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Characterization of Transforming Growth Factor-ß Receptors in the Human Endometrium

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September, 1995

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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Abstract

Transforming growth factor-ß (TGF-ß) is a multifunctional polypeptide growth factor which is believed to play an important role in the growth and differentiation of uterine cells. Although the expression of TGF-ß in the uterus has been previously described, the receptors for TGF-ß in this tissue have not been characterized. In the present study, the cell surface receptors for TGF-ß were characterized on cultures of stromal cells prepared from human endometrial biopsies, and on a human endometrial epithelial cell line (RL95-2) using affinity labeling techniques. On stromal cells, five TGF-ß binding proteins were identified. Analysis of the sensitivity of these proteins to dithiothreitol and phosphatidylinositol-specific phospholipase C, together with results from immunoprecipitations with anti-TGF-ß receptor antibodies, confirmed that three of these binding proteins correspond to the cloned type I, II, and III TGF-ß receptors. The other two binding proteins exhibited characteristics of isoform-specific glycosyl-phosphatidylinositol-anchored TGF-ß binding proteins. On RL95-2 cells, three TGF-ß binding proteins, corresponding to the type I, II, and III TGF-ß receptors, were identified. The number of receptors on endometrial cells and their relative affinity for TGF-ß was estimated by Scatchard analysis. These receptors are responsive to physiological concentrations of TGF-ß as demonstrated by the effect of TGF-ß on DNA synthesis in these cells. Accordingly, they have the potential to respond to TGF-ß expressed in the endometrium in an autocrine and/or paracrine manner.

Résumé

Le TGF-ß est un facteur de croissance polypeptide multifonctionnel qui joue un rôle important dans la croissance et la différenciation des cellules utérines. Malgré l'expression du TGF-ß dans l'utérus, les récepteurs du TGF-ß n'ont pas encore été caractérisés dans ce tissu. Dans la présente étude, les récepteurs du TGF-ß ont été caractérisés sur des cellules stromales préparées à partir de biopsies endométriales humaines, ainsi que sur une lignée cellulaire endométriale épithéliale (RL95-2) par marquage d'affinité. Sur les cellules stromales, cinq protéines liant le TGF-ß ont été identifiées. L'analyse de la sensibilité de ces protéines au dithiothréitol et à la phospholipase C spécifique au phosphatidylinositol, ainsi que les résultats des immunoprécipitations utilisant des anticorps contre les récepteurs du TGF-ß, ont confirmé que trois de ces protéines correspondent aux récepteurs de type I, II, et III du TGF-ß. Les deux autres protéines identifiées démontrent des caractéristiques de protéines ancrées à la membrane par liaison covalente au glycosyl-phoshatidylinositol. Sur les cellules RL95-2, les récepteurs de type I, II et III du TGF-ß ont été identifiés. Le nombre de ces récepteurs sur les cellules endométriales ainsi que leur affinité pour le TGF-ß ont été estimés en analysant les données de liaisons par la méthode de Scatchard. L'effet du TGF-ß sur la synthèse de l'ADN dans les cellules endométriales démontre que ces récepteurs répondent à des concentrations physiologiques de TGF-ß. Ils ont donc le potentiel de répondre au TGF-ß exprimé dans l'endomètre de manière autocrine et/ou paracrine.

Dedication

To my parents for their love and support,

but above all, for always being there

Acknowledgements

I would like to thank my supervisor, Anie Philip and co-supervisor, Beverley Pearson-Murphy, for giving me the opportunity to carry out this research project, and for their continuous support from start to finish. I am especially grateful to Anie for her time, patience and guidance, and for encouraging me to present my work at numerous scientific meetings.

Very special thanks to Maureen O'Connor-McCourt for giving me the privilege of working in her laboratory, and for her valuable advice and guidance throughout the project. I am indebted to everyone in Maureen's group for sharing their time and expertise with me, and for making BRI an excellent environment to work and learn in.

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Finally, I would like to express my sincere gratitude to Patrice Bouchard for all his help, support and encouragement, but above all, for his friendship which I will always treasure.

Preface

The contents of a manuscript which has already been published has been incorporated into this thesis:

Dumont, N., O'Connor-McCourt, M.D. and Philip, A. (1995) Transforming growth factor-ß receptors on human endometrial cells: identification of the type I, II, and III receptors and glycosyl-phosphatidylinositol anchored TGF-ß binding proteins. Molec. Cell. Endocrinol. 111, 57-66.

A reprint of the manuscript, along with permission from Elsevier Science Ireland Ltd. is included in the addendum.

All the work presented in this thesis was carried out by myself except for the iodination of TGF-ß which was performed by Josée Plamondon.

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List of Abbreviations

B_{max}	Maximal binding
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GPI	Glycosyl-phosphatidylinositol
GS domain	Glycine serine rich domain
K_d	Dissociation constant
LAP	Latency associated peptide
mRNA	Messenger ribonucleic acid
PI-PLC	Phosphatidylinositol-specific phospholipase C
pRB	Retinoblastoma gene product
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TGF-ß	Transforming growth factor-ß

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1. Introduction

1.1 The TGF-ß family

Transforming growth factor-ß (TGF-ß) is a 25 kDa disulphide-linked dimeric peptide which was first isolated from human platelets (Assoian *et al.*, 1983). It was cloned from a human complementary deoxyribonucleic acid (cDNA) library (Derynck *et al.*, 1985), and later named TGF-ß1 (Cheifetz *et al.*, 1987). Five TGF-ß isoforms, denoted TGF-ß1 to TGF-ß5, each encoded by a different gene, have now been identified in vertebrates (Massagué, 1990; Roberts and Sporn, 1990). Of the five isoforms, TGF-ß1, -ß2, and -ß3 are found in mammals, while TGF-ß4 and -ß5 are found in chick and frog, respectively (Jakowlew *et al.*, 1988; Kondaiah *et al.*, 1990).

The three mammalian TGF-ß isoforms, which show approximately 70 to 80% amino acid homology (Massagué, 1990), are initially synthesized as larger, biologically inactive precursor polypeptides that differ markedly in sequence outside of the C-terminal TGF-ß motif (Derynck *et al.*, 1987). The TGF-ßs may be secreted either as a small latent complex which is composed of the active C-terminal TGF-ß dimer linked in a non-covalent association with a dimer of the N-terminal remnant of the TGF-ß precursor, referred to as latency associated peptide (LAP), or as a large latent complex which, in addition to the active TGF-ß and LAP, consists of a third component denoted latent TGF-ß binding protein (Miyazono *et al.*, 1993; Fig. 1).

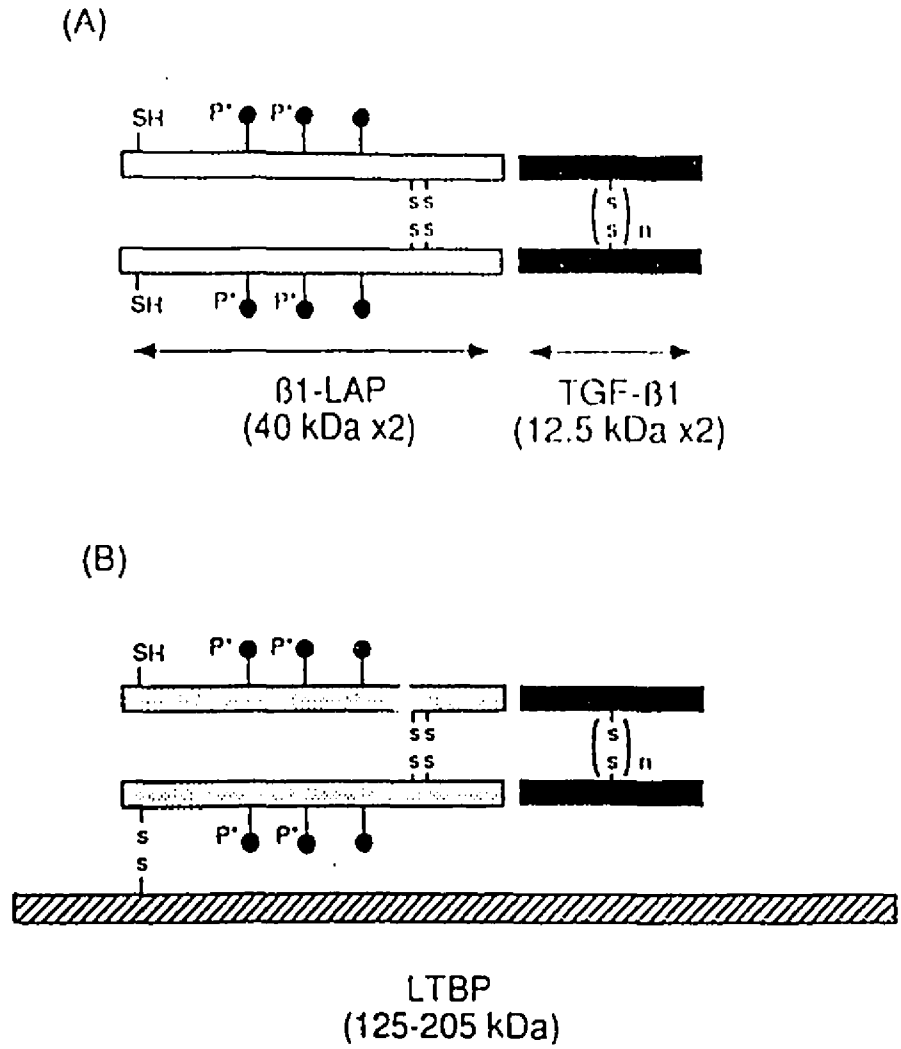


Fig. 1. Schematic representation of the small and large latent TGF-ß1 complex. (A) Small latent TGF-ß1 complex. ß1-LAP is the N-terminal remnant of the TGF-ß1 precursor. Two of the N-linked carbohydrates may contain mannose 6-phosphate (shown by P'). The number of interchain disulphide bonds in the mature TGF-ß1 is not known. (B) Large latent TGF-ß1 complex. A single molecule of latent TGF-ß binding protein (LTBP) is bound to ß1-LAP by disulphide bonds. Reproduced from Miyazono et al. (1993), with modifications.

TGF-ß can be activated by the dissociation of these latent complexes *in vitro* by transient acidification, heating, urea, sodium dodecyl sulfate (SDS), or by treatment with proteases such as plasmin and cathepsin D (Lyons *et al.*, 1988; 1990). Although a variety of mechanisms have been proposed (Dennis and Rifkin, 1991; Kojima *et al.*, 1993; Schultz-Cherry and Murphy-Ullrich, 1993), the conditions that activate latent TGF-ß under physiological circumstances *in vivo* are not known. Whatever the physiological mechanism for activation may be, it represents an important potential control step for the regulation and localization of the effects of TGF-ß.

The TGF-ß isoforms often exhibit similar actions and overlapping patterns of expression. Although their biological potencies are similar in many *in vitro* assays (Cheifetz *et al.*, 1987, 1988b; Seyedin *et al.*, 1987), marked differences have been observed in certain cases (Cheifetz *et al.*, 1988b, 1990; Ohta *et al.*, 1987; Rosa *et al.*, 1988; Tsunawaki *et al.*, 1988). Additionally, there is evidence indicating that each isoform may have distinct activities *in vivo* (Rosa *et al.*, 1988; Joyce *et al.*, 1990). In support of this idea, immunohistochemical analysis using isoform-specific anti-peptide antibodies to the TGF-ßs has recently shown that the protein expression patterns for each isoform are both temporally and spatially unique throughout the processes of embryonic development (Pelton *et al.*, 1991), wound repair (Levine *et al.*, 1993), and carcinogenesis (Gold *et al.*, 1992; Gorsch *et al.*, 1992). This implies cell-specific functions and complex gene regulation for TGF-ß isoforms *in vivo*.

1.2 Biological activities of TGF-ß

Although originally defined for its ability to cause reversible phenotypic transformation and anchorage-independent growth of normal fibroblasts (Anzano *et al.*, 1983), a wide spectrum of biological activities is now attributed to TGF-ß, one of the most notable of which is TGF-ß's potent regulatory effects on cell growth and differentiation (Massagué, 1990; Roberts and Sporn, 1990, 1993). In this respect, TGF-ß is bifunctional, that is, it may act as either a positive or negative regulator of cell division (Moses *et al.*, 1990). In general, TGF-ß stimulates the proliferation of mesenchymal stromal cells, but inhibits the proliferation of epithelial cells. Whether cells are stimulated or inhibited to proliferate by TGF-ß may also depend on their state of differentiation.

In addition to its complex growth-regulatory activities on essentially all cell types, compelling evidence has accumulated documenting the role of TGF-ß as a potent immunomodulatory molecule. Studies have shown that TGF-ß induces monocyte-macrophage chemotaxis and growth factor production (Wahl *et al.*, 1987, 1990; McCartney-Francis *et al.*, 1990), but suppresses their release of both superoxide and nitric oxide (Tsunawaki *et al.*, 1988; Ding *et al.*, 1990). TGF-ß also suppresses the activity of natural killer cells (Rook *et al.*, 1986) as well as the proliferation and function of lymphocytes (Kehrl *et al.*, 1986a, 1986b). Moreover, it antagonizes the effects of inflammatory effector cytokines such as interleukin-1ß, tumor necrosis factor-α, and interferon-γ (Ruscetti and Palladino, 1991; Wahl, 1992).

Thus, TGF-ß plays a central role in the modulation of immune response.

Another important function of TGF-ß is in the regulation of extracellular matrix formation. Studies have shown that TGF-ß increases the synthesis of proteins such as collagen, fibronectins, and cell surface integrins; decreases the synthesis of enzymes such as collagenase that catalyse degradation of extracellular matrix components; and increases levels of inhibitors of these degradative enzymes (Massagué, 1990; Roberts and Sporn, 1990). Hence, the net effect of these activities is an increase in production and deposition of extracellular matrix which may be important in various processes such as tissue repair and wound healing. This hypothesis has been strengthened by findings that TGF-ß enhances wound healing in animal models (Mustoe *et al.*, 1987, 1991; Quaglino *et al.*, 1991; Cox *et al.*, 1992), and that anti-TGF-ß antibodies reduce scarring (Shah *et al.*, 1992).

TGF-ß is also involved in numerous other physiological processes including angiogenesis, myogenesis, osteogenesis, and steroidogenesis (Roberts and Sporn, 1990). In addition, TGF-ß has been detected as early as the four-cell stage of preimplantation development (Rappolee *et al.*, 1988), and all three mammalian TGF-ß isoforms have been identified in embryonic tissues suggesting an important role for TGF-ß in development (Akhurst *et al.*, 1991; Pelton *et al.*, 1991). In fact, there is evidence that TGF-ß is involved in embryonic hematopoiesis as well as in the formation of the palate and in the development of the eye, heart, nervous system, skeletal system, and adrenal cortex (Pelton *et al.*, 1990, 1991; Akhurst *et al.*, 1991,

1992; Johnson *et al.*, 1993).

Despite evidence for the role of TGF-ß in developmental processes, transgenic mice deficient in TGF-ß1 show no gross developmental abnormalities, but they succumb to a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response which leads to organ failure and death shortly after weaning (Schull *et al.*, 1992; Kulkarni *et al.*, 1992). Interestingly, genotyping of pups born from intercrosses of mice heterozygous for the TGF-ß1 mutation revealed that less than half of the expected TGF-ß1 homozygotes are born. It is unclear why the majority of TGF-ß1 homozygotes die during embryonic development, but since there is evidence that TGF-ß is present in murine breast milk, and that it can cross the placenta, some have suggested that maternal TGF-ß may "rescue" some of the TGF-ß1 homozygote mutant mice (Letterio *et al.*, 1994). The hypothesis that TGF-ß may be transferred to mutant mice via the mother's milk, and the onset of inflammatory dysfunction at weaning emphasize the important role of TGF-ß as an anti-inflammatory protein.

The creation of mice that overexpress TGF-ß has also been attempted. Although such attempts have often led to embryonic lethality, restriction of TGF-ß expression to specific organs using appropriate promoters has been successful (Sporn and Roberts, 1992). For example, overexpression of TGF-ß in the mammary gland impairs the development and secretory function of mammary tissue during sexual maturation and pregnancy (Jhappan *et al.*, 1993; Pierce *et al.*, 1993), while

overexpression of TGF-ß in the liver causes multiple tissue lesions, including hepatic fibrosis and extensive glomerulonephritis (Sanderson *et al.*, 1995).

1.3 TGF-ß receptors and binding proteins

Once active, TGF-ß elicits its effects through binding to specific cell surface receptors. Studies have shown that TGF-ß binds with high affinity to many cell types (Wakefield *et al.*, 1987). Affinity labeling studies using chemical cross-linking agents have revealed that the type I (53 kDa), II (70-85 kDa), and III (200-400 kDa) TGF-ß receptors are the most widely distributed cell surface molecules that bind TGF-ß (Massagué, 1992). All three receptors have been cloned (López-Casillas *et al.*, 1991; Wang *et al.*, 1991; Lin *et al.*, 1992; Franzén *et al.*, 1993). The type I and II receptors are transmembrane serine/threonine kinases (Lin *et al.*, 1992; Franzén *et al.*, 1993) which are thought to form a signaling complex (Wrana *et al.*, 1992). Although the details of the signal transduction pathway remain to be established, studies with TGF-ß resistant cell mutants have shown that the type I receptor requires the presence of the type II receptor to bind ligand (Laiho *et al.*, 1991; Wrana *et al.*, 1992), while the type II receptor requires the presence of the type I receptor for signaling (Wrana *et al.*, 1992), but not for ligand binding (Lin *et al.*, 1992). Thus, both the type I and II receptors appear to be necessary for signal transduction. According to the current model for the initiation of signaling by the TGF-ß receptor (Wrana *et al.*, 1994; see Fig. 2), TGF-ß binds directly to the type

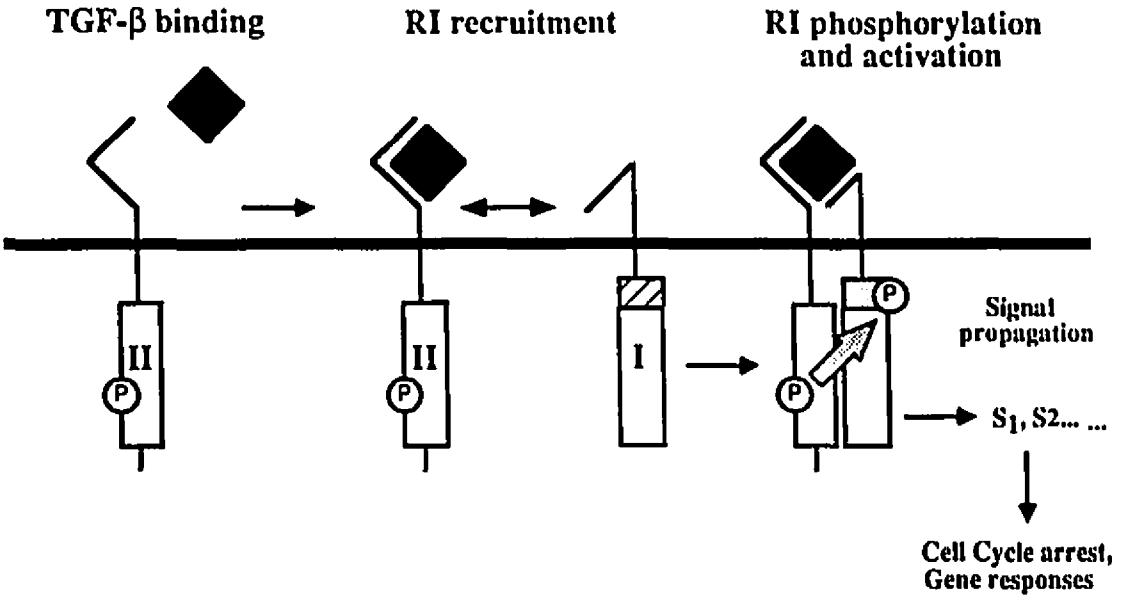


Fig. 2. **A general model for the initiation of signaling by the TGF-ß receptor.** Receptor II is the primary TGF-ß receptor and is a constitutively active serine/threonine kinase that recruits receptor I by means of bound TGF-ß (diamond). Subsequent phosphorylation of of the GS domain (Glycine Serine rich domain, striped box) by receptor II allows the receptor I kinase to propagate the signal to downstream substrates that mediate antiproliferative as well as gene responses. Reproduced from Wrana *et al.* (1994).

II receptor which is a constitutively active kinase. Bound TGF-ß is then recognized by the type I receptor which is recruited to the complex and becomes phosphorylated by the type II receptor. Phosphorylation allows the type I receptor to propagate the signal to downstream substrates.

The type III receptor, also known as betaglycan, is a transmembrane proteoglycan (Cheifetz et al, 1988a). It contains both heparan and chondroitin sulfate glycosaminoglycan chains which cause it to migrate heterogenously upon gel electrophoresis as a protein in the range of 200-400 kDa (Cheifetz *et al.*, 1988a; Segarini and Seyedin, 1988). Studies have shown that these glycosaminoglycan chains are not required for the folding, targeting, or TGF-ß binding activity of the receptor (Cheifetz and Massagué, 1989; Pépin *et al.*, 1994). In contrast to the type I and II receptors, the type III receptor has a short cytoplasmic domain with no apparent signaling motif (López-Casillas *et al.*, 1991; Wang *et al.*, 1991). In fact, many TGF-ß responsive cells lack the type III receptor (Segarini *et al.*, 1989). Although this receptor appears to be dispensable for signaling, its expression is believed to enhance binding of TGF-ß ligands to the type II receptor (Wang *et al.*, 1991). There is also evidence suggesting that the type III receptor forms a stable non-covalent heteromeric complex with the type II receptor (López-Casillas *et al.*, 1993; Moustakas *et al.*, 1993). Hence, this receptor has been proposed to regulate access of TGF-ß to the signaling receptors. Soluble forms of the type III receptor which bind TGF-ß similarly to the membrane-bound form have also been detected

(Andres *et al.*, 1989). It appears as though the soluble form of betaglycan is derived from the membrane-bound form by a hydrolytic process (López-Casillas *et al.*, 1991).

On most cells, the type I and II receptors display a higher affinity for TGF-ß1 than for TGF-ß2, while the type III receptor has been reported to bind both isoforms with either a similar affinity (Cheifetz *et al.*, 1987, 1988b, 1990; Segarini *et al.*, 1989), or a somewhat higher affinity for TGF-ß2 (Mitchell and O'Connor-McCourt, 1991). The observed dissociation constant (K_d) values range from 2 to 50 pM for the type I and II receptors, and from 50 to 300 pM for the type III receptor (Segarini *et al.*, 1987; Cheifetz *et al.*, 1990; Massagué *et al.*, 1992). The receptor number is tissue- and species-specific and varies from as low as 380 receptors/cell on human monocytes (Wahl *et al.*, 1987) to 120,000 receptors/cell on mouse embryo fibroblasts (Andres *et al.*, 1989). Furthermore, there appears to be an inverse relationship between the number of receptors expressed per cell and their affinity for TGF-ß (Wakefield *et al.*, 1987). It has been speculated that the expression of multiple forms of TGF-ß that have varying degrees of affinity for the different types of cell surface receptors may provide a means to finely tune the response of multicellular systems to these polypeptides (Cheifetz *et al.*, 1987; Ohta *et al.*, 1987).

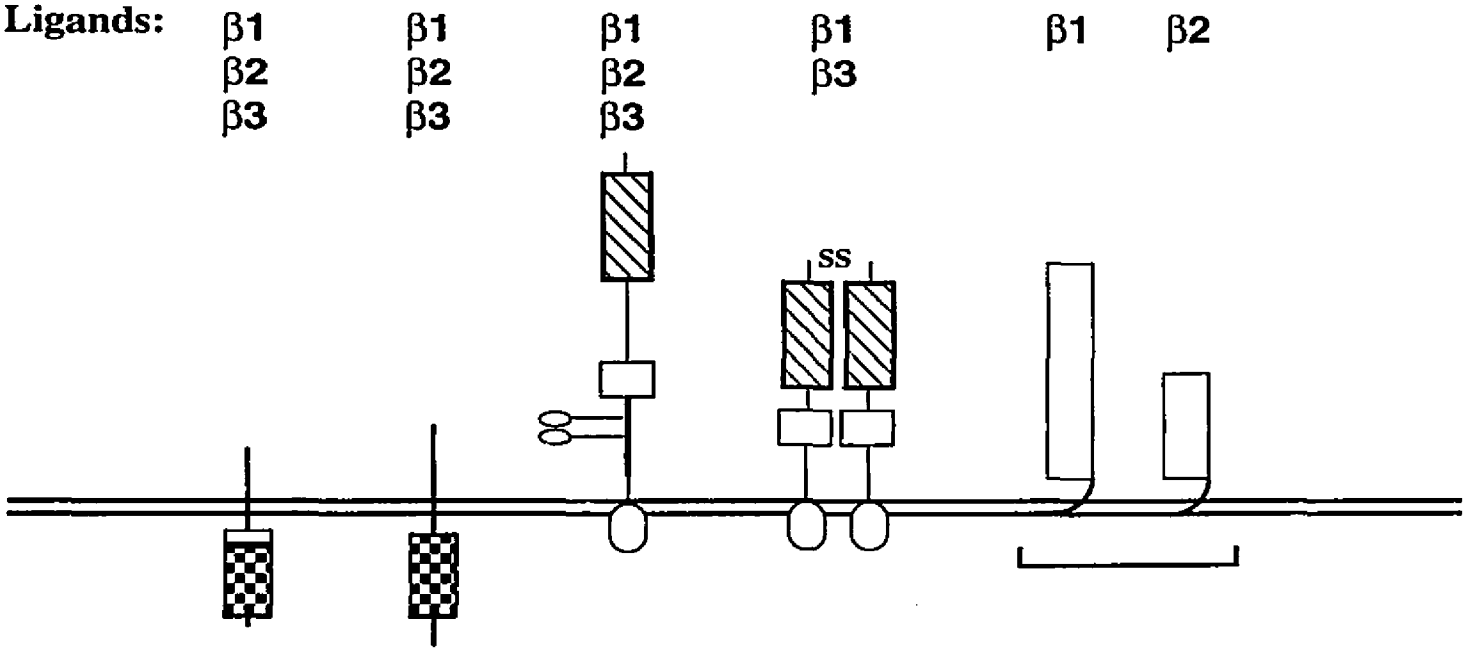
In addition to the three well characterized TGF-ß receptors, other membrane-bound proteins have been shown to bind TGF-ß in an isoform-specific manner. For example, proteins with membrane attachments sensitive to degradation by

phosphatidylinositol-specific phospholipase C (PI-PLC), which have high affinity for either TGF-ß1 or TGF-ß2, have been identified in some cell lines (Cheifetz and Massagué, 1991). These proteins are unique in that they appear to be attached to the membrane via a glycosyl-phosphatidylinositol (GPI)-anchor. Although they display affinity constants for TGF-ß in the subnanomolar range, their affinity for TGF-ß is lower than that of the type I and II receptors (Cheifetz and Massagué, 1991; see also Fig. 3). Another protein, known as endoglin, which is predominantly expressed in vascular endothelial cells, has been shown to bind TGF-ß1 and TGF-ß3, but not TGF-ß2 (Cheifetz *et al.*, 1992). Endoglin is a homodimeric membrane glycoprotein composed of disulfide-linked subunits of 95 kDa (Gougos and Letarte, 1990). Its cytoplasmic domain is remarkably similar (71% amino acid sequence identity) to that of the type III receptor, and there are also two regions of weaker similarity in the ectodomains of these proteins (Cheifetz *et al.*, 1992). As no signal transducing structure is discernible in its cytoplasmic domain, a role similar to that of the type III receptor has been proposed for endoglin (Yamashita *et al.*, 1994). However, its functions as well as those of the GPI-anchored TGF-ß binding proteins remain to be elucidated. For a summary of the membrane-bound TGF-ß binding proteins described in this section including the TGF-ß receptors, see Fig. 3.

1.4 TGF-ß in normal uterine growth and function

In addition to its multiple effects in many cell types, emerging evidence

Fig. 3. **Schematic representation of TGF-ß receptors and other membrane-bound TGF-ß binding proteins.** The type I, II, and III TGF-ß receptors as well as endoglin have been cloned. The type I and II receptors are transmembrane serine/threonine kinases (checkered boxes). The GS domain of the type I receptor is indicated by an empty rectangle. The type III receptor is a membrane proteoglycan which contains both heparan and chondroitin sulfate glycosaminoglycan chains (side chains + oval). Endoglin is a disulfide-linked dimeric peptide which binds TGF-ß1 and TGF-ß3, but not TGF-ß2. The three principal regions of homology between the type III receptor and endoglin are illustrated by the similar oval, shaded, and striped domain structures which represent regions of 71%, 48%, and 28% amino acid sequence identity, respectively. The GPI-anchored proteins are unique in that they display high affinity for only one of the TGF-ß isoforms and they can be released from the cell surface by treatment with PI-PLC. All these proteins display affinity constants for TGF-ß in the subnanomolar range.



Name:	**I**	**II**	**III**	**Endoglin**	**GPI-anchored**	
	53 K	**70-85 K**	**200-400 K**	**2 x 95 K**	**180 K**	**60 K**
K_d:	**2-50 pM**	**2-50 pM**	**50-300 pM**	**50 pM**	**0.2 nM**	**0.5-1 nM**

suggests that TGF-ß is a potential regulator of uterine growth and function (Tabibzadeh, 1991; Guidice, 1994; Giudice and Saleh, 1995). Studies have shown that all three mammalian TGF-ß isoforms are expressed in the mouse uterus during the periimplantation period suggesting that TGF-ß may play important roles in blastocyst implantation and in the decidualization of the endometrial stroma (Tamada *et al.*, 1990; Das *et al.*, 1992). In agreement with these findings is the immunohistochemical localization of TGF-ß in human implantation sites (Selick *et al.*, 1994), and evidence for the role of TGF-ß as a mediator of trophoblastic invasion of the endometrium (Lala and Graham, 1990). TGF-ß has also been identified at the fetal-maternal interface (Graham *et al.*, 1992), and both TGF-ß messenger ribonucleic acid (mRNA) and receptors have been identified in the placenta (Dungy *et al.*, 1991; Mitchell and O'Connor-McCourt, 1991; Mitchell *et al.*, 1992). These results provide further evidence for the role of TGF-ß in the regulation of endometrial-trophoblast interaction during implantation.

Recent studies have shown that human uterine cells of all types also express mRNA and protein for all three mammalian TGF-ß isoforms (Chegini *et al.*, 1994b; Tang *et al.*, 1994). According to these studies, there appears to be considerable variation in the expression of TGF-ß mRNAs and proteins in endometrial tissue throughout the menstrual cycle with the highest levels being expressed during the late proliferative and early to midsecretory phases. Hence, it has been suggested that TGF-ß may contribute to inhibition of cellular proliferation during periods in

which cellular differentiation is dominant, as is the case during the secretory phase of the menstrual cycle. There is also evidence that TGF-ß depresses both protein and DNA synthesis in endometrial epithelial cells, but stimulates DNA synthesis in endometrial stromal cells (Hammond *et al.*, 1993; Marshburn *et al.*, 1994; Tang *et al.*, 1994; Whitworth *et al.*, 1994). All these findings taken together suggest that TGF-ß may participate in the regulation of endometrial cell proliferation and differentiation *in vivo*.

TGF-ß may also affect uterine growth and function indirectly by regulating other growth factors and their receptors. For example, Casey *et al.* (1992) have reported that TGF-ß stimulates parathyroid hormone-related protein expression and secretion in human endometrial stromal cells. Although the physiological role for this hormone is currently undefined in the endometrium, Casey and co-workers (1992) have suggested that it may promote placental calcium transport. Since there is evidence that parathyroid hormone acts on human placental trophoblasts to promote an increase in epidermal growth factor receptors (Alsat *et al.*, 1991), parathyroid hormone-related protein may also interact with other growth factor systems.

1.5 TGF-ß in abnormal uterine growth and function

In addition to evidence for the role of TGF-ß in normal uterine growth and function, studies suggest that TGF-ß may also implicated in uterine

pathophysiologies such as endometriosis, endometrial hyperplasia, and endometrial cancer (Chegini *et al.*, 1994a; Gold *et al.*, 1994; Murphy, 1994).

Endometriosis is a disease characterized by the presence of viable endometrial cells and stroma outside their normal location within the uterine cavity. Although several theories relating to the etiology of endometriosis have been proposed, the development and progression of the disease remain poorly understood (Dmowski, 1991; Rock and Markham, 1992; Olive and Schwartz, 1993). There is, however, emerging evidence for the role of both growth factors (Surrey and Halme, 1991; Tabibzadeh, 1991; Zhang *et al.*, 1991), and the immune system (Hill, 1992) in the endometriotic process. Recent studies indicate that TGF-ß may be one of the growth factors involved in this disease process. Using a model of surgically induced endometriosis in the rat, Chegini and co-workers (1994a) have reported that ectopic endometrial implants contain immunoreactive TGF-ßs 1-3. There is also evidence that TGF-ß activity is increased in peritoneal fluid from women with endometriosis compared to both fertile and infertile control groups (Oosterlynck *et al.*, 1994). Interestingly, some of the effects of TGF-ß on the immune system, namely, its induction of macrophage growth factor production, its suppression of natural killer cell activity, and its inhibition of lymphocyte function and proliferation, are all effects also observed in patients with endometriosis (Halme *et al.*, 1987; Zhang *et al.*, 1991; Oosterlynck *et al.*, 1991, 1992; Gilmore *et al.*, 1992). This suggests that TGF-ß may be responsible for the alterations in immune function associated with

this disease.

In addition, TGF-ß regulates other processes such as extracellular matrix formation, chemotaxis, and angiogenesis which may also be relevant to the development and maintenance of endometriotic implants. Since extracellular matrices perform many important functions in controlling attachment, migration, proliferation, and differentiation of cells (Cybulsky *et al.*, 1990; Koochekpour *et al.*, 1995), TGF-ß's potent stimulatory effect on extracellular matrix protein synthesis may promote the attachment and growth of endometrial cells in the peritoneal cavity, while its angiogenic properties may promote maintenance and further outgrowth of these ectopic endometrial implants by enhancing vascularization.

TGF-ß has also been implicated in other uterine pathologies including endometrial hyperplasia and endometrial cancer. It is widely accepted that prolonged unopposed estrogenic stimulation of the uterus, whether as a result of endogenous estrogen overproduction or exogenous estrogen given as hormone replacement therapy, often leads to endometrial hyperplasia which may then progress to endometrial carcinoma (Silverberg, 1988; Fu *et al.*, 1990; Mencaglia *et al.*, 1990). Since the endometrium is a target tissue for the action of cytokines and growth factors, Gold and co-workers (1994) have recently investigated the role of TGF-ß in this process, and have found that the glandular epithelium demonstrates a significant stepwise increase in the expression of all three mammalian TGF-ß isoforms with progression from the normal proliferative endometrium to simple

hyperplasia and on to complex hyperplasia. Interestingly, no further increase in TGF-ß expression was observed from pre-neoplastic complex hyperplasia to carcinoma. Nonetheless, TGF-ß is expressed in many endometrial cancer cells (Boyd and Kaufman, 1990; Murphy *et al.*, 1991), and malignant transformation is often associated with reduced sensitivity to the inhibitory effects of TGF-ß. In fact, resistance to the growth inhibitory effects of TGF-ß appears to increase with malignant transformation and in some cancer cell lines, TGF-ß actually stimulates cell proliferation (Boyd and Kaufman, 1990; Anzai *et al.*, 1992; Croxtall *et al.*, 1992; Sakata *et al.*, 1993).

Despite the identification of TGF-ß in the human uterus (Kauma *et al.*, 1990; Chegini *et al.*, 1994b; Marshburn *et al.*, 1994), and evidence for the role of TGF-ß in both normal and abnormal uterine growth and function (see sections 1.4, 1.5, and references therein), the receptors for TGF-ß in this tissue have not been well defined. Although TGF-ß binding proteins have been purified from porcine uterus membrane preparations (Ichijo *et al.*, 1991), and specific ^{125}I-TGF-ß1 binding sites have recently been detected in the mouse uterus (Takahashi *et al.*, 1994), the only evidence for TGF-ß receptors in the human uterus comes from a study recently conducted by Chegini and co-workers (1994b) in which mRNA and protein for the type II receptor was identified by *in situ* hybridization and immunohistochemistry. Thus, the objective of this study was to identify and characterize the different types of TGF-ß receptors as well as any other TGF-ß binding proteins expressed on

human endometrial cells so as to better understand the role of the mammalian TGF-ß isoforms in uterine physiology and pathophysiology.

2. Materials and Methods

2.1 Cell culture

Human endometrial biopsies were obtained from premenopausal women undergoing diagnostic laparoscopy for unexplained infertility or suspected endometriosis. The biopsies collected from women with no obvious endometrial pathologies were minced into small pieces (~1 mm^3) using a razor blade, and were then dissociated by enzymatic digestion in nutrient medium containing 0.25% collagenase/dispase (Boehringer Mannheim, W. Germany). After a one hour incubation with agitation at 37°C, the suspension produced by collagenase digestion consisted of single stromal cells and fragments of epithelial glands. These two populations were separated by differential sedimentation at unit gravity as previously described by Kariya *et al.* (1991), with some modifications. Briefly, the cell suspension was placed in a 15 ml polypropylene conical tube and centrifuged at 300 x g for 10 minutes. The cells were then resuspended in 10 ml of nutrient medium and left in an upright position for 30 minutes to allow epithelial glands and cell aggregates to precipitate to the bottom. The supernatant, consisting of single

stromal cells, was transferred to a new tube, centrifuged at 300 x g for 10 minutes, and resuspended in as small a volume of nutrient medium as possible (~3-5 ml) so as to obtain a concentrated population of cells. The cells were then plated into 25 cm^2 plastic culture flasks. Erythrocytes and contaminating epithelial cells were removed by changing the medium within one hour of plating (after stromal cells had adhered to the flask). The human endometrial epithelial cell line, RL95-2 was obtained from the American Type Culture Collection (Rockville, MD). All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air using nutrient medium consisting of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10 μg/ml bovine insulin, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B (Gibco, Burlington, Ontario).

2.2 Immunofluorescence and flow cytometric studies

The purity and homogeneity of the stromal cell preparations was assessed by immunofluorescence microscopic studies using a mouse monoclonal antibody to human Thy-1 (Serotec, Toronto, Ontario), and a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody to human cytokeratin (Dako, Mississauga, Ontario) as follows. Coverslip cultures of purified stromal cells were rinsed twice with cold Dulbecco's phosphate-buffered saline (D-PBS), fixed in 100% methanol for 20 min at -20 °C, and permeabilized with acetone for 1-2 minutes at the same

temperature. After permeabilization, cultures were rinsed twice with D-PBS containing 1% bovine serum albumin (BSA), and incubated in D-PBS containing 3% BSA for 30 minutes at room temperature to block non-specific binding. Coverslips were then transferred to a humidified chamber and incubated with appropriate dilutions (1:100) of the above-mentioned antibodies for 1 hour. Following three washes with D-PBS containing 1% BSA, the cultures previously incubated with anti-Thy-1 were incubated for an additional hour in the dark using rat anti-mouse IgG-phycoerythrin (Biomeda Corp., Foster City, CA) as the secondary antibody. After extensive washing, all cultures were mounted on glass microscope slides and examined on a Leitz microscope equipped with fluorescence. Routine controls included incubation with normal serum in place of the primary antibody, and omission of the primary antibody to control for non-specific binding of the conjugate.

Flow cytometric analysis was carried out using an EPICS$^{(R)}$ Profile Analyzer. For detection of the Thy-1 cell surface glycoprotein, live cell suspensions were incubated with the above-mentioned anti-human Thy-1 antibody or an ascites fluid control at a concentration of 5 $\mu g/10^6$ cells for 1 hour at 4°C. The cells were then pelleted by centrifugation at 300 x g for 10 minutes, washed with D-PBS, and incubated for an additional hour at 4°C with rat anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) at a concentration of 5 $\mu g/10^6$ cells. After two washes with D-PBS, the cells were resuspended in 500 μl of D-

PBS and analyzed. For detection of intracellular cytokeratins, thoroughly washed cells in D-PBS were fixed by adding absolute ethanol (-20°C) drop by drop to a final concentration of 70% ethanol in D-PBS while slowly vortexing, and incubated overnight at -20°C. The cells were then washed with D-PBS, pelleted, and resuspended in D-PBS containing 4% heat-inactivated FBS. Non-specific binding was blocked by incubating the cells with mouse IgG whole molecule (50 μg/10^6 cells) for 30 minutes at room temperature. Cells were then incubated either with the above-mentioned FITC-conjugated mouse anti-human cytokeratin or an FITC-conjugated mouse IgG1 negative control for 1 hour at room temperature with continuous gentle agitation. Following extensive washing, cells were resuspended in D-PBS and analyzed.

2.3 Iodination of TGF-ß and affinity labeling of cells

Recombinant TGF-ß1 (Bristol Myers-Squibb Pharmaceutical Research Institute, Seattle, WA) and recombinant TGF-ß2 (Celtrix Pharmaceuticals Inc., Santa Clara, CA) were iodinated using the chloramine-T method of Frolik *et al.* (1984) as previously described by Philip and O'Connor-McCourt (1991). Affinity labeling studies were performed as described by Mitchell *et al.* (1992). Briefly, cells from stock culture flasks were trypsinized and plated into 12-well plates at a density of 4-6 x 10^5 cells/ml. Approximately 48 hours after plating, confluent cell monolayers were washed three times with ice-cold binding buffer (D-PBS with 0.5 mM Ca^{+2} and

1 mM Mg^{+2}, pH 7.4, containing 0.1% BSA) over 30 minutes, and incubated with either 100 pM ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2, in the absence or presence of varying concentrations of unlabeled TGF-ß1 or TGF-ß2 for 3 hours - the time required for the formation of ligand-receptor complexes under equilibrium binding conditions. This incubation was carried out at 4°C with continuous gentle agitation. The receptor-ligand complexes were then cross-linked with 400 µl of 1 mM Bis(sulfosuccinimidyl)suberate (Pierce, Rockford, Il) on ice. After 10 minutes, the reaction was stopped with the addition of 100 µl of 500 mM glycine and a further 5 minute incubation. Cells were washed twice with D-PBS, and solubilized in 20 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 10 µg/ml benzamidine (solubilization buffer). Solubilized material was recovered from each well. One-fifth volume of 5x electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, and trace bromophenol blue) was added to each sample. The samples were then divided into equal volumes and analyzed under both reducing and non-reducing conditions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. SDS-PAGE was performed by the method of Laemmli (Laemmli and Favre, 1973), with 3-11% polyacrylamide gradient gels. Following electrophoresis, gels were stained with Coomassie Brilliant Blue, destained, dried, and exposed to Kodak Omat AR film at -80°C with use of Dupont Cronex Lightning Plus intensifying screens. The apparent relative molecular weights

of the proteins were estimated using ^{14}C-labeled molecular mass standards which included lysozyme (14 kDa), ß-lactoglobulin (18 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (68 kDa), phosphorylase B (97 kDa), and myosin heavy chain (200 kDa).

2.4 Treatment of cells with PI-PLC or dithiothreitol (DTT)

Confluent cell monolayers were washed three times over 30 minutes with D-PBS containing 0.5% BSA, twice with protein-free D-PBS, and then incubated at 37°C with either 0.3 U/ml of PI-PLC (Boehringer Mannheim, W. Germany) for 30 minutes or 1mM DTT for 5 minutes. Following incubation, the cells were washed twice with ice-cold D-PBS containing 0.1% BSA, and affinity-labeled with ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2 as described above.

2.5 Immunoprecipitation of TGF-ß receptors

Anti-peptide antibodies against the type II and type III TGF-ß receptors were a gift from Dr. A. Moustakas (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). Immunoprecipitations were carried out as follows. Cells were affinity labeled with 200 pM of either ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2 as described above. After a 30 minute incubation with solubilization buffer, cell lysates were centrifuged at 5000 x g for 10 minutes. Aliquots of the supernatants were incubated overnight at 4°C with antibodies specific for either the type II or

type III TGF-ß receptors in the absence or presence of equimolar amounts (3 µg/ml) of the respective immunizing peptides. Other supernatant aliquots were boiled 5 minutes in the presence of 1% SDS prior to incubation with the antibodies. Immune complexes were then incubated with 50 µl of a protein A-Sepharose slurry (50% packed beads in D-PBS containing 0.2% Triton X-100) at 4°C for 2 hours. The beads were pelleted by centrifugation and washed thoroughly with D-PBS containing 0.2% Triton X-100. The immune complexes were resuspended in 1x sample buffer containing 5% ß-mercaptoethanol, boiled for 5 minutes, and analyzed by SDS-PAGE and autoradiography.

2.6 Pre-incubation of cells with TGF-ß

Cells from stock culture flasks were trypsinized and plated into 12-well plates. After 24 hours, the cells were washed twice with D-PBS and incubated under both serum and insulin free conditions in the absence or presence of either 100 pM of TGF-ß1 or TGF-ß2 for 16 hours. All incubations were carried out at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Appropriate vehicle controls were included in each experiment. Following pre-incubation with TGF-ß, the cells were washed extensively with D-PBS containing 0.1% BSA to allow dissociation of any pre-bound TGF-ß. The cells were then affinity labeled with either 100 pM of ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2 as described above (section 2.3).

2.7 Equilibrium saturation binding assay and Scatchard analysis

Equilibrium saturation binding assays were carried out in a manner similar to the affinity labeling assays described above (section 2.3), with the following modifications. Cell monolayers in 24-well plates were washed three times over 30 minutes with ice cold binding buffer (D-PBS, pH 7.4, containing 0.3% BSA), and incubated with varying concentrations of either ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2 ranging between 1 and 500 pM. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled TGF-ß. Duplicate wells were used for each condition. At the end of a 3 hour incubation period at 4°C, the binding buffer was collected to measure the concentration of free ^{125}I-TGF-ß, and the cells were quickly washed with D-PBS. Bound counts were then solubilized in 300 µl of 20 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100, 10% glycerol, and 1 mM EDTA, for 30 minutes at 4°C with gentle agitation. Cell number was determined using a hemocytometer on wells treated as above except that trypsinization substituted for the solubilization step. Binding data were analyzed by the method of Scatchard (1949) using GraphPad Prism software. The equation Y=Bmax*X/(Kd+X) was used for analysis of one site binding, while the equation Y=Bmax1*X/(Kd1+X) + Bmax2*X(Kd2+X) was used for analysis of two site binding. Goodness-of-fit for both linear and non-linear regressions of Scatchard transformations was assessed by the value r^2.

2.8 [^{3}H]-Thymidine incorporation assay

Human endometrial stromal cells and RL95-2 cells were plated into 24-well dishes at a density such that at the time of TGF-ß addition the cells were semi-confluent. Approximately 24 hours after plating, the cells were washed with D-PBS and incubated under serum and insulin free conditions in the absence and presence of varying concentrations (0.1-500 pM) of TGF-ß1 or TGF-ß2, in triplicate, for either 24 hours (RL95-2) or 48 hours (stromal). [Methyl-^{3}H]-thymidine (Amersham, Life Science, 2 µCi/ml of medium) was added to RL95-2 cells during the last 3 hours of the 24 hour test period, and to stromal cells either during the last 24 hours of the 48 hour test period, or during the entire 48 hour test period in the presence of 2% FBS. At the end of labeling, the cells were placed on ice and washed three times with 200 mM NaCl - 50 mM Tris buffer, three times with 10% ice cold trichloroacetic acid, and finally, three times with 95% ethanol. The cells were then air dried, solubilized with 1% SDS, and the incorporated radioactivity measured in a liquid scintillation counter using UniverSol™ scintillation cocktail (ICN Biomedicals, Montréal, Québec).

3. Results

3.1 Evaluation of stromal cell preparations

The purity and homogeneity of the stromal cell preparations was assessed by immunofluorescence microscopic studies using a mouse monoclonal antibody to human Thy-1 which labels a cell surface glycoprotein present on stromal cells, but not on epithelial cells ernández-Shaw *et al.*, 1992), and a mouse monoclonal antibody to human cytokeratin which labels a wide range of intermediate filaments (including keratin Nos 5, 6, 8, 17 and 19) in epithelial cells, but not in stromal cells (Matthews *et al.*, 1992). As indicated in Fig. 4, the stromal cell preparation stains positively for the Thy-1 stromal marker. Although specific cell surface staining is not apparent on fixed cells such as these, such staining was observed on live cell preparations (data not shown). No staining was observed with the cytokeratin antibody, indicating the absence of epithelial contamination (data not shown).

These results were confirmed quantitatively by flow cytometry which indicates that 99.9 % of the stromal cell population is positive for the stromal marker, Thy-1 (Fig. 5, gate 3), while 97.9 % is negative for cytokeratin (Fig. 6, gate 1). The RL95-2 endometrial epithelial cell line was used as a positive control for cytokeratin staining, and as demonstrated in Fig. 7 (gate 2), 99.2 % of this cell population is positive for cytokeratin.

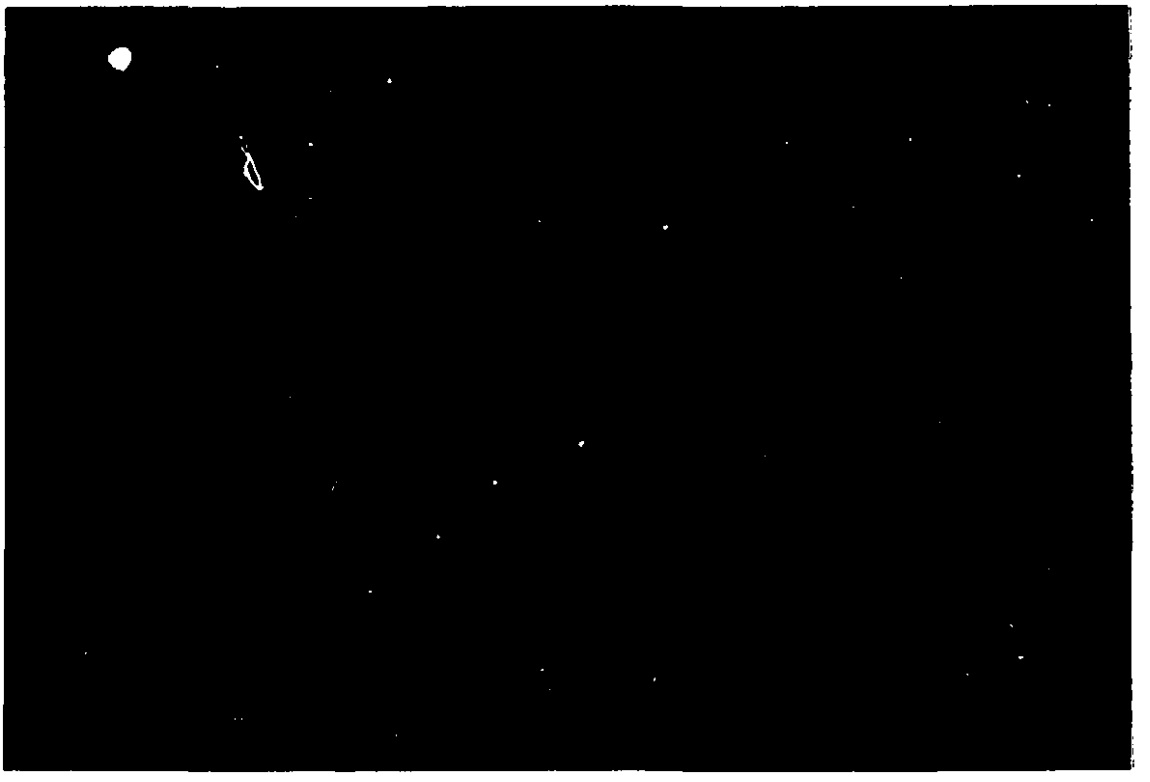
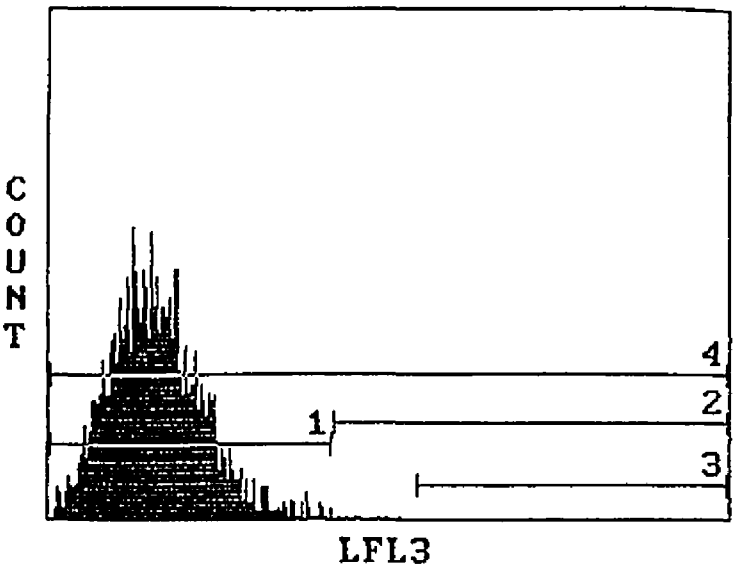


Fig. 4. **Immunofluorescence micrograph of human endometrial stromal cells stained with an antibody to human Thy-1, x250.**

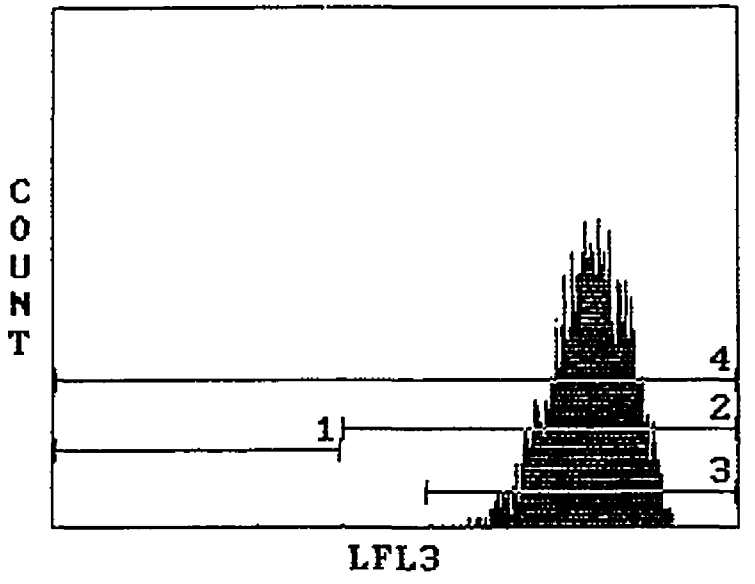
Fig. 5. **Flow cytometric analysis of human endometrial stromal cells using a stromal marker.** Live cell suspensions were labeled either with an ascites-FITC control (A), or with anti-human Thy-1-FITC (B) and analyzed on an EPICS$^{(R)}$ Profile Analyzer.

A.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	4.806	9971	99.7	0.442	0.295	17.4
2	4.810	1023.	29	0.3	6.555	1.553	0.38
3	15.49	1023.	0	0.0			
4	0.102	1023.	10000	100.0	0.446	0.304	17.4

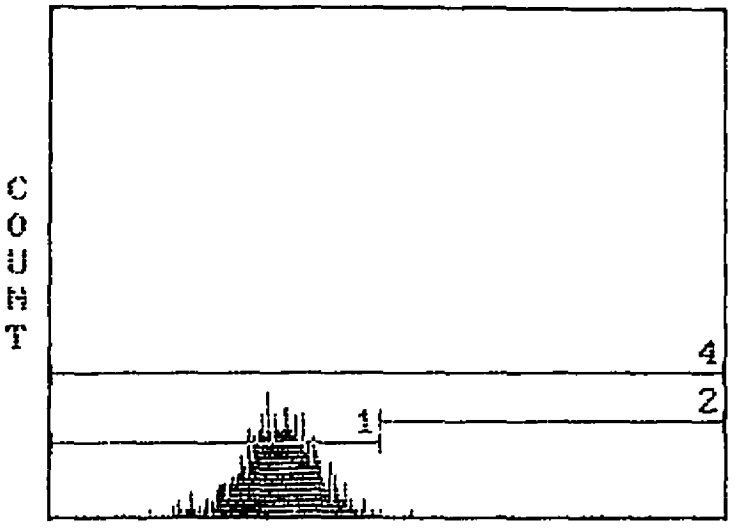
B.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	4.806	2	0.0	0.413	0.577	0.38
2	4.810	1023.	9998	100.0	139.3	72.6	27.5
3	15.49	1023.	9991	99.9	139.5	72.0	27.5
4	0.102	1023.	10000	100.0	139.1	73.5	27.5

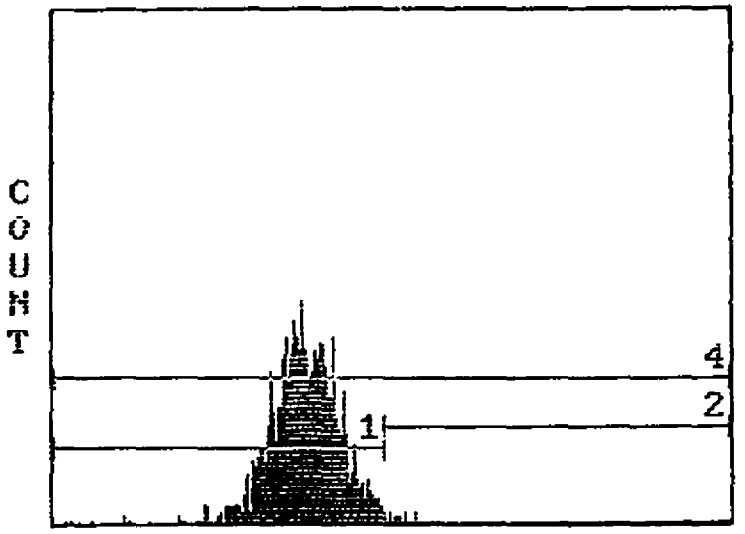
Fig. 6. **Flow cytometric analysis of human endometrial stromal cells using an epithelial marker.** Fixed cell suspensions were labeled either with an IgG1-FITC control (A), or with anti-human cytokeratin-FITC (B) and analyzed on an EPICS$^{(R)}$ Profile Analyzer.

A.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	9.351	3493	98.1	2.313	1.280	11.7
2	9.359	1023.	67	1.9	37.19	54.21	0.38
4	0.102	1023.	3560	100.0	2.437	1.704	11.7

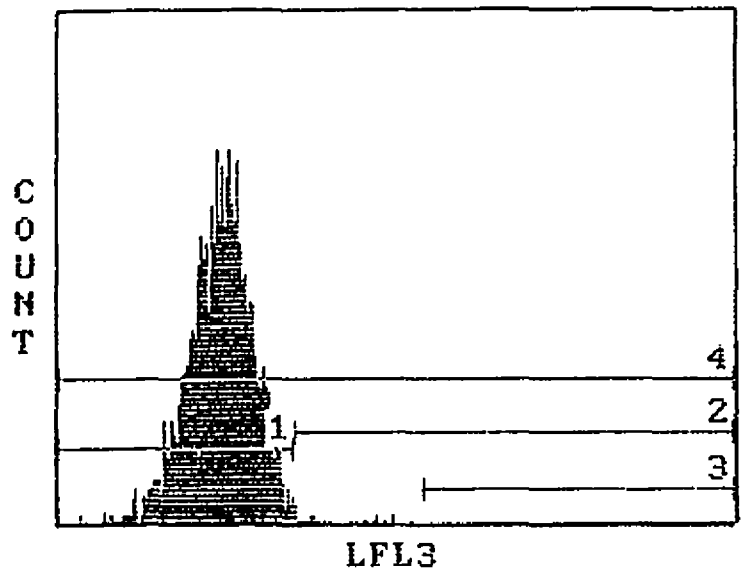
B.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	9.351	5927	97.9	3.071	1.552	26.4
2	9.359	1023.	129	2.1	15.63	14.60	0.52
4	0.102	1023.	6056	100.0	3.179	1.815	26.4

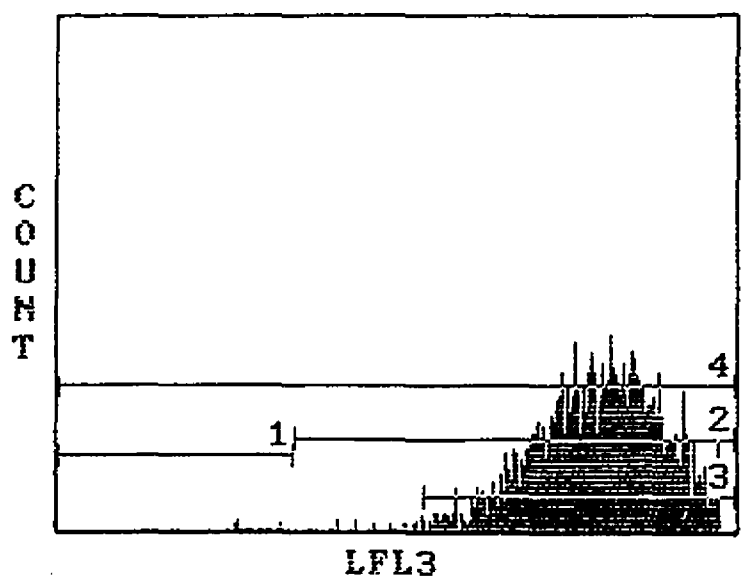
Fig. 7. **Flow cytometric analysis of RL95-2 cells using an epithelial marker.** Fixed cell suspensions were labeled either with an IgG1-FITC control (A), or with anti-human cytokeratin-FITC (B) and analyzed on an EPICS$^{(R)}$ Profile Analyzer.

A.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	2.561	9908	99.1	0.931	0.416	20.0
2	2.609	1023.	84	0.8	4.042	1.810	93.0
3	15.49	1023.	0	0.0			
4	0.102	1023.	10000	100.0	0.943	0.441	20.0

B.



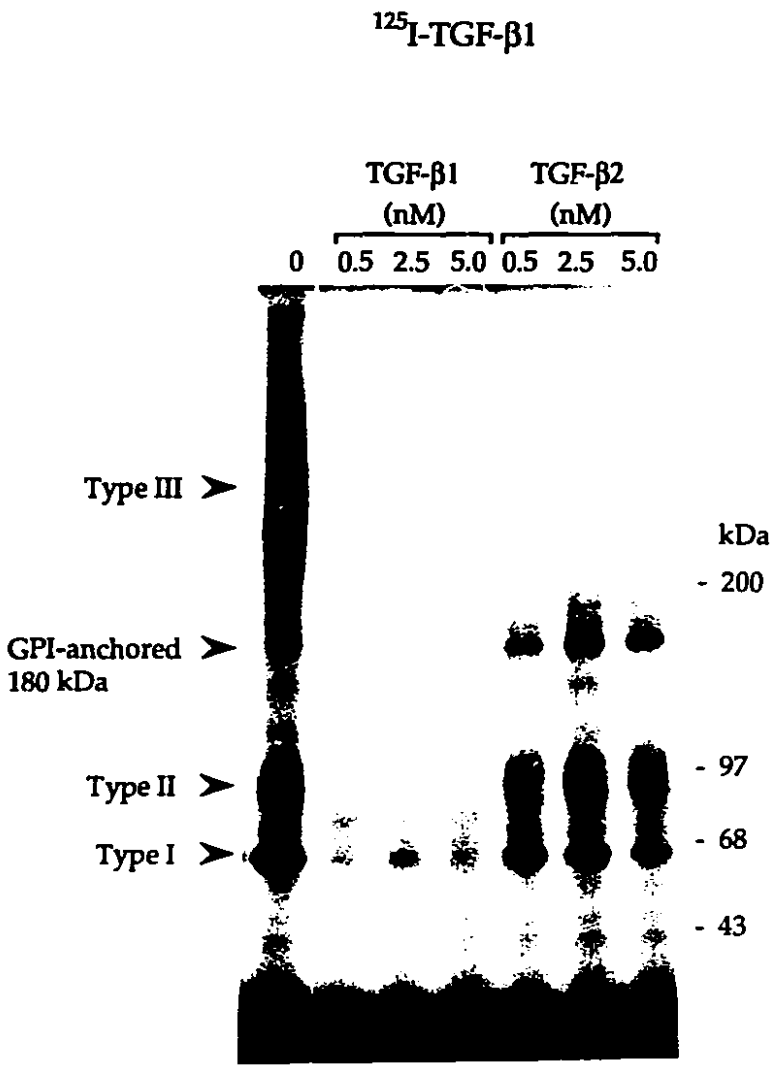
	MIN	MAX	COUNT	PERCENT	MEAN	SD	%HPCV
1	0.102	2.561	81	0.8	1.088	0.797	0.48
2	2.609	1023.	9917	99.2	156.8	153.7	16.7
3	15.49	1023.	9691	96.9	168.0	144.4	16.7
4	0.102	1023.	10000	100.0	150.5	164.5	16.7

3.2 Affinity labeling of cell surface TGF-ß binding proteins

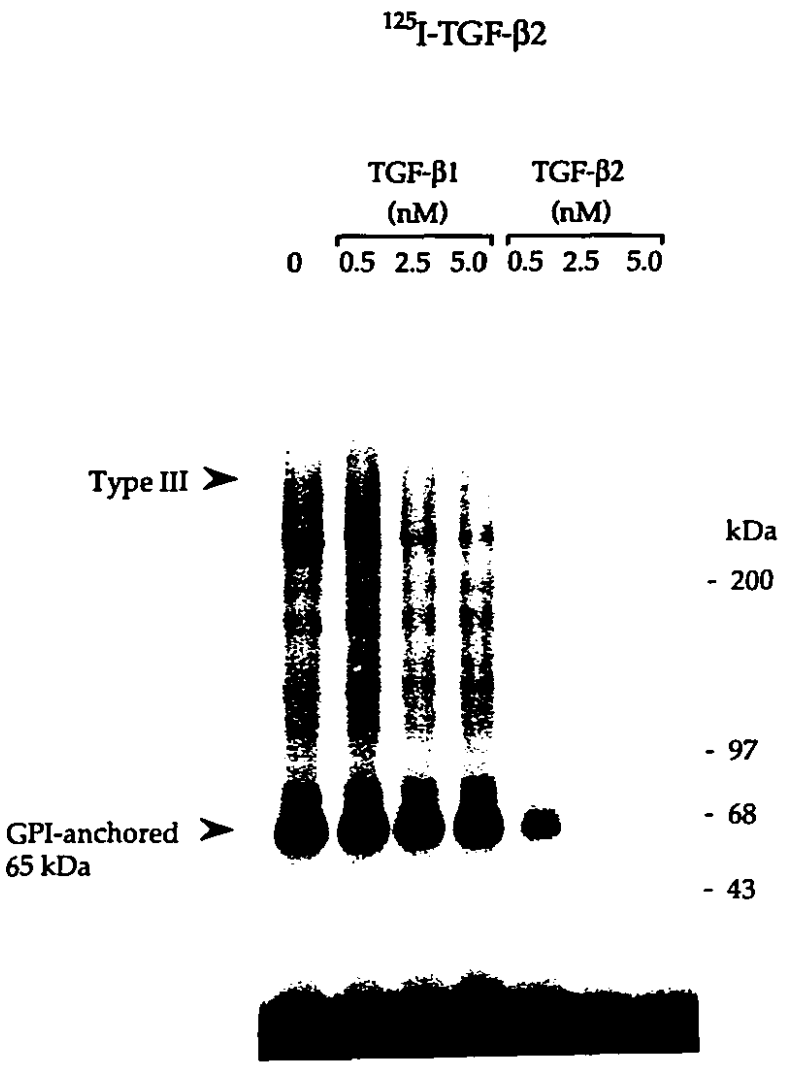
Affinity labeling of human endometrial stromal cells with 100 pM of ^{125}I-TGF-ß1 reveals four binding complexes with apparent relative molecular weights of 65, 85, 180 and 200-300 kDa. As illustrated in the autoradiogram in Fig. 8A, competition experiments using unlabeled TGF-ß1 and TGF-ß2 indicate that 0.5 nM of unlabeled TGF-ß1 markedly blocks the labeling of the 65 and 85 kDa binding complexes while ten times the amount (5 nM) of unlabeled TGF-ß2 only slightly blocks their labeling. This indicates that these two binding complexes display a much higher affinity for TGF-ß1 than for TGF-ß2. In contrast, the 200-300 kDa binding complex binds strongly to both TGF-ß isoforms. The apparent relative molecular weights, isoform specificity, and migration patterns of the 65, 85, and 200-300 kDa binding complexes are characteristic of the type I, II and III TGF-ß receptors that have been previously described on most cell types (Cheifetz *et al.*, 1986, 1988b; Segarini *et al.*, 1989; Massagué, 1990). Under reducing conditions, the type I receptor/TGF-ß complex migrates as a binding complex of approximately 65 kDa (53 kDa for the receptor plus 12.5 kDa for the TGF-ß monomer). Also noteworthy is the diffuse banding pattern of the 200-300 kDa binding complex which is a distinctive feature of the type III receptor proteoglycan (Cheifetz *et al.*, 1988a; Massagué *et al.*, 1992). In addition, the competition experiments demonstrate that the 180 kDa binding complex, later identified as a GPI-anchored protein (see section 3.3), binds TGF-ß1 but not TGF-ß2.

Fig. 8. **Affinity labeling of TGF-ß binding proteins on human endometrial stromal cells.** Cells were affinity-labeled with 100 pM ^{125}I-TGF-ß1 (A) or 100 pM ^{125}I-TGF-ß2 (B), in the absence and presence of the indicated concentrations of unlabeled TGF-ß1 or TGF-ß2. Samples were analyzed by SDS-PAGE under reducing conditions. The type I, II, and III TGF-ß receptors, the 65 and 180 kDa GPI-anchored TGF-ß binding proteins, and the molecular weight standards are indicated in the margins.

A.


^{125}I-TGF-β1
TGF-β1 (nM)
TGF-β2 (nM)
0 0.5 2.5 5.0 0.5 2.5 5.0
Type III
GPI-anchored 180 kDa
Type II
Type I
kDa
- 200
- 97
- 68
- 43

B.


^{125}I-TGF-β2
TGF-β1 (nM)
TGF-β2 (nM)
0 0.5 2.5 5.0 0.5 2.5 5.0
Type III
GPI-anchored 65 kDa
kDa
- 200
- 97
- 68
- 43

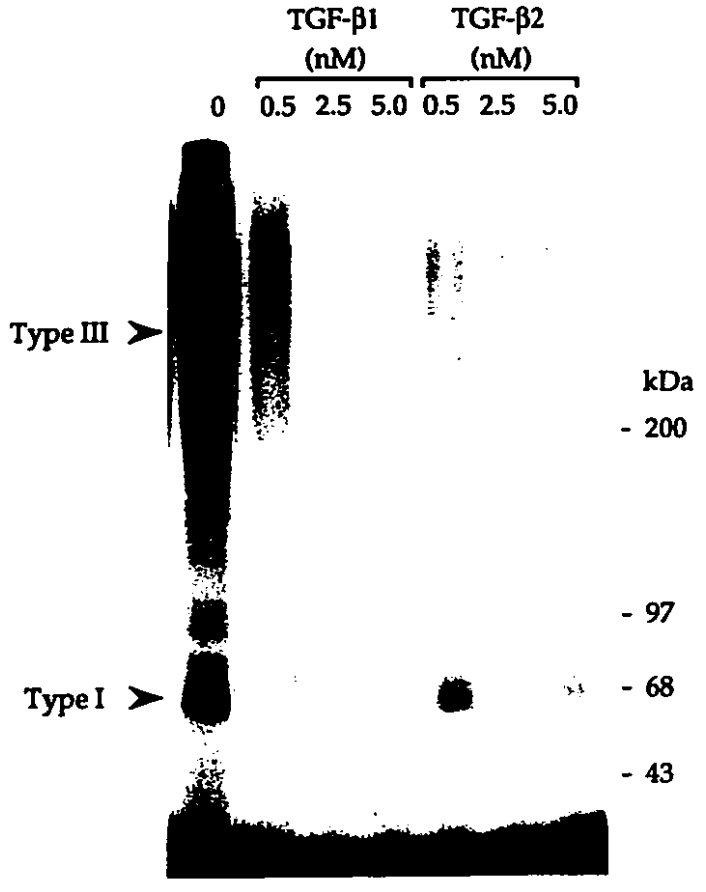
When ^{125}I-TGF-ß2 is used as the radiotracer, a prominent binding complex of 65 kDa, and a weakly labeled binding complex of 200-300 kDa are observed (Fig. 8B). The 85 and 180 kDa binding complexes are not detectable, which is consistent with their low affinities for TGF-ß2. Although the 200-300 kDa binding complex displays similar affinity for both TGF-ß isoforms when it is labeled with ^{125}I-TGF-ß1, it binds TGF-ß2 preferentially when ^{125}I-TGF-ß2 is used as a radiotracer. This suggests that there may be two distinct subtypes of the type III receptor with different affinities for TGF-ß1 and TGF-ß2 expressed on stromal cells. Interestingly, subtypes of the type I, II, and III TGF-ß receptors have been observed on human placental trophoblast cells (Mitchell et al., 1992). As for the 65 kDa binding complex, when it is labeled with ^{125}I-TGF-ß2, it behaves differently from the type I receptor since it binds TGF-ß2 but not TGF-ß1, as demonstrated by the inability of TGF-ß1 to compete for binding. In fact, this protein was later identified as a GPI-anchored TGF-ß binding protein (see section 3.3).

On the human endometrial epithelial cell line, RL95-2, affinity labeling with both ^{125}I-TGF-ß1 (Fig. 9A) and ^{125}I-TGF-ß2 (Fig. 9B) reveals two binding complexes with apparent relative molecular weights of 65 and 200-300 kDa. As on the stromal cell preparations, the competition experiments show that the 200-300 kDa binding complex displays similar affinity for both TGF-ß isoforms when it is labeled with ^{125}I-TGF-ß1, but binds TGF-ß2 preferentially when ^{125}I-TGF-ß2 is used as a radiotracer.

Fig. 9. **Affinity labeling of TGF-ß binding proteins on RL95-2 cells.** Cells were affinity-labeled with 100 pM ^{125}I-TGF-ß1 (A) or 100 pM ^{125}I-TGF-ß2 (B), in the absence and presence of the indicated concentrations of unlabeled TGF-ß1 or TGF-ß2. Samples were analyzed by SDS-PAGE under reducing conditions. The type I and III TGF-ß receptors, and the molecular weight standards are indicated in the margins.

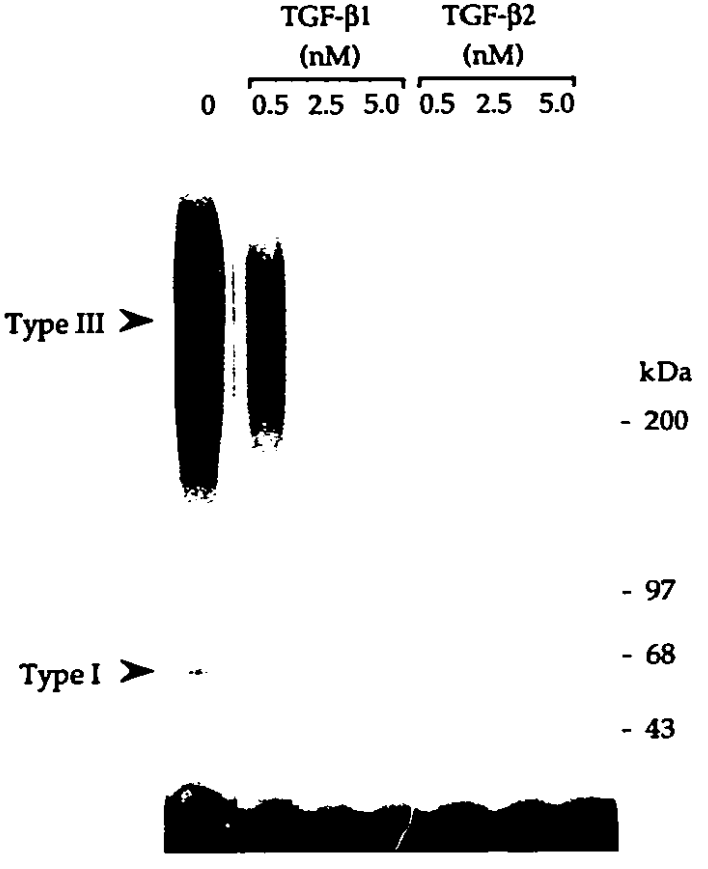
A.

^{125}I-TGF-β1



B.

^{125}I-TGF-β2



The 65 kDa binding complex, when it is labeled with ^{125}I-TGF-ß1, displays higher affinity for TGF-ß1 than for TGF-ß2. In contrast to the stromal cells, the 65 kDa binding complex is very poorly labeled when ^{125}I-TGF-ß2 is used as the radiotracer, as expected for the type I receptor.

The autoradiograms shown in Figures 8 and 9 are of polyacrylamide gels run under reducing conditions. There were no changes in the affinity labeling patterns of either cell type when the gels were run under non-reducing conditions (data not shown). Therefore, all subsequent autoradiograms shown are of reducing SDS-PAGE.

3.3 Effect of PI-PLC and DTT on affinity labeling of TGF-ß binding proteins

Although most membrane proteins are inserted into the membrane via a transmembrane domain, a number of proteins have been described which have a GPI anchor (Low, 1989). GPI anchors are phospholipid attachments sensitive to degradation by PI-PLC. Thus, treatment with PI-PLC results in the cleavage of the phospholipid anchor and the release of such proteins from the cell surface. TGF-ß binding proteins with GPI anchors, including ones of 60 and 180 kDa, have been described in some cell lines (Cheifetz and Massagué, 1991). Therefore, in order to determine whether any of the TGF-ß binding proteins identified on human endometrial cells are anchored to the membrane in this manner, the cells were treated with PI-PLC prior to affinity labeling. In addition, since brief treatment of

cells with 1 mM DTT has been shown to completely prevent labeling of the type I TGF-ß receptor without decreasing labeling of the type II or III receptors (Cheifetz and Massagué, 1991), the effect of DTT on affinity labeling of TGF-ß binding proteins was also assessed. As illustrated by the autoradiogram in Fig. 10A, when endometrial stromal cells are labeled with ^{125}I-TGF-ß1, the 180 kDa binding complex is sensitive to PI-PLC treatment. None of the other ^{125}I-TGF-ß1 labeled complexes are affected by the PI-PLC treatment. Similarly, the only binding complex sensitive to DTT is the 65 kDa complex. In contrast, when the cells are labeled with ^{125}I-TGF-ß2 (Fig. 10B), the observed 65 kDa binding complex is insensitive to DTT, but sensitive to PI-PLC. The ^{125}I-TGF-ß2 labeled 200-300 kDa binding complex is unaffected by either treatment. Thus, human endometrial stromal cells express the type I, II, and III TGF-ß receptors, a 180 kDa GPI-anchored TGF-ß1 binding protein, and a 65 kDa GPI-anchored TGF-ß2 binding protein which co-migrates with the type I receptor.

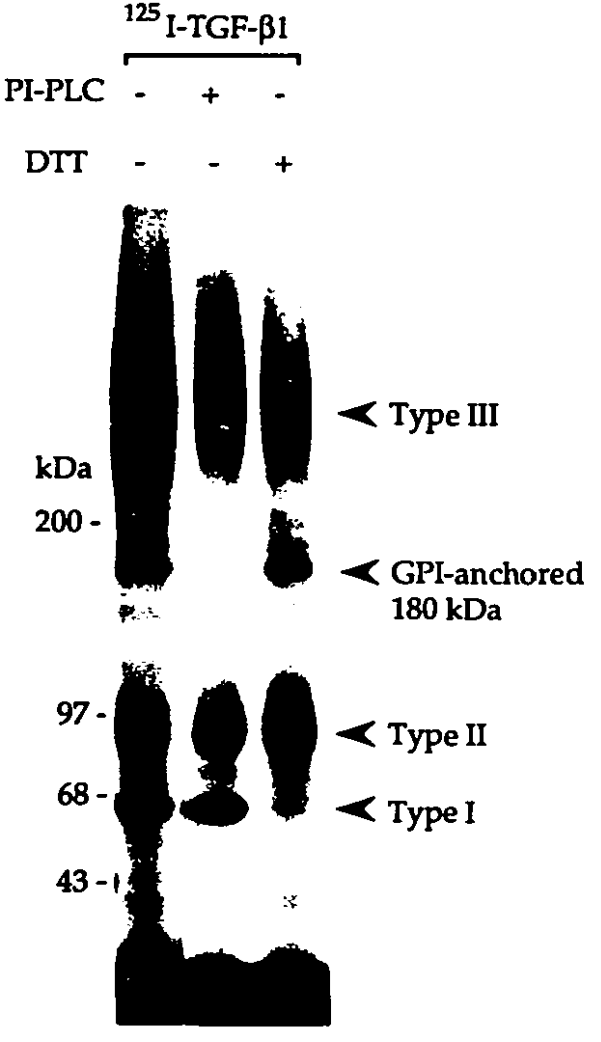
As shown in Fig. 11A & B, when RL95-2 cells are labeled with either ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2, neither the 65 or 200-300 kDa complexes are PI-PLC sensitive indicating that they are not GPI-anchored TGF-ß binding proteins. The 65 kDa complex is DTT sensitive, regardless of which isoform is used for labeling.

3.4 Immunoprecipitation of TGF-ß receptors

In order to confirm that the 85 and 200-300 kDa binding complexes observed

Fig. 10. **Effect of PI-PLC and DTT on affinity labeling of TGF-ß binding proteins in human endometrial stromal cells.** Cells were pretreated with PI-PLC or DTT, as indicated, and affinity-labeled with 100 pM ^{125}I-TGF-ß1 (A), or 100 pM ^{125}I-TGF-ß2 (B). Samples were analyzed by SDS-PAGE under reducing conditions. The type I, II, and III TGF-ß receptors, the 65 and 180 kDa GPI-anchored TGF-ß binding proteins, and the molecular weight standards are indicated in the margins.

A.


125 I-TGF-β1
PI-PLC - + -
DTT - - +
Type III
kDa
200 -
GPI-anchored
180 kDa
97 -
Type II
68 -
Type I
43 -

B.

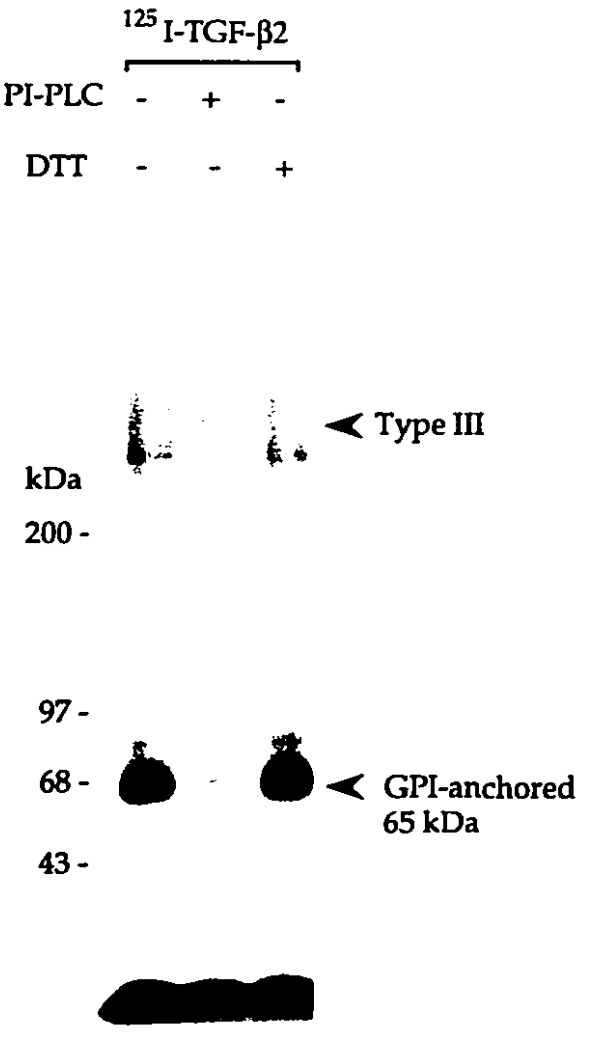
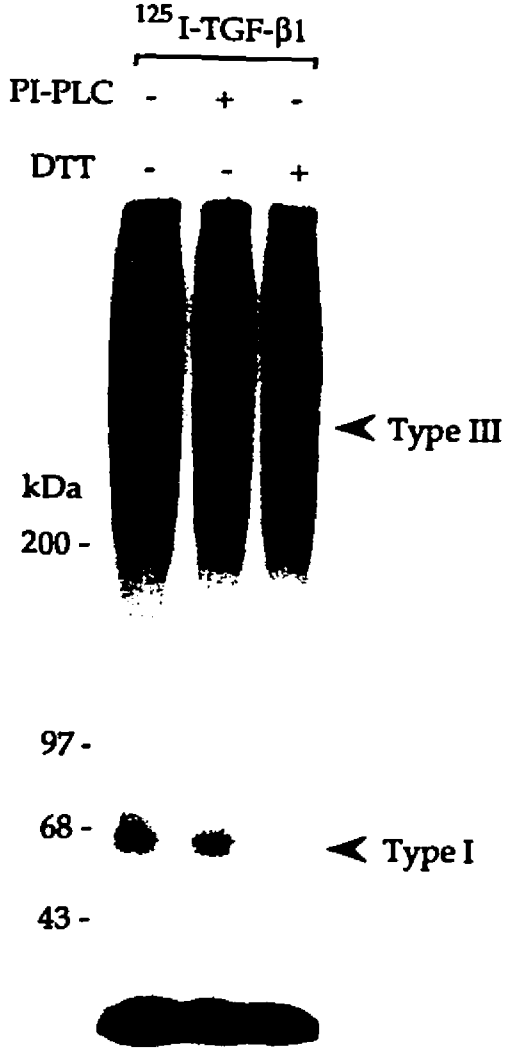

125 I-TGF-β2
PI-PLC - + -
DTT - - +
Type III
kDa
200 -
97 -
68 -
GPI-anchored
65 kDa
43 -

Fig. 11. **Effect of PI-PLC and DTT on affinity labeling of TGF-ß binding proteins in RL95-2 cells.** Cells were pretreated with PI-PLC or DTT, as indicated, and affinity labeled with 100 pM ^{125}I-TGF-ß1 (A), or 100 pM ^{125}I-TGF-ß2 (B). Samples were analyzed by SDS-PAGE under reducing conditions. The type I and III TGF-ß receptors, and the molecular weight standards are indicated in the margins.

A.


125I-TGF-β1
PI-PLC - + -
DTT - - +
Type III
kDa
200 -
97 -
68 -
Type I
43 -

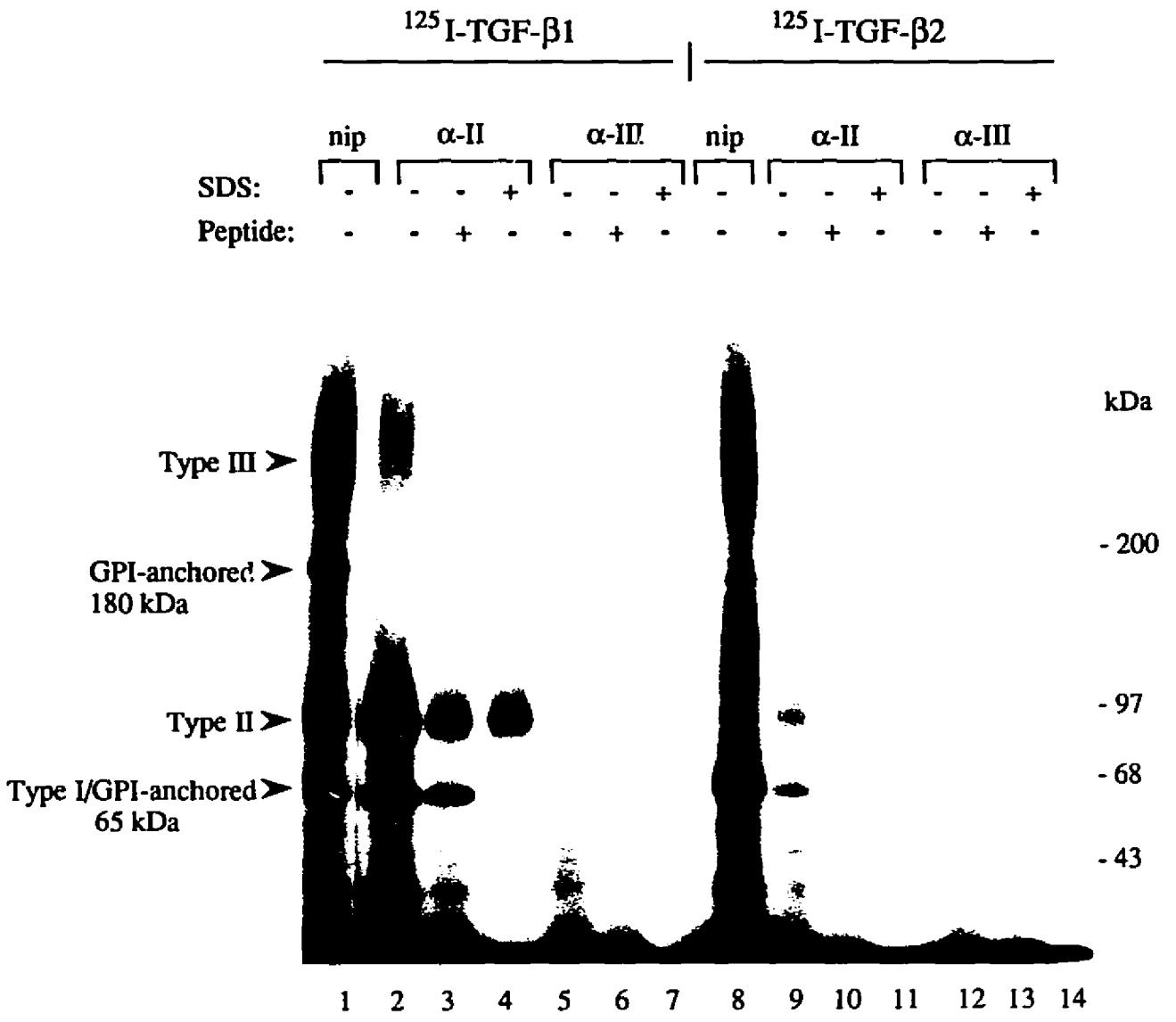
B.


125I-TGF-β2
PI-PLC - + -
DTT - - +
Type III
kDa
200 -
97 -
68 -
Type I
43 -

on human endometrial stromal cells do in fact correspond to the type II and III TGF-ß receptors, proteins cross-linked to ^{125}I-TGF-ß1 and ^{125}I-TGF-ß2 were immunoprecipitated with specific anti-type II and anti-type III TGF-ß receptor antibodies. As illustrated in Fig. 12 (lane 2), an anti-type II receptor antibody, which was generated against a C-terminal epitope of the type II TGF-ß receptor, immunoprecipitates the ^{125}I-TGF-ß1 labeled type I and type III TGF-ß receptors in addition to the 85 kDa type II receptor. Immunoprecipitation in the presence of equimolar amounts (3 µg/ml) of the immunizing peptide reduces the amount of protein being immunoprecipitated indicating that the complexes are being immunoprecipitated specifically (Fig. 12, lane 3). As illustrated in Fig. 12 (lane 4), denaturation of cell extracts with SDS prior to immunoprecipitation prevents the precipitation of the type I and III receptors along with the type II receptor. This result indicates that the 85 kDa type II receptor is being immunoprecipitated directly, while the type I and III receptors are being co-immunoprecipitated as heteromeric complexes with the type II receptor.

When the cells are labeled with ^{125}I-TGF-ß2, the anti-type II TGF-ß receptor antibody also immunoprecipitates the type II and III receptors, and an ^{125}I-TGF-ß2 labeled 65 kDa binding complex (Fig. 12, lane 9). Since there is evidence indicating that the 65 kDa GPI-anchored protein observed in other cells does not co-precipitate with the type II receptor (Morello *et al.*, 1995), the ^{125}I-TGF-ß2 labeled 65 kDa binding complex immunoprecipitated by the anti-type II receptor probably

Fig. 12. **Immunoprecipitation of affinity labeled human endometrial stromal TGF-ß binding proteins with anti-type II and anti-type III TGF-ß receptor antibodies.** Cells were affinity labeled with 200 pM ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2. Solubilized cell extracts were either not immunoprecipitated (nip), or immunoprecipitated with anti-type II (α-II) or anti-type III (α-III) TGF-ß receptor antibodies as indicated. In lanes labeled peptide (+), the immunoprecipitations were carried out in the presence of equimolar amounts (3 μg/ml) of the respective immunizing peptides. In lanes labeled SDS (+), the cell extracts were boiled 5 minutes in the presence of 1% SDS prior to immunoprecipitation. The type I, II, and III TGF-ß receptors, the 65 and 180 kDa GPI-anchored binding proteins, and the molecular weight standards are indicated in the margins. Note that lane 1 was exposed 8 days while the remaining lanes were exposed 14 days.

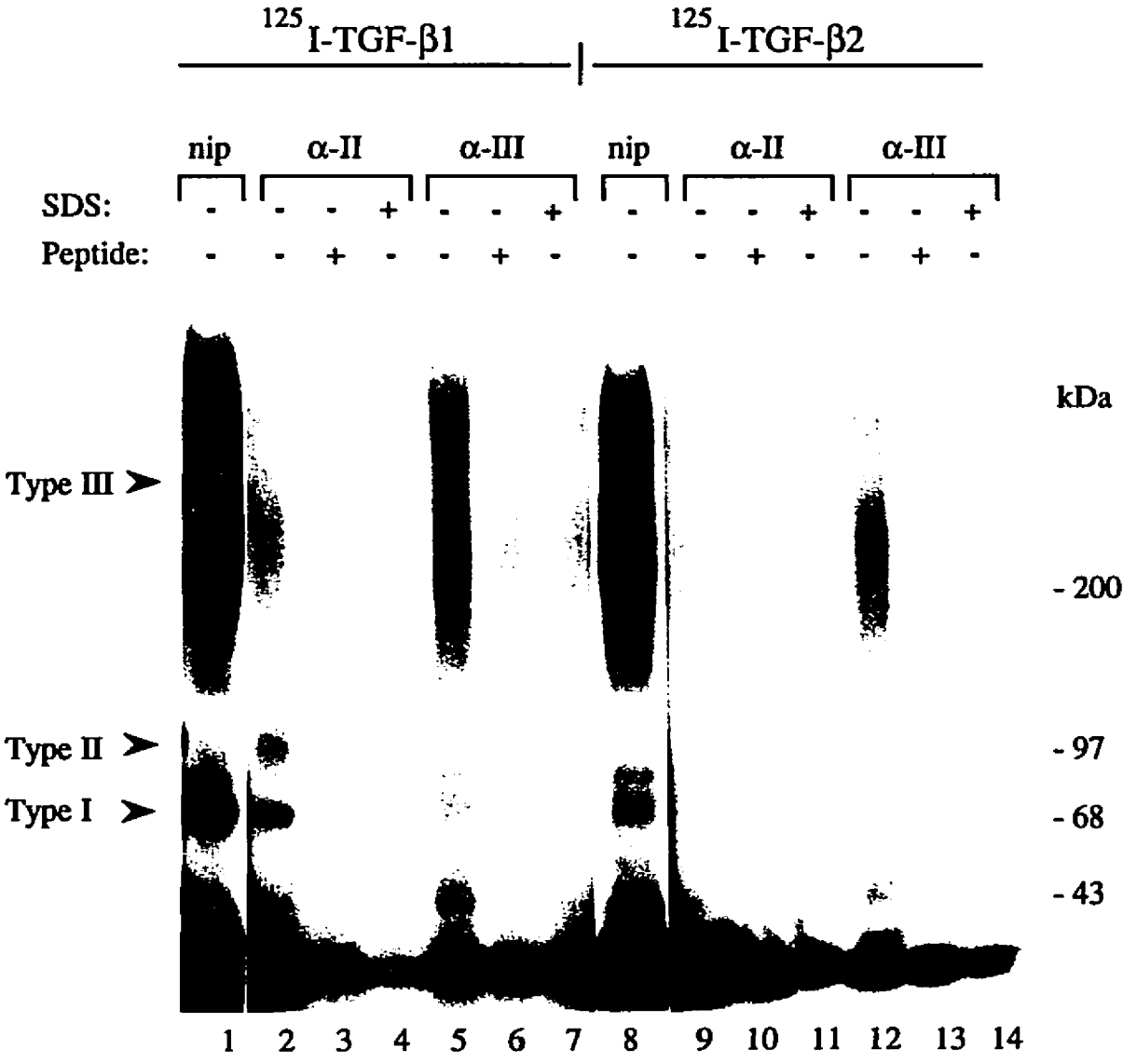

125I-TGF-β1
125I-TGF-β2
nip
α-II
α-III
nip
α-II
α-III
SDS:
Peptide:
Type III
GPI-anchored
180 kDa
Type II
Type I/GPI-anchored
65 kDa
kDa
- 200
- 97
- 68
- 43
1 2 3 4 5 6 7 8 9 10 11 12 13 14

corresponds to the type I receptor weaklylabeled with ^{125}I-TGF-ß2.

Immunoprecipitation of both ^{125}I-TGF-ß1 and ^{125}I-TGF-ß2 affinity labeled proteins on stromal cells with an anti-type III antibody, which was generated against a C-terminal epitope of the type III TGF-ß receptor, confirmed the presence of the type III receptor on these cells (Fig. 12, lanes 5 and 12). The immunoprecipitation of the type III receptor is specific since no cross-linked proteins are precipitated when equimolar amounts (3 µg/ml) of the immunizing peptide are included in the immunoprecipitation reaction mixtures (Fig. 12, lanes 6 and 13).

In order to confirm the presence of the type III receptor on RL95-2 cells, and to determine whether our inability to detect the type II receptor on these cells is due to the lack of sensitivity of the cross-link labeling technique, affinity labeled RL95-2 cell TGF-ß binding proteins were immunoprecipitated with the anti-type II and anti-type III TGF-ß receptor antibodies. As illustrated in Fig. 13, the results obtained are similar to those obtained with human endometrial stromal cells. Thus, the presence of the type I and III TGF-ß receptors on these cells was confirmed, while the previously undetected type II receptor was revealed. Denaturation of cell extracts with SDS demonstrates that the type I and III receptors form heteromeric complexes with the type II receptor on these cells as well.

Fig. 13. **Immunoprecipitation of affinity labeled RL95-2 cell TGF-ß binding proteins with anti-type II and anti-type III TGF-ß receptor antibodies.** Cells were affinity labeled with 200 pM ^{125}I-TGF-ß1 or ^{125}I-TGF-ß2. Solubilized cell extracts were either not immunoprecipitated (nip), or immunoprecipitated with anti-type II (α-II) or anti-type III (α-III) TGF-ß receptor antibodies as indicated. In lanes labeled peptide (+), the immunoprecipitations were carried out in the presence of equimolar amounts (3 μg/ml) of the respective immunizing peptides. In lanes labeled SDS (+), the cell extracts were boiled 5 minutes in the presence of 1% SDS prior to immunoprecipitation. The type I, II, and III TGF-ß receptors, and the molecular weight standards are indicated in the margins. Note that lanes 1 and 8 were exposed 6 days while the remaining lanes were exposed 18 days.


125I-TGF-β1
125I-TGF-β2
nip
α-II
α-III
nip
α-II
α-III
SDS:
Peptide:
Type III
Type II
Type I
kDa
- 200
- 97
- 68
- 43
1 2 3 4 5 6 7 8 9 10 11 12 13 14

3.5 Effect of pre-incubation with TGF-ß on affinity labeling of TGF-ß binding proteins

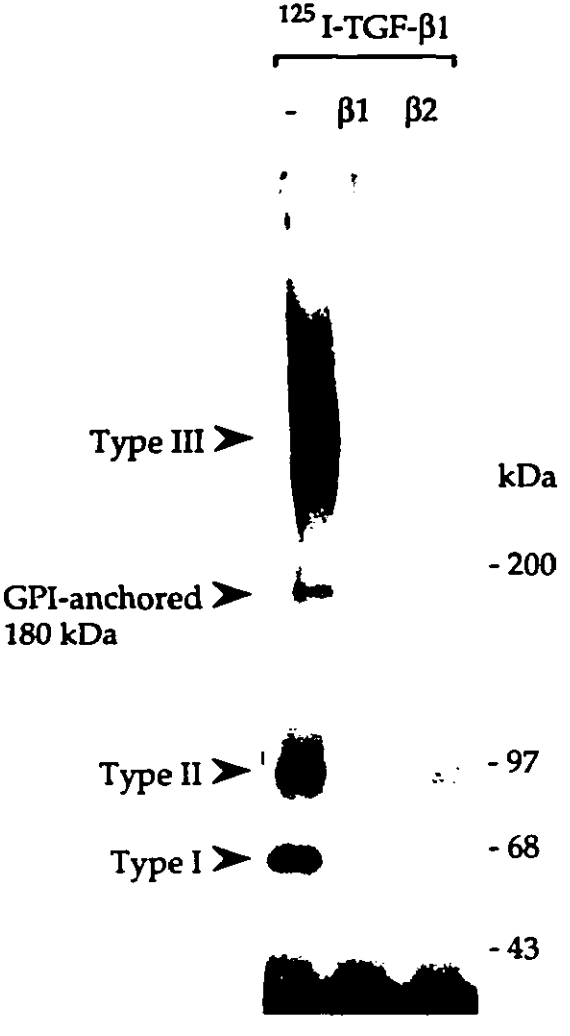
Studies have shown that the type I, II, and III TGF-ß receptors undergo marked downregulation following a 16 hour incubation with TGF-ß (MacKay and Danielpour, 1991). In order to determine whether the two GPI-anchored TGF-ß binding proteins identified on human endometrial stromal cells also undergo ligand-induced downregulation, stromal cells were pre-incubated with 100 pM of unlabeled TGF-ß1 or TGF-ß2, and affinity labeled with ^{125}I-TGF-ß1 (Fig. 14A) or ^{125}I-TGF-ß2 (Fig. 14B). The results indicate that both TGF-ß isoforms block the labeling of the type I, II, and III receptors, but have little or no effect on the labeling of either the 180 kDa GPI-anchored TGF-ß1 binding, or the 65 kDa GPI-anchored TGF-ß2 binding protein. Thus, GPI-anchored TGF-ß binding proteins are not only distinct from the previously identified TGF-ß receptors in their sensitivity to PI-PLC and in their affinity for only one of the TGF-ß isoforms, they are also unique in their failure to downregulate.

3.6 Scatchard Analysis

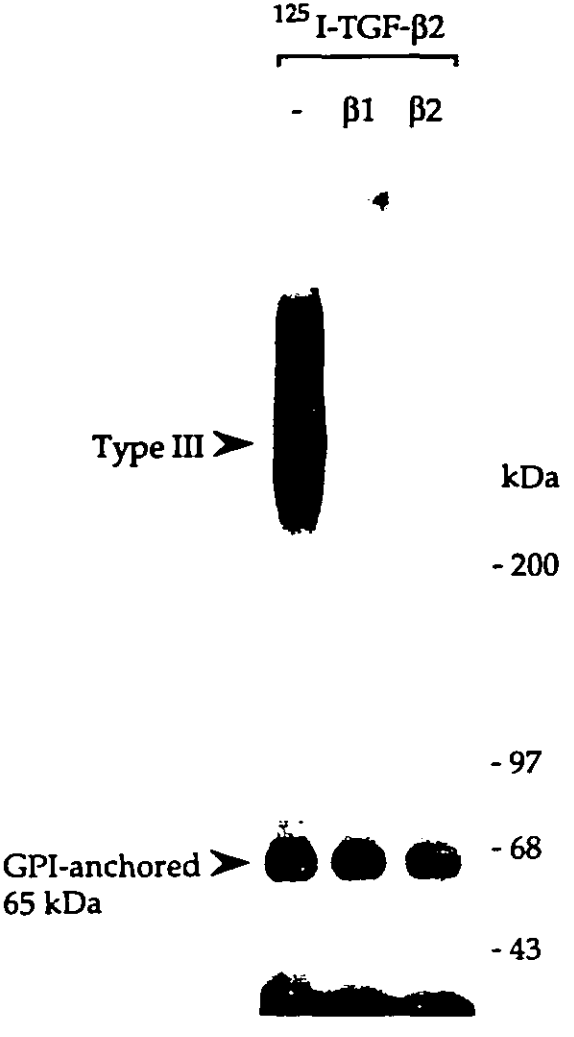
In order to get an idea of the number of TGF-ß receptors present on human endometrial cells, equilibrium saturation binding experiments with ^{125}I-TGF-ß1 and ^{125}I-TGF-ß2 were performed and analyzed by the method of Scatchard. Specific ^{125}I-TGF-ß1 binding to endometrial stromal cells indicated that saturation of TGF-ß

Fig. 14. **Effect of pre-incubation with TGF-ß on affinity labeling of TGF-ß binding proteins on human endometrial stromal cells.** Cells were pre-treated with 100 pM of TGF-ß1 or TGF-ß2, as indicated, for 16 hours and affinity labeled with 100 pM ^{125}I-TGF-ß1 (A), or 100 pM ^{125}I-TGF-ß2 (B). Samples were analyzed by SDS-PAGE under reducing conditions. The type I, II, and III TGF-ß receptors, the 65 and 180 kDa GPI-anchored TGF-ß binding proteins, and the molecular weight standards are indicated in the margins.

A.


125 I-TGF-β1
-
β1
β2
Type III
kDa
- 200
GPI-anchored
180 kDa
Type II
- 97
Type I
- 68
- 43

B.


125 I-TGF-β2
-
β1
β2
Type III
kDa
- 200
- 97
GPI-anchored
65 kDa
- 68
- 43

receptors at 4°C is achieved at a TGF-ß concentration of 100 pM (Fig. 15). Scatchard analysis of the binding data resulted in a linear plot suggesting the presence of one single class of high affinity binding sites with a K_d of 14 pM and approximately 3000 binding sites per cell (Fig. 15, inset).

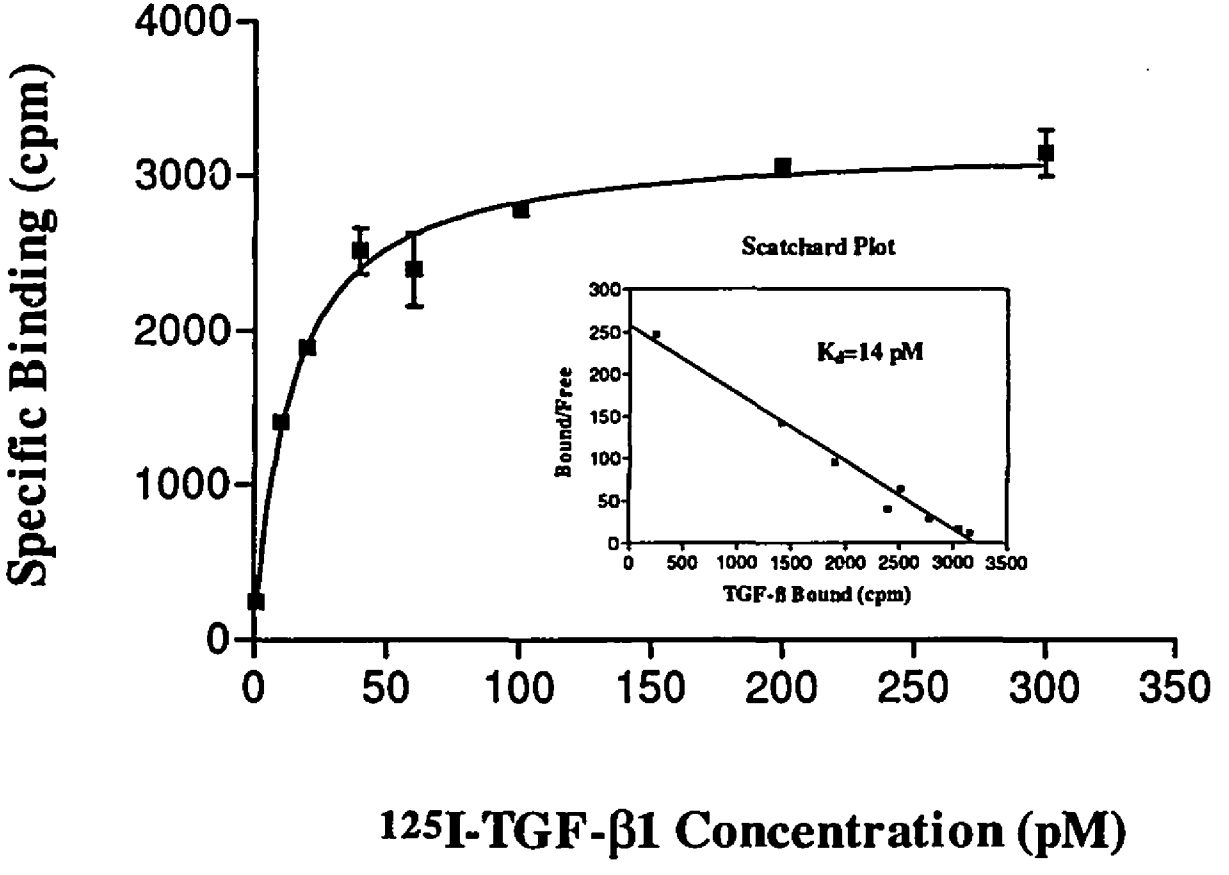
Similar experiments with RL95-2 cells using ^{125}I-TGF-ß1 indicated that saturation of TGF-ß receptors is achieved within the same range of TGF-ß concentrations required to saturate receptors on stromal cells (~100 pM), and Scatchard analysis also resulted in a linear plot with a K_d of 20 pM (Fig. 16). Although the K_d value observed for these cells is similar to that observed for stromal cells (20 versus 14 pM, respectively), the number of binding sites on RL95-2 cells is approximately four times lower than that observed on stromal cells (700 versus 3000 binding sites/cell, respectively).

When equilibrium saturation binding experiments were performed with ^{125}I-TGF-ß2, the results obtained were quite different. On stromal cells, Scatchard analysis of the binding data resulted in a curvilinear plot (Fig. 17) characteristic of at least two populations of receptors with distinct affinities for TGF-ß. The K_d and maximal binding (B_{max}) values obtained indicate that there are approximately 500 high affinity sites per cell with a K_d of 4 pM, and 9000 low affinity binding sites per cell with a K_d of 226 pM.

Similar experiments with ^{125}I-TGF-ß2 binding to RL95-2 cells also resulted in a curvilinear Scatchard plot with K_d values of 14 and 2850 pM and corresponding

Fig. 15. **Saturation curve of ^{125}I-TGF-ß1 binding to human endometrial stromal cells.** Cells were incubated with increasing concentrations of ^{125}I-TGF-ß1 (1-300 pM) to determine total binding. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled TGF-ß1. Duplicate wells were used for each condition. The curve shown corresponds to specific ^{125}I-TGF-ß1 binding (cpm) as a function of free ligand concentration (pM), where ^{125}I-TGF-ß1 specifically bound is the difference between the total and non-specific binding. The inset shows the corresponding Scatchard plot. For the linear regression of the Scatchard, r^2=0.9797. The K_d value obtained is shown, and corresponds to 3000 binding sites per cell.

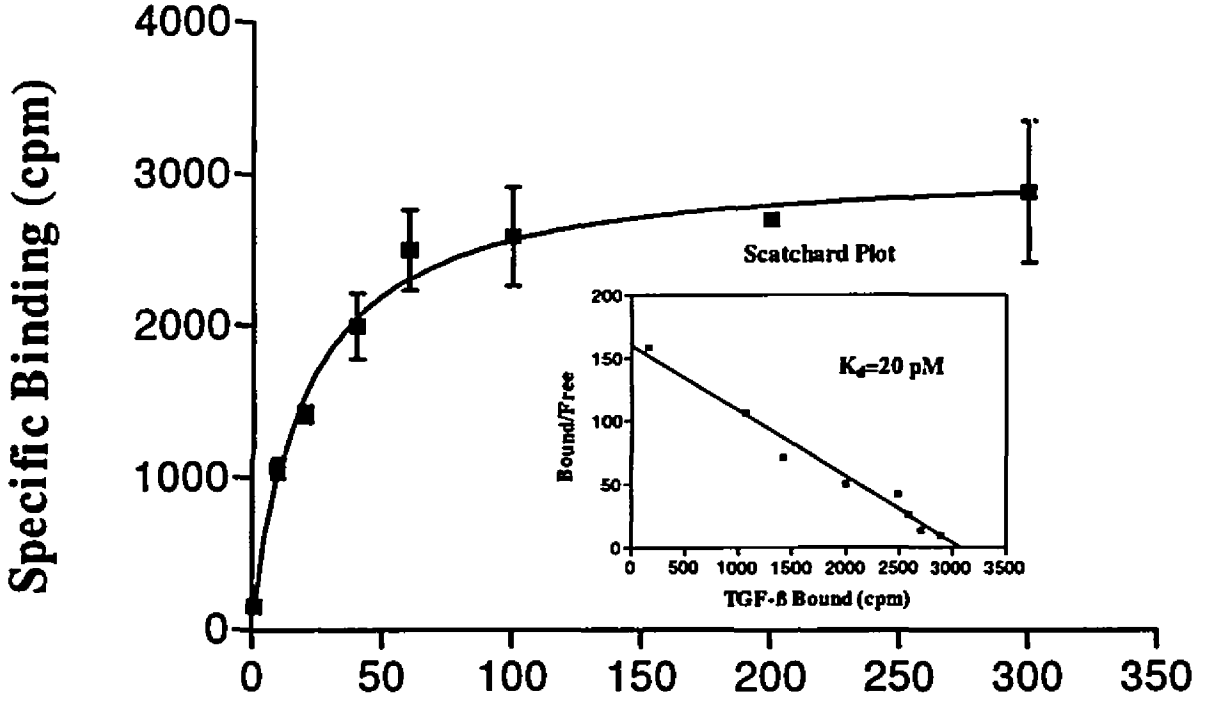
Saturation Binding Isotherm (stromal)



^{125}I-TGF-β1 Concentration (pM)

Fig. 16. **Saturation curve of ^{125}I-TGF-ß1 binding to RL95-2 cells.** Cells were incubated with increasing concentrations of ^{125}I-TGF-ß1 (1-300 pM) to determine total binding. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled TGF-ß1. Duplicate wells were used for each condition. The curve shown corresponds to specific ^{125}I-TGF-ß1 binding (cpm) as a function of free ligand concentration (pM), where ^{125}I-TGF-ß1 specifically bound is the difference between the total and non-specific binding. The inset shows the corresponding Scatchard plot. For the linear regression of the Scatchard, r^2=0.9737. The K_d value obtained is shown, and corresponds to 700 binding sites per cell.

Saturation Binding Isotherm
(RL95-2)



^{125}I-TGF-β1 Concentration (pM)

Fig. 17. **Saturation curve of ^{125}I-TGF-ß2 binding to human endometrial stromal cells.** Cells were incubated with increasing concentrations of ^{125}I-TGF-ß2 (1-500 pM) to determine total binding. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled TGF-ß2. Duplicate wells were used for each condition. The curve shown corresponds to specific ^{125}I-TGF-ß2 binding (cpm) as a function of free ligand concentration (pM), where ^{125}I-TGF-ß2 specifically bound is the difference between the total and non-specific binding. The inset shows the corresponding Scatchard plot. For the non-linear regression of the Scatchard, r^2=0.9386. The K_d values obtained are shown, and correspond to 500 and 9000 binding sites per cell, respectively.

Saturation Binding Isotherm (stromal)

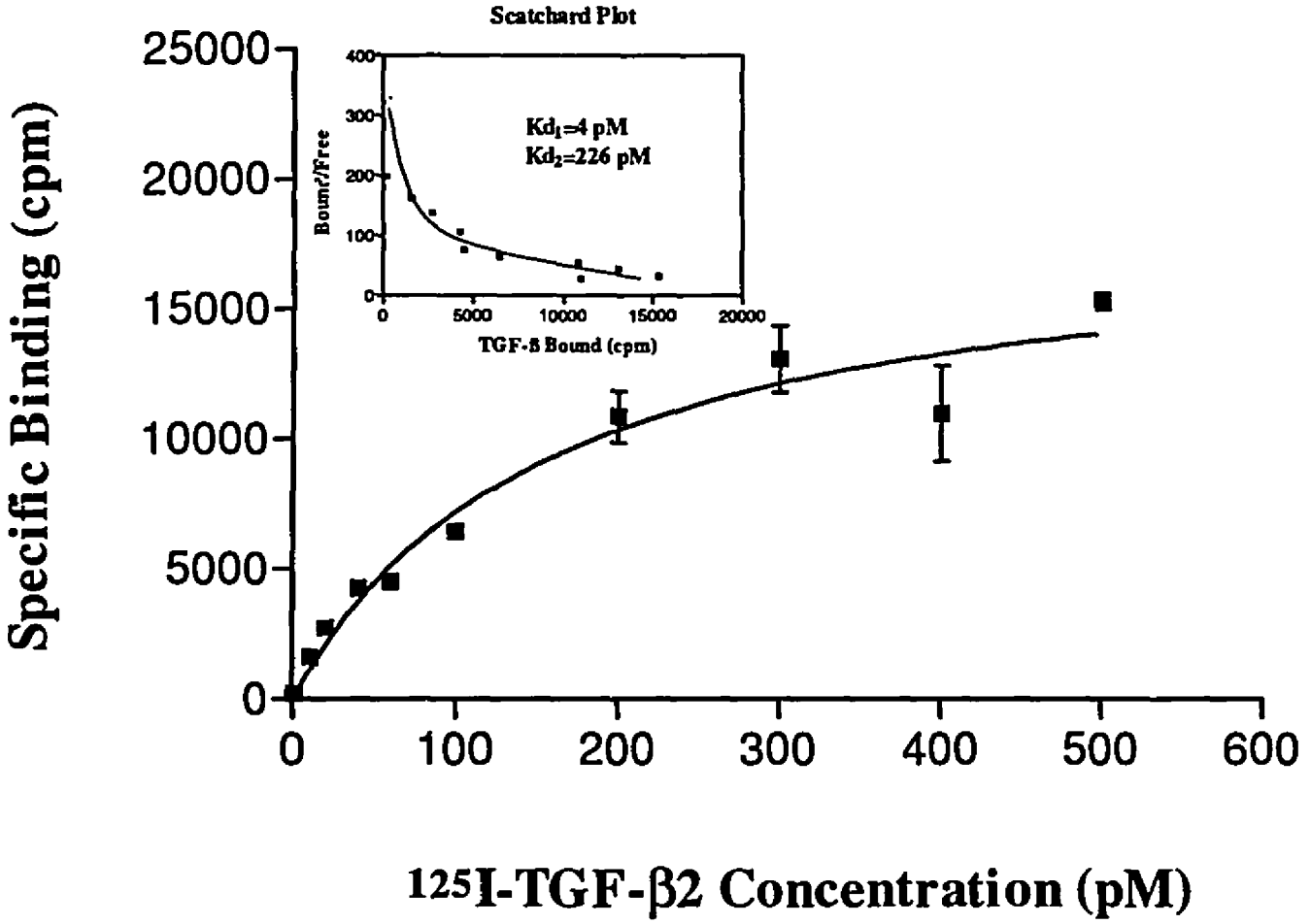
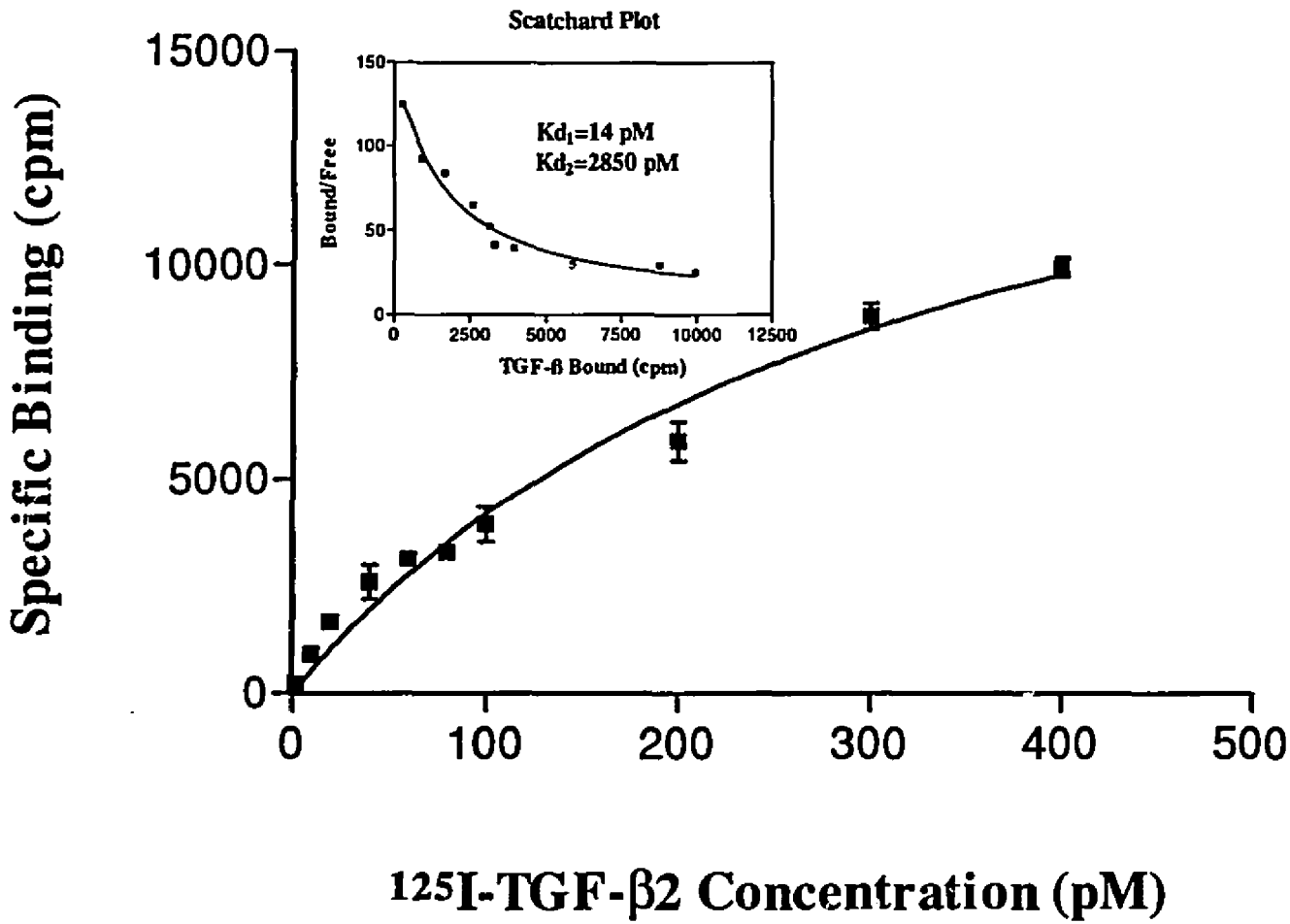


Fig. 18. **Saturation curve of ^{125}I-TGF-ß2 binding to RL95-2 cells.** Cells were incubated with increasing concentrations of ^{125}I-TGF-ß2 (1-400 pM) to determine total binding. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled TGF-ß2. Duplicate wells were used for each condition. The curve shown corresponds to specific ^{125}I-TGF-ß2 binding (cpm) as a function of free ligand concentration (pM), where ^{125}I-TGF-ß2 specifically bound is the difference between the total and non-specific binding. The inset shows the corresponding Scatchard plot. For the non-linear regression of the Scatchard, r^2=0.9865. The K_d values obtained are shown, and correspond to 1000 and 30,000 binding sites per cell, respectively.

Saturation Binding Isotherm (RL95-2)



receptor numbers of 1000 and 30,000 per cell (Fig. 18). Interestingly, although the affinity labeling profile with both TGF-ß isoforms is quite similar on these cells, Scatchard analyses of the binding data result in a linear plot for TGF-ß1 labeling, but a curvilinear plot for TGF-ß2 labeling. Thus, in both cell types, it appears as though TGF-ß2 is binding to two different classes of receptors while TGF-ß1 is binding to one class of high affinity receptors which probably correspond to the type I, II, and III receptors.

3.7 Effect of TGF-ß on DNA synthesis in human endometrial cells

Having characterized the receptors for TGF-ß on human endometrial cells, the responsiveness of these cells to TGF-ß was then examined by studying the effect of TGF-ß1 and TGF-ß2 on DNA synthesis in endometrial cells. Stromal and RL95-2 cells were incubated under serum free conditions with varying concentrations of TGF-ß1 or TGF-ß2 (0.1-500 pM). DNA synthesis was measured using a [^{3}H]-thymidine incorporation assay. The results obtained indicate that TGF-ß1 and TGF-ß2 inhibit DNA synthesis in RL95-2 cells (Fig. 19). This inhibitory effect is concentration-dependent, and the IC_{50} is approximately 50 pM for both TGF-ß1 and TGF-ß2. No significant difference in potency was detected between the two TGF-ß isoforms over the range of concentrations tested. In contrast, TGF-ß had no significant effect on DNA synthesis in stromal cells under serum free conditions (data not shown), but had a slight stimulatory effect on quiescent stromal cells in the

Fig. 19. **Effect of TGF-ß1 and TGF-ß2 on DNA synthesis in RL95-2 cells.** Cells were plated into 24-well plates, grown for 24 hours, and incubated under both serum and insulin free conditions with varying concentrations of TGF-ß1 (●) or TGF-ß2 (■) (0-500 pM), in triplicate, for 24 hours. [^{3}H]-thymidine was added directly to each well during the last 3 hours of the TGF-ß incubation. The average [^{3}H]-thymidine incorporation for each concentration tested is plotted as percent incorporation in the absence of TGF-ß (% control). The standard error within triplicates was calculated for each concentration tested.

Effect of TGF-ß on DNA Synthesis in RL95-2 Cells

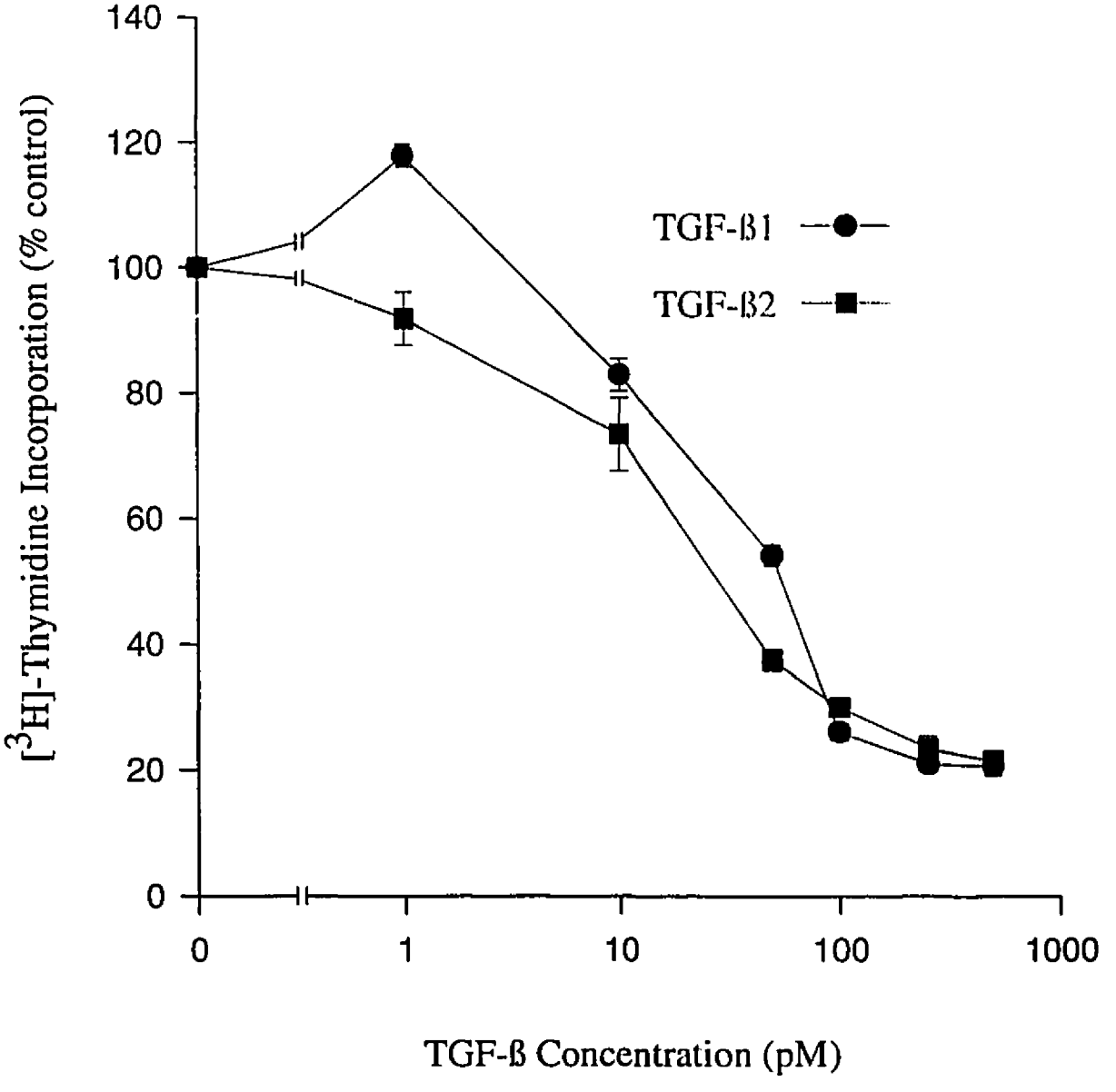
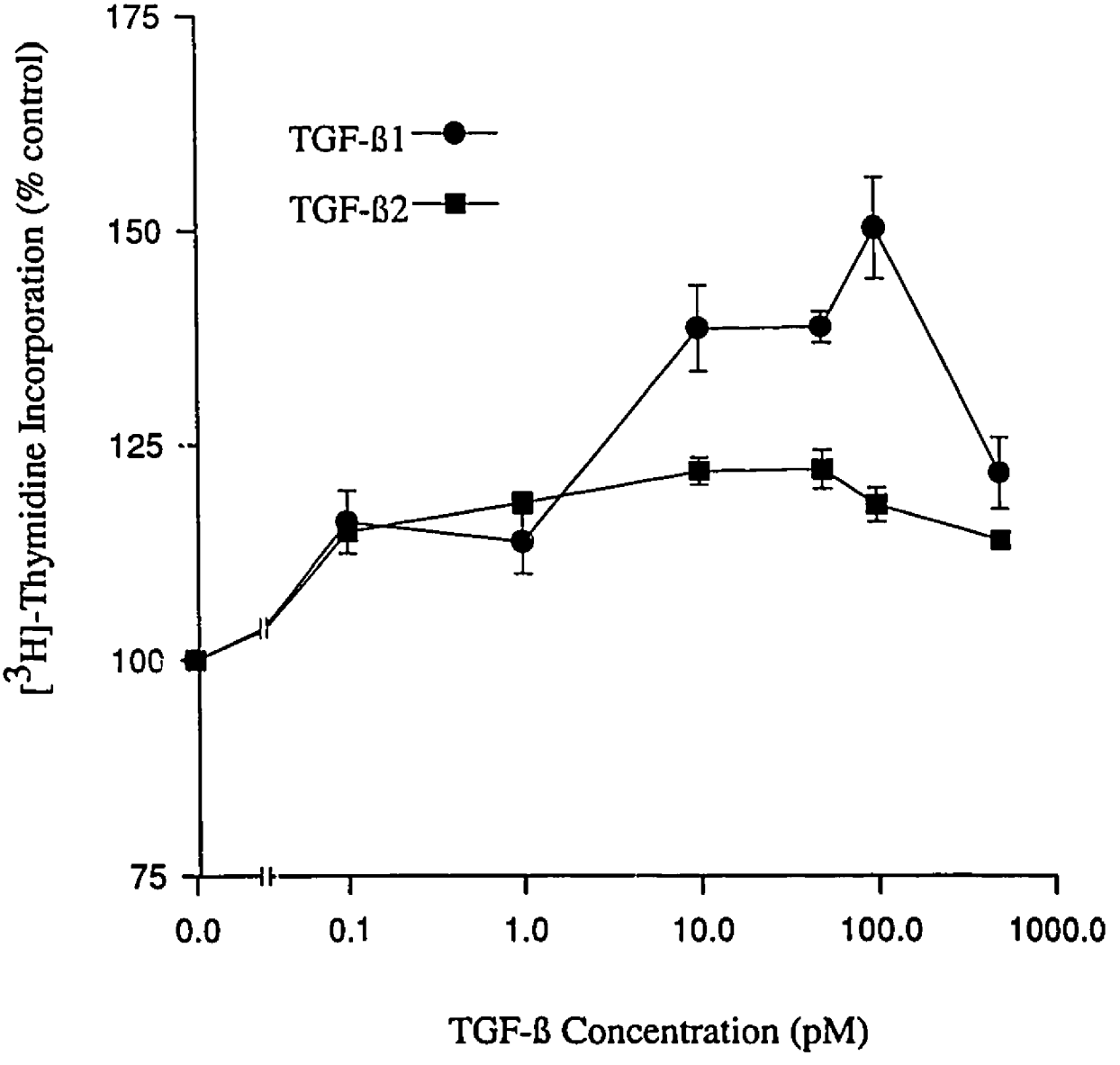


Fig. 20. **Effect of TGF-ß1 and TGF-ß2 on DNA synthesis in human endometrial stromal cells.** Cells were plated into 24-well plates, grown for 24 hours, serum-deprived for 48 hours, and incubated with varying concentrations of TGF-ß1 (●) or TGF-ß2 (■) (0-500 pM) in the presence of 2% FBS and 2μCi/ml of [^{3}H]-thymidine, in triplicate, for 48 hours. The average [^{3}H]-thymidine incorporation for each concentration tested is plotted as percent incorporation in the absence of TGF-ß (% control). The standard error within triplicates was calculated for each concentration tested.

Effect of TGF-ß on DNA Synthesis in Stromal Cells



presence of 2% fetalbovine serum (Fig. 20). Although, this stimulatory effect was observed in some stromal cell preparations, it was not reproduced in all stromal cell preparations. Thus, with respect to DNA synthesis, the responsiveness of these primary cell cultures to TGF-ß appears to be variable.

4. Discussion

Using affinity cross-link labeling techniques, five TGF-ß binding proteins have been identified on human endometrial stromal cells. Analysis of the affinity of these proteins for TGF-ß1 and TGF-ß2, their sensitivity to DTT, together with results from immunoprecipitations with specific antibodies against the type II and III TGF-ß receptors, confirmed that three of these, the 65, 85, and 200-300 kDa binding complexes, correspond to the cloned type I, II, and III TGF-ß receptors, respectively. Analysis of affinities and PI-PLC sensitivity indicate that the other two binding proteins observed represent a 180 kDa GPI-anchored TGF-ß1 binding protein and a 65 kDa GPI-anchored TGF-ß2 binding protein. Thus, there are two distinct co-migrating 65 kDa binding complexes on stromal cells, namely, an ^{125}I-TGF-ß1 labeled DTT-sensitive type I receptor and an ^{125}I-TGF-ß2 labeled GPI-anchored binding protein.

Interestingly, the mRNA for endoglin, a membrane glycoprotein that is predominantly expressed in endothelial cells and which binds TGF-ß1 and TGF-ß3

but not TGF-ß2, has recently been identified in murine uterine stromal cells (St.-Jacques *et al.*, 1994). However, this protein was not detected by affinity labeling in either the human endometrial stromal cell preparations or the RL95-2 cell line. After chemical cross-linking with ^{125}I-TGF-ß1, dimeric endoglin migrates as a 180 kDa complex under non-reducing conditions, while under reducing conditions the endoglin monomer co-migrates with the type II receptor (Cheifetz *et al.*, 1992). The 180 kDa TGF-ß1 binding protein which was observed on the stromal cells was not affected by reducing and non-reducing conditions, suggesting that this protein does not correspond to endoglin. The sensitivity of this binding protein to PI-PLC treatment indicates that it is similar to the 180 kDa GPI-anchored protein described by Cheifetz and Massagué (1991).

A 65 kDa GPI-anchored TGF-ß2 binding protein was also identified on endometrial stromal cells. Although TGF-ß binding proteins with membrane attachments sensitive to degradation by PI-PLC have been described in some cell lines (Cheifetz and Massagué, 1991; Mackay, 1993), this is the first report of GPI-anchored TGF-ß binding proteins in primary cell cultures. Unlike the type I, II, and III receptors, these GPI-anchored TGF-ß binding proteins identified on stromal cells fail to downregulate following a 16 hour incubation with TGF-ß. Their failure to downregulate, together with their sensitivity to PI-PLC and their high affinity for only one of the TGF-ß isoforms, distinguishes them from all other TGF-ß binding proteins.

Interestingly, there is evidence that GPI-anchored proteins in other cell types can become sequestered in small membrane invaginations called caveolae that have been implicated in the transport of folate and other small molecules (potocytosis) and in intracellular signaling processes (Mayor *et al.*, 1994; Lisanti *et al.*, 1994). Studies have also shown that antibodies directed against GPI-anchored proteins can co-immunoprecipitate Src-related tyrosine kinases (Shenoy-Scaria *et al.*, 1992; Stefanová *et al.*, 1993; Thomas and Samelson, 1992). This has led to the suggestion that GPI-anchored proteins may function as signaling molecules by associating with these kinases. Since GPI-anchored proteins and Src-related kinases are restricted to opposite leaflets of the membrane bilayer and cannot bind each other directly, a transmembrane "linker" protein is proposed to mediate the interaction between the two (Brown, 1993).

GPI-anchored proteins may also play an important role in cell-cell adhesion (Vestal and Ranscht, 1992; Rader *et al.*, 1993). In this respect, rapid lateral mobility due to the lipid anchor might facilitate recruitment of the protein into areas of contact, while subsequent cleavage of the anchor by a specific phospholipase could be a useful mechanism for breaking adhesion between cells. Alternatively, cleavage of GPI anchors resulting in the rapid release of proteins from the cell surface might be advantageous in regulating the concentration of proteins as well as their rate of secretion (Low and Saltiel, 1988). Moreover, once released the proteins could acquire altered or enhanced enzymatic function or behave as paracrine and/or

autocrine factors. There is indeed evidence suggesting that a placenta-derived 34 kDa polypeptide with autocrine growth factor properties is initially anchored to the membrane via phosphatidylinositol (Roy-Choudhury *et al.*, 1988). Thus, GPI-anchored TGF-ß binding proteins may be involved in a wide variety of functions, but their precise physiological role in endometrial stromal cells remains to be elucidated.

On RL95-2 cells, three TGF-ß binding proteins were identified. Two of these, namely, the 65 and 200-300 kDa binding complexes, were detected by affinity cross-link labeling. The sensitivity of the 65 kDa complex to DTT indicated that this binding protein corresponds to the type I receptor, while immunoprecipitation with the type III TGF-ß receptor antibody confirmed that the 200-300 kDa binding complex corresponds to the type III receptor. Although the type II receptor was not detected by affinity labeling, immunoprecipitation with an anti-type II TGF-ß receptor antibody revealed that the type II receptor is also present on RL95-2 cells. This suggests that the type II receptor observed on these cells is poorly cross-linked and/or present at low concentrations. In fact, others have reported that antiserum against type II receptors predominantly precipitates labeled type I receptor, suggesting that the efficiency of cross-linking of the type I receptor is higher than that of the type II receptor (Franzén *et al.*, 1993).

Interestingly, although the type III receptor is the major binding protein observed on RL95-2 cells, it is only weakly labeled on stromal cells suggesting that

the number of type III receptors might be lower in these cells. In contrast, the type I and II receptors are more prominent on stromal cells than on RL95-2 cells. In both cell types, the type II and I receptors, and the type II and III receptors form heteromeric complexes as evidenced by the ability of the anti-type II TGF-ß receptor antibody to precipitate labeled type I and III receptors in addition to the type II receptor. These findings are consistent with previous studies which provide evidence for heteromeric complex formation between the TGF-ß receptors (Attisano *et al.*, 1993; Franzén *et al.*, 1993; Moustakas *et al.*, 1993), and support the theory that the type III receptor binds TGF-ß and then presents it to the type II receptor, forming a high affinity ternary complex (López-Casillas *et al.*, 1993). It is also interesting to note that the ^{125}I-TGF-ß1 labeled 180 kDa GPI-anchored binding protein does not co-immunoprecipitate with the type II receptor. Thus, this protein does not appear to be involved in the heteromeric complex formation between the type I, II, and III receptors.

Unlike the anti-type II TGF-ß receptor antibody, the anti-type III receptor antibody does not co-immunoprecipitate the type II / type III heteromeric complex. A possible explanation for this is that the amino acid sequence recognized by the anti-type III receptor antibody becomes shielded upon interaction of the type III receptor with the type II receptor. Thus, the fraction of type III receptors engaged in heteromeric complex formation with type II receptors is not recognized by this antibody. This would also explain the apparent inefficiency of the type III receptor

antibody in precipitating the the type III receptor.

Scatchard analysis of ^{125}I-TGF-ß1 binding to both stromal and RL95-2 cells resulted in a linear Scatchard plot suggesting the presence of one class of high affinity receptors for TGF-ß. However, affinity labeling experiments and immunoprecipitations with TGF-ß receptor antibodies clearly demonstrate that all three TGF-ß receptors are expressed in both cell types, and that stromal cells also express GPI-anchored TGF-ß binding proteins. Thus, the observation of one class of high affinity receptors suggests that the interactions between the receptors which result from events such as the presentation of ligand by the type III receptor to the type II receptor, and the formation of a signaling complex between the type I and II receptors, render the detection of each individual receptor impossible through saturation binding experiments. In fact, previous studies have also shown linear Scatchard plots for many cell lines despite the presence of multiple receptors for TGF-ß on these cells (Massagué and Like, 1985; Segarini *et al.*, 1987; Wakefield *et al.*, 1987). The K_d values obtained for both stromal (14 pM) and RL95-2 (20 pM) cells, are within the same range of K_d values previously described for the type I and II receptors (Cheifetz *et al.*, 1987; 1990). Thus, despite the lower affinity of the type III receptor, the affinity of all three receptors collectively, is similar to that of the type I and II signaling receptors.

In contrast, Scatchard analysis of ^{125}I-TGF-ß2 binding to both cell types resulted in a curvilinear plot suggesting the presence of two classes of receptors with

distinct affinities for TGF-ß. Since the affinity labeling profile with ^{125}I-TGF-ß2 binding to stromal cells reveals that the GPI-anchored 65 kDa binding complex is by far the most prominent ^{125}I-TGF-ß2-labeled binding complex, and since studies have shown that the GPI-anchored TGF-ß binding proteins observed on cell lines display a lower affinity for TGF-ß than do the type I, II, and III receptors (Cheifetz and Massagué, 1991), the low affinity binding site for which there are a higher number of binding sites per cell probably corresponds to the 65 kDa GPI-anchored TGF-ß2 binding protein. This would mean that the type III receptor corresponds to the higher affinity site for which there are only 500 binding sites per cell. However, since the type III receptor does not usually display such high affinity for TGF-ß (Massagué at al., 1992), and since immunoprecipitations with TGF-ß receptor antibodies indicate that the type I and II receptors are also present on stromal cells, it seems more likely that the higher affinity site corresponds to a combination of the type I, II, and III receptors.

The observation of a curvilinear Scatchard plot on RL95-2 cells is intriguing since the affinity labeling profile with ^{125}I-TGF-ß2 is very similar to the affinity labeling profile with ^{125}I-TGF-ß1 which resulted in a linear Scatchard plot. Thus, there may be a large number of low affinity TGF-ß binding sites on these cells which were not detected with the affinity labeling protocol used. These binding sites might be revealed by raising the concentration of ^{125}I-TGF-ß used to label from a picomolar to a nanomolar range. However, since most cells are responsive to

picomolar concentrations of TGF-ß (Massagué, 1990; Roberts and Sporn, 1990) which would not effectively bind this class of receptors, the functional significance of such low affinity sites is unclear. Perhaps these sites play a role in as yet unidentified cellular events involving TGF-ß.

The effect of TGF-ß on DNA synthesis in endometrial cells is consistent with the effects of TGF-ß on other cell types. A number of studies have indicated that TGF-ß inhibits DNA synthesis in epithelial cells, but stimulates DNA synthesis in mesenchymal stromal cells (Moses *et al.*, 1990). Similarly, in this study, both TGF-ß isoforms inhibited DNA synthesis in the RL95-2 endometrial epithelial cell line. Studies have reported that TGF-ß generally interrupts the cell cycle in mid-to-late G1 phase, preventing induction of DNA synthesis and progression into S phase. Although the mechanism by which TGF-ß causes this cell cycle arrest is not yet known, interesting correlations have emerged. For example, the retinoblastoma gene product (pRB) becomes hyperphosphorylated upon initiation of S phase, but following addition of TGF-ß to proliferating cells, pRB remains underphosphorylated, possibly causing the block in progression to S phase (Laiho *et al.*, 1990). TGF-ß also decreases RB gene expression (Yan *et al.*, 1992), and reduces transcription of the c-myc gene (Pietenpol *et al.*, 1990), an effect that correlates with inhibition of cell proliferation.

No TGF-ß effect was detected on DNA synthesis in stromal cells under serum free conditions. This lack of effect was also observed by Tang *et al.* (1994)

under similar conditions, however, both this study and their's revealed that in the presence of 2% FBS, TGF-ß isoforms stimulate DNA synthesis in quiescent endometrial stromal cells. In contrast to these findings, others have reported a stimulatory effect of TGF-ß on DNA synthesis in human endometrial stromal cells even in the absence of serum (Hammond *et al.*, 1993; Marshburn *et al.*, 1994).

With the exception of the type III receptor, all TGF-ß binding proteins observed on both types of endometrial cells display a higher affinity for only one of the TGF-ß isoforms. The fact that the effect of TGF-ß on DNA synthesis in endometrial cells, like the type III receptor affinity labeling profile, does not display any isoform specificity, supports the idea that the type III receptor "delivers" TGF-ß to the signaling receptors, thereby enhancing cell responsiveness to TGF-ß and eliminating marked biological differences between TGF-ß isoforms. However, while this may be the case for the DNA synthesis response *in vitro*, other responses which have not been assayed may show isoform specificity.

5. Summary, Conclusion, and Future Prospects

In summary, the analysis of TGF-ß receptors in the two major cell types of the endometrium demonstrates that several cell surface receptors and binding proteins are co-expressed in this tissue. These proteins show high affinity for TGF-ß with apparent K_d values in the picomolar range, and appear to be functional as

evidenced by the effect of TGF-ß on DNA synthesis in these cells. The results of this study, together with the identification of all three mammalian TGF-ß isoforms in the human endometrium (Kauma *et al.*, 1990; Chegini *et al.*, 1994a, 1994b; Tang *et al.*, 1994) provide a basis for defining the role of TGF-ß in uterine physiology and pathophysiology.

The cellular localization of TGF-ß in the endometrium supports autocrine and/or paracrine roles for TGF-ß in this tissue, while its cycle-dependent expression suggests steroidal regulation of expression. Indeed, there is now substantial evidence that the expression of growth factors in reproductive tissues is hormonally regulated, and that the effects of the various hormones on cellular proliferation might be mediated in part by these peptides (Roberts and Sporn, 1992). For example, the antiproliferative effects of progestins on human endometrial adenocarcinoma cells appear to be due to the modulation of expression and action of TGFs α and ß (Gong *et al.*, 1991). Likewise, the ability of estrogens to stimulate and of antiestrogens to inhibit the growth of breast cancer cell lines has been correlated with their opposite effects on cellular secretion of TGF-ß (Knabbe *et al.*, 1987; Wakefield *et al.*, 1990; Jeng and Jordan, 1991). The synthetic progestin, gestodene, which inhibits the growth of several breast cancer cell lines, has also been shown to induce a large increase in the secretion of TGF-ß (Colletta *et al.*, 1991).

These observed effects of steroids on TGF-ß *in vitro*, have also been

demonstrated *in vivo*. In ovariectomized mice, injection of estradiol has been reported to cause a rapid increase in TGF-ß2 mRNA levels in the uterus (Das *et al.*, 1992). Studies have also shown that androgen deprivation, as a result of castration, leads to enhanced expression of both TGF-ß and TGF-ß receptors in rat ventral prostate (Kyprianou and Isaacs, 1988), indicating that TGF-ß is an important negative regulator in prostate cell proliferation.

Given this evidence, it would be interesting to determine whether the expression of TGF-ß receptors in the endometrium is regulated by steroid hormones, as is TGF-ß itself, and whether this expression pattern parallels the cycle-dependent ligand expression pattern. This would provide valuable insight into the role of TGF-ß in endometrial physiology during the menstrual cycle and in early pregnancy. Moreover, the identification of endogenous regulatory molecules responsible for the selective expression of the different TGF-ß isoforms and receptors may be an important step toward identifying analogs or antagonists of these agents which would allow manipulation of TGF-ß expression and activity in a target-specific manner.

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7. Claims of Originality

The following findings presented in this thesis represent original contributions to knowledge:

1. Identification and characterization of the type I, II, and III TGF-ß receptors in the human endometrium.

2. Identification of GPI-anchored TGF-ß binding proteins in primary cultures of human endometrial stromal cells.

3. Use of ligand-induced downregulation studies as a means of distinguishing GPI-anchored TGF-ß binding proteins from TGF-ß receptors in primary cultures of human endometrial stromal cells.

4. Demonstration of ligand-induced TGF-ß receptor downregulation in human endometrial stromal cells.

5. Demonstration of the failure of the GPI-anchored TGF-ß binding proteins expressed on human endometrial stromal cells to undergo ligand-induced downregulation.

6. Identification of heteromeric complex formation between the type I, II, and III TGF-ß receptors in human endometrial stromal cells and RL95-2 cells.

7. Estimate of the number and relative affinity of TGF-ß receptors in the human endometrium.

8. Addendum

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Transforming growth factor-β receptors on human endometrial cells: identification of the type I, II, and III receptors and glycosyl-phosphatidylinositol anchored TGF-β binding proteins

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Abstract

In the present study, we have characterized the cell surface receptors for transforming growth factor-β (TGF-β) on monolayer cultures of stromal cells prepared from human endometrial biopsies, and on a human endometrial epithelial cell line (RL95-2) using affinity cross-link labeling techniques. On the stromal cells, five TGF-β binding proteins were identified. Analysis of the sensitivity of these proteins to dithiothreitol and phosphatidylinositol-specific phospholipase C, together with results from immunoprecipitations with antibodies against the type II and III TGF-β receptors, confirmed that three of these binding proteins correspond to the cloned type I, II, and III TGF-β receptors. The other two binding proteins observed exhibit the characteristics of isoform-specific GPI-anchored TGF-β binding proteins. On RL95-2 cells, three TGF-β binding proteins, corresponding to the type I, II, and III TGF-β receptors, were identified. The receptors which we have characterized on endometrial cells are responsive to physiological concentrations of TGF-β as demonstrated by the effect of TGF-β on endometrial cell proliferation. Accordingly, these receptors have the potential to respond to the TGF-β isoforms which have recently been detected in the endometrium in an autocrine and/or paracrine manner.

Keywords: Transforming growth factor-β; Receptors; Endometrium

1. Introduction

Transforming growth factor-β (TGF-β) is a 25 kDa dimeric peptide which was first isolated from human platelets (Assoian et al., 1983). Although originally defined for its ability to cause reversible phenotypic transformation and anchorage independent growth of normal fibroblasts (Anzano et al., 1983), a wide spectrum of biological activities are now attributed to TGF-β, the most notable of which include TGF-β's potent regulatory effects on cell growth and differentiation, extracellular matrix protein synthesis, and immune function (for reviews see Massagué, 1990; Roberts and Sporn, 1990, 1993). There are five closely related TGF-β isoforms in vertebrates, three of which, TGF-β1, -β2, and -β3, are known in mammals. Although the biological potencies of these isoforms are similar in many in vitro assays (Cheifetz et al., 1987), marked differences have been observed in certain cases (Cheifetz et al., 1990). Additionally, there is evidence indicating that each isoform may have distinct activities in vivo (Rosa et al., 1988; Joyce et al., 1990).

TGF-β binds with high affinity to many cell types (Wakefield et al., 1987). Affinity labeling studies using chemical cross-linking agents have revealed that the type I (53 kDa), II (70–85 kDa), and III (200–400 kDa) TGF-β receptors (Massagué, 1992) are the most widely distributed cell surface molecules that bind TGF-β. The type I and II receptors are transmembrane serine/threonine kinases (Lin et al., 1992; Franzén et al., 1993) which are thought to form a signaling complex (Wrana et al., 1992). Although the details of the signal transduction pathway remain to be established, studies with TGF-β resistant cell

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mutants have shown that the type I receptor requires the presence of the type II receptor to bind ligand (Laiho et al., 1991; Wrana et al., 1992), while the type II receptor requires the presence of the type I receptor for signaling (Wrana et al., 1992), but not for ligand binding (Lin et al., 1992). Thus, both the type I and II receptors appear to be necessary for signal transduction. In contrast, the type III receptor, which is a transmembrane proteoglycan, has a short cytoplasmic domain with no apparent signaling motif (López-Casillas et al., 1991; Wang et al., 1991). In fact, many TGF-β responsive cells lack the type III receptor (Segarini et al., 1989). However, expression of the type III receptor is believed to enhance binding of TGF-β ligands to the type II receptor (Wang et al., 1991). There is also evidence suggesting that the type III receptor forms a stable non-covalent heteromeric complex with the type II receptor (López-Casillas et al., 1993; Moustakas et al., 1993). Hence, this receptor has been proposed to regulate access of TGF-β to the signaling receptors. On most cells, the type I and II receptors display a higher affinity for TGF-β1 than for TGF-β2, while the type III receptor has been reported to bind both isoforms with either a similar affinity (Cheifetz et al., 1987, 1988b, 1990; Segarini et al., 1989), or a somewhat higher affinity for TGF-β2 (Mitchell and O'Connor-McCourt, 1991).

In addition to the three well-characterized TGF-β receptors, several other membrane-bound proteins have been shown to bind TGF-β in an isoform-specific manner. For example, proteins with membrane attachments sensitive to phosphatidylinositol-specific phospholipase C (PI-PLC), which have high affinity for either TGF-β1 or TGF-β2, have been identified in various cell lines (Cheifetz and Massagué, 1991). Endoglin, a homodimeric protein which is expressed in endothelial cells and which shows sequence similarity to the type III TGF-β receptor, has been shown to bind TGF-β1 and TGF-β3, but not TGF-β2 (Cheifetz et al., 1992). The functional significance of these TGF-β binding proteins remains to be elucidated.

Emerging evidence suggests that TGF-β is a potential regulator of uterine growth and function (Guidice, 1994). Recent studies have shown that human uterine cells of all types express mRNA and protein for all three mammalian TGF-β isoforms as well as for the type II TGF-β receptor (Chegini et al., 1994b; Tang et al., 1994). According to these studies, there appears to be considerable variation in the expression of TGF-β mRNAs and proteins in endometrial tissue during the menstrual cycle. Additionally, it has been suggested that TGF-β may play a role in implantation and placentation (Tamada et al., 1990; Das et al., 1992; Graham et al., 1992; Mitchell et al., 1992), as well as in the pathogenesis of uterine cancer and endometriosis (Chegini et al., 1994a; Murphy, 1994).

Although the expression of TGF-β in the human uterus has been previously described (Kauma et al., 1990; Chegini et al., 1994b), the receptors for TGF-β in this tissue are poorly documented. In the present study, we have characterized the cell surface receptors for TGF-β on monolayer cultures of stromal cells prepared from human endometrial biopsies, and on a human endometrial epithelial cell line, RL95-2. In addition, we have evaluated the growth regulatory effect of TGF-β on these cells.

2. Materials and methods

2.1. Cell culture

Human endometrial biopsies obtained from patients undergoing laparoscopy were dissociated by enzymatic digestion in nutrient medium containing 0.25% collagenase/dispase (Boehringer Mannheim, Germany). After a 1-h incubation at 37°C, the suspension produced by collagenase digestion consisted of single stromal cells and fragments of epithelial glands. These two populations were separated by differential sedimentation at unit gravity as previously described (Kariya et al., 1991). Endometrial stromal cells were grown as monolayers in 75 cm^2 plastic culture flasks. Erythrocytes and contaminating epithelial cells were removed by changing the medium within 1 h of plating (after stromal cells had adhered to the flask). The purity and homogeneity of the stromal cell preparations was assessed by immunofluorescence microscopic studies using a mouse monoclonal antibody to human Thy-1 (Serotec Toronto, Ontario), a stromal cell specific marker (Fernandez-Shaw et al., 1992) and a mouse monoclonal antibody to human cytokeratin (Dako, Mississauga, Ontario), an epithelial cell specific marker. Immunofluorescence data were confirmed quantitatively by flow cytometry which indicated that 99.9% of our stromal cell population is positive for the stromal marker, Thy-1 (data not shown). The human endometrial epithelial cell line, RL95-2 was obtained from the American Type Culture Collection (Rockville, MD). All cells were maintained in medium consisting of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μg/ml bovine insulin (Gibco, Burlington, Ontario).

2.2. Iodination of TGF-β and affinity labeling of cells

Recombinant TGF-β1 (Bristol Myers-Squibb Pharmaceutical Research Institute, Seattle, WA) and recombinant TGF-β2 (Celtrix Pharmaceuticals Inc., Santa Clara, CA) were iodinated as previously described (Philip and O'Connor-McCourt, 1991). Affinity labeling studies were performed as described by Mitchell et al. (1992). Briefly, cells from stock culture flasks were trypsinized and plated in 12-well plates at a density of (4–6) $\times$ 10^5 cells/ml. Approximately 48 h after plating, confluent cell monolayers were washed three times with ice-cold binding buffer (Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (D-PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA)) over 30 min, and incubated with

either 100 pM ^{125}I-labeled TGF-β1 or ^{125}I-labeled TGF-β2, in the absence or presence of varying concentrations of unlabeled TGF-β1 or TGF-β2 for 3 h at 4°C with continuous gentle agitation. The receptor-ligand complexes were then cross-linked with 400 μl of 1 mM bis(sulfosuccinimidyl)suberate (BS^3) (Pierce, Rockford, IL) on ice. After 10 min, the reaction was stopped with the addition of 100 μl of 500 mM glycine and a further 5 min incubation. Cells were washed twice with D-PBS, and solubilized in buffer containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 20 mM Tris–HCl (pH 7.4), and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, and 25 mM benzamidine. Solubilized material was recovered from each well. One-fifth volume of 5× electrophoresis sample buffer (0.25 M Tris–HCl, pH 6.8, 5% SDS, 50% glycerol, and trace bromophenol blue) was added to each sample. The samples were then divided into equal volumes and analyzed under both reducing and non-reducing conditions using 3–11% SDS-PAGE and autoradiography as previously described (Mitchell and O'Connor-McCourt, 1991).

2.3. Treatment of cells with PI-PLC or dithiothreitol (DTT)

Confluent cell monolayers were washed three times over 30 min with D-PBS containing 0.5% BSA, twice with protein-free D-PBS, and then incubated at 37°C with either 0.3 U/ml of PI-PLC (Boehringer Mannheim, Germany) for 30 min or 1 mM DTT for 5 min. Following incubation, the cells were washed twice with ice-cold D-PBS containing 0.1% BSA, and affinity-labeled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2 as described above.

2.4. Immunoprecipitation of TGF-β receptors

Anti-peptide antibodies against the type II and type III TGF-β receptors were a gift from Dr. A. Moustakas (Whitehead Institute for Biomedical Research, Cambridge, MA). Immunoprecipitations were carried out as follows. Cells were affinity labeled with 200 pM of either ^{125}I-TGF-β1 or ^{125}I-TGF-β2 as described above. After a 30 min incubation with solubilization buffer, cell lysates were centrifuged at 5000 × g for 10 min. Aliquots of the supernatants were incubated overnight at 4°C with antibodies specific for either the type II or type III TGF-β receptors in the absence or presence of equimolar amounts (3 μg/ml) of the respective immunizing peptides. Other supernatant aliquots were boiled 5 min in the presence of 1% SDS prior to incubation with the antibodies. Immune complexes were then incubated with 50 μl of a protein A-Sepharose slurry (50% packed beads in D-PBS containing 0.2% Triton X-100) at 4°C for 2 h. The beads were pelleted by centrifugation and washed thoroughly with D-PBS containing 0.2% Triton X-100. The immune complexes were resuspended in 1× sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and analyzed by SDS-PAGE and autoradiography.

2.5. [^{3}H]Thymidine incorporation assay

Human endometrial stromal cells and RL95-2 cells were plated into 24-well dishes in the above-mentioned medium at a density such that at the time of TGF-β addition the cells were semi-confluent. Approximately 24 h after plating, the cells were washed with Ca^{2+}-Mg^{2+} free PBS and incubated under serum and insulin free conditions in the absence and presence of varying concentrations (1–500 pM) of TGF-β1 or TGF-β2, in triplicate, for either 24 h (RL95-2) or 48 h (stromal). [*methyl*-^{3}H]-Thymidine (Amersham, Life Science, 2 μCi/ml of medium) was added to RL95-2 cells during the last 3 h of the 24-h test period, and to stromal cells during the last 24 h of the 48-h test period. At the end of labeling, the wells were washed three times with 200 mM NaCl/50 mM Tris buffer, three times with 10% ice-cold trichloroacetic acid, and finally, three times with 95% ethanol. The wells were then air-dried, solubilized with 1% SDS, and the incorporated radioactivity measured in a liquid scintillation counter.

3. Results

3.1. Affinity labeling of cell surface TGF-β binding proteins on human endometrial cells

Affinity labeling of human endometrial stromal cells with 100 pM ^{125}I-TGF-β1 reveals four binding complexes with apparent relative molecular weights of 65, 85, 180 and 200–300 kDa. As illustrated by the autoradiogram in Fig. 1A, competition experiments using unlabeled TGF-β1 and TGF-β2 show that the 65, 85 and 180 kDa binding complexes display much higher affinity for TGF-β1 than for TGF-β2, i.e. binding of ^{125}I-TGF-β1 is markedly blocked by 0.5 nM unlabeled TGF-β1, but only slightly blocked by 5.0 nM TGF-β2. In contrast, the 200–300 kDa binding complex binds strongly to both TGF-β isoforms when ^{125}I-TGF-β1 is used as a radiotracer, while it preferentially binds TGF-β2 when ^{125}I-TGF-β2 is used. Although 2.5 nM TGF-β1 competed as expected for the type II and III receptors, and the 180 kDa GPI-anchored binding protein, it was not as effective as 0.5 nM TGF-β1 in competing for the type I receptor in the same lane. The reason for this discrepancy is unclear at this time, however, positive cooperativity in the presence of excess unlabeled ligand has been observed previously for TGF-β receptors (Segarini et al., 1992). The apparent molecular weights, isoform specificity, and migration patterns of the 65, 85, and 200–300 kDa binding complexes are characteristic of the type I, II and III TGF-β receptors that have been previously described on most cell types (Cheifetz et al., 1986, 1988b; Segarini et al., 1989; Massagué, 1990). The diffuse banding pattern of the 200–300 kDa binding complex is a distinctive feature of the type III receptor

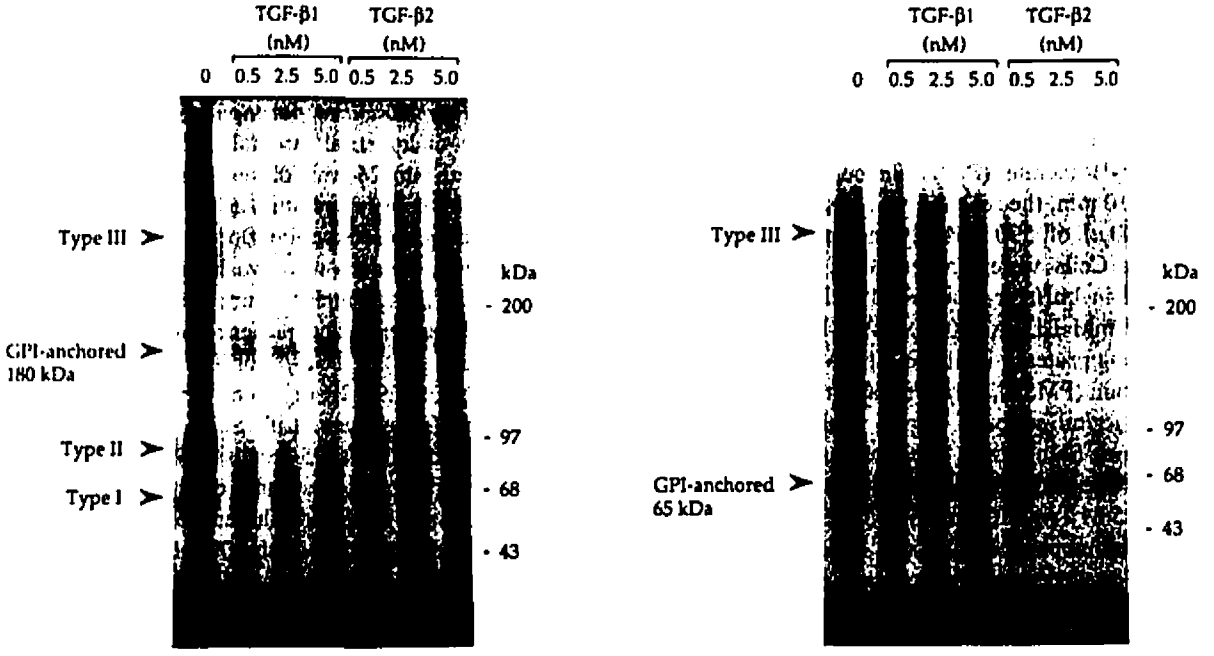

Fig. 1. Affinity labeling of TGF-β binding proteins on human endometrial stromal cells. Cells were affinity-labeled with 100 pM ^{125}I-TGF-β1 (A) or 100 pM ^{125}I-TGF-β2 (B), in the absence and presence of the indicated concentrations of unlabeled TGF-β1 or TGF-β2. Samples were analyzed by SDS-PAGE under reducing conditions. The type I, II, and III TGF-β receptors, the 65 and 180 kDa GPI-anchored TGF-β binding proteins, and the molecular weight standards are indicated in the margins. The autoradiogram shown is representative of over 25 experiments.

proteoglycan (Cheifetz et al., 1988a; Massagué et al., 1992). The competition experiments also demonstrate that the 180 kDa binding complex, later identified as a GPI-anchored protein (see Section 3.2), binds TGF-β1 but not TGF-β2.

When ^{125}I-TGF-β2 is used as the radiotracer, a prominent binding complex of 65 kDa, and a weakly labeled binding complex of 200–300 kDa are observed (Fig. 1B; also lane 8, Fig. 5). The 85 and 180 kDa binding complexes are not detectable, which is consistent with their

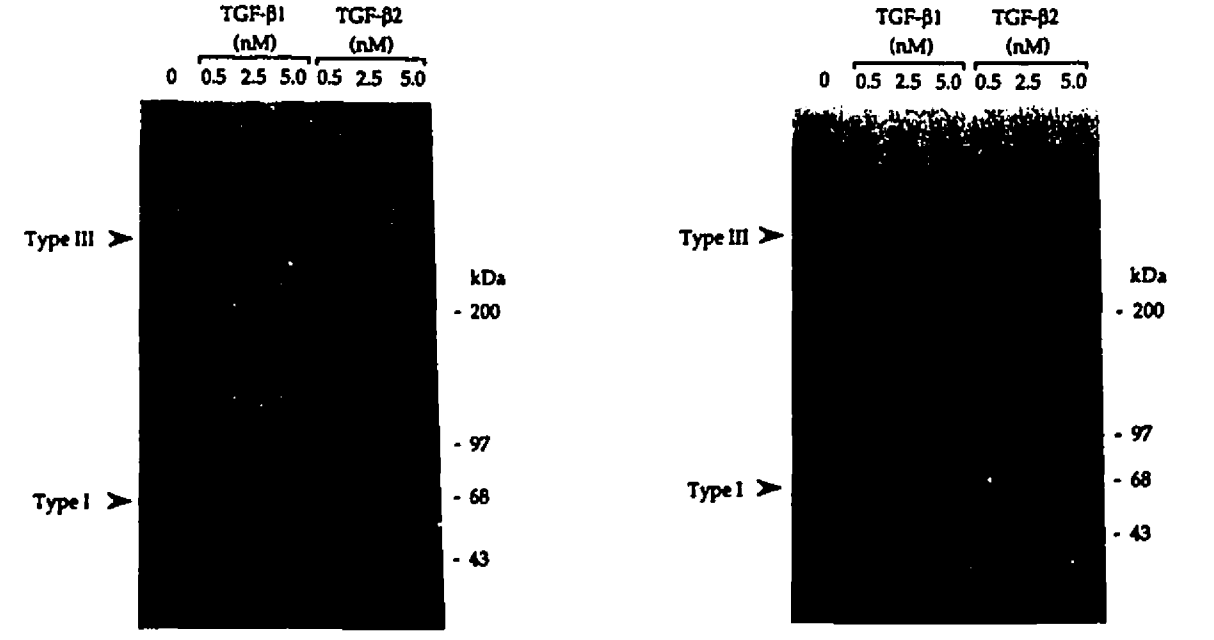

Fig. 2. Affinity labeling of TGF-β binding proteins on RL95-2 cells. Cells were affinity-labeled with 100 pM ^{125}I-TGF-β1 (A) or 100 pM ^{125}I-TGF-β2 (B), in the absence and presence of the indicated concentrations of unlabeled TGF-β1 or TGF-β2. Samples were analyzed by SDS-PAGE under reducing conditions. The type I and III TGF-β receptors, and the molecular weight standards are indicated in the margins. The autoradiogram shown is representative of over 10 experiments.

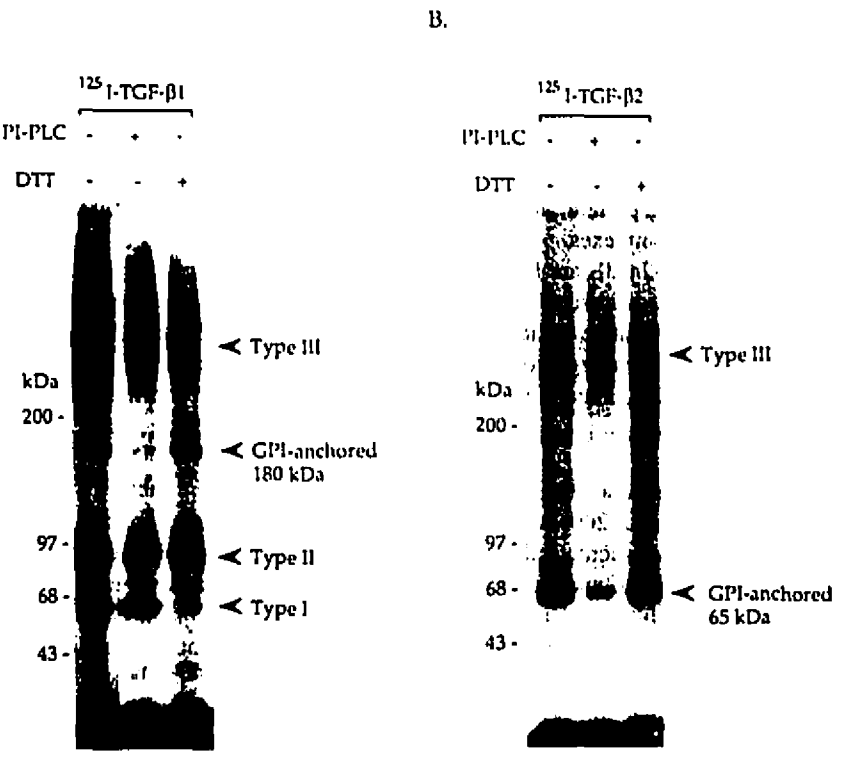

Fig. 3. Effect of PI-PLC and DTT on affinity labeling of TGF-β binding proteins in human endometrial stromal cells. Cells were pretreated with PI-PLC or DTT, as indicated, and affinity-labeled with 100 pM ^{125}I-TGF-β1 (A), or 100 pM ^{125}I-TGF-β2 (B). Samples were analyzed by SDS-PAGE under reducing conditions. The type I, II, and III TGF-β receptors, the 65 and 180 kDa GPI-anchored TGF-β binding proteins, and the molecular weight standards are indicated in the margins.

low affinities for TGF-β2. The ^{125}I-TGF-β2 labeled 200–300 kDa binding complex displays a similar affinity for both TGF-β isoforms, as expected for the type III receptor. However, the 65 kDa binding complex, when it is labeled with ^{125}I-TGF-β2, behaves differently from the type I receptor, since it is competed by TGF-β2 but not by TGF-β1. In fact, as demonstrated in Section 3.2, this protein is a GPI-anchored TGF-β binding protein.

On the human endometrial epithelial cell line, RL95-2, affinity labeling with both ^{125}I-TGF-β1 (Fig. 2A) and ^{125}I-TGF-β2 (Fig. 2B) reveals two binding complexes with apparent relative molecular weights of 65 and 200–300 kDa. As on the stromal cell preparations, the competition experiments show that the 200–300 kDa binding complex, when it is labeled with either TGF-β isoform, displays equal affinity for TGF-β1 and TGF-β2, as expected for the type III receptor. The 65 kDa binding complex, when it is labeled with ^{125}I-TGF-β1, displays higher affinity for TGF-β1 than for TGF-β2. In contrast to the stromal cells, the 65 kDa binding complex is very poorly labeled when ^{125}I-TGF-β2 is used as the radiotracer.

The autoradiograms shown in Figs. 1 and 2 are of polyacrylamide gels run under reducing conditions. There were no changes in the affinity labeling patterns of either cell type when the gels were run under non-reducing conditions (data not shown). Therefore, all figures shown are of reducing SDS-PAGE.

3.2. Effect of PI-PLC and DTT on affinity labeling of TGF-β binding proteins

Although most membrane proteins are inserted into the membrane via a transmembrane domain, a number of proteins have been described which have a GPI anchor (Low, 1989). TGF-β binding proteins with such anchors, including ones of 65 and 180 kDa, have been described in some cell lines (Cheifetz and Massagué, 1991). We therefore examined whether any of the TGF-β binding proteins which we have identified on human endometrial cells are anchored in this manner by treating the cells with PI-PLC prior to affinity labeling. In addition, since brief treatment of cells with 1 mM DTT has been shown to completely prevent labeling of the type I TGF-β receptor without decreasing labeling of the type II or III receptors (Cheifetz and Massagué, 1991), the effect of DTT on affinity labeling of TGF-β binding proteins was also assessed. As illustrated by the autoradiogram in Fig. 3A, when endometrial stromal cells are labeled with ^{125}I-TGF-β1, the 180 kDa binding complex is sensitive to PI-PLC treatment. None of the other ^{125}I-TGF-β1 labeled complexes were affected by the PI-PLC treatment. The only binding complex sensitive to DTT is the 65 kDa complex.

In contrast, when the cells are labeled with ^{125}I-TGF-β2 (Fig. 3B), the observed 65 kDa binding complex is insensitive to DTT, but sensitive to PI-PLC. The ^{125}I-TGF-β2 labeled 200–300 kDa binding complex is unaffected by either treatment. Thus, human endometrial stromal cells express the type II and III TGF-β receptors, a 180 kDa GPI-anchored TGF-β1 binding protein, and two distinct co-migrating 65 kDa binding complexes.

As shown in Fig. 4A,B, when RL95-2 cells are labeled with either ^{125}I-TGF-β1 or ^{125}I-TGF-β2, neither the 65 or 200–300 kDa complexes are PI-PLC sensitive indicating that they are not GPI-anchored TGF-β binding proteins. The 65 kDa complex is DTT sensitive, regardless of which isoform is used for labeling.

3.3. Immunoprecipitation of TGF-β receptors

In order to confirm that the 85 and 200–300 kDa binding complexes observed on human endometrial stromal cells do in fact correspond to the type II and III TGF-β receptors, proteins cross-linked to ^{125}I-TGF-β1 and -β2 were immunoprecipitated with specific anti-type II and anti-type III TGF-β receptor antibodies. As illustrated in Fig. 5 (lane 2), an anti-type II receptor antibody, which was generated against a C-terminal epitope of the type II TGF-β receptor, immunoprecipitates not only the 85 kDa type II receptor, but also immunoprecipitates the ^{125}I-TGF-β1 labeled type I and type III TGF-β receptors. The complexes are immunoprecipitated specifically since no cross-linked proteins are precipitated when equimolar amounts (3 μg/ml) of the immunizing peptide are included in the immunoprecipitation reactions (Fig. 5, lane 3). As illustrated in Fig. 5 (lane 4), denaturation of cell extracts with SDS prior to immunoprecipitation does not affect precipitation of the type II receptor, while precipitation of the type I and III receptors is prevented. This result indicates that the 85 kDa type II receptor band is being immunoprecipitated directly, while the type I and III receptors are being co-immunoprecipitated as heteromeric complexes with the type II receptor.

When the cells are labeled with ^{125}I-TGF-β2, the anti-type II TGF-β receptor antibody also immunoprecipitates the type II and III receptors, and an ^{125}I-TGF-β2 labeled 65 kDa binding complex (Fig. 5, lane 9). Since we have evidence that the 65 kDa GPI-anchored protein observed in other cells does not co-precipitate with the type II receptor, the ^{125}I-TGF-β2 labeled 65 kDa binding complex immunoprecipitated by the anti-type II receptor probably corresponds to ^{125}I-TGF-β2 labeled type I receptor.

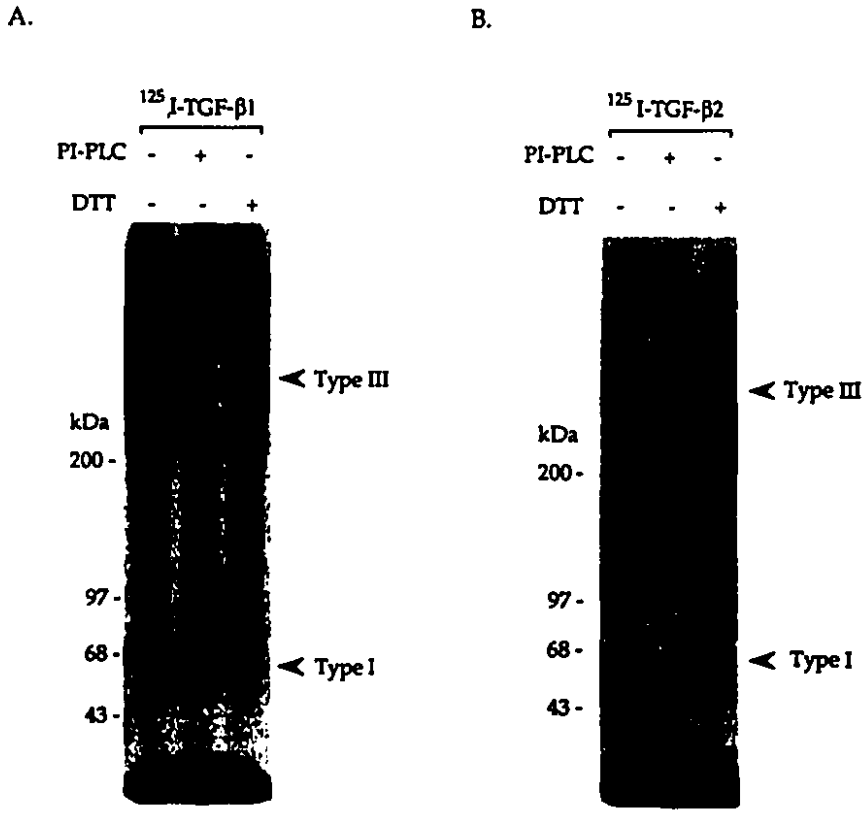

Fig. 4. Effect of PI-PLC and DTT on affinity labeling of TGF-β binding proteins in RL95-2 cells. Cells were pretreated with PI-PLC or DTT, as indicated, and affinity labeled with 100 pM ^{125}I-TGF-β1 (A), or 100 pM ^{125}I-TGF-β2 (B). Samples were analyzed by SDS-PAGE under reducing conditions. The type I and III TGF-β receptors, and the molecular weight standards are indicated in the margins.

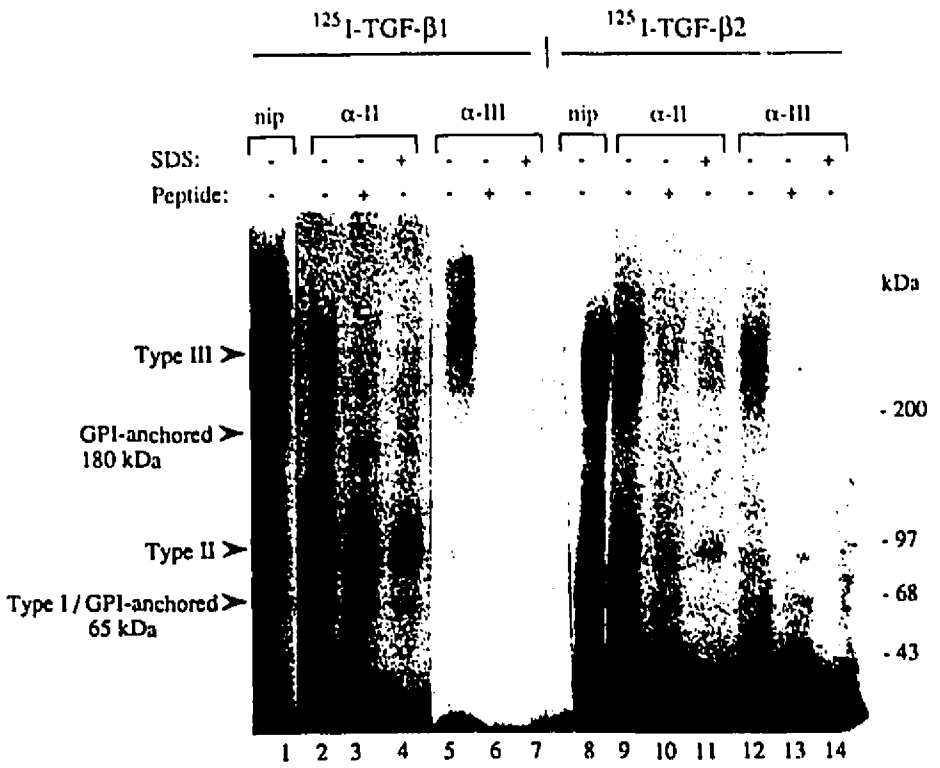

Fig. 5. Immunoprecipitation of affinity labeled human endometrial stromal TGF-β binding proteins with anti-type II and anti-type III TGF-β receptor antibodies. Cells were affinity labeled with 200 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Solubilized cell extracts were either not immunoprecipitated (nip), or immunoprecipitated with anti-type II (α-II) or anti-type III (α-III) TGF-β receptor antibodies as indicated. In lanes labeled peptide (+), the immunoprecipitations were carried out in the presence of equimolar amounts (3 μg/ml) of the respective immunizing peptides. In lanes labeled SDS (+), the cell extracts were boiled for 5 min in the presence of 1% SDS prior to immunoprecipitation. The type I, II, and III TGF-β receptors, the 65 and 180 kDa GPI-anchored binding proteins, and the molecular weight standards are indicated in the margins. Note that lanes 1 and 8 were exposed for 6 days while the remaining lanes were exposed for 16 days.

Immunoprecipitation of both ^{125}I-TGF-β1 and -β2 affinity labeled proteins on stromal cells with an anti-type III antibody, which was generated against a C terminal epitope of the type III TGF-β receptor, confirmed the presence of the type III receptor on these cells (Fig. 5, lanes 5 and 12). The type III receptor is immunoprecipitated specifically since no cross-linked proteins are precipitated when equimolar amounts (3 μg/ml) of the immunizing peptide are included in the immunoprecipitation reaction mixtures (Fig. 5, lanes 6 and 13).

In order to confirm the presence of the type III receptor on RL95-2 cells, and to determine whether our inability to detect the type II receptor by affinity labeling is due to the sensitivity of the method, affinity labeled RL95-2 cell TGF-β binding proteins were immunoprecipitated with the anti-type II and anti-type III TGF-β receptor antibodies. As illustrated in Fig. 6, the results obtained are similar to those obtained with human endometrial stromal cells. Thus the presence of the type I and III TGF-β receptors on these cells was confirmed, while the previously undetected type II receptor was revealed. Denaturation of cell extracts with SDS demonstrates that the type I and III receptors form heteromeric complexes with the type II receptor on these cells.

3.4. Effect of TGF-β on human endometrial cellular proliferation

Having characterized the receptors for TGF-β on human endometrial cells, we then examined the responsiveness of these cells to TGF-β by studying the effect of TGF-β1 and TGF-β2 on endometrial cell growth. Stromal and RL95-2 cells were incubated under serum free conditions with varying concentrations of TGF-β1 or TGF-β2 (1–500 pM). Cellular proliferation was measured using a [^{3}H]thymidine incorporation assay. Our results indicate that TGF-β1 and TGF-β2 inhibit RL95-2 cellular proliferation (Fig. 7). This growth-inhibitory effect is concentration-dependent, and the IC_{50} is approximately 50 pM for both TGF-β1 and TGF-β2. No significant difference in potency was detected between the two TGF-β isoforms over the range of concentrations tested. In contrast, TGF-β had no significant effect on stromal cell proliferation under serum-free conditions, but had a slight stimulatory effect on quiescent stromal cells in the presence of 2% fetal bovine serum (data not shown).

4. Discussion

Using affinity cross-link labeling techniques, we have

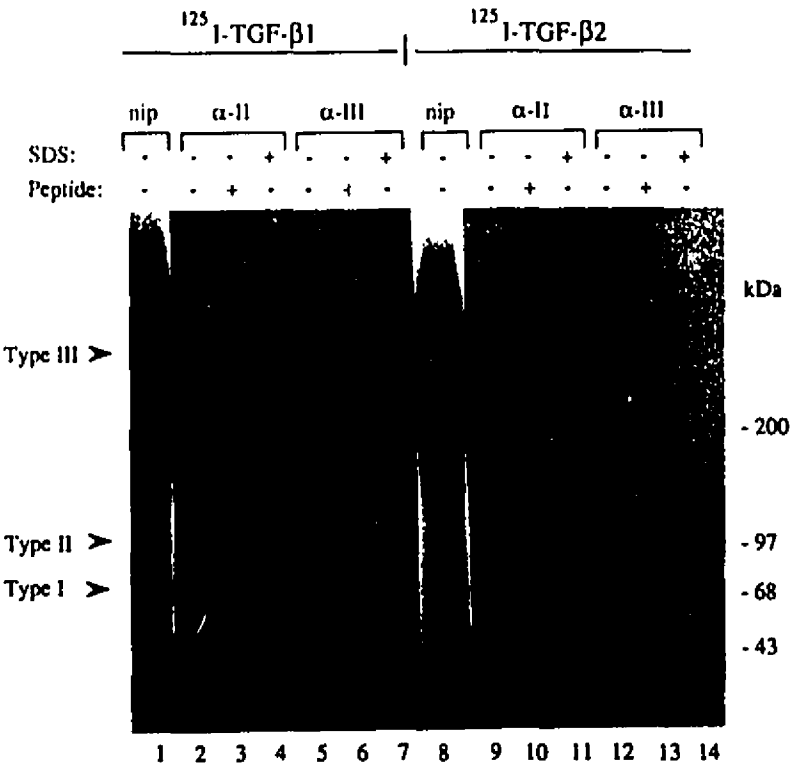

Fig. 6. Immunoprecipitation of affinity labeled RL95-2 cell TGF-β binding proteins with anti-type II and anti-type III TGF-β receptor antibodies. Cells were affinity labeled with 200 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Solubilized cell extracts were either not immunoprecipitated (nip), or immunoprecipitated with anti-type II (α-II) or anti-type III (α-III) TGF-β receptor antibodies as indicated. In lanes labeled peptide (+), the immunoprecipitations were carried out in the presence of equimolar amounts (3 μg/ml) of the respective immunizing peptides. In lanes labeled SDS (+), the cell extracts were boiled for 5 min in the presence of 1% SDS prior to immunoprecipitation. The type I, II, and III TGF-β receptors, and the molecular weight standards are indicated in the margins. Note that lanes 1 and 8 were exposed 6 days while the remaining lanes were exposed 18 days.

identified five TGF-β binding proteins on human endometrial stromal cells. Analysis of the affinity of these proteins for TGF-β1 and TGF-β2, their sensitivity to DTT, and their immunoprecipitability with specific antibodies against the type II and III TGF-β receptors, confirmed that three of these, the 65, 85, and 200–300 kDa binding complexes, correspond to the cloned type I, II, and III TGF-β receptors, respectively. Analysis of affinities and PI-PLC sensitivity indicate that the other two binding proteins observed include a 180 kDa GPI-anchored TGF-β1 binding protein and a 65 kDa GPI-anchored TGF-β2 binding protein. Thus, there are two distinct co-migrating 65 kDa binding complexes on stromal cells, namely, an ^{125}I-TGF-β1 labeled DTT-sensitive type I receptor and an ^{125}I-TGF-β2 labeled GPI-anchored binding protein.

Interestingly, the mRNA for endoglin, a membrane glycoprotein that is predominantly expressed in endothelial cells and which binds TGF-β1 and TGF-β3 but not TGF-β2, has recently been identified in murine uterine stromal cells (St.-Jacques et al., 1994). However, we were unable to detect this protein by affinity labeling in our human endometrial cells. After chemical cross-linking with ^{125}I-TGF-β1, dimeric endoglin migrates as a 180 kDa complex under non-reducing conditions, while under reducing conditions the endoglin monomer co-migrates with the type II receptor (Cheifetz et al., 1992). The 130 kDa TGF-β1 binding protein which we observed on the stromal cells was not affected by reducing and non-reducing conditions, suggesting that this protein does not correspond to endoglin. The sensitivity of this binding protein to PI-PLC treatment indicates that it is similar to the 180 kDa GPI-anchored protein described by Cheifetz and Massagué (1991).

A 65 kDa GPI-anchored TGF-β2 binding protein was also identified on endometrial stromal cells. Although TGF-β binding proteins with membrane attachments sensitive to PI-PLC have been described in some cell lines (Cheifetz and Massagué, 1991; Mackay, 1993), this is the first report of GPI-anchored TGF-β binding proteins on primary cells. There is evidence suggesting that GPI-anchored proteins can function as signaling molecules by associating with Src-related protein tyrosine kinases (Stefanová et al., 1993), and that they can become sequestered in small membrane invaginations called caveolae (Lisanti et al., 1994; Mayor et al., 1994). Whether the GPI-anchored TGF-β binding proteins observed on endometrial stromal cells also become seques-

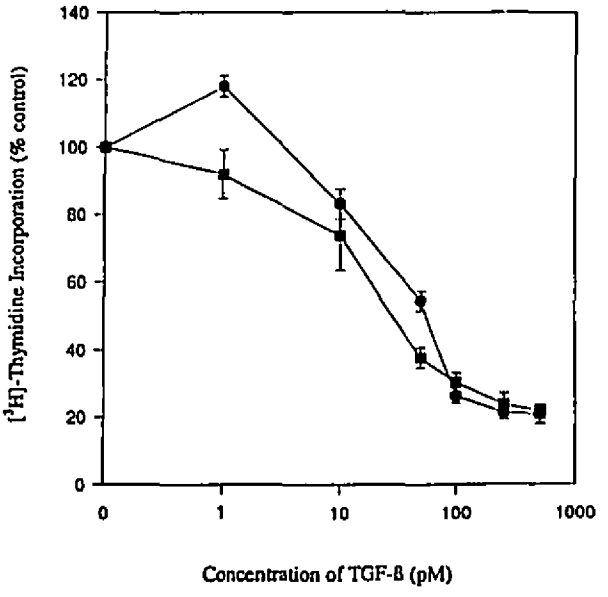

Fig. 7. Effect of TGF-β1 and TGF-β2 on RL95-2 cellular proliferation. Cells were plated into 24-well plates, grown for 24 h, and incubated under serum free and insulin free conditions with varying concentrations of TGF-β1 (●) or TGF-β2 (■) (0–500 pM), in triplicate, for 24 h. [^{3}H]Thymidine was added directly to each well during the last 3 h of the TGF-β incubation. The average [^{3}H]thymidine incorporation for each concentration tested is plotted as percent incorporation in the absence of TGF-β (% control). The standard deviation within triplicates was calculated for each concentration tested. The curves shown are representative of five experiments.

tered to caveolae, and whether they have any signaling capacity remains to be determined.

On RL95-2 cells, three TGF-β binding proteins were identified. Two of these, namely, the 65 and 200–300 kDa binding complexes, were detected by affinity cross-link labeling. The sensitivity of the 65 kDa complex to DTT indicated that this binding protein corresponds to the type I receptor, while immunoprecipitation with the type III TGF-β receptor antibody confirmed that the 200–300 kDa binding complex corresponds to the type III receptor. Although the type II receptor was not detected by affinity labeling, immunoprecipitation with an anti-type II TGF-β receptor antibody revealed that the type II receptor is also present on RL95-2 cells. This suggests that the type II receptor observed on these cells is poorly cross-linked and/or present at low concentrations. In fact, others have reported that antiserum against type II receptors predominantly precipitates labeled type I receptor, suggesting that the efficiency of cross-linking of the type I receptor is higher than that of the type II receptor (Franzén et al., 1993).

Interestingly, although the type III receptor is the major binding protein observed on RL95-2 cells, it is only weakly labeled on stromal cells suggesting that the number of type III receptors might be lower in these cells. In contrast, the type I and II receptors are more prominent on stromal cells than on RL95-2 cells. In both stromal cells and the RL95-2 cell line, the type II and I receptors, and the type II and III receptors form heteromeric complexes since the antibody specific for the type II TGF-β receptor precipitates labeled type I and type III receptors in addition to the type II receptor. Unlike the anti-type II TGF-β receptor antibody, the anti-type III antibody does not co-immunoprecipitate the type II/type III heteromeric complex. A possible explanation for this is that the amino acid sequence recognized by the anti-type III antibody becomes shielded upon interaction of the type III receptor with the type II receptor. It is interesting to note that the ^{125}I-TGF-β1 labeled 180 kDa GPI-anchored binding protein does not co-immunoprecipitate with the type II receptors.

The growth regulatory effect of TGF-β on endometrial cells is consistent with the effects of TGF-β on other cell types. A number of studies have indicated that TGF-β acts as a negative growth regulator of epithelial cells, and a positive growth regulator of mesenchymal stromal cells (Moses et al., 1990). Similarly, we found that both TGF-β1 and TGF-β2 inhibit proliferation of the RL95-2 endometrial epithelial cell line with similar potencies. We were unable to detect any effect of TGF-β on stromal cell proliferation under serum-free conditions. This lack of effect was also observed by Tang et al. (1994) under similar conditions, however, both we and they have found that in the presence of 2% fetal bovine serum, TGF-β isoforms stimulate the proliferation of quiescent endometrial stromal cells.

With the exception of the type III receptor, all TGF-β binding proteins observed on both types of endometrial cells display a higher affinity for one of the TGF-β isoforms. The fact that the growth regulatory effect of TGF-β on endometrial cells, like the type III receptor affinity labeling profile, does not display any isoform specificity, supports the idea that the type III receptor 'delivers' TGF-β to the signaling receptors, thereby enhancing cell responsiveness to TGF-β and eliminating marked biological differences between TGF-β isoforms. However, while this may be the case for growth response in vitro, other responses which have not been assayed may show isoform specificity.

In summary, our analysis of TGF-β receptors on two cell types from the endometrium demonstrates that several cell surface receptors and binding proteins are co-expressed in this tissue. In situ hybridization and immunohistochemical observations have indicated that mRNAs for the three TGF-β isoforms are expressed in endometrial tissues throughout the menstrual cycle, with the highest level expressed during the late proliferative and early midsecretory phases (Chegini et al., 1994b). It would be interesting to determine whether the TGF-β receptor expression pattern parallels the cycle-dependent ligand expression pattern. Our results, together with the identification of TGF-β isoforms in the endometrium (Kauma et al., 1990; Chegini et al., 1994a,b; Tang et al., 1994) pro-

vide a basis for the definition of TGF-β function in the human uterus.

Acknowledgements

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