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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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, α6A, 6B (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 Mv1Lu 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 used 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 × 10^6 cells/100-mm tissue culture dish. TGF-β1 was added to the culture for 50 µm. 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 presence of TGF-β1 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 sequencing analysis.

Yeast Affinity-binding Assay—Appropriate strains of yeast (α strain for library, α strain for library) were transformed with pGBD-IIIcyto (containing the cytoplasmic domain of the type III receptor) or pGAD-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 Trp–Leu–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+–Adr 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 αFLAG antibody.

TGF-β Binding and Cross-linking—Radioligand binding and cross-linking of [125I]TGF-β to Mv1Lu, L6, L6-III, or L6-III-DEL cell lines was performed by incubating subconfluent cells with KRH buffer (50 mM Hapes, pH 7.5, 130 mM NaCl, 5 mM MgSO4, 1 mM CaCl2, and 5 mM KCl) containing 0.5% BSA and then with 100 pM [125I]TGF-β for 3 h at 4 °C. [125I]TGF-β was cross-linked with 0.5 mg/ml disuccinimidyl suberate for 15 min and quenched with 20 mM glycine. Cells were then washed with KRH buffer and lyzed in radioimmunoprecipitation buffer, and the type III receptor was immunoprecipitated with αHA antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE and phosphorimaging.

Western Blot Analysis—Protein extracts were obtained from cell lines by lysing equal numbers of cells directly in boiling 2× sample buffer. Samples were resolved on 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose at 4 °C. Western blots analysis was performed using the M2-FLAG monoclonal antibody (Sigma) and horse-radish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech) with ECL detection (Amersham Pharmacia Biotech).

[3H]Thymidine Incorporation Assay—Cells were plated in 24-well plates at 2 × 10^4 cells/ml, transfected with GIPC, and then treated with 0–200 pM TGF-β1 or TGF-β2. After 48 h of incubation, cells were treated with 10 µCi of [3H]thymidine (Amersham Pharmacia Biotech) for 4 h. Cells were washed with phosphate-buffered saline and 5% trichloroacetic acid before harvesting cells with 0.1 N NaOH. The amount of [3H]thymidine incorporated was analyzed by liquid scintillation counting. Growth inhibition was calculated as the ratio of radioactivity with TGF-β treatment/radioactivity in the absence of TGF-β treatment.

Transcription Reporter Luciferase Assays—3 × 10^4 cells/well were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and plated in a 24-well plate. Cells were transfected with a pE2.1 vector that contains the luciferase gene under the regulation of a promoter based on the TGF-β-inducible promoter, PAI-1 (two tandem repeats of nucleotides −586 to −551 of the PAI-1 promoter), the pSVβ vector encoding β-galactosidase to control for transfection 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-β1 (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 (Beyotime), using an automated luminometer. The luciferase activity was expressed as the fold induction over no TGF-β treatment after adjusting for β-galactosidase expression.

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 (GαIP interacting protein, C terminus) (Fig. 1A). GIPC, also known as Tax1P2, Glut1CIP, SEMCAP-1, Neuropilin-1-P, and synectin, was previously cloned out of yeast two-hybrid screens using RGS-GAIP (9), Tax (8), Glut-1 (10), SemaF (11), neuropilin (12), syndecan (13), tyrosinase-related protein-1 (14), and integrin α6 (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 (acyl 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 pGPD vector (pGPD-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 pGPD-IIIcyto or pGAD-GIPC vector allowed growth under these conditions; however, yeast mated and selected to carry both pGPD-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 (pGPD-IIIcyto-DEL). Indeed, yeast mated and selected to carry both pGPD-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

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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 the 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. 1C). To verify that this interaction was a direct interaction between GIPC and the cytoplasmic domain of the type III receptor, we expressed 35S-labeled GIPC by in vitro transcription/translation and assayed for its interaction with GST-IIIcyto. Indeed, GST-IIIcyto but not GST alone was able to bind and pull down 35S-labeled GIPC, verifying a direct interaction between GIPC and the type III receptor (data not shown). Taken together, these results determine that the type III receptor and GIPC interact and that this interaction depends on the Class I PDZ binding motif of the type III receptor.

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-
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The type III receptor and III-DEL are expressed at physiological levels in L6 myoblasts, and GIPC specifically decreases expression of transiently expressed type III receptor. A, equal numbers of L6 cells either transiently expressing HA-epitope-tagged type III receptor (L6+III) or HA epitope-tagged type III receptor without the Class I PDZ binding motif (L6+III-DEL) or stably expressing these receptors (L6-III, L6-III-DEL) and Mv1Lu cells were affinity-labeled with [125I]TGF-β1, immunoprecipitated with the αHA antibody or the α820 antibody to the cytoplasmic domain of the type III receptor (for Mv1Lu cells), and analyzed on 7.5% SDS-PAGE gels. The wild-type and mutant type III receptors are expressed at levels similar to the endogenous receptor in Mv1Lu cells whether stably or transiently expressed. B, L6 cells transiently expressing HA epitope-tagged type III receptor (Type III) or HA epitope-tagged type III receptor without the Class I PDZ binding motif (Type III-DEL) and increasing amounts of GIPC (0–8 μg) were lysed, analyzed on 7.5% SDS-PAGE gels, and detected by Western blot analysis with the αFLAG antibody. Increasing the quantity of the GIPC expression vector increases the amount of FLAG epitope-tagged GIPC expressed. The arrow indicates GIPC, and the asterisk indicates a nonspecific band demonstrating equal protein loading across the lanes. C and D, L6 cells transiently expressing HA epitope-tagged type III receptor (Type III) or HA epitope-tagged type III receptor without the Class I PDZ binding motif (Type III-DEL), and increasing amounts of GIPC (0–8 μg) were either affinity-labeled with [125I]TGF-β1, immunoprecipitated with the αHA antibody, and analyzed on 7.5% SDS-PAGE gels (C) or were immunoprecipitated with the αHA antibody, analyzed on 7.5% SDS-PAGE gels, and detected by Western blot analysis with the αHA antibody (D). C, GIPC decreases the cell surface expression of the type III receptor, but not type III-DEL, in a dose-dependent manner. The bracket delineates the type III receptor. D, GIPC decreases the total cellular expression of the type III receptor, but not type III-DEL, in a dose-dependent manner. The arrow indicates the type III receptor. Molecular mass markers (in kDa) are indicated on the right.

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 affected 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 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) 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 effected by the expression of GIPC (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 cell 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 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 proteasome, 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 proteasome, 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 proteasome-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 thymidine incorporation assays. Paradoxically, expression of GIPC in the original resistant Mv1Lu clones conferred no enhanced resistance to TGF-β as measured by thymidine incorporation assays (data not shown). Similar results have been reported for MDM2, also pulled out in our screen and shown previously to confer resistance to TGF-β after prolonged exposure 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 pAI-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. L6-III cells stably expressing HA epitope-tagged type III receptor and transiently expressing GIPC (4 μg) in the presence of the indicated concentrations of MG-132 and lactacystin were affinity-labeled with [125I]TGF-β1, immunoprecipitated with the oHA antibody, and analyzed on 7.5% SDS-PAGE gels. A, MG-132 (10 μM) treatment for 14 h accentuates the ability of GIPC to increase the cell surface expression of the stably expressed type III receptor. The bracket delineates the type III receptor. B, MG-132 and lactacystin treatment enhances type III receptor expression in a dose-dependent fashion. The first 0 μM dose given is in the absence of GIPC and the rest in the presence of GIPC. The bracket delineates the type III receptor. C, MG-132 (5 μM) and lactacystin (5 μM) treatment enhances type III receptor expression with a maximum effect at 18 h of treatment. The first 0 h time point is taken in the absence of GIPC, and the rest are done in the presence of GIPC. The bracket delineates the type III receptor. Molecular mass markers (in kDa) are indicated on the right.](image-url)
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, demonstrating that the effect of GIPC was specific for its interaction with the type III receptor. These results determine that increasing type III re-

![Graph A](image1.png)

**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 ptg TGF-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 ptg TGF-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 ptg TGF-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 affect 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 signaling. The 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 increase 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 (tyrosinase-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 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

**TABLE I**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GIPC mRNA expression</th>
<th>Type III receptor mRNA expression</th>
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<tr>
<td>Adipose</td>
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<td>–</td>
</tr>
<tr>
<td>Aorta</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
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</table>
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 Regulating Expression of the Type III Receptor in Tumorigenesis—Although the type III receptor is ubiquitously expressed, there are cells and tissues in the body that lack expression of this receptor, including smooth muscle cells, endothelial cells, and hematopoietic cells. In these cells, the absence of type III receptor expression may be partially compensated for by the expression of a related receptor, endoglin, as noted above. Type III receptor expression is also regulated during tumorigenesis, with decreased expression of the type III receptor reported in breast cancer cell lines (30) and in pancreatic cancers (31). Indeed, we have found that the type III receptor is regulated at the mRNA level in a substantial proportion of breast, colon, and renal cell cancers. What is the role of regulating GIPC protein expression in the regulation of endogenous levels of the type III receptor protein? We have found no consistent pattern between GIPC expression and type III receptor expression at the mRNA level, with most tissues expressing mRNA for both, and some tissues expressing mRNA for GIPC but not for the type III receptor (Table I). Although these results may suggest that GIPC expression is not critical for the regulation of type III receptor expression, GIPC and type III receptor mRNA levels do not necessarily correlate with GIPC and type III receptor protein levels, and there are many potential mechanisms for regulating type III receptor expression, with GIPC representing only one of the potential mechanisms. Indeed, as expected, GIPC expression is unable to induce expression of the type III receptor in cells that do not normally express type III receptor mRNA or protein (L6 myoblasts, data not shown). Confirmation of the role of GIPC in vivo awaits the determination of the physiological circum-

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2 G. C. Blobe, unpublished observations.