A Ligand-inducible Epidermal Growth Factor Receptor/Anaplastic Lymphoma Kinase Chimera Promotes Mitogenesis and Transforming Properties in 3T3 Cells*

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Oncogenic rearrangements of the anaplastic lymphoma kinase (ALK) gene, encoding a receptor type tyrosine kinase, are frequently associated with anaplastic large cell lymphomas. Such rearrangements juxtapose the intracellular domain of ALK to 5′-end sequences belonging to different genes and create transforming fusion proteins. To understand how the oncogenic versions of ALK contribute to lymphomagenesis, it is important to analyze the biological effects and the biochemical properties of this receptor under controlled conditions of activation. To this aim, we constructed chimeric receptor molecules in which the extracellular domain of the ALK kinase is replaced by the extracellular, ligand-binding domain of the epidermal growth factor receptor (EGFR). Upon transfection in NIH 3T3 fibroblasts, the EGFR/ALK chimera was correctly synthesized and transported to the cell surface, where it was fully functional in forming high ɛνεs low affinity EGF-binding sites and transducing an EGF-dependent signal intracellullarly. Overexpression of the EGFR/ALK chimera in NIH 3T3 was sufficient to induce the malignant phenotype; the appearance of the transformed phenotype was, however, conditionally dependent on the administration of EGF. Moreover, the EGFR/ALK chimera was significantly more active in inducing transformation and DNA synthesis than the wild type EGFR when either was expressed at similar levels in NIH 3T3 cells. Comparative analysis of the biochemical pathways implicated in the transduction of mitogenic signals did not show any increased ability of the EGFR/ALK to phosphorylate PLC-γ and MAPK compared with the EGFR. On the contrary, EGFR/ALK showed to have a consistently greater effect on phosphatidylinositol 3-kinase activity compared with the EGFR, indicating that this enzyme plays a major role in mediating the mitogenic effects of ALK in NIH 3T3 cells.

The ALK1 proto-oncogene encodes a 200-kDa membrane-spanning tyrosine kinase receptor that is most closely related to leukocyte tyrosine kinase, a member of the insulin receptor superfamily (1, 2). Only very recently pleiotrophin (PTN) has been identified as a high affinity ligand for ALK (3). No function has been ascribed for ALK; however, its restricted expression in neural tissue suggests that this receptor might play an important role in the normal development and function of the nervous system (1, 2).

Oncogenic rearrangements of ALK have been detected with a high frequency in anaplastic large cell lymphoma (ALCL), a subgroup of non-Hodgkin’s lymphoma (4–8). Over half of these lymphomas are characterized by the chromosomal translocation (t;2;5)(p23;q35) that results in the production of an 80-kDa chimeric protein in which the N-terminal region of the nuclear protein nucleophosmin (NPM) (9, 10) is fused to the intracellular region of ALK, including the tyrosine kinase domain (7). Similarly to other genes responsible for activating receptor tyrosine kinase oncogenes, NPM is constitutively expressed in all tissues and activates the catalytic domain of ALK through dimerization (11). p80

*The abbreviations used are: ALK, anaplastic lymphoma kinase; ALCL, anaplastic large cell lymphoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PLC-γ, phospholipase C-γ; PI3-K, phosphatidylinositol 3-kinase; PTN, pleiotrophin; Tyr(P), phosphotyrosine; NPM, nucleophosmin; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; LTR, long terminal repeat; FBS, fetal bovine serum; FFU, focus-forming units; BSA, bovine serum albumin; TBS, Tris-buffered saline; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; IRS-1, insulin receptor substrate-1.
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In NIH 3T3 cells, activation of a chimeric receptor possessing the intracellular domain of ALK induces neurite outgrowths, suggesting a role in the transduction of differentiative signals (21). The possibility that ALK is involved in the regulation of cell proliferation is suggested by the observation that adoptive expression of either NPM/ALK or ATIC/ALK in an interleukin-3-dependent murine pro-B lymphocyte cell line is sufficient to bypass the events normally regulating cell division, causing sustained proliferation in the absence of interleukin-3 (17, 22). Finally, the recent identification of PTN as the ligand for ALK has clearly proved the ability of this receptor to deliver mitogenic signals inside the cells.

In contrast to the situation for most of the receptor tyrosine kinases, the biochemical events involved in the signal transduction triggered by normal ALK are largely unknown, except for some evidence deriving from studies of ALK oncocenes (11, 12, 22–24). This line of investigations, relevant for the comprehension of the molecular basis of ALK-associated diseases, has been so far hampered by the lack of a known ligand for the ALK proto-oncogene. A strategy to circumvent this problem derives from the engineering of chimeric molecules containing the extracellular ligand-binding domain of the epidermal growth factor receptor (EGFR) and the intracellular region of ALK. Such an approach has been successfully used to analyze the signaling properties of ligand-orphan receptors and the structure-function of several receptor tyrosine kinases (25–31).

We have therefore engineered a chimeric EGFR/ALK molecule to study ALK biological and biochemical activities in a fibroblast target cell. We demonstrate that overexpression of such a chimera is associated with a ligand-dependent expression of the transformed phenotype. In addition, the EGFR/ALK chimera was significantly more efficient at inducing cell transformation and DNA synthesis than the wild-type EGFR when activated by EGF. Comparative analysis of the biochemical pathways implicated in the transduction of mitogenic signals did not show any increased ability of the EGFR/ALK to phosphorylate PLC-γ and MAPK compared with the EGFR. On the contrary, EGFR/ALK was much more efficient than EGFR in inducing PI3-K activity; the up-regulation of PI3-K activity following activation of ALK can explain, at least in part, the stronger mitogenic potency of ALK in NIH 3T3 cells.

MATERIALS AND METHODS

Engineering of Eukaryotic Expression Vectors—The EGFR/ALK expression vector was engineered starting from a previously described modified version of the long terminal repeat (LTR)-EGFR 5M vector in which a novel SaI site was generated in the sequence of the EGFR cDNA immediately following the transmembrane region (32). The SaI site and a unique MluI site located in the LTR vector downstream of the natural stop codon of the EGFR cDNA were used for the recombination. To obtain the EGFR/ALK chimera, we initially amplified, by recombinant PCR, the entire intracellular domain of ALK in the region comprising amino acids 1058–1362 by amplification with unique XhoI and MluI sites, at the 5′- and 3′-ends of the PCR product, respectively. The sequence of the primers used were GGAAGATCTTGGGACGCTGGTCTG (5′ primer) and ATCCTCTAGAGCGAGCCAGCTGTCTG (3′ primer). After digestion with XhoI and MluI, the PCR-amplified fragment was cloned into the homologous sites of the LTR-2 vector. This recombination yielded to the LTR-NPM/ALK expression vector.

The LTR-ALK/PDGF, LTR-EGFR/ALK, and LTR-NPM/ALK expression vectors were sequenced in both strands of the regions that underwent genetic manipulations to verify that the predicted structures were achieved after the recombination procedure.

Cell Culture and Transfection Assays—NIH 3T3 and NR6 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) (Invitrogen) and transfected with circular DNA according to the calcium phosphate precipitation technique (35) as modified by Wigler et al. (36). Transformed foci were scored at 2–3 weeks. Where indicated, EGF (Upstate Biotechnology, Inc., Lake Placid, NY) (20 ng/ml) was added at day 14, and foci were scored at day 21. Transferring efficiency was calculated in focus-forming units/pmol of added DNA (FFU/pmol) after correction for the relative molecular weight of the respective plasmid and normalization for the efficiency of colony formation in parallel dishes subjected to selection in mycophenolic acid-containing medium. The expression vectors used in the transfection experiments were LTR-EGFR/ALK (described above), LTR-EGFR 5M (32) and LTR-NP/ALK (described above). All the vectors contained the eukaryotic resistance marker Escherichia coli gpt. Cells expressing the E. coli gpt gene were selected for their ability to grow in the presence of mycophenolic acid (37).

The ^3H incorporation assay was performed as described previously (26). Briefly, NIH 3T3 transfectants, grown to confluence in 24-well poly-L-lysine-treated plates (Costar), were serum-starved for 22 h in Dulbecco’s modified Eagle’s medium, containing transferrin (5 μg/ml) (Becton Dickinson) and Na2SeO3 (10 μM) and then stimulated for 22 h with either 1% (v/v) FBS or EGF at the indicated concentrations in the presence of 4 μCi/well of [methyl-^3H]thymidine (Amersham Pharmacia Biotech). Background was measured in parallel assays in which cells were treated with [methyl-^3H]thymidine in the absence of mitogens. Trichloroacetic acid-precipitable radioactivity was extracted and determined as described above. Data are presented as mitogenic index calculated as the fraction of stimulation obtained in the presence of EGF with respect to the stimulation obtained in the presence of nonsaturating concentrations of an optimal mitogen (1% FBS). The mitogenic index was calculated as follows: ([EGF cpm − background cpm]/[1% serum cpm − background cpm]) × 100.

When appropriate, 100 μM wortmannin (Sigma) was added to the serum-saturated cells 1 h before the addition of EGF and maintained at the same concentration throughout the experiments. Stock solution of the inhibitor was prepared in Me2SO and diluted so that the final concentration of Me2SO in culture medium never exceeded 0.02%.

Soft Agar Assay—Single cell suspensions from NIH 3T3 transfectants were plated at 10-fold serial dilutions in semisolid medium containing 10% FBS and 0.5% sea plaque agarose (FMC BioProducts). Visible colonies forming 100 cells were scored at 14 days. Where indicated, EGF was added at a concentration of 20 ng/ml.

Protein Analysis—Cells grown to subconfluence in poly-L-lysine-coated dishes were incubated overnight in serum-free medium supplemented with transferrin (5 μg/ml) and Na2SeO3 (10 μM) and then exposed to growth factors at the indicated concentrations for the indicated lengths of time at 37°C (see figure legends). Lysis was performed in a buffer containing 1% Triton X-100, 50 μM HEPES, pH 7.5, 150 mM NaCl, 10 mM glycerol, 1.5 mM MgCl2, 5 mM EGTA, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotonin, and 10 mM NαP. For immunoprecipitation procedures, cellular lysates were incubated with appropriate concentrations of antibodies for 2 h at 4°C with gentle agitation. The immunoprecipitates were recovered by adsorption to protein G-bound to Sepharose beads (Amersham Pharmacia Biosciences). After several washes with buffer containing 0.1% Triton X-100, 20 mM HEPES, 10% glycerol, and 150 mM NaCl, SDS-PAGE sample buffer (30% glycerol, 5% SDS, 0.1 M Tris, pH 6.8, 8% 2-mercaptoethanol, and 0.01% bromphenol blue) was added, and samples were boiled for 5 min. Lysates or immunoprecipitated proteins were then...
analyzed by SDS-PAGE and transferred onto nitrocellulose filters. For Western blotting procedures, filters were incubated for 2 h at 4°C with TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5% (w/v) bovine serum albumin (BSA). Filters were then incubated with a primary antibody at adequate dilutions in TBS containing 0.5% BSA for 2 h at room temperature. Blots were then extensively washed in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then immunodetected either with 125I-protein A (Amersham Pharmacia Biotech) (0.2 μCi/ml) or with 125I-labeled anti-mouse-IgG (0.2 μCi/ml) (Amersham Pharmacia Biotech) in TBS containing 0.5% BSA.

In some experiments, filters were incubated with biotin-labeled GST or GST fusion protein (5 × 10−10 M) in 0.5% BSA for 2 h at room temperature. Filters were then extensively washed in TBS and then incubated with horseradish peroxidase-neutravidin (Pierce) at 0.025 μg/ml and then subjected to ECL reaction (Pierce) according to the manufacturer’s directions.

The monoclonal antibody recognizing the intracellular domain of ALK (ALKc) was kindly provided by Prof. B. Falini (Institute of Hematology, University of Perugia, Italy) and Prof. P. G. Pelicci (European Institute of Oncology, Milan, Italy) and was previously described (38). Ab-1, the monoclonal antibody directed against the extracellular domain of EGFR, was purchased from Calbiochem. The rabbit anti-pep-Ab-1, the monoclonal antibody directed against the extracellular domain of EGFR, was previously described (39). A commercially available polyclonal antibody directed against the extracellular domain of ALK (ALKc) was kindly provided by Prof. B. Falini (Institute of Hematology, University of Perugia, Italy) and was previously described (38).

RESULTS

Construction and Expression of an EGFR/ALK Chimeric Receptor—To test the biological and biochemical activity of the ALK kinase under conditions of controlled ligand-induced activation, we engineered an EGFR/ALK chimeric molecule possessing the extracellular and transmembrane domain of EGFR and the intracellular portion of ALK (see “Materials and Methods” and Fig. 1). The EGFR/ALK recombinant molecule was placed under the transcriptional control of the Moloney murine leukemia virusLTR promoter into the LTR–2 expression vector (34), which also contains the E-ecogpt transcription unit and thus allows selection of transfected cells in mycophenolic acid (37). The EGFR/ALK expression vector, which had the potential to encode a 1245-amino acid-long protein with a predicted molecular mass of ~140 kDa, was transfected into NIH 3T3 cells. For comparison, we transfected the same cells with the LTR–EGFR 5M expression vector (32).

Mass cell populations (NIH-EGFR/ALK and NIH-EGFR), derived after marker selection, were subjected to immunoprecipitation with Ab-1, a monoclonal antibody that recognizes the extracellular domain of human EGFR, and analyzed by immunoblot using antibodies specific either for the cytoplasmic domain of EGFR (Fig. 2A) or for the intracellular domain of ALK (Fig. 2B) or for anti-Tyr(P) (Fig. 2C, α-PY7). Results have shown that the EGFR/ALK chimera was efficiently expressed in the transfecteds as a 150–160-kDa protein, and it became heavily phosphorylated on tyrosine in a ligand-dependent manner. In another series of experiments, we compared Tyr(P)-containing proteins in NIH-EGFR and NIH-EGFR/ALK transfecteds. As shown in Fig. 2D, there were qualitative differences in the pattern of Tyr(P)-containing proteins observed upon EGFR triggering of the two cell lines. These data clearly indicate that the EGFR/ALK chimera was correctly synthesized and expressed to the cell surface, where it was capable of interacting with EGF and transducing an EGF-mediated signal via the ALK-specific signaling pathway.

To confirm the expression of EGFR/ALK on the cell surface and to characterize its affinity to EGF, we have evaluated the EGF binding properties of the EGFR/ALK and wild-type EGFR. As shown in Table I, both NIH-EGFR/ALK and NIH-
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EGFR cells displayed two classes of receptors, with dissociation constants ($K_d$) in the range of 6.2–6.4 nM (low affinity) and 0.09–0.1 nM (high affinity). In addition, the quantitative partition of high versus low affinity sites in these two marker-selected mass cell populations were similar; therefore, they were selected for further analysis.

Biological Activity of EGFR/ALK—We next analyzed the biological effects of the described EGFR/ALK chimera on the phenotype of NIH 3T3 fibroblasts. It has been reported previously that NPM/ALK is capable of transforming NIH 3T3 cells (12), Fr3T3 (11), and Rat-1 (22) fibroblasts. Under our experimental conditions, the LTR-NPM/ALK expression vector displayed readily detectable transforming activity in NIH 3T3 cells, inducing about $8 \times 10^5$ PFU/pmol of added DNA (Table II). The EGFR/ALK expression vector was also capable of inducing malignant transformation of NIH 3T3 cells; the appearance of the transformed phenotype was, however, conditionally dependent on the administration of EGF. In fact, in the absence of exogenously added EGF, the EGFR/ALK expression vector showed an efficiency of transformation of about 20 FFU/pmol of DNA; when the assay was performed in the presence of EGF, the transforming activity of the EGFR/ALK chimera was strongly increased to about $5 \times 10^3$ FFU/pmol of DNA. It is noteworthy that the LTR/EGFR was much less potent than the EGFR/ALK expression vector, despite comparable levels of protein expressions (data not shown).

As an independent approach toward assessing the conditional nature of the growth alterations induced by the LTR-EGFR/ALK construct, we investigated the ability of transfected NIH 3T3 cells to display anchorage-independent growth, a property known to correlate well with the malignant phenotype (43). Following transfection and marker selection, a mass cell population expressing a comparable number of receptors (see Table I) was suspended in semisolid medium. In the absence of EGF supplement, NIH-EGFR/ALK displayed only a low colony-forming ability of around 1.85% (Table II). However, upon the addition of EGF at the concentration of 20 ng/ml, we observed a dramatic increase of colony formation (16.9%) with a shift toward large, progressively growing colonies. In comparison, NIH-EGFR-transfected cells displayed a clonogenic capability in semisolid medium of around 12.73% in the presence of EGF (Table II).

The results in the transformation and soft agar assays were paralleled by those obtained in a DNA synthesis assay. For these experiments, we used two marker-selected mass populations displaying a comparable number of EGF-binding sites (see Table I). Fig. 3 shows the EGF dose-response profiles of these two cell lines; NIH-EGFR/ALK cells exhibited a 2.5–3-fold increase in maximal mitogenic response relative to NIH-EGFR. However, both cell lines displayed comparable $ED_{50}$ for EGF (about 0.1 nM), indicating that different levels of mitogenic signaling were achieved by the same receptor occupancy and most likely due to different ability of ALK and EGFR to couple with signaling pathways. Similar results were obtained when EGFR and EGFR/ALK were expressed in NR6 cells, which are devoid of endogenous EGFR (44) (data not shown).

PLC-γ Phosphorylation by the EGFR/ALK—As an approach to investigating the biochemical basis for the greater mitogenic potency of EGFR/ALK than EGFR in NIH 3T3 cells, we compared their ability to couple with known pathways implicated in mitogenic signal transduction. One such pathway, leading to hydrolysis of inositol phospholipid, is initiated by tyrosine phosphorylation of PLC-γ by receptor tyrosine kinases. Complex formation of NPM/ALK and PLC-γ in vivo has been shown previously by coimmunoprecipitation experiments in large cell anaplastic lymphoma cells (22). Therefore, we investigated the ability of EGFR/ALK chimera to phosphorylate PLC-γ on tyrosine residues. NIH-EGFR/ALK and NIH-EGFR cells expressing comparable levels of receptors were treated with EGF for 10 min at 37 °C prior to lysis. Equal amounts of cellular proteins from either cell lines were immunoprecipitated with a mixture of anti-PLC-γ monoclonal antibodies and then subjected to immunoblot analysis with anti-Tyr(P) antibodies. As shown in Fig. 4, EGF stimulation of NIH-EGFR/ALK induced tyrosine phosphorylation of PLC-γ. However, the extent of PLC-γ tyrosine phosphorylation in NIH-EGFR/ALK cells was much lower than that of NIH-EGFR under the same conditions of ligand stimulation. Since neither the level of immunoprecipitated PLC-γ nor the levels of receptors were different in the two cell lines examined, we concluded that the EGFR/ALK chimera was less efficient in phosphorylating PLC-γ than wild-type EGFR.

Effects of ALK Activation on MAPK—An important metabolic cascade regulating cell proliferation is started by activa-
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**TABLE I**

Analysis of the $^{125}$I-EGF-binding properties of NIH-EGFR and NIH-EGFR/ALK cells

| Receptor          | High affinity | Low affinity |
|-------------------|---------------|--------------|
|                   | No. of receptors/cell | $K_d$ | No. of receptors/cell | $K_d$ |
| Wild-type EGFR    | $2.3 \times 10^5$ | 0.09 | $2.7 \times 10^6$ | 6.2 |
| EGFR/ALK          | $1.8 \times 10^5$ | 0.1 | $2.5 \times 10^6$ | 6.4 |

*Single cell suspensions were plated at 10-fold serial dilution in 0.5% sea plaque-agarose medium plus 10% fetal bovine serum. EGF (20 ng/ml) was added after 14 days. Focus forming activity was scored at day 21 on duplicate plates subjected to marker selection. Results are the means of three experiments performed in duplicate.*

**TABLE II**

Biological activity of EGFR/ALK chimera in NIH 3T3

| Transfected DNA          | Transforming efficiency$^a$ | Growth in soft agar$^b$ |
|--------------------------|-----------------------------|-------------------------|
|                          | $-EGF$ | $+EGF$ | $-EGF$ | $+EGF$ |
|                          | FFU/pmol DNA % | % | % | % |
| LTR-EGFR/ALK             | $0.2 \times 10^2$ | $5 \times 10^3$ | 1.85 | 16.90 |
| LTR-NPM/ALK              | $8 \times 10^3$ | $8 \times 10^3$ | 17.50 | 17.89 |
| LTR-EGFR                 | $<10^3$ | $0.8 \times 10^3$ | 1.03 | 12.73 |
| LTR vector               | $<10^3$ | $<10^3$ | $<0.01$ | $<0.01$ |

*Transfection of NIH 3T3 cells was performed with 40 µg of calf thymus DNA as carrier by means of the calcium phosphate precipitation technique (36). Where indicated, EGF (20 ng/ml) was added after 14 days. Focus forming activity was scored at day 21 on duplicate plates transfected with serial 10-fold dilutions of the DNAs of interest. The specific activity is adjusted to FFU/pmol of cloned DNA added, based on the relative molecular weights of the respective plasmids. Transforming activity is corrected for the efficiency of transfection calculated in parallel plates subjected to marker selection. Results are the means of three independent experiments.*

**FIG. 4.** In vivo tyrosine phosphorylation of PLC-γ by EGFR/ALK. NIH-EGFR/ALK and NIH-EGFR cell populations expressing comparable levels of receptors (see Table I) were serum-starved for 16 h and then stimulated for 10 min at 37°C with 100 ng/ml EGF (+) or mock-treated (−) and lysed thereafter. Total cellular protein (3 mg) was immunoprecipitated with an anti-phospho-(Tyr) antibody. Right panel, the remainder of each immunoprecipitate was analyzed by immunoblotting with an anti-PLC-γ antibody. Molecular mass markers are indicated in kilodaltons. Comparable results were obtained in three independent experiments.
EGF or PDGF BB, as well as in PDGF BB-stimulated NIH-EGFR/ALK. Conversely, background levels of activated MAPK were revealed in NIH-EGFR/ALK following EGF stimulation (Fig. 5B, upper panel). Since the observed differences were not due to sample variations (Fig. 5B, lower panel), we concluded that EGFR/ALK kinase is inefficient at causing activation of MAPK in NIH 3T3 fibroblasts.

Effects of EGFR/ALK on PI3-K Activity—One of the immediate cellular responses to stimulation by different growth factor receptors is the activation of PI3-K. This enzyme catalyzes the production of 3-phosphoinositides, which act intracellularly as important second messengers (47).

We therefore analyze the ability of the EGFR/ALK chimera to couple with the PI3-K-activated signaling pathway in comparison with that of EGFR expressed at similar levels. In addition, since both NIH-EGFR/ALK and NIH-EGFR cells expressed PDGF receptors at comparable levels (data not shown), we used PDGF-BB stimulation as an internal control.

As shown in Fig. 6, PI3-K activity was readily detectable in anti-Tyr(P) immunoprecipitates of both NIH-EGFR/ALK and NIH-EGFR cells treated with PDGF-BB, in agreement with the notion that the PDGF receptor is very efficient at coupling with the PI3-K-activated signaling pathway (48). Anti-Tyr(P) immunoprecipitates obtained from EGFR-treated NIH-EGFR/ALK revealed high levels of enzymatic activity comparable with those observed following stimulation of the same cells with PDGF-BB. In contrast, a low level of PI3-K activity was detected in anti-Tyr(P) immunoprecipitates from EGF-treated NIH-EGFR (around 7.19-fold stimulation over unstimulated NIH-EGFR cells). From these results, we conclude that the ALK kinase can couple with the PI3-K signaling pathway much more efficiently than the EGFR.
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Fig. 7. Effect of the PI3-K inhibitor wortmannin on EGF-induced mitogenesis in NIH-EGFR/ALK cells. Cells were serum-starved for 72 h and then either left untreated (●) or pretreated for 1 h with 100 μM wortmannin (□) or with MeSO as control (○) before stimulation with the indicated dose of EGF in the presence of 4 μCi of [methyl-3H]thymidine/well for 22 h. Results are expressed as a mitogenic index calculated as indicated under “Materials and Methods.” Proliferation of NIH EGFR/ALK cells in the presence of 1% FBS is referred to as 100% proliferation. Results are representative of four independent experiments, performed with triplicate wells for each of the indicated EGF concentrations as well as for the controls. Similar results were obtained with the PI3-K inhibitor LY 294002 (data not shown).

Receptors. A large body of evidence indicates that the biological properties of such molecules depend on their cytoplasmic domain, and therefore they faithfully replicate the behavior of their wild-type counterparts from which the intracellular domain was derived (25–31). In addition, many constructed chimeras possessing the extracellular domain of EGFR have been shown to be expressed at the cell surface as two classes of receptors with high and low affinity for EGF binding comparable with those of wild-type EGFR (26, 27, 50, 51).

In the present study, we sought to analyze the selective advantage, if any, that overexpression of a ligand-inducible ALK receptor might confer to cells in a model system. For our studies, we used the established mouse NIH 3T3 fibroblast cell line, a system widely employed to assess the transforming and biological activities of oncogenes; furthermore, it has been already shown that these cells express only few EGFRs/cell and therefore are essentially not responsive to EGF (52). We constructed chimeric EGFR/ALK receptor molecules and have shown that they were correctly localized to the plasma membrane of NIH 3T3 cells, where they bound EGF with dissociation constants (Kd) in the range of 6.2–6.4 nM (low affinity) and 0.09–0.1 nM (high affinity), as has been previously reported for cells expressing wild type EGFR (53, 54). We also demonstrated that EGF induced tyrosine phosphorylation of EGFR/ALK as well as a specific set of cellular proteins; therefore, the EGFR/ALK chimera allows us to dissect ALK catalytic function in an inducible system. Moreover, the availability of cell lines expressing similar levels of either EGFR/ALK or EGFR allowed us to analyze the biological and biochemical activities of these kinases under conditions in which the cellular responses to EGF were solely dependent on the coupling of either kinase with its own signaling pathways.

The EGFR/ALK chimera was much more potent at inducing cell transformation than the EGFR when activated by EGF; in addition, we demonstrated that the EGFR/ALK chimera conferred upon the recipient cells a 3-fold-increased responsive-

ness to EGF compared with EGFR. However, the ED50 of EGF was comparable in the two cell lines, indicating that different levels of mitogenic signaling were achieved by the same receptor occupancy. Thus, the greater ability of the ALK kinase to function as a mitogenic signal transducer provides a mechanistic basis of its greater transforming activity than the EGFR.

A number of studies have described the association of the oncprotein NPM/ALK with P13-K, PLC-γ, Shc, IRS-1, and Grb-2. These studies have indicated the importance of PLC-γ and P13-K for the oncogenic potential of NPM/ALK, whereas association with Shc and IRS-1 seems to be dispensable (11, 12, 22, 23, 24). Specific interaction with Shc and Grb-2 have also been shown for ATIC/ALK, a different ALK oncprotein (16). Conversely, the role of the cytoplasmic molecules involved in the control of the mitogenic signaling mediated by native, membrane-bound ALK are completely unknown. While this work was in progress, Stoica et al. (3) reported that PTN acts as a high affinity ligand for ALK; in this work, the authors also showed tyrosine phosphorylation of IRS-1, Shc, PLC-γ, and P13-K following PTN stimulation of SW-13 human adrenal carcinoma cells overexpressing wild type ALK. Obviously, further investigations are required to evaluate the molecular relevance of these intracellular substrates in mediating the mitogenic action of ALK under conditions of controlled ligand-induced activation.

Using our model system, we showed, indeed, that the EGFR/ALK chimera was able to phosphorylate PLC-γ in response to EGF stimulation. However, in NIH-EGFR/ALK, the level of PLC-γ tyrosine phosphorylation was much lower compared with that obtained in similarly treated NIH-EGFR cells. Therefore, despite its stronger mitogenic and transforming ability in fibroblasts, ALK did not show any increased ability to phosphorylate PLC-γ compared with the EGFR. Obviously, our results do not address directly whether phosphorylation of PLC-γ is necessary for the mitogenic action of ALK; however, the low stoichiometry of PLC-γ phosphorylation described for ligand-activated wild-type EGFR renders this possibility very unlikely (26). This conclusion seems to contrast with previous results reported by Bai et al. (22), which suggested the importance of PLC-γ activation for the mitogenic activity of ALK.
since knock-out of this single pathway was sufficient to significantly inhibit NPM-ALK-mediated transformation in BA/F3 cells and Rat-1 fibroblasts. However, it is worthwhile noting that a PLC-γ binding-deficient NPM-ALK mutant had no effect on transformation activity in Fr3T3 fibroblasts (11). Thus, it has been suggested that PLC-γ activation depends on the cell type specificity; our results are in agreement with this possibility.

Surprisingly, in our model system, the EGFR/ALK chimera does not induce activation of the MAPK pathway, since we have not been able to detect EGF-stimulated MAPK phosphorylation in NIH-EGFR/ALK cells under conditions in which MAPK phosphorylation was easily detected in EGF-treated NIH-EGFR. In addition, PDGF treatment resulted in a complete phosphorylation was easily detected in EGF-treated NIH-EGFR/ALK cells under conditions in which MAPK has been suggested that PLC-γ/alk takes place indirectly through the formation of ternary complexes, which might involve additional proteins other than Shc. 2

Analysis of human neoplasia suggested that quantitative alterations in the expression levels of receptor tyrosine kinases might suffice to overcome normal growth regulation and in this way contribute to malignant transformation (59). These findings have established a direct causal link between growth factor receptors overexpression and cellular transformation. Our present study provides a mechanistic basis for ALK gene amplification in human malignancies. These results acquire a particular interest in view of the recent observation that coexpression of PTN and ALK occurs in different human cancer cell lines (60). Therefore, analysis of PTN and ALK coexpression in cancer cell lines should be further characterized to evaluate the role of ALK in the genesis of neoplasms other than ALCCL.

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