A Novel Mechanism for Regulating Transforming Growth Factor β (TGF-β) Signaling

FUNCTIONAL MODULATION OF TYPE III TGF-β RECEPTOR EXPRESSION THROUGH INTERACTION WITH THE PDZ DOMAIN PROTEIN, GIPC*

Received for publication, July 19, 2001, and in revised form, August 10, 2001
Published, JBC Papers in Press, August 23, 2001, DOI 10.1074/jbc.M106831200

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Transforming growth factor β (TGF-β) mediates its biological effects through three high-affinity cell surface receptors, the TGF-β type I, type II, and type III receptors, and the Smad family of transcription factors. Although the functions of the type II and type I receptors are well established, the precise role of the type III receptor in TGF-β signaling remains to be established. While expression cloning signaling molecules downstream of TGF-β, we cloned GIPC (GAIP-interacting protein, C terminus), a PDZ domain-containing protein. GIPC binds a Class I PDZ binding motif in the cytoplasmic domain of the type III receptor resulting in regulation of expression of the type III receptor at the cell surface. Increased expression of the type III receptor mediated by GIPC enhanced cellular responsiveness to TGF-β both in terms of inhibition of proliferation and in plasminogen-activating inhibitor (PAI)-based promoter gene induction assays. In all cases, deletion of the Class I PDZ binding motif of the type III receptor prevented the type III receptor from binding to GIPC and abrogated the effects of GIPC on type III receptor expressing cells. These results establish, for the first time, a protein that interacts with the cytoplasmic domain of the type III receptor, determine that expression of the type III receptor is regulated at the protein level and that increased expression of the type III receptor is sufficient to enhance TGF-β signaling. These results further support an essential, non-redundant role for the type III receptor in TGF-β signaling.

Transforming growth factor β (TGF-β) is a member of a family of growth factors that regulate cellular proliferation, cellular differentiation, embryonic development, wound healing, and angiogenesis in a cell-specific manner (1). TGF-β regulates this diverse array of cellular processes through binding three high-affinity cell surface receptors, the TGF-β type I, type II, and type III receptors. The type I and II receptors contain serine/threonine protein kinases in their intracellular domain. TGF-β initiates cellular signaling by either binding to type III receptors, which then presents TGF-β to type II receptors, or binding to type II receptors directly. Once activated by TGF-β, the type II receptor recruits, binds, and transphosphorylates the type I receptor, thereby stimulating its protein kinase activity. The activated type I receptor phosphorylates Smad2 or Smad3 that then bind to Smad4. The resulting Smad complex then translocates into the nucleus where it interacts in a cell-specific manner with numerous transcription factors to regulate the transcription of TGF-β-responsive genes.

How this simplistic pathway regulates the diverse array of biology attributed to TGF-β remains to be elucidated. Numerous proteins that interact with the type I or type II receptors and the Smad proteins to modulate TGF-β signaling have been described (2). Another method by which diversity may be generated is through the formation of distinct receptor complexes that could then utilize distinct TGF-β pathways. Indeed, Smad-independent signaling and signaling through mitogen-activated protein kinase and other cellular signaling pathways have been reported recently (3–7).

In the process of retroviral expression cloning screens to identify additional members of the downstream signaling pathway for TGF-β, we cloned GIPC, a PDZ domain-containing protein. This protein had been cloned previously by several groups using the yeast two-hybrid system as a protein that interacted with Class I PDZ binding motifs in Tax (8), RGS-GAIP (9), Glut-1 (10), SemaF (11), neuropilin (12), syndecan (13), tyrosinase-related protein-1 (14) and integrins α5, α6β1, and α6β1 (15). Inspection of the TGF-β receptors revealed that the type III receptor contained a Class I PDZ binding motif in the cytoplasmic domain. Indeed, GIPC bound to the type III receptor in vivo and in vitro. In MvILu cells, binding of the type III receptor to GIPC resulted in enhanced expression of the type III receptor at the cell surface. In L6 myoblasts, which normally do not express the type III receptor, GIPC decreased the expression of transiently expressed type III receptor but increased the expression of stably expressed type III receptor. Increased expression of the type III receptor was due to stabilization at the cell surface and was sufficient to enhance cellular responsiveness to TGF-β both in terms of inhibition of proliferation and induction of PAI-based promoter-driven gene expression. The type III receptor lacking the Class I PDZ binding motif did not bind GIPC and was not regulated by the expression of GIPC. Taken together, these results, establish for the first time the existence of a type III receptor-binding pro-
tein, that the type III receptor expression is regulated at the protein level, and that this altered expression is sufficient to modulate TGF-β signaling. These results have implications for the role of the type III receptor in TGF-β signaling and the role of GIPC as well as other PDZ domain proteins in regulating cell surface receptors as discussed.

MATERIALS AND METHODS

Retroviral Cloning—Generation of a retroviral cDNA library from NIH3T3 cells was described previously. (16) High-titer retrovirus stock was prepared by transient transfection of BOSC23 packaging cell line as described previously. (17) The supernatant was then utilized to infect 5 million L20 cells (Mv1Lu cells expressing the murine ecotropic receptor). Infected cells were then expanded and seeded at a concentration of 2 × 10⁵ cells/100-mm tissue culture dish. TGF-β1 was added to the culture for 50 pm. The cells were incubated in the presence of TGF-β1 for 3 weeks with medium changes once a week. Cell clones that grew in the culture at 50 pm were isolated using cloning rings and expanded for further analysis. Retroviral insertions that conferred resistance to the antiproliferative effects of TGF-β were recovered using a pair of polymerase chain reaction primers spanning the multiple cloning site as described previously. (16) The identity of the retroviral clone was determined by sequence analysis.

Yeast Two-Hybrid—Appropriate strains of yeast (a strain for bait, α strain for library) were transformed with pGBD-IIIcyto (containing the cytoplasmic domain of the type III receptor) or pGBD-IIIcyto-DEL (containing the cytoplasmic domain of the type III receptor lacking the Class I PDZ binding motif) and pGAD-GIPC (encoding full-length GIPC) respectively. These yeast were then mated overnight in YPAD medium (yeast extract, peptone, adenine, and dextrose) at 30 °C, plated on YPD plates, and incubated at 30 °C for 3–5 days to allow diploid cells to form visible colonies. Colonies were then replica-plated on His+ or His− Ade plates to assay for interaction.

GST Affinity-binding Assay—Cells were lysed with 1% Triton X-100 lysis buffer and precleared with glutathione-agarose beads. GST fusion protein of the cytoplasmic domain of the type III receptor (GST-III) or the type III receptor lacking the Class I PDZ binding motif (GST-III-DEL) complexed with glutathione-agarose beads were incubated with FLAG epitope-tagged GIPC, harvested by centrifugation, and washed three times with lysis buffer. Binding proteins were analyzed by SDS-PAGE and Western blot analysis with aFLAG antibody.

RESULTS

Isolation of Murine GIPC—While performing retroviral expression cloning screens to identify members of the downstream signaling pathway for TGF-β, we isolated a clone encoding almost the entire coding region of the PDZ domain-containing protein, GIPC (GAIP interacting protein, C terminus) (Fig. 1A). GIPC, also known as TaxIP2, Glut1CIP, SEMCAP-1, Neurphilin-1, and tyrosinase-related protein-1 (14), and integrin α5β1 (15) as baits. GIPC is a 333-amino acid protein with a predicted molecular mass of 36 kDa. In addition to the centrally located PDZ domain, GIPC contains an ACP (acetyl carrier protein) domain at the carboxyl terminus, and several consensus protein kinase C and casein kinase II phosphorylation sites (Fig. 1A). GIPC has been shown previously to interact specifically with a Class I PDZ binding motif comprising the last three amino acids at the carboxyl terminus of these proteins via its PDZ domain (Fig. 1B). Although GIPC has been suggested to alter the subcellular localization of these interacting proteins or mediate binding to other proteins, the functional roles of GIPC have not been elucidated.

Interaction of GIPC with the Type III Receptor—As the portion of GIPC we cloned contained a PDZ domain, we sought to identify whether GIPC could interact via its PDZ domain with a member of the TGF-β family. Upon inspection of the receptors for TGF-β, we identified a Class I PDZ binding motif at the carboxyl terminus of the type III receptor, which was similar to the Class I PDZ motif found in the other interacting proteins for GIPC (Fig. 1B). This feature was unique to the type III receptor, as neither the type II receptor nor the type I receptor contained a similar motif.

To investigate the potential for GIPC and the cytoplasmic domain of the type III receptor to interact, we utilized the yeast two-hybrid mating system of James and colleagues (18). The entire cytoplasmic domain of the type III receptor was cloned into the pGAD vector (pGAD-IIIcyto) in frame with the Gal4 AD, and full-length GIPC was cloned into the pGAD vector (pGAD-GIPC) in frame with the Gal4 DNA binding domain. Yeast transformed with these vectors were then mated, and the yeast grown in Ade+ His+ conditions selecting for interacting proteins. Neither the pGAD-IIIcyto or pGAD-GIPC vector allowed growth under these conditions; however, yeast mated and selected to carry both pGAD-IIIcyto and pGAD-GIPC vectors grew, demonstrating that these proteins interact in the yeast two-hybrid system (Fig. 1B, data not shown). To investigate whether the last three amino acids of the type III receptor were essential for this interaction, a bait was made in which the last three amino acids of the type III receptor were deleted (pGAD-IIIcyto-DEL). Indeed, yeast mated and selected to carry both pGAD-IIIcyto-DEL and pGAD-GIPC vectors did not grow (Fig. 1B, data not shown), indicating that these proteins did not interact in the yeast two-hybrid system and that the Class I PDZ binding motif of the type III receptor was essential for this interaction, consistent with the results with other GIPC-interacting proteins (Fig. 1B).

To investigate the interaction of the type III receptor and GIPC in vivo via co-immunoprecipitation and co-localization efficiency, and varying amounts of pEXL-GIPC expressing full-length GIPC. After 24 h, the cells were washed with Dulbecco’s modified Eagle’s medium before incubation with TGF-β (100 pm) for an additional 24-h period. After the last incubation, the cells were lysed in luciferase lysis buffer (Promega). The luciferase activity was read after the addition of luciferin (Bio), and the results were expressed as the -fold induction over no TGF-β treatment after adjusting for β-galactosidase expression.
studies, we utilized HA-tagged type III receptors and FLAG epitope-tagged GIPC. Although we could express and detect expression of either the type III receptor or GIPC individually, we could not detect expression of the type III receptor in the presence of GIPC (data not shown). To circumvent this difficulty, we expressed FLAG epitope-tagged GIPC in COS-7 cells and utilized a GST fusion protein of either the cytoplasmic domain of type III receptor (GST-IIIcyto) or the cytoplasmic domain with the Class I PDZ binding motif deleted (the last three amino acids in the cytoplasmic domain, GST-IIIcyto-DEL) to attempt to pull down GIPC. GST-IIIcyto, but not GST alone or GST-IIIcyto-DEL, was able to pull down GIPC in this assay, verifying that GIPC and the type III receptor interact and that this interaction depends on the Class I PDZ binding motif of the type III receptor (Fig. 1).

Effect of GIPC on Type III Receptor Expression—Our inability to detect the type III receptor in the presence of GIPC expression suggested that GIPC effects type III receptor expression. To determine whether GIPC was effecting expression of the type III receptor, we examined the cell surface expression of HA-tagged type III receptor in the presence and absence of GIPC expression in the L6 myoblast cell line by binding and cross-linking with 125I-TGF-β. The L6 myoblast cell line was utilized as it normally does not express the type III receptor, allowing us to express and analyze effects of the wild-type type III receptor and the mutant type III receptor lacking the Class I PDZ binding motif. Initially we transiently transfected HA-tagged type III re-
ecptor in L6 myoblasts with and without co-transfection with GIPC and immunoprecipitated the type III receptor with the αHA antibody as this mimicked the conditions we had utilized in our co-immunoprecipitation and co-localization studies. When GIPC was expressed, there was a significant decrease in the amount of the type III receptor that was expressed at the cell surface, consistent with our inability to detect the type III receptor with GIPC expression in previous experiments (Fig. 2C). This was a dose-dependent effect, as increasing the level of expression of GIPC (Fig. 2B) relative to the expression of the type III receptor was able to progressively decrease the expression of the type III receptor (Fig. 2C). To confirm that the effect of GIPC was dependent on the interaction of the type III receptor with GIPC, we analyzed the effect of GIPC on expression of the type III receptor, which does not bind GIPC because of deletion of the Class I PDZ binding motif (type III receptor-DEL). Type III receptor-DEL was transiently expressed in the presence of GIPC. The type III receptor-DEL was expressed at the cell surface, and bound TGF-β. However, the expression of the type III receptor-DEL was not effected by the expression of GIPC even when increasing the amount of GIPC expressed (Fig. 2, B and C). These studies determined that expression of GIPC decreases the expression of transiently expressed type III receptor at the cell surface and that this effect is dependent on the binding of GIPC to the type III receptor. Similar results were found in transiently transfected COS-7 cells expressing only the type III receptor and GIPC, determining that significant levels of the type II receptor or the type I receptor were not necessary for the role of GIPC in regulating the type III receptor expression (data not shown). To establish the mechanism by which GIPC abrogates expression of the type III receptor at the cell surface, we examined the effect of GIPC on total cellular expression of the type III receptor in L6 by immunoprecipitation and Western blot analysis with αHA antibody. We were able to detect expression of the HA-tagged type III receptor, both as the 180–300-kDa proteoglycan and predominately as the unmodified core, which migrated at 130 kDa (Fig. 2D, data not shown). When HA-tagged type III receptor was expressed in the presence of increasing levels of GIPC, the expression of the type III receptor was markedly decreased (Fig. 2D). When HA-tagged type III receptor-DEL was analyzed in a similar fashion, GIPC had no effect (Fig. 2D). These studies determine that GIPC effects total cellular expression of transiently expressed type III receptor, not just cellular surface expression, and thus may be acting during biosynthesis, processing, and trafficking of the type III receptor to the cell surface.

Although the effect of GIPC on the type III receptor was specific to the type III receptor able to bind GIPC (as the type III receptor-DEL was not effected) and these results explained our inability to detect the type III receptor in the presence of GIPC in our transient expression assays, we sought to determine whether GIPC regulated endogenous type III receptor expression in a physiological manner. To make this evaluation, we analyzed the expression of the TGF-β receptors in the original Mv1Lu clones (which constitutively express the type III receptor and have been retrovirally infected and selected to stably express GIPC). Although the GIPC-expressing Mv1Lu clones expressed identical levels of the type I and type II receptors compared with the parental Mv1Lu cell line, surprisingly, these cells expressed significantly higher levels of the type III receptor (Fig. 3A).

Two potential reasons for the discrepant effects of GIPC on the type III receptor expression between the Mv1Lu cell line and the L6 myoblast and COS-7 cell lines are: 1) the type III receptor is constitutively expressed in the Mv1Lu cell line and transiently expressed at higher levels in the L6 and COS-7 cell lines (Fig. 2A, data not shown); and 2) the GIPC is expressed after the type III receptor has been expressed, processed, and transported to the cell surface in the Mv1Lu cell line but before or while those same processes are occurring in the L6 and COS-7 cell lines. To determine whether stable expression of the type III receptor influenced the effect of GIPC, the HA-tagged type III receptor was stably expressed in L6 myoblasts (L6-III). The L6-III cells were then transfected with GIPC and the type III receptor immunoprecipitated by the αHA antibody. As
shown in Fig. 3D, GIPC expression in the L6-III cells induced a significant increase in the amount of the type III receptor that was expressed at the cell surface in a dose-dependent manner, consistent with the effect of GIPC on the type III receptor in the Mv1Lu cells. The effect of GIPC was dependent on the interaction of the type III receptor with GIPC, as expression of stably expressed type III receptor-DEL (L6-III-DEL), which does not bind GIPC, was not affected by the expression of GIPC even when increasing the amount of GIPC expressed (Fig. 3D). We then examined the effect of GIPC on total cellular expression of the stably expressed type III receptor in L6 cells. Again, we were able to detect expression of the HA-tagged type III receptor or HA-tagged type III receptor-DEL, both as the 180–300 kDa proteoglycan and predominately as the unmodified core, which migrated at 130 kDa (Fig. 3E). When HA-tagged type III receptor was stably expressed in the presence of increasing levels of GIPC, the total cellular expression of the type III receptor was unchanged (Fig. 3E). When HA-tagged type III receptor-DEL was analyzed in a similar fashion, as expected, GIPC had no effect (Fig. 3E). To confirm whether the effect of GIPC on the TGF-β pathway was specific to the type III receptor, we analyzed the effect of GIPC expression on the expression of the type I and type II TGF-β receptors in the stable L6 myoblast cell lines as well. As expected, in L6-III and L6-III-DEL cell lines, GIPC had no effect on the expression of the type I and type II TGF-β receptors (Fig. 3, B and C). These results demonstrate that altered expression of the type III receptor by GIPC does not alter the expression of
the type II or type I receptor indirectly. To ensure that immunoprecipitation of these stably expressed receptors was not altering the results, we performed similar studies on the L6-III and L6-III-DEL cell lines and directly analyzed total cellular lysates. As shown in Fig. 3C, GIPC had similar dose-dependent effects on the stably expressed type III receptor but not on III-DEL, the type II receptor, or the type I receptor. Finally, to determine the levels of type III receptor expressed in these stable cell lines as well as in our transiently expressed systems, we analyzed receptor expression in equal numbers of L6-III and L6-III-DEL cells, L6 cells transfected with L6-III or L6-III-DEL, and Mv1Lu cells as a control. As shown in Fig. 2A, although transient expression does result in slightly higher expression for both the type III receptor (L6+III versus L6-III) and III-DEL (L6+III-DEL versus L6-III-DEL), in all the cases the levels of the expression are within the same range as endogenously expressed type III receptor (Mv1Lu), confirming that the results are obtained with physiologically relevant levels of type III receptor expression. These studies establish that GIPC specifically regulates cell surface expression of the stably expressed type III receptor without altering total cellular expression, suggesting that GIPC regulates the stability of the type III receptor at the cell surface.

Mechanism for GIPC Effect on Type III Receptor Expression: Role of Proteosome Degradation—The ubiquitin/proteosome pathway has been implicated in the targeted degradation of a number of members of the TGF-β family (19–22). As GIPC increases cellular surface expression of endogenous or stably expressed type III receptor and interacts directly with the type III receptor at the protein level, we wondered whether GIPC was mediating the access of the type III receptor to the ubiquitin/proteosome pathway. To investigate this possibility, we assayed the effect of GIPC in the presence of the potent reversible inhibitor of the 26-S proteosome, MG-132. In the presence of MG-132, GIPC was still able to enhance expression of stably expressed type III receptor at the cell surface. Indeed, exposure to MG-132 synergized with GIPC to dramatically increase the expression of stably expressed type III receptor at the cell surface (Fig. 4A). To further investigate this effect, we assayed the ability of MG-132, and lactacystin, a highly specific irreversible inhibitor of the 20-S proteosome, to alter the expression of the type III receptor in the presence of GIPC. Both MG-132 and lactacystin were able to increase the expression of the type III receptor in the presence of GIPC in a dose- and time-dependent fashion with both inhibitors inducing a maximum cell surface expression of the type III receptor after 18 h, with maximum effects at 3 μM for MG-132 and 5 μM for lactacystin (Fig. 4, B and C). These results suggest that proteosome-mediated degradation is involved in determining the level of cell surface expression of the type III receptor and that one role of GIPC is to protect the type III receptor from degradation.

Effect of GIPC on TGF-β-mediated Biological Responses—As the primary effect of GIPC on the TGF-β signaling pathway under physiological conditions is to increase type III receptor expression, we examined whether this increased expression of the type III receptor was sufficient to induce acute changes in TGF-β-mediated biological responses. We initially examined the response of Mv1Lu cells to TGF-β in terms of acute inhibition of proliferation as measured by colony formation (23) but not to TGF-β-mediated acute inhibition of proliferation as measured by cell cycle analysis (24). Thus, prolonged exposure to TGF-β and selection for resistant colonies appears to select for other mutations that confer resistance to TGF-β-mediated growth inhibition but not to the acute effects of TGF-β on inhibition of proliferation/cell cycle progression. To further characterize the effect of GIPC on acute changes in TGF-β-mediated biological responses, we examined the response of the Mv1Lu clones to TGF-β-induced gene expression. For these studies, the original viral clones expressing GIPC were re-expressed in Mv1Lu cells stably expressing pE2.1-luciferase, a luciferase reporter gene under the control of the TGF-β-responsive FAV-1-based promoter. The ability of the Mv1Lu cells expressing the GIPC clone to form colonies in the presence of TGF-β was confirmed (data not shown), and TGF-β-mediated gene induction was assayed by measuring luciferase activity. Mv1Lu cells that expressed GIPC had an enhanced response to TGF-β (both TGF-β1 and TGF-β2) with a consistent 2-fold increased induction (Fig. 5A). This increased TGF-β activity was in accord with the enhanced type III receptor expression in the Mv1Lu cells expressing GIPC, demonstrating that the TGF-β signaling pathway in the cells expressing GIPC remained intact.

To further evaluate the effect of GIPC on TGF-β-mediated biological responses, and establish the specificity of this re-

![Fig. 4. Effect of proteosome inhibitors on type III TGF-β receptor expression.](http://www.jbc.org/)
response, we utilized the L6-III and L6-III-DEL stable cell lines in thymidine incorporation assays and pE2.1-luciferase reporter gene induction assays in the presence and absence of GIPC. The TGF-β2 isoform was utilized because this isoform cannot bind the type II receptor directly and thus depends on the presence of the type III receptor to signal. As shown in Fig. 5B, the parental L6 myoblast cell line is largely insensitive to the TGF-β2 isoform; however, expression of the full-length type III receptor in the L6-III cell line or the type III receptor lacking the Class I PDZ binding motif in the L6-III-DEL cell line restored sensitivity to TGF-β2. When GIPC was expressed in the L6-III cells, the cells became even more responsive to TGF-β, consistent with their increased expression of the type III receptor. In contrast, expression of GIPC with the type III-DEL receptor in the L6-III-DEL cell line failed to enhance sensitivity of L6-III-DEL cells to TGF-β2, determining that the effect of GIPC was specific for its interaction with the type III receptor. These results determine that increasing type III re-

**FIG. 5. GIPC expression is sufficient to alter cellular responses to TGF-β.** A, Mv1Lu cells stably expressing the pE2.1-luciferase reporter gene construct were infected with retrovirus expressing GIPC (Mv1Lu + GIPC), and TGF-β-mediated gene induction was assayed. 200 μTGF-β1 or TGF-β2 was able to induce an ~8-fold induction in luciferase activity in Mv1Lu cells. Expression of GIPC in Mv1Lu cells increased this to a 14–15-fold induction. B, L6, L6-III, and L6-III-DEL cell lines were transfected with GIPC (4 μg) and treated with 200 μTGF-β2. Cells were then assayed for TGF-β-induced inhibition of proliferation as measured by thymidine incorporation assays. Expression of the type III receptor in the L6-III and L6-III-DEL cell lines enhances cellular response to TGF-β. Expression of GIPC specifically enhances TGF-β-induced inhibition of proliferation in the L6-III cell line but has no effect on L6 cells not expressing the type III receptor or on the L6-III-DEL cell line expressing the type III receptor, which does not bind GIPC. C, L6-III and L6-III-DEL cell lines were transfected with GIPC, and the pE2.1-luciferase reporter gene construct and TGF-β-mediated gene induction were assayed. 200 μTGF-β2 induced a 6-fold induction in luciferase activity in L6-III and L6-III-DEL cells. Expression of GIPC in L6-III increased this to an 11-fold induction but did not effect induction in the L6-III-DEL cells.
Receptor expression was sufficient to mediate increased responsiveness to TGF-β in terms of inhibition of proliferation. To see whether this effect was specific to inhibition of proliferation, the L6-III and L6-III-DEL cell lines were assayed for their response to TGF-β in of the pE2.1-luciferase reporter gene induction assay. As shown in Fig. 5C, expression of GIPC was also able to increase responsiveness in terms of TGF-β-induced gene expression, and this effect was specific for the type III receptor, as increased induction was not seen in the L6-III-DEL cell line. Taken together, these results determine that GIPC specifically increases the expression of the type III receptor and that this effect is sufficient to increase cellular responses to TGF-β.

**DISCUSSION**

The role of the type III receptor TGF-β receptor in TGF-β signaling has not been well characterized. In the present study, we have determined that GIPC, a PDZ domain-containing protein, binds to the type III receptor via a Class I PDZ binding motif in the cytoplasmic domain of the type III receptor. GIPC binding to the type III receptor results in altered expression of the type III receptor with GIPC decreasing the expression of transiently expressed type III receptor but increasing the expression of stably expressed type III receptor. GIPC-induced increases in type III receptor expression were sufficient to increase TGF-β responsiveness both in terms of TGF-β-mediated inhibition of proliferation and PAI-based promoter TGF-β-mediated gene induction. These studies, for the first time, define a type III receptor-binding protein, define that the expression of the type III receptor is regulated at the protein level, and establish that increasing levels of type III receptor expression is sufficient to enhance TGF-β signaling. Finally, these studies suggest that similar to other members of the TGF-β signaling pathway, expression of the type III receptor is regulated by the ubiquitin/proteosome pathway.

GIPC was isolated initially in a screen for proteins that, when over-expressed, conferred resistance to TGF-β-mediated growth inhibition as measured by colony formation after several weeks of exposure to TGF-β. Indeed, upon retroviral rescue and transfer to new Mv1Lu cells, GIPC expression was able to confer a similar phenotype, confirming the specificity of this function for GIPC. In contrast to this finding, when the acute effect of GIPC on TGF-β-mediated growth inhibition as measured by thymidine incorporation assays was analyzed, no effect of GIPC was observed (data not shown), and when PAI-1 induction was assayed, GIPC actually increased TGF-β activity in concordance with the enhanced type III receptor expression. One of the other proteins identified in this screen, MDM2, was previously identified as a protein that induced TGF-β resistance after long-term exposure to TGF-β (23). Nevertheless, MDM2 was also unable to confer acute resistance to TGF-β-mediated growth inhibition as measured by cell cycle analysis, suggesting that prolonged exposure to TGF-β selects for other alterations in the cell that confer TGF-β resistance (24). As GIPC increases the expression of the type III receptor (Fig. 3A), we hypothesize that this results in enhanced sensitivity to TGF-β (Fig. 5A). However, during prolonged exposure to TGF-β, this enhanced sensitivity increases the selective pressure for other mutations to occur, and these mutations confer resistance to TGF-β-mediated growth inhibition.

**Mechanism for Effect of GIPC on Type III Receptor Expression**—GIPC has a clearly demonstrated effect on the regulation of the expression of the type III receptor at the cell surface. This effect is dependent on GIPC binding to the type III receptor, as the type III receptor without the Class I PDZ binding motif (III-DEL) does not bind GIPC and is not regulated by GIPC. The discrepant effects of GIPC on transiently expressed and stably expressed type III receptor, along with the differential effects on total cellular levels of the type III receptor, suggest that GIPC regulates the processing and trafficking of the type III receptor to the cell surface (explaining the ability of GIPC to decrease the expression of transiently expressed type III receptor in the cell and on the cell surface) and then regulates the stability of the type III receptor at the cell surface (explaining the ability of GIPC to increased the expression of stably expressed type III receptor on the cell surface without effecting total cellular expression). The effect of the proteosome inhibitors suggest that, similar to other members of the TGF-β signaling pathway, the type III receptor is subject to regulation by the ubiquitin/proteosome pathway; and the ability of proteosome inhibitors to enhance the effect of GIPC further suggests that GIPC modulates this process. Recently, GIPC has been demonstrated to bind specifically to newly synthesized gp75 (tyrosine-related protein-1) in the juxtanuclear Golgi, suggesting that it plays a role in the biosynthetic sorting of gp75 in melanocytes (14). Studies are currently under way to establish the precise mechanism for the effect of GIPC on type III receptor expression.

**Role of the Type III Receptor in TGF-β Signaling**—The type III receptor is the most abundant TGF-β receptor and was the first TGF-β receptor cloned 10 years ago. However, because the subsequent cloning of the type II and type I receptors, and the identification of serine/threonine protein kinase domains in their intracellular domains, the type III receptor with its short cytoplasmic domain has been largely ignored. Indeed, a review of Medline reveals that there have been more than 1500 publications on the type II and type I receptors but less than 150 publications on the type III receptor since their initial characterization.

The type III receptor is classically thought to have a role in presenting the TGF-β ligand to the type II receptor. The presentation role for the type III receptor was suggested by the somewhat lower affinity of the type III receptor for TGF-β ligands, 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. Indeed, the type III receptor has been demonstrated to enhance TGF-β binding to the type II receptor and to...
enhance TGF-β signaling. Although this may be one role of the type III receptor, recent results are beginning to challenge this model and to establish a larger role for the type III receptor in TGF-β signaling. For example, cells that do not express the type III receptor, including hematopoietic and endothelial cells, express the closely related receptor endoglin, which shares significant homology (70%) with the type III receptor in the cytoplasmic domain. These cells continue to respond to TGF-β1, 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 (25). The type III receptor also has an essential, non-redundant role in TGF-β signaling, mediating the effects of TGF-β on mesenchymal transformation in chick embryonic heart development (26), and the loss of functional type III receptor expression on intestinal goblet cells is sufficient to mediate resistance to TGF-β (27). The type III receptor has also been shown to bind and regulate signaling by another TGF-β superfamily member, inhibin (28).

We have recently demonstrated a specific interaction of the cytoplasmic domain of the type III receptor with autophosphorylated, activated type II receptor, resulting in the phosphorylation of the type III receptor by the type II receptor and the dissociation of the type III receptor from the active signaling complex of the type II receptor and the type I receptor (29). This interaction has an essential role in mediating TGF-β signaling, as deletion of the cytoplasmic domain abrogates type III receptor function. Here we demonstrate another function of the cytoplasmic domain of the type III receptor, namely to bind to GIPC. This interaction specifically regulates the expression of the type III receptor, and this altered expression is sufficient to alter the responsiveness of cells to TGF-β. Taken together, these studies define a vital role for the type III receptor in mediating and regulating TGF-β signaling.

Role of GIPC and Other PDZ Domain-containing Proteins on Cell Surface Receptors—An increasing number of PDZ domain-containing proteins have been cloned, with over 75 currently known, and estimates from the human genome suggesting that there are a total of 162 genes encoding PDZ domain-containing proteins. (32) That nearly half of the PDZ domain-containing proteins have been identified can be attributed to the ease with which these proteins are identified in yeast two-hybrid screens. Although in most cases the precise function of these PDZ domain-containing proteins has not been elucidated, two principles have emerged: 1) PDZ domain-containing proteins interact predominately with membrane-associated proteins and proteins involved in signal transduction; and 2) the PDZ domain-containing proteins are usually restricted in their localization to specific subcellular or cell surface domains. Thus, PDZ domain-containing proteins are thought to have important roles in localizing proteins on the cell surface to discrete domains and in bringing components of signal transduction pathways in proximity, forming the framework for efficient signal transduction. GIPC has been demonstrated to interact with 11 distinct proteins. Ten of these proteins are cell surface receptors, nine of them have a single transmembrane region, and all of them possess a relatively short cytoplasmic domain of 11–146 amino acids (Fig. 1B). The ability of GIPC to regulate the expression of the type III receptor and to bind specifically to newly synthesized gp75 (TRP-1) in melanocytes suggests that it may perform a similar role in regulating the biosynthetic sorting and processing of its other cellular binding partners. GIPC has also been noted to interact with itself and with other proteins including myosin VI, and α-actinin-1 through regions other than its PDZ domain. This suggests that GIPC may also function to cluster the transmembrane receptors with which it interacts to specific domains on the cell membrane or to facilitate their interactions with signaling molecules (10, 33). It is particularly intriguing that one of the other binding partners of GIPC, syndecan-4, shares several structural and functional properties with the type III receptor, as both are ubiquitously expressed proteoglycan receptors that act as co-receptors for signal transduction molecules: basic fibroblast growth factor for syndecan-4 and TGF-β for the type III receptor. Whether GIPC modulates the function of syndecan-4 in a similar manner remains to be established. Finally, another TGF-β receptor, endoglin, has a short cytoplasmic domain that is 70% homologous to the cytoplasmic domain of the type III receptor. The cytoplasmic domain of endoglin also contains a Class I PDZ binding domain. Whether GIPC binds and regulates the endoglin receptor remains to be established.

Acknowledgments—We thank R&D Systems, Inc. for the generous supply of TGF-β1 and Dr. Marilyn G. Farquhar for the generous supply of full-length mouse GIPC cDNA.

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A Novel Mechanism for Regulating Transforming Growth Factor β (TGF-β) Signaling: FUNCTIONAL MODULATION OF TYPE III TGF- β RECEPTOR EXPRESSION THROUGH INTERACTION WITH THE PDZ DOMAIN PROTEIN, GIPC

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J. Biol. Chem. 2001, 276:39608-39617.
doi: 10.1074/jbc.M106831200 originally published online August 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106831200

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