Cell-surface proteoglycans have been shown to modulate transforming growth factor-β (TGF-β) responsiveness in epithelial cells and other cell types. However, the proteoglycan (heparan sulfate or chondroitin sulfate) involved in modulation of TGF-β responsiveness and the mechanism by which it modulates TGF-β responsiveness remain unknown. Here we demonstrate that TGF-β1 induces transcriptional activation of plasminogen activator inhibitor-1 (PAI-1) and growth inhibition more potently in CHO cell mutants deficient in heparan sulfate (CHO-677 cells) than in wild-type CHO-K1 cells. 125I-TGF-β binding to TβR-II and TβR-I, respectively. Receptor-bound 125I-TGF-β undergoes nystatin-inhibitable rapid degradation in CHO-K1 cells but not in CHO-677 cells. In Mv1Lu cells (which, like CHO-K1 cells, exhibit nystatin-inhibitable rapid degradation of receptor-bound 125I-TGF-β), treatment with heparitinase or a heparan sulfate biosynthesis inhibitor results in a change from a low (<1) to a high (>1) ratio of 125I-TGF-β binding to TβR-II and TβR-I and enhanced TGF-β-induced transcriptional activation of PAI-1. Sucrose density gradient analysis indicates that a significant fraction of TβR-I and TβR-II is localized in caveolae/lipid-raft fractions in CHO-K1 and Mv1Lu cells whereas the majority of the TGF-β receptors are localized in non-lipid-raft fractions in CHO-677 cells. These results suggest that heparan sulfate negatively modulates TGF-β responsiveness by decreasing the ratio of TGF-β1 binding to TβR-II and TβR-I, facilitating caveolae/lipid-raft-mediated endocytosis and rapid degradation of TGF-β1, thus diminishing non-lipid-raft-mediated endocytosis and signaling of TGF-β1 in these epithelial cells.

TGF-β is a family of 25-kDa disulfide-linked dimeric proteins. It has three members in mammals (TGF-β1, TGF-β2, and TGF-β3), which share ~70% sequence homology (1–3). TGF-β exhibits bifunctional growth regulation; it inhibits growth of most cell types, including epithelial cells, endothelial cells, and lymphocytes, and stimulates proliferation of mesenchymal cells such as fibroblasts. The growth regulatory activity of TGF-β has been implicated in many physiological and pathological processes (e.g. embryonic development, morphogenesis, carcinogenesis, autoimmune diseases, and Alzheimer disease) (1–3). One other prominent activity of TGF-β is transcriptional activation of extra-cellular matrix synthesis-related genes, which has been implicated in tissue fibrosis. TGF-β also exhibits chemotactic activity toward monocytes and neutrophils and is involved in the process of inflammation (1–3).

The various biological activities of TGF-β are mediated by specific cell-surface type I, type II, type III, and type V TGF-β receptors (TβR-I, TβR-II, TβR-III, and TβR-V) (4–7). TβR-I is localized in caveolae/lipid-raft fractions in CHO-K1 and Mv1Lu cells whereas the majority of the TGF-β receptors are localized in non-lipid-raft fractions in CHO-677 cells. These results suggest that heparan sulfate negatively modulates TGF-β responsiveness by decreasing the ratio of TGF-β1 binding to TβR-II and TβR-I, facilitating caveolae/lipid-raft-mediated endocytosis and rapid degradation of TGF-β1, thus diminishing non-lipid-raft-mediated endocytosis and signaling of TGF-β1 in these epithelial cells.

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2 The abbreviations used are: TGF, transforming growth factor; MES, (2-N-morpholinoethanesulfonic acid); CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; DSS, dисuccinimidyl suberate; PAI-1, plasminogen activator inhibitor-1; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
HEPARAN SULFATE MODULATES TGF-β RESPONSIVENESS

EXPERIMENTAL PROCEDURES

Materials—Na$^{251}$I (17 Ci/mg), [α$^{32}$P]ATP, and [methyl-$^{3}$H]thymidine (67 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). Molecular mass protein standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa), nystatin, SDS, Dulbecco’s modified Eagle’s medium (DMEM), DMEM-F-12 medium, heparitinase, phenylmethanesulfonyl fluoride, bovine serum albumin, trichloroacetic acid, and MES, peroxidase-conjugated anti-rabbit IgG, and disuccinimidyl suberate (DSS) were obtained from Sigma. The prestained protein ladder (64, 49, 37, 26, and 20 kDa) was obtained from Invitrogen. TGF-β was purchased from Austral Biologicals (San Ramon, CA). The ECL system and rabbit polyclonal antibodies to TβR-I, TβR-II, and TβR-III were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Wild-type Chinese hamster ovary (CHO) cells (CHO-K1 cells) and mutant CHO cells (CHO-677 cells) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM-F-12 medium containing 50 µg/mL of streptomycin and 10% fetal calf serum. CHO-677 cells (pgpD 677 cells) specifically lacked heparan sulfate (20). The mutation in CHO-677 cells affected both GlcNAc and GlcA transferase activity required for heparan sulfate polymerization (20). Mink lung epithelial cells (Mv1Lu cells) were maintained in DMEM containing 50 µg/mL streptomycin and 10% fetal calf serum.

125I-TGF-β, Affinity Labeling of Cell-surface TGF-β Receptors in CHO and Mv1Lu Cells—CHO cells (CHO-K1 and CHO-677 cells) and Mv1Lu cells were grown on 6-well cluster dishes in DMEM-F-12 medium and DMEM containing 10% fetal calf serum, respectively, as described (16, 17, 20). 125I-TGF-β, affinity labeling of cell-surface TGF-β receptors was performed using the bifunctional cross-linking agent DSS as described previously (11, 14, 16, 17). The affinity-labeled TGF-β receptors were then analyzed by 5 and 7.5% SDS-PAGE under reducing conditions and autoradiography. In some experiments, the affinity-labeled TGF-β receptors were immunoprecipitated by specific antibodies to TβR-I, TβR-II, and TβR-III as described previously (17). The immunoprecipitates were then analyzed by 7.5% SDS-PAGE under reducing conditions and autoradiography.

[methyl-$^{3}$H]Thymidine Incorporation and Northern Blot Analyses—Cells were grown to near confluence on 24-well cluster dishes and then treated with several concentrations of TGF-β, at 37 °C for 18 or 2 h for [methyl-$^{3}$H]thymidine incorporation into cellular DNA or for plasmidogen activator inhibitor-1 (PAI-1) expression analysis, respectively. [methyl-$^{3}$H]Thymidine incorporation into cellular DNA and Northern blot analysis of PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in CHO-K1, CHO-677, and Mv1Lu cells were carried out as described previously (16, 17). The relative level of PAI-1 mRNA was estimated based on the ratio of PAI-1 mRNA and G3PDH mRNA intensities on the autoradiogram. The relative intensities of both mRNAs on the autoradiogram were quantitated by a Phosphoimager.

Treatment of Mv1Lu Cells with Heparitinase or a Heparan Sulfate Biosynthesis Inhibitor—Mv1Lu cells were grown near confluence on 6-well cluster dishes, washed twice with serum-free DMEM, and treated with vehicle only, heparitinase (100 units/ml) (11), or a heparan sulfate biosynthesis inhibitor p-nitrophényl-β-D-xilopyranoside (3 mM) in DMEM containing 0.1% fetal calf serum (21). After 3 h at 37 °C (for heparitinase) and 72 h at 37 °C (for the inhibitor) in serum-free DMEM containing 1% bovine serum albumin, 125I-TGF-β, affinity labeling of cell-surface TGF-β receptors and Northern blot analysis of PAI-1 or G3PDH mRNA expression was performed as described above.

Western Blot Analysis of TβR-I and TβR-II in CHO Cells—Cell lysates of CHO-K1 and CHO-677 cells (~50 µg of protein) were subjected to 7.5% SDS-PAGE under reducing conditions and then electrotransferred to nitrocellulose membranes. After being incubated with 5% nonfat milk in Tris-buffered saline plus Tween (TBST) (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature, the membranes were further incubated with specific polyclonal antibodies to TβR-I and TβR-II in TBST/nonfat milk at room temperature for 1 h and washed three times with TBST for 10 min each. Bound antibodies were detected using peroxidase-conjugated anti-rabbit IgG and visualized using the ECL system (Santa Cruz Biotechnology).

Cellular Degradation of Receptor-bound 125I-TGF-β, in CHO-K1, CHO-677, and Mv1Lu Cells—Cells grown on 24-well cluster dishes were pretreated with 25 µg/ml nystatin at 37 °C for 1 h in serum-free DMEM-F-12 medium and then incubated with 100 µM 125I-TGF-β, in the presence and absence of 100-fold excess unlabeled TGF-β. The presence of 100-fold-excess unlabeled TGF-β was used to estimate nonspecific binding. After 2.5 h at 0 °C, cells were washed and warmed to 37 °C in DMEM-F-12 medium (for CHO cells) or DMEM (for Mv1Lu cells) containing 0.2% bovine serum albumin (22). After several time periods in the presence and absence of nystatin (25 µg/ml), the conditioned medium was precipitated with 10% trichloroacetic acid at 4 °C for 0.5 h. The trichloroacetic acid-soluble radioactive material, which contained the degradation products (e.g. amino acids or peptides) of cell-surface bound 125I-TGF-β, after internalization and degradation and were released from cells, was counted. The trichloroacetic acid-soluble radioactive material derived from the specific binding of 125I-TGF-β, was estimated as the percentage of the total specific binding of 125I-TGF-β, determined before incubation at 37 °C.

Sucrose Density Gradient Analysis—Sucrose density gradient analysis was performed at 4 °C as described previously (23). Briefly, cells were grown to near confluence in 100-mm Petri dishes. After washing with ice-cold HEPES buffer (16, 17), cells were scraped into 0.85 ml of 500 mM sodium carbonate, pH 11.0. The cell pellets were homogenized with a tight-fitting Dounce homogenizer followed by three 20-s bursts of ultrasonic disintegrator on ice. The homogenates were adjusted to 45% sucrose using 90% sucrose in MES-buffered saline, pH 6.5 (25 mM MES and 0.15 mM NaCl) and placed at the bottom of an ultracentrifuge tube. Two solutions (1.7 ml each) of 35 and 5% sucrose were laid sequentially on the top of the 45% sucrose solution. After ultracentrifugation at 35,000 rpm with Beckman SW Ti55 rotor for 16–20 h, 10 0.5-ml fractions were collected from the top of the tubes, and a portion of each fraction was analyzed by SDS-PAGE followed by Western blot analysis using specific antibodies to TβR-I, TβR-II, or caveolin-1.

RESULTS

Deficiency in Heparan Sulfate Augments TGF-β Responsiveness in CHO Cells—CHO wild-type and mutant cells, which are epithelial cells defective in heparan sulfate and chondroitin sulfate synthesis, have provided an excellent system for defining the roles of proteoglycans in cellular responses to stimuli in CHO cells (20, 24). To define the roles of heparan sulfate and chondroitin sulfate in TGF-β-induced cellular responses (TGF-β responsiveness) in CHO cells, we determined the effects of increasing concentrations of TGF-β, on cell growth (as determined by measurement of DNA synthesis and cell number) and PAI-1 expression in CHO-K1 and CHO-677 cells. CHO-K1 cells are wild-type CHO cells. CHO-677 cells are CHO mutant cells, which are defective in heparan sulfate synthesis but not chondroitin sulfate synthesis (20). As shown in Fig. 1A, TGF-β, inhibited DNA synthesis in CHO-677 cells more potently than in CHO-K1 cells. At 25 pM, TGF-β, inhibited DNA synthesis by ~60% in CHO-677 cells and by ~40% in CHO-K1 cells (Fig. 1A, panel a). TGF-β, also inhibited cell growth more potently in

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CHO-677 cells than in CHO-K1 cells. TGF-β1 (100 pM) inhibited cell growth by ~75 and ~25% in CHO-677 and CHO-K1 cells, respectively (Fig. 1A, panel b). CHO-677 cells also responded more strongly to TGF-β1-induced expression of PAI-1 when compared with wild-type CHO-K1 cells (Fig. 1B, panel a). At 20 pM, TGF-β1 stimulated PAI-1 expression by ~2.2-fold in CHO-677 cells (Fig. 1B, panel b). In contrast, TGF-β1 at the same concentration only slightly stimulated PAI-1 expression by 1.2-fold in CHO-K1 cells (Fig. 1B, panel b). These results suggest that the defect in heparan sulfate biosynthesis augments TGF-β1 responsiveness in CHO cells.

**Deficiency in Heparan Sulfate Increases the Ratio of TGF-β1 Binding to TBR-II and TBR-I in CHO Cells**—125I-TGF-β1 affinity labeling of cells (125I-TGF-β1 binding and cross-linking with the bifunctional reagent DSS) followed by SDS-PAGE has been commonly used for determining 125I-TGF-β1 binding to individual cell-surface TGF-β receptor types (11, 14, 17). To determine the effect of the deficiency in heparan sulfate on 125I-TGF-β1 binding to TGF-β receptors in CHO cells, CHO-K1 and CHO-677 cells were affinity-labeled with 125I-TGF-β1 (100 pM) and analyzed by 7.5% SDS-PAGE under reducing conditions and autoradiography. As shown in Fig. 2A, in CHO-677 cells, 125I-TGF-β1 affinity-labeled TBR-III exhibited a distinct band with molecular mass of ~120 kDa (TBR-III*), representing the 125I-TGF-β1 affinity-labeled core protein of TBR-III (Fig. 2A, panel a, lane 3). In CHO-K1 cells, 125I-TGF-β1 affinity-labeled TBR-III (~270–300 kDa) migrated at the top of the separating gel on 7.5% SDS-PAGE (Fig. 2A, panel a, lane 2). 125I-TGF-β1 affinity-labeled TBR-I and TBR-II migrated as distinct bands with molecular masses of 68 and 88 kDa, respectively, on SDS-PAGE in CHO cell lines including CHO-LRP-1 cells are CHO mutant cells lacking TBR-V (22) (Fig. 2A, lane 1). The lack of TBR-V appeared to affect 125I-TGF-β1 binding to other TGF-β receptor types in CHO cells. Quantitative analysis of 125I-TGF-β1 affinity labeling of TGF-β receptors in these CHO cells revealed that CHO-677 cells exhibited an increase of TGF-β1 binding to TBR-II and a decrease of 125I-TGF-β1 binding to TBR-I when compared with CHO-K1 cells (Fig. 2A, panel b). The ratio (~1.4) of 125I-TGF-β1 affinity-labeled TBR-II and TBR-I appeared to be higher in CHO-677 cells than that (0.6) in wild-type cells (CHO-K1 cells) (Fig. 2A, panel c). This result suggests that the deficiency in heparan sulfate alters binding of TGF-β1 to TBR-1 and TBR-II. To further define this, CHO-677 and CHO-K1 cells were incubated with increasing concentrations of 125I-TGF-β1. 125I-TGF-β1 affinity labeling of TGF-β receptors in these cells was then performed and analyzed by 5% (Fig. 2B) and 7.5% (Fig. 2C). SDS-PAGE under reducing conditions and autoradiography. As shown in Fig. 2, B and C, 125I-TGF-β1 bound to TBR-I, TBR-II, and TBR-III or TBR-III in a concentration-dependent manner in CHO-K1 cells (lanes 1–5) and CHO-677 cells (lanes 6–10). The half-maximum concentration (~50 pM) of 125I-TGF-β1 has been commonly used for determining 125I-TGF-β1 binding to individual cell-surface TGF-β receptor types (11, 14, 17). To determine the effect of the deficiency in heparan sulfate on 125I-TGF-β1 binding to TGF-β receptors in CHO cells, CHO-K1 and CHO-677 cells were affinity-labeled with 125I-TGF-β1 (100 pM) and analyzed by 7.5% SDS-PAGE under reducing conditions and autoradiography. As shown in Fig. 2A, in CHO-677 cells, 125I-TGF-β1 affinity-labeled TBR-III exhibited a distinct band with molecular mass of ~120 kDa (TBR-III*), representing the 125I-TGF-β1 affinity-labeled core protein of TBR-III (Fig. 2A, panel a, lane 3). In CHO-K1 cells, 125I-TGF-β1 affinity-labeled TBR-III (~270–300 kDa) migrated at the top of the separating gel on 7.5% SDS-PAGE (Fig. 2A, panel a, lane 2). 125I-TGF-β1 affinity-labeled TBR-I and TBR-II migrated as distinct bands with molecular masses of 68 and 88 kDa, respectively, on SDS-PAGE in CHO cell lines including CHO-LRP-1 cells are CHO mutant cells lacking TBR-V (22) (Fig. 2A, lane 1). The lack of TBR-V appeared to affect 125I-TGF-β1 binding to other TGF-β receptor types in CHO cells. Quantitative analysis of 125I-TGF-β1 affinity labeling of TGF-β receptors in these CHO cells revealed that CHO-677 cells exhibited an increase of TGF-β1 binding to TBR-II and a decrease of 125I-TGF-β1 binding to TBR-I when compared with CHO-K1 cells (Fig. 2A, panel b). The ratio (~1.4) of 125I-TGF-β1 affinity-labeled TBR-II and TBR-I appeared to be higher in CHO-677 cells than that (0.6) in wild-type cells (CHO-K1 cells) (Fig. 2A, panel c). This result suggests that the deficiency in heparan sulfate alters binding of TGF-β1 to TBR-1 and TBR-II. To further define this, CHO-677 and CHO-K1 cells were incubated with increasing concentrations of 125I-TGF-β1. 125I-TGF-β1 affinity labeling of TGF-β receptors in these cells was then performed and analyzed by 5% (Fig. 2B) and 7.5% (Fig. 2C). SDS-PAGE under reducing conditions and autoradiography. As shown in Fig. 2, B and C, 125I-TGF-β1 bound to TBR-I, TBR-II, and TBR-III or TBR-III in a concentration-dependent manner in CHO-K1 cells (lanes 1–5) and CHO-677 cells (lanes 6–10). The half-maximum concentration (~50 pM) of 125I-TGF-β1
TGF-β₁ for binding to the core protein (~120 kDa) of TβR-III (TβR-III*) in CHO-677 cells was similar to that of ¹²⁵I-TGF-β₁ for binding to TβR-III (~270–300 kDa) in CHO-K1 cells (Fig. 2B, panels a and b). CHO-K1 and CHO-677 cells also did not exhibit significant differences in the half-maximum concentrations (~50 pM) of ¹²⁵I-TGF-β₁ for binding to TβR-I and TβR-II (Fig. 2C, panels b and c). This suggests that the affinities of TGF-β₁ for binding to TβR-I and TβR-II are similar in CHO-K1 and CHO-677 cells. However, the total binding of ¹²⁵I-TGF-β₁...
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FIGURE 3. Western blot analysis of TβR-I and TβR-II in CHO-K1 and CHO-677 cells. Cells were grown to confluence on 24-well cluster dishes in DMEM/F-12 medium. The cell lysates (200 μg of protein) were analyzed by Western blot analysis. The brackets indicate the locations of TβR-I, TβR-II, and β-actin.

TGF-β to TβR-II (determined as the [125I]-TGF-β, affinity labeled TβR-II) increased in CHO-677 cells as compared with that in CHO-K1 cells (Fig. 2C, panel a, lanes 6→10 versus lanes 1→5 and Fig. 2C, panel c versus panel b). The binding of [125I]-TGF-β to TβR-I ([125I]-TGF-β, affinity-labeled TβR-I) decreased concomitantly in CHO-677 cells as compared with that in CHO-K1 cells (Fig. 2B, panel a, and Fig. 2C, panel a, lanes 6→10 versus lanes 1→5 and Fig. 2C, panel c versus panel b). At 100 pM [125I]-TGF-β, the ratio of [125I]-TGF-β, affinity-labeled TβR-II and TβR-I in CHO-677 cells was estimated to be ~2 (Fig. 2C, panel d). In contrast, at the same concentration of [125I]-TGF-β, the ratio of [125I]-TGF-β, affinity-labeled TβR-II and TβR-I was estimated to be 0.7 in CHO-K1 cells (Fig. 2C, panel d). CHO-677 and CHO-K1 cells exhibited similar ratios of TβR-II and TβR-I protein levels as determined by Western blot analysis (Fig. 3, lane 2 versus lane 1). The multiple bands of TβR-II shown in Fig. 3 were also demonstrated previously (25). These results suggest that the deficiency in heparan sulfate alters the total binding of TGF-β to TβR-I and TβR-II without significantly affecting the affinities of TGF-β, binding to TβR-I and TβR-II and their protein expression in these CHO cells. The similar half-maximum concentrations of [125I]-TGF-β, for binding to TβR-III and TβR-III* (Fig. 2B, panel b) in CHO-K1 and CHO-677 cells suggest that the binding affinities of [125I]-TGF-β, for TβR-III and TβR-III* are similar in these cells. Since TβR-III is known to present the ligand TGF-β to TβR-II and then TβR-I, and since TβR-III is also known to form hetero-oligomeric complexes with TβR-I and TβR-II in the presence of TGF-β (11, 26, 27), the lack of heparan sulfate in TβR-III* (in CHO-677 cells) may affect the formation of the hetero-oligomeric complexes of TβR-III/TβR-II/TβR-I, which contain different percentages of TβR-I and TβR-II (28). To test this possibility, we performed immunoprecipitation using specific antibodies to TβR-I, TβR-II, and TβR-III following [125I]-TGF-β, affinity labeling of CHO-677 and CHO-K1 cells. As shown in Fig. 4, CHO-677 cells exhibited more [125I]-TGF-β, affinity-labeled TβR-II and TβR-I in the TβR receptor complexes when compared with CHO-K1 cells (lanes 2, 4, and 6 versus lanes 1, 3, and 5).

Enzymic Removal of Heparan Sulfate Increases the Ratio of TGF-β, Binding to TβR-II and TβR-III and Augments TGF-β-induced PAI-1 Expression in Mv1Lu Cells—To see if absent or decreased heparan sulfate alters TGF-β, binding to TβR-I and TβR-II and resultant TGF-β responsiveness in other epithelial cell systems, mink lung epithelial cells (Mv1Lu cells) were treated with heparitinase for 3 h at 37 °C or with a heparan sulfate biosynthesis inhibitor p-nitrophenyl-β-d-xylopyranoside for 72 h at 37 °C and then affinity-labeled with [125I]-TGF-β, at 0 °C. The [125I]-TGF-β, affinity-labeled TGF-β receptors were analyzed by 7.5% (Fig. 5A) and 5% (Fig. 5B) SDS-PAGE under reducing conditions and autoradiography. The brackets indicate the locations of [125I]-TGF-β, TβR-I, [125I]-TGF-β, TβR-II, and [125I]-TGF-β, TβR-III complexes. The [125I]-TGF-β, TβR-III complex migrated at the top of the separating gel.

FIGURE 4. Immunoprecipitation of [125I]-TGF-β, affinity-labeled TGF-β receptor complexes in CHO-K1 and CHO-677 cells. Cells were affinity-labeled with 100 pM [125I]-TGF-β, in the presence of DSS. The [125I]-TGF-β, affinity-labeled cell lysates were subjected to immunoprecipitation with specific antibodies to TβR-I, TβR-II, and TβR-III (α-TβR-I, α-TβR-II, and α-TβR-III, respectively). The immunoprecipitates were then analyzed by 7.5% SDS-PAGE under reducing conditions and autoradiography. The brackets indicate the locations of [125I]-TGF-β, TβR-I, [125I]-TGF-β, TβR-II, and [125I]-TGF-β, TβR-III complexes.
Cells Lacking Heparan Sulfate or Treated with Heparitinase Exhibit Decreased Cellular Degradation of TGF-β—TGF-β receptor-mediated signaling is known to occur in endosomes (29–31). The TGF-β receptor complex with a higher ratio of 125I-TGF-β1 binding to TβR-II and TβR-I (e.g. in CHO-677 cells and Mv1Lu cells treated with heparitinase) is hypothesized to undergo clathrin-mediated endocytosis and transduces signaling in endosomes (28–31). On the other hand, the TGF-β receptor complex with a low ratio (<1) of 125I-TGF-β1 binding to TβR-II and TβR-I (e.g. in CHO-K1 and Mv1Lu cells) is hypothesized to undergo rapid degradation via caveolae/lipid-raft-mediated endocytosis (28–31). To test this hypothesis, we examined the cellular degradation of 125I-TGF-β1 bound to cell-surface receptors in CHO-677 cells, CHO-K1 and Mv1Lu cells. As shown in Fig. 7, 125I-TGF-β1 bound to cell-surface TGF-β receptors underwent rapid degradation, as determined by measurement of specific trichloroacetic acid-soluble radioactive material in the conditioned medium, in wild-type CHO cells (Fig. 7A) as compared with CHO-677 cells (Fig. 7B). The cellular degradation of 125I-TGF-β1 bound to cell-surface TGF-β receptors appeared to be inhibited by nystatin in CHO-K1 and Mv1Lu cells but not in CHO-677 cells (Fig. 7, A and C versus B). Nystatin is a cholesterol-binding compound and has been used to inhibit caveolae/lipid-raft-mediated endocytosis/degradation (29–32). This result suggests that caveolae/lipid-raft-mediated endocytosis is involved in rapid degradation of TGF-β receptor complexes in CHO-K1 and Mv1Lu cells, whereas clathrin-mediated endocytosis/signaling/degradation, which is not inhibited by nystatin, mainly occurs in CHO-677 cells. To test this, we performed sucrose density gradient analysis of TβR-I and TβR-II in CHO-K1, CHO-677, and Mv1Lu cells. As shown in Fig. 8, a significant fraction of TβR-I and TβR-II was localized in caveolae/lipid-raft fractions (fractions 4 and 5) in CHO-K1 and Mv1Lu cells. The TβR-I localization in Mv1Lu cells was not
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FIGURE 7. Cellular degradation of receptor-bound ¹²⁵I-TGF-β₁ in CHO-K1 (A), CHO-677 (B), and Mv1Lu (C) cells. Cells were pretreated with nystatin (25 μg/ml) at 37°C for 1 h and then incubated with ¹²⁵I-TGF-β₁ (100 pM) in the presence and absence of 100-fold excess unlabeled TGF-β₁ (for estimating nonspecific binding of ¹²⁵I-TGF-β₁). After 2.5 h at 0°C, cells were washed and warmed to 37°C. After several time periods (0.5, 1, and 1.5 h) in the presence or absence of nystatin (25 μg/ml), 10% trichloroacetic acid-soluble radioactive material (derived from the specific binding of ¹²⁵I-TGF-β₁) in the medium was determined and expressed as the percentage of the total specific binding of ¹²⁵I-TGF-β₁. The fraction no. (n = 4). * p < 0.001, nystatin-treated cultures versus vehicle-treated cultures.

shown because the antibody to TBR-I used in the experiment did not react well with mink TBR-I antigen. By contrast, the majority of TBR-I and TBR-II were localized in non-lipid-raft fractions (fractions 7 and 8) in CHO-677 cells. These results are consistent with the notion that caveolae/lipid-raft-mediated endocytosis facilitates rapid degradation of TGF-β and attenuates TGF-β responsiveness (28–31).

DISCUSSION

Members of the TGF-β family are among the most potent growth factors or cytokines known. They are active at subpicomolar concentrations. TGF-β is regulated at several levels including: 1) transcription (1–3, 10), 2) activation of the latent form of TGF-β (33, 34), 3) endocytosis of TGF-β receptor complexes (28–31), and 4) post-TGF-β receptor signaling (35–38). Among these, the molecular mechanisms underlying the regulation of endocytosis and signaling of TGF-β receptor complexes have been less studied until recently. TGF-β-induced signaling has recently been shown to occur in endosomes (29–31). Potassium chloride depletion, which inhibits clathrin-mediated endocytosis, has been shown to attenuate TGF-β receptor internalization and TGF-β-induced cellular responses in Mv1Lu cells (29–31). TGF-β receptors at the cell surface undergo ligand-independent internalization and recycling via clathrin-coated and caveolin-positive vesicles (28–31). In the absence of ligand, TGF-β receptors undergo constitutive internalization and recycling (30). Following ligand binding, ligand-activated TGF-β receptor complexes are internalized into endosomes (where they mediate signaling) and caveolin-positive vesicles (where they are subjected to rapid degradation). The biochemical properties of TGF-β receptor complexes designated for endosomes and caveosomes (caveolin-1-positive vesicles) are unknown (29–31). Here we demonstrate that cells defective in biosynthesis of heparan sulfate or treated with a heparan sulfate biosynthesis inhibitor respond more strongly to TGF-β₁-induced cellular responses than wild-type cells or untreated cells. These cells exhibit a higher ratio (>1) of TGF-β₁ binding to TBR-II and TBR-I and a decrease of degradation of TGF-β₁ bound to TGF-β receptors as compared with those observed in wild-type or untreated cells. These results support the hypothesis that the ratio of TBR-II and TBR-I in the TGF-β receptor complex provides a signal for determining TGF-β responsiveness (28)

A model is proposed to demonstrate how the formation of distinct TGF-β receptor complexes at the cell surface determine the cellular response to TGF-β₁. In this model (Fig. 9) modified from the models reported previously (28,32), TBR-III presents TGF-β₁ to TBR-I at the cell surface. TBR-I is recruited to form TBR-III-TBR-I-TBR-I ternary complexes that have two forms: I and II. Complex I, which contains more TBR-II than TBR-I (as determined by ¹²⁵I-TGF-β₁ affinity labeling), undergoes clathrin-mediated endocytosis and transduces signaling in endosomes. Complex II, which contains more TBR-I than TBR-II, undergoes caveolae lipid-raft-mediated endocytosis and rapid degradation. The formation of these complexes can be regulated by the following: 1) the proteoglycan moiety of TBR-III: larger proteoglycan moieties in TBR-III facilitate the formation of Complex I (13), and no proteoglycan or a small proteoglycan moiety in TBR-III facilitates the formation of Complex I (13); 2) altered expression of endoglin: endoglin is a TGF-β₁-binding, proteoglycan-containing membrane protein and shares with TBR-III a limited amino acid sequence homology (39), and the increased expression of endoglin facilitates the formation of Complex II, leading to the attenuated TGF-β₁-induced cellular responses (40); and 3) altered expression of TBR-I or TBR-II. Increased expression of TBR-II or TBR-I facilitates the formation of Complex I or II and enhances or attenuates TGF-β₁-induced cellular responses, respectively (40–45). Blobe et al. (12) reported that stable transfection of L6 myoblasts with TBR-III cDNA yields a higher ratio (>1) of TGF-β₁ binding to TBR-II and TBR-I and enhances TGF-β responsiveness.

FIGURE 8. Sucrose density gradient analysis of TBR-I and TBR-II in CHO-K1, CHO-677, and Mv1Lu cells. Cells were subjected to sucrose density gradient ultracentrifugation as described (24). The fractions were analyzed by Western blot analysis using antibodies to TBR-I, TBR-II, and caveolin-1. The arrows indicate the locations of TBR-I, TBR-II, and caveolin-1. Fractions 4 and 5 were caveolar/lipid-raft fractions whereas fractions 7 and 8 were non-lipid-raft (or clathrin) fractions.
Eickelberg et al. (13) demonstrated that, in LLC-PK1 (which are epithelial cells, unlike L6 myoblasts), overexpression of TβR-III produces a lower ratio (<1) of TGF-β binding to TβR-II and TβR-I and attenuates TGF-β responsiveness. Since TβR-III in LLC-PK1 cells exhibits higher molecular weight glycosaminoglycan chains than that in L6 cells, they suggested that the sizes of glycosaminoglycan chains in the TβR-III molecule affect the ratio of TGF-β binding to TβR-II and TβR-I and thus affect TGF-β responsiveness. However, which proteoglycan (heparan sulfate or chondroitin sulfate) is involved in modulating TGF-β responsiveness has not previously been determined. Here we demonstrate that epithelial cells deficient in heparan sulfate or treated with heparitinase or a heparan sulfate biosynthesis inhibitor exhibit a higher ratio of TGF-β binding to TβR-II and TβR-I, decreased degradation of TGF-β, and enhanced TGF-β responsiveness. This suggests that heparan sulfate negatively modulates TGF-β responsiveness by facilitating formation of Complex II in epithelial cells.

In murine embryonic fibroblasts, the TβR-III gene ablation does not significantly affect the ratio of TGF-β binding to TβR-II and TβR-I in TβR-III-null murine embryonic fibroblasts (44), which is >1.0.4 This suggests that the formation of Complex I (with a high ratio of TGF-β binding to TβR-II and TβR-I) is a default process and that the formation of Complex II (e.g. induced by heparan sulfate) is a regulatory event. TGF-β is known to stimulate the expression of proteoglycans, which may play a feedback regulatory role in TGF-β actions (45, 46). Certain carcinoma cells may acquire resistance to TGF-β growth suppression by up-regulation of heparan sulfate expression (47–49).

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