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Cloning and characterization of a transmembrane serine kinase that acts as an activin type I receptor

(activin/transforming growth factor β/rat activin type I receptor/activin type II receptor)

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ABSTRACT **Activin type II receptors are transmembrane protein-serine/threonine kinases. By using a reverse-transcription PCR assay to screen for protein kinase sequences, we isolated a cDNA clone, activin X1 receptor, from rat brain that encodes a 55-kDa transmembrane protein-serine kinase which is structurally related to other receptors in this kinase subfamily. The predicted protein consists of 509 amino acids, and the kinase domain shows 40% and 37% identity to the activin and transforming growth factor β type II receptors, respectively. No activin-binding was observed when activin X1 receptor was expressed alone in COS-M6 cells; however, coexpression with type II activin receptors gave rise to a 68-kDa affinity-labeled complex in addition to the 85-kDa type II receptor complex. The size of this cross-linked band is consistent with the size of the type I activin receptor; furthermore, activin X1 receptor associated with type II receptors, as judged by coimmunoprecipitation with type II receptor antibodies. These data suggest that activin X1 receptor can serve as an activin type I receptor and that the diverse biological effects of activins may be mediated by a complex formed by the interaction of two transmembrane protein-serine kinases.**

Activins are members of a large family of polypeptide growth and differentiation factors, of which the prototypic members are the transforming growth factors β (TGF-β) (1, 2). Activins bind to cell-surface receptors of 50–55 kDa and 70–75 kDa, known as type I and type II receptors (ActRI and ActRII, respectively) (3, 4). Two closely related type II ActRs have been cloned (ActRII and ActRIIB; refs. 3–5) and belong to the recently defined family of transmembrane protein-serine/threonine kinases, which includes the *daf-1* gene product of *Caenorhabditis elegans* (6) and the type II TGF-β receptor (TβRII) (7). Intrinsic kinase activity for the ActRIIs has been reported both *in vitro* (8) and *in vivo* (9). These molecules are involved in activin signal transduction; disruption of normal ActRII or ActRIIB expression in *Xenopus* embryos alters the developmental program (5, 10, 11).

Receptors I and II can be visualized by chemical cross-linking of ^{125}I-labeled activin A to cells, yielding affinity-labeled complexes of 60–65 and 85–90 kDa. ActRI has been observed to associate with ActRII, based on the immunoprecipitation of both affinity-labeled complexes by an antibody against ActRII (9). A similar relationship has been observed for TGF-β receptors I and II (TβRII) (12, 13); furthermore, both TGF-β receptors are apparently required for signal transduction (12–14). The formation of a heteromeric complex between receptors I and II suggested that ActRI could be structurally related to the type II receptors. To test this hypothesis, we have undertaken reverse-transcription PCR with degenerate primers deduced from the conserved serine/threonine kinase domains of activin receptors and TβRII. We have isolated another kinase that is a member of the type II receptor family, activin X1 receptor (ActX1R), from a rat brain cDNA library.§ The sequence of the mouse (named Tsk 7L; ref. 15) and human (named SKR1; ref. 16) homologues of ActX1R have been reported during the preparation of this manuscript. The former has been proposed to be a TGF-β type I receptor. We report here that the rat ActX1R fulfills the criteria of the ActRI, raising the possibility that a common type I receptor could interact with both ActRII and TβRII.

MATERIALS AND METHODS

Materials. Recombinant human activin A and inhibin A were provided by J. Mather (Genentech). Human TGF-β_1 was from R & D Systems.

Reverse Transcription and PCR. A mixture of oligo(dT)-primed cDNAs prepared from 5 μg of total RNA isolated from adult rat brain was used as a template for PCR. The following degenerate primers were used: H1 (5′-CGGGATC-CGTNGCNGTNAARATHTTYCC-3′; sense primer that corresponds to the amino acid sequence VAVKIFP in kinase subdomain II), H2 (5′-CGGGATCCRTTYTTNSWYTTDA-TRTCNCKRTG-3′; antisense primer that corresponds to the amino acid sequence HRDIKSKN in kinase subdomain VIB), and H3 (5′-CGGGATCCYTCNGGNGCCATR-TANCKYCTNGTNCC-3′; antisense primer that corresponds to the amino acid sequence GTRRYMAPE in kinase subdomain VIII). N represents all four nucleotides. All three primers have *Bam*HI sites at the 5′ termini to facilitate subcloning the PCR products. Two-step PCR amplification was done according to the following schedule; an initial denaturation step at 94°C for 5 min, 35 cycles of 94°C for 1 min, 46°C for 2 min, and 72°C for 3 min, and a final incubation for 10 min at 72°C. An aliquot of the PCR reaction using primers H1 and H3 was electrophoresed on a 5% polyacrylamide gel. The amplified DNA (≈450 bp) was purified from the gel and subjected to a second PCR using primers H1 and H2; the conditions were the same as above, except the annealing temperature was 55°C. The final PCR products (≈320 bp) were purified and subcloned into pBluescript SK after digestion with *Bam*HI. Ten out of 14 clones sequenced were ActRII or IIB; two clones (designated ActX1R) were found to have a different kinase motif. The same PCR products were obtained when pituitary gland and testis RNAs were used for the templates of reverse transcriptase-PCRs.

cDNA Cloning. Adult Sprague–Dawley rat whole-brain poly(A)$^+$ RNA was used for the synthesis of a cDNA library.

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Abbreviations: ActRI, activin type I receptor; ActRII, activin type II receptor; ActRIIB, activin type IIB receptor; TGF-β, transforming growth factor β; TβRII, TGF-β type II receptor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L19341).

Double-stranded cDNA was ligated to *Eco*RI–*Not* I adaptors (Pharmacia LKB) and cDNAs >2 kbp were ligated into the λZAPII vector (Stratagene) as described (17). Clones (5×10^5) were screened by hybridization with the 320-bp cDNA fragment of ActX1R. Two positive clones were isolated and rescued into pBluescript SK. The cDNA inserts of these two clones showed identical restriction enzyme patterns, and one of these two was selected for further sequence analysis.

Binding and Chemical Cross-Linking. ActX1R was subcloned into the mammalian expression vector pcDNA1 (Invitrogen) and transfected into COS-M6 cells alone or with mouse ActRII (ActRII) subcloned into the same vector by the DEAE-dextran method as described (3). Cells (1.5×10^6) were plated on 150-mm dishes and transfected with 10 μg of total DNA, either 5 μg of specific receptor DNA and 5 μg of vector DNA or 5 μg of each receptor DNA. Recombinant human activin A was iodinated as described (3). For receptor-binding assays, transfected cells were plated in 24-well dishes, incubated with ^{125}I-labeled activin A in 0.25 ml of Hepes-buffered saline in the presence or absence of nonlabeled competitors, washed, and solubilized in 0.5 M NaOH, and cell-associated radioactivity was quantitated. For cross-linking, cells were mechanically released, bound with ^{125}I-labeled activin A, washed, incubated with 500 μM disuccinimidyl suberate at 0°C, and solubilized with 1% Triton X-100. Affinity-labeled receptors were subjected to immunoprecipitation by using affinity-purified antibody 199D, directed against the COOH terminus of ActRII, as described (9). Final concentration of SDS during immunoprecipitation was 0.07%.

RNA Blot Hybridization Analysis. Poly(A)$^+$ RNAs were isolated, and RNA blot hybridization analysis was done by using 3 μg of each poly(A)$^+$ RNA as described (18). The PCR product for ActX1R and the 1.1-kb fragment that represents the intracellular kinase domain of mActRII were used as probes.

RESULTS

cDNA Cloning and Structure of ActX1R. To isolate and characterize additional members of the activin/TGF-β type II receptor serine kinase family, we performed reverse transcriptase-PCR with degenerate primers based on sequences in the conserved kinase regions (3, 7). By using adult rat brain cDNA as a template, we identified several different fragments containing the protein-serine/threonine kinase motif (19). We then isolated a full-length cDNA clone of one of these, ActX1R, from a rat brain cDNA library by using this PCR product as a hybridization probe. ActX1R encodes 509-amino acid residues (Fig. 1); there is a 20-amino acid signal peptide at the N terminus (22) and a single putative membrane-spanning domain between residues 124 and 146. Thus, the mature peptide is predicted to be a 489-amino acid membrane protein with a 103-amino acid extracellular domain, a 23-amino acid transmembrane domain, and a 363-amino acid intracellular domain. The predicted molecular mass is ≈55 kDa. There is also one potential site of N-linked glycosylation in the extracellular domain (Asn-102). The kinase domain of ActX1R shows 38–40% sequence identity to ActRII and -IIB and shows 37% identity to TβRII (Fig. 1). The extracellular domains of these receptors share little sequence similarity, although 8 out of 10 cysteine residues can be aligned fairly well.

Binding Properties of ActX1R. To determine whether ActX1R could bind activin, COS-M6 cells were transiently transfected with ActX1R, either alone or in the presence of ActRII, and receptor-binding assays were performed. Activin binding to untransfected COS cells was not detectable by using these assay conditions. ActX1R expressed alone in COS cells did not increase activin binding (Fig. 2*A*); furthermore, coexpression of ActX1R with ActRII did not affect the affinity of binding of either activin (Fig. 2 *A* and *B*) or inhibin, or TGF-β (Fig. 2 *C* and *D*) when compared with cells expressing ActRII alone (Fig. 2 *B–D*). Cells expressing ActRII, alone and in the presence of ActX1R, bound activin with a K_d of ≈500 pM (Fig. 2*B*); that value is in the range reported both for native and cloned ActRIIs (3–5, 23, 24). The total amount of activin binding to transfected cells was reduced ≈30% when ActX1R was expressed with ActRII (Fig. 2*B*).

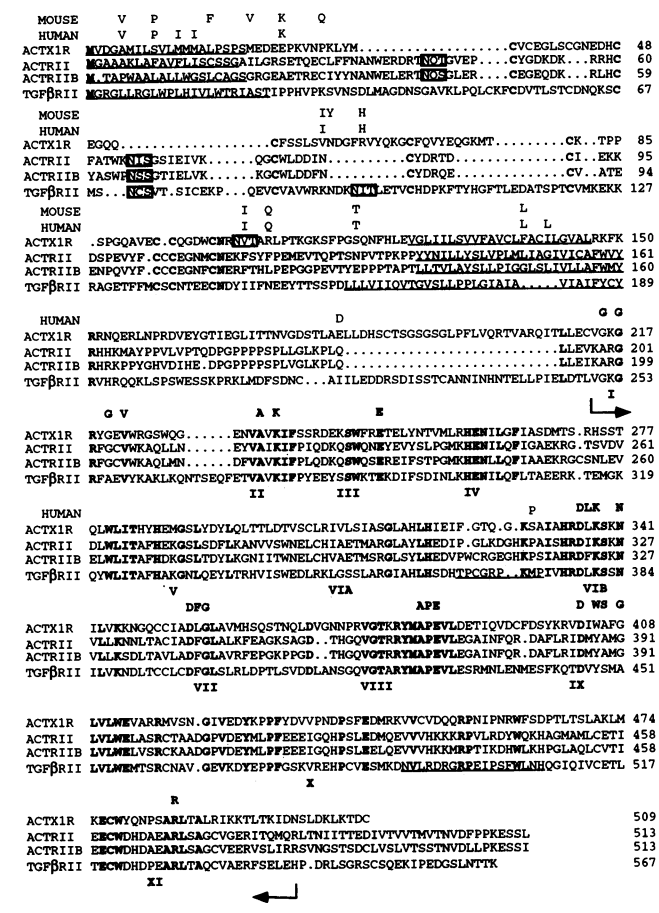

FIG. 1. Amino acid sequence of ActX1R. The deduced sequence of rat ActX1R is compared with mouse (15) and human (16) ActX1R, rat ActRII (20), rat ActRIIB (21), and rat TβRII (17). In mouse and human ActX1R, only the amino acids different from those of rat ActX1R are shown above the alignment. Amino acids conserved in all four proteins are shown in boldface type. The signal peptide, transmembrane domain, and two kinase inserts are shown with a single underline. Putative N-linked glycosylation sites are boxed. The kinase domain is delineated with arrows. Highly conserved amino acids in the serine/threonine kinase subfamily and subdomains in the kinase domain are shown in boldface type, above and below the alignment, respectively.

To test whether ActX1R could, nevertheless, be a component of a receptor complex, we performed chemical cross-linking on COS cells transfected with ActX1R and ActRII, either independently or in combination. ActRII, when expressed alone, gave rise to a broad affinity-labeled complex of ≈85 kDa, as has been described (3, 9) (Fig. 3). In accord with the receptor assay data, cells expressing ActX1R alone did not reveal any activin-binding proteins; however, coexpression of ActX1R with ActRII resulted in the appearance of an additional affinity-labeled complex of 68 kDa that was not observed when ActRII was expressed alone (Fig. 3). The size of this band is consistent with the size of the type I ActR in AtT20 and other activin-responsive cells. As with the

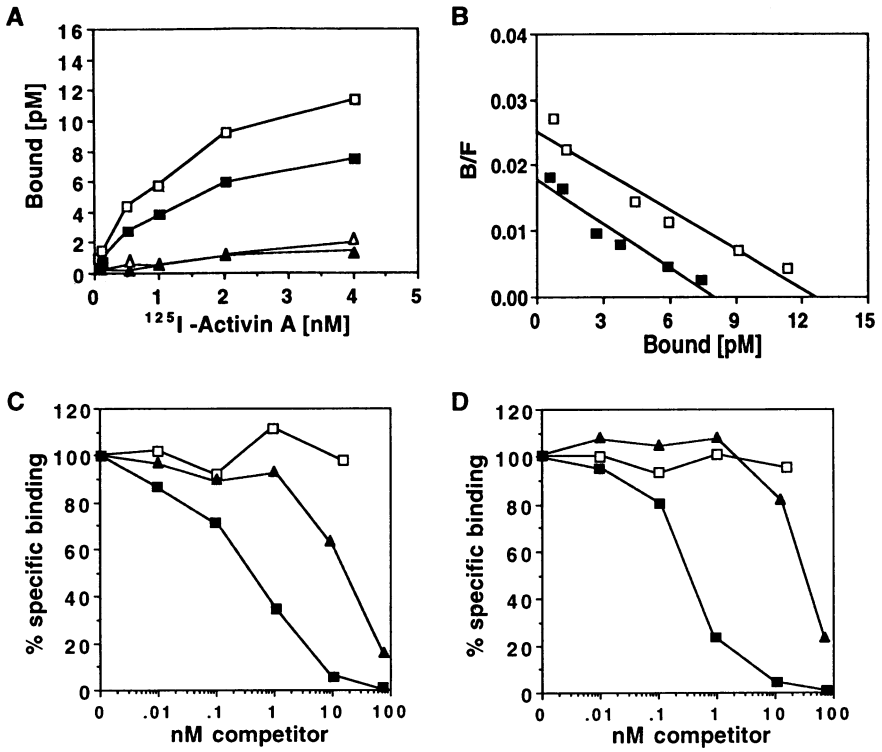

FIG. 2. Binding of ^{125}I-labeled activin A to untransfected and transfected COS cells. (*A*) Untransfected COS cells (△) and COS cells transfected either with ActX1R (▲), ActRII (□), or ActX1R and ActRII (■) were bound with various concentrations of ^{125}I-labeled activin A in the absence or presence of 81 nM unlabeled activin A in a 0.25-ml vol. The values are the mean of triplicate determinations. (*B*) Scatchard plot analysis of the data shown in *A*. B/F, bound/free. (*C* and *D*) COS cells transfected with ActRII (*C*) or ActX1R and ActRII together (*D*) were bound with 100 pM of ^{125}I-labeled activin A in the presence of various concentrations of unlabeled activin A (■), inhibin A (▲), or TGF-β_1 (□), as described. Data are expressed as percent specific binding; 100% specific binding and nonspecific binding represented 3.2% and 0.7% of input cpm, respectively, for *C* and represented 2.8% and 0.6% of input cpm, respectively, for *D*.

receptor-binding data, the overall amount of activin binding in the type II receptor complex was reduced in cells expressing both molecules.

The formation of both the type II and the putative type I affinity-labeled complexes could be prevented by incubation with cold activin, partially blocked by cold inhibin, and was not affected by the presence of unlabeled TGF-β_1 (Fig. 4). Because ActRI could be precipitated by an antibody against ActRII (9), we immunoprecipitated lysates from cells expressing ActX1R and ActRII. The 68-kDa affinity-labeled band from ActX1R expression was coprecipitated with ActRII by antibody 199D, which was raised against a peptide predicted from the COOH terminus of ActRII; this association between receptors I and II was abolished when the lysates were denatured by heating with 1% SDS before incubation with the antibody (Fig. 4). The same association

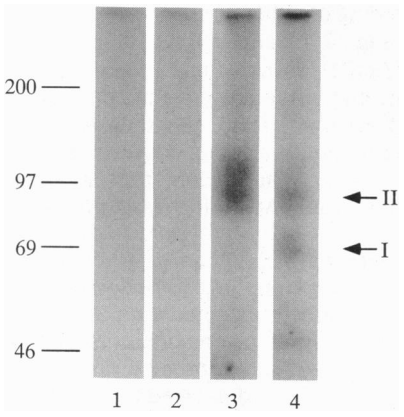

FIG. 3. Affinity labeling of transfected COS cells. Untransfected COS cells (lane 1) or COS cells transfected with ActX1R (lane 2), ActRII (lane 3), or ActRII and ActX1R together (lane 4) were bound with 2 nM ^{125}I-labeled activin A and cross-linked with disuccinimidyl suberate; the receptor-containing membrane fraction was solubilized, and the products were resolved by SDS/PAGE under reducing conditions. Molecular mass standards are indicated in kDa.

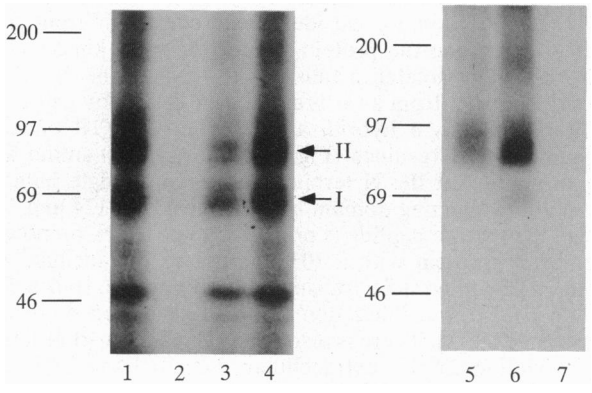

FIG. 4. Specificity and immunoprecipitation of affinity-labeled activin receptors. COS cells transfected with both ActX1R and ActRII were bound with ^{125}I-labeled activin A without (lane 1) or with 36 nM activin A (lane 2), 36 nM inhibin A (lane 3), or 18 nM TGF-β_1 (lane 4), cross-linked with disuccinimidyl suberate, and the detergent-soluble fraction was resolved by SDS/PAGE under reducing conditions. For immunoprecipitation analysis, affinity-labeled receptors were precipitated by an antibody (199D) against ActRII without (lanes 5 and 6) or with (lane 7) competing amounts of the peptide immunogen. For the sample in lane 5, the cell lysate was heated at 100°C for 5 min in 1% SDS before immunoprecipitation. Molecular mass standards are indicated in kDa.

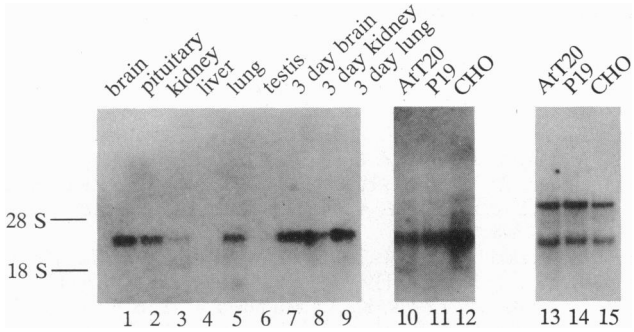

FIG. 5. Blot hybridization analysis of ActX1R mRNA. Poly(A)$^+$ RNAs prepared from adult rat brain, pituitary gland, kidney, liver, lung, and testis; 3-day-old rat brain, kidney, and lung; and AtT20 (mouse corticotropic pituitary tumor), P19 (mouse embryonal carcinoma), and CHO (Chinese hamster ovary) cells (3 μg each) were resolved on a denaturing agarose gel and analyzed by blot hybridization with either an ActX1R cDNA probe (lanes 1–12) or an ActRII cDNA probe (lanes 13–15). Positions of rRNAs (28S and 18S) are indicated.

was seen when immunoprecipitation of lysates from cells expressing ActRIIB and ActX1R was done with the antibody raised against a peptide predicted from the COOH terminus of ActRIIB (ref. 9; K.T. and W.W.V., unpublished data).

Tissue Distribution of ActX1R. Expression of ActX1R mRNA was analyzed by RNA blot hybridization analysis of poly(A)-selected RNAs. A single 3.5-kb transcript was detected in multiple adult tissues (Fig. 5); in kidney, an additional 2.2-kb transcript was also detected. Expression was high in brain, pituitary gland, and lung; moderate in kidney; and low in liver and testis. Three-day-old tissues of brain, kidney, and lung also expressed this mRNA at a level comparable to adult tissues. In addition, a number of activin-responsive cell lines, including AtT20, P19, and CHO cells were observed to express mRNA for ActX1R (Fig. 5); these cell types all expressed comparable levels of mRNA for ActRII (Fig. 5).

DISCUSSION

In this study, we describe the cloning and characterization of an additional member of the transmembrane protein-serine/threonine kinase receptor family. By several criteria, we conclude that the cDNA reported here encodes an ActRI. (*i*) When coexpressed with ActRII in mammalian cells, ActX1R gave rise to a 68-kDa affinity-labeled band, which is consistent with the size of ActRI (3, 4, 9). (*ii*) The ActX1R affinity-labeled complex could be immunoprecipitated with an antibody against ActRII; this association was abolished by denaturation of the proteins before incubation with the antibody and is characteristic of ActRI in activin-responsive cells (9). (*iii*) ActX1R was expressed in activin-responsive cells and tissues where ActRII is also expressed (3); it is thus possible that the two molecules could form a complex *in vivo*.

While this manuscript was in preparation, the murine homologue of ActX1R was proposed to be a type I TGF-β receptor on the basis of a similar interaction with TβRII (15). In that report, coexpression of mouse ActX1R with TβRII resulted not only in the appearance of a type I TGF-β receptor complex, but also in the inhibition of the formation of the type II complex. We also observed an inhibition of binding to mouse ActRII after coexpression of rat ActX1R; the decrease in binding was dose-dependent (unpublished data). This result is due, in part, to decreased amounts of ActRII protein in the transfected cells (L.S.M., unpublished data), although ActX1R may also block the ligand–receptor interaction, either directly or indirectly. This inhibition is probably not an artefact of the transient transfection system because coexpression of ActX1R with the growth hormone receptor did not affect growth hormone binding (L.S.M., unpublished data). Analysis of cell lines stably expressing receptors I and II will be required to determine the mechanism of inhibition and to assess whether the decreased cell-surface binding is functionally important. Our observation raises the interesting possibility that a common type I receptor could interact with ActRII, ActRIIB, and TβRII, although it is not formally established whether ActX1R serves as a signaling receptor for activin, TGF-β, or both. Thus, we propose that ActX1R should be called TARI (for TGF-β receptor/ActRI). A shared receptor could explain, in part, the common activities of activin and TGF-β in several systems. For example, both factors are thought to inhibit cell proliferation by blocking the phosphorylation of the retinoblastoma gene product, pRB (25–27). Furthermore, TβRII can mimic the action of the ActRIIs in early *Xenopus* development; expression of human TβRII in *Xenopus* embryos allows TGF-β_1 to induce mesoderm tissues (A. Bhushan and C. R. Kintner, personal communication).

The use of a common signaling receptor subunit is well characterized in several other systems. Interleukin 6, leukemia inhibitory factor, and oncostatin M each have ligand-specific, low-affinity receptors, which dimerize with a single related molecule, gp130, to form a high-affinity receptor complex; gp130 is essential for signal transduction (28). Similarly, interleukins 3 and 5 and granulocyte–macrophage colony-stimulating factor have specific α subunits and share a common β subunit to form high-affinity, signaling receptor complexes (28). Activin receptors I and II are unique in the sense that the intrinsic kinase activity suggests that both molecules have signaling capability. Whether a common type I receptor mediates similar effects of activin and related ligands, whereas ligand-specific type II receptors mediate other effects (29), remains to be determined.

The nature of the interaction between the type I receptor, the type II receptor, and dimeric activin remains unknown. A heteromeric association of all three is suggested by the fact that the ActX1R affinity-labeled complex could be immunoprecipitated with an antibody against ActRII or ActRIIB, even though there are no similarities between the sequences of ActX1R and the peptide immunogens used to generate antibodies toward ActRII and ActRIIB (ref. 9; Fig. 1). Although activin crosslinks to the type I receptor, there is no evidence, as yet, that they bind to each other in the absence of the type II receptor. The type II receptor might participate in the processing or trafficking of the type I receptor, or the type I receptor might only recognize activin when associated with the type II receptor. The observation, however, that coexpression of ActX1R with ActRII did not affect the affinity of activin binding relative to cells expressing ActRII alone, argues that binding of activin within a putative ActRI–ActRII complex is noncoordinate. ActRI might not bind ligand directly but to an ActRII–activin complex and might thereby be affinity-labeled by activin by virtue of juxtapositioning within the heteromer. The elucidation of the stoichiometry of the ligand–receptor(s) interaction will be important for clarifying the mechanism of generation of the transmembrane signal.

It is well established that binding of ligands such as epidermal growth factor, which signals through a receptor tyrosine kinase, leads to receptor dimerization and transphosphorylation, both of which are required for signaling. Dimerization is clearly important for the receptor serine kinases as well because truncated forms of ActRIIB (11) and TβRII (29, 30) can serve as dominant negative mutants. These results imply that functional dimer formation is necessary for signaling. It is of interest that in Mv1Lu cells, the truncated TβRII inhibited only a subset of the TGF-β re-

sponses (29), implying that different receptor complexes activate different signaling pathways. In accord with that observation, we have identified several other kinases related to type I receptors that are expressed tissue-specifically (K.T. and W.W.V., unpublished data), suggesting that combinatorial association of receptor subunits could account for the diverse effects of these ligands.

Note Added in Proof. After communication of this manuscript, the sequence of rat ActX1R was also reported by He *et al.* (31), who studied its developmental expression but did not establish its function. Ebner *et al* (32), in agreement with our findings, have found that the mouse homologue of rat ActX1R meets criteria expected of an activin type I receptor.

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