Activation of Anaplastic Lymphoma Kinase Receptor Tyrosine Kinase Induces Neuronal Differentiation through the Mitogen-activated Protein Kinase Pathway*

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Anaplastic lymphoma kinase (ALK) is a novel neuronal orphan receptor tyrosine kinase that is essentially and transiently expressed in specific regions of the central and peripheral nervous systems, suggesting a role in its normal development and function. To determine whether ALK could play a role in neuronal differentiation, we established a model system that allowed us to mimic the normal activation of this receptor. We expressed, in PC12 cells, a chimeric protein in which the extracellular domain of the receptor was replaced by the mouse IgG 2b Fc domain. The Fc domain induced the dimerization and oligomerization of the chimeric protein leading to receptor phosphorylation and activation, thus mimicking the effect of ligand binding, whereas the wild type ALK remained as a monomeric nonphosphorylated protein. Expression of the chimera, but not that of the wild type ALK or of a kinase inactive form of the chimera, induced the differentiation of PC12 cells. Analysis of the signaling pathways involved in this process pointed to an essential role of the mitogen-activated protein kinase cascade. These results are consistent with a role for ALK in neuronal differentiation.

The common structural features of a receptor tyrosine kinase (RTK)§ include an extracellular ligand binding region, a hydrophobic membrane-spanning segment, and a cytoplasmic domain that carries the catalytic function. Following ligand binding, the RTK dimerizes and autophosphorylates (1). The activated RTK initiates signal transduction cascades through binding of SH2 domain-containing proteins to specific receptor phosphorytyrosine residues (2). RTKs can regulate a wide variety of cellular processes involved in cell division, differentiation, survival, and motility. A number of RTKs play essential roles during the development of the nervous system by contributing to neuronal differentiation, survival, and function (reviewed in Ref. 3). Most of these receptors have specific or shared ligands called neurotrophic factors that have been identified (reviewed in Ref. 3). However, for some of them, named orphan receptors, their ligands are still unknown (4–6).

Anaplastic lymphoma kinase (ALK), a novel orphan neuronal receptor, was originally identified as a member of the insulin receptor subfamily of receptor tyrosine kinases that acquires transforming capability when truncated and fused in the t(2;5) chromosomal rearrangement associated with the non-Hodgkin lymphoma (7). This translocation produces a fusion gene that encodes a soluble chimeric transforming protein comprised of the N-terminal portion of the phosphoprotein nucleophosmin (NPM), a highly conserved RNA-binding nucleolar protein, linked to the cytoplasmic portion of ALK (7). The NPM-ALK fusion protein was localized within both the cytoplasm and the nucleoplasm and also within the nucleoli of t(2;5)-translocation-positive lymphoma cells (8). However, whereas the NPM sequence is essential for the transforming activity (9), the nuclear localization, occurring via the shuffling activity of NPM (10), is not required for oncogenesis (11). It has been demonstrated that the NPM portion was responsible for the dimerization of the fusion protein leading to the constitutive activation of the kinase and to the transforming activity (8).

Human and mouse cDNAs encoding full-length ALK have been characterized (5, 6). The deduced amino acid sequences revealed that ALK is a novel RTK having an extracellular domain, a single transmembrane domain, and an intracellular domain containing the tyrosine kinase activity. The open reading frame encodes a 1620-amino acid protein that is most closely related to leukocyte tyrosine kinase (4, 12). Surface labeling studies indicated that the mature form of the receptor is a 200-kDa glycoprotein exposed at the cell membrane (5), consistent with the prediction that ALK serves as the receptor for yet unidentified ligand(s). In situ hybridization analysis showed that ALK RNA is essentially and transiently expressed in specific regions of the central and peripheral nervous systems such as the thalamus, mid-brain, olfactory bulb, and peripheral ganglia and that it localizes mostly in neuronal cells (5). The neonatal brain showed the highest expression, suggesting a possible involvement of ALK in development of the nervous system when axon sprouting and retraction are occurring. Because ALK expression is maintained, albeit at a lower level, in the adult brain, it may also play a role in synapse formation and maintenance (5). Thus, ALK is a novel orphan receptor tyrosine kinase that might play an important role in the normal development and function of the nervous system.

The ligand of ALK is unknown. Therefore, to investigate whether ALK can play a role in neuronal differentiation, we...
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generated a constitutively active transmembrane form of ALK by substituting the extracellular domain of the receptor by the Fc fragment of mouse IgG 2b. We show here that the ALK.Fc protein expressed in PC12 cells dimerized, oligomerized, and was tyrosine-phosphorylated. Furthermore, we show that transient expression of ALK.Fc induced neuronal differentiation through the mitogen-activated protein (MAP) kinase pathway.

**MATERIALS AND METHODS**

*Reagents*—PDP98059 was obtained from New England BioLabs (Beverly, MA). Wortmannin was from Sigma (St. Quentin-Fallavier, France). ET-18-OCH₃ was purchased from Calbiochem (San Diego, CA). Nerve growth factor (NGF) was from Life Technologies (Cergy Pontoise, France) and basic fibroblast growth factor (bFGF) was a gift from F. Mascarélli (Inserm U450, Paris). Rabbit anti-ALK antibody was purchased from Accurate Chemicals Co. (Westbury, NY). Horseradish peroxidase-conjugated anti-mouse IgG was from Dako (Copenhagen, Denmark). Goat anti-P-MAPK antibody was obtained from UBI (Lake Placid, NY). Rabbit anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY). Rabbit anti-SGC10 antibody was a gift from S. Ozon (Inserm U440, Paris).

*Plasmid Constructions*—The full-length human ALK cDNA in pBluescript was obtained from the American Tissue Culture Collection (ATCC). The cDNA, cut with XhoI and blunt-ended, was inserted at the EcoRV site of the mammalian expression vector pcDNA3.1 (Invitrogen, Groningen, The Netherlands) generating the pcDNA-ALK.wt construct. The cDNA, 6226 bp, covered the entire coding sequence of the protein. The ATG start codon is located at nucleotide position 912, the sequence coding for the transmembrane domain is located between nucleotides 4020 and 4083, and the stop codon is at position 5774. There is an HincII site at position 1079 and a PshAI site at position 3882. These sites were used to delete a major part of the ALK extracellular domain to generate the pcDNA-ALK.wt construct (see below).

The 864-bp PstI-EcoRV fragment of the mouse IgG 2b cDNA corresponding to the Fc fragment (GenBank accession number M61367) in pBluescript was a gift from Dr. N. Doyen (Institut Pasteur, Paris). To construct the cDNA coding for the chimeric protein (pcDNA-ALK.Fc) containing extracellularly the mouse IgG 2b Fc domain linked to the membrane-spanning segment and the whole cytoplasmic domain of ALK, a polymerase chain reaction product containing the entire ALK extracellular domain was PCR-amplified by using a 5’-cII site at position 1079 and a 3’-PshAI site at position 3882. These sites were used to delete a major part of the ALK extracellular domain to generate the pcDNA-ALK.wt construct with its HincII-PshAI segment deleted.

We prepared a kinase-defective form of the chimera (designated ALK*F.C, and the corresponding construct was pcDNA-ALK*Fc) in which the invariant lysine residue located in the ATP-binding portion of the catalytic domain was changed to arginine. This lysine residue, originally identified as residue 210 of the NPM-ALK fusion protein (7, 8), is located at position 1150 in ALK (5, 6). The mutation was generated originally identified as residue 210 of the NPM-ALK fusion protein (7, 8), and the catalytic domain was changed to arginine. This lysine residue, which the invariant lysine residue located in the ATP-binding portion of ALK, was deleted.

Because the ALK ligand is unknown, we generated a constitutively active form of ALK to study the biological function(s) of this receptor. We thus chose a strategy in which the extracellular domain of ALK was substituted by the mouse IgG 2b Fc domain that we expected would dimerize the resulting chimeric protein through disulphide bond formation between cysteine residues of the Fc domain. pcDNA-ALK.Fc was expressed in PC12 cells transiently transfected with the different constructs (pcDNA, pcDNA-ALK.wt, pcDNA-ALK.Fc, or pcDNA-ALK*Fc) were grown on glass coverslips for 72 h, washed in PBS, fixed for 10 min at room temperature with 4% formaldehyde in PBS, and then washed 3 x 5 min with PBS, 50 mM NH₄Cl. After 1 h of blocking in PBS, containing 5% BSA, cells were incubated in the same buffer with an FITC-conjugated goat anti-mouse IgG antibody (1/500 dilution; Jackson Laboratories, West Grove, PA) to visualize ALK.Fc or ALK*-Fc-expressing cells. After washing 5 x 5 min with PBS, cells were mounted in Citifluor (UKC Chemical Laboratory, Canterbury, UK) before viewing on a conventional fluorescence microscope (Praxis, Olympus). To analyze and localize SGC10, a rabbit anti-SGC10 antibody was added to the cells after blocking with PBS, 3% bovine serum albumin, 0.05% saponin for 1 h at room temperature. After washing 3 x 5 min with PBS, 0.05% saponin, cells were incubated with an FITC-conjugated anti-rabbit IgG (Jackson Laboratories, West Grove, PA) for 1 h, washed 5 x 5 min in PBS, mounted, and visualized as described above.

**RESULTS**

Because the ALK ligand is unknown, we generated a constitutively active form of ALK to study the biological function(s) of this receptor. We thus chose a strategy in which the extracellular domain of ALK was substituted by the mouse IgG 2b Fc domain that we expected would dimerize the resulting chimeric protein through disulphide bond formation between cysteine residues of the Fc domain.

Fig. 1 shows the structures of the pcDNA-ALK.wt, pcDNA-ALK.Fc, and pcDNA-ALK*Fc constructs. The pcDNA-ALK.wt construct encodes the membrane-bound wild type receptor (see introduction and below). The pcDNA-ALK.Fc construct codes for a transmembrane protein that contains the 30 N-terminal amino acids of ALK, the Fc fragment and a juxtamembrane portion of the extracellular domain (42 amino acids), the transmembrane, and the entire intracellular domain of ALK. The pcDNA-ALK*Fc construct codes for a kinase-defective form of the chimera in which the invariant lysine residue located in the ATP-binding portion of the catalytic domain was changed to arginine (see "Materials and Methods"). This mutation, which has previously been shown to completely inhibit the transforming capability of NPM-ALK (8).

Expression of ALK.Fc and ALK.wt in PC12 Cells—The pcDNA-ALK.wt and the pcDNA-ALK.Fc constructs were transiently expressed in PC12 cells, and the proteins they encoded were analyzed by SDS-PAGE and Western blotting with anti-ALK antibody or anti-mouse IgG (Fig. 2). Under both reducing and nonreducing conditions, the ALK.wt receptor migrated as a single 200-kDa band, in agreement with previous reports (5). In contrast, the ALK.Fc protein migrated as a single band of 120 kDa under reducing conditions and mainly as a doublet of about 240 and 360 kDa under nonreducing conditions. The
ALK Induces Neuronal Differentiation of PC12 Cells—

Expression ALK.Fc Induced Neuronal Differentiation of PC12 Cells—PC12 cells transiently expressing the pcDNA-ALK.Fc construct exhibited neurite extensions, whereas cells expressing the pcDNA-ALK.wt construct, or those that were transfected with the pcDNA-ALK*.Fc vector or the empty pcDNA3 vector, did not (Fig. 3A). The neurites were visible as soon as 24 h post-electroporation and reached the size of severalfold the cell body size at 48 h post-electroporation. To visualize cells overexpressing the ALK.Fc protein, immunofluorescence staining was performed with an FITC-conjugated goat anti-mouse IgG. As shown in Fig. 3B, only neurite-bearing cells stained with the antibody and were therefore expressing ALK.Fc. Immunostaining for the neuronal marker SCG10 (Fig. 3C) revealed an increased expression and a perinuclear localization of the protein (probably in the Golgi network) of the neurite-bearing cells as previously demonstrated for PC12 cells induced to differentiate with NGF (14, 15). In contrast, cells expressing the ALK*.Fc kinase-inactive form of the chimera ALK.Fc failed to extend neurites, although they clearly expressed the corresponding protein, as demonstrated by immunostaining with FITC-conjugated goat anti-mouse IgG (Fig. 3B). One can note that the staining appeared concentrated at the periphery of the transfected cells, suggesting a plasma membrane localization of the protein. No specific immunoreactivity was detected in cells electroporated with both the pcDNA vector or the pcDNA-ALK.wt construct (not shown).

When cells transiently transfected with the pcDNA-ALK.Fc construct were maintained in the presence of 500 μM of the reducing agent β-mercaptoethanol, the neurite extension process was almost completely blocked (Fig. 4, A and B). This indicated that the neurite outgrowth process was due to ALK.Fc receptor dimerization and oligomerization through disulfide bond formation, because neurite extension stimulated by either bFGF or NGF was not affected by the presence of β-mercaptoethanol in the culture medium. These results demonstrate that overexpression of ALK.Fc induced neuronal differentiation of PC12 cells.

We were unable to isolate stable transfectants from pcDNA-ALK.Fc-transfected cultures, probably because sustained activation of ALK leads essentially to neuronal differentiation and not to cell proliferation in PC12 cells. However, we easily isolated stable transfectants from pcDNA-ALK.wt cultures (not shown). These cells could be good tools for the isolation of the ALK ligand(s) and for further studies on the neurotrophic activity of ALK.

ALK.Fc-induced PC12 Neuronal Differentiation Was Blocked by the MEK-1 Inhibitor PD98059—To analyze the signal transduction cascade involved in the neuronal differentiation process induced by ALK.Fc, we used pharmacological inhibitors targeting the major signaling pathways coupled to RTKs. The
MEK1 (a MAP kinase kinase) inhibitor PD98059, at a concentration of 10 μM, completely blocked the neurite outgrowth process induced by ALK.Fc (Fig. 5, A and B). In contrast, the Phosphoinositide-3 kinase (PI3K) inhibitor wortmannin and the phospholipase C\(\gamma\) (PLC\(\gamma\)) inhibitor ET-18-OCH\(_3\), used at their active concentrations, 20 nM for each inhibitor, had no apparent effect on this process (Fig. 5, A and B). Thus, these data indicate that the PC12 neuronal differentiation induced by ALK.Fc involves mainly the MAP kinase pathway.

**Expression of ALK.Fc Induced Tyrosine Phosphorylation and Activation of the MAP Kinases ERK1 and ERK2**—To determine whether ALK.Fc was autophosphorylated and tyrosine phosphorylated downstream signaling molecules, cell extracts from PC12 cells transiently electroporated with the empty vector, the pcDNA-ALK.wt, or the pcDNA-ALK.Fc constructs were subjected to immunoprecipitation with a rabbit anti-phospho-tyrosine antibody, and the immunoprecipitated proteins were visualized by Western blotting with the same antibody. Although the ALK.wt protein was highly tyrosine-phosphorylated, the ALK.Fc protein showed no apparent phosphorylation (Fig. 6A), indicating that dimerization and/or oligomerization of the receptor via the Fc fragment induced its autophosphorylation on tyrosines. Several other tyrosine-phosphorylated proteins, most probably downstream signaling molecules, with molecular masses in the range 50–110 kDa coimmunoprecipitated with ALK.Fc (Fig. 6A).

To confirm the activation of the MAP kinase cascade by ALK.Fc, cell extracts from PC12 cells transiently electroporated by the empty vector, the pcDNA-ALK.wt, or the pcDNA-ALK.Fc DNAs were analyzed by immunoblotting with an anti-phospho-MAP kinase antibody (i.e. an antibody reacting with the active forms of MAP kinases). The results in Fig. 6B show indeed that the amount of active forms of the MAP kinases (phospho-ERK1 and -ERK2) were higher in cell extracts from PC12/ALK.Fc cells than in extracts from PC12/ALK.wt and PC12/pcDNA cells. Thus activation of the ALK receptor induced by the Fc fragment led to the activation of the MAP kinase pathway.

**DISCUSSION**

ALK is a novel orphan receptor tyrosine kinase that is essentially and transiently expressed in the nervous system (mostly in neuronal cells), suggesting an important role for this receptor during normal development and function of the nervous system. However, because the ligand of this receptor has...
not yet been identified, the normal biological functions of ALK are still unknown. In particular, it remains to be shown whether it could act as a functional membrane receptor and whether activation of its kinase activity could induce neuronal differentiation.

To answer these questions, and to study the signal transduction pathways involved in the potential neurotrophic activity of ALK, we established a model system that allowed us to mimic the normal activation of this receptor. We generated an ALK.Fc chimera containing extracellularly the mouse IgG 2b Fc domain linked to the membrane-spanning segment and the whole cytoplasmic domain of ALK and expressed it into PC12 cells. PC12 cells are a widely used and well established in vitro model system for the study of neuronal differentiation, because they can differentiate to neuron-like cells upon exposure to neurotrophic factors. The MEK1, PI3K, and PLCγ inhibitors (PD98059, wortmannin, ET-18-OCH3) were added 2 h after electroporation and used at concentrations of 10 μM, 20 nM, and 20 nM, respectively. Addition of another PI3K inhibitor, Ly290042, gave results similar to those of wortmannin (data not shown).

Additional proof for the specificity of the neurite elongation process driven by ALK.Fc in PC12 cells was given by transient expression of the pcDNA-ALK.Fc construct in epithelial COS cells.
the activation of the MAP kinase pathway, because the amount be required for the prevention from apoptosis (27) but not for ration supports this assertion.

instance, stimulation of PC12 cells with NGF or bFGF led to induction of a marked and sustained activation (22–25). Nevertheless, when insulin or EGF receptors induced by various neurotrophic factors in PC12 cells. For instance, stimulation of PC12 cells expressing the pcDNA-ALK.Fc construct versus the pcDNA-ALK.wt construct. Thus, these results indicated for the first time that the MAP kinase pathway can be involved in ALK-mediated oncogenicity in lymphocytes (20).

Using pharmacological inhibitors of the classical pathways coupled to RTKs, we found that the ALK.Fc-induced neurite extension process requires the MAP kinase but not the PI3K and PLCγ activities. Studies at the molecular level confirmed the activation of the MAP kinase pathway, because the amount of active forms of ERK1 and ERK2 was found to be higher in PC12 cells expressing the pcDNA-ALK.Fc construct versus the pcDNA-ALK.wt construct. Thus, these results indicated for the first time that the MAP kinase pathway can be involved in ALK signaling and that it was necessary for the promotion of neurite extension induced by this receptor.

In addition, these data call several remarks:

First of all, sustained activation of the MAP kinase signaling cascade seems to be essential for the differentiation processes induced by various neurotrophic factors in PC12 cells (21). For instance, stimulation of PC12 cells with NGF or bFGF led to sustained activation of MAP kinase and to neuronal differentiation (22). In contrast, stimulation of the same cells with insulin or EGF triggered transient activation of MAP kinase and did not lead to neuronal differentiation but to cell proliferation (22–25). Nevertheless, when insulin or EGF receptors were overexpressed, sustained activation of MAP kinase and neuronal differentiation was obtained with the respective ligands (17, 18), indicating that the level of receptor expression is critical for the induction of a marked and sustained activation of MAP kinase and neuronal differentiation. In our experiments, the constitutive activation of ALK.Fc probably led to sustained activation of MAP kinase. The fact that we were able to detect MAP kinase activation as late as 72 h post-electroporation supports this assertion.

Second, the PI3K pathway has been shown to be nonessential for neuronal differentiation (26) and has been proposed to be required for the prevention from apoptosis (27) but not for neurite extension (28, 29) promoted by NGF. Thus, these results are consistent with our data showing that PI3K is not involved in the neurite extension induced by ALK.Fc.

Third, mutations at either the PLCγ or Shc binding site on TrkA (the high affinity NGF receptor) showed no defects in NGF-induced neurite outgrowth and MAP kinase activation, but mutations at both sites did (30). These results indicate that both PLCγ and Shc trigger the MAP kinase cascade and that they can substitute for each other in NGF signaling. In agreement with our data, these results point to a pivotal role of the MAP kinase cascade in neuritogenesis and can explain why the sole inhibition of PLCγ activity with ET-18-OCH3 did not block the neurite extension process induced by ALK.Fc. Therefore, it seems that PLCγ activation is crucial for the mediation of the oncogenic potential of NPM-ALK (see above) (20) but nonessential for the neuronal differentiating activity of ALK.

In conclusion, our results showed that activation of the ALK receptor tyrosine kinase led to neuronal differentiation and this differentiating effect was mainly achieved through the MAP kinase signaling pathway. Thus, these results suggest that ALK could be involved in neuronal differentiation and present the first example for a biological role assigned to ALK. 

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