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Phosphorylation of Ser165 in TGF-β type I receptor modulates TGF-β1-induced cellular responses

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Transforming growth factor-β (TGF-β) signals via an oligomeric complex of two serine/threonine kinase receptors denoted TGF-β type I receptor (TβR-I) and type II receptor (TβR-II). We investigated the *in vivo* phosphorylation sites in TβR-I and TβR-II after complex formation. Phosphorylation of TβR-II was observed at residues in the C-terminus (Ser549 and Ser551) and at residues in the juxtamembrane domain (Ser223, Ser226 and Ser227). TGF-β1 induced *in vivo* phosphorylation of serine and threonine residues in the juxtamembrane domain of TβR-I in a region rich in glycine, serine and threonine residues (GS domain; Thr185, Thr186, Ser187, Ser189 and Ser191), and more N-terminal of this region (Ser165). Phosphorylation in the GS domain has been shown previously to be involved in activation of the TβR-I kinase. We show here that phosphorylation of TβR-I at Ser165 is involved in modulation of TGF-β1 signaling. Mutations of Ser165 in TβR-I led to an increase in TGF-β1-mediated growth inhibition and extracellular matrix formation, but, in contrast, to decreased TGF-β1-induced apoptosis. A transcriptional activation signal was not affected. Mutations of Ser165 changed the phosphorylation pattern of TβR-I. These observations suggest that TGF-β receptor signaling specificity is modulated by phosphorylation of Ser165 of TβR-I.

Keywords: serine-threonine kinase/signal transduction/receptor/transforming growth factor-β

Introduction

Transforming growth factor-β (TGF-β) isoforms (TGF-β1, -β2 and -β3) belong to a large superfamily of structurally related dimeric proteins that regulate cell proliferation, apoptosis, differentiation and motility of many different cell types (Moses *et al.*, 1990; Roberts and Sporn, 1990). The effect induced by TGF-β is dependent on cell type and on the microenvironment surrounding the cell. TGF-β is thought to play important roles in many physiological processes such as the immune response, inflammation, embryogenesis, angiogenesis and wound healing.

TGF-β exerts its effects through interaction with multiple receptors and cell surface binding proteins (Lin and Lodish, 1993; Derynck, 1994; Massagué *et al.*, 1994; Yingling *et al.*, 1995; ten Dijke *et al.*, 1996), of which TGF-β type I and type II receptors (TβR-I and TβR-II) are essential for signal transduction (Wrana *et al.*, 1994). TβR-I and TβR-II are widely expressed on many cell types and bind TGF-β with dissociation constants in the low picomolar range (Massagué *et al.*, 1990). Cloning and sequencing of TβR-I and TβR-II cDNAs revealed that they are single transmembrane serine/threonine kinases with cysteine-rich extracellular domains (Lin *et al.*, 1992; Franzén *et al.*, 1993; Bassing *et al.*, 1994). The two kinase domains have 41% amino acid sequence similarity and each contains two short kinase inserts. TβR-II binds ligand directly, whereas TβR-I can recognize TGF-β only when complexed with TβR-II (Wrana *et al.*, 1992).

Overexpressed TβR-II is a constitutively active kinase, which transphosphorylates the ligand-recruited TβR-I on serine and threonine residues, predominantly located in a region of the juxtamembrane domain containing a GSGSGS motif (the GS domain) (Wrana *et al.*, 1994). Mutation of multiple serine and threonine residues in the GS domain impairs TGF-β responses, illustrating their importance in signal transduction (Wrana *et al.*, 1994; Franzén *et al.*, 1995; Wieser *et al.*, 1995). Ser172 and Thr176 in TβR-I were found to be dispensable for extracellular matrix (ECM) formation, but essential for the growth inhibition by TGF-β (Saitoh *et al.*, 1996). However, phosphorylation of Ser172 and Thr176 has not been reported, and a molecular mechanism remains to be elucidated. The notion that TβR-I acts downstream of TβR-II and that TβR-I phosphorylation and activation is essential and sufficient for most TGF-β-mediated responses is supported by data showing that a mutation in TβR-II which inactivates its ability to recognize TβR-I as a substrate does not signal (Cárcamo *et al.*, 1995), and that a constitutively active TβR-I mutant (threonine residue at 204 replaced by an aspartic acid residue) can signal in the absence of ligand and TβR-II (Wieser *et al.*, 1995). TGF-β induces the formation of a heteromeric complex of TβR-I and TβR-II (Wrana *et al.*, 1992; Franzén *et al.*, 1993), most likely a heterotetramer of two molecules each of TβR-I and TβR-II (Yamashita *et al.*, 1994b). Recent complementation studies between kinase-defective TβR-I and activation-defective TβR-I indicate a cooperative interaction between multiple TβR-I molecules that is essential for signal transduction (Weiss-Garcia and Massagué, 1996).

TβR-I and TβR-II have an intrinsic affinity for each other (Ventura *et al.*, 1994) and, upon overexpression, they may form a ligand-independent heteromeric complex in which both receptors are phosphorylated (Ventura *et al.*, 1994; Chen and Weinberg, 1995). Moreover, it has been reported that overexpression of TβR-I alone to a high level can lead to its constutive autophosphorylation (Chen and Weinberg, 1995).

Our understanding of the signaling mechanisms that

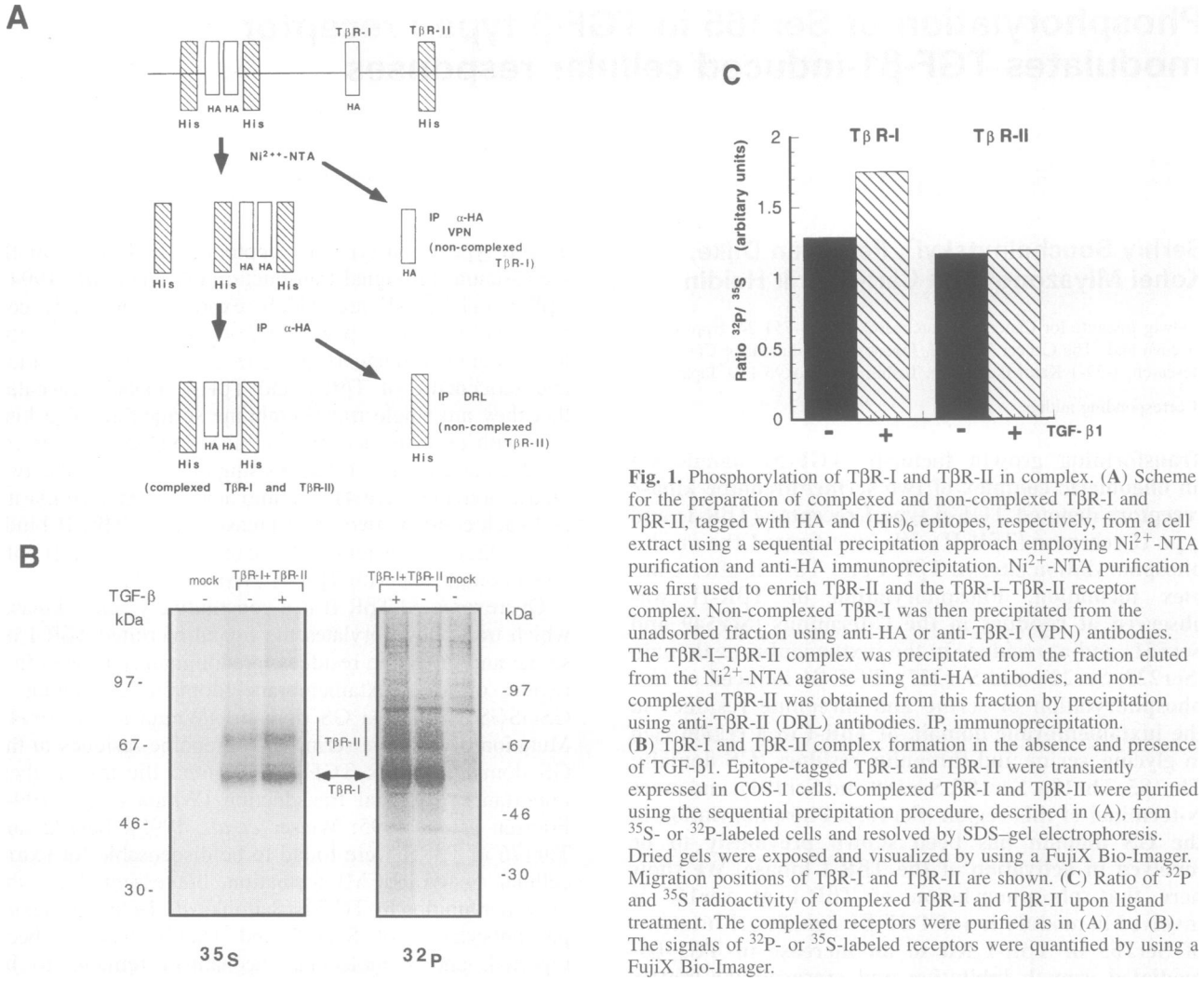

Fig. 1. Phosphorylation of TβR-I and TβR-II in complex. (**A**) Scheme for the preparation of complexed and non-complexed TβR-I and TβR-II, tagged with HA and $(His)_6$ epitopes, respectively, from a cell extract using a sequential precipitation approach employing Ni^{2+}-NTA purification and anti-HA immunoprecipitation. Ni^{2+}-NTA purification was first used to enrich TβR-II and the TβR-I–TβR-II receptor complex. Non-complexed TβR-I was then precipitated from the unadsorbed fraction using anti-HA or anti-TβR-I (VPN) antibodies. The TβR-I–TβR-II complex was precipitated from the fraction eluted from the Ni^{2+}-NTA agarose using anti-HA antibodies, and non-complexed TβR-II was obtained from this fraction by precipitation using anti-TβR-II (DRL) antibodies. IP, immunoprecipitation. (**B**) TβR-I and TβR-II complex formation in the absence and presence of TGF-β1. Epitope-tagged TβR-I and TβR-II were transiently expressed in COS-1 cells. Complexed TβR-I and TβR-II were purified using the sequential precipitation procedure, described in (A), from ^{35}S- or ^{32}P-labeled cells and resolved by SDS–gel electrophoresis. Dried gels were exposed and visualized by using a FujiX Bio-Imager. Migration positions of TβR-I and TβR-II are shown. (**C**) Ratio of ^{32}P and ^{35}S radioactivity of complexed TβR-I and TβR-II upon ligand treatment. The complexed receptors were purified as in (A) and (B). The signals of ^{32}P- or ^{35}S-labeled receptors were quantified by using a FujiX Bio-Imager.

provide a link between activated TGF-β receptors and downsteam effects of TGF-β, e.g. growth inhibition and increased ECM formation, is incomplete. Using a yeast two-hybrid interaction screen, FKBP-12 (Wang *et al.*, 1994) and farnesyl-protein transferase α (Kawabata *et al.*, 1995) have been isolated as molecules that interact with TβR-I. In addition, TRIP-1 has been isolated as a protein interacting with TβR-II using a similar method (Chen *et al.*, 1995). However, the functional significance of these interactions remains to be determined.

In the present study we describe that one of the major ligand-dependent phosphorylation sites in TβR-I is Ser165. Whereas the phosphorylation of Ser165 is not essential for TGF-β1 signaling, its mutation increases the ECM formation and growth inhibition, but decreases the apoptotic response induced by TGF-β1 in mink lung epithelial (Mv1Lu) cells. Phosphorylation of Ser165 therefore appears to modulate TGF-β1 signaling.

Results

Phosphorylation of TβR-I and TβR-II

TGF-β signals through a heteromeric complex of TβR-I and TβR-II (Lin and Lodish, 1993; Derynck, 1994; Massagué *et al.*, 1994; Yingling *et al.*, 1995; ten Dijke *et al.*, 1996). To determine the phosphorylation sites in TGF-β receptors, we used a sequential immunoprecipitation approach, by which TβR-I and TβR-II can be isolated in a heteromeric complex and in non-complexed forms from a cell extract (Wrana *et al.*, 1994) (Figure 1A). In agreement with previous data (Chen and Weinberg, 1995), ligand-independent complex formation was observed between TβR-I and TβR-II when both receptor types were overexpressed in COS-1 cells (Figure 1B). Endogenously produced TGF-β did not contribute significantly to complex formation as addition of anti-TGF-β neutralizing antibodies had no effect on complex formation. Overexpression of receptors in Mv1Lu cells also promoted complex formation in the absence of ligand (data not shown). TβR-I and TβR-II in complex were found to be phosphorylated, as revealed by isolation of the complex from [^{32}P]orthophosphate-labeled cells; addition of ligand led to an increase in complex formation and to a concomitant increase in phosphorylation of the receptors, as determined by comparison with parallel immunoprecipitation of ^{35}S-labeled receptors. Ligand addition led to an increase of the phosphorylation level of TβR-I but not of TβR-II (Figure 1C).

Consistent with previous reports (Wrana *et al.*, 1994; Chen and Weinberg, 1995), we found that the non-complexed overexpressed TβR-II was phosphorylated and that this phosphorylation was not dependent on the pres-

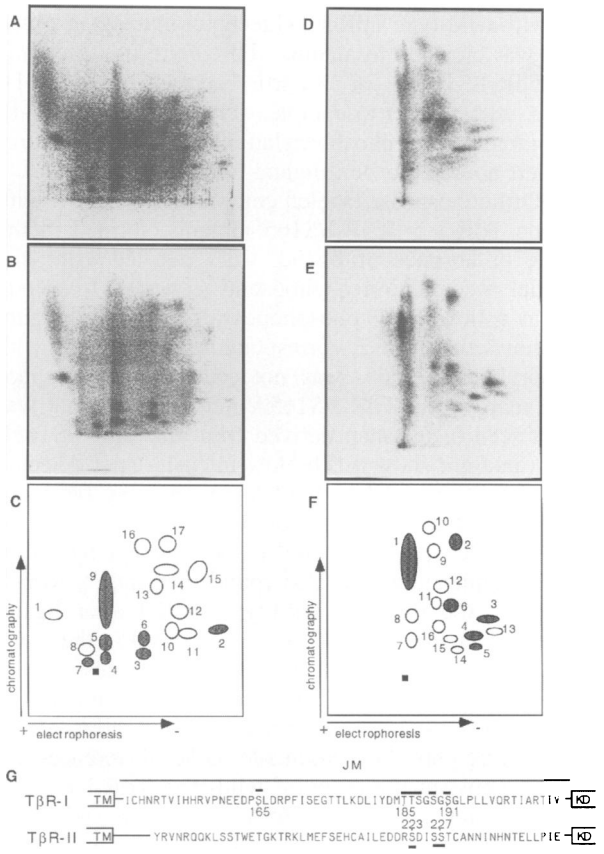

Fig. 2. *In vivo* phosphorylation sites in TβR-I and TβR-II. Epitope-tagged TβR-I and TβR-II were transiently expressed in COS-1 cells. The complexed receptors were purified, digested with trypsin and peptides were resolved by high-voltage electrophoresis and thin-layer chromatography. Plates were exposed and analyzed by using a FujiX Bio-Imager. The phosphopeptide maps of complexed TβR-II from non-treated cells (**A**) or cells treated with TGF-β1 (**B**) and a schematic presentation of the phosphopeptide map of TβR-II (**C**) are shown, as well as the phosphopeptide maps of TβR-I purified from non-treated cells (**D**) or cells treated with TGF-β1 (**E**) and a schematic presentation of the phosphopeptide maps of TβR-I (**F**). The phosphopeptide spots of type I and II receptors used for Edman degradation and phosphoamino acid analysis are shown in gray. Sample application points are shown by small black squares. (**G**) Schematic illustration of the sequences of TβR-I and TβR-II with the juxtamembrane domains (JM) *in vivo* phosphorylation sites indicated. Transmembrane domains (TM) and kinase domains (KD) of the receptors are indicated. Serine and threonine residues that are phosphorylated are indicated by bold lines.

ence of ligand. In contrast, non-complexed TβR-I expressed in transfected COS-1 and Mv1Lu cells was found not to be phosphorylated in most experiments. However, in some experiments when TβR-I was over-expressed in COS-1 cells to very high levels, non-complexed TβR-I was found in a phosphorylated form (data not shown).

Mapping of in vivo phosphorylation sites in TβR-I and TβR-II

Tryptic phosphopeptide mapping of TβR-II phosphorylated *in vivo* in COS-1 cells and isolated in a complex with TβR-I revealed 17 spots of different intensities (Figure 2A and B). We found no change in the distribution of TβR-II-derived phosphopeptides in the absence (Figure 2A) or presence (Figure 2B) of ligand; TβR-II not complexed with TβR-I also gave a similar phosphopeptide map (data not shown). Phosphoamino acid analysis of the peptides shown in gray (Figure 2C; spots 2, 3, 4, 5, 6, 7 and 9) revealed that these peptides were phosphorylated only on serine residues (data not shown). The positions of the phosphorylated serine residues were determined by release of radioactivity upon Edman degradation; alignment with serines that are present in tryptic peptides, as predicted from the TβR-II cDNA sequence, indicated that Ser549 and Ser551 in the C-terminal tail of TβR-II (Figure 2C; spot 4), and Ser223, Ser226 and Ser227 in the juxtamembrane region of TβR-II (Figure 2C; spots 6 and 7), were phosphorylated. Two phosphorylation patterns in the juxtamembrane region were observed, either Ser223, Ser226 and Ser227 were phosphorylated (spot 7), or Ser223 and Ser227 (spot 6; see Figure 2G).

Tryptic phosphopeptide maps of TβR-I phosphorylated *in vivo* in COS-1 cells and isolated in a complex with TβR-II revealed 16 spots of different intensity. In contrast to what was found for TβR-II, ligand binding and complex formation induced changes in the phosphopeptide maps of TβR-I. Whereas non-complexed TβR-I was not phosphorylated appreciably, TβR-I in complex with TβR-II was phosphorylated on several sites (Figure 2D, E and F). The phosphorylation of five peptides (spots 1, 2, 3, 4 and 5) increased further upon stimulation with TGF-β1. The different tryptic phosphopeptides were subjected to phosphoamino acid analysis and Edman degradation; by comparing the results with the predicted tryptic peptides from the TβR-I cDNA sequence, the location of several phosphorylated serine and threonine residues in the juxtamembrane region of TβR-I could be determined (Figure 2G).

Based on the release of ^{32}P radioactivity upon Edman degradation, the peptide of the major broad migrating ligand-stimulated spot (spot 1) could be aligned to the GS domain of TβR-I. This broad spot 1 consisted of multiple slightly overlapping components. Phosphoamino acid analysis revealed that peptides of spot 1 were phosphorylated both on serine and threonine residues (data not shown). We found three different patterns of phosphorylation in the GS domain, i.e. phosphorylation of Thr186, of Ser189 and Ser191, and of Thr185, Thr186, Ser187, Ser189 and Ser191 (Figure 2G). It remains to be determined if the multiple phosphorylation sites are present in one peptide or if several peptides phosphorylated at different positions co-migrate in one spot. The phosphoamino acid analysis of another major site of ligand-stimulated phosphorylation (spot 2) showed phosphorylation on serine residues (Figure 3A). The appearance of a phosphorylated residue after eight cycles in Edman degradation sequencing indicates that Ser165 in the tryptic peptide consisting of amino acid residues 158–178 was phosphorylated, since this is the only serine residue eight residues downstream of a lysine or an arginine as predicted from the TβR-I cDNA sequence (Figure 3B). The alignment data of Ser165 as a phosphorylation site were confirmed by the immunoprecipitation of radioactivity from this spot with VPN antibodies, which are made against a peptide corresponding to amino acid residues 158–178 in TβR-I (Figure 3C).

TβR-II was found to phosphorylate TβR-I at Ser165; when baculovirally expressed complexed wild-type or kinase-inactive TβR-I were purified and subjected to

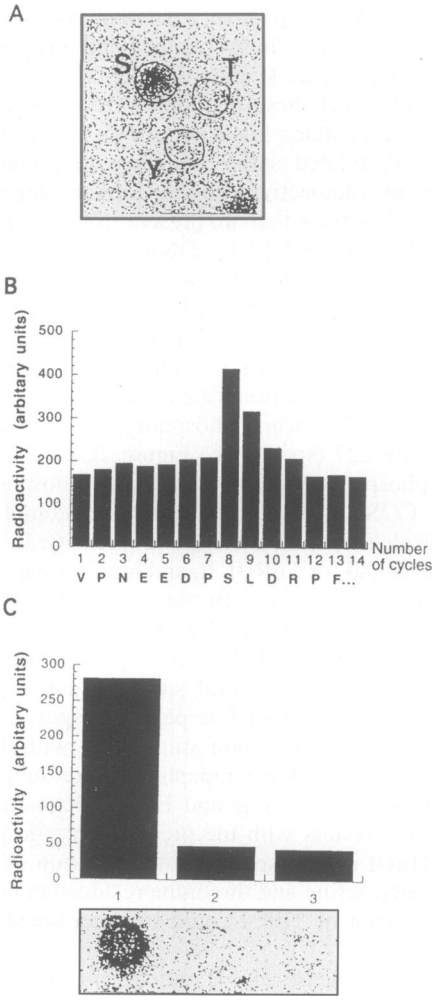

Fig. 3. Identification of Ser165 as a phosphorylation site in TβR-I. **(A)** The peptide corresponding to spot 2 of TβR-I was subjected to phosphoamino acid analysis. The migration of phosphorylated serine (S), threonine (T) and tyrosine (Y), used as standards, is shown. **(B)** The same peptide was also subjected to Edman degradation; the elution positions of ^{32}P-labeled amino acids are shown and aligned to the sequence of a putative tryptic peptide (VPN), the only one with a serine eight residues downstream of a lysine or an arginine residue. **(C)** The peptide corresponding to spot 2 was precipitated by VPN antibodies, directed against the VPN peptide (residues 158–178 in TβR-I), spotted onto a thin-layer chromatography plate and quantified by using a FujiX Bio-Imager. Radioactivities, immunoprecipitated from peptide of spot 2 by VPN antibodies (1), VPN antibodies in the presence of excess VPN peptide (2), and non-immune serum (3), and their quantitations are shown.

in vitro kinase assay followed by phosphopeptide mapping, Ser165 was phosphorylated in both cases (Figure 4A and B). That Ser165 was phosphorylated was confirmed by the observation that radioactivity from the predicted spot could be immunoprecipitated with VPN antibodies, but not by pre-immune serum. Phosphorylation of Ser165 in kinase-inactive TβR-I (Figure 4B) indicates that the phosphorylation of this residue is not dependent on TβR-I kinase activity, and thus most likely is phosphorylated directly by TβR-II.

In order to determine the functional importance of the phosphorylation of Ser165 in TβR-I, this residue was mutated to alanine, glutamic acid or aspartic acid residues. No differences were found between COS-1 cells transfected with wild-type TβR-I and receptor mutants in which Ser165 was changed to alanine (TβR-I/S165A), glutamic acid (TβR-I/S165E) or aspartic acid (TβR-I/S165D) residues, with respect to complex formation with TβR-II, overall intensities of phosphorylation (*in vivo* and *in vitro*) or in their abilities to bind ligand (data not shown).

[^{32}P]Orthophosphate-labeled complexes of TβR-II with wild-type TβR-I or TβR-I/S165 mutants, formed in the presence or absence of ligand, were purified using the sequential precipitation method and subjected to tryptic digestion followed by phosphopeptide mapping. Figure 4C–F shows that spot 2, corresponding to a peptide with phosphorylated Ser165, was not seen in the phosphopeptide map of the TβR-I/S165A mutant. This spot was also not seen in the map derived from the TβR-I/S165E mutant (data not shown). The low intensity spot detected in this area of the TβR-I/S165A mutant may be a co-migrating peptide from another part of the receptor, of which the intensity is too low to be detected by radiochemical sequencing. We also found that spot 4, which appeared in the maps of wild-type TβR-I after ligand stimulation (Figure 4C and D), was detected in the maps of the TβR-I/S165A mutant even in the absence of ligand (Figure 4E and F); however, the low amount of radioactivity in peptide 4 precluded determination of the corresponding phosphorylation site. Other differences are also seen between the maps of wild-type TβR-I (Figure 4C and D) and the TβR-I/S165A (Figure 4E and F) or TβR-I/S165E (data not shown) mutants. However, in a series of four different experiments in which the separation conditions were varied, these other differences were not found to be reproducible.

Mutation of Ser165 in TβR-I modulates TGF-β1 signaling

To determine the role of Ser165 phosphorylation in TGF-β signal transduction, we tested TGF-β1 responses in a cell line lacking a functional endogenous TβR-I (clone R4.2 of Mv1Lu cells; Laiho *et al.*, 1990), after stable transfection with wild-type or mutant TβR-I. In TβR-I mutants Ser165 was replaced with other amino acid residues (TβR-I/S165A, TβR-I/S165E or TβR-I/S165D) or with a kinase-inactive variant of TβR-I (TβR-I/K232R) under transcriptional control of the inducible human metallothionein IIA promoter. All transfectants that were used expressed TβR-I or TβR-I mutants to the same level (Figure 5A). We found that phosphorylation of Ser165 is not essential for the transduction of the effect of TGF-β1 on cell growth, since cells transfected with TβR-I/S165A or TβR-I/S165E responded to TGF-β1 addition with growth inhibition (Figure 5B). In fact, the TGF-β1-induced inhibition of cell proliferation was more pronounced in cells transfected with TβR-I/S165A, and especially TβR-I/S165E, than in cells with wild-type TβR-I (ED_{50} values of 0.41, 0.06 and 0.54 ng/ml, respectively).

TGF-β1 stimulated the biosynthesis of plasminogen activator inhibitor-1 (PAI-1) and fibronectin and their deposition in ECM of wild-type TβR-I transfected cells (Figure 6). The level of PAI-1 induction was higher in cells expressing TβR-I/S165A or TβR-I/S165E than in cells expressing wild-type TβR-I (Figure 6A and B). These differences were detected already after 4–5 h of TGF-β1 treatment (data not shown), but the effect was

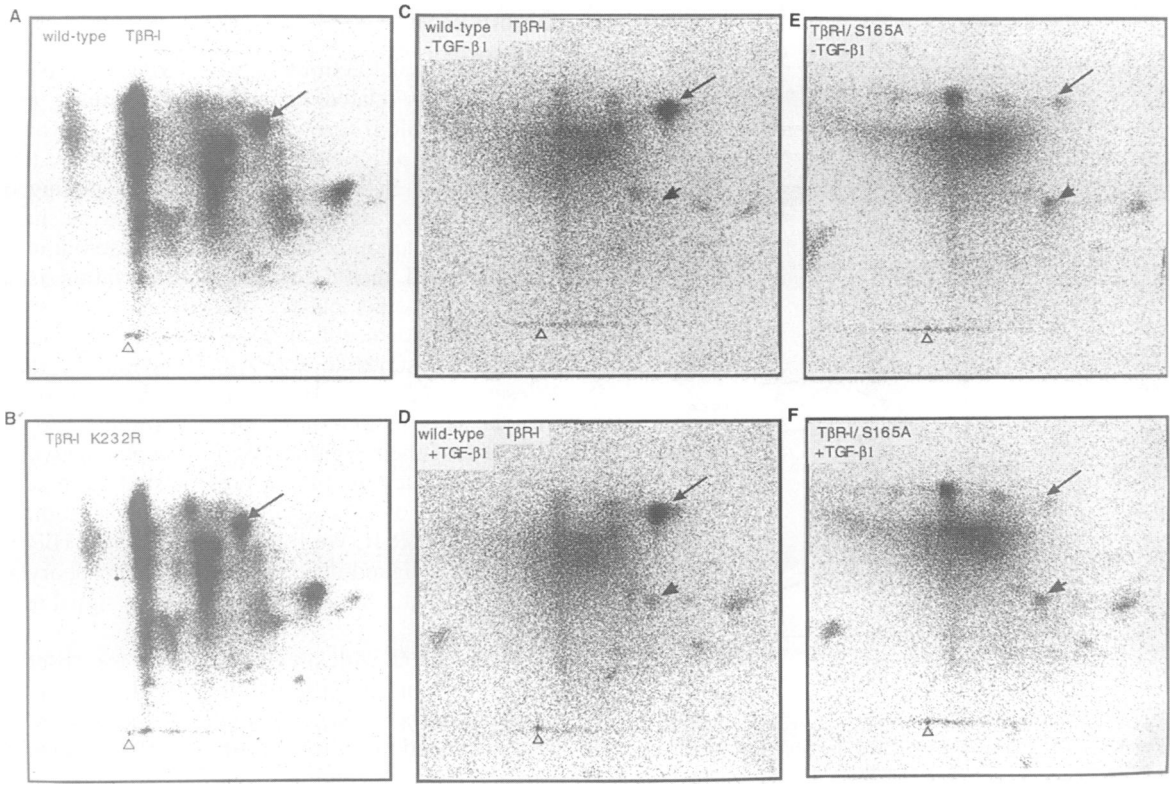

Fig. 4. Phosphopeptide maps of complexed mutated and wild-type TβR-I. Baculovirally expressed wild-type (**A**) and kinase-inactive (**B**) TβR-I, complexed with TβR-II, were purified, subjected to *in vitro* kinase assay, digested with trypsin and phosphopeptide maps were obtained. Complexes of TβR-II with wild-type TβR-I (**C** and **D**) or with TβR-I/S165A (**E** and **F**), phosphorylated *in vivo* were purified from COS cells, digested by trypsin and subjected to two-dimensional analysis. The arrows show the migration position of the Ser165-containing peptide. The arrowheads show the migration positions of a peptide, the phosphorylation of which is dependent on mutation of Ser165. Cells were treated (A, B, D and F) or not (C and E) with 10 ng/ml of TGF-β1. Sample application points are marked with open arrowheads.

more pronounced after 16–18 h of ligand treatment (Figure 6A and B). A similar pattern of response, albeit much weaker, was found for TGF-β1 stimulation of newly synthesized fibronectin, incorporated in ECM (Figure 6C and D); the cells with Ser165 of TβR-I mutated incorporated 1.8–2.0 times more fibronectin into ECM than did wild-type TβR-I cells. In this assay, no difference between TβR-I/S165A and TβR-I/S165E cells was observed. TGF-β1 had no effect on ECM formation in non-transfected cells or TβR-I/K232R cells (Figure 6 and data not shown). To investigate the effect of the Ser165 mutations in TβR-I on cell behavior in dense cultures, we cultured cells transfected with inducible vectors encoding wild-type TβR-I, TβR-I/K232R, TβR-I/S165A, TβR-I/S165D or TβR-I/S165E for 3 days to confluency. Thereafter, cells were cultured with or without $ZnCl_2$ to induce the expression of the receptors, and with various concentrations of TGF-β1 for 7–10 days with fresh media replacement every day. No differences were observed for the wild-type and mutant receptor-transfected cells in the absence of induction of TβR-I expression (data not shown). Upon induction of receptor expression, TGF-β1 showed no effect on cells with wild-type TβR-I (Figure 7A and B), TβR-I/S165A (Figure 7C and D) or TβR-I/K232R cells (data not shown). However, cells with TβR-I/S165E (Figure 7E–G) or TβR-I/S165D (data not shown) showed TGF-β1-dependent cell overgrowth and formation of three-dimensional structures after 4–5 days in dense cultures. The shape of the structures is not regular and it is not likely to be derived from a single cell (Figure 7G). The appearence of these structures may be related to the effect of Ser165 mutants on ECM formation.

The effects of TGF-β1 on growth inhibition, fibronectin biosynthesis and deposition in ECM, and PAI-1 induction, suggest that the mutations of Ser165 to alanine, and especially to glutamic acid, give TβR-I a higher signal transducing potential in these assays. However, no difference was found between wild-type TβR-I, TβR-I/S165A and TβR-I/S165E cells in the TGF-β1 induction of luciferase expression, controlled by PAI-1 promoter and TPA-responsive elements in transfected Mv1Lu and COS-1 cells (Figure 8), and in TGF-β1-mediated inhibition of cell migration as measured by the migration of cells into a defined area in dense cultures from which cells have been scraped off (data not shown).

Expression of wild-type TβR-I in R4.2 cells restored ligand-induced apoptosis (Figure 9, lanes 13–16). Interestingly, the TGF-β1-stimulated accumulation of apoptotic bodies and DNA ladder formation occurred less efficiently for TβR-I/S165A cells and especially for TβR-I/S165E cells, when compared with wild-type TβR-I cells, and was not seen at all in TβR-I/K232R cells (Figure 9, lanes 1–12). Thus, the mutation of Ser165 to alanine or glutamic acid residues weakens the effect of TGF-β1 on apoptosis, whereas the effect on growth inhibition and ECM formation is strengthened.

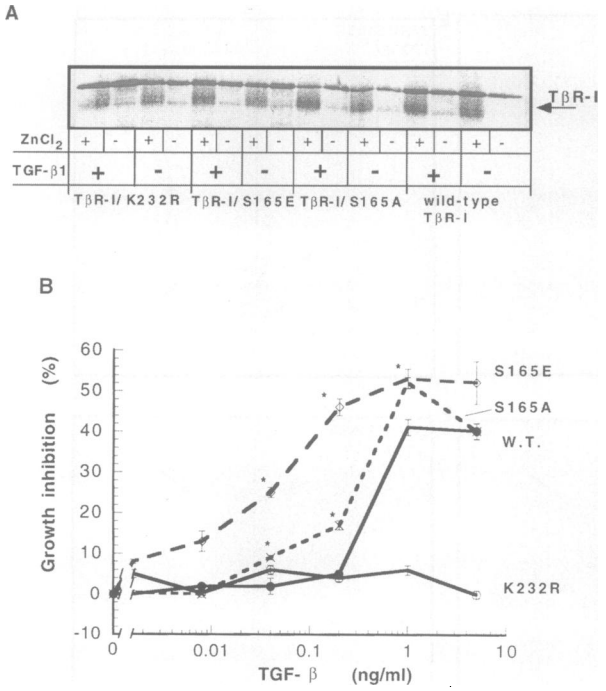

Fig. 5. Analysis of TGF-β1 signaling in cells transfected with TβR-I and TβR-I mutants. (**A**) Expression of TβR-I in stably transfected cells. R4.2 cells were stably transfected with different constructs of TβR-I in pMEP4 vector. Chosen cell pools were ^{35}S-labeled, treated with 50 μM $ZnCl_2$ and/or TGF-β1 (10 ng/ml), and TβR-I were immunoprecipitated with anti-HA antibodies. Immunoprecipitates were resolved by SDS–gel electrophoresis, followed by visualization and quantitation using a FujiX Bio-Imager. (**B**) Analysis of growth inhibition in response to TGF-β1. R4.2 cells stably transfected with wild-type TβR-I and TβR-I mutants were subjected to a [^{3}H]thymidine assay in the presence or absence of different concentrations of TGF-β1. The percentage of growth inhibition was calculated by measuring decrease relative to the [^{3}H]thymidine incorporation into cells in the absence of TGF-β1. * $P<0.001$.

Discussion

TGF-β exerts its cellular signals through cooperation of two distantly related serine/threonine kinase receptors, TβR-I and TβR-II (Lin and Lodish, 1993; Derynck, 1994; Massagué *et al.*, 1994; Yingling *et al.*, 1995; ten Dijke *et al.*, 1996). Subsequent to the cDNA cloning of both receptor types, our understanding of the mechanism of signal transduction of TGF-β has increased dramatically. The proposed model for receptor activation is distinct from that of other classes of cytokine receptors in that phosphorylation occurs in a sequential manner with TβR-I acting as a substrate for TβR-II (Wrana *et al.*, 1994; Wieser *et al.*, 1995). However, little is yet known about the phosphorylation sites that are present in TβR-I and TβR-II and their significance in receptor activation and signal transduction. In the present study, we report the identification of several *in vivo* phosphorylation sites in TβR-I and TβR-II. We found that Ser165 in TβR-I is one of the major ligand-dependent sites of phosphorylation in TβR-I and that it has an important role in signal regulation of TGF-β1.

In agreement with previous reports, we observed that overexpression of TβR-I and TβR-II induced ligand-independent heteromeric complex formation, which has been attributed to an intrinsic affinity of TβR-I and TβR-II for each other (Ventura *et al.*, 1994; Chen and Weinberg, 1995). TGF-β1 addition led to a further increase in complex formation, probably by stabilizing the interaction between the receptors in the complex. In the complex, both TβR-I and TβR-II were found to be phosphorylated, whereas non-complexed TβR-I was found not to be phosphorylated unless it was overexpressed to very high levels. In contrast, TβR-II was found to be phosphorylated also in non-complexed form. These observations are consistent with previous data showing that TβR-II forms a homodimer (Chen and Derynck, 1994; Henis *et al.*,

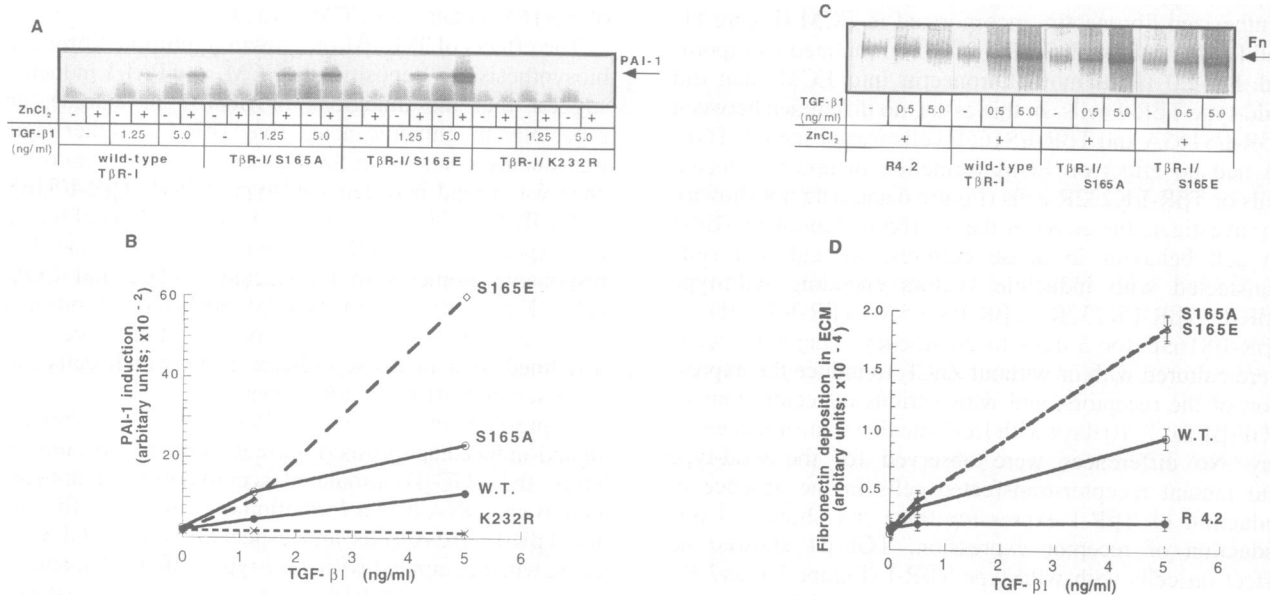

Fig. 6. Mutation of Ser165 in TβR-I leads to an increase of TGF-β1-stimulated extracellular matrix formation. Cells stably transfected with wild-type or mutant TβR-I constructs were treated with 50 μM $ZnCl_2$ to induce expression of receptors and then treated with TGF-β1 for 16–18 h. Cells were ^{35}S-labeled with 'ProMix' for the last 2 h of incubation and proteins in extracellular matrix were resolved by SDS–gel electrophoresis, visualized and quantitated using a FujiX Bio-Imager. TGF-β1 induction of PAI-1 biosynthesis (**A**) and fibronectin incorporated into extracellular matrix (**C**) by cells expressing wild-type TβR-I or TβR-I mutants are shown; quantitation of data is presented in (**B**) and (**D**), respectively.

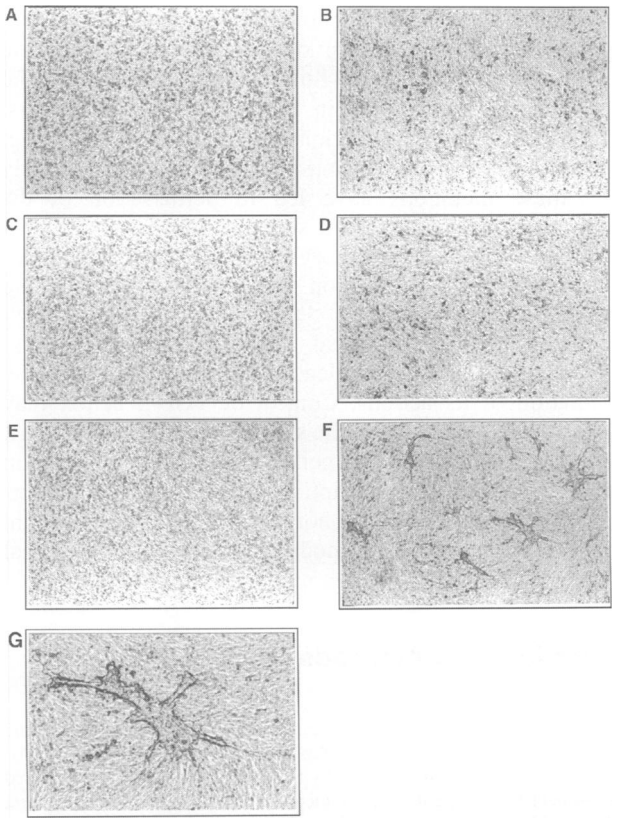

Fig. 7. TGF-β1 induces cell overgrowth in TβR-I/S165E cells. Cells stably transfected with wild-type TβR-I (**A** and **B**), TβR-I/S165A (**C** and **D**) or TβR-I/S165E (**E**, **F** and **G**) were kept in dense cultures; the medium was changed every day in the absence (A, C and E) or presence (B, D, F and G) of TGF-β1 (5 ng/ml) and 50 μM $ZnCl_2$. Photographs were taken after 5 days of culturing. Representative photographs from four experiments are shown. (G) represents a picture of one of the structures, formed by cell overgrowth, at higher magnification.

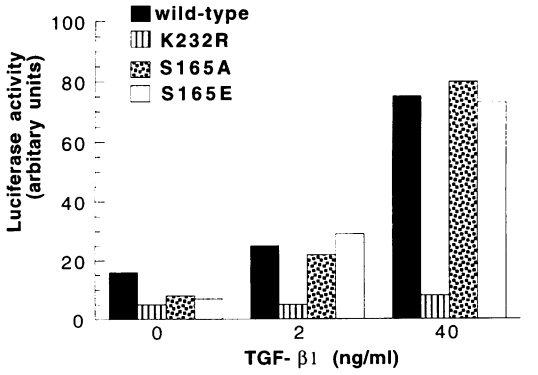

Fig. 8. Effect of TβR-I Ser165 mutations on gene expression. R4.2 cells, stably transfected with wild-type TβR-I or TβR-I mutants, were transiently transfected with p3TP–Lux plasmid, and receptor expression was induced by treatment with $ZnCl_2$. Luciferase activity was determined after stimulation with different concentrations of TGF-β1, as described in Materials and methods (representative of five experiments).

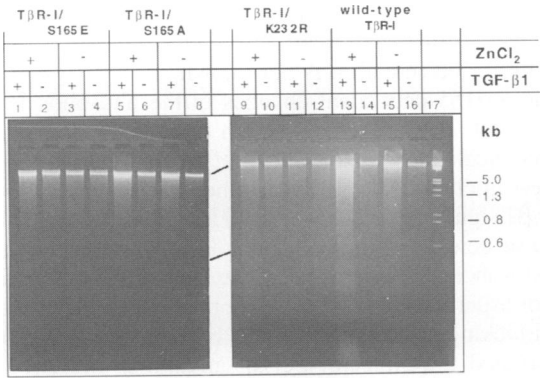

Fig. 9. Mutation of Ser165 in TβR-I decreases the ability of TGF-β1 to stimulate apoptosis. R4.2 cells expressing wild-type TβR-I (lanes 13–16), TβR-I/K232R (lanes 9–12), TβR-I/S165A (lanes 5–6) or TβR-I/S165E (lanes 1–4) were treated or not with $ZnCl_2$ to induce receptor expression. Cells were then stimulated or not with 5 ng/ml TGF-β1. DNA prepared from apoptotic bodies was analyzed by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining. Migration of DNA molecular weight standards is shown in lane 17. Essentially the same results were obtained in six different experiments and in experiments with two different cell preparations.

1994) and is a constitutively active kinase in the absence of ligand (Wrana *et al.*, 1994). Identical tryptic phosphopeptide maps were found for TβR-II whether it was stimulated or not with ligand, and whether it occurred in complex with TβR-I or not. Phosphoamino acid analysis of isolated tryptic fragments from TβR-II showed phosphorylation only on serine residues, consistent with previous findings (Wrana *et al.*, 1994). In TβR-II, Ser549 and Ser551, located in the C-terminal tail, were identified as phosphorylation sites. Wieser *et al.* (1993) found that the TβR-II C-terminal tail was dispensable for signal transduction, as a TβR-II mutant without this domain, and thus lacking both Ser549 and Ser551, was capable of restoring TGF-β sensitivity to cells lacking TβR-II (DR mutant cells) after transfection (Wieser *et al.*, 1993).

Phosphorylation sites were also identified in the juxtamembrane domain of both TβR-I and TβR-II. In TβR-II, phosphorylation was detected at Ser223, Ser226 and Ser227, which are located a similar distance from the kinase domain as Thr186, Ser187, Ser189 and Ser191 that were identified as phosphorylation sites in the GS domain of TβR-I (Figure 2G). The number of phosphorylated residues, as opposed to their particular position in the GS domain, appears to be of importance for TβR-I signal transduction (Franzén *et al.*, 1995; Wieser *et al.*, 1995). This suggests that the phosphorylated residues have a role in the activation of the kinase domain of TβR-I by charge-induced conformational changes rather than providing docking sites for downstream elements. Consistent with this notion, we found three patterns of GS domain phosphorylations *in vivo*, which may correspond to different levels of TβR-I kinase activation. Phosphorylation in the juxtamembrane domain of TβR-II also occurs in multiple patterns and may also have a role in activation of the kinase.

Ligand treatment of cells not only led to an increase in TβR-I phosphorylation through increased complex formation, but also induced qualitative changes in the tryptic phosphopeptide pattern of TβR-I (Figure 2D–F). The latter changes may be of particular importance for full activation of TβR-I. For TβR-I, the serine and threonine residues in the GS domain, as well as Ser165, were identified as major ligand-dependent *in vivo* phosphorylation sites (Figure 2G). The mechanism behind this change and the significance for TGF-β receptor signaling remain to be elucidated. In addition, mutation of Ser165 also led to

Table I. Comparison of the biological effects of wild-type and Ser165 mutants on TβR-I signal transduction

Assay	TβR-I		
	Wild-type	Ser165Ala	Ser165Glu/ Ser165Asp
Growth inhibition	+	++	+++
ECM production (fibronectin, PAI-1)	+	+	++
Cell overgrowth	–	–	+
Induction of gene expression	+	+	+
Cell migration	+	+	+
Apoptosis	++	(+)	(+)

Response: –, none; (+), weak; +, intermediate; ++, strong; +++, very strong.

ligand-independent appearance of spot 4 in the tryptic phosphopeptide maps of TβR-I, which for wild-type TβR-I appeared only after ligand treatment. Whether this change reflects an influence of Ser165 on the TβR-I phosphorylation pattern or is due to structural changes as a result of the introduced mutation is not known.

The Ser165-dependent changes in the phosphorylation patterns of TβR-I (Figure 4C–F) and the strong ligand dependence of phosphorylation of Ser165 increased our interest in elucidating its involvement in the regulation of TGF-β1 signaling. We mutated Ser165 of TβR-I to alanine, glutamic acid or aspartic acid residues, thereby abrogating the phosphorylation of this residue. Mutations to glutamic acid or aspartic acid residues were made with the intention of mimicking the negative charge of the phosphate group. We studied the effect of Ser165 mutations in TβR-I on growth inhibition, gene expression, ECM production, contact inhibition, cell migration and apoptosis induced by TGF-β1 using Mv1Lu cells. In none of these assays did we find an abrogation of the TGF-β1 response or ligand-independent signal in any of the Ser165 mutations. However, we observed that Ser165 mutations can modulate the effects of TGF-β1 in a positive or negative way, depending on the particular response that was measured (Table I). The mutation of Ser165 led to higher signaling potential for TGF-β1 in the growth inhibition assay (Figure 5B) and in ECM production (Figure 6). In addition, in cells with TβR-I/S165E or TβR-I/S165D, but not in cells with wild-type TβR-I, TGF-β1 was able to induce cell overgrowth (Figure 7). A certain threshold appears necessary for this response, because in TβR-I/S165A cells TGF-β1 showed no effect. The observed cell overgrowth may be related to the stimulation of substrate-independent proliferation of NRK cells by TGF-β (Roberts *et al.*, 1981), in which induction of ECM formation plays an important role.

In contrast, we found a decrease in TGF-β1-stimulated apoptosis in cells with a Ser165 mutant of TβR-I compared with wild-type TβR-I cells (Figure 9). Thus, TβR-I phosphorylation at Ser165 appears important for signal regulation of the activated TGF-β receptor. We did not detect any differences between wild-type TβR-I and TβR-I/Ser165 mutants in their cell migration and induction of gene expression using the p3TP–Lux reporter construct (Figure 8), which suggests that the signaling of these effects is not regulated by Ser165 phosphorylation. The appearence of effects of Ser165 mutants on PAI-1 protein synthesis and the absence of differences in luciferase expression from the p3TP–Lux reporter construct may be explained by differences in respective gene promoters. The fact that Ser165 mutations affects TGF-β1 signaling, without affecting TβR-I kinase activity, makes it unlikely that these mutations have led to perturbation of the conformation of TβR-I and supports the possibility that phosphorylation–dephosphorylation of Ser165 has a regulatory role in the interaction with downstream signaling molecules.

TGF-β is multifunctional and its effects depend on the cellular context, implicating a need for coordinate regulation of its actions. Control of TGF-β action may exist at different levels in the signal transduction pathway, from regulation of TGF-β gene expression to modulation of TGF-β-induced transcriptional responses. Our results suggest that Ser165 phosphorylation plays an important role in this process by modulating multiple biological effects of TGF-β1.

Materials and methods

Cell lines

COS-1 and Mv1Lu cells were obtained from American Type Culture Collection. Mv1Lu cells that lack functionally active TβR-I (R4.2 cells; Laiho *et al.*, 1990), were provided by Dr J.Massagué. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) with 10% fetal bovine serum (FBS; Gibco-BRL), 100 U/ml of penicillin and 50 μg/ml of streptomycin. *Spodoptera frugiperda* H5 cells were cultured in IPL-41 medium (JRH) containing 10% FBS supplemented with tryptose phosphate broth.

Constructs and cell transfection

cDNAs for TβR-I tagged with a hemagglutinin epitope and TβR-II tagged with $(His)_6$, cloned in the pCMV-5 vector (Wrana *et al.*, 1994), were obtained from Dr J.Massagué. Mutant forms of receptors were generated by PCR with mutagenic primers using receptor DNAs as template. All mutant constructs were sequenced over the regions that were exchanged with the parental receptor plasmids. COS-1 cells were transiently transfected with 1 μg of TβR-I vector and 7 μg of TβR-II vector per 40 mm dish by the calcium phosphate precipitation method using the MBS mammalian transfection kit (Stratagene), following the manufacturer's protocol. For stable transfection in R4.2 cells, all tagged receptor cDNAs were cloned in pMEP4 (Invitrogen) under transcriptional control of the metallothionein promoter. Transfection was done using the calcium phosphate precipitation method. Selection of transfectants was performed in the presence of 100 U/ml of hygromycin. We obtained four cell pool cultures, transfected with wild-type TβR-I, TβR-I/K232R, TβR-I/S165A and TβR-I/S165E, respectively, and 60 individual clones, 12 for every above-mentioned TβR-I variant and 12 for TβR-I/S165D. All these cultures and clones were tested for induction of TβR-I expression upon $ZnCl_2$ treatment. The one pool cell culture and two clones for every mutant with the same level of the receptor expression were used in biological assays.

Purification of TGF-β receptors

Cells were washed three times with phosphate-buffered saline (PBS) and lysed in lysis buffer [LB; 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin]. TβR-I and TβR-II heteromeric complex and non-complexed $(His)_6$-tagged TβR-II were precipitated from clarified extract with Ni^{2+}-NTA agarose (Qiagen). Non-precipitated non-complexed HA-tagged TβR-I subsequently was immunoprecipitated from the unadsorbed fraction using anti-HA antibodies (12CA5, Babco) or anti-TβR-I (VPN) antibodies (Franzén *et al.*, 1993). Immunocomplexes were collected on protein A–Sepharose beads (Immunosorb; EC Diagnostics AB). Ni^{2+}-NTA agarose beads were washed three times with 25 mM imidazole in LB and bound receptors were eluted in 250 mM imidazole in LB. The eluate was subjected to immunoprecipitation with anti-HA antibodies to

enrich for the TβR-I and TβR-II heteromeric complex and with rabbit peptide antisera against TβR-II (DRL) (Yamashita *et al.*, 1994a) to enrich for the non-complexed TβR-II.

[^{32}P]Orthophosphate labeling of cells, tryptic phosphopeptide mapping and two-dimensional phosphoamino acid analysis

Cells were labeled in phosphate-free medium supplemented with 0.5% dialyzed FBS, 15 mM HEPES, pH 7.2, and 1.0 mCi/ml of [^{32}P]orthophosphate for 3 h. During the last 15 min of incubation, TGF-β1 was added as mentioned in the figure legends. Cells were lysed and receptors isolated as described above, separated by SDS–gel electrophoresis and electrotransferred to a nitrocellulose filter (Hybond C-extra; Amersham). For tryptic phosphopeptide mapping, receptor bands were localized by exposure on a FujiX Bio-Imager (Fuji), excised from the filter and digested *in situ* with trypsin (modified sequencing grade; Promega). Two-dimensional phosphopeptide mapping was done using the Hunter thin-layer electrophoresis apparatus (HTLE-7000; CBS Scientific), essentially as described by Boyle *et al.* (1991). First dimension electrophoresis was performed in pH 1.9 buffer (formic acid/glacial acetic acid/double-distilled water; 44:156:1800 by vol.) for 35 min at 2000 V, and second dimension ascending thin-layer chromotography in isobutyric acid buffer (isobutyric acid/*n*-butanol/pyridine/glacial acetic acid/double-distilled water; 1250:38:96:58:558 by vol.). After exposure, phosphopeptides were eluted from the plates in pH 1.9 buffer and lyophilyzed. The fractions were then subjected to two-dimensional phosphoamino acid analysis and, in parallel, automated Edman degradation. For Edman degradation, phosphopeptides were coupled to Sequelon-AA membranes (Millipore) according to the manufacturer's instructions and sequenced on an Applied Biosystems Gas Phase Sequencer Model 470A. Released phenylthiohydantoin amino acid derivatives from each cycle were spotted onto thin-layer chromatography plates. The radioactivity in each spot was quantitated by exposure to a FujiX Bio-Imager.

For phosphopeptide mapping, H5 cells at ~80% confluency were co-infected with TβR-I and TβR-II baculoviruses at an m.o.i. of 10, and 5 p.f.u./cell. Cells were used 48 h post-infection. Purification of receptors was done as described above, purified receptors were subjected to *in vitro* kinase assay as described (Franzén *et al.*, 1995), and tryptic phosphopeptide mapping was done.

Growth inhibition assay

Cells were seeded at a density of 1×10^4 cells per well in 24-well plates in DMEM with 10% FBS. Receptor expression was stimulated by incubation of the cells in medium with 0.1% FBS with 50 μM $ZnCl_2$ for 5 h. Subsequently, medium containing 3% FBS with or without 50 μM $ZnCl_2$ was added and cells were incubated with different concentrations of TGF-β1 for 22–24 h; during the last 2 h cells were labeled with 1 μCi/ml [^{3}H]thymidine (Amersham Corp.). Thereafter, the cells were fixed in 5% ice-cold trichloroacetic acid (TCA) for 20 min, washed three times with TCA, and solubilized with 1 M NaOH. The cell extract was neutralized with 1 M HCl and ^{3}H radioactivity was measured in a liquid scintillation β-counter using Ecoscint (National Diagnostics).

Extracellular matrix formation assay

Cells were seeded in 6-well plates at a density of 5×10^4 cells per well. After 18–24 h, the medium was changed to DMEM supplemented with 0.1% FBS, with or without 50 μM $ZnCl_2$. After 5 h, TGF-β1 was added to the cells and incubation prolonged for 16–18 h. To label the newly synthesized proteins, the cells were incubated in methionine-free medium MCDB (SVA, Sweden) with 50 μCi/ml of ^{35}S-labeling mixture 'ProMix' (Amersham Corp.) during the last 3 h of TGF-β1 treatment. Aliquots of the medium (100 μl) or secreted proteins bound to gelatin–Sepharose (Pharmacia) were analyzed by SDS–gel electrophoresis. For ECM isolation, the cells were removed by washing on ice once in PBS, three times in 10 mM Tris–HCl, pH 8.0, 0.5% sodium deoxycholate, and 1 mM PMSF, twice in 20 mM Tris–HCl, pH 8.0, and once in PBS. ECM proteins were scraped off and extracted into SDS sample buffer containing 10 mM dithiothreitol. Secreted proteins and ECM proteins were analyzed by SDS–gel electrophoresis, followed by fluorography using Amplify (Amersham Corp.). The gels were exposed and quantified using a FujiX Bio-Imager. PAI-1 was identified as a 45 kDa protein in the ECM fraction (Laiho *et al.*, 1991). Fibronectin was identified as a 230 kDa protein (Laiho *et al.*, 1991) and was present both in the soluble fraction and in the ECM fraction.

Transcriptional response assay

Stable transfectants of TβR-I were transiently transfected with p3TP–Lux (Cárcamo *et al.*, 1995), as described above. The following day, the cells were washed extensively with PBS to remove calcium phosphate precipitates. Subsequently, the cells were incubated in DMEM with 10% FBS for 16–20 h. Thereafter, the receptor expression was induced by treatment of the cells with 50 μM $ZnCl_2$ in DMEM supplemented with 0.1% FBS for 5 h, after which TGF-β1 was added. Luciferase activity in the cell lysate was measured after 22–24 h using the luciferase assay system (Promega Biotec), according to the manufacturer's protocol using an LKB Luminometer (LKB-Bromma).

Cell overgrowth assay

Cells stably transfected with wild-type TβR-I, TβR-I/S165A, TβR-I/S165E, TβR-I/S165D or TβR-I/K232R were cultured for 3 days to reach confluency and, thereafter, in the presence or absence of 50 μM $ZnCl_2$ and TGF-β1. The medium was changed every day for 7–10 days. Cells were examined regularly by microscope.

Apoptosis assay

Cell seeding, induction of receptor expression and treatment with TGF-β1 were done as defined for the growth inhibition assay, except that cells were incubated in medium with 0.1% FBS for 13 h before harvesting the detached material. Apoptotic bodies were treated with 0.5 mg/ml proteinase K (Sigma) for 1 h at 50°C and 0.2 mg/ml RNase (Sigma) for 20 min at 60°C to remove protein and RNA, respectively. Apoptotic DNA was analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

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References

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 β type I receptor that signals to activate gene expression. *Science*, **263**, 87–89.

Boyle,W.J., Van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Cárcamo,J., Zentella,A. and Massagué,J. (1995) Disruption of transforming growth factor β signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol. Cell. Biol.*, **15**, 1573–1581.

Chen,F. and Weinberg,R.A. (1995) Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases. *Proc. Natl Acad. Sci. USA*, **92**, 1565–1569.

Chen,R.-H. and Derynck,R. (1994) Homomeric interactions between type II transforming growth factor-β receptors. *J. Biol. Chem.*, **269**, 22868–22874.

Chen,R.-H., Miettinen,P.J., Maruoka,E.M., Choy,L. and Derynck,R. (1995) A WD-domain protein that is associated with and phosphorylated by the type II TGF-β receptor. *Nature*, **377**, 548–552.

Derynck,R. (1994) TGF-β-receptor-mediated signaling. *Trends Biochem. Sci.*, **19**, 548–553.

Franzén,P., ten Dijke,P., Ichijo,H., Yamashita,H., Schulz,P., Heldin,C.-H. and Miyazono,K. (1993) Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. *Cell*, **75**, 681–692.

Franzén,P., Heldin,C.-H. and Miyazono,K. (1995) The GS domain of the transforming growth factor-β type I receptor is important in signal transduction. *Biochem. Biophys. Res. Commun.*, **207**, 682–689.

Henis,Y.I., Moustakas,A., Lin,H.Y. and Lodish,H.F. (1994) The types II and III transforming growth factor-β receptors form homo-oligomers. *J. Cell Biol.*, **126**, 139–154.

Kawabata,M., Imamura,T., Miyazono,K., Engel,M.E. and Moses,H.L. (1995) Interaction of the transforming growth factor-β type I receptor with farnesyl-protein transferase-α. *J. Biol. Chem.*, **270**, 29628–29631.

Laiho,M., Weis,F.M.B. and Massagué,J. (1990) Concomitant loss of transforming growth factor (TGF)-β receptor types I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. *J. Biol. Chem.*, **265**, 18518–18524.

Laiho,M., Rönnstrand,L., Heino,J., DeCaprio,J.A., Ludlow,J.W., Livingston,D.M. and Massagué,J. (1991) Control of JunB and extracellular matrix protein expression by transforming growth factor-β1 is independent of SV40 T antigen-sensitive growth-inhibitory events. *Mol. Cell. Biol.*, **11**, 972–978.

Lin,H.Y. and Lodish,H.F. (1993) Receptors for the TGF-β superfamily. *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-β type II receptor, a functional transmembrane serine/threonine kinase. *Cell*, **68**, 775–785.

Massagué,J., Cheifetz,S., Boyd,F.T. and Andres,J.L. (1990) TGF-β receptors and TGF-β binding proteoglycans: recent progress in identifying their functional properties. *Ann. N.Y. Acad. Sci.*, **593**, 59–72.

Massagué,J., Attisano,L. and Wrana,J.L. (1994) The TGF-β family and its composite receptors. *Trends Cell Biol.*, **4**, 172–178.

Moses,H.L., Yang,E.Y. and Pietenpol,J.A. (1990) TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell*, **63**, 245–247.

Roberts,A.B. and Sporn,M.B. (1990) The transforming growth factor-βs. In Sporn,M.B. and Roberts,A.B. (eds), *Peptide Growth Factors and Their Receptors, Part I.* Springer-Verlag, Berlin, 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. *Proc. Natl Acad. Sci. USA*, **78**, 5339–5343.

Saitoh,M., Nishitoh,H., Amagasa,T., Miyazono,K., Takagi,M and Ichijo,H. (1996) Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-β. *J. Biol. Chem.*, **271**, 2769–2775.

ten Dijke,P., Miyazono,K. and Heldin,C.-H. (1996) Signaling via hetero-oligomeric complex of type I and type II serine/threonine kinase receptors. *Curr. Opin. Cell Biol.*, **8**, 139–145.

Ventura,F., Doody,J., Liu,F., Wrana,J.L. and Massagué,J. (1994) Reconstitution and transphosphorylation of TGF-β receptor complexes. *EMBO J.*, **13**, 5581–5589.

Wang,T., Donahoe,P.K. and Zervos,A.S. (1994) Specific interaction of type I receptors of the TGF-β family with the immunophilin FKBP-12. *Science*, **265**, 674–676.

Weiss-Garcia,F. and Massagué,J. (1996) Complementation between kinase-defective and activation-defective TGF-β receptors reveals a novel form of receptor cooperativity. *EMBO J.*, **15**, 276–289.

Wieser,R., Attisano,L., Wrana,J.L. and Massagué,J. (1993) Signaling activity of transforming growth factor β type II receptors lacking specific domains in the cytoplasmic region. *Mol. Cell. Biol.*, **13**, 7239–7247.

Wieser,R., Wrana,J.L. and Massagué,J. (1995) GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex. *EMBO J.*, **14**, 2199–2208.

Wrana,J.L., Attisano,L., Cárcamo,J., Zentella,A., Doody,J., Laiho,M., Wang,X.-F. and Massagué,J. (1992) TGFβ signals through a heteromeric protein kinase receptor complex. *Cell*, **71**, 1003–1014.

Wrana,J.L., Attisano,L., Wieser,R., Ventura,F. and Massagué,J. (1994) Mechanism of activation of the TGF-β receptor. *Nature*, **370**, 341–347.

Yamashita,H., Ichijo,H., Grimsby,S., Morén,A., ten Dijke,P. and Miyazono,K. (1994a) Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-β. *J. Biol. Chem.*, **269**, 1995–2001.

Yamashita,H., ten Dijke,P., Franzén,P., Miyazono,K. and Heldin,C.-H. (1994b) Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. *J. Biol. Chem.*, **269**, 20172–20178.

Yingling,J.M., Wang,X.-F. and Bassing,C.H. (1995) Signaling by the transforming growth factor-β receptors. *Biochim. Biophys. Acta*, **1242**, 115–136.

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