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Activation of Signalling by the Activin Receptor Complex

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Activin exerts its effects by simultaneously binding to two types of protein serine/threonine kinase receptors, each type existing in various isoforms. Using the ActR-IB and ActR-IIB receptor isoforms, we have investigated the mechanism of activin receptor activation. ActR-IB and -IIB are phosphoproteins with demonstrable affinity for each other. However, activin addition strongly promotes an interaction between these two proteins. Activin binds directly to ActR-IIB, and this complex associates with ActR-IB, which does not bind ligand on its own. In the resulting complex, ActR-IB becomes hyperphosphorylated, and this requires the kinase activity of ActR-IIB. Mutation of conserved serines and threonines in the GS domain, a region just upstream of the kinase domain in ActR-IB, abrogates both phosphorylation and signal propagation, suggesting that this domain contains phosphorylation sites required for signalling. ActR-IB activation can be mimicked by mutation of Thr-206 to aspartic acid, which yields a construct, ActR-IB(T206D), that signals in the absence of ligand. Furthermore, the signalling activity of this mutant construct is undisturbed by overexpression of a dominant negative kinase-defective ActR-IIB construct, indicating that ActR-IB(T206D) can signal independently of ActR-IIB. The evidence suggests that ActR-IIB acts as a primary activin receptor and ActR-IB acts as a downstream transducer of activin signals.

Activin was discovered for its ability to regulate follicle-stimulating hormone production by pituitary cells and is among the first identified members of the transforming growth factor β (TGF-β) family (reviewed in reference 50). Activin acts on many cell types, regulating hormone production in placental cell cultures (43), inducing differentiation in erythroblasts (14) and osteoblasts (38), and inhibiting proliferation of gonadal cell lines (17), endothelial cells (35), lung epithelial cells (7), and hepatocytes (60). When added to *Xenopus* embryo animal cap explants at low concentrations activin induces ventrolateral mesoderm tissues such as mesenchyme, muscle, and kidney, and at higher concentrations it induces dorsal mesoderm structures such as notochord (reviewed in references 24, 25, and 45). When added to chicken blastula hypoblasts, activin induces formation of axial structures including notochord and somites (36). Furthermore, injection of dominant negative activin receptor mRNA into *Xenopus* early embryos interferes with mesoderm induction, suggesting that the endogenous mesoderm inducer signals through activin receptors (21). Collectively, these observations indicate that signalling through activin receptors is important in the regulation of cell fate in vertebrates.

Activin exists in three isoforms that are homo- and heterodimers of βA and βB chains encoded by separate genes (reviewed in reference 50), and additional isoforms might arise from a putative activin, βC, recently cloned from human cells (21a). A family of transmembrane serine/threonine kinases that act as receptors for these and other TGF-β group factors have been identified in mammals, *Xenopus laevis*, and *Drosophila melanogaster* (reviewed in references 5, 25, and 37). These receptors fall into two distinct subfamilies known as type I and type II receptors that are distinguished by the level of sequence homology of their kinase domains and by other structural and functional features. Type I and type II receptors act cooperatively to bind ligand and transduce signals. Type II receptors for activin, as those for TGF-β, bind ligand on their own, whereas the type I receptors bind ligand only when coexpressed with the corresponding type II receptors (3, 13, 16, 22, 56). Genetic and biochemical evidence indicates that coexpression of type I and type II receptors in the same cell is required for signalling (26, 56). In the TGF-β receptor system, a basis for this phenomenon has been recently provided by the demonstration that the TGF-β type II receptor is a constitutively active kinase that upon ligand binding associates with and phosphorylates the TGF-β type I receptor, leading to its activation and to signal propagation (57). These features are distinct from the phenomenon of ligand-induced autophosphorylation, which is central to tyrosine kinase receptor activation (20, 49).

Mammalian receptors that bind activin in transfection assays include the type I receptors ActR-I, ActR-IB, and TSR-I (3, 7, 13, 19, 34, 47, 48) and the type II receptors ActR-II and ActR-IIB (4, 31, 33). Some of these receptors may be shared with other members of the TGF-β family, in particular the bone morphogenetic proteins (BMPs). BMP-7 (also known as OP-1) can interact with ActR-I and ActR-II in addition to interacting with the receptors BMPR-I and BMPR-II (28, 59). The *Drosophila* punt/Atr-II gene product (11) can bind activin in concert with the Atr-I gene product (58) and Dpp (a *Drosophila* BMP homolog) in concert with the thick veins and saxophone gene products (27), further illustrating the complex combinatorial potential of this receptor family.

In order to determine how activin receptors generate a signal, we have used a mink lung epithelial cell line that becomes highly responsive to activin upon transfection of activin receptors (7). Using this system and the activin receptors ActR-IB and IIB, we show here that these receptors are activated by the same basic mechanism as TGF-β receptors, suggesting the generality of this process. Furthermore, we have created a

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constitutively active ActR-IB mutant that can generate cellular responses in the absence of activin or in the presence of a dominant negative activin type II receptor. The properties of this constitutively active receptor argue that ActR-IB is a downstream signalling component in the activin receptor system.

MATERIALS AND METHODS

Expression vectors and reporter constructs. Activin receptor constructs were cloned in the pCMV5 vector for expression in transient assays (2). To construct epitope-tagged versions of the activin receptors, the HA1 epitope of influenza virus hemagglutinin or six histidine residues were introduced into pCMV5-ActRIIB2 (4) and pCMV5-ActR-IB (7), respectively, at the C terminus by using a PCR-based strategy as described previously (3, 56). Generation of mutations in pCMV5-ActR-IIB/His(K217R), pCMV5-ActR-IB/HA(K234R), pCMV5-ActR-IB/HA(ST187-206AV), and pCMV5-ActR-IB/HA(T206D) by PCR were as described previously for the TGF-β receptors (56). All mutations were verified by sequencing the PCR-amplified region. The construction of wild-type TβR-II/His, TβR-I/HA, and the TGF-β inducible reporter construct p3TP-Lux has been described previously (8, 56).

Cell lines and transfections. The mink lung epithelial cell line derivative R-1B (L-17 clone) was maintained in histidine-free minimal essential medium (MEM) containing 0.5 mM histidinol, 10% fetal bovine serum, and nonessential amino acids (3). R-1B (L-17 clone) cells were transiently transfected with the appropriate vectors by using a DEAE-dextran method. Exponentially growing cells were plated at approximately 80% confluence in one or three 100-mm dishes for purification of single receptors or complexes, respectively. Cells were washed once with MEM and then incubated in MEM containing 2 μg of plasmid DNA per ml, 125 μg of DEAE-dextran per ml, and 100 μM chloroquine at 37°C for 3.5 h. Cultures were then shocked for 2 min with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS), washed once in MEM containing 10% fetal bovine serum, amphotericin B, and gentamicin, and incubated overnight in this medium. Cells were then concentrated threefold by trypsinization and replating and were assayed the next day.

Luciferase assay. For luciferase assays, cells were plated in 60-mm dishes and transfected with 3 μg of the 3TP-Lux plasmid and a total of 1 μg of DNA consisting of pCMV5-ActR-IB (0.004 μg), ActR-IIB (0.1 μg) and/or the pCMV5 vector alone (unless otherwise indicated) in a final volume of 2 ml. One day after transfection, cells were seeded into 12-well plates, allowed to attach to the plastic for 4 h, incubated in MEM plus 0.2% fetal bovine serum for 3 to 4 h, and then incubated in the presence or absence of 500 pM activin A (Genentech, Inc.) in MEM plus 0.2% fetal bovine serum for 14 to 16 h at 37°C. Cells were harvested, and luciferase activity in cell lysates was determined by using a luciferase assay system (Promega) as suggested by the manufacturer. Total light emission was measured during the initial 20 s of the reaction in a luminometer (Berthold Lumat LB 9501).

Immunoprecipitations and purification of receptor complexes. For [^{35}S]methionine labelling, transiently transfected R-IB (L17 clone) cells were washed twice and incubated in methionine-free medium for 15 min at 37°C, followed by incubation for 2.5 h in the same medium with 50 μCi of [^{35}S]methionine (Trans [^{35}S]-label; ICN) per ml in the presence or absence of 1 nM activin A in the last 20 min. For pulse-chase experiments, cells were incubated for 10 min in 200 μCi of [^{35}S]methionine per ml and then incubated for the indicated times in MEM containing methionine (15 mg/liter). In vivo phosphorylation studies were carried out by washing and incubating transfected cells with phosphate-free MEM for 15 min. Cells were then incubated in the same medium with 1 mCi of [^{32}P]phosphate per ml (3,000 Ci/mmol; Amersham) for 2 h at 37°C, followed by 20 min at 37°C with or without 1 nM activin A. [^{35}S]methionine- or [^{32}P]phosphate-labelled cell monolayers were washed once with ice-cold PBS and lysed for 20 min in lysis buffer (20 mM Tris-Cl [pH 7.4], 150 mM NaCl, 0.5% [vol/vol] Triton X-100) containing 50 mM NaF, 10 mM Na pyrophosphate, 1 mM Na orthovanadate, and protease inhibitors. To isolate receptor complexes, a two-step procedure was used. The cell extracts were clarified by centrifugation, brought to 25 mM imidazole, incubated with Ni^{2+}-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, precipitated with anti-HA antibody 12CA5 (BAbCo), and adsorbed to protein A-Sepharose (Pharmacia). Immunoprecipitates were washed twice with lysis buffer, once with RIPA buffer (10 mM Tris-HCl, 1% sodium deoxycholate, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate [SDS] [pH 7.5]), and once in lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography using various exposure times for easy visualization. For quantitation of relative levels of phosphate incorporation, the ratio of [^{32}P]phosphate to [^{35}S]methionine was determined with a Molecular Dynamics Phosphorimager. For analysis of ActR-IB/HA alone, cell lysates were directly subjected to immunoprecipitations with anti-HA antibody as described above. For analysis of ActR-IIB/His alone, Ni^{2+}-NTA-Agarose-bound receptors were eluted and then precipitated with polyclonal antisera raised against bacterially expressed ActR-IIB. For rabbit immunizations with bacterially expressed protein, the entire cytoplasmic domain of ActR-IIB1 (4) was purified as a glutathione *S*-transferase fusion protein by using pGEX2T. Rabbits were injected four times with 100 μg of protein before collection of sera as described elsewhere (18).

RESULTS

Basal phosphorylation state of activin type I and type II receptors. MvlLu mink lung epithelial cells have low levels of activin type I and type II receptors and very limited responsiveness to activin. However, these cells show strong transcriptional and antiproliferative responses to activin when transfected with appropriate activin receptors (3, 7), thus providing an ideal system to study the functional properties and interactions of these receptors. To this end, we created constructs encoding wild-type and kinase-defective forms of human activin type I and type II receptors, each with a C-terminal sequence tag for efficient retrieval of the proteins from cell lysates. The type I receptor ActR-IB was tagged with the HA influenza virus hemagglutinin epitope (55) that binds to Sepharose-coupled HA antibody, whereas the type II receptor ActR-IIB was tagged with a hexahistidine sequence that binds to nickel-agarose beads. These tags were chosen because their attachment to the C termini of TGF-β type I and type II receptors does not disturb the functional properties of these receptors as determined by their ability to restore TGF-β responsiveness to receptor-defective cell mutants (56).

Mink lung cells (clone R-1B-L17) were transiently transfected with tagged ActR-IB and ActR-IIB under conditions that allow approximately half of the cells to take up foreign DNA (7). After isolation from cells metabolically labelled with [^{35}S]methionine, each receptor migrated on SDS electrophoresis gels as a sharp band and a broad, more slowly migrating band. Receptors isolated from cells briefly pulsed with [^{35}S]methionine and then incubated in the presence of excess unlabelled methionine demonstrated that, with increasing chase times, the faster-migrating sharp band was chased into the broader, more slowly migrating band (Fig. 1A). These results suggested that the sharper band corresponds to a biosynthetic precursor of the other product, which is presumably the mature post-Golgi receptor. Antibody controls showed that the lower-molecular-weight bands in ActR-IIB precipitates were nonspecific (data not shown).

To examine the phosphorylation state of individually transfected receptors, cells transfected with only ActR-IB or only ActR-IIB were labelled with [^{32}P]phosphate. Precipitation of transfected receptors showed that both receptors were phosphorylated in the absence of activin (Fig. 1B). Cell incubation with activin A (the isoform used throughout these studies) did not alter the basal phosphorylation level of individually transfected ActR-IB or IIB (Fig. 1B). Notably, this basal level of phosphorylation in ActR-IB is much higher than that detected for TβR-I transfected into these cells (57; data not shown). The basal phosphorylation of ActR-IIB observed here confirms previous studies which additionally showed that the constitutive phosphorylation of ActR-IIB is in part catalyzed by its own kinase activity (32).

Ligand-dependent and -independent receptor complexes. Since ActR-IIB and ActR-IB are known to form a heteromeric complex in the presence of ligand, the effect of activin on the association and phosphorylation state of activin receptors was examined. A two-step precipitation protocol that involves binding of cell lysates to nickel-agarose, elution of bound material with imidazole, and binding of the eluate to anti-HA Sepharose was used (Fig. 2A). This procedure results in the isolation of double-tagged receptor complexes away from free individual components (57). Lysates from [^{35}S]methionine-la-

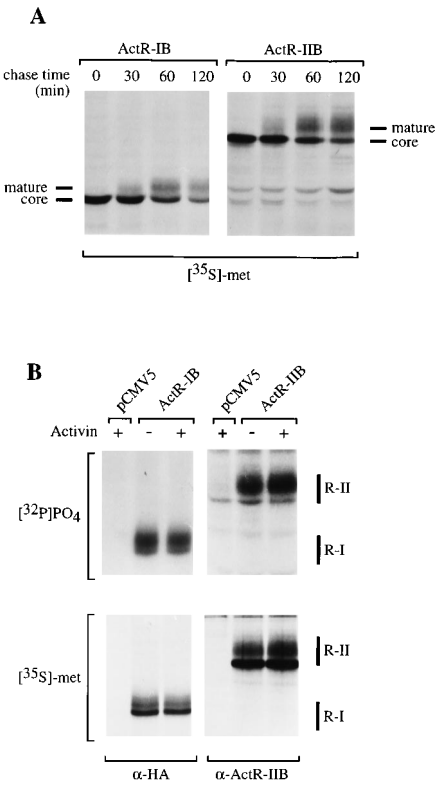

FIG. 1. (A) Pulse-chase analysis of metabolically labelled activin receptors. R-1B cells were transiently transfected with HA-tagged ActR-IB or ActR-IIB as indicated. Cells were pulsed with [^{35}S]methionine for 10 min and then chased for the indicated times in the presence of excess unlabelled methionine. Receptors were immunoprecipitated with the indicated antibodies, resolved by SDS-PAGE, and visualized by fluorography. Bands corresponding to the core and mature forms of ActR-IB (47 and 52 kDa, respectively) and ActR-IIB (65 and 75 kDa, respectively) are indicated. (B) Phosphorylation of ActR-IB and ActR-IIB. R-1B cells were transiently transfected with ActR-IB, ActR-IIB, or vector alone (pCMV5). Cells were labelled with [^{32}P]phosphate (upper panel) or [^{35}S]methionine (lower panel) in the presence (+) or absence (−) of 2 nM activin A. Receptors were purified and visualized as described above. The positions of ActR-IB (R-I) and ActR-IIB (R-II) are indicated.

belled cells cotransfected with ActR-IB and -IIB yielded activin receptor complexes in the absence of added activin (Fig. 2), suggesting an inherent affinity between ActR-IB and ActR-IIB. However, the receptor forms precipitated from cells not exposed to activin were predominantly biosynthetic precursors, as determined by the appearance of the bands on electrophoresis gels. Addition of activin for 20 min at 37°C caused a marked increase in the yield of the mature receptor complex with only a small effect on the yield of immature forms (Fig. 2B). Cotransfection of ActR-IB with ActR-II gave results similar to those for ActR-IB cotransfected with ActR-IIB (data not shown).

To examine the specificity of receptor and ligand interactions, various combinations of activin and TGF-β type I and type II receptors were cotransfected. Whereas TβR-II forms a ligand-dependent complex with TβR-I (Fig. 2B) in agreement with previous findings (57), neither ActR-IB or ActR-IIB cotransfected with TβR-II or TβR-I, respectively, yielded a ligand-induced receptor complex (Fig. 2B). The presence of only immature receptor forms in these incorrectly partnered receptors suggests a propensity of the overexpressed receptors to form ligand-independent complexes.

Previous ligand binding results (3, 4, 7, 31, 33, 47) indicate that ActR-IIB can bind activin on its own whereas ActR-IB binds only when coexpressed with a type II receptor. The ligand dependence of the activin receptor complexes observed here suggests that ActR-IB interacts with activin that is bound to ActR-IIB and, as a result, becomes incorporated into a receptor complex. However, Phosphorimager quantitation of free ActR-IB and ActR-IIB-associated ActR-IB recovered by single or double immunoprecipitation, respectively, showed that only approximately 5% of the metabolically labelled mature ActR-IB was recovered as part of the ligand-induced complex under these conditions. Although this low proportion might be due to losses incurred during the two-step precipitation procedure, we have previously determined that the additional Ni chromatography step used in this procedure is highly efficient (3). The low recovery of the ligand-induced receptor complex rather suggests that this complex may be transient.

Dependence of signalling on receptor kinase activity. In order to assess the functional consequences of activin addition to the receptors, we first determined the level of activin signalling generated by diverse receptor combinations of interest. The set of transcriptional responses induced by activin in mink lung cells transfected with the appropriate receptors include activation of the p3TP-Lux reporter construct (3, 7). This construct contains three tetradecanoyl phorbol acetate response elements and a portion of the PAI-1 promoter cloned upstream of a luciferase reporter gene (8) and was used in the present studies in order to efficiently survey the signalling capacity of various wild-type and mutant activin receptor combinations.

Cells transfected with empty vector or ActR-IIB alone had a low level of luciferase activity that was increased little if at all by activin addition (Fig. 3). In comparison, cells transfected with ActR-IB alone expressed a reproducibly higher level of basal luciferase activity that was further increased by activin addition (Fig. 3). Endogenous type II receptor kinase activity was required for signalling by transfected ActR-IB since cotransfection of the dominant negative construct ActR-IIB (K217R) decreased luciferase activity. The lysine residue mutated in this construct, which is conserved in all protein kinases, is involved in ATP phosphate coordination, and its mutation to arginine strongly diminishes or eliminates the kinase activity (46). The luciferase response to activin was also dependent on the kinase activity of ActR-IB since the kinase-defective construct ActR-IIB(K234R) was unable to signal either alone or in combination with ActR-IIB (Fig. 3). Thus, the kinase activities of both receptor components are required for this activin response.

These results additionally suggested that ligand-induced interaction of transfected ActR-IB with the small amount of endogenous ActR-II expressed in these cells (7) is sufficient to generate a signal. Furthermore, cells cotransfected with ActR-IB and -IIB showed high luciferase levels even in the absence of added activin (Fig. 3). This ligand-independent response might be mediated by the ligand-independent interactions between the overexpressed receptors (Fig. 2), in agreement with an inherent affinity between ActR-IB and -IIB and a requirement for a ligand in order to promote complex formation at physiological receptor concentrations.

Receptor transphosphorylation. The effect of activin on ActR-IB phosphorylation within a complex was determined by precipitating receptors from [^{32}P]phosphate-labelled cells in the two-step procedure. Cells were transfected with wild-type or kinase-deficient forms of ActR-IB and ActR-IIB. The small amounts of receptors recovered in the absence of added activin were composed predominantly of phosphorylated biosynthetic precursors (Fig. 4A). As determined by [^{35}S]methionine label-

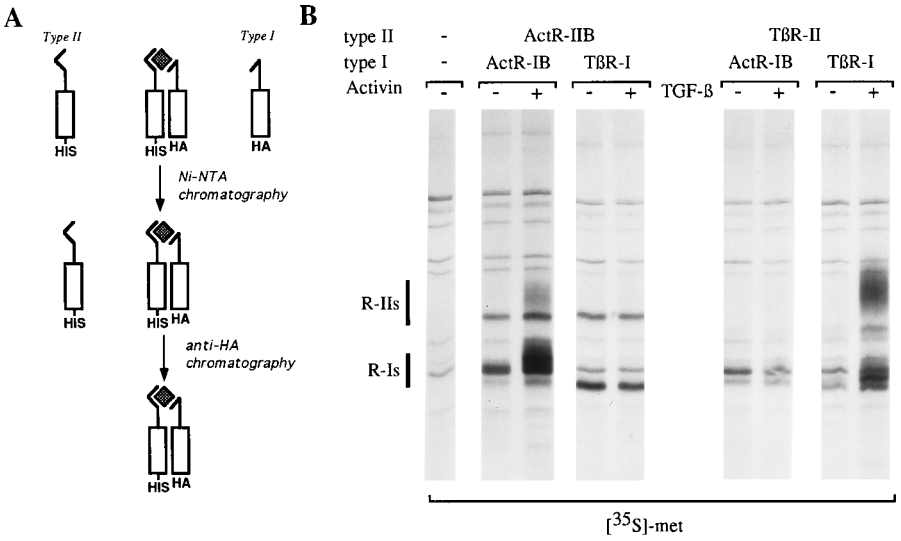

FIG. 2. Ligand-dependent complex formation. (A) Protocol for isolation of type I and type II heteromeric complexes. ActR-IIB and ActR-IB tagged at the carboxy terminus with hexahistidine (HIS) or a hemagglutinin epitope (HA) respectively, are purified by using a two-step procedure. HIS-tagged receptors are collected by incubation with Ni^{2+}-NTA-Agarose and eluted under nondenaturing conditions, and type I or type II receptor complexes are isolated by immunoprecipitation with anti-HA antibody. (B) Ligand-dependent complex formation. R-1B cells were transiently transfected with the type I receptor ActR-IB/HA or TβR-I/HA together with the type II receptor ActR-IIB/HIS or TβR-II/HIS. Cells were labelled with [^{35}S]methionine and then incubated in the presence (+) or absence (−) of 2 nM activin A or TGF-β1. Receptor complexes were purified as described for panel A, resolved by SDS-PAGE, and visualized by fluorography. The labelled type I (R-I) and type II (R-II) receptors are indicated.

ling, activin addition led to the appearance of mature receptor complexes at levels that were the same regardless of the presence or absence of kinase activity in the receptors (Fig. 4A, right panel). On the basis of the ratio of the ^{32}P signal to the ^{35}S signal, the phosphorylation level of ActR-IB was higher when this protein was coprecipitated with ActR-IIB than when it was coprecipitated with ActR-IIB(K217R) (Fig. 4A, left panel). Additionally, this increased phosphorylation was accompanied by an upward mobility shift that is often indicative of protein phosphorylation. ActR-IB recovered from complexes with ActR-IIB contained phosphoserine and phosphothreonine at a 8:1 ratio and no phosphotyrosine, as determined by phosphoamino acid analysis of material excised from electrophoresis gels (data not shown).

The phosphorylation level and gel mobility of either receptor component in the receptor complex were the same regardless of whether the transfected ActR-IB was wild type or kinase defective (Fig. 4A). This suggested that ActR-IB did not increase its own phosphorylation or that of ActR-IIB in response to activin. The residual phosphorylation observed when both ActR-IB and ActR-IIB were kinase defective is presumably catalyzed by kinases of unknown identity.

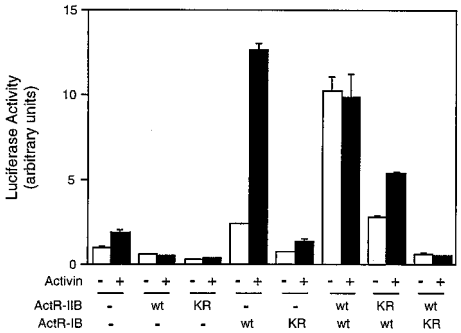

FIG. 3. Ligand-dependent and -independent signalling by activin receptors. Requirement of activin type I and type II receptor kinase activities for signalling. R-1B cells were transiently transfected with p3TP-Lux and the indicated combinations of wild-type (wt) and kinase-deficient (KR) versions of ActR-IIB or ActR-IB. Cells were incubated overnight with 0.5 nM activin A (filled bars) or no activin (open bars). Luciferase activity in cell lysates was plotted as the average and standard deviation for triplicate determinations.

The relative phosphorylation level of ActR-IB isolated as part of a complex was compared with levels in the total ActR-IB pool isolated in a single-step anti-HA immunoprecipitation from the same cotransfected cells or from cells transfected with ActR-IB alone. The phosphorylation level of ActR-IB under these conditions was quantitated by determining the level of ^{32}P incorporation relative to the level of [^{35}S]methionine incorporation. Single precipitation of ActR-IB showed that the basal phosphorylation level of this receptor was not increased by activin addition even in cells cotransfected with ActR-IIB (Fig. 4B). In contrast, the phosphorylation level on ActR-IB coprecipitated with ActR-IIB after activin addition was approximately fourfold higher than the phosphorylation level on total (mostly uncomplexed) ActR-IB isolated from the same cells or from cells transfected with ActR-IB alone (Fig. 4B). Presumably, when the total pool of ActR-IB is examined, the more highly phosphorylated state present in this complex eludes detection because of the limited proportion (~5%) of receptors present in this complex. The results of these phosphorylation experiments suggest that, in the activin-induced receptor complex, ActR-IB is in a highly phosphorylated state that requires the kinase activity of ActR-IIB.

Involvement of the GS domain. Mutational analysis and proteolytic phosphopeptide mapping of TβR-I have shown that in vitro and in intact cells this receptor is phosphorylated by TβR-II at the GS domain (51, 57), a 30-amino-acid sequence adjacent to the N-terminal boundary of the TβR-I kinase domain and highly conserved in all other known type I receptors but not in type II receptors (30). The GS domain in ActR-IB contains one threonine and four serines in the core sequence STSGSGSG and two additional downstream threonines (Fig. 5A). Mutation of the threonine and serines in this

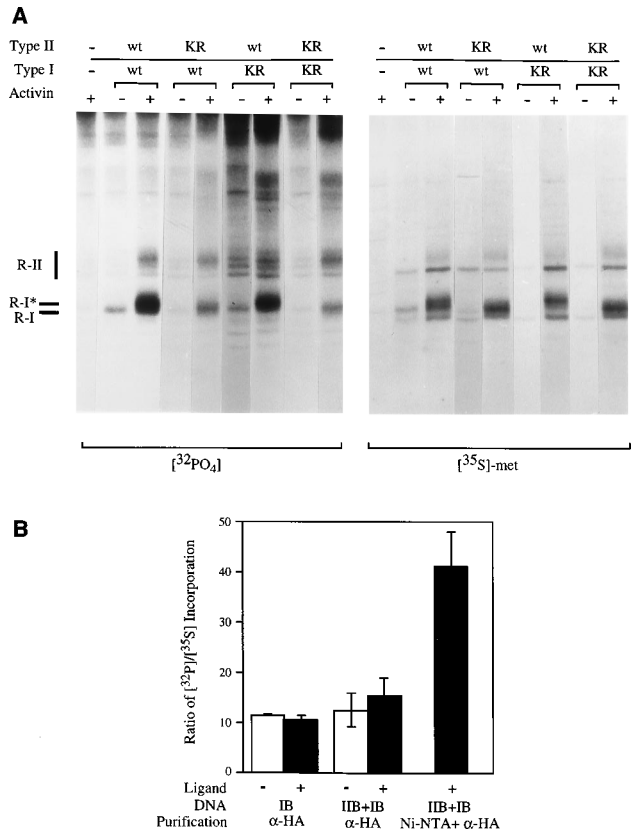

FIG. 4. Phosphorylation of ActR-IB. (A) Phosphorylation of ActR-IB and ActR-IIB in receptor complexes of wild-type and kinase-deficient receptors. R-1B cells were transiently transfected with the indicated combinations of wild-type (wt) or kinase-deficient (KR) versions of ActR-IIB/His (type II) and ActR-IB/HA (type I). Cells were labelled with [^{35}S]methionine (right panel) to control for receptor protein levels or with [^{32}P]phosphate (left panel) with 1 nM activin A (+) or without activin (−). Receptor complexes were purified, resolved by SDS-PAGE, and visualized by autoradiography or fluorography. The positions of hyperphosphorylated (R-I*) and hyposphosphorylated (R-I) ActR-IB forms are indicated. The signal intensity of [^{35}S]methionine-labelled proteins shows uniform receptor levels in all transfectants. This experiment was repeated three times with similar results. (B) Relative phosphorylation levels in ActR-IB expressed alone or in a complex with ActR-IIB. ActR-IB was precipitated in a single step (anti-HA [α-HA]) or in a two-step procedure which yields complexed receptors (Ni-NTA+ α-HA) from cells transfected with the indicated receptors (DNA) in the presence (+) or absence (−) of activin. The ratio of [^{32}P]phosphate incorporation to [^{35}S]methionine incorporation was determined by quantitation with a Molecular Dynamics Phosphorimager. The data shown are the averages with standard deviations for three experiments.

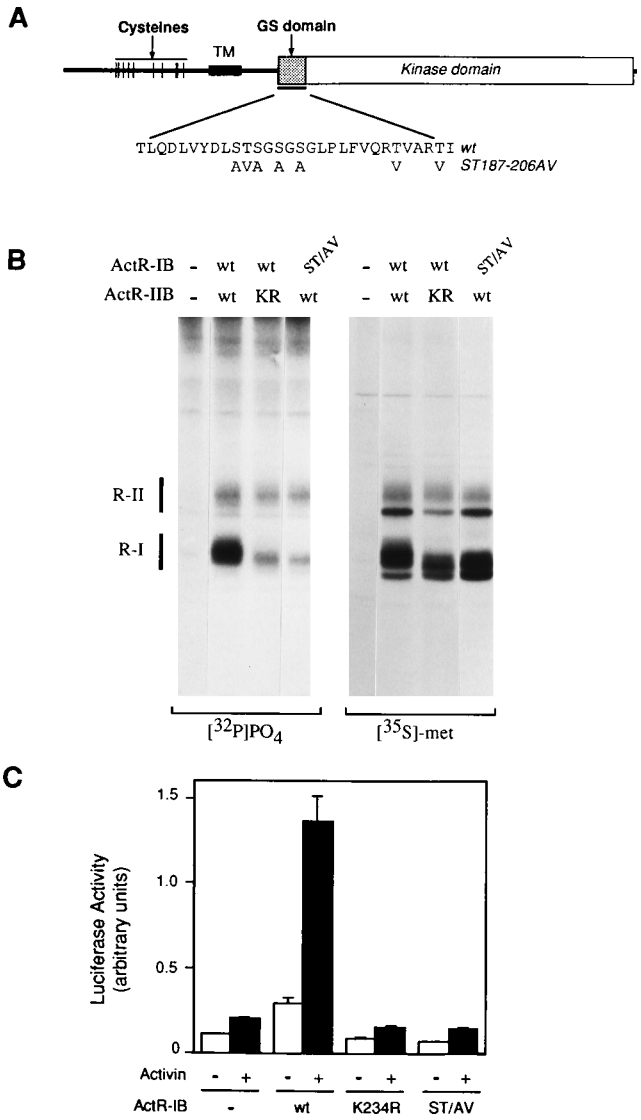

FIG. 5. Role of the GS domain. (A) Schematic representation of ActR-IB. The GS domain, transmembrane domain (TM), kinase domain, and extracellular cysteines are indicated. The amino acid sequence of the wild-type GS domain and the changes introduced in the mutant ActR-IB(ST187-206AV) are indicated. (B) Phosphorylation of wild-type ActR-IB and a GS domain mutant of ActR-IB. R-1B cells were transiently transfected with the indicated combinations of wild-type (wt) or kinase-deficient (KR) versions of ActR-IIB/His and wild-type (wt) or GS domain mutant (ST/AV) versions of ActR-IB/HA. Cells were labelled with [^{35}S]methionine (right panel) to control for receptor expression or with [^{32}P]phosphate (left panel) and then incubated in the presence of 2 nM activin A. Receptor complexes were purified, resolved by SDS-PAGE, and visualized by autoradiography or fluorography. The positions of ActR-IB (R-I) and ActR-IIB (R-II) are indicated. (C) Requirement of an intact GS domain for signalling. R-1B cells were transiently transfected with p3TP-Lux and either the wild type (wt), the kinase-deficient construct ActR-IB(K234R), or the GS domain mutant ActR-IB(ST/AV). Cells were incubated overnight with 0.5 nM activin A (filled bars) or no activin (open bars). Luciferase activity in cell lysates was plotted as the average and standard deviation for triplicate determinations.

domain to valine and alanines, respectively, yielded a construct [ActR-IB(ST187-206AV); Fig. 5A] that formed a complex with ActR-IIB but was not phosphorylated in response to ligand (Fig. 5B). The phosphorylation level and electrophoretic mobility of this product were similar to those of ActR-IB complexed with kinase-defective ActR-IIB(K217R) (Fig. 5B). Furthermore, ActR-IB(ST187-206AV) transfection did not confer activin responsiveness to mink lung cells (Fig. 5C). This suggested that the ability to phosphorylate ActR-IB, presumably at the GS domain, was required for activin signal propagation.

Constitutively active ActR-IB mutant. The preceding results with activin receptors and previous results with TGF-β receptors (57) can be interpreted to suggest that phosphorylation by receptor II allows receptor I to propagate the signal to downstream substrates via its kinase domain. This model posits that the ligand and receptor II should act as upstream activators of receptor I, and predicts that an activating mutation in ActR-IB should allow this receptor to signal in the absence of activin and receptor II. A recent search for this type of mutation in the TGF-β receptor system showed that substitution of the last threonine in the GS domain by aspartic acid constitutively activates TβR-I (54). We therefore introduced an analogous mutation in ActR-IB [construct ActR-IB(T206D)] and tested it signalling activity in the p3TP-Lux activation assay. Although

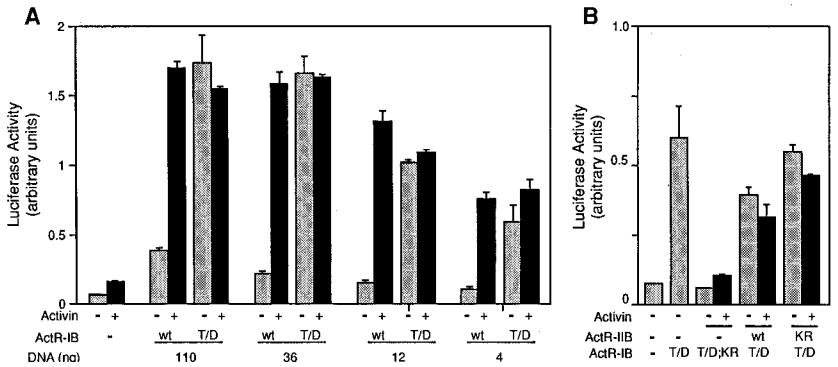

FIG. 6. Signalling by a constitutively active ActR-IB mutant. (A) R-1B cells were transiently transfected with p3TP-Lux and the indicated amounts of vector DNA encoding wild-type ActR-IB (wt) or the ActR-IB(T206D) mutant (T/D). Cells were incubated overnight with 0.5 nM activin (solid bars) or no activin (open bars). Luciferase activity was measured in cell lysates and plotted in arbitrary units as the average and standard deviation for triplicate determinations. (B) Requirements for signalling by ActR-IB(T206D). R-1B cells were transiently transfected with p3TP-Lux and the indicated combinations of wild-type (wt) or kinase-deficient (KR) ActR-IIB and the single mutant ActR-IB(T206D) (T/D) or the double mutant ActR-IB(T206D; K234R) (T/D;KR). The amount of plasmid DNA used in the transfections was 4 ng in the case of ActR-IB-related vectors and 0.1 μg in the case of ActR-IIB-related vectors. Luciferase activity in the absence or presence of added activin was measured as described above.

transfection of wild-type ActR-IB required the presence of activin to activate the 3TP-Lux reporter construct, transfection of ActR-IB(T206D) generated a full response in the absence of added activin (Fig. 6A). The amount of signal obtained was dependent upon the amount of plasmid DNA in the transfection (Fig. 6A). Integrity of the kinase domain of this constitutively active receptor was essential for signalling since the double mutant ActR-IB(T206D, K234R) did not signal even in the presence of activin (Fig. 6B). On the other hand, the kinase-deficient construct ActR-IIB(K217R) did not exert a dominant-negative effect on ActR-IB(T206D) (Fig. 6B).

These results suggest that ActR-IB(T206D) is a constitutively active receptor capable of signalling without requiring either activin or activin type II receptor. Although the mutation of Thr-206 to an acidic residue could act by mimicking the presence of a phosphate group (12, 29, 40), we note that this threonine and the corresponding threonine in TβR-I are not known to be phosphorylation sites (54) and their mutation might act through a conformational change that activates the kinase. The properties of this construct demonstrate that the type I receptor can be a downstream signalling component of the activin receptor complex.

DISCUSSION

Activin exerts diverse effects on a wide range of tissues and organisms by acting through serine/threonine receptors that form heteromeric complexes. In order to determine how these receptors transduce activin signals, we have investigated the biochemical and signalling functions of each component in an activin receptor complex. We chose the receptor isoforms ActR-IB and ActR-IIB for these studies because they appear to have higher affinity and higher selectivity for activin than other receptor isoforms (4, 28, 59) and together they can generate readily measurable responses in Mv1Lu cells (7). Our results show that the activation process of these receptors is analogous to that of the TGF-β receptors but has several significant differences that provide additional insights into the molecular interactions of this receptor family.

Like other related receptors, ActR-IIB binds activin free in the media, whereas ActR-IB interacts with ligand only in the presence of a type II activin receptor (7, 47). Activin addition to cells coexpressing ActR-IB and IIB promotes the association of these receptors. Although these complexes result from an association of ActR-IB with activin-bound ActR-IIB, it is not known whether ActR-IB recognizes a certain conformation of bound activin, the type II receptor, or a structural determinant constituting portions of both molecules. ActR-IB must come in close proximity to activin in this complex since the two can be cross-linked by exposure to the 11-Å (1.1-nm) agent disuccinimidyl substrate (7). Complex formation is ligand specific, since activin-bound ActR-IIB associates with ActR-IB but not TβR-I and TGF-β-bound TβR-II associates with TβR-I but not ActR-IB. Importantly, the amount of ActR-IB or ActR-IIB recovered as a ligand-induced complex is only a small fraction (~5%) of the total receptor present in cotransfected cells, suggesting that the ligand-induced complex may be a transient state during the activation process.

The present results reveal a high propensity of ActR-IB and -IIB to interact in a ligand-independent manner when overexpressed by cotransfection. The TGF-β receptors TβR-I and TβR-II also have an affinity for each other since they can form ligand-independent complexes when transfected in COS cells and 293 cells (9, 10, 52), when expressed via baculovirus in insect cells (51), when coincubated as recombinant proteins in vitro (51), or when the TβR-I and TβR-II cytoplasmic domains are tested in a yeast two-hybrid system (51). By using the yeast two-hybrid system, interactions have also been detected between the cytoplasmic domains of TβR-II and Tsk7L (the murine ActR-I) (10) and between the cytoplasmic domains of TβR-I and the bone morphogenetic protein type II receptor BMPR-II (23, 28). When transiently transfected, TGF-β receptors can reach levels that exceed the cell's capacity to properly process the receptor protein, and the accumulating immature receptors are often the predominant forms observed in ligand-independent complexes (52). However, in Mv1Lu cells expressing receptors at closer to physiological levels, formation of stable TGF-β- or BMP-receptor complexes is highly ligand dependent (28, 57). The activin-induced receptor complexes observed in the present work involve mostly biosynthetically mature receptors, whereas ligand-independent complexes involve predominantly immature receptor forms. Although some of the interactions observed under these various experimental conditions may not be physiological, they nonetheless reveal an intrinsic affinity between receptors. This affinity may prime the formation of ligand-dependent complexes. The ligand may be essential to promote complex formation at physiological recep-

tor concentrations and to enforce specificity in this combinatorial receptor system.

The ability of ActR-IB and ActR-IIB to interact in a ligand-independent manner is also shown by their signalling activities when they are cotransfected in the Mv1Lu assay system. ActR-IB transfected alone signals in response to added activin, and this requires a ligand-induced interaction with endogenous type II receptors since cotransfection of a kinase-defective ActR-IIB construct blocks these responses. However, when ActR-IB is cotransfected with ActR-IIB, which by itself does not elicit a response, luciferase expression from the 3TP-Lux reporter gene is very high and no longer increased by activin. This signalling activity is still dependent on the kinase activities of both receptors components.

ActR-IIB is constitutively autophosphorylated (32), and its phosphorylation is not decreased by mutations that inactivate the kinase activity of a cotransfected ActR-IB (this study). In contrast, ActR-IB becomes hyperphosphorylated when in a ligand-induced complex with ActR-IIB, and this phosphorylation is dependent on ActR-IIB kinase activity. Interestingly, the increased phosphorylation of ActR-IB in the receptor complex does not lead to a detectable increase in the phosphorylation of the total ActR-IB pool, much of which is in free form and phosphorylated independently of activin addition to these cells. This phenomenon may be explained by the fact that only a small proportion of ActR-IB is detected as a receptor complex after cell incubation with activin. The basal phosphorylation level of transiently transfected ActR-IB is higher than that observed with TβR-I stably transfected in Mv1Lu cells (57), although a high level of TβR-I phosphorylation has been observed in transiently transfected COS cells (9). The cause of the basal ActR-IB phosphorylation is not known; however, one possibility is that transient interactions between transfected ActR-IB and endogenous or transfected ActR-IIB are occurring. In agreement with this possibility, we observed that cotransfection of kinase-inactive ActR-IIB decreases the basal phosphorylation of ActR-IB.

By using recombinant TGF-β receptors, it has been shown that TβR-II can directly phosphorylate TβR-I (9, 51, 57), and phosphorylation occurs on serine and threonine residues in the TTSGSGSG sequence within the GS domain of TβR-I (54, 57). Mutation of all the serine and threonine residues in the GS domain of ActR-IB, including the STSGSGSG cluster, prevents ActR-IB phosphorylation. By analogy to the TGF-β receptor system, ActR-IIB is likely to phosphorylate ActR-IB directly rather than through intermediary kinases, a point that needs confirmation using purified activin receptors. In any case, signalling activity is blocked by mutations that prevent ActR-IB phosphorylation by disrupting the kinase activity of ActR-IIB or eliminating putative phosphorylation sites.

The evidence supports a model in which activin and ActR-IIB act as upstream activators of ActR-IB, and ActR-IB propagates the signal by phosphorylating cytoplasmic substrates. The kinase activity of ActR-IIB is required for ActR-IB phosphorylation, which is essential for signalling. Signal transduction also requires the kinase activity of ActR-IB, whose substrates are unknown. A key prediction of this model is that, once activated, the type I receptor should be able to signal independently of ligand and primary receptor. This prediction is confirmed by the properties of the ActR-IB(T206D) construct. Thr-206 resides right at the N-terminal boundary of the kinase domain, and its mutation to aspartic acid leads to constitutive activation of ActR-IB in the absence of added activin. Importantly, the signalling activity of ActR-IB(T206D) is not disturbed by cotransfection of the kinase-defective, dominant negative ActR-IIB(K217R) mutant. Therefore, signalling by ActR-IB(T206D) appears to be independent of type II receptor activity.

One important feature of the activin receptor system is its combinatorial nature. Distinct activin type I receptors are available for recruitment into a complex with ActR-IIB (or ActR-II). According to the receptor activation model presented here, the type I receptor plays a central role in transducing a specific signal, and each type I receptor may lead to different signals. This is consistent with previous observations that ActR-I and ActR-IB, which are activin type I receptors with considerably divergent kinase domains, have different signalling capacities, with only ActR-IB mediating growth-inhibitory responses in Mv1Lu cells (7). A similar example is provided by the Dpp receptor system in *D. melanogaster*. Dpp is involved in many aspects of embryogenesis and larva development in the fly, including embryo dorsalization and formation of the gut, eyes, and appendages (15, 39, 41, 53). The ubiquitously expressed Dpp type II receptor punt (11, 27, 44) interacts with thick veins and saxophone, two different type I receptors of partially overlapping expression patterns (1, 6, 42) that jointly control an array of distinct developmental events (27).

In summary, the basic aspects of the activin receptor activation process revealed by the present studies shows clear similarities as well as interesting differences with respect to those previously delineated for the TGF-β receptors (57). In both systems, a transmembrane serine/threonine kinase acts as a primary receptor that associates with and causes the phosphorylation of a related kinase that then transduces signals to downstream substrates. At physiological receptor concentrations, ligand binding to the primary receptor is required for a productive interaction with the signal transducer type I receptor. This requirement may also help enforce specificity in the combinatorial interactions of this receptor system. The similarities between the TGF-β and activin signalling mechanisms suggest that this mechanism may be general among members of the receptor serine/threonine kinase family.

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