Positive and negative regulation of type II TGF- β receptor signal transduction by autophosphorylation on multiple serine residues

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The type II transforming growth factor- β (TGF- β) receptor Ser/Thr kinase (T β RII) is responsible for the initiation of multiple TGF- β signaling pathways, and loss of its function is associated with many types of human cancer. Here we show that T β RII kinase is regulated intricately by autophosphorylation on at least three serine residues. Ser213, in the membrane-proximal segment outside the kinase domain, undergoes intra-molecular autophosphorylation which is essential for the activation of T β RII kinase activity, activation of T β RI and TGF- β -induced growth inhibition. In contrast, phosphorylation of Ser409 and Ser416, located in a segment corresponding to the substrate recognition T-loop region in a three-dimensional structural model of protein kinases, is enhanced by receptor dimerization and can occur via an inter-molecular mechanism. Phosphorylation of Ser409 is essential for T β RII kinase signaling, while phosphorylation of Ser416 inhibits receptor function. Mutation of Ser416 to alanine results in a hyperactive receptor that is better able than wild-type to induce T β RI activation and subsequent cell cycle arrest. Since on a single receptor either Ser409 or Ser416, but not both simultaneously, can become autophosphorylated, our results show that T β RII phosphorylation is regulated intricately and affects TGF- β receptor signal transduction both positively and negatively.

Keywords: autophosphorylation/growth inhibition/serine-threonine kinase/signal transduction/type II TGF- β receptor

Introduction

Transforming growth factor- β (TGF- β) is a multi-potent cytokine that regulates many biological functions, including arrest of the cell cycle in G₁, production of extracellular matrix components and differentiation of many cell types (Roberts and Sporn, 1990). The signaling cell surface type I (T β RI) (Franzen *et al.*, 1993; Bassing *et al.*, 1994) and type II (T β RII) (Lin *et al.*, 1992) receptors for TGF- β , as well as receptors for activins (Mathews and Vale, 1991; Attisano *et al.*, 1992, 1993; Mathews *et al.*, 1992; ten Dijke *et al.*, 1994a) and bone morphogenic proteins (Estevez *et al.*, 1993; Koenig *et al.*, 1994; ten Dijke *et al.*, 1994b; Liu *et al.*, 1995; Rosenzweig *et al.*, 1995), form a family of proteins with a cysteine-rich extracellular

domain, a single transmembrane segment and a cytoplasmic segment with a serine/threonine kinase domain. T β RII exists as a homodimer on the cell surface even in the absence of ligand (Chen and Derynck, 1994; Henis *et al.*, 1994). However, in the absence of T β RI, T β RII homodimers are insufficient for TGF- β signal transduction (Luo and Lodish, 1996). The Ser/Thr kinase activity of T β RII is constitutively active and the receptor is autophosphorylated (Lin *et al.*, 1992). Binding of TGF- β 1 to the extracellular domain of T β RII does not affect receptor kinase activity (Wrana *et al.*, 1994; Chen and Weinberg, 1995). Rather, it causes the formation of a heteromeric complex containing T β RI and T β RII (Wrana *et al.*, 1992) followed by transphosphorylation of T β RI by the T β RII kinase (Wrana *et al.*, 1994). Phosphorylation of T β RI by T β RII is thought to activate its kinase activity and allow it to phosphorylate and activate downstream substrate(s). Both T β RI and T β RII kinase activities are required for signal transduction (Lin *et al.*, 1992; Wrana *et al.*, 1992; Franzen *et al.*, 1993; Bassing *et al.*, 1994). Mink lung cells expressing T β RII with a point mutation that disrupts its ability to transphosphorylate T β RI are insensitive to TGF- β stimulation (Carcamo *et al.*, 1995).

Mutations or deletions in T β RII can result in a loss of sensitivity to TGF- β , a process that frequently accompanies the progression of malignancy, and may contribute to the development of many types of human cancer (Kadin *et al.*, 1994; Park *et al.*, 1994; Sun *et al.*, 1994; Markowitz *et al.*, 1995; Mazars *et al.*, 1995; Knaus *et al.*, 1996). For example, colon cancer cells with microsatellite instability (RER+) harbor mutations in the T β RII gene which cluster within small repeated sequences in the T β RII gene and which are accompanied by loss of T β RII surface expression. This inactivation of T β RII is likely to be a critical step in tumor progression, rather than a simple correlate of the RER+ phenotype (Markowitz *et al.*, 1995). Truncations of T β RII or point mutations resulting in a dominant-negative T β RII were also found in gastric cancer and lymphoma (Park *et al.*, 1994; Knaus *et al.*, 1996). Therefore, T β RII is a tumor suppressor.

Here we focus on the regulation of T β RII activity by autophosphorylation, since autophosphorylation has been recognized as an important regulatory mechanism for protein kinases. Autophosphorylation on tyrosine, serine or threonine residues often induces critical structural changes around the catalytic center of the kinase that result in the activation of kinase activity. This mechanism is employed in the activation of both the insulin receptor tyrosine kinase (Hubbard *et al.*, 1994) and MAP kinase ERK2 (Zhang *et al.*, 1994). These activating autophosphorylation sites usually are located in the T-loop, within the kinase domain in close proximity to the catalytic loop and putative substrate-binding site. Autophosphorylation of tyrosine residues in kinases such as the platelet-derived

growth factor (PDGF) or epidermal growth factor (EGF) receptors also generates docking sites for downstream signal transduction proteins containing either SH2 domains or PTB domains (Marengere and Pawson, 1994; van der Geer and Pawson, 1995). Finally, autophosphorylation of certain tyrosine residues is required for endocytosis and down-regulation of many receptor protein tyrosine kinases (Cadena *et al.*, 1994; Nesterov *et al.*, 1995).

Virtually nothing is known about T β RII autophosphorylation; we do not know which residues are autophosphorylated nor the functional consequences of T β RII autophosphorylation. In particular, we do not know whether autophosphorylation regulates signal transduction by T β RII. Here we report the identification of three major autophosphorylation sites in T β RII and show that they play important roles in both positive and negative regulation of the kinase activity and signal transduction of T β RII. Two of these are located near the putative catalytic loop and putative substrate-binding site, in a segment corresponding to the T-loop in other kinases. Thus, T β RII activity is regulated intricately by autophosphorylation and possibly by phosphorylation by other cellular kinases.

Results

The T β RII kinase undergoes autophosphorylation on multiple serine residues in the cytoplasmic domain

T β RII kinase undergoes autophosphorylation on similar sites when expressed in mammalian cells and in bacteria (Chen and Weinberg, 1995). We therefore used bacterial expressed T β RII to map the sites of autophosphorylation. When expressed in and purified from *Escherichia coli* as a GST fusion protein, the complete cytoplasmic domain of T β RII became phosphorylated *in vitro* in the presence of [γ - 32 P]ATP (Figure 1A, lane 1). The kinase-inactive K277R mutant of the T β RII cytoplasmic domain (unable to bind ATP) failed to become labeled with [γ - 32 P]ATP (Figure 1A, lane 2), indicating that phosphorylation of T β RII resulted from autophosphorylation. Phosphoamino acid analysis of the autophosphorylated T β RII cytoplasmic domain indicated that autophosphorylation occurred predominantly on serine residues (Lin *et al.*, 1992) (Figure 1B). Two-dimensional tryptic peptide mapping revealed multiple spots (Figure 1C), suggesting that there may be more than one site of autophosphorylation.

Ser213 is a major site of autophosphorylation

To map the autophosphorylation sites in T β RII, the 32 P-labeled cytoplasmic domain of T β RII, removed from GST by thrombin digestion, was digested with trypsin and the resulting peptides separated by HPLC (Figure 2A). Only two adjacent HPLC fractions contained 32 P-labeled peptides (Figure 2B). Amino acid sequencing of the two fractions revealed that they both contained a mixture of two peptides that differed only by one amino acid at the amino-terminus (Figure 2C). Since trypsin does not cleave clusters of basic amino acid residues efficiently, it is likely that the longer of the two peptides resulted from a partial tryptic digestion. These peptides contained only one serine residue: Ser213. To confirm that Ser213 was indeed phosphorylated, radio-sequencing of the two HPLC fractions was carried out. This assay monitored the release of

Fig. 1. Autophosphorylation of T β RII. The cytoplasmic domain of T β RII was expressed in and purified from *E. coli* as a GST fusion protein (GST-T β RIIc), and then subjected to *in vitro* kinase assay in the presence of [γ - 32 P]ATP. (A) Autophosphorylation assay. The reaction mixtures were analyzed by SDS-PAGE and autoradiography. The position of GST-T β RIIc is indicated by an arrow. Lane 1, wild-type T β RII cytoplasmic domain; lane 2, T β RII(K277R) cytoplasmic domain. (B) The autophosphorylated T β RII cytoplasmic domain was gel purified and then subjected to phosphoamino acid analysis as described in Materials and methods. (C) The gel-purified, autophosphorylated T β RII was digested with trypsin, and the resulting peptides resolved by two-dimensional peptide mapping as described in Materials and methods. The arrow indicates the origin of sample loading.

32 P_i from the peptide mixture after each cycle of Edman degradation. As expected, 32 P_i was released after five cycles of Edman degradation, presumably from the shorter peptide, and reached the highest level after the sixth cycle due to the presence of the longer peptide with its N-terminal arginine residue (Figure 2D).

To confirm biochemically that Ser213 is phosphorylated, Ser213 was mutated to Ala (S213A mutant) and Asp (S213D mutant), respectively. The S213A mutant mimics an unphosphorylated Ser213, and the S213D mutant, with its negatively charged side chain at position 213, resembles phosphorylated Ser213. The mutations were introduced into both the full-length T β RII and a chimeric receptor (EpoR-T β RII) containing the extracellular domain of the erythropoietin receptor (EpoR) and the cytoplasmic domain of T β RII (Luo and Lodish, 1996). When expressed transiently in 293T cells, S213A displayed markedly weakened autophosphorylation, as evidenced by both an *in vitro* autophosphorylation assay (Figure 2E) and two-dimensional tryptic mapping (Figure 2G). In contrast, the S213D mutants underwent autophosphorylation (Figure 2E). Two-dimensional tryptic peptide mapping of the 32 P-labeled S213D mutant revealed efficient phosphorylation of spots b and c in a manner indistinguishable from the wild-type receptor (Figure 2G). However, spots a and a' were missing, indicating that they correspond to the two tryptic peptides containing phosphorylated Ser213. Mutation of Ser213 to alanine impaired the kinase activity, and phosphorylation on spots b and c was greatly reduced.

Therefore, Ser213 is a major site of T β RII autophosphorylation. The negative charge conferred by the phosphorylation of this site, which is mimicked by the S213D

A Separation of peptides by HPLC **B Radioactivity counting of HPLC fractions** **C Amino acid sequencing** **D Radio-sequencing** **E** **F** **G**

Fig. 2. Identification of Ser213 as a major autophosphorylation site. The T β RII cytoplasmic domain was autophosphorylated *in vitro*, as in Figure 1, removed from GST by thrombin digestion and then digested with trypsin. (A) HPLC fractionation of tryptic peptides. The top panel depicts the eluted peptides; individual fractions were collected and (B) 32 P-labeled peptides were detected by scintillation counting. The arrows in (A) indicate the two fractions containing the 32 P-labeled peptides that were subjected to amino acid sequencing (C) and radio-sequencing (D). (E) *In vitro* autophosphorylation assay. Wild-type, S213A or S213D mutant E-RIs were expressed by transient transfection in 293T cells, then isolated by immunoprecipitation with antibodies to the extracellular domain of the EpoR. The immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of [γ - 32 P]ATP, and the reaction mixtures analyzed by SDS-PAGE and autoradiography. The position of E-RII is indicated by an arrow. (F) Ba/F3 cells expressing wild-type or S213D mutant E-RIs were stimulated with Epo for the indicated period of time. E-RIs were then isolated by immunoprecipitation and subjected to an *in vitro* kinase assay in the presence of [γ - 32 P]ATP. The reaction mixtures were analyzed by SDS-PAGE and autoradiography (upper panel). As a control for protein expression (lower panel), cells were labeled with 35 S-Express and stimulated with Epo. E-RIs were then isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography in parallel with the 32 P-labeled samples. Lanes 1–3: wild-type E-RII; lanes 4–6: E-RII(S213D). (G) Gel-purified E-RIs from (E) were digested with trypsin and analyzed by two-dimensional peptide mapping. The arrow indicates the origin of sample loading.

mutation, is required for kinase activity and autophosphorylation of other serine residues represented by spots b and c. Ser213 is located in the membrane-proximal region of T β RII outside the kinase domain.

Phosphorylation of Ser213 in T β RII is required for transducing TGF β growth inhibitory signals

To investigate the functions of autophosphorylation at Ser213 in TGF- β receptor-mediated growth inhibition,

T β RIIs containing the S213A or S213D mutations were introduced into two different mammalian cell lines and analyzed for their ability to mediate TGF- β -induced growth inhibition.

Hep3B hepatoma cells express T β RI and T β RII as well as the type III (betaglycan) TGF- β receptor, and undergo growth inhibition in response to TGF- β stimulation (Figure 3A, left panel: mock). A mutant Hep3B cell line, Hep3B.TR, lacks endogenous T β RII and therefore is unresponsive to TGF- β (Figure 3A, right panel: mock) (Inagaki *et al.*, 1993). Expression of wild-type T β RII in Hep3B.TR cells restored growth inhibition to TGF- β (Inagaki *et al.*, 1993 and Figure 3A, right panel). In contrast, expression of T β RII(S213A) in Hep3B.TR cells failed to restore growth inhibition to TGF- β (Figure 3A, right panel), even though the level of expression of T β RII(S213A) was comparable with, if not higher than, that of a similarly expressed exogenous wild-type T β RII (data not shown). Interestingly, when introduced into the TGF- β -responsive Hep3B cells containing wild-type endogenous T β RII, the mutant T β RII(S213A) receptor blocked TGF- β -induced growth inhibition (Figure 3A, left panel), suggesting that the S213A mutant is functioning in a dominant-negative manner.

In contrast, the T β RII(S213D) mutant was similar to the wild-type T β RII in its ability to restore growth inhibition to Hep3B.TR cells (Figure 3A, right panel). Expression of T β RII(S213D) in Hep3B cells had no effect on the growth response to TGF- β (Figure 3A, left panel).

Previously we constructed and analyzed chimeric receptors containing the extracellular domain of the EpoR and the transmembrane and cytoplasmic domains of T β RII (E-RII) or T β RI (E-RI) (Luo and Lodish, 1996). When co-expressed in TGF- β -responsive Ba/F3 cells, E-RI and E-RII mediate Epo-induced growth inhibition in a manner similar to that mediated by the endogenous TGF- β receptors in these cells in response to TGF- β stimulation (Luo and Lodish, 1996) (Figure 3B). Consistent with the results in Hep3B/TR cells, co-expression of E-RII(S213A) with E-RI did not result in Epo-induced growth inhibition. In contrast, co-expression of E-RII(S213D) with E-RI did result in Epo-induced growth inhibition, though not to the same extent as with wild-type E-RII (Figure 3B).

Mutation of Ser213 in T β RII does not affect its ability to form a heteromeric complex with T β RI on the cell surface

The inability of the mutant T β RII(S213A) to mediate growth inhibitory responses may be due to a deficiency in cell surface expression, to an inability to form a heteromeric complex with T β RI or to a lack of kinase activity necessary for the activation of T β RI. To investigate these possibilities, 293T cells co-expressing T β RI and S213A or S213D mutant T β RII receptors were incubated with [125 I]TGF- β 1 followed by cross-linking and immunoprecipitation with an antibody specific for the cytoplasmic domain of T β RII. Similarly to wild-type T β RII (Figure 4, lane 2), both S213A and S213D mutant receptors were expressed on the cell surface and were capable of binding to ligand. Furthermore, all could form normal heteromeric complexes with T β RI, as evidenced by co-immunoprecipitation of [125 I]TGF- β 1 cross-linked to T β RI by anti-T β RII antibodies (Figure 4, lanes 3 and 4).

Mutation of Ser213 in T β RII decreases its ability to transphosphorylate the T β RI cytoplasmic domain

Since mutation of Ser213 to alanine in T β RII decreased its ability to autophosphorylate, we next asked whether the S213A mutation affected the ability of T β RII to transphosphorylate T β RI. Since Hep3B cells express too low a level of TGF- β receptors to allow biochemical analyses, Ba/F3 cells expressing chimeric EpoR-TGF- β Rs were used in this experiment. Our previous experiments established that Epo induced transphosphorylation of E-RI by E-RII in a manner similar to TGF- β -induced transphosphorylation of T β RI by T β RII (Luo and Lodish, 1996) (Figure 5A, lanes 1 and 2). In cells expressing the E-RII(S213A) mutant receptor, Epo-induced transphosphorylation was reduced to a level <20% of that observed in cells expressing wild-type E-RII (Figure 5A, lanes 3 and 4), even though similar amounts of E-RI were expressed in these cells (Figure 5A, lower panel). E-RII(S213D) transphosphorylated E-RI in response to Epo stimulation to an extent comparable with that of the wild-type E-RII (Figure 5A, lanes 5 and 6). These experiments suggest that the S213A mutant has a reduced ability to phosphorylate the T β RI cytoplasmic domain, a step crucial to the activation of and subsequent signal transduction by T β RI.

Homodimerization of the RII cytoplasmic domain enhances its autophosphorylation

Since T β RII exists as a homodimer on the cell surface, an important question is whether dimerization of T β RII has any effect on its autophosphorylation and whether autophosphorylation occurs by an inter- or intra-molecular mechanism. Previously it has been impossible to study this question since dimerization of T β RII is constitutive even in the absence of ligand. With the EpoR-T β RII chimeric receptor, which exists as a monomer normally and requires Epo to dimerize, we could control the dimerization process and address these questions.

Figure 2F shows that homodimerization of the RII cytoplasmic domain by Epo enhances its ability to undergo autophosphorylation. In this study, Ba/F3 cells expressing E-RII or E-RII(S213D) were treated with Epo to induce dimerization, and autophosphorylation of RII was examined by an *in vitro* kinase assay. Stimulation with Epo induced a rapid increase in the autophosphorylation of both E-RII and E-RII(S213D), reaching the maximal level within 1 min after stimulation (Figure 2F). Since autophosphorylation of E-RII(S213D) was also increased by Epo-induced dimerization, the dimerization-enhanced autophosphorylation occurs on serine residues other than Ser213. Two-dimensional tryptic peptide mapping of 32 P-labeled E-RII(S213D) before and after Epo stimulation did not reveal any new spots (data not shown), suggesting that the increased autophosphorylation occurs on the peptides represented by spots b and c.

Ser409 and Ser416 are additional sites of autophosphorylation

The identity of these additional autophosphorylation sites could not be determined using the HPLC and peptide sequencing approach described above for the identification of Ser213 because peptides containing these sites could not be recovered from the HPLC column (data not shown).

Fig. 3. Growth inhibition assays. **(A)** Growth inhibition assays in Hep3B cells. Hep3B or Hep3B.TR cells stably expressing the indicated TGF- β receptors were incubated with 50 pM TGF- β 1 for 5 days. The growth of unstimulated cells was assigned a value of 100%. **(B)** Ba/F3 cells co-expressing E-RI and the indicated E-RIs were incubated with increasing concentrations of Epo for 4 days. The growth of cells was measured by cell counting as described in Materials and methods. **(C)** Growth inhibition assay of Ba/F3 cells stably expressing the S409A, S409D, S416A, S416D and S409,416A mutant E-RIs. The growth of cells was measured by cell counting as in (B).

We therefore generated point mutations in the cytoplasmic domain of T β R β II, changing 24 out of a total of 29 serine residues individually to alanine (for details, see Materials and methods). The mutant receptors were transfected transiently into 293T cells and isolated by immunoprecipit-

ation followed by *in vitro* autophosphorylation and two-dimensional tryptic mapping to determine the identity of spots b and c. Surprisingly, none of the single serine to alanine substitutions exhibited any alteration in phosphorylation of spot b (data not shown). These data

Fig. 4. Mutations of autophosphorylation sites do not affect the ability of T β RII to form a heteromeric complex with T β RI on the cell surface. 293T cells co-transfected with T β RI and various T β RIIs were affinity labeled with [125 I]TGF- β 1 and cross-linked as described in Materials and methods. T β RII and the associated T β RI were isolated by immunoprecipitation with antibodies to the cytoplasmic domain of T β RII. Lane 1, T β RI only; lane 2, T β RI plus wild-type T β RII; lane 3, T β RI plus T β RII(S213A); lane 4, T β RI plus T β RII(S213D); lane 5, T β RI plus T β RII(S416A); lane 6, T β RI plus T β RII(S409A); lane 7, T β RI plus T β RII(S409,416A).

indicated that spot b was probably composed of peptides containing multiple serine residues which are capable of being phosphorylated. We therefore analyzed mutants containing multiple point mutations.

The experiment shown in Figure 6 suggested that spot b represents a peptide containing either phosphorylated Ser409 or phosphorylated Ser416. The maps shown in this and subsequent experiments focus on the area containing spots b and c and do not include spots a and a'. When both Ser409 and Ser416 were mutated simultaneously to alanine (S409,416A), spot b was not labeled with 32 P after *in vitro* phosphorylation (Figure 6A). In contrast, mutation of either Ser409 or Ser416 individually to alanine did not affect the pattern. In particular, we did not observe a peptide with the mobility expected of one in which both Ser409 and Ser416 were phosphorylated, nor was any phosphopeptide missing in either the S409A or S416A mutants (data not shown). Thus, either Ser409 or Ser416 can become phosphorylated, but not both simultaneously on any one receptor. Importantly, mutations at Ser409 and Ser416 did not decrease the kinase activity of RII significantly (Figure 6B), and all receptors underwent autophosphorylation at spot c (Figure 6A) as well as at spots a and a' (data not shown). Mutation of Ser416 to alanine even enhanced autophosphorylation of the receptor slightly (Figure 6B).

Phosphorylation of Ser409 is essential for T β RII signaling while phosphorylation of Ser416 is inhibitory

To examine the functions of phosphorylation of Ser409 and Ser416 in T β RII signal transduction, Ba/F3 cells

stably expressing E-RI together with a chimeric E-RII receptor containing either an S409A, S409D, S416A, S416D mutation or the S409,416A double mutation were stimulated with Epo, and the growth of these cells was examined 4 days later. Mutation of Ser409 to alanine markedly reduced the ability of E-RII to mediate growth inhibition in the presence of E-RI (Figure 3C). In contrast to the S213D mutant, which exhibited a partial growth inhibitory response, the S409D mutant failed to mediate cell cycle arrest when co-expressed with E-RI (Figure 3C). This suggests that phosphorylated Ser409 affects the function of RII in a manner different from that of phosphorylated Ser213.

Importantly, mutation of Ser416 to alanine rendered E-RII hypersensitive in that the growth of Ba/F3 cells containing this mutant E-RII was inhibited to a greater extent than was that of the cells expressing wild-type E-RII (Figure 3C). These results suggest that phosphorylation at Ser409 enhances the ability of E-RII to mediate growth inhibition, but that phosphorylation at Ser416 inhibits the ability of E-RII to transduce a growth inhibitory signal. Therefore, Ser409 appears to be a positive regulatory site while Ser416 is an inhibitory site. Consistent with the inhibitory function of Ser416, mutation of Ser416 to aspartic acid, which mimicked fully phosphorylated Ser416, completely abolished the ability of the E-RII receptors to mediate growth inhibition (Figure 3C). Interestingly, when both Ser409 and Ser416 were mutated to alanine, the resultant E-RII(S409,416A) could transduce growth inhibitory signals, albeit at a level lower than normal E-RII (Figure 3C). Thus, it appears that E-RII(S409,416A) functions as an 'average' of the two single Ser $\rightarrow$ Ala mutations.

Mutations of Ser409 and Ser416 affect the transphosphorylation of the T β RI cytoplasmic domain

Mutation of Ser409 or Ser416 did not affect their ability to form a heteromeric complex with T β RI on the cell surface (Figure 4, lanes 5–7). Rather, they affect the ability of RII kinase to transphosphorylate the T β RI cytoplasmic domain. In cells co-expressing E-RI and E-RII(S409A), we consistently observed an elevated level of E-RI phosphorylation even in the absence of ligand (Figure 5B, lane 3). However, this phosphorylation of T β RI did not result in the transduction of growth inhibitory signals. Importantly, addition of Epo did not significantly increase the phosphorylation of E-RI by E-RII(S409A) (Figure 5B, lane 4). Similar results were observed in cells expressing E-RII(S409D) (Figure 5B, lanes 9 and 10). These results suggest that phosphorylation of T β RI does not always lead to activation of its kinase activity and signal transduction. It is likely that this ligand-independent phosphorylation of T β RI occurs on a site different from those phosphorylated in a ligand-dependent manner in cells expressing wild-type E-RII.

In contrast, cells expressing the mutant E-RII(S416A) receptor exhibited an increased Epo-induced phosphorylation of E-RI (about twice as much as in cells expressing E-RII, as evidenced by the ratio of 32 P incorporated relative to the amount of 35 S-labeled protein), and consequently the mobility of the phosphorylated E-RI in cells expressing E-RII(S416A) was slower than that in cells expressing

Fig. 5. Mutations of the autophosphorylation sites in T β RII affect the ability of the T β RII kinase to transphosphorylate RI. Ba/F3 cells stably co-expressing 'normal' E-RI and various mutant E-RIIs were stimulated with Epo for 2 min (+) or left unstimulated (–). Then E-RI and associated E-RII were isolated by immunoprecipitation and subjected to an *in vitro* kinase assay as described in Materials and methods. The phosphorylated E-RI and E-RII in the complex were then recovered by immunoprecipitation with antibodies to the extracellular domain of the EpoR, and resolved by SDS–PAGE (upper panels). As a control for protein expression (lower panels), in parallel the cells were biosynthetically labeled with ^{35}S -Express and then stimulated or not with Epo. The E-RI and E-RII were isolated by immunoprecipitation in the same manner as for the kinase assays, and resolved by SDS–PAGE followed by autoradiography. (A) The effect of mutations of Ser213 on the ability of the T β RII kinase to transphosphorylate RI. Lane 1, wild-type E-RII plus E-RI, without Epo; lane 2, wild-type E-RII plus E-RI, with Epo; lane 3, E-RII(S213A) plus E-RI, without Epo; lane 4, E-RII(S213A) plus E-RI, with Epo; lane 5, E-RII(S213D) plus E-RI, without Epo; lane 6, E-RII(S213D) plus E-RI, with Epo. (B) The effects of mutation of Ser409 and Ser416 on the ability of the T β RII kinase to transphosphorylate RI. Lane 1, wild-type E-RII plus E-RI, without Epo; lane 2, wild-type E-RII plus E-RI, with Epo; lane 3, E-RII(S409A) plus E-RI, without Epo; lane 4, E-RII(S409A) plus E-RI, with Epo; lane 5, E-RII(S416A) plus E-RI, without Epo; lane 6, E-RII(S416A) plus E-RI, with Epo; lane 7, E-RII(S409,416A) plus E-RI, without Epo; lane 8, E-RII(S409,416A) plus E-RI, with Epo; lane 9, E-RII(S409D) plus E-RI, without Epo; lane 10, E-RII(S409D) plus E-RI, with Epo; lane 11, E-RII(S416D) plus E-RI, without Epo; lane 12, E-RII(S416D) plus E-RI, with Epo.

wild-type E-RII (compare lanes 2 and 6 in Figure 5B). This enhancement in E-RI phosphorylation is consistent with the increased ability of the E-RII(S416A) receptor to mediate growth inhibition (Figure 3C). The double mutant E-RII(S409,416A) phosphorylated E-RI in a manner that appears to be an average of the single mutants S409A and S416A. In particular, both an elevated level of ligand-independent phosphorylation of E-RI (similar to that seen with the S409A mutant) (Figure 5B, lane 7), and an increase in ^{32}P -labeled E-RI with a slower mobility (similar to that seen with the S416A mutant) (Figure 5B, lane 8) were observed. Since the E-RII(S409,416A) double mutant was weaker than wild-type E-RII and ERII(S416A) in inducing cell cycle arrest, and since ERII(S409A) is defective in signal transduction, the ligand-independent phosphorylation of E-RI induced by E-RII(S409A) is probably inhibitory.

Interestingly, mutation of Ser416 to Asp abolished the ability of E-RII to autophosphorylate, and also to phosphorylate E-RI (Figure 5B, lanes 11 and 12). Since the S416D mutant mimics E-RII with a phosphorylated Ser416, this result further supports the conclusion that phosphorylation at Ser416 is inhibitory to the kinase activity of T β RII.

Autophosphorylation of T β RII occurs by both intra- and inter-molecular mechanisms

Studies with other protein serine and tyrosine kinases demonstrate both intra- and inter-molecular mechanisms

of autophosphorylation. An intra-molecular mechanism involves phosphorylation of the same peptide chain, while an inter-molecular mechanism involves the kinase domain of one polypeptide chain phosphorylating amino acid residues on another of the same type. To examine which mechanism is involved in T β RII autophosphorylation, two mutant EpoR–T β RIIs were co-transfected into 293T cells (Figure 7A). One is the kinase-inactive E-RII(K277R), whose ATP-binding site was destroyed by the Lys→Arg mutation. This mutant still retains all serine phosphorylation sites. The second mutant, E-RII(S409,416A), has lost the Ser409 and Ser416 autophosphorylation sites, but still retains kinase activity. If autophosphorylation occurs by an inter-molecular mechanism, the kinase-active E-RII(S409,416A) will phosphorylate serine residues on E-RII(K277R), which can be detected by two-dimensional tryptic mapping. In contrast, if autophosphorylation only occurs intra-molecularly, no phosphorylation of Ser409 or Ser416 will be observed because E-RII(K277R) cannot phosphorylate itself and E-RII(S409,416A) lacks these phosphorylatable serine residues.

Figure 7B shows that autophosphorylation at Ser409 or Ser416 can occur via an inter-molecular mechanism. Expression of E-RII(K277R) alone did not result in receptor autophosphorylation (data not shown). As shown in Figure 6A and also in Figure 7B, mutation of both Ser409 and Ser416 to alanine resulted in the loss of ^{32}P -labeled spot b (Figure 7B). However, when E-RII(K277R) was co-expressed with E-RII(S409,416A), stimulation by

Fig. 6. Mapping of Ser409 and Ser416 autophosphorylation sites. (A) Sequence of the tryptic peptide containing Ser409 and Ser416. Wild-type E-RII or E-RII(S409,416A) were isolated from transfected 293T cells by immunoprecipitation, labeled *in vitro* by autophosphorylation and analyzed by two-dimensional tryptic mapping. The arrow indicates the origin of sample loading. (B) Wild-type and mutant E-RIs were isolated from transfected 293T cells by immunoprecipitation and labeled *in vitro* by autophosphorylation. The reaction mixtures were analyzed by SDS-PAGE and autoradiography; the position of E-RII is indicated by an arrow.

Epo resulted in the phosphorylation of Ser409 and Ser416 on E-RII(K277R), as evidenced by the radiolabeling of spot b (Figure 7B). This indicates that phosphorylation of Ser409 and/or Ser416 can occur via an inter-molecular mechanism.

In a similar experiment, we co-transfected 293T cells with the E-RII(K277R) together with the E-RII(S213D) receptor. Stimulation with Epo did not result in the radiolabeling of spots a and a' (data not shown), suggesting that autophosphorylation at Ser213 occurred only by an intra-molecular mechanism.

The finding that phosphorylation on Ser409 and/or Ser416 can occur inter-molecularly is consistent with the enhancement of phosphorylation at Ser409 or Ser416 upon dimerization of the RII cytoplasmic domain (Figure 2F). In contrast, phosphorylation at Ser213 does not depend on receptor dimerization, and in this sense can be considered constitutive.

Discussion

Here we show that TβRII kinase is regulated intricately by autophosphorylation on at least three serine residues

Fig. 7. Inter-molecular autophosphorylation of Ser409 or Ser416. (A) The two mutant receptors co-expressed in 293T cells. (B) Receptors were isolated from transfected 293T cells, labeled *in vitro* by autophosphorylation and analyzed by two-dimensional tryptic mapping. The arrow indicates the origin of sample loading.

Table I. Summary of TβRII autophosphorylation

Site	Mutation	Growth inhibition	Phosphorylation	
			Auto	TβRI
Wild-type RII		+	+	+
S213	A	-	-	-
	D	+	+	+
S409	A	-	+	-
	D	-	+	-
S416	A	++	++	++
	D	-	-	-

(Table I): Ser213, in the membrane-proximal segment outside the kinase domain, and Ser409 and Ser416, located in the substrate-binding site in a segment corresponding to the T-loop in a three-dimensional structural model of protein kinases (Figure 8). Phosphorylation of Ser213 is required for the kinase activity of TβRII and its ability to phosphorylate TβRI. In contrast, phosphorylation of Ser409 does not significantly affect the kinase activity of TβRII since the S409A mutant can still undergo autophosphorylation, yet its ability to signal growth inhibitory signals is impaired dramatically. Therefore, the stimulatory effects of (phospho) Ser213 and Ser409 are achieved through distinct mechanisms. Phosphorylation of Ser416, on the other hand, inhibits the kinase activity of TβRII, since mutation of this serine to alanine enhances ligand-induced phosphorylation of TβRI and results in a

Fig. 8. Model of the structure of the PKA Ser/Thr kinase domain bound to the inhibitor PKI (Knighton *et al.*, 1991). The amino- and carboxy-termini of the kinase domain are indicated by the letters N and C, respectively and the substrate is colored red. The locations of Ser409 and Ser416 in the T β RII kinase domain were identified by aligning the sequences of the two kinases and are indicated by arrows. Ser213 is located in a region amino-terminal to the kinase domain.

hyperactive receptor that is more able than wild-type to induce T β R1 activation and subsequent cell cycle arrest. Also, the S416D mutant, which mimics the fully phosphorylated Ser416, is deficient in autophosphorylation as well as phosphorylation of T β R1. Since on a single receptor either Ser409 or Ser416, but not both simultaneously, can become autophosphorylated, our results show that T β RII phosphorylation is regulated intricately and affects TGF- β receptor signal transduction both positively and negatively.

Autophosphorylation at Ser213 also occurs via a different mechanism from that at Ser409 and Ser416. Phosphorylation at Ser213 occurs intra-molecularly while Ser409 and Ser416 can be phosphorylated via an inter-molecular mechanism. This accounts for the enhancement of autophosphorylation at Ser409 and Ser416 by dimerization. More importantly, it suggests an important function for a T β RII homodimer on the cell surface (Chen and Derynck, 1994; Henis *et al.*, 1994). Despite the fact that T β RII homodimer alone is not sufficient for signal transduction, homodimerization of T β RII may play an important role in the regulation of T β R1 activation. Dimerization of the RII cytoplasmic domain leads to increased phosphorylation of Ser409 or Ser416, which, in turn, modulates T β R1 activation. Since phosphorylation of the two serine residues has opposite effects and since both are potential substrates for other cellular kinases, it is possible that, by regulating the ratio of phosphorylation

at these two serines in response to an ever-changing cellular environment, the extent of substrate phosphorylation can be fine-tuned in an accurate and reversible manner. In particular, the ratio of phosphorylation at Ser409 and Ser416 could alter in response to TGF- β stimulation and this may provide a mechanism for the up- or down-regulation of receptor signal transduction.

Phosphorylation at Ser213

Ser213 is the primary site of autophosphorylation in T β RII and is required for the kinase activity of T β RII. Unlike many stimulatory autophosphorylation sites commonly seen in other protein kinases, which are located in the kinase domain, Ser213 is located outside the kinase domain, in a 56 amino acid segment between the membrane-spanning and the kinase domain. This segment is poorly if at all conserved among receptors of the TGF- β receptor family, in terms of both its length and amino acid sequence. Therefore, Ser213 could be a regulatory autophosphorylation site specific to T β RII. Phosphorylation at Ser213 could lead to conformational changes in the kinase domain of T β RII, resulting in the activation of its kinase activity. Since phosphorylation at Ser213 occurs via an intra-molecular mechanism, it will be interesting to determine the three-dimensional structure of the complete cytoplasmic domain of T β RII and learn how it folds such that Ser213 becomes accessible to the catalytic region of the kinase domain in the same molecule to be autophosphorylated. The three-dimensional structure of the T β RII cytoplasmic domain may also tell us how phosphorylation at Ser213 activates the kinase activity of T β RII.

Phosphorylation at serines 409 and 416

Ser409 and Ser416 are only six amino acid residues apart, yet they exert opposite effects on the signal transduction of T β RII. Phosphorylation of Ser409 is stimulatory while phosphorylation of Ser416 is inhibitory to T β RII function. Since Ser409 and Ser416 are found in the same tryptic peptide, we are not able to determine the relative phosphorylation on these residues. However, we speculate that Ser409 normally is phosphorylated to a higher extent than Ser416, since E-RII(S409,416A), lacking both serine residues, was much less able to mediate growth inhibitory responses than was the wild-type E-RII chimeric receptor.

By homology modeling with cAMP-dependent protein kinase (PKA) (Knighton *et al.*, 1991), Ser409 and Ser416 are located within the kinase domain in the so-called T-loop region that is involved directly in binding to the substrate peptide (Figure 8). Comparison of amino acid sequences of this region between T β RII and PKA reveals an 'insert' of seven amino acids that is specific for T β RII (amino acid residues 408–414), and this sequence could play an important role in the ability of T β RII to bind specific substrates. Deletion of this peptide insert markedly decreased the kinase activity of T β RII, and the resultant mutant receptor was defective in mediating growth inhibitory responses by TGF- β (data not shown). Ser409 is located within this insert, while Ser416 is just outside it.

The T-loop region is also the location where conserved autophosphorylation sites are found in many protein serine and tyrosine kinases, including PKA (Knighton *et al.*, 1991), ERK2 (Zhang *et al.*, 1994) and the insulin receptor (Hubbard *et al.*, 1994). Autophosphorylation of one or

more of these sites is required for activation of kinase activity. However, mutation of Ser409 to alanine did not significantly affect T β RII kinase activity, since the receptor still underwent autophosphorylation. Furthermore, although mutation of Ser409 to aspartate mimics phosphorylated serine, S409D could not activate T β RI for downstream signal transduction. This indicates that while phosphorylated Ser409 is essential for receptor signal transduction, it is not only the negative charge of the phosphate that is important. Phosphorylation at Ser409 could change the conformation of this region of the kinase, thereby affecting the binding of its substrates, but this change is not (solely) due to the negative charge of the phosphate group. Alternatively, phosphorylated Ser409 could be involved directly in the recruiting and binding of the substrates in a manner similar to that of phosphotyrosine binding to SH2 or PTB domains (Marengere and Pawson, 1994; van der Geer and Pawson, 1995). This latter hypothesis becomes especially intriguing following the recent discoveries that phosphoserine can mediate protein-protein interactions between the Raf1 and 14-3-3 proteins (Muslin *et al.*, 1996). Phosphorylated Ser409 could be involved directly in the binding and activation of T β RI, or it could bind and titrate cellular inhibitors away from T β RI. It may also mediate binding and consequently phosphorylation of other proteins that transduce signals from the TGF- β receptors.

In contrast, phosphorylation of Ser416 could lead to an unfavorable conformation of the catalytic region for autophosphorylation and/or substrate phosphorylation. This negative effect could be mimicked fully by mutating Ser416 to aspartate. Elimination of this effect by changing Ser416 to alanine increased both T β RII autophosphorylation and transphosphorylation of T β RI. Neither on receptors isolated from transfected cells nor on GST-kinase fusion proteins were we able to detect a phosphopeptide corresponding to one in which both Ser409 and Ser416 were phosphorylated; spot b on our two-dimensional maps corresponds to a peptide in which either Ser409 or Ser416 are phosphorylated. A key question, therefore, is how the relative phosphorylation of these two serine residues is controlled. Since these could be phosphorylated by an inter-molecular mechanism, the precise geometry by which the cytosolic domains of two T β RII receptors are juxtaposed in a homodimer could be important.

In cells expressing the mutant S409A or S409D receptors, we consistently observed an elevated level of T β RI phosphorylation in the absence of ligand stimulation. However, this phosphorylation of T β RI did not result in any significant inhibition of growth, since the cells grew normally. In contrast, in cells expressing the S416A mutant, ligand stimulation resulted in enhanced phosphorylation of T β RI and enhanced growth inhibition. These results suggest that phosphorylation of E-RI occurs on different residues when induced by wild-type, S416A or S409A E-RIIs and with different consequences. T β RI phosphorylation may not always lead to activation of receptor signal transduction.

Phosphorylation at multiple serine residues

Besides Ser213, Ser409 and Ser416, there are additional autophosphorylation sites in T β RII, in particular, those corresponding to spot 'c' on the two-dimensional tryptic

maps. Therefore, the regulation of T β RII is not as simple as had been thought before. Multiple autophosphorylation events have been observed in receptor tyrosine kinases including PDGF receptor (Kashishian *et al.*, 1992) and colony-stimulating factor-1 receptor (van der Geer and Hunter, 1993). These phosphorylated tyrosine residues mediate interactions with different downstream signaling molecules and thus provide a mechanism that enables one receptor to initiate multiple signaling pathways. Since TGF- β receptor can mediate complex biological responses, autophosphorylation on multiple serine residues may serve as a possible mechanism for the activation of different signal transduction pathways leading to these responses. Although identified as autophosphorylation sites, these serine residues may also serve as targets for other cellular kinases, and thus the activity of T β RII could be up- or down-modulated in response to an array of intracellular signals. The identification of these important serine residues serves as a starting point to dissect further the regulatory mechanisms and signal transduction pathways of the TGF- β receptors.

Materials and methods

Cells

Ba/F3, a pro-B cell line, was grown in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 10% WEHI-3 cell-conditioned medium as a source of interleukin-3. 293T, a human kidney cell line, and Bosc and Bing, two 293T cell-based retroviral packaging cell lines (Pear *et al.*, 1993) were maintained in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with 10% FBS. Hep3B, a human hepatoma cell line, was grown in minimum Eagle's medium (MEM) supplemented with 10% FBS. The Hep3B.TR cell line (Inagaki *et al.*, 1993) was derived from Hep3B cells and lacks endogenous T β RII. It was maintained in the same medium as Hep3B.

Antibodies

A polyclonal antibody directed against the extracellular domain of the EpoR (anti-EpoR) was raised in rabbits against a GST fusion protein containing the EpoR extracellular domain (Klingmuller *et al.*, 1995). A polyclonal rabbit antiserum against a peptide corresponding to a C-terminal sequence of the human T β RII (anti-RII) and a polyclonal antiserum specific for a cytoplasmic peptide in human ALK5 (anti-RI) were described previously (Moustakas *et al.*, 1995).

DNA constructs

The following serine residues in the cytoplasmic domain of T β RII were mutated to alanine: Ser198, 199, 213, 225, 228, 229, 268, 286, 295, 343, 352, 353, 363, 382, 383, 401, 409, 416, 432, 441, 449, 502, 548 and 553. Point mutations of serine residues of T β RII were introduced by PCR with *Pfu* polymerase. All constructs were confirmed by sequencing.

Transfection and infection

The pLXSN and pBABEpuro DNA constructs were transfected into 293T, Bosc or Bing cells using the Ca²⁺ phosphate precipitation method described previously (Pear *et al.*, 1993). Infection of Ba/F3 cells was performed using procedures described previously (Luo and Lodish, 1996). Infected cells were selected in a 24-well cluster plate in growth medium containing either 600 μ g/ml G418 (Gibco) for the chimeric E-RII cDNAs or 1.5 μ g/ml puromycin (Sigma) for the chimeric E-RI cDNAs. Infected Hep3B and TR cells were selected in medium containing 600 μ g/ml G418.

Growth inhibition assay

Thirty thousand Ba/F3 cells were grown for 4 days in 2 ml of medium containing varying concentrations of growth factors in one well of a 24-well culture plate. Viable cells were then counted in a Coulter Counter. The extent of growth inhibition by the various factors was calculated from the ratio of the number of viable cells compared with cultures without any added factor. Similarly, 5 $\times$ 10⁴ Hep3B cells were seeded in each well of a 6-well cluster plate. Then, 24 h later, various concentrations

of TGF- β 1 were added and the growth of the cells was evaluated 5 days later by cell counting.

Radioiodination of TGF- β and binding-cross-linking assay

Iodination of TGF- β 1 and the binding and cross-linking assay were carried out as described previously (Moustakas *et al.*, 1995).

Immunoprecipitations

Ba/F3 cells expressing the chimeric receptors were washed, resuspended at a concentration of 5×10^7 cells/ml and stimulated with 100 U of Epo (Amgen) at room temperature for 1–10 min. Cells were then lysed in NP-40 lysis buffer [1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 μ g/ml aprotinin and 2 mM phenylmethylsulfonyl fluoride (PMSF)] at a concentration of 1×10^7 cells/ml for 20 min at 4°C. Immunoprecipitation and deglycosylation with PNGase F were carried out as described previously (Luo and Lodish, 1996).

In vitro kinase assay

In vitro kinase reactions using immunoprecipitates of the chimeric receptors bound to protein A–Sepharose were carried out in 20 μ l of kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂) in the presence of 10 μ Ci [γ -³²P]ATP (3000 Ci/mmol) for 10 min at 30°C. The reaction was terminated by the addition of 5 mM EDTA, and the immune complexes were eluted from the beads by boiling in SDS gel sample buffer. The labeled chimeric receptors were then re-immunoprecipitated with antibodies to the extracellular domain of the EpoR.

Phosphoamino acid analysis and two-dimensional tryptic peptide mapping

T β RII or E-RII were labeled *in vitro* with [γ -³²P]ATP, separated by SDS–PAGE and transferred to a PVDF or a nitrocellulose membrane. Phosphoamino acid analysis was performed on proteins immobilized on PVDF membranes as described (Kamps, 1991). Tryptic digestion and two-dimensional phosphopeptide mapping were carried out with protein bound to nitrocellulose as described previously (Luo *et al.*, 1991).

Mapping of autophosphorylation sites

Bacterially expressed GST–RII, a fusion protein of GST and the T β RII cytoplasmic domain (Luo *et al.*, 1995), was digested with trypsin and the resulting peptides separated by reverse phase HPLC using a C18 column. Peptide fractions were collected based on absorbance at 210 nm and counted for the presence of ³²P. The ³²P-labeled peptides were then subjected to amino acid sequencing and radio-sequencing. To monitor the release of ³²P_i from the peptide during radio-sequencing, fractions collected after each cycle of Edman degradation were spotted on a TLC plate, and the TLC plate was exposed to an X-ray film.

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