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TGF-β Receptor Expression on Human Keratinocytes: A 150 kDa GPI-Anchored TGF-β1 Binding Protein Forms a Heteromeric Complex With Type I and Type II Receptors

Betty Y.Y. Tam,[1] Lucie Germain,[2] and Anie Philip[1]*

[1]Division of Plastic Surgery, Montreal General Hospital and Department of Surgery, McGill University, Montreal, Quebec, Canada
[2]Loex Laboratory, Hopital du Saint Sacrement and Universite Laval, Sainte-Foy, Quebec, Canada

Abstract Keratinocytes play a critical role in re-epithelialization during wound healing, and alterations in keratinocyte proliferation and function are associated with the development of various skin diseases. Although it is well documented that TGF-β has profound effects on keratinocyte growth and function, there is a paucity of information on the types, isoform specificity and complex formation of TGF-β receptors on keratinocytes. Here, we report that in addition to the types I, II, and III TGF-β receptors, early passage adult and neonatal human keratinocytes display a cell surface glycosylphosphatidylinositol (GPI)-anchored 150 kDa TGF-β1 binding protein. The identities of the four proteins were confirmed on the basis of their affinity for TGF-β isoforms, immunoprecipitation with specific anti-receptor antibodies, sensitivity to phosphatidylinositol specific phospholipase C and dithiothreitol, and 2-dimensional electrophoresis. Interestingly, the antitype I TGF-β receptor antibody immunoprecipitated not only the type I receptor, but also the type II receptor and the 150 kDa component, suggesting that the 150 kDa component form heteromeric complexes with the signalling receptors. In addition, two-dimensional (nonreducing/reducing) electrophoresis confirmed the occurrence of a heterotrimeric complex consisting of the 150 kDa TGF-β1 binding protein, the type II receptor, and the type I receptor. This technique also demonstrated the occurrence of types I and II heterodimers and type I homodimers of TGF-β receptors on keratinocytes, supporting the heterotetrameric model of TGF-β signalling proposed using mutant cells and cells transfected to overexpress these receptors. The keratinocytes responded to TGF-β by markedly downregulating all four TGF-β binding proteins and by potently inhibiting DNA synthesis. The demonstration that the 150 kDa GPI-anchored TGF-β1 binding protein forms a heteromeric complex with the TGF-β signalling receptors suggests that this GPI-anchored protein may modify TGF-β signalling in human keratinocytes. J. Cell. Biochem. 70:573–586, 1998.

Key words: skin; signaling; wound healing; skin diseases; receptor regulation; cell proliferation

The transforming growth factor-β (TGF-β) is a family of multifunctional proteins that have widespread effects on many aspects of growth and development [Roberts and Sporn, 1990]. Three distinct isoforms, TGF-β1, -β2, and -β3, have been found in mammals. The TGF-β isoforms have profound effects on the skin. They have been shown to play critical roles in the process of wound healing [O'Kane and Ferguson, 1997] and skin development [Pelton et al., 1991; Sellheyer et al., 1993] and have been implicated in the development of pathological conditions such as psoriasis [Cai et al., 1996; Wataya-Kaneda et al., 1996] and skin cancer [Glick et al., 1994]. A large number of studies have demonstrated the efficacy of exogenously administered TGF-β isoforms in enhancing wound healing in animal models [Quaglino et al., 1987; Ksander et al., 1993; Wu and Mustoe, 1995]. In addition, manipulation of the ratios of TGF-β isoforms has been shown to diminish scarring [Shah et al., 1995; O'Kane and Ferguson, 1997]. During wound healing, the repair process starts with re-epithelialization, which involves the recruitment of epidermal stem cells

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*Correspondence to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-177, Montreal, Quebec, Canada H3G 1A4. E-mail: mcap@musica.mcgill.ca

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and formation of an epithelial tongue of migrating keratinocytes at the very edge of the wound, phenotypic alteration of keratinocytes, and keratinocyte proliferation [Clark, 1996]. TGF-β has been demonstrated to promote the migration of epidermal cells from organ cultures [Hebda, 1988], to be chemotactic to keratinocytes, and to induce fibronectin and vitronectin receptors on migrating epithelium [Zambruno et al., 1995]. Despite its promigratory effect on keratinoytes, it is a potent inhibitor of keratinocyte proliferation *in vitro* [Pientenpol et al., 1990] and *in vivo* [Glick et al., 1993]. The loss of expression of TGF-β in epidermis and epidermal tumors is associated with hyperproliferation and a high risk for malignant conversion [Glick et al., 1993]. In addition, targeted deletion of the TGF-β1 gene in keratinocytes causes rapid progression to squamous cell carcinoma [Glick et al., 1994].

The actions of TGF-β are mediated by specific cell surface receptors that are present in almost all cell types analyzed [Wakefield et al., 1987]. Most cells have three types of TGF-β receptors designated as type I, type II, and type III [Massague, 1990; Roberts and Sporn, 1990]. The types I [Franzen et al., 1993] and II receptors [Lin et al., 1992] are transmembrane serine/threonine kinases, which have been shown to be directly involved in signal transduction by forming a signaling complex [Wrana et al., 1992]. The type III receptor is a membrane proteoglycan that is believed to regulate TGF-β binding to the signaling receptors [Lopez-Casillas et al., 1991; Wang et al, 1992]. The type III receptor has been reported to form a noncovalent heteromeric complex with the type II receptor and has been proposed to "present" TGF-β to the type II receptor [Lopez-Casillas et al., 1993]. In addition to these three cloned TGF-β receptors, several other cell surface proteins have been shown to bind to TGF-β in different cell types. These include endoglin [Cheifetz et al., 1992], which shows high homology to the type III receptor, and glycosyl phosphatidylinositol (GPI) anchored proteins that others [Cheifetz and Massague, 1991] and we [Dumont et al., 1995; Tam and Philip, 1998] have identified. The functional significance of GPI-anchored proteins remains to be elucidated. It is not known whether the GPI-anchored proteins modulate TGF-β binding to the signalling receptors or whether they are involved in TGF-β signaling in some other manner. The formation of a heteromeric complex between the type I and type II TGF-β receptors has been shown to be important for TGF-β signal transduction. However, it is uncertain as to how many molecules of type I and type II receptors are involved in the heteromeric complex. The current heteromeric model of TGF-β receptors is derived using cell mutants resistant to TGF-β growth response or using cell lines transfected to overexpress the native or chimeric receptors [Franzen et al., 1993; Bassing et al., 1994; Luo and Lodish, 1996]. Since nonphysiological receptor concentrations or nonnative receptors were used in these studies, the question as to whether homo- or heterodimers of native TGF-β receptors occur on the surface of normal TGF-β responsive cells with physiological levels and ratios of receptors, remains to be answered. Furthermore, it is not known whether the GPI-anchored TGF-β binding proteins form heteromeric complexes with the types I, II, or III TGF-β receptors.

Although the critical role of keratinocytes in cutaneous tissue repair and in the development of various skin diseases is well established, and the profound effects of TGF-β in modulating keratinocyte function are well documented, there is limited information on the types, isoform specificity, and complex formation of TGF-β receptors in this cell type. It is not known whether these cells express novel TGF-β receptors other than the commonly expressed types I, II, and III receptors. Defining the TGF-β receptor profiles and the formation of oligomeric receptor complexes in skin cells is important for understanding the mechanisms underlying the alterations in TGF-β function in various skin disease states. In the present study, we report that normal human adult and neonatal keratinocytes display on their cell surface a 150 kDa GPI-anchored TGF-β1 binding protein in addition to the types I, II, and III TGF-β receptors. In addition, we demonstrate the occurrence of heterotrimeric complexes consisting of the 150 kDa GPI-anchored component and the types I and II TGF-β receptors, and heterodimeric complexes of types I and II TGF-β receptors on this normal TGF-β responsive cell type. Our results indicate that the 150 kDa GPI-anchored TGF-β1 binding component interact with the TGF-β signaling receptors and may modulate TGF-β signaling on human keratinocytes.

MATERIALS AND METHODS

Cell Culture

Early passage human adult keratinocytes were cultured from skin tissue obtained at breast reduction surgery. Neonatal keratinocytes were cultured from tissue obtained at newborn male circumcision. Both were prepared as described in Germain et al. [1993]. The adult and neonatal keratinocytes were cultured in keratinocyte growth medium (Clonetics, San Diego, CA, or Gibco, Burlington, Ontario) containing 0.1 ng/ml hEGF, 5μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 μg/ml amphotercin-B, and 0.15 mM calcium. The cultures were maintained at 37°C in an atmosphere of 5% CO_2/air. Cells of third to fifth passages were used for experiments. An immortalized human keratinocyte cell line, HaCaT (obtained from Dr. Boukamp, Heidelberg, Germany) was cultured in MEM (Minimal Essential Medium) containing 5% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (all from Gibco).

Affinity Labeling of Cells

Iodination of TGF-β1 and β2 was done as previously described [Philip and O'Connor-McCourt, 1991]. Affinity-labeling technique was performed as detailed previously [Dumont et al., 1995]. Briefly, monolayers of cells were washed with ice cold binding buffer (Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+}, pH 7.4, (D-PBS) containing 0.1% bovine serum albumin (BSA) and were incubated with 100 pM of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 in the presence or absence of varying concentrations of nonradioactive TGF-β1, -β2, or -β3 (obtained from Dr. O'Connor-McCourt, Montreal; Genzyme, Cambridge, MA, or R & D Systems, Minneapolis, MN, respectively).The receptor-ligand complexes were cross-linked with Bis-(Sulfocsuccinimidyl) suberate (BS3, Pierce). The reaction was stopped by the addition of glycine and the cells were solubilized. The solubilized samples were run on a 3%-11% SDS-PAGE under nonreducing or reducing (in the presence of 5% β-mercaptoethanol) conditions and analyzed by autoradiography.

Immunoprecipitation of TGF-β Receptors

For immunoprecipitation studies, cells were affinity labeled with 200 pM ^{125}I-TGF-β1, and the cell extracts were incubated with 3 μg/ml of anti type I or II receptor antibodies in the presence or absence of their respective immunizing peptides. Immune complexes were then incubated with a protein A-Sepharose (Pharmacia-Biotech) slurry and the beads were pelleted by centrifugation. The immune complexes were analyzed by SDS-PAGE under reducing conditions followed by autoradiography . The anti-type I TGF-β receptor antibody specific to an epitope corresponding to amino acids 158–179 of the type I receptor was from Santa Cruz Biotechnology (Santa Cruz, CA). Antipeptide antibodies against the type II and type III TGF-β receptors were a gift from Dr. O'Connor-McCourt (Montreal, Quebec). The peptide sequences used for the types II and III receptors and the procedure used for the preparation of these antibodies were the same as described by Moustakas et al. [1993]. In comparison studies, these antibodies displayed the same specificity as those obtained from Dr. Moustakas (Cambridge, MA).

Treatment of Cells With Dithiothreitol (DTT) or Phosphatidylinositol Specific Phospholipase C (PI-PLC)

The procedure was the same as described previously [Dumont et al., 1995]. Briefly, monolayers were washed with D-PBS containing 0.1% BSA and then with protein free D-PBS. Cells were incubated with 10 mM DTT (Bio-Rad, Mississauga, Ontario) for 5 minutes at 37°C or with 0.3 u/ml of PI-PLC (Boehringer-Mannheim, Mannheim, Germany) for 1 hour at 37°C. Thereafter, the cells were washed twice with D-PBS containing 0.1% BSA and affinity labeled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2 as described above. In some cases, cells were affinity labeled with ^{125}I-TGF-β1 and then treated with PI-PLC. The supernatants were then collected, proteins precipitated with acetone, and analysed by SDS-PAGE to determine the migration pattern of cleaved GPI-anchored protein(s).

Two-Dimensional (nonreducing/reducing) Gels

Two-dimensional gel electrophoresis was performed as described by MacKay et al. [1990] except that 3-mercaptopropionic acid was omitted in the second dimension. Solubilized extracts of cells affinity labeled with ^{125}I-TGF-β1 were first electrophoresed on a 0.75-mm-thick 3–11% gradient gel under nonreducing conditions in the first dimension and on a 1.5-mm-

thick 3–11% gradient gel under reducing conditions for the second dimension. The gel was then subjected to autoradiography.

Autoregulation of TGF Receptors by TGF-β Isoforms

Monolayers were left untreated or treated with 100 pM TGF-β1, TGF-β2, or TGF-β3 in keratinocyte growth medium for 4 hours at 37°C in an atmosphere of 5% CO_2/air and the regulation of TGF-β binding sites was determined. The cells were washed with D-PBS containing 0.1% BSA, three times over 30 minutes, followed by a single 5-minute wash with 0.1% glacial acetic acid to remove any endogenous/added TGF-β as described by Glick et al [1990]. The cells were then incubated with ^{125}I-TGF-β1 in the absence (total binding) or presence of 40 times excess unlabeled TGF-β1, TGF-β2, or TGF-β3 (nonspecific binding). The radiolabeled TGF-β1 bound to the cells was solubilized without cross-linking and the radioactivity determined using a gamma counter. Specific binding was determined as "total" minus "nonspecific." The regulation of receptor subtypes was determined by affinity cross-link labeling and SDS-PAGE analyses as described above. Any alteration in total protein content in cultures due to treatment with TGF-β was monitored by determining protein concentrations using a protein assay based on the Bradford method (Bio-Rad, Mississauga, Ont). All lanes were from the same experiment in which cells pretreated with the three isoforms were incubated with the same preparation of ^{125}I-TGF-β1, and equal amounts of protein were loaded and run on the same gel.

Determination of Growth Inhibition

Growth inhibition was determined using ^{3}H-thymidine incorporation assay. Keratinocytes were plated at 30–40% confluency and cultured for 24 hours and then treated with TGF-β1, TGF-β2, or TGF-β3 at varying concentrations (2–100 pM) under normal conditions for 48 hours. ^{3}H-thymidine (1 μCi/mL, Amersham) was added to each well for the final 12 hours of TGF-β treatment. The cells were washed three times with phosphate- buffered saline and once with 10% trichloroacetic acid. The cells were then solubilized in 1% SDS and the incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

TGF-β Receptor Profiles on Early Passage Human Adult Keratinocytes

The TGF-β receptor profiles on keratinocytes affinity labeled with ^{125}I-TGF-β1 and ^{125}I-TGF-β2 are illustrated in (a) and (b), respectively, of Figure 1.

On keratinocytes affinity labeled with ^{125}I-TGF-β1 (Fig. 1a), in the absence of unlabeled TGF-β, four major binding complexes of relative molecular weights of 65 kDa, 85 kDa, 150 kDa, and 200–300 kDa are observed when analyzed under reducing conditions. The apparent molecular weights, and the isoform specificity of the 65 kDa, 85 kDa and 200–300 kDa binding complexes are characteristic of the cloned type I, II, and III TGF-β receptors [Segarini et al., 1989]. It is noteworthy that the the relative concentration of type III receptors on keratinocytes are low compared to that observed on most other cell types [Segarini et al., 1989; Dumont et al., 1995]. Competition experiments using increasing concentrations of unlabeled TGF-β isoforms show that the 150 kDa complex has virtually no affinity for TGF-β2, has high affinity for TGF-β1 and a somewhat lower affinity for TGF-β3. The types I, II, and III receptors display much higher affinity for TGF-β1 than for TGF-β2 and an intermediate affinity for TGF-β3.

On cells labeled with ^{125}I-TGF-β2, three binding complexes, two weakly labeled complexes of 65 kDa and 85 kDa and a 200–300 kDa complex, which correspond to the types I, II, and III TGF- β receptors are detectable (Fig. 1b). Interestingly, the affinity of the types I and II receptors for TGF-β2 is now similar to that for TGF-β1 and -β3, while the affinity of the type III receptor for TGF-β2 is even higher than for TGF-β1 and -β3. This suggests that a portion of the types I, II, and III receptors exists on keratinocytes in a state in which they can bind TGF-β2 with high affinity. The 150 kDa complex is not detected reflecting its low affinity for TGF-β2.

Affinity labeling and competition studies of the human keratinocyte cell line, HaCaT, and human neonatal keratinocytes with ^{125}I-TGF-β1 and ^{125}I-TGF-β2 revealed that the TGF-β receptor profiles and the isoform specificities of the receptor subtypes are virtually identical to those of the human adult keratinocytes (data not shown).

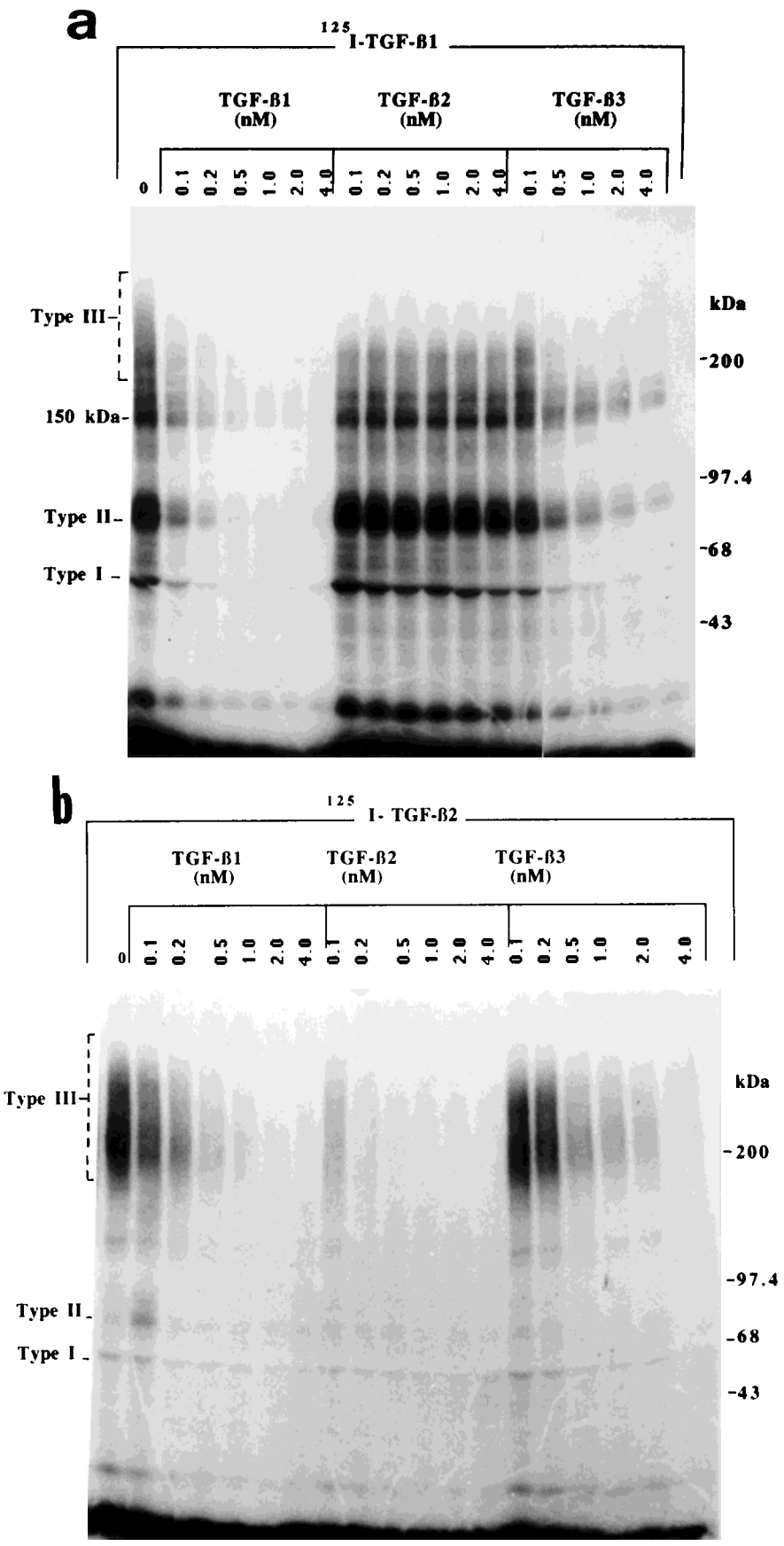


Fig. 1. Affinity labeling of keratinocytes with ^{125}I-TGF-β1 **(a)** or ^{125}I-TGF-β2 **(b)**. Confluent monolayers of early passage adult keratinocytes were affinity labeled with 100 pM ^{125}I- TGF-β1 or ^{125}I-TGF-β2 in the absence or presence of the indicated concentrations of unlabeled TGF-β1, TGF- β2, or TGF-β3. Cell extracts were analyzed on SDS-PAGE under reducing conditions.

Immunoprecipitation of TGF-β Receptors With Specific Antireceptor Antibodies

The identities of types I and II TGF-β receptors observed on the keratinocytes were confirmed by immunoprecipitation with specific antitype I and antitype II TGF-β receptor antibodies (Fig. 2). On cells affinity cross-link labeled with ^{125}I-TGF-β1, the antitype I antibody (anti-TGF-βR1) immunoprecipitated not only the type I receptor but also the types II and III TGF-β receptors and the 150 kDa GPI-anchored protein. The immunoprecipitation is specific, since no labeled proteins were precipitated when the immunizing peptide was included in the immunoprecipitation reaction (anti-TGF-βR1 + peptide). An antibody specific to a C-terminal epitope of the type II TGF-β receptor (anti-TGF-βRII) precipitated the type II receptor and co-immunoprecipitated the types I and III receptors. The addition of immunizing peptide specific to this antibody blocked this reaction (anti-TGF-βRII + peptide). A possible explanation for the failure for the antitype II receptor antibody to coimmunoprecipitate detectable amounts of the 150 kDa is that the epitope on the type II receptor recognized by the antitype II antibody is masked upon complex formation with the 150 kDa protein.

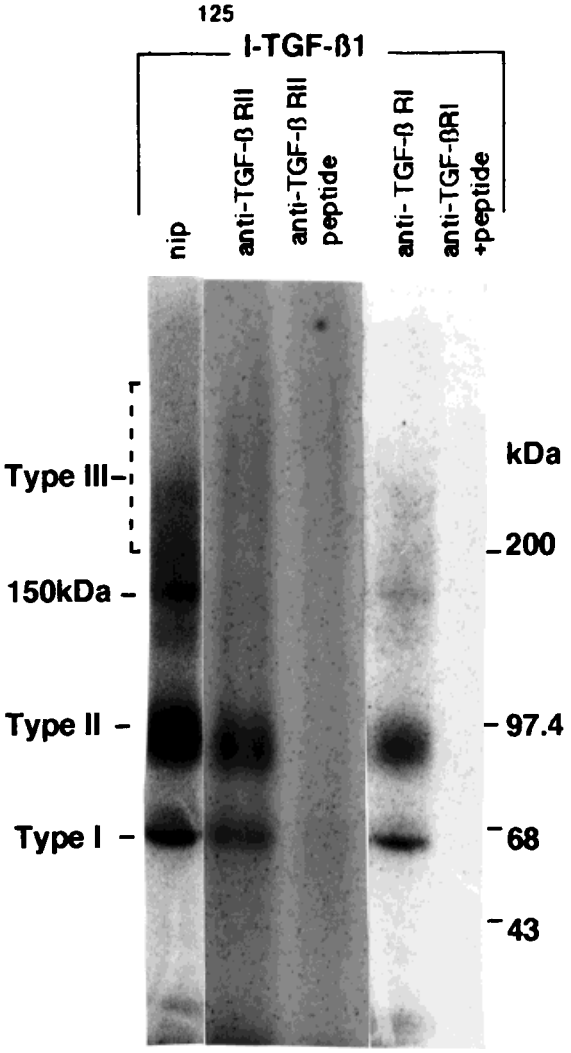


Fig. 2. Immunoprecipitation of TGF-β binding proteins on keratinocytes. Early passage adult keratinocytes were affinity labeled with ^{125}I-TGF-β1 and solubilized cell extracts were not immunoprecipitated (nip) or were treated with 3 μg/ml of anti-type I or anti-type II TGF-β receptor antibodies in the absence and presence of their corresponding immunizing peptide. The complexes were analyzed by SDS-PAGE under reducing conditions.

Sensitivity of Cell Surface TGF-β Binding Proteins to PI-PLC and DTT

It has been demonstrated by others on cell lines [Cheifetz and Massague, 1991] and more recently by us on primary cells [Dumont et al., 1995; Tam and Philip, 1998], that certain cell surface proteins that use a GPI anchor for membrane insertion can bind TGF-β. We examined whether any of the TGF-β binding proteins detected on adult keratinocytes have such an anchor, by pretreating the cells with the enzyme, PI-PLC, prior to affinity labeling with ^{125}I-TGF-β1 and SDS-PAGE analysis. In addition, we assessed the effect of pretreating the cells with DTT since the type I receptor, but not the type II or type III receptor, has been shown to be sensitive to this agent [Cheifetz and Massague, 1991]. When cells were pretreated with PI-PLC, the 150 kDa binding complex is the only ^{125}I-TGF-β1 labeled complex that is sensitive to the PI-PLC treatment (Fig. 3a). The 65 kDa complex is sensitive to DTT, which demonstrates that it is indeed the type I receptor. Identical results were obtained using neonatal keratinocytes and the HaCaT keratinocyte cell line (data not shown).

To determine whether any GPI-anchored proteins can be released into the supernatant, cells were affinity labeled with ^{125}I-TGF-β1 and then treated with PI-PLC. Results shown in Figure 3b indicate that the 150 kDa TGF-β1 binding protein is released into the supernatant of PI-PLC treated, but not untreated cells. The low molecular weight material seen under both conditions probably correspond to ^{125}I-TGF-β1 aggregates.

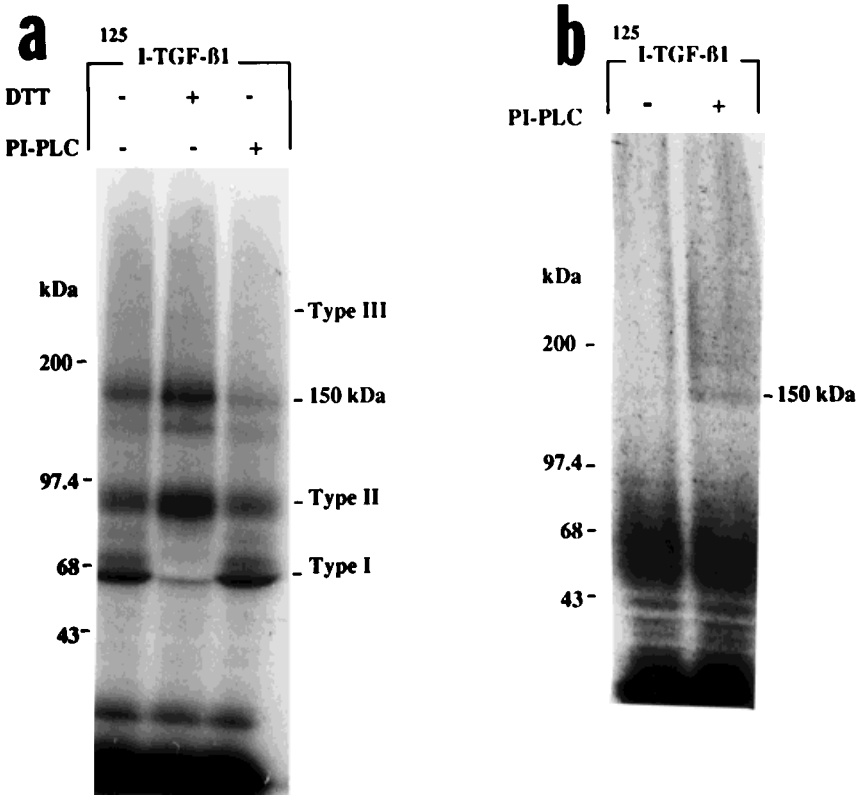


Fig. 3. **(a)** Effect of DTT and PI-PLC on affinity labeling of keratinocytes. Confluent monolayers of early passage adult keratinocytes were treated with 10 mM DTT or 0.3 u/ml of PI-PLC prior to affinity labelling with 100 pM of ^{125}I-TGF-β1. Control lanes received neither treatment (-). Cell extracts were analyzed by SDS-PAGE under reducing conditions. (**b**) Confluent monolayers of keratinocytes were affinity labeled with 100 pM of ^{125}I-TGF-β1 and then treated with 0.3 u/ml of PI-PLC. Supernatants were collected, proteins precipitated with acetone and analyzed by SDS-PAGE under reducing conditions. Control lanes received no PI-PLC treatment (-).

Evidence for Heteromeric Formation of TGF-β Receptor Complexes on Human Keratinocytes by Two-dimensional Gel Electrophoresis

To further characterize the ^{125}I-TGF-β1 binding complexes on keratinocytes two-dimensional gel electrophoresis was performed under nonreducing conditions in the first dimension and thereafter, under reducing conditions in the second dimension (Fig. 4a). When keratinocytes labelled with ^{125}I-TGF-β1 were analyzed under nonreducing conditions, in addition to the types I, II, and III receptors and the 150 kDa complex, two other complexes of 140 kDa and ~240 kDa were also detectable (Fig. 4b). The relative molecular weight of the ~240 kDa is only an approximation since it was obtained by extrapolation. Both the 140 kDa and ~240 kDa complexes are specific TGF-β binding complexes since unlabeled TGF-β1 and β2 compete with ^{125}I-TGF-β1 for binding to these complexes (Fig. 4b). The results illustrated in Figure 4a indicate that two spots with identical mobilities as those of the type I (65 kDa) and type II (85 kDa) TGF-β receptors fell from the 140 kDa complex, after reduction. This strongly suggests that the 140 kDa complex represents a heterodimer consisting of type I and type II TGF-β receptors where these receptors are cross-linked to different subunits of the same ^{125}I-TGF-β1 dimer. The reducing agent disrupts the disulphide bond between the two subunits of the TGF-β dimer. Thus after reduction, complexes composed of one type I or type II molecule cross-linked to a TGF-β monomer are seen in the second dimension. The data shown in Figure 4a also illustrate that three spots with identical mobilities as those of the type I (65 kDa), type II (85 kDa), and the GPI-anchored 150 kDa proteins, fell from the ~240kDa complex, after reduction. This indicates that the GPI-anchored 150kDa TGF-β1 binding protein forms a hetero-oligomeric com-

plex with the TGF-β signalling receptors. For example, the type I or the type II receptor cross-linked to the opposite arms of the ^{125}I-TGF-β1 dimer may get cross-linked to a second ^{125}I-TGF-β1 dimer in which one subunit is cross-linked to the 150 kDa GPI-anchored protein. Although the ~240 kDa complex appears to be a heterotrimer composed of the types I and II TGF-β receptors and the 150 kDa protein, it may have been derived from a heterotetramer or higher order complex. In some experiments, a 65 kDa spot (mobility same as that of the type I receptor) fell from the 115 kDa position, in addition to the spots that fell from 140 kDa and the ~240 kDa positions (Fig. 4c). This indicates that type I TGF-β receptor homodimers occur on keratinocytes. In control experiments where gel electrophoresis was performed under nonreducing conditions in both dimensions, no spots were visible under the 115kDa, 140 kDa and the ~240 kDa positions (Fig. 4d).

Regulation of TGF-β Receptors

It was of interest to test whether the TGF-β receptors on keratinocytes are autoregulated by the three TGF-β isoforms. The regulatory effects of TGF-β1, -β2, and -β3 on specific TGF-β1 binding of human adult keratinocytes are shown in Figure 5a. Pretreatment of these cells with 100 pM of TGF-β1, -β2, and β3 at 37°C for 4 hours significantly decreased ^{125}I-TGF-β1 specific binding, expressed as a percent of what was observed for unpretreated cells ($P<0.05$, <0.05, and <0.01, respectively). In experiments where cells were preincubated with ^{125}I-TGF-β1 instead of unlabeled TGF-β, at 37°C for 4 hours, the washing method, which included a mild acid wash, was sufficient to allow dissociation of >95% prebound ^{125}I-TGF-β1 (data not shown). In addition, control experiments showed that this washing procedure did not diminish the ability of receptors to subsequently bind TGF-β (data not shown). This is consistent with the results obtained by others who used similar washing procedures to analyze TGF-β binding of other cell types [Coffey et al., 1987; Glick et al., 1990]. These results indicate that the downregulation observed is not due to competition with prebound TGF-β or due to detrimental effects of the washing procedure on the receptors.

Figure 5b shows the autoregulation of receptor types by TGF-β1, -β2, and -β3, as analyzed by SDS-PAGE under reducing conditions after affinity cross-link labeling with ^{125}I-TGF-β1. The results indicate that the types I, II, and III TGF-β receptors and the 150 kDa TGF-β1 binding protein are differentially downregulated by the three isoforms. Densitometric analyses of autoradiograms reveal that pretreatment with TGF-β1and -β3 decreased the intensity of the types I and II receptors to approximately 20% of control. In contrast, TGF-β2 was less effective in downregulating the type I receptor (40% of control) and essentially ineffective in downregulating the type II receptor (90% of control). The GPI-anchored 150 kDa protein was downregulated by TGF-β1 and -β3 to 65% and 75% of control respectively, but only to 85% of control by TGF-β2.

Growth Regulation of Human Keratinocytes by TGF-β Isoforms

We then examined the responsiveness of these cells to TGF-β by studying the effect of TGF-β1, -β2, and-β3 on the proliferation of these cells as measured using thymidine incorporation assay. The results in Figure 6 illustrate that all three isoforms at concentrations of 10 pM and higher significantly inhibited DNA synthesis in human keratinocytes ($P<0.0005$ or less). The inhibitory effect is concentration-dependent with maximal inhibition being observed by 50 pM for all three isoforms.

DISCUSSION

Keratinocytes play an important role in the cutaneous tissue repair process and alterations in keratinocyte proliferation and function are associated with the development of a variety pathological skin conditions such as psoriasis and malignancy. Although it is well documented that TGF-β has profound effects on keratinocyte growth and function, there is a paucity of information on the types, specificity, and oligomeric complex formation of TGF-β receptors on human keratinocytes. Also it has not been known whether these cells express novel TGF-β receptors other than the commonly expressed types I, II, and III receptors. In the present study, we identify a cell surface 150 kDa GPI-anchored TGF-β1 binding protein, in addition to the types I, II, and III TGF-β receptors on normal adult and neonatal human keratinocytes. Furthermore, we demonstrate that the 150 kDa TGF-β1 binding component forms a heterotrimeric complex with the types I and II receptors on keratinocytes. In addition, we

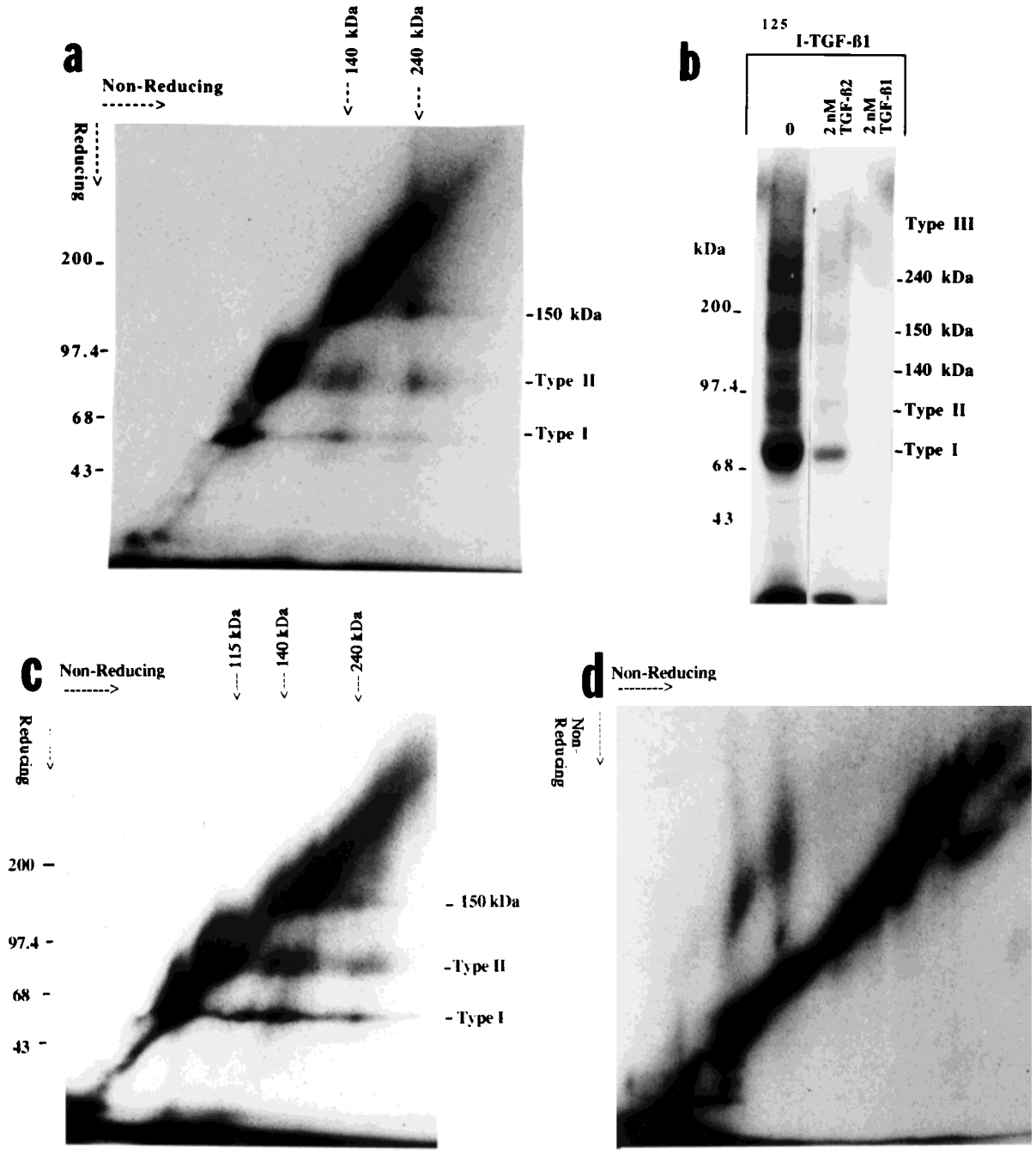
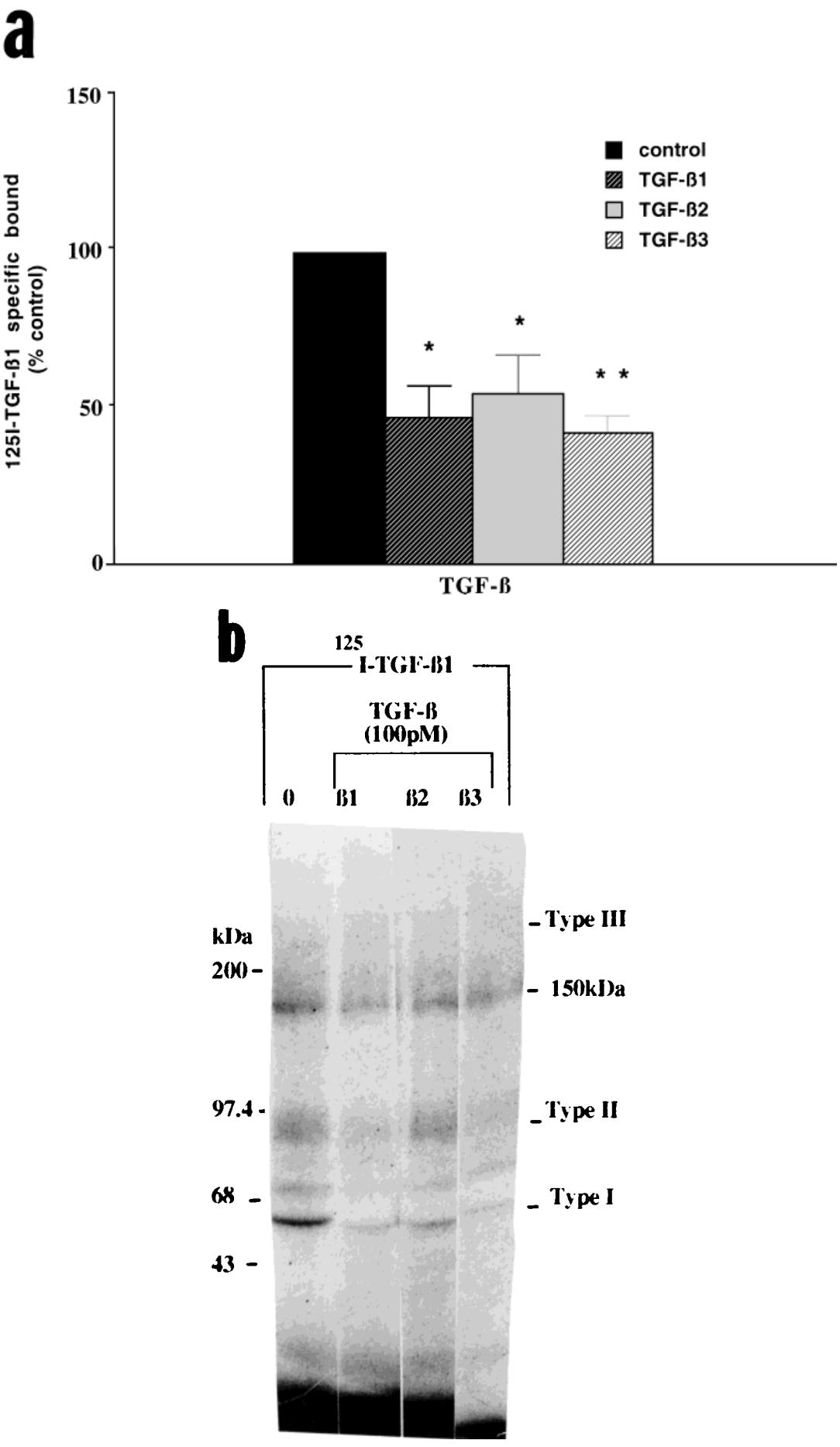


Fig. 4. **(a, c, d)**: Two-dimensional gel electrophoresis of TGF-β binding complexes on keratinocytes. Confluent monolayers of early passage adult keratinocytes were affinity labeled with 100 pM ^{125}I-TGF-β1. Samples were analyzed on SDS-PAGE under nonreducing conditions in the first dimension. The individual lane was cut and laid horizontally and subjected to SDS-PAGE under reducing conditions in the second dimension (a,c). In control experiments, the second dimension was also nonreducing (d). (**b**) Affinity labeling of keratinocytes as analyzed by SDS-PAGE under nonreducing conditions to demonstrate the competition by unlabeled TGF-β1 or TGF-β2 for ^{125}I- TGF-β1-binding receptor oligomeric complexes. Early passage adult keratinocytes were affinity labelled with 100 pM ^{125}I- TGF-β1 in the absence or presence of 2 nM of unlabelled TGF-β1 or TGF-β2. Cell extracts were analyzed by SDS-PAGE under nonreducing conditions.


a
150
100
50
0
125I-TGF-ß1 specific bound
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control
TGF-ß1
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200
97.4
68
43
Type III
150kDa
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Figure 5.

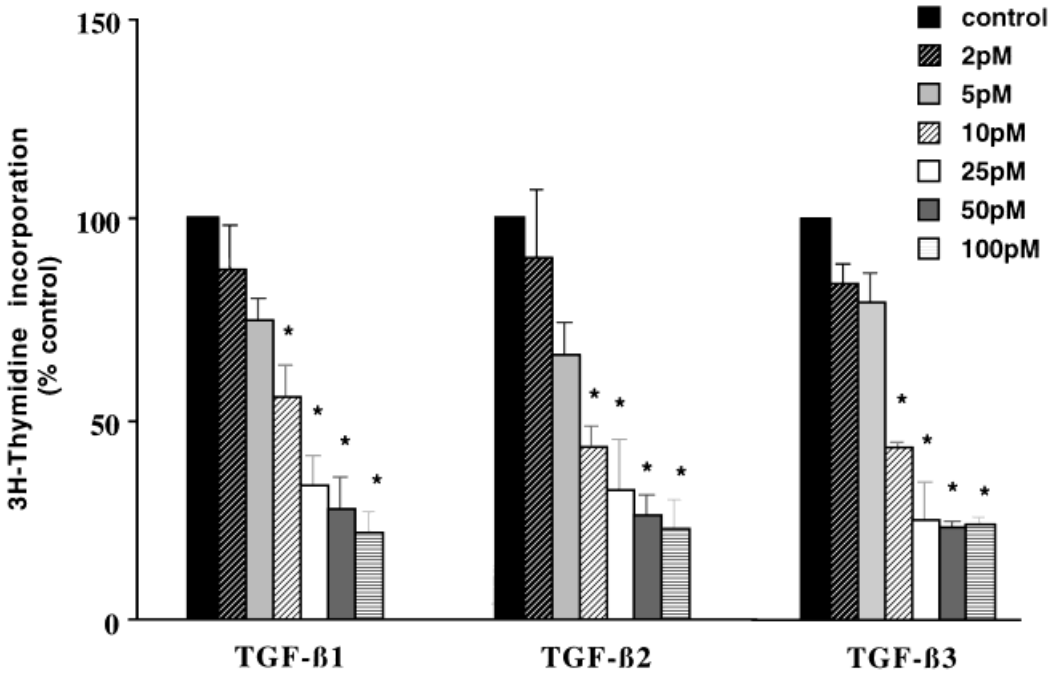

Fig. 6. Inhibition of thymidine incorporation in keratinocytes by TGF-β isoforms. Subconfluent monolayers of early passage adult keratinocytes were treated with TGF-β1, TGF-β2, or TGF-β3 at indicated concentrations for 48 hours. ^{3}H-thymidine (1μCi/mL), was added for the final 12 hours of TGF- β treatment and the incorporated radioactivity was determined by liquid scintillation counting. *$P<0.0005$ or less.

show the differential binding and downregulation of the four TGF-β binding proteins by the three TGF-β isoforms. The demonstration that the 150 kDa GPI-anchored TGF-β1 binding protein forms a heteromeric complex with the TGF-β signaling receptors suggests that this may modify TGF-β signaling on human keratinocytes. This is the first time that a GPI-anchored protein has been shown to form a hetero-oligomeric complex with the TGF-β signaling receptors.

The identities of the four TGF-β binding proteins on human keratinocytes were confirmed on the basis of their relative molecular weights, affinity for TGF-β isoforms, immunoprecipitability with specific antibodies against the types I and II TGF-β receptors, and sensitivity to DTT and PI-PLC. It is not possible to infer reliable quantitative estimation of the various TGF-β receptors and binding proteins identified in the present study since the efficiency of cross-linking to these proteins is not known. The 150 kDa TGF-β1 binding complex is not affected by reducing and nonreducing conditions, indicating that it does not represent endoglin, a reductant sensitive 180 kDa TGF-β1 binding protein predominantly expressed on endothelial cells [Cheifetz et al., 1992]. The question as to whether this protein is related to the GPI-anchored TGF-β1 binding proteins of 180 kDa and 90–100 kDa described by Cheifetz and Massague [1991] on cell lines and the 180 kDa protein we identified on primary cells [Dumont et al., 1995; Tam and Philip, 1998] remains to be answered.

The evidence for the interaction of the 150 kDa GPI-anchored component with the types I and II signalling receptors is based on the (1) co-immunoprecipitation of the 150 kDa component with the types I and II receptors by the

Fig. 5. **(a)** Regulation of TGF-β binding on keratinocytes by TGF-β1, TGF-β2, TGF-β3. Early passage adult keratinocytes were pretreated with 100 pM TGF-β1, β2, or -β3 at 37°C for 4 hours. The cells were then incubated with ^{125}I-TGF-β1 at 4°C in the absence (total binding) or the presence of 40 times excess of unlabeled TGF-β1 (nonspecific binding). ^{125}I-TGF-β1 specifically bound was determined as (total minus nonspecific) binding and expressed as percentages of ^{125}I-TGF-β1 specifically bound in the absence of pretreatment with TGF-β isoforms. *$P < 0.05$; **$P < 0.01$. (**b**) Regulation of TGF-β receptor subtypes by TGF-β1, β2, β3 on keratinocytes as detected by affinity labeling and SDS-PAGE analysis. Early passage adult keratinocytes were pre-treated with 100 pM of TGF-β1, β2, or β3 for 4 hours at 37°C and subjected to affinity cross-link labeling and SDS-PAGE analysis as described in Materials and Methods.

antitype I receptor antibody and (2) occurrence of a heteromeric complex consisting of the 150 kDa component, the type II receptor, and the type I receptor, as detected by two-dimensional gel electrophoresis under non-reducing (first dimension) and reducing (second dimension) conditions. Under nonreducing conditions, multiple receptor molecules cross-linked to the opposite arms of the TGF-β dimers can exist. Under reducing conditions, the reducing agent will disrupt the disulphide bond between the opposite arms of the TGF-β dimer and the major cross-linked complexes will consist of the monomer of the ligand cross-linked to the monomeric receptor. The most likely reason for the low amounts of hetero-oligomeric complexes detected is the low efficiency of forming multiple cross-links per complex.

Although an increasing number of GPI-anchored membrane proteins have been identified on mammalian cells, a common biological function connecting these proteins has not been described [Turner, 1994]. GPI-anchored proteins have been suggested to participate in signal transduction by associating with src-related protein tyrosine kinases [Stefanova et al., 1993] or by becoming sequestered in small membrane invaginations called caveolae where it may interact with signaling receptors [Mayor et al., 1994]. Whether the GPI-anchored 150 kDa TGF-β1 binding protein has a similar function remains to be determined. Alternatively, the GPI-anchored protein may sequester TGF-β on the cell surface or present TGF-β to the signaling receptors, thus playing a similar role as the type III receptor. Unlike the type III receptor, the 150 kDa protein may perform those functions in an isoform specific manner since it displays high affinity for TGF-β1, an intermediate affinity for TGF-β3 and virtually no affinity for TGF-β2. Given the diversity of the biological effects of TGF-β, it is possible that the this protein may be involved in certain keratinocyte specific TGF-β actions.

In addition to confirming the identity of the type II receptor, the immunoprecipitation studies using specific antitype II receptor antibodies, provide evidence for the formation of heteromeric complexes between types I and II receptors and between the types II and III receptors on keratinocytes. Co-immunoprecipitation of types I and II receptors have been well documented and form the basis of the notion that TGF-β signal transduction involves the formation of heteromeric complexes of type I and type II receptors [Franzen et al., 1993; Bassing et al., 1994]. The detection of heteromeric complexes between types II and III receptors in primary cells confirm previous reports on the formation of such complexes on cell lines [Lopez-Casillas et al., 1993; Moustakas et al., 1993]. The demonstration that types I and II TGF-β receptors form ligand-induced heterodimers, and homodimers on normal human keratinocytes (present study) and skin fibroblasts (Tam and Philip, 1998), allow the expansion of the heteromeric TGF-β receptor signaling paradigm proposed using cell mutants, cells overexpressing these receptors or cells expressing chimeric receptors [Laiho et al., 1991; Henis et al., 1994; Luo and Lodish, 1996], to include normal TGF-β responsive cells with physiological receptor concentrations and ratios [Tam and Philip, 1998].

The data from the two-dimensional electrophoresis demonstrating the occurrence of heterotrimeric and of hetero- and homodimeric complexes of TGF-β binding proteins are consistent with the results from the immunoprecipitation studies demonstrating on the existence of oligomeric TGF-β receptor complexes. Also, it is noteworthy that endoglin, another cell surface TGF-β binding protein, has been shown to form a heteromeric complex with the signaling receptors on porcine aortic endothelial cells [Yamashita et al., 1994]. These data, taken together, point to the possibility of TGF-β inducing, in parallel, the formation of receptor complexes of different receptor subtypes and multimeric units such as dimers, trimers, or tetramers. Formation of receptor complexes containing different receptor subtypes of differing composition and or ratios may represent modes of regulating diverse actions of TGF-β.

That the keratinocytes studied are responsive to TGF-β is shown by the potent inhibitory effects of TGF-β isoforms on DNA synthesis in these cells. The differences observed in the relative binding affinities of the three isoforms are reflected in their differential downregulation of the receptor subtypes, but not in their ability to inhibit growth. The failure to observe marked differences in the response between the three isoforms may be explained by the data, which suggest that a portion of the types I, II, and III receptors exist in a state in which they can bind TGF-β2 with high affinity. This receptor state

becomes detectable when TGF-β2 is used to compete with ^{125}I-TGF-β2 (Present study-Figure 1b; Cheifetz et al., 1990). Although it remains to be determined how this receptor state is produced, the fact that the type III receptor display a higher affinity for TGF-β2 under these conditions suggests that the type III receptor (via presentation of ligand and oligomerization) may be responsible for the type I and II receptor states with high affinity for TGF-β2 and thus the obliteration of the differences in biological response. It is, however, possible that biological responses other than proliferation may exhibit isoform specificity.

In conclusion, the present results show that human keratinocytes display a 150 kDa GPI-anchored cell surface TGF-β1 binding protein in addition to the types I, II, and III TGF-β receptors. More importantly, we demonstrate that this protein interact with the types I and II TGF-β signalling receptors by forming hetero-oligomeric complexes. These findings suggest that the 150 kDa GPI-anchored protein may modify TGF-β signaling on human keratinocytes. Future studies will be aimed at elucidating the significance of this protein in TGF-β signaling and in TGF-β induced biological responses in keratinocytes.

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