Functional Roles for the Cytoplasmic Domain of the Type III Transforming Growth Factor β Receptor in Regulating Transforming Growth Factor β Signaling*

Transforming growth factor β (TGF-β) signals through three high affinity cell surface receptors, TGF-β type I, type II, and type III receptors. The type III receptor, also known as betaglycan, binds to the type II receptor and is thought to act solely by “presenting” the TGF-β ligand to the type II receptor. The short cytoplasmic domain of the type III receptor is thought to have no role in TGF-β signaling because deletion of this domain has no effect on association with the type II receptor, or with the presentation role of the type III receptor. Here we demonstrate that the cytoplasmic domains of the type III and type II receptors interact specifically in a manner dependent on the kinase activity of the type II receptor and the ability of the type II receptor to auto-phosphorylate. This interaction results in the phosphorylation of the cytoplasmic domain of the type III receptor by the type II receptor. The type III receptor with the cytoplasmic domain deleted is able to bind TGF-β, to bind the type II receptor, and to enhance TGF-β binding to the type II receptor but is unable to enhance TGF-β2 signaling, determining that the cytoplasmic domain is essential for some functions of the type III receptor. The type III receptor functions by selectively binding the autophosphorylated type II receptor via its cytoplasmic domain, thus promoting the preferential formation of a complex between the autophosphorylated type II receptor and the type I receptor and then dissociating from this active signaling complex. These studies, for the first time, elucidate important functional roles of the cytoplasmic domain of the type III receptor and demonstrate that these roles are essential for regulating TGF-β signaling.

* This work was supported by a Howard Hughes Medical Institute postdoctoral research fellowship for physicians (to G. C. B.) and by Grants CA73161-01 from the NCI, National Institutes of Health (to W. P. S.) and CA63260 from the National Institutes of Health (to H. F. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; GST, glutathione S-transferase; KD, kinase-dead; HA, hemagglutinin.

Received for publication, January 9, 2001, and in revised form, April 25, 2001
Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M100189200
the cell cycle/proliferation (4, 5). In addition, Smad-independent signaling, and signaling through mitogen-activated protein kinase and other cellular signaling pathways has been reported (6–10).

To explore the involvement of TGF-β receptor complexes in TGF-β signaling, we examined the complexes formed by the type III receptor and the role of the type III receptor in TGF-β signaling. The type III receptor forms complexes individually with either the type II receptor or the type I receptor, as well as with complexes with the type II and type I receptors together. Current evidence suggests that the interaction of the type III receptor with the type II receptor occurs through their respective extracellular domains because deletion of the cytoplasmic domain of the type III receptor does not alter the ability of these receptors to interact (11). Indeed, no functional role for the cytoplasmic domain of the type III receptor has been established. The studies described herein establish a role for a specific, functional, and biologically significant interaction between the cytoplasmic domains of the type II and type III receptors. The protein kinase activity of the type II receptor and autophosphorylation of the type II receptor are both shown to be essential for this interaction, which results in the phosphorylation of the cytoplasmic domain of the type III receptor by the type II receptor. The type III receptor with its cytoplasmic domain deleted is able to bind TGF-β, the type II receptor, and enhance TGF-β binding to the type II receptor but is unable to enhance TGF-β2 signaling. The type III receptor functions by selectively binding autophosphorylated type II receptor via its cytoplasmic domain, preferentially promoting the formation of a complex between the autophosphorylated type II receptor and the type I receptor, and then dissociating from this active signaling complex. These studies elucidate important functions for the cytoplasmic domain of the type III receptor and demonstrate that these functional roles are essential for regulating TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The COS-7 and L6 cell lines were obtained from ATCC. These cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were transiently transfected using expression vectors for the type III receptor and type II or type I receptors using FuGene6 reagent under conditions described by the manufacturer (Roche). The L6-III and L6-III-cyto stable cell lines were selected in 0.6 mg/ml G418 and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 0.3 mg/ml G418. All cells were grown in 5% CO2 at 37 °C in a humidified atmosphere.

TGF-β Binding and Cross-linking—Radioligand binding and cross-linking of 125I-TGF-β1 to L6, L6-III, L6-III-cyto, or transfected COS-7 cells were performed by incubating subconfluent cells with KRH buffer (50 mM Heps, pH 7.5, 130 mM NaCl, 5 mM MgSO4, 1 mM CaCl2, and 5 mM KCl) containing 0.1% bovine serum albumin for 30 min at 30 °C, then with 100 μM 125I-TGF-β1 for 3 h at 4 °C. 125I-TGF-β1 was cross-linked with 0.5 ng/ml disuccinimidyl suberate for 15 min and quenched with 20 mM glycine. Cells were then washed with KRH buffer, lysed in RIPA lysis buffer, immunoprecipitated with the indicated antibodies, and analyzed by SDS-polyacrylamide gel electrophoresis and phosphorimaging analysis of dried gels. Quantitation of bands was performed using ImageQuant version 1.2 software from Molecular Dynamics.

Phosphorylation Assays in Vivo—COS-7 and 293T cells were transfected with the type II receptors or the type II receptor-KD (kinase-dead), and the HA epitope-tagged type III receptor. After 48 h, the cells were washed in phosphate-free medium and labeled with 1.0 mM/ml 32P, for 4 h. Cells were washed with phosphate-buffered saline, lysed with RIPA lysis buffer, immunoprecipitated with αHA antibody and protein G-Sepharose, analyzed on 10% SDS-polyacrylamide gels, and detected by phosphorimaging analysis of the dried gels.

RESULTS

Interaction of the Cytoplasmic Domains of the Type III Receptor and the Type II Receptor—The type I, II, and III TGF-β receptors have been demonstrated to associate in various complexes through both co-immunoprecipitation studies (12–16) and in live cells using co-patching experiments (17, 18). Antibodies to either the type II or type III receptor are capable of co-immunoprecipitate both receptors, indicating that they form a complex in the presence of ligand.

We have developed a polyclonal antibody to a small peptide (amino acids 833–847) in the cytoplasmic domain of the type III receptor (α277). In contrast to other antibodies to the type III receptor, we have observed that the α277 antibody is unable to co-immunoprecipitate the type II receptor (14). In addition, while examining the expression of the type III receptor in endothelial cell lines, we have observed that the α277 antibody was increasingly unable to immunoprecipitate the type III receptor in cell lines with higher levels of expression of the type II receptor relative to the type III receptor (19). These observations suggested that the cytoplasmic domains of the receptors interact with one another, with the type II receptor preventing immunoprecipitation of the type III receptor by the α277 antibody.

To establish the mechanism and relevance of this phenomenon, we analyzed the ability of the α277 antibody to immunoprecipitate or co-immunoprecipitate wild type or HA epitope-tagged type II and type III receptors expressed in COS-7 cells by transient transfection. COS-7 cells were utilized because they express low levels of endogenous receptors that could confound analysis. Expression of these receptors was detected by binding and cross-linking the receptors with iodinated TGF-β1. As expected, the affinity-labeled type III receptor appears as a band at 95 kDa (Fig. 1). The α277 antibody was able to immunoprecipitate the type III receptor, however, co-expression of the type II receptor with the type III receptor abolished the ability of the α277 antibody to immunoprecipitate the type III receptor (Fig. 1A). Similar results were obtained when untagged versions of the receptor were utilized (data not shown). This effect was dose-dependent, as increasing the expression of the type II receptor relative to the expression of the type III receptor was able to inhibit progressively the ability of the α277 antibody to immunoprecipitate the type III receptor (Fig. 1B). We were also able to detect increased expression of the type II receptor because this was not co-immunoprecipitated by the α277 antibody. These findings demonstrate that the α277 antibody immunoprecipitates only the type III receptor that is not complexed with type II receptors and suggest that the effect of the type II receptor is the result of an interaction of its cytoplasmic domain with the cytoplasmic domain of the type III receptor.

To determine whether this effect was specific to the α277 antibody we performed a number of control experiments. First, the ability of the type II receptor to affect immunoprecipitation...
mass standards (in kDa) are indicated on the right. The molecular mass band from 180–300 kDa characteristic of the type III receptor was precipitated with the indicated antibodies, and analyzed on 10% SDS-polyacrylamide gels. The positions of the type III and type II receptors and molecular mass standards (in kDa) are indicated on the right.

Fig. 1. Interaction of the cytoplasmic domain of the type II TGF-β receptor with the cytoplasmic domain of the type III TGF-β receptor. Panel A, COS-7 cells were transiently transfected with the HA-tagged type III TGF-β receptor with (+) or without (−) the type II TGF-β receptor. Cells were affinity labeled with 125I-TGF-β1, immunoprecipitated (IP) with the indicated antibodies, and analyzed on 10% SDS-polyacrylamide gels. The bracket delineates the high molecular mass band from 180–300 kDa characteristic of the type III receptor. The arrow indicates the position of the type II receptor. Molecular mass standards (in kDa) are indicated on the right. Panel B, dose dependence of the type II receptor interaction with the type III receptor. COS-7 cells were transiently transfected with 2 μg of the HA-tagged type III TGF-β receptor and increasing amounts (0.5–8 μg) of the type II TGF-β receptor or 0.5–8 μg of the kinase-dead type II TGF-β receptor (type II-KD). Cells were affinity labeled with 125I-TGF-β1, immunoprecipitated with the indicated antibodies, and analyzed on 10% SDS-polyacrylamide gels. The positions of the type III and type II receptors and molecular mass standards (in kDa) are indicated on the right.

of the HA-tagged type III receptors with the αHA antibody was analyzed. The αHA antibody was able to immunoprecipitate the affinity-labeled type III receptor, and co-expression of the type II receptor did not block this ability (Fig. 1A). In addition, when increasing the expression of the type II receptor relative to the type III receptor, the αHA antibody was able to co-immunoprecipitate the type II receptor and to detect the increased expression of the type II receptor (Fig. 1B). Second, an antibody to the intracellular domain of the type II receptor (α260) was able to immunoprecipitate the type II receptor and co-immunoprecipitate the type III receptor and, as expected, did not co-immunoprecipitate the type III receptor unless the type II receptor was expressed (Fig. 1A). Finally, the ability of an antibody to the entire cytoplasmic domain of the type III receptor was analyzed (α277). This antibody was able to immunoprecipitate the type III receptor, and expression of the type II receptor had no effect on this ability (Fig. 1A). In addition, the type II receptor was co-immunoprecipitated by this antibody (Fig. 1A). These studies confirm that the ability of the type II receptor to interfere with the immunoprecipitation of the type III receptor is specific to the α277 antibody, establishing this α277 antibody immunoprecipitation assay as a measure of a specific interaction between the cytoplasmic domains of the type II receptor and the type III receptor.

Specificity of the type II TGF-β Receptor—To determine whether the ability of the cytoplasmic domain of the type II TGF-β receptor to interact with the cytoplasmic domain of the type III receptor was specific to the type II TGF-β receptor, we analyzed the effect of expression of the type II TGF-β receptor as well as the type II receptors for activin and BMP, respectively. As shown in Fig. 2, the type I TGF-β receptor, the kinase-dead type I TGF-β receptor, the kinase-dead type II TGF-β receptor, and the type II BMP receptor were unable to interact with the cytoplasmic domain of the type III receptor. These results demonstrate that the ability of the type II TGF-β receptor to interact with the cytoplasmic domain of the type III receptor is specific to the type II TGF-β receptor.

Role of the Cytoplasmic Domain of the Type II Receptor on Type II-Type III Interactions—The α277 antibody immunopre-
Role of the Cytoplasmic Domain of the Type III Receptor—We have demonstrated previously that a series of deletion mutants of the extracellular region of the type III receptor which contain the membrane proximal region retains the ability to bind TGF-β, and a series of deletion mutants lacking the indicated amino acids from the extracellular domains (Δ44–303, Δ44–382, Δ44–402, Δ44–420, Δ44–564, Δ44–575) and either the type II TGF-β receptor (type II) or the type II TGF-β receptor without the cytoplasmic domain (type II Δcyto) were transiently transfected with the HA-tagged type III TGF-β receptor or mutants lacking the indicated amino acids from the extracellular domains. We were able to detect not only the monomer but also oligomeric forms of these mutants (Fig. 3). In all cases, these mutants interacted with the type II receptor, but not with the type II-cyto60 (type II(Δcyto)) mutant (Fig. 3). These results demonstrate that the type II receptor can interact with the type III receptor and suggest that the type II receptor interacts with the cytoplasmic domain of the type III receptor to block immunoprecipitation of the type III receptor (Fig. 3). These studies confirm that, as expected, these regions of the extracellular domain of the type III receptor were not necessary for the interactions of the cytoplasmic domains of these receptors. In addition, we were able to detect the oligomers of the type III receptor with the α277 antibody, demonstrating that the interaction of the cytoplasmic domain of the type III receptor with itself does not interfere with access of the α277 antibody epitope, in contrast to the situation with the heterocomplex of the type II and type III receptors.

Role of the Type III Receptor in TGF-β Signaling—The cytoplasmic domain of the type III receptor contains a serine/threonine protein kinase that functions by autophosphorylating the type II receptor and by phosphorylating and activating the type I receptor. The inability of the type II-cyto60 or the type III receptor in homodimers to block immunoprecipitation suggests that the type II receptor interacts with the cytoplasmic domain of the type III receptor in a nonsteric manner. To determine whether the kinase activity of the type II receptor mediates the ability of the type II receptor to interact with the type III receptor, we utilized a kinase-dead version of the type II receptor (type II-KD). The type II-KD was effectively co-immunoprecipitated with the type III receptor, the type II-KD mutant was effectively co-immunoprecipitated with the type III receptor (Fig. 1B). In contrast with the type II receptor, the type II-KD mutant was effectively co-immunoprecipitated with the α277 antibody, indicating that the α277 antibody could detect the type III receptor complexed with the type II-KD mutant receptor (Fig. 1B). These studies demonstrate that the kinase activity of the type II receptor is essential for the ability of the type II receptor to interact with the cytoplasmic domain of the type III receptor.

In addition, we are able to detect not only the monomer but also oligomeric forms of these mutants (Fig. 3). In all cases, this was true for mutants lacking amino acids from the extracellular domains (Δ44–303, Δ44–382, Δ44–402, Δ44–420, Δ44–564, Δ44–575) and either the type II TGF-β receptor (type II) or the type II TGF-β receptor without the cytoplasmic domain (type II Δcyto). Cells were affinity labeled with [125I]TGF-β, immunoprecipitated with the α277 antibody, and analyzed on 10% SDS-polyacrylamide gels. The bracket delineates the type III receptor, the arrow delineates the type III receptor oligomeric (oligo) and monomeric (mono) complexes, the type III receptor core, and the TGF-β ligand as indicated. The molecular mass standards (in kDa) are indicated on the right.
Phosphorylation of the Type III Receptor by the Type II Receptor—Two mechanisms can be proposed for why the kinase activity of the type II receptor is essential for its interaction with the cytoplasmic domain of the type III receptor. Either phosphorylation of the type III receptor by the type II receptor directly prevents the $\alpha_{277}$ antibody from binding its epitope on the type III receptor, or autophosphorylation of the type II receptor indirectly blocks the epitope of the $\alpha_{277}$ antibody either by inducing a close association between the type III and type II receptors or by recruiting an adaptor protein that binds to the autophosphorylated type II receptor and/or the phosphorylated type III receptor cytoplasmic domain.

The cytoplasmic domain of the type III receptor is rich in serine and threonine residues and is phosphorylated (21, 22); however, the kinase(s) responsible for phosphorylating the type III receptor in vivo has not been elucidated. To determine whether the type II receptor was the protein kinase responsible for phosphorylating the type III receptor, we initially investigated whether the cytoplasmic domain of the type III receptor could be a substrate for the type II receptor. For these studies, a GST fusion of the cytoplasmic domain of the type III receptor was utilized. A GST fusion of the cytoplasmic domain of the closely related receptor, endoglin, was utilized as a control substrate, and the type II-KD receptor was utilized as a control kinase source. As expected, the type II receptor was able to autophosphorylate, whereas type II-KD was not (data not shown). The type II receptor was also able to phosphorylate the GST-type III cytoplasmic domain significantly, with a 3-fold induction relative to type II-KD (data not shown). The GST-endoglin cytoplasmic domain was also phosphorylated significantly by the type II receptor relative to type II-KD (with a 2-fold induction) but not to the same extent as GST-type III cytoplasmic domain. These studies demonstrate that the type II receptor can phosphorylate the cytoplasmic domain of the type III receptor in vitro.

To analyze whether this phosphorylation occurred in vivo, the type III receptor was expressed in the presence and absence of either the type II receptor or the type II-KD receptor, the cells were labeled with $^{32}$P, and the phosphorylation state of the type III receptor was analyzed by immunoprecipitation. As

\[ \text{Fig. 4. The type II receptor phosphorylates the type III receptor in vivo.} \]

COS-7 cells were transiently transfected with the HA-tagged type III TGF-\(\beta\) receptor and either the type II TGF-\(\beta\) receptor (Type II-WT) or the kinase-dead type II TGF-\(\beta\) receptor (Type II-KD), labeled with \(P_{\gamma}\), and immunoprecipitated (IP) with the \(\alpha\)-HA antibody. Panel A, the products were analyzed on 10% SDS-polyacrylamide gels. The bracket delineates the type III receptor, and molecular mass standards (in kDa) are indicated on the right. Panel B, the phosphorimaging data were quantified utilizing Image Gauge version 3.0, and the data are expressed in arbitrary units. Panel C, the $\alpha_{277}$ antibody is able to immunoprecipitate the phosphorylated type III receptor. The GST fusion protein of the cytoplasmic domain of the type III receptor (GST-III) was phosphorylated by the type II receptor in vitro. This reaction was then immunoprecipitated with the $\alpha_{277}$ antibody and analyzed on 10% SDS-polyacrylamide gel.
shown in Fig. 4, the phosphorylation state of the type III receptor was increased 7-fold by co-expression of the type II receptor but not by the type II-KD receptor or in the absence of an expressed type II receptor. The ability of the type II receptor to phosphorylate the type III receptor directly in vitro, together with its ability to enhance the phosphorylation of the type III receptor in vivo, demonstrates that the type III receptor is a physiological substrate for the type II receptor.

To determine whether phosphorylation of the type III receptor by the type II receptor was the mechanism by which the type II receptor interacts with the type III receptor to block TGF-β signaling, we investigated whether phosphorylation of the cytoplasmic domain of the type III receptor by the type II receptor altered the ability of the α277 antibody to immunoprecipitate the type III receptor. When the GST-type III cytoplasmic domain was phosphorylated by the type II receptor in vitro, the α277 antibody was able to immunoprecipitate and detect this phosphorylation (Fig. 4C). In addition, when the type III receptor was expressed in the presence and absence of the type II receptor, immunoprecipitated by the αHA antibody and analyzed by Western blot with the α277 antibody, the α277 antibody was able to detect the type III receptor that had been expressed alone or with the type II receptor (data not shown). These results demonstrate that phosphorylation of the type III receptor by the type II receptor is not responsible for the inability of the α277 antibody to detect the type III receptor in the presence of the type II receptor.

We then investigated whether autophosphorylation of the type II receptor was responsible for the dependence on type II kinase activity by testing autophosphorylation site mutants. The type II receptor is autophosphorylated on at least three serine residues in the cytoplasmic domain, Ser-213, Ser-409, and Ser-416, with Ser-213 being the major site (23). Mutational analysis of these autophosphorylation sites (from serine to alanine) demonstrated that Ser-213 was important for maximal kinase activity, whereas Ser-409 and Ser-416 were not, and that Ser-409 was important for TGF-β signaling. As shown in Fig. 5A, when either Ser-213 or Ser-416 was mutated to alanine, there was no effect on the ability of the type II receptor to interact with the cytoplasmic domain of the type III receptor. In contrast, mutation of Ser-409 to alanine either alone, or in combination with Ser-416, completely blocked the ability of the type II receptor to interact with the cytoplasmic domain of the type III receptor. To investigate the ability of these autophosphorylation mutants of the type II receptor to phosphorylate the type III receptor and autophosphorylate in vivo, the type III receptor was expressed in the presence of either the type II receptor or the autophosphorylation site mutants of the type II receptor, the cells were labeled with 32P, and the phosphorylation state of the type III receptor and type II receptor was analyzed by immunoprecipitation. As shown in Fig. 5B, all of the type II receptors with the exception of the type II-KD were able to phosphorylate the type III receptor with varying ability (S416A > S213A > wild type > S409A). However, only the S213A and S416A mutants of the type II receptor were able to autophosphorylate and their ability to autophosphorylate (S416A > S213A > wild type) correlated directly with their ability to interact with the cytoplasmic domain of the type III receptor (Fig. 5). These results demonstrate that kinase activity of the type II receptor is necessary but not sufficient for its interaction with the type III receptor and that autophosphorylation of the type II receptor is required as well. As the cytoplasmic domain of type III receptor interacted selectively with the autophosphorylated form of the type II receptor and autophosphorylation of the type II receptor is required for type II receptor activation and subsequent down-stream TGF-β signaling, these results suggest a mechanism by which this interaction could functionally regulate TGF-β signaling.
Fig. 6. Requirement for the cytoplasmic domain of the type III receptor for type III receptor-mediated signaling. Stable cell lines of the L6 myoblast cell line that express the HA-tagged type III receptor (L6-III) or the HA-tagged type III receptor lacking the cytoplasmic domain (L6-III-cyto) were made. Panel A, full-length type III receptor and the type III receptor lacking the cytoplasmic domain are expressed and bind the type II receptor to a similar degree. The L6-III and L6-III-cyto cell lines were affinity labeled with 125I-TGF-β1, immunoprecipitated (IP) with the αHA antibody or the α260 (type II receptor) antibody, and analyzed on 10% SDS-polyacrylamide gels. The bracket delineates the type III receptor, and the arrow indicates the type II receptor. The data were quantified using ImageQuant software, and the data are expressed in arbitrary units. Panel B, full-length type III receptor and the type III receptor lacking the cytoplasmic domain enhance TGF-β1 binding to the type II receptor to a similar degree. The L6, L6-III, and L6-III-cyto cell lines were affinity labeled with 12.5–100 pM 125I-TGF-β1 and analyzed directly on 10% SDS-polyacrylamide gels (upper image). The L6-III and L6-III-cyto cell lines were treated with 12.5–100 pM TGF-β1, and extracts were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibody to the type II TGF-β receptor (lower image). The data were quantified using ImageQuant software, and the data are expressed arbitrary units, normalized to the expression of the type II TGF-β receptor. Panel C, the L6-III and L6-III-cyto cell lines were utilized in thymidine incorporation assays in the presence of 200 pM TGF-β2. Results are the average of four experiments done in triplicate, with S.E. indicated.

Functional Role of the Cytoplasmic Domain of the Type III Receptor in TGF-β Signaling—Although previous studies have suggested that the cytoplasmic domain of the type III receptor is not essential for formation of a complex between the type II and type III receptors or for the presentation role of the type III receptor, the present studies suggested that the cytoplasmic domain of the type III receptor plays an important role in regulating TGF-β signaling. To investigate the role of the cytoplasmic domain of the type III receptor in TGF-β signaling, we analyzed the ability of the type III receptor and mutants of the type III receptor lacking the cytoplasmic domain to bind TGF-β, bind the type II receptor, present TGF-β to the type II receptor, and mediate TGF-β signaling. The type III receptor and a mutant of the type III receptor which lacks the entire cytoplasmic domain (III-cyto) were transfected into the L6 myoblast cell line, which normally lacks expression of the type III receptor, and selected for stable expression. As shown in Fig. 6A, the type III receptor and the type III-cyto receptor in these stable cell lines were both expressed at the cell surface and bound TGF-β to a similar degree. In addition, both receptors bound the type II receptor as demonstrated by the ability of both receptors to co-immunoprecipitate the type II receptor using the αHA antibody and the ability of both receptors to be co-immunoprecipitated with the type II receptor using the α260 antibody for the type II receptor (Fig. 6A). Both the type III receptor and the type III-cyto receptor also increased binding of TGF-β to the type II receptor relative to the parental L6 cell line to a similar degree (Fig. 6B). The stable cell lines expressing the type III receptor and the type III-cyto receptor were then utilized in [3H]thymidine incorporation assays to investigate the effect of these type III receptors on TGF-β signaling. The TGF-β2 isofrom was utilized because this isofrom cannot bind the type II receptor directly and thus depends on the presence of the type III receptor to signal. As shown in Fig. 6C, the parental L6 myoblast cell line is largely insensitive to the TGF-β2 isofrom; however, expression of the full-length type III receptor restores sensitivity to TGF-β2. In contrast, expression of the type III-cyto receptor failed to restore sensitivity of L6 cells to TGF-β2. To our knowledge, this is the first demonstration that the cytoplasmic domain of the type III receptor is essential for mediating TGF-β2 signaling and that the type III receptor does more than present TGF-β ligand to the type II receptor.

Mechanism of Action of the Type III Receptor Cytoplasmic Domain—The effects of the cytoplasmic domain of the type III receptor on TGF-β signaling occur downstream of the type III receptor binding TGF-β, associating with the type II receptor and presenting the TGF-β ligand to the type II receptor. The
results with the autophosphorylation site mutants determined further that the cytoplasmic domain of the type III receptor selectively interacts with and regulates autophosphorylated/activated type II receptor. After delivery of the TGF-β ligand to the type II receptor, the type III receptor delivers the type II receptor to the type I receptor; however, the fate of the type III receptor in these complexes is unknown. Either the type III receptor remains associated with the type II receptor and the type I receptor, or the type III receptor dissociates from the active “signaling complex” between the type II receptor and the type I receptor. To determine which of these possibilities was the case, the effect of the cytoplasmic domain of the type III receptor on interactions among the type III receptor, the type II receptor, and the type I receptor was investigated. To approximate most closely the physiological situation where the type II receptor and the type III receptor form a complex with each other and then the type I receptor is recruited to this complex, increasing amounts of the type I receptor were added to a fixed quantity of the type II receptor and the HA-tagged type III receptor or the HA-tagged type III-cyto receptor. The complexes among the type III receptor, the type II receptor, and the type I receptor were then detected by immunoprecipitation with the α277 antibody (panel A) or the αHA antibody (panel B), and analyzed on 10% SDS-polyacrylamide gels. The brackets delineate the type III receptor, the type II receptor, and the type I receptor. Molecular mass standards (in kDa) are indicated on the right. Panel C, the data were quantified using ImageQuant software, and the data are expressed as a percent of control.

Fig. 7. Mechanism of action for the cytoplasmic domain of the type III receptor. COS-7 cells were transiently transfected with 2 μg of the HA-tagged type III TGF-β receptor with (III) or without (III-cyto) the cytoplasmic domain, 2 μg of the type II TGF-β receptor, and increasing amounts (0.5–8 μg) of the type I TGF-β receptor. Cells were affinity labeled with 125I-TGF-β1, immunoprecipitated (IP) with the α277 antibody (panel A) or the αHA antibody (panel B), and analyzed on 10% SDS-polyacrylamide gels. The brackets delineate the type III receptor, the type II receptor, and the type I receptor. Molecular mass standards (in kDa) are indicated on the right. Panel C, the data were quantified using ImageQuant software, and the data are expressed as a percent of control.
not only to bring autophosphorylated/activated type II receptor into the complex, but also to dissociate the type III receptor from the activated signaling complex between the type II receptor and type I receptor. As the type II receptor phosphorylates the type III receptor on the cytoplasmic domain, this phosphorylation represents a potential mechanism by which the type III receptor dissociates from the active signaling complex between the type II receptor and the type I receptor (Fig. 8).

DISCUSSION

TGF-β mediates a vast array of biology through an apparently simplistic signaling pathway. A number of factors that interact with the TGF-β signaling pathway, including the type I and type II receptor-interacting proteins, TRIP-1, STRAP, FKBP-12, PP2A, and SARA, and transcription factors that interact with the Smad transcription factors including NF-1, Sp-1, AP-1, CREB, TFE3, and FAST1/2, have been described. These proteins increase the complexity of the pathway and may serve to regulate the TGF-β pathway (1). Alternative mechanisms for the generation of complexity are through interactions among the numerous cell surface receptors for TGF-β as well as through potentially novel downstream signaling pathways from these receptors.

Here we establish strong evidence for an essential and biologically significant role of the type III receptor in TGF-β signaling mediated by a functional interaction of the cytoplasmic domains of the type III receptor with the type II receptor. This interaction is demonstrated by 1) the ability of cytoplasmic domain of the type II receptor to interact with the cytoplasmic domain of the type III receptor as measured by the a277 antibody immunoprecipitation assay (i.e. binding of the type III receptor to the a277 antibody and the type II receptor are mutually exclusive); 2) the requirement for kinase activity of the type II receptor for this interaction; 3) the ability of the type II receptor to phosphorylate the cytoplasmic domain of the type III receptor; and 4) the specificity of the cytoplasmic domain of the type III receptor for autophosphorylated type II receptor. The significance of this interaction is demonstrated by the inability of mutants of the type III receptor lacking the cytoplasmic domain to carry out a type III receptor function, namely enhancing TGF-β2 signaling. The mechanism of action of the type III receptor in mediating signaling involves the specific ability of the cytoplasmic domain of the type III receptor to bind autophosphorylated/activated type II receptor and the ability of the type III receptor to dissociate from the active signaling complex comprised of the type II receptor and the type I receptor.
Role of the Type III Receptor in TGF-β Superfamily Signaling—The type III receptor has previously been thought to have a nonessential role in TGF-β signaling, acting only to “present” ligand to the signaling type I and type II receptors. In this presentation role, the type III receptor binds TGF-β and then the type II receptor and presents TGF-β to the type II receptor. The type II receptor is activated by binding TGF-β and recruits the type I receptor into a complex and phosphorylates it to activate further downstream signaling (Fig. 8). The presentation role for the type III receptor was suggested by the somewhat lower affinity of the type III receptor for TGF-β ligands (30–300 pm for the type III receptor versus 25–50 pm for the type II receptor), the lack of an obvious signaling motif in the short cytoplasmic domain of the type III receptor, and the ability of cells to respond to TGF-β in the absence of type III receptor expression.

Recent results have begun to challenge this model. Many of the cells and cell lines that do not express the type III receptor, including hematopoietic and endothelial cells, express the closely related receptor, endoglin, which shares highest homology (70%) to the type III receptor in the cytoplasmic domain. These cells do respond to TGF-β but are unresponsive to TGF-β2, as endoglin does not bind TGF-β2. Sensitivity to TGF-β2 can be restored by ectopic expression of the type III receptor, supporting an essential role for the type III receptor in TGF-β2 signaling (24). The type III receptor has also been shown to have an essential nonredundant role in TGF-β signaling, mediating the effects of TGF-β (TGF-β1 or TGF-β2) on mesenchymal transformation in chick embryonic heart development (25). In addition, the loss of functional type III receptor expression on intestinal goblet cells is sufficient to mediate resistance to TGF-β (26). Finally, the type III receptor was shown to bind and modulate signaling by another TGF-β superfamily member, inhibin (27).

The present results demonstrating an interaction of the cytoplasmic domain of the type III receptor with the type II receptor, the phosphorylation of the type III receptor by the type II receptor, and the essential role of the cytoplasmic domain of the type III receptor in mediating TGF-β signaling establish that the type III receptor is essential for mediating the effects of TGF-β, particularly for the TGF-β2 isof orm. The type III receptor without the cytoplasmic domain can bind the type II receptor and increase TGF-β binding to the type II receptor, but this is insufficient to enhance signaling. Thus, the type III receptor does more than simply present ligand. We propose a model depicted in Fig. 8. The type III receptor selectively engages the formation of the active signaling complex between the autophosphorylated type II receptor and the type I receptor. The type III receptor carries out this function by selectively binding the autophosphorylated, activated type II receptor via its cytoplasmic domain, mediating the specific interaction of the autophosphorylated type II receptor with the type I receptor, and then dissociating itself from the activated signaling complex between the type II receptor and the type I receptor. This dissociation may involve the phosphorylation of the type III receptor by the type II receptor as discussed below.

Roles for the Cytoplasmic Domain of the Type III Receptor—Although the cytoplasmic domain of the type III receptor is highly conserved across species and with the related receptor, endoglin, no function has been described for this domain. Indeed, deletion of the cytoplasmic domain was reported to have no effect on the ability of the type III receptor to bind TGF-β, to bind the type II receptor, or to enhance TGF-β binding to the type II receptor (11). Although we find that the type III receptor lacking its cytoplasmic domain can bind TGF-β, associate with the type II receptor, and present TGF-β to the type II receptor, its ability to mediate TGF-β2 signaling in a biological assay is affected. The previous observations regarding the role of the cytoplasmic domain of the type III receptor were based on experiments done in transiently transfected COS cells. In addition, although the ability of the type III receptor without the cytoplasmic domain to bind the type II receptor and enhance TGF-β binding was investigated, the ability to enhance TGF-β signaling was not (11). The functions ascribed here to the cytoplasmic domain of the type III receptor, namely phosphorylation by the type II receptor, associating specifically with the cytoplasmic domain of the autophosphorylated type II receptor and dissociating the type III receptor from the complex of the type II receptor and the type I receptor, appear to be essential for the role of the type III receptor in TGF-β signaling. Indeed, phosphorylation of the type III receptor by the type II receptor may be the mechanism by which the type III receptor is released from the active signaling complex. The vital role of the cytoplasmic domain is supported further by evidence that the extracellular domains of the type II and type III receptors do not bind TGF-β2 in an cooperative manner and that the binding of the extracellular domain of the type III receptor to TGF-β2 does not promote binding of the extracellular domain of the type II receptor to TGF-β2.2

The association of the cytoplasmic domain of the type III receptor with the type II receptor may either be direct or via an adaptor protein that binds to the autophosphorylated type II receptor and/or the phosphorylated type III receptor cytoplasmic domain. We have been unable to demonstrate a direct interaction of the cytoplasmic domain of the type III receptor with the type II receptor in vitro using GST pull-down assays.3 In addition, yeast two-hybrid screens with the cytoplasmic domain of the type III receptor did not yield any clones encoding the type II receptor.3

In other investigations, we have established a protein that does bind the cytoplasmic domain of the type III receptor, GIPC, a PDZ domain-containing protein, binds to a class I PDZ binding motif in the cytoplasmic domain of the type III receptor.4 Mutating the Class I PDZ binding motif of the type III receptor abolishes binding of GIPC to the type III receptor, but does not affect the interaction between the type III receptor and the type II receptor studied here, establishing that this protein is not an adaptor protein in this interaction. GIPC does regulate the expression of the type III receptor and the response of cells to TGF-β, further establishing the importance of the cytoplasmic domain of the type III receptor in TGF-β signaling.

Oligomeric Structure of TGF-β Receptors on the Cell Surface—The type III receptor exists as homodimers and oligomers in vivo. We were able to detect these homodimers and oligomers with the a277 antibody, indicating that the a277 epitope is accessible when the type III receptor is complexed with other type III receptors. The accessibility of the type III receptor cytoplasmic domain suggests that these oligomeric complexes may be able to interact with the type II receptor. This is supported further by the finding that type II receptor expression inhibits immunoprecipitation of the homo-oligomers of the 44–564 and 44–575 mutants of the type III receptor by the a277 antibody (Fig. 5). Although the precise stoichiometry of TGF-β receptor complexes at the cell surface has not been elucidated, these results support a model in which oligomers of the type III receptor form complexes with one or

2 De Crescenzo, G., Grothe, S., Zwaagstra, J., Tsang, M., and O’Connor-McCourt, M. D. (May 29, 2001) J. Biol. Chem. 10.1074/jbc.M009765200
3 G. C. Blobe and H. F. Lodish, unpublished observations.
4 G. C. Blobe, X. Liu, and H. F. Lodish, manuscript in preparation.
more type II receptors. The type II receptor has been found in complexes with either the type I receptor or the type III receptor, as well as in complexes with both the type I and III receptors. We had observed previously that only a minority of the type II receptor is complexed with a minority of the type III receptor in vivo (17). The present results, which demonstrate that the type III receptor binds preferentially to autophosphorylated and activated type II receptor and dissociates from the type II receptor complex when the type I receptor is recruited, are consistent with this finding.

Implications for Endoglin Signaling—The high degree of homology between the cytoplasmic domain of the type III receptor and the related receptor, endoglin, points to a conserved role for the cytoplasmic domain of these receptors. The present results suggest that one of these roles is to interact either directly or indirectly with the cytoplasmic domain of the autophosphorylated type II receptor. The epitope recognized by the α277 antibody is critical for this interaction, which is consistent with the high degree of conservation of this region. The region of high homology between the type III receptor and endoglin can be divided into three domains: box 1 (94%), which includes part of the transmembrane domain and the first five amino acids in the cytoplasmic domain, and box 2 (88%), which is composed of the last 17 amino acids of the cytoplasmic domain (including all of the epitope for the α277 antibody). The 22–24-amino acid sequence linking these two regions is much less conserved (30%). In contrast to the α277 antibody, the polyclonal antibody to the entire cytoplasmic domain of the type III receptor (α820) and a polyclonal antibody to the linker region of endoglin are both able to immunoprecipitate the type II receptor along with either endoglin or the type III receptor, respectively (16, 28). Thus, it appears that within the type II-type III receptor complex, box 2 is shielded from antibody interaction, whereas the other regions remain accessible for antibody binding. Interestingly, box 2, which associates with the type II receptor, is absent in a splice variant of endoglin which is truncated after box 1 (29). This presents the intriguing possibility that one difference between these splice variants will be in their ability to interact with the type II receptor and the TGF-β signaling pathway. Indeed, in the one report in which the role of these splice variants was investigated, it was shown that full-length endoglin was able to antagonize TGF-β signaling, whereas the truncated version was not (30). The role of the type II receptor interactions with endoglin and the relevance of this to TGF-β signaling will require further evaluation.

Acknowledgments—We thank Josee Plamondon for expert technical assistance, Dr. Joan Massague for the generous supply of the α820 antibody, Dr. Kunxin Luo for the type II receptor autophosphorylation site mutant constructs, and R & D Systems, Inc. for generous supply of TGF-β1.

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