Phosphorylation and Activation of Transforming Growth Factor Beta (TGF-ß) Receptor Kinases

by

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M. S. Biology

Beijing University, 1989

Submitted to the Department of Biology
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

at the
Massachusetts Institute of Technology

June 1996

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April 8, 1996

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I was born not knowing
and have only had a little time to change that here and there.

Richard Feynman

Das Sein ist ewig; denn Gesetze
Bewahren die lebend'gen Schätze
Aus welchen sich das All geschmückt.

GOETHE

Being is eternal; for laws there are to conserve
the treasures of life on which the Universe draws for beauty.

(English translation from "WHAT IS LIFE?", by Erwin Schrödinger)

To my mother and father,
You-kun Quan and Guo-ying Chen

and
For all my mentors

ACKNOWLEDGMENTS

The many best years of my life at MIT and the Whitehead Institute have been a very valuable time; I have learned so much!

-- I thank my advisor, Bob Weinberg, for his intellectual example, for his personal guidance, and for the privilege to grow up in such an exhilarating environment where I have met so many excellent colleagues and warm friends whom I will treasure forever.

-- I thank the members of my thesis committee: Phil Sharp for his scientific excellence and inspiration, Harvey Lodish for his continuous encouragement and support, Jackie Lees for her patience and technical directions, and Patricia Donahoe for her interest and willingness.

-- I thank Drs. Richard Hynes and Frank Solomon and many other MIT faculty members who have influenced me professionally and personally during my graduate education.

-- I thank my former mentors in China, Professors Zhonghei Zhai, Mingxiao Ding, Weichun Pan, Suyun Wang and Zhangliang Chen, for the opportunity to pursue my graduate training at MIT.

People in the Weinberg laboratory, at the Whitehead Institute and MIT, have shaped me in many ways; I will not be who I am today without them.

-- I thank those who helped smooth my transition into the lab: Tyler, Phil, Sybella, Jeff, Charlie, Sean, Peter, David, Steve, Masanori, Shuntaro and Qiang.

-- I thank those who came after with diverse interests and talents: Yan, Claude, Yin, Maricarmen, Cathrin, Julie, Bill, Matthew and Chris.

-- I thank my ideal labmates: Ralph for his wisdom, Tomi for always being there, Rene for his optimism, Felix for computer assistance, Julie for showing me the efficient ways of working at the bench, and all the fun time.

-- I thank Riki, a wonderful colleague and special friend, for going through the most exciting and difficult times together along our collaborative research endavors.

-- I thank the joint TGF-ß group between the Weinberg and Lodish labs, all the past and present members, Xiao-fan, Herb, Elinor, Andrew, Dick, Aris, Petra, Yoav, Toru, Carlos, Becky, Haya, Kunxin and Xuedong, for many stimulating lunchs together.

-- I thank Chris in the office, for her warm smile and helping hands.

-- I thank the Mulligan lab, especially Connie, for being an excellent neighbor.

-- I thank my accompanying graduate students: Gene, Bart, Amin, Sang-Ho, David, Mary, Sachiko, Anne and Brenda, for all the precious memories by the Charles.

-- And in particular, I thank Matthew and Riki for critical reading this thesis.

Graduate school is a long path; studying abroad is a challenge.

-- I thank many friends who have made my time in Boston more enjoyable and memorable, especially, Liming, Hong, Fei, Joy, Hai, Bin, Liuzhong, Chuangjie, Yongshu, Fang, Xiaoquan, Donna, Beth, Fahah and Pam.

-- I thank my wonderful family, Grandma, Mom, Dad, Sister and Brother, for their forever affection and support, that I often take for granted and that make makes me never feel being away from home.

Finally, I thank Glen, for his love and trust, for impressing upon me his appreciation of nature and sharing with me his perspectives in life.

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ABSTRACT

Transforming growth factor-beta (TGF-ß) exerts broad biological effects in the control of growth, differentiation, development and pathogenesis. TGF-ß signals through a receptor complex containing the type I (TGF-ßRI) and type II (TGF-ßRII) receptors. My research has focused on biochemical studies of the early events in the TGF-ß signaling pathway, especially the phosphorylation and activation of the TGF-ß receptor kinases.

TGF-ßRII is highly phosphorylated when expressed alone in COS1 cells; its autophosphorylation occurs via an intramolecular (cis-) mechanism that is independent of ligand binding (Chapter 2). TGF-ßRI is also highly phosphorylated when expressed alone in COS1 cells. While TGF-ßRI does not seem to affect the phosphorylation pattern of TGF-ßRII, both wild type TGF-ßRI and a kinase-deficient mutant thereof are transphosphorylated by the co-expressed TGF-ßRII kinase in a ligand-independent fashion in these cells (Chapter 3). Furthermore, a truncated TGF-ß RII lacking both the extracellular and transmembrane regions was constructed and expressed in COS1 cells. In spite of its inability to bind ligand, this truncated TGF-ßRII not only maintains its autophosphorylation characteristics, but also is able to transphosphorylate the full length TGF-ß RI co-expressed in COS1 cells, indicating an intrinsic affinity of type I and II kinase domains for each other independent of ligand (Chapter 4).

Taken together, these data suggest that the association of TGF-ß type I and II receptors, induced either by ligand binding or by overexpression, leads to transphosphorylation of TGF-ßRI by the TGF-ßRII kinase. This in turn may allow one or both of the associated kinases to direct their activities toward downstream substrates. Such novel mechanisms of receptor activation are distinct from those of tyrosine kinase receptors and may well apply to other serine/threonine kinase receptors.

Finally, the constitutive phosphorylation of TGF-ßRII was also demonstrated by examining the endogenous receptors of two cell lines whose growth is either inhibited (HaCat) or stimulated (HER14) in response to TGF-ß. This provides a useful system to establish the biological significance of the biochemical events in TGF-ß signal transduction (Chapter 5).

Thesis Supervisor: Robert A. Weinberg, American Cancer Society Professor of Biology.

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ABBREVIATIONS

TGF-ß	transforming growth factor ß
TGF-ßR	transforming growth factor ß receptor
TGF-ßRI	type I receptor for transforming growth factor ß
TGF-ßRII	type II receptor for transforming growth factor ß
TGF-ßRIII	type III receptor for transforming growth factor ß
ActR	receptor for Activin
BMP	bone morphogenetic protein
dpp	decapentaplegic
MIS	Müllerian Inhibiting Substance
HA	hemagglutinin
DSS	disuccinimidyl suberate
endoH	endoglycosidase H
PNGaseF	peptide N-glycosidase F
GST	glutathione S-transferase
EGFR	epidermal growth factor receptor
PDGF	platelet-derived growth factor
CS	calf serum
DMEM	Dulbecco's modified Eagle's medium
Mv1Lu	mink lung epithelial cell(s)
PAI	plasminogen activator inhibitor
ELISA	enzyme-linked immunosorbent assay
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis

Chapter 1

Introduction

I. THE TGF-ß LIGANDS

1.1 The Physiology of TGF-ß

Effects on Cell Growth and Differentiation

Proteins belonging to the TGF-ß family are prototypes of a large superfamily of polypeptides that have pivotal control functions in many physiological and pathological processes (Roberts, 1990). The first member of the family, TGF-ß1 (transforming growth factor ß1), was isolated more than a decade ago. It was named because of its ability to induce a transformed phenotype in mesenchymal cells (Moses et al., 1981; Roberts et al., 1981; 1983). Following the initial purification and characterization of TGF-ß1 (Assoian et al., 1983; Frolik et al., 1983), there has been an exponential growth of our knowledge about this versatile molecule. To date, more than two dozen relatives of TGF-ß1 have been identified from a variety of organisms defining the TGF-ß superfamily (Kingsley, 1994). This large group of secreted signaling molecules appear to play important roles in regulating cell growth, differentiation and tissue organization (Kingsley, 1994; Massagué et al., 1994). Clearly, the original definition of TGF-ß based solely on its "transforming" activity is rather narrow and somewhat misleading. Indeed, a cell context-dependent, multifunctional nature is characteristic of TGF-ß, in that it is able to stimulate as well as inhibit cell proliferation and more strikingly, both responses can be observed in the same cells depending upon their growth phases and conditions (Massagué, 1990; Moses et al., 1990).

No clear evidence exists indicating that TGF-ß is able to directly activate any of the known mitogenic pathways. It seems to operate indirectly by regulating the mitogens and their receptors or extracellular matrix components and cell adhesion receptors (Guadagno et al., 1993; Massagué, 1990). Since these effects of TGF-ß are shared with other growth factors, they are unlikely to account for its specific

actions; however, the mechanism by which the cross-talk occurs is still of great interest. Interestingly, the ability of TGF-ß to modulate expression of receptors for other mitogens appears to be cell-specific and therefore, is likely to be related to its mode of action on those cells. For example, in NRK cells, TGF-ß increases synthesis of EGF receptors, which correlates well with the growth-stimulating effects of TGF-ß on these cells (Assoian et al., 1984). In AKR cells, TGF-ß causes delayed mitogenesis, which is perhaps mediated indirectly by induction of c-sis mRNA and secretion of PDGF-like proteins; also induced were the PDGF-regulated genes c-myc and c-fos (Leof et al., 1986; Shipley et al., 1985). In endothelial cells, TGF-ß induces expression of both the A and B chains of PDGF (Daniel et al., 1987; Starksen et al., 1987). However, in some leukemia cell lines, TGF-ß selectively increases expression of the PDGF A chain (Mäkelä et al., 1987).

The best documented effect of TGF-ß on cells is its ability to act as a potent growth-inhibitory compound for a wide variety of cell types, particularly pronounced in epithelial, endothelial and hematopoietic cells (Roberts, 1990). Also attributed to its antiproliferative activity, at least in part, is the immunosuppressive effect of TGF-ß on lymphocytes (Wahl, 1994). In addition, the *in vivo* anti-mitogenic action of TGF-ß has been demonstrated by its inhibition of growth and morphogenesis in mammary tissue (Silberstein and Daniel, 1987).

Studies on the cell cycle of a variety of cell types growth-suppressed by TGF-ß indicate that the inhibitory effects of TGF-ß result in arrest of these cells in middle to late G1 phase, delaying entry into S phase (Alexandrow and Moses, 1995). Interestingly, this is the period preceding the "restriction point" defined by Pardee as a discrete time point during the transition from a serum-dependent to serum-independent state (Pardee, 1989). Once cells pass beyond the "restriction point" as they approach the end of G1 phase, they become committed to completing the division cycle autonomously in a fashion that is

indifferent to mitogens; it is at this stage that cells can no longer be inhibited by TGF-ß even with its receptors and transcription responses still operative (Laiho et al., 1990).

The mechanism underlying the antiproliferative effect of TGF-ß has been linked to pRB, the product of the retinoblastoma tumor suppressor gene, which plays central roles in regulating the cell cycle clock apparatus. pRB exists in two distinct phosphorylation states which correlate with its growth-inhibitory functions in a cell cycle-dependent manner: during the early G1 phase of the cell division cycle, pRB is hypophosphorylated and active in binding and sequestering E2F transcription factors and other components required for the execution of S phase; in contrast, pRB becomes hyperphosphorylated and therefore inactive in mid to late G1, causing the release of these factors. The cyclin-dependent kinases (CDKs), in conjunction with their G1 cyclin partners as regulatory subunits, have been established as the key enzymes controlling pRB phosphorylation. Indeed, TGF-ß prevents pRB hyperphosphorylation, thus maintaining pRB in an active growth-inhibitory state that is incompatible with cell cycle progression (Weinberg, 1995).

Two major molecular mechanisms have been proposed to explain how TGF-ß blocks pRB phosphorylation. First, TGF-ß directly down-regulates certain G1 cyclins and CDKs depending on the cell type; for example, cyclin E, Cdk2 and Cdk4 in human keratinocytes, Cdk4 in mink lung epithelial cells and cyclin E in breast epithelial cells, respectively (Ewen et al., 1993; Geng and Weinberg, 1993; Slingerland et al., 1994). While these effects are largely at the transcriptional level, TGF-ß does dramatically reduce the Cdk4 protein levels in mink lung cells without affecting its mRNA levels (Ewen et al., 1993; 1995).

TGF-ß may alternatively act through recently discovered CDK inhibitors (CDIs). Two classes of CDIs with distinct molecular structures and function modes have been identified to date. p27 interacts with G1 cyclin/Cdk complexes containing Cdk2, Cdk4 or Cdk6, and inhibits their activation by CAK (Polyak et al., 1994a; Slingerland et al., 1994; Kato et al., 1994; Nourse et al., 1994; Toyoshima and Hunter, 1994). Expression levels

of p27 are not affected by TGF-ß, yet its association with Cdk2 has been found in TGF-ß treated cells, this suggests the possible regulation of its activity at a posttranslational level (Ewen et al., 1993; 1995; Polyak et al., 1994b). p21, which shares limited homology with p27 in the regions that bind and inhibit CDKs, is upregulated by the p53 tumor suppressor gene product in response to DNA damage (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). Mice lacking p21CIP/WAF1 undergo normal development, but are defective in G1 checkpoint control of the cell cycle (Deng et al., 1995). Interestingly, it has also been shown that TGF-ß induces p21 through a p53-independent mechanism (Datto et al., 1995a; 1995b).

While both p21 and p27 target various cyclin/Cdk complexes, another class of CDI including p15 and p16, seem to target only Cdk4 and its close homologue Cdk6 (Hannon and Beach, 1994; Serrano et al., 1993). p15 expression is induced by TGF-ß (Hannon and Beach, 1994) and the inhibitory effects of both p15 and p16 on cell proliferation require functional pRB (Guan et al., 1994; Medema et al., 1995; Serrano et al., 1995). Taken together, a "threshold model" has been proposed in which an adjustable level of CDI determines the level of CDK necessary for cell cycle progression by stoichiometrically inhibiting amounts of CDK below this threshold. Accordingly, cell cycle inhibition can be accomplished by increasing CDI levels and/or decreasing CDK levels (Massagué and Polyak, 1995).

It is worth mentioning that the p16 as well as p15 genes have been implicated as tumor suppressor genes based on their involvement in a various human malignancies (Fynan and Reiss, 1993; Kamb et al., 1994; Nobori et al., 1994). However, the p21 and p27 genes seem to be mutated infrequently in human tumors, if at all. These observations have led to the hypotheses that perhaps p21 and p27 are essential for the normal cell growth; that loss of their functions will perhaps be incompatible with cell viability; and that cells harboring mutations of p21 and/ or p27 will be eliminated and escape detection.

Another protein, c-myc, has also been implicated in playing a role in the TGF-ß-mediated inhibition of the cell cycle. TGF-ß1 treatment rapidly down-regulates c-myc expression at both the RNA and protein levels in several different cell lines, and c-myc expression has been shown to be required for cell growth, particularly at the G1-S transition (Coffey et al., 1988; Gai et al., 1989; Pietenpol et al., 1990a; 1990b; Ruegemer et al., 1990; Takehara et al., 1987). One possible mechanism by which c-myc affects the cell cycle progression is at least indirectly by regulating the expression of G1 cyclins including cyclins E, A and D1 (Jansen-Durr et al., 1993; Philipp et al., 1994; Shibuya et al., 1992). Taken together, it has been hypothesized that TGF-ß1 may block cell growth by suppression of the c-myc gene product. Indeed, recent studies have demonstrated that overexpression of c-myc protein can block the inhibition by TGF-ß1 of two cell lines into S phase (Alexandrow et al., 1995; Selvakumaran et al., 1994).

The rapid progress made in the past few years in dissecting the cell cycle machinery and characterizing the membrane receptors for TGF-ß has provided insights into the mechanism(s) of TGF-ß functions. However to date, the cytoplasmic network connecting the TGF-ß receptors to their nuclear targets (CDIs/Cyclins/CDKs) remains elusive. Some possible candidates which have been suggested to be the components in the TGF-ß signaling pathway will be discussed later (section 4 of Chapter 6).

Effects on Extracellular Matrix (ECM)

Both *in vitro* and *in vivo* evidence suggests that TGF-ß causes increased synthesis of extracellular matrix proteins. It was first reported that TGF-ß treatment induced increased levels of type I collagen and fibronectin in many fibroblasts (Ignotz and Massagué, 1986; Roberts et al., 1986; Wrana et al., 1986). There are also observations that some other matrix proteins, including type II, III, IV and V collagen (Galera et al., 1992; Heckmann et al., 1992; Konig and Bruckner-Tuderman, 1992), thrombospondin

(Murphy-Ullrich et al., 1992), tenascin (Pearson et al., 1988) and proteoglycans (Morales and Roberts, 1988; Nugent and Edelman, 1992) are induced by TGF-ß.

TGF-ß acts at multiple levels in controlling the matrix interactions of cells. First, TGF-ß activates gene transcription and increases synthesis and secretion of matrix proteins. Several studies have shown that TGF-ß elevates mRNA levels encoding types I, III, VI and V collagen and fibronectin (Armendariz-Borunda et al., 1992; Hansch et al., 1995; Kikuchi et al., 1992). Experiments have demonstrated that TGF-ß can directly stimulate the activity of the mouse α2(I) collagen promoter and the fibronectin promoter (Dean, 1989; Dean et al., 1988; Greenwel et al., 1995; Rossi et al., 1988). In addition, under certain growth conditions, increased mRNA levels of the matrix proteins also result, in part, from the TGF-ß effects on stabilization of the mRNA (Penttinen et al., 1988; Raghow et al., 1987).

Secondly, TGF-ß decreases synthesis of proteolytic enzymes that degrade matrix proteins and increases synthesis of protease inhibitors that block the activity of these enzymes. There is substantial evidence supporting a direct role of TGF-ß in altering mRNA levels of affected genes. Depending on the cell type, these target genes include plasminogen activator inhibitor (Laiho et al., 1986; 1987; Lund et al., 1987), collagenase (Overall et al., 1989), metalloproteinase (Overall, 1995), and a thiol protease (major excreted protein) (Chiang and Nilsen-Hamilton, 1986).

Yet a third way by which TGF-ß acts to increase the interaction of cells with extracellular matrix is to increase the transcription, translation, and processing of cellular receptors for matrix proteins. TGF-ß can specifically and selectively regulate the expression of both the α and ß subunits of integrins which serve as the fibronectin receptor complex (Heino et al., 1989; Ignotz and Massagué, 1987). The coordinate regulation by TGF-ß of synthesis of both fibronectin and its receptor complex is necessary for assembly of fibronectin into the pericellular matrix (McDonald et al., 1987). As a result, TGF-ß

treatment has been shown to promote increased adhesion to fibronectin and collagen substrates in a variety of cell types (Ignotz et al., 1989; Ignotz and Massagué, 1987).

Effects on Pathogenesis

Given the multifunctional nature of TGF-ß, it is not surprising that it has been found to be involved in many pathogenetic processes. For instance, due to its potent inductive effects on the extracellular matrix, TGF-ß has significant implications in wound healing and the pathogenesis of diseases such as arthritis and atherosclerosis (Border et al., 1992; Border and Ruoslahti, 1992)

However, the hypothesis that loss of growth regulation by TGF-ß may be an important step in carcinogenesis has attracted the most attention in the TGF-ß field for the past decade. It has long been found that many cultured malignant or transformed cell lines frequently have lost their ability to respond to TGF-ß, which often correlates with the absence of TGF-ß surface receptors. Retinoblastoma cells, which are resistant to growth inhibition by TGF-ß, do not express TGF-ß receptors on their cell surface (Kimchi et al., 1988). Gastric cancer cells that are resistant to TGF-ß have been shown to have truncations or undetectable levels of the type II TGF-ß receptor (Park et al., 1994). A hepatoma cell line (Hep 3B-TR) which is refractory to TGF-ß has lost both alleles of the TGF-ßRII gene (Inagaki et al., 1993). It has also been reported that certain human bladder carcinoma cells (the EJ cell) and colon adenocarcinoma cells (SW480) are nonresponsive to TGF-ß (Geiser et al., 1992). Furthermore, loss of receptors for TGF-ß has been reported in human T-cell malignancies (Kadin et al., 1994).

Another line of evidence supporting the role of TGF-ß in tumor progression has come from experiments using animal models. Glick described a system of multistage carcinogenesis to study the contribution of autocrine and paracrine TGF-ß1 to tumor progression, in which keratinocytes with a targeted deletion of the TGF-ß1 gene were

initiated in vitro with the v-Ha-ras oncogene. Their *in vivo* tumorigenic properties were determined by grafting initiated cells onto the skin of athymic mice in combination with either wild-type or null dermal fibroblasts. They demonstrated that autocrine TGF-ß1 suppresses the frequency and rate of malignant progression, and that autocrine and paracrine TGF-ß1 can have opposing effects on tumor cell proliferation (Glick et al., 1993; 1994). Consistent with this, overexpression of TGF-ß1 in the epidermis of transgenic mice caused inhibition of skin development (Sellheyer et al., 1993). Mammary tumor suppression by TGF-ß transgene expression has also been reported (Pierce et al., 1995).

The third, perhaps the most exciting line of research to date in this aspect came from the recent discovery that human colon cancer cell lines with high rates of microsatellite instability harbor mutations in the TGF-ßRII gene. Different mutations were found clustered within small repeated sequences in the TGF-ßRII gene, accompanied by the absence of cell surface TGF-ßRII expression and usually associated with small amounts of TGF-ßRII transcript. It is intriguing that these TGF-ßRII mutations, by inducing the escape of cells from TGF-ß-mediated growth control, link DNA repair defects with a specific pathway of tumor progression (Markowitz et al., 1995; Wang et al., 1995)

Effects on Development

The potent effects of TGF-ß in the control of cell growth, differentiation, and extracellular matrix, the central role of TGF-ß in wound healing and carcinogenesis, and the almost universal distribution of TGF-ß receptors on cells, strongly implicate TGF-ß in embryonic development (Roberts, 1990). It has been demonstrated that TGF-ß is localized in a unique pattern, not only spatially, but also temporally, in the developing mouse embryo, correlating with specific morphogenetic and histogenetic events (Heine et al., 1987; Jakowlew et al., 1994; Roelen et al., 1994).

Interestingly, however, mice having a disruption of the TGF-ß1 gene by homologous recombination show no gross developmental abnormalities and survive until about 20 days after birth, when they succumb to a wasting syndrome accompanied by multifocal inflammatory diseases (Kulkarni et al., 1993; Shull and Doetschman, 1994; Shull et al., 1992). This is probably due to the compensation of other TGF-ß isoforms or the contribution of maternal TGF-ß which can be transmitted across the placenta (Letterio et al., 1994). Subsequent analysis has shown that the immune dysregulation in TGF-ß-deficient mice is lymphocyte-mediated (Christ et al., 1994).

1.2 The Superfamily of TGF-ß

Since TGF-ß1, the prototype of the TGF-ß superfamily, was discovered more than a decade ago, a large body of knowledge about its biology has been accumulated. Over two dozen TGF-ß related factors have been identified in flies, worms , frogs, birds and mammals. These TGF-ß superfamily members account for roughly one-fifth of all known cytokines and have a remarkable range of activities (Kingsley, 1994).

Family Members

As it will be discussed later (see 1.3 of this chapter) in more detail, the biologically active form of TGF-ß1 is a 25-Kd homodimer . The amino acid sequences of all members of the TGF-ß superfamily share homology with that of TGF-ß1, particularly in the mature region where the most conserved feature is the spacing of seven cysteines. These structural similarities set the basic criteria for dividing the TGF-ß subfamilies and have led to the identification of new family members. Other family members were discovered from screens for specific biological activities, for example, induction of bone and cartilage by BMPs, and were then assigned to a certain subfamily based on their structural properties.

Historically, the TGF-ß-related factors have been divided into four subfamilies as listed in Table 1.1: transforming growth factor-ßs, the activins/inhibins, Müllerian inhibiting substance, and the decapentaplegic/Vg-1 related (DVR) factors. With recent additions, the borders of these groups have become blurry, and some new members are only distantly related to the existing subfamilies. However, to a certain extent, this classification does reflect the structural similarities and functional conservation, and is very helpful in serving as a basis for discussion of biological functions of this large family of versatile factors.

Table 1.1 Ligands of the TGF-ß Superfamily

TGF-ß subfamily

- TGF-ß1
- TGF-ß2
- TGF-ß3
- TGF-ß5

Activin/Inhibin subfamily

- Activin A (ßA.ßA)
- Activin AB (ßA.ßB)
- Inhibin A (α. ßA)
- Inhibin B (α.ßB)

Dpp/Vg-1 Related (DVR) subfamily

- decapentaplegic (dpp)
- Vg1
- Vg2
- nodal
- 60A
- dorsalin
- BMP2
- BMP3
- BMP4
- BMP5
- BMP6
- BMP7
- BMP8

Müllerian Inhibiting Substance (MIS) subfamily

- MIS

Biological Functions

For a long time, knowledge of the roles of TGF-ß and related members of the superfamily has primarily come from two different experimental approaches. First, the expression pattern of a certain factor indicates spatial and temporal availability, which often correlates with its biological activities. Secondly, examination of the purified and recombinant forms of some factors when exogenously applied to cultured cells or animals has suggested the possible normal functions of these factors. Taken together, these studies have led to many interesting hypotheses. However, the most valuable information on the normal functions of TGF-ß superfamily in key development events has only been recently learned from genetic studies in a variety of organisms (Kingsley, 1994).

Transforming Growth Factor-ß Subfamily There are at least four genes in the TGF-ß superfamily which are much more closely related to TGF-ß1 than to other members. These were initially named TGF-ß2 through TGF-ß5 and collectively termed "TGF-ß". They are actually found in several different species. TGF-ß1, TGF-ß2 and TGF-ß3 were first identified in mammalian species (de Martin et al., 1987; Derynck et al., 1985; 1988; ten Dijke et al., 1988) TGF-ß5 was discovered in Xenopus (Kondaiah et al., 1990); and TGF-ß4 is now considered as the chick homologue of mammalian TGF-ß1 taking into account evolutionary divergence (Burt and Jakowlew, 1992). While the various members of this subfamily share sequence identity as high as about 80% with each other, a given isoform is almost entirely conserved at the amino acid level between different species.

As has been detailed earlier (see this chapter 1.1), TGF-ß has a broad range of biological functions. Although it has been suggested that most of the TGF-ß molecules have similar activities in different systems (Massagué, 1990), one should be aware that supporting data were often derived from cultured cells. Indeed, it is worth mentioning that

TGF-ß1, TGF-ß2 and TGF-ß3 do have distinct expression patterns during murine development and mice harboring null mutations of each of these isoforms show distinct phenotypes, which strongly argue that they play specific roles during mouse development (T. Doetschman, personal communication; Cox, 1995).

Activin/Inhibin Subfamily Activins are homo- or hetero- dimeric molecules including activin A (ßA: ßA), activin B (ßB: ßB) and activin AB (ßA: ßB). The ßA and ßB subunits can also form dimers with another more distantly related subunit called inhibin a, and therefore compose inhibin A (α: ßA) and inhibin B (α: ßB). Interestingly, the activins and inhibins often have opposite effects on many biological systems (Ying, 1989).

To date, genes for all three subunits (ßA, ßB, α) of activins and inhibins have been disrupted in mice using homologous recombination in embryonic stem (ES) cells. Mutation of the ßB subunit leads to defects in eyelid development and female reproduction (Vassalli et al., 1994). Elimination of the ßA subunit, on the other hand, causes abnormal craniofacial development (Matzuk et al., 1995a). Mice lacking both ßB and ßA subunits do not show any additional abnormalities beyond the defects of the individual mutants alone, indicating the non-redundant functions of these two proteins during embryogenesis. Activins have been shown to be essential for mesoderm formation in Xenopus and other lower vertebrates, but surprisingly this is not the case in mice (Matzuk et al., 1995a; 1995b).

Inhibins (α: ßA and α: ßB dimers) were originally identified as inhibitors of follicle-stimulating hormone (FSH) production in the pituitary by providing negative feedback signals (Haisenleder et al., 1990). Consistent with this, inactivation of the inhibin α gene, which should disrupt both forms of inhibin, has lead to elevated FSH levels and infertility of mutant animals although without any other developmental abnormalities. In addition, all mutant animals develop gonadal tumors, an observation

which suggests that inhibin plays a critical role in regulating gonadal stromal cell proliferation and in principle is a tumor suppressor gene (Matzuk et al., 1992).

Müllerian Inhibiting Substance Subfamily As the only member in this group, MIS is rather distantly related to other members of the TGF-ß superfamily, sharing only about 25% amino acid identity. It was originally identified becasue of its ability to induce the regression of embryonic Müllerian duct that would otherwise develop into oviducts and uterus (Lee and Donahoe, 1993). Consistent with its biological activities, MIS has been found to be expressed strictly in the Sertoli cells of the male testes during mouse embryonic development (Munsterberg and Lovell-Badge, 1991) and in the granulosa cells of the adult ovary in developing follicles (Takahashi et al., 1986). Inactivation of MIS in human leads to persistence of Müllerian duct in males; and conversely, ecotopic overexpression of MIS in female transgenic mice inhibits differentiation of Müllerian duct and therefore blocks the formation of normal female reproductive system (Behringer et al., 1990). MIS-deficient male mice develop as pseudohermaphrodites and have Leydig cell hyperplasia. More interestingly, adult inhibin/MIS-deficient male mice have been shown to accumulate Leydig cell tumors and large amount of uterine fluid, indicating the synergistic effects of inhibins and MIS on testicular tumorigenesis (Behringer et al., 1994; Matzuk et al., 1995c).

Decapentaplegic/Vg-1 Related (DVR) Subfamily Named after decapentaplegic (dpp), a protein involved in embryonic morphogenesis in *Drosophila melenogaster* (Padgett et al., 1987), and Vg-1, a gene whose mRNA is specifically localized to the vegetal pole of Xenopus (Weeks and Melton, 1987), this is the largest family with more than a dozen members identified from a variety of different organisms. They have profound implications in both the basic body pattern formation and the more specific morphogenesis during development.

Drosophila offers an excellent system for genetics, and in this sense, the dpp locus is the best-studied among all the genes specifying members of the TGF-ß superfamily. Mutation analysis has suggested that the expression level of dpp is crucial for normal dorsal-ventral axis formation (Ferguson and Anderson, 1992a; 1992b; Wharton et al., 1993). Ectopic expression (Wharton et al., 1993) and RNA injection experiments (Ferguson and Anderson, 1992a; 1992b) have reinforced the notion that dpp may act through an activity gradient to control the specific cell fate determination at different concentrations, assuming that different concentration thresholds exist for different cellular responses (Slack, 1993). In addition to its role in laying down the body plan during fly embryogenesis, dpp is also required for gut formation (Immergluck et al., 1990; Panganiban et al., 1990), and for imaginal discs to develop normally into adult appendages such as eyes, wings, legs, antennae and genitals (Spencer et al., 1982). Moreover, a series of *cis*-acting elements that control these different dpp functions are assembled as a mosaic structure in different regions of the gene (Blackman et al., 1991; Hursh et al., 1993; Masucci et al., 1990), suggesting an interesting mode of regulating its diverse functions. Another single Drosophila gene, 60A, was isolated as a TGF-ß homolog and named after its chromosomal map location yet lacks known functions (Doctor et al., 1992; Wharton et al., 1991).

Another founding member in this subfamily is the Vg1 gene, originally isolated due to its asymmetric distribution in Xenopus oocytes (Weeks and Melton, 1987). Subsequently, Vg1, like several other TGF-ß superfamily members including activins and BMPs, has been shown to have dramatic effects on the induction and patterning of mesoderm tissue of embryos, a function consistent with its strictly vegetal localization in developing oocytes. Proteolytic processing of the Vg1 precursor protein has been suggested as an important mechanism in controlling its specific localization (Thomsen and Melton, 1993).

The mammalian homologs in the Dpp/Vg-1 Related (DVR) group are mostly involved in osteogenesis, as suggested by their names, BMPs (Bone Morphogenetic Protein). Homologous members from different species not only share a high degree of structural similarity, but can also exchange their functions to some extent. For example, with 75% identity to Drosophila DPP, human BMP4 sequences have been shown to rescue the dorsal-ventral axis defects in dpp mutant flies (Padgett et al., 1993); conversely purified DPP proteins can induce bone and cartilage formation in mammals (Sampath et al., 1993).

Genetic evidence for the roles of this family in vertebrate development comes from the characterization of two interesting mouse mutants. BMP5 has been mapped to the mouse "short ear" locus, a classical mutation causing abnormal skeletal development including a much smaller external ear. This finding indicates that BMP5 is essential for normal bone and cartilage development of specific skeletal elements, and also leads to the hypothesis that different genes are utilized in building the skeleton in the different regions of body (Kingsley, 1994). nodal, a mutation derived from a retroviral insertion, on the other hand, appears to play an important role in mesoderm formation in mice. Inactivation of nodal severely interferes with primitive streak formation in early embryogenesis (Zhou et al., 1993).

More extensive screening for additional mammalian members of TGF-ß superfamily has led to the recent discovery of Vgr2/GDF3 (Vg-related gene 2/Growth Differentiation Factor 3), GDF1 and GDF9 with still unknown functions (Lee, 1991; McPherron and Lee, 1993). Dorsalin was identified in the developing chick nervous system (Basler et al., 1993). GDNF (Glial-Derived Neurotropic Growth Factor) was isolated via its ability to stimulate the differentiation and survival of dopaminergic neurons form the midbrain (Lin et al., 1993).

In summary, studies of this large group of diverse factors has revealed valuable information on the roles of TGF-ß superfamily in normal development. Indeed, they have contributed greatly to establishing the principles for resolving some long-lasting mysteries. They also provide excellent systems for genetic analysis and developmental manipulations for the future studies of the TGF-ß superfamily. And most importantly, they lead to the realization that similar signaling pathways and mechanisms operate in vastly different organisms during development.

1.3 The Structure of TGF-ß

Although the various members of the TGF-ß superfamily have a broad range of biological activities, they do share common structural features with TGF-ß1, the prototype of the family. Not only do they show sequence similarity to TGF-ß1, they also follow similar pathways to become bioactive forms from latent precursors, as detailed below.

Processing of TGF-ß

TGF-ß is secreted from most cell types, but in a biologically inactive form that is further processed into a bioactive dimer. The ligand is initially synthesized as a large precursor with an apparent molecular weight of about 100 kDa. After dimerization, its C-terminal region of 112 amino acids is then cleaved out at a defined site with a cluster of basic residues and results in the mature TGF-ß dimer of about 25 kDa (Massagué, 1990; Roberts, 1990). The remaining 75kDa N-terminal portion of the precursor, called the pro-region or ß-LAP for "TGF-ß latency associated protein", may remain noncovalently bound to the bioactive domain (Gentry and Nash, 1990) and together with the bioactive region yields an inactive complex referred as "latent TGF-ß". In some cases, another latent

TGF-ß binding protein, named LTBP, has also been found in association with this inactive TGF-ß complex, although its function remains unclear. It has been shown that the TGF-ß latent complex is unable to bind receptors and is therefore biologically inactive until later activation in the extracellular medium (Miyazono et al., 1993)

The cleavage of the TGF-ß precursor and the release of the mature dimeric form are considered to be important mechanisms in regulating TGF-ß activity. *In vitro*, a number of methods have been developed to activate the latent TGF-ß: chemical treatment at low pH (usually under 4), heating, or alternatively, enzymatic digestion with plasmin or cathepsin D (Lyons et al., 1988). However, the physiological mechanisms for TGF-ß activation are still largely unknown, although many hypotheses have been proposed. In particular, a number of extracellular matrix components have been implicated in regulating this process (McCaffrey et al., 1992; Miyazono et al., 1993; Ruoslahti and Pierschbacher, 1986; Wakefield et al., 1990).

Crystal Structure of TGF-ß2

Recently, the crystal structure of TGF-ß2 has been solved. It has revealed many very interesting features which are consistent with previous studies based on amino acid sequence, NMR (nuclear magnetic resonance), and protein domain swap analyses (Daopin et al., 1993; Schlunegger and Grutter, 1993). It has long been noticed that the most conserved feature among all the TGF-ß superfamily members is the spacing of seven cysteine residues. The crystallography studies have now shown that six of these seven cysteines are closely arranged in a rigid central structure called the "cysteine knot". This knot links several twisted ß-sheets in forming an extended structure like two slightly curved fingers, opposite to the heel of a hand, a structure formed by an α-helix. The remaining cysteine residue (Cys77) in each monomer then bridges two monomers with an additional disulfide bond. The overall structure is rather open and is very similar to the structure

proposed for TGF-ß1 based on the NMR studies (Archer et al., 1993). A TGF-ß1 mutant with Cys77 replaced by serine, [Ser77]TGF-ß1 has been expressed in CHO (Chinese hamster ovary) cells and is reported to act as a full agonist in both growth inhibition and transcription activation assays. It is 20% as potent as native TGF-ß1 in the induction of plasminogen activator inhibitor (PAI) promoter expression in mink lung cells (Mv1Lu), while it is less than 1% as potent as native TGF-ß1 in inhibition of growth in the same cell line. [Ser77]TGF-ß1, an expected monomer as revealed on the SDS-PAGE, binds to TGF-ßRII with lower affinity than that of the native TGF-ß1. Interestingly, it binds to soluble TGF-ßRIINt and competes with native TGF-ß1; however, in Mv1Lu cells, the mutant TGF-ß1 shows preferential crosslinking to the type I rather than the type II TGF-ßR (Amatayakul-Chantler et al., 1994).

The crystal structure of TGF-ß2 has provided valuable information on the specificity of different TGF-ß family members. For example, TGF-ß1 is 100-fold more potent than TGF-ß2 in a growth inhibition assay on endothelial cells. A 42-amino-acid region responsible for the different potencies has been identified by domain swap between TGF-ß1 and ß2; this includes the longest α-helix outside the rigid "cysteine knot" in the monomer as viewed in the TGF-ß2 crystal structure (Qian et al., 1992). Based on their amino acid sequences, differences between members of the TGF-ß superfamily have been mapped to several distinct regions of these molecules; in particular, dramatic variations are found in their N-terminal portions. The amino terminus of TGF-ß2 forms an α-helix, which is bridged to the core structure of the monomer by an additional disulfide bond that is missing in most of the other members (Schlunegger and Grutter, 1993). The crystal structure of TGF-ß2 has also offered some predictions on the regions of the TGF-ß ligand that may be involved in interacting with its receptors, but further structural and functional studies are needed to define these domains.

Since the primary sequences of the superfamily members are well conserved, it is expected that they also share similar three-dimensional structures. Interestingly, the novel

"cysteine knot" motif identified in TGF-ß2 crystal structure has also been discovered in the monomer structure of members of the nerve growth factor (NGF) and platelet-derived growth factor (PDGF) families despite the fact that they show less than 10% amino acid sequence similarity. On the other hand, the further arrangement between the monomeric subunits are very different among the dimers of TGF-ß, NGF and PDGF (McDonald and Hendrickson, 1993). This might suggest that all these three growth factor families are derived from a common ancestor, yet have evolved separately to give rise to differences in the higher order structure of the factors and interactions with their receptors.

II. THE TGF-ß RECEPTORS

Here I will focus on the characterization and molecular cloning of cell surface receptors for TGF-ß. While the basic structural features will also be discussed, many functional studies will be summarized later (this chapter, III. TGF-ß SIGNALING).

2.1 The TGF-ß Binding Proteins

Knowing that TGF-ß plays an important role in a variety of biological processes, it has been of great interest to understand the mechanisms by which it signals such diverse actions. Information on the TGF-ß ligands has made it possible to start searching for TGF-ß interacting proteins on the cell surface , which presumably are the transmembrane mediators of TGF-ß signals. Starting in the early 1980s, various methods were developed for affinity labeling of radioiodinated TGF-ß to cells (Frolik et al., 1984; Massagué and Like, 1985; Tucker et al., 1984; Wakefield et al., 1987). These resulted in major progress in identifying the TGF-ß binding proteins on the cell surface. Hundreds of different cell types and cell lines have been screened and, with only very few exceptions, nearly all cells bind TGF-ß with affinities in the range of picomolar concentration (Roberts, 1990).

Further analyses using chemical cross-linking procedures have revealed three distinct classes of cell membrane proteins that are the most widely expressed and bind TGF-ß specifically with high affinity (Massagué, 1990). They are called the type I, type II, and type III receptors for TGF-ß, with molecular weights of 50-55kDa, 70-85kDa and 200-400kDa, respectively. As detailed in a later section of this chapter (III. TGF-ß SIGNALING), evidence for the functional relevance of these TGF-ß binding proteins in TGF-ß signaling came mainly from a set of mutant cell lines derived from Mv1Lu epithelial

cells, whose loss of TGF-ß surface receptors correlates with their resistance to TGF-ß (Laiho et al., 1991).

Other cell-surface TGF-ß binding proteins include the following. The type IV receptor, a 70kDa transmembrane protein found in GH3 pituitary cells, binds to TGF-ß as well as activin and inhibin (Cheifetz et al., 1988) and recently has been cloned as a dual specificity type I receptor (Takumi et al., 1995). It has also been reported that a type V receptor, having a molecular weight of 400 kDa, binds to TGF-ß1. Interestingly, the purified type V receptor from bovine liver membranes has serine/threonine kinase activity (O'Grady et al., 1991; 1992).

In addition, a number of soluble proteins have been found to interact with TGF-ß, such as α2-macroglobulin (O'Connor-McCourt and Wakefield, 1987), the thrombospondin (Murphy-Ullrich et al., 1992), α-fetoprotein and ß-amyloid precursor protein (Attisano et al., 1994; Miyazono and Heldin, 1992). More importantly, there is increasing evidence that the soluble betaglycans decorin and biglycan are involved in regulating the availability of TGF-ß ligand for the signaling receptors (Border and Ruoslahti, 1992). They bind to TGF-ß and therefore can sequester the ligand and only release it in response to certain activating signals; alternatively they can serve as accessory molecules that present ligands to their signaling receptors, a role that is also proposed for the type III TGF-ß receptor, as discussed below.

2.2 The Type III Receptor

Compared to the type I and type II receptors for TGF-ß, the type III receptor is unique in a number of ways: it is a large protein with a broad range of molecular weight due to the heterogeneous heparin sulfate and chondroitin sulfate glycosaminoglycan chains; it also has broader specificity for all isoforms of TGF-ß, meaning that it binds TGF-ß1, TGF-ß2 and TGF-ß3 with high affinity while the type I and II receptors seem to bind

poorly to TGF-ß2; lastly, unlike the two other types of TGF-ß receptors that are lost in some tumor cells, the presence of type III does not appear to correlate with the tumorigenicity of these cells (Massagué, 1990; Roberts, 1990).

Using the powerful expression cloning technique, the type III receptor was the first TGF-ß receptor cloned. Sequence analysis of this betaglycan revealed that it has a single transmembrane domain, a large extracellular domain containing complex sulfate chains, and a short cytoplasmic tail with no discernible structure involved in signal transduction (Lopez-Casillas et al., 1991; Wang et al., 1991). However, it is curious that this tiny domain with only 43 amino-acid is very rich in serines and threonines. It shares significant sequence similarity with endoglin, which is a dimeric membrane glycoprotein that was originally identified in endothelial cells and also binds to TGF-ß (Cheifetz et al., 1992; Gougos and Letarte, 1990). Interestingly, it has been shown that endoglin is differentially expressed on fetal and adult hematopoietic cells in human bone marrow and mutations of endoglin are associated with hereditary haemorrhagic telangiectasia (McAllister et al., 1994; Rokhlin et al., 1995).

There has been considerable controversy regarding the roles of the proteoglycan type III receptor in TGF-ß action. Experimental data generated by using the cloned receptor have illustrated at least several important points. When expressed in cells, betaglycan increases the binding of free TGF-ß in the cell culture medium to its signaling receptors. This effect is particularly obvious when TGF-ß2 is examined, likely due to its otherwise very low affinity for the type I and II receptors (Lopez-Casillas et al., 1991). This suggests a role for the membrane-anchored betaglycan as an accessory receptor in presenting TGF-ß to the signaling receptors. In contrast, however, the extracellular domain of betaglycan produced in insect cells acts as an antagonist of TGF-ß binding and function. Because the soluble form of this betaglycan normally exists in the medium, it was proposed that betaglycan might regulate the actions of TGF-ß *in vivo* via the balance

between its two opposite roles in interfering with ligand binding (Andres et al., 1989; Lopez-Casillas et al., 1991; 1994).

2.3 The Type II Receptor

In the past few years, genes specifying receptors for various members of the TGF-ß superfamily have been isolated. Progress in cloning the receptor genes has also led to the discovery of a novel class of transmembrane receptors having a serine/threonine kinase domain within their cytoplasmic region and set a new mode of the signal transduction through the cell surface (Kingsley, 1994; Lin and Lodish, 1993; Massagué, 1992; 1994).

The first indication that the growth factors in the TGF-ß superfamily may employ the transmembrane ser/thr kinases to transduce their signals came from the isolation of the activin type II receptor gene, ActRII (Mathews and Vale, 1991). Until then, only two other putative transmembrane serine-specific protein kinases had been described, encoded by the nematode daf-1 gene (Georgi et al., 1990) and the ZmPK1 gene of maize (Walker and Zhang, 1990), neither with known ligands. It is from here that a family of new receptor kinases started emerging. Subsequently, another type II activin receptor gene, ActRIIB, was identified in a PCR-based screen for new members of the protein serine/threonine kinase receptor family (Attisano et al., 1992). Interestingly, in mouse, ActRIIB exists as at least four isoforms generated from alternative splicing, varying in their affinity for activin (Attisano et al., 1992; Mathews et al., 1992). Furthermore, evidence has been provided that the different isoforms of ActRIIB may be responsible for the remarkable concentration dependence of the activin ligand in cells (Green et al., 1992).

The type II receptor for TGF-ß, TGF-ßRII or TßRII, was also identified using an expression cloning approach (Lin et al., 1992). Sequence analysis revealed that the overall structure of TßRII is composed of a cysteine-rich extracellular domain, a single

hydrophobic transmembrane domain, and a predicted cytoplasmic serine/threonine kinasae domain that fits well into the new family of ser/thr kinase receptors. With very limited homology at their extracellular regions, TGF-ßRII show extensive homology with ActRII (45%) and Daf-1 (33%) at their intracellular kinase regions. It shares about 20% homology with most cytoplasmic ser/thr kinases when a kinase family tree was constructed based on their evolutionary relatedness (Hanks et al., 1988; Lin et al., 1992).

Other type II receptors for the TGF-ß superfamily include C14, Atr-II, daf-4 and BMPRII as listed in Table 1.2. Since C14 has a restricted expression pattern, it has been considered as a candidate for the MIS receptor (Baarends et al., 1994). Atr-II, an activin receptor, is the first member of this family of growth factors that has been cloned in Drosophila (Childs et al., 1993). The product of the daf-4 gene in *C. elegans* is the type II receptor for BMPs (Estevez et al., 1993), while it has been shown that daf-1 gene actually encodes a type I receptor for activin in the worm (Georgi et al., 1990). BMPRII, the human type II receptor for BMPs was recently cloned by a yeast two-hybrid and a PCR-based screen (Liu et al., 1995; Rosenzweig et al., 1995).

Table 1.2 Receptors of the TGF-ß Superfamily

Type I subfamily

TSRI / ALK-1 / R3

ActRI / ALK-2/ R1 / Tsk7L / SKR1

BRK-1 / ALK-3

ActRIB / ALK-4 / R2

TGF-ßRI / ALK-5 / R4 / RPK2

DAF-1

AtrI

RPK-1

Type II subfamily

ActRII

ActRIIB

ArtII

TGF-ßRII

BMPRII

DAF-4

C14

2.4 The Type I Receptor

The history of the cloning of the type I receptors of the TGF-ß superfamily actually reflects nicely the differences between the type I and the type II receptors. Originally, a number of transmembrane ser/thr kinases were isolated from the search for new receptors of various members of the TGF-ß superfamily (Franzen et al., 1993; He et al., 1993). However, they remained orphan receptors, named "ALKs" for activin-like kinases, since no relevant ligands could be identified in the binding-crosslinking assays when expressed alone. Later when they were co-expressed with a variety of different type II receptors and tested for the corresponding ligands, it was realized that the type I receptors can not bind to ligands without associating with type II receptors and that their specificity is indeed determined by the type II partners (Attisano et al., 1993; Bassing et al., 1994; Ebner et al., 1993a; 1993b; Franzen et al., 1993). To date, there are at least eight members of this subgroup as listed in Table 1.2.

In comparison with the type II receptors, the type I receptors have a unique "GS domain" characterized by a conserved SGSGSG motif which is located just N-terminal to the kinase region. Also, the type I receptors have a shorter extracellular domain and lack a tail sequence on the C-terminus of the kinase domain. In addition, members of this subgroup display 60-70% identity among their kinase domains and therefore are closely related to each other, composing a distinct kinase subfamily (Attisano et al., 1993; Franzen et al., 1993).

Having identified the major players in the TGF-ß signaling pathway as a novel class of ser/thr receptor kinases , a fascinating journey then begins with the hope of discovering the underlying mechanisms by which these kinases transduce signals across the membrane.

III. TGF-ß SIGNALING

It has long been acknowledged that cells respond to a broad range of extracellular signals through membrane receptors containing protein kinases within their cytoplasmic domains. Receptors bearing tyrosine kinase activity are among the best studied of these (Schlessinger and Ullrich, 1992; Taniguchi, 1995; Ullrich and Schlessinger, 1990). As reviewed earlier in this chapter, the identification of receptors for the TGF-ß superfamily members has led to the discovery of a new class of receptor kinases with specificity toward serine/threonine residues. The fact that TGF-ß superfamily members and their receptors are found to control many important biological processes in species ranging from worms to human has attracted a great interest in the mode of the signal transduction of these ser/thr kinase receptors.

3. 1 A Heterodimeric Model for the TGF-ß Receptors

As mentioned earlier, the first insight into the requirement of both the type I and type II TGF-ß receptors (TGF-ßRI and TGF-ßRII) for TGF-ß signal transduction came from the study on a set of mutant Mv1Lu mink lung epithelial cells. They were originally generated from chemical mutagenesis using ethyl methanesulfonate (EMS, (Lebkowski et al., 1986) and selected for resistance to TGF-ß. In contrast to the parent cells, these mutant cell lines show non-responsiveness to TGF-ß when assayed for growth inhibition and transcriptional activation, which correlates with their loss of TGF-ß receptors on the cell surface (Laiho et al., 1991).

These mutant Mv1Lu cells were subsequently grouped into three categories based on genetic complementation studies and their deficiency in TGF-ß receptors was characterized: R mutants lack binding of TGF-ß to the type I receptor; DR (double receptor) mutants lack binding of TGF-ß to both the type I and type II receptors; and S

(signaling) mutants have normal display of both types of the TGF-ß receptors yet fail to response to TGF-ß. When the R cells and DR cells were fused, the resulting hybrids showed surface type I receptor, and more importantly, restored responsiveness to TGF-ß. These data suggest that the type I receptor may require the type II for ligand binding and that both receptors are essential for TGF-ß signaling (Laiho et al., 1991; Weis-Garcia and Massagué, 1996; Wrana et al., 1992). Similar observations have also been made in a hepatoma cell line (Hep 3B-TR) and certain colon adenocarcinoma cells (SW480) (Geiser et al., 1992; Inagaki et al., 1993).

Molecular cloning of TGF-ßRII and TGF-ßRI has allowed further characterization of defective TGF-ß receptors in these mutant cell lines. Two clones from the "DR" group were found to have either a missense mutation in the extracellular domain or a nonsense mutation in the transmembrane domain of the TGF-ßRII that abolish normal TGF-ß ligand binding to the type II receptor. Transfection of a wild type TGF-ßRII cDNA not only restored the TGF-ß binding to the surface TGF-ßRII, but also the binding of ligand to the endogenous TGF-ßRI. More interestingly, the TGF-ß response as measured by inhibition of cell growth and induction of extracellular matrix protein expression was also restored. The physical association of the type I and II TGF-ß receptors was demonstrated by coimmunoprecipitation upon binding and crosslinking to the iodinated TGF-ß ligand.

Together, these observations have led to a proposed model of TGF-ßR operation, in that the type I and type II receptors associate as a heterodimeric complex; TGF-ßRI requires TGF-ßRII to bind TGF-ß; and TGF-ßRII requires TGF-ßRI to signal (Wrana et al., 1992). Subsequently, a heterodimeric complex model similar to that of TGF-ßRII and TGF-ßRI has also been shown to exist in the signaling pathways of other TGF-ß superfamily members, including activin (Carcamo et al., 1994; Liu et al., 1995).

3.2 Oligomerization of the TGF-ß Receptors

Functional analysis of complex formation of the TGF-ß receptors relied on a mutant TGF-ßRII lacking the intracellular kinase domain. When expressed in TGF-ß-responsive epithelial (Mv1Lu) or cardiac cell lines, it acted in a dominant-negative fashion on both the growth inhibition and transcriptional activation assays in each case (Brand and Schneider, 1995; Carcamo et al., 1994). A similarly structured dominant-negative TGF-ßRI mutant was also tested in a cardiac cell line and was shown to have less prominent dominant-negative effect than that of the TGF-ßRII mutant (Brand and Schneider, 1995). However, another group has reported conflicting results in Mv1Lu cells utilizing a similar TGF-ßRII dominant negative mutant and has argued for an alternative model in which TGF-ßRII and TGF-ßRI separately mediate growth inhibition and transcription activation, respectively (Chen et al., 1993). This raised two questions: (1) Are both the TGF-ßRII and TGF-ßRI kinases needed for regulating both cell growth and transcriptional responses? (2) Does TGF-ß function exclusively through the heterodimeric complex of TGF-ßRI and TGF-ßRII, or alternatively, via homodimers of each type of TGF-ß receptor?

Biochemical evidence for the existence of both heteromeric and homomeric complex of the TGF-ßRs has been reported using a variety of approaches. Homo-oligomers of the type II and III receptors were demonstrated in COS-7 cells transiently transfected with differentially epitope-tagged receptors, using both antibody-mediated co-patching approach and affinity-labeling followed by sequential immunoprecipitation (Henis et al., 1994). Homodimerization of the TGF-ßRII transfected in 293 cells was also suggested by double immunoprecipitation analyses of the metabolically labeled receptor proteins (Chen and Derynck, 1994). The stoichiometry of the heteromeric complex of the TGF-ßRs was determined by affinity labeling and covalently crosslinking with iodinated TGF-ß1, followed by immunoprecipitation and 2-D gel electrophoresis under non-reducing (1st) and reducing (2nd) conditions; despite the limitations of the methodology, a heterotetramer

complex containing two molecules each of TGF-ßRI and TGF-ßRII was favored (Yamashita et al., 1994).

3.3 Phosphorylation and Activation of the TGF-ß Receptors

Members of the TGF-ß superfamily are dimeric molecules that can bind to multiple receptors co-existing in the same cells; therefore, it is conceivable that different oligomeric combinations will give rise to a broad array of ligand-receptor complexes. On the one hand, this will generate the signaling capacity that accounts for the multifunctional nature of TGF-ß superfamily members; on the other, the mechanism by which these receptors trigger a specific signaling pathways is still elusive. To this end, it is important to establish the consequences of the oligomerization process and to understand the activation process of the receptor kinases.

As the prototype of this superfamily of cytokines, TGF-ß has attracted the most investigation, and therefore serves as the focus of my work presented in this thesis. The type II TGF-ß receptor was believed to act as a primary receptor which initiates the first signaling step upon TGF-ß simulation, given the fact that it can bind ligand by itself, while the type I receptor can not bind to the ligand alone (Wrana et al., 1992). Also, because the type II receptor was among the first cloned ser/thr kinase receptors, my research described in this thesis thus began with TGF-ßRII.

The structural feature of TGF-ßRII strongly suggests that phosphorylation plays an important role in TGF-ß function. Indeed, a GST fusion protein containing the intracellular domain of TGF-ßRII expressed and purified in E. *coli* could autophosphorylate *in vitro* (Lin et al., 1992). A number of important questions then followed. First, does TGF-ßRII also have a ser/thr kinase activity toward itself in living cells? In other words, did the results with recombinant TGF-ßRII produced in bacteria truly reflect its function *in vivo*? Second, what are the biochemical mechanisms underlying activation of the TGF-ß receptor

kinases? Does ligand binding cause increased autophosphorylation of TGF-ßRII? Third, do TGF-ß receptors employ a mechanism of phosphorylation and activation similar to that of the tyrosine kinase receptors? For example, does autophosphorylation of the kinase receptor TGF-ßRII also occur in a trans- manner characteristic of tyrosine kinase receptors? And more importantly, does ligand induce receptor complex formation and lead to activation of TGF-ß receptor kinases? My hypothesis behind this was that the ser/thr kinase receptors do resemble the overall structure design of that observed with well characterized tyrosine kinase receptors although they have distinct kinase specificity. It is these questions and hypotheses that laid the foundation upon which this thesis project was built (see Chapter 2).

It was shown that TGF-ßRII could bind ligand when expressed alone and that its kinase activity is necessary for TGF-ß signaling in Mv1Lu cells (Wrana et al., 1992). However, controversy remained concerning whether TGF-ßRII was sufficient to transduce TGF-ß signals by itself (Brand and Schneider, 1995; Carcamo et al., 1994; Chen et al., 1993). Thus, the theme of my research presented in Chapter 3 focused on interactions between the type I and II TGF-ß receptor kinases.

Finally, when a growth factor or cytokine works through a signaling complex consisting of multiple receptor subunits, different mechanisms can be employed to initiate the activation step. For instance, the ligand may be required to induce formation of the complex, or it may simply stabilize the pre-existing complex and perhaps activate the receptor kinases by causing some conformational changes. These decisions may depend on the affinity of each receptor subunits for the other, and may also be affected by their density or expression levels on the cell surface. Therefore, the mode by which TGF-ß elicits its signals and the relationship of TGF-ßRI to TGF-ßRII in this process are the issues subsequently addressed in Chapter 4 and Chapter 5.

IV. OVERVIEW

The aim of the research presented in this thesis is to understand the biochemical mechanism of the early events in the TGF-ß signaling pathway; this may well serve as a paradigm for other serine/threonine kinase receptors. In particular, this work has focused on the phosphorylation and activation of TGF-ß receptor kinases upon ligand binding.

To begin with, I established a COS cell overexpression system to examine whether TGF-ß receptors signal in a mode analogous to that of the tyrosine kinase receptors (Chapter 2). This led me to the discovery of a novel mechanism of phosphorylation and activation of TGF-ß receptors distinct from that of tyrosine kinase receptors (Chapters 3 and 4). Finally, I extended my findings to the TGF-ß responsive cell lines to study the endogenous TGF-ß receptors (Chapter 5).

V. AUTHOR'S NOTE

During the past few years as this project was proceeding, the TGF-ß field has experienced fascinating advances. Accordingly, in each of the following chapters, I will try to provide more specific background of the field at the time.

Chapter 2

In vivo Phosphorylation of TGF-ß RII in Transiently Transfected COS1 Cells

PREFACE

Most of this work has been published (Chen and Weinberg, 1995).

When this work began, only the TGF-ßRII was cloned (Lin et al., 1992) while the molecular identity of the TGF-ßRI was still a mystery. The experiments in this chapter revealed significant differences in the biochemical mechanism(s) of phosphorylation of serine/threonine kinase TGF-ßRII from those of tyrosine kinase receptors.

RESULTS

2.1 Construction of expression vectors and generation of monoclonal antibodies against TGF-ßRII.

The human TGF-ßRII cDNA (H2-3FF clone, Lin et al., 1992) was subcloned into the expression vector Rc/CMV (Invitrogen, San Diego, CA). The epitope tag sequence (YPYDVPDYA) encoding the influenza virus hemagglutinin (HA) was inserted between codons Pro26 and His27 in the reading frame encoding TGF-ßRII (Henis et al., 1994). The kinase-deficient mutant receptor, TGF-ßRIIK277-->R was generated by site-directed mutagenesis using PCR-based strategies. The coding regions containing the mutations and epitope tags were verified by DNA sequencing.

pGEX-RIICt encoding a GST fusion protein containing the intracellular domain of TGF-ßRII was constructed by inserting the HapI/EcoRI fragment of human TGF-ßRII cDNA into pGEX-2T vector. GST fusion protein containing TGF-ßRIICt was purified from *E. coli* and subsequently used as antigen to generate antibodies against TGF-ßRII as described in Chapter 7. One of the monoclonal antibodies, named 5c, which specifically reacted with TGF-ßRIICt, was further characterized and used in this study.

The expression level of TGF-ß receptors in mammalian cells is normally very low, complicating most biochemical studies. For this reason, both full-length TGF-ßRII and mutant derivatives were transiently overexpressed in COS1 African green monkey kidney cells using CMV-based vectors and metabolically labeled with ^{35}S-methionine. Immunoprecipitates with mAb 5c yielded a heterogeneously migrating band of 70-90 kDa upon gel electrophoresis, a size that fits well with that of the polypeptide core of the type II receptor (~65kD) plus its three predicted carbohydrate side chains (Fig 2.1. lanes 3, 4). In order to distinguish between autophosphorylation of this receptor and its phosphorylation by other cellular kinases, a kinase-deficient mutant receptor was also created by

constructing a single amino acid substitution (K277-->R) at the ATP-binding site of the type II receptor kinase domain. Both wild-type (TGF-ßRIIwt) and mutant (TGF-ßRIIKR) receptors were equally well expressed, indicating that the point mutation did not affect receptor synthesis and post-translational processing detectably (Fig 2.1. lanes 1, 2). We note that mAb 5c could also precipitate the endogenous TGF-ßRII of these cells in spite of its much lower expression level (Fig 2.1. lanes 5,6).

2.2 TGF-ßRII is constitutively phosphorylated when expressed alone in COS1 cells.

To determine the phosphorylation state of these TGF-ßRII proteins, they were immunoprecipitated from COS1 cells that were [^{32}P]-labeled (Fig. 2.2). Unexpectedly, even in the absence of ligand, the wild type TGF-ßRII appeared as a highly phosphorylated species that co-migrated with the [^{35}S]-labeled form (Fig 2.2. lane 10). In contrast, precipitates from cells expressing the mutant TGF-ß RIIKR exhibited only barely detectable receptor-associated label. Indeed, the level of this radiolabel was consistent with its derivation from the endogenous receptor proteins of these cells (Fig 2.2. lanes 2 and 12), indicating that the kinase activity of TGF-ßRII is required for its own phosphorylation.

To measure further the effects of ligand binding on the phosphorylation of TGF-ßRII, transfected COS1 cells were treated with 400pM TGF-ß1 for various time intervals prior to lysis and immunoprecipitation. As shown in Fig 2.2, lanes 6, 8 and 10, no detectable change in the overall phosphate content of TGF-ßRII was observed, suggesting that the TGF-ß type II receptor functions as a constitutively active, ligand-independent kinase *in vivo* .

2.3 *In vivo* phosphorylation of TGF-ß RII in transiently transfected COS1 cells is primarily due to its autophosphorylation.

Although the overall level of TGF-ßRII phosphorylation appeared to be unaffected by the presence of ligand, it remained possible that TGF-ß was able to induce some changes in the spectrum of sites phosphorylated on the receptor. Therefore, I subjected immunoprecipitated, [^{32}P]-labeled TGF-ßRII proteins to tryptic phosphopeptide analysis. Initial analysis of TGF-ßRII in the absence of ligand revealed eight major phosphopeptides, as shown in Fig 2.3D, with spots 2, 3 and 5 having higher intensities than the others. I repeatedly noted that spot 1 disappeared when cells had been treated with TGF-ß prior to lysis and immunoprecipitation (Fig 2.3A, 2.3B), but this observation was not pursued further.

Previous work had shown that a GST fusion protein containing only the intracellular domain of the TGF-ßRII (GST-RIICt) was capable of autophosphorylation *in vitro* (Lin et al., 1992). This afforded me the opportunity of comparing the residues of the TGF-ßRII labeled *in vitro* with those phosphorylated in the living cell. As shown in Fig 2.3C, the major phosphopeptides seen upon *in vitro* labeling- spots 2, 3, 4 and 5 - are very similar to those derived from the full length TGF-ßRII phosphorylated *in vivo* . I also noted the absence of spots 1 and 6 in GST-RIICt. This reinforced my earlier conclusion that most of the phosphorylation of the TGF-ßRII observed *in vivo* is due to autophosphorylation. When TGF-ßRII is expressed in COS1 cells, the phosphorylation of a minority of residues (represented by spot 1) may well be influenced by the presence of ligand.

2.4 Autophosphorylation of TGF-ßRII occurs via an intramolecular (cis) mechanism that is independent of ligand binding.

The apparent autophosphorylation of the TGF-ßRII intracellular domain may in fact be explained by two distinct mechanistic models. On the one hand, two TGF-ßRII kinase domains brought in close juxtaposition may be able to phosphorylate one another in a bimolecular reaction. Such transphosphorylation is postulated to play a central role in the ligand-mediated activation of tyrosine kinase mitogen receptors. Alternatively, a single kinase domain may be able to phosphorylate itself in a monomolecular reaction.

In order to resolve between these two possibilities, I attached an epitope tag to the inactive mutant kinase as described earlier and studied the interactions between this tagged mutant receptor and the wild type receptor. Immunoprecipitation using the mAb 5c, which recognizes equally well both the tagged mutant (IIKR-HA) and untagged wild-type (IIWT) receptor, revealed equivalent, high levels of both proteins in transfected COS1 cells (Fig 2.4, lanes 1 and 6). Only a small portion of each of the two receptor forms was digestible by Endo-H, indicating that the side chains of both were efficiently processed into complex carbohydrate-containing forms (Fig 2.4, lanes 2, 3 and 4, 5). The slower migration rate of the tagged receptor allowed me to resolve it from the wild type receptor upon gel electrophoresis. Treatment with N-glycosidase F resulted in a sharpening of the bands representing the two receptor forms of TGF-ßRII (Fig 2.5) and is used in most of the following experiments.

Cells expressing the untagged wild type and/or the tagged mutant receptors were labeled metabolically with ^{32}P-orthophosphate. Lysates were then immunoprecipitated with either mAb5c (α RII) or 12CA5 (α HA). As shown in Fig 2.6, HA-tagged type II receptors could be specifically recognized by 12CA5

(lanes 3 to 8), but only the wild type TGF-ßRII was autophosphorylated (lanes 3,4 and 7, 8). Importantly, the HA-tagged mutant receptor was not phosphorylated by the co-expressed wild type receptor, even in the presence of ligand (Fig 2.6, lanes 9 and 10). Control experiments (lanes 13 to 16) indicated that addition of an HA-tag to the wild type receptor had no effect on its state of phosphorylation. Moreover, other amino acid substitutions in the ATP-binding site of TGF-ßRII yielded identical results (data not shown).

Taken together, these results strongly suggested that autophosphorylation of the TGF-ßRII occurs via a cis- (intramolecular) mechanism even though the TGF-ßRII is capable of homo-oligomerization when overexpressed in COS1 cells (Chen and Derynck, 1994; Henis et al., 1994; Yamashita et al., 1994). This indicates fundamental differences that distinguish the mechanism of activation of the TGF-ßRII from that used by mitogen-activated tyrosine kinase receptors.

DISCUSSION

Unlike the mitogen-activated tyrosine kinase receptors of similar structure, the type II TGF-ß receptor is able to autophosphorylate in a ligand-independent manner. This is in agreement with a recent study using Mv1Lu cells in which the constitutive autophosphorylation of TGF-ßRII was also observed (Wrana et al., 1994). Indeed, ligand-independent autophosphorylation of the activin type II receptor has also been previously reported (Mathews and Vale, 1993), suggesting that this mechanism will apply to all receptors of this class. Use of both epitope tagging and site-directed mutagenesis has yielded the first evidence providing strong indication that TGF-ßRII autophosphorylation occurs as an intramolecular reaction, a process quite distinct from that of tyrosine kinases, in which bimolecular transphosphorylation appears to operate (Canals, 1992; Lammers et al., 1990).

We have also found that TGF-ß ligand does not affect the process of TGF-ßRII autophosphorylation but does cause a decrease in the phosphorylation of the receptor presumably by other cellular kinases. This represents the first indication that the type II receptor undergoes a functional change in response to ligand. The physiologic significance of this in the signaling process is unclear at present.

Site-directed mutagenesis analysis of the TGF-ßRII also supports the notion that its activation may employ a mechanism that differs from that of the tyrosine kinase receptors (Wieser et al., 1993) Upon ligand binding, mitogenic growth factor receptors having tyrosine kinase activity dimerize and autophosphorylate on tyrosine residues, which then act as docking sites for interacting with the cytoplasmic substrates. It has been shown that most of the "substrate-binding" phosphotyrosines reside at the C-termini of the receptor kinases or within the kinase insert regions, which are essential for signaling (Schlessinger and Ullrich, 1992; Ullrich and Schlessinger, 1990). In contrast, the corresponding regions of the TGF-ßRII can be deleted without affecting ligand-induced growth inhibition or transcriptional activation responses (Wieser et al., 1993).

ACKNOWLEDGMENTS

I thank Dr. P. Segarini for generous gifts of TGF-ß1 and neutralizing antibodies against TGF-ßs; Dr. R. Goldsby for his advice and assistance in generating antibodies against TGF-ß type II receptor; Dr. Y. Henis for his help in making epitope-tagged construct TGF-ßRII-HA; and members of the Weinberg and H.F. Lodish laboratories, especially R. Perlman, T. P. Mäkelä, C. Sardet, R. E. Herrera and R. Medema for their stimulating discussions and continuous help.

Figure 2.1 Characterization of TGF-ßRII transfected in COS1 cells

Immunoprecipitation of ^{35}S-met labeled TGF-ßRII with monoclonal antibody 5c (+) or controls (-).

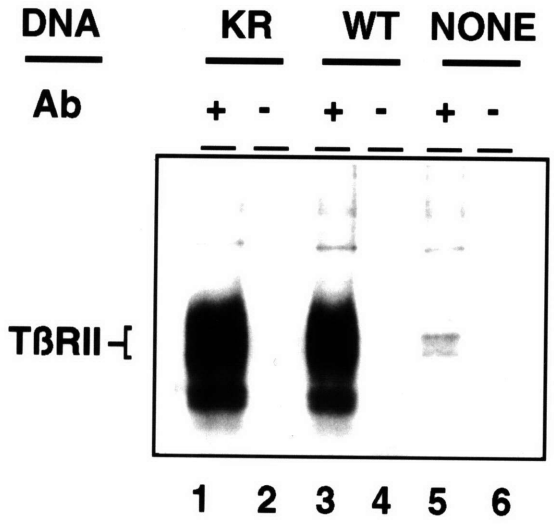

DNA
KR
WT
NONE
Ab
+ - + - + -
TßRII
1 2 3 4 5 6

Figure 2.2 Autophosphorylation of TGF-ßRII transfected in COS1 cells

Immunoprecipitation of ^{32}P-orthophosphate labeled TGF-ßRII after treatment with TGF-ß1 for various time periods.

KR: TGF-ßRIIKR, WT: TGF-ßRIIWT,
NONE: mock transfection.

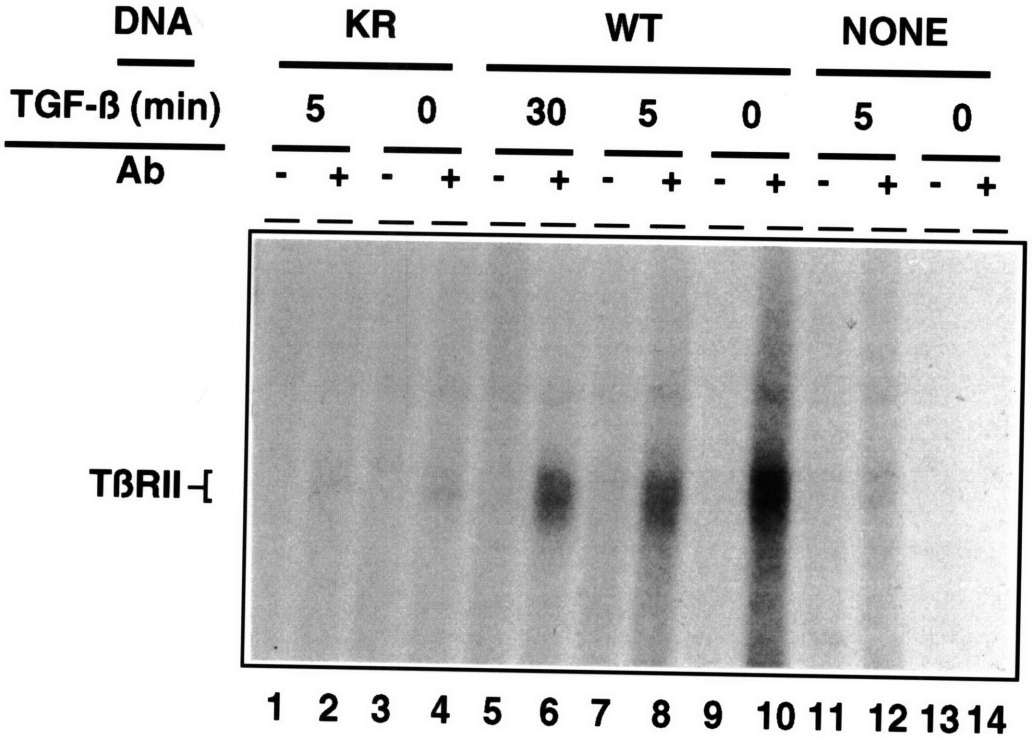

DNA
KR
WT
NONE
TGF-ß (min)
5
0
30
5
0
5
0
Ab
- + - + - + - + - + - + - +
TßRII
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 2.3 Tryptic phosphopeptide maps of TGF-ßRII

(A). COS1 cells transfected with wild-type TGF-ßRII were labeled with ^{32}P-orthophosphate, immunoprecipitated with mAb 5c, and subjected to phosphopeptide analysis (PPA).

(B) Same as in (A), except that cells were treated with TGF-ß1 at 400 pM for 5 minutes before lysis and immunoprecipitation.

(C) GST fusion protein containing kinase domain of TGF-ßIIR was purified from *E.coli*, an in vitro kinase assay was then performed, and 32P-labeled GST-TGF-ßRIICt was subjected to PPA.

(D) Schematic indicates the specific phosphopepetides referred to in the text.

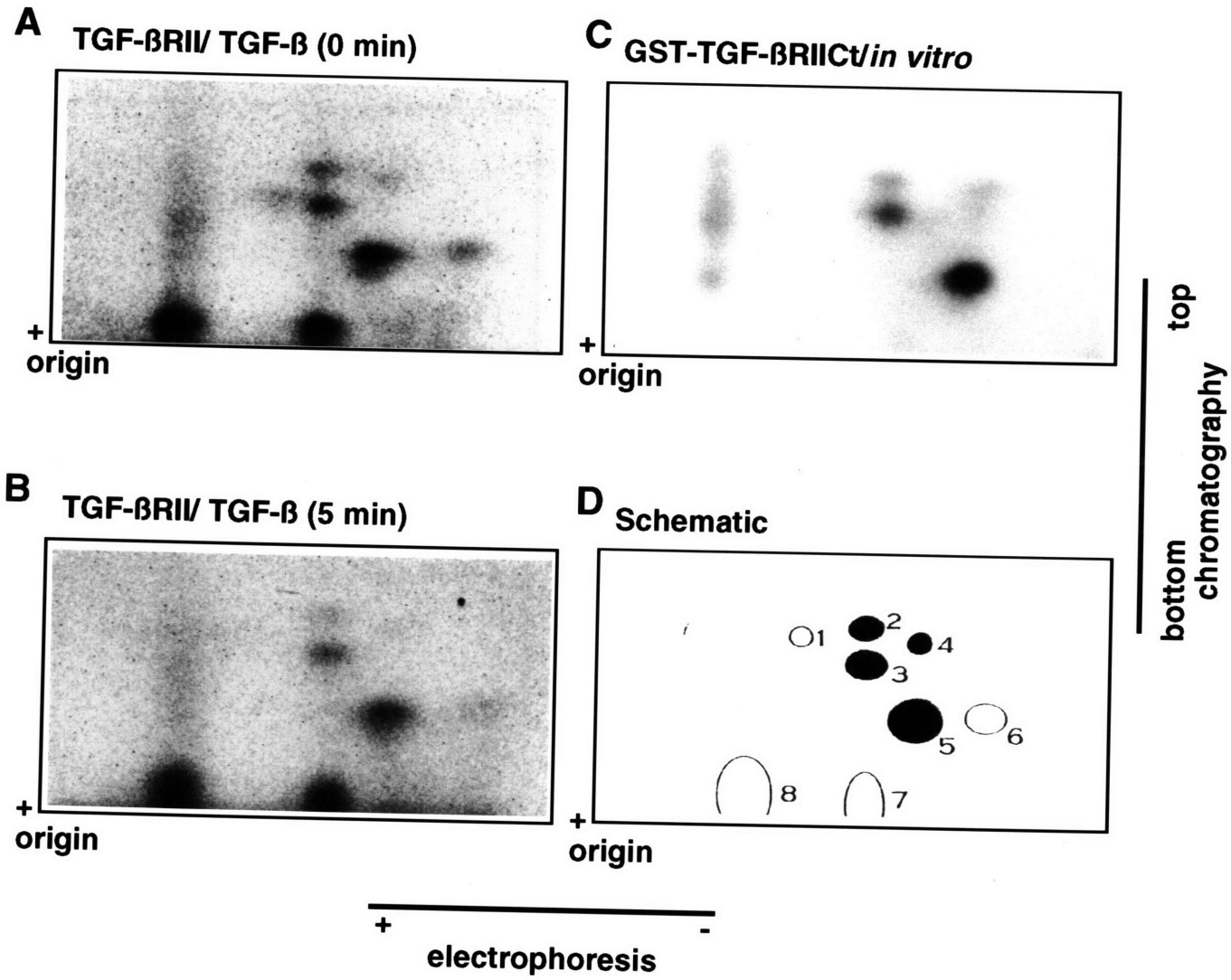

A
TGF-ßRII/ TGF-ß (0 min)
+
origin
C
GST-TGF-ßRIICt/in vitro
+
origin
top
bottom
chromatography
B
TGF-ßRII/ TGF-ß (5 min)
+
origin
D
Schematic
1
2
3
4
5
6
7
8
+
origin
+
-
electrophoresis

Figure 2.4 Characterization of HA-tagged TGF-ßRII variants

Immunoprecipitates of TGF-ßRIIWT and TGF-ßRIIKR-HA from ^{35}S-met labeled COS1 cells using mAb 5c, were treated with (+) or without (-) Endo-H as indicated.

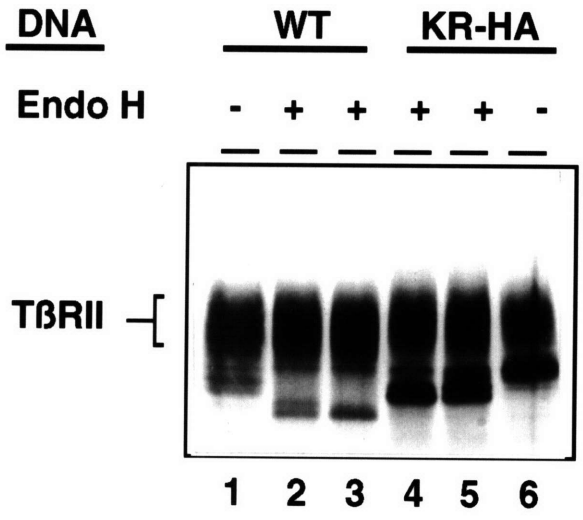

DNA
WT
KR-HA
Endo H
- + + + + -
TßRII
1 2 3 4 5 6

Figure 2.5 Co-expression of TGF-ßRII variants in COS1 cells

Co-expression of TGF-ßRIIWT and TGF-ßRIIKR-HA assayed by immunoprecipitation with monoclonal antibodies 12CA5 (α-HA) or 5c (α-RII) from ^{35}S-met labeled COS1 cells.

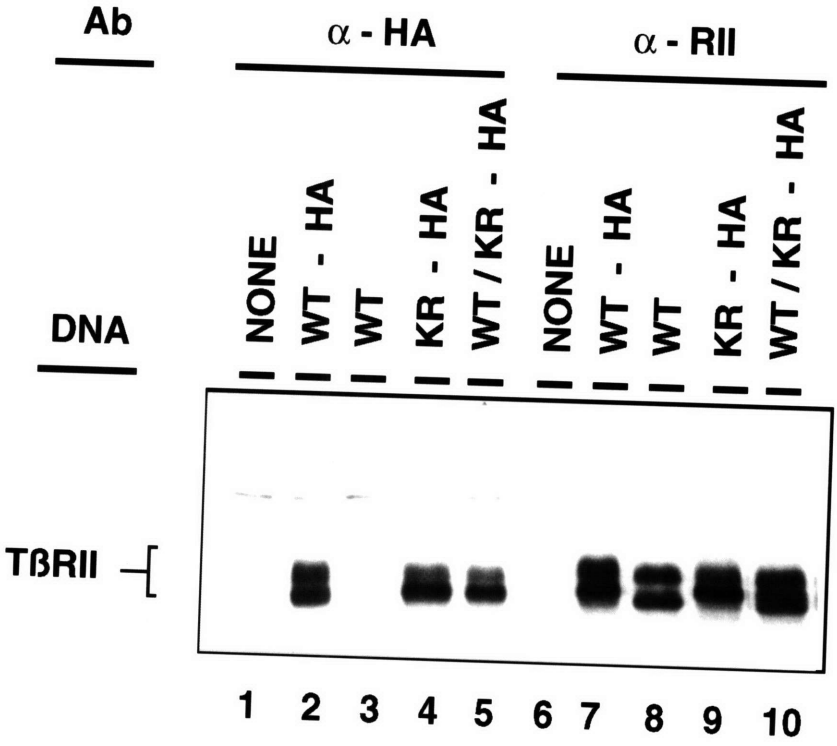
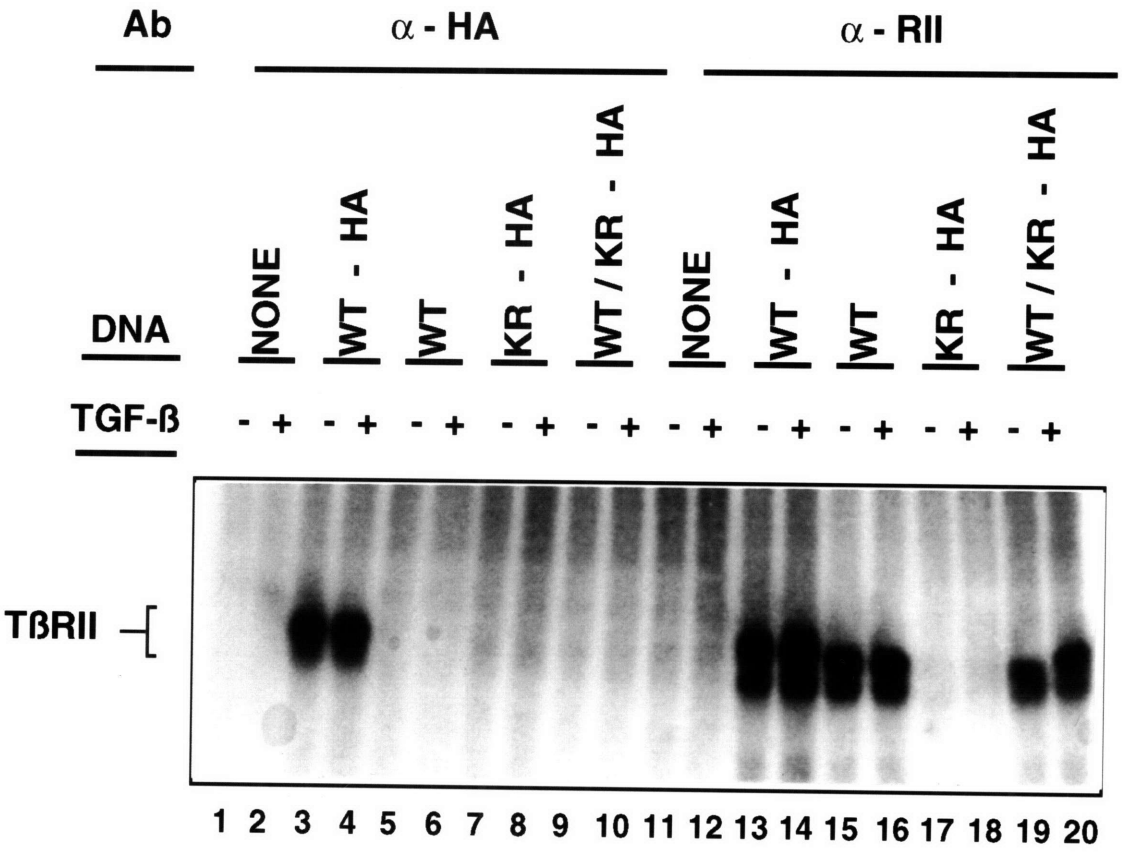

Ab
α - HA
α - RII
DNA
NONE
WT - HA
WT
KR - HA
WT / KR - HA
NONE
WT - HA
WT
KR - HA
WT / KR - HA
TβRII
1 2 3 4 5 6 7 8 9 10

Figure 2.6 Biochemical mechanism of TGF-ßRII autophosphorylation

In vivo phosphorylation of TGF-ßRIIWT and TGF-ßRIIKR in the presence (+ : 10 min) or absence (- : 0 min) of TGF-ß1 (400 pM).


Ab
α - HA
α - RII
DNA
NONE
WT - HA
WT
KR - HA
WT / KR - HA
NONE
WT - HA
WT
KR - HA
WT / KR - HA
TGF-ß
- + - + - + - + - + - + - + - + - + - +
TßRII
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Chapter 3

Transphosphorylation of TGF-ßRI and TGF-ßRII in COS1 Cells

PREFACE

Part of the work presented here has been published (Chen and Weinberg, 1995).

Data from this chapter led us to propose a non-reciprocal hetero-oligomerization model of the type I and type II TGF-ß receptors, in that TGF-ßRII is able to transphosphorylate TGF-ßRI while the reverse mechanism does not seem to operate.

INTRODUCTION

Genetic studies on chemically mutagenized cells (Laiho et al., 1990; Wrana et al., 1992) and tumor cell lines (Inagaki et al., 1993) resistant to TGF-ß have suggested that both functional type I and type II receptors are essential for TGF-ß signal transduction. In addition, a physical association between type I and type II receptors has been demonstrated by co-precipitation of both receptors after chemical crosslinking with radiolabeled TGF-ß (Attisano et al., 1993; Franzen et al., 1993; Inagaki et al., 1993; Wrana et al., 1992). The recent cloning of the type I receptor has enabled us and others to study the interactions between receptor I and II in detail. This cloning has revealed that the type I TGF-ß receptor (TGF-ßRI) also has a short extracellular domain, a single transmembrane segment, and a cytoplasmic region containing serine/threonine kinase domain (Attisano et al., 1993; Franzen et al., 1993; He et al., 1993). Others have reported that a GST fusion protein containing only the intracellular kinase domain of the TGF-ßRI is capable of autophosphorylation *in vitro* (Bassing et al., 1994).

RESULTS

3.1 Construction of expression vectors.

The human TGF-ßRI cDNA, ALK-5 clone (Franzen et al., 1993)was subcloned into the expression vector CMV7 (Andersson et al., 1989). The kinase-deficient mutant receptor, TGF-ßRIK232-->R, was generated by introducing a single amino acid substitution at its ATP-binding site using a PCR-based site-directed mutagenesis strategy. Both the wild-type and mutant receptors (TGF-ßRIWT and TGF-ßRIKR) were HA-tagged at their C-termini. The coding regions containing the mutation and epitope tag were verified by DNA sequencing.

3.2 TGF-ßRI is autophosphorylated when expressed alone in COS1 cells.

As shown in Fig 3.1, when expressed in COS cells, only the wild type TGF-ßRI was highly phosphorylated, indicating that TGF-ßRI, like TGF-ßRII, is capable of autophosphorylation *in vivo* (lanes 1, 2 and 3). Furthermore, the specific activity of the TGF-ßRI autophosphorylation was comparable to that of TGF-ßRII when the ^{32}P-orthophosphate and ^{35}S-methionine labeling intensities were compared (data not shown). These data are in agreement with the observation that recombinant protein containing only the kinase domain of the TGF-ßRI is capable of autophosphorylation *in vitro* (Bassing et al., 1994). Later, it was also demonstrated that TGF-ßRI expressed in insect cells infected with recombinant baculovirus can undergo autophosphorylation (Ventura et al., 1994) However, these data contrast with a report that the level of phosphorylation of TGF-ßRI singly transfected in Mv1Lu R-1B cells was very low (Wrana et al., 1994).

3.3 The wild type TGF-ßRI is transphosphorylated by the co-expressed TGF-ßRII kinase in a ligand-independent fashion in COS cells.

Others have shown that TGF-ßRI can not bind ligand in the absence of TGF-ßRII (Attisano et al., 1993; Franzen et al., 1993; Inagaki et al., 1993; Wrana et al., 1992). When co-expressed with wild type TGF-ßRII, the mutant and wild type TGF-ßRI receptors were found to bind ligand equally well, indicating that the kinase activity of TGF-ßRI is not required for its binding to ligand in concert with TGF-ßRII (data not shown).

The presence of either the mutant or wild type TGF-ßRI had no apparent effect on the overall level of TGF-ßRII phosphorylation (lanes 9-16 in Fig 3.2 and Fig 3.3). As discussed in Chapter 2, TGF-ßRII is a constitutively active kinase and can

autophosphorylate itself independent of the presence of ligand (Fig 2.2). A similar pattern of TGF-ßRII autophosphorylation was observed when the TGF-ßRIKR mutant was co-transfected (lanes 9 and 10 in Fig 3.3); the light bands in lanes 11 and 12 are due to the endogeneous TGF-ßRII in COS cells that are immunoprecipitated by mAb 5c (α RII), which specifically recognizes the cytoplasmic domain of TGF-ßRII. Furthermore, co-transfected TGF-ßRIWT does not change the constitutive autophosphorylation of TGF-ßRII in the same cells (lanes 9 and 10 in Fig 3.2). I concluded that TGF-ßRII is unlikely to be a substrate of the TGF-ßRI kinase.

I then attempted to explore the alternative possibility, namely that TGF-ßRI can be phosphorylated by the TGF-ßRII kinase. As shown in Fig 3.3, TGF-ßRIWT-HA was co-expressed with either TGF-ßRIIWT or TGF-ßRIIKR in COS1 cells. Lysates were prepared from either ^{32}P-orthophosphate or ^{35}S-methionine-labeled cells treated with TGF-ß1 (400pM, 10 min) or control buffer, split and immunoprecipitated with either mAb 5c (α RII) or mAb 12CA5 (α HA). While TGF-ßRI is autophosphorylated when expressed alone in COS cells as shown in Fig 3.1, the kinase-deficient TGF-ßRIIKR did not alter the autophosphorylation pattern of TGF-ßRI detectably (Fig 3.2, lanes 3 and 4), suggesting that the kinase activity of TGF-ßRII was not required for the autophosphorylation of TGF-ßRI.

Interestingly, co-expression of wild type TGF-ßRII gave rise to a slowly migrating form of TGF-ßRI in addition to its autophosphorylated species, either in the presence or absence of ligand (Fig 3.2, lanes 1 and 2), indicating that TGF-ßRI could be transphosphorylated by the TGF-ßRII kinase. I also noted that the autophosphorylation of wild type TGF-ßRI was reduced upon co-expression of wild type TGF-ßRII (Fig 3.2, lanes 1 to 4), suggesting that the type II receptor is capable of suppressing the intrinsic autophosphorylation activity of the type I receptor. One possible explanation for this is that the TGF-ßRI kinase can serve as a substrate both for itself in an autophosphorylation

reaction and for the TGF-ßRII kinase in a transphosphorylation reaction; accordingly, what I observed here may represent a competition between the two kinases.

3.4 A kinase-deficient mutant of TGF-ßRI is transphosphorylated by the co-expressed TGF-ßRII kinase in a ligand-independent fashion in COS cells.

To eliminate the background level of TGF-ßRI autophosphorylation in the experiment described in Fig 3.2, the kinase-deficient TGF-ßRIKR-HA was used to evaluate the possibility of transphosphorylation. It is clear that TGF-ßRIKR-HA could be transphosphorylated by wild type TGF-ßRII even in the absence of ligand (Fig. 3.3, lanes 1 and 2), indicatingthat, as before, TGF-ßRI could serve as a substrate of TGF-ßRII kinase. I also noted a decrease in the phosphate content of TGF-ßRIKR-HA when co-expressed with TGF-ßRIIKR compared to that of TGF-ßRIKR-HA when expressed alone in COS1 cells (lanes 3 and 4 in Fig. 3.3, lane 3 in Fig 3.1). This phenomenon, which requires further study, suggests a dominant-negative effect of TGF-ßRIIKR on the endogenous TGF-ßRII in COS1 cells, reducing its ability to transphosphorylate TGF-ßRIKR.

Importantly, co-expressed TGF-ßRI and TGF-ßRII could co-immunoprecipitate under rather stringent conditions of cell lysis both in the presence and absence of ligand; moreover, the kinase activities of the two receptors were not required for this association (Fig 3.2 and 3.3). Taken together, these data indicate the autophosphorylation of both types of TGF-ß receptors and the unidirectional transphosphorylation of the TGF-ßRI by the TGF-ßRII kinase.

Although there were no observed quantitative change on the transphosphorylation of TGF-ßRI by the type II receptor kinase upon TGF-ß treatment, it remained possible that alteration of some specific phosphorylation sites might have not been detected in this assay (Fig 3.2 and 3.3). To further address this question, phosphopeptide analysis (PPA) was

applied to the TGF-ßRIKR that had been transphosphorylated by the wild-type TGF-ßRII in the presence or absence of ligand. As shown in Fig. 3.4, identical maps were obtained for the transphosphorylated TGF-ßRIKR in both cases. I conclude that the transphosphorylation of TGF-ßRI by the TGF-ßRII kinase was not affected by ligand binding when both receptors were overexpressed in COS1 cells.

DISCUSSION

The two components involved in initiating TGF-ß signal transmission, TGF-ßRI and TGF-ßRII, appear to interact asymmetrically, in that the type II is able to transphosphorylate type I receptors while the reverse mechanism does not seem to operate (Fig 3.2 and Fig 3.3). This contrasts with the behavior of the tyrosine kinase receptors, either homodimeric receptors like EGFR, FGFR (Schlessinger and Ullrich, 1992; Ullrich and Schlessinger, 1990) or closely related receptors of mixed subunit composition such as InsR/EGFR (Lammers et al., 1990) and Neu/EGFR (Qian et al., 1994), whose activation appears to involve bidirectional transphosphorylation by both associated partners. This evidence provides support for the recent work in Mv1Lu cells by others (Wrana et al., 1994), which described this unique mode of signal transduction by these ser/thr kinase receptors.

My results are discordant with the report by Wrana et al. (1994) in two important respects. I have found that TGF-ßRI is autophosphorylated *in vivo* when expressed alone in COS1 cells (Fig 3.1). Indeed, the specific activity of TGF-ßRI autophosphorylation was comparable to that of TGF-ßRII when expressed alone in these cells. In contrast, in transfected Mv1Lu R-1B cells, the basal level of phosphorylation of TGF-ßRI expressed alone was reported to be very low (Wrana et al., 1994). We are unable to explain this discrepancy at present.

Secondly, this other report provided evidence that the transphosphorylation of the type I receptor by the type II kinase occurs only in a heteromeric complex that is assembled and stabilized by ligand (Wrana et al., 1994). However, we have observed the ligand-independent complex formation of type I and type II receptors and associated ligand independent transphosphorylation of TGF-ßRI by the TGF-ßRII kinase (Fig 3.2 and 3.3). The other studies (Wrana et al., 1994) depended upon analysis of only a fraction of the receptor proteins that are purified as detergent-resistant heteromeric complexes through sequential chromatography in the presence of ligand. Since such complexes do not form in the absence of ligand, the ligand-independent transphosphorylation of type I receptors by the type II kinase could not be measured. Indeed, such analysis can not exclude the possibility that the type I receptors that are not present in such detergent-resistant complexes are transphosphorylated by the type II kinase in a ligand-independent manner. In contrast, our analysis examined the bulk population of the TGF-ß receptors in transfected COS1 cells.

I have been puzzled that no obvious ligand-induced effects on the association and transphosphorylation of TGF-ß receptors were observed even when receptor-expressing COS1 cells were treated with high concentration of TGF-ß1. This phenomenon might be explained in two ways. (a) The cell culture medium used to propagate the COS1 cells might contain sufficient background levels of TGF-ß to activate the receptors, even in the absence of added TGF-ß. This background level of ligand might derive from latent forms of TGF-ß present in the serum or from TGF-ß secreted by the COS1 cells. However, experiments I performed using a TGF-ß neutralizing antibody, 1D11.16 (Dasch et al., 1989), did not support these possibilities (data not shown). (b) Alternatively, the substantial overexpression of TGF-ß receptors in the COS1 cells might also be responsible for their constitutive autophosphorylation; just such an effect has been observed with the erbB2/neu receptor (Di Fiore et al., 1987; Samanta et al., 1994).

Others have previously reported that both types of TGF-ß receptors are required for downstream signaling (Inagaki et al., 1993; Laiho et al., 1990; 1991; Wrana et al., 1992). Moreover, type I receptor appears to be capable of binding ligand only in the presence of type II receptor (Attisano et al., 1993; Franzen et al., 1993). Together, these observations indicate that TGF-ß induces ternary (or higher order) complexes that include ligand and receptor subunits of both types. At low physiologic levels of receptor expression, TGF-ß may be required to bring the two receptor molecules into close proximity to assemble and stabilize the complexes; such association may be achieved in a ligand-independent fashion at the high levels of receptor expression used in our assays. I note that other work in my laboratory using a yeast two-hybrid expression system indicates an intrinsic affinity of type I and type II kinase domains for each other in the absence of extracellular domains and ligand (R.Perlman and R. A. W., unpublished observations).

My present results cause me to propose that the association of the TGF-ß type I and II receptors, induced either by ligand binding or overexpression, leads to transphosphorylation of TGF-ßRI by the TGF-ßRII kinase. This in turn may allow one or both of the associated kinases to direct their activities toward downstream substrates. Such a mechanism will only be fully vadidated when we are able to measure receptor kinase activities using appropriate substrate(s) and the phosphorylation of receptor molecules when they are expressed at physiologic levels.

To date, at least three lines of evidence have suported this heterodimeric complex model for TGF-ßRI and TGF-ßRII. First, physical interaction between the type I and II TGF-ß receptors has been demonstrated both in a yeast two-hybrid system and by coimmunoprecipitation from mammalian cells that are co-transfected with differentially epitope-tagged receptors (Chen et al., 1995a; Yamashita et al., 1994). Second, amino acid substitutions in both receptors that interfere with the trans-phosphorylation of TGF-ßRI by the TGF-ßRII kinase have been characterized. A type II receptor having a point mutation that changes proline 525 to leucine in its kinase domain XI (TßRII P525L) has

that changes proline 525 to leucine in its kinase domain XI (TßRII P525L) has autophosphorylating activity similar to that of the wild-type TGF-ßRII and can also support ligand binding to TGF-ßRI via complex formation; however, it fails to transphosphorylate the associated TGF-ßRI and therefore is unable to signal TGF-ß responses (Carcamo et al., 1995). Conversely, a type I receptor bearing a point mutation that changes Thr 204 to an acidic residue Asp in its GS domain is a constitutively active kinase and is able to introduce TGF-ß signal in the absence of ligand and TGF-ßRII (Wieser et al., 1995) The GS domain is a glycine- and serine-rich region immediately preceding the kinase domain and has been shown to be transphosphorylated by TGF-ßRII kinase in the ligand-induced heterodimeric complex (Wrana et al., 1994). Indeed, mutation of two or more of the serine and threonine residues within GS domain of TGF-ßRI results in decreased phosphorylation and impaired signaling (Franzen et al., 1995; Wieser et al., 1995). Finally, this model for activation of the type I and type II TGF-ß receptors has been extended to other members of the TGF-ß superfamily; it has been reported that activin and BMP are also signaling through a heterodimeric complex (Carcamo et al., 1995; Letsou et al., 1995; Liu et al., 1995; Penton et al., 1994).

ACKNOWLEDGMENTS

We thank Drs. C.-H. Heldin and K. Miyazono for providing us with TGF-ß type I receptor cDNA (ALK5).

Figure 3.1 Characterization of TGF-ßRI expressed alone in COS1 cells

COS1 cells transfected with HA-tagged TGF-ßRIWT or mutant TGF-ßRIKR alone were metabolically labeled with ^{35}S-met or ^{32}p-orthophosphate, and immunoprecipitation was performed using indicated antibodies.

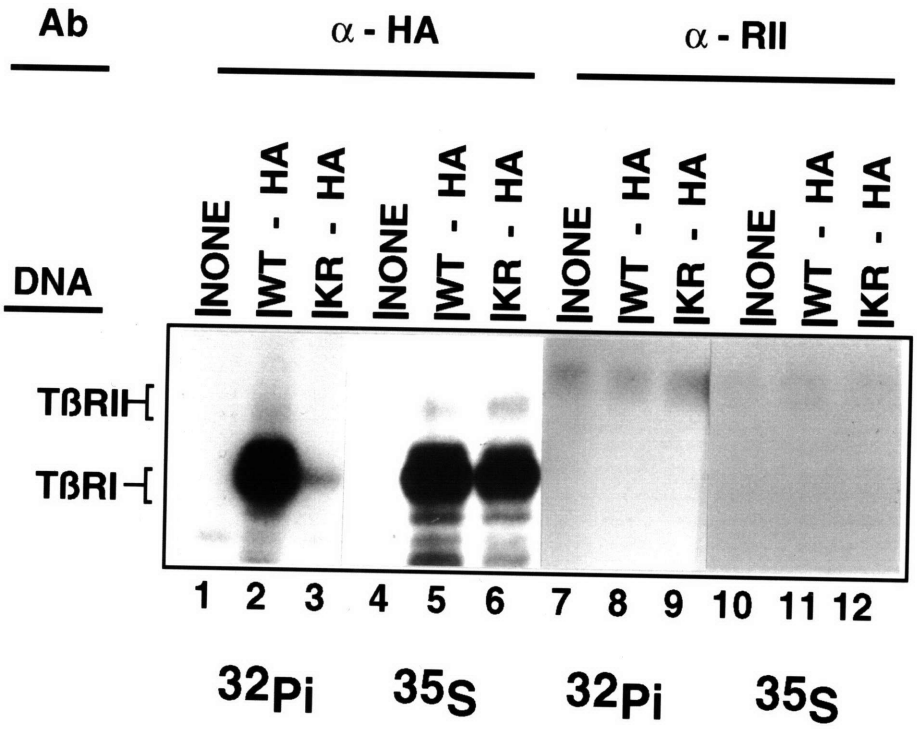

Ab
α - HA
α - RII
DNA
NONE
WT - HA
KR - HA
NONE
WT - HA
KR - HA
NONE
WT - HA
KR - HA
NONE
WT - HA
KR - HA
TβRII
TβRI
1 2 3 4 5 6 7 8 9 10 11 12
32Pi
35S
32Pi
35S

Figure 3.2 Transphosphorylation of wild type TGF-ßRI by the co-expressed TGF-ßRII kinase in COS1 cells

HA-tagged wild type TGF-ßRI was co-transfected with either wild type (WT) or kinase-deficient (KR) TGF-ßRII in COS1 cells. Metabolic labeling was followed by TGF-ß1 treatment (400 pM; - : 0 min; + : 10 min) and immunoprecipitation as indicated.

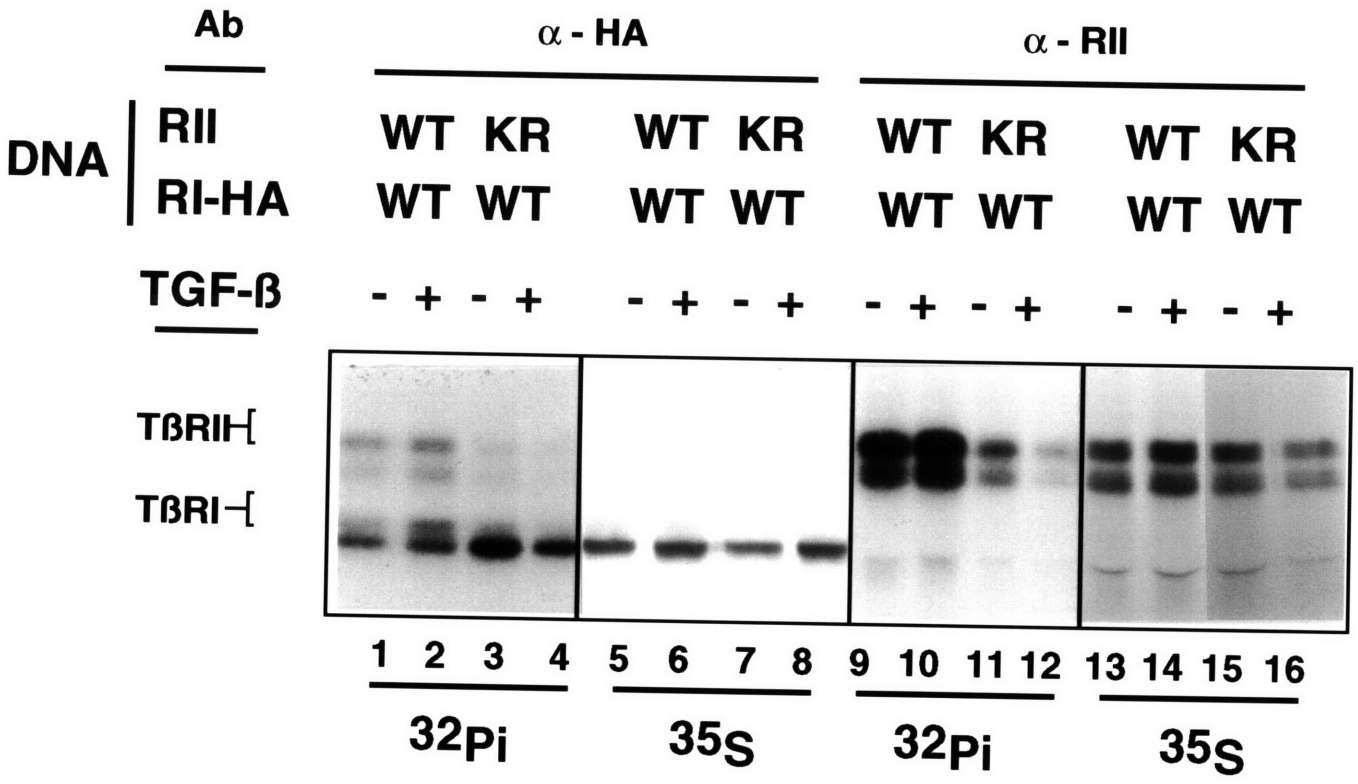

Ab
α - HA
α - RII
DNA
RII
RI-HA
WT KR WT KR WT KR WT KR
WT WT WT WT WT WT WT WT
TGF-ß
- + - + - + - + - + - + - + - +
TßRII
TßRI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
32Pi
35S
32Pi
35S

Figure 3.3 Transphosphorylation of kinase-deficient mutant TGF-ßRI by the co-expressed TGF-ßRII kinase in COS1 cells

Same as in Fig 3.2, except that HA-tagged kinase-deficient TGF-ßRIKR was co-transfected with either wild type (WT) or kinase-deficient (KR) TGF-ßRII in COS1 cells.

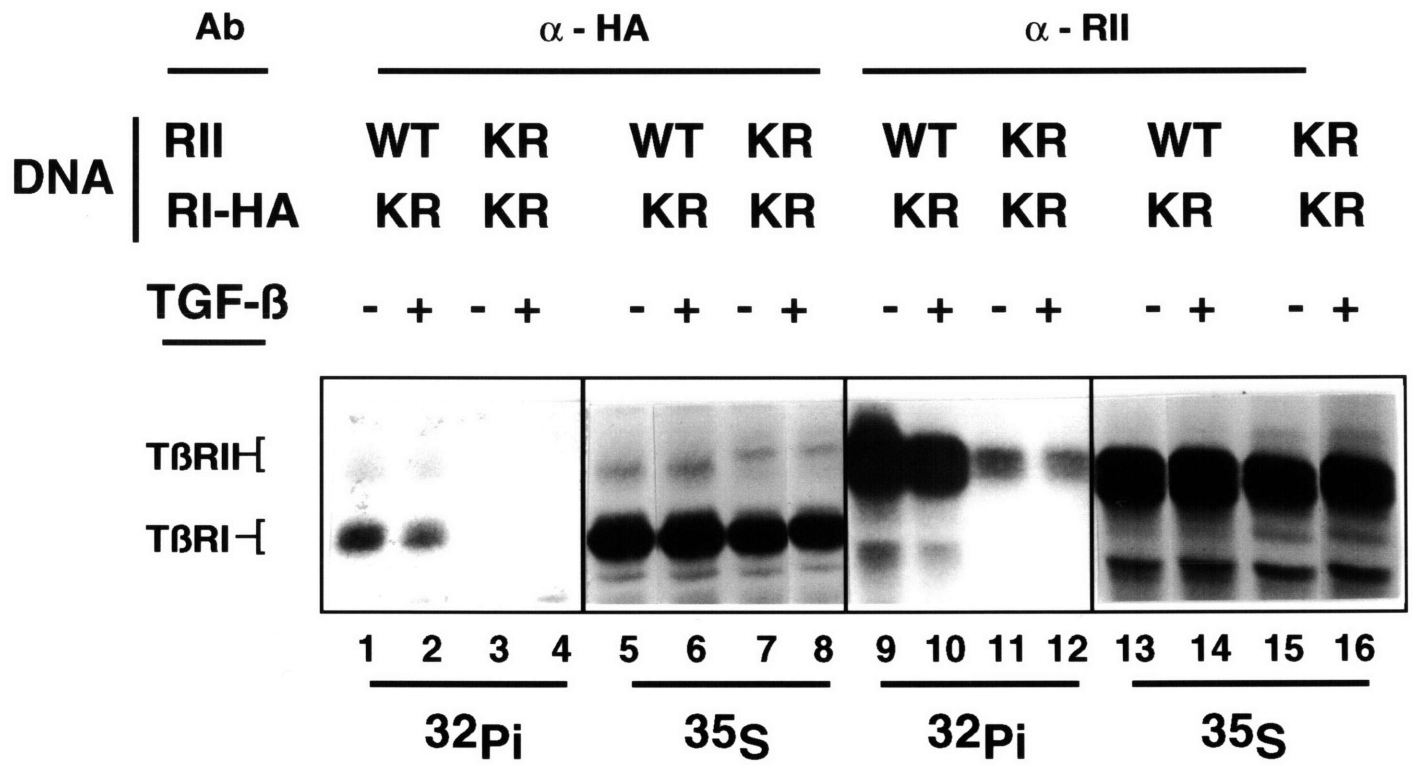
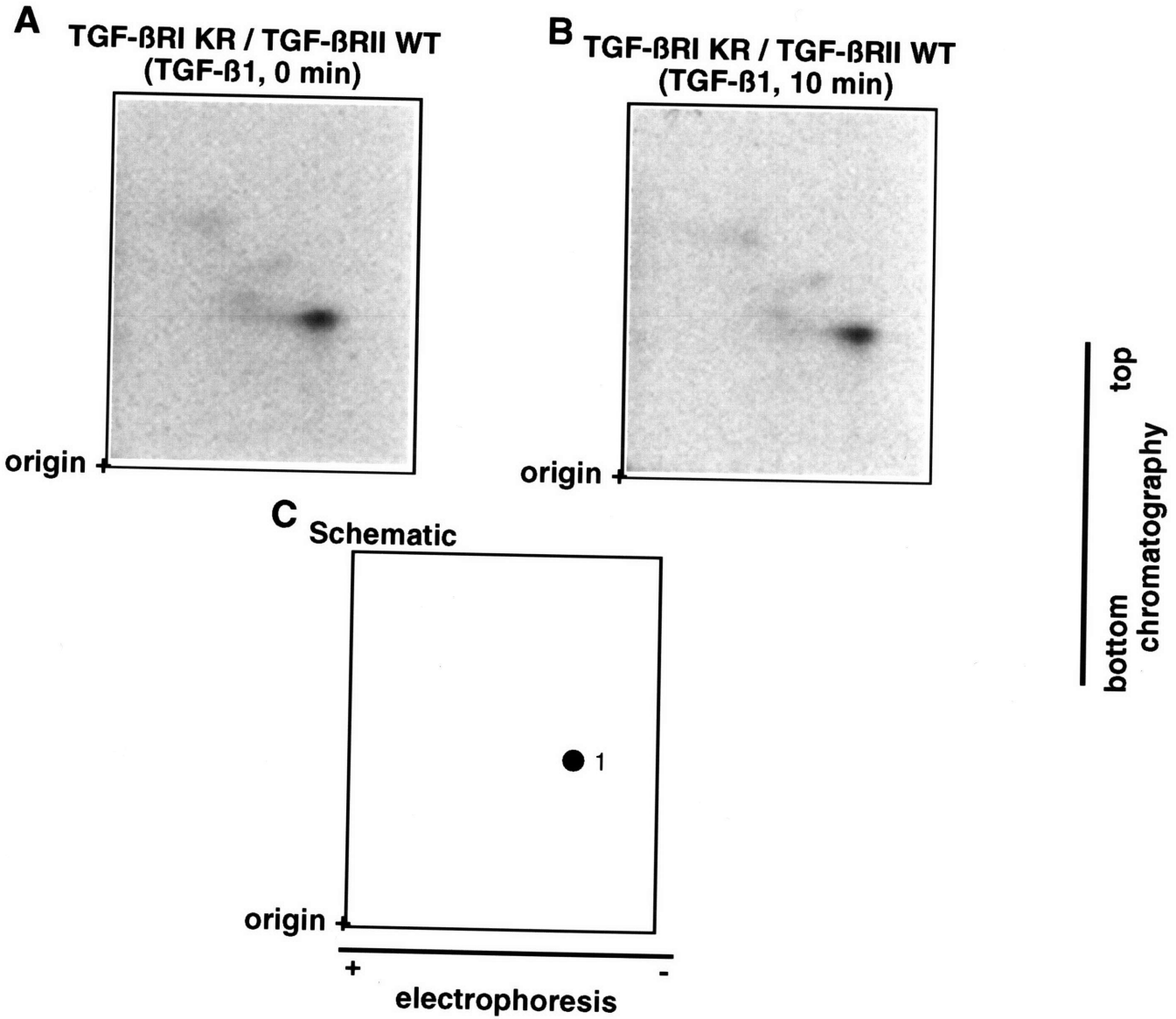

Ab
α - HA
α - RII
DNA
RII
RI-HA
WT KR WT KR WT KR WT KR
KR KR KR KR KR KR KR KR
TGF-ß
- + - + - + - + - + - + - + - +
TßRII
TßRI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
32Pi
35S
32Pi
35S

Figure 3.4 Tryptic phosphopeptide maps of TGF-ßRI

(A). COS1 cells co-transfected with an HA-tagged kinase-deficient mutant TGF-ßRIKR and wild-type TGF-ßRII were labeled with ^{32}P-orthophosphate, immunoprecipitated with mAb 12CA5, and subjected to phosphopeptide analysis.

(B) Same as in (A), except that cells were treated with TGF-ß1 at 400 pM for 10 minutes before lysis and immunoprecipitation.

(C) Schematic indicates the specific phosphopepetides referred to in the text.


A
TGF-βRI KR / TGF-βRII WT
(TGF-β1, 0 min)
origin
B
TGF-βRI KR / TGF-βRII WT
(TGF-β1, 10 min)
origin
top
bottom
chromatography
C
Schematic
1
origin
+
-
electrophoresis

Chapter 4

Phosphorylation and Interaction of the Cytoplasmic Domains of TGF-ßRII and TGF-ßRI

PREFACE

The goal of this set of experiments is to directly demonstrate the ligand-independent complex formation and phosphorylation of TGF-ßRII and TGF-ßRI, indicating the intrinsic interaction of their cytoplasmic domains. All the data in this chapter have not yet been published.

INTRODUCTION

Previous work described in Chapter 2 and Chapter 3 led me to propose that the association of the TGF-ß type I and II receptors, induced either by ligand binding or overexpression, leads to transphosphorylation of the TGF-ßRI by the TGF-ßRII kinase. This may be the key step in the activation of the TGF-ß receptor kinases and may therefore allow one or both of the associated kinases to direct their activities toward downstream substrates. Such a mechanism differs from that of the tyrosine kinase receptors, in that (1) TGF-ßRII, the primary receptor which binds to ligand, is a constitutively autophosphorylated kinase, and (2) the two subunits in the heterodimeric complex interact with each other asymmetrically (Chen and Weinberg, 1995).

The biochemical mechanism by which TGF-ßRI and TGF-ßRII form a heterodimeric complex and subsequently become activated is still largely unknown. Since it was shown that the type I TGF-ß receptor (TGF-ßRI) could not bind and be chemically cross-linked to the ligand unless TGF-ßRII was present (Ebner et al., 1993b; Franzen et al., 1993; Wrana et al., 1994), one model has been proposed based on work from mink lung epithelial cells: that TGF-ß binds to the type II receptor first and that only the TGF-ßRII-bound ligand (or ligand-bound TGF-ßRII) can then be recognized by the TGF-ßRI. Accordingly, the TGF-ß ligand is essential to recruit the TGF-ßRI to the TGF-ßRII in order to form a complex (Wrana et al., 1992; 1994). However, I have observed ligand-independent transphosphorylation of TGF-ßRI by the TGF-ßRII kinase, which indicates an intrinsic interaction of these two receptors in the absence of ligand. This led me to hypothesize an alternative mechanism of the TGF-ß receptor complex formation and activation: TGF-ßRI and TGF-ßRII may indeed form a complex in the absence of ligand binding due to their intrinsic affinity toward each other; TGF-ß ligand may trigger the signaling across the membrane by either stabilizing this otherwise transient complex or by inducing a conformational change that is required for the receptor complex

to interact with downstream substrates. According to this model, TGF-ß does not simply bridge two receptors by bringing them into proximity, but rather acts as an activation switch either quantitatively (by stabilizing a complex) or qualitatively (by inducing a specific conformational change).

To test this hypothesis, it was critical to characterize further the ligand-independent interaction between TGF-ßRI and TGF-ßRII. One major concern was that the possible existence of a latent or active form of TGF-ß in the cell culture medium might cause the transphosphorylation of TGF-ßRI by the TGF-ßRII even in the absence of exogenously added TGF-ß. I addressed this question earlier using a neutralizing antibody against TGF-ß and concluded that this was not the case (Chen and Weinberg, 1995). However, it was unclear whether the depletion of ligand was complete under this condition. More importantly, I wished to learn whether the intracellular domains of the TGF-ß receptors alone could interact with one another, or whether the extracellular and transmembrane domains are also required for the ligand-independent transphosphorylation of TGF-ßRI by the TGF-ßRII. Knowing that TGF-ßRI does not bind to TGF-ß ligand unless TGF-ßRII is also present, I reasoned that COS cells co-expressing a truncated TGF-ßRII, without the extracellular domain and transmembrane domain, together with an intact TGF-ßRI would provide a clean system in which the ligand effects could be completely eliminated and the interaction of receptor cytoplasmic domains could be directly analyzed.

RESULTS

4.1 Construction of expression vectors

A truncated TGF-ßRII mutant receptor, TGF-ßRIICtwt, was constructed by deleting the entire extracellular domain and transmembrane domain. Specifically, a convenient restriction site (HpaI) in the human TGF-ßRII cDNA sequence (H2-3FF clone)

(Lin et al., 1992) immediately following the transmembrane domain was used to subclone the DNA fragment of TGF-ßRII encoding the intact intracellular domain into an expression vector Rc/CMV (Invitrogen, San Diego, CA). The kinase-deficient mutant receptors, TGF-ßRIICtKR, was generated by site-directed mutagenesis using PCR-based strategies as described before (Chapter 2). The coding region containing the mutation was verified by DNA sequencing. Indeed, TGF-ßRIICt contains precisely the same portion of the type II TGF-ß receptor as is expressed by pGEX-RIICt which encodes a GST fusion protein containing the intracellular domain of TGF-ßRII as described in Chapter 2.

4.2 The cytoplasmic domain of TGF-ßRII is an active kinase in transiently transfected COS1 Cells.

As an initial step, I characterized the expression and autophosphorylation of the cytoplasmic domain of TGF-ßRII (TGF-ßRIICt). As shown in Fig 4.1, either the wild-type truncated receptor (TGF-ßRIICtwt) or the kinase-deficient mutant (TGF-ßRIICtKR) was transiently transfected in COS cells. 36-48 hours after transfection, cells were metabolically labeled with either ^{35}S-met or ^{32}P-orthophosphate and immunoprecipitation was performed using mAb5c, which specifically recognizes the intracellular domain of TGF-ßRII as described before (Chapter 2).

Both truncated receptors, the kinase-active TGF-ßRIICtwt and the kinase-deficient TGF-ßRIICtKR mutant, were equally well expressed in COS cells, indicating that the point mutation does not affect receptor synthesis and post-translational processing detectably (lanes 1 and 2 in Fig 4.1). They both migrated on SDS-PAGE gels with an apparent molecular weight of about 35-40 kD consistent with the calculated molecular mass predicted from the DNA sequence of TGF-ßRII. As expected, the TßRIICtwt could autophosphorylate itself just as the full-length TGF-ßRII, while the kinase-deficient mutant TGF-ßRIICtKR could not (lanes 3 and 4, Fig 4.1).

4.3 Transphosphorylation of TGF-ßRI by the cytoplasmic domain of TGF-ßRII kinase

Having characterized its expression in COS1 cells, I then investigated whether the TGF-ßRIICt could also transphosphorylate TGF-ßRI as had been demonstrated with the full-length type II receptor. Since the TGF-ßRIICt no longer has the extracellular region that is responsible for binding to ligand, such transphosphorylation assay would provide a direct biochemical evidence for an interaction between the intracellular domain of TGF-ßRII and TGF-ßRI in the absence of ligand.

Constructs for the wild-type truncated receptor (TGF-ßRIICtwt) and the kinase deficient truncated mutant (TGF-ßRIICtKR) were transfected in COS1 cells either by themselves or together with an epitope-tagged kinase-deficient mutant type I TGF-ß receptor (TßRI-HA). 36-48 hours after transfection, cells were metabolically labeled with ^{32}P-orthophosphate and immunoprecipitation was performed with either monoclonal antibody 5c which specifically recognizes the TGF-ßRII (Fig 4.2) or monoclonal antibody 12CA5 which specifically reacts with the HA-tag attached to the kinase-deficient mutant TßRI-HA (Fig 4.3).

As shown in Fig 4.3, the truncated receptor TGF-ßRIICtwt phosphorylated the kinase-deficient mutant TGF-ßRIKR as well as did the full-length TGF-ßRII (lanes 4 and 5). In contrast, the kinase-deficient mutant TGF-ßRIICtKR did not phosphorylate the TGF-ßRIKR (the faint phosphate-labeled band seen in lane 6 is most likely due to phosphorylation by the endogenous TGF-ßRII in COS cells). The wild-type TGF-ßRI was autophosphorylated when expressed alone in COS cells, as described in Chapter 3. TGF-ßRI could also be transphosphorylated when it was co-transfected with the truncated receptor TGF-ßRIICtwt but not the TGF-ßRIICtKR although this was less apparent due to the background of its autophosphorylation (lanes 7 and 8).

In order to detect the transphosphorylation signal of TGF-ßRIKR (lanes 5 and 6 in Fig 4.3), the autoradiograph had to be exposed for a relatively long time; with the same exposure time, the other half of the autoradiograph showing the phosphorylation of TGF-ßRII was apparently overexposed (Fig 4.2). The dark band at the bottom of lane 8 in Fig 4.3 is actually from the adjacent lane on the gel analyzing the autophosphorylation of the TGF-ßRIICtwt (lane 1 in Fig 4.2). Indeed, I noted that the cytoplasmic domain of TGF-ßRII showed higher kinase activity toward itself than did the full length TGF-ßRII (lanes 1 and 3 in Fig 4.2, bottom panel); this phenomenon was not further explored in this thesis.

4.4 Tryptic phosphopeptide analysis of a kinase-deficient mutant TGF-ßRI transphosphorylated by the cytoplasmic domain of TGF-ßRII

To study further the kinase specificity of the TGF-ßRIICt expressed in COS cells, I compared the tryptic phosphopeptide maps of the truncated TGF-ßRIICt and the full-length TGF-ßRII when they were expressed alone in COS cells and autophosphorylated. The tryptic phosphopeptide analyses (PPA) of the full length TGF-ßRII (Fig 4.4, panel A) and the truncated TGF-ßRIICt (Fig 4.4, panel B) were derived from lane 3 and lane 1 in Fig 4.2, respectively. It is clear that the overall pattern of these two tryptic phosphopeptide maps are rather similar, and that the major sites 5, 7 and 8 as indicated in the schematic drawing are identical (Fig 4.4, panel C). The differences of other spots between the two maps may result from the different migration rate due to their distinct protein sizes.

The specific sites on TGF-ßRI that are transphosphorylated by the cytoplasmic domain of TGF-ßRII were also analyzed. As revealed in Fig 4.5, the phosphopeptide analysis of TßRIKR-HA co-transfected with the full-length wild type TGF-ßRII (panel A) or with the truncated TGF-ßRIICt (panel B) were derived from lane 4 and lane 5 in Fig 4.3, respectively. Apparently, when expressed in COS cells, both the full length and the

truncated TGF-ßRII transphosphorylated the TGF-ßRIKR on a similar site as indicated in Fig 4.5 (panel C).

DISCUSSION

A truncated TGF-ßRII mutant receptor (TGF-ßRIICtwt) was constructed by deleting the entire extracellular domain and transmembrane domain. Hence, it no longer was able to bind TGF-ß nor could it support the TGF-ßRI in ligand binding. However, not only did it maintain the autophosphorylation characteristics of the full length TGF-ßRII, but it also transphosphorylated the TGF-ßRI co-expressed in COS1 cells with the same specificity as that of the full length TGF-ßRII. This provided direct biochemical evidence for the intrinsic ability of the TGF-ßRI and the TGF-ßRII to interact with each other in the absence of ligand.

Consistent with my work presented in this chapter, it has also been reported that the cytoplasmic domains of the TGF-ßRI and TGF-ßRII can be co-immuprecipitated from ^{35}S-labeled cells transfected with both TGF-ß receptors (Chen et al., 1995a). In addition, I note that work using yeast two-hybrid expression systems indicates an inherent affinity of type I and II kinase domains for each other in the absence of extracellular domains and ligand (R.Perlman and R. A. W., unpublished results; Chen et al., 1995a) Taken together, these observations strongly suggest that TGF-ßRI and TGF-ßRII can indeed form a complex due to their intrinsic affinity toward each other. Ligand binding may stabilize this otherwise transient pre-complex or induce a conformational change that in turn will activate the receptor complex and trigger the downstream signaling cascade. This mechanism can well explain the ligand-independent association and phosphorylation of the TGF-ßRI and TGF-ßRII I observed in chapter 2 and 3, and is different from the alternative model in that TGF-ß is needed to bring two receptors into proximity in order for the heterodimeric complex to form (Wrana et al., 1994).

I have shown that the cytoplasmic domain of the TGF-ßRII can transphosphorylate the TGF-ßRI in a ligand-independent manner. However, further studies are still needed to test whether this truncated TGF-ßRII, in concert with TGF-ßRI, is capable of triggering growth inhibition and transcriptional activation which can normally be induced by TGF-ß in cells with both type I and type II receptors. One approach would be to co-transfect the wild-type cytoplasmic domain of the TGF-ßRII into a cell line that lacks the TGF-ß type II receptors (for example, the "DR" group of mutant mink lung cells) and to assay whether growth inhibition and transcriptional activation can be restored independent of ligand. If the key function of the TGF-ßRII in the heterodimeric receptor complex is to transphosphorylate the TGF-ßRI on the specific sites, it is predicted that the cytoplasmic domain of the TGF-ßRII should be able to activate the downstream pathway of TGF-ß through its immediate substrate TGF-ßRI. Indeed, a single amino acid substitution (Thr204 --> Asp204) of one of the predicted phosphorylation sites regulated by the TGF-ß type II kinase on its GS domain yields a mutant TGF-ß type I receptor that is constitutively active and can mediate anti-proliferative and transcriptional responses in the absence of TGF-ßRII and ligand when introduced into TGF-ßRI-deficient cells (Wieser et al., 1995).

This also raised a related question: what are the contributions of the different domains of TGF-ßRI and TGF-ßRII to complex formation and activation? While the intrinsic interaction of the cytoplasmic domains of the TGF-ßRI and TGF-ßRII has been demonstrated in yeast two-hybrid systems and transfected mammalian cells, there is also biochemical evidence for the direct association between the extracellular domains of these two receptors. The truncated type I and type II receptors lacking their cytoplasmic domains could be coimmunoprecipitated when co-transfected in mammalian cells, indicating that their extracellular and transmembrane domains display intrinsic affinity toward each other (Chen et al., 1995a). In fact, it has been shown that the truncated TGF-ßRII lacking the intracellular domain is sufficient to associate with TGF-ßRI and support its binding to the ligand (Brand and Schneider, 1995; Chen et al., 1993; Wieser et al., 1993). Therefore, it

is conceivable that multiple interaction points may be involved in the formation and stabilization of the heterodimeric receptor complex for an optimal TGF-ß signaling.

Finally, it is worth mentioning that most of the data regarding the TGF-ß receptor complex formation and activation has primarily come from investigations utilizing cells that are transfected with TGF-ß receptor cDNAs and therefore likely have receptor levels higher than those at physiological conditions. This issue will be further addressed in chapter 5.

ACKNOWLEDGMENTS

I thank Dr. Riki Perlman for sharing unpublished information with me.

Figure 4.1 Characterization of the cytoplasmic domain of TGF-ßRII in transiently transfected COS1 Cells

COS1 cells transfected with the cytoplasmic domain of wild-type or kinase-deficient mutant were metabolically labeled with ^{35}S-met or ^{32}P-orthophosphate, and immunoprecipitation was performed using monoclonal antibody 5c, which specifically recognizes the intracellular domain of TGF-ßRII.

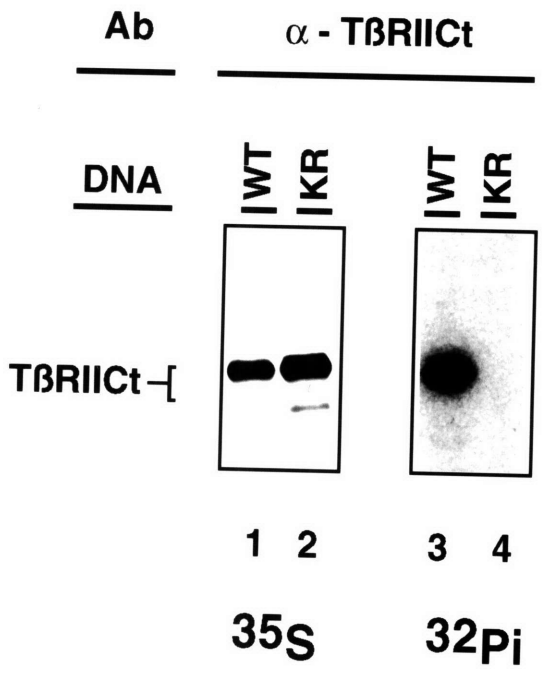

Ab
α - TßRIICt
DNA
WT
KR
WT
KR
TßRIICt
1 2 3 4
35S
32Pi

Figure 4.2 Transphosphorylation of kinase-deficient mutant TGF-ßRI by the cytoplasmic domain of TGF-ßRII kinase (A)

An epitope-tagged kinase-deficient mutant type I TGF-ß receptor (TßRI-HA) was co-transfected with either wild type full-length TGF-ßRII (TßRII) or the cytoplasmic domain of TGF-ßRII (TßRIICt) in COS1 cells. Cells were metabolically labeled with ^{32}P-orthophosphate, and immunoprecipitation was performed using monoclonal antibody 5c. The top panel is the same exposure as that in Figure 4.3 and they are from the same set of experiment ; the bottom panel is a lighter exposure of the same gel in the top panel.

wt: wild-type TGF-ß receptors; R: kinase-deficient mutant receptor

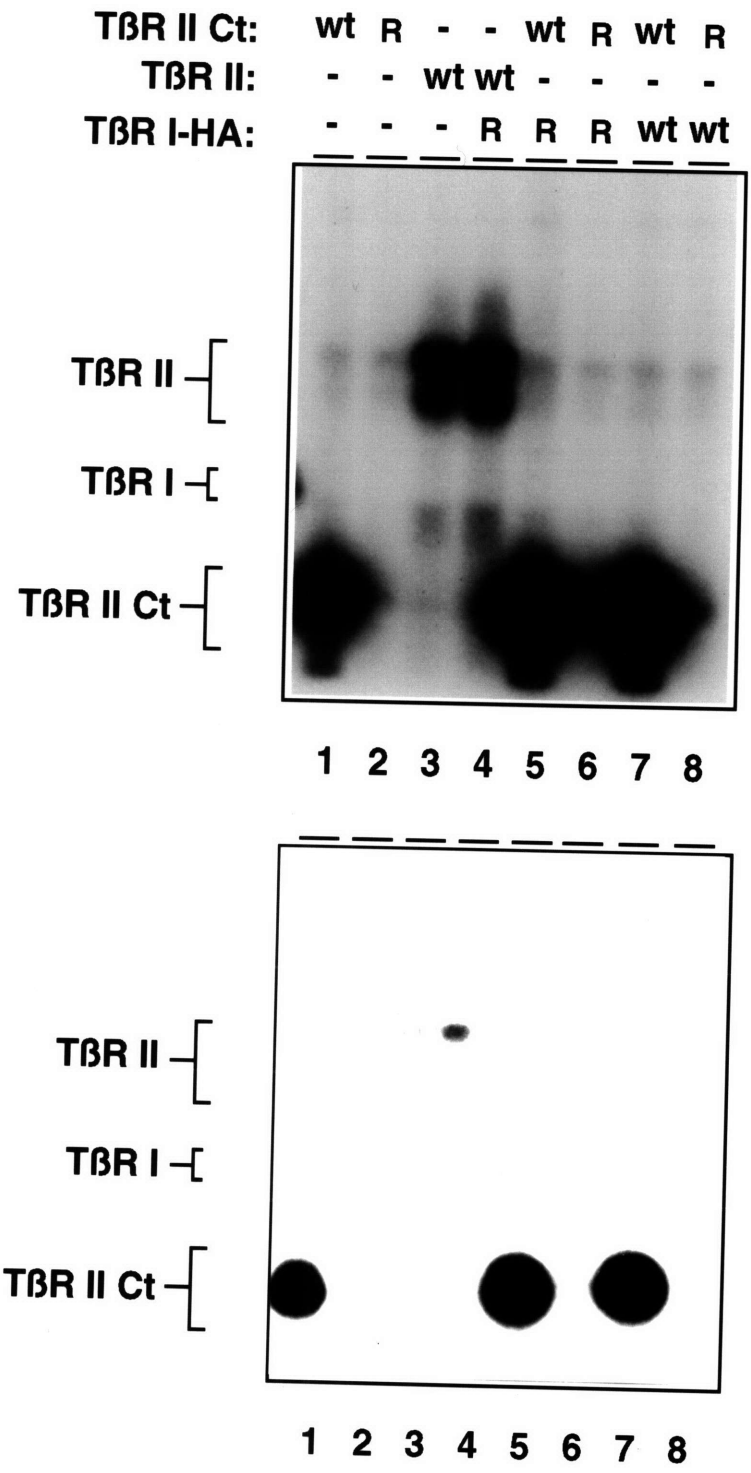

TβR II Ct: wt R - - wt R wt R
TβR II: - - wt wt - - - -
TβR I-HA: - - - R R R wt wt
TβR II
TβR I
TβR II Ct
1 2 3 4 5 6 7 8
TβR II
TβR I
TβR II Ct
1 2 3 4 5 6 7 8

Figure 4.3 Transphosphorylation of kinase-deficient mutant TGF-ßRI by the cytoplasmic domain of TGF-ßRII kinase (B)

Same as in Figure 4.2, an epitope-tagged kinase-deficient mutant type I TGF-ß receptor (TßRI-HA) was co-transfected with either wild type full-length TGF-ßRII (TßRII) or the cytoplasmic domain of TGF-ßRII (TßRIICt) in COS1 cells. Cells were metabolically labeled with ^{32}P-orthophosphate, however, immunoprecipitation was performed using anti-HA antibody (12CA5).

wt: wild-type TGF-ß receptors; R: kinase-deficient mutant receptor

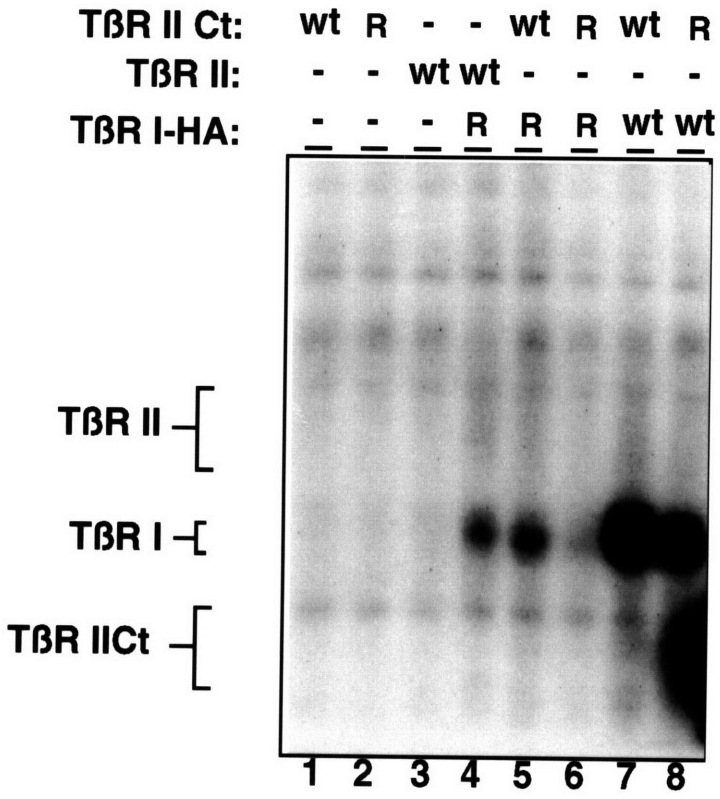

TβR II Ct: wt R - - wt R wt R
TβR II: - - wt wt - - - -
TβR I-HA: - - - R R R wt wt
TβR II
TβR I
TβR IICt
1 2 3 4 5 6 7 8

Figure 4.4 Tryptic phosphopeptide maps of the full-length TGF-ßRII and its cytoplasmic domain

(A). COS1 cells transfected with wild type full-length TGF-ßRII (TßRII) were labeled with ^{32}P-orthophosphate, immunoprecipitated with mAb 5c and subjected to phosphopeptide analysis.

(B) Same as in (A), except that cells were transfected with the cytoplasmic domain (TßRIICt) of wild-type TGF-ßRII.

(C) Schematic indicates the specific phosphopepetides referred in the text.

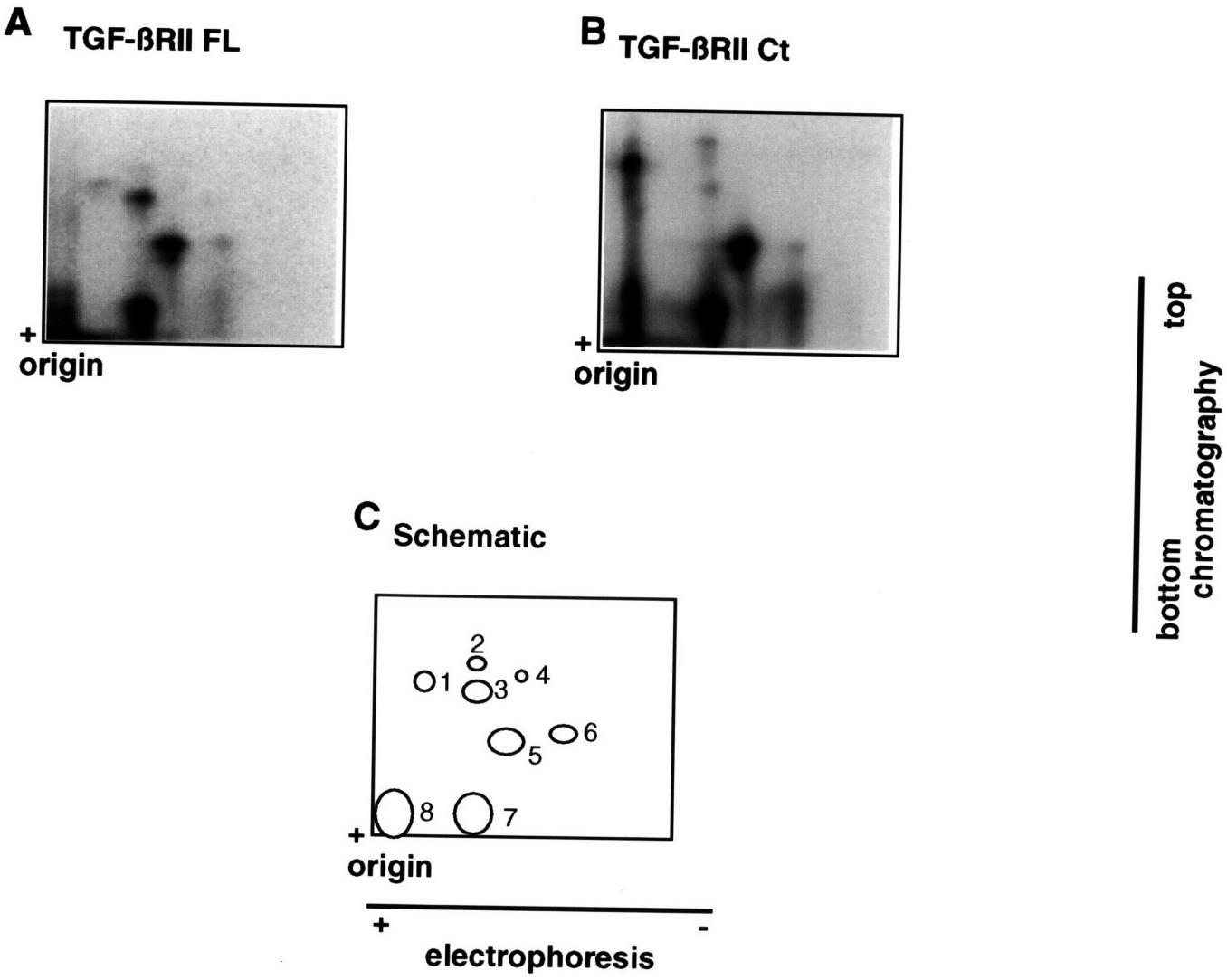

A
TGF-βRII FL
+
origin
B
TGF-βRII Ct
+
origin
top
bottom
chromatography
C
Schematic
2
1
3
4
5
6
8
7
+
origin
+
-
electrophoresis

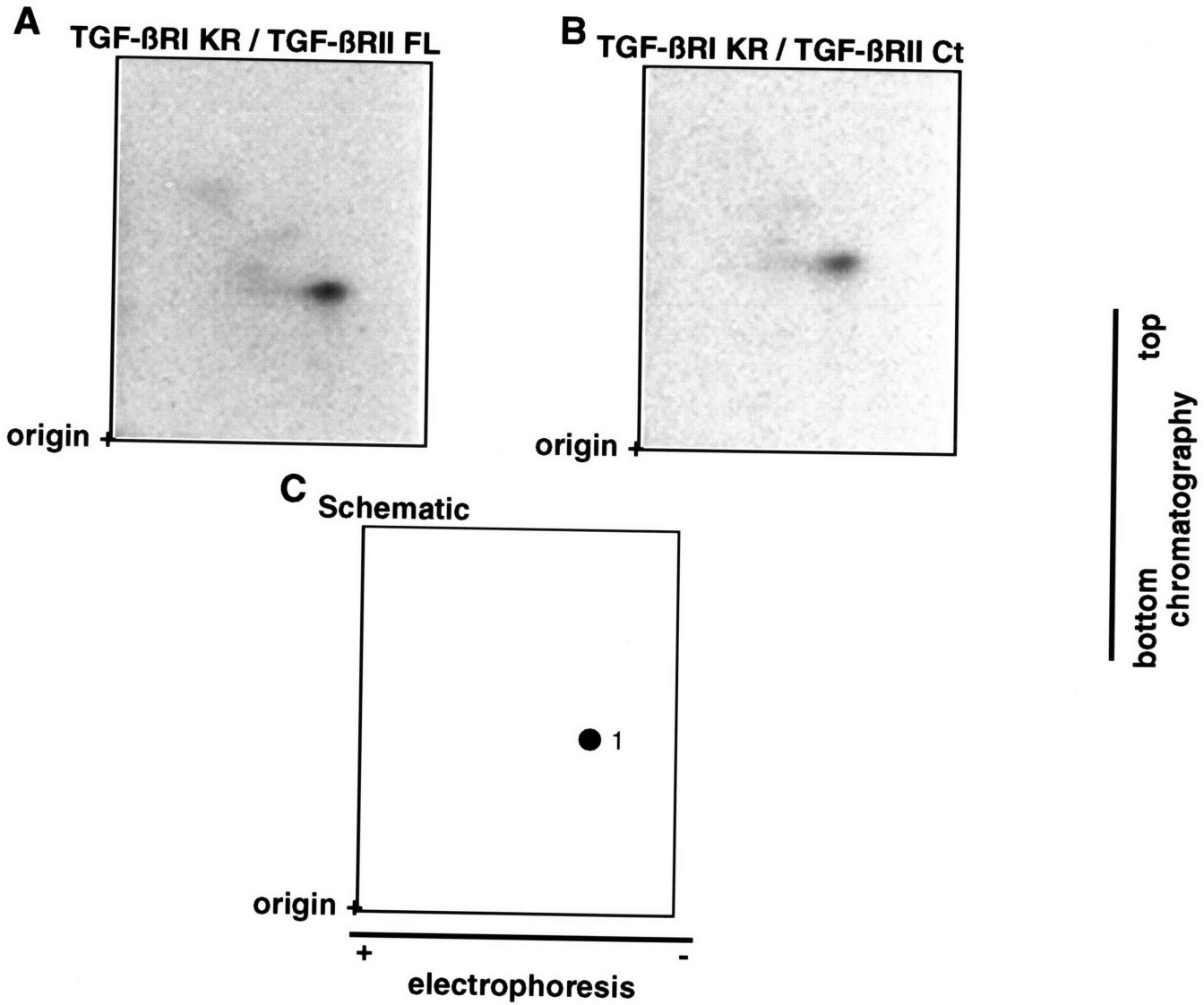
Figure 4.5 Tryptic phosphopeptide maps of kinase-deficient mutant TGF-ßRI transphosphorylated by the full-length TGF-ßRII and its cytoplasmic domain

An epitope-tagged kinase-deficient mutant type I TGF-ß receptor (TßRI-HA) was co-transfected with either wild type full-length TGF-ßRII (TßRII) or the cytoplasmic domain of TGF-ßRII (TßRIICt) in COS1 cells. Cells were metabolically labeled with ^{32}P-orthophosphate, and immunoprecipitation was performed using anti-HA antibody (12CA5).

(A). Phosphopeptide analysis of TßRI-HA co-transfected with the full-length wild type TGF-ßRII (TßRII).

(B) Phosphopeptide analysis of TßRI-HA co-transfected with the cytoplasmic domain of wild-type TGF-ßRII (TßRIICt).

(C) Schematic indicates the specific phosphopepetides referred in the text.


A
TGF-βRI KR / TGF-βRII FL
origin
+
B
TGF-βRI KR / TGF-βRII Ct
origin
+
top
bottom
chromatography
C
Schematic
1
origin
+
+
-
electrophoresis

Chapter 5

Phosphorylation of Endogenous TGF-ßRII in TGF-ß Responsive Cells

PREFACE

The results discussed in this chapter have not yet been unpublished.

The purpose of the experiments described here is to analyze complex formation and phosphorylation of the endogenous TGF-ßRII and TGF-ßRI in TGF-ß-responsive cells. Two cell lines, one that is growth-stimulated by TGF-ß (HER14) and one that is growth-inhibited by TGF-ß (HaCat) were chosen for this research.

INTRODUCTION

Studies with COS cells described in the preceding chapters, suggest that the association of TGF-ß type I and II receptors, induced either by ligand binding or overexpression, leads to transphosphorylation of TGF-ßRI by the TGF-ßRII kinase. This represents a novel mechanism of receptor activation distinct from that of the tyrosine kinase receptors. My work, together with that of others, has further demonstrated the existence of a ligand-independent receptor pre-complex due to the intrinsic affinity of TGF-ßRI for TGF-ßRII.

However, all the observations on the TGF-ß receptor complex formation and activation have been made using cell lines that have been transfected with TGF-ß receptor cDNAs and therefore have overexpressed TGF-ß receptors. In order to further understand the mechanism of TGF-ß signaling, it is important to relate these biochemical studies to the physiological roles played by the ligand in TGF-ß-responsive cells in which the TGF-ß receptors are expressed at physiological levels.

One of the most puzzling observations is that the TGF-ßRII seems to be constitutively active and to remain autophosphorylated independent of the presence of ligand. This is in contrast to the tyrosine kinase receptors, which undergo ligand-induced autophosphorylation to activate the receptor kinases as well as to provide docking sites for interaction by downstream substrates (Schlessinger and Ullrich, 1992; Ullrich and Schlessinger, 1990).

There are two reservations about the existing observations: First, serum supplements in cell culture medium may contain active TGF-ß that is responsible for the constitutive autophosphorylation of TGF-ßRII. Second, overexpression of the TGF-ßRII may cause a deregulated receptor phosphorylation that is normally ligand-dependent. I will address these issues in two cell lines that exhibit opposite growth responses to TGF-ß.

The expression level of TGF-ß receptors in mammalian cells is normally very low, complicating most biochemical studies. During the course of generating cell lines that could stably express TGF-ßRII, I and others had noted that cells that were growth-inhibited by TGF-ß could not maintain high expression levels of transfected TGF-ßRII. This observation suggested to me that these cell lines may suffer a disadvantage of growth due to the constitutive activation of the TGF-ß receptors caused by their overexpression or by the presence of active TGF-ß in tissue culture medium. Since TGF-ß has different effects on cell proliferation depending upon the cell type, I reasoned that cell lines growth-stimulated by TGF-ß might offer a better starting point for studying endogenous TGF-ßRII.

Indeed, earlier work from others has shown that TGF-ß functions as a mitogenic factor for certain mesenchymal cells (Shipley et al., 1985) and potentiates the action of EGF in some fibroblasts (Goldkorn and Mendelsohn, 1992; Massagué, 1985; Roberts and Sporn, 1987). Therefore, the fibroblast cell line HER14, an NIH3T3 cell expressing endogenous PDGF receptor and transfected human EGF receptor, was chosen for the initial study (Honegger et al., 1987; Xu et al., 1994). These cells not only might provide a system for studying the mechanism of the growth-stimulatory effects of TGF-ß, but would also allow me to investigate the possible crosstalk between the TGF-ß pathway and other mitogenic pathways such as that activated by EGF.

I also extended my research to HaCat cells, an established line of human keratinocytes, which is well studied for its responsiveness to TGF-ß's growth-inhibitory effects (Geng and Weinberg, 1993). I wished to investigate more than one cell line so that the possible bias from any specific cell line could be eliminated. In particular, I choose two cell lines with opposite responsiveness to TGF-ß's growth-regulating effects so that I could compare whether the phosphorylation of the TGF-ß receptors differs in these two cell lines.

RESULTS

5.1 Effects of TGF-ß and growth factors on HER14 cell proliferation

HER14 cells were originally established by transfecting constructs encoding human EGF receptor (EGFR) into cultured NIH3T3 cells lacking the endogenous EGFR (Honegger et al., 1987; Xu et al., 1994). To study the activation mechanism of TGF-ßRs in this cell line, I first characterized its responsiveness to TGF-ß in conjunction with serum and other growth factors. HER14 cells were seeded in 24-well tissue culture plates, grown to subconfluence, and starved in culture medium containing 0.1% calf serum (CS) for 48 hours. Different growth factors were then added and ^{3}H-thymidine incorporation was measured 18 hours after restimulation. As illustrated in Fig 5.1, when 100 pM TGF-ß1 was added alone to the starved cells, an approximately two-fold increase in ^{3}H-thymidine incorporation was observed (compare columns 6 and 2). In addition, TGF-ß1 appeared to synergize with EGF but not with PDGF in promoting growth of HER14 cells (compare columns 7, 8 and 3, 4). Taken together, these experiments indicate that TGF-ß stimulates growth-arrested HER14 cells to reenter the cell cycle and interestingly, that EGF and TGF-ß synergize in their growth-promoting effects.

To further explore TGF-ß effects on HER14 cells, I titrated the concentration of TGF-ß1 in comparison with serum (Fig 5.2). While the TGF-ß stimulatory effect was readily detected in this assay at concentrations less than 10 pM, the optimal concentration of about 50 pM was just as potent mitogenically as 10% calf serum. I concluded that TGF-ß positively regulates HER14 cell proliferation in a dose-dependent manner and with a potency comparable to the serum normally present in cell culture medium. Moreover, I designed an experiment to define the synergism of TGF-ß and EGF, as shown in Fig 5.3. It is clear that in the presence of different concentration of EGF (0 to 10 ng/ml), TGF-ß could potentiate HER14 cell growth in a dose-dependent fashion (0 to 50 pM). This

suggested that the synergism between TGF-ß and EGF in stimulating HER14 cell proliferation was determined by the concentrations of both factors.

5.2 Characterization of TGF-ß receptors of HER14 cells

Subconfluent HER14 cells were incubated with 100 pM iodinated TGF-ß1 (^{125}I-TGF-ß) and a chemical crosslinking assay was conducted using disuccinimidyl suberate (DSS) as described (Massagué and Like, 1985). Cells were then lysed in detergent-containing buffer and subjected to SDS-PAGE directly. Fig 5.4, lane 1 demonstrates that HER14 cells express all three types of the TGF-ßRs. The same amount of lysates were also used for immunoprecipitation using antibodies against TßRII or TßRI, and a complex composed of type I, type II and type III receptors were detected (Fig 5.4, lanes 2 to 4). Two different antibodies specifically recognizing the intracellular domain of TGF-ßRI were used (Fig 5.4, lanes 3 and 4).

Having learned about the basic repertoire of TGF-ß receptors on the cell surface of HER14, I next attempted to detect TGF-ßRII in these cells by metabolic labeling with ^{35}S-methionine. In this case, a key reagent is the monoclonal antibody 5c (mAb5c) I generated, which specifically recognizes the intracellular portion of the TGF-ßRII and has been extensively characterized in preceding chapters. Two criteria were established to ensure that the protein species recognized by mAb5c was indeed endogenous TGF-ßRII: (1) Endogenous TGF-ßRII is a glycoprotein that should exhibit a mobility shift on the SDS-PAGE upon glycosidase treatment. (2) The core protein recognized by mAb5c should have a molecular mass of about 60 to 65 kDa as predicted by the TGF-ßRII cDNA sequence and previous studies with transfected COS cells. As shown in Fig 5.5, both criteria were fulfilled in detecting the endogenous TGF-ßRII of COS cells and HER14 cells (lanes 5 to 8), and the doublet observed after deglycosylation with PNGaseF was typical of TGF-ßRII (Chapter 2, Fig 2.5). This result was further confirmed when COS cells transfected with

TßRII-HA were used as controls and immunoprecipitation was performed either with mAb5c that reacts with TGF-ßRII, or with mAb 12CA5 which recognizes the HA-tag (Fig 5.5, lanes 1 to 4). This was a critical step that made it possible to examine the *in vivo* phosphorylation status of TGF-ßRII in HER14 cells.

5.3 *In vivo* phosphorylation of TGF-ßRII in HER14 cells

The observation that TGF-ßRII was constitutively autophosphorylated when transfected in COS cells (Chapter 2) stimulated me to set up two essential controls for the experiments designed to analyze the causes of observed phosphorylation of the endogenous TGF-ßRII in HER14 cells. I wished to explore here the possible presence of active TGF-ß in the cell culture medium. First, I did ^{32}P-orthophosphate labeling and subsequent TGF-ß treatment either in the absence (Fig 5.6, lanes 3 and 4) or presence (Fig 5.6, lanes 5 and 6) of serum which may contain TGF-ßs and therefore lead to the activation of TGF-ß receptors. Second, in the cases where cells were not treated with TGF-ß (Fig 5.6, lanes 3 and 5), a neutralizing antibody, specifically recognizing TGF-ß1, ß2 and ß3, was included in the medium throughout the metabolic labeling; this was intended to eliminate the effects of TGF-ßs that might be secreted by the HER14 cells.

Subconfluent HER14 cells were metabolically labeled with ^{32}P-orthophosphate in a phosphate-free medium alone (-) or in one containing 10% dialyzed fetal calf serum (+) as described above. Cells were then treated either with (+) or without (-) 100 pM TGF-ß1 for 10 minutes before they were lysed in a buffer containing inhibitors of proteases and phosphatases as detailed in Materials and Methods. Immunoprecipitation was performed using the mAb5c anti-TßRII antibody and the purified immunoprecipitates were treated with PNGaseF. Samples were separated by SDS-PAGE and subjected to phosphoimager analysis.

As shown in Fig 5.6, the phosphorylated endogenous type II TGF-ß receptor in HER14 cells migrated with an apparent molecular weight of 60-65kDa, the same size as the polypeptide core of the type II receptor (lanes 3 to 6). Importantly, no difference was detected in the intensity of the TGF-ßRII phosphorylation with or without TGF-ß treatment (lanes 3 and 4), indicating that the endogenous TGF-ßRII in HER14 cells is constitutively phosphorylated independent of exogenously added ligand. Samples from lanes 3 and 4 were prepared from cells labeled with ^{32}P-orthophosphate in the absence of serum and a neutralizing antibody against TGF-ß was included in lane 3; therefore, the phosphorylation of TGF-ßRII observed here was indeed free from the possible existing TGF-ß in the culture medium. Furthermore, when serum remained in the cell culture medium during ^{32}P-orthophosphate labeling and TGF-ß treatment, no difference was observed on the TGF-ßRII phosphorylation in HER14 cells either (lanes 5 and 6). These results indicate that growth factors present in the serum do not affect the phosphorylation of TGF-ßRII in this assay.

Based on the earlier characterization of endogenous TGF-ßRII in HER14 cells metabolically labeled with ^{35}S-methionine (Fig 5.5), two additional controls were designed here to further confirm the molecular identity of the endogenous TGF-ßRII in ^{32}P-orthophosphate labeled HER14 cells: (1) Without PNGaseF treatment, immunoprecipitates with mAb 5c yielded a heterogeneously migrating band of approximately 70-90kDa upon gel electrophoresis, just as predicted for a TGF-ßRII as a glycoprotein with three carbohydrate side chains (Fig 5.6, lane 7). (2) An unrelated monoclonal antibody, 12CA5, which reacts with HA-tag as described before in Chapter 2, was used to show the specificity of the mAb5c in recognizing the phosphorylated endogenous type II TGF-ß receptor in HER14 cells (Fig 5.6, lane 2). Taken together, these observations demonstrated for the first time that the endogenous TGF-ßRII is constitutively phosphorylated independent of ligand or serum treatment in a cell line that is growth-stimulated by TGF-ß.

5.4 *In vivo* phosphorylation of TGF-ßRII in HaCat cells

In order to determine if the constitutive autophosphorylation of TGF-ßRII in HER14 cells indeed reflected an intrinsic property of this kinase and was not simply due to some peculiar feature of the HER14 cell line, I extended my observation to an established line of human keratinocytes. HaCat is one of the widely characterized epithelial cell lines that is strongly growth-inhibited by TGF-ß and has served as a model system for many cell cycle studies (Geng and Weinberg, 1993; Hannon and Beach, 1994).

As with HER14 cells, I first characterized the TGF-ß receptors of HaCat cells by binding-crosslinking experiments with ^{125}I-TGF-ß1. As shown in Fig 5.7, HaCat cells have all three types of TGF-ß receptors, yet with a pattern distinct from that of HER14 cells: more type I receptors were present in this assay, while both the type II and III receptors were much less abundant. Perhaps because of its low level in HaCat cells, I failed to detect the endogenous TGF-ßRII by immunoprecipitation from cells metabolically labeled with ^{35}S-methionine. However, I was able to visualize the endogenous type II TGF-ß receptor in HaCat cells as a heterogeneous glycoprotein with correct molecular weight following ^{32}P-orthophosphate labeling (Fig 5.8). Interestingly, just as in HER14 cells, TGF-ßRII in HaCat cells was autophosphorylated *in vivo* even in the absence of ligand and serum, strongly indicating that the constitutive phosphorylation of this type of TGF-ß is not an artifact resulting from the overexpression in transfected COS cells, nor is it a peculiar feature of HER14 cells. Instead, it is an inherent property of this receptor kinase.

DISCUSSION

In vivo phosphorylation of the type II TGF-ß receptor was studied at the endogenous level in cell lines that are growth-regulated by TGF-ß. I observed constitutive

phosphorylation of the TGF-ßRII in cell lines that are either growth-stimulated by TGF-ß (HER14) or growth-inhibited by TGF-ß (HaCat). In an assay using ^{32}P-orthophosphate labeling and immunoprecipitation with a monoclonal antibody specifically recognizing TGF-ßRII, no quantitative changes of *in vivo* phosphorylation of the type II TGF-ß receptor kinase were detected upon TGF-ß treatment in the presence or absence of serum in both cell lines. Therefore, it was unambiguously established that TGF-ßRII is a constitutively active kinase under physiological conditions in TGF-ß responsive cell lines. These observations also suggested that the growth-stimulating and growth-inhibiting effects of TGF-ß on different cells were not regulated at the level of TGF-ß receptor phosphorylation. Although the downstream pathways of TGF-ß signaling need to be further characterized, studies described here represent an encouraging starting point to work with endogenous TGF-ß receptors under biologically relevant situations.

The existing results could not distinguish whether the constitutive phosphorylation of TGF-ßRII in HER14 and HaCat cells represented the constitutive autophosphorylation of this type of receptor kinase or was due to transphosphorylation by other cellular kinases. However, I have shown earlier (Chapter 2) that in transfected COS cells, the in *vivo* phosphorylation of TGF-ßRII resulted primarily from its autophosphorylation, based on studies with a kinase-deficient mutant TGF-ßRIIKR (Fig 2.2) and a GST fusion protein containing the kinase portion of TGF-ßRII (Fig 2.3). I have also demonstrated that the autophosphorylation of TGF-ßRII transfected in these cells was ligand-independent and most likely via an intramolecular (cis-) mechanism that is different from tyrosine kinase receptors (Fig 2.4 and Fig 2.5). Taken together, these results suggest us the constitutive phosphorylation of the endogenous TGF-ßRII is a consequence of the constitutive activity of the receptor kinase.

It is possible that while most phosphorylation sites on TGF-ßRII are constitutively phosphorylated, some particular sites may be phosphorylated in a ligand-dependent manner. In this case, the subtle changes of TGF-ßRII phosphorylation upon TGF-ß and

serum treatment might not be revealed in the immunoprecipitation assay described in this chapter. To achieve higher resolution on this subject, more detailed tryptic phosphopeptide analysis will be necessary. However, the very low expression levels of the endogenous TGF-ßRII have made this a rather difficult task.

Another important issue will be to test whether the kinase activity of TGF-ßRII towards exogenous substrates is altered upon ligand binding in addition to its autophosphorylation. Transphosphorylation of the type I TGF-ß receptor (TGF-ßRI) by the type II receptor kinase (TGF-ßRII) will first be examined once the reagents become available to detect the endogenous TGF-ßRI metabolically labeled in HER14 and HaCat cells. Moreover, future efforts should be directed to identify other substrates for TGF-ßRII so that its kinase activity can be measured quantitatively before and after TGF-ß treatment.

Over the past few years, the biochemical studies of TGF-ß receptors have been primarily focused on cells that are transiently transfected with TGF-ßRs cDNAs (mostly COS and 293 cells) or that are growth-inhibited by TGF-ß such as Mv1Lu cells. Work with HER14 cells in this chapter provided the first example of the phosphorylation of endogenous TGF-ß receptors in a cell line whose growth is stimulated by TGF-ß in a dose-dependent manner. To confirm that the stimulation effects on HER14 cell proliferation truly derive from TGF-ß, I tested different isoforms of the ligand and observed very similar results with either TGF-ß1, TGF-ß2 or TGF-ß3 (Fig 5.1 to Fig 5.3 and data not shown). It is also worth mentioning that the TGF-ß ligands used in these studies were recombinant proteins secreted by engineered CHO cells and purified by sequential HPLC. More importantly, a neutralizing antibody against TGF-ß (1D11.16) (Dasch et al., 1989) abolished the TGF-ß stimulating effects on HER14 cells when included in the assay at a concentration of 10 µg/ml (data not shown). Therefore, the growth-promoting effects on HER14 cells were unlikely to be due to other contaminating growth factors in the ligand preparations but instead reflected an intrinsic biological function of TGF-ß.

Unexplained by this work is the mechanism underlying the TGF-ß growth-promoting effects. It is possible that TGF-ß stimulates HER14 cell growth as a secondary effect via actions of other growth factors or extracellular matrix (ECM). It has been shown that TGF-ß can stimulate proliferation of cultured smooth muscle cells (SMCs) as well as human fibroblasts and chondrocytes by inducing autocrine PDGF-AA secretion (Battegay et al., 1990). In NRK fibroblasts, TGF-ß has a synergistic effect with EGF that is likely due to the increase in the number of plasma membrane receptors for EGF (EGFR) in response to TGF-ß (Assoian et al., 1984). It has been reported that TGF-ß can modulate phosphorylation of the EGFR and proliferation of the A431 epidermoid carcinoma cell line (Goldkorn and Mendelsohn, 1992). In addition, the extracellular matrix (ECM) has also been implicated as a mediator of the growth-stimulating effect of TGF-ß since both cell adhesion proteins and their receptors are regulated by TGF-ß (Heino et al., 1989; Ignotz and Massagué, 1987; Morton and Barrack, 1995).

In addition, there is increasing evidence that TGF-ß may act as a direct mitogen and the mitogen-activated protein kinase (MAPK) pathway has been suggested to be one of the candidate targets. Indeed, TAK1 (designated for TGF-ß activated kinase), a distinct member of the MAPKKK family, was shown to participate in regulation of transcription by TGF-ß and its kinase activity was stimulated rapidly in response to TGF-ß and another TGF-ß superfamily member BMP (bone morphogenenic protein) (Yamaguchi et al., 1995). There is also a report on the rapid activation of certain MAP kinases by TGF-ß in a colon carcinoma cell line that is sensitive to TGF-ß stimulation (Yan et al., 1994). Furthermore, a recent study has demonstrated that TGF-ß may promote the proliferation of vascular smooth muscle cells (VSMCs) by activating the PKC-δ-dependent signaling pathways (Saltis and Bobik, 1996).

Yet another possibility is that TGF-ß regulates cell proliferation by acting at a level distal from the early mitogenic signaling transduction pathway (Diaz-Meco et al., 1992; Like and Massagué, 1986). For example, cyclin-dependent kinase inhibitors (CDIs) such

as p15, p16, p21 and p27 may be differentially regulated by TGF-ß and other proliferation signals in a cell-type-dependent manner and therefore affect the cell cycle progression (Massagué and Polyak, 1995) To distinguish all the possible mechanisms of TGF-ß action on cell proliferation of HER14 cells, the key issue is to establish the kinetics of TGF-ß-induced growth stimulation; efforts can then be focused on either the direct early effects of TGF-ß or its secondary roles through other growth stimuli.

ACKNOWLEDGMENTS

I thank Drs. J. Schlessinger and Wei Li for providing the HER14 cells.

Figure 5.1 Effects of TGF-ß and mitogenic growth factors on HER14 cell proliferation

HER14 cells were growth-arrested by starvation in culture medium containing 0.1% CS for 48 hours. Different growth factors were then added and ^{3}H-thymidine incorporation was measured 18 hours post restimulation. CS: calf serum.

All assays were performed at least three times, with triplicates in each experiment. Data are mean ± SD of triplicates from a representative experiment.

Effects of TGF-ß and Growth Factors on HER 14 Cells

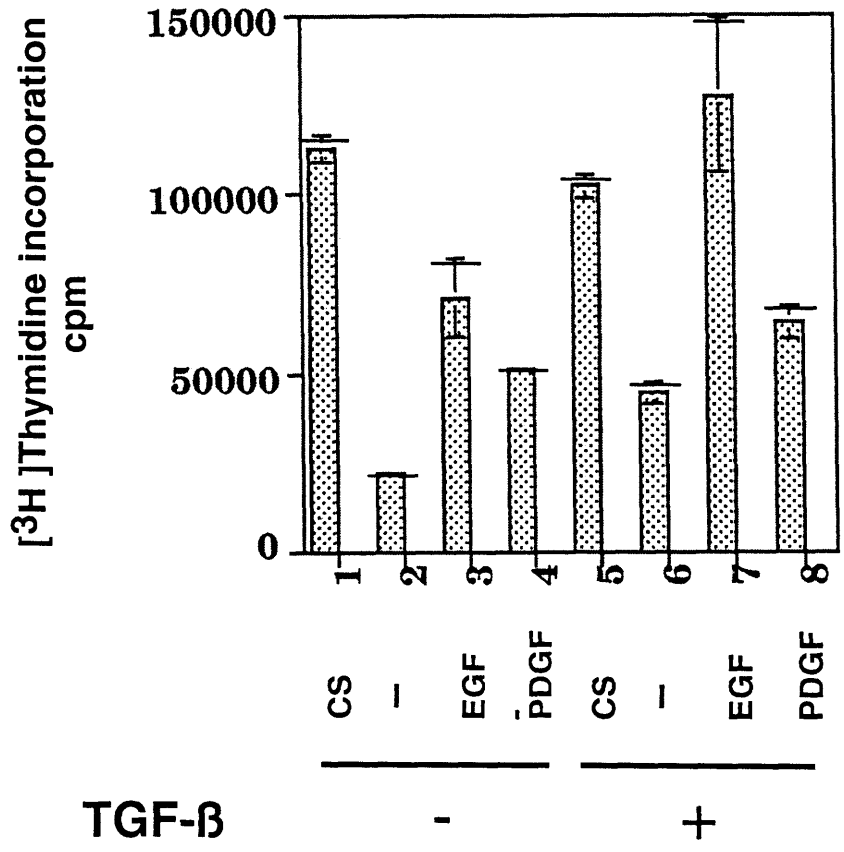


Figure 5.2 Comparison of TGF-ß and serum effects on HER14 cell proliferation

HER14 cells were growth-arrested by starvation in culture medium containing 0.1% CS for 48 hours. Different concentrations of TGF-ß1 or serum were then added and ^{3}H-thymidine incorporation was measured 18 hours post restimulation.

CS: calf serum.

TGF-ß and Serum Effects on HER14 Cells

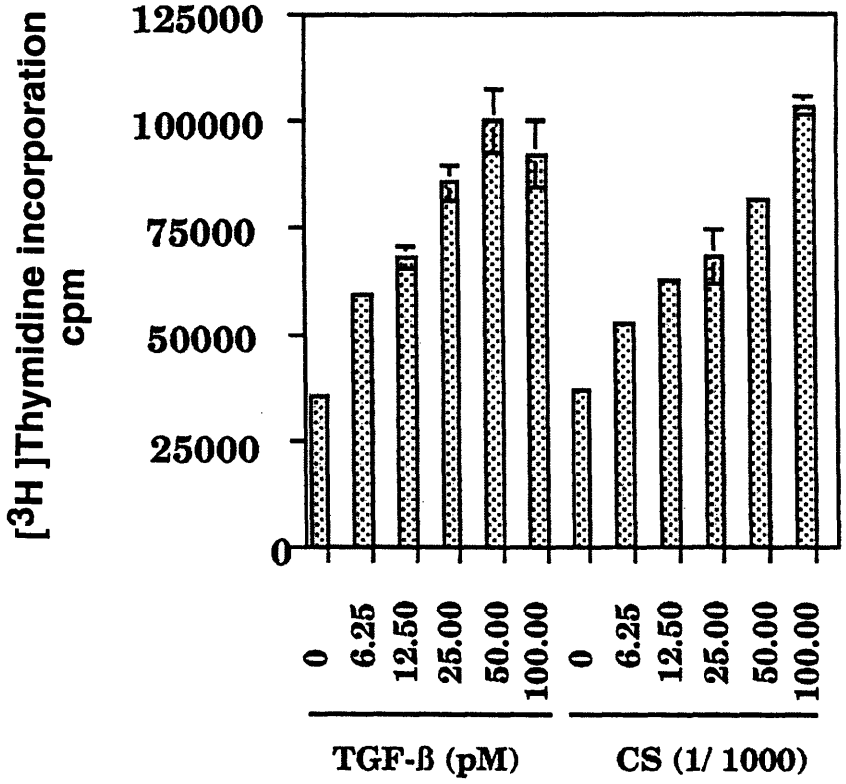


Figure 5.3 Synergism of TGF-ß and EGF on HER14 cell proliferation

HER14 cells were growth-arrested by starvation in culture medium containing 0.1% CS for 48 hours. Different concentrations of TGF-ß1 in combination with EGF were then added and ^{3}H-thymidine incorporation was measured 18 hours post restimulation.

CS: calf serum.

TGF-ß and EGF Effects on HER14 Cells

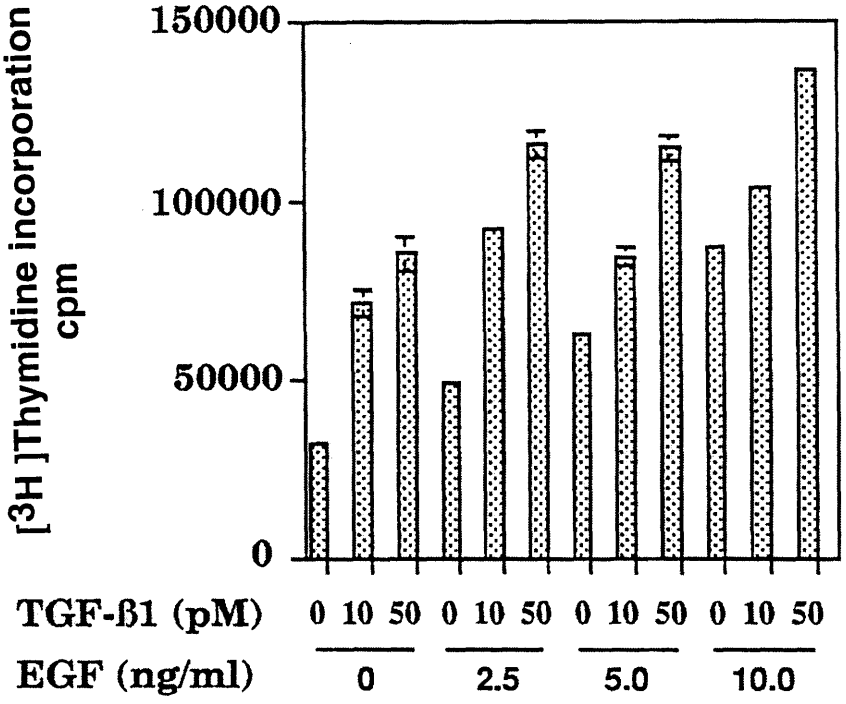


Figure 5.4 Characterization of TGF-ß receptors of HER14 cells (A) -- Binding and crosslinking to ^{125}I-TGF-ß

Subconfluent HER14 cells were incubated with 100 pM iodinated TGF-ß1 and applied to a chemical crosslinking assay as described. Cells were then lysed and immunoprecipitation was performed using antibodies against TßRII (labeled as II) or TßRI (labeled as I).

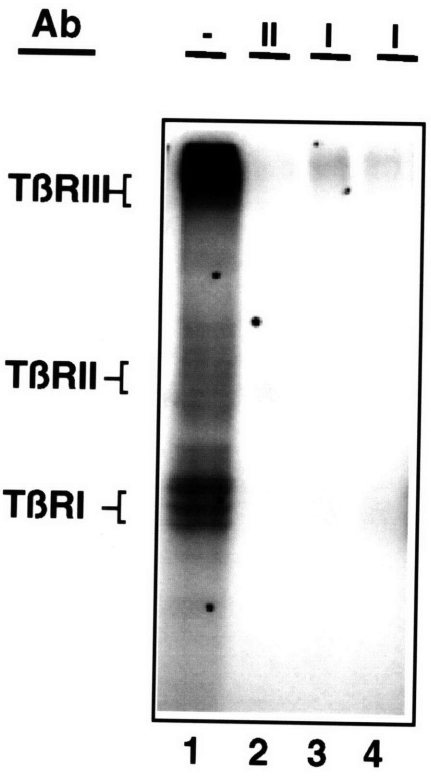

Ab
-
II
I
I
TβRIII
TβRII
TβRI
1
2
3
4

Figure 5.5 Characterization of TGF-ß receptors of HER14 cells (B)
-- Metabolic labeling with ^{35}S-methionine

Subconfluent HER14 cells (lanes 7 and 8) and COS cells (lanes 5 and 6) were metabolically labeled with ^{35}S-met and immunoprecipitation was performed using monoclonal antibody 5c. COS cells transfected with TßRII-HA (lanes 1 to 4) were used as controls and immunoprecipitation was performed using indicated antibodies: 5c that is specifically against TßRII or 12CA5 that recognizes HA-tag.

TGF-ßRII*: deglycosilated TGF-ßRII core protein.

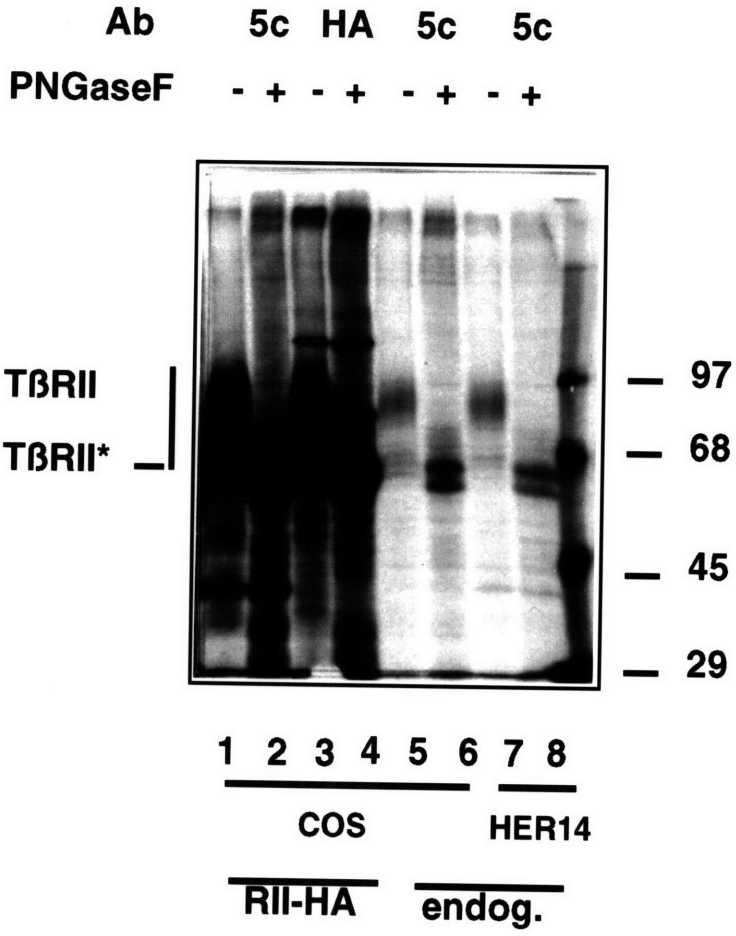

Ab
5c
HA
5c
5c
PNGaseF
- + - + - + - +
TßRII
TßRII*
97
68
45
29
1 2 3 4 5 6 7 8
COS
HER14
RII-HA
endog.

Figure 5.6 *In vivo* phosphorylation of TGF-ßRII in HER14 cells

HER14 cells were metabolically labeled with ^{32}P-orthophosphate and immunoprecipitation was performed using anti-TßRII antibody (5c). Labeling was performed either in the presence (+) or absence (-) of 10% calf serum in the cell culture medium. Cells were treated either with (+) or without (-) TGF-ß1 before lysis.

m: a mixture of equal amount of lysates from cells treated differently with serum and TGF-ß1 (lane 3 through lane 6) was used in lanes 2 and 7 for controls.

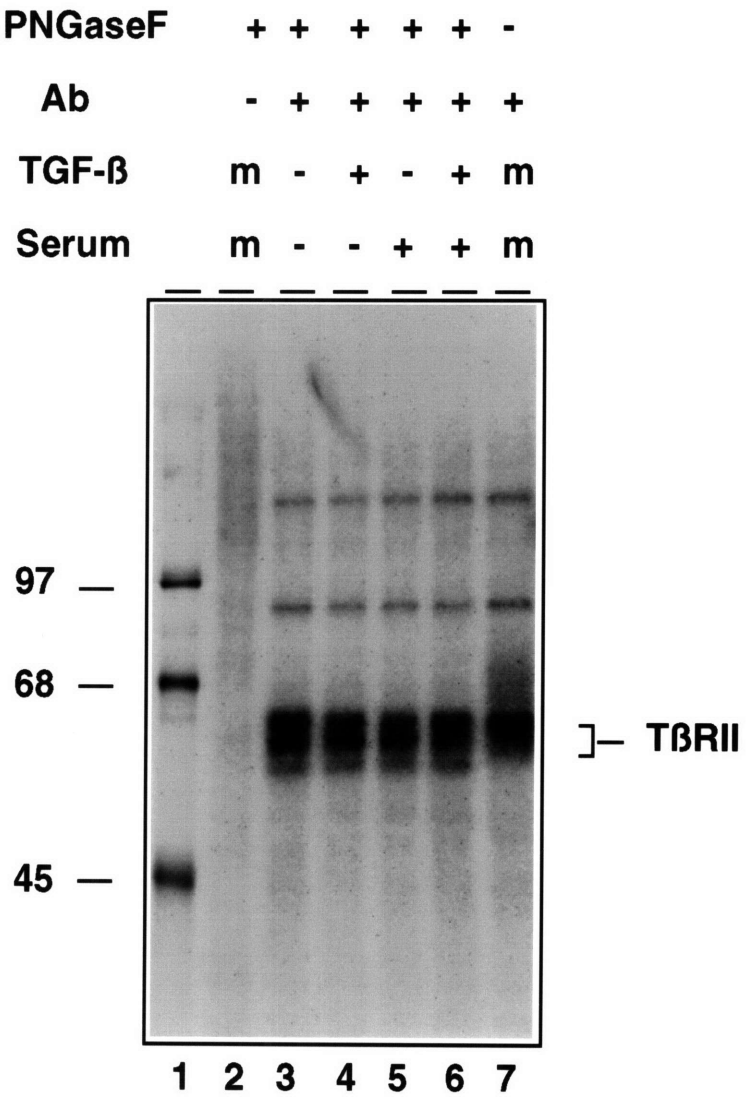

PNGaseF + + + + + -
Ab - + + + + +
TGF-ß m - + - + m
Serum m - - + + m
97
68
45
TßRII
1 2 3 4 5 6 7

Figure 5.7 Characterization of TGF-ß receptors of HaCat cells
-- Binding and crosslinking to ^{125}I-TGF-ß

Subconfluent HaCat cells were incubated with 100 pM iodinated TGF-ß1 and applied to chemically crosslinking assay as described. Cells were then lysed and immunoprecipitation was performed using antibodies against TßRII (labeled as II) or TßRI (labeled as I).

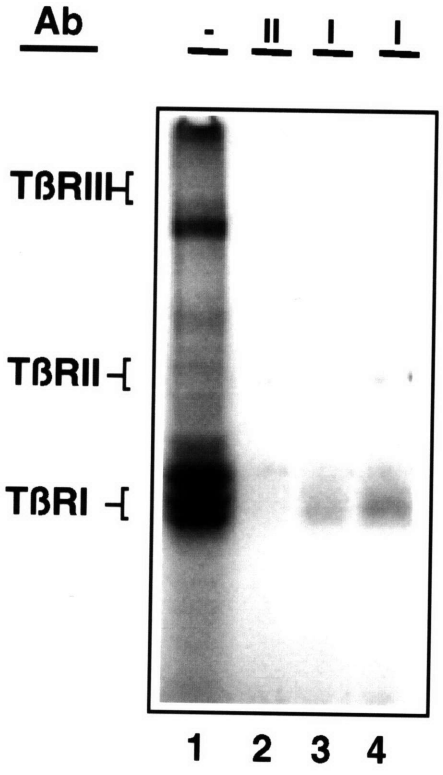
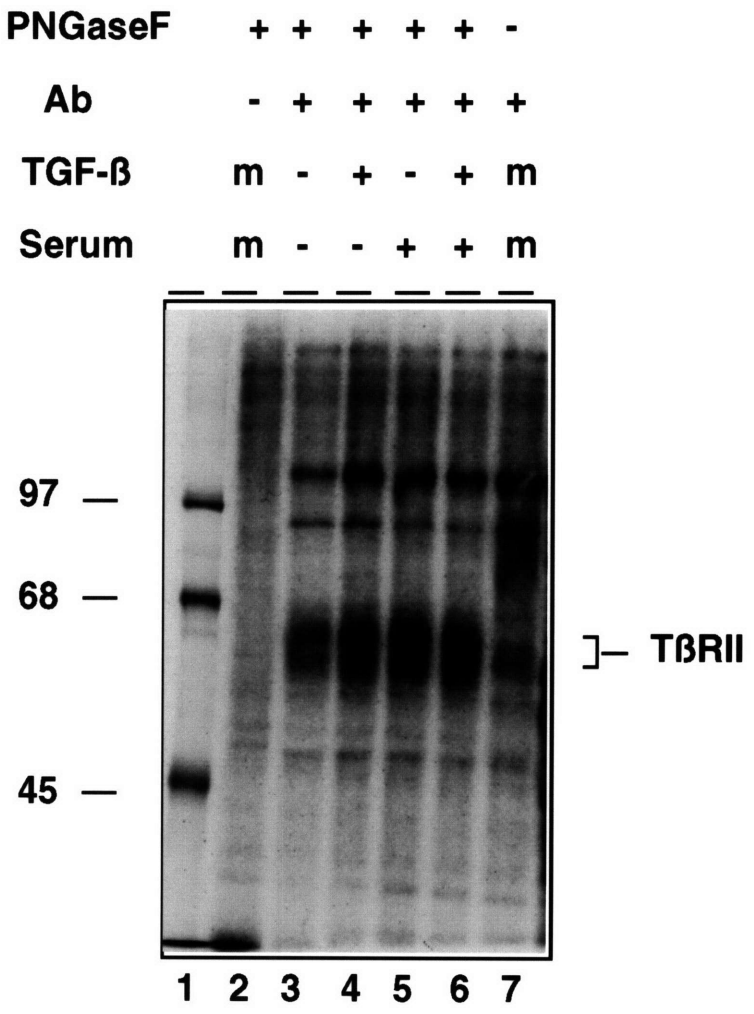

Ab
-
II
I
I
TßRIII
TßRII
TßRI
1
2
3
4

Figure 5.8 *In vivo* phosphorylation of TGF-ßRII in HaCat cells

HaCat cells were metabolically labeled with ^{32}P-orthophosphate and immunoprecipitation was performed using anti-TßRII antibody (5c). Labeling was performed either in the presence (+) or absence (-) of 10% calf serum in the cell culture medium. Cells were treated either with (+) or without (-) TGF-ß1 before lysis.

m: a mixture of equal amount of lysates from cells treated differently with serum and TGF-ß1 (lanes 3 to 6) was used in lanes 2 and 7 for controls.


PNGaseF + + + + + -
Ab - + + + + +
TGF-ß m - + - + m
Serum m - - + + m
97
68
45
TßRII
1 2 3 4 5 6 7

Chapter 6

Conclusions and Prospects

Research presented in this thesis has focused on the biochemical mechanism of the early events in the TGF-ß signal transduction pathway. I have studied the phosphorylation and biochemical interaction of the type I and type II TGF-ß receptors that were transiently overexpressed in COS cells, as well as of the endogenous receptors in TGF-ß responsive cell lines. This has revealed a novel mechanism of activation of TGF-ß receptors distinct from that of most mitogenic growth factor receptors.

I will now summarize my own work and other exciting findings in the TGF-ß field during the past few years with the hope of providing an overview on our current understanding of the mechanisms underlying TGF-ß action. I will also discuss new issues raised from recent advances. I will focus on TGF-ß, just as did my research project; however, what has been learned will most likely apply to other members of the TGF-ß superfamily.

1. Why Does TGF-ß Require Two Receptor Kinases to Signal?

As detailed earlier in Chapter 1, cloning of the receptors for the TGF-ß superfamily members led to the discovery of a novel family of kinases, the transmembrane ser/thr receptor kinases and has laid a foundation for further dissecting the signaling pathways downstream from those receptors. Biochemical and genetic evidence has supported the notion that the signaling complex for TGF-ß is composed of two interdependent subunits, the type I and type II receptors. Similar receptor complexes have also been reported for activin, BMP (Attisano et al., 1993; Carcamo et al., 1995; Letsou et al., 1995; Liu et al., 1995; Penton et al., 1994) and other members of the TGF-ß superfamily.

Both type I and type II receptors for TGF-ß superfamily members are transmembrane proteins with an amino-terminal signal peptide, an extracellular region, a single hydrophobic transmembrane helix, and a cytoplasmic domain having serine/threonine kinase activity. The cloned type I receptors constitute a distinct subfamily

whose kinase domains share 60-70% homology with each other , but are quite divergent from those of the type II receptors (with about 30-40% homology). Also unique to the type I receptors is the presence of a 'GS box' that contains a characteristic series of tandem Ser/Gly residues and other conserved amino acids immediately preceding the kinase domain. In addition, the type I receptors lack a tail sequence on the C-terminus of the kinase domain that can be found in the type II receptors (Kingsley, 1994; Massagué et al., 1994).

Importantly, the extracellular regions of the type I and type II ser/thr kinase receptors feature different structural designs and therefore have different capacities for ligand binding. Aside from the conserved "cysteine box" close to the membrane among all receptors of this class, the distribution of cysteines in the extracellular domains of type II receptors differs depending upon the ligand they recognize and the pattern of extracellular cysteines is likely to be the key in determining ligand binding specificity. On the contrary, all the type I receptors for various ligand have a similar localization of extracellular cysteines (Kingsley, 1994; Massagué et al., 1994). In fact, the type I receptors usually can not bind ligand unless the type II receptors are also present on the cell surface; conversely, the type II receptors are not sufficient to transduce signal although they alone can bind ligand. This dual requirement of both receptor kinases for TGF-ß signaling has been best studied in Mv1Lu cells (Wrana et al., 1992; 1994).

To understand the molecular mechanism underlying the TGF-ß action, it will be beneficial to make a comparison with those better studied signaling pathways triggered by other growth factors. The tyrosine kinase receptors bear a similar structural design to that of the ser/thr kinase receptors. However, their distinct kinase specificity implicates the distinct modes of signaling. Indeed, the tyrosine kinase receptors undergo autophosphorylation on specific tyrosine residues (often located in the C-terminal tail or in kinase insert regions) upon ligand binding; these autophosphorylation sites then mediate interaction with downstream signaling molecules. On the other hand, the type II TGF-ß

receptor is constitutively autophosphorylated independent of ligand binding, and a mutagenesis study has suggested that its C-terminal tail and the kinase inserts are dispensable for TGF-ß signaling (Wieser et al., 1993). Moreover, the tyrosine kinase receptors signal through homodimers or dimers of closely related isoforms (Dougall et al., 1994; Schlessinger and Ullrich, 1992), whereas two distinct TGF-ß receptor kinases form a heterodimeric signaling complex (Wrana et al., 1992).

The ligand-induced homo- or hetero- oligomerization of receptor components is believed to be the key step in the initiation of signal transduction from immune and hematopoietic cytokines including erythropoietin (EPO), granulocyte/macrophage-colony-stimulating factor (GM-CSF), interferon (IFN) and various interleukins (Ihle, 1995; Kishimoto et al., 1994). What distinguishes these other cytokines from TGF-ß is that their receptors do not contain intrinsic kinase activity; instead, they achieve transmembrane signaling through association with other cytoplasmic tyrosine kinases, especially the *Janus* kinases (JAKs). Ligand binding mediates the aggregation of multiple receptor subunits and the concomitant homo- or hetero- oligomerization of JAKs. As a consequence, the JAKs phosphorylate themselves or each other to activate the kinase complex, phosphorylate the associated receptors to create docking sites for the downstream interacting proteins, and phosphorylate the recruited substrates, including the signal transducers and activators of transcription (STATs). The specificity of signaling from individual cytokine is accomplished by a strategy for activating a subset of the multiple receptors, JAKs and STATs (Ihle, 1995; Kishimoto et al., 1994).

In this context, TGF-ß is a unique growth factor, in that its predominant effect at the cellular level is growth-inhibitory, whereas ligands for many tyrosine kinase receptors are mitogenic. TGF-ß is also a unique cytokine which has diverse functions in different cell types through its rather ubiquitously expressed receptors. Perhaps then, the dual requirement for type I and type II receptor kinases reflects the need for a mechanism that can provide the flexibility of delicate regulation. It is conceivable that two interdependent

receptors with distinct extracellular and kinase domains will generate a variety of combination in TGF-ß signals differing both qualitatively and quantitatively. I will discuss this point more explicitly below, referring to complex formation and activation of the TGF-ß receptor kinases, their interaction with downstream substrates, and also the implications for other TGF-ß superfamily members.

2. How Do the TGF-ß Receptors Form Complexes?

Research from different avenues has characterized the interaction among different types of TGF-ß receptors and led to the recognition that the receptor complex formation is a key event in initiating TGF-ß signaling. The first biochemical evidence for the oligomerization of the type I, type II and type III TGF-ß receptors came from the observations that all three receptors could be coimmunoprecipitated upon binding and crosslinking to ^{125}I-TGF-ß on the cell surfaces (Moustakas et al., 1993; Wrana et al., 1992). Based on these results and also on genetic studies in mink lung cells (Mv1Lu), a model was proposed for the ligand-induced heterodimeric complex containing the type I and type II TGF-ß receptors (Wrana et al., 1992). Although the potential role of type III TGF-ß receptor in signaling was appreciated, more detailed analysis of its interaction with the other two receptor kinases is currently lacking.

Work in this thesis demonstrated that the type I TGF-ß receptor (TGF-ßRI) could associate and become phosphorylated by the type II kinase (TGF-ßRII) even in the absence of ligand when cDNAs for both receptors were cotransfected in COS cells (chapter 3), suggesting that the TGF-ßRI and TGF-ßRII could interact in a ligand-independent manner due to their intrinsic affinity to one another. Indeed, the cytoplamic kinase domain of the TGF-ßRII could still phosphorylate the cotransfected TGF-ßRI although it could not bind TGF-ß (chapter 4), providing direct biochemical evidence for the ligand-independent interaction between TGF-ßRI and TGF-ßRII.

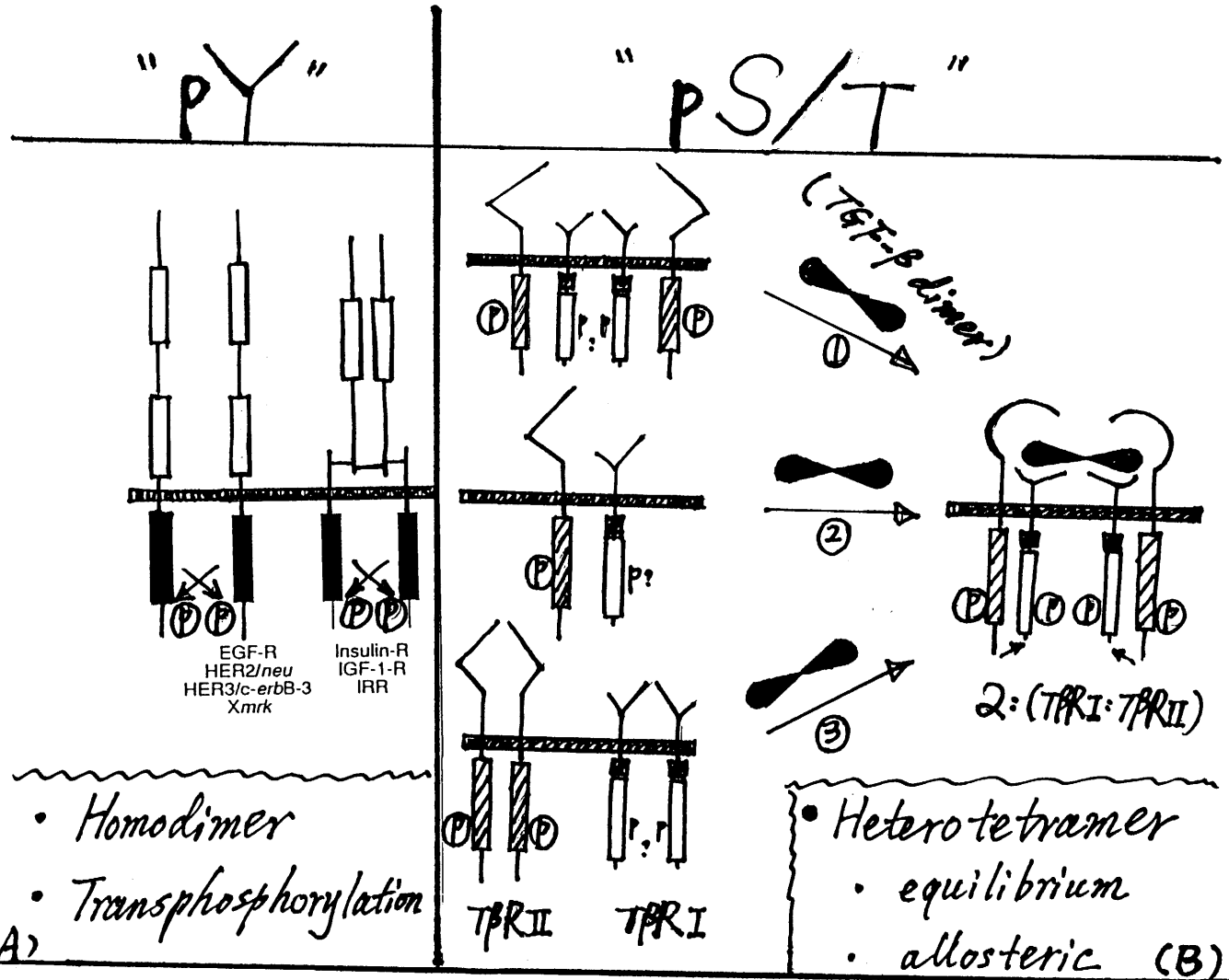


Fig 6.1 Activation of Tyrosine Kinases (A) and Ser/Thr Kinases (B)

Consistent with my work, others have shown that the intracellular domains of the TGF-ßRI and TGF-ßRII could interact in a yeast two-hybrid system, as did their extracellular domains (Perlman, unpublished observation; Chen et al., 1995a). Similar results were also obtained in mammalian cells using transient transfection systems (Chen et al., 1995a). Therefore, the type I and type II TGF-ß receptors may interact through multiple contact points to form the signaling complex.

Taken together, I propose that TGF-ß may not be required to bring two receptor subunits into proximity as stated above in the heterodimeric model (Wrana et al., 1992), and that instead the ligand may stabilize a pre-existing complex consisting of both receptors and induce the conformational changes needed for signaling (Figure 6.1B). This model is based on the fact that the TGF-ß receptors are ubiquitously expressed on the cell surface of many different cell types (Roberts, 1990), and that all the lipid molecules and most integral membrane proteins are laterally mobile in the plasma membrane (Lodish et al., 1995); therefore, the interaction between the TGF-ßRI and TGF-ßRII subunits may be considered to be a dynamic process that reaches a certain equilibrium under different physiological conditions.

In the absence of ligand, the equilibrium between the monomeric subunits of the TGF-ßRI and/or TGF-ßRII and their oligomeric forms is mainly driven by two factors: the affinity of the receptor subunits towards each other and the abundance of individual receptors on the cell surface. Accordingly, there will be at least three different possible compositions of the pre-existing TGF-ß receptor complexes independent of ligand binding as illustrated in Figure 6.1B: homodimeric TGF-ßRI and homodimeric TGF-ßRII (case 3 at the bottom), heterodimeric TGF-ßRI and TGF-ßRII (case 2 in the middle), and heterotetramer in which two molecules each of TGF-ßRI and TGF-ßRII are involved (case 1 on the top). As discussed before in Chapter 4, the homodimer of TGF-ßRI, the homodimer of TGF-ßRII , and the heterodimer of TGF-ßRI/TGF-ßRII have indeed been observed in different experimental systems, while the higher order complexes of these

receptors have also been suggested (Chen and Derynck, 1994; Henis et al., 1994; Yamashita et al., 1994). Interestingly, TGF-ßRI was identified as an interacting protein for the cytoplasmic domain of TGF-ßRII from a yeast two-hybrid screen (Perlman, unpublished data); conversely, the type II receptor for another TGF-ß superfamily member, BMPRII, was identified from another yeast two-hybrid screen using the cytoplasmic domain of TGF-ßRI as a bait (Liu et al., 1995). These results strongly suggest that the cytoplasmic domains of TGF-ßRI and TGF-ßRII have high affinity towards each other and that the hetero-oligomer formation is important for TGF-ß signaling.

In the presence of ligand, it is likely that two molecules of TGF-ßRII in the pre-existing complex will bind the disulfide-linked homodimer of TGF-ß and that the allosteric changes which occur in the receptor complex will then allow the TGF-ßRI to contact its ligand. As a result, a heterotetramer of TGF-ß receptors is stabilized in a favorable configuration (shown on the right part of Figure 6.1B) that will trigger the phosphorylation and activation of their intracellular kinases (as detailed later in next section).

According to this model, the TGF-ß receptor complex formation is distinct from that of the most tyrosine receptor kinases which form homodimers upon ligand binding (Figure 6.1A) (Schlessinger and Ullrich, 1992). However, the existence of hybrid complexes between structurally very similar receptors has also been demonstrated, such as alpha and beta type PDGF receptors, EGF receptor and HER2/neu, or receptors for insulin (InsR) and insulin-like growth factor 1 (IGF-1R) (Figure 6.1A, Hammacher et al., 1989; Qian et al., 1994; Soos and Siddle, 1989). In the last example, the InsR and IGF-1R each contain an extracellular α subunit and transmembrane ß subunit which are disulfide-linked to form "half receptors"; the two αß halves are also stabilized by a disulfide bridge. In this case, ligand binding induces allosteric interaction of two αß halves that are already covalently associated (Figure 6.1A, Riedel et al., 1989).

The stoichiometry of the TGF-ß type I and type II receptor complex is still an open question and the final determination may have to wait for the resolution of the crystal structure of both receptors. However, I favor the heterotetramer model for the formation of oligomeric receptor complexes (Figure 6.1B) because this is consistent with all the data reported to date.

(1) The crystal structure of the TGF-ß2 has revealed that the biologically active ligand is a homodimer (Daopin et al., 1993; Schlunegger and Grutter, 1992).

(2) While TGF-ßRII binds to ligand by itself, TGF-ßRI contacts ligand only when TGF-ßRII is also present in the same cell; but importantly, both receptors can then bind and be crosslinked to TGF-ß (Bassing et al., 1994; Franzen et al., 1993; Wrana et al., 1992).

(3) Genetic studies on TGF-ß receptor-defective cell lines have demonstrated that both the type I and type II receptors are essential for TGF-ß signaling (Laiho et al., 1990; Wrana et al., 1992).

(4) Biochemical evidence for the existence of both homodimer and heterodimer of TGF-ßRI and TGF-ßRII has been derived from several different approaches, including chemical binding and crosslinking of surface receptors to ^{125}I-TGF-ß, coimmuno-precipitation from metabolically labeled cells, antibody-mediated co-patching of surface receptors with differential epitope-tags, and *in vivo* transphosphorylation of the TGF-ßRI by TGF-ßRII (Chen and Weinberg, 1995; Chen and Derynck, 1994; Henis et al., 1994; Wrana et al., 1994; Yamashita et al., 1994).

(5) The most appealing evidence for the functional requirement of the TGF-ßRI homodimer comes from a complementation study on different TGF-ßRI mutant receptors in chemically-mutagenized Mv1Lu cells (Weis-Garcia and Massagué, 1996). Two groups of mutant TGF-ßRI were characterized: one mutation disrupted the kinase activity while the other mutation prevented ligand-induced TGF-ßRI transphosphorylation by TGF-ßRII and thus activation. Neither the kinase-defective TGF-ßRI nor the activation-defective

TGF-ßRI had signaling ability. However, when co-transfected in a mutant Mv1Lu cell line (R-1B), the two classes of mutant TGF-ßRI could restore both anti-proliferative and transcriptional responsiveness to TGF-ß, strongly suggesting that ligand-induced receptor complex contained two or more TGF-ßRI molecules (Weis-Garcia and Massagué, 1996).

Although it remains to be discovered how many TGF-ß ligand molecules are in the receptor complex, the fact that more than one molecule of each of multiple receptor subunits is found favors the existence of a ligand-independent pre-complex. Ligand binding is unlikely to bring together all four components of the heterotetramer, but rather it may stabilize this otherwise transient complex or induce specific conformational changes necessary for the activation. In this model, it is conceivable that various combinations of the type I and type II receptors with distantly related extracellular and intracellular domains can generate different specificity for ligand binding and substrate interaction that is in accord with the diverse function of TGF-ß superfamily members.

Another important issue to be further explored is the involvement of different domains of the TGF-ß receptors in signaling complex formation. To date, the functional assignment of individual domains of the TGF-ß type I and type II receptors has mainly come from studies of dominant-negative receptors generated by deleting their cytoplasmic regions and of chimeric receptors constructed by switching their extracellular and intracellular regions (Brand and Schneider, 1995; Chen et al., 1993; Okadome et al., 1994; Vivien et al., 1995; Wieser et al., 1993). A truncated TGF-ßRII lacking the intracellular domain has been shown to have dominant-negative effects on both growth inhibition and transcriptional activation in mink lung epithelial cells and in cardiac myocytes (Brand and Schneider, 1995; Wieser et al., 1993). On the other hand, contradictory results have been reported in mink lung cells where only the anti-proliferative pathway but not the early gene activation pathway is impaired by this truncated TGF-ßRII (Chen et al., 1993). A similar dominant-negative mutant type I TGF-ß receptor has also been described to impair the

TGF-ß-dependent transcription activation although less completely than the type II mutant receptor (Brand and Schneider, 1995).

Interestingly, when the extracellular domain of TGF-ßRII was fused to the transmembrane and cytoplasmic domain of TGF-ßRI, it yielded TGF-ßRII/I that was shown to act in a dominant-negative fashion against both the wild-type TGF-ßRII and TGF-ßRI (Vivien et al., 1995): it abolished the restoration of TGF-ß-dependent growth inhibition and transcriptional activation by a co-transfected TGF-ßRII in TGF-ßRII-defective cell line (Mv1Lu, DR clone) or by a co-transfected TGF-ßRI in TGF-ßRI-defective cell line (Mv1Lu, R-1B clone). The reciprocal chimeric receptor TGF-ßRI/II constructed by fusing the extracellular domain of TGF-ßRI to the transmembrane and cytoplasmic domain of TGF-ßRII behaved similarly. Neither TGF-ßRII/I when co-transfected with TGF-ßRI nor TGF-ßRI/II when co-transfected with TGF-ßRII could restore the TGF-ß responsiveness in TGF-ß receptor-defective cell lines (Vivien et al., 1995).

It has also been reported that another set of chimeras including "TGF-ßR1.2" containing the extracellular and transmembrane domains of TGF-ßRI fused to the cytoplasmic domain of TGF-ßRII and its reciprocal partner "TGF-ßR2.1" (Okadome et al., 1994). Similarly, neither "TGF-ßR1.2" when co-transfected with TGF-ßRII nor "TGF-ßR2.1" when co-transfected with TGF-ßRI could restore the TGF-ß responsiveness in TGF-ß receptor-defective cell lines. Surprisingly, however, when "TGF-ßR1.2" and "TGF-ßR2.1" were co-expressed in mutant Mv1Lu (R-1B), they could restore the TGF-ß responsiveness (Okadome et al., 1994). I note that fusion of the latter set of chimeras were made intracellularly at the transmembrane and cytoplasmic junction while the former ones were fused at the extracellular and transmembrane junction. Nevertheless, they both raised important issues regarding the stoichiometry of the TGF-ßRI and TGF-ßRII complex and the possible distinct roles of the homodimers and the heterodimers of these receptors.

To understand further the mechanism of TGF-ß receptor complex formation, much effort is needed to dissect the detailed interaction among all components involved (TGF-ß, TGF-ßRI and TGF-ßRII), and crystallography and mutagenesis should provide valuable information. It is also unclear whether the homodimers of TGF-ß receptors are able to signal and what unique functions each type of the TGF-ß receptors can be assigned.

Attempts to answer these questions have been complicated in two ways: the lack of assays for diverse TGF-ß action and the lack of cellular systems with only individual TGF-ß receptors expressed. Therefore, although the homodimers of TGF-ßRI or TGF-ßRII were scored negative for growth inhibition and transcriptional activation in Mv1Lu cells, they might function in generating other TGF-ß responses, assays for which are not yet available (Okadome et al., 1994; Vivien et al., 1995).

3. How Do the TGF-ß Receptor Kinases Become Activated?

As discussed above, members of the TGF-ß superfamily signal through the complexes of two distantly related transmembrane serine/threonine kinases in different combinations. Binding and crosslinking experiments using radiolabeled ligands have yielded data on the ligand binding specificity of different receptor subunits. With this assay, each type II receptor can interact with more than one type I receptor and support their binding to given factors; for example, ActR-II can complex with both ActR-I (also called R1, ALK2, SKR1 or Tsk7L depending on the original cloning) and ActR-IB (also called R2 or ALK4) (Massagué et al., 1994). Conversely, some type I receptors can interact with several type II receptors; for instance, TSR-I (also called R3 or ALK1) has been shown to complex wtih TGF-ßRII in TGF-ß binding or with ActR-II in activin binding (Attisano et al., 1993).

It has been acknowledged that not all the receptor complexes observed in the binding and crosslinking experiments can actually mediate biological responses in the

available functional assays. Indeed, the complex of TGF-ßRI and TGF-ßRII could mediate growth inhibition and transcriptional activation in response to TGF-ß, and so could the complex of ActR-I and Act-RII in response to activin. However, TSR-I could not transduce these signals in concert with TGF-ßRII nor Act-RII (Carcamo et al., 1994). In the light of these observations, a "perfect match" of two receptor subunits seems to be required to generate a productive signaling complex for a given ligand. Hence, at least two other levels of regulation should exist in addition to their capacities to bind ligands: (1) whether the kinase activity of the receptor complex can be triggered by ligand binding; and (2) whether the activated receptor kinase(s) can then interact with the specific downstream substrate(s) necessary for certain biological response. I will focus on the activation of the receptor complexes in the rest of this section.

It appears to be a universal phenomenon among growth factors and cytokines that ligand-induced receptor oligomerization results in elevated protein tyrosine kinase activity and therefore permits the transmission of the extracellular signals across the cytoplasmic membrane barrier. The single transmembrane receptors with an intracellular tyrosine kinase domain form homodimers or hybrid complexes between structurally very similar receptors upon ligand binding. Two kinase subunits transphosphorylate one another on specific tyrosine residues which can then serve as docking sites for downstream interacting proteins; meanwhile, the dimerized receptors possess increased kinase activity to catalyze the phosphorylation of exogenous substrates (Figure 6.1A, Schlessinger and Ullrich, 1992). As mentioned earlier, cytokine receptors involved in lymphoid and myeloid cell function are lacking their own catalytic activity. Instead, aggregation of various members of the cytokine receptor superfamily upon ligand binding leads to their association with and subsequent activation of other cytoplasmic tyrosine kinases, especially those in the JAK family (Ihle, 1995; Kishimoto et al., 1994).

As yet another unique variation on the common theme of receptor dimerization and activation, I propose here a model for the activation of TGF-ß receptor kinases, which will

presumably extend to the ser/thr kinase receptors for the other TGF-ß superfamily members. First of all, it is worth emphasizing that TGF-ßRI and TGF-ßRII can interact with one another via multiple contact regions independent of ligand binding (Chapter 3 and 4). These observations have demonstrated the intrinsic affinity of these two receptors and suggest that TGF-ß most likely acts through introducing allosteric interaction among different receptor subunits in the pre-existing complex (Figure 6.1B).

One distinct feature regarding to the phosphorylation and activation of TGF-ß receptor kinases is that TGF-ßRII remains constitutively phosphorylated not only when overexpressed in COS cells (Chapter 2) but also when endogenously expressed in TGF-ß responsive cells such as HaCat and HER14 (Chapter 5). Most if not all of the observed ligand-independent phosphorylation of TGF-ßRII depends on its own kinase activity; and furthermore, this autophosphorylation does not appear to operate in a trans-manner as does that of the tyrosine kinase receptors but is rather via an intramolecular mechanism (Chapter 2). Therefore, TGF-ßRII presumably stays in a phosphorylated form even in the pre-existing receptor complex prior to ligand binding (Figure 6.1B)

Another unique aspect is the asymmetric pattern of the interaction between TGF-ßRI and TGF-ßRII, meaning that TGF-ßRII can transphosphorylate TGF-ßRI and presumably liberate its kinase activity, but TGF-ßRI does not appear to affect the phosphorylation of TGF-ßRII. This suggests that TGF-ßRI is the immediate downstream substrate of TGF-ßRII, but the reciprocal mechanism does not seem to operate (Chapter 3 and Figure 6.1B). Consistent with this notion are the previous reports demonstrating that TGF-ßRI could not bind ligand when expressed alone on the cell surface as did the TGF-ßRII (Attisano et al., 1993; Franzen et al., 1993). In addition, it has been shown that TGF-ßRI and ActR-IB with closely related kinase domains (90% amino acid sequence identity) could transduce similar growth-inhibitory and transcriptional responses to TGF-ß and activin although their type II receptor partners have very different kinase domains (42% amino acid sequence identity) (Carcamo et al., 1994). Furthermore, a mutant TGF-ßRI has

been generated that could mediate growth-inhibitory and transcriptional responses in the absence of ligand and TGF-ßRII (Wieser et al., 1995).

While the constitutive autophosphorylation of TGF-ßRII is a well-established phenomenon as discussed above, the nature of TGF-ßRI autophosphorylation remains to be further explored. In the model illustrated in Figure 6.1B, I indicate that it is not yet clear whether the TGF-ßRI is normally autophosphorylated in the absence of ligand binding. However, if it is assumed that the TGF-ßRI autophosphorylates itself via a trans-mechanism which is common for most of the receptor kinases, it is unlikely to be autophosphorylated due to its low abundance on the cell surface and relatively low affinity to form homodimers. Only when the ligand and TGF-ßRII are present, the two TGF-ßRI molecules in the heterotetramer complex can undergo certain allosteric changes which then lead to their transphosphorylation one another. This in turn may activate the kinase activity of TGF-ßRI towards downstream substrates or create docking sites to recruit cytoplasmic interacting proteins.

Obviously, the first question to be answered is whether the autophosphorylation of TGF-ßRI does occur in cells. Work in this thesis using COS cells has shown that TGF-ßRI is autophosphorylated when overexpression is achieved (Chapter 3, Chen and Weinberg, 1995). Similar results have been obtained when TGF-ßRI was reconstituted in baculovirus (Ventura et al., 1994) and when the kinase domain of TGF-ßRI was expressed and purified as recombinant proteins from bacteria (Bassing et al., 1994). In contrast however, the basal level of phosphorylation of TGF-ßRI was reported to be very low when expressed alone in Mv1Lu R-1B cells (Wrana et al., 1994). This discrepancy can be explained in at least two aspects: (1) The different cell systems used in these experiments may result in different level expression of TGF-ßRI and therefore its different phosphorylation pattern. (2) The latter studies (Wrana et al., 1994) depended upon a two-step precipitation protocol that would analyze only a fraction of receptor proteins purified as

detergent-resistant heteromeric complexes induced by the ligand. Indeed, it is possible that the ligand-independent phosphorylation of TGF-ßRI proteins could not be scored.

In the case that TGF-ßRI autophosphorylation does occur, its trans- or cis-mechanism can be analyzed using the approach described in Chapter 2 for studying TGF-ßRII autophosphorylation. Interestingly, the autophosphorylation of TGF-ßRI is apparently not required for its association with TGF-ßRII or its transphosphorylation by TGF-ßRII since both the wild-type and kinase-deficient TGF-ßRI could serve as substrates for TGF-ßRII (Chapter 3; Chen and Weinberg, 1995; Wrana et al., 1994). Perhaps then the autophosphorylation of TGF-ßRI contributes to the activation of TGF-ß receptor complex by activating its own kinase activity towards exogenous substrates or creating specific sites for intracellular targets to bind. Indeed, the level of TGF-ßRI autophosphorylation correlates with its ability to phosphorylate casein *in vitro* (see Appendix); the constitutively active TGF-ßRI mutant was also shown to have elevated *in vitro* kinase activity (Wieser et al., 1995).

However, it is worth pointing out that the autophosphorylaiton of TGF-ßRI is not sufficient for TGF-ß signaling; its transphosphorylation by TGF-ßRII is also essential for TGF-ß transduction. The most appealing evidence in supporting this notion is that mutations harbored by TGF-ßRI (G322D) preventing its ligand-induced phosphorylation (and thus activation) by TGF-ßRII could not mediate TGF-ß signal even if the mutant TGF-ßRI was more active than the wild-type TGF-ßRI when the *in vitro* kinase activity was assayed. Interestingly, this activation-deficient TGF-ßRI mutant can be functionally complemented by a kinase-deficient TGF-ßRI, indicating that two or more TGF-ßRI molecules may be presented in the ligand-induced receptor complex and that they work in trans to cooperate with one another in interacting with TGF-ßRII and trigger the TGF-ß signaling pathway (Weis-Garcia and Massagué, 1996).

My own work and many other recent findings in the field have laid the groundwork for understanding the mechanism underlying the activation process of the TGF-ß receptor kinases, but they have also raised many interesting questions for future study.

(1) Assays for the kinase activity of TGF-ß receptors toward exogenous substrates.

It has been proposed that the transphosphorylation of the TGF-ßRI by the TGF-ßRII kinase upon their association induced either by ligand binding or overexpression may allow one or both of the associated kinases to direct their activities toward downstream substrates. However, there is no direct biochemical evidence in supporting this model due to the lack of identified *in vivo* substrates, as will be detailed below.

An initial effort was made in screening a variety of recombinant proteins available which may serve as putative *in vitro* substrates for the quantitative measurement of the catalytic activity of TGF-ß receptors and casein was among the most promising candidates (Chapter 9). Others have developed combinatorial peptide libraries to determine the specificity of these kinases and shown that both the TGF-ßRI and TGF-ßRII share the optimal substrate KKKKKK(S/T)XXX; here, K stands for lysine, S/T for either serine or threonine, and X for one of any genetically encoded amino acids except serine and threonine (Luo et al., 1995). To date , there is still an immediate need to develop sensitive assays for the presumably variable kinase activity of the TGF-ß receptors in the different oligomerization and phosphorylation states.

(2). Analysis of critical phosphorylation sites on TGF-ß receptors.

Both the TGF-ßRI and TGF-ßRII have multiple phosphorylation sites on their intracellular regions and their biological function are largely unassigned (Chapters 2 and 3; Wrana et al., 1994). Mutagenesis analysis has focused on the GS domain which was shown to be phosphorylated by TGF-ßRII; and it has been suggested that the signal transduction by TGF-ßRI is not affected by mutation of any particular serine or threonine residue, but rather depends on the number of these two types of residue remaining intact in this region (Franzen et al., 1995; Wieser et al., 1995). On the other hand, there is evidence

for other region(s) of the TGF-ßRI to be the critical transphosphorylation sites by the type II kinase instead of or in addition to the GS domain (K. Luo, personal communication). Therefore, it is still not clear at present whether any of the phosphorylation site of TGF-ßRI will have a specific effect on its signaling capacity by providing a docking site for a particular downstream substrate.

The detailed characterization and functional analysis of the phosphorylation sites on the TGF-ßRII have not yet been reported. It is interesting that TGF-ßRII contains two kinase inserts and a serine/threonine rich C-terminal tail and that the corresponding regions in most tyrosine kinase receptors have been shown to harbor the phosphorylation sites important for signaling. However, mutant TGF-ßRII with deletion of these putative phosphorylation site-containing regions did not abolish its signaling ability (Wieser et al., 1993). It is still unknown which phosphorylation site(s) of TGF-ßRII may be responsible for specific interaction with downstream substrates and which for regulating its kinase activity. One potential complication on this issue is that these two functions may be difficult to separate if the autophosphorylation of TGF-ßRII is indeed in a cis- manner (Chapter 2). Further understanding on this subject will greatly benefit from site-directed mutagenesis and crystallography studies.

(3) Crosstalk between TGF-ß receptors and other growth factor receptor kinases.

As discussed earlier (Chapter 4), TGF-ß displays synergistic effects with EGF and some other growth factors depending on the cells used for study. It has been suggested that TGF-ß may regulate the phosphorylation of EGFR in A431 epidermoid carcinoma cell line (Goldkorn and Mendelsohn, 1992). However, it is not clear whether the phosphorylation and/or the activity of TGF-ß receptor kinases can also be modulated by EGFR and other tyrosine kinases involved in growth factor and cytokine signaling. An excellent example of such kind is the recent report on the crosstalk between receptors for stem-cell factor (SCF) and erythropoietin (EPO), two cytokines working in concert to regulate the proliferation and maturation of colony-forming unit erythroid (CFU-Es)

(Wu et al., 1995).

(4) Down-regulation of the TGF-ß receptor kinases.

While much effort has been directed to investigate the activation of TGF-ß receptor kinases, little is known about the other side of the coin: how is their signaling capacity being down-regulated? Only when the positive and negative modulation reach a balance (qualitatively and quantitatively), can the delicate TGF-ß action take place. There are at least three potential ways to down-modulate the activity of TGF-ß receptors: first, by affecting biosynthesis, internalization, and degradation; second, through dephosphorylation of the TGF-ß receptor kinases, just as that of the cytokine receptors by hematopoietic cell phosphatase (HCP, or PTP-1C, or SH2-PTP1) (Ihle, 1995); or third, via specific phosphorylation on the TGF-ß receptors, like the negative regulation of EGFR by protein kinase C (PKC) (Davis and Czech, 1985; Hunter et al., 1984).

4. What Are the Downstream Targets for the TGF-ß Receptor Kinases?

While the diverse biological functions of TGF-ß have been intensively described over the past decade, the signaling pathways that underlie these effects only started to come to light upon the molecular identification of its receptors (Franzen et al., 1993; He et al., 1993; Lin et al., 1992; Wang et al., 1991). Since this identification, the search for the downstream targets for the TGF-ß receptors has been the main focus of the field. Although the signaling cascades that mediate TGF-ß action have not yet been thoroughly characterized , some interesting pieces of these chains have been discovered. I will now summarize four areas in which rapid progress have been made in the recent years.

(1) Cell cycle studies.

A real breakthrough in understanding the growth-inhibitory effect of TGF-ß took place when the cell cycle machinery was recognized as the ultimate target of this anti-mitogen. As I have detailed earlier (Chapter 1), TGF-ß has utilized at least two different

strategies to down-regulate the cyclin-dependent kinase (cdk) activities that are essential for the growth suppression function of pRB (Massagué and Polyak, 1995; Weinberg, 1995). It has been shown that TGF-ß can negatively modulate the expression of certain G1 cyclins and CDKs in some cells (Ewen et al., 1993; 1995; Geng and Weinberg, 1993; Slingerland et al., 1994). More importantly, a novel family of CDK inhibitors (CDIs) has been found to mediate TGF-ß signaling: p15 and p21 could be rapidly induced at the transcriptional level in HaCat cells upon TGF-ß treatment (Datto et al., 1995a; Hannon and Beach, 1994), while p27 might be regulated at a posttranslational level (Ewen et al., 1993; 1995; Polyak et al., 1994a; 1994b). Other genes whose transcription can also be regulated by TGF-ß include c-myc and those encoding extracellular matrix proteins. However, the biochemical design to transduce the TGF-ß signal from the cytoplasmic membrane to the nucleus is still unclear at present and is attracting much effort.

(2). Yeast genetics.

The yeast two-hybrid system has led to the recent discovery of a few potential downstream interacting proteins for the type I and type II TGF-ß receptors. The first report was on the isolation of the immunophilin FKBP-12, an intracellular target of rapamycin and FK506, as a specific interactor with TGF-ßRI (or R4) and other type I receptors for TGF-ß superfamily (Wang et al., 1994). FKBP-12 shares the same binding sites with TGF-ßRI and with the macrolide FK506 and has been proposed to negatively regulate TGF-ßRI-mediated signaling (Wang et al., 1996). Future study is still needed to understand the physiological roles of FKBP12 in TGF-ß action.

Another group of interacting proteins identified from this screen are two variants of $p21^{RAS}$ farnesyltransferase α subunit (FNTA), an enzyme playing a critical role in the activation of RAS by establishing its association with the cell membrane (Kawabata et al., 1995; Wang et al., 1996). In contrast to FKBP12, FNTA only specifically interact with the TGF-ßRI and ActRI and interestingly, FNTA was released from the TGF-ßRI which was transphosphorylated by the TGF-ßRII in a ligand-induced complex (Wang et al.,

1996). Apparently there is much to be learned about this novel mechanism of interaction between FNTA and the TGF-ßRI under different phosphorylation states; and the potential roles of TGF-ß in regulating RAS pathway are worth further investigation.

A WD-domain-containing protein has been identified as a specific substrate of the TGF-ßRII from yeast two-hybrid screen and named TRIP-1 (for TGF-ß-receptor interacting protein-1) (Chen et al., 1995b). It could be phosphorylated *in vitro* on serine and threonine by the type II TGF-ß receptor kinase, but did not interact with the type II activin receptor nor the type I receptors. WD proteins are built of highly conserved repeated modules usually ending with Trp-Asp (WD) (Neer et al., 1994). Since WD domains are implicated in multiple protein interaction, it has been speculated that TRIP-1 may function as a "GRB-2 type" adapter to introduce conformational changes necessary for the recruitment of downstream proteins (Chen et al., 1995b).

Based on a mitogen-activated protein kinase (MAPK) pathway in yeast, TAK1 (designated for TGF-ß-activated kinase) was identified from a genetic complementation screen as a distinct member of the MAPKKK family. TAK1 was shown to be involved in the transcriptional regulation by TGF-ß and its kinase activity was enhanced in response to TGF-ß and BMP (Yamaguchi et al., 1995). However, the biochemical evidence for its association with TGF-ß receptors is currently lacking. Therefore, it may act as a signaling mediator in the kinase cascade without direct interaction with the receptors.

(3). Signal transduction.

In searching for the downstream targets of TGF-ß receptors, efforts have also been directed to re-examine the well-characterized signaling molecules such as those in the RAS and MAPK pathway. To date, it has been reported that $p21^{ras}$ can be rapidly activated by TGF-ß (Atfi et al., 1994; Mulder and Morris, 1992); so can several MAPK (Yan et al., 1994) and PKC-δ (Saltis and Bobik, 1996). In addition, a 78-kDa protein serine/threonine kinase was shown to be activated by TGF-ß in an in-gel kinase assay and its molecular identity is unclear at present (Atfi et al., 1995). It is worth mentioning that results from

these studies have been highly cell-type-dependent and somewhat contradictory depending on the research groups. I also note that there has not been any reported candidate interacting proteins for the TGF-ß receptors which were identified by applying biochemical methods similar to those used for tyrosine kinase receptors such as EGFR (Schlessinger and Ullrich, 1992). In addtion to the possible techanical difficultities due to their low expression, this may also reflect the unique nature of the TGF-ß receptor kinases and perhaps the intrinsic different mechanisms of phosphorylation and interaction for ser/thr kinase receptors and tyrosine kinase receptors.

(4). TGF-ß superfamily.

Members of the TGF-ß superfamily have been found in many distantly related organisms and shown to be involved in diverse biological processes (Kingsley, 1994). It is intriguing that a highly conserved protein family (called dwarfins) has been suggested from genetic studies of three different systems as the universal components required for TGF-ß-like signaling. However, the cellular function of dwarfins is currently unclear since their predicted polypeptides lack known protein motifs (Savage et al., 1996).

The first member of the dwarfins family was identified in *Drosophila melanogaster* as a gene whose mutation enhanced the mutant phenotype of *decapentaplegic (dpp)* that belongs to the TGF-ß superfamily; hence it was named *Mothers against dpp (Mad)* (Raftery et al., 1995; Sekelsky et al., 1995). Subsequently, three genes (*sma-2, sma-3* and *sma-4*) were characterized in *C. elegans* based on the fact that they all display mutant phenotypes similar to those of *daf-4* , a gene encoding the type II receptor kinase for BMP in the TGF-ß superfamily. Multiple dwarfins were soon identified from mice and human indicating that this novel protein family universally exist in both invertebrates and vertebrates (Savage et al., 1996).

More importantly, recent search for candidate tumor suppressor genes on human chromosome 18q has led to the discovery of DPC4 (designated for homozygously deleted in pancreatic carcinoma, locus 4), a gene that is lost or inactivated in 50% of pancreatic

tumors and that shows sequence similarity to the members of dwarfins family (Hahn et al., 1996). These findings strongly suggested that DPC4 is involved in TGF-ß growth-inhibitory pathway and therefore is a candidate tumor suppressor gene.

5. What Regulates the Diversified Functions of TGF-ß-Related Factors?

Members of the TGF-ß superfamily regulate many key events in the growth and development of a wide range of different organisms. They not only share the conserved structural features, but also appear to employ a similar mechanistic design to exhibit their diverse biological functions. For example, the mammalian bone morphogenetic proteins 4 (BMP4) is about 75% identical to the *Drosophila dpp* gene and they are functionally exchangeable, in that human BMP4 sequences can rescue the *dpp* mutant phenotype in fly (Padgett et al., 1993) and conversely, purified DPP protein can induce bone and cartilage in mammals (Sampath et al., 1993).

This structural and functional similarity can also be extended to receptors for the TGF-ß family. In *Xenopus* embryos, activin can induce mesoderm formation in the animal cap assay while TGF-ß1 can not, due to the possible lack of TGF-ß receptors. However, when mRNA encoding the human TGF-ßRII (having 50% homology with ActRII) was injected into the *Xenopuss* embryos, the exogenously added TGF-ß1 could then show mesoderm induction in the animal cap assay just as did activin (Bhushan et al., 1994).

At the cellular level, closely related type I receptors for the TGF-ß superfamily members could signal a common set of growth-inhibitory and transcriptional responses in concert with their corresponding type II receptors and ligands. For instance, in Mv1Lu epithelial cells, TGF-ßRI and TGF-ßRII could mediate elevated expression of extracellular matrix proteins in response to TGF-ß and so did ActRIB and ActRII in response to activin (Carcamo et al., 1994); the same was true for the BMP receptors (Liu et al., 1995).

Taken together, these findings have suggested that members of the TGF-ß superfamily in distantly related organisms indeed share similar downstream targets for their diverse activities. If true, this will certainly facilitate the studies in dissecting their signaling pathways since information from different experimental systems can be integrated into a general theme. On the other hand, a specific biological system will be essential to define the *in vivo* function of a given ligand or receptor.

The striking nature of members in the TGF-ß superfamily is that they can trigger distinct physiological responses depending on the cell type and cell context. Then a real issue will be how the TGF-ß-like factors display vastly divergent biological activities if they appear to utilize similar downstream targets as I discussed above. It is clear that such actions will have to be delicately regulated.

The first level at which such a regulation can be exercised is the ligand accessibility, and a number of ways have been employed by the TGF-ß superfamily. (1) Within the superfamily, there are factors which are more closely related to one another and clustered to form a subfamily. For example, TGF-ß subfamily includes at least four isoforms in mammals, TGF-ß1, -ß2, -ß3 and -ß5. While all these isoforms appear to behave similarly *in vitro*, they play distinct roles *in vivo* (Guenard et al., 1995; Proetzel et al., 1995; Shull and Doetschman, 1994). This may partly be explained by the differential modulation of their expression via specific *cis*-acting regulatory elements of these genes. In *Drosophila*, the different functions of *dpp* in various tissues during development have been mapped to separate control regions of the gene (Huang et al., 1993; Hursh et al., 1993; Masucci et al., 1990). (2) All ligands in the TGF-ß family are synthesized as latent precursor proteins and the subsequent cleavage process is highly regulated to give rise to the bioactive mature forms (Miyazono et al., 1993). (3) Other accessory molecules, either soluble proteins like follistatin or transmembrane polypeptides such as TGF-ßRIII and endoglin also modulate the binding of TGF-ß-like factors to their signaling receptors. Together, gradients of the

activity of these factors may be generated for a particular cell type or at a specific developmental stage.

The second level of regulation focuses on the receptors and their combinatorial features define the ability of cells to respond to given factors in the TGF-ß family. (1) For an individual ligand, the type I and type II receptors can be further specified by alternative splicing as reported for activin. In mouse, one of the type II activin receptors, ActR-IIB, has four isoforms that arise from alternative splicing and show different binding affinities for the ligand (Attisano et al., 1992). (2) The expression of receptor subunits for the TGF-ß family is controlled in a different manner. While TGF-ß receptors exist ubiquitously in many cell types, receptors for some other factors such as MIS are narrowly expressed (Baarends et al., 1994; He et al., 1993). (3) As I have mentioned before, the specificity of ligand binding and downstream signaling will undoubtedly attribute to the combinatorial interactions of multiple receptor subunits with different molecular identities at different ratios.

Yet another level of regulation will have to rely on the diversity of downstream substrates in the signaling pathways initiated by receptors of the TGF-ß family members, which we have just begun to understand. Nevertheless, the signaling specificity and strength of receptors for the TGF-ß superfamily members, in conjunction with the gradients of ligand activity and different concentration thresholds for cellular responses will lead to the delicately modulated translation of these quantitatively variable biochemical signals into qualitatively distinct physiological responses.

Summary

Rapid progresses have been made in this fascinating field over the past few years, especially in the identification of receptors for various members of the TGF-ß superfamily. However, we still do not know the mode of signaling of the dual kinases TGF-ßRI and TGF-ßRII: do they phosphorylate two different substrates; or do they phosphorylate one common substrate yet on two (or more) separate sites; or do they actually work in a linear order such that TGF-ßRII phosphorylates the TGF-ßRI and TGF-ßRI in turn phosphorylates the downstream substrate(s)?

Neither can we predict what kinds of downstream interacting proteins are mediating the action of TGF-ß receptors: do they serve as the adapter-type protein docking units just like the "SH2/SH3 domains" utilized by tyrosine kinase signaling; or do they sit in the kinase cascades similar to those constructed in the MAPK pathway; or do they translocate into the nucleus as transcription factors "mimicking" STATs in the cytokine signaling?

We can not even speculate on the logic basis for TGF-ß signaling: does ligand binding induce the intrinsic activity of the receptor kinases; or does it simply liberate the receptor kinases by triggering release of the "inhibitors" which suppress their activity; in another words, is the default state of TGF-ß receptor kinases OFF or ON?

These questions are expected to be better understood in the near future with our realization that similar interacting molecules and pathways appear to underlie the diverse biological activities of the TGF-ß superfamily members in a wide range of organisms. The powerful combination of various tools in genetics, biochemistry and developmental biology will lead to new advances in this remarkable field and these in turn should greatly enrich our knowledge on many key issues in cell signaling and organism development.

Chapter 7

Materials and Methods

Construction of Expression Vectors

The human TGF-ßRII cDNA (H2-3FF clone) was subcloned into expression vector Rc/CMV (Invitrogen, San Diego, CA). The tag sequence (YPYDVPDYA) encoding the influenza virus hemagglutinin (HA) was inserted between codons His27 and Pro26 in the open reading frame encoding TGF-ßRII. The human TGF-ßRI cDNA (ALK-5 clone) was subcloned into expression vector CMV7 and a HA-tag was attached at the its C-terminus using a PCR-based strategy.

The kinase-deficient mutant receptors, TGF-ßRIIK277R and TGF-ßRIK232R, were generated by site-directed mutagenesis using PCR-based strategies. All the coding regions of TGF-ßRI and TGF-ßRII manipulated through PCR were verified by DNA sequencing.

pGEX-RIICt encoding a GST fusion protein containing the intracellular domain of TGF-ßRII has been described previously. pET-RIICt encoding a pET fusion protein containing the same intracellular portion of TGF-ßRII was generated using the vector pET-5 (Novagen, Madison, WI).

Other constructs were made using similar strategies and are described in the "RESULTS" sections of individual chapters.

Cell Culture and Transfections

COS1 cells (CRL 1650; American Type Culture Collection, Rockville, MD) were propagated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% CS, 100U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL, Gaithersburg, MD). They were transiently transfected with the expression vectors described above using a DEAE-dextran method (Warren and Shields, 1984). 36-48 hours after transfection, cells were metabolically labeled as indicated.

Generation of Monoclonal Antibodies

GST fusion protein containing TGF-ßRIICt was purified from *E. coli* as described by (Smith and Johnson, 1988) and subsequently used to immunize BALB/c mice. pET fusion protein consisting the same portion of the TGF-ßRIICt was purified from inclusion bodies and applied in western blotting to test tail bleeds. Hybridomas were made using standard techniques and screened by ELISA.

Immunoprecipitaton and Deglycosylation

Monoclonal antibodies, 5c (α RII) decribed above and 12CA5 (α HA) purchased from BAbCO (Berkeley, CA) were used for immunoprecipitation of TGF-ß receptors. For ^{35}S-labeling, the transfected COS1 cells were labeled with 100 µCi/ml ^{35}S-methionine at 37 ^{o}C for 4 hrs. At the end of the labeling, cells were treated with or without TGF-ß1 at 400 pM for the indicated time and lysed in 1 ml ice-cold buffer A (50 mM Tris-Cl (pH = 7.4), 150 mM NaCl, 1% NP-40, 0.5% DOC and 0.1% SDS, 10 µg/ml aprotinin and leupeptin, 1 mM PMSF, 1 µg/ml pepstatin A, 10 µg/ml soybean trypsin inhibitor, 1 mM DTT, and 0.25% BSA). Lysates were precleared and incubated with appropriate antibodies at 4 ^{o}C for 2 hrs. Immune-complexes were then recovered on protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) which had been saturated with rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc), and subjected to 7.5 % SDS-PAGE and autoradiography. For ^{32}P-labeling, the transfected COS1 cells were incubated in phosphate-free medium containing 500 µCi/ml ^{32}P-orthophosphate at 37 ^{o}C for 4 hrs. Cells were lysed and immunoprecipitated as described above except with the addition of phosphatase inhibitors (10 mM ß- glycerophosphate, 5 mM NaF, 10 mM pNpp, 100mM Na pyrophosphate, and 500 µM Na_3VO_4).

For deglycosylation, immunoprecipitates were heated in 20 µl denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) at 100 ^{o}C for 10 min, then brought to a buffer containing 50 mM sodium phosphate, pH 7.5, and 1% Nonidet P-40, incubated with 250

units of peptide N-glycosidase F or 150 units of Endo-H (New England Biolabs) for 1 hr at 37 ^{o}C.

Tryptic Phosphopeptide Mapping

Tryptic phosphopeptide mapping was performed as described by Boyle et al. on LKB 2117 Mutiphor II Electrophoresis Unit (Bromma, Sweden). In brief, purified ^{32}P-labeled TGF-ßRII proteins were digested with L-TPCK-treated trypsin (Boehringer, Germany). Resulting peptides were resolved by thin-layer electrophoresis at pH 1.9, followed by chromatography in phosphochromo buffer on the second dimension.

^{3}H-Thymidine Incorporation

For ^{3}H-thymidine labeling, HER14 cells were growth-arrested by starvation in culture medium containing 0.1% calf serum (CS) for 48 hours. Different growth factors were then added for certain period of time. At the end of re-stimulation, cells were labelled with imCi / ml ^{3}H-thymidine in DMEM minus serum for 1 hour. Cells were treated with ice-cold 5% trichloroacetic acid (TCA) for 30 minutes and extensively washed with H_2O. Cells were then lysed with 0.5M NaOH and scintillation counting was performed.

Iodination of TGF-ß

TGF-ß was iodinated using the chloramine-T method as described (Cheifetz et al., 1988). Briefly, 2 to 3 micrograms of TGF-ß were mixed with equal volume of 1.5 M NaPO4 plus Na^{125}I. Chloramine-T was then added and followed by N-acytyltyrosine, KI, and an acidic urea solution as described previously (Frolik and De Larco, 1987). Aliquots were moved to estimate the specificity by seperating free iodine from labeled protein using chromatography. The reaction mixture was then desalting on Sephadex G25 spin columns and fractions with high specificity were collected.

Binding and Crosslinking

Cells were incubated with ^{125}I-labeled TGF-ß in binding buffer (Krebs-Ringer buffered with 20 mM HEPES (pH 7.5), 5 mM MgSO4, 0.5% BSA), and washed extensively. Crosslinking was performed with DSS and terminated by addition of 7% sucrose. Cells were then collected and lysed for 7.5 % SDS-PAGE and autoradiography as described before (Lin et al., 1992).

Immuno-Complex Kinase Assay

Monoclonal antibody 12CA5 (a HA) purchased from BAbCO (Berkeley, CA) were used for immunoprecipitation of HA-tagged TGF-ß receptors in the "Appendix". One 10 cm dish of cells were washed in ice-cold PBS$^-$ and lysed in 1 ml ice-cold buffer K (50 mM Tris-Cl (pH = 7.4), 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.25% BSA) with the addition of proteinase and phosphatase inhibitors as described above.

The absorbed immunocomplexes were washed with lysis buffer as above and kinase buffer (50 mM Tris-Cl (pH = 7.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1mM DTT). Then 3 µl of kinase mixture containing 3 µCi/µl of gamma -^{32}P-ATP in 100 µM ATP with or without 10 µg/µl casein was added to the immunoprecipitate, which was incubated for 30 minutes at 30 ^{o}C. Sample buffer (1% SDS, 2 mM EDTA, 1 mM DTT) was added at the end of kinase reaction and the samples were subjected to 10 % SDS-PAGE and autoradiography.

Chapter 8

References

Alexandrow, M. G., Kawabata, M., Aakre, M., and Moses, H. L. (1995). Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. Proc Natl Acad Sci U S A *92*, 3239-43.

Alexandrow, M. G., and Moses, H. L. (1995). Transforming growth factor beta and cell cycle regulation. Cancer Res *55*, 1452-7.

Amatayakul-Chantler, S., Qian, S. W., Gakenheimer, K., Bottinger, E. P., Roberts, A. B., and Sporn, M. B. (1994). [Ser77]transforming growth factor-beta 1. Selective biological activity and receptor binding in mink lung epithelial cells. J Biol Chem *269*, 27687-91.

Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989). Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem *264*, 8222-9.

Andres, J. L., Stanley, K., Cheifetz, S., and Massagué, J. (1989). Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. J Cell Biol *109*, 3137-45.

Archer, S. J., Bax, A., Roberts, A. B., Sporn, M. B., Ogawa, Y., Piez, K. A., Weatherbee, J. A., Tsang, M. L., Lucas, R., Zheng, B. L., and et al. (1993). Transforming growth factor beta 1: NMR signal assignments of the recombinant protein expressed and isotopically enriched using Chinese hamster ovary cells. Biochemistry *32*, 1152-63.

Armendariz-Borunda, J., Katayama, K., and Seyer, J. M. (1992). Transcriptional mechanisms of type I collagen gene expression are differentially regulated by interleukin-1 beta, tumor necrosis factor alpha, and transforming growth factor beta in Ito cells. J Biol Chem *267*, 14316-21.

Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M., and Sporn, M. B. (1984). Transforming growth factor-beta controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell *36*, 35-41.

Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., and Sporn, M. B. (1983). Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem *258*, 7155-60.

Atfi, A., Drobetsky, E., Boissonneault, M., Chapdelaine, A., and Chevalier, S. (1994). Transforming growth factor beta down-regulates Src family protein tyrosine kinase signaling pathways. J Biol Chem *269*, 30688-93.

Atfi, A., Lepage, K., Allard, P., Chapdelaine, A., and Chevalier, S. (1995). Activation of a serine/threonine kinase signaling pathway by transforming growth factor type ß. PNAS *92*, 12110-12114.

Attisano, L., Carcamo, J., Ventura, F., Weis, F. M., Massagué, J., and Wrana, J. L. (1993). Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. Cell *75*, 671-80.

Attisano, L., Wrana, J. L., Cheifetz, S., and Massagué, J. (1992). Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell *68*, 97-108.

Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massagué, J. (1994). TGF-beta receptors and actions. Biochim Biophys Acta *1222*, 71-80.

Baarends, W. M., van Helmond, M. J., Post, M., van der Schoot, P. J., Hoogerbrugge, J. W., de Winter, J. P., Uilenbroek, J. T., Karels, B., Wilming, L. G., Meijers, J. H., and et al. (1994). A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the mullerian duct. Development *120*, 189-97.

Basler, K., Edlund, T., Jessell, T. M., and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. Cell *73*, 687-702.

Bassing, C. H., Yingling, J. M., Howe, D. J., Wang, T., He, W. W., Gustafson, M. L., Shah, P., Donahoe, P. K., and Wang, X. F. (1994). A transforming growth factor beta type I receptor that signals to activate gene expression. Science *263*, 87-9.

Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F., and Ross, R. (1990). TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell *63*, 515-24.

Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D., and Brinster, R. L. (1990). Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. Nature *345*, 167-70.

Behringer, R. R., Finegold, M. J., and Cate, R. L. (1994). Müllerian-inhibiting substance function during mammalian sexual development. Cell *79*, 415-25.

Bhushan, A., Lin, H. Y., Lodish, H. F., and Kintner, C. R. (1994). The transforming growth factor beta type II receptor can replace the activin type II receptor in inducing mesoderm. Mol Cell Biol *14*, 4280-5.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T., and Gelbart, W. M. (1991). An extensive 3' cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. Development *111*, 657-66.

Border, W. A., Noble, N. A., Yamamoto, T., Harper, J. R., Yamaguchi, Y., Pierschbacher, M. D., and Ruoslahti, E. (1992). Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. Nature *360*, 361-4.

Border, W. A., and Ruoslahti, E. (1992). Transforming growth factor-beta in disease: the dark side of tissue repair. J Clin Invest *90*, 1-7.

Brand, T., and Schneider, M. D. (1995). Inactive type II and type I receptors for TGF beta are dominant inhibitors of TGF beta-dependent transcription. J Biol Chem *270*, 8274-84.

Burt, D. W., and Jakowlew, S. B. (1992). Correction: a new interpretation of a chicken transforming growth factor-beta 4 complementary DNA. Mol Endocrinol *6*, 989-92.

Carcamo, J., Weis, F. M., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massagué, J. (1994). Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. Mol Cell Biol *14*, 3810-21.

Carcamo, J., Zentella, A., and Massagué, J. (1995). Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol Cell Biol *15*, 1573-81.

Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J., and Massagué, J. (1988). Heterodimeric transforming growth factor beta. Biological properties and interaction with three types of cell surface receptors. J Biol Chem *263*, 10783-9.

Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeu, C., Massagué, J., and Letarte, M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem *267*, 19027-30.

Cheifetz, S., Ling, N., Guillemin, R., and Massagué, J. (1988). A surface component on GH3 pituitary cells that recognizes transforming growth factor-beta, activin, and inhibin. J Biol Chem *263*, 17225-8.

Chen, F., and Weinberg, R. A. (1995). Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor beta receptor kinases. Proc Natl Acad Sci U S A *92*, 1565-9.

Chen, R. H., and Derynck, R. (1994). Homomeric interactions between type II transforming growth factor-beta receptors. J Biol Chem *269*, 22868-74.

Chen, R. H., Ebner, R., and Derynck, R. (1993). Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. Science *260*, 1335-8.

Chen, R. H., Moses, H. L., Maruoka, E. M., Derynck, R., and Kawabata, M. (1995a). Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II transforming growth factor-beta receptors. J Biol Chem *270*, 12235-41.

Chen, R. H., Miettinen, P. J., Maruoka, E. M., Choy, L., and Derynck, R. (1995b). A WD-domain protein that is associated with and phosphorylated by the type II TGF-beta receptor. Nature *377*, 548-52.

Chiang, C. P., and Nilsen-Hamilton, M. (1986). Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-beta on the production of secreted proteins by murine 3T3 cells and human fibroblasts. J Biol Chem *261*, 10478-81.

Childs, S. R., Wrana, J. L., Arora, K., Attisano, L., O'Connor, M. B., and Massagué, J. (1993). Identification of a Drosophila activin receptor. Proc Natl Acad Sci U S A *90*, 9475-9.

Christ, M., McCartney-Francis, N. L., Kulkarni, A. B., Ward, J. M., Mizel, D. E., Mackall, C. L., Gress, R. E., Hines, K. L., Tian, H., Karlsson, S., and et al. (1994). Immune dysregulation in TGF-beta 1-deficient mice. J Immunol *153*, 1936-46.

Coffey, R. J., Jr., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E., and Moses, H. L. (1988). Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. Mol Cell Biol *8*, 3088-93.

Daniel, T. O., Gibbs, V. C., Milfay, D. F., and Williams, L. T. (1987). Agents that increase cAMP accumulation block endothelial c-sis induction by thrombin and transforming growth factor-beta. J Biol Chem *262*, 11893-6.

Daopin, S., Li, M., and Davies, D. R. (1993). Crystal structure of TGF-beta 2 refined at 1.8 A resolution. Proteins *17*, 176-92.

Dasch, J. R., Pace, D. R., Waegell, W., Inenaga, D., and Ellingsworth, L. (1989). Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. J Immunol *142*, 1536-41.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995a). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A *92*, 5545-9.

Datto, M. B., Yu, Y., and Wang, X. F. (1995b). Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem *270*, 28623-8.

Davis, R. J., and Czech, M. P. (1985). Tumor-promoting phorbol diesters cause the phosphorylation of epidermal growth factor receptors in normal human fibroblasts at threonine-654. Proc Natl Acad Sci U S A *82*, 1974-8.

de Martin, R., Haendler, B., Hofer-Warbinek, R., Gaugitsch, H., Wrann, M., Schlusener, H., Seifert, J. M., Bodmer, S., Fontana, A., and Hofer, E. (1987). Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. Embo J *6*, 3673-7.

Dean, D. C. (1989). Expression of the fibronectin gene. Am J Respir Cell Mol Biol *1*, 5-10.

Dean, D. C., Newby, R. F., and Bourgeois, S. (1988). Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. J Cell Biol *106*, 2159-70.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675-84.

Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B., and Goeddel, D. V. (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. Nature *316*, 701-5.

Derynck, R., Lindquist, P. B., Lee, A., Wen, D., Tamm, J., Graycar, J. L., Rhee, L., Mason, A. J., Miller, D. A., Coffey, R. J., and et al. (1988). A new type of transforming growth factor-beta, TGF-beta 3. Embo J *7*, 3737-43.

Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987). erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science *237*, 178-82.

Diaz-Meco, M. T., Dominguez, I., Sanz, L., Municio, M. M., Berra, E., Cornet, M. E., Garcia de Herreros, A., Johansen, T., and Moscat, J. (1992). Phospholipase C-mediated hydrolysis of phosphatidylcholine is a target of transforming growth factor beta 1 inhibitory signals. Mol Cell Biol *12*, 302-8.

Doctor, J. S., Jackson, P. D., Rashka, K. E., Visalli, M., and Hoffmann, F. M. (1992). Sequence, biochemical characterization, and developmental expression of a new member of the TGF-beta superfamily in Drosophila melanogaster. Dev Biol *151*, 491-505.

Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. (1994). The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. Oncogene *9*, 2109-23.

Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R., and Derynck, R. (1993a). Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. Science *260*, 1344-8.

Ebner, R., Chen, R. H., Lawler, S., Zioncheck, T., and Derynck, R. (1993b). Determination of type I receptor specificity by the type II receptors for TGF-beta or activin. Science *262*, 900-2.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-25.

Estevez, M., Attisano, L., Wrana, J. L., Albert, P. S., Massagué, J., and Riddle, D. L. (1993). The daf-4 gene encodes a bone morphogenetic protein receptor controlling C. elegans dauer larva development. Nature *365*, 644-9.

Ewen, M. E., Oliver, C. J., Sluss, H. K., Miller, S. J., and Peeper, D. S. (1995). p53-dependent repression of CDK4 translation in TGF-beta-induced G1 cell-cycle arrest. Genes Dev *9*, 204-17.

Ewen, M. E., Sluss, H. K., Whitehouse, L. L., and Livingston, D. M. (1993). TGF beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. Cell *74*, 1009-20.

Ferguson, E. L., and Anderson, K. V. (1992a). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the Drosophila embryo. Cell *71*, 451-61.

Ferguson, E. L., and Anderson, K. V. (1992b). Localized enhancement and repression of the activity of the TGF-beta family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. Development *114*, 583-97.

Franzen, P., Heldin, C. H., and Miyazono, K. (1995). The GS domain of the transforming growth factor-beta type I receptor is important in signal transduction. Biochem Biophys Res Commun *207*, 682-9.

Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H., and Miyazono, K. (1993). Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. Cell *75*, 681-92.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., and Sporn, M. B. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. Proc Natl Acad Sci U S A *80*, 3676-80.

Frolik, C. A., and De Larco, J. E. (1987). Radioreceptor assays for transforming growth factors. Methods Enzymol *146*, 95-102.

Frolik, C. A., Wakefield, L. M., Smith, D. M., and Sporn, M. B. (1984). Characterization of a membrane receptor for transforming growth factor-beta in normal rat kidney fibroblasts. J Biol Chem *259*, 10995-1000.

Fynan, T. M., and Reiss, M. (1993). Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. Crit Rev Oncog *4*, 493-540.

Gai, X. X., Rizzo, M. G., Valpreda, S., and Baserga, R. (1989). Regulation of c-myc mRNA levels by insulin or platelet-poor plasma. Oncogene Res *5*, 111-20.

Galera, P., Vivien, D., Pronost, S., Bonaventure, J., Redini, F., Loyau, G., and Pujol, J. P. (1992). Transforming growth factor-beta 1 (TGF-beta 1) up-regulation of collagen type II in primary cultures of rabbit articular chondrocytes (RAC) involves increased mRNA levels without affecting mRNA stability and procollagen processing. J Cell Physiol *153*, 596-606.

Geiser, A. G., Burmester, J. K., Webbink, R., Roberts, A. B., and Sporn, M. B. (1992). Inhibition of growth by transforming growth factor-beta following fusion of two nonresponsive human carcinoma cell lines. Implication of the type II receptor in growth inhibitory responses. J Biol Chem *267*, 2588-93.

Geng, Y., and Weinberg, R. A. (1993). Transforming growth factor beta effects on expression of G1 cyclins and cyclin-dependent protein kinases. Proc Natl Acad Sci U S A *90*, 10315-9.

Gentry, L. E., and Nash, B. W. (1990). The pro domain of pre-pro-transforming growth factor beta 1 when independently expressed is a functional binding protein for the mature growth factor. Biochemistry *29*, 6851-7.

Georgi, L. L., Albert, P. S., and Riddle, D. L. (1990). daf-1, a C. elegans gene controlling dauer larva development, encodes a novel receptor protein kinase. Cell *61*, 635-45.

Glick, A. B., Kulkarni, A. B., Tennenbaum, T., Hennings, H., Flanders, K. C., O'Reilly, M., Sporn, M. B., Karlsson, S., and Yuspa, S. H. (1993). Loss of expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc Natl Acad Sci U S A *90*, 6076-80.

Glick, A. B., Lee, M. M., Darwiche, N., Kulkarni, A. B., Karlsson, S., and Yuspa, S. H. (1994). Targeted deletion of the TGF-beta 1 gene causes rapid progression to squamous cell carcinoma. Genes Dev *8*, 2429-40.

Goldkorn, T., and Mendelsohn, J. (1992). Transforming growth factor beta modulates phosphorylation of the epidermal growth factor receptor and proliferation of A431 cells. Cell Growth Differ *3*, 101-9.

Gougos, A., and Letarte, M. (1990). Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem *265*, 8361-4.

Green, J. B., New, H. V., and Smith, J. C. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. Cell *71*, 731-9.

Greenwel, P., Hu, W., Kohanski, R. A., and Ramirez, F. (1995). Tyrosine dephosphorylation of nuclear proteins mimics transforming growth factor beta 1 stimulation of alpha 2(I) collagen gene expression. Mol Cell Biol *15*, 6813-9.

Gu, Y., Turck, C. W., and Morgan, D. O. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature *366*, 707-10.

Guadagno, T. M., Ohtsubo, M., Roberts, J. M., and Assoian, R. K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression [published erratum appears in Science 1994 Jan 28; 263(5146):455]. Science *262*, 1572-5.

Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev *8*, 2939-52.

Guenard, V., Rosenbaum, T., Gwynn, L. A., Doetschman, T., Ratner, N., and Wood, P. M. (1995). Effect of transforming growth factor-beta 1 and -beta 2 on Schwann cell proliferation on neurites. Glia *13*, 309-18.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-3.

Haisenleder, D. J., Ortolano, G. A., Jolly, D., Dalkin, A. C., Landefeld, T. D., Vale, W. W., and Marshall, J. C. (1990). Inhibin secretion during the rat estrous cycle: relationships to FSH secretion and FSH beta subunit mRNA concentrations. Life Sci *47*, 1769-73.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42-52.

Hannon, G. J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature *371*, 257-61.

Hansch, G. M., Wagner, C., Burger, A., Dong, W., Staehler, G., and Stoeck, M. (1995). Matrix protein synthesis by glomerular mesangial cells in culture: effects of transforming growth factor beta (TGF beta) and platelet-derived growth factor (PDGF) on fibronectin and collagen type IV mRNA. J Cell Physiol *163*, 451-7.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-16.

He, W. W., Gustafson, M. L., Hirobe, S., and Donahoe, P. K. (1993). Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor-beta type II receptor family. Dev Dyn *196*, 133-42.

Heckmann, M., Aumailley, M., Chu, M. L., Timpl, R., and Krieg, T. (1992). Effect of transforming growth factor-beta on collagen type VI expression in human dermal fibroblasts. FEBS Lett *310*, 79-82.

Heine, U., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H. Y., Thompson, N. L., Roberts, A. B., and Sporn, M. B. (1987). Role of transforming growth factor-beta in the development of the mouse embryo. J Cell Biol *105*, 2861-76.

Heino, J., Ignotz, R. A., Hemler, M. E., Crouse, C., and Massagué, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J Biol Chem *264*, 380-8.

Henis, Y. I., Moustakas, A., Lin, H. Y., and Lodish, H. F. (1994). The types II and III transforming growth factor-beta receptors form homo-oligomers. J Cell Biol *126*, 139-54.

Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1987). Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell *51*, 199-209.

Huang, J. D., Schwyter, D. H., Shirokawa, J. M., and Courey, A. J. (1993). The interplay between multiple enhancer and silencer elements defines the pattern of decapentaplegic expression. Genes Dev *7*, 694-704.

Hunter, T., Gould, K. L., and Cooper, J. A. (1984). Tyrosine protein kinases, viral transformation and the control of cell proliferation. Biochem Soc Trans *12*, 757-9.

Hursh, D. A., Padgett, R. W., and Gelbart, W. M. (1993). Cross regulation of decapentaplegic and Ultrabithorax transcription in the embryonic visceral mesoderm of Drosophila. Development *117*, 1211-22.

Ignotz, R. A., Heino, J., and Massagué, J. (1989). Regulation of cell adhesion receptors by transforming growth factor-beta. Regulation of vitronectin receptor and LFA-1. J Biol Chem *264*, 389-92.

Ignotz, R. A., and Massagué, J. (1987). Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell *51*, 189-97.

Ignotz, R. A., and Massagué, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem *261*, 4337-45.

Ihle, J. N. (1995). Cytokine receptor signalling. Nature *377*, 591-4.

Immergluck, K., Lawrence, P. A., and Bienz, M. (1990). Induction across germ layers in Drosophila mediated by a genetic cascade. Cell *62*, 261-8.

Inagaki, M., Moustakas, A., Lin, H. Y., Lodish, H. F., and Carr, B. I. (1993). Growth inhibition by transforming growth factor beta (TGF-beta) type I is restored in TGF-beta-resistant hepatoma cells after expression of TGF-beta receptor type II cDNA. Proc Natl Acad Sci U S A *90*, 5359-63.

Jakowlew, S. B., Ciment, G., Tuan, R. S., Sporn, M. B., and Roberts, A. B. (1994). Expression of transforming growth factor-beta 2 and beta 3 mRNAs and proteins in the developing chicken embryo. Differentiation *55*, 105-18.

Jansen-Durr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G., and Eilers, M. (1993). Differential modulation of cyclin gene expression by MYC. Proc Natl Acad Sci U S A *90*, 3685-9.

Kadin, M. E., Cavaille-Coll, M. W., Gertz, R., Massagué, J., Cheifetz, S., and George, D. (1994). Loss of receptors for transforming growth factor beta in human T-cell malignancies. Proc Natl Acad Sci U S A *91*, 6002-6.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S. r., Johnson, B. E., and Skolnick, M. H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science *264*, 436-40.

Kawabata, M., Imamura, T., Miyazono, K., Engel, M. E., and Moses, H. L. (1995). Interaction of the transforming growth factor-beta type I receptor with farnesyl-protein transferase-alpha. J Biol Chem *270*, 29628-31.

Kikuchi, K., Hartl, C. W., Smith, E. A., Le Roy, E. C., and Trojanowska, M. (1992). Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGF beta 1 stimulation. Biochem Biophys Res Commun *187*, 45-50.

Kimchi, A., Wang, X. F., Weinberg, R. A., Cheifetz, S., and Massagué, J. (1988). Absence of TGF-beta receptors and growth inhibitory responses in retinoblastoma cells. Science *240*, 196-9.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev *8*, 133-46.

Kishimoto, T., Taga, T., and Akira, S. (1994). Cytokine signal transduction. Cell *76*, 253-62.

Kondaiah, P., Sands, M. J., Smith, J. M., Fields, A., Roberts, A. B., Sporn, M. B., and Melton, D. A. (1990). Identification of a novel transforming growth factor-beta (TGF-beta 5) mRNA in Xenopus laevis. J Biol Chem *265*, 1089-93.

Konig, A., and Bruckner-Tuderman, L. (1992). Transforming growth factor-beta stimulates collagen VII expression by cutaneous cells in vitro. J Cell Biol *117*, 679-85.

Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M., and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A *90*, 770-4.

Laiho, M., De Caprio, J. A., Ludlow, J. W., Livingston, D. M., and Massagué, J. (1990). Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. Cell *62*, 175-85.

Laiho, M., Saksela, O., and Keski-Oja, J. (1986). Transforming growth factor beta alters plasminogen activator activity in human skin fibroblasts. Exp Cell Res *164*, 399-407.

Laiho, M., Saksela, O., and Keski-Oja, J. (1987). Transforming growth factor-beta induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. J Biol Chem *262*, 17467-74.

Laiho, M., Weis, F. M., Boyd, F. T., Ignotz, R. A., and Massagué, J. (1991). Responsiveness to transforming growth factor-beta (TGF-beta) restored by genetic complementation between cells defective in TGF-beta receptors I and II. J Biol Chem *266*, 9108-12.

Laiho, M., Weis, M. B., and Massagué, J. (1990). Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction. J Biol Chem *265*, 18518-24.

Lammers, R., Van Obberghen, E., Ballotti, R., Schlessinger, J., and Ullrich, A. (1990). Transphosphorylation as a possible mechanism for insulin and epidermal growth factor receptor activation. J Biol Chem *265*, 16886-90.

Lebkowski, J. S., Miller, J. H., and Calos, M. P. (1986). Determination of DNA sequence changes induced by ethyl methanesulfonate in human cells, using a shuttle vector system. Mol Cell Biol *6*, 1838-42.

Lee, M. M., and Donahoe, P. K. (1993). Müllerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev *14*, 152-64.

Lee, S. J. (1991). Expression of growth/differentiation factor 1 in the nervous system: conservation of a bicistronic structure. Proc Natl Acad Sci U S A *88*, 4250-4.

Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., Di Corleto, P. E., and Moses, H. L. (1986). Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci U S A *83*, 2453-7.

Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., Massagué, J., and et al. (1995). Drosophila Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF beta receptor family. Cell *80*, 899-908.

Letterio, J. J., Geiser, A. G., Kulkarni, A. B., Roche, N. S., Sporn, M. B., and Roberts, A. B. (1994). Maternal rescue of transforming growth factor-beta 1 null mice. Science *264*, 1936-8.

Like, B., and Massagué, J. (1986). The antiproliferative effect of type beta transforming growth factor occurs at a level distal from receptors for growth-activating factors. J Biol Chem *261*, 13426-9.

Lin, H. Y., and Lodish, H. F. (1993). Receptors for the TGF-ß superfamily: multiple polypeptides and serine/threonine kinases. Trends Cell Biol. *3*, 14-19.

Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992). Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase [published erratum appears in Cell 1992 Sep 18; 70(6):following 1068]. Cell *68*, 775-85.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science *260*, 1130-2.

Liu, F., Ventura, F., Doody, J., and Massagué, J. (1995). Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol Cell Biol *15*, 3479-86.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S. L., Matsudaira, P., and Darnell, J. (1995). Molecular Cell Biology, 3rd Edition (New York: Scientific American Books), pp.595-633.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massagué, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell *67*, 785-95.

Lopez-Casillas, F., Payne, H. M., Andres, J. L., and Massagué, J. (1994). Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol *124*, 557-68.

Lund, L. R., Riccio, A., Andreasen, P. A., Nielsen, L. S., Kristensen, P., Laiho, M., Saksela, O., Blasi, F., and Dano, K. (1987). Transforming growth factor-beta is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. Embo J *6*, 1281-6.

Luo, K., Zhou, P., and Lodish, H. F. (1995). The specificity of the transforming growth factor beta receptor kinases determined by a spatially addressable peptide library. Proc Natl Acad Sci U S A *92*, 11761-5.

Lyons, R. M., Keski-Oja, J., and Moses, H. L. (1988). Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. J Cell Biol *106*, 1659-65.

Mäkelä, T. P., Alitalo, R., Paulsson, Y., Westermark, B., Heldin, C. H., and Alitalo, K. (1987). Regulation of platelet-derived growth factor gene expression by transforming growth factor beta and phorbol ester in human leukemia cell lines. Mol Cell Biol *7*, 3656-62.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., and et al. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science *268*, 1336-8.

Massagué, J. (1992). Receptors for the TGF-beta family. Cell *69*, 1067-70.

Massagué, J. (1990). The transforming growth factor-beta family. Annu Rev Cell Biol *6*, 597-641.

Massagué, J. (1985). Transforming growth factor-beta modulates the high-affinity receptors for epidermal growth factor and transforming growth factor-alpha. J Cell Biol *100*, 1508-14.

Massagué, J., Attisano, L., and Wrana, J. (1994). The TGF-ß family and its composite receptors. Trends In cell Biology *4*, 174-178.

Massagué, J., and Like, B. (1985). Cellular receptors for type beta transforming growth factor. Ligand binding and affinity labeling in human and rodent cell lines. J Biol Chem *260*, 2636-45.

Massagué, J., and Polyak, K. (1995). Mammalian antiproliferative signals and their targets. Curr Opin Genet Dev *5*, 91-6.

Masucci, J. D., Miltenberger, R. J., and Hoffmann, F. M. (1990). Pattern-specific expression of the Drosophila decapentaplegic gene in imaginal disks is regulated by 3' cis-regulatory elements. Genes Dev *4*, 2011-23.

Mathews, L. S., and Vale, W. W. (1993). Characterization of type II activin receptors. Binding, processing, and phosphorylation. J Biol Chem *268*, 19013-8.

Mathews, L. S., and Vale, W. W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell *65*, 973-82.

Mathews, L. S., Vale, W. W., and Kintner, C. R. (1992). Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. Science *255*, 1702-5.

Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J., and Bradley, A. (1992). Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature *360*, 313-9.

Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. (1995a). Functional analysis of activins during mammalian development. Nature *374*, 354-6.

Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995b). Different phenotypes for mice deficient in either activins or activin receptor type II. Nature *374*, 356-60.

Matzuk, M. M., Finegold, M. J., Mishina, Y., Bradley, A., and Behringer, R. R. (1995c). Synergistic effects of inhibins and Müllerian-inhibiting substance on testicular tumorigenesis. Mol Endocrinol *9*, 1337-45.

McAllister, K. A., Grogg, K. M., Johnson, D. W., Gallione, C. J., Baldwin, M. A., Jackson, C. E., Helmbold, E. A., Markel, D. S., McKinnon, W. C., Murrell, J., and et al. (1994). Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet *8*, 345-51.

McCaffrey, T. A., Falcone, D. J., and Du, B. (1992). Transforming growth factor-beta 1 is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-beta 1. J Cell Physiol *152*, 430-40.

McDonald, J. A., Quade, B. J., Broekelmann, T. J., La Chance, R., Forsman, K., Hasegawa, E., and Akiyama, S. (1987). Fibronectin's cell-adhesive domain and an amino-

terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J Biol Chem *262*, 2957-67.

McDonald, N. Q., and Hendrickson, W. A. (1993). A structural superfamily of growth factors containing a cystine knot motif. Cell *73*, 421-4.

McPherron, A. C., and Lee, S. J. (1993). GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. J Biol Chem *268*, 3444-9.

Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995). Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc Natl Acad Sci U S A *92*, 6289-93.

Miyazono, K., and Heldin, C. H. (1992). Structure, function and possible clinical application of transforming growth factor-beta. J Dermatol *19*, 644-7.

Miyazono, K., Ichijo, H., and Heldin, C. H. (1993). Transforming growth factor-beta: latent forms, binding proteins and receptors. Growth Factors *8*, 11-22.

Morales, T. I., and Roberts, A. B. (1988). Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem *263*, 12828-31.

Morton, D. M., and Barrack, E. R. (1995). Modulation of transforming growth factor beta 1 effects on prostate cancer cell proliferation by growth factors and extracellular matrix. Cancer Res *55*, 2596-602.

Moses, H. L., Branum, E. L., Proper, J. A., and Robinson, R. A. (1981). Transforming growth factor production by chemically transformed cells. Cancer Res *41*, 2842-8.

Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990). TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell *63*, 245-7.

Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O'Connor-McCourt, M. D., and Lodish, H. F. (1993). The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. J Biol Chem *268*, 22215-8.

Mulder, K. M., and Morris, S. L. (1992). Activation of p21ras by transforming growth factor beta in epithelial cells. J Biol Chem *267*, 5029-31.

Munsterberg, A., and Lovell-Badge, R. (1991). Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. Development *113*, 613-24.

Murphy-Ullrich, J. E., Schultz-Cherry, S., and Hook, M. (1992). Transforming growth factor-beta complexes with thrombospondin. Mol Biol Cell *3*, 181-8.

Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. F. (1994). The ancient regulatory-protein family of WD-repeat proteins [published erratum appears in Nature 1994 Oct 27; 371(6500):812]. Nature *371*, 297-300.

Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature *368*, 753-6.

Nugent, M. A., and Edelman, E. R. (1992). Transforming growth factor beta 1 stimulates the production of basic fibroblast growth factor binding proteoglycans in Balb/c3T3 cells. J Biol Chem *267*, 21256-64.

O'Connor-McCourt, M. D., and Wakefield, L. M. (1987). Latent transforming growth factor-beta in serum. A specific complex with alpha 2-macroglobulin. J Biol Chem *262*, 14090-9.

O'Grady, P., Kuo, M. D., Baldassare, J. J., Huang, S. S., and Huang, J. S. (1991). Purification of a new type high molecular weight receptor (type V receptor) of transforming growth factor beta (TGF-beta) from bovine liver. Identification of the type V TGF-beta receptor in cultured cells. J Biol Chem *266*, 8583-9.

O'Grady, P., Liu, Q., Huang, S. S., and Huang, J. S. (1992). Transforming growth factor beta (TGF-beta) type V receptor has a TGF-beta-stimulated serine/threonine-specific autophosphorylation activity. J Biol Chem *267*, 21033-7.

Okadome, T., Yamashita, H., Franzen, P., Moren, A., Heldin, C. H., and Miyazono, K. (1994). Distinct roles of the intracellular domains of transforming growth factor-beta type I and type II receptors in signal transduction. J Biol Chem *269*, 30753-6.

Overall, C. M. (1995). Repression of tissue inhibitor of matrix metalloproteinase expression by all-trans-retinoic acid in rat bone cell populations: comparison with transforming growth factor-beta 1. J Cell Physiol *164*, 17-25.

Overall, C. M., Wrana, J. L., and Sodek, J. (1989). Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. J Biol Chem *264*, 1860-9.

Padgett, R. W., St.Johnston, R. D., and Gelbart, W. M. (1987). A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. Nature *325*, 81-4.

Padgett, R. W., Wozney, J. M., and Gelbart, W. M. (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. Proc Natl Acad Sci U S A *90*, 2905-9.

Panganiban, G. E., Reuter, R., Scott, M. P., and Hoffmann, F. M. (1990). A Drosophila growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development *110*, 1041-50.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. Science *246*, 603-8.

Park, K., Kim, S. J., Bang, Y. J., Park, J. G., Kim, N. K., Roberts, A. B., and Sporn, M. B. (1994). Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. Proc Natl Acad Sci U S A *91*, 8772-6.

Pearson, C. A., Pearson, D., Shibahara, S., Hofsteenge, J., and Chiquet-Ehrismann, R. (1988). Tenascin: cDNA cloning and induction by TGF-beta. Embo J *7*, 2977-82.

Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassill, J. A., Massagué, J., and Hoffmann, F. M. (1994). Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a decapentaplegic receptor. Cell *78*, 239-50.

Penttinen, R. P., Kobayashi, S., and Bornstein, P. (1988). Transforming growth factor beta increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. Proc Natl Acad Sci U S A *85*, 1105-8.

Philipp, A., Schneider, A., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Alitalo, K., and Eilers, M. (1994). Repression of cyclin D1: a novel function of MYC. Mol Cell Biol *14*, 4032-43.

Pierce, D. F., Jr., Gorska, A. E., Chytil, A., Meise, K. S., Page, D. L., Coffey, R. J., Jr., and Moses, H. L. (1995). Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A *92*, 4254-8.

Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. (1990a). TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell *61*, 777-85.

Pietenpol, J. A., Holt, J. T., Stein, R. W., and Moses, H. L. (1990b). Transforming growth factor beta 1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci U S A *87*, 3758-62.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994a). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59-66.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massagué, J., Roberts, J. M., and Koff, A. (1994b). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev *8*, 9-22.

Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W., and Doetschman, T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet *11*, 409-14.

Qian, S. W., Burmester, J. K., Merwin, J. R., Madri, J. A., Sporn, M. B., and Roberts, A. B. (1992). Identification of a structural domain that distinguishes the actions of the type 1 and 2 isoforms of transforming growth factor beta on endothelial cells. Proc Natl Acad Sci U S A *89*, 6290-4.

Qian, X., Le Vea, C. M., Freeman, J. K., Dougall, W. C., and Greene, M. I. (1994). Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. Proc Natl Acad Sci U S A *91*, 1500-4.

Raftery, L. A., Twombly, V., Wharton, K., and Gelbart, W. M. (1995). Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. Genetics *139*, 241-54.

Raghow, R., Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., and Kang, A. H. (1987). Transforming growth factor-beta increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. J Clin Invest *79*, 1285-8.

Roberts, A. B., Sporn, M. B. (1990). Peptide Growth Factors and Their Receptors, M. B. Sporn, Roberts, A. B., ed. (Heidelberg: Springer), pp.419-472

Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., and Sporn, M. B. (1981). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci U S A *78*, 5339-43.

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., Pan, Y. C., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C., and et al. (1983). Purification and properties of a type beta transforming growth factor from bovine kidney. Biochemistry *22*, 5692-8.

Roberts, A. B., and Sporn, M. B. (1987). Transforming growth factor-beta: potential common mechanisms mediating its effects on embryogenesis, inflammation-repair, and carcinogenesis. Int J Rad Appl Instrum [B] *14*, 435-9.

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotta, L. A., Falanga, V., Kehrl, J. H., and et al. (1986).

Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A *83*, 4167-71.

Roelen, B. A., Lin, H. Y., Knezevic, V., Freund, E., and Mummery, C. L. (1994). Expression of TGF-beta s and their receptors during implantation and organogenesis of the mouse embryo. Dev Biol *166*, 716-28.

Rokhlin, O. W., Cohen, M. B., Kubagawa, H., Letarte, M., and Cooper, M. D. (1995). Differential expression of endoglin on fetal and adult hematopoietic cells in human bone marrow. J Immunol *154*, 4456-65.

Rosenzweig, B. L., Imamura, T., Okadome, T., Cox, G. N., Yamashita, H., ten Dijke, P., Heldin, C. H., and Miyazono, K. (1995). Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A *92*, 7632-6.

Rossi, P., Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. B., and de Crombrugghe, B. (1988). A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. Cell *52*, 405-14.

Ruegemer, J. J., Ho, S. N., Augustine, J. A., Schlager, J. W., Bell, M. P., McKean, D. J., and Abraham, R. T. (1990). Regulatory effects of transforming growth factor-beta on IL-2- and IL-4-dependent T cell-cycle progression. J Immunol *144*, 1767-76.

Ruoslahti, E., and Pierschbacher, M. D. (1986). Arg-Gly-Asp: a versatile cell recognition signal. Cell *44*, 517-8.

Saltis, J., and Bobik, A. (1996). Role of protein kinase C-δ on the mitogenic activity of transforming growth factor-ß1 in vascular smooth muscle cells. In Proceedings of the 8th Lorne Cancer Conference, pp. 14.

Samanta, A., Le Vea, C. M., Dougall, W. C., Qian, X., and Greene, M. I. (1994). Ligand and p185c-neu density govern receptor interactions and tyrosine kinase activation. Proc Natl Acad Sci U S A *91*, 1711-5.

Sampath, T. K., Rashka, K. E., Doctor, J. S., Tucker, R. F., and Hoffmann, F. M. (1993). Drosophila transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci U S A *90*, 6004-8.

Savage, C., Das, P., Finelli, A. L., Townsend, S. R., Sun, C. Y., Baird, S. E., and Padgett, R. W. (1996). Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. Proc Natl Acad Sci U S A *93*, 790-4.

Schlessinger, J., and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. Neuron *9*, 383-91.

Schlunegger, M. P., and Grutter, M. G. (1993). Refined crystal structure of human transforming growth factor beta 2 at 1.95 A resolution. J Mol Biol *231*, 445-58.

Schlunegger, M. P., and Grutter, M. G. (1992). An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-beta 2. Nature *358*, 430-4.

Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H., and Gelbart, W. M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics *139*, 1347-58.

Sellheyer, K., Bickenbach, J. R., Rothnagel, J. A., Bundman, D., Longley, M. A., Krieg, T., Roche, N. S., Roberts, A. B., and Roop, D. R. (1993). Inhibition of skin development by overexpression of transforming growth factor beta 1 in the epidermis of transgenic mice. Proc Natl Acad Sci U S A *90*, 5237-41.

Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994). The novel primary response gene MyD118 and the proto-oncogenes myb, myc, and bcl-2 modulate transforming growth factor beta 1-induced apoptosis of myeloid leukemia cells. Mol Cell Biol *14*, 2352-60.

Serrano, M., Gomez-Lahoz, E., De Pinho, R. A., Beach, D., and Bar-Sagi, D. (1995). Inhibition of ras-induced proliferation and cellular transformation by p16INK4. Science *267*, 249-52.

Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704-7.

Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K., and Taniguchi, T. (1992). IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. Cell *70*, 57-67.

Shipley, G. D., Tucker, R. F., and Moses, H. L. (1985). Type beta transforming growth factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S phase after a prolonged prereplicative interval. Proc Natl Acad Sci U S A *82*, 4147-51.

Shull, M. M., and Doetschman, T. (1994). Transforming growth factor-beta 1 in reproduction and development. Mol Reprod Dev *39*, 239-46.

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature *359*, 693-9.

Silberstein, G. B., and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-beta. Science *237*, 291-3.

Slack, J. M. (1993). Embryonic induction. Mech Dev *41*, 91-107.

Slingerland, J. M., Hengst, L., Pan, C. H., Alexander, D., Stampfer, M. R., and Reed, S. I. (1994). A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor beta-arrested epithelial cells. Mol Cell Biol *14*, 3683-94.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31-40.

Spencer, F. A., Hoffmann, F. M., and Gelbart, W. M. (1982). Decapentaplegic: a gene complex affecting morphogenesis in Drosophila melanogaster. Cell *28*, 451-61.

Starksen, N. F., Harsh, G. R. t., Gibbs, V. C., and Williams, L. T. (1987). Regulated expression of the platelet-derived growth factor A chain gene in microvascular endothelial cells. J Biol Chem *262*, 14381-4.

Takahashi, M., Hayashi, M., Manganaro, T. F., and Donahoe, P. K. (1986). The ontogeny of Müllerian inhibiting substance in granulosa cells of the bovine ovarian follicle. Biol Reprod *35*, 447-53.

Takehara, K., Le Roy, E. C., and Grotendorst, G. R. (1987). TGF-beta inhibition of endothelial cell proliferation: alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. Cell *49*, 415-22.

Takumi, T., Moustakas, A., Lin, H. Y., and Lodish, H. F. (1995). Molecular characterization of a type I serine-threonine kinase receptor for TGF-beta and activin in the rat pituitary tumor cell line GH3. Exp Cell Res *216*, 208-14.

Taniguchi, T. (1995). Cytokine signaling through nonreceptor protein tyrosine kinases. Science *268*, 251-5.

ten Dijke, P., Hansen, P., Iwata, K. K., Pieler, C., and Foulkes, J. G. (1988). Identification of another member of the transforming growth factor type beta gene family. Proc Natl Acad Sci U S A *85*, 4715-9.

Thomsen, G. H., and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. Cell *74*, 433-41.

Tucker, R. F., Branum, E. L., Shipley, G. D., Ryan, R. J., and Moses, H. L. (1984). Specific binding to cultured cells of 125I-labeled type beta transforming growth factor from human platelets. Proc Natl Acad Sci U S A *81*, 6757-61.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-12.

Vassalli, A., Matzuk, M. M., Gardner, H. A., Lee, K. F., and Jaenisch, R. (1994). Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev *8*, 414-27.

Ventura, F., Doody, J., Liu, F., Wrana, J. L., and Massagué, J. (1994). Reconstitution and transphosphorylation of TGF-beta receptor complexes. Embo J *13*, 5581-9.

Vivien, D., Attisano, L., Wrana, J. L., and Massagué, J. (1995). Signaling activity of homologous and heterologous transforming growth factor-beta receptor kinase complexes. J Biol Chem *270*, 7134-41.

Wahl, S. M. (1994). Transforming growth factor beta: the good, the bad, and the ugly. J Exp Med *180*, 1587-90.

Wakefield, L. M., Smith, D. M., Masui, T., Harris, C. C., and Sporn, M. B. (1987). Distribution and modulation of the cellular receptor for transforming growth factor-beta. J Cell Biol *105*, 965-75.

Wakefield, L. M., Winokur, T. S., Hollands, R. S., Christopherson, K., Levinson, A. D., and Sporn, M. B. (1990). Recombinant latent transforming growth factor beta 1 has a

longer plasma half-life in rats than active transforming growth factor beta 1, and a different tissue distribution. J Clin Invest *86*, 1976-84.

Walker, J. C., and Zhang, R. (1990). Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of Brassica. Nature *345*, 743-6.

Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L. E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J. K., and et al. (1995). Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. J Biol Chem *270*, 22044-9.

Wang, T., Danielson, P. D., Li, B., Shah, P. C., Kim, S. D., and Donahoe, P. K. (1996). The $p21^{ras}$ farnesyltransferase α subunit in TGF–ß and activin signaling. Science *271*, 1120-1122.

Wang, T., Donahoe, P. K., and Zervos, A. S. (1994). Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. Science *265*, 674-6.

Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991). Expression cloning and characterization of the TGF-beta type III receptor. Cell *67*, 797-805.

Warren, T. G., and Shields, D. (1984). Expression of preprosomatostatin in heterologous cells: biosynthesis, posttranslational processing, and secretion of mature somatostatin. Cell *39*, 547-55.

Weeks, D. L., and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-beta. Cell *51*, 861-7.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-30.

Weis-Garcia, F., and Massagué, J. (1996). Complementation between kinase-defective and activation-defective TGF-ß receptors reveals a novel form of receptor cooperativity essential for signaling. EMBO Journal *15*, 276-289.

Wharton, K. A., Ray, R. P., and Gelbart, W. M. (1993). An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the Drosophila embryo. Development *117*, 807-22.

Wharton, K. A., Thomsen, G. H., and Gelbart, W. M. (1991). Drosophila 60A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. Proc Natl Acad Sci U S A *88*, 9214-8.

Wieser, R., Attisano, L., Wrana, J. L., and Massagué, J. (1993). Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. Mol Cell Biol *13*, 7239-47.

Wieser, R., Wrana, J. L., and Massagué, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. Embo J *14*, 2199-208.

Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massagué, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003-14.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGF-beta receptor. Nature *370*, 341-7.

Wrana, J. L., Sodek, J., Ber, R. L., and Bellows, C. G. (1986). The effects of platelet-derived transforming growth factor beta on normal human diploid gingival fibroblasts. Eur J Biochem *159*, 69-76.

Wu, H., Klingmuller, U., Besmer, P., and Lodish, H. F. (1995). Interaction of the erythropoietin and stem-cell-factor receptors. Nature *377*, 242-6.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701-4.

Xu, J., Rockow, S., Kim, S., Xiong, W., and Li, W. (1994). Interferons block protein kinase C-dependent but not-independent activation of Raf-1 and mitogen-activated protein kinases and mitogenesis in NIH 3T3 cells. Mol Cell Biol *14*, 8018-27.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science *270*, 2008-11.

Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C. H. (1994). Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem *269*, 20172-8.

Yan, Z., Winawer, S., and Friedman, E. (1994). Two different signal transduction pathways can be activated by transforming growth factor beta 1 in epithelial cells. J Biol Chem *269*, 13231-7.

Ying, S. Y. (1989). Inhibins, activins and follistatins. J Steroid Biochem *33*, 705-13.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L., and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature *361*, 543-7.

Appendix

In Vitro Kinase Assays for TGF-ßRII and TGF-ßRI in COS Cells

PREFACE

As discussed earlier in Chapter 6, there is a immediate need for *in vitro* kinase assays for TGF-ßRII and TGF-ßRI so that their activation can be measured quantitatively. Due to the lack of physiological substrates, I attempted to screen a panel of available purified proteins which potentially can be used as *in vitro* substrates.

RESULTS

COS1 cells transfected with wild-type or kinase-deficient TGF-ßRI or TGF-ßRII in different combinations and immuno-complex kinase assays were performed as described in Chapter 7. Among all the purified proteins I tested (that were either commercially available products or recombinant proteins made by colleagues in our laboratory), casein was the only one that could be specifically phosphorylated by the TGF-ß receptor kinases.

A.1 Autophosphorylation

As shown in Figure A.1, both wild-type TGF-ßRI and TGF-ßRII could phosphorylate themselves in this assay while their kinase-deficient mutant could not, suggesting both kinase receptors can autophosphorylate *in vitro*. I also noted that the autophosphorylation of TGF-ßRIwt was decreased when a mutant TGF-ßRIIKR was co-expressed (Fig A.1, lane 5 and 6), consistent with the model that two types of receptors can complex with each other and their activity can then be regulated.

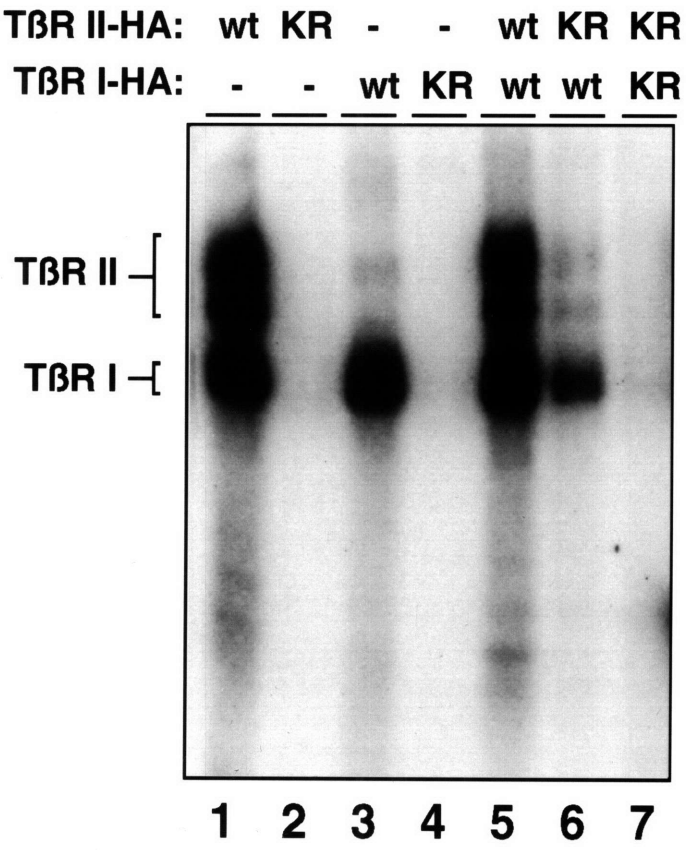
A.2 Casein as exogenous substrate

As shown in Figure A.2, both wild-type TGF-ßRI and TGF-ßRII could phosphorylate casein in this assay. Interestingly, the activity of TGF-ßRIwt toward casein was also affected by the co-transfected TGF-ßRIIKR (Figure A.2, lanes 5 and 6). However, no quantitative increase of TGF-ßRI kinase activity upon coexpression of TGF-ßRIIwt could be observed (lanes 3 and 5 in Figure A.1 and A.2). Casein has also been used to measure the kinase activity of mutant TGF-ßRI with single or multiple amino acid substitutions in its GS domain mutants (Franzen et al., 1995).

Figure A.1 *In vitro* kinase assays for TGF-ßRII and TGF-ßRI transfected in COS cells (A)

-- Autophosphorylation

Autophosphorylation of TGFß receptors

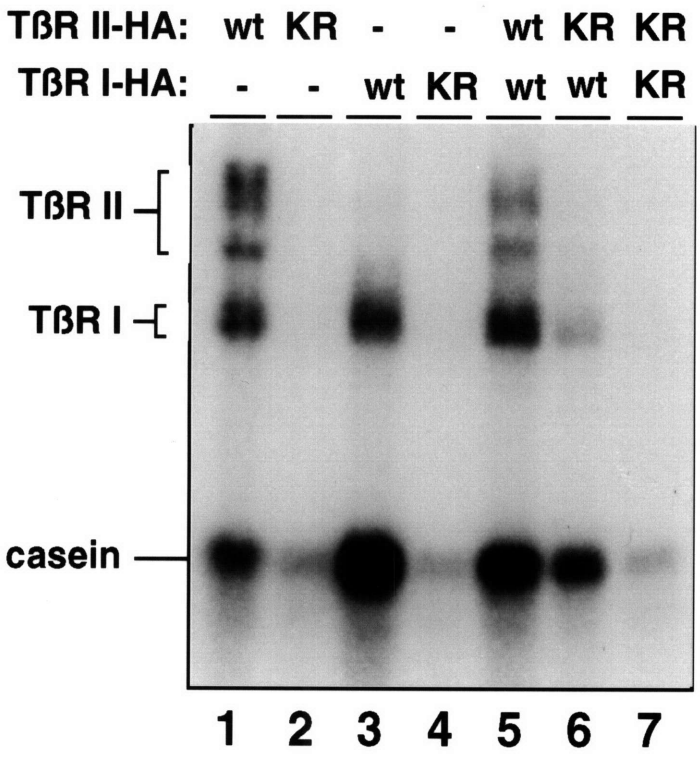


α-HA IP-kinase assay

Figure A.2 *In vitro* kinase assays for TGF-ßRII and TGF-ßRI transfected in COS cells (B)

-- Casein as exogenous substrate

Casein as a TGFß receptor substrate



α-HA IP-kinase assay

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1. Z. Zhai, **F. Chen,** J. A. Nickerson, and S. Penman. Association of Adenovirus DNA Transcription Activity with Nuclear Matrix of Host Cells, *Scientia Sinica (B)*, 32, p176 (1988).
2. **F. Chen,** and Z. Zhai. The Nuclear Matrix (review), *Chinese Journal of Cell Biology*, 11, p49 (1989).
3. **F. Chen,** L. Xu, M. Ding and Z. Zhai. Replicating Adenovirus DNA is Associated with the Nuclear Matrix of Host Cells, *Acta Biologae Experimetnalis Sinica*, 23 (2), p227 (1990).
4. **F. Chen,** and R. A. Weinberg. Biochemical Evidence for the Autophosphorylation and Transphosphorylation of Transforming Growth Factor ß Receptor Kinases, *Proc. Natl. Acad. Sci. USA*, 92, p1565-1569 (1995).
5. C. Rodriguez, **F. Chen,** R. A. Weinberg and H. F. Lodish. Cooperative Binding of Transforming Growth Factor (TGF)-ß2 to the Types I and II TGF-ß Receptors, *J. Biol. Chem.*, 270 (27), p15919-15922 (1995).
6. **F. Chen,** R. Perlman and R. A. Weinberg. Phosphorylation and Interaction of the Cytoplasmic Domains of the Type I and II Transforming Growth Factor (TGF)-ß Receptors, in preparation (1996).
7. **F. Chen,** and R. A. Weinberg. *In Vivo* Phosphorylation of the Transforming Growth Factor (TGF)-ß Receptors in HaCat and HER14 Cells, in preparation (1996).