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In contrast to agonist monoclonal antibodies, both C-terminal truncated form and full length form of Pleiotrophin failed to activate vertebrate ALK (anaplastic lymphoma kinase)?

Thomas Mathivet, Pierre Mazot, Marc Vigny *

INSERM, UMR-S 839, Institut du Fer à Moulin, 17 rue du Fer à Moulin; and UPMC, 4 Place Jussieu, Paris, F-75005, France

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Abstract

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase essentially and transiently expressed during development in specific regions of the central and peripheral nervous system. ALK expression persists at a lower level in the adult brain. Thus, it might play an important role in both the normal development and function of the nervous system. The nature of the cognate ligand of this receptor in vertebrates is still a matter of debate. Pleiotrophin and Midkine have been proposed as ligands of ALK but several independent studies do not confirm this hypothesis. Interestingly, a recent study proposed that a C-terminal truncated form of Pleiotrophin (Pleiotrophin.15) and not the full length form (Pleiotrophin.18) promotes Glioblastoma proliferation in an ALK-dependent fashion. These data were obviously a strong basis to conciliate the conflicting results so far reported in the literature. In the present study, we first purified to homogeneity the two forms of Pleiotrophin secreted by HEK 293 cells. In contrast to agonist monoclonal antibodies, both Pleiotrophin.15 and Pleiotrophin.18 failed to activate ALK in Neuroblastoma and Glioblastoma cells expressing this receptor. Thus, for our point of view, ALK is still an orphan receptor is vertebrates.

Keywords: ALK; Pleiotrophin; ERK kinase; Glioblastoma; Neuroblastoma

1. Introduction

Anaplastic lymphoma kinase (ALK) 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 (see [1] for a review). Human, mouse and *Drosophila* cDNAs encoding full length ALK have been characterized [2–4]. The deduced amino acid sequences revealed that ALK is a RTK having an extracellular domain, a single transmembrane domain, and an intracellular domain containing the tyrosine kinase activity. *In situ* hybridization analysis showed that ALK RNA is essentially and transiently expressed during development in specific regions of the central and peripheral nervous systems and that it is present mostly in neuronal cells. This expression profile suggests a possible involvement of ALK in the development of the nervous system, and a recent report indicates that *Caenorhabditis elegans* ALK may play a role in presynaptic differentiation of the neuromuscular junctions [5]. ALK expression is maintained at a lower level in the adult brain. Thus, it could also play a role in the maintenance of the adult nervous system. In addition, ALK has been recently proposed to be a novel member of the growing family of dependence receptors [6]. These receptors induce or increase apoptosis when the ligand is absent. This latter property of ALK could be essential to further understand its biological activities. Pleiotrophin (Pleiotrophin) and Midkine are developmentally regulated proteins forming a family of heparin-binding molecules with functions during cell growth and differentiation [see for review [7]]. Strong evidence reported by the Wellstein group [8–10] supported the hypothesis that Pleiotrophin and Midkine

Abbreviations: ALK; Anaplastic Lymphoma Kinase; RPTPβ; Receptor Tyrosine Phosphatase Beta; mAb; monoclonal antibody; Pleiotrophin; Pleiotrophin; FN; Fibronectin.

* Corresponding author. Tel.: +33 1 45 87 61 40; fax: +33 1 45 87 61 32.
E-mail address: vigny@fer-a-moulin.inserm.fr (M. Vigny).

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were the cognate ligands of ALK, but recent studies performed by different groups [6,11–13] including ours [14] do not confirm this hypothesis. In addition, the protein jelly belly (Jeb) has been identified as the ligand of *Drosophila* ALK [15,16] and Jeb is distinct from the *Drosophila* homologs of Pleiotrophin and Midkine, Miple 1 and 2 [15]. Finally, no obvious vertebrate homolog has been identified for Jeb in the sequence databases. It is noteworthy that Pleiotrophin also signals through the inactivation of a second receptor, the receptor tyrosine phosphatase RPTPβ [see for a review [17]], Pleiotrophin being the first natural ligand to be discovered for any of the transmembrane tyrosine phosphatase receptors.

Interestingly, Lu et al. [18] recently described the isolation and characterization of two naturally occurring forms of Pleiotrophin with migratory masses of 18 and 15 kDa, named Pleiotrophin.18 and Pleiotrophin.15. Pleiotrophin.15 results from a post-translational processing of 12 C-terminal amino acids of the full length form Pleiotrophin.18. In this study, the authors reported that Pleiotrophin.15 specifically promotes Glioblastoma proliferation in an ALK-dependent fashion, whereas Pleiotrophin.18 promotes Glioblastoma migration in a RPTPβ-dependent fashion. Conversely Pleiotrophin.18 was unable to activate ALK and Pleiotrophin.15 failed to stimulate the receptor RPTPβ. These data were obviously a strong basis to conciliate the conflicting results so far reported in the literature concerning the exact nature of the Pleiotrophin receptors. The presence or absence of the truncated Pleiotrophin.15 form in the different Pleiotrophin batches used could be critical to trigger or not the activation of the ALK receptor.

Recently, we produced a panel of monoclonal antibodies (mAbs) directed against the extracellular domain of the human receptor [14]. Two mAbs strongly activated the receptor in the nM range. In contrast, other mAbs presented the characteristics of potential antagonists. These latter mAbs will be useful tools as blocking antibodies of the cognate ligand when its identity will be fully confirmed. Thus, in the absence of clearly established ligand(s) in vertebrates, these mAbs allowed the control activation or inhibition of the receptor and could be essential for a better understanding of the biological roles of ALK.

In this paper, we first analyzed the kinetics of activation of ALK and of the downstream signaling pathways triggered by our agonist mAbs in human Neuroblastoma (SH-SY5Y) cells endogenously expressing ALK. We then purified to homogeneity the two forms of Pleiotrophin processed and secreted by HEK 293 cells. In contrast to our agonist mAbs, both Pleiotrophin.15 and Pleiotrophin.18 failed to activate ALK in SH-SY5Y cells. Similar results were obtained with the Glioblastoma cell lines expressing ALK either endogenously (at the very low level compared to the SH-SY5Y cell line) or at higher level after transfection. It is noteworthy that in Glioblastoma cell lines the level of endogenous expression of ALK appeared very low. This expression is not (easily) compatible with a strong activation of the transduction pathways downstream of the receptor after its activation either with the cognate ligand when it will be identified or with ligand substitutes such as the agonist mAbs. Finally we confirmed that Pleiotrophin.18 and not Pleiotrophin.15 promoted cell migration in a Glioblastoma cell line expressing the RPTPβ receptor. Thus, from our point of view, ALK is still an orphan receptor in vertebrates.

2. Materials and methods

2.1. Cell lines and tissue culture

Human Neuroblastoma cell line SH-SY5Y and the human Glioblastoma cell lines LN229 and U138MG were purchased from the American Type Culture Collection and maintained in minimum essential medium-(MEM) supplemented with 10% fetal calf serum (FCS), 1× non-essential amino acids, 1 mM sodium pyruvate. In addition, the human Glioblastoma cell lines LN229, U87MG, GM1600 and U138MG were a kind gift of Dr. P. Mischel.

2.2. Reagents and antibodies

Rabbit anti-insulin receptor phosphospecific (pY/pY1162/1163) was purchased from Biomol (Plymouth Meeting, PA). Mouse anti-phosphotyrosine antibody 4G10 and rabbit anti-AKT phosphoserine-473 were from Cell Signaling Technology (Beverly, MA). Mouse anti-phospho-ERK1/2 and monoclonal anti-βtubulin were from Sigma. Goat affinity-purified anti-recombinant human Pleiotrophin was from R&D systems Inc (Minneapolis MN). Rabbit anti-ERK1/2 was from Upstate (Charlottesville, VA, USA).

Rabbit polyclonal antibodies (named RECA) and monoclonal antibodies (mAb) 30, 46 and 48 to the extracellular domain of the ALK human receptor have been previously described [6,14,19].

2.3. Origin of the different human Pleiotrophins used in this study

Constructs in pCDNA3.1 coding for the full length human Pleiotrophin (Pleiotrophin.18) was a kind gift of Dr J. Delbé (Creteil, France). Mutation introducing a stop codon 36 bases upstream of the endogenous stop codon was generated with the QuikChange site-directed mutagenesis kit (Stratagene Europe, Amsterdam, the Netherlands). Mutagenesis was verified by sequencing (Genset, Paris, France).

The resulting constructs were further subcloned into the pCEP4 vector (Invitrogen) to generate pCEP4-Pleiotrophin.18 and pCEP4-Pleiotrophin.15. The human embryonic kidney HEK-293 cell line stably transfected with the EBNA-1 gene (Invitrogen) was cultured in DMEM containing 10% FCS and 0.4 mg/ml geneticin at 37 °C in 5% CO2. HEK-293 cells, plated at 5.10^3 cells/cm^2 for 2 days, were transfected by electroporation with the pCEP4 constructs. Thirty-six hours after transfection, medium was changed and hygromycin (Sigma) was added to the medium at 0.5 mg/ml. After 10 days of selection, the medium was changed to the serum-free AIM-V synthetic medium (Invitrogen). The AIM-V production media were collected every 2–3 days. The secreted Pleiotrophins were purified to homogeneity through a heparin–Sepharose column [20].

Commercial Pleiotrophin expressed in SF1 insect cells was obtained from Sigma.

2.4. Cell transfection

U138MG cells were transfected using calcium phosphate co-precipitation of 3 μg DNA (pcDNA-ALK.wt construct [21]) adjusted to 14 μg per 10-cm Petri dish with pBluescript carrier DNA. Two days after transfection, cells were selected for their geneticin resistance, allowing the selection of stable cells expressing the ALK receptor.

2.5. Immunoblotting and immunoprecipitation analysis

Cell extracts were prepared by lysing the cells in a RIPA buffer (10 mM NaPi buffer, pH 7.8, 60 mM NaCl, 1% Triton X100, 0.5% deoxycholic acid, 0.1% SDS, 10% glycerol, 25 mM β-glycerol phosphate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate and protease inhibitor mixture “complete”, Roche) and analyzed by direct immunoblotting