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Disruption of Transforming Growth Factor β Signaling by a Mutation That Prevents Transphosphorylation within the Receptor Complex

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TβR-II (transforming growth factor β [TGF-β] type II receptor) is a transmembrane serine/threonine kinase that acts as the primary TGF-β receptor. Ligand binding to TβR-II leads to the recruitment and phosphorylation of TβR-I, a distantly related transmembrane kinase that acts as a downstream signaling component. TβR-I phosphorylation by TβR-II is shown here to be essential for signaling. A mutant TβR-II that binds ligand but lacks signaling activity was identified. This mutant was identified by screening with a TGF-β-inducible vector a series of mink lung epithelial cell clones that have normal TGF-β binding activity but have lost antiproliferative and transcriptional responses to TGF-β. When transiently cotransfected with TβR-II, one of these cell lines, S-21, recovered TGF-β responsiveness. cDNA cloning and sequencing of TβR-II from S-21 cells revealed a point mutation that changes proline 525 to leucine in kinase subdomain XI. A recombinant receptor containing this mutation, TβR-II(P525L), is similar to wild-type TβR-II in its abilities to bind ligand, support ligand binding to TβR-I, and form a complex with TβR-I in vivo. TβR-II(P525L) has autophosphorylating activity in vitro and in vivo; however, unlike the wild-type receptor, it fails to phosphorylate an associated TβR-I. These results suggest that TβR-II(P525L) is a catalytically active receptor that cannot recognize TβR-I as a substrate. The close link between TβR-I transphosphorylation and signaling activity argues that transphosphorylation is essential for signal propagation via TβR-I.

Transforming growth factor β (TGF-β) is prototypic of a large family of cytokines that regulate a wide variety of cellular processes, including cell proliferation, differentiation, motility, and organization (18, 25, 33). TGF-β generates these responses by interacting with an oligomeric receptor complex that consists of components known as TGF-β receptor types I and II (TβR-I and TβR-II) (3, 22, 25, 30, 43). Both components are transmembrane proteins with a short, cysteine-rich extracellular region and a cytoplasmic region whose principal element is a serine/threonine kinase domain. Despite the similarity in overall structure, TβR-I and TβR-II are very different at the amino acid sequence level, with less than 40% identity in the kinase domain and even less similarity in other regions (5, 15). TβR-II and other type II receptors for the TGF-β family contain a serine/threonine-rich C-terminal tail not present in type I receptors. TβR-I and other type I receptors contain a conserved region preceding the kinase domain and characterized by the sequence Ser-Gly-Ser-Gly-Ser-Gly, hence the designation of this region as the GS domain (45).

Type I and II receptors differ in their ligand-binding properties. Type II receptors can bind ligand directly from the medium, whereas type I receptors recognize ligand that is bound to type II receptors but generally do not recognize free ligand in the medium (1, 5, 8, 15, 36–38, 43, 45). Although both TβR-I and TβR-II contain a ligand-binding domain and a catalytic domain, formation of a ligand-induced complex between the two is required for generation of antiproliferative and transcriptional responses to TGF-β (44). Type II receptors can each interact with a small repertoire of type I receptors, providing a potential mechanism by which TGF-β and related cytokines can generate separate sets of responses, depending on the availability of receptor partners in a given cell type (1, 5, 8, 13, 15).

To understand how the effects of TGF-β-related factors on development, tissue repair, and disease occur, it is critical to understand in detail the activation process of their receptors. Important clues about this process have emerged from recent studies with TGF-β receptors (44). TβR-II is a constitutively active kinase whose activity is not increased by ligand binding. Ligand binding to TβR-II induces recruitment of TβR-I into a stable complex. Once this complex is formed, TβR-II phosphorylates TβR-I at serine and threonine residues in the GS domain, a process observed both in vivo (44) and with reconstituted receptor complexes in vitro (40). TβR-I does not phosphorylate itself or the associated TβR-II; however, its kinase activity is essential for signaling (5, 8, 45). Thus, TβR-I is a TβR-II substrate and appears to function as the downstream component in this first step of the TGF-β signal transduction pathway. Consistent with this model, a constitutively active form of TβR-I can generate antiproliferative and transcriptional responses in the absence of TGF-β and TβR-II (42).

Previous evidence suggested that phosphorylation of TβR-I by TβR-II might be crucial for receptor activation. A mutation that destroys the kinase activity of TβR-II, thus preventing TβR-I phosphorylation, blocks signaling by the receptor complex (44). Likewise, mutations that eliminate phosphorylation sites in the GS domain of TβR-I prevent phosphorylation and block signaling (42, 44). However, the invasive nature of these mutations left doubts about the essential nature of the transphosphorylation event per se. Here, we describe the identification of a TβR-II mutation that does not prevent autophosphorylation but blocks transphosphorylation of TβR-I. These results show that TβR-I phosphorylation by TβR-II is essential for signal propagation.

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MATERIALS AND METHODS

Receptor constructs. Receptor constructs were generated by the PCR using TβR-II as a template and appropriate oligonucleotide primers. The TβR-II(P525L) and TβR-II(P525A) receptor constructs were verified by sequencing. TβR-II(P525L) and TβR-II(P525A) were tagged at the N terminus with the influenza virus hemagglutinin epitope (HA) or at the C terminus with a hexahistidine sequence as previously described (44). The HA-tagged and His-tagged constructs were subcloned into pCMV5 by using convenient restriction sites. The construction of wild-type TβR-II, TβR-II(K277R), TβR-I, and TβR-I(K232R) and of their HA-tagged or hexahistidine-tagged derivatives was previously described (1, 43, 45).

Cell lines and transfections. Cell lines R-1B, DR-26, and S-21 were derived in earlier studies by chemical mutagenesis of the mink lung epithelial cell line Mv1Lu (6, 21). The Mv1Lu cell line (CCL-64; American Tissue Culture Collection) and the mutant cell lines DR-26 and S-21 were maintained in minimal essential medium (MEM) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. R-1B (L17 clone) cells were maintained in histidine-free MEM containing 0.5 mM histidinol, 10% FBS, and nonessential amino acids. COS-1 cells were maintained in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% FBS. Transient transfection of COS-1 cells with the appropriate receptor vectors was done by the DEAE-dextran method (43), and cells were assayed 48 to 72 h after transfection.

Parental and mutant Mv1Lu cell lines were screened for TGF-β responsiveness and dependence on transfected receptors by using the TGF-β-inducible reporter construct p3TP-Lux and appropriate receptor expression vectors. Transient transfections into control and test cells were done as previously described (43). Briefly, cells were plated into six-well plates at 75,000 per well and grown for 2 days until they were about 50% confluent. They were then washed twice with MEM containing amphotericin B (Fungizone) and gentamicin (MEM-f/g) and incubated for 2 min in 1 ml of MEM-f/g containing 10 μM chloroquine. Then 170 μl of phosphate-buffered saline (PBS) containing 3 μg of p3TP-Lux DNA and 1 μg of receptor vector (or pCMV5) DNA and 170 μl of a solution containing 1 mg of dextran per ml were added. Cells were incubated at 37°C for 3.5 h and then with 10% dimethyl sulfoxide in PBS for 2 min, washed in MEM-f/g containing 10% FBS, and incubated overnight in MEM-f/g containing 10% FBS.

Reverse transcription-PCR analysis of TβR-II. RNA was prepared from S-21 cells as previously described (43). Total RNA (1 μg) was reverse transcribed into cDNA by using oligo(dT) priming and random priming. cDNAs were amplified on a Perkin-Elmer Thermal Cycler 9600 by using the GeneAmp PCR kit (Perkin-Elmer) as previously described (43). The cycling parameters were as previously described for degenerated primers (2). Three independent amplified products were separated by agarose electrophoresis and isolated and sequenced with appropriate primers as previously described (43).

Receptor affinity labeling assays. TGF-β1 from porcine platelets (R&D Systems) was labeled with ^{125}I as previously described (9, 10). Cell monolayers were incubated for 3 h at 4°C with 250 pM ^{125}I-labeled TGF-β1. Receptors were cross-linked to bound ligands with disuccinimidyl suberate (Pierce Chemical Co.) and solubilized in the presence of Triton X-100 as previously described (9, 24). Cell extracts were clarified by centrifugation and subjected to immunoprecipitation with anti-HA antibodies and/or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (1).

DNA replication assays. Cells were seeded in 24-well plates at 50,000 per well. They were incubated overnight in regular medium and then placed in MEM containing 0.2% FBS. After 5 h, cells were incubated for 18 h with appropriate concentrations of TGF-β1 and then incubated with 1 μCi of ^{125}I-deoxyuridine per ml for an additional 4 h, washed three times with cold PBS, and fixed with 95% methanol for 1 h at 4°C. Fixed cells were washed with PBS and extracted with 1 N NaOH for 10 min. The extracts were collected, and radiation was measured with a gamma counter.

PAI-1 and fibronectin assays. Cultures at 50% confluency in six-well plates were incubated overnight in MEM–10% FBS and then for 4 h in 1 ml of methionine-free MEM containing 100 pM TGF-β1. During the final 2 h of this incubation, 40 μCi of [^{35}S]methionine (Trans-^{35}S-label; ICN) was added. Cells were washed once with ice-cold PBS, three times with a buffer containing 10 mM Tris-HCl (pH 8), 0.5% deoxycholate, and 1 mM phenylmethylsulfonyl fluoride, twice with 2 mM Tris-HCl (pH 8), and once with PBS. Extracellular matrix proteins were extracted from the plates by being scraped into electrophoresis sample buffer (19). The samples were subjected to SDS-PAGE, and plasminogen activator inhibitor 1 (PAI-1) was identified by its characteristic mobility and induction by TGF-β.

To measure fibronectin production, cells grown in medium containing 0.2% FBS were incubated with 100 pM TGF-β for 15 h and labeled with 50 μCi of [^{35}S]methionine per ml in methionine-free MEM for 2 h. The culture medium was collected, and fibronectin was isolated by being bound to gelatin-Sepharose (Pharmacia) and analyzed as previously described (43).

Northern (RNA) blot assays. Total RNA was isolated with RNAzol B (Biotex Laboratories), separated on 1% agarose–morpholinepropanesulfonic acid (MOPS) formaldehyde gels, and transferred to Nytran membranes. Specific *junB* mRNA was detected with a human *junB* cDNA probe (34) as previously described (32). Hybridization signals were detected by autoradiography and quantified.

Luciferase assay. One day after transfections, cells were seeded into 12-well plates, allowed to attach to the plastic for 4 h, incubated in MEM-f/g plus 0.2% FBS for 6 h, and then incubated in MEM-f/g plus 0.2% FBS for 14 h. During this final incubation, cultures received TGF-β1 as indicated in the figure legends. Cells were harvested, and luciferase activity in cell lysates was determined by using a luciferase assay system (Promega) as described by the manufacturer. Total light emission was measured during the initial 20 s of the reaction in a luminometer.

Purification of receptor complexes. Transiently transfected R-1B cells (L17 clone) were labeled with [^{35}S]methionine by being washed and incubated for 30 min in methionine-free medium and then incubated for 2.5 h in this medium containing 50 μCi of [^{35}S]methionine (Trans-^{35}S-label; ICN) per ml. Phosphorylation in intact cells was carried out by washing and incubating the cells with phosphate-free media for 1 h and then incubating them in this medium containing 1 mCi of [^{32}P]phosphate (3,000 Ci/mmol; Amersham) per ml for 3 h at 37°C. Labeled cell monolayers were washed once with ice-cold PBS and lysed for 20 min in lysis buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 0.5% [vol/vol] Triton X-100) containing 50 mM NaF, 10 mM Na PP_i, 1 mM Na orthovanadate, and protease inhibitors. To isolate receptor complexes, cell extracts were clarified by centrifugation, brought to 25 mM imidazole, incubated with Ni^{2+}-nitrilotriacetic acid (NTA)-agarose (Qiagen) for 1 h at 4°C, and rinsed briefly three times and then twice for 15 min with 20 mM imidazole in lysis buffer. Ni^{2+}-NTA-agarose-bound receptors were eluted with 250 mM imidazole in lysis buffer. Eluates were diluted threefold with lysis buffer and precipitated with an anti-HA antibody (12CA5; Babco) and then adsorbed to protein G-sepharose (Pharmacia). Immunoprecipitates were washed three times with lysis buffer and three times with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, 1% sodium deoxycholate, 0.5% Triton X-100, 0.1% SDS [pH 7.5]) and subjected to SDS-PAGE and autoradiography.

In vitro autophosphorylation assays. HA-tagged TβR-II constructs were transiently transfected into COS-1 cells. On the second day after transfection, cells were lysed in TNE buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA) containing 0.5% (vol/vol) Triton X-100 and immunoprecipitated with an anti-HA antibody (12CA5; Babco) and protein G-Sepharose. The immunoprecipitates were washed four times with TNE containing 0.1% Triton X-100 and once with kinase assay buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM $CaCl_2$). The beads were incubated at 37°C for 10 min in kinase buffer containing 60 μM ATP and 5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) in a total volume of 40 μl. Beads were washed three times with TNE containing 0.1% Triton X-100 and then twice with RIPA buffer. Proteins were eluted by being boiled in SDS-PAGE sample buffer and separated on a 7.5% polyacrylamide gel.

In vitro transphosphorylation assay. R-1B cells (L17 clone) were transiently cotransfected with His-tagged TβR-II constructs and HA-tagged TβR-I. Cells were incubated with 1 nM TGF-β1 for 30 min at 4°C and lysed, and receptors were collected by being bound to Ni^{2+}-NTA-agarose. These samples were incubated for 10 min at 37°C in kinase assay buffer containing 5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) in a total volume of 60 μl. Bound protein was eluted from the beads by incubation with a 100 mM EDTA solution for 15 min at room temperature. Receptor complexes in these eluates were dissociated by being boiled for 5 min in the presence of 0.5% SDS. These samples were then diluted 10-fold in TNE buffer containing 0.1% Triton X-100 and 1% sodium deoxycholate, and TβR-I was precipitated with anti-HA antibody and displayed by SDS-PAGE and autoradiography.

Tryptic phosphopeptide mapping of TβR-II. Bands of SDS-separated ^{32}P-TβR-II were excised from the gel and digested for 13 h with trypsin. Phosphopeptides were resolved by thin-layer electrophoresis followed by chromatography on thin-layer chromatography plates with the Hunter thin-layer electrophoresis system (HTLE7000) and visualized by autoradiography (7).

RESULTS

Identification of a TGF-β receptor defective in signaling function. S-21 cells are TGF-β-resistant clones derived from the mink lung epithelial line Mv1Lu by mutagenesis with ethyl methanesulfonate and selection for proliferation in the presence of TGF-β (6, 21). S-21 cells do not show typical TGF-β responses, including inhibition of DNA synthesis (Fig. 1A), elevation of PAI-1 production (Fig. 1B), elevation of fibronectin production (Fig. 1C), or elevation of *junB* mRNA (Fig. 1D). However, affinity labeling of S-21 cells by cross-linking to bound ^{125}I-TGF-β1 showed a normal profile of TGF-β receptors I, II, and III (Fig. 1E), suggesting that S-21 cells are defective in a downstream component of the TGF-β signaling pathway or in a receptor domain involved in signaling but not in ligand binding.

To distinguish between these possibilities, we determined the ability of transfected wild-type receptors to restore respon-

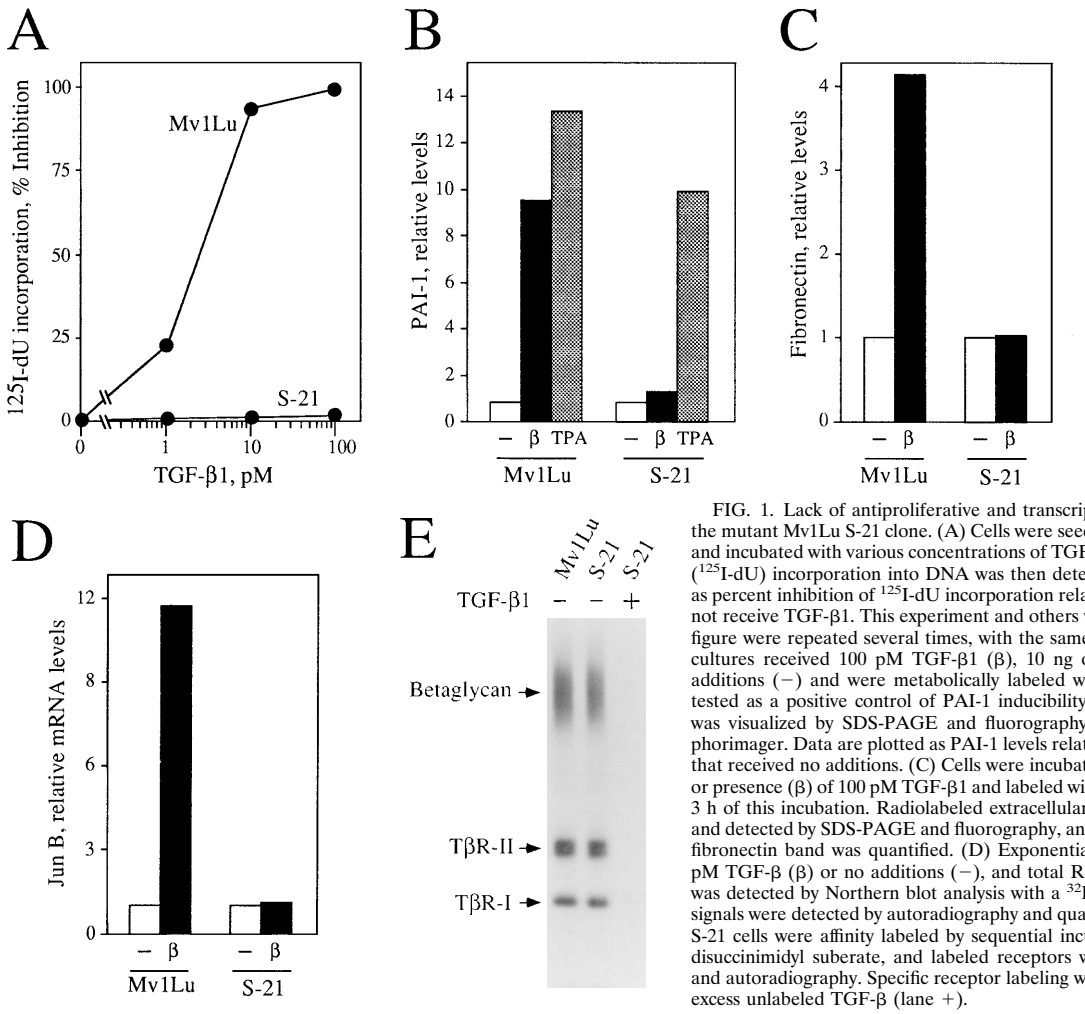

FIG. 1. Lack of antiproliferative and transcriptional responses to TGF-β in the mutant Mv1Lu S-21 clone. (A) Cells were seeded sparsely into 24-well plates and incubated with various concentrations of TGF-β1 for 18 h. ^{125}I-deoxyuridine (^{125}I-dU) incorporation into DNA was then determined. Results are expressed as percent inhibition of ^{125}I-dU incorporation relative to that of cultures that did not receive TGF-β1. This experiment and others whose results are shown in this figure were repeated several times, with the same results each time. (B) Sparse cultures received 100 pM TGF-β1 (β), 10 ng of TPA per ml (TPA), or no additions (−) and were metabolically labeled with [^{35}S]methionine. TPA was tested as a positive control of PAI-1 inducibility. Secreted radiolabeled PAI-1 was visualized by SDS-PAGE and fluorography and quantified with a Phosphorimager. Data are plotted as PAI-1 levels relative to those of control cultures that received no additions. (C) Cells were incubated for 16 h in the absence (−) or presence (β) of 100 pM TGF-β1 and labeled with [^{35}S]methionine for the final 3 h of this incubation. Radiolabeled extracellular matrix proteins were isolated and detected by SDS-PAGE and fluorography, and the signal associated with the fibronectin band was quantified. (D) Exponentially growing cells received 100 pM TGF-β (β) or no additions (−), and total RNA was isolated. *junB* mRNA was detected by Northern blot analysis with a ^{32}P-labeled probe. Hybridization signals were detected by autoradiography and quantified. (E) Parental Mv1Lu or S-21 cells were affinity labeled by sequential incubation with ^{125}I-TGF-β1 and disuccinimidyl suberate, and labeled receptors were displayed by SDS-PAGE and autoradiography. Specific receptor labeling was inhibited by the presence of excess unlabeled TGF-β (lane +).

siveness to TGF-β. An efficient method of screening Mv1Lu cells for responsiveness to TGF-β involves cotransfection of receptors with the TGF-β-inducible construct p3TP-Lux. This construct contains three tetradecanoyl phorbol acetate (TPA) response elements and a TGF-β-responsive segment of the human PAI-1 promoter (17) cloned upstream of a luciferase reporter gene (Fig. 2A). p3TP-Lux construction (43), its response to TGF-β in Mv1Lu cells (41, 43), and its use to identify TGF-β receptors (1, 5, 8) have been previously described. A more detailed characterization of this reporter system revealed that the three TPA response elements conferred responsiveness to TGF-β that could be augmented by inclusion of the PAI-1 promoter region more than by inclusion of the dyad symmetry element (DSE) from the c-*fos* promoter (39) (Fig. 2A). Induction of luciferase expression from p3TP-Lux in Mv1Lu cells was half maximal with 5 pM TGF-β1 (Fig. 2B) and was detectable 4 h after TGF-β addition and continued to rise 36 h after TGF-β addition (Fig. 2B). TGF-β2 and TPA in combination with epidermal growth factor were also strong activators of p3TP-Lux in Mv1Lu cells, whereas TPA, epidermal growth factor, and fetal calf serum added separately were intermediate activators and forskolin and activin A were weak activators (Fig. 2C). A survey of TGF-β-responsive cell lines showed a cell-dependent ability to mediate p3TP-Lux induction. Epithelial Mv1Lu, A549, and HepG2 cells are strong responders, showing a luciferase activity induction of 10- to 100-fold over the basal level, whereas fibroblastic NIH 3T3, CHO, and Rat 1 cells show weaker inductions of approximately five- or sixfold over the basal level (data not shown).

Transfection of TβR-II restored TGF-β responsiveness in S-21 cells, whereas transfection of TβR-I did not, as determined by the ability of these receptors to mediate induction of cotransfected p3TP-Lux vector (Fig. 3). This suggested that the lack of TGF-β responsiveness in these cells was caused by a mutation in the endogenous TβR-II. TGF-β induction of luciferase activity in TβR-II-transfected S-21 cells was somewhat lower than that in wild-type Mv1Lu cells (Fig. 3).

A point mutation in TβR-II kinase subdomain XI. The S-21 TβR-II cDNA was amplified by reverse transcription-PCR, and the products of three independent reactions were sequenced with identical results. Comparison with the wild-type mink TβR-II sequence (43) revealed that S-21 TβR-II contains a single mutation in its entire length. This is a point mutation (P525L) that converts the proline 525 codon (CCA) into a leucine codon (CTA). According to the canonical subdomain division of protein kinases (16), Pro-525 falls in subdomain XI (Fig. 4A). Except for a universally conserved arginine (16), this domain is widely divergent among protein kinases. However, this domain is highly conserved in type I and type II receptors for TGF-β and related cytokines (Fig. 4B). Interestingly, the kinases in this receptor family have a conserved Cys-Trp se-

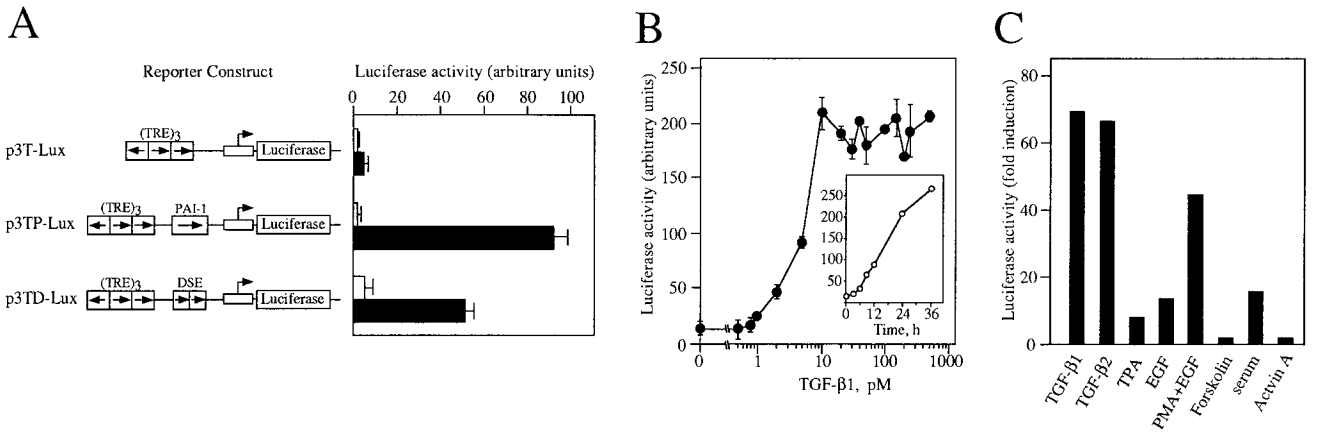

FIG. 2. Functional characterization of the TGF-β-inducible construct p3TP-Lux. (A) Schematic representation of the reporter plasmids showing the relative positions and orientation of the TPA response elements (TRE), PAI-1 promoter region, DSE, and luciferase reporter gene. Mv1Lu cells transiently transfected with the indicated reporter constructs were incubated for 20 h in the presence (closed bars) or absence (open bars) of 240 pM TGF-β1. Cell extracts were prepared and luciferase activity was measured in a luminometer. Data are the averages of triplicate determinations ± standard deviations. (B) Mv1Lu cells transiently transfected with the p3TP-Lux reporter construct were incubated with the indicated concentrations of TGF-β1 for 20 h or with 240 pM TGF-β1 for the indicated periods (inset). Luciferase activity was measured in cell lysates in triplicate. (C) Mv1Lu cells transiently transfected with the p3TP-Lux construct were incubated for 18 h with 240 pM TGF-β1, 240 pM TGF-β2, 1 nM TPA, 1 nM epidermal growth factor (EGF) alone or with phorbol myristate acetate (PMA), 10 μM forskolin, 20% fetal calf serum, or 250 pM activin A. Luciferase activity was measured and is plotted as fold induction relative to that of controls that received no additions.

quence in domain XI that is present in most tyrosine kinases but not in other serine/threonine kinases. Pro-525 is conserved in most type I receptors and in the anti-Müllerian hormone receptor (4, 12) but is substituted by alanine in other type II receptors, including mammalian activin receptors ActR-II (26) and ActR-IIB (2, 28), their *Drosophila melanogaster* counterpart Atr-II (45), and the *Caenorhabditis elegans* DAF-4 receptor that binds bone morphogenic protein 2 (14).

Signaling deficiency and dominant-negative phenotype of TβR-II(P525L). To study the properties of a receptor with a signaling deficiency, a P525L mutation was introduced into the human TβR-II cDNA and the resulting construct, TβR-II(P525L), was tested for its ability to restore TGF-β responsiveness to a TβR-II-deficient cell line. We used the Mv1Lu cell line derivative DR-26, which harbors a nonsense mutation in the transmembrane region of the endogenous TβR-II and therefore lacks this membrane receptor (21, 43). As previously reported (43), DR-26 cells transfected with wild-type TβR-II recovered TGF-β responsiveness, showing a strong increase in luciferase activity upon addition of TGF-β (Fig. 5A). In con-

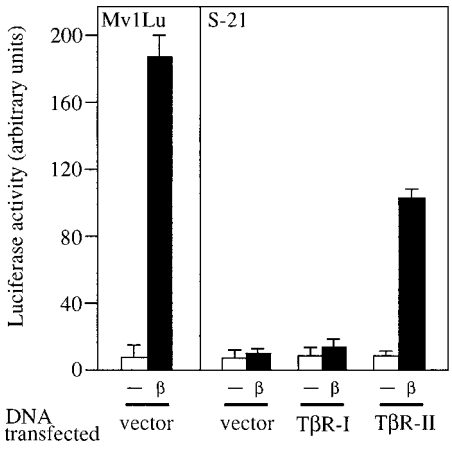

FIG. 3. Transfection of TβR-II restores TGF-β responsiveness in S-21 cells. Mv1Lu or S-21 cells were transiently cotransfected with p3TP-Lux and either empty pCMV5 vector (vector), TβR-I vector, or TβR-II vector. Transfectants were incubated for 20 h in the presence (β) or absence (−) of 250 pM TGF-β1. Luciferase activity in cell lysates was determined. Data are the averages of triplicate determinations ± standard deviations.

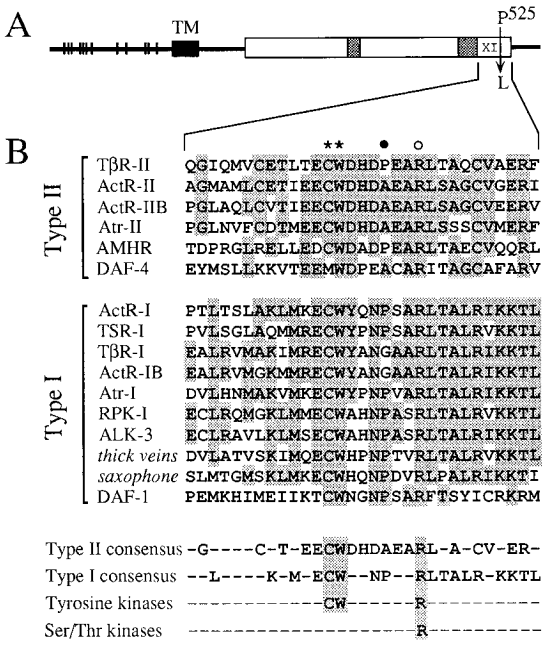

FIG. 4. S-21 TβR-II contains a point mutation in the kinase subdomain XI. (A) Schematic representation of TβR-II. Shown are the extracellular cysteines (vertical lines), transmembrane region (TM), kinase domain (open box), and kinase inserts (shaded boxes). Subdomain XI and the position of the proline-to-leucine mutation (P^{525}) are indicated. (B) Amino acid sequence alignment of protein kinase subdomains XI from TGF-β family receptors. Residues appearing in at least three of the six type II receptor sequences or in at least four of the 10 type I receptor sequences are shaded. Consensual sequences in the type I and type II receptor subgroups are shown below. The proline that was mutated in S-21 TβR-II (closed circle), the arginine universally conserved in protein kinases (open circle), and the cysteine-tryptophan sequence conserved in tyrosine kinases (asterisks) are indicated.

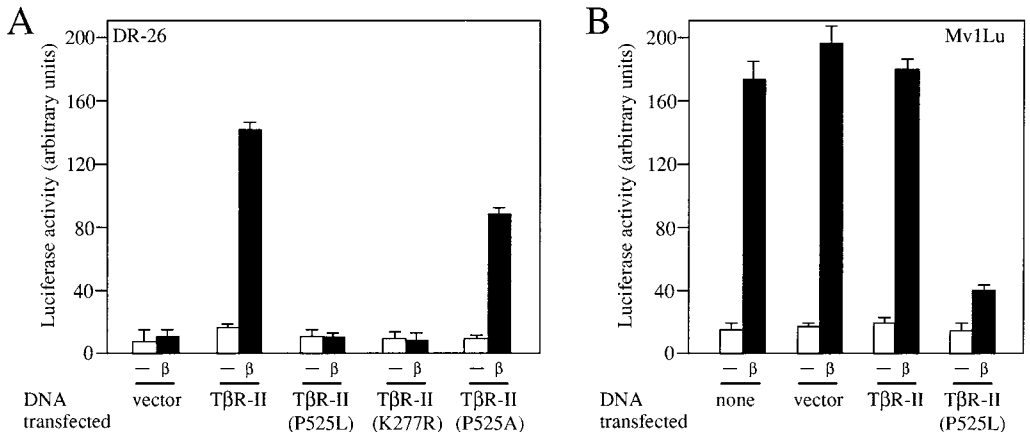

FIG. 5. Signaling deficiency and dominant-negative phenotype of TβR-II(P525L). (A) DR-26 cells were transiently transfected with either empty pCMV5 (vector), wild-type TβR-II, TβRII(P525L), TβR-II(K277R), or TβR-II(P525A), together with the p3TP-Lux reporter construct. Transfectants were incubated for 20 h with 250 pM TGF-β1 (β) or no additions (−), and luciferase activity was measured in cell lysates in triplicate. (B) Mv1Lu cells were transiently cotransfected with either empty pCMV5 (vector), wild-type TβR-II, or TβR-II(P525L) and p3TP-Lux. Transfectants were incubated in the presence (β) or absence (−) of 250 pM TGF-β1. Transfectants were incubated for 20 h with 250 pM TGF-β1 (closed bars) or no additions (open bars), and luciferase activity was measured in cell lysates in triplicate.

trast, cells transfected with vector alone or TβR-II(P525L) did not respond to TGF-β (Fig. 5A). TβR-II(P525L) was as ineffective as TβR-II(K277R) (Fig. 5A), a kinase-defective construct containing a mutation that disrupts the ATP binding site (8, 43).

The inactivating effect of substituting Pro-525 by the apolar residue leucine was surprising, because this position is replaced by the apolar residue alanine in all other type II receptors. Therefore, we generated and tested the construct TβR-II(P525A), containing an alanine residue at position 525. This construct restored TGF-β responsiveness to DR-26 cells, albeit less efficiently than did wild-type TβR-II (Fig. 5A). Thus, the presence of the somewhat bulkier leucine side chain at position 525 was sufficient to disrupt signal transduction.

We also tested the effect of transfecting TβR-II(P525L) into wild-type Mv1Lu cells. Transfection of vector alone or TβR-II did not perturb the p3TP-Lux response to TGF-β, whereas transfection of the mutant receptor strongly decreased this response (Fig. 5B). This dominant-negative effect suggests that the TβR-II(P525L) competes with endogenous receptor for a limiting component in the TGF-β signaling pathway, presumably TβR-I.

Ligand binding, TβR-I association, and autophosphorylation in TβR-II(P525L). Wild-type TβR-II binds ligand when expressed alone and allows ligand binding to a coexpressed TβR-I, forming a ligand-induced complex with this receptor (44). To confirm that these activities were preserved in TβR-II(P525L), this receptor or wild-type TβR-II was transfected alone or with TβR-I into COS-1 cells. Affinity labeling of these transfectants with ^{125}I-TGF-β1 showed that TβR-II(P525L) bound ligand and allowed ligand binding to a cotransfected TβR-I (Fig. 6A). These experiments were done with TβR-II constructs tagged at the C terminus with influenza virus HA (29). Precipitation with anti-HA antibody demonstrated an association of TβR-I with TβR-II(P525L) (Fig. 6A).

The P525L mutation might prevent signaling by disrupting the kinase activity of TβR-II. This possibility was tested with an in vitro kinase assay based on immunoprecipitation of the receptor from transfected COS-1 cells and incubation of the immune complexes with [γ-^{32}P]ATP (8, 44). HA-tagged versions of wild-type TβR-II and TβR-II(P525L) and the kinase-defective mutant TβR-II(K277R) as a negative control were subjected to this assay. Under these conditions, TβR-II and TβR-II(P525L) became phosphorylated to similar extents, whereas, as previously reported (41), TβR-II(K277R) was not phosphorylated (Fig. 6B). The same pattern was observed at various times during the linear phase of the autophosphorylation reaction (Fig. 6C). These results indicated that the autophosphorylating activity of the receptor was not severely affected by the P525L mutation.

Failure to transphosphorylate TβR-I. TβR-II is a constitutively active kinase that phosphorylates TβR-I after the two form a complex in vivo (44) or in vitro (40). The ligand dependence of this complex can be established by using a two-step precipitation assay in which TβR-II constructs tagged at the C terminus with a hexahistidine sequence that binds to nickel-NTA-agarose are cotransfected with TβR-I tagged at the C terminus with HA (44). These modifications do not perturb the ability of TβR-I and TβR-II to restore responsiveness in receptor-defective cells (3, 8, 30, 43, 44). Transfections were done in R-1B (L17) cells, a highly transfectable cell line defective in endogenous TβR-I (1, 6). R-1B cells transfected with TβR-I–HA together with wild-type or mutant TβR-II–His constructs were labeled with [^{35}S]methionine and incubated with TGF-β to generate receptor complexes. Cell lysates were first bound to nickel beads, the beads were eluted with imidazole under conditions that do not dissociate the receptor complex (44), and the eluate was precipitated with anti-HA antibody to isolate receptor complexes. Equal amounts of complex were recovered with TβR-II and TβR-II(P525L), and in both cases the complexes were ligand dependent (Fig. 7A).

TβR-I in complex with wild-type TβR-II was highly phosphorylated, as determined by isolation of receptor complexes from [^{32}P]phosphate-labeled cells (Fig. 7B) (44). TβR-I in complex with TβR-II(P525A) was also phosphorylated (data not shown). TβR-I was not phosphorylated when in a complex with the kinase-defective TβR-II(K277R) construct (Fig. 7B), as previously described (43). Most importantly, TβR-I was not phosphorylated when in a complex with TβR-II(P525L). This result indicated that the P525L mutation prevented TβR-I transphosphorylation even though it did not disrupt TβR-II autokinase activity.

To confirm this point, we conducted in vitro phosphorylation assays under conditions that allow TβR-I transphosphorylation by TβR-II (44). Cells were cotransfected with HA-tagged TβR-I and either TβR-II, TβR-II(K277R), or TβR-II(P525L)

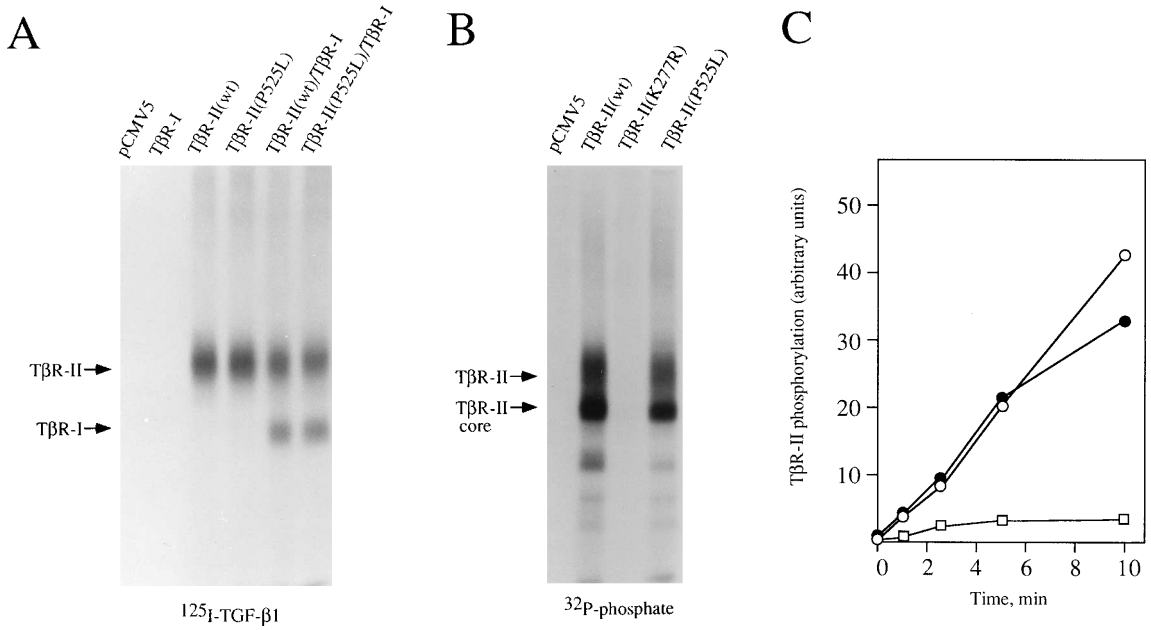

FIG. 6. Ligand binding and kinase activities of TβR-II(P525L). (A) COS-1 cells transiently transfected with the indicated receptor combinations or empty pCMV5 were affinity labeled by incubation with ^{125}I-TGF-β1 and cross-linking with disuccinimidyl suberate. The wild-type (wt) and mutant TβR-II receptors were tagged with HA at the N terminus. Cell lysates were precipitated with a monoclonal antibody against HA, and the precipitates were subjected to SDS-PAGE and autoradiography. (B) Lysates from COS-1 cells transiently transfected with the indicated HA-tagged receptor constructs were precipitated with anti-HA antibody. The immunoprecipitates were incubated for 10 min with [γ-^{32}P]ATP in kinase buffer, and the phosphorylated products were visualized by SDS-PAGE and autoradiography. Separate experiments indicated that the faster migrating of the two major species corresponds to partially glycosylated immature receptor core that accumulates in COS-1 cells because of receptor overexpression. (C) HA-tagged TβR-II (open circles), TβR-II(K277R) (open squares), and TβR-II(P525L) (closed circles) were immunoprecipitated from transfected COS-1 cells and incubated with [γ-^{32}P]ATP for the indicated times. The phosphorylated products were separated by SDS-PAGE, and the radioactivity associated with each receptor product was quantitated with a Phosphorimager.

or were transfected with vector alone. Cell lysates were bound to nickel-NTA-agarose via hexahistidine sequences engineered into the various TβR-II derivatives. After incubation of these complexes with [γ-^{32}P]ATP in a kinase reaction buffer (44), the complexes were eluted from the beads by incubation in EDTA solution and dissociated in SDS solution. TβR-I isolated from these samples by precipitation with anti-HA antibody was strongly phosphorylated when derived from complexes with TβR-II but not when derived from complexes with TβR-II(K277R) or TβR-II(P525L) (Fig. 7D). Parallel controls showed that the yields of TβR-I protein were the same with all three TβR-II forms (Fig. 7D).

TβR-II is phosphorylated by itself at several sites and by cellular kinases at other sites (44). The lower level of phosphorylation observed in TβR-II(K277R) (Fig. 7B and C) is due to loss of autophosphorylation (44). The relative phosphorylation level of TβR-II(P525L) after normalization based on metabolic labeling of the protein with [^{35}S]methionine was similar to that of wild-type TβR-II (Fig. 7C). This result was consistent with the presence of autophosphorylating activity in TβR-II(P525L) in vitro (Fig. 6B). TβR-II(P525L) autophosphorylation in vivo was confirmed by phosphopeptide mapping of receptor isolated from [^{32}P]phosphate-labeled cells (Fig. 8). Four phosphopeptides in this map (Fig. 8) are due to autophosphorylation, since they are missing in maps of receptor from TβR-II(K277R) (44). The weakest of these phosphopeptides appeared to be missing in TβR-II(P525L), whereas the other autophosphorylated peptides were present in TβR-II(P525L), although their relative intensities were somewhat different from those of the wild-type TβR-II (Fig. 8). These results indicated that TβR-II(P525L) is active as a kinase but is unable to recognize TβR-I as a substrate, providing further evidence that TβR-I transphosphorylation by TβR-II is essential for TGF-β signal propagation.

DISCUSSION

The recent identification of type I and type II receptors for various members of the TGF-β family has provided important information about the primary structure and ligand-binding properties of these receptors, and clues about the mode of activation of this class of receptors have begun to emerge. The TGF-β receptor complex is formed when ligand binds to TβR-II and contacts TβR-I. Recruitment into the complex leads to TβR-I phosphorylation by TβR-II, a unidirectional process since TβR-I does not phosphorylate itself or the associated TβR-II (44). Since the kinase activity of TβR-I is required for signaling (5, 8), it is thought that this activity phosphorylates as-yet-unknown substrates that propagate the signal (44). Thus, TβR-II acts as the primary TGF-β receptor and TβR-I acts as its substrate and downstream signaling component. Furthermore, a constitutively active mutant of TβR-I can generate antiproliferative and transcriptional signals in the absence of ligand and TβR-II (42), arguing that TβR-II acts upstream of TβR-I.

The present studies underscore the importance of receptor transphosphorylation in this activation process. We have identified a missense mutation in TβR-II that prevents transphosphorylation of an associated TβR-I both in vitro and in vivo, and this defect is accompanied by a loss of TGF-β responses. The antiproliferative response, the extracellular matrix response (elevation of fibronectin and PAI-1 expression levels), and the *junB* response are all lost in cells harboring TβR-II

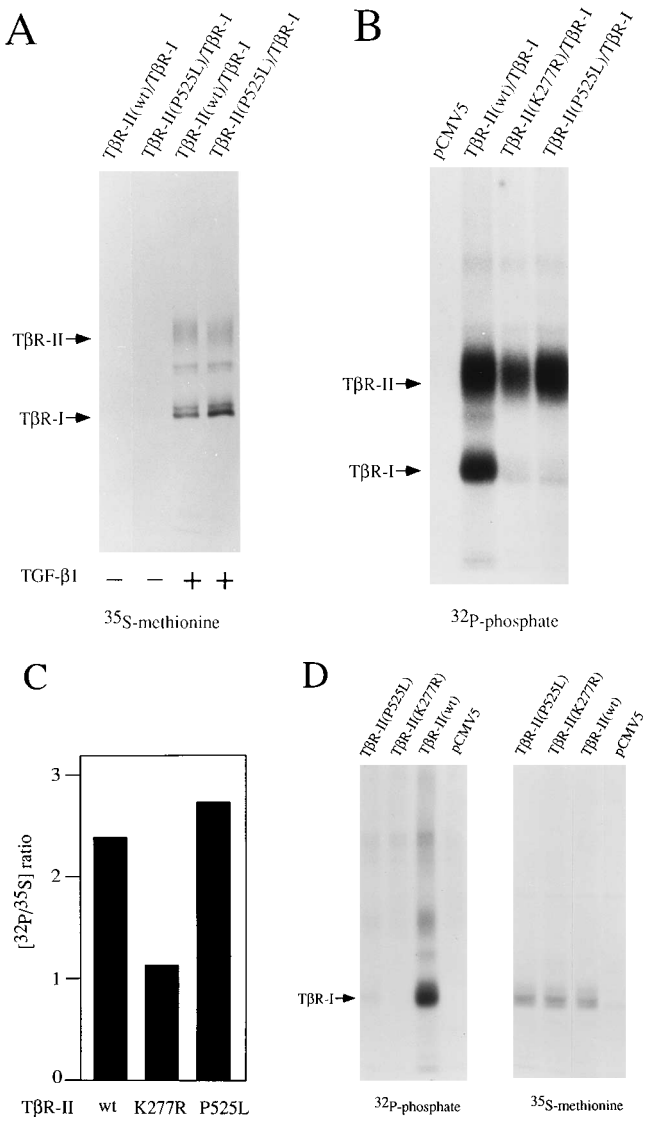

FIG. 7. TβR-II(P525L) is unable to phosphorylate TβR-I. (A) R-1B cells transiently transfected with the indicated receptors were labeled with [^{35}S]methionine and incubated with (+) or without (−) 1 nM TGF-β1 for 10 min at 4°C. Wild-type and mutant TβR-II constructs were tagged with a hexahistidine sequence at the C terminus, and TβR-I was tagged with HA at the C terminus. Receptor complexes were isolated by a two-step precipitation procedure with Ni^{2+}-NTA-agarose and anti-HA antibody beads. Precipitates were visualized by SDS-PAGE and autoradiography. (B) R-1B cells transiently transfected with the indicated receptor combinations were labeled with $^{32}P_i$ for 3 h and incubated with 1 nM TGF-β1 for 10 min. Receptor complexes were isolated by the two-step procedure, subjected to SDS-PAGE, and visualized by autoradiography. (C) The signal associated with the TβR-II, TβR-II(K277R), or TβR-II(P525L) bands in the experiment whose results are shown in panel B was normalized on the basis of signal associated with the corresponding bands metabolically labeled with [^{35}S]methionine in parallel cultures. wt, wild type. (D) Receptor complexes from R-1B cells transiently cotransfected with His-tagged TβR-II constructs and HA-tagged TβR-I(K232R) were bound to Ni^{2+}-NTA-agarose. Beads were incubated with [γ-^{32}P]ATP for 10 min. The receptor complexes were disrupted by being boiled in SDS, and TβR-I was isolated by precipitation with anti-HA antibody (right panel). Parallel dishes of cotransfected cells were metabolically labeled with [^{35}S]methionine and subjected to the same protocol but with the kinase reaction step omitted. This control showed that the amounts of recovered TβR-I were the same in all cotransfections.

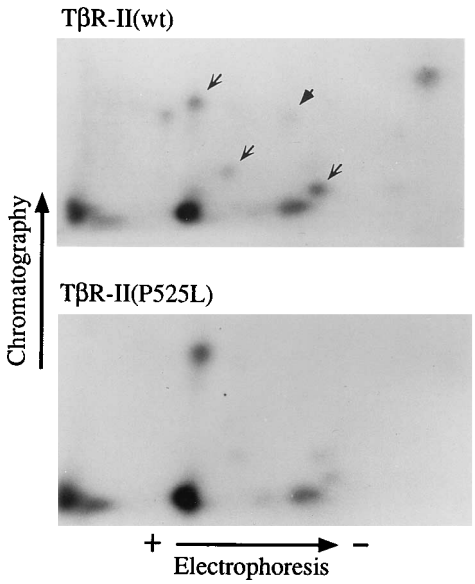

FIG. 8. Two-dimensional tryptic phosphopeptide mapping of wild-type (wt) and mutant TβR-II. TβR-II receptors from ligand-induced ^{32}P-labeled receptor complexes formed in transfected R-1B cells were isolated from SDS-PAGE gels and subjected to trypsin digestion. Phosphopeptides were resolved by thin-layer electrophoresis followed by chromatography on the second dimension. The typical positions of four autophosphorylation peptides (arrows) in the TβR-II map (41) are indicated, one of which (filled arrowhead) was not detectable in TβR-II(P525L) maps.

with the P525L mutation. TβR-II(P525L) can bind ligand and form a stable complex with TβR-I and is active as an autophosphorylating kinase. However, TβR-II(P525L) fails to transphosphorylate an associated TβR-I. The loss of signaling function in TβR-II(P525L) is therefore attributable to its inability to transphosphorylate TβR-I.

Identification of a TβR-II mutant defective in signaling function was made possible by the availability of a rapid screening assay for TGF-β responsiveness. This assay is based on transient transfection of TGF-β-unresponsive cells with either TβR-I or TβR-II together with the TGF-β-inducible vector p3TP-Lux. This vector was initially developed to demonstrate that TGF-β signals through a heteromeric receptor complex (43) and has been subsequently used to identify functional type I receptors for TGF-β and activin (1, 5, 8) and to determine the signaling activity of mutant receptor forms (41, 44). A more detailed characterization of p3TP-Lux in the present studies shows that its inducibility by TGF-β in mink lung epithelial cells is sustained for at least 3 days, and among different cell lines tested, the response is stronger in epithelial cells (Mv1Lu, A549, and HepG2) than in mesenchymal cells (NIH 3T3 and Rat 1).

Using this assay, we screened TGF-β-resistant Mv1Lu cell clones classified in previous studies as S (signaling) mutants (6, 20). These clones lack TGF-β responses but present normal TGF-β binding activity as determined by affinity labeling with radiolabeled ligand. Some S mutants, including the previously described S-1A and S-1B clones (6), are not complemented by transfection of either receptor, suggesting that they are defective in a downstream component of the TGF-β signaling pathway (40b). However, several mutants were complemented by transfection of TβR-I (40b) or TβR-II (this study), indicating the presence of defects in the signaling domain of these receptors and demonstrating the usefulness of this screening method.

Of the TGF-β-resistant clones that were rescued by TβR-II, we focused on S-21 because its endogenous TβR-II contains a missense mutation in a residue whose functional importance was not anticipated. Proline 525 is located in kinase subdomain

XI at the C terminus of the kinase domain. Although conserved in members of the TGF-β receptor family, kinase subdomain XI is very divergent in other protein kinases except for the presence of a highly conserved arginine. Interestingly, domain XI in TGF-β family receptors contains a Cys-Trp sequence that is also present in many tyrosine kinases but not in serine/threonine kinases (16). Phosphorylation of tyrosine as well as serine and threonine was reported for activin type II receptor preparations isolated from cultured cells (31). However, only serine and threonine phosphorylation has been reported so far for the cloned receptors (5, 8, 23, 27, 44).

In the crystal structures of cyclic AMP-dependent protein kinase (35), cell cycle-dependent protein kinase 2 (11), and the mitogen-activated protein kinase Erk2 (46), the domain XI arginine forms an ion pair with a highly conserved glutamic residue in domain VIII. Proline 525 is located 3 residues upstream of this arginine in TβR-II and is conserved in various members of the TGF-β receptor family, being substituted in others by the small apolar amino acids alanine and glycine (Fig. 4B). Mutation of Pro-525 to alanine in TβR-II does not prevent signaling, whereas mutation to leucine does. Although the imido group of proline has the characteristic of restricting the angle of the peptide backbone, in this particular case the higher degree of freedom imparted by an alanine residue does not disturb kinase activity. Since the proline and alanine side chains are otherwise similar, in size and hydrophobicity, we speculate that the bulkier leucine residue alters the tertiary structure of the receptor in such a way that it prevents recognition of TβR-I as a substrate without inactivating the intrinsic kinase activity of TβR-II. Such a defect is likely to spare the ion pairing of the conserved arginine in domain XI, since it would otherwise eliminate receptor kinase activity.

Although the P525L mutation preserved the kinase activity of TβR-II, it altered somewhat the relative autophosphorylation levels of certain sites in vivo. However, mapping of these sites and site-directed mutagenesis experiments suggest that TβR-II autophosphorylation is not essential for signaling (40a). The altered autophosphorylation pattern in TβR-II(P525L) may result from a conformational alteration in domain XI affecting phosphorylation of vicinal sites.

The properties of TβR-II(P525L) support the conclusion that TβR-I transphosphorylation is an essential part of the signal propagation process. Until recently, this conclusion rested on evidence obtained with mutations that are more disruptive than P525L. One such mutation is the TβR-II(K277R) mutation, which eliminates the kinase activity of this receptor (44). Signaling and TβR-I phosphorylation are also concomitantly disrupted by mutation of seven serine and threonine residues in the GS domain of TβR-I that are potential phosphorylation sites (32, 44). However, these mutations could have a marked effect on the overall structure of the GS domain. The ability to prevent TβR-I phosphorylation and TGF-β signaling with the less invasive P525L mutation argues strongly that these two events are causally linked. Therefore, the evidence leads to the conclusion that phosphorylation of TβR-I by TβR-II is essential for propagation of various antiproliferative and transcriptional responses to TGF-β.

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