The Type V Transforming Growth Factor β Receptor Is the Putative Insulin-like Growth Factor-binding Protein 3 Receptor

Sandra M. Leal, Qianjin Liu, Shuan Shian Huang, and Jung San Huang†

From the Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104

Insulin-like growth factor-binding protein 3 (IGFBP-3) has been shown to inhibit cell growth by IGF-dependent and -independent mechanisms. The putative cell-surface IGFBP-3 receptor that mediates the IGF-independent growth inhibition has not been identified. Here we show that recombinant human IGFBP-3 inhibits 125I-transforming growth factor (TGF)-β1 binding to the type V TGF-β receptor (M, 400,000) in mink lung epithelial cells. We also demonstrate that the 400-kDa 125I-IGFBP-3 affinity-labeled putative IGFBP-3 receptor is immunoprecipitated by specific antiserum to the type V TGF-β receptor. The 125I-IGFBP-3 affinity labeling of the putative receptor and IGFBP-3-induced growth inhibition as measured by DNA synthesis in these cells is blocked by a TGF-β1 peptide antagonist. The 125I-IGFBP-3 affinity-labeled putative receptor can only be detected in cells expressing the type V TGF-β receptor, but not in cells lacking the type V TGF-β receptor. These results indicate that the type V TGF-β receptor is the putative IGFBP-3 receptor and that IGFBP-3 is a functional ligand for the type V TGF-β receptor.

The type V transforming growth factor β (TGF-β) receptor is a 400-kDa non-protoglycan membrane glycoprotein that co-expresses with the type I, type II, and type III TGF-β receptors in most cell types (1–5). The type V TGF-β receptor as well as the type I and type II TGF-β receptors are Ser/Thr-specific protein kinases and belong to the new class of membrane receptors associated with a Ser/Thr-specific protein kinase activity (1–6). The type I and type II TGF-β receptors have been shown to be important in TGF-β-induced cellular responses (1–6), but the role of the type V TGF-β receptor in these responses has not been defined (3–5). Recently, we have demonstrated that the type V TGF-β receptor mediates TGF-β-induced growth inhibition and that both type I and type II TGF-β receptors are required for mediating maximal growth inhibition (7).

Insulin-like growth factor-binding protein 3 (IGFBP-3) is the most abundant insulin-like growth factor-binding protein in the circulation (8–11). In human plasma, IGFBP-3 forms an ~140-kDa ternary complex with IGFs and an acid-labile subunit (12). This complex serves as a reservoir for IGFs (12). IGFBP-3 is produced by a variety of cell types (12) and appears to inhibit cell growth by IGF-dependent and -independent mechanisms (13–15). Although several small cell membrane-associated IGFBP-3 binding proteins have recently been reported (16–18), the putative IGFBP-3 receptor that mediates the IGF-independent growth inhibition has not been identified.

IGFBP-3 has been implicated as a mediator of the actions of TGF-β, retinoic acid, and the tumor suppressor gene p53 (19–21). Since the type V TGF-β receptor appears to play an important role in TGF-β-induced growth inhibition (7), we tested the hypothesis that IGF-independent actions of IGFBP-3 are mediated by the type V TGF-β receptor. In this communication, we demonstrate that IGFBP-3 inhibits 125I-labeled TGF-β1 (125I-TGF-β1) binding to the type V TGF-β receptor in mink lung epithelial cells. We also show that 125I-labeled IGFBP-3 (125I-IGFBP-3) affinity-labeled putative cell-surface IGFBP-3 receptor is immunoprecipitated by specific antiserum to the type V TGF-β receptor and that the 125I-IGFBP-3-putative receptor complex is detected only in cells expressing the type V TGF-β receptor. Finally, we show that 125I-IGFBP-3 affinity labeling of the putative IGFBP-3 receptor and IGFBP-3-induced growth inhibition can be blocked by a TGF-β1 peptide antagonist.

EXPERIMENTAL PROCEDURES

Materials—Na125I (17 Ci/mg) and [methyl-3H]Thymidine (67 Ci/mmol) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA). High molecular mass protein standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa) and other chemical reagents were obtained from Sigma. D Accord imidyl suberate (DSS) was obtained from Pierce. TGF-β1 was purchased from Celtrix Pharmaceutical Inc. (Santa Clara, CA). 125I-TGF-β1 and 125I-IGFBP-3 were prepared as described previously (5, 26) except 0.2 M sodium phosphate buffer, pH 7.4, was used as the solvent for Sephadex G-25 column chromatography to separate 125I-IGFBP-3 from free 125I. The specific radioactivity of 125I-TGF-β1 and 125I-IGFBP-3 was 1–4 × 107 cmpg/d. The antigen used to prepare specific rabbit antiserum to the type V TGF-β receptor was thyroglobulin-conjugated to a hexadecapeptide whose amino acid sequence was derived from the partial amino acid sequence of bovine type V TGF-β receptor (7). The antiserum specifically reacted with the type V TGF-β receptor from different species, including mink, rat, mouse, cow, and human (7). This antiserum did not react with the type I, type II, and type III TGF-β receptors on Western blot analysis and in immunoprecipitation (7). TGF-β1 and TGF-β3 peptide antagonists were synthetic pentacosapeptides whose amino acid sequences were derived from those of TGF-β1 and TGF-β3, respectively.2 The IC50 values of TGF-β1 and TGF-β3 peptide antagonists for inhibiting 125I-TGF-β1 (0.1 nM) binding to TGF-β receptors in mink lung epithelial cells are ~1–2 and ~20–30 nM, respectively.3 Human colorectal carcinoma cells transfected with neo vector only and with vector expressing type II TGF-β receptor CDNA.

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Cells were plated on 24-well clustered dishes at near confluence and incubated with various concentrations of IGFBP-3 or 10 pM of TGF-β1 (Fig. 2A, lanes 4 and 5). The specific binding of 125I-TGF-β1 was calculated by subtracting the total binding from the nonspecific binding obtained in the presence of 100-fold excess of unlabeled TGF-β1 or 10 μM TGF-β1 peptide antagonist. The 125I-TGF-β1 binding affinity of cell-surface TGF-β receptors was carried out using DSS as the cross-linking agent (3, 26).

125I-IGFBP-3 Binding and Affinity Labeling in Mink Lung Epithelial Cells and Human Colorectal Carcinoma Cells—The 125I-IGFBP-3 binding and affinity labeling were carried out as described previously (3, 26). The specific binding of 125I-IGFBP-3 was determined by a liquid scintillation counter. For RNA analysis, cells were detached and lysed in 100 μl of 100 mM Tris-HCl, pH 7.4, 0.2% Triton X-100, and autoradiography. The blots were washed 10-fold with Triton X-100-free buffer and incubated with anti-IGFBP-3 antibody, followed by immunoprecipitation with specific antiserum to the type V TGF-β receptor specifically immunoprecipitated the 125I-IGFBP-3-type V TGF-β receptor complex (Fig. 2A, lanes 5 and 6). The autoradiogram was quantitated by a Phosphorimage.

The relative intensity of 125I-IGFBP-3-type V TGF-β receptor complexes was determined by liquid scintillation counting. For analysis, cells grown on 12-well cluster dishes were treated with various concentrations of 125I-IGFBP-3 or 10 μg of Triton-X-100-free buffer and incubated with anti-IGFBP-3 antibody, followed by immunoprecipitation with specific antiserum to the type V TGF-β receptor specifically immunoprecipitated the 125I-IGFBP-3-type V TGF-β receptor complex (Fig. 2A, lanes 5 and 6). The autoradiogram was quantitated by a Phosphorimage.

RESULTS AND DISCUSSION

TGF-β is the most potent known polypeptide growth inhibitor for epithelial cells and other cell types (23–25). Our recent studies have indicated that the type V TGF-β receptor, a 400-kDa membrane glycoprotein which co-expresses with the type I, type II, and type III TGF-β receptors in most cell types (2–4, 28), plays an important role in mediating TGF-β-induced growth inhibition in mink lung epithelial cells (7). To see if the IGFBP-3 binding affinity labeling of IGFBP-3 is mediated by other TGF-β receptors, we investigated the effect of IGFBP-3 on the binding of 125I-TGF-β1 to mink lung epithelial cells, for which IGFBP-3 is also a growth inhibitor. As shown in Fig. 1A, IGFBP-3 inhibited the specific binding of 125I-TGF-β1 in a concentration-dependent manner. At 16 μg/ml (~500 nM) or higher of IGFBP-3, a maximal ~50% inhibition was observed. This partial inhibition implies that IGFBP-3 competes with 125I-TGF-β1 for binding to specific TGF-β receptor types. To identify which TGF-β receptor types are responsible for IGFBP-3 binding, we performed 125I-TGF-β1 affinity labeling of cell-surface TGF-β receptors after incubation of the cells with 125I-TGF-β1 in the presence of 16 μg/ml unlabeled IGFBP-3 or 10 μM of TGF-β1 peptide antagonist. The TGF-β1 peptide antagonist is a synthetic pentacosa-peptide whose amino acid sequence was derived from that of TGF-β1 (7). As shown in Fig. 1B, the type I, type II, type III, and type V TGF-β receptors were all affinity-labeled with 125I-TGF-β1 in the presence of the cross-linking agent DSS (lane 2). Unlabeled IGFBP-3 (~500 nM) appeared to completely block 125I-TGF-β1 affinity labeling of the type V TGF-β receptor, and to a much lesser extent (30–40% inhibition), the type III TGF-β receptor (Fig. 1B, lane 3). In the control experiment, the TGF-β1 peptide antagonist completely blocked 125I-TGF-β1 affinity labeling of all TGF-β receptor types (Fig. 1B, lane 1). These results suggest that IGFBP-3 strongly competes with 125I-TGF-β1 for binding to the type V TGF-β receptor.

To further confirm that IGFBP-3 binds to the type V TGF-β receptor with high affinity or that the type V TGF-β receptor is the putative receptor for IGFBP-3, we performed the binding and cross-linking of 125I-labeled recombinant nonglycosylated human IGFBP-3 (125I-IGFBP-3, 5 nM) to its putative cell-surface receptor, followed by immunoprecipitation with specific antiserum to the type V TGF-β receptor (7). At 5 nM, 125I-IGFBP-3 was found to bind to the type V TGF-β receptor but not other TGF-β receptor types. As shown in Fig. 2A, 125I-IGFBP-3 was cross-linked to an ~400-kDa putative receptor on the cell surface of mink lung epithelial cells (lane 1). This 125I-IGFBP-3 binding and subsequent cross-linking was blocked by 100-fold excess of unlabeled IGFBP-3 or 10 μM TGF-β1 peptide antagonist but not by 10 μM TGF-β3 peptide antagonist (Fig. 2A, lanes 2, 3, and 4, respectively). The TGF-β3 peptide antagonist, a pentacosapeptide whose amino acid sequence was derived from TGF-β3, has a lower affinity to the type V TGF-β receptor.2 The antisera to the type V TGF-β receptor specifically immunoprecipitated the ~400-kDa 125I-IGFBP-3-purative receptor complex (Fig. 2A, lanes 5 and 7). Two, ~70-kDa and ~64 kDa, 125I-IGFBP-3 complexes were

FIG. 1. Effect of IGFBP-3 on 125I-TGF-β1 binding (A) and 125I-TGF-β1 affinity labeling (B) of the type V TGF-β receptor in mink lung epithelial cells. A, cells were incubated with 0.1 mM 125I-TGF-β1, in the presence of various concentrations of IGFBP-3 with or without 100-fold excess of unlabeled TGF-β1. The specific binding of 125I-TGF-β1 to the cells was determined. The specific binding of 125I-TGF-β1 in the absence of IGFBP-3 was taken as 0% inhibition (4,537 ± 250 cpm/well). The error bars are means ± S.D. of triplicate cultures. B, after incubation of cells with 0.1 mM 125I-TGF-β1, in the absence (lane 2) and presence of 16 μg/ml of IGFBP-3 (lane 3) or 10 μM TGF-β1 peptide antagonist (lane 1), the cell-surface TGF-β receptors were affinity-labeled and analyzed by 5% SDS-polyacrylamide gel electrophoresis and autoradiography. The brackets indicate the locations of the 125I-TGF-β1 affinity-labeled type I, type II, and type III TGF-β receptors (TGF-I, TGF-II, and TGF-III). The arrow indicates the location of the 125I-TGF-β1, affinity-labeled type V TGF-β receptor (TGF-V).
also found in the cell lysates and in the immunoprecipitates (Fig. 2A, lanes 1, 4, 5, and 7). Since the preparation of 125I-IGFBP-3 (apparent Mr, \( \sim 35,000 \)) on SDS-polyacrylamide gel electrophoresis) used in the experiments was found to contain proteolytic products (apparent Mr \( \leq 32,000 \)), and since 125I-IGFBP-3 has been shown to form a dimer in solution \((17)\), these 125I-IGFBP-3 complexes may be cross-linked dimers of 125I-IGFBP-3 and its proteolytic products. In the control experiments, no 125I-IGFBP-3-putative receptor complex was found in the immunoprecipitates when the cells were incubated with 125I-IGFBP-3 in the presence of 10 \( \mu \)M TGF-\( \beta \), peptide antagonist prior to cross-linking and immunoprecipitation (Fig. 2A, lane 6). Non-immune serum did not immunoprecipitate the 125I-IGFBP-3-putative receptor complex (Fig. 2A, lane 8).

These results suggest that 125I-IGFBP-3 specifically binds to the type V TGF-\( \beta \) receptor in mink lung epithelial cells. To further characterize the binding of 125I-IGFBP-3 to the type V TGF-\( \beta \) receptor, we determined the specific binding of various concentrations of 125I-IGFBP-3 to the type V TGF-\( \beta \) receptor in mink lung epithelial cells. As shown in Fig. 2B, 125I-IGFBP-3 bound to the type V TGF-\( \beta \) receptor in these cells in a concentration dependent manner (lanes 1–5). The Scatchard plot analysis of the binding revealed that the apparent \( K_d \) for 125I-IGFBP-3 binding to the type V TGF-\( \beta \) receptor was 6 ± 2 nM (data not shown). Since IGFBP-3 is known to bind IGFs with high affinity, and since it contains a heparin-binding site near its C-terminal end (8–11), we determined the effects of the IGF-I complex and heparin on the binding of 125I-IGFBP-3 to type V TGF-\( \beta \) receptor in mink lung epithelial cells. As shown in Fig. 2C, at a mol:1 mol stoichiometry of IGF-I and 125I-IGFBP-3, approximately 80% of the 125I-IGFBP-3-specific binding to the type V TGF-\( \beta \) receptor was inhibited. Heparin at 100 \( \mu \)g/ml inhibited \( \sim 80\% \) of the 125I-IGFBP-3 binding to the type V TGF-\( \beta \) receptor (Fig. 2D, lane 9 versus lane 1). The control (Fig. 2D, lane 1) was overexposed to show the 125I-IGFBP-3-type V TGF-\( \beta \) receptor complex in lanes 2, 3, and 8. These results suggest that both the 125I-IGFBP-3-IGF-1 complex and the 125I-IGFBP-3-heparin complex are not capable of binding to the type V TGF-\( \beta \) receptor. As a control, TGF-\( \beta \), peptide antagonist (3 \( \mu \)M) strongly inhibited \( >95\% \) of the 125I-IGFBP-3 binding to the type V TGF-\( \beta \) receptor (Fig. 2D, lane 2 versus lane 1). To further demonstrate that the type V TGF-\( \beta \) receptor is the putative IGFBP-3 receptor, we performed the 125I-IGFBP-3-affinity labeling of its putative cell-surface receptor in cells expressing and lacking the type V TGF-\( \beta \) receptor. As shown in Fig. 3, human colorectal carcinoma cells (R-II-37 cells and HCT 116 Neo cells), which lack the type V TGF-\( \beta \) receptor (7, 27), did not show the \( \sim 400-kDa \) 125I-IGFBP-3-putative receptor complex (Fig. 3, lanes 3–6). In contrast, NIH 3T3 cells, which are known to express the type V TGF-\( \beta \) receptor (26), showed the \( \sim 400-kDa \) 125I-IGFBP-3-putative receptor complex (Fig. 3, lanes 1 and 2). The formation of the \( \sim 400-kDa \) 125I-IGFBP-3-putative receptor complex in NIH 3T3 cells was blocked in the presence of 100-fold excess of unlabeled IGFBP-3 or 10 \( \mu \)g/ml TGF-\( \beta \), peptide antagonist (data not shown).

Since the type V TGF-\( \beta \) receptor has been shown to mediate the growth inhibitory response in mink lung epithelial cells (7), we examined the effect of IGFBP-3 on the proliferation of wild-type and type I and type II TGF-\( \beta \) receptor-defective mutant mink lung epithelial cells (Mv1Lu, R-1B, and DR26 cells, respectively) (22, 28–30). All Mv1Lu, R-1B, and DR26 cells have been shown to express the type V TGF-\( \beta \) receptor (7). IGFBP-3 should be a specific ligand to test the function of the type V TGF-\( \beta \) receptor, because it does not bind to the type I, type II, or type III TGF-\( \beta \) receptor with high affinity. As shown in Fig. 4, IGFBP-3 (0.6 \( \mu \)g/ml or \( \sim 20 \) nM) induced a similar growth inhibitory response as measured by DNA synthesis.

\(^{3}\) S. M. Leal, Q. Liu, S. S. Huang, and J. S. Huang, unpublished results.
shown). TGF-β on the transcription of PAI-1 in these epithelial cells (data not shown). The effect of IGFBP-3 on the transcriptional expression of PAI-1 cells do not respond to TGF-β carcinoma cells lacked the type V TGF-β receptor expression in cells stably transfected with the type II TGF-β receptor-defective mutant mink lung epithelial cells (HCT 116 Neo and RII-37 cells) that do not express the type V TGF-β receptor (27). Stable transfection of these cells could be blocked in the presence of TGF-β peptide antagonist (Fig. 4). These results indicate that IGFBP-3 induces a growth inhibitory response in cells expressing the type V TGF-β receptor. These results also support the hypothesis that the type V TGF-β receptor can mediate the growth inhibitory response (7).

In a previous study (26), we reported that many types of carcinoma cells lacked the type V TGF-β receptor and that such cells do not respond to TGF-β stimulation, as measured by growth inhibition (7). Recently, hereditary human colorectal carcinoma cells (HCT 116 cells) were shown to be deficient in the type II TGF-β receptor (27). Stable transfection of these carcinoma cells with the type II TGF-β receptor cDNA was found to rescue the transcriptional response but failed to restore the growth inhibitory response to exogenous TGF-β stimulation (27). This appears to be due to the lack of the type V TGF-β receptor expression in cells stably transfected with the neo vector only (HCT 116 Neo cells) or with vector expressing the type II TGF-β receptor cDNA (RII-37 cells) (7, 27). As would be expected, IGFBP-3 also failed to inhibit growth in these HCT 116 Neo and RII-37 cells that do not express the type V TGF-β receptor (data not shown).

TGF-β elicits a variety of biological activities in different cell types (23–25). In addition to growth inhibitory activity, the other prominent activity of TGF-β is transcriptional activation of fibronectin, collagen, and PAI-1 genes (23–25). To see if IGFBP-3 and TGF-β share similar activities, we determined the effect of IGFBP-3 on the transcriptional expression of PAI-1 in mink lung epithelial cells. IGFBP-3 showed little if any effect on the transcription of PAI-1 in these epithelial cells (data not shown). TGF-β has been shown to be a bifunctional growth regulator: a growth inhibitor for epithelial cells, endothelial cells, and other cell types, and a mitogenic factor for mesenchymal cells (23–25). We therefore determined the effect of IGFBP-3 on DNA synthesis in NIH 3T3 cells, for which TGF-β is a mitogen. IGFBP-3 did not stimulate DNA synthesis of NIH 3T3 cells at concentrations of 0.1–100 nM, suggesting that IGFBP-3 is a partial agonist of TGF-β. These results also support the hypothesis that the type V TGF-β receptor preferentially mediates the growth inhibitory response in responsive cells (7).

IGFBP-3 has been implicated as a mediator of the actions of TGF-β, retinoic acid, and p53 (19–21). Antisense deoxoygycylanucleotide to IGFBP-3 has been shown to diminish the growth inhibitory response induced by TGF-β and retinoic acid in human mammary carcinoma cells (19–21). The functional role of IGFBP-3 in TGF-β-induced growth inhibition in other cell types is unknown. We speculate that the IGFBP-3 expression increases IGFs from binding to IGF-I receptor in IGF-responsive cells and by propagating the growth inhibitory response mediated by the type V TGF-β receptor in IGF-unresponsive cells. It is important to note that upon ligand activation, the type V TGF-β receptor may also decrease IGF-II concentration in the extracellular compartment by increasing the internalization and recycling of the cell surface mannose 6-phosphate/IGF-II receptor.4 This effect on IGF-II may also contribute to the growth inhibitory response mediated by the type V TGF-β receptor.

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