Glycosylphosphatidylinositol (GPI)-anchored proteins have been demonstrated to bind transforming growth factor-β (TGF-β) in certain cell lines. However, the identity of these GPI-anchored proteins and the role they may play in TGF-β signaling remain unknown. We have previously reported the presence of GPI-anchored TGF-β-binding proteins on human skin fibroblasts and keratinocytes (Tam, B. Y. Y., and Philip, A. (1998) J. Cell. Physiol. 176, 553–564; Tam, B. Y. Y., Germain, L., and Philip, A. (1998) J. Cell. Biochem. 70, 573–586). On human keratinocytes, we identified a 150-kDa GPI-anchored TGF-β-binding protein (r150) and demonstrated that it can form a heteromeric complex with the type I and II TGF-β signaling receptors. To explore whether GPI-anchored proteins modulate TGF-β signaling in keratinocytes that express keratinocyte-derived PEDF as a natural inhibitor, we identified the r150 gene encoding the GPI M, GPI mutant; GPI NM, GPI non-mutant; STAT, signal transducer and activator of transcription.

Transforming Growth Factor-β (TGF-β) signaling in keratinocytes (Tam, B. Y. Y., and Philip, A. (1998) J. Cell. Biochem. 70, 573–586). On human keratinocytes, we identified a 150-kDa GPI-anchored TGF-β-binding protein (r150) and demonstrated that it can form a heteromeric complex with the type I and II TGF-β signaling receptors. To explore whether GPI-anchored proteins modulate TGF-β signaling in keratinocytes that express keratinocyte-derived PEDF as a natural inhibitor, we identified the r150 gene encoding the GPI M, GPI mutant; GPI NM, GPI non-mutant; STAT, signal transducer and activator of transcription.

TGF-β is a 25-kDa multifunctional growth factor that plays an important role in cell growth and differentiation, extracellular matrix deposition, cell adhesion, and immunomodulation. It is a member of the TGF-β superfamily, which includes activin, inhibin, bone morphogenetic protein, growth and differentiation factor-1, and glial derived neurotropic factor. Three distinct isoforms of TGF-β (TGF-β1, -β2, and -β3) have been described in mammals. There is ~70% homology between the isoforms, but they appear to have distinct activities in vivo as suggested by their differences in receptor binding, potencies; developmental expression; and the dissimilar phenotypes exhibited by mice null for TGF-β1, -β2, or -β3 genes (3, 4).

TGF-β signaling is transduced by a pair of transmembrane serine/threonine kinases known as type I and type II receptors, which are present in almost all cell types analyzed. The type I receptor does not bind TGF-β in the absence of the type II receptor. According to the current model, the binding of TGF-β to the type II receptor, a constitutively active kinase, results in the recruitment and phosphorylation of the type I receptor in its "GS sequence" upstream of the kinase domain, resulting in its activation. The activated type I receptor then phosphorylates its intracellular substrates, Smad2 and Smad3, which are released from the receptor and form a complex with Smad4. This heteromeric Smad complex then translocates to the nucleus and interacts with DNA to regulate target gene expression by recruiting transcriptional coactivators such as CBP (cAMP-responsive element-binding protein-binding protein)/p300 and corepressors such as TGF-β-induced factor, Ski, and SmoN (5–7).

Other cell-surface TGF-β-binding proteins that have been identified include betaglycan (type III TGF-β receptor) and endoglin (CD105), which have a more limited tissue distribution and are considered to be accessory receptors. Betaglycan, a membrane proteoglycan, binds all three TGF-β isoforms with high affinity and is thought to facilitate TGF-β binding to the type II TGF-β receptor (8, 9). Endoglin shows 70% homology to betaglycan and binds TGF-β1 and TGF-β3 with high affinity through its association with the type II receptor (10, 11). Furthermore, endoglin appears to interact with activin and bone morphogenetic protein in the presence of their cognate receptors (12).

Interestingly, overexpression of endoglin attenuates TGF-β responses in monocytes and myoblasts (13), whereas overexpression of betaglycan enhances TGF-β responses in these cells (9). Glycosylphosphatidylinositol (GPI)-anchored proteins, which lack transmembrane and cytoplasmic domains, have also been shown to bind TGF-β. These proteins have been reported in several cell lines (14–17), but the identity of these GPI-anchored proteins and the role they may play in TGF-β signaling remain unknown. We have shown recently that GPI-anchored proteins bind TGF-β in early passage human endometrial stromal cells (15), skin fibroblasts (16), and keratinocytes (17). In addition, we have characterized a 150-kDa GPI-anchored TGF-β-binding protein on human keratinocytes (designated as r150) that can form a heteromeric complex with the type I and II TGF-β receptors (17). In the present study, we
have generated a GPI anchor mutant form of a human keratinocyte cell line (HaCaT) and have demonstrated that these cells display reduced levels of r150 on their cell surface relative to parental HaCaT cells. Furthermore, these cells show an enhanced responsiveness to TGF-β1. Our results suggest that GPI-anchored proteins regulate TGF-β1 signaling and implicate r150 as an inhibitor of TGF-β1 signal transduction in human keratinocytes.

EXPERIMENTAL PROCEDURES

Plasmids—The 3TP-lux and pCMV-βgal constructs were gifts from Dr. M. D. O’Connor-McCourt (Biotechnology Research Institute, Montreal, Quebec, Canada). The 3TP-lux construct contains three Dol-tetradecanoylphorbol-13-acetate-responsive elements and TGF-β-responsive elements from the PAI-1 promoter fused in tandem to a luciferase reporter (18). (CAGA)12-lux and the empty vector MLP-lux were gifts from Dr. S. Huet (Laboratoire Glaxo Wellcome, France). (CAGA)12-lux is a luciferase construct specific for Smad3/4-driven signaling and contains 12 repeats of the sequence CAGA upstream of the MLP of adenovirus and a luciferase gene (19, 20). Dominant-negative Smad3 and its empty vector pDNA3 were gifts from Dr. J. J. Lebrun (McGill University). Dominant-negative Smad3 has its C-terminal region deleted (21).

Cell Culture—HaCaT cells were obtained from Dr. P. Boukamp (German Cancer Research Center, Heidelberg, Germany). HaCaT cells are a spontaneously immortalized cell line and show no major differences in differentiation compared with normal keratinocytes. They exhibit a transformed phenotype, but are not tumorigenic (22). HaCaT cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 units/ml human recombinant insulin (BD Biosciences, Mississauga). Cell sorting was repeated twice. At the indicated times, cells were trypsinized and counted on a hemocytometer.

Affinity Labeling of Cells with 125I-TGF-β—Affinity cross-link labeling was performed as described by Dumont et al. (15). Briefly, cell monolayers were washed three times for 10 min each with ice-cold binding buffer (PBS with Ca2+ and Mg2+, pH 7.4 (Dulbecco's PBS) containing 0.1% bovine serum albumin). Cells were then incubated at 4 °C for 3 h with 100 μM 125I-TGF-β1. Incubations were also done in the presence of excess nonradioactive TGF-β1 (Genzyme Corp., Cambridge, MA) to confirm specific binding (data not shown). The receptor-ligand complexes were then cross-linked with 1 mM bis(sulfosuccinimidyl)suberate (BS3, Pierce). After 10 min, the reaction was stopped by the addition of 500 mM glycine (final concentration of 10 mM) for 5 min. The supernatant was aspirated, and cells were washed twice with Dulbecco's PBS. Cells were lysed with solubilization buffer (20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, 200 μg/ml bovine serum albumin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml pepstatin). The solubilized material was mixed with 0.2 volume of 5× electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, and trace bromphenol blue). Samples were resolved by 3–11% SDS-PAGE under nonreducing or reducing (in the presence of 5% β-mercaptoethanol) conditions. The gels were analyzed by autoradiography, and radioactive bands were quantitated by densitometry.

Isolation and Cloning of GPI Anchor Mutant Keratinocytes—The preparation and selection of cells mutated in GPI biosynthesis was performed as described by Stevens (23). HaCaT cells grown to 50–60% confluence in a 75-cm2 flask (Falcon) were treated with 300 μg/ml EMS for 24 h at 37 °C in a humidified atmosphere of 5% CO2 and air. Cells were allowed to recover for 48 h in normal growth medium. FACS was performed to select cells that lost expression of GPI-anchored proteins on their cell surface. GPI anchor mutant cells were negatively sorted for their cell surface. GPI anchor mutant cells were negatively sorted for their cell surface. GPI anchor mutant cells were negatively sorted for their cell surface. GPI anchor mutant cells were negatively sorted for their cell surface.

RESULTS

Isolation and Cloning of HaCaT Cells Mutated in GPI Anchor Biosynthesis—HaCaT cells were treated with 300 μg/ml EMS for 24 h as described under "Experimental Procedures." EMS is an ethylating agent that has been used to cause mu-
GPI-anchored Proteins Regulate TGF-β Signaling

Tagenogenesis of genes, including those involved in the biosynthesis of the GPI anchor (23, 24). CD59, a 21-kDa GPI-anchored protein expressed on keratinocytes, was used as a marker for the cloning of GPI anchor-deficient cells. CD59 is an inhibitor of the membrane attack complex (25, 26). After EMS treatment, cells were stained with fluorescein isothiocyanate-conjugated anti-CD59 antibody and negatively sorted by FACS analysis. Cells selected from CD59-negative populations were cloned using the FACS Vantage automated cell deposition unit. Ten clones survived and were reanalyzed for CD59 expression by flow cytometry. Two clones displayed reduced expression of CD59 on their cell surface and were presumed to be mutated in GPI anchor biosynthesis. These clones were designated as GPI M and GPI M1. Fig. 1A illustrates the flow cytometric analysis of parental HaCaT and GPI M cells. Although the expression of CD59 was not completely abolished in GPI M cells, the immunofluorescence intensity of CD59 was approximately half of that obtained for HaCaT cells (56.3 ± 7.4%, p < 0.001). Similar results were obtained with GPI M1 cells (data not shown).

To confirm that the cloned GPI mutant cells display reduced levels of r150 on their cell surface, we affinity-labeled HaCaT and GPI M cells with 125I-TGF-β1, and r150 levels were compared by autoradiography. Fig. 1B shows a significant reduction in r150 levels in GPI M cells relative to parental cells, whereas the relative amounts of type I, II, and III receptors bound to 125I-TGF-β1 in HaCaT versus GPI M cells remained equivalent.

Cell Growth and Morphology Are Unchanged in GPI Anchor Mutant Cells—A deficiency or loss of GPI-anchored proteins as a result of EMS mutagenesis has no reported effects on cell morphology or cell cycle progression in other cell types (23). To confirm that these parameters are not altered in GPI M cells, the doubling times and cell morphologies of HaCaT and GPI M cells were compared. Fig. 2A demonstrates that HaCaT and GPI M cells have similar growth rates with equivalent doubling times (~16 h). In addition, the morphologies of HaCaT (Fig. 2B) and GPI M (Fig. 2C) cells are similar.

GPI-anchored Proteins Inhibit TGF-β1-induced Transcriptional Activity—The 3TP-lux transcriptional reporter contains TGF-β-responsive elements from the PAI-1 promoter and is widely used to assess TGF-β signaling requirements (28). To examine the potential effect(s) of GPI-anchored proteins on TGF-β signaling in keratinocytes, we transfected HaCaT and GPI M cells with the 3TP-lux reporter construct and compared luciferase activities after 4 and 16 h of TGF-β1 treatment. A HaCaT cell clone subjected to the same cloning procedure but not mutated in GPI anchor biosynthesis (GPI NM) was included as a control. GPI M cells exhibited a 30% increase in luciferase activity relative to HaCaT cells following a 4-h treatment with 10 pM TGF-β1 (p < 0.05) (Fig. 3A). At 100 pM TGF-β1, GPI M cells showed an even greater (50%) increase in luciferase activity relative to HaCaT cells (p < 0.003). Interestingly, the fold induction of luciferase activity in HaCaT cells treated with 10 and 100 pM TGF-β1 remained similar (3.3 and 3.9, respectively), whereas GPI M cells displayed a dose response with 4.9- and 8.4-fold increases, respectively.

Similarly, after 16 h of TGF-β1 treatment, GPI M cells displayed a markedly enhanced response to 100 pM TGF-β1 compared with HaCaT cells (Fig. 3B). GPI M cells exhibited a 91.2-fold induction, which is approximately twice that of HaCaT (50.2-fold) and GPI NM (51.3-fold) cells (p < 0.01 for GPI M versus HaCaT) in response to 100 pM TGF-β1. In contrast, HaCaT, GPI NM, and GPI M cells showed no significant difference in response to 100 pM TGF-β2 (Fig. 3B). In addition, both HaCaT and GPI M cells displayed a dose-dependent response to 10 and 100 pM TGF-β1 at this time point (data not shown). After treatment with 10 pM TGF-β1 for 16 h, GPI M cells displayed a 21.4-fold stimulation of luciferase activity, with HaCaT and GPI NM cells showing 13.6- and 10.6-fold

Fig. 1. A, GPI anchor mutant keratinocytes show decreased expression of CD59. Shown is a representative histogram demonstrating the expression of CD59 in a GPI anchor-deficient clone, GPI M (solid white peak), and in parental HaCaT cells (solid black peak) as assessed by flow cytometry using fluorescein isothiocyanate-conjugated anti-CD59 antibody. The immunofluorescence intensity of CD59 in GPI anchor-deficient cells was ~50% of that obtained in HaCaT cells. Flow cytometric analysis of HaCaT cells was also performed in the absence of antibody (dashed white peak). B, 125I-TGF-β1 affinity labeling of HaCaT (normal [Norm]) and GPI M cell proteins. GPI M cells showed a marked reduction in r150 expression relative to HaCaT cells. Affinity-labeled proteins were resolved by SDS-PAGE under reducing conditions and analyzed by autoradiography.
increases in luciferase activities, respectively (p < 0.005 for GPI M versus HaCaT).

**GPI Anchor Mutant Cells Display Enhanced Smad2 Phosphorylation at Low Doses of TGF-β1**—To determine whether the loss of GPI-anchored proteins has any effect on the phosphorylation of endogenous Smad2, HaCaT and GPI M cells were treated with 1–50 pM TGF-β1 for 20 min, and immunoblotting was performed with anti-phospho-Smad2 antibody. As demonstrated in Fig. 4A, GPI M cells exhibited enhanced Smad2 phosphorylation in response to low (2 and 5 pM), intermediate (10 pM), and higher (50 pM) doses of TGF-β1. Immunoblotting with an anti-Smad2 antibody that detects both unphosphorylated and phosphorylated forms of Smad2 demonstrated equivalent protein loading. Equal protein loading of HaCaT and GPI M cell lysates was also confirmed upon immunoblotting with anti-STAT3 antibody because the STAT3 level is not affected by TGF-β treatment during the time period used

**FIG. 2. Loss of GPI-anchored proteins in keratinocytes cells has no effect on cell growth or morphology.** A, HaCaT and GPI M cells showed equivalent doubling times of ~16 h. HaCaT and GPI M cells were seeded at 8.0 × 10^5 cells in 60-mm dishes in duplicate, and cell numbers were determined after 14, 24, 28, 36, 46, 52, and 72 h by counting cells on a hemocytometer. B and C, HaCaT and GPI M cells showed similar cell morphologies. Shown are microscopic images (magnification ×10) of HaCaT (B) and GPI M (C) cells under normal culture conditions.

**FIG. 3. GPI anchor mutant keratinocytes show enhanced TGF-β1-stimulated transcriptional response.** HaCaT, GPI M, and GPI NM (non-mutant control) cells were transiently transfected with 1.0 μg each of 3TP-lux and pCMV-β-gal and then treated with 0, 10, or 100 pM TGF-β1 or TGF-β2 as indicated for 4 h (A) or 16 h (B) as described under "Experimental Procedures." Luciferase activity was normalized to β-galactosidase activity, and data are expressed as fold induction of luciferase activity. The data are representative of at least three independent experiments. Error bars represent S.D.
Importantly, TGF-β2 treatment did not result in enhanced phosphorylation of Smad2 in GPI M cells compared with HaCaT cells, as GPI M and HaCaT cells demonstrated the same weak pattern of TGF-β2-induced Smad2 phosphorylation (Fig. 4B).

Elevated Smad2 Phosphorylation Is Sustained in GPI Anchor Mutant Cells—We further examined whether the GPI anchor-deficient cells exhibited enhanced Smad2 phosphorylation upon prolonged exposure to TGF-β1 (Fig. 5A). In response to 100 pM TGF-β1, maximal stimulation of Smad2 phosphorylation in HaCaT cells occurred at 10 min and was maintained for 180 min. In comparison with HaCaT cells, GPI M cells displayed significantly elevated Smad2 phosphorylation, which was evident at 45 min and was sustained for up to 180 min. The second GPI anchor mutant clone (GPI M1) also exhibited enhanced Smad2 phosphorylation in response to TGF-β1 (Fig. 5B). In this experiment, GPI M1 cells displayed an elevated level of TGF-β1-induced Smad2 phosphorylation compared with HaCaT cells after 20 min, and this was sustained for up to 180 min. On the other hand, Smad2 phosphorylation in GPI NM control cells in response to TGF-β1 was similar to that in HaCaT cells at all time periods studied (Fig. 5C). Immunoblotting with anti-STAT3 antibody demonstrated equal protein loading of the cell lysates. These results show that the GPI anchor-deficient cells (GPI M and GPI M1), but not GPI NM control cells, exhibit enhanced TGF-β1-stimulated phosphorylation of endogenous Smad2 in comparison with parental HaCaT cells.

GPI Anchor Mutant Cells Display Enhanced Smad3 Activation at Low Doses of TGF-β1—Although both Smad2 and Smad3 act as mediators of cellular responses elicited by TGF-β, they have been shown to have differing functions (28, 29).
Smad3 binds DNA directly, whereas Smad2 does not (19). Because the experiments described above demonstrate that GPI mutant cells displayed enhanced Smad2 phosphorylation, it was of interest to determine whether these cells demonstrate augmented Smad3 activation. To analyze Smad3 activation, we transfected GPI M and HaCaT cells with the (CAGA)12-lux reporter construct specific for Smad3-driven signaling and compared luciferase activity in response to TGF-β1 treatment. Fig. 6 shows that GPI M cells had a higher luciferase activity than HaCaT cells at 0, 5, and 50 pM TGF-β1 (p < 0.05, p < 0.0006, and p < 0.001, respectively).

Stimulation of TGF-β1-induced Transcriptional Activity in GPI Anchor Mutant Cells Involves Smad3—We then determined whether Smad3 is involved in the stimulation of PAI-1 transcriptional activity induced by TGF-β1 in GPI M cells. The PAI-1 promoter present in the 3TP-lux reporter construct contains three consensus binding sites for Smad3, but is also responsive to other transcription factors such as c-Jun (30). To determine whether the enhanced 3TP-lux reporter activity in GPI M cells involves Smad3, we tested whether dominant-negative Smad3 blocks this activity. Fig. 7 illustrates that cotransfection of dominant-negative Smad3 decreased 3TP-lux activity in both GPI M and HaCaT cells by ~70%. The data are representative of at least three independent experiments. Error bars represent S.D.

**DISCUSSION**

To examine whether GPI-anchored proteins modulate TGF-β signaling in keratinocytes, we created keratinocyte defective in GPI anchor biosynthesis (GPI M cells) by chemical mutagenesis of HaCaT cells. In comparison with parental HaCaT cells, GPI M cells demonstrated significantly lower expression of the GPI-anchored TGF-β1-binding protein r150 on their cell surface. Furthermore, GPI M cells showed no alteration in type I and II TGF-β receptor expression or in their abilities to bind TGF-β1. In addition, GPI M cells did not show any differences in cell morphology or doubling time. Importantly, GPI M cells demonstrated enhanced Smad2 and Smad3 activation in a dose-dependent manner in response to TGF-β1 (but not TGF-β2) treatment. Also, these cells displayed markedly increased TGF-β1-responsive gene transcriptional activity. Taken together, our results indicate that GPI-anchored protein(s) negatively modulate TGF-β responses in human keratinocytes and implicate r150 as the GPI-anchored protein responsible for this inhibition in these cells.

In the isolated GPI anchor-deficient keratinocytes, the cell-surface expression of the GPI-anchored protein marker CD59 was still detectable by flow cytometry, thus indicating that the cells did not display a complete abrogation of GPI-anchored
proteins. However, a decrease in immunofluorescence intensity of ∼50% exhibited by GPI M cells is comparable to the loss of GPI-anchored proteins on the cell surface after phosphatidylinositol-specific phospholipase C treatment (31). In addition, 125I-TGF-β1 affinity cross-link labeling of GPI M cells demonstrated a significant loss of r150 on their cell surface relative to parental HaCaT cells. That GPI anchor biosynthesis is not completely abolished in these cells is likely due to the presence of GPI anchor biosynthetic genes that are resistant to EMS mutagenesis (32). The use of EMS to cause mutagenesis of genes, including those involved in the biosynthesis of the GPI anchor, is well documented (29, 34). EMS mutagenesis of Chinese hamster ovary cells has recently been used to study the role of GPI-anchored proteins in the development of Alzheimer's disease (33) and in the “cross-talk” between caveolae and GPI-enriched lipid microdomains (34).

Because the expression of all GPI-anchored proteins may be affected in GPI mutant cells, it is possible that the loss of GPI proteins other than r150 may have an impact on TGF-β signaling. However, because r150 is the only GPI-anchored protein that binds to TGF-β1 in keratinocytes, and it has the ability to interact with the type I and II TGF-β receptors in these cells, it is likely that the loss of this protein is responsible for the enhanced TGF-β1 signaling in GPI mutant cells. None of the mammalian GPI-anchored proteins whose identities are known have been shown to bind TGF-β or have been implicated in TGF-β signaling (35, 36). Unidentified mammalian GPI-anchored TGF-β-binding proteins that have been demonstrated in various cell lines include a 180-kDa TGF-β1-binding protein and a 65- and 140-kDa TGF-β2 binding protein (14). However, early passage human keratinocytes and HaCaT cells do not appear to express any GPI-anchored TGF-β-binding complexes with the above-mentioned relative molecular masses (17). Also, they have not been shown to interact with the type I and II TGF-β receptors (16). Thus, it is likely that the observed augmentation in TGF-β1-induced cellular responses exhibited by the two isolated GPI mutant clones is due to the loss of r150.

The conclusion that GPI-anchored proteins inhibit TGF-β signaling is based on the enhanced phosphorylation of endogenous Smad2 and activation of Smad3-specific signaling exhibited by the GPI anchor-deficient cells compared with parental HaCaT cells. This phenomenon is reproducible in a dose-dependent manner in both of the GPI anchor mutants that were cloned (GPI M and GPI M1). Furthermore, GPI M cells displayed increased PAI-1 transcriptional activity upon 4 and 16 h of TGF-β1 treatment. The involvement of Smad3 in the enhanced PAI-1 promoter activity in these cells is demonstrated by a marked decrease in the activity in the presence of dominant-negative Smad3. The activation of both Smad2 and Smad3 likely contributes to the enhanced transactivation of the PAI-1 promoter in the GPI anchor-deficient cells. This is consistent with previous reports that have shown that both Smad2 and Smad3 are involved in TGF-β-mediated PAI-1 upregulation (29). Interestingly, the loss of GPI-anchored proteins may not impact TGF-β2 responsiveness because treatment with TGF-β2 did not result in enhanced Smad2 phosphorylation or PAI-1 promoter activity. This is not surprising because r150 has virtually no affinity for TGF-β2 (17).

The molecular mechanisms by which r150 regulates TGF-β signaling remain to be determined. Because r150 forms a heteromeric complex with the TGF-β signaling receptors, it may modulate the serine/threonine kinase activity of the type I or II receptors. The autophosphorylation of key residues in the type II receptor is important in the regulation of kinase function (37). Our experiments using the in vitro kinase assay with anti-type II receptor antibody indicate that there was no marked difference in the type II autophosphorylation in GPI anchor-deficient cells compared with HaCaT cells in the absence or presence of TGF-β1 (Fig. 8). Alternatively, the interaction of r150 with the type II receptor may exert a negative regulatory effect on TGF-β signaling, as is seen with TRIP-1, a tryptophan-aspartic acid protein that specifically associates with the type II receptor and suppresses TGF-β-induced response (38). Also, it is possible that r150 may interact with the type I receptor to hamper its phosphorylation by the type II receptor and hence activation of the type I kinase. This would be similar to the action of FKBP12, an immunophilin that interacts with the type I kinase and exerts an inhibitory effect on TGF-β-mediated cellular responses (39). Our attempts to assess the phosphorylation of the type I kinase using the in vitro kinase assay were unsuccessful. It is unknown if r150 preferentially interacts with the type I or II receptor.

Our previous work indicates that soluble r150 retains its ability to bind TGF-β1 in the absence of the type I and II receptors and of an intact membrane (40). Hence, it is likely that r150 binds to TGF-β1 independently of the TGF-β1 signaling receptors when attached to the cell surface. This is in contrast to the type I receptor and endoglin, which recognize TGF-β only when bound to the type II receptor (12, 18, 25). Thus, the membrane-anchored form of r150 may regulate TGF-β1 binding to its receptors and hence signaling. The strong dose-dependent response in the 3TP-lux luciferase activity displayed by the GPI mutant cells (4.9-fold versus 8.4-fold) relative to parental HaCaT cells (3.3-fold versus 3.9-fold) upon treatment with 10 and 100 pM TGF-β1 suggests that r150 may play a role in dampening large fluctuations in TGF-β signaling. Therefore, unlike the type III receptor, whose role is to facilitate binding of TGF-β to its signaling receptors (8), r150 may restrict the access of TGF-β1 to its receptors. Because TGF-β1 (but not TGF-β2) treatment results in enhanced TGF-β1 signaling, r150 may act to sequester TGF-β1 away from the signaling receptors. Thus, in the absence of r150, the heteromerization of the type I and II receptors and thus the activation of the type I kinase may occur more rapidly and at lower concentrations of TGF-β1 to reach the signaling threshold.

r150 may potentially have a role in the cellular “compartmentalization” of the type I and II receptors and of the receptor-regulated Smad proteins. There is an emerging theme in signal transduction biology whereby signaling molecules can be compartmentalized into membrane entities known as caveolae and “lipid rafts.” These are organized lipid microdomains that serve as centers in which signaling molecules of various pathways can effectively interact (41, 42). GPI-anchored proteins have been detected in caveolae or lipid rafts in association with signaling molecules such as Src-like kinases, G-proteins, protein kinase C, and the platelet-derived growth factor receptor (41, 43–45). The association of r150, a GPI-anchored protein, with the type I and II receptors supports the notion that the TGF-β receptors may also be organized in these plasmaemmal entities. The type I TGF-β receptor was recently shown to co-localize and associate with caveolin-1, the main caveolar scaffolding protein, in caveola-enriched membrane fractions (46). Furthermore, recent evidence indicates that TGF-β receptors internalize via both the lipid raft-caveolar pathway and the clathrin-dependent EEA1 endosomal pathway and that whereas the EEA1 endosomal pathway promotes TGF-β signaling, the caveolar pathway enhances receptor turnover and inhibition of signaling (47). This raises the interesting possibility that r150 as a GPI-anchored protein may be an important determinant of the compartmentalization of TGF-β receptors into caveolae. The loss of r150 will thus lead to poor receptor...
internalization via the lipid raft-caveolar pathway and diminished receptor turnover, resulting in enhanced signaling.

GPI-anchored proteins are implicated in the maintenance of skin homeostasis. Targeted deletion of the GPI anchor biosynthetic gene *PIG-A* in the epidermis may result in smaller pups exhibiting wrinkled skin and a thickened stratum corneum with death within 1–3 days after birth (48). Interestingly, transgenic mice overexpressing TGF-β1 in the epidermis also demonstrate a compact stratum corneum with a significant reduction in the number of proliferating epidermal cells (49). Death of these transgenic mice is also attributed to abnormal skin development. Thus, the phenotype of transgenic mice overexpressing TGF-β1 in the epidermis appears to mimic that of the *PIG-A* deletion. It is possible to envision that the ablation of r150 expression in the epidermis as a consequence of hyperactivity of TGF-β1 signaling in the skin. Hence, r150 may play an essential role in skin development as a key regulator of TGF-β function in epidermal differentiation and homeostasis.

In conclusion, studies of TGF-β responses in GPI anchor-deficient keratinocytes have provided important insight into the role of GPI-anchored proteins in the regulation of TGF-β signaling. Our results strongly suggest that GPI-anchored proteins negatively modulate TGF-β action in human keratinocytes. It is conceivable that the novel GPI-anchored TGF-β1-binding protein r150 may directly modulate type I and II kinase activities through its interaction with the signaling receptors. Alternatively, r150 may determine the compartmentalization and turnover of the signaling receptors, thus modulating their biological response. In addition, due to the ability of r150 to bind TGF-β1 on its own, membrane-bound and soluble r150 may regulate ligand availability by acting as a scavenger receptor. Delineation of the structure of r150 is necessary to elucidate the precise molecular mechanisms by which this novel accessory receptor regulates TGF-β signaling.

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