A Novel Type I Receptor Serine-Threonine Kinase Predominantly Expressed in the Adult Central Nervous System*

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Receptor serine-threonine kinases (RSTK) mediate inhibitory as well as stimulatory signals for growth and differentiation by binding to members of the transforming growth factor-β (TGF-β) superfamily. Over 12 different RSTKs have been isolated so far, displaying wide expression in peripheral tissues and in the nervous system. Here we report the isolation and characterization of a novel type I RSTK termed activin receptor-like kinase-7 (ALK-7) that, unlike other members of this receptor family, is predominantly expressed in the adult central nervous system. The ALK-7 gene encodes a 55-kDa cell-surface protein that exhibits up to 78% amino acid sequence identity in the kinase domain to previously isolated type I receptors for TGF-β and activin. In the extracellular domain, however, ALK-7 is more divergent, displaying comparable similarities with all members of the ALK subfamily. RNase protection and *in situ* hybridization studies demonstrated a highly specific mRNA distribution restricted to neurons in several regions of the adult rat central nervous system, including cerebellum, hippocampus, and nuclei of the brainstem. Receptor reconstitution and cross-linking experiments indicated that ALK-7 can form complexes with type II RSTKs for TGF-β and activin in a ligand-dependent manner, although direct binding of ALK-7 to ligand in these complexes could not be demonstrated. The specific expression pattern of ALK-7, restricted to the postnatal central nervous system, indicates that this receptor may play an important role in the maturation and maintenance of several neuronal subpopulations.

TGF-β[1] superfamily members are pleiotropic growth factors with a wide range of activities in many different tissues. They can be subdivided, based on their structural and biological similarities, into three subfamilies, the TGF-βs (TGF-β1 to β3),

¹ The abbreviations used are: TGF-β, transforming growth factor-β; RSTK, receptor serine-threonine kinases; BMP, bone morphogenetic proteins; GDF, growth and differentiation factors; GDNF, glial cell line-derived neurotrophic factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); ALK-7, activin receptor-like kinase-7; PTU, propylthiouracil.

the activins, and the heterogeneous decapentaplegic- and Vg1-related group. The latter includes the mammalian bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs). Other more distantly related members include glial cell line-derived neurotrophic factor (GDNF) and Müllerian inhibiting substance (for review see Ref. 1). Several members of the TGF-β superfamily are expressed in the developing as well as the mature nervous system, among others TGF-β2, TGF-β3 (2–5), BMP-6 (6), GDF-1 (7), and GDNF (8, 9). In the nervous system, TGF-βs control proliferation of neural progenitors, neuronal survival, and differentiation and responses to nervous injury (10). In particular, TGF-β2 and -β3 were recently shown to act as survival factors on embryonic midbrain dopaminergic neurons (8). Activins and BMPs have more limited effects, restricted to only certain neuronal subpopulations (11, 12). GDNF has raised much interest due to its trophic activities on dopaminergic neurons of the substantia nigra, motor neurons, and central noradrenergic neurons (for a recent review, see Ref. 13).

TGF-βs mediate their effects by binding to three classes of cell surface proteins termed TGF-β type I receptor (TβR-I), TGF-β type II receptor (TβR-II), and TGF-β type III receptor (TβR-III) according to their apparent electrophoretic mobility (14). Two structurally related forms of TβR-III are known, betaglycan (15, 16) and endoglin (17), both with short cytoplasmic domains. Betaglycan has been shown to facilitate TGF-β signaling by ligand binding and presentation to the TβR-I and TβR-II receptors (18). Endoglin may also have a ligand presentation function, as it has been shown to form a heteromeric complex with TβR-I and TβR-II (19). TβR-I and TβR-II are glycoproteins of 55 and 75 kDa, respectively, consisting of a short extracellular domain, a single transmembrane span, and a large intracellular region, containing a serine-threonine kinase domain (20, 21). TβR-II is a constitutively active kinase that can bind TGFβ-1 in the absence of other receptor subunits, whereas TβR-I requires TβR-II for ligand binding (22, 23). Binding of ligand induces the formation of heteromeric complexes between TβR-I and TβR-II, which leads to the phosphorylation and subsequent activation of TβR-I (22). Phosphorylation of TβR-I by TβR-II takes place predominantly in a 30-amino acid long, Gly-Ser-rich juxtamembrane domain that is highly conserved among different type I RSTKs. The importance of this domain in TβR-I activation has recently been demonstrated by site-directed mutagenesis (24).

Although most studies on RSTK signaling have focused on type I and type II receptors for TGF-βs, recent data indicate that this two-receptor model may also apply to receptors for activins (25, 26) and BMPs (27, 28). Different members of the TGF-β superfamily interact with specific sets of type I and type II RSTKs that display a broad pattern of expression throughout

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most developing and mature tissues (1, 14). To date, six type I receptors (also termed activin receptor-like kinases, ALKs (29)) and five type II receptors have been isolated in mammals, each one displaying a distinct ligand specificity. Here, we report the isolation and characterization of a novel seventh member of the type I receptor subfamily, ALK-7, which, unlike previous members of this receptor family, is predominantly expressed in the adult central nervous system.

EXPERIMENTAL PROCEDURES

DNA Cloning—Degenerate primers were designed based on two conserved amino acid stretches contained in subdomains II (VAVKIF) and VIII (YMAPE) of the kinase region of type I and type II receptors (21). The upstream primer was 5′GCGGATCCGT(AT/GC)GCIGT(CT/AG)AA(AG)AT(ACT)TT3′, and the downstream primer was 5′CGGAATTC(AT/GC)GGIGCCAT(AG)TA3′. PCRs were performed for 40 cycles, annealing temperatures were 37 °C (first cycle), 42 °C (second and third cycle), and 50 °C for 37 cycles. Melting and elongation steps were performed at 94 and 72 °C, respectively. Fragments were gel-purified using QIAEX beads (QIAGEN), subcloned into pBluescript KS+ (Stratagene), and sequenced using the dedeoxy chain termination method.

mRNA Expression—Fragments of ALK-7 derived from the kinase region were subcloned into pBluescript KS+. A 310-bp-long fragment was used for RNase protection assays and a 320-bp-long fragment for *in situ* hybridization. cRNA probes were generated from linearized plasmids using either T7 or T3 RNA polymerase. For ribonuclease protection assay, 5 μg of total RNA was used and analyzed with [α-^{32}P]CTP-labeled cRNA probes according to manufacturer's instructions (Ambion Inc.; Austin, TX). Samples were controlled for differences in RNA loading using a 160-bp-long rat glyceraldehyde-3-phosphate dehydrogenase riboprobe (kindly provided by Dr. J. M. Blanchard, Institut de Genetique Moleculaire, Montpellier, France) as described previously (30). For *in situ* hybridization, brains from Sprague-Dawley rats, ranging from E13 to adulthood were dissected, immediately frozen on dry ice, and mounted. Sections were cut at 14 μm in a cryostat (Leitz), thawed onto silane-coated slides, and fixed in 4% paraformaldehyde for 15 min. After washing in phosphate-buffer saline (PBS, pH 7.5) and distilled water, sections were treated with 0.1 M HCl for 10 min and acetylated for 20 min with 0.25% acetic anhydride in 0.1 M ethanolamine. Following ethanol dehydration, sections were air-dried. Hybridization was performed overnight in a humidified chamber with 100 μl of hybridization buffer per slide (hybridization buffer: 50% formamide, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 0.3 mM NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly(A) RNA (Sigma), 1 × Denhardt's solution, and 10% dextran sulfate) containing 2.5 × 10^6 cpm/ml ^{35}S-UTP-labeled cRNA probe. After hybridization, sections were washed once in 1 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate (pH 7.0)) at 48 °C for 45 min, treated with RNase (10 μg/ml) in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA at 37 °C for 30 min, and washed twice with 0.5 × SSC and twice with 0.1 × SSC for 10 min each at 60 °C. Slides were then coated with NT-B3 emulsion (Kodak), exposed for 6 weeks, developed, and counterstained with cresyl-violet. To control for unspecific labeling, sense probes were used in parallel on adjacent slides.

Primary Cell Culture—Granular cell cultures were prepared using cerebella from P8 and P10 rats. Briefly, cerebella were minced, washed in PBS, and trypsinized for 15 min at 37 °C under gentle mixing (10 mg/ml trypsin in PBS, Sigma). Trypsin was inactivated by addition of basal medium Eagle's media (Sigma) containing 10% fetal calf serum. After a brief centrifugation cells were resuspended in BME containing 10 mg/ml DNase solution (Pharmacia Biotech Inc.) and left for 10 min at 37 °C. Cells were then gently dissociated, pelleted, and resuspended in BME supplemented with 10% fetal calf serum, 25 mM KCl, and antibiotics. 1.4 × 10^6 cells were plated on 100-mm poly(D-lysine)-coated dishes and grown for 5–7 days at 37 °C.

Receptor Tagging, Expression Vectors, and Transient Transfections— The HA1 epitope of influenza virus hemagglutinin protein was introduced into the C terminus of ALK-7 by PCR as described previously (22). ALK-7-HA was subcloned into pBSKS+ and used for *in vitro* translation with [^{35}S]Cys using a kit of reagents from Promega. Products were immunoprecipitated with a monoclonal antibody 12CA5 (Boehringer Mannheim), directed toward the HA epitope. The ALK-7-HA construct was subcloned into expression vectors pRcCMV and pcDNA3 (Invitrogen) and used in transient transfections on COS cells. Transfections were performed using either the DEAE dextran/chloro-quine method (31) or the calcium phosphate precipitation method with the MBS mammalian transfection kit (Stratagene). Expression plasmids encoding ALK-2-HA, ALK-5-HA, BMPR-II, TβR-II, and ActR-II were previously described (23, 25, 27, 29). ^{125}I-Surface labeling was performed on COS cells transfected with ALK-7-HA/pRcCMV by the lactoperoxidase method. Briefly, cells from one 10-cm culture plate were resuspended in 200 μl of PBS after which 1 mCi of ^{125}I, 10 μg of lactoperoxidase and H$_2$O$_2$ to a final concentration of 0.001% were added. Following a 30-min incubation at room temperature, cells were pelleted and washed three times with TBS (150 mM NaCl, 10 mM Tris-HCl (pH 7.5)) after which they were lysed with 1% Triton lysis buffer supplemented with protease inhibitors. Immunoprecipitation was performed with 12CA5, and samples were separated on SDS-PAGE gels, dried, and exposed to Kodak X-Omat films using intensifying screens at −70 °C.

Chemical Cross-linking and Immunoprecipitation—TGF-β1 and -β2 were obtained from R&D systems and from Kirin Brewery Co., activin A from Ajinomoto Co., and OP-1/BMP-7 from Creative Biomolecules Inc. GDNF was produced and purified as described previously (30). Factors were ^{125}I-labeled using the chloramine-T method (32). Bindings and chemical cross-linkings were performed in cell culture plates as described previously (33) using sulfosuccinimidyl suberate and disuccinimidyl suberate (Pierce) as cross-linkers. Receptor complexes were immunoprecipitated with antibodies directed toward HA (either 12CA5 or YPY, the latter was generated against the peptide sequence, YPYDVPDYAGYPYDVPDYA) or type II receptors. Polyclonal antibodies directed toward endogenous ALK-7 (RQC) were generated by immunizing rabbits with an *in vitro* synthesized peptide with the sequence RQCTYRKTKRHNVEEPLAEYS. The peptide was coupled to keyhole limpet hemocyanin using glutaraldehyde. Immunoprecipitates were separated by SDS-PAGE, followed by autoradiography. In order to dissociate ligand-bound type I and type II complexes, immunoprecipitates were boiled for 5 min and re-immunoprecipitated.

RESULTS AND DISCUSSION

ALK-7 Is a Novel Type I Receptor Serine-Threonine Kinase—In order to isolate novel members of the RSTK family expressed in brain, we used degenerate primers based on conserved regions in the kinase domains of type I and type II receptors and performed PCR on postnatal day 1 (P1) rat brain cDNA. DNA sequence analysis of over 150 independent PCR subclones showed that the majority of the isolated fragments corresponded to previously characterized sequences for type I and type II RSTKs. However, three subclones represented a novel sequence that was related but distinct from previously isolated type I receptors. This fragment was used to screen a rat brain P7 cDNA library from which a 1.7-kilobase fragment was isolated. Its nucleotide sequence revealed an open reading frame of 1482 bp coding for 494 amino acids, with an initiator codon surrounded by favorable consensus sequences for initiation of translation (34). In the deduced amino acid sequence, all the conserved motifs of type I receptors were present (Fig. 1*A*), including a cysteine-rich region in the extracellular domain with a putative glycosylation site and a conserved spacing of cysteine residues. In the cytoplasmic region, a serine-threonine kinase region was preceded by a juxtamembrane domain rich in Gly and Ser (GS domain), previously described in other type I receptors. We named this receptor ALK-7, for activin receptor-like kinase-7, according to the nomenclature of ten Dijke *et al.* (29). Comparison of the predicted amino acid sequence of the ALK-7 kinase domain to those of other known ALK receptors showed up to 78% sequence identity to type I receptors for activin and TGF-β (also known as ALK-4 and ALK-5, respectively). The similarity of ALK-7 to other members of the ALK subfamily was lower (Fig. 1*B*).

In vitro translation of a hemagglutinin (HA)-tagged ALK-7 cDNA revealed a major product of 55 kDa, in accordance with the size predicted by the ALK-7 open reading frame (Fig. 2*A*, *lane 1*). Additional species of smaller molecular weights were also seen after *in vitro* translation, most likely corresponding to initiation at internal Met codons. Cell surface ^{125}I labeling of COS cells that had been transiently transfected with an ALK-7

A

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MTPASRSALSLALLLVALASDLAAGLKCVCLLCDSSNFTCQTEGACWASV    50

MLTNGKEQVIKSCVSLPELNAQVFCHSSNNVTKTECCFTDFCNNITLHLP   100

TASPDAPRLGPTELTVVITVPVCLLSIAAMLTIWACQDRQCTYRKTKRHN   150

VEEPLAEYSLVNAGKTLKDLIYDATASGSGSGLPLLVQRTIARTTVLQEI   200

VGKGRFGEVWHGRWCGEDVAVKIFSSRDERSWFREAETYQTVMLRHENIL   250

GFIAADNKDNGIWTQLWLVSEYHEQGSLYDYLNRNIVTVAGMVKLALSIA   300

SGLAHLHMEIVGTQGKPAIAHRDIKSKNILVKKCDTCAIADLGLAVKHDS   350

IMNTIDIPQNPKVGTKRYMAPEMLDDIMNVNIFESFKRADIYSVGLVYWE   400

IARRCSVGGLVEEYQLPYYDMVPSDPSIEEMRKVVCDQKLRPNLPNQWQS   450

CEALRVMGRIMRECWYANGAARLTALRVKKTISQLCVKEDCKA*        494
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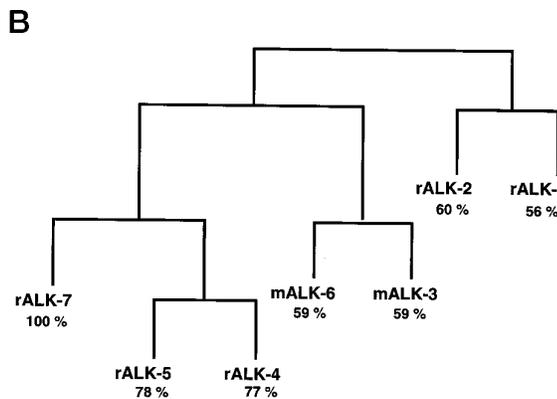
B

FIG. 1. **ALK-7 clone and comparison to other ALKs.** *A*, predicted amino acid sequence of ALK-7. Putative transmembrane domain and GS domain are *underlined* (*bold* and *double lines,* respectively). Primers used for PCR are *boxed*. *B*, evolutionary comparison of the serine-threonine kinase domains of rat ALK-1, -2, -4, -5, and mouse ALK-3 and -6 to ALK-7. *Numbers* indicate percentage identity in the kinase region. Amino acid sequences were aligned using the program PILEUP of the Genetics Computer Group of the University of Wisconsin. The alignment was edited with LINEUP, and evolutionary relationships were calculated with DISTANCES using default algorithms. The plot was generated with GROWTREE.

expression construct also revealed a major product of 55 kDa, demonstrating that the ALK-7 cDNA encoded a surface protein (Fig. 2*A, lane 2*). Production of ALK-7 protein in transfected COS cells was further demonstrated after ^{35}S-metabolic labeling and immunoprecipitation with anti-HA antibodies (Fig. 2*B*). The majority of ALK-7 showed an electrophoretic mobility that was slower than those of either ALK-2 or ALK-5 in SDS-PAGE, despite their similar number of amino acids (Fig. 2*B*). Co-transfection with expression plasmids for different type II receptors revealed that ALK-7 was capable of associating with BMPR-II, a type II RSTK for bone morphogenetic proteins but not with TβR-II in the absence of ligand (Fig. 2*B*, compare *lanes 5* and *4*, respectively). Other members of the ALK subfamily, including ALK-5, have also been shown to associate with BMPR-II in the absence of ligand (27). An anti-peptide antisera specific for a sequence from the juxtamembrane domain of ALK-7 (RQC) also immunoprecipitated ^{35}S-metabolically labeled ALK-7 from COS cells. This band was not recovered from COS cells transfected with an empty vector (Fig. 2*C*) or if the RQC antiserum was preincubated with an excess of the peptide antigen (not shown).

ALK-7 mRNA Is Predominantly Expressed in the Postnatal Central Nervous System—Expression of ALK-7 mRNA was

studied in central and peripheral tissues of the developing and adult rat by RNase protection analysis using a riboprobe derived from the ALK-7 kinase domain. With the exception of low levels in 2-week-old ovary and adult kidney, no ALK-7 mRNA could be detected in peripheral tissues, including liver, spleen, muscle, and skin, at any developmental stage (Fig. 3*A* and data not shown). In contrast, broad ALK-7 mRNA expression was detected in the adult central nervous system, with particularly high levels in the cerebellum (Fig. 3*B*). In most regions examined, including cerebellum (Fig. 3*C*), hippocampus, and ventral midbrain (data not shown), ALK-7 mRNA expression increased during postnatal development reaching maximal levels in the adult. In contrast to the more restricted pattern of expression of ALK-7, other ALKs, including ALK-2, ALK-4, and ALK-5, showed a broader expression pattern, both during development and in the adult, in all tissues examined (data not shown).

The developmental regulation of ALK-7 mRNA expression suggested that this receptor may be important for the maturation of several regions of the central nervous system. The thyrostatic drug propylthiouracil (PTU) induces a hypothyroid state, affecting the development of several thyroxin-sensitive tissues such as the cerebellum. Animals subjected to a hypothyroid state during embryonic development show a delayed maturation of the cerebellum, including deficits in synaptogenesis and in the arborization of Purkinje cells (35). We then investigated expression levels of ALK-7 mRNA in cerebellar RNA isolated from control and PTU-treated rats. All these animals showed the characteristic features of hypothyroidism, including uncertain gait, delay in eye-opening, retarded behavioral development, and weight reduction. RNase protection assays of cerebellar RNA from these animals revealed a delay in the appearance of ALK-7 mRNA expression after PTU treatment (Fig. 3*D*), in support of a role for ALK-7 in cerebellar maturation. Interestingly, similar observations have recently been made with other neurogenic receptors, including members of the Trk family of neurotrophin receptors (36).

A detailed *in situ* hybridization analysis was also performed on adult rat brain that confirmed and extended the results from the RNase protection analyses. In the cerebellum, ALK-7 mRNA was detected in Purkinje cells, and a lower but detectable expression was also found in the granular cell layer (Fig. 4*A*). In the hippocampus, neurons in the dentate gyrus and CA3 regions were found to express ALK-7 mRNA (not shown). Neurons of brainstem nuclei, including the mesencephalic nucleus of the trigeminal nerve (Fig. 4*B*), the facial nucleus (Fig. 4*C*), the spinal vestibular nucleus (Fig. 4*D*), and the locus coeruleus (not shown) also expressed ALK-7 mRNA. Lower but significant expression of ALK-7 mRNA was also seen in other regions, including the substantia nigra (not shown).

Summarizing our mRNA expression studies, ALK-7 appears to be a neuron-specific RSTK expressed at very low levels during embryonic and early postnatal development, reaching maximal levels of expression during adulthood. With the possible exception of C14, a putative type II receptor for Müllerian inhibiting substance (37), previously characterized RSTKs have been found to be expressed more broadly and both during embryonic and postnatal development (38).

Ligand-dependent Interaction of ALK-7 with Type II Receptors TβR-II and ActR-II—We investigated the interaction of ALK-7 with different members of the TGF-β superfamily by binding and chemical cross-linking of radiolabeled ligands to ALK-7-expressing cells. We screened various cell lines of neuronal origin for ALK-7 mRNA expression, including the human neuroblastoma SY5Y, the rat pheochromocytoma PC12, the raphe nucleus precursor line RN33B (39), and the motor neuron hybrid cell line 2F.10.14 (40). However, no detectable levels
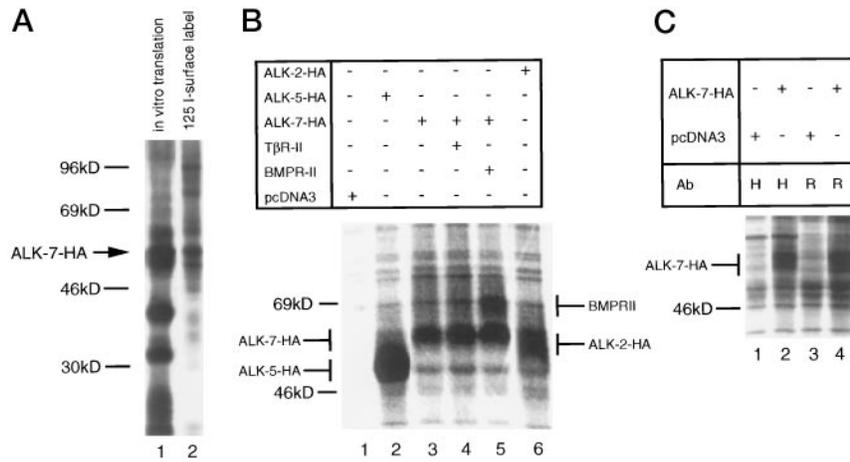
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FIG. 2. **ALK-7 is a cell-surface protein.** *A, in vitro* translation of ALK-7-HA. Reactions were immunoprecipitated with an anti-HA antibody (12CA5). Size markers and ALK-7 protein are indicated (*lane 1*). COS cells transfected with the ALK-7-HA construct were surface-labeled with ^{125}I. Cells were lysed and immunoprecipitated with 12CA5. The migration of ALK-7 protein is indicated (*lane 2*). *B*, COS cells were transfected with the indicated constructs and metabolically labeled with [^{35}S]Cys. Cells were lysed and immunoprecipitated with 12CA5 antibodies. Receptors are indicated. Note the slower mobility of ALK-2 and -7 compared with ALK-5. In cotransfections with type II receptors, ALK-7 associated with BMPR-II, but not TβR-II, in the absence of ligand (*lanes 5* and *4*, respectively). *C*, COS cells were transfected with 50 μg of either ALK-7-HA construct or pcDNA3 and metabolically labeled with [^{35}S]Cys. Immunoprecipitations were performed with the anti-HA (*H*) or a polyclonal anti-ALK-7 (*R*, see "Experimental Procedures") antisera as indicated. Products of the expected size were only detected in ALK-7-HA-transfected cells.
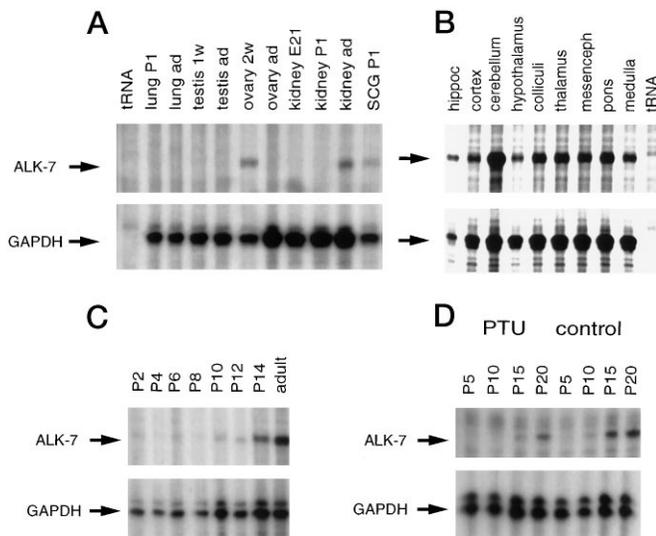

FIG. 3. **ALK-7 is predominantly expressed in the central nervous system.** ALK-7 mRNA expression analyzed by RNase protection assays. *Arrows* indicate ALK-7 mRNA signal. Yeast tRNA was used as a negative control. A glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was included in each reaction to control for differences in RNA loading (*lower panels*). *A*, representative peripheral tissues are shown. Note the absence of ALK-7 expression in most tissues, with the exception of 2-week-old ovary (*2w*) and adult (*ad*) kidney. *B*, a widespread and elevated expression was found in various regions of the adult rat brain. Note the high signal in cerebellum. *C*, developmental expression pattern of ALK-7 mRNA in cerebellum. Expression levels during the first postnatal week were at the detection limit. Maximum levels were reached in adulthood. *D*, analysis of ALK-7 mRNA expression in cerebella from rats treated with propylthiouracil (*PTU*) compared with control rats. Levels of ALK-7 expression were lower and developmentally delayed in PTU-treated rats.

of ALK-7 mRNA expression could be found in any of these cells (data not shown), perhaps due to their early developmental origin. We then tested ligand binding to ALK-7 in primary cultures of early postnatal cerebellar granular cells, which express moderate levels of ALK-7 mRNA (not shown). However, while receptor complexes containing TGF-β and activin could be recovered after immunoprecipitation with anti-ALK-5 or anti-ActR-II antibodies, respectively, no cross-linked com-

plexes could be detected with anti-ALK-7 antibodies (not shown). Since early postnatal granular cells express only moderate levels of ALK-7 mRNA as assessed by a very sensitive RNase protection assay, these results do not conclusively rule out the possibility that TGF-βs or activins may interact with ALK-7.

Because of the unavailability of cells with substantial levels of endogenous ALK-7 expression, we investigated ligand binding to ALK-7 in COS cells with reconstituted receptor complexes by transient transfection of cDNAs encoding different type I and type II RSTKs. For this, we used HA-tagged constructs of ALK-2 (an activin receptor, also termed ActR-I (26, 29, 41)), ALK-5 (a TGFβ receptor, also termed TβR-I (21)), and ALK-7, together with type II receptors for activin (ActR-II), TGF-βs (TβR-II), or BMPs (BMPR-II). Transfected COS cells were used in binding experiments with different iodinated ligands, including TGF-β1, TGF-β2, activin A, OP-1/BMP-7, and GDNF, followed by chemical cross-linking and immunoprecipitation using antibodies directed against the HA tag or type II receptors ActR-II (ARC) (42), TβR-II (DRL) (43), or BMPR-II (28). All type I receptors isolated so far have been shown to bind ^{125}I-TGF-β1, albeit with variable affinities, when co-expressed with the TGF-β type II receptor TβR-II (44). We therefore tested binding of TGF-β1 to ALK-7 in COS cells transfected with HA-tagged ALK-7 in the presence or absence of TβR-II. Cells transfected with ALK-5-HA were used as positive control. The TβR-II-specific antiserum (DRL) immunoprecipitated type III, type II, and type I receptor complexes in mock-transfected cells (Fig. 5*A, lane 6*), reflecting the presence of endogenous TGF-β receptors in COS cells. No receptor complexes were immunoprecipitated by the anti-HA antiserum in mock-transfected cells or in cells transfected with cDNAs for ALK-5-HA or ALK-7-HA (Fig. 5*A, lanes 1, 2*, and *4*). The latter was in agreement with the requirement of type II receptor co-expression for ligand binding to type I receptors (22). In cells co-transfected with ALK-5-HA and TβR-II expression plasmids, type I and type II receptor complexes were readily detected after immunoprecipitation with either anti-HA or anti TβR-II antibodies (Fig. 5*A, lanes 3* and *8*). After co-transfection of ALK-7-HA and TβR-II, a type II receptor complex could be immunoprecipitated with anti-HA antibodies (Fig. 5*A, lane 5*). HA antibodies
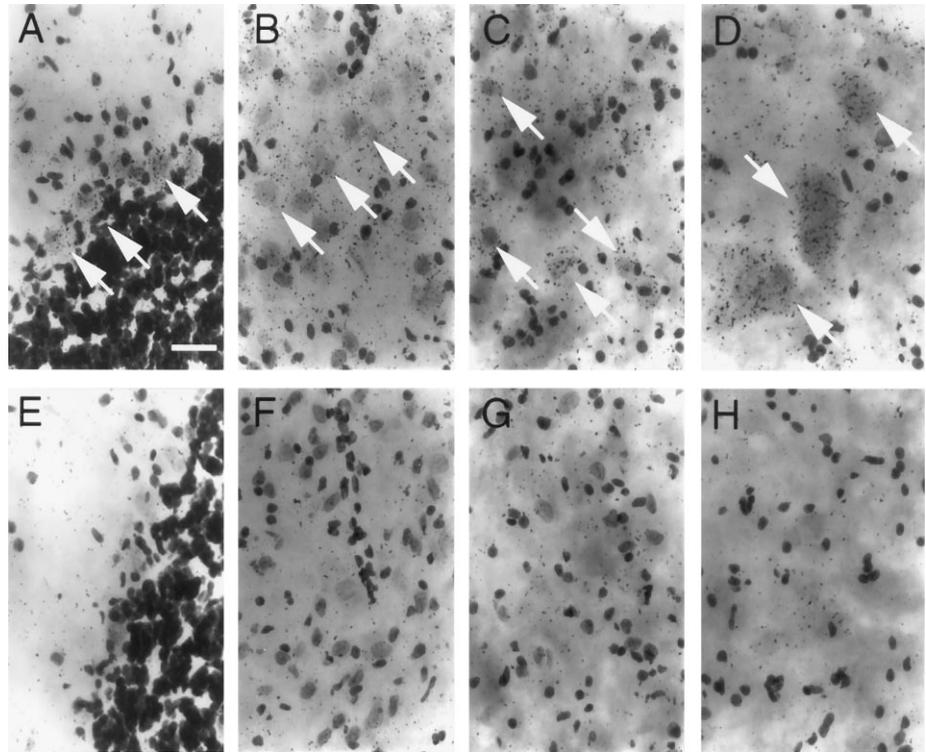
Fig. 4. **Neuronal expression of ALK-7.** ^{35}S-Riboprobe *in situ* hybridization analysis of ALK-7 in the central nervous system of the adult rat. Sections from different brain regions were hybridized with an antisense ALK-7 probe (*A--D*) or a sense probe (*E–H*). Specific labeling on neurons is seen in sections hybridized with antisense probe (*arrows*). *A* and *E*, cerebellum. Note the elevated labeling in Purkinje cells. *Scale bar* is 40 μm. *B* and *F*, mesencephalic nucleus of the trigeminal nerve. *C* and *G*, facial nucleus. *D* and *H*, spinal vestibular nucleus.

could not immunoprecipitate TβR-II in the absence of ALK-7-HA (not shown). Since TβR-II could not be co-precipitated with ALK-7 in the absence of ligand (see Fig. 2*B, lane 4*), the co-precipitation of ALK-7 with TβR-II after cross-linking to TGF-β1 suggests a ligand-dependent complex formation between ALK-7 and TβR-II in transfected cells. Similar results were obtained using ^{125}I-TGF-β2 (data not shown). Although the association between ALK-7 and TβR-II was only seen in the presence of ligand, direct binding of TGF-β1 to ALK-7 was not observed (Fig. 5*A, lanes 5* and *9*). Because the high electrophoretic mobility of ALK-7 could have made its complex difficult to distinguish from that of TβR-II, we performed a double-immunoprecipitation analysis with a denaturation step in between to recover only type I receptor complexes. After boiling, affinity labeled ALK-5, but not ALK-7, could be recovered after cotransfection with TβR-II (Fig. 5*B*, compare *lanes 3* and *4* with *1* and *2*). Thus, although ALK-7 is capable of forming a complex with TβR-II in the presence of ligand, its affinity for TGF-βs may be too low to allow efficient cross-linking.

Binding and cross-linking experiments were also performed with iodinated activin A and COS cells transfected with ALK-7-HA or ALK-2-HA, alone or in combination with ActR-II. As seen before with TβR-II, affinity-labeled ActR-II could be co-immunoprecipitated using anti-HA antibodies (Fig. 5*C, lane 8*), although direct binding of activin to ALK-7 was not detected. This indicated that ALK-7 can also form complexes with the activin type II receptor in the presence of ligand. ALK-7 appeared to be more efficient than ALK-2 in associating with ActR-II (compare *lanes 4* and *8* in Fig. 5*C*). Interestingly, ALK-5, did not associate with ActR-II in this assay (Fig. *5, lane 6*), suggesting that the interaction between ALK-7 and ActR-II was specific and may be physiologically relevant. No binding of OP-1/BMP-7 or GDNF to ALK-7 could be detected after cotransfection with different type II receptors (not shown).

Taken together, our data indicate that ALK-7 can interact in a ligand-dependent manner with type II RSTKs for TGF-β and activin. Current models of TGF-β receptor signaling indicate that the interaction between type I and type II receptors is essential and sufficient for downstream signaling (23). By selectively interfering with TGF-β binding to type I receptors, Vivien and Wrana (45) have recently shown that ligand binding to TβR-I is not required for complex formation with ligand-bound TβR-II or for type I receptor phosphorylation, the first steps in the TGF-β signaling pathway. Together with our results, this evidence suggests that the ligand-dependent association of ALK-7 with type II receptors may have physiological significance. On the other hand, several of the previously isolated ALKs display similar association properties when co-expressed with TβR-II or ActR-II in COS-cells, although only a subset of the type I receptors bind ligand in responsive cells and mediate intracellular signals (44). Therefore, the possibility remains that ALK-7 may require another not yet identified type II receptor for ligand binding or additional ligand-presenting receptors. In this respect, it has been shown that TGF-β2, but not TGF-β1 or TGF-β3, requires the presence of betaglycan for efficient binding to type I and type II receptors (18, 46). The current unavailability of cell lines with high levels of endogenous ALK-7 expression presents a limitation to the analysis of candidate physiological ligands for ALK-7.

The developmental expression pattern of ALK-7 suggests a role for this receptor in neuronal maturation. This is supported by the changes in ALK-7 expression observed in the cerebellum of PTU-treated animals. The activities of ALK-7 in this brain structure could involve neuronal arborization, synapse formation or elimination, as well as inhibition of proliferation and/or differentiation, all processes that are known to occur during the postnatal development of the cerebellum. Several members of the TGF-β superfamily are differentially expressed in the nervous system; putative ALK-7 ligands could be expected to have a partially overlapping spatial and temporal expression pattern with this receptor. Interestingly, the pattern of expression of TGF-β2 mRNA in postnatal and adult murine brain shows a close similarity with that of ALK-7, including expression in cerebellar Purkinje cells and hippocampal neurons (5). In addition, TGF-β2 has been shown to inhibit the proliferation of small postnatal cerebellar neurons *in vitro*. Another member of

Cloning of a Novel Receptor Serine-Threonine Kinase

A

ALK-7-HA	-	-	-	+	+	-	-	-	+
ALK-5-HA	-	+	+	-	-	-	-	+	-
TβR-II	-	-	+	-	+	-	+	+	+
pcDNA3	+	-	-	-	-	+	-	-	-
Antibody	H	H	H	H	H	D	D	D	D

TβR-III ⊣

— 96 kD

TβR-II ⊣
ALK-5 ⊣

— 69 kD

— 46 kD

1 2 3 4 5 6 7 8 9

B

ALK-7-HA	+	+	-	-
ALK-5-HA	-	-	+	+
TβR-II	+	+	+	+
Boiling	-	+	-	+

TβR-II ⊣

⊢ ALK-5

1 2 3 4

C

ALK-2-HA	-	-	+	+	-	-	-	-	-	-	-	+	-
ActR-II	-	+	-	+	-	+	-	+	-	+	+	+	+
ALK-7-HA	-	-	-	-	-	+	+	-	-	-	-	-	+
ALK-5-HA	-	-	-	+	+	-	-	-	-	-	+	-	-
pcDNA3	+	-	-	-	-	-	-	+	-	-	-	-	-
Antibody	H	H	H	H	H	H	H	H	A	A	A	A	A

96 kD —
69 kD —
46 kD —

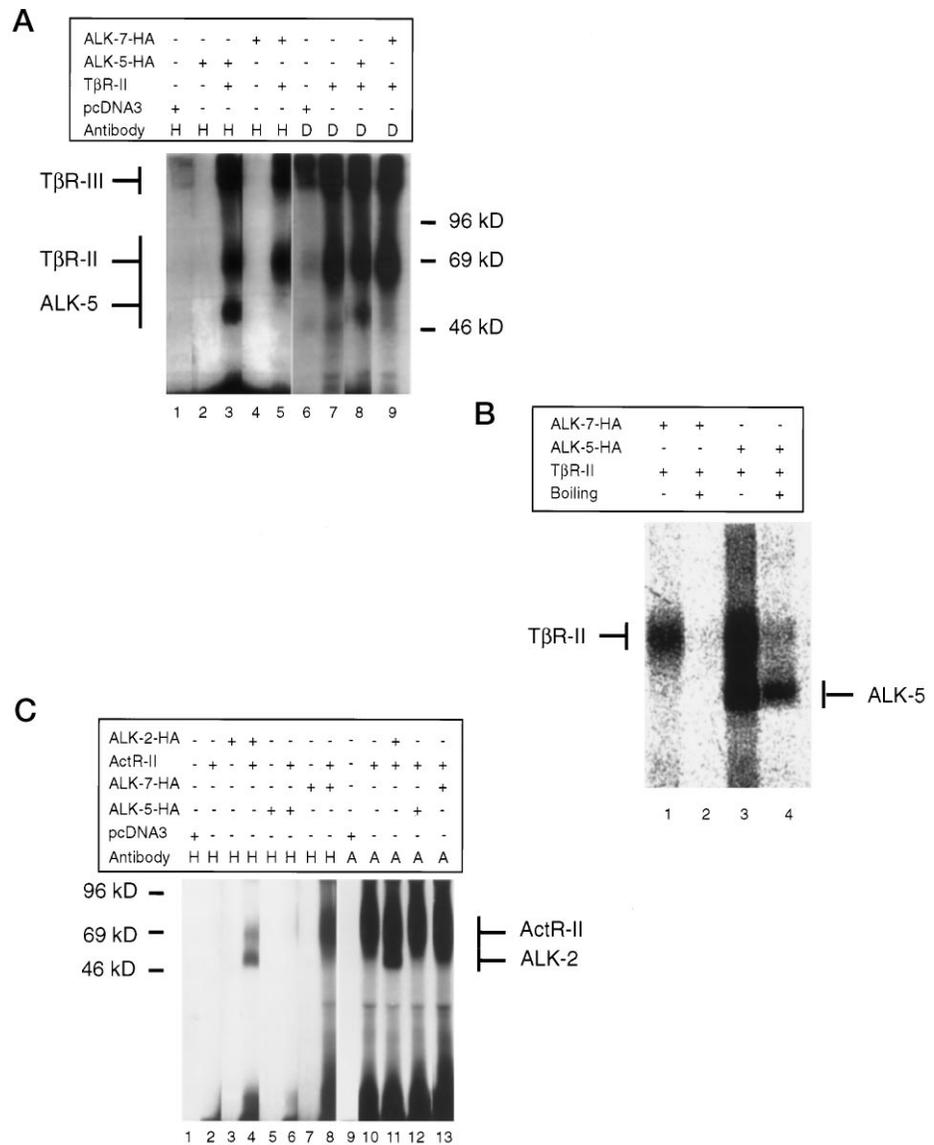ActR-II
ALK-2

1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 5. **Interaction of ALK-7 with type II receptors.** COS cells were transfected with the indicated constructs and affinity labeled with 5 ng/ml radioiodinated factor. Following chemical cross-linking, detergent extracts were immunoprecipitated with the indicated antibodies (anti-HA (*H*), anti-TβR-II (*D*) or anti-ActR-II (*A*)), separated by SDS-PAGE and visualized by autoradiography. *A*, ^{125}I-TGFβ-1 binding ALK-5-HA. TβR-II and TβR-III complexes are indicated. *B*, detergent extracts were separated after a boiling step in between two immunoprecipitations with HA antibodies. This step dissociates the type I-type II receptor-complex. Note that an ALK-5-TGFβ complex is seen both before and after boiling (*lanes 3* and *4*) indicating true ligand-receptor cross-linking. In contrast, no type I receptor complex can be detected in cells transfected with ALK-7-HA. *C*, ^{125}I-activin A binding; ALK-2 and ActR-II complexes are indicated.

the TGF-β superfamily, GDF-1, is specifically expressed in the developing and adult nervous system, particularly in cerebellum, brain stem, and spinal cord (7). Moreover, in peripheral non-neuronal tissues, GDF-1 mRNA has been detected primarily in ovary, one of the few peripheral sites of ALK-7 mRNA expression. Thus, TGF-β2 and GDF-1 could, among other TGF-β superfamily members, be possible endogenous ligands for ALK-7.

Conclusions—ALK-7 is a novel type I RSTK predominantly expressed in the adult central nervous system. Receptor reconstitution experiments indicate that ALK-7 can interact with type II receptors for TGF-β and activin in a ligand-dependent manner. The function of ALK-7 remains to be elucidated. Its mRNA expression pattern, restricted to the mature central nervous system, indicates that ALK-7 may be important during postnatal maturation and/or maintenance of several neuronal subpopulations, particularly in the cerebellum. Putative endogenous ligands of ALK-7 may be members of the TGF-β superfamily with neurotrophic activities.

REFERENCES

1. Kingsley, D. M. (1994) *Genes Dev.* **8,** 133–146
2. Pelton, R. W., Dickinson, M. E., Moses, H. L., and Hogan, B. L. (1990) *Development* **110,** 609–620
3. Pelton, R. W., Saxena, B., Jones, M., Moses, H. L., and Gold, L. I. (1991) *J. Cell Biol.* **115,** 1091–1105
4. Flanders, K. C., Ludecke, G., Engels, S., Cissel, D. S., Roberts, A. B., Kondaiah, P., Lafyatis, R., Sporn, M. B., and Unsicker, K. (1991) *Development* **113,** 183–191
5. Constam, D. B., Schmid, P., Aguzzi, A., Schachner, M., and Fontana, A. (1994) *Eur. J. Neurosci.* **6,** 766–778
6. Tomizawa, K., Matsui, H., Kondo, E., Miyamoto, K., Tokuda, M., Itano, T., Nagahata, S., Akagi, T., and Hatase, O. (1995) *Mol. Brain Res.* **28,** 122–128
7. Lee, S. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4250–4254
8. Poulsen, K. T., Armanini, M. P., Klein, R. D., Hynes, M. A., Phillips, H. S., and Rosenthal, A. (1994) *Neuron* **13,** 1245–1252
9. Arenas, E., Trupp, M., Åkerud, P., and Ibáñez, C. F. (1995) *Neuron* **15,** 1465–1473
10. Krieglstein, K., Rufer, M., Suter-Crazzolara, C., and Unsicker, K. (1995) *Int. J. Dev. Neurosci.* **13,** 301–315
11. Mehler, M. F., Xu, H., and Kessler, J. A. (1993) *Soc. Neurosci. Abstr.* **19,** 416
12. Sawchenko, P. E., Plotsky, P. M., Pfeiffer, S. W., Cunningham, E. T., Vaughan, J., Rivier, J., and Vale, W. (1988) *Nature* **334,** 615–617
13. Arenas, E. (1996) *Mol. Psychiatry* **1,** 179–182
14. Attisano, L., Wrana, J. L., López-Casillas, F., and Massagué, J. (1994) *Biochim. Biophys. Acta* **1222,** 71–80
15. Wang, X.-F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991) *Cell* **67,** 797–805
16. López-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and

Massagué, J. (1991) *Cell* **67,** 785–795

17. Gougos, A., and Letarte, M. (1990) *J. Biol. Chem.* **265,** 8361–8364
18. López-Casillas, F., Wrana, J. L., and Massagué, J. (1993) *Cell* **73,** 1435–1444
19. Yamashita, H., Ichijo, H., Grimsby, S., Morén, A., ten Dijke, P., and Miyazono, K. (1994) *J. Biol. Chem.* **269,** 1995–2001
20. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992) *Cell* **68,** 775–785
21. Franzén, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H., and Miyazono, K. (1993) *Cell* **75,** 681–692
22. Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F., and Massagué, J. (1992) *Cell* **71,** 1003–1014
23. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994) *Nature* **370,** 341–347
24. Wieser, R., Wrana, J. L., and Massagué, J. (1995) *EMBO J.* **14,** 2199–2208
25. Cárcamo, J., Weis, F. M. B., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massagué, J. (1994) *Mol. Cell. Biol.* **14,** 3810–3821
26. Attisano, L., Cárcamo, J., Ventura, F., Weis, F. M. B., Massagué, J., and Wrana, J. L. (1993) *Cell* **75,** 671–680
27. Liu, F., Ventura, F., Doody, J., and Massagué, J. (1995) *Mol. Cell. Biol.* **15,** 3479–3486
28. Rosenzweig, B. L., Imamura, T., Okadome, T., Cox, G. N., Yamashita, H., ten Dijke, P., Heldin, C. H., and Miyazono, K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7632–7636
29. ten Dijke, P., Ichijo, H., Franzén, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H., and Miyazono, K. (1993) *Oncogene* **8,** 2879–2887
30. Trupp, M., Rydén, M., Jörnvall, H., Timmusk, T., Funakoshi, H., Arenas, E., and Ibáñez, C. (1995) *J. Cell Biol.* **130,** 137–148

31. Luthman, H., and Magnusson, G. (1983) *Nucleic Acids Res.* **11,** 1295–1305
32. Cheifetz, S., Andres, J. L., and Massagué, J. (1988) *J. Biol. Chem.* **263,** 16984–16991
33. Massagué, J. (1987) *Methods Enzymol.* **146,** 174–195
34. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148
35. Legrand, J. (1979) *Trends Neurosci.* **2,** 234–236
36. Neveu, I., and Arenas, E. (1996) *J. Cell Biol.* **133,** 631–646
37. Baarends, W. M., van Helmond, M. J. L., Post, M., van der Schoot, P. J. C. M., Hoogerbrugge, J. W., de Winter, J. P., Uilenbroek, J. T. J., Karels, B., Wilming, L. G., Meijers, J. H. C., Themmen, A. P. N., and Grootegoed, J. A. (1994) *Development* **120,** 189–197
38. He, W. W., Gustafson, M., Hirobe, S., and Donahoe, P. (1993) *Dev. Dyn.* **196,** 133–142
39. Whittemore, S. R., and White, L. A. (1993) *Brain Res.* **615,** 27–40
40. Salazar-Gruesco, E., Kim, S., and Kim, H. (1991) *Neuroreport* **2,** 505–508
41. Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., López, A. R., and Derynck, R. (1993) *Science* **260,** 1344–1348
42. Ichijo, H., Yamashita, H., ten Dijke, P., Eto, Y., Heldin, C. H., and Miyazono, K. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1508–1514
43. Yamashita, H., ten Dijke, P., Franzén, P., Miyazono, K., and Heldin, C.-H. (1994) *J. Biol. Chem.* **269,** 20172–20178
44. ten Dijke, P., Yamashita, H., Ichijo, H., Franzén, P., Laiho, M., Miyazono, K., and Heldin, C. H. (1994) *Science* **264,** 101–104
45. Vivien, D., and Wrana, J. L. (1995) *Exp. Cell Res.* **221,** 60–65
46. López-Casillas, F., Payne, H. M., Andres, J. L., and Massagué, J. (1994) *J. Cell Biol.* **124,** 557–568