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The Orphan Receptor Serine/Threonine Kinase ALK7 Signals Arrest of Proliferation and Morphological Differentiation in a Neuronal Cell Line*

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The signaling capabilities and biological functions of activin receptor-like kinase 7 (ALK7), a type I receptor serine/threonine kinase predominantly expressed in the nervous system, are unknown. We have constructed a cell line derived from the rat pheochromocytoma PC12 in which expression of a constitutively active mutant of ALK7 (T194D) is under the control of a tetracycline-inducible promoter. For comparison, another cell line was engineered with tetracycline-regulated expression of a constitutively active variant of the transforming growth factor-β type I receptor ALK5. Expression of activated ALK7 in PC12 cells resulted in activation of Smad2 and Smad3, but not Smad1, as well as the mitogen-activated protein kinases extracellular signal-regulated kinase and c-Jun N-terminal kinase. Reporter assays demonstrated that ALK7 activation stimulates transcription from the Smad-binding element of the Jun-B gene, the plasminogen activator inhibitor-1 gene, and AP-1 elements. In addition, ALK7 activation induced expression of endogenous gene products, including Smad7, c-*fos* mRNA, and plasminogen activator inhibitor-1. Thymidine incorporation assays revealed an anti-proliferative effect of ALK7 activation in PC12 cells, which correlated with increased transcription from the promoters of cycline-dependent kinase inhibitors p15^{INK4B} and p21. Unexpectedly, ALK7 signaling produced a remarkable change in cell morphology characterized by cell flattening and elaboration of blunt, short cell processes. Interestingly, no such changes were observed upon induction of activated ALK5. The alterations in cell morphology upon ALK7 activation were more pronounced in cultures grown in full serum, were accompanied by rearrangements of actin filaments, and were maintained for several days after withdrawal of treatment. PC12 cultures that had been "primed" in this way showed an accelerated and augmented differentiation response to nerve growth factor. These results indicate that ALK7 may participate in the control of proliferation of neuronal precursors and morphological differentiation of postmitotic neurons.

The transforming growth factor (TGF)[1] β superfamily constitutes the largest group of polypeptide growth factors known, including the TGF-βs, activins, bone morphogenetic proteins (BMPs), nodal, and growth and differentiation factors (GDFs). Members of the TGF-β superfamily exhibit an extensive array of biological activities, regulating proliferation, lineage determination, differentiation, migration, adhesion and apoptosis during development, homeostasis, and repair in practically all tissues from flies to humans. TGF-β ligands signal through a complex of two transmembrane receptor serine/threonine kinases belonging to two distinct subfamilies: the type I receptors of ~55 kDa and the type II receptors of ~70 kDa. Both receptors cooperate with ligand binding, type II receptors phosphorylate type I receptors, and type I receptors activate members of the Smad family of signal transducers, which then translocate to the nucleus where they take part in a number of DNA binding complexes (1–3). Receptor-specific Smads transiently interact with and become phosphorylated by type I receptors. Smad2 and 3 participate in downstream signaling from TGF-β/activin receptors, and Smad1, 5, and 8 participate in downstream signaling from BMP/GDF receptors. Smad4 form complexes with receptor-specific Smads and is a common mediator of the nuclear responses of TGF-β/activin and BMP/GDF. Smad 6 and 7, on the other hand, are inhibitory Smads that compete with receptor-specific Smads for association with type I receptors. Once in the nucleus, activated Smads may bind DNA on their own or, most commonly, in association with other DNA-specific factors. A number of general transcription factors, coactivators, and corepressors have been found to associate with Smads during TGF-β signaling (1–3). TGF-β has also been shown to activate several other cytoplasmic signaling pathways in addition to the Smads, including several members of the MAP kinase family such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (4–8). The signaling events leading from the activated receptors to these signaling components remain for the most part undefined.

Activin receptor-like kinase 7 (ALK7) is an orphan member of the type I subfamily of receptor serine/threonine kinases (9–11). It is predominantly expressed in distinct subpopulations of central neurons during postnatal development and adulthood (9, 12). Presently, not only the ligand of ALK7 but also its corresponding type II receptor partner are unknown.

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[1] The abbreviations used are: TGF, transforming growth factor; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ALK7, activin receptor-like kinase 7; NGF, nerve growth factor; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MAP, mitogen-activated protein.

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ALK7 is highly similar in its intracellular domain to ALK5 (78% identity) and ALK4 (77% identity), type I receptors for TGF-βs and activins, respectively. Despite this similarity, the extracellular domain of ALK7 is very divergent from other type I receptors, indicating that it binds a distinct member of the TGF-β superfamily.

In the present work, we have investigated the signaling and biological activities of ALK7 using a constitutively active form of this receptor in an inducible expression system in PC12 cells. Taking advantage of the ability of these cells to adopt a neuronal phenotype after differentiation with nerve growth factor (NGF), we have also studied the interactions between this well characterized cell system of neuronal differentiation and ALK7 signaling.

EXPERIMENTAL PROCEDURES

Subcloning and Mutagenesis— Single-stranded DNA from rat ALK7 subcloned in pBS KS+ (Stratagene) was used for oligonucleotide-based site-directed mutagenesis as described previously (13).

Cell Culture and Tetracycline System—The rat pheochromocytoma cell line PC12 was cultured in Dulbecco's modified Eagle's medium containing 10% horse serum, 5% fetal bovine serum, 2 mM L-glutamine, and 60 μg/ml gentamycin (Life Technologies, Inc.) at 37 °C in a 5% CO_2 humidified atmosphere. All serum was screened for absence of tetracycline using the CHO-AA8-Luc Tet-Off control cell line from CLONTECH. Low serum experiments where done using one-twentieth of the serum concentrations specified above. Perfect Lipid 4 (Invitrogen) was used for cell transfections according to the manufacturer's instructions. PC12 cells were transfected with an expression construct of the reverse tetracycline transactivator (pUHD172–1neo) and constitutively active His_6-tagged T194D-ALK7 or hemagglutinin (HA)-tagged T204D-ALK5 under the control of the tet-operon and a minimal cytomegalovirus promoter (pUHD10-3) (14). Stably transfected cells were selected with 200 μg/ml geneticin (G-418) and 1 μg/ml puromycin (Life Technologies, Inc. and Sigma, respectively), expanded, and individually screened for low leakage and high tetracycline inducibility by RNase protection assay (ALK7) or Western blotting (ALK5). The tetracycline analogue doxycycline was used in all inductions at 1 μg/ml or at the concentrations indicated.

RNase Protection Assay—RNA isolation was done by guanidinium isothiocyanate lysis and acid phenol extraction as previously described (9). For RNase protection assays, a 310-bp-long *Hinc*II-*Pvu*II fragment of rat ALK7 cDNA was subcloned into pBS KS+, linearized, and used as template for T7 RNA polymerase. The rat c-*fos* probe has been previously described (15). A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe was used for normalization. 10 μg of total RNA was hybridized to [α-^{32}P]CTP-labeled cRNA probes using a kit from Ambion according to the manufacturer's instructions. Protected bands were visualized and quantified using a STORM840 PhosphorImager and ImageQuant software (Molecular Dynamics).

Western Blotting, JNK in Vitro Kinase Assay, and PAI-1 Assay—For protein analyses, cells were lysed in 1% Nonidet P-40 with 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM NaF, 2 mM Na_3VO_4, and Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). The supernatant after mild centrifugation constituted the cytosolic fraction. The nuclear pellet was then further treated by trituration through a 23-gauge needle and boiling in SDS to lyse the nuclei and solubilize nuclear proteins. The fractions were then separated by SDS-PAGE. Bands were electroblotted onto Amersham Hybond-P polyvinylidene difluoride transfer membranes and further processed using standard Western protocols. Protein bands were visualized using enhanced chemifluorescence (Amersham Pharmacia Biotech) and a STORM840 fluorimaging system and quantification software as described above. Monoclonal anti-HA (12CA5) and anti-His_6 were from BAbCo; anti-P-ERK, anti-ERK, and anti-JNK antibodies were from New England Biolabs; polyclonal antibodies against ALK7 (RQC), Smad1, phospho-Smad1, Smad2, phospho-Smad2, Smad3, and Smad7 have been previously described (9, 16–18).

For JNK *in vitro* kinase assays, total cell lysates were incubated with glutathione-Sepharose beads that had been previously loaded with a purified c-Jun-glutathione *S*-transferase fusion protein (Upstate Biotechnology). After three washings in lysis buffer, the beads were further washed once in 0.1 M Tris, pH 7.5, 5 mM LiCl and once in kinase buffer (25 mM HEPES, pH 7.0, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM dithiothreitol) and then incubated at 37 °C for 20 min in 30 μl of kinase buffer containing 2 μCi of [γ-^{32}P]ATP, 20 μM rATP, and 5 μM protein kinase A inhibitor. The reaction was stopped by adding SDS/β-mercaptoethanol sample buffer, and the samples were then boiled, fractionated by SDS-PAGE, and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The membranes were exposed to phosphorscreens and subsequently scanned in a STORM840 PhosphorImager (Molecular Dynamics). JNK activity was quantified using ImageQuant software. After exposure, the membranes were probed with anti-JNK antibodies.

Levels of PAI-1 were determined by SDS-PAGE of extracellular matrix proteins of metabolically labeled cells as previously described (19, 20). Nearly confluent 35-mm wells were stimulated with doxycycline at 1 μg/ml in full serum Dulbecco's modified Eagle's medium. After 24 h the cells were washed once in phosphate-buffered saline (PBS), and the medium was replaced with serum-free Dulbecco's modified Eagle's medium without methionine and cysteine. After 2 h, ^{35}S-labeled methionine and cysteine (Amersham Pharmacia Biotech; PRO-MIX, SJQ0079) were added at 40 μCi/ml, and the cells were incubated for an additional 2 h. The cells were then removed by washing on ice once in PBS, three times in 10 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, two times in 2 mM Tris-HCl, pH 8.0, and once in PBS. SDS samble buffer containing β-mercaptoethanol were added, and the remaining extracellular matrix proteins on the plate were transferred to an Eppendorf tube for subsequent SDS-PAGE. After fixing in 40% methanol, 7% acetic acid, the gel was dried, and the 45-kDa PAI-1 band was visualized using a STORM840 PhosphorImager.

Luciferase Reporter Assays—Transcriptional response assays with luciferase reporters were analyzed using the Dual-Luciferase Reporter Assay System kit from Promega. Briefly, 6-well dishes of cells at 50% confluence were transfected as indicated above with 2 μg of the appropriate firefly luciferase reporter construct. For internal control of cell number and transfection efficiency, 50 ng of *Renilla* luciferase under a minimal cytomegalovirus promoter (pRL-CMV, Promega) was included in the transfection. After transfection, full medium was added with or without doxycycline at 1 μg/ml, and the cultures were incubated for an additional 48 h before cell lysis as indicated by the manufacturer. Firefly luciferase activity was normalized to the *Renilla* luciferase activity, and fold induction was calculated relative to the luciferase activity in the absence of doxycycline for each reporter construct and cell line. Luciferase expression was quantified on a 1450 MicrobetaJet luminescence counter (Wallac).

Thymidine Incorporation Assay—Proliferation assays were done on cells cultured on poly-D-lysine-coated 12-well plates at an density of 10×10^3 cells/well. For cell viability assays, 24 h after plating, cells were pulsed for 4 h with 2.0 μCi/ml [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech), washed, and then incubated for an additional 48 h with or without doxycycline at 1 μg/ml. For DNA synthesis assays, the 4 h pulse with [methyl-^{3}H]thymidine was done after the 48-h treatment with doxycycline. At the end of either type of experiment, the medium was replaced by 0.25% trypsin and 10 mM EDTA in PBS and, after a 30-min incubation at 37 °C, the cells were harvested onto filters mats using a cell harvester (Skatron Instruments). [^{3}H]Thymidine incorporation was quantified on a 1450 Microbeta liquid scintillation counter (Wallac).

Filamentous Actin Staining and Morphological Studies—F-actin was stained using a fluorescent phallotoxin from Molecular Probes (Alexa 488 phalloidin, A-12379) according to manufacturer's instructions. Briefly, cells were plated on poly-D-lysine-coated chamber slides (Lab-Tek) and left to attach overnight. The medium was then changed according to the experimental parameters, and the cells were incubated for additional 4.5 days before washing twice with PBS and fixing in 3.7% formaldehyde solution in PBS for 10 min at room temperature. After two additional washes in PBS, each slide was stained at room temperature for 20 min with one unit of Alexa 488 phalloidin in PBS. Following the staining, the slides were washed twice in PBS and mounted under a coverslip in a 1:1 solution of PBS and glycerol and examined by fluorescence microscopy. Morphological studies were performed in PDL-coated 6-well tissue culture plates in a similar fashion. Cells were always washed with PBS in between changes of medium to avoid carry over. For neuronal differentiation, NGF (Promega) was used at 50 ng/ml in low serum medium (*i.e.* $1/20^{th}$ of its normal strength). Cultures were examined and photographed under phase contrast illumination in a Zeiss Axiovert microscope.

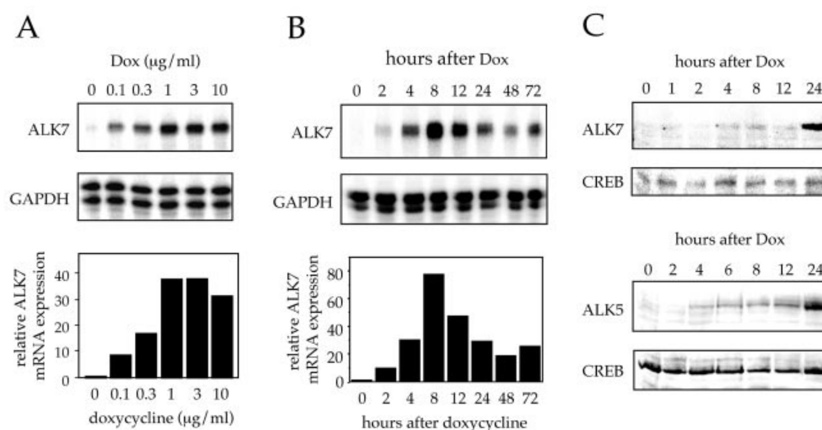

FIG. 1. **Inducible expression of constitutively active ALK7 and ALK5 receptors in PC12 cells.** *A*, dose-response to doxycycline (*Dox*) of the induction of ALK7 mRNA expression in ALK7-PC12 cells analyzed by RNase protection assay 48 h after doxycycline treatment (*top panel*). The *middle panel* shows the signal obtained with a GAPDH riboprobe and demonstrates comparable amounts of RNA in all the lanes. The histogram (*bottom panel*) shows the quantification of the ALK7 mRNA signals relative to GAPDH obtained by phosphorimaging. *B*, time course of the induction of ALK7 mRNA expression in ALK7-PC12 cells treated with 1 μg/ml doxycycline analyzed by RNase protection assay (*top panel*). The *middle panel* shows the signal obtained with a GAPDH riboprobe and demonstrates comparable amounts of RNA in all the lanes. The histogram (*bottom panel*) shows the quantification of the ALK7 mRNA signals relative to GAPDH obtained by phosphorimaging. *C*, time course of the induction of ALK7 (*upper panels*) and ALK5 (*lower panels*) expression in PC12 cells treated with 1 μg/ml doxycycline analyzed by Western blotting with anti-His_6 antibodies (ALK7) or anti-HA antibodies (ALK5). The *lower blots* in each panel show reprobing of the same filters with anti-CREB antibodies and demonstrate comparable amounts of protein in all the lanes.

RESULTS

PC12 Cell Lines with Inducible Expression of Constitutively Active ALK7 and ALK5 Receptors—In the absence of the ALK7 ligand and its corresponding type II receptor partner, an alternative system was developed to study the downstream signaling mechanism and potential biological activities of the ALK7 receptor. Type I receptor serine/threonine kinases can be activated in a ligand- and type II receptor-independent way by replacing an acidic residue for a specific threonine within the juxtamembrane region of the intracellular domain, a segment known to be involved in kinase regulation (21). An equivalent mutation in the ALK7 receptor, corresponding to replacement of Asp for Thr^{194} (T194D), also results in constitutive activation of ALK7 as evaluated using TGF-β-specific reporter assays (10).[2] Signaling by TGF-β ligands is known to have profound effects on cell proliferation, survival, and differentiation. To avoid potential problems caused by constitutive signaling during clone expansion and to allow the study of acute effects of ALK7 signaling, an inducible expression system based on the tetracycline-responsive operon (14) was utilized to generate stable cell lines. As a cellular host for inducible expression of constitutively active ALK7, we chose the rat pheochromocytoma PC12. This cell line has been extensively used as model system in studies of signal transduction, primarily because of its ability to develop a striking neuronal phenotype upon treatment with NGF (22). For comparison with a better characterized signaling system, we also generated PC12 cells with inducible expression of a constitutively active form of the TGF-β type I receptor ALK5, generated, as previously described, by replacement of Asp for Thr^{204} (T204D) (21). A C-terminal His_6 tag was placed in ALK7, and a C-terminal HA tag was placed in ALK5. It should be noted that parental PC12 cells do not express ALK7 and express only moderate levels of endogenous ALK5 (data not shown).

Under basal conditions, ALK7-PC12 or ALK5-PC12 cells did not express any detectable levels of the introduced ALK7 or ALK5 receptors, respectively (Fig. 1). However, treatment with the tetracycline analogue doxycycline resulted in a marked increase in the expression of the respective receptors (Fig. 1).

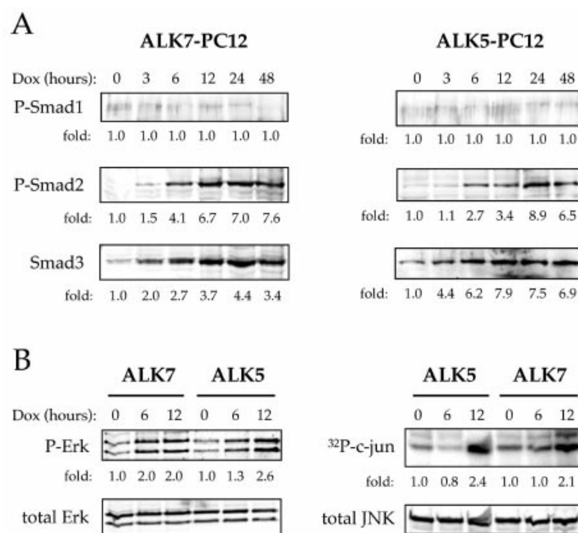

FIG. 2. **Activation of Smad proteins and ERK and JNK kinases in response to ALK7 and ALK5 signaling in PC12 cells.** *A*, nuclear extracts of KAL7- and ALK5-PC12 cells treated with doxycycline (*Dox*) for the indicated periods of time were analyzed by Western blotting with antibodies against phospho-Smad1, phospho-Smad2, and Smad3 as indicated. Lysates were normalized in protein content prior to the analysis. The *numbers* below the lanes indicate the fold increase relative to control. ALK7, similar to ALK5, causes accumulation of phospho-Smad2 and Smad3 in the nucleus but not phospho-Smad1. *B*, total extracts of KAL7- and ALK5-PC12 cells treated with doxycycline for the indicated periods of time were analyzed by Western blotting with antibodies against P-ERK (*left panels*) or by a JNK *in vitro* kinase assay (*right panels*). *Lower panels* show reprobing of the corresponding blots with the indicated antibodies and demonstrate comparable amounts of protein in all the lanes. The *numbers* below the lanes indicate the fold increase relative to control normalized to the total level of ERK or JNK in each lanes. *P-*, phospho-.

Induction of ALK7 mRNA expression was dose-dependent over a wide range of doxycycline concentrations (Fig. 1*A*), with a peak 8 h after doxycycline treatment followed by a slow decline (Fig. 1*B*). Expresion of ALK7 and ALK5 protein could be first detected 4 h after doxycycline stimulation and was maximal at 24 h (Fig. 1*C*). The delay observed between the time course of induction of protein and mRNA expression following Dox treat-

[2] H. Jörnvall, A. Blokzijl, P. ten Dijke, and C. F. Ibáñez, unpublished results.

ment is unclear and could be due to differences in the sensitivity of the two methods. As shown below, several ALK7-induced signaling events can be detected as early as 3 and 6 h following Dox treatment, indicating that levels of ALK7 protein must in fact be present at these early time points. Expression of the exogenous receptors declined 5 days after uninterrupted doxycycline treatment, perhaps because of autoinhibition following continuous signaling (data not shown).

Activation of Smad Proteins and MAP Kinases in Response to ALK7 Signaling in PC12 Cells—A universal signal transduction event following activation of ALK receptors is the phosphorylation and nuclear translocation of members of the Smad protein family. Phosphorylation and nuclear accumulation of receptor specific Smads after ALK7 activation was investigated in nuclear fractions of ALK7- and ALK5-PC12 cells treated with doxycycline using specific antibodies for Smad1, Smad2, and Smad3. Activation of ALK7 resulted in phosphorylation and nuclear translocation of Smad2 but not Smad1 (Fig. 2*A*). Smad3 was also found to accumulate in the nuclei of ALK7-PC12 cells treated with doxycycline (Fig. 2*A*). Similar results were obtained in ALK5-PC12 cells in agreement with previous observations. In agreement with the kinetics of ALK receptor expression, activation of Smad2 and Smad3 could first be detected 3 h after doxycycline stimulation and was maximal at 24 h (Fig. 2*A*).

We also looked for activation of the MAP kinases ERK and JNK in ALK7- and ALK5-PC12 cells after doxycycline treatment. Increased phosphorylation of ERK1 and ERK2 could be detected 6 and 12 h following doxycycline stimulation in both ALK7- and ALK5-expressing cells (Fig. 2*B*). Using a c-Jun-glutathione *S*-transferase fusion protein as substrate, we assayed the activity of JNK in cell lysates of ALK7- and ALK5-PC12 cells after doxycycline treatment. JNK activity was significantly elevated in both cell lines after 12 h of doxycycline treatment (Fig. 2*B*).

Thus, in agreement with the structural similarity between the kinase domain of ALK7 and those of the TGF-β and activin receptors ALK5 and ALK4, signal transduction by the ALK7 receptor also utilizes the pathway-specific Smads 2 and 3, as well as the MAP kinases ERK and JNK.

Activation of TGF-β-responsive Promoter Elements Following ALK7 Signaling—Downstream transcriptional responses to ALK7 signaling were investigated using the luciferase reporter gene coupled to regulatory elements from different genes known to be responsive to TGF-β superfamily proteins, including the genes of cycline-dependent kinase (cdk) inhibitors $p15^{INK4B}$ (23) and p21 (24), the PAI-1 gene (*i.e.* p3TP-Lux, which also includes three TPA-responsive sequences) (25), four tandem copies of the Smad-binding element from the Jun-B gene ($(SBE)_4$) (26), AP-1 elements, and nine tandem copies of the Smad-binding element from the PAI-1 promoter $(CAGA)_9$ (27). $(SBE)_4$ is activated by both TGF-β/activin and BMP ligands, whereas $(CAGA)_9$ is only activated by Smad3-mediated signaling (27). ALK7-PC12, ALK5-PC12, and parental PC12 cells were transiently transfected with reporter plasmids, and reporter gene activity was assayed in cell lysates 48 h following doxycycline treatment. ALK7 signaling stimulated transcription from all the reporters (Fig. 3*A*). Similar results were obtained in an independently isolated ALK7-PC12 cell clone (data not shown). With the exception of the p21 cyclin inhibitor reporter, which appeared down-regulated after doxycycline

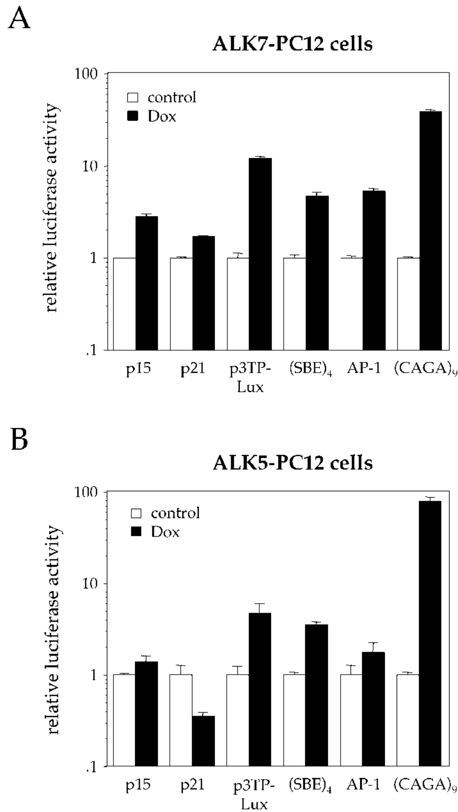

FIG. 3. **Activation of TGF-β-responsive promoter elements by ALK7 signaling.** Luciferase reporter constructs as indicated were transiently transfected into ALK7-PC12 cells (*A*) or ALK5-PC12 cells (*B*). Samples from control (*open bars*) or doxycycline-treated cells (*solid bars*) were assayed 48 later. The results indicate corrected luciferase values expressed relative to control (averages ± S.D.) and were reproduced in several other independent experiments. Dox, doxycycline.

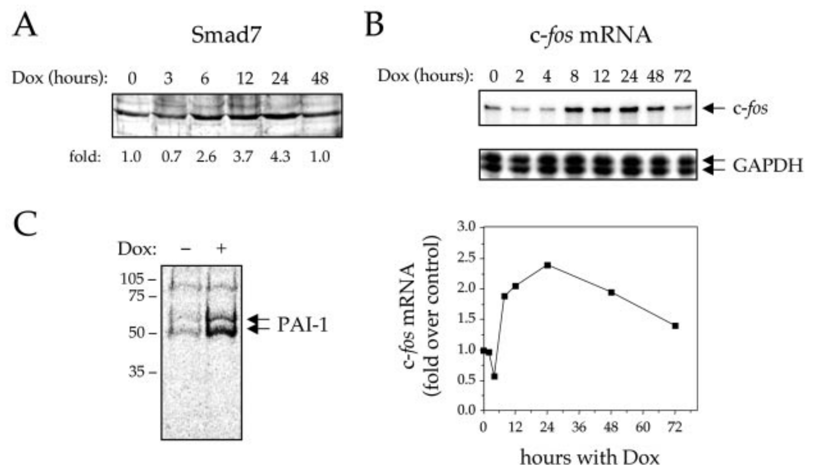

FIG. 4. **Induction of endogenous genes by ALK7 signaling in PC12 cells.** *A*, induction of Smad7 expression was assessed by Western blotting of total extracts of cells stimulated with doxycycline for the indicated periods of time. *B*, induction of c-*fos* mRNA expression was determined by RNase protection assay. A GAPDH probe was included in the same reaction as loading control. *C*, induction of PAI-1 expression was assessed by direct SDS-PAGE and autoradiography of cell matrix extracts prepared from metabolically labeled cells. The two most prominent bands (*arrows*) correspond to the PAI-1 protein. Dox, doxycycline.

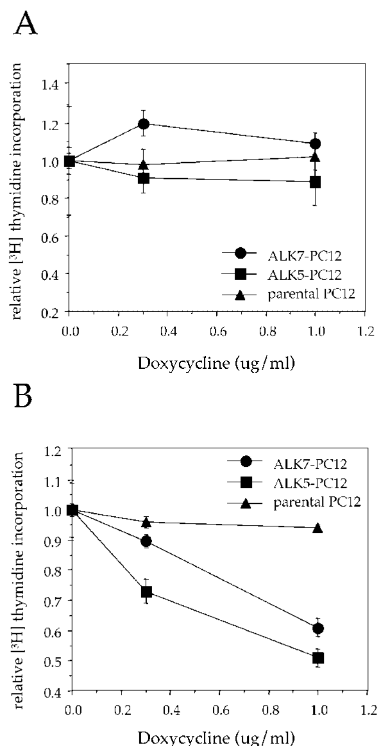

FIG. 5. **Anti-proliferative effects of ALK7 activation in PC12 cells.** *A*, assay of cell viability. The indicated cells were pulsed with [^{3}H]thymidine and then cultured for 48 h with the indicated concentrations of doxycycline. The results are expressed relative to control as averages ± S.D. *B*, assay of DNA synthesis. The indicated cells were cultured for 48 h with the indicated concentrations of doxycycline and then pulsed with [^{3}H]thymidine. The results are expressed relative to control as averages ± S.D.

treatment, similar results were also observed in ALK5-expressing cells (Fig. 3*B*). No changes were detected in parental PC12 cells after doxycycline stimulation (data not shown). Thus, with the possible exception of p21, ALK7 signaling resembles that of the type I TGF-β receptor ALK5 in PC12 cells, including the robust activation of the Smad3-specific reporter $(CAGA)_9$.

Regulation of Endogenous Gene Expression by ALK7 Signaling in PC12 Cells—We also investigated whether ALK7 is able to regulate endogenous genes known to be targets of TGF-β and Activin signaling pathways. Expression of the inhibitory Smad7 is induced by TGF-β via direct interaction of Smad3 and 4 with the *smad7* gene promoter (17). In PC12 cells, ALK7 activation induced expression of Smad7 with a peak of protein expression between 12 and 24 h following Dox treatment (Fig. 4*A*), indicating that, similar to other TGF-β family members, ALK7 signaling is also regulated through a feedback loop of inhibitory Smad proteins. Expression of immediate early genes is also regulated by TGF-β signaling, and the fact that activation of ALK7 induced phosphorylation of Erk proteins prompted us to examine the levels of c-*fos* mRNA in these cells. Doxycycline stimulation stimulated c-*fos* mRNA levels in ALK7-PC12 with a peak at 24 h of treatment, declining back to basal levels by 72 h (Fig. 4*B*). Finally, we examined the expression of PAI-1, an extracellular matrix component which is up-regulated by TGF-β and activin in several cell types (19). We found that in ALK7-PC12 cells treated with doxycycline, the levels of PAI-1 were elevated compared with untreated cells (Fig. 4*C*), indicating that ALK7 activation regulates target genes responsible for some of the phenotypic changes induced by TGF-β proteins.

Anti-proliferative Effect of ALK7 Activation—Thymidine incorporation experiments were performed to investigate the effects of ALK7 signaling on cell survival and proliferation. In the first set of experiments, the cells were pulsed at the beginning with [^{3}H]thymidine for 4 h and then cultured for another 48 h in the presence or absence of doxycycline. Under these conditions, there was no change at the end of the experiment between parental PC12, ALK7-PC12, or ALK5-PC12 cells (Fig. 5*A*), indicating that neither ALK7 nor ALK5 signaling affected the viability of PC12 cells. In the second experiment, the cells were first incubated for 48 h in the presence or absence of doxycycline and then pulsed for 4 h with [^{3}H]thymidine prior to harvesting. In this experiment, however, both ALK7 and ALK5 signaling produced a pronounced decrease in thymidine incorporation in a dose-dependent manner (Fig. 5*B*), indicating that activation of ALK7, like the TGF-β receptor ALK5, has an anti-proliferative effect on PC12 cells.

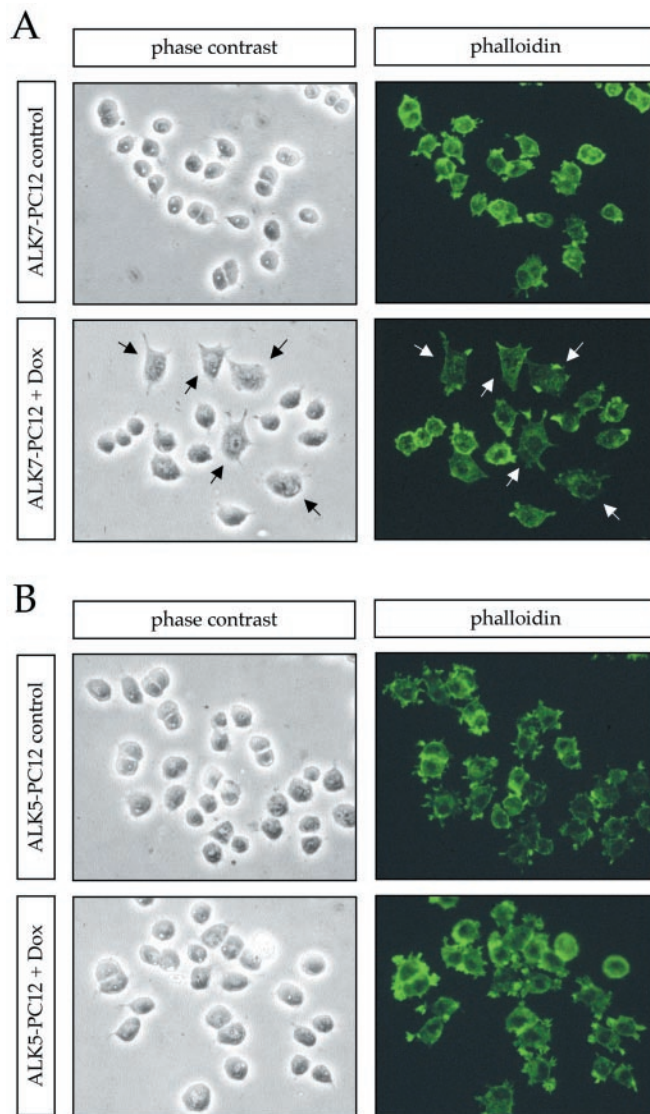

FIG. 6. **Effects of ALK7 signaling on PC12 cell morphology, actin filaments, and neuronal differentiation.** *A*, ALK7-PC12 cells were cultured for 4 days in the absence (*control*) or presence (*+Dox*) of doxycycline and then visualized by phase contrast illumination (*left panels*) or stained with Alexa488-conjugated phalloidin (*right panels*). Note the marked effects on cell morphology produced by ALK7 activation (*black arrows*, *left*), paralleled by a decrease in actin filament staining in the same cells (*white arrows*, *right*). *B*, ALK5-PC12 cells, in contrast, do not show alterations in cell morphology or actin filament staining 4 days after doxycycline treatment.

Effects of ALK7 Signaling on PC12 Cell Morphology, Actin Cytoskeleton, and Neuronal Differentiation—During the course

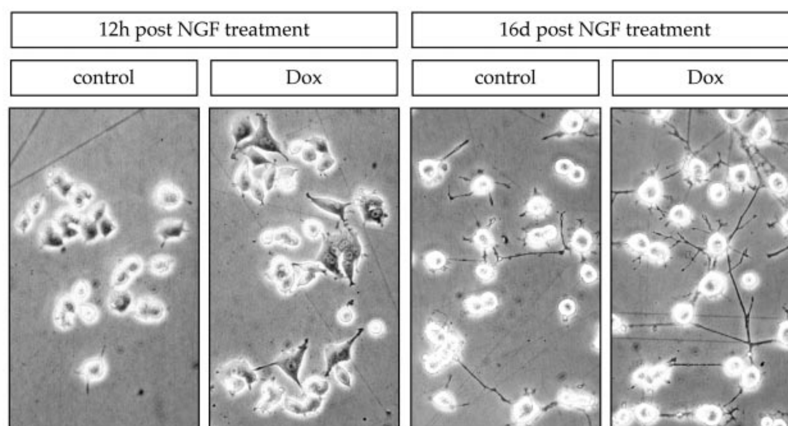

FIG. 7. **Accelerated neuronal differentiation in response to NGF after activation of ALK7 signaling.** ALK7-PC12 cells were cultured for 4 days in the absence (*control*) or presence (*Dox*) of 1 μg/ml doxycycline prior to stimulation with 50 ng/ml NGF for 12 h (*left panels*) or 16 days (*right panels*). Note that many more cells (50%) had already initiated neuronal differentiation in doxycycline-treated cultures 12 h after NGF addition compared with controls (10%). The extent of differentiation 16 days after NGF treatment was also more pronounced in cells exposed to ALK7 activity.

of the studies described above, it became evident that ALK7 signaling had very characteristic effects on cell morphology. Under control conditions, ALK7-PC12 cells were indistinguishable from parental cells. However, within 12 h of doxycycline treatment, ALK7-PC12 cells flattened out and began extending blunt, short cell processes. Four days after doxycycline stimulation, ALK7-PC12 cells were unequivocally different from parental cells (Fig. 6*A*). Conditioned medium from doxycycline-treated ALK7-PC12 cells had no biochemical or morphological effects on naïve PC12 cells (not shown), indicating that the changes induced in ALK7-PC12 cells were produced by a direct, cell autonomous mechanism and not via the production of a secondary signal. The effects of ALK7 on PC12 cell morphology were most evident in the presence of serum. Although cultures grown in low serum did show some changes, these were not as pronounced and mostly restricted to a few cells (not shown). Intriguingly, the morphological changes induced by ALK7 activation were still evident several days after removal of doxycycline, suggesting a long lasting alteration. The effects of ALK7 activation on PC12 cell morphology were also reflected in the distribution of actin filaments, as revealed by phalloidin staining (Fig. 6*A*). ALK7 signaling resulted in an overall reduction in actin staining, indicating actin filament depolimerization (Fig. 6*A*). Loss of cortical actin staining, characteristic of round, phase bright parental PC12 cells, was also seen in several of the most affected cells (Fig. 6*A*). Similar effects in cell morphology could also be observed in an independently isolated PC12 cell clone overexpressing constitutively active ALK7 (data not shown). Surprisingly, ALK5 activation had no effect on the morphology or actin filament distribution of PC12 cells (Fig. 6*B*), suggesting that, despite their apparently similar signaling mechanisms, ALK5 and ALK7 have the capacity to transmit distinct biological responses.

Although the effects of ALK7 signaling on cell morphology were clearly different from those induced by NGF, both processes shared several features, including a reduction in proliferation rate and the elaboration of cell processes, albeit short in the case of ALK7. In the absence of doxycycline, ALK7-PC12 cells responded to NGF just like parental cells, differentiating at the same speed and to the same extent after comparable treatments (data not shown). On the other hand, cultures that were pretreated with doxycycline showed an accelerated differentiation in response to NGF, already evident 12 h after the onset of NGF stimulation (Fig. 7). At this time, more than 50% of the ALK7-PC12 cells stimulated with doxycycline and NGF showed signs of undergoing neuronal differentiation, whereas only a smaller proportion (10%) did in the cultures that received only NGF (Fig. 7). Two weeks into the differentiation process, ALK7-PC12 cells treated with doxycycline showed in addition a more robust phenotype with longer neurites and a greater percentage of differentiated cells (Fig. 7). A similar, albeit less pronounced effect was obtained if the cultures were simultaneously exposed to both doxycycline and NGF (data not shown). Again, no accelerated neuronal differentiation in response to NGF could be seen in ALK5-PC12 cells that had been stimulated with doxycycline (data not shown).

DISCUSSION

We have devised a system to study the function of type I serine/threonine kinase receptors that bypasses the ligand and the type II receptor. We have used this system to obtain insights into the signaling capabilities and biological activities of the orphan type I receptor ALK7.

The high similarity in the primary sequence of the kinase domain of ALK7 with type I receptors for activin and TGF-β, *i.e.* ALK4 and ALK5, anticipated further similarities in the signaling mechanisms of these receptors. In agreement with this, we saw very comparable signaling profiles following ALK7 and ALK5 activation in PC12 cells, both in the type of Smad proteins and MAP kinases activated as well as in the transcriptional responses elicited on a variety of TGF-β responsive reporters. A recent study failed to detect activation of Smad2 by a constitutively active ALK7 receptor (28), which is surprising given the universal role played by this Smad in downstream signaling by receptors of the TGF-β and activin subfamily. In addition to the cell type used, a major difference between the present and previous studies that utilized constitutively active forms of type I receptors lies in our ability to control the onset of receptor activity by stimulation with doxycycline. This has allowed us to study the acute effects of ALK7 activation, something that has not been possible in previous experiments that relied on heterogeneous populations of transiently transfected cells.

In agreement with its ability to induce the promoters of cycline-dependent kinase inhibitors $p15^{INK4B}$ and p21, ALK7 activation had an anti-proliferative effect on PC12 cells. In this regard, ALK7 shares with other type I receptors of the TGF-β superfamily the ability to control the cell cycle and inhibit cell division. Arrest of proliferation is in many neuronal systems a prerequisite for cell differentiation. The effects seen here on PC12 cells suggest that ALK7 signaling may play a similar role *in vivo*, for example, facilitating the differentiation of mitotically active neuronal precursors. Intriguingly, a site of abundant expression of ALK7 is the developing postnatal cerebellum (9), where the onset of ALK7 expression coincides with cessation of mitosis and neuronal differentiation of granule and Purkinje cells. Together, these observations suggest the possibility that one or more cell types in the developing cerebellum may be a source of ALK7 ligand that, upon activation of the ALK7 receptor complex on precursor cells, induces or facilitates

cell cycle exit and neuronal differentiation.

In line with a role in neuronal differentiation, ALK7 activation in PC12 cells induced a marked and long lasting morphological change, characterized by cell flattening, reduction of actin polymerization, loss of phase brightness and cortical actin filaments, and elaboration of short, blunt cell processes. In particular, the effects of ALK7 activity on the actin cytoskeleton suggest cross-talk with signaling components that regulate actin reorganization, such as the small G proteins of the Rho subfamily. Interestingly, JNK, a downstream effector of the Rho family proteins Rac and cdc42, was also activated by ALK7. Of note, TGF-β has recently been found to regulate the levels of Rho GTPases, and inhibition of Rho function was shown to block the ability of TGF-β1 to induce cytoskeletal reorganization in transformed fibroblasts (29). Although the precise mechanisms by which TGF-β proteins affect the cytoskeleton remain unknown, these and other studies offer possible biochemical links between type I receptor signaling and actin reorganization.

The morphological changes induced by ALK7 activity were quite different from those elicited by NGF, which does not induce cell flattening but stimulates the growth of round, phase bright cell bodies and long, thin neuritic processes. Nevertheless, ALK7 collaborated with NGF in promoting neuronal maturation, as demonstrated by an acceleration of the differentiation process in cells exposed to both signals. In this regard, the effects of ALK7 activation in PC12 cells resemble the "priming" effect of a transient exposure to NGF, which also results in accelerated differentiation during a subsequent encounter with this factor (30). Although a direct extrapolation of the effects of ALK7 on PC12 cells to other cell types expressing endogenous ALK7 *in vivo* is limited at present, our observations do suggest that this receptor may have effects on cell differentiation beyond the control of proliferation, in particular, in the regulation of cytoskeletal rearrangements and cell morphology.

Unexpectedly, despite the similarities in signaling between ALK7 and ALK5, the latter was unable to induce the kind of morphological changes promoted by ALK7 in PC12 cells. This difference could not have been due to different levels of expression or signaling intensity between the two systems, because all other effects of ALK7 and ALK5 activation were fully comparable in magnitude and duration across several different assays, including Smad and MAP kinase activation, induction of reporter activity, and inhibition of DNA synthesis. On the other hand, the evidence obtained suggests a qualitative difference in the signaling of these two receptors in PC12 cells. Although the underlying mechanism is unclear at the moment, these results suggest that different type I receptors may have distinct biological effects, even if they activate the same complement of Smad proteins.

In conclusion, the orphan receptor serine/threonine kinase ALK7 appears to utilize many of the same signaling mechanisms activated by TGF-β and activin receptors. The biological effects of ALK7 signaling in PC12 cells suggest, however, that some of the signals transmitted by this receptor are distinct, as illustrated by its unique ability to induce morphological and cytoskeletal changes in these cells. Together with its predominantly neuronal pattern of expression, these findings indicate that ALK7 may participate in the control of proliferation of neuronal precursors and morphological differentiation of postmitotic neurons in several regions of the central nervous system.

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