Glioblastoma multiforme is the most common highly aggressive human brain cancer, and receptor tyrosine kinases have been implicated in the progression of this malignancy. We have recently identified anaplastic lymphoma kinase (ALK) as a tyrosine kinase receptor for pleiotrophin, a secreted growth factor that is highly expressed during embryonic brain development and in tumors of the central nervous system. Here we report on the contribution of pleiotrophin-ALK signaling to glioblastoma growth. We found ALK overexpressed in human glioblastoma relative to normal brain and detected ALK mRNA in glioblastoma cell lines. We reduced the endogenous ALK in glioblastoma cells by ribozyme targeting and demonstrated that this prevents pleiotrophin-stimulated phosphorylation of the anti-apoptotic protein Akt. Furthermore, this depletion of ALK reduced tumor growth of xenografts in athymic nude mice and prolonged survival of the animals because of increased apoptosis in the tumors. These findings directly implicate ALK signaling as a rate-limiting factor in the growth of glioblastoma multiforme and suggest potential utility of therapeutic targeting of ALK.

Tumors of glial origin including astrocytomas, oligodendrogliomas, and ependymomas account for almost 80% of all primary brain malignancies. Glioblastoma multiforme is both the single most common glial tumor and the most lethal with a mean survival of only 1 year despite aggressive treatment (1). Although these tumors exhibit multiple genetic alterations, including loss or mutation of the tumor suppressors PTEN (2), p53 (3), and INK4a-ARF (4), receptor tyrosine kinase (RTK) signaling seems to play a particularly important role in tumor development and growth. Glioblastomas and glioblastoma cell lines have been shown to overexpress the tyrosine kinase receptors for epidermal growth factor (5), platelet-derived growth factor (PDGF) (6), hepatocyte growth factor (7), platelet-derived growth factor (PDGF) (6), hepatocyte growth factor (7), nerve growth factor (8), and vascular endothelial growth factor (9). In addition, these tumors frequently overexpress the ligands for these RTKs, suggesting a potential role for autocrine RTK signaling in glioblastoma growth. The importance of RTK signaling is supported by the finding that the combined activation of two downstream targets of RTK signaling (Ras and Akt) in neural progenitor cells induces glioblastoma-like tumors in mice (10).

Pleiotrophin is a secreted heparin-binding growth factor highly expressed in the developing nervous system and down-regulated in the adult. Relative to normal brain, pleiotrophin expression is increased following acute ischemic injury (11) and in tumors (12). This suggests a potential role of pleiotrophin as a tumor growth factor insofar as the reactivation of a developmentally regulated signaling pathway may provide a tumor with a powerful growth signal. In fact, pleiotrophin expression induces tumor growth and metastasis of NIH3T3 cells (13) and has a rate-limiting role both as an angiogenic factor (14) and a tumor growth factor for different tumors including melanoma and choriocarcinoma (15–17) (reviewed in Ref. 12). In addition, pleiotrophin activates both the Ras-MAPK and the PI3K-Akt signaling axes (18), and both pathways are implicated in glial tumorigenesis (10). Recently we identified ALK, an orphan RTK, as the receptor for pleiotrophin (19). The ALK tyrosine kinase was originally discovered as a fusion protein with nucleophosmin due to a t(2;5) translocation (20). This fusion resulted in constitutive activation of the intracellular ALK kinase domain and was shown to induce anaplastic lymphoma (20). More recently, the full-length ALK receptor has been shown to be highly expressed in the developing nervous system and down-regulated postnatally (21), very similarly to the expression profile of its ligand, pleiotrophin (19). Here we report that this tyrosine kinase receptor is overexpressed in human glioblastoma and is rate-limiting for the growth of a xenograft model of glioblastoma.

MATERIALS AND METHODS

Cell Culture—Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in improved minimal essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum.

RNase Protection Assay—[32P]-UTP-labeled riboprobes were prepared using linearized DNA templates to generate antisense transcripts of human ALK (364 nt) and glyceraldehyde-3-phosphate dehydrogenase (102 nt or 36B4 (300 nt). 60 μg of total RNA from appropriate cells was mixed with riboprobes (ALK, 50,000 dpm; glyceraldehyde-3-phosphate dehydrogenase and 36B4, 2,000 dpm) and allowed to hybridize overnight at 50 °C following denaturation by boiling. The next day unprotected RNA was digested with RNase A (500 ng/ml) and run on a precast 6% acrylamide Tris borate/EDTA-urea gel (Invitrogen). The gel was subsequently dried and left to expose a film at −70 °C. Expected sizes of protected fragments are 237 nt (ALK), 104 nt and 84 nt (36B4).

Western Blotting—5–8 × 10^5 cells were plated per well in 6-well plates. Cells were serum-starved for 48 h and treated with growth factors (pleiotrophin generated as in Ref. 22 or PDGF-BB, Upstate Biotechnology, Lake Placid, NY), usually for 5 min. Where noted, cells

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were treated with wortmannin or LY294002 (Sigma) for 1 h prior to stimulation with growth factors. Cells were harvested in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 40 mM β-glycerophosphate, 0.25% sodium deoxycholate, 1% Nonidet P-40, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 100 µg/ml Pefabloc), rocked at 4°C for 20 min, and cleared by centrifugation at 4°C. Protein was quantitated with the BCA protein assay (Pierce), and from nucleotides 1104 to 1341 and from nucleotides 1352 to 1852 (data not shown). Consecutive sections (1:500) at 4°C were incubated with anti-digoxigenin alkaline phosphatase-conjugated antibodies (1:500) at 4°C. The next day, sections were washed for 5 min in 2×SSC, 0.5×SSC, 0.1×SSC, and finally 0.1×SSC. Sections were blocked in 2% horse serum in Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) followed by overnight incubation with anti-digoxigenin alkaline phosphatase-conjugated antibodies (1:500) at 4°C. The reaction was stopped in 1 mM EDTA, and after drying sections were mounted with a xylene-based medium. For in situ hybridization of tumor cell lines, cells were allowed to attach to glass slides overnight and were fixed in methanol:acetic acid (3:1) and 10% formaldehyde. The hybridization and washes were carried out as with tumor sections, omitting washes of 0.5×SSC and 0.1×SSC. Two pairs of digoxigenin-labeled riboprobes were generated to correspond to sense and antisense orientations of the ALK mRNA from nucleotides 1104 to 1341 and from nucleotides 1352 to 1852 (data not shown). Consecutive sections were hybridized with all probes, and congruency of antisense staining was confirmed.

Immunodetection of PTN and ALK—For immunohistochemistry, formalin-fixed paraffin-embedded sections were kept overnight at 55°C and for 1 h at 65°C and finally were heated in the microwave for 3 min.
The sections were deparaffinized and incubated with 10 μm citrate buffer (pH 7.4) at 90 °C for 20 min. After incubation with 0.3% hydrogen peroxide at 4 °C for 20 min and three washes with PBS/0.1% Tween 20, nonspecific binding was blocked with 10% horse or goat serum in PBS/0.1% Tween 20/2% bovine serum albumin for PTN or ALK staining, respectively, for 1 h at room temperature. After an additional three washes slides were incubated with the respective primary antibodies (monoclonal anti-PTN 4B7 1:20 (23); rabbit anti-ALK-ECD 1:20 (21); goat anti-T18 N-terminal peptide (Santa Cruz Biotechnologies, San Diego, CA)) in PBS/0.1% Tween 20% bovine serum albumin at 4 °C in a wet chamber overnight. After washing, bound antibody was detected using the appropriate biotinylated secondary antibodies and commercially available detection reagents for visualization (Vector Labs, Burlingame, CA), and the tissues were counterstained with hematoxylin. Tumor and normal tissues were provided by the tumor bank of the Lombardi Cancer Center.

Ribozyme-expressing Cell Lines—Ribozymes targeted to hybridize the human ALK mRNA at either 5'-ggactggtcgatagcttcc-3' (Rz 1) or 5'-ggactggtcgatagcttcc-3' (Rz 2) and cleave the target just 3' to gtc were directionally cloned into pRC/CMV vectors as described (15). Cells were transfected with these constructs and selected in 0.5 mg/ml G418 for 2 weeks. Clones were picked from colonies grown from the initial transfections and expanded in selective medium.

In Vivo Tumorigenicity—Plated cells were washed in 1× phosphate-buffered saline and brought into suspension with PBS containing 2 mM EDTA. The concentration of cells was brought to 20 million cells/ml with media supplemented with 10% fetal calf serum. For each cell line, four (pRC/CMV and Rz1–2, Rz2–9, and Rz2–10) NU/NU mice were injected with 0.1 ml of cell suspension in parallel experiments with four additional cases of glioblastoma (not shown). Anti-ALK antibodies raised against different anti-ALK gen's, i.e. the extracellular domain (ECD) of ALK and ECD-derived peptides gave the same distinctive result (not shown). In cultured cell lines we found ALK mRNA expressed in three of seven human glioma or glioblastoma lines by RNase protection. For the tumor samples we used in situ hybridization to identify ALK mRNA and immunohistochemistry to identify the ALK protein, and we assessed the expression relative to normal brain. Astrocytoma, glioblastoma multiforme, and oligodendroglioma tumor sections all showed expression of ALK mRNA (Fig. 1, A–L), whereas normal brain showed no expression of ALK mRNA (Fig. 1, A–C). Glioblastoma multiforme also showed a strong staining for ALK protein, whereas the signal was below detection in normal adjacent brain tissues (Fig. 1, M–P). This was confirmed in parallel experiments with four additional cases of glioblastoma (not shown). Anti-ALK antibodies raised against different antigens, i.e. the extracellular domain (ECD) of ALK and ECD-derived peptides gave the same distinctive result (not shown).

A control for the specificity of the human ALK probe shows no cross-reaction with rodent ALK mRNA (C6 and SW-13/murine ALK-transfected cells; Fig. 1Q). Rodent 36B4 mRNA is also not recognized by the human probe in C6 cells. Furthermore, we found pleiotrophin mRNA expressed in the ALK-positive cell lines (not shown) supporting a role of pleiotrophin as an autocrine and/or paracrine stimulator.

Pleiotrophin Signal Transduction in Glioblastoma Cells via PI3-Kinase—We utilized the U87MG cell line to study the contribution of the pleiotrophin-ALK axis to the malignant phenotype of glioblastoma. U87MG cells are a well-characterized model system to study tumorigenesis and signaling in glioblastoma (see Refs. 24 and 25) and to express different receptor tyrosine kinases and their ligands such as epidermal growth factor receptor (26) and PDGF receptor (6). Furthermore, U87MG cells contain a mutated and inactive form of the tumor suppressor PTEN (Δ exon 3) (27, 28), and expression of an active form of PTEN suppresses tumorigenicity and tumor angiogenesis (24, 25). The lipid phosphatase PTEN controls signaling pathways that involve PI3-kinase activity (28), and we thus initially examined whether pleiotrophin signals through PI3-kinase in the U87MG cells. For this we monitored phosphorylation of the downstream target molecule Akt, an anti-apoptotic effector of PI3-kinase signaling that is activated by phosphorylation (28, 29). Pleiotrophin induced Akt phosphorylation at serine 473 in a dose-dependent manner and reached saturation at a very low concentration of the ligand (<1 ng/ml) after 5 min of treatment (Fig. 2A). The phosphorylation signal was maintained for at least 1 h (Fig. 2B). The rapid response suggested to us that Akt phosphorylation is not caused by a secondary effect of pleiotrophin signaling but rather is due to a close link between the activated receptor of pleiotrophin and PI3-kinase. Pleiotrophin-induced Akt phosphorylation was inhibited by pretreatment of U87MG cells with the inhibitors LY294002 and wortmannin, confirming that this effect is indeed mediated by PI3-kinase (Fig. 2C). Interestingly, the MAPK pathway in U87MG cells is activated constitutively, and no increase in phosphorylation was observed after treatment with pleiotrophin (not shown). This suggested to us that the glioblastoma cells predominantly use the PI3-kinase pathway for pleiotrophin-ALK signaling in contrast to epithelial cells in which ALK stimulation by pleiotrophin results in activation of both the MAPK and PI3-kinase pathways (18, 19).

Ribozyme-mediated Reduction of Endogenous ALK Specifically Reduces Pleiotrophin Signaling—To assess whether pleiotrophin signaling through ALK is rate-limiting for the malignant phenotype of U87MG cells, we generated derivative
FIG. 3. Effect of ALK depletion by ribozymes on pleiotrophin signaling. A and B, detection of ALK mRNA by RNase protection assay in empty vector (pRC/CMV) and different ribozyme transfected U87MG cells. Autoradiogram (A) and quantitation by PhosphorImager analysis (B) are shown relative to control (pRC/CMV). Results are representative of two independent experiments. C, in situ hybridization of pRC/CMV and Rz1–7 cells confirming high and low expression of ALK mRNA, respectively. Dose-response of pleiotrophin and PDGF-BB on pRC/CMV cells (squares), Rz1–7 cells (triangles), and Rz2–2 cells (circles) is shown. F, comparison of the effect of pleiotrophin (0.5 ng/ml, open circles) or PDGF-BB (20 ng/ml, closed circles) as a function of ALK levels in the different cell lines from B. Densitometric analyses of results of the immunoblots are presented as the mean of three independent experiments ± S.E. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIG. 4. Xenograft tumor growth. A, size of pRC/CMV (squares), Rz1–2 (diamonds), Rz1–7 (triangles), and Rz2–9 (circles) xenograft tumors as a function of time after tumor cell inoculation. B, tumor size at day 13 of different cell lines (see Fig. 3B) as a function of residual ALK levels. C, survival curves of mice from A. Results are representative of two independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIG. 5. Analysis of mitosis and apoptosis in tumor xenografts. A, high power (400×), hematoxylin and eosin (H&E) (left column), and TUNEL (right column) stained sections from size-matched pRC/CMV (upper row) and Rz1–2 (lower row) xenograft tumors. B, number of mitotic figures (open circles) and TUNEL positive cells (closed circles) as a function of relative ALK levels. Results are presented as the mean of 20 high power (400×) fields ± S.E.
cell lines in which the endogenous ALK is reduced by constitutive expression of ALK mRNA targeted ribozymes. We have previously applied this approach to evaluate the contribution of different gene products to the malignant phenotype (15–17, 30) (reviewed in Ref. 31), and we initially screened different ALK-targeted ribozymes for efficacy. From this we selected two ribozyme expression vectors that target sequences in the 5′ and 3′ ends of the ALK mRNA (Rz1 and Rz2, respectively) and generated a panel of stably transfected U87MG cell lines with high, medium, and low residual ALK mRNA levels (Fig. 3, A and B). Real-time quantitative PCR (not shown) as well as in situ hybridization of pRC/CMV and Rz1–7 cells confirmed high and low residual ALK mRNA levels, respectively (see Fig. 3C). In these cell lines the ability of pleiotrophin to stimulate Akt phosphorylation was reduced in parallel with the reduced endogenous ALK mRNA (Fig. 3, D and F). In contrast, Akt phosphorylation induced through an independent tyrosine kinase receptor (PDGF-R) that is expressed in U87MG cells (6) was unaffected by the reduction of ALK (Fig. 3, E and F). From these results we concluded that the ability of pleiotrophin to induce Akt phosphorylation is strictly dependent on ALK.

**Reduction of ALK, Xenograft Tumor Growth, and Animal Survival**—Interestingly, the derivative U87MG cells with different residual levels of ALK showed no significant difference in proliferation rate or colony formation in soft agar (not shown). This suggests that under in vitro growth conditions, ALK is not a rate-limiting factor. To determine whether the reduction of pleiotrophin-ALK signaling affects the in vivo tumor growth of the U87MG cells, we next grew the different cell lines as tumor xenografts in nude mice. The control cells with the highest residual levels of ALK formed rapidly growing tumors (Fig. 4A) at a rate that was indistinguishable from wild-type cells (not shown). In contrast, tumor growth of the ALK-depleted cells was significantly reduced (Fig. 4A). As with the responsiveness to pleiotrophin induction of Akt phosphorylation, the tumor size of the xenografts grown from the different cell lines was directly dependent on the level of ALK expression in a gene dose-dependent manner (Fig. 4B). This gene dose effect was observed with clonal and mass-transfected cell lines (not shown). Finally, the difference in tumor growth also resulted in a shift of the survival curve; although all of the control mice died by the 20th day after injection, most mice injected with the ALK-depleted cells survived at least twice as long (Fig. 4C), and the median survival time correlated with the residual ALK levels. The increased survival time of mice injected with the ALK-depleted cells Rz1–2 and Rz1–7 was statistically significant compared with those mice injected with the control cells pRC/CMV (p = 0.0027 and p = 0.0058, respectively).

**ALK Levels, Mitotic Index, and Apoptosis in Tumors**—In light of the fact that pleiotrophin signaling through ALK activates a pathway known to promote cell survival, namely PI3-kinase and Akt, we decided to investigate the rate of apoptosis in the xenograft tumor specimens and compare this with the mitotic index in the same samples. Using sections from size-matched tumors, we examined hematoxylin and eosin- and TUNEL-stained sections for mitotic and apoptotic cells, respectively (Fig. 5A). We found no significant difference in the number of mitotic figures but a striking difference in the number of TUNEL positive cells. In fact, the number of TUNEL positive cells correlated directly with the reduction of ALK levels (Fig. 5B). Recent studies suggested a role of PTEN in tumor angiogenesis (25), and thus we also assessed whether the reduction of ALK in the U87MG cells influenced the in vitro production of endothelial cell growth factors as well as the extent of tumor angiogenesis in vivo. Neither the endothelial cell growth stimulatory activity present in the supernatants of the different U87MG cell lines nor the extent of tumor angiogenesis measured in the tumor samples was significantly affected by the reduction of ALK (not shown). Overall, these findings suggest that the pleiotrophin-ALK signaling provides an essential survival signal that is rate-limiting for tumor growth of U87MG cells.

**DISCUSSION**

We recently identified ALK, an orphan RTK, as the receptor for the growth factor pleiotrophin (19). Previous studies have shown that a t(2;5) translocation can generate a fusion protein of the intracellular ALK kinase domain with nucleophosmin and that this constitutively active kinase can induce anaplastic lymphoma (20). In contrast with this limited role of the nucleophosmin/ALK fusion protein for lymphoid neoplasms, the full-length ALK receptor has been shown to be expressed in the developing nervous system and by virtue of its expression pattern is implicated with nervous system physiology (21). We now report that one of the highly malignant tumors of the brain, glioblastoma, overexpresses ALK, and we demonstrate that the pleiotrophin-ALK pathway is essential for tumor growth of one of the commonly used model systems, U87MG cells. Furthermore, our initial studies on the expression of ALK in cancers of epithelial origin showed ALK expression in one-third of breast cancer cell lines (7 of 18) and in a little over one-half of breast cancer tissues from patients (8 of 15). Still, the expression levels of ALK in breast cancers relative to normal tissues were not as prominent as in glioblastoma, and we thus focused the studies reported here on this latter tumor type. Interestingly, depletion of ALK from HaS587T breast cancer cells that express high levels of the ALK receptor as well as pleiotrophin significantly inhibited tumor growth in animals very similar to the data reported here for the U87MG cells. This suggests a potentially rate-limiting role of ALK for tumor types other than glioblastoma that utilize this growth factor receptor pathway.

As outlined above, the major pathway affected by the pleiotrophin-ALK interaction is apoptosis, and this begs the question as to whether the activity of apoptosis-inducing cytotoxic drugs might cooperate with this pathway. To test this hypothesis we studied the efficacy of cisplatinum, an alkylating agent used to treat glioblastoma, in the U87MG cell panel. Indeed, cells with reduced residual levels of endogenous ALK become more sensitive to cisplatinum-induced apoptosis. This suggests the potential for a positive interaction between ALK tyrosine kinase-targeted and conventional cytotoxic therapies.

In the current paper, we have utilized ribozyme targeting of ALK to generate a panel of cells with a range of residual endogenous expression of this receptor tyrosine kinase. The residual receptor levels correlated with 1) pleiotrophin induction of Akt phosphorylation, 2) tumor growth, 3) median survival, and 4) apoptosis in a gene dose-dependent manner. Finally, the tumor cells became more sensitive to a cytotoxic agent, cisplatinum, upon the reduction of ALK. This suggests to us that drugs that specifically inhibit the pleiotrophin-stimulated ALK kinase activity hold the promise to be efficacious against tumors that utilize this growth stimulatory axis.

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Pleiotrophin Signaling through Anaplastic Lymphoma Kinase Is Rate-limiting for Glioblastoma Growth
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