The EMBO Journal vol.14 no.10 pp.2199–2208, 1995

GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex

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Communicated by J.Pouysségur

The TGF-β type II receptor (TβR-II) is a transmembrane serine/threonine kinase that, upon ligand binding, recruits and phosphorylates a second transmembrane kinase, TβR-I, as a requirement for signal transduction. TβR-I is phosphorylated by TβR-II in the GS domain, a 30 amino acid region preceding the kinase domain and conserved in type I receptors for other TGF-β-related factors. The functional role of seven serines and threonines in the TβR-I GS domain was investigated by mutational analysis. Five of these residues are clustered (TTSGSGSG) in the middle of the GS domain. Mutation of two or more of these residues impairs phosphorylation and signaling activity. Two additional threonines are located near the canonical start of the kinase domain, and their individual mutation to valine strongly inhibits receptor phosphorylation and signaling activity. Replacement of one of these residues, Thr204, with aspartic acid yields a product that has elevated *in vitro* kinase activity and signals anti-proliferative and transcriptional responses in the absence of ligand and TβR-II. The identification of constitutively active TβR-I forms confirms the hypothesis that this kinase acts as a downstream signaling component in the TGF-β receptor complex, and its activation by TβR-II or by mutation is necessary and sufficient for propagation of anti-proliferative and transcriptional responses.

Key words: receptor/serine threonine kinases/signal transduction/TGF-β

Introduction

Protein kinases involved in signal transduction are activated by different mechanisms depending on their nature and position in a signaling pathway. In the case of membrane tyrosine kinase receptors, activation is by ligand-induced dimerization and autophosphorylation of tyrosines in the cytoplasmic domain. Once phosphorylated, these sites bind cytoplasmic signal transducers that become activated as a result of recruitment to the membrane (Ullrich and Schlessinger, 1990; Koch *et al.*, 1991; Montminy, 1993; Ziemiecki *et al.*, 1994). Signals generated in this manner reach cytoplasmic serine/threonine kinases that act in sequence with one phosphorylating and activating the next. Phosphorylation of these particular kinases often occurs in a linker region of their catalytic domain and eliminates steric constraints to substrate binding (Taylor and Radzio-Andzelm, 1994; Zhang *et al.*, 1994).

In the case of transmembrane kinases that act as receptors for TGF-β and related factors, activation is by a different mechanism. These receptors have a short extracellular region, a single transmembrane span and a cytoplasmic region dominated by a serine/threonine kinase domain. Based on structural and functional considerations, they are subdivided into type I receptors (Attisano *et al.*, 1993; Ebner *et al.*, 1993; Franzén *et al.*, 1993; ten Dijke *et al.*, 1993, 1994b; Bassing *et al.*, 1994; Brummel *et al.*, 1994; Koenig *et al.*, 1994; Nellen *et al.*, 1994; Penton *et al.*, 1994; Wrana *et al.*, 1994b; Xie *et al.*, 1994) and type II receptors (Mathews and Vale, 1991; Attisano *et al.*, 1992; Lin *et al.*, 1992; Mathews *et al.*, 1992; Estevez *et al.*, 1993). TGF-β and the related activins and bone morphogenetic proteins each interact with specific sets of type I and type II receptors (Attisano *et al.*, 1994; Kingsley, 1994; Massagué *et al.*, 1994). The kinase domains of different type I receptors are more similar to each other than to the kinase domains of type II receptors (<40% amino acid sequence identity). Type II receptors bind ligand on their own, however, most type I receptors bind ligand only in the presence of an appropriate type II receptor, the two receptor types then forming a heteromeric complex. Observations made with TGF-β receptors indicate that formation of this heteromeric receptor complex is required for signaling (Wrana *et al.*, 1992; Franzén *et al.*, 1993; Bassing *et al.*, 1994; Cárcamo *et al.*, 1994).

Type I receptors from human through *Drosophila* contain a highly conserved 30 amino acid region known as the 'GS domain' located immediately upstream of the kinase domain (Wrana *et al.*, 1994b). Interest in the GS domain arises from recent insights into the mechanism of TGF-β receptor activation. The TGF-β type II receptor, TβR-II, is a constitutively active kinase that does not require ligand for activation (Wrana *et al.*, 1994a). TβR-II binds ligand free in the medium, or presented by the accessory receptor betaglycan (López-Casillas *et al.*, 1993; Moustakas *et al.*, 1993). Ligand bound to TβR-II is recognized by TβR-I which cannot contact the ligand otherwise (Wrana *et al.*, 1992; 1994a; Franzén *et al.*, 1993). Once recruited into this complex, TβR-I becomes phosphorylated by TβR-II on serine and threonine residues in the GS domain, a reaction observed *in vivo* (Wrana *et al.*, 1994a) and with recombinant receptors *in vitro* (Ventura *et al.*, 1994). Mutations that prevent TβR-I phosphorylation by eliminating the kinase activity of TβR-II (Wrana *et al.*, 1992), disrupting the ability of TβR-II to recognize TβR-I as a substrate (Cárcamo *et al.*, 1995) or altering the GS domain of TβR-I (Wrana *et al.*,

1994a), prevent signaling by the receptor complex. These observations support a model in which TGF-β binding allows a constitutively active transmembrane kinase (receptor II) to capture and phosphorylate another transmembrane kinase (receptor I) as a requisite for signal propagation.

How signals are carried beyond the TGF-β receptor complex is not known, but various observations raise the possibility that signal propagation to downstream substrates is via the type I receptor. TβR-I does not phosphorylate itself or the associated TβR-II, yet its kinase activity is required for signaling (Bassing *et al.*, 1994; Cárcamo *et al.*, 1994). Furthermore, the responses mediated by TGF-β or activin receptor complexes depend on the particular type I receptor isoform they contain (Cárcamo *et al.*, 1994). To seek further evidence that TβR-I is the downstream signaling component of the TGF-β receptor complex, we conducted a mutational analysis of the phosphorylation sites in the TβR-I GS domain. This led to the identification of a constitutively active form of TβR-I that signals typical TGF-β responses without requiring ligand or TβR-II. Identification of a constitutively active TβR-I places this kinase downstream of TβR-II in the TGF-β signaling pathway.

Results

Mutational analysis of GS domain phosphorylation sites

Proteolytic mapping of TβR-I phosphorylated *in vivo* indicates that all the sites for TβR-II phosphorylation reside in the GS domain (Wrana *et al.*, 1994a). This domain contains the core sequence T185TSGSGSG and two additional threonines, Thr200, which is conserved in all type I receptors, and Thr204, which is often replaced by a glutamine in other type I receptors (Figure 1). Mutation of all seven residues to alanines or valines [construct TβR-I(185–204)] does not perturb the ability of TβR-I to bind ligand in concert with TβR-II, but inactivates its ability to restore TGF-β responsiveness in receptor defective cells (Wrana *et al.*, 1994a; see Figure 2). To analyze the importance of individual serines and threonines in the GS domain, we mutated these residues individually or in various combinations (Figure 1). These constructs were tested by transfection into R-1B cells, a TβR-I defective cell line obtained by mutagenesis of the mink lung epithelial cell line Mv1Lu (Boyd and Massagué, 1989). Transfection of wild type TβR-I into R-1B cells restores anti-proliferative and transcriptional responses to TGF-β (Franzén *et al.*, 1993; Bassing *et al.*, 1994; Cárcamo *et al.*, 1994), making this system ideal for assaying the signaling activity of TβR-I variants.

We first analyzed the effect of eliminating potential phosphorylation sites in the TTSGSGSG sequence. TβR-I constructs of interest were transiently co-transfected with p3TP-Lux, a reporter construct that directs luciferase expression in response to TGF-β (Cárcamo *et al.*, 1994). When co-transfected with p3TP-Lux into R-1B cells, wild type TβR-I can mediate a 50-fold increase in luciferase activity in response to TGF-β (Figure 2A). In contrast, the construct TβR-I(VVAAA), which has all threonines and serines in the TTSGSGSG sequence replaced with

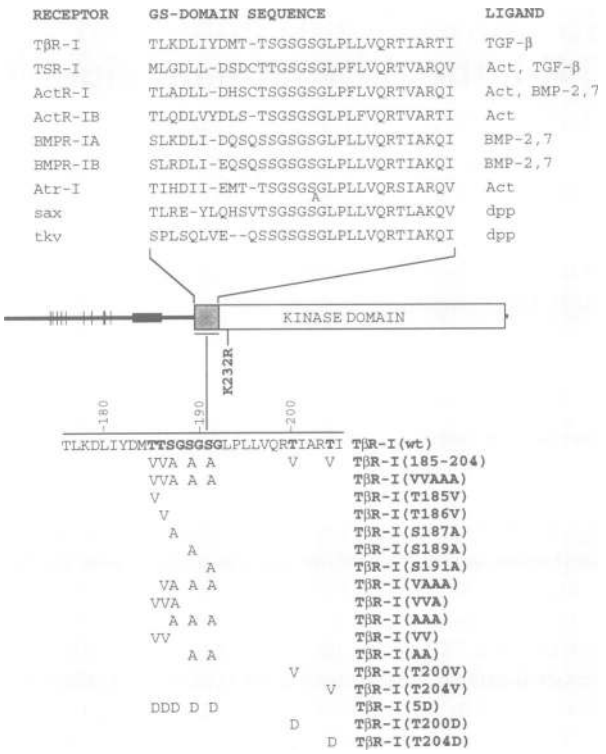

Fig. 1. The GS domain. Top, sequence comparison of the GS domains of different type I receptors for the TGF-β superfamily and bottom, mutations in the TβR-I GS domain generated in this study. The sequences of TβR-I (Franzén *et al.*, 1993), TSR-I (Attisano *et al.*, 1993), ActR-I (Attisano *et al.*, 1993), ActR-IB (Cárcamo *et al.*, 1994) and ALK3 (ten Dijke *et al.*, 1993) are human, ALK6 (ten Dijke *et al.*, 1994a) is murine, and Atr-I (Wrana *et al.*, 1994b), sax and tkv (Brummel *et al.*, 1994; Nellen *et al.*, 1994; Penton *et al.*, 1994; Xie *et al.*, 1994) are from *Drosophila melanogaster*. Act, activin.

valines or alanines, respectively, did not mediate luciferase expression in response to TGF-β (Figure 2A).

Receptor affinity labeling by crosslinking to bound [^{125}I]TGF-β confirmed that TβR-I(VVAAA) and all other constructs described below were similar to wild type TβR-I in their ability to be expressed, reach the cell surface and bind ligand in complex with endogenous TβR-II (data not shown). Therefore, the failure of TβR-I(VVAAA) to restore TGF-β responsiveness is attributable to disruption of its signaling activity by the mutations. The construct TβR-I(VAAA) was also completely inactive in this functional assay (Figure 2A). However, constructs with only three, two or one mutated residues showed weak, intermediate or full signaling activity, respectively, compared with wild type receptor (Figure 2A). The activity of the various double mutants was similar irrespective of which residues were mutated, and the same was observed with the two triple mutants.

These results suggested that the signaling activity of TβR-I did not depend on the availability of any particular serine or threonine in the TTSGSGSG sequence but rather on how many of such residues were intact. To confirm this point more quantitatively and with a different TGF-β response, all constructs were stably transfected into R-1B cells, and inhibition of DNA synthesis was determined in transfectant pools with a range of TGF-β concentrations (Figure 2B and C). Over a range or TGF-β concentrations, TβR-I forms containing singly mutated serine or threonine

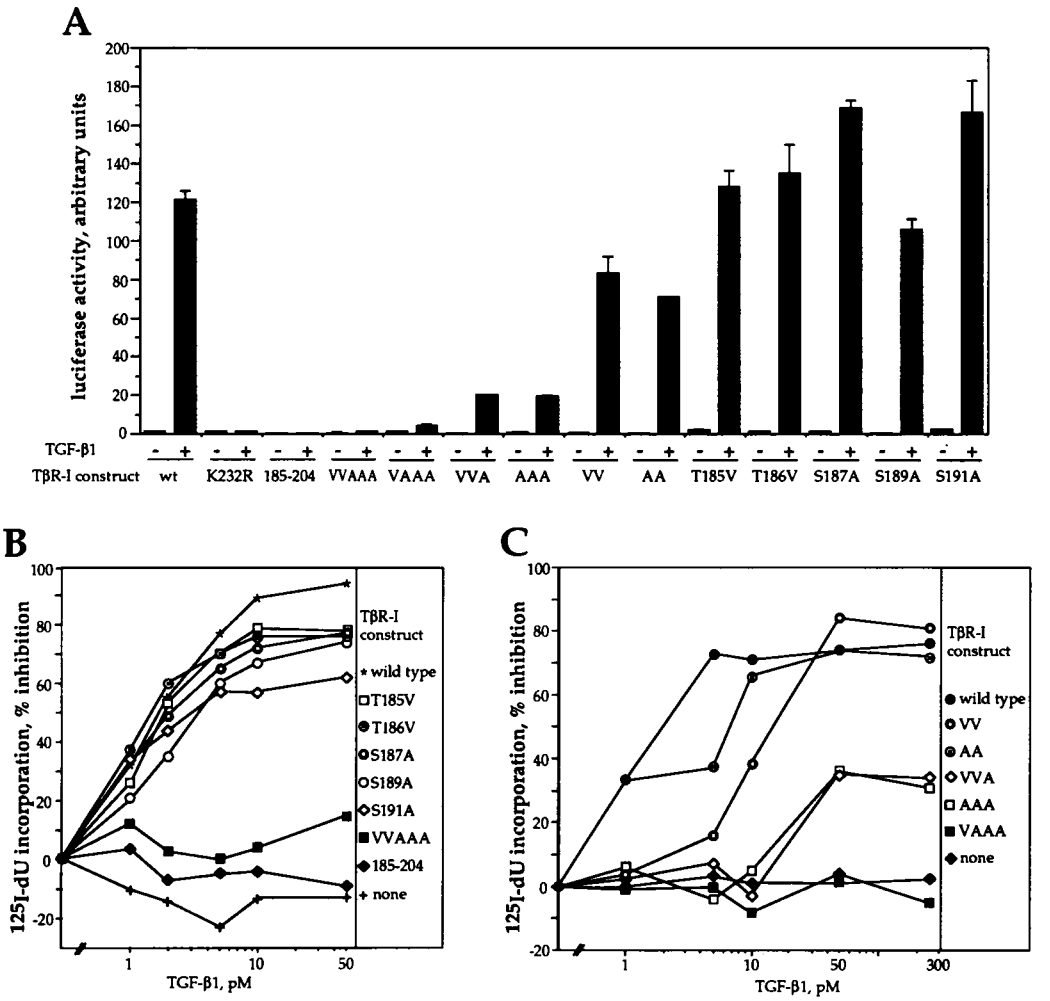

Fig. 2. Biological effects of mutations in the TTSGSGSG motif. (**A**) Luciferase activity in transiently transfected R-1B cells. Three micrograms of p3TP-Lux and 1 μg of each receptor construct were co-transfected into R-1B cells. On the next day, media were changed to low serum media, and TGF-β1 was added at 100 pM. 20 h later, cells were lysed and luciferase activity was determined. Each assay was carried out in duplicate in at least two experiments. (**B**) and (**C**) Inhibition of DNA synthesis in stably transfected R-1B cells. Cells were seeded sparsely into 24-well plates and incubated in low serum media in the presence of the indicated concentrations of TGF-β for 24 h. [^{125}I]deoxyuridine incorporation into DNA was then determined. Data are the average of triplicate determinations from one representative experiment. Standard deviations were <10% of each value. The data are presented as percent incorporation relative to transfectants that did not receive TGF-β.

residues in the TTSGSGSG sequence were essentially as active as the wild type receptor (Figure 2B), whereas the double and triple mutants were 3- to 10-fold and 50-fold less active, respectively (Figure 2C), and the four- and five-residue mutants were inactive (Figure 2B and C). A similar pattern was observed when another TGF-β response, elevation of fibronectin expression (Ignotz and Massagué, 1986; Wieser *et al.*, 1993), was measured (data not shown).

Phosphorylation of TTSGSGSG motif mutants

We verified that the partial loss of signaling activity in TβR-I containing two or more mutations in the TTSGSGSG sequence correlated with a reduction in phosphorylation by TβR-II. *In vivo* phosphorylated receptor complexes were retrieved by using tagged receptors and a two-step precipitation protocol that depends on the presence of both receptors in the complex (Wrana *et al.*, 1994a). TβR-II was tagged at the C-terminus with a hexahistidine sequence that binds to nickel–NTA–agarose (Bugge *et al.*, 1992) and the various TβR-I constructs were tagged at the C-terminus with a HA influenza virus hemagglutinin epitope (Meloche *et al.*, 1992). These modifications do not perturb the ability of TβR-I and TβR-II to restore responsiveness in receptor-defective cells (Wrana *et al.*, 1992, 1994a; Cárcamo *et al.*, 1994). R-1B cells transfected with TβR-II–His together with wild type or mutant TβR-I–HA constructs were labeled in separate dishes with [^{32}P]phosphate or [^{35}S]-methionine and incubated with TGF-β to generate receptor complexes. Cell lysates were bound to nickel beads, eluted with imidazole under conditions that do not dissociate the receptor complex (Wrana *et al.*, 1994a), and receptor complexes were precipitated from the eluate with HA antibody.

The yields of complex obtained with the different TβR-I constructs were comparable, as determined by analysis of ^{35}S-labeled receptors (Figure 3A). The phosphorylation level of TβR-I in the complex decreased as the number of mutant residues increased (Figure 3A). TβR-I(VVAAA) had a very low phosphorylation level which was comparable with that of TβR-I obtained from complexes con-

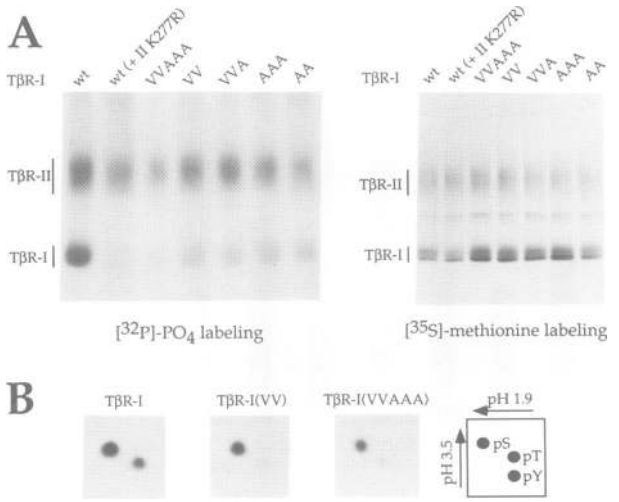

Fig. 3. TβR-I phosphorylation in complexes with TβR-II. (**A**) R-1B cells were transiently co-transfected with the indicated TβR-I constructs carrying a HA tag at the C-terminus, and either TβR-II or the kinase-defective construct TβR-II(K277R) both tagged with hexahistidine at the C-terminus. Cells were labeled with [^{32}P]orthophosphate or [^{35}S]methionine, incubated with TGF-β, and receptor complexes purified by sequential nickel chromatography and anti-HA immunoprecipitation. Phosphorylated products were visualized by SDS–PAGE and autoradiography. (**B**) Phosphoamino acid analysis. Selected TβR-I receptors phosphorylated by TβR-II in (A) were eluted from the gel and subjected to phosphoamino acid analysis. The amount of material loaded was four times as much for TβR-I(VV) and TβR-I(VVAAA) as for wild type receptor.

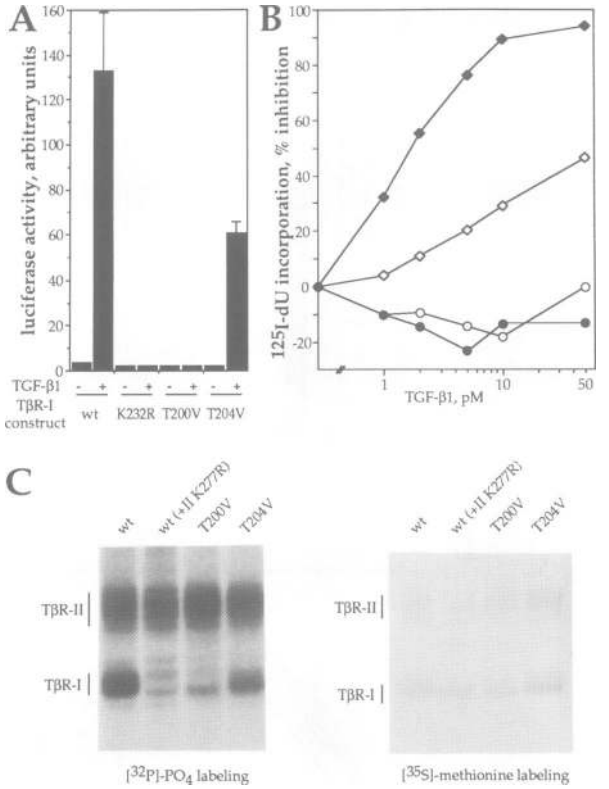

Fig. 4. Effects of Thr200 and 204 mutations. (**A**) and (**B**) Luciferase assays and DNA synthesis inhibition assays were carried out in R-1B cells transfected with the indicated constructs as described in the legend to Figure 2. Closed circles, untransfected cells; closed diamonds, wild type TβR-I transfectants; open diamonds, TβR-I(T204V) transfectants; open circles, TβR-I(T200V) transfectants. (**C**) Ligand-dependent TβR-I phosphorylation in complexes with TβR-II was determined with the indicated constructs as decribed in the legend to Figure 3A. As controls, parallel dishes were processed in the same way, except that cells were metabolically labeled with [^{35}S]methionine instead of [^{32}P]orthophosphate.

taining a kinase-inactive TβR-II construct [TβR-II(K277R); Wrana *et al.*, 1992]. Relative phosphorylation levels were determined by quantifying the ^{32}P-signal with a PhosphorImager and normalizing it based on the ^{35}S-signal from receptor samples isolated in parallel. Compared with the value obtained with wild type TβR-I (considered 100%), the phosphorylation level was 41% in TβR-I(VV), 67% in TβR-I(AA), 37% in TβR-I(VVA) and 61% in TβR-I(AAA).

Phosphoamino acid analysis of type I receptor protein eluted from this gel showed that mutation of Thr185 and 186 to valine led to a loss of phosphothreonine and to a lesser extent of phosphoserine, whereas the TβR-I(VVAAA) construct showed a marked loss in both phosphoserine and phosphothreonine (Figure 3B; note the differences in sample loading described in the legend). The presence of residual phosphoserine in this construct is attributable in part to basal TβR-I phosphorylation which is mostly on serine (data not shown). Collectively, these results suggest that all serines and threonines in the GS core contribute to receptor phosphorylation, and their mutation may also affect phosphorylation of vicinal sites.

Mutation of threonines adjacent to the kinase domain

Both Thr200 and 204 are located in RTI sequences at the end of the GS domain (Figure 1). These threonines were separately mutated to valines yielding the constructs TβR-I(T200V) and TβR-I(T204V) (Figure 1). Immunoprecipitation from metabolically labeled cells and affinity-labeling by crosslinking to bound [^{125}I]TGF-β both demonstrated that these constructs were expressed and bound ligand as efficiently as did wild type TβR-I (data not shown). However, when tested for their ability to activate p3TP-Lux expression in response to TGF-β in transiently transfected R-1B cells (Figure 4A), or to mediate an anti-proliferative response in stably transfected R-1B cells (Figure 4B), TβR-I(T200V) was completely inactive and TβR-I(T204V) was 10- to 20-fold less active than wild type receptor. These two mutants were also tested for phosphorylation by TβR-II in intact cells as described above. Mutation of Thr204 to valine caused a partial reduction in TβR-I phosphorylation whereas mutation of Thr200 to valine completely prevented ligand-induced phosphorylation (Figure 4C). Since ligand-induced phosphorylation of TβR-I by TβR-II is at serines and threonines in the GS domain, the T200V mutation must interfere with phosphorylation of serines and possibly threonines in the TTSGSGSG sequence.

A constitutively active TβR-I mutant

The crucial role of GS domain phosphorylation in signaling raised the possibility of creating constitutively active TβR-I versions by mutating hydroxyamino acids to acidic amino acids, thus simulating the presence of negatively charged phosphate groups. We created TβR-I(T200D) and

TβR-I(T204D) with Thr200 and 204, respectively, mutated to aspartic acid, and TβR-I(5D) with all five serines and threonines in the TTSGSGSG sequence mutated to aspartic acid. When assayed for induction of p3TP-Lux expression upon transient transfection into R-1B cells, TβR-I(T200D) and TβR-I(5D) were essentially inactive even in the presence of TGF-β (Figure 5A). In contrast, TβR-I(T204D) transfection reproducibly elevated the basal luciferase activity 20-fold compared with transfection of wild type TβR-I in the absence of TGF-β (Figure 5A). TGF-β addition to TβR-I(T204D) transfectants further increased luciferase expression to a maximal level similar to that reached in TβR-I transfectants stimulated with TGF-β (Figure 5A). Similar results were obtained with the construct TβR-I(T204E), in which Thr204 was replaced with glutamic acid (data not shown). These results suggested that mutation of Thr204 to an acidic residue constitutively activated the receptor.

To determine whether TβR-I(T204D) was also able to constitutively signal anti-proliferative responses, R-1B cells were stably transfected with this construct subcloned into the pMEP-4 vector which is inducible with Zn^{2+} via a metallothionein promoter. Similar transfectants were also created with wild type TβR-I and TβR-I(T200D). In the absence of Zn^{2+}, cell pools stably transfected with these constructs showed a similar rate of [^{125}I]deoxyuridine incorporation into DNA (Figure 5B). TGF-β addition to uninduced TβR-I or TβR-I(T204D) transfectants showed a concentration-dependent inhibition of DNA synthesis (Figure 5B). This response is attributed to leaky expression of functional receptors from the uninduced pMEP vector. TGF-β addition to uninduced or Zn-induced TβR-I(T200D) transfectants had no effect on DNA synthesis, confirming that this receptor is inactive (Figure 5B).

Addition of Zn^{2+} decreased the rate of [^{125}I]deoxyuridine incorporation in TβR-I(T204D) transfectants considerably (by 63%) but only slightly in TβR-I or TβR-I(T200D) transfectants (Figure 5B). TGF-β addition to Zn-induced TβR-I transfectants caused a concentration-dependent inhibition of [^{125}I]deoxyuridine incorporation. The lowest incorporation rate in these transfectants (seen with >10 pM TGF-β; Figure 5B) was similar to the incorporation in Zn-induced TβR-I(T204D) transfectants not exposed to TGF-β (Figure 5B). The Zn-induced anti-proliferative response in TβR-I(T204D) transfectants was therefore consistent with the presence of constitutive signaling activity in this construct.

Since the T204D mutation renders TβR-I capable of signaling independently of TGF-β, we asked whether it would also confer independence from TβR-II. For this purpose we used DR-26 cells, a TGF-β resistant derivative of Mv1Lu that is devoid of TβR-II because it harbors a nonsense mutation in the transmembrane domain of this receptor (Wrana *et al.*, 1992). TβR-II transfection into DR-26 cells restores TGF-β responsiveness whereas TβR-I transfection does not (Wrana *et al.*, 1992; Cárcamo *et al.*, 1994). Transient transfection of DR-26 cells with TβR-I did not activate the p3TP-Lux reporter gene whereas transfection with TβR-I(T204D) induced p3TP-Lux expression with an intensity proportional to the amount of plasmid DNA in the transfection (Figure 5C). At the highest plasmid concentration tested, luciferase activity exceeded the level observed in TβR-II transfectants treated with TGF-β (Figure 5C). Thus, an activated TβR-I was able to signal in the absence of TGF-β and TβR-II, confirming that TβR-I is a downstream effector of TβR-II.

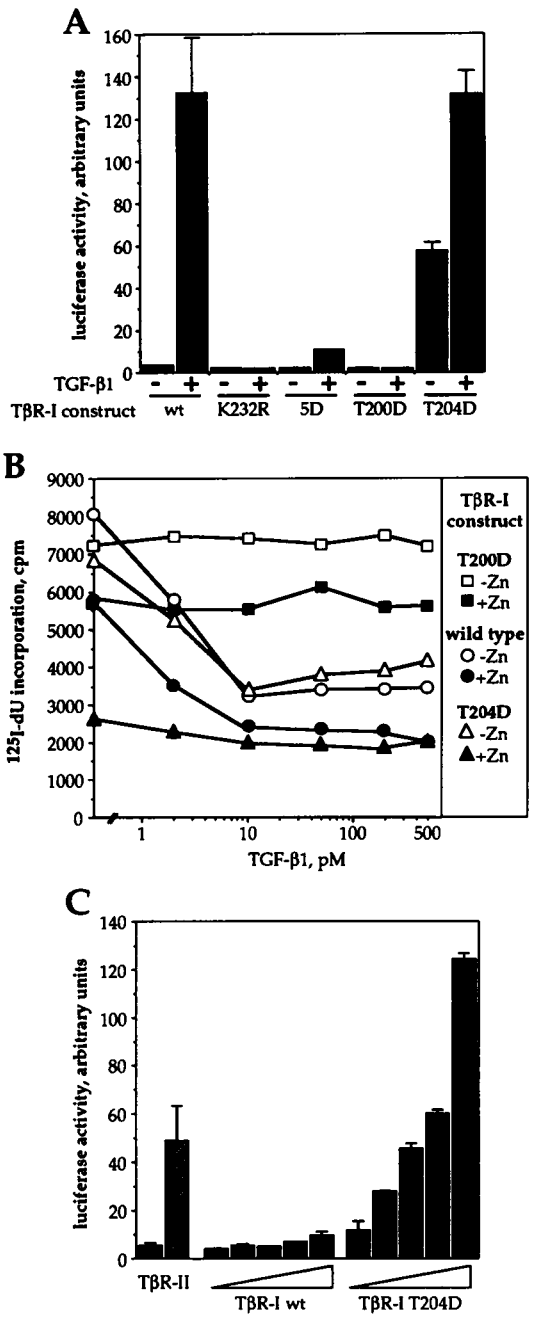

Fig. 5. Biological effects of the mutations to aspartic acid. (**A**) Luciferase assays were carried out in R-1B cells transfected with the indicated constructs as described in the legend to Figure 2. Data are the average of duplicate determinations ± SD. (**B**) The indicated constructs subcloned in the Zn-inducible vector pMEP were stably transfected into R-1B cells. [^{125}I]deoxyuridine incorporation into DNA was determined in the presence of the indicated TGF-β1 concentrations, with and without Zn induction. Data are the average of triplicate determinations. (**C**) The TβR-II-defective cell line DR-26 was transiently co-transfected with the p3TP-Lux reporter construct and TβR-II or increasing concentrations of TβR-I or TβR-I(T204D) vector. Some TβR-II transfectants (stippled bar) were incubated with TGF-β before lysis as described in Figure 2. Luciferase activity was then determined. Data are the average of duplicate determinations ± SD.

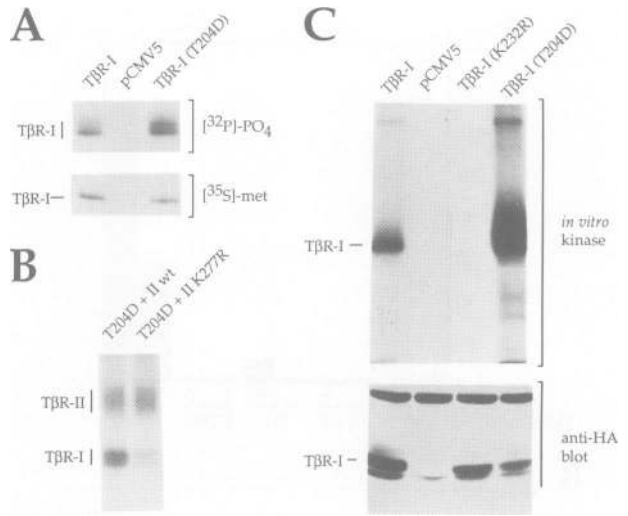

Fig. 6. (**A**) Basal phosphorylation of TβR-I(T204D) in intact cells. R-1B cells transiently transfected with the indicated HA-tagged constructs were labeled in parallel dishes with [^{35}S]methionine or [^{32}P]orthophosphate. Receptors were immunoprecipitated and analyzed by SDS–PAGE and autoradiography. (**B**) TβR-II-dependent phosphorylation of TβR-I(T204D) in ligand-induced receptor complexes. Labeling of transiently transfected R-1B cells with [^{32}P]orthophosphate and purification of receptor complexes were carried out as described in the legend to Figure 3A. (**C**) *In vitro* kinase assay. The indicated HA-tagged receptor constructs were immunoprecipitated from transiently transfected COS-1 cells and incubated with [γ-^{32}P]ATP. Phosphorylated immunoprecipitates were analyzed by SDS–PAGE and autoradiography (top). To control for the amount of receptor protein present in the assays, separate aliquots of the cell lysates were resolved by SDS–PAGE and immunoblotted with anti-HA antibody (bottom).

Phosphorylation of wild type and activated TβR-I

We determined the phosphorylation level of TβR-I(T204D) *in vivo* by immunoprecipitating this transfected protein from [^{32}P]phosphate-labeled R-1B cells. The basal phosphorylation of this protein was reproducibly 2-fold higher than that of wild type TβR-I (Figure 6A). However, as with wild type TβR-I, TβR-I(T204D) phosphorylation was further increased when this receptor formed a ligand-induced complex with TβR-II. This was determined by isolating TβR-I(T204D) from ligand-induced complexes with a co-transfected TβR-II or kinase-negative TβR-II(K277R) (Figure 6B). The phosphorylation level of TβR-I(T204D) was higher when this protein was in a complex with TβR-II than when it was in a complex with TβR-II(K277R). This result suggested that despite its elevated basal phosphorylation, TβR-I(T204D) was susceptible to ligand-induced hyperphosphorylation by an associated TβR-II kinase.

In vitro kinase activity of wild type and activated TβR-I

To determine whether the T204D mutation enhanced the ability of TβR-I to act as a kinase, we conducted *in vitro* kinase assays. Although the TβR-I kinase activity is clearly required for signaling (Bassing *et al.*, 1994; Wrana *et al.*, 1994a), the identity of its *in vivo* substrates is not known (Wrana *et al.*, 1994a). Therefore, the possibility that the T204D mutation might act by elevating the kinase activity of this receptor towards exogenous substrates could not be determined. However, it has been shown that recombinant TβR-I isolated from transfected COS cells (Cárcamo *et al.*, 1994) or its cytoplasmic region produced in bacteria (Bassing *et al.*, 1994) have autophosphorylating activity *in vitro*. We therefore used this type of assay to determine whether the T204D mutation conferred increased kinase activity. Even though autophosphorylation under these artificial conditions does not represent an activity observed in intact cells, it provides a useful indicator of the status of the receptor kinase (Cárcamo *et al.*, 1994). Therefore, wild type TβR-I, TβR-I(T204D) and the kinase-deficient construct TβR-I(K232R) as a negative control, were transiently transfected into COS-1 cells, quantitated by immunoblotting with anti-HA antibody, immunoprecipitated and incubated with [γ-^{32}P]ATP in kinase reaction buffer. The results demonstrated TβR-I phosphorylation that was eliminated by the K232R mutation and markedly elevated by the T204D mutation (Figure 6C).

Phosphopeptide maps of wild type and activated TβR-I

The phosphorylation states of TβR-I and TβR-I(T204D) were compared by tryptic phosphopeptide mapping. The phosphopeptide maps of these two proteins phosphorylated *in vivo* in the absence of ligand were similar (Figure 7A). It has been previously shown that the basal phosphorylation of TβR-I is mediated by cellular kinases of unknown identity since similar maps were obtained from the kinase-defective TβR-I(K232R) construct or from TβR-I in complex with the kinase-inactive TβR-II(K277R) (Wrana *et al.*, 1994a). TβR-I and TβR-I(T204D) isolated as part of ligand-induced complexes with co-transfected TβR-II yielded equivalent maps (Figure 7B) and contained one major phosphopeptide (peptide 6) that was previously shown to correspond to the phosphorylated GS domain (Wrana *et al.*, 1994a).

The phosphopeptide maps of TβR-I and TβR-I(T204D) phosphorylated *in vitro* were also similar to each other (Figure 7C), except for a relatively higher phosphorylation level in one of the peptides in TβR-I(T204D) (Figure 7C, peptide c). These maps resembled those of TβR-I and TβR-I(T204D) basally phosphorylated *in vivo* (compare Figure 7A and C) and lacked the phosphopeptide corresponding to the phosphorylated GS domain (compare Figure 7B and C). These results suggest that the TβR-I sites autophosphorylated *in vitro* are not those transphosphorylated by TβR-II *in vivo*. Nonetheless, the elevated autophosphorylation of TβR-I(T204D) *in vitro* and the ability of this receptor to signal in the absence of TGF-β and TβR-II suggest that the T204D mutation leads to constitutive activation of the TβR-I kinase *in vivo*.

TβR-I activation via a heterologous GS domain

ActR-I is one of several identified activin type I receptors (Attisano *et al.*, 1993; Ebner *et al.*, 1993; ten Dijke *et al.*, 1993). The amino acid sequence and biological activity of ActR-I differ from those of TβR-I (ten Dijke *et al.*, 1993; Cárcamo *et al.*, 1994). To further investigate the role of GS domains in receptor activation, we asked whether replacement of the GS domain in TβR-I with the corresponding domain from ActR-I would still allow TGF-β-dependent activation of the resulting mosaic receptor, TβR-I(GS^{ActR-I}). Transfection of this construct into R-1B cells resulted in a 15-fold elevation of luciferase activity

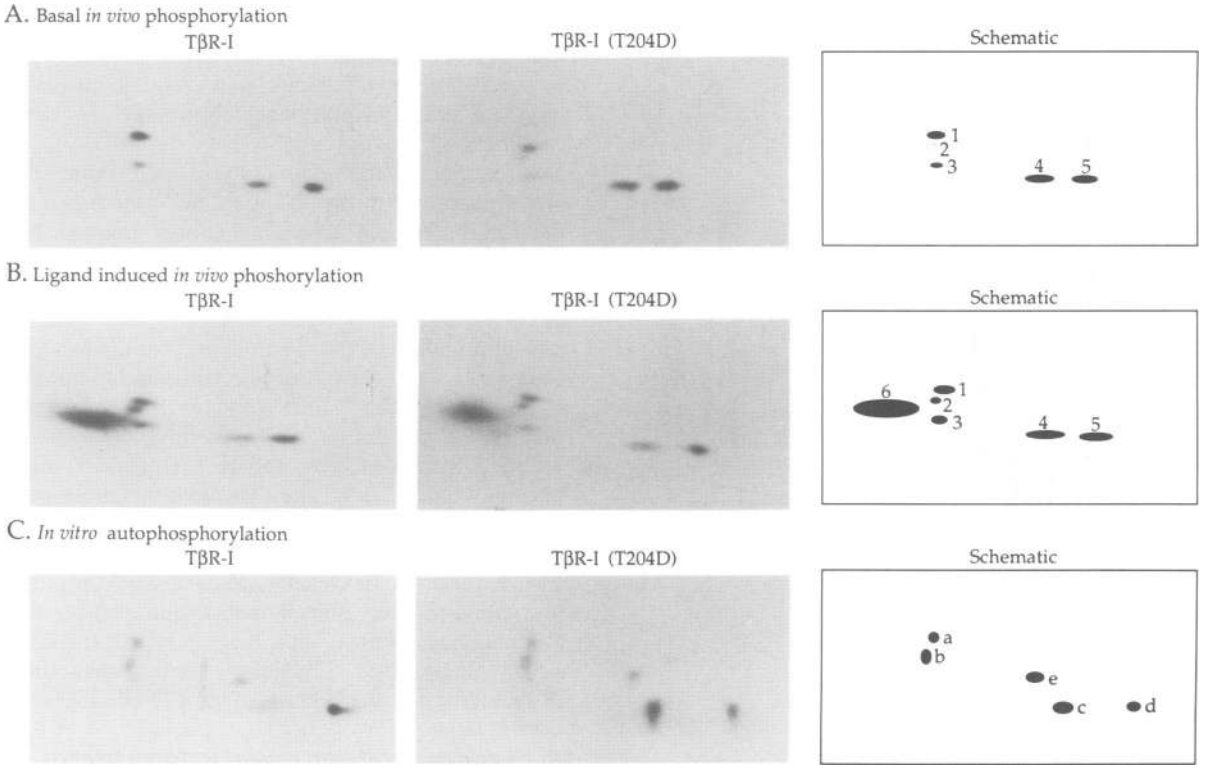

Fig. 7. Tryptic phosphopeptide maps of TβR-I and TβR-I(T204D) phosphorylated *in vivo* and *in vitro*. (**A**) Basally phosphorylated receptors were isolated by direct immunoprecipitation from [^{32}P]phosphate-labeled R-1B transfectants. (**B**) Receptors phosphorylated in response to ligand were isolated by two-step precipitation of receptor complexes after TGF-β addition to R-1B transfectants. (**C**) *In vitro* phosphorylated receptors were prepared from transfected COS cells as described in Figure 6C. Phosphorylated TβR-I and TβR-I(T204D) were transferred from SDS−PAGE gels onto nitrocellulose membranes, digested with trypsin and the resulting peptides resolved by thin layer electrophoresis followed by thin layer chromatography in the second dimension.

(Figure 8A), demonstrating its ability to restore TGF-β mediated activation of the p3TP-lux reporter gene. This response was less intense than the response mediated by TβR-I (~30-fold elevation; Figure 8A), however, it was much stronger than the 3-fold elevation in p3TP-lux activity mediated by ActR-I in response to activin (Attisano *et al.*, 1993).

In some members of the type I receptor family, including ActR-I, the position corresponding to Thr204 is occupied by a glutamine (see Figure 1). Since the ActR-I GS domain could functionally replace the TβR-I GS domain, and the resulting mosaic receptor mediated an easily measurable response, we tested the effect of mutating Gln204 to aspartic acid in the ActR-I GS domain of the TβR-I(GS^{ActR-I}) construct. Transfection of R-1B cells with the resulting construct, TβR-I(GS^{ActR-I}, Q204D), elevated p3TP-Lux expression in a TGF-β-independent manner (Figure 8A), and TGF-β addition caused only a limited (<2-fold) further increase in luciferase activity. The constitutive activation of luciferase expression by TβR-I(GS^{ActR-I}, Q204D) compared with TβR-I(GS^{ActR-I}) transfectants could not be explained by differences in receptor expression levels since these were similar (Figure 8B). Thus, TβR-I was constitutively activated by introduction of an acidic residue in the penultimate position of its GS domain regardless of whether the mutated residue was a threonine, as in wild type TβR-I, or a glutamine, as in TβR-I(GS^{ActR-I}).

Discussion

Ligand-induced formation of the TGF-β receptor complex allows TβR-II to phosphorylate TβR-I at the GS domain, a step required for signal propagation (Wrana *et al.*, 1994a; Cárcamo *et al.*, 1995). In the present studies, we have conducted a mutational analysis to determine the functional relevance of individual residues in this region. The results show that certain mutations in the GS domain inactivate the receptor whereas others yield constitutively active receptor forms. The properties of these mutants provide new insights into the significance of TGF-β receptor phosphorylation and the role of TβR-I as a downstream signaling component in the receptor complex.

Inactivating mutations in two distinct motifs

Previous work showed that simultaneous mutation of all seven serines and threonines in the GS domain of TβR-I prevents phosphorylation and abolishes signaling (Wrana *et al.*, 1994a). These residues are located in two distinct motifs, the characteristic TTSGSGSG sequence and the repeated RTI sequence adjacent to the canonical start of the kinase domain. By individually mutating these potential phosphorylation sites, we have determined the distinct character of the two motifs.

TβR-I is phosphorylated at serines and threonines in the GS domain (Wrana *et al.*, 194a), and the present results indicate that such residues in the TTSGSGSG

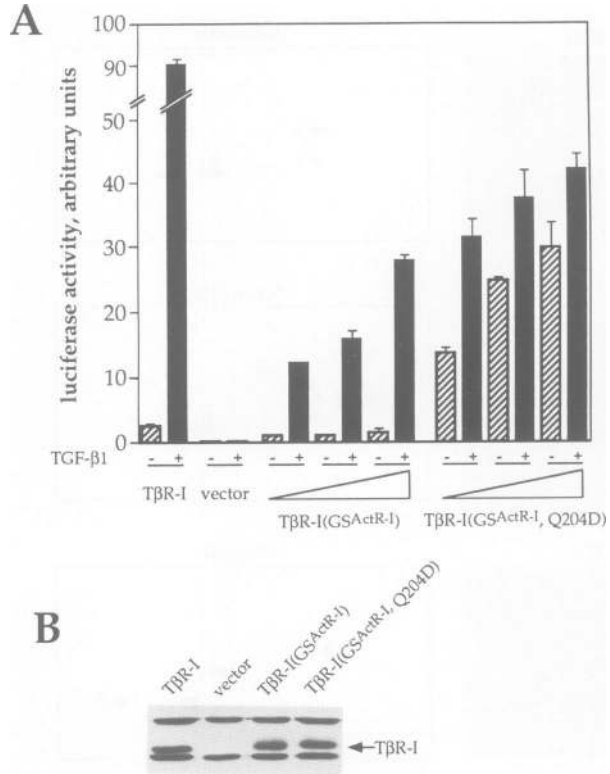

Fig. 8. Ligand-dependent activation of TβR-I via a heterologous GS domain, and constitutive activation of the chimeric receptor TβR-I(GS^{ActR-I}) by mutation of Gln204 to Asp. (**A**) R-1B cells were transiently co-transfected with luciferase reporter and either wild type TβR-I or increasing amounts of TβR-I(GS^{ActR-I}) or TβR-I(GS^{ActR-I}, Q204D). Luciferase activity was determined as described in the legend to Figure 2A. (**B**) R-1B cell cultures transfected in parallel with the ones used to measure luciferase activity were lysed and proteins analyzed by immunoblotting with HA antibody.

motif undergo ligand-induced phosphorylation. The only serines in the GS domain are contained within this motif, and their mutation to alanine indeed decreases TβR-I phosphorylation. Likewise, mutation of the threonines in this motif to valine induces a selective loss in ligand-induced threonine phosphorylation. Compared with wild type TβR-I, the level of ligand-induced phosphorylation is markedly decreased by mutation of any two or three hydroxyamino acids in this motif, and is further decreased by mutation of all five. Mutation of two or more serines or threonines impairs signaling, the severity of the effect being proportional to the number of mutated residues. Double mutants are 3- to 10-fold less active than wild type TβR-I, triple mutants are ~50-fold less active, and mutation of four or five hydroxyamino acids in this region essentially abolishes signaling activity. None of these mutations alters receptor expression or interaction with ligand and TβR-II.

The clustering of phosphorylation sites in the TTSGSGSG sequence raises the possibility that their phosphorylation might be processional, with phosphorylation of one residue being required for phosphorylation of the next. However, if this were the case, mutation of the first residue to be phosphorylated should prevent phosphorylation of all the others. Instead, we observed that any one serine or threonine in the TTSGSGSG sequence can be mutated to an apolar residue with little loss of phosphorylation or signaling activity. Thus, a certain level of phosphorylation in the TTSGSGSG region is necessary for signaling, however, the availability of any four potential phosphorylation sites can satisfy this requirement.

In contrast to the relatively innocuous nature of single site mutations in the TTSGSGSG sequence, mutation of Thr200 or 204 to valine causes marked losses in ligand-induced phosphorylation and signaling. The T200V mutation in particular causes a nearly complete loss of ligand-induced phosphorylation. Since ligand-induced phosphorylation of the GS domain is at serines as well as threonines (3:1 phosphoserine:phosphothreonine; Wrana *et al.*, 1994a), the T200V mutation must prevent phosphorylation of serines, and possibly threonines, in the TTSGSGSG motif. Are the RTI sequences themselves phosphorylated? If so, they should generate tryptic phosphopeptides containing phosphothreonine but not phosphoserine. However, the ligand-induced phosphorylation of TβR-I appears to be concentrated in a single tryptic peptide that contains both phosphoserine and phosphothreonine (Wrana *et al.*, 1994a). Thus, it could be that the RTI motifs are not themselves phosphorylated but their integrity is essential for phosphorylation of the TTSGSGSG sequence.

Activating mutations reveal a downstream signaling kinase

The functional consequences of protein phosphorylation can be mimicked in certain cases by mutating phosphorylation sites to acidic residues (Mansour *et al.*, 1994; Pagès *et al.*, 1994). Therefore, we studied the functional consequences of mutating putative phosphorylation sites in the GS domain of TβR-I to acidic residues. Mutation of the five hydroxyamino acids in the TTSGSGSG motif to aspartic acid inactivates the receptor, as does the T200D mutation. However, mutation of Thr204 to aspartic or glutamic acid yields constitutively active receptors that can signal anti-proliferative and transcriptional responses in the absence of TGF-β. Transfected TβR-I(T204D) generates these responses in cells that are defective in endogenous TβR-I or TβR-II, arguing that constitutively active TβR-I does not require interaction with endogenous receptors for signaling.

The constitutively active phenotype of TβR-I(T204D) appears to involve activation of the TβR-I kinase. This mutant receptor displays elevated autophosphorylating activity when compared with wild type TβR-I in an *in vitro* assay of recombinant receptors immunoprecipitated from COS cells. The reaction measured in this assay is dependent on the TβR-I kinase, as shown by the lack of phosphorylation in the kinase-defective construct TβR-I(K232R). Although this reaction may not represent a physiological event—wild type TβR-I is not autophosphorylated *in vivo* (Wrana *et al.*, 1994a) or after isolation from insect cells (Ventura *et al.*, 1994)—it is nonetheless a useful indicator of the kinase activity present in TβR-I. It is interesting that in the absence of ligand, TβR-I(T204D) shows a somewhat higher level of basal phosphorylation *in vivo* than does TβR-I. This increase could result from higher susceptibility to a cellular kinase, lower susceptibility to a phosphatase, or acquisition of a limited ability to autophosphorylate. The actual mechanism could not be established since the increase in basal phosphoryla-

tion was relatively small. TβR-I(T204D) still underwent TβR-II-dependent phosphorylation at the GS domain once incorporated into the ligand-induced heteromeric receptor complex.

It is not clear that the T204D mutation acts by mimicking phosphorylation of this site. As mentioned above, Thr204 phosphorylation could not be confirmed and furthermore this residue is replaced by glutamine in the GS domain of most other type I receptors (see Figure 1). It is possible that uncharged polar residues such as threonine and glutamine in this position are compatible with receptor function, whereas apolar residues (e.g., as in the T204V mutant) are unfavorable and acidic residues cause constitutive activation. Support for this hypothesis comes from our results with a TβR-I construct containing the GS domain of ActR-I. This activin type I receptor shows considerable sequence divergence from TβR-I and has a glutamine as the penultimate residue of the GS domain. Unlike TβR-I or ActR-IB, ActR-I does not mediate anti-proliferative responses in mink lung cells, and its ability to mediate activation of the p3TP-Lux reporter gene in these cells is very limited (a 3-fold induction) compared with the strong effects of TβR-I and ActR-IB (Attisano *et al.*, 1993; Cárcamo *et al.*, 1994). Despite these differences, a TβR-I receptor construct containing the GS domain of ActR-I is able to mediate TGF-β responses. Furthermore, mutation of Gln204 to aspartate in the ActR-I GS domain of this protein yields a constitutively active form that signals in a ligand-independent manner. These results suggest that the ActR-I GS domain can be recognized by a heterologous type II receptor kinase (TβR-II), can support signal propagation by a heterologous type I receptor kinase (TβR-I) and causes constitutive receptor activation when its penultimate residue is mutated to aspartic acid.

The identification of constitutively active versions of TβR-I provides key support for the conclusion that TβR-I acts downstream of TβR-II in the TGF-β signal transduction pathway. The results are consistent with previous proposals that TβR-I is rate limiting for signaling (Wrana *et al.*, 1992) and is a mediator of both extracellular matrix production and anti-proliferative responses (Wieser *et al.*, 1993; Cárcamo *et al.*, 1994). Constitutively active TβR-I can generate these responses in the absence of ligand or functional TβR-II, arguing that TβR-I is a downstream signaling kinase in the TGF-β receptor complex. Activating mutations analogous to the ones described here should help establish the responses specified by other members of this receptor family and identify substrates for these protein kinases.

Materials and methods

DNA constructs

Mutations in the TβR-I cDNA as well as the chimaeric receptor TβR-I(GS^{ActR-I}) were generated by two-step PCR using mutagenic primers as previously described (Wieser *et al.*, 1993). The resulting PCR fragments were subcloned into TβR-I−HA (Cárcamo *et al.*, 1994) using convenient internal restriction sites, and each construct was verified by DNA sequencing (Sequanase 2.0 kit, USB). Constructs were subcloned into pMEP4 (Invitrogen) for stable transfection and pCMV5 (Andersson *et al.*, 1989) for transient transfection.

Cell lines and transfections

R-1B cells were maintained in minimal essential medium (MEM) with non-essential amino acids (NEAA) and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Stable transfectants were generated using Lipofectin (GIBCO-BRL) and selected in 300 μg/ml hygromycin B (400 U/mg, Calbiochem). Transient transfections were conducted using the DEAE–dextran protocol and a highly transfectable R-1B cell subclone (L17) (Cárcamo *et al.*, 1994).

Functional assays

The functional assays used to characterize signaling by receptor mutants have been described previously (Wieser *et al.*, 1993). Briefly, for luciferase assays, six-well dishes of R-1B cells or DR26 cells were transiently transfected with 3 μg of luciferase reporter DNA and 1 μg of either pCMV5 or pMEP4 containing the receptor construct per well. To demonstrate the dose-dependence of the activating mutation T204D in DR26 cells, DNA concentrations from 0.05 to 2 μg/well were used. For the experiment showing the dose-dependent activation of the luciferase reporter by TβR-I(GS^{ActR-I}, Q204D) in R-1B cells, DNA concentrations were varied from 0.07 to 0.7 μg/well. Following transfection, cells were trypsinized and split into two wells of a 12-well plate. After 4 h, cells were changed to media containing 0.2% FBS (and 50 μM $ZnCl_2$ for pMEP4 constructs). After an additional 4 h, media were replaced with fresh media containing 100 pM TGF-β1 or no additions. Twenty hours later, cells were lysed and luciferase activity determined in a luminometer (Berthold Lumat LB 9501) using luciferin (Luciferase assay kit, Promega) as a substrate.

For DNA synthesis inhibition assays, actively proliferating cells were seeded sparsely into 24-well plates. After attachment (~4 h), cell layers were changed to media containing 0.2% FBS with or without 50 mM $ZnCl_2$, as indicated. 4–5 h later, media were replaced with fresh media containing the indicated concentrations of TGF-β1. Cells were labeled 20–24 h later with [^{125}I]deoxyuridine for 2 h, fixed with methanol, extracted with 0.2 N NaOH, and [^{125}I]deoxyuridine incorporation was determined in a gamma counter. All assays were carried out in triplicate or quadruplicate.

Fibronectin production in stably transfected cell lines was determined by seeding cells into 12-well plates and allowing them to grow for at least 24 h. None or 50 pM TGF-β were added in low serum media containing 50 μM $ZnCl_2$. After 20 h, cells were labeled with [^{35}S]methionine for 2 h. Media were collected and proteins separated by SDS−PAGE and visualized by fluorography. Fibronectin was detected as a typical 200 kDa band.

Metabolic labeling and in vivo phosphorylation assays

Labeling of intact cells with [^{35}S]methionine or [^{32}P]phosphate, isolation of receptor complexes, phosphoamino acid analysis, and tryptic phosphopeptide mapping were carried out as described previously (Boyle *et al.*, 1991; Luo *et al.*, 1991; Wrana *et al.*, 1994a). Briefly, actively growing R-1B cells were co-transfected with 1–1.5 μg/ml of hexahistidine-tagged TβR-II (TβR-II−His) and the same amount of HA-tagged TβR-I (TβR-I−HA) using the DEAE–dextran method. On the next day, cells were trypsinized and split into two dishes which were metabolically labeled on the following day with either [^{32}P]phosphate or [^{35}S]methionine. After 2–3 h of labeling, TGF-β was added at a final concentration of 1 nM for 10–15 min. Cells were then lysed and complexes between TβR-I and TβR-II purified by sequential Ni−NTA agarose chromatography and anti-HA immunoprecipitation (Wrana *et al.*, 1994a). Proteins were separated by SDS−PAGE on 8% gels and visualized by autoradiography.

For phosphoamino acid analysis, bands corresponding to TβR-I were then excised from the gel, and proteins eluted and subjected to partial acid hydrolysis by incubating in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional thin layer electrophoresis on cellulose plates (Merck) using pH 1.9 buffer (50 ml 88% formic acid, 156 ml glacial acetic acid, 1794 ml H_2O) in the first dimension and pH 3.5 buffer (100 ml glacial acetic acid, 10 ml pyridine, 1890 ml H_2O) in the second dimension.

For tryptic peptide mapping, ^{32}P-labeled TβR-I products were electrotransferred from SDS−PAGE gels onto nitrocellulose and digested for 13 h with 20 μg of trypsin per sample. Phosphopeptides were resolved by thin layer electrophoresis using the Hunter thin layer electrophoresis system (HTLE7000) followed by chromatography on TLC plates, and visualized by autoradiography.

In vitro kinase assays

In vitro kinase assays of TβR-I immunoprecipitated from COS-1 cells were carried out as described (Cárcamo *et al.*, 1994). Briefly, 2 days after transfection, COS-1 cells were lysed. One aliquot of the lysate was used for immunoblotting with HA antibody and a horseradish peroxidase-conjugated secondary antibody, followed by detection with the ECL

system (Amersham). Another aliquot was immunoprecipitated with HA antibody. Immunoprecipitates were washed several times and assayed for kinase activity by incubating for 15 min at 37°C in kinase assay buffer (Cárcamo *et al.*, 1994) containing 40 μM ATP and 30 μCi [γ-^{32}P]ATP. Autophosphorylated receptors were washed, separated by SDS–PAGE and visualized by autoradiography.

Acknowledgements

We thank C.-H.Heldin and K.Miyazono for the TβR-I cDNA. This work was supported by grants from the National Institutes of Health to J.M. and to Memorial Sloan-Kettering Cancer Center. R.W. is an Erwin Schrödinger postdoctoral fellow and an Olson fellow. J.L.W. is a Medical Research Council of Canada postdoctoral fellow. J.M. is a Howard Hughes Medical Institute Investigator.

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Received on September 29, 1994; revised on January 16, 1995