Journal of Cellular Biochemistry 83:494–507 (2001)

Characterization of a 150 kDa Accessory Receptor for TGF-β1 on Keratinocytes: Direct Evidence for a GPI Anchor and Ligand Binding of the Released Form

B.Y.Y. Tam,[1] D. Larouche,[2] L. Germain,[2] N.M. Hooper,[3] and A. Philip[1]

[1]Division of Plastic Surgery, Montreal General Hospital and Department of Surgery, McGill University, Montreal, Quebec, H3G 1A4, Canada
[2]LOEX Laboratory, Hpital Saint Sacrement and Department of Surgery, Université Laval, Sainte Foy, Quebec, Canada
[3]School of Biochemistry and Molecular Biology, University of Leeds, Leeds, United Kingdom

Abstract Transforming growth factor-β (TGF-β) is a key modulator of epidermal development and homeostasis, and has been shown to potently regulate keratinocyte migration and function during wound repair. There are three cloned TGF-β receptors termed type I, type II, and type III that are found on most cell types. The types I and II are the signaling receptors, while the type III is believed to facilitate TGF-β binding to the types I and II receptors. Recently, we reported that in addition to these receptors, human keratinocytes express a 150 kDa TGF-β1 binding protein (r150) which forms a heteromeric complex with the TGF-β signaling receptors. This accessory receptor was described as glycosyl phosphatidylinositol-specific anchored based on its sensitivity to phosphatidylinositol phospholipase C (PIPLC). In the present study, we demonstrate that the GPI-anchor is contained in r150 itself and not on a tightly associated protein and that it binds TGF-β1 with an affinity similar to those of the types I and II TGF-β signaling receptors. Furthermore, the PIPLC released (soluble) form of this protein is capable of binding TGF-β1 independently from the signaling receptors. In addition, we provide evidence that r150 is released from the cell surface by an endogenous phospholipase C. Our observation that r150 interacts with the TGF-β signaling receptors, together with the finding that the soluble r150 binds TGF-β1 suggest that r150 in either its membrane anchored or soluble form may potentiate or antagonize TGF-β signaling. Elucidating the mechanism by which r150 functions as an accessory molecule in TGF-β signaling may be critical to understanding the molecular mechanisms underlying the regulation of TGF-β action in keratinocytes. J. Cell. Biochem. 83: 494–507, 2001.

Key words: TGF-β; receptors; keratinocytes; GPI anchor; skin

Transforming growth factor-β (TGF-β) is a 25 kDa multi functional growth factor which plays a central role in the wound healing process [Roberts and Sporn, 1990; O'Kane and Ferguson, 1997]. It is an important regulator of the immune response [Letterio and Roberts, 1998], angiogenesis, reepithelialization [Roberts and Sporn, 1990], extracellular matrix synthesis, and remodeling [Peltonen et al., 1991; Yamamoto et al., 1994]. During wound healing, re-epithelialization initiates the repair process which is characterized by recruitment of epidermal stem cells, keratinocyte proliferation, and the formation of an epithelial tongue of migrating keratinocytes at the wound edge [Clark, 1996]. TGF-β is chemotactic to keratinocytes and induces the expression of integrins on the migrating epithelium [Hebda, 1988; Zambruno et al., 1995]. In spite of its promigratory effect on keratinocytes, TGF-β is a potent inhibitor to epithelial cell proliferation in vitro [Pietenpol et al., 1990] and in vivo [Glick et al., 1993]. Targeted deletion of the TGF-β1 gene in keratinocytes causes rapid progression to squamous cell carcinoma [Glick et al., 1994]. In addition, the epidermis of transgenic mice expressing a dominant negative TGF-β receptor exhibits a hyperplastic and hyperkeratotic

Grant sponsor: Medical Research Council of Canada to AP; Grant sponsor: Chercheur Boursier salary support award from FRSQ, Quebec to AP.

*Correspondence to: Anie Philip, Department of Surgery, McGill University Health Centre, 1650, Av. Cedar, H3G 1A4 Montreal, Quebec, Canada.

Received 1 February 2000; Accepted 29 November 2000

DOI 10.1002/jcb.1074

phenotype [Wang et al., 1997]. These results support the importance of proper expression of TGF-β and regulation of its function in epidermal development and homeostasis.

There are three widely distributed TGF-β receptors, type I, type II, and type III, all of which have been cloned [Roberts and Sporn, 1990; Massague, 1998]. The types I and II receptors are both transmembrane serine/threonine kinases that are essential for TGF-β signal transduction. The type III receptor, also known as betaglycan, is a high molecular weight proteoglycan which is not required for signaling, but is believed to play a role in presenting the ligand to the type II receptor [Lopez-Casillas et al., 1993]. Endoglin, is another TGF-β receptor predominantly expressed on endothelial cells [Gougos and Letarte, 1990]. According to the present model of TGF-β signal transduction, binding of TGF-β to the type II receptor which is a constitutively active kinase, leads to the recruitment and phosphorylation of the type I receptor [Wrana et al., 1994]. The activated type I kinase phosphorylates its intracellular substrates Smad2 and Smad3 which allows them to form a complex with Smad4. This heteromeric complex then translocates into the nucleus where it regulates expression of target genes [Heldin et al., 1997].

Although the types I and II receptors are central to TGF-β signaling, it is possible that accessory receptors interacting with the signaling receptors modify TGF-β responses. For example, both endoglin and type III receptor which form heteromeric complexes with the type II receptor have been reported to modulate TGF-β function. When overexpressed in myoblasts, endoglin inhibited while type III receptor enhanced TGF-β responses [Letamendia et al., 1998]. In addition, endoglin was shown to antagonize TGF-β mediated growth inhibtion of human vascular endothelial cells [Li et al., 2000]. Similarly, the newly identified type I-like receptor BAMBI which associates with TGF-β family receptors can inhibit signaling [Onichtchouk et al., 1999].

Recently, we reported the identification of a 150 kDa glycosyl phosphatidylinositol (GPI)-anchored TGF-β1 binding protein (r150) that can form a heteromeric complex with the types I and II TGF-β receptors on human keratinocytes [Tam et al., 1998]. GPI-anchored proteins lack transmembrane and cytoplasmic domains, and are attached to the cell membrane via a lipid anchor in which the protein is covalently linked to a glycosyl phosphatidylinositol moiety. GPI-anchored proteins have been reported to associate with cholesterol and glycosphingolipid-rich membrane microdomains [Brown and London, 1998; Hooper, 1999], and to have roles in intracellular sorting [Rodriguez-Boulan and Powell, 1992] and in transmembrane signaling [Brown, 1993]. Also, the GPI anchor enables a protein to be selectively released from the membrane by phospholipases [Metz et al., 1994; Movahedi and Hooper, 1997]. We characterized r150 as GPI-anchored, based on its sensitivity to phosphatidylinositol phospholipase C (PIPLC). However, it is important to rule out other possibilities, namely, (i) r150 is not itself GPI-anchored, but is tightly associated with a protein that is GPI-anchored, and therefore is susceptible to release by PIPLC; (ii) r150 is a complex of two lower molecular weight proteins which became inadvertently cross-linked during the affinity labeling procedure.

In the present study, we demonstrate that the GPI-anchor is contained in r150 itself and not on a tightly associated protein and that it binds TGF-β1 with an affinity comparable to those of the signaling receptors. Furthermore, the released (soluble) form of this protein binds TGF-β1 independent of the types I and II receptors. In addition, we provide evidence that r150 is released from the cell surface by an endogenous phospholipase C. These findings, taken together with the observation that r150 forms a heteromeric complex with the signaling receptors, suggest that this accessory receptor in either its membrane anchored or soluble form may potentiate or antagonize TGF-β responses in human keratinocytes.

METHODS

Cell Culture

Neonatal keratinocytes were prepared from foreskin tissue obtained at newborn male circumcision as described by Germain et al. [1993]. The keratinocytes were cultured in serum free keratinocyte medium (Gibco, Burlington, Ontario) and cells of third to fifth passage were used for experiments. The immortalized keratinocyte cell line, HaCaT, was obtained from Dr. Boukamp (Heidelberg, Germany), and the mink lung epithelial cells (Mv1Lu) were from ATCC. Both cell types were

maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 5% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Gibco, Burlington, Ontario). All cells were maintained at 37°C in an atmosphere of 5% CO_2/air.

Affinity Labeling of Cells

Iodination of TGF-β1 (Collaborative Biomedical) was done as described [Philip and O' Connor-McCourt, 1991]. Affinity labeling technique was performed as detailed previously [Dumont et al., 1995]. Briefly, cell monolayers were washed with ice-cold binding buffer (DPBS or Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+}, pH 7.4) containing 0.1% bovine serum albumin (BSA). Cells were incubated with 100–200pM of ^{125}I-TGF-β1 for 3 h at 4°C. In some experiments, incubations were done in the absence or presence of increasing concentrations of unlabeled TGF-β isoforms to determine the competition profiles of the receptors. The receptor-ligand complexes were cross-linked with 1mM Bis- (Sulfosuccinimidyl) suberate (BS^3, Pierce). The reaction was stopped by the addition of glycine and the cells were solubilized, and separated on 3–11% polyacrylamide SDS gel. The results were analyzed by using autoradiography, followed by quantitative densitometry (Gel-Cypher, Lightools Inc, Encinitas, CA).

Temperature Induced Phase Separation in Triton X-114 of r150 and Hydrolysis by PIPLC

Temperature induced phase separation in Triton X-114 and PIPLC treatment was performed as described previously with modifications [Bordier, 1981]. Keratinocytes were affinity labeled with 150 pM of ^{125}I-TGF-β1 and lysed in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% Triton X-114, 1 mM phenylmethylsufonyl flouride and protease inhibitor cocktail (200 μg/ml BSA, 1 μg/ml leupeptin, 10 μg/ml benzamide, 10 μg/ml soyabean trypsin inhibitor, and 2 μg/ml pepstatin) for 60 min at 4°C. The cell lysates were centrifuged at 13,000*g* for 15 min at 4°C. The Triton X-114 soluble material was incubated at 30°C for 10 min followed by a 10 min centrifugation at 13 000*g* at room temperature to separate the detergent rich phase from the aqueous detergent poor phase. An aliquot (20%) from each phase was precipitated with ethanol/acetone, and analyzed by SDS-PAGE and autoradiography. The remaining 80% of the detergent phase was utilized to determine the effect of PIPLC on the detergent solubility of r150 using the method of Lisanti et al. [1988]. Briefly, the GPI-anchored protein enriched detergent phase was incubated with or without 0.6 U/ml of PIPLC (Roche Diagnostics) for 1 h at 37°C with mild agitation. Temperature induced phase separation was then repeated. Both the aqueous and detergent phases were precipitated by adding ethanol/acetone, and subjected to SDS-PAGE and autoradiography.

Affinity Labeling of Soluble r150

Neonatal keratinocytes were harvested by treating confluent monolayers with Hanks' balanced salt solution containing 5 mM EDTA (pH 7.5). The cell pellet was washed with DPBS and treated with 0.6 U/ml of PIPLC or left untreated, for 1 h at 37°C with mild agitation. The supernatant containing the released GPI-anchored proteins was collected and concentrated by Centricon 30 (Amicon). Aliquots of the concentrated supernatant were affinity labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β (7.5 nM) and analyzed by SDS-PAGE as described above except that the solubilization step was omitted.

^{125}I-TGF-β1 Binding to Mv1Lu Cells

To test whether soluble r150 regulates the binding of TGF-β to its receptors, the supernatant obtained from PIPLC treated HaCaT cells were used in a ^{125}I-TGF-β1 Mv1Lu binding assay. The HaCaT cells, which display the r150 with identical properties as the neonatal keratinocytes [Tam et al., 1998], were grown in T-25cm^2 tissue culture flasks (Falcon) were left untreated or treated with PIPLC, and the resulting supernatants were concentrated by Centricon 30 (Amicon). Mv1Lu cells were incubated with 50 pM of ^{125}I-TGF-β1 in the absence or presence of increasing doses of the concentrated supernatant for 3 h at 4°C. The cells were washed, solubilized and the bound radioactivity was determined by a gamma counter.

To rule out the possibility that any alteration in ^{125}I-TGF-β1 binding caused by the supernatants was not due to the presence of TGF-β1, the above binding assay was also done in the presence of the supernatant treated with an anti-TGF-β1 antibody. The supernatant was

incubated with the antibody (15 μg/ml) overnight at 4°C. It was then precleared of immune complexes and excess antibody that may interfere with the assay by incubating with a protein A Sepharose slurry (PharmaciaBiotech) for 2 h before addition to the assay. The TGF-β1 antiserum [obtained from Dr. M. O'Connor-McCourt; Moulin et al., 1997] was purified on a HiTrap Protein G column (PharmaciaBiotech) following standard procedures. The specificity of the antibody was verified by Western Blot analysis.

Determination of TGF-β Concentration in HaCaT Supernatant

The concentration of TGF-β1 was quantitated in the supernatants from HaCaT cells left untreated or treated with PIPLC using a mink lung epithelial cell-luciferase assay as described by Nunes et al. [1996]. This quantitative bioassay for TGF-β is based on the ability of TGF-β to induce the expression of plasminogen activator inhibitor type 1 (PAI-1) gene. The mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the luciferase reporter gene were provided by Dr. D.B. Rifkin (New York University Medical Center). Briefly, these cells were incubated with varying doses of HaCaT supernatant, and the luciferase activity (expressed as relative light units) was quantitated by a Berthold Luminometer. Recombinant TGF-β1 (Austral Biochemicals; 0.5 pM–50 pM) was used to create a standard curve.

Immunoaffinity Chromatography and Immunoblotting of r150

Neonatal keratinocytes were harvested by treating confluent monolayers with Hanks' balanced salt solution containing 5 mM EDTA (pH 7.5). The cell pellet was washed with DPBS and treated with 0.6 U/ml of PIPLC for 1 h at 37°C with mild agitation. The supernatant containing the released GPI-anchored proteins was purified through a TGF-β1 affinity column (made available by Dr. M. O'Connor-McCourt, Montreal, Quebec). The column was prepared by incubating 1 mg of TGF-β1 in a 200 mM HCO_3 (pH 8.3) buffer containing 30% (v/v) of n-propanol with 5 mg of Reacti-gel (Pierce) for 72 h at 4°C. The reaction was stopped by the addition of 200 μl of 2 M TBS (pH 7.4), and the gel was washed to remove any unbound TGF-β1. The supernatant containing the GPI-anchored medium was loaded on the column equilibrated with 50 mM Tris (pH 7.4) and eluted with a 10 mM citrate/300 mM NaCl buffer (pH 2.5). Fractions of 0.5 ml were collected, and each fraction was analyzed for binding to TGF-β1, and immunoblotted for the presence of GPI anchor.

To verify binding to TGF-β1, an aliquot from each fraction was affinity labeled with 150 pM of ^{125}I-TGF-β1 and analyzed by SDS-PAGE and autoradiography as described above, except that the solubilization step was omitted.

The fraction containing r150 and adjacent fractions were immunoblotted with the anti-CRD antibody to detect the GPI anchor. The anti-CRD antibody is specific to the inositol 1,2 cyclic monophosphate moiety, known as the 'cross-reacting determinant' (CRD) which is exposed in GPI-anchored proteins that have been hydrolyzed by PIPLC. The antibody obtained from Oxford GlycoSystems (Wakefield, MA), was raised against the inositol 1,2 cyclic monophosphate moiety of the trypanosome variant surface glycoprotein (VSG). Samples were analyzed on 3–11% polyacrylamide gradient SDS gels and transferred to nitrocellulose membrane. The membrane was blocked in TBS-T (30 mM Tris, 150 mM NaCl, pH 7.5, 0.5% Tween 20) containing 5% non-fat dry milk and was incubated overnight with 4 μg/ml of the anti-CRD antibody at 4°C. The blots were washed in TBS-T and incubated for 3 h at room temperature with the alkaline phosphatase conjugated secondary antibody (1:1500) (Roche Diagnostics). The membrane was then subjected to chemiluminescence analysis (CDP-Star) as detailed by the manufacturers (Roche Diagnostics).

Immunoprecipitation of r150

Two different anti-CRD antibodies (i) Oxford GlycoSystems antibody and (ii) an antibody raised against a mammalian GPI-anchored pig membrane dipeptidase (MDP) were used for immunoprecipitation studies. The latter antibody was isolated from the bulk of the anti-MDP antiserum by fractionation on a column of the immobilized form of the trypanasome VSG. Both anti-CRD antibodies are specific to the inositol 1,2-cyclic monophosphate and have been well characterized [Zamze et al., 1988; Broomfield and Hooper, 1993]. Cells were affinity labeled with 200 pM ^{125}I-TGF-β1 and cell extracts were incubated with the anti-CRD

TABLE I. Concentration of Unlabelled TGF-β1, β2, and β3 at Which Half-Maximal Inhibition of ^{125}I-TGF-β1 Binding to TGF-β Receptors on Keratinocytes was Observed

		Concentration at half maximal inhibition (pM)		
Radioactive Ligand	Competing Ligand	150 kDa	Type I	Type II
^{125}I-TGF-β1	TGF-β1	220	180	165
	TGF-β2	–	–	–
	TGF-β3	1950	300	360

antibody. The resulting immune complexes were treated with protein A Sepharose (Pharmacia-Biotech) slurry and the beads were pelleted by centrifugation, and were analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Binding Affinity of r150 for TGF-β Isoforms

We have previously reported that in addition to the types I, II, and III receptors, keratinocytes express a novel GPI-anchored TGF-β1 binding protein r150 which forms a heteromeric complex with the TGF-β signaling receptors [Tam et al., 1998]. Since this protein has the potential to regulate TGF-β signaling, we further characterized this protein. Here we determined the relative affinity of r150 for the three TGF-β isoforms and report that its affinity for TGF-β1 approximates that of the TGF-β signaling receptors, and that r150 is predominantly a TGF-β1 binding protein. Keratinocytes affinity labeled with ^{125}I-TGF-β1 in the absence or presence of increasing concentrations of unlabeled TGF-β1, -β2, or -β3, were analyzed by SDS-PAGE and autoradiography (Fig. 1A), and competition curves for r150, type I and type II receptors were created from the autoradiogram using quantitative densitometry (Fig. 1B, C, D). r150 is not sensitive to reducing agents since its migration pattern is identical when the SDS-PAGE analysis is done under non-reducing (Fig. 1A) or reducing conditions [Tam et al., 1998]. The half-maximal inhibition of ^{125}I-TGF-β1 binding was determined from the competition curves as the TGF-β isoform concentration at which the inhibition was 50% of that observed when no unlabeled ligand was present (Table I). The concentration of unlabeled TGF-β1 required for half maximal inhibition of ^{125}I-TGF-β1 binding by r150 is only 1.2 and 1.3 times higher than that required by type I and II receptors respectively. Although r150 also binds TGF-β3, it does so with a much lower affinity as compared to the types I and II receptors since it requires a six-fold higher concentration of TGF-β3 to reach half-maximal inhibition of ^{125}I-TGF-β1 binding than the types I or II receptors. Unlabeled TGF-β2, even at 40 times excess concentrations minimally inhibited ^{125}I-TGF-β1 binding of r150.

Partitioning of the Membrane Bound and Released r150 in Triton X-114

In order to ascertain that the membrane bound r150 is hydrophobic as expected of a GPI-anchored protein and that the released r150 behaves as a hydrophilic soluble protein, we used the temperature dependent phase separation property of the non-ionic detergent Triton X-114. Phase separation using Triton-X 114 results in the partitioning of hydrophilic proteins into the aqueous detergent poor phase while integral membrane proteins and lipid attached proteins partition into the detergent rich phase. This procedure has been useful in distinguishing between the amphipathic (membrane bound) and hydrophilic (released from cell surface] forms of GPI-anchored proteins [Hooper, 1992].

Affinity labeled keratinocytes were subjected to Triton X-114 partitioning and the detergent rich phase containing hydrophobic proteins and the detergent poor phase containing the hydrophilic proteins were analyzed by SDS-PAGE. As expected of a GPI-anchored protein, r150 partitioned predominantly into the detergent rich phase, along with the transmembrane type I, II, and III receptors (Fig. 2A). We then tested the partitioning of soluble r150 in Triton X-114. When the detergent rich phase containing the membrane bound affinity labeled r150 was left untreated or treated with PIPLC, and the temperature-induced phase separation was repeated, the aqueous phase of the sample treated with PIPLC was enriched in r150 while that of the sample left untreated contained only low amounts of r150 (Fig. 2B). These results

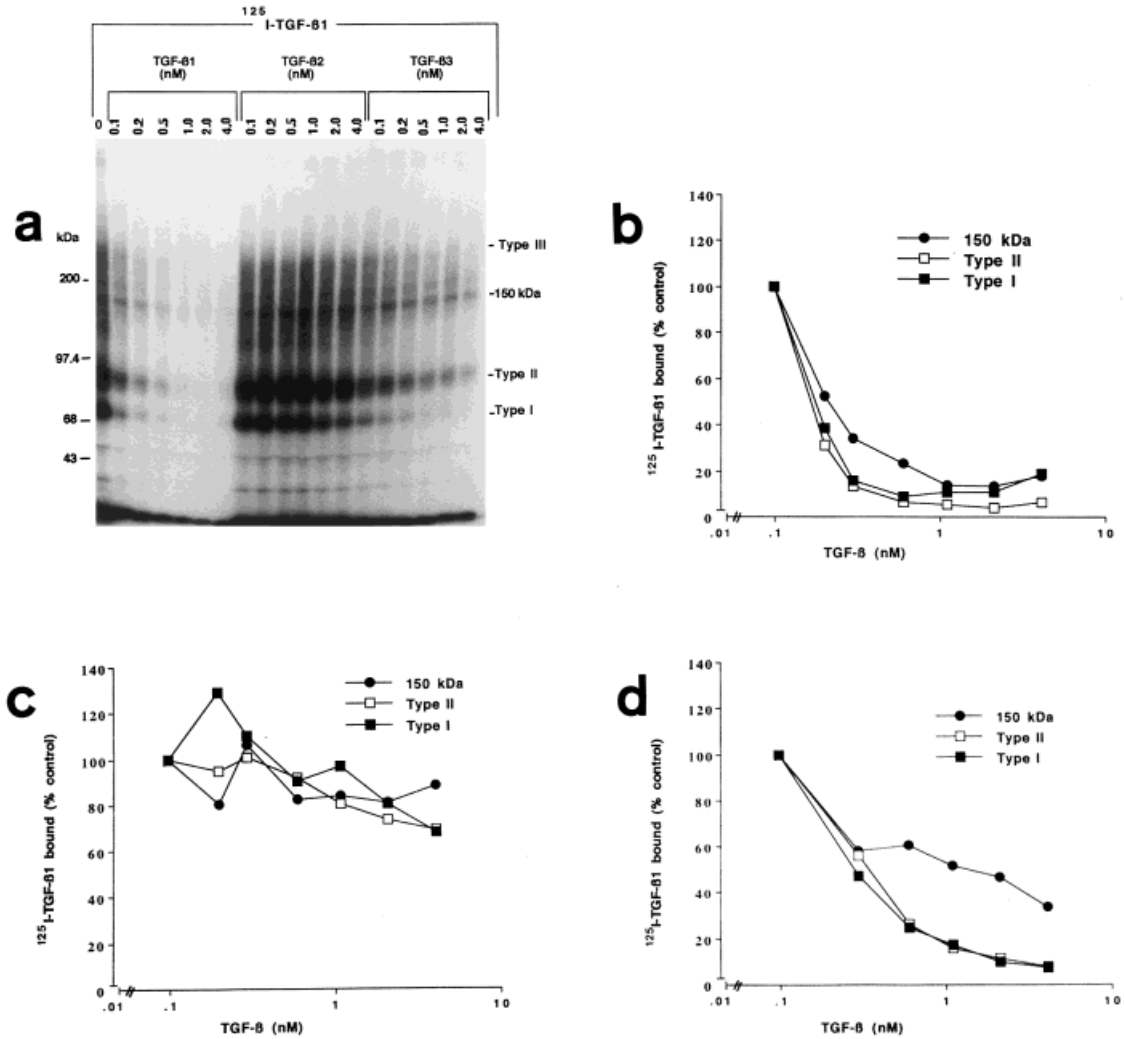


Fig. 1. Affinity cross-link labeling of human neonatal keratinocytes with ^{125}I-TGF-β1. Confluent monolayers were affinity labeled with 100 pM ^{125}I-TGF-β1 in the absence or presence of unlabeled TGF-β1, -β2, or -β3. Solubilized cell extracts were analyzed by SDS-PAGE under non reducing conditions and autoradiography **(a):** Competition curves for r150 and the types I and II TGF-β receptors were derived by densitometric analysis of a typical autoradiogram. The data for each binding complex are expressed as a percent of the value in control wells incubated with ^{125}I-TGF-β1 alone and are plotted against the concentration of unlabeled TGF-β1 **(b)**, -β2 **(c)**, or -β3 **(d)**. The autoradiogram and competition curves are representative of three different experiments.

strongly indicate that the PIPLC-released r150 is indeed hydrophilic. In contrast, the detergent phase of samples left untreated with PIPLC contained the major portion of r150 while the detergent phase of samples treated with PIPLC contained minimal amounts of r150 (data not shown).

Soluble r150 Binds TGF-β1

We next examined whether r150 released from the cell surface is capable of binding TGF-β1. Data shown in Figure 3A demonstrate that soluble r150 in the supernatant obtained from keratinocytes treated with PIPLC could be affinity labeled with ^{125}I-TGF-β1. This binding was specific since it was markedly reduced when the labeling was done in the presence of unlabeled TGF-β1, unlabeled TGF-β1 did not exhibit any competition for these complexes. The low molecular weight bands below 97.4 kDa appear to be nonspecific since unlabeled TGF-β1 did not exhibit competition for these complexes in a reproducible manner. The fact that released r150 binds TGF-β1 indicates that r150 is

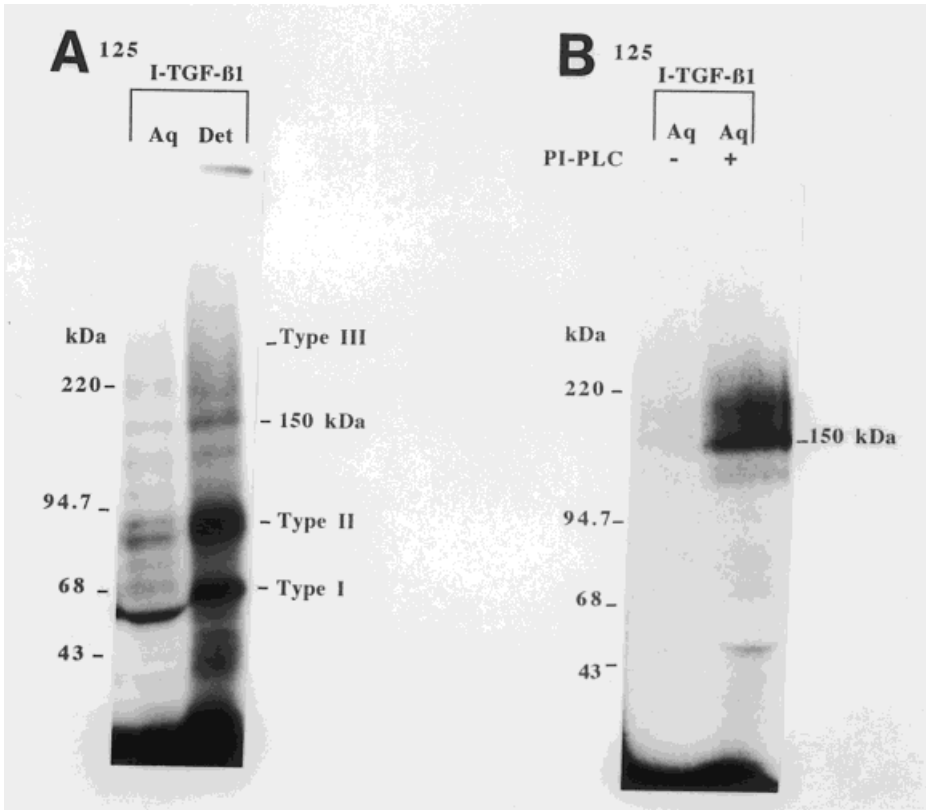
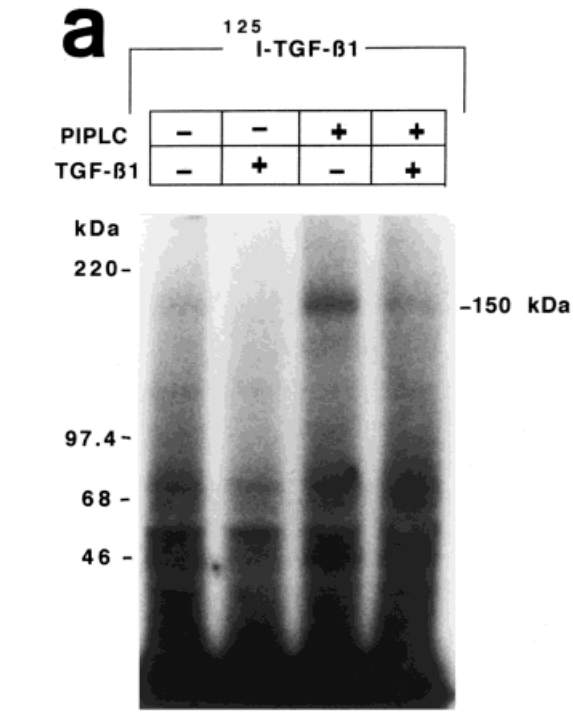


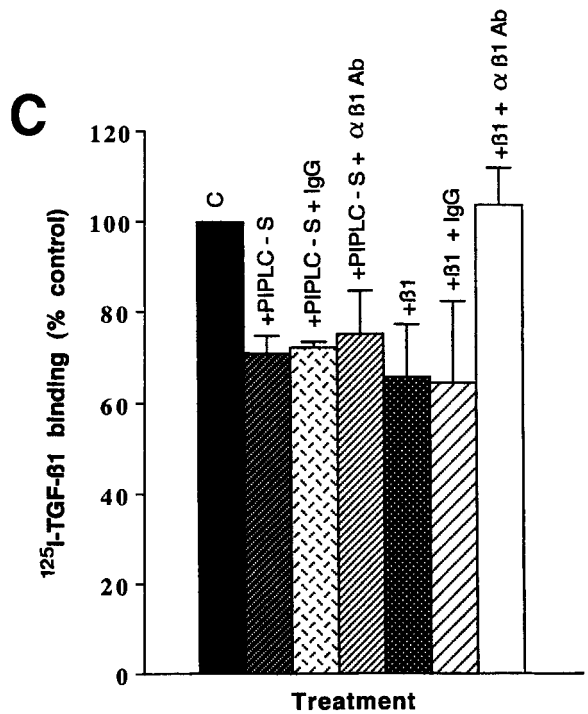
Fig. 2. Temperature induced phase separation in Triton X-114 of TGF-β binding proteins on human neonatal keratinocytes. Keratinocytes affinity labeled with ^{125}I-TGF-β1 were lysed in 1% Triton X-114. The Triton X-114 soluble material was incubated at 30°C for 10 min followed by centrifugation at room temperature to induce phase separation of the detergent rich phase and the aqueous detergent poor phase. **(a):** An aliquot (20%) from each phase was precipitated with ethanol/acetone, and analyzed by SDS-PAGE under reducing conditions. ^{125}I-TGF-β1 labeled proteins in the aqueous (Aq) and the detergent rich (Det) phases, representative of three different experiments, are shown. **(b):** The remaining 80% of the detergent phase was utilized to determine the effect of PIPLC treatment on the partitioning of r150 in Triton X-114. The detergent phase was incubated in the absence (−) or presence (+) of 0.6 U/ml of PIPLC followed by temperature induced phase separation and ethanol/acetone precipitation as above, to distinguish between the hydrophilic and amphipathic forms of the proteins. Analysis of the ^{125}I-TGF-β1 labeled proteins in the aqueous phases of PIPLC treated (+) and untreated (−) samples, by SDS-PAGE under reducing conditions are shown. The results shown are representative of two different experiments.

capable of binding the ligand in the absence of type I, II, and III TGF-β receptors or an intact membrane structure. Interestingly, detectable amounts of r150 were observed in the supernatant not treated with PIPLC, which led us to suspect that there might be an endogenous phospholipase capable of releasing r150.

That the soluble r150 can inhibit TGF-β1 binding to TGF-β receptors was demonstrated using a binding assay. As seen in Figure 3B, the supernatant from PIPLC treated keratinocytes competed in a dose dependent fashion for ^{125}I-TGF-β1 as seen by decreased binding to MvLu1 cells. The supernatant from a T-25cm^2 flask treated with PIPLC inhibited binding by 33% ($P < 0.005$) and 50% ($P < 0.04$) at doses of 1 and 2 respectively (approximately 1×10^6 cells, represented as an arbitrary unit of "1" in Fig. 3B). The inhibition of binding with the supernatant from cells not treated with PIPLC is consistent with the observation that detectable amounts of r150 is present in this supernatant, alluding to the presence of an endogenous phospholipase capable of releasing r150 (Fig. 3A; also see below, Fig. 5). This inhibition of ^{125}I-TGF-β1 binding corresponded to 15% and 31% ($P < 0.03$ in both cases), respectively for doses 1 and 2. In the supernatants obtained by PIPLC treatment, the inhibition of binding at doses 1 and 2, however, was significantly higher ($P < 0.03$ in both cases) than in the untreated supernatants.

To rule out the possibility that the competition observed by PIPLC treated supernatant was due to TGF-β, the supernatant was neutralized with anti-TGF-β1 antibody prior to





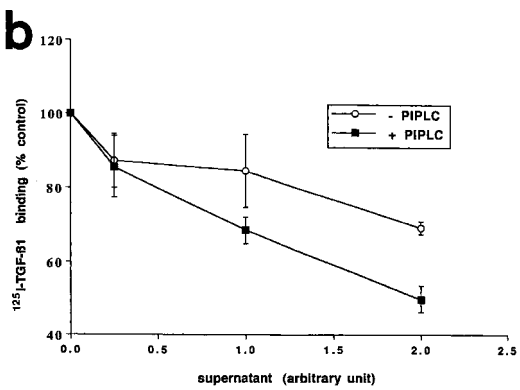


Fig. 3. **(a):** Affinity labeling of soluble r150 with ^{125}I-TGF-β1. To verify that the soluble form of the r150 can bind to TGF-β1, human keratinocytes (HaCaT) were left untreated (−) or treated with 0.6 U/ml of PIPLC (+). The GPI-anchored proteins released into the supernatant were concentrated and an aliquot was affinity cross-link labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β1 and subjected to SDS-PAGE under reducing conditions. The result shown is representative of four different experiments. **(b):** Inhibition of ^{125}I-TGF-β1 binding to TGFβ receptors by soluble r150. Confluent monolayers of HaCaT cells grown in T-25cm^2 culture flasks were left untreated or treated with 0.6 U/ml of PIPLC for 60 min at 37°C. The supernatants were collected and concentrated by Centricon. MvLu1 cells were affinity labeled with 50 pM ^{125}I-TGF-β1 in the absence or presence of indicated doses of supernatants and ^{125}I-TGF-β1 specifically bound was plotted as a function of the amount of supernatant used. The arbitrary unit of "1" is equivalent to a dose of supernatant from a T-25cm^2 flask (approximately 1×10^6 cells). **(c):** Effect of anti-TGF-β1 on the inhibition of TGF-β1 binding to TGF-β receptors. Confluent monolayers of MvLu1 cells were affinity labeled with 50 pM ^{125}I-TGF-β1 in the absence (C), or presence of PIPLC treated supernatant (+PIPLC - S), or PIPLC-S pretreated with non-immune rabbit IgG (15μg/ml), or PIPLC-S pretreated with anti-TGF-β1 antibody (15μg/ml). To demonstrate that the anti-TGF-β1 antibody effectively neutralizes TGF-β, experiments were also performed with 100 pM of TGF-β1 (+β1), β1 pretreated with non-immune rabbit IgG (15μg/ml), or β1 pretreated with anti-TGF-β1 antibody (15μg/ml). The values shown in (b) and (c) are the mean (± S.D.) of at least three to five different experiments.

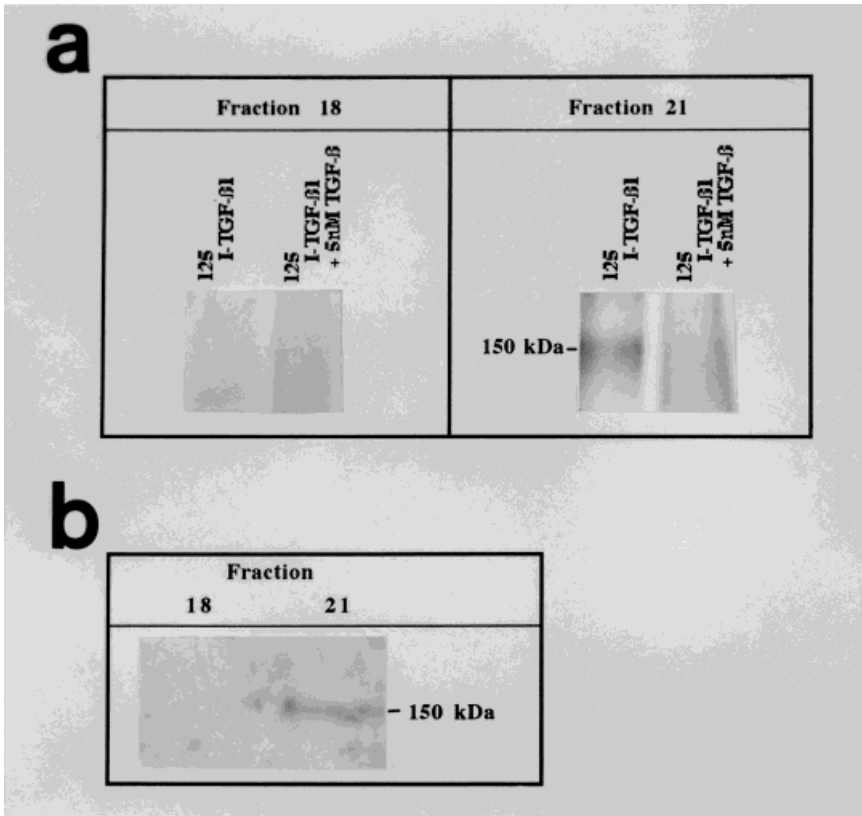


Fig. 4. Identification of a GPI-anchor in r150. Human neonatal keratinocytes were harvested and treated with PIPLC (as described in Materials and Methods). The supernatant containing the GPI-anchored proteins were purified using a TGF-β1 affinity column (see Materials and Methods for details). After the addition of the sample to the column, 0.5 ml fractions were collected during washing and elution. **(a)**: An aliquot from each fraction was affinity labeled with 150 pM of ^{125}I-TGF-β1 in the absence or presence of excess unlabeled TGF-β1 and samples were analyzed by SDS-PAGE under reducing conditions. Only fraction 21 demonstrated an affinity labeled protein at 150 kDa while in adjacent fractions no 150 kDa band was detectable. Affinity labeling pattern obtained for fraction 21 and 18 are shown. **(b)**: Selected fractions were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and the samples were immunoblotted with an anti-CRD antibody (Oxford GlycoSystems). A 150 kDa protein was detected in fraction 21 but not in adjacent fractions (fraction 18). Immunoblotting with the anti-CRD antibody was performed twice and the affinity cross-link labeling experiments of soluble r150 was done at least three times.

being used in the binding assay. Neutralization with this antibody had no effect on the inhibition by the r150 enriched supernatant (Fig. 3C). In contrast, 100 pM TGF-β1 markedly inhibited ^{125}I-TGF-β1 binding to the cells and this binding could be neutralized by anti-TGF-β1 antibody, but not by non-immune IgG. Furthermore, using a PAI-luciferase assay no TGF-β was detected in the supernatants of cells untreated or treated with PIPLC (data not shown). Taken together, these results suggest that the released form of r150 is capable of binding to TGF-β1 and modulating ligand binding to TGF-β receptors.

Identification of a GPI-Anchor in r150

Although r150 is sensitive to PIPLC, it is possible that it is not itself GPI-anchored, but is associated with a protein that is GPI anchored. Also, it is conceivable that it is a complex of two lower molecular weight proteins which became inadvertently cross-linked during the affinity labeling procedure. In order to eliminate these possibilities, we performed Western blot analysis of r150 after its release from the cell membrane using an anti-CRD antibody specific for an epitope which becomes exposed in GPI anchored proteins only upon treatment with PIPLC.

Keratinocytes were treated with PIPLC and the supernatant was purified on a TGF-β1 affinity column. Analysis of fractions by affinity labeling and SDS-PAGE demonstrated that the fraction 21, but not adjacent fractions (represented by fraction 18) contained a 150 kDa TGF-β1 binding protein (Fig. 4A). The binding of ^{125}I-

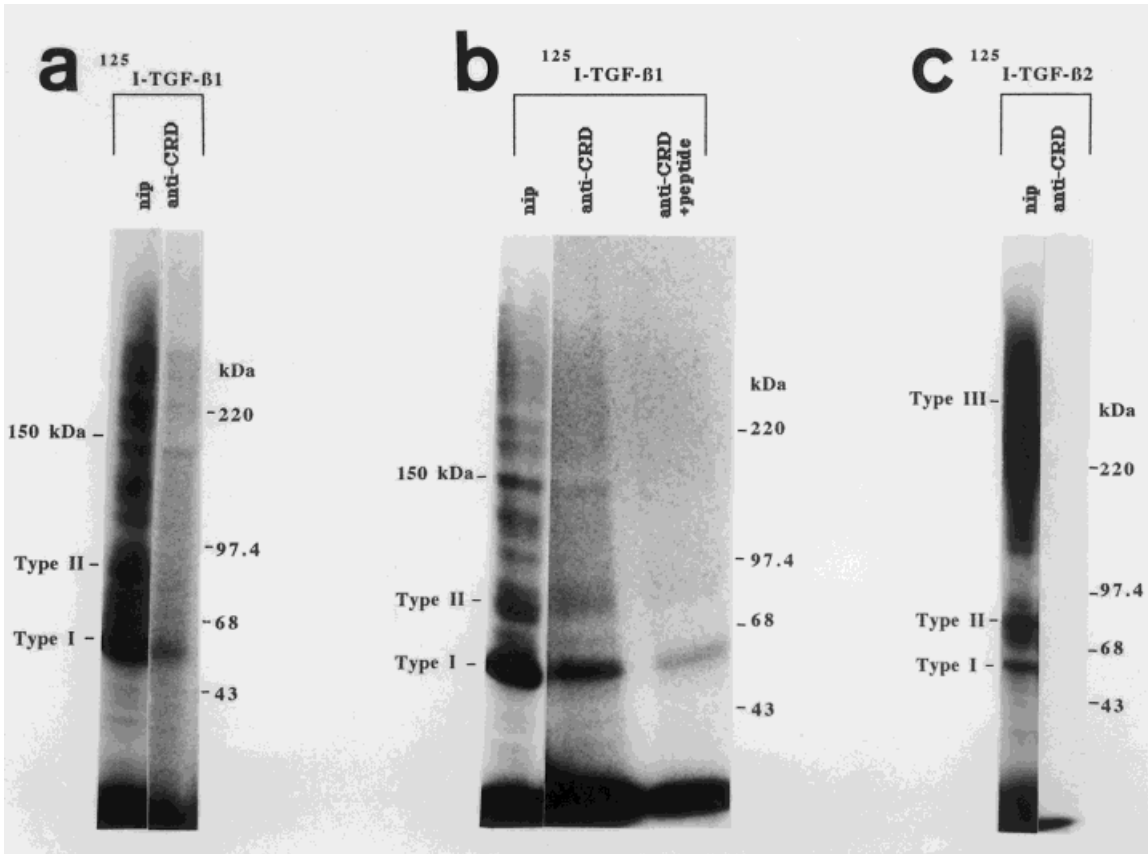


Fig. 5. Immunoprecipitation of affinity labeled TGF-β binding complexes on human neonatal keratinocytes with the anti-CRD antibodies. Keratinocytes not treated with PIPLC were affinity labeled with 100 pM ^{125}I-TGF-β1 **(a & b)** or ^{125}I-TGF-β2 **(c):** and were not immunoprecipitated (nip) or subjected to immunoprecipitation with an anti-CRD antibody against trypanosomal sVSG (Oxford GlycoSystems) (a & c), or with an anti-CRD antibody against porcine membrane dipeptidase [Broomfield and Hooper, 1993] (c). In the lane marked ''+peptide'' the immunoprecipitation was carried out using the anti-CRD antibody which was preincubated with PIPLC treated membrane dipeptidase. Immune complexes were subjected to SDS-PAGE under reducing conditions and analyzed by autoradiography. The results shown are representative of at least four to five experiments.

TGF-β1 to this protein is specific since it was blocked in the presence of 5 nM TGF-β1. These results confirm that soluble r150 binds TGF-β1.

When the fractions were analyzed by Western blotting with the anti-CRD antibody, it was revealed that fraction 21, but not other fractions contained a protein of relative molecular weight of 150 kDa which was recognized by the anti-CRD antibody. Detection of a 150 kDa protein by the anti-CRD antibody in the absence of chemical cross-linking demonstrates that r150, but not an associated protein, contains a GPI-anchor and, that r150 does not represent two smaller proteins which got inadvertently cross-linked (Fig. 4B). Figure 4B also shows that r150 was not detectable in an adjacent fraction (fraction 18).

Evidence to Indicate That an Endogenous Phospholipase C Releases r150 in Human Keratinocytes

Next, we tested whether the anti-CRD antibody can immunoprecipitate r150 and/or coprecipitate the types I and II TGF-β receptors. During the course of these studies, we observed that the anti-CRD antibody immunoprecipitated r150 from ^{125}I-TGF-β1 labeled keratinocytes, even in the absence of PIPLC treatment (Fig. 5). This result was reproducible using two different anti-CRD antibodies: the Oxford GlycoSystems antibody that is raised against the CRD epitope of variant surface glycoprotein of *Trypanosoma brucei* (Oxford GlycoSystems), and the antibody specific for the CRD epitope

of the porcine membrane dipeptidase [MDP, Broomfield and Hooper, 1993]. Since both anti-CRD antibodies are specific for the inositol 1,2-cyclic monophosphate epitope exposed only upon PIPLC treatment, recognition by the two antibodies in the absence of PIPLC treatment indicates that an endogenous phospholipase C cleaved r150 to expose this epitope. The anti-CRD antibody not only precipitated the r150, but also coprecipitated the types I and II receptors. Interestingly, the intensities of the types I and II bands were much stronger than that of the r150 itself. The coimmunoprecipitation of the types I and II receptors demonstrates the heteromeric complex formation of r150 with those receptors (Fig. 5a,b) which confirms our previous finding [Tam et al., 1998]. The immunoprecipitation with the anti-CRD antibody is specific because the precipitation of labeled complexes is efficiently blocked when the PIPLC hydrolyzed form of MDP which contains the epitope to which the antibody was raised against was included in the reaction (Fig. 5b). These complexes are not detected when the cells were affinity labeled with ^{125}I-TGF-β2 because r150 has a much lower affinity for TGF-β2 than for the TGF-β1 and TGF-β3 isoforms in these cells (Fig. 5c).

DISCUSSION

In a previous report we have shown that a novel 150 kDa TGF-β1 accessory receptor (r150) forms a heteromeric complex with the TGF-β signaling receptors on human keratinocytes [Tam et al., 1998]. This accessory receptor was described as GPI-anchored based on its sensitivity to PIPLC. Here we demonstrate that the GPI-anchor is contained in r150 itself and not on an associated protein and that it binds TGF-β1 with an affinity similar to those of the types I and II TGF-β receptors. In addition, we provide evidence that r150 is released from the cell surface by an endogenous phospholipase C. The most important finding in the present study is that the released (soluble) form of r150 binds TGF-β1 independent of the signaling receptors.

We characterized r150 as GPI-anchored, based on its sensitivity to phosphatidylinositol specific phospholipase C (PIPLC). In order to prove that the GPI-anchor is present in the r150 itself, it was necessary to rule out other possibilities, namely: (i) r150 is not itself GPI-anchored, but is tightly associated with a protein that is GPI-anchored. Upon PIPLC treatment, the associated GPI-anchored protein is cleaved which results in the release of both the proteins into the supernatant. This has been shown to be the case for lipoprotein lipase which was initially identified as GPI-anchored protein based on its sensitivity to PIPLC; but it was later found that its PIPLC sensitivity was a result of close association with a GPI-linked heparan sulfate proteoglycan [Chajek-Shaul et al., 1989]. (ii) r150 is a noncovalently associated complex of two lower molecular weight proteins whose combined molecular weights equate 150 kDa, of which one component is GPI-anchored. During affinity labeling, the two proteins get inadvertently crosslinked by the chemical crosslinker BS^3, and thus upon analysis by SDS-PAGE, the cross-linked complex is detected at 150 kDa.

By immunoblotting the purified, soluble form of the r150 with the anti-CRD antibody that can specifically recognize the epitope exposed by the cleavage of the GPI-anchor by PIPLC, the above two possibilities were eliminated. Elution from a TGF-β1 affinity column and detection as a 150 kDa protein in the absence of cross-linking, together with its ability to be recognized by the anti-CRD antibody, prove that r150 has a relative molecular weight of 150 kDa and that the GPI-anchor is contained in r150 itself. There are two GPI-anchored proteins expressed in mammalian tissues that have similar molecular weights as r150. These include an isoform of NCAM (140 kDa) [Rosen et al., 1992] and ceruloplasmin (135 kDa) [Patel and David, 1997]. However, immunoprecipitation with antibodies specific to these proteins did not immunoprecipitate r150 affinity labeled with ^{125}I-TGF-β1 (data not shown).

The soluble r150 is capable of binding to TGF-β1 as shown by affinity labeling of the released protein, and retention on the TGF-β1 affinity column. This suggests that r150 can bind TGF-β in the absence of types I, II, and III receptors or an intact membrane. That soluble r150 has the potential to modulate TGF-β binding to its receptors was demonstrated by its ability to inhibit ^{125}I-TGF-β1 binding to receptors on mink lung cells. Although studies of the inhibition of TGF-β binding to its receptors by r150 used cellular supernatant and not purified r150, this inhibition is most likely due to r150 itself, since there was no measurable TGF-β and neutralizing anti-TGF-β antibody had no effect on this inhibition. The inhibition obtained by

the supernatant not treated by PIPLC is likely due to endogenous release of r150 (Fig. 5). As expected, exogenous addition of PIPLC resulted in significantly higher inhibition of binding since more r150 will be released. In addition, r150 is the major TGF-β binder released by PIPLC (Fig. 2). The other potential binder, α2-macroglobulin is unlikely to be released in sufficient quantity during the 1 h incubation.

That soluble r150 binds TGF-β1 is reminiscent of what is observed of the ectodomain of type III receptor which has been shown to be released by a not yet characterized mechanism [Lopez-Casillas et al., 1994; Philip et al., 1999]. The soluble r150 may act as an antagonist by preventing the binding of TGF-β to the signaling receptors as has been suggested for the soluble type III receptor [Lopez-Casillas et al., 1994]. This is supported by our finding which suggests that the soluble r150 inhibits ^{125}I-TGF-β1 binding to receptors on mink lung cells. But unlike the type III receptor, r150 would antagonize TGF-β1 activity in an isoform specific manner, since it has a low affinity for TGF-β2 and a moderate affinity for TGF-β3, as determined by competition experiments using unlabeled TGF-β isoforms. Furthermore, affinity cross-link labeling of keratinocytes with ^{125}I-TGF-β2 or ^{125}I-TGF-β3 did not demonstrate labeling of r150 (data not shown). Recently, a soluble type I receptor has been cloned from a rat kidney cDNA library [Choi, 1999]. In contrast to the soluble type III receptor, the soluble type I receptor requires the co-expression of the type II receptor in order to bind TGF-β. However, it appeared to potentiate TGF-β signal transduction and the author has suggested that this potentiation may be due to the stabilization of the heteromeric TGF-β signaling receptor complex. The observation that the soluble r150 can bind the ligand independently of signaling receptors, and modulate TGF-β binding to its receptors (present study), together with fact that the membrane bound r150 binds TGF-β1 and forms heteromeric complex with the type I and II receptors [Tam et al., 1998] raise the possibility that r150 in its membrane bound or soluble form may act as an agonist or an antagonist of TGF-β signaling by regulating ligand availability, or stability of the signaling receptor complex or by directly affecting the signal transduction process.

We predict that r150 has dual roles in TGF-β signaling depending on whether it exists as a cell surface anchored protein or as the soluble form. Consequently, a potential mechanism for the regulation of its action would be the hydrolysis of the GPI-anchor. Both the release of the protein from the cell surface and the ability of the soluble form to sequester TGF-β may modulate TGF-β receptor function. Such a possibility in vivo is supported by our observation that an endogenous phospholipase C releases r150 from the cell surface. Since the anti-CRD antibody only recognizes GPI-proteins released from the cell surface by PIPLC, and the cells were not pretreated with PIPLC, the results indicate that there is an endogenous phospholipase C in keratinocytes capable of hydrolyzing r150 at the same site as the PIPLC. The soluble r150 identified in the cellular extract is not due to protease activity since proteolytic cleavage will result in a protein of lower molecular weight. Neither can it be due to failure of cells to add the GPI anchor during synthesis, since the antibody will not recognize that protein. It is of interest to note here that r150 was detectable upon overexposure of films in the PIPLC untreated aqueous fractions obtained by Triton X-114 partitioning (data not shown).

Although the presence of a mammalian PIPLC has not been definitively established, the activity of PIPLC-like enzymes have been implicated in the insulin signaling pathway in rat liver [Saltiel, 1996]. Furthermore, Movahedi and Hooper [1997] have demonstrated that the insulin stimulated release of GPI-anchored proteins from differentiated 3T3-L1 adipocytes occurs via the action of an endogenous phospholipase C. We choose the general term of phospholipase C for the enzyme that releases the r150 in keratinocytes, since the identity and specificity of the enzyme is not confirmed. Nevertheless, our results suggest that the release of r150 involves an enzyme that hydrolyzes r150 at the same site as PIPLC. PIPLD hydrolyze the GPI-anchor at a different site from PIPLC which does not result in the formation of the inositol 1,2-cyclic monophosphate, and therefore the anti-CRD antibodies that we used in this study cannot recognize PIPLD cleaved proteins [Broomfield and Hooper, 1993]. However, PIPLD is expressed in mammalian serum and has been shown to be present in keratinocytes [Xie et al., 1993]. It is possible that the activity of both enzymes may be involved in regulating the cell

surface expression of r150 on human keratinocytes.

GPI-anchored proteins have been reported to bind TGF-β on certain cell lines [Cheifetz and Massague, 1991]. More recently, we reported the presence of GPI-anchored TGF-β binding proteins on early passage human endometrial stromal cells [Dumont et al., 1995] and human skin fibroblasts [Tam and Philip, 1998]. Both endometrial stromal cells and skin fibroblasts displayed a 180 kDa GPI-anchored TGF-β1 binding protein and a 65 kDa TGF-β2 binding protein. However, whether the GPI anchor is present in the proteins themselves has not been ascertained for any of them, and the identities of these proteins remain unknown. Interestingly, GPI-anchored proteins have been implicated in the maintenance of the epidermis. When the expression of GPI-anchored proteins was abrogated in the skin by the tissue specific deletion of the *PIG-A* gene, a gene essential for GPI-protein synthesis, mutants died shortly after birth and their skin was wrinkled and scaly in comparison to that of the wild type [Tarutani et al., 1997]. Since TGF-β has an important role in epidermal homeostasis, it is conceivable that the GPI-function which is compromised in these mutants is related to dysregulated TGF-β action due to the loss of r150.

Our results from the competition experiments demonstrating that r150 has high affinity for the TGF-β1 isoform suggest that it is an endogenous ligand for this protein. Whether the membrane bound or soluble form of r150 acts as scavenger receptors regulating ligand availability, whether they participate directly in the modulation of downstream signaling, or if the release of the soluble form is a regulated event in vivo, remain to be determined. Identification of its structure will facilitate resolution of these issues. Elucidating the mechanism by which r150 functions as an accessory molecule in TGF-β signaling in keratinocytes may be critical to understanding the molecular mechanisms underlying the regulation of TGF-β action.

ACKNOWLEDGMENTS

The authors thank Dr. Maureen O'Connor-McCourt for the use of the TGF-β1 affinity column. We gratefully acknowledge Alan Forster for photography. We are grateful to Dr. H. Brown and the Division of Plastic Surgery for administrative support.

REFERENCES

Bordier C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256:1604–1607.

Broomfield SJ, Hooper NM. 1993. Characterization of an antibody to the cross-reacting determinant of the GPI-anchor of human membrane dipeptidase. Biochim Biophys Acta 1145:212–218.

Brown D. 1993. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. Curr Opin Immunol 5:349–354.

Brown DA, London E. 1998. Functions of lipid rafts in biological membranes. Ann Rev Cell Dev Biol 14:111–136.

Chajek-Shaul T, Halimi O, Ben-Naim M, Stein O, Stein Y. 1989. Phosphatidylinositol-specific phospholipase releases lipoprotein lipase from the heparin releasable pool in rat heart cell cultures. Biochim Biophys Acta 1014:178–183.

Cheifetz S, Massague J. 1991. Isoform-specific TGF-β binding proteins with membrane attachments sensitive to PI-PLC. J Biol Chem 266:20767–20772.

Choi ME. 1999. Cloning and characterization of a naturally occurring soluble form of TGF-β type I receptor. Am J Physiol 276:F88–F95.

Clark RAF. 1996. Wound Repair: overview and general considerations. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press. pp. 3–35.

Dumont N, O'Connor-McCourt MD, Philip A. 1995. Transforming growth factor-β on human endometrial cells: identification of the type I, II and III receptors, and glycosyl-phosphatidyl inositol anchored TGF-β binding proteins. Mol Cell Endocrinol 111:57–66.

Germain L, Rouabhia M, Guignard R, Carrier L, Bouvard V, Auger FA. 1993. Improvement of human keratinocyte isolation and culture using thermolysin. Burns 19:99–104.

Glick AB, Kulkarni A, Tannenbaum T, Hennings H, Flanders KC, O' Reilly M, Sporn MB, Karlsson S, Yuspa SH. 1993. Loss of expression of TGF-β in skin and skin tumors is associated with hyper proliferation and high risk of malignant conversion. Proc Natl Acad Sci USA 90:6076–6080.

Glick AB, Lee MM, Darwiche N, Kulkarni AB, Karlsson S, Yuspa SH. 1994. Targeted deletion of TGF-β1 gene causes rapid progression to squamous cell carcinoma. Genes and Dev 8:2429–2440.

Gougos A, Letarte M. 1990. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem 265:8361–8364.

Hebda PA. 1988. Stimulatory effects of TGF-β and EGF on epidermal cell outgrowth and porcine skin explant cultures. J Invest Dermatol 91:440–445.

Heldin CH, Miyazono K, ten Dijke P. 1997. TGF-β signaling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471.

Hooper NM. 1992. Identification of glycosyl-phosphatidylinositol anchor on membrane proteins. In: Hooper NM, Turner AJ, editor. Lipid modification of proteins: a practical approach. New York: IRL Press. pp. 89–115.

Hooper NM. 1999. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae. Mol Memb Biol 16:145–156.

Letamendia A, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C. 1998. Role of endoglin in cellular responses to TGF-β. J Biol Chem 273:33011–33019.

Chengang Li, Hampson IN, Hampson L, Kumar P, Bernabeu C, Kumar S. 2000. CD105 antagonizes the inhibitory signaling of transforming gowth factor-β1 on human vascular endothelial cells. FASEB J 14:55–64.

Lisanti MP, Sargiacomo M, Graeve L, Saltiel AR, Rodriguez-Boulan E. 1988. Polarized apical distribution of glycosyl phosphatidylinositol-anchored proteins in renal epithelial cell lines. Proc Natl Acad Sci USA 85:9557–9561.

Letterio JJ, Roberts AB. 1998. Regulation of immune responses by TGF-β. Ann Rev Immun 16:137–161.

Lopez-Casillas F, Wrana JL, Massague J. 1993. Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73:1435–1444.

Lopez-Casillas F, Payne HM, Andres JL, Massague J. 1994. Betaglycan can act as a dual modulator of TGF-β access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol 124:557–568.

Massague J. 1998. TGF-β Signal Transduction. Ann Rev Biochem 67:753–791.

Metz CN, Brunner G, Choi-Muira NH, Nguyen H, Gabrilove J, Caras IW, Altszuler N, Rifkin DB, Wilson EL, Davitz MA. 1994. Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. EMBO J 13:1741–1751.

Moulin V, Auger FA, O'Connor-McCourt M, Germain L. 1997. Fetal and postnatal sera differentially modulate human dermal fibroblast phenotypic and functional features in vitro. J Cell Physiol 171:1–10.

Movahedi S, Hooper NM. 1997. Insulin stimulates the release of the GPI-anchored membrane dipeptidase from 3T3-L1 adipocytes through the action of a phospholipase C. Biochem J 326:531–537.

Nunes I, Kojima S, Rifkin DB. 1996. Effects of endogenously activated TGF-β on growth and differentiation of retinoic acid treated HL-60 cells. Cancer Res 56:495–499.

O'Kane S, Ferguson MWJ. 1997. Transforming growth factor-βs and wound healing. Int J Biochem Cell 29:63–78.

Onichtchouk D, Chen Y-G, Dosch R, Gawantka V, Dellus H, Massague J, Niehrs C. 1999. Silencing of TGF-β signaling by the psuedoreceptor BAMBI. Nature 401:480–484.

Patel BN, David S. 1997. A Novel Glycosyl phosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. J Biol Chem 272:20185–20190.

Peltonen J, Hsaio LL, Jaakkola S, Solberg S, Aumailley M, Timpl R, Chu ML, Uitto J. 1991. Activation of collagen gene expression in keloids: co-localization of type I and type IV collagen and TGF-β1 mRNA. J Invest Dermatol 97:240–248.

Philip A, O' Connor-McCourt MD. 1991. Interaction of TGF-β with alpha 3-macroglobulin: role in transforming growth factor-β clearance. J Biol Chem 266:22290–22296.

Philip A, Hannah R, O'Connor-McCourt M. 1999. Ectodomain cleavage and shedding of the type III transforming growth factor-β receptor in lung membranes. Eur J Biochem 261:618–628.

Pietenpol JA, Holt JT, Stein RW, Moses HL. 1990. TGF-β1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci USA87:3758–3762.

Roberts AB, Sporn MB. 1990. The transforming growth factor-βs. In: Sporn MB, Roberts AB, editors. Peptide growth factors and their receptors I. New York: Springer-Verlag. pp. 419–472.

Rodriguez-Boulan E, Powell SK. 1992. Polarity of epithelial and neuronal cells. Ann Rev Cell Biol 8:395–427.

Rosen CL, Lisanti MP, Salzer JL. 1992. Expression of unique sets of GPI-linked proteins by different primary neurons in vitro. J Cell Biol 117:17–627.

Saltiel AR. 1996. Diverse signaling pathways in the cellular action of insulin. Am J Physiol 270:E375–E382.

Tam BYY, Philip A. 1998. Transforming growth factor-β receptor expression on human skin fibroblasts: dimeric complex formation of type I and type II receptors and identification of glycosyl phosphatidylinositol-anchored TGF-β binding proteins. J Cell Physiol 176:553–564.

Tam BYY, Germain L, Philip A. 1998. TGF-β receptor expression on human keratinocytes: a 150 kDa GPI-anchored TGF- β binding protein forms a heteromeric complex with the types I and II receptors. J Cell Biochem 70:573–586.

Tarutani M, Itami S, Okabe M, Ikawa M, Tezuka T, Yoshikawa K, Kinoshita T, Takeda J. 1997. Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. Proc Natl Acad Sci USA94:7400–7405.

Wang XJ, Greenlaugh DA, Bikenbach JR, Jiang A, Bundman DS, Kreig T, Derynck R, Roop DR. 1997. Expression of a dominant-negative type II TGF-β receptor in the epidermis of transgenic mice blocks TGF-β-mediated growth inhibition. Proc Natl Acad Sci USA94:2386–2391.

Wrana JL, Attisano L, Wieser R, Venturs F, Massague J. 1994. Mechanism of activation of the TGF-β receptor. Nature 370:341–347.

Xie M, Sesko AM, Low M. 1993. Glycosyl phosphatidylinositol-specific phospholipase D. Am J Physiol 265:C1156–C1166.

Yamamoto T, Noble NA, Miller DE, Border WA. 1994. Sustained expressed of TGF-β1 underlies development of progressive kidney fibrosis. Kidney Int 45:916–927.

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Mechiori A, Giannett A, DeLuca M. 1995. TGF-β1 modulates β1 and 5 integrin receptors and induces the de novo expression of the alphaV6 heterodimer in normal human keratinocytes for wound healing. J Cell Biol 129:853–865.

Zamze SE, Ferguson MAJ, Collins R, Dwek RA, Rademacher TW. 1988. Characterization of the cross-reacting determinant (CRD) of the glycosylphosphatidyl membrane anchor of Trypanosoma brucei variant surface glycoprotein. Eur J Biochem 176:527–534.