per 35-mm dish with αMEM/10% calf serum. After attachment, the cells were rinsed with PBS, and fresh αMEM without serum was added for 24 hr. The medium was collected and centrifuged to remove cell debris.

Heterotypic cultures. BAE and BSM cells were seeded together at a 4:1 BAE/BSM ratio in αMEM/10% calf serum. A total of 1×10^6 cells per 60-mm dish or 4×10^5 cells per 35-mm dish was used. After 2 hr, cells were rinsed with PBS and serum-free medium was added. After 24 hr, the medium was collected and centrifuged.

Acidification of CM. CM from homotypic or heterotypic cultures was acidified to pH 2.0 with 1 M HCl for 1 hr at room temperature and then neutralized with NaOH.

Plasmin Treatment of CM. Plasmin (final concentration, 1 unit/ml) was added to CM from homotypic or heterotypic cultures for 2 hr at 37°C. Aprotinin (37.5 μg/ml) was then added for 0.5 hr at 37°C to inhibit further proteolysis.

Plasminogen Activator (PA) Assays. Confluent cultures of BAE cells were incubated overnight with the indicated additions. After incubation, the cells were washed twice with PBS and extracted with 0.5% Triton X-100/0.1 M Tris·HCl, pH 8.1. Cell extracts were then assayed for PA activity by using a ^{125}I-labeled fibrin plate assay (26).

RESULTS

Effect of Man-6-*P* on TGF-β Production in Cocultures. Because LTGF-β is known to bind to the Man-6-*P*/IGF-II receptor (17, 18), we examined the possible inhibitory effect of Man-6-*P* on activation of LTGF-β by cocultures of BAE and BSM cells (Fig. 1). In this system, the generation of TGF-β can be measured by its ability to inhibit cell migration in wounded cultures of BAE cells. The observed inhibition of BAE migration by coculture CM has been shown to be alleviated by neutralizing TGF-β antibodies (24). Thus, when fresh medium was added to a BAE wound, 92 cells migrated from the initial wound edge (column 1). The addition of CM from homotypic BSM cultures to wounded BAE cell cultures had no effect on cell migration (column 2). Addition of coculture CM to a wounded culture inhibited BAE cell migration by 48% (column 3). The inhibition caused by coculture CM was abrogated by adding anti-TGF-β at 10 μg/ml to the BAE wound (column 4), whereas adding non-

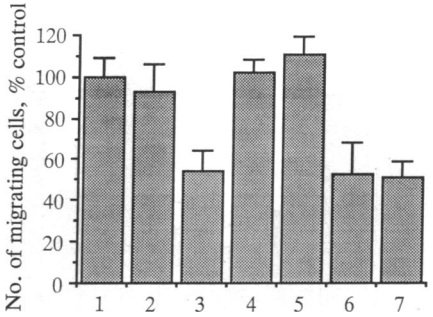

FIG. 1. Specific effect of Man-6-*P* on inhibition of BAE-cell migration by coculture CM. CM was prepared as described from homotypic cultures or heterotypic cultures. Fresh medium (column 1) or 25% CM (columns 2–7) was added to wounded BAE monolayers, and cell migration was measured as described. Columns: 2, homotypic BSM-CM; 3, BAE/BSM coculture CM; 4, BAE/BSM coculture CM plus anti-TGF-β at 10 μg/ml added to the BAE wound; 5–7, BAE/BSM coculture CM conditioned with 100 μM Man-6-*P* (column 5), 100 μM mannose 1-phosphate (column 6), or 100 μM glucose 6-phosphate (column 7). Total number of cells that moved in the control (column 1) was 92.

immune IgG at 10 μg/ml had no effect (data not shown). Inclusion of 100 μM Man-6-*P* in the original coculture medium also abrogated the inhibition of migration seen with coculture CM (column 5), whereas the inclusion of mannose 1-phosphate (column 6) and glucose 6-phosphate (column 7) in the coculture medium did not block the inhibition.

The extent of BAE-cell migration varied with the Man-6-*P* concentration originally added to the medium of BAE/BSM cocultures. At a Man-6-*P* concentration of 10 μM, the inhibitory effect of coculture CM on BAE-cell migration was reduced from 50% to 67% of control. At a Man-6-*P* concentration of 100 μM in the original coculture medium, BAE migration was 97% of control. Higher concentrations of Man-6-*P* (1 and 10 mM) inhibited migration. This result was not explored but may relate to the observation of Miyazono and Heldin (16) that high concentrations of Man-6-*P* directly activate TGF-β.

Specificity of the Effect of Man-6-*P*. The following experiments were done to ensure that Man-6-*P* blocked LTGF-β activation and not TGF-β action. As shown previously, addition of anti-TGF-β at 10 μg/ml to the wounded BAE monolayer in the presence of coculture CM reversed the inhibition of migration. If coculture CM prepared with Man-6-*P* was transiently acidified (a process known to activate LTGF-β), an inhibition of migration (44% of control) was seen. This effect was specifically reversed by adding anti-TGF-β to the wound (data not shown). This result indicates that CM collected in the presence of Man-6-*P* contained LTGF-β that can be activated by transient acidification and that the inhibition of migration observed in the wound assays was from the generation of active TGF-β.

A series of experiments were designed to test the effect of Man-6-*P* on the BAE-cell migration assay as well as on LTGF-β activation (Fig. 2). Comparison of cell migration seen in fresh medium (column 1) and fresh medium plus Man-6-*P* (column 2) revealed that basal-level BAE-cell migration was not affected by 100 μM Man-6-*P*. BAE-cell migration was inhibited when exogenous TGF-β (2 ng/ml) was added to cultures of wounded BAE cells (column 3), but addition of 100 μM Man-6-*P* to medium containing TGF-β (2 ng/ml) did not affect the inhibition caused by TGF-β (column 4). We tested whether Man-6-*P* interfered with the plasmin-mediated activation of LTGF-β because plasmin is critical for generation of active TGF-β in coculture systems (19). The normally observed inhibition of BAE-cell migration with coculture CM is illustrated in column 5, whereas column 6

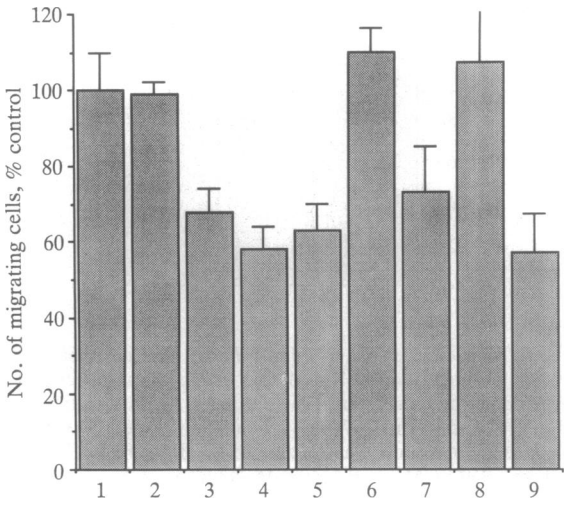

FIG. 2. Man-6-*P* control experiments. BAE wound assays were performed as before with the following additions: columns: 1, fresh medium; 2, fresh medium plus 100 μM Man-6-*P*; 3, TGF-β at 2 ng/ml; 4, TGF-β at 2 ng/ml plus 100 μM Man-6-*P*; 5, 25% BAE/BSM coculture CM; 6, 25% coculture medium conditioned with 100 μM Man-6-*P*; 7, plasmin activation of 25% coculture medium conditioned with 100 μM Man-6-*P*; 8, 25% coculture medium conditioned from hr 24 to 48 with 100 μM Man-6-*P*; 9, acid activation of 25% coculture medium conditioned from hr 24 to 48 with 100 μM Man-6-*P*. The total number of cells that moved in the control (column 1) was 72.

shows the lack of inhibition of BAE-cell migration seen when coculture CM collected with 100 μM Man-6-*P* was assayed. LTGF-β in this coculture CM containing Man-6-*P* could be activated, as medium exposed to plasmin inhibited BAE-cell migration (column 7). The inhibition seen with plasmin activation of LTGF-β in coculture CM containing Man-6-*P* was identical to that seen when LTGF-β in homotypic culture CM was plasmin activated (data not shown). The activation of LTGF-β by plasmin was less effective than activation by transient acidification, consistent with earlier results (13). That plasmin activates TGF-β less effectively in a test tube than on the surface of cells attests to the fact that conditions on the cell surface probably optimize the activation reaction in a way that we do not understand.

Finally, we tested whether Man-6-*P* interfered with the release of LTGF-β from cells. Medium from cocultures was collected in the presence of 100 μM Man-6-*P* as before. The medium was replaced at 24 hr with fresh medium containing 100 μM Man-6-*P*. We reasoned that because most cells secrete LTGF-β constitutively, an effect of Man-6-*P* on the release of LTGF-β might be more apparent in CM collected after 24-hr exposure to Man-6-*P*. Man-6-*P* continued to inhibit activation of LTGF-β by cocultured BAE/BSM cells during a second 24-hr period (column 8). When CM used in the sample from column 8 was transiently acidified, inhibition of BAE-cell migration was seen due to activation of LTGF-β (column 9). This result indicated that the release of LTGF-β by cocultured BAE/BSM cells was not affected by Man-6-*P*. Overall, these results show that Man-6-*P* does not alter the basal migration of BAE cells, the activity of exogenously added TGF-β, the activation of LTGF-β by plasmin, or the release of LTGF-β from cells. Therefore, we conclude that Man-6-*P* probably blocks activation of LTGF-β by interfering with its binding to the Man-6-*P*/IGF-II receptor.

TGF-β decreases the PA production in endothelial cells (27). Therefore, we used PA production by BAE cells treated with different media as a second assay to corroborate the inhibitory effect of Man-6-*P* on TGF-β activation. Fig. 3 demonstrates the results of these assays. The basal PA level in BAE cells is illustrated in column 1. Addition of BAE/

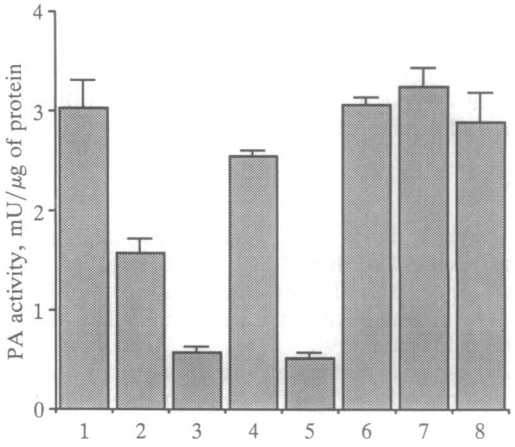

FIG. 3. Effect of Man-6-*P* and coculture CM on PA production by BAE cells. The following samples were added to confluent BAE cells overnight, and 24 hr later the PA levels in the cell extracts were measured (23). Columns: 1, fresh medium; 2, 25% BAE/BSM coculture CM; 3, 25% BAE/BSM coculture CM after transient acidification; 4, 25% BAE/BSM medium conditioned with 100 μM Man-6-*P*; 5, 25% BAE/BSM medium conditioned with 100 μM Man-6-*P*, followed by transient acidification; 6–8, same as columns 2, 3, and 5, respectively, only anti-TGF-β at 10 μg/ml was added to the wounds. mU, Milliunits.

BSM coculture CM decreases the basal PA level by 50% (column 2). Further inhibition of basal PA activity was seen when the CM was transiently acidified, thereby activating residual LTGF-β (column 3). The inhibition by coculture CM of PA production was greatly diminished when coculture medium conditioned in the presence of 100 μM Man-6-*P* was assayed (column 4). The incomplete blocking of the inhibitory activity may indicate that some TGF-β was activated in this experiment. Furthermore, acidification of the CM assayed in column 4 inhibited PA activity 80%, indicating that LTGF-β was present in the CM (column 5). Addition of anti-TGF-β (columns 6–8) to the samples assayed in columns 2, 3, and 5, respectively, abrogated the observed inhibition. In contrast, addition of nonimmune IgG to the samples assayed in columns 2–5 did not alter PA production (data not shown). This result indicated that the inhibition of PA activity was from TGF-β.

Effect of Anti-Man-6-*P*R on LTGF-β Activation. To confirm our hypothesis that binding to the cation-independent Man-6-*P*/IGF-II receptor is required for LTGF-β activation, we tested the effect of anti-Man-6-*P*R in our coculture system. The inclusion of anti-Man-6-*P*R during preparation of coculture CM led to a dose-dependent blockage of inhibition of BAE-cell migration (Fig. 4). The ED_{50} was ≈30 μg/ml, and the maximum level of migration, or greatest inhibition of

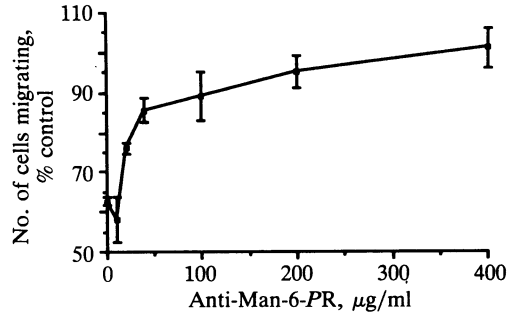

FIG. 4. Dose-dependent inhibition of LTGF-β activation by anti-Man-6-*P*R. Anti-Man-6-*P*R was added to cocultures of BAE/BSM cells at the indicated concentrations, and 24 hr later the CM was assayed in wound assays. Cell migration was measured as before. The total number of cells that migrated in the control was 59.

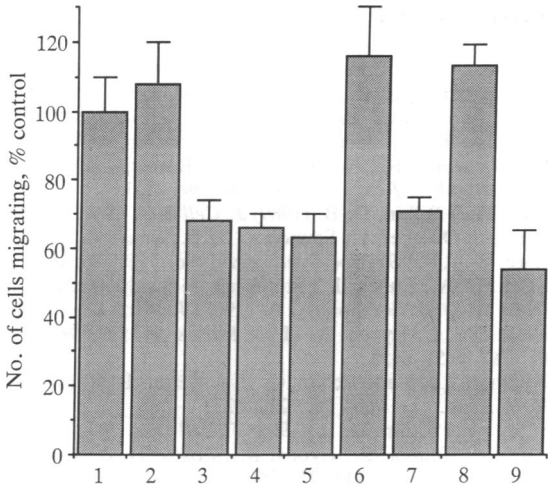

FIG. 5. Anti-Man-6-*P*R control experiments. BAE wound assays were done as before, with the indicated additions. Columns: 1, fresh medium; 2, fresh medium plus anti-Man-6-*P*R at 400 μg/ml; 3, TGF-β at 2 ng/ml; 4, TGF-β at 2 ng/ml plus anti-Man-6-*P*R at 400 μg/ml; 5, 25% BAE/BSM coculture CM; 6, 25% coculture medium conditioned with anti-Man-6-*P*R at 400 μg/ml; 7, plasmin activation of 25% coculture medium conditioned with anti-Man-6-*P*R at 400 μg/ml; 8, 25% coculture medium conditioned from hr 24 to 48 with anti-Man-6-*P*R at 400 μg/ml; 9, acid activation of 25% coculture medium conditioned from hr 24 to 48 with anti-Man-6-*P*R at 400 μg/ml. The total number of cells that moved in one field in column 1 was 69.

LTGF-β activation, occurred at a concentration of 400 μg/ml. In addition, two other antisera against the cation-independent Man-6-*P*/IGF-II receptor (from G. Sahagian, Tufts University School of Medicine, and S. Kornfeld, Washington University School of Medicine) were equally effective in blocking the inhibition of BAE-cell migration caused by coculture CM (data not shown).

Several experiments were done to ensure that anti-Man-6-*P*R did not affect processes that might precede or follow LTGF-β activation (Fig. 5). Comparison of the effect of medium alone (column 1) and medium containing anti-Man-6-*P*R (column 2) on migration of BAE cells revealed that addition of anti-Man-6-*P*R at 400 μg/ml did not affect the basal level of BAE-cell migration. In addition, anti-Man-6-*P*R had no effect on the activity of exogenously added TGF-β, as shown in columns 3 (medium containing TGF-β) and 4 (medium containing TGF-β and anti-Man-6-*P*R). The inhibition of migration seen when coculture CM is added to a wound (column 5) was not seen with coculture media conditioned in the presence of anti-Man-6-*P*R (column 6). Anti-Man-6-*P*R did not interfere with activation of LTGF-β by plasmin because plasmin addition to the CM used in column 6 inhibited migration (column 7). To assess the effect of anti-Man-6-*P*R on the release of LTGF-β, an experiment was done as described earlier (Fig. 2, columns 8 and 9). Coculture medium collected during hr 24–48 of incubation with anti-Man-6-*P*R did not inhibit BAE-cell migration. However, inhibition of BAE-cell migration was seen when this CM was transiently acidified (Fig. 5, column 9). This inhibition of cell migration was prevented by adding anti-TGF-β (data not shown). These experiments indicate that anti-Man-6-*P*R does not affect the basal migration of BAE cells, the activity of exogenously added TGF-β, the activation of LTGF-β by plasmin, or the release of LTGF-β from cells.

DISCUSSION

The activation of LTGF-β in cocultures has a number of requirements. A species specificity appears to exist, the two cell types must be either in contact or in very close proximity, and the activation requires PA and plasmin (19, 24). In several respects, this reaction resembles the surface-catalyzed activation of coagulation factors in which zymogens and cofactors bind to specific cell-surface molecules, forming assemblages in which the catalytic efficiency of activation is increased by orders of magnitude. The effective concentrations of enzyme and substrate are increased by binding to cell-surface sites, thus accelerating the reaction. For LTGF-β activation, the participation of proteases and their potential surface localization appears important (19). However, activation of soluble LTGF-β by this mechanism would probably be inefficient because the concentration of substrate (LTGF-β) would remain low. The occurrence of a cell-surface-binding protein for LTGF-β would serve to increase the concentration of the substrate at the surface where activation occurs.

In this paper we have described a requirement for cell-surface localization of LTGF-β during its activation. We have determined that both Man-6-*P* and anti-Man-6-*P*R inhibit the activation of LTGF-β in BAE/BSM cocultures without affecting TGF-β activity, plasmin activation of LTGF-β, LTGF-β release, or BAE-cell basal migration. The most likely explanation for the effect of Man-6-*P* and anti-Man-6-*P*R is that they inhibit binding of the Man-6-*P*-containing TGF-β precursor to cell-surface Man-6-*P*/IGF-II receptors. Although we have not formally demonstrated binding of LTGF-β to Man-6-*P*/IGF-II receptors, Kovacina *et al.* (18) showed that both recombinant LTGF-β and platelet-derived LTGF-β bind to the cation-independent Man-6-*P*/IGF-II receptor. Indeed both BAE and BSM cells express cell-surface Man-6-*P*/IGF-II receptors (P.A.D., unpublished observations). Although Man-6-*P* and anti-Man-6-*P*R could inhibit the activation of LTGF-β by blocking the binding of other Man-6-*P*-containing molecules necessary for LTGF-β activation to cell-surface Man-6-*P*/IGF-II receptors, we feel this explanation is unlikely.

Another potential feature of this binding requirement is that it effectively makes the activation reaction irreversible. Others have recently reported that the LAP can recombine with TGF-β after acid activation to reconfer latency upon this TGF-β molecule (28). If the LAP were bound to the Man-6-*P*/IGF-II receptor after activation, there would be little opportunity for this species to recombine with TGF-β liberated in a soluble form. However, the fate of the LAP in LTGF-β activation in coculture remains to be established.

Most workers activate LTGF-β by transient acid treatment. In this regard, we note that Kovacina *et al.* (18) reported the rapid internalization of ^{125}I-labeled LTGF-β bound to the cell surface. Because internalized ligands and receptors encounter endosomal/lysosomal compartments that are acidic, activation of LTGF-β could occur in this milieu as has been proposed (29). We feel that this setting may be unlikely, however, because this would eliminate a requirement for plasmin and PA as well as fail to prevent the reassociation of the LAP with TGF-β once pH had been neutralized.

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