Identification of Anaplastic Lymphoma Kinase as a Receptor for the Growth Factor Pleiotrophin*

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Gerald E. Stoica‡§, Angera Kuo‡§, Achim Aigner‡, Iruvanti Sunitha‡, Boussad Soutou‡, Claudia Malerczyk†, Dana J. Caughey†, Duanzhi Wen†, Alex Karavanov†, Anna T. Riegel‡, and Anton Wellstein***

From the ‡Lombardi Cancer Center, Georgetown University, Washington, D.C. 20007, §AMGEN, Thousand Oaks, California 91320, and ¶Ciphergen Biosystems, 490 San Antonio Road, Palo Alto, California 94306

Pleiotrophin (PTN) is a secreted growth factor that induces neurite outgrowth and is mitogenic for fibroblasts, epithelial, and endothelial cells. During tumor growth PTN can serve as an angiogenic factor and drive tumor invasion and metastasis. To identify a receptor for PTN, we panned a phage display human cDNA library against immobilized PTN protein as a bait. From this we isolated a phage insert that was homologous to an amino acid sequence stretch in the extracellular domain (ECD) of the orphan receptor tyrosine kinase anaplastic lymphoma kinase (ALK). In parallel with PTN, ALK is highly expressed during perinatal development of the nervous system and down-modulated in the adult. Here we show in cell-free assays as well as in radioligand receptor binding studies in intact cells that PTN binds to the ALK ECD with an apparent $K_d$ of $32 \pm 9$ pm. This receptor binding is inhibited by an excess of PTN, by the ALK ECD, and by anti-PTN and anti-ECD antibodies. PTN added to ALK-expressing cells induces phosphorylation of both ALK and of the downstream effector molecules IRS-1, Shc, phospholipase C-γ, and phosphatidylinositol 3-kinase. Furthermore, the growth stimulatory effect of PTN on different cell lines in culture coincides with the endogenous expression of ALK mRNA, and the effect of PTN is enhanced by ALK over-expression. From this we conclude that ALK is a receptor that transduces PTN-mediated signals and propose that the PTN-ALK axis can play a significant role during development and during disease processes.

Polypeptide growth factors induce their effects by interacting with cell surface receptors. Frequently these receptors are composed of an extracellular ligand-binding domain (ECD), a single transmembrane region and may contain an intracellular kinase domain. Upon binding, ligands induce protein kinase activity and signal via intracellular adaptor or effector molecules that are recruited to the intracellular domain and modulate phosphorylation of downstream targets (1, 2). Here we identify a receptor for pleiotrophin (PTN), a growth factor initially described a decade ago and shown to induce mitogenesis in cultured cells of epithelial, endothelial, and mesenchymal origin as well as neurite outgrowth in neuronal cells (3–8). Animal studies demonstrated that PTN can serve as a rate-limiting angiogenic factor during tumor growth, invasion, and metastasis (8–12). Clinical studies showed elevated serum levels and tumor expression of PTN in samples from patients with colon, stomach, pancreatic, and breast cancer (5, 13). Furthermore, PTN has been implicated in neonatal brain development as well as in neurodegenerative disorders (reviewed in Ref. 14). Obviously, understanding of PTN-mediated signal transduction as well as identification of a receptor for PTN would enhance studies on the biology and pathology of this growth factor family.

Our previous studies have shown that the activation of mitogen-activated protein kinase and PI 3-kinase pathways is required for mitogenic activity of PTN, and we had found that the adaptor molecule Shc participated in signal transduction (15). Based on the work of different laboratories in various cell types, it was hypothesized that proteins of 170–220 kDa that are tyrosine-phosphorylated in response to PTN could be part of the receptor complex (15–17). More recently, several cell membrane-located proteins were shown to bind PTN at low affinity and serve as potential coreceptors or modulators of signal transduction (18–21), but none of these molecules carried the hallmarks of a signal transducing receptor predicted from the earlier work.

To identify a receptor for PTN, we rationalized that panning of a phage display cDNA library against immobilized PTN as a bait would allow us to isolate phage containing a ligand binding fragment of the receptor on their surface. Because of the high levels of expression of PTN during the perinatal development of the nervous system, we hypothesized that fetal brain would most likely also express a PTN receptor. We therefore panned a human fetal brain cDNA phage display library over several rounds against purified PTN that had been tested for biological activity (15). From this we isolated a phage insert homologous to an amino acid sequence stretch in the ECD of the receptor tyrosine kinase anaplastic lymphoma kinase (ALK), a recently described orphan receptor with an apparent molecular mass of

C-γ, PTN, pleiotrophin; SELDI, surface-enhanced laser desorption/ionization; PCR, polymerase chain reaction; RT, reverse transcriptase; PBS, phosphate-buffered saline; NPM, nucleophosmin.
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200–220 kDa (22, 23). Similar to PTN, ALK is highly expressed during perinatal development of the nervous system and down-modulated in the adult (3, 22, 23).

The ALK tyrosine kinase domain was originally discovered because of its oncogenic activity resulting from a t(2;5) translocation and fusion of the ALK intracellular domain with nucleophosmin (24) (see Fig. 1α). More recently, the full-length ALK protooncogene was cloned as a transmembrane receptor (22, 23) of an apparent molecular mass of ~220 kDa. The closest homologue of ALK is leukocyte-tyrosine kinase (LTK) (25), a smaller (100 kDa) transmembrane protein that lacks 60% of the N terminus of the ECD of ALK. We now describe PTN as an activating ligand for the ALK orphan tyrosine kinase and show in receptor binding studies in intact cells as well as in cell-free binding studies with PTN and the ALK ECD that ALK is a receptor for PTN.

MATERIALS AND METHODS

Phage Display Cloning—An M13 phage display library of human fetal brain cDNA (EasyMATCH Phage Display) was obtained from CLONTECH. The human cDNA fragments are located downstream of the phage gene III leader sequence to generate gene III fusion proteins that are exposed on the phage surface. Phages containing candidate PTN receptor cDNA fragments as inserts were selected by repeated panning of the library with purified PTN (~1 µg/well) (6, 15) that had been immobilized in the wells of a 96-well plate. Panning of selected clones against fibroblast growth factor-2 was used as a negative control. Several rounds of panning against purified, biologically active PTN resulted in the isolation of two distinct phases that bound to PTN. One of the phage insert was homologous to a cDNA of unknown function that was identified during screening of metastatic melanoma cells (GenBank™ D50525; see Ref. 26) and contains only a very short open reading frame. The other phage contained a cDNA insert that encoded for a peptide sequence homologous to a region in the ECD of the orphan tyrosine kinase receptor ALK (22, 23), and we report on this below.

Cell Lines, Transfections, and Plasmid Constructs—Stable transfections of SW-13 human adrenal carcinoma cells (6) and of 32D murine pre-B lymphocytes (22) were created using plasmid pCAGGS-alk (anti-ALK) as an insert. From this antiserum, IgGs were affinity-purified overnight with 0.25 mCi of [35S]cysteine in cysteine-free Dulbecco’s modified Eagle’s medium with 2% fetal calf serum (further details in Refs. 5 and 15). The concentration of PTN protein in the ligand preparations was measured by Silverstain and Western blot using commercially available PTN (Sigma) as a standard and an affinity-purified 10-PTN monoclonal antibody (R&D) as described below.

Receptor Binding Studies in Intact Cells—32D cells (ALK-transfected or vector controls; 15 million in 2 ml of growth medium with 10% fetal calf serum) were incubated with radioligand or the respective additions for 1 h at 37 °C. Cells were then pelleted, the supernatant was aspirated, and after two additional washes in 10 ml of growth medium, bound radioactivity in the final cell pellet was detected by scintillation counting. The radiolabeled PTN was purified from supernatants of PTN-overexpressing SW-13 cells (clone 8, see Ref. 5) that had been metabolically labeled overnight with 0.25 mCi of [35S]cysteine in cysteine-free Dulbecco’s modified Eagle’s medium with 2% fetal calf serum (further details in Refs. 5 and 15). The concentration of PTN protein in the ligand preparations was measured by Silverstain and Western blot using commercially available PTN (Sigma) as a standard and an affinity-purified 10-PTN monoclonal antibody (R&D) as described below.

Cell Growth, Immunoprecipitation, and Immunoblotting—For metabolic labeling experiments cells were plated at ~15% confluence in T-162 flasks and starved in serum-free medium for 2 days with one intermittent media change. Cells were then incubated for 2 h in phosphate-free Iscove’s modified Eagle’s medium (Biofluids) and subsequently incubated in 7 ml of phosphate-free Iscove’s modified Eagle’s medium with 0.5 mCi of [35S]orthophosphate (64014L; ICN Biomedical, Irvine, CA) for 4 h and stimulated for the indicated times with PTN (10 ng/ml) (15). Cell lysates were then prepared, and a total of 3 mg of cellular proteins were subjected to immunoprecipitation as described (15). For uncoupled antibodies, Sepharose-bound protein G (Gamba-bind plus, Amersham Pharmacia Biotech) was used to precipitate immunocomplexes. Antibodies were anti-phosphotyrosine (agarose-purified R-PTN antibody; R&D Systems, Minneapolis, MN), anti-ALK (a mixture of 1 µg/ml each of N-19, T-18, and C-19 from Santa Cruz Biotechnology, Santa Cruz, CA and p80 from Accurate Chemicals, Westburg, NY), anti-IRS-1 (3 µg of rabbit IgG, gift of Dr. L.M. Wang, NCI, National Institutes of Health). The resulting precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Experimental procedures for immunoblots for phosphotyrosine, IR, AKT, and TAK-1 (anti-PLC-γ, Upstate Biotechnology Inc.) and PI 3-kinase (Upstate Biotechnology Inc.) using unlabeled cells were as described earlier (15).

RT-PCR—Total RNA was prepared from cell lines as described (9). Random-primed cDNAs were generated using avian moloney virus reverse transcriptase and specific fragments were amplified using Taq polymerase (Life Sciences, Inc., St. Petersburg, FL) and primers from the region coding for the ECD of human or murine ALK (human sense: 5′-CCA GAG TGG AAG GAT CAT-3′; murine sense: 5′-CCA CAA CAG TGG AAG AGA-3′; and antisense human and murine 5′-GTC CCA TTC CAA CAA GTG AAG AGA-3′). To amplify the ALK DNA fragment, the sample was initially heated at 94 °C for 2 min followed by 30 cycles consisting of heating at 94 °C for 30 sec, cooling at 60 °C for 45 sec, and extension at 72 °C for 45 sec. After separation in agarose gels, the PCR products were blotted onto nitrocellulose, and specific products were visualized by hybridization with nested, radiolabeled oligonucleotides (5′-ACT CCA GAG AAT GGT TGG-3′ or 5′-GGA TGT TCC TTC ACT GCA GTT C-3′). Hybridization was overnight at 42 °C in 5× SSC, 5× Denhardt’s, 0.5% SDS, 0.1 mg/ml denaturated salmon sperm DNA. After washes at 42 °C twice in 2× SSC, 0.1% SDS and once in 1× SSC, 0.1% SDS membranes were exposed to Hyperfilm MP (Amersham Pharmacia Biotech).

Data Analysis—The Prism-Graphpad software was used for data analysis by chi square, linear, or nonlinear regression analysis. For saturation binding studies the equation $B = B_{\text{max}} \times L/(L + K_d) + a \times L$ was applied. In this equation, $B$ is the amount of ligand bound, $B_{\text{max}}$ is the receptor binding capacity, $L$ is the ligand concentration, $K_d$ is the estimated dissociation constant, and $a\times L$ is the estimated ligand-independent binding.
equilibrium dissociation constant, and \( a \) is the slope of linearly increasing, nonspecific binding.

RESULTS

Identification of ALK as a Candidate Receptor for PTN—To identify a receptor for PTN, we used immobilized human PTN protein as a bait to screen a phage display library for a ligand binding fragment of the putative receptor on its surface. Fibroblast growth factor receptor-2 was used as a negative control. Several rounds of panning against purified, biologically active PTN resulted in the isolation of a phage cDNA insert that encoded for a peptide sequence homologous to a region in the ECD of a diverse family of transmembrane proteins (Prosite database PDOC 00604, MAM domain). The intracellular domain (ICD) containing the tyrosine kinase (TK) and the translocation site in the juxtamembrane region for fusion with nucleophosmin (t(2,5) NPM-ALK; Ref. 24) are also depicted. Consensus binding sites for insulin receptor substrate-1 (IRS-1), Shc, and PLC-\( \gamma \) are indicated by the arrows. b, binding of the ALK ECD to PTN. The purified PTN protein used as a bait for phage display was immobilized on a nitrocellulose membrane, incubated without (+) or with (-) ECD-Fc fusion protein, and bound ECD was visualized by immunodetection. c, concentration-dependent binding of PTN to the immobilized ALK ECD. The inset shows the experimental setup as well as a representative immunoblot of the binding of the highest concentration of PTN relative to control. The binding isotherm from repeated measurements at different concentrations are shown. Data represent the means \( \pm \) S.E. of triplicate readings at each concentration. The experiment was repeated twice.

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cells are dependent on interleukin-3 for their growth in suspension culture and have been used extensively for the study of tyrosine kinase receptors (e.g. Refs. 27, 28, and 31). The 32D cells do not express ALK mRNA as assessed by RT-PCR (see below), and we hence stably expressed the ALK cDNA to compare radioligand binding in 32D/control and 32D/ALK-transfected cells. PTN radioligand was purified from supernatants of PTN-overexpressing cells that were metabolically labeled with [35S]cysteine (5). As expected for a protein, the PTN radioligand showed background binding to 32D/control cells that increased linearly with increasing radioligand concentrations and was not saturated within the concentration range used (Fig. 2a, open symbols, 32D/ctrl). This binding to the 32D/control cells was also not competed by excess cold PTN or by anti-PTN antibodies (not shown) and is hence considered nonspecific binding. In contrast, ALK-expressing 32D cells showed significantly increased binding of the PTN radioligand (Fig. 2a, filled symbols, 32D/ALK) in comparison with the 32D/control cells. The ALK-dependent binding was saturated within the concentration range of PTN used and an equilibrium dissociation constant \( K_d \) for PTN of \( 32 \pm 9 \text{ pm} \) (0.5 ng/ml) was calculated from nonlinear regression analysis of these data. At PTN concentrations equivalent to this high affinity \( K_d \), the nonspecific binding to the 32D/control cells was \( \sim 25\% \) of the binding to 32D/ALK cells (Fig. 2a; 0.5 ng/ml of PTN). In addition to the evaluation by nonlinear regression, the binding data were also subjected to the more traditional Scatchard analysis (Fig. 2b). For this, nonspecific binding of the PTN radioligand to 32D/control cells was subtracted from the total binding to 32D/ALK cells to obtain the amount of PTN bound to ALK at different concentrations of PTN. These data were plotted against the respective ratio of bound/free PTN (Fig. 2b). A linear regression analysis of the Scatchard plot showed that all of the data points were within the 95% confidence interval (dotted lines in Fig. 2b) and resulted in a \( K_d \) value (36 \pm 8 \text{ pm}) indistinguishable from the value derived by nonlinear regression analysis of the direct binding data (see above). This evaluation of PTN radioligand binding using isogenic cells (32D/ALK and 32D/control cells) demonstrates that ALK can serve as a high affinity receptor for PTN in intact cells.

In addition to the direct radioligand binding studies, we used distinct competitors to provide independent evidence that the high affinity PTN binding to 32D/ALK cells is due to PTN binding to the ALK receptor expressed in these cells. First, an excess of added cold PTN as well as anti-PTN antibodies was able to compete for radioligand binding (Fig. 2c), whereas fibroblast growth factor-2 did not compete (not shown). This supports ligand specificity of the receptor binding. Second, the comparison between 32D/ALK and 32D/control cells already showed that high affinity binding of PTN is only observed after the expression of ALK (see above). In support of this receptor specificity, added ALK ECD protein was also able to inhibit the PTN binding (Fig. 2c). This finding further corroborates the results of the in vitro protein/protein binding studies that had suggested a high affinity interaction between the ALK ECD protein and the PTN ligand (Fig. 1, b–d). Third, from the phage display screen we had derived a putative ligand binding domain in the ALK ECD (Fig. 1a, diamond). We raised antibodies against a fusion protein containing this domain (anti-ECD; see “Materials and Methods”) and found that these IgGs inhibit high affinity binding of PTN to the 32D/ALK cells (Fig. 2c). Unrelated IgGs did not compete for binding (not shown). This suggests that the putative binding domain in the ALK ECD participates in the PTN receptor binding. We conclude from this series of experiments that PTN specifically binds to the ALK orphan receptor as a high affinity ligand at least in part via the putative ligand binding domain described above. Of note, biologically effective concentrations of PTN are within the range of the \( K_d \) values derived from these receptor binding studies supporting a role of ALK for PTN-induced effects (see below and Refs. 5 and 6).

**PTN-induced Signal Transduction through ALK**—To assess how ALK participates in PTN signal transduction and affects the growth response to PTN, we used SW-13 cells. These cells form colonies in soft agar in response to exogenously added PTN protein (5) and were used as indicator cells to purify and N-terminally sequence biologically active PTN from the supernatants of human breast cancer cells (6). Furthermore, after expression of PTN SW-13 cells become clonogenic in vitro (6, 9) and tumorigenic in mice (6). From these previous findings we reasoned that SW-13 cells contain the complete machinery required for PTN effects and could thus be used in signal transduction as well as in growth studies of a candidate PTN receptor. Expression studies showed that SW-13 cells express...
only low levels of endogenous ALK mRNA that is detectable by RT-PCR (Table I) and below detection by Northern analysis (not shown). We thus generated ALK-overexpressing SW-13 cells (SW-13/ALK cells) for further analysis of signal transduction pathways (Fig. 3) as well as comparative growth response to PTN (Fig. 4). Immunoblot analysis showed that the endogenous ALK is below detection in control SW-13 cells and readily found in cell extracts from SW-13/ALK cells (Fig. 4, inset).

The response to PTN was assessed by counting cells (mitogenesis) or soft agar colony formation as described (6) or published in the respective references.

**Table I**  
Expression of ALK mRNA in different cell lines in comparison with their growth in response to PTN.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression of ALK mRNA</th>
<th>Growth in response to PTN</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial: HUVEC</td>
<td>+</td>
<td>+</td>
<td>Ref. 6</td>
</tr>
<tr>
<td>Fibroblast: NIH 3T3</td>
<td>+</td>
<td>+</td>
<td>Ref. 8</td>
</tr>
<tr>
<td>Adrenal carcinoma: SW-13</td>
<td>+</td>
<td>+</td>
<td>Refs. 5 and 6</td>
</tr>
<tr>
<td>Pancreatic cancer: Colo357</td>
<td>+</td>
<td>+</td>
<td>Ref. 40</td>
</tr>
<tr>
<td>Squamous cell: ME-180</td>
<td>+</td>
<td>+</td>
<td>Unpubl.</td>
</tr>
<tr>
<td>Glioblastoma: U87</td>
<td>+</td>
<td>+</td>
<td>Unpubl.</td>
</tr>
<tr>
<td>Breast cancer: MDA-MB 231</td>
<td>+</td>
<td>+</td>
<td>Ref. 49</td>
</tr>
<tr>
<td>Breast cancer: MCP-7</td>
<td>+</td>
<td>+</td>
<td>Unpubl.</td>
</tr>
<tr>
<td>Choriocarcinoma: JEG-3</td>
<td>+</td>
<td>+</td>
<td>Ref. 11</td>
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<td>−</td>
<td>Unpubl.</td>
</tr>
<tr>
<td>Glioblastoma: U87</td>
<td>+</td>
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*Unpubl., A. Wellstein, unpublished data.

Fig. 3. Signal transduction of PTN through ALK.  
a, effect of PTN on ALK phosphorylation in SW-13 cells. SW-13 cells were stimulated for 5 min with PTN (10 ng/ml). Cell lysates were then prepared and subjected to immunoprecipitation with antibodies to ALK (IP α-ALK). The resulting precipitates were analyzed by subsequent Western blot with an anti-phosphotyrosine antibody (WB α-PY). The arrow indicates the position of the ALK protein.  
b, effect of addition of the ALK ECD-Fc fusion protein (0.7 μg/ml) or an affinity-purified α-PTN antibody (2.5 μg/ml) on PTN-induced tyrosine phosphorylation in SW-13/ALK cells. SW-13/ALK cells were stimulated for 5 min with PTN that had been preincubated with α-PTN or with the ALK ECD. An α-phosphotyrosine Western blot (WB α-PY) of immunoprecipitates with an α-PY antibody (IP α-PY) is shown.  
c, identification of phosphoproteins after PTN-stimulation of SW-13/ALK cells. Cells were stimulated with PTN for different times, and extracts were subjected to immunoprecipitation with an α-PY antibody and subsequent Western blots with the antibodies indicated. PI 3-K refers to the p85 subunit of PI 3-kinase. Details are under “Materials and Methods.”  
d, SW-13 control or SW-13/ALK-transfected cells were metabolically labeled with [32P]orthophosphate and stimulated for the indicated times with PTN (10 ng/ml). Cell lysates were then prepared and subjected to immunoprecipitation with antibodies to phosphotyrosine (α-PY), ALK, or IRS-1. The resulting precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Changes in phosphoproteins that were analyzed further are indicated by arrows.
with antiphosphotyrosine antibodies. This approach showed that ALK is indeed phosphorylated in response to PTN (Fig. 3a, arrow), although the endogenous receptor level in SW-13 cells is low (see above), and the resulting phosphorylation signal hence is only small. Overexpression of ALK enhances protein phosphorylation of SW-13 cells in response to PTN (Fig. 3, b–d), and we utilized the SW-13/ALK cells for a more detailed analysis of signal transduction.

In one experimental approach, tyrosine-phosphorylated proteins were immunoprecipitated from PTN-stimulated SW-13/ALK cell extracts and further analyzed by immunoblotting (Fig. 3, b and c). As an alternative approach, the SW-13/ALK cells as well as the respective control cells were metabolically labeled with $[^{32}P]$orthophosphate, and protein phosphorylation in response to PTN stimulation was analyzed after immunoprecipitation with different antibodies (α-PY, α-ALK, or α-IRS-1; Fig. 3d). Analysis of the time course of the PTN effect shows that induction of phosphorylation is obvious at the earliest time point, i.e. 1 min after addition of PTN (Fig. 3d, right panels). The anti-phosphotyrosine antibody (15) precipitated several distinct phosphoproteins from SW-13/ALK cells after $[^{32}P]$orthophosphate labeling (arrowheads in Fig. 3d; α-PY), and the induction of protein phosphorylation was maintained at all three time points tested (1, 2, and 10 min). Furthermore, detection of tyrosine-phosphorylated proteins by α-PY immunoprecipitation followed by α-PY immunoblot detected a similar pattern of phosphoproteins after ligand stimulation (Fig. 3b, two leftmost lanes). In contrast with this dramatic induction of protein phosphorylation in SW-13/ALK cells by PTN, control SW-13 cells showed only very small increases in overall protein phosphorylation (Fig. 3d, left panel).2

To evaluate the specificity of the ligand/receptor interaction in the SW-13/ALK cells, we included an α-PTN antibody and an excess of the ALK-ECD protein in the phosphorylation studies. As shown in Fig. 3b, the PTN-stimulated tyrosine phosphorylation of proteins in SW-13/ALK cells was inhibited significantly by preincubation of the ligand with the recombinant ALK-ECD protein or with the α-PTN antibody. We conclude from this set of data that the ligand-induced protein phosphorylation in intact cells occurs rapidly and is specific to the PTN ligand as well as the ALK receptor.

Immunoprecipitation of SW-13/ALK cell extracts with antibodies directed against ALK revealed that a number of phosphoproteins are associated with the receptor after PTN stimulation of the $[^{32}P]$orthophosphate-labeled cells (Fig. 3d; α-ALK). Thus, we analyzed signaling proteins for which a consensus binding site was found in the intracellular domain of ALK (Fig. 1a); i.e. Shc, IRS-1, and PLC-γ. Furthermore, our previous studies had shown that PI 3-kinase was a target for PTN-induced phosphorylation (15), and we included this in our analysis. Immunoprecipitation of $[^{32}P]$-labeled proteins with an α-IRS-1 antibody showed that the adaptor molecule IRS-1 is phosphorylated upon PTN stimulation of SW-13/ALK cells (Fig. 3d, rightmost panel). Furthermore, immunoprecipitation with an α-PY antibody and subsequent Western blots revealed that PLC-γ, PI 3-kinase, and Shc are also phosphorylated after PTN stimulation of SW-13/ALK cells (Fig. 3c). These findings in conjunction with our earlier studies (15), suggest that PTN induces the phosphorylation of ALK and subsequent signal propagation via the adaptor molecules IRS-1 and Shc as well as the enzymes PLC-γ, ERK, and PI 3-kinase.

**Effect of ALK Overexpression on the Growth of Cells**—As described in the previous section, PTN induces ALK phosphorylation in SW-13 cells, although the relatively low levels of ALK protein expression in SW-13 cells is reflected in only a very small induction of overall protein phosphorylation in response to PTN. This PTN-induced protein phosphorylation is strongly enhanced in the SW-13/ALK cells (Fig. 3). In parallel with this increase in phosphorylation, PTN-induced soft agar growth of SW-13/ALK versus control cells is also enhanced significantly (an 18–20-fold stimulation versus a 3–6-fold stimulation, respectively). In addition, the sensitivity of the SW-13/ALK cells is shifted to 3-fold lower PTN concentrations (Fig. 4). We conclude from this that overexpression of ALK enhances PTN-induced growth stimulation.

**Expression of ALK in Different Cell Lines and Their Response to PTN**—The ALK protooncogene was described as an orphan receptor that is highly expressed in the developing brain and down-regulated in the adult (22, 23). This tissue expression pattern of ALK coincides with the expression pattern of PTN (14), supporting the notion that PTN and ALK could function as a ligand/receptor pair. In cultured cells of different lineage we found ALK mRNA expressed in 7 of 10 cell lines. The expression of ALK (and the lack thereof) correlated significantly with the growth effect of PTN on the same cells (Table I; $p = 0.0016$, chi square test). These data lend further support to the notion of ALK as a receptor for PTN.

**DISCUSSION**

A number of laboratories have been studying the signal transduction of the PTN growth factor family (reviewed in Ref. 14) and have sought after a receptor for this growth factor. Our current studies provide evidence that PTN is a ligand for the orphan receptor ALK (anaplastic lymphoma kinase). The receptor binding and signal transduction studies reported here were initiated after a phage display screen of a human fetal brain cDNA library with the PTN protein as the bait. This screen resulted in the isolation of a phage containing an insert homologous to a small fragment in the ECD of the protooncogene ALK. The oncogenic ALK kinase was originally discovered in a chromosomal (2, 5) translocation associated with anaplastic large cell lymphomas. In this translocation the 3'-half of the ALK gene (i.e. the intracellular portion of ALK with the kinase domain) is fused to the 5'-portion of the nucleosomin (NPM) gene (Fig. 1a and Ref. 24). This fusion generates a constitutively active ALK kinase because of the dimerization of NPM-ALK via the NPM region (32, 33). Several more recent studies have suggested that genes other than NPM can be fused to the ALK kinase and may serve as dimerization and activation domains (34, 35). The activated ALK kinase can act as an oncogene in different cell systems and induce malignant transformation of fibroblasts (32, 36, 37) as well as Bu/F3 murine

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2. A. Wellstein, unpublished data.
pro-B lymphoid cells (36). In addition, retroviral transduction of murine bone marrow with an expression vector for NPM-ALK resulted in B-cell lymphoma in animals transplanted with the transduced bone marrow (38). More recently, the ALK kinase sequence from the NPM-ALK oncogene was used by two different laboratories to clone the full-length ALK receptor (22, 23). This work showed that this receptor contains a transmembrane region and a large ECD that comprises over 60% of the protein (Fig. 1a).

A number of independent studies showed that PTN is expressed in a tightly regulated manner during perinatal organ development and in selective populations of neurons and glia in the adult (reviewed in Ref. 14). Northern blot analysis of different tissues showed that the ALK mRNA is mostly expressed in the brain and spinal cord. In situ hybridization studies revealed that ALK expression in mice initiates late during embryonic development (around embryonic day 11), peaks during the neonatal period, but persists into adulthood in a few select portions of the nervous system such as the thalamus, midbrain, and ganglionic cells of the gut. Immunoblotting with anti-ALK antibodies corroborated the tissue expression and developmental regulation of ALK at the protein level (22, 23).

Interestingly, more recent immunohistochemistry studies with human tissues showed that the ALK protein is expressed in the normal central nervous system, in particular in some neurons as well as glial cells and is also found in endothelial cells in these tissues (39). The expression profiles of ALK and PTN suggest that this growth factor/receptor pair may play an important role in the normal development and function of the nervous system. However, the expression of ALK in endothelial cells in tissues (39) as well as in cell culture (Table I) suggests a potentially broader role of PTN/ALK.

A comparison of ALK mRNA expression and PTN response of 10 different cultured cell lines indicates that cells that lack ALK expression also fail to show a growth response to PTN and vice versa (Table I). Based on the lack of a PTN response of ALK-negative cells, it is tempting to speculate that there are only few distinct ALK-related receptors for PTN. To find potential ALK homologues in addition to the known LTK (22, 23), we used the ALK ECD protein sequence for a search of currently available data bases. From this search only sequences coding for ALK in (different vertebrate and invertebrate species) and LTK were identified. This search was done in different modes against the protein data bases as well as with the broader “blastn” algorithm that searches the nucleotide data bases (nr, EST, htgs, and Drosophila) and reveals any DNA sequences that code for homologous protein fragments. Interestingly, the Drosophila ALK contains the putative ligand binding region in the human ALK ECD and also shows the same signature sequence (MAM domains; Fig. 1a) in the vicinity of this region. In contrast to ALK, the human and murine LTK proteins only comprise short ECDs and lack this putative receptor binding portion present in the ALK ECD, and we speculate that LTK might act as a coreceptor for ALK. The Drosophila genome contains at least one gene homologous to the PTN growth factor family ("nipple"; GenBank accession number AF149800), suggesting that a PTN/ALK interaction is also possible in invertebrates.

In our studies we have mostly focused on the role of PTN and ALK in human cancers. We and others demonstrated that PTN can be a rate-limiting factor for tumor growth, invasion, angiogenesis, and metastasis (8–12, 40). ALK could be involved in the activity of PTN as an angiogenic factor, because ALK was found expressed in endothelial cells in culture (Table I) as well as by immunohistochemistry in the endothelium of human tissues (39). Furthermore, ALK might be a target for the stimulation of stromal and mesenchymal responses to tumor-derived PTN because of its expression in fibroblasts as well as glial cells (Table I and Refs. 22, 23, and 39). Finally, coexpression of PTN and of ALK in cancer cell lines (e.g. Colo357 pancreatic cancer, Hs578T breast cancer, and U87 glioblastoma; Refs. 14 and 40) indicates that PTN and ALK could form an autocrine loop of growth stimulation (41) of the tumor cells. The intracellular domain of ALK is highly homologous to the LTK kinase and to the protooncogene ras as well as the insulin receptor/insulin-like growth factor-1 receptor kinases (22). Furthermore, earlier studies have demonstrated that IRS-1 associates with NPM-ALK (32) as well as with LTK (42–44) and appears to be required for growth stimulatory or transforming activity. Finally, our finding that IRS-1 is one of the signal transduction molecules that is phosphorylated in response to PTN (Fig. 3d) also assigns the ALK receptor more to the insulin receptor family. Although the phenotypic significance of IRS-1 for PTN/ALK-mediated signal transduction remains to be tested, it is likely that the IRS-1 docking protein will play a role in PTN growth or survival signals. This would add the PTN/ALK receptor to the insulin/insulin-like growth factor-1/interleukin-4 group of receptors that utilize IRS-1 as a major docking protein (for a recent review see Ref. 2).

In addition to IRS-1, the docking protein Shc was shown to be utilized by LTK for signaling through the Ras pathway (44). We demonstrate here that Shc is phosphorylated by PTN signaling through ALK (Fig. 3c), and we showed earlier that Shc is phosphorylated in response to PTN (15). In contrast to Shc, IRS-1 not only mediates Ras signaling initiated by LTK activation via a chimeric EGF-R/LTK receptor but also cell survival through PI 3-kinase (44). This finding coincides with our earlier studies on the signal transduction of PTN through PI 3-kinase (15) as well as the PTN/ALK-mediated phosphorylation of the p85 subunit of PI 3-kinase (Fig. 3c). Finally, we demonstrate phosphorylation of PLC-γ in response to PTN stimulation of SW-13/ALK cells (Fig. 3c). This finding corroborates PLC-γ binding to the activated NPM-ALK kinase in Ba/F3 and Rat-1 cells (36). Furthermore, PLC-γ activation was suggested as a crucial step for mitogenic activity of the ALK and potentially the LTK kinase (36, 45). Typically PLC-γ is activated by fibroblast growth factor, hepatocyte growth factor, or platelet-derived growth factor in a variety of cell lines (46). Although the sequence homology of the kinase domain and IRS-1 signaling assigns the ALK/LTK kinases to the insulin receptor family, a role of PLC-γ suggests an overlap with other growth factor receptors and would be in line with a potential of PTN on cell lines of distinct germ layer origin (Table I; reviewed in Refs. 2 and 47).

In conclusion, the identification of ALK as a receptor for PTN will be the basis for studies into the physiological as well as pathological functions attributed to this growth factor. PTN can induce neurite outgrowth and is thought to play a role during neonatal brain development and maintenance of neuronal function (Ref. 4; reviewed in Ref. 14). Furthermore, dysregulation of PTN has been described in neurodegenerative disease processes (48), and it is conceivable that studies of ALK in conjunction with PTN will generate novel insights into the mechanisms underlying these diseases. Beyond a role in the nervous system, PTN plays a significant role for tumor growth, invasion, angiogenesis, and metastasis in some of the most aggressive human cancer types, e.g. melanoma and pancreatic cancer (10, 11, 40). Also, ALK is expressed in a significant portion of human cancer cell lines, and it is tempting to speculate that blockade of ALK could be of therapeutic benefit in cancers or other diseases in which PTN plays a pathological role.
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Identification of Anaplastic Lymphoma Kinase as a Receptor for the Growth Factor Pleiotrophin

Gerald E. Stoica, Angera Kuo, Achim Aigner, Iruvanti Sunitha, Boussad Souttou, Claudius Malerczyk, Dana J. Caughey, Duanzhi Wen, Alex Karavanov, Anna T. Riegel and Anton Wellstein

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