The Phosphorylation of EphB2 Receptor Regulates Migration and Invasion of Human Glioma Cells

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ABSTRACT

Eph receptor tyrosine kinases and their ligands, ephrins, mediate neurodevelopmental processes such as boundary formation, axon guidance, vasculogenesis, and cell migration. We determined the expression profiles of the Eph family members in five glioma cell lines under migrating and nonmigrating conditions. EphB2 mRNA was overexpressed in all five during migration (1.2–2.8-fold). We found abundant EphB2 protein as well as strong phosphorylation of EphB2 in migrating U87 cells. Confocal imaging showed EphB2 localized in lamellipodia of motile U87 cells. Treatment with ephrin-B1/Fc chimera stimulated migration and invasion of U87, whereas treatment with a blocking EphB2 antibody significantly inhibited migration and invasion. Forced expression of EphB2 in U251 cells stimulated cell migration and invasion and diminished adhesion concomitant with the tyrosine phosphorylation of EphB2. U251 stably transfected with EphB2 showed more scattered and more pronounced invasive growth in an ex vivo rat brain slice. In human brain tumor specimens, EphB2 expression was higher in glioblastomas than in low-grade astrocytomas or normal brain; patterns of phosphorylated EphB2 matched the expression levels. Laser capture microdissection of invading grade astrocytomas or normal brain; patterns of phosphorylated EphB2 confirmed the overexpression of EphB2 in glioblastomas. Immunohistochemistry showed EphB2 localized primarily in glioblastoma cells (56 of 62 cases) and not in normal brain. This is the first demonstration that migrating glioblastoma cells overexpress EphB2 in vitro and in vivo; glioma migration and invasion are promoted by activation of EphB2 or inhibited by blocking EphB2. Dysregulation of EphB2 expression or function may underlie glioma invasion.

INTRODUCTION

Eph kinases constitute the largest family of receptor protein tyrosine kinases, consisting of 14 distinct members. They are dichotomized into EphA (EphA1 to EphA8) and EphB (EphB1 to EphB6) according to their sequence homologies and ligand-binding specificities (1). Eph ligands are also expressed mainly on cell surfaces and are termed ephrins. The ephrin-A (ephrin-A1 to ephrin-A5) group are ligands of EphA and are glycosylphosphatidylinositol-anchored proteins. The ephrin-B (ephrin-B1 to ephrin-B3) group are ligands of EphB and are transmembrane proteins. When ligands bind to Eph receptors, the kinase domain is phosphorylated, which leads to phosphorylation of many cytoplasmic substrate proteins. The transmembrane ephrin-B ligands can also function as reciprocal receptors for EphB molecules and transduce signals into cells (2). One major effect of this bidirectional activation of Eph receptors and ephrin ligands is cell repulsion. Present understanding of the Eph signaling axis is that the phosphorylation of Eph receptors and ephrins, including vascular development, tissue-border formation, cell migration, axon guidance, and synaptic plasticity (4–7).

There is increasing evidence that the B-family of Eph receptors and ephrins are functionally involved in the organization of the central nervous system during development (3, 4). EphB–ephrin-B signaling is critical for establishing boundaries between segments of the vertebrate hindbrain (8). Early-migrating EphB2-expressing neural crest cells are repelled by ephrin-B ligands expressed in the caudal somatic compartment (9, 10). Recently it was demonstrated that EphB2 and ephrin-B2 signaling mediates glial scarring after spinal cord injury (11), suggesting EphB and ephrin-B are also involved in pathological conditions in the central nervous system.

A characteristic of malignant astrocytic tumors is their ability to infiltrate and invade the surrounding normal brain tissue. This phenotype makes their complete surgical resection difficult and focal therapy ineffective. Thus, understanding the mechanisms of astrocytic tumor cell invasion in the brain is essential to developing new strategies to control this malignancy. The process of glioma cell invasion may include similar mechanisms directing cell movement during neural development, suggesting that signaling proteins that contribute to migration of neural crest cells may be associated with glioma invasion. Among Eph family members, EphA5 expression has been described in glioblastoma cells (12); however, little is known about the role of the other family in this disease.

In this study we report a role for EphB receptors in invasive glioma cells. Expression of the EphB family was examined in migrating glioma cells. We demonstrate induction of both EphB2 mRNA and protein in actively migrating human glioma cell lines in vitro and in vivo. Phosphorylation of EphB2 is correlated with migration and invasion, whereas blocking of EphB2 activation inhibits glioma invasion in vitro. These results suggest a functional role for EphB2 in the invasive behavior of malignant astrocytic tumors.

MATERIALS AND METHODS

Cell Culture Conditions and Extracellular Matrix (ECM) Preparation.

Human embryonic kidney 293T cells and the human astrocytoma cell lines U87, T98G, U251 (American Type Culture Collection, Manassas, VA), SF767, and G112 (13) were maintained in DMEM supplemented with 10% fetal bovine serum. Astrocytoma-derived ECM was prepared as described previously (13). Briefly, a confluent monolayer of SF767 cells was maintained in culture for 3–5 days for ECM deposit. The cells were lysed from the ECM with 0.5% Triton X-100 for 30 min at 25°C followed by incubation with 0.1 M NH4OH for 5 min at 25°C, and the residual ECM was thoroughly rinsed with PBS.

Antibodies and Reagents.

Antiphosphotyrosine monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-EphB2 polyclonal antibody, which recognizes the extracellular domains of EphB2 and ephrin-B1/Fc chimera, was purchased from R&D systems (Minneapolis, MN). α-Tubulin monoclonal antibody was obtained from Oncogene Research (Boston, MA). Control mouse IgG and Fe fragment of mouse IgG were purchased from Sigma (St. Louis, MO) and Jackson ImmunoResearch (West Grove, PA), respectively.
Migration Assays. Cellular migration was quantified by a micron scale radial migration assay as described previously (14). Briefly, astrocytoma-derived ECM was prepared on 10-well slides (Erie Scientific, Portsmouth, NH) as described above. Approximately 3000 serum-starved glioma cells were plated in each well by use of a cell sedimentation manifold (CSM Inc., Phoenix, AZ) to establish a confluent monolayer 1 mm in diameter. The diameter of the circle circumscribing the cells was measured by use of an inverted microscope (Axiovert; Zeiss, Thornwood, NY) and image analysis equipment (Scion Image, Frederick, MD). Cells were then allowed to migrate for 24 h in serum-free medium, and the diameter of the circle circumscribing the population of cells was again measured. The average migration rate of 10 replicates was calculated from the increase in diameter of the circle circumscribing the cells as a function of time.

To investigate the influence of EphB2 phosphorylation on glioma cell motility, we seeded U87 cells in the migration assay format and allowed them to adhere. The medium was then exchanged for serum-free medium containing 0.2–2 μg/ml recombinant ephrin-B1/Fc chimera, anti-EphB2 antibody, or control mouse IgG, and the migration rate was evaluated for 24 h.

Real-Time Quantitative Reverse Transcription-PCR (QRT-PCR). QRT-PCR was performed in a LightCycler (Roche Diagnostics, Indianapolis, IN) with SYBR green fluorescence signal detection after each cycle of amplification as described previously (15). PCR was performed with the following primers:

- EphB1 (NM_004441): sense, 5'-AGAGGAGGAAAAGGACCGAG-3'; antisense, 5'-GGTTTCCCGAGGATCTC-3' (amplicon size, 183 bp);
- EphB2 (AF025304): sense, 5'-AAAAAGGCCTGGGAGATTCAT-3'; antisense, 5'-GTCCTCATTCTGCTGTTAG-3' (amplicon size, 215 bp);
- EphB3 (NM_004443): sense, 5'-GCTGGGCTTCTTCTTGTTG-3'; antisense, 5'-CCCTGGCAACTCCCGAGA-3' (amplicon size, 184 bp);
- EphB4 (NM_004444): sense, 5'-GGCTGCTGCCAACCCTCTG-3'; antisense, 5'-CCACATCACAATCCCGTAAC-3' (amplicon size, 211 bp);
- EphB6 (NM_004445): sense, 5'-GAGAAAGGGAGGAGGACTA-3'; antisense, 5'-CCACGGAGGACCCATAG-3' (amplicon size, 241 bp);
- Histone H3.3 (NM_002107): sense, 5'-CACCACAGCTCTTGATTCGC-3'; antisense, 5'-GGCTGTCTAGCTGTAGGTCT-3' (amplicon size, 215 bp).

The nucleotide number and amplicon size for each primer are presented in the parentheses. The PCR data were analyzed with the LightCycler analysis software as described previously (15).

Immunoprecipitation and Immunoblot Analysis. Immunoprecipitation and immunoblot analysis were performed as described previously (16). To detect the phosphorylation of EphB2, we stimulated U87 cells with ephrin-B1/Fc for 15 min and treated them with EphB2 antibody for 30 min at 37°C before cell lysis extraction.

Immunohistochemistry and Immunofluorescent Microscopy. Immunohistochemistry was performed using avidin-biotin immunoperoxidase technique as described previously (16). Anti-EphB2 antibody was used at a dilution of 1:100.

For immunofluorescence, after the migration interval, cells were fixed and permeabilized. After washing with PBS, cells were blocked with 2% BSA and 3% goat serum and incubated with anti-EphB2 antisera (1:100 dilution) for 1 h at 25°C. Negative controls were stained with a 1:50 dilution of preimmune serum (Sigma Chemicals Co., St. Louis, MO). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label the cell nuclei.

Cell Invasion Assays. Cell invasion assays were carried out using modified Boyden chambers consisting of Transwell with precoated Matrigel membrane filter inserts in 24-well tissue culture plates (BD Biosciences Discovery Labware, Bedford, MA) as described previously (17). Serum-deprived cells (2 × 10^5) suspended in 100 μl of DMEM containing 1 mg/ml BSA and 0.5% serum were added to each Transwell. After 16 h, noninvading cells were removed by wiping the upper side of the membrane, and the invading cells were fixed with methanol and stained with crystal violet (Sigma). The number of invading cells was quantified by counting of at least six random fields (total magnification, ×200) per filter. In certain experiments, ephrin-B1/Fc, EphB2 antibody, or control Fc fragment of mouse IgG was applied to the upper chamber.

Expression Plasmids and Cell Transfection. The expression plasmid for EphB2 was constructed as follows. The cDNA fragment encoding EphB2 was PCR-amplified using 293T cDNA as a template; the fragment was then inserted into pEAK plasmid (18). EphB2 cDNA cloned into pEAK, which contains a puromycin-resistant gene, was stably transfected into U251 cells by the calcium phosphate method, and stable transfectants were selected in the presence of 1.5 μg/ml puromycin (Sigma) as described previously (19). As a control, cells were transfected with the empty plasmid vector. U251 cells cotransfected with green fluorescent protein (GFP) and EphB2 or pEAK were obtained by transfecting GFP expression plasmid and EphB2 or pEAK into U251 cells with a selectable neomycin-resistant gene. Cells were then cultured in the presence of 800 μg/ml G418 (Sigma) and 1.5 μg/ml puromycin to select for stable dual transfectants.

Cell Adhesion Assay. The cell adhesion assay was carried out as described previously (20). Briefly, 96-well plastic plates were precoated with astrocytoma-derived ECM. Control dishes were prepared by blocking with BSA alone. Cells were then plated at 1 × 10^4 cells/well and allowed to adhere to the dishes for 2 h at 37°C before staining with 1% crystal violet. After the plate was washed with PBS, the crystal violet bound to the cells was eluted with 10% acetic acid and measured by the absorbance at 590 nm (Spectrafluor Plus; TECAN, Durham, NC). For ligand stimulation experiments, the EphB2-stably transfected U251 cells were treated for 15 min with 2 μg/ml preclustered ephrin-B1/Fc or control Fc fragment before seeding into the wells.

Ex Vivo Invasion Assay on Rat Brain Slices. A brain slice model system of rat whole cerebrum was modified according to the organotypic culture methods reported previously (21, 22). Brain tissue was retrieved from 4-week-old male Wistar rat [Crl:WI(BR); Charles River Lab, Wilmington, MA] after halothane anesthesia. The cerebrum was immediately removed and cut vertically to the base in 400-μm-thick sections with a vibratome (1000 Plus; The Vibratome Co., St. Louis, MO). A pinhole was created with a micropipette on the putamen of the brain slice, and 1 × 10^3 U251 glioma cells cotransfected with GFP and EphB2 or pEAK were gently placed in the pinhole (0.5-μl transfer volume). Typically, six brain slices were used in each experiment. The protocol was approved by the Institutional Animal Care and Use Committee. Fluorescent stereomicroscopic imaging of the specimens was performed at ×10 magnification using a Macro-Fluorescent Imaging System (SZX12-RFL3; Olympus, Tempe, AZ) with a GFP barrier filter (DP50; Olympus) at 0, 24, and 72 h after seeding of the cells.

To quantitate glioma cell invasion into the brain slice, we used an inverted laser confocal microscope to observe GFP-labeled cells on a tissue insert with the micropore filter membrane, and serial sections were obtained every 20 μm downward from the surface plane to the bottom of the slice. The invasion rate was calculated as described previously (23).

Clinical Samples and Histology. Under an Institutional Review Board-approved protocol, fresh human brain tumor tissues were obtained from 26 patients who underwent therapeutic removal of astrocytic brain tumors. Non-neoplastic control brain tissues were identified from the margins of the tumors when possible. Histological diagnosis was made by standard light-microscopic evaluation of the sections stained with H&E. The classification of human brain tumors used in this study is based on the revised WHO criteria for tumors of the central nervous system (24). The 26 astrocytic tumors consisted of 7 low-grade astrocytomas, 8 anaplastic astrocytomas, and 11 glioblastomas. All of the tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotherapy or radiation therapy before resection.

Laser Capture Microdissection. Cryopreserved glioblastoma specimens from seven patients were cut in serial 6–8-μm sections and mounted on uncoated slides treated with diethyl pyrocarbonate. We collected 3000 individual cells for QRT-PCR analysis as described previously (15). Laser capture microdissection was performed with a PixCell II Microscope (Arcturus Engineering, Inc., Mountain View, CA). Neoplastic astrocytes in the invasive rim ~1 cm from the edge of the tumor core were identified according to the criteria of nuclear atypia ( coarse chromatin, nuclear pleomorphism, and multinucleation) and, whenever possible, according to nuclear and/or cytoplasmic similarity with the glioblastoma cells in the core. Reactive astrocytes were identified by morphology and were avoided.

Statistics. Statistical analyses were performed with the χ² test and the two-tailed Mann-Whitney U test. P < 0.05 was considered significant.
RESULTS

Actively Migrating Glioma Cells Had Increased Expression of EphB2. A microscale radial monolayer migration assay was used to assess the intrinsic migration rate of various glioma cell lines on astrocytoma-derived ECM. Astrocytoma-derived ECM has previously been shown to enhance the motility behavior of glioma cells (13). U87 cells migrated at a significantly higher rate (mean ± SD, 19.25 ± 0.99 μm/h; *P < 0.01), whereas U251 migrated more slowly (9.04 ± 1.67 μm/h; *P < 0.05) than the other glioma cell lines (Fig. 1A).

Because the EphB family has been described to play a role in cell motility (3), we characterized the expression of the various EphB receptors in accordance to cell motility in five glioma cell lines. Glioma cells were deposited on 10-well glass slides precoated with astrocytoma-derived ECM. After 24 h of cell motility, cells actively migrating at the rim and migration-restricted cells at the core were mechanically collected as separate populations. RNA was isolated from the two cell populations, and the expression levels of EphB family members were determined by real-time QRT-PCR. To compare the EphB gene expression level across the different cell lines, the expression level of core U87 mRNA was arbitrarily chosen to normalize the data. Among all receptors of the EphB family, only EphB2 was overexpressed in the migrating cells in all cell lines (1.2–2.8-fold) relative to the migration-restricted cells at the core (Fig. 1B). EphB2 expression is highest in U87, which is the most rapidly migrating cell line (Fig. 1, A and B).

Fig. 1. Migration of glioma cell lines and profiles of EphB mRNA expression in glioma cell lines at the core and rim as determined in the migration assay. A, migration rate obtained after 24 h. Bars, SD. *, *P < 0.05; **, *P < 0.01 versus T98G, SF767, and G112. B, relative mRNA expression levels of EphB family genes (target mRNA:histone H3.3 mRNA ratios) in the cells at core (columns C) and rim (columns R) of the migration assay were analyzed by quantitative reverse transcription-PCR. mRNA levels are expressed as a proportion of the U87 core mRNA level, which was given a value of 1.

Actively Migrating Glioma Cells Had Increased Expression of Activated EphB2. Because EphB2 mRNA levels are elevated during glioma migration, we assessed the protein levels of EphB2. Immunoblot analysis of total cellular lysates detected EphB2 protein expression in three of five glioma cell lines (Fig. 2A, Lanes U87, U251, and T98G). The most motile cell line, U87, had the highest protein expression. To determine whether the EphB2 receptor is active in glioma cells, we immunoblotted the EphB2 immunoprecipitates with a monoclonal antibody recognizing phosphorylated tyrosine residues. Tyrosine-phosphorylated EphB2 receptor was detected only in the highly migratory U87 cells (Fig. 2A).

We also assessed whether EphB2 phosphorylation increases in the course of activated cell migration. Consistent with the changes in mRNA expression, we observed a 2-fold increase in EphB2 protein expression in the total lysates of actively migrating U87 cells relative to that from migration-restricted cells (Fig. 2B). As expected, more EphB2 protein was immunoprecipitated in the actively migrating cells and displayed increased tyrosine phosphorylation (Fig. 2B). The subcellular localization of EphB2 in migrating glioma cells was determined by immunofluorescent microscopy. Migrating glioma cells displayed increased staining for EphB2 relative to migration-restricted cells (Fig. 2C). In addition, EphB2 staining localized predominantly within lamellipodia at the leading edge of cell migration (Fig. 2C, Rim). These data suggest that EphB2 is active in migrating glioma cells and that it localizes in lamellipodia.

EphB2 Phosphorylation Correlates with Migration and Invasion of U87 Cells in Vitro. To investigate whether the activation of EphB2 stimulates glioma cell migration, we used a recombinant ephrin-B1 ligand to activate endogenous EphB2 in U87. As shown in

Fig. 2. Production, phosphorylation, and localization of EphB2 in human glioma cell lines. A, immunoprecipitation (IP) using total cell lysate from each of the indicated cell lines. Equal amounts of cell lysates were immunoprecipitated with anti-EphB2 antibody. The immunoprecipitates were probed by immunoblotting (IB) with the antibody indicated. PT, phosphotyrosine. Whole cell lysates (WCL) were also immunoblotted with EphB2 or α-tubulin antibody to control for equal protein loading of the five cell lines. B, U87 migration-restricted cells and migration-activated glioma cells collected after the migration assay and immunoprecipitation and immunoblotting were performed as above. C, U87 cells plated in the migration format on astrocytoma-derived extracellular matrix-coated slides, allowed to migrate overnight, and then fixed. The slide was processed for EphB2 immuncytochemistry. Arrows in panel Rim indicate more intense staining for EphB2 at lamellipodia in migration activating cells. Bar, 20 μm.
Fig. 3A, phosphorylation of EphB2 was induced by addition of ephrin-B1/Fc chimera. EphB2 phosphorylation in U87 cells was inhibited in a dose-dependent manner by addition of a functional blocking antibody to EphB2; treatment with control IgG had no effect.

Migration assay and invasion studies were performed in the absence or presence of 0.2–2 μg/ml recombinant ephrin-B1/Fc chimera, EphB2 antibody, or control mouse IgG. Ephrin-B1/Fc chimera (2.0 μg/ml) effectively stimulated migration of U87 glioma cells (mean ± SD, 23.84 ± 0.61 μm/h; P < 0.05) ~1.24 fold relative to cells treated with control IgG (Fig. 3B). The migration of U87 was significantly suppressed by the addition of 2.0 μg/ml EphB2 antibody (15.57 ± 1.42 μm/h; P < 0.05). Similar outcomes were obtained in the migration of U251 and T98G, which express EphB2 (data not shown).

Invasion assay data also indicated that ephrin-B1/Fc chimera stimulated invasion of U87 cells (160 ± 10% of control; P < 0.01). EphB2 antibody inhibited the invasion of U87 cells [87 ± 12% of control (P < 0.05) and 72 ± 10% of control (P < 0.01)] as concentrations of EphB2 antibody increased (Fig. 3C), whereas control IgG had no effect on U87 cells invasion. Taken together, these data indicate that activation or interference of EphB2 accelerates or retards glioma cell migration and invasion, respectively.

EphB2 Phosphorylation Inhibits Cell Adhesion and Promotes Cell Migration or Invasion in U251 Cells. To examine the functional effects of EphB2, we evaluated U251 cells transfected with EphB2 or control vector (pEAK) constructs in adhesion, migration, and invasion assays. Forced expression of the EphB2 receptor in U251 cells resulted in phosphorylation of the transreceptor (Fig. 4A). EphB2 activation led to dramatic changes in cell morphology; cells expressing the activated receptor partially detached from the substrate and rounded up (Fig. 4B). In contrast, cells transfected with empty vector remained spread and spindle-shaped (Fig. 4B). Immunocytochemistry showed that EphB2 localized to the cell membrane (Fig. 4B), suggesting that the transgene functions normally in the transfected cells. These cells grew with doubling times comparable to those for the parental cells; cells expressing exogenous EphB2 did not display any cytotoxic effects as determined by changes in trypan blue exclusion, propidium iodide uptake, 4′,6′-diamidino-2-phenylindole hydrochloride staining, and immunostaining with anti-activated caspase 3 antibodies (data not shown). In addition, cells in which activation of the EphB2 receptor could be stimulated by treatment with an ephrin ligand (Fig. 4C) showed reduced cell substrate adhesion on treatment with chimeric ephrin-B1/Fc chimera (mean ± SD, 0.30 ± 0.025; P < 0.05; Fig. 4D). There was no difference after addition of control Fc relative to EphB2 transfectants (0.68 ± 0.07; Fig. 4D).

In addition, overexpression of EphB2 in U251 induced a small but measurable increase in migration rate (11.57 ± 1.67 μm/h; P < 0.05) relative to pEAK-transfected cells (9.6 ± 0.91 μm/h). The migration rate was enhanced by addition of ephrin-B1/Fc chimera in the EphB2 transfectants (13.0 ± 2.01 μm/h; P < 0.05), whereas addition of control Fc had no measurable effect (11.4 ± 1.34 μm/h; Fig. 4E).

As shown in Fig. 4F, cell invasion through membranes coated with Matrigel was increased in cells expressing EphB2 (mean ± SD, 228 ± 58% of control; P < 0.05) and in the cells treated with ephrin-B1/Fc chimera (473 ± 53% of control; P < 0.01), whereas we observed no significant change between EphB2 and that produced by addition of control Fc (224 ± 46% of control), indicating that the phosphorylation of EphB2 is required for stimulation of cell invasion.

To evaluate the effects of EphB2 on invasion through a more physiologically relevant matrix, we examined U251 stably cotransfected with control or EphB2 and GFP expression plasmids for their growth and dispersion within an ex vivo organotypic rat brain slice. Overexpression of EphB2 in U251-GFP-EphB2 cells increased phosphorylation of the transgene product (data not shown). To compare the growth of mock transfectants with that of EphB2 stable transfectants, we implanted aggregations of each of these engineered cells in the putamen on contralateral sides of the same rat brain slice (Fig. 5A); images were taken at 0, 24, and 72 h after the implantation. The U251-EphB2 cells displayed greater migration and invasion into the organotypic rat brain slice than did the less invasive mock-transfectant cells (Fig. 5B). To quantify cell invasion, serial optical sections were obtained every 20 μm downward (Z axis) from the basal plane to the bottom using confocal microscopy. The U251-EphB2 cells penetrated further into the brain slices (mean ± SD, 51.8 ± 14.9 μm/72 h) than the mock cells (33.8 ± 10.35 μm/72 h; P < 0.05; Fig. 5C). These data suggest that EphB2 plays a role in invasion both in vitro and in vivo.

Overexpression and Phosphorylation of EphB2 in Invading Cells of Glioblastoma. To evaluate a potential role for EphB2 in the malignant behavior of human gliomas, the expression level of EphB2 was evaluated as a function of tumor grade. Levels of EphB2 mRNA in human brain tumors were evaluated by QRT-PCR using histone H3.3 mRNA as an internal reference for normalization. The mRNA levels of the EphB2 gene (EphB2 mRNA:histone H3.3 mRNA ratios) were significantly higher in glioblastoma tissues (mean ± SD, 0.391 ± 0.25; n = 11) than those in normal brain tissues (0.129 ± 0.03; P < 0.05; n = 3) and low-grade astrocytoma tissues (0.136 ± 0.082; P < 0.01, n = 7; Fig. 6A). The expression levels of EphB2 increased significantly as tumor grade increased. To investigate the potential role of EphB2 in invasion in vivo, we collected invading glioblastoma cells and cells in the tumor core by laser capture microdissection of seven glioblastoma surgical specimens and performed QRT-PCR of the isolated RNA. EphB2 was overexpressed in invading glioblastoma cells (1.5–3.5-fold) relative to the cells in the tumor core in all seven biopsy specimens (Fig. 6B).

Consistent with the QRT-PCR results, protein levels and tyrosine phosphorylation of EphB2 were increased in glioblastoma tissue.
Fig. 4. Morphological change, cell adhesion, migration, and invasion of U251 cells expressing EphB2. A, activation of EphB2 in stably transfected U251 cells. EphB2 was immunoprecipitated (IP) from cells transfected with EphB2 or pEAR (Mock). The immunoprecipitates were probed by immunoblotting (IB) as indicated. B, morphological changes caused by expression of activated EphB2 in U251 cells. pEAK vector was used as control. Upper panels, phase-contrast microscopy; lower panels, immunofluorescence (IF) for EphB2. EphB2 is localized on the membrane. Bars, 50 μm. C, activation of EphB2 by ephrin-B1 ligand. EphB2 was immunoprecipitated (IP) from U251 cells that had been stably transfected with EphB2 and treated with soluble ephrin-B1/Fc chimera at the indicated concentration or left untreated. The immunoprecipitates were probed by immunoblotting (IB) as indicated. D, cells were plated on dishes coated with astrocytoma-derived extracellular matrix (ECM) in the absence (−) or presence (+) of control Fe(Fc) or 2.0 μg/ml ephrin-B1/Fc chimera (ephrin-B1/Fc) and then incubated for 2 h at 37°C. Crystal-violet-stained cells attached to dishes were dissolved in 10% acetic acid, and the absorbance at 590 nm was measured spectrophotometrically. The mean absorbance value from U251-Mock cells attached to dishes coated with astrocytoma-derived ECM is shown as 1. Bars, SD. **, P < 0.01 versus Mock. E, cells were plated on 10-well glass slides precoated with astrocytoma-derived ECM and incubated as above after attachment of the cells to the dishes. Cell migration was assessed over 24 h. Bars, SD. *, P < 0.05 versus Mock. F, cells were treated as above and then used in the invasion assay. Mean cell counts from at least six fields and four experiments are shown. Bars, SD. *, P < 0.05; **, P < 0.01 versus Mock.

relative to normal brain, low-grade astrocytoma, and anaplastic astrocytoma (Fig. 6C).

EphB2 was immunolocalized predominantly in the neoplastic astrocytes in the majority of all glioblastoma specimens (56 of 62 cases; Fig. 6D, panel a). Invading neoplastic cells also contained significant staining for EphB2 (Fig. 6D, panel b). Neoplastic astrocytes were identified by nuclear atypia and H&E-stained sections and were confirmed by positive glial fibrillary acidic protein staining (data not shown). Reactive astrocytes and occasional endothelial cells of blood vessels also demonstrated positive immunostaining for EphB2. In anaplastic astrocytomas, some atypical cells were weakly immunostained, but no staining was seen in the normal brains (Fig. 6D, panel c) or when the primary antibody was substituted for normal serum (Fig. 6D, panel d). The immunohistochemistry results are consistent with those obtained by QRT-PCR and immunoprecipitation analysis.

DISCUSSION

The evidence presented here supports a role for EphB2 in glioma cell motility in vitro and in vivo. Expression of EphB2 was elevated in all five glioma cell lines tested on adaption of a migratory phenotype. The fastest migrating glioma cell line, U87, contained the highest level of total EphB2 protein as well as the highest concentration of tyrosine-phosphorylated EphB2 protein; additionally, EphB2 was localized to the leading edge at sites of lamellipodia formation. The activity of migration and invasion in U87 was correlated with the phosphorylation of EphB2. Forced expression of EphB2 in U251, which was the least invasive of the five glioma cell lines and showed low constitutive expression of EphB2, transformed U251 to a more highly invasive phenotype as assessed by in vitro and ex vivo assays. The expression and phosphorylation of EphB2 were up-regulated in glioblastoma, particularly in the invading cells. These results support a role for EphB2 in glioblastoma tumor invasion.

EphB expression has been reported in various human cancers, including carcinomas of the lung (25), colon (26), and endometrium (27), as well as osteosarcoma (28) and neuroblastoma (29). EphB2 expression has been confirmed in carcinomas of the colon, stomach, esophagus (30), and neuroblastoma (31); however, its role in the invasion or metastasis processes in human carcinomas has not been described. Thus, the present study is the first to demonstrate that EphB2 receptor is involved in tumor invasion activity and possibly malignant progression in human brain tumors.

EphB2 shares ~49–66% amino acid homology with other members of the EphB subgroup, but the homology between human and mouse EphB2 is >99% (30). This high degree of homology suggests an important conserved function of EphB2. Kiyokawa et al. (30) reported that EphB2 was expressed in fetal brain but not in adult brain, providing evidence that it may be associated with the neurodevelopmental process. This finding corroborates our QRT-PCR data of its low expression in the normal brain. Considerable efforts have been made in recent years to elucidate the biological function of EphB family receptors in neural development. To date, EphB2 has been implicated in proper formation of the anterior commissure (32), regulation of fluid homeostasis in the semicircular canal (33), and activation of dendritic spine morphogenesis (34). Unlike other members of the Eph family, EphB2 has both kinase-dependent and -independent effects (4). In the U87 and U251 stable transfectant data, we observed a strong correlation between the phosphorylation level of EphB2 and adhesion, migration, and invasion rates. These data indicate that the kinase activity of EphB2 is important for the invasive phenotype of glioblastoma cells.

In U87 glioma cells, the engineered soluble ligand, ephrin-B1/Fc chimera, induced EphB2 tyrosine phosphorylation that correlated with increased migration and invasion activity. This is consistent with previous data obtained with an endothelial cell line (35). Because our
Overexpression of EphB2 increased migration and invasion as measured with a monolayer radial migration assay, Boyden chambers, and an ex vivo organotypic rat brain slice model. The rat brain slice model is likely to be a more physiologically relevant matrix for invasion than the monolayer assay or Boyden chamber; it may also be subject to artifacts due to interspecies variations in ECM, cell surface molecules, receptor ligands, and unspecific physiological changes accompanying the ex vivo preparation. To date, cross-species comparisons of three members of the ephrin-B family have been reported, with deduced amino acid homologies of 96, 98, and 100% for human ephrin-B1/rat ephrin-B1, human ephrin-B2/mouse ephrin-B2, and human ephrin-B3/mouse ephrin-B3, respectively (44–48). The ephrin-B1 gene is expressed in rat brain throughout its life (45). It is therefore possible that ephrin-B constitutively present in the rat brain slice provides a ligand for activation of the transfected human EphB2
in U251 glioma cells. However, because the basal levels of EphB2 phosphorylation were readily detected in U251 cells transfected with EphB2 absent addition of activating ligand ephrin-B1/Fc, the stimulus of ephrin-B ligand in brain may not explain the heightened invasion of these glioma cells. We suspect that overexpression of EphB in glioma cells may suffice for activation by autophosphorylation.

The present studies demonstrate that levels of EphB2 mRNA and protein and the tyrosine-phosphorylated form of the protein are significantly higher in glioblastoma tissue than in normal brain or low-grade astrocytoma. The increase in EphB2 mRNA and protein in tumor tissue is ascribed to astrocytic tumor cells because EphB2 immunolocalized predominantly to glioma cells. Because invading cells overexpressed EphB2, as demonstrated by the QRT-PCR data, and the production of EphB2 was confirmed by immunohistochemistry in invading glioblastoma cells, it seems likely that production of EphB2 is up-regulated in the invading cells as observed in our in vitro experiments. Together, the in vitro and in vivo data suggest that up-regulation of EphB2 expression in glioma tissues may contribute to the malignant behavior of glioblastomas and may drive invasion of glioma cells into normal brain tissue.

In conclusion, this study reveals the biological significance of EphB2 expression in glioma and illustrates that phosphorylation of EphB2 is associated with heightened invasive activity of glioma cells. An understanding of the function and regulation of the EphB2 may lead to the development of effective therapies for patients with glioma.

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