Neuregulin-1 Enhances Motility and Migration of Human Astrocytic Glioma Cells*

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Gliomas are the most frequently diagnosed adult primary brain malignancy. These tumors have a tendency to invade diffusely into the surrounding healthy brain tissue, thereby precluding their successful surgical removal. In this report, we examine the potential for the neuregulin-1/erbB receptor signaling network to contribute to this process by modulating glioma cell motility. Neuregulin-1 is expressed throughout the immature and adult central nervous system and has been demonstrated to influence the migration of a variety of cell types in the developing brain. In addition, erbB2, an integral member of the heterodimeric neuregulin-1 receptor, has been shown to be overexpressed in human glioma biopsies. Using antibodies specific for erbB2 and erbB3, we show that these receptors localize preferentially in regions of the plasma membrane which are involved in facilitating cellular movement. Here, erbB2 colocalizes and coimmunoprecipitates with members of the focal complex including β1-integrin and focal adhesion kinase. Further, erbB receptor activation by neuregulin-1 enhances cell motility in two-dimensional scratch motility assays and stimulates cell invasion in three-dimensional Transwell migration assays. These effects of neuregulin-1 appear to involve the activation of focal adhesion kinase, which occurs downstream from erbB2 receptor stimulation. Taken together these data suggest that neuregulin-1 plays an important modulatory role in glioma cell invasion.

Primary brain tumors are derived from the various types of cells of the central nervous system and include gliomas, neuroblastomas, ependymomas, and meningiomas. The most common adult primary brain tumor is the glioma, which is thought to arise from central nervous system glial cells. Of the gliomas, those classified as astrocytic tumors are the most frequently diagnosed (1). Implicit in this classification is the assumption that astrocytic tumors arise from astrocytic glial cells. However, their precise lineage relationship is unknown; it is unclear whether these tumors arise from differentiated astrocytes or from their undifferentiated precursors. Indeed, there are striking similarities between neural precursors and gliomas such as their ability to migrate, often over long distances, through the brain (for review, see Ref. 2). Successful surgical treatment of patients diagnosed with a glioma is difficult because of this migratory phenotype. In particular, it is believed that cells at the tumor margin migrate away from their site of origin to colonize distant sites within the brain (3, 4). The molecular mechanisms underlying this aggressive phenotype are poorly understood. However, a number of factors have been implicated in promoting tumor cell invasion. These include the remodeling of the extracellular environment through the secretion of matrix proteases such as matrix metalloprotease-2 (5), the deposition of extracellular matrix proteins such as laminins that facilitate tumor cell migration (6), and the mutation and/or overexpression of growth factor receptors capable of initiating intracellular signaling cascades that enhance cellular motility (7, 8).

One example of a growth factor receptor that has received particular attention in other malignancies is the erbB2 receptor. Specifically, in human breast cancer cell lines, ligand-dependent activation of erbB2 has been shown to promote cellular motility and invasiveness via activation of matrix proteases (9, 10), phosphorylation of paxillin (11), modulation of focal adhesion kinase (FAK) (12) and of a related kinase, Pyk2 (13), and regulation of the actin cytoskeleton (14). ErbB2 is a member of the type 1 family of receptor tyrosine kinases which also includes erbB1, erbB3, and erbB4. Ligand-dependent activation of erbB receptors results in homo- or heterodimerization. This stimulates receptor auto- and/or transphosphorylation on cytoplasmic tyrosine residues creating binding sites for adaptor proteins, kinases, and phosphatases. The downstream signaling cascades initiated depend on the identity of the erbB heterodimers, which is determined, in part, by the specific erbB receptor ligand (15).

Although erbB2 has no known ligand, it can be activated in a ligand-dependent manner via heterodimerization with other erbB receptors (16). Ligands for erbB1 are numerous and include transforming growth factor-α and epidermal growth factor. ErbB3 and erbB4 bind with low and high affinity, respectively, to a family of polypeptide growth factors called the neuregulins (17). In the central nervous system, neuregulin-1 (NRG-1) is expressed by both neurons and glia (18–20). Moreover, the extracellular domain of type I and type II NRG-1 isoforms contains an Ig-like motif that enables them to adhere to the extracellular environment (21). This allows them to function in a paracrine manner. Interestingly, recent findings (22) demonstrate that glioma cells can produce NRG-1 isoforms. Furthermore, erbB2 has been repeatedly reported to be overexpressed in human glioma biopsy samples and in a number of human glioma cell lines (22–26). Despite this evidence, relatively little is known regarding the role of the NRG-1/erbB receptors in glioma cell biology.

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The abbreviations used are: FAK, focal adhesion kinase; ECM, extracellular matrix; MAB, migration assay buffer; NRG-1, neuregulin-1; PBS, phosphate-buffered saline; PP, protein phosphatase.
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In an attempt to fill this void, we examined the potential for NRG-1 to modulate glioma cell motility. Our results demonstrate that the NRG-1 receptors erbB2 and erbB3 localize in regions of the plasma membrane involved in cellular movement. In addition, their activation by a recombinant form of NRG-1, NRG-1β, stimulates a functional association between erbB2 and FAK, thereby facilitating glioma cell migration.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The U251-MG (U251) human glioma cell line was a gift from the laboratory of Dr. Y. Gillespie (University of Alabama at Birmingham). U87-MG and U118-MG human glioma cells were purchased from ATCC. Cells were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Recombinant NRG-1β was produced by the laboratory of Dr. S. Carroll (University of Alabama at Birmingham). Inhibitors Tyrphostin AG 825 (AG 825) and PP2 were purchased from Calbiochem (La Jolla, CA). Recombinant NRG-1α was produced by the laboratory of Dr. S. Carroll (University of Alabama at Birmingham). Cells were maintained as described previously (27).

Immunocytochemistry—For our initial erbB receptor immuno- staining, U251 cells on uncoated glass coverslips were rinsed with sterile PBS and fixed for 10 min with 4% paraformaldehyde in PBS. Fixed cells were rinsed three times with PBS, then placed in a blocking buffer, which consisted of PBS, 5% normal goat serum, and 0.1% Triton X-100. After 30 min, cells were incubated with the appropriate primary antibody diluted in blocking buffer overnight at 4 °C. Polyclonal antibodies for the erbB receptors (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1 µg/ml (1:200). The following day, the cells were rinsed three times for 10 min with blocking buffer and then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. Fluorescent images were visualized using a Leica DMRB fluorescence microscope, Heerbrugg, Switzerland, and digitally imaged (Spot RT, Diagnostic Instruments, Sterling Heights, MI).

To study erbB2 localization in actively migrating glioma cells, U251 cells were fixed after a migration assay (see below). The cells and cellular processes on the undersurface of the Transwell filter were immunostained for erbB2 as above. The filter was then removed from the polycarbonate filter, creating a liquid diffusion barrier effectively dividing the well into two chambers, upper and lower, to which factor and/or inhibitors were added. Subconfluent U251 cells in 10-cm dishes were serum starved overnight. On the day of the experiment, they were lifted from the plate using 0.5 mM EDTA in PBS, pelleted, and resuspended in MAB to attain a concentration of 100,000 cells/ml. Equal numbers of cells (40,000/ filter) were aliquoted onto the top surface of the Transwell filter and allowed to adhere for 30 min prior to the addition of any drugs. Inhibitors Tyrphostin AG 825, PP2, and PP3 were added for 30 min prior to the addition of NRG-1β. The cells were returned to the incubator for 4–6 h, rinsed with PBS, fixed, and stained with an ethanol-based crystal violet solution. Cells on the upper surface of the filter were removed using a cotton-tipped applicator. Cells on the lower surface of the filter were visualized and imaged as above for immunostaining. Experiments were repeated at least four times.

Motility and Migration Assays—The scratch motility assay was used to measure two-dimensional movement. U251 cells were grown to confluency in 24-well plates. A scratch was then made on the monolayer using a sterile 200-µl pipette tip. The monolayer was rinsed three times with migration assay buffer (MAB) consisting of serum-free medium plus 0.1% fatty-acid-free bovine serum albumin and placed in MAB medium with 1 µg/ml NRG-1α. At the initiation of the experiment (t = 0) a digital image of the scar was taken at a magnification of ×10 (Axon Imaging, Foster City, CA). 24 h later (t = 24) the same region of the scar was imaged again. The images were imported into the Scion imaging program (available online at scioncorp.com). This software allowed us to quantify the two-dimensional movement of the cells by measuring the surface area of the scratch at t = 0 and comparing it with the surface area at t = 24. Experiments were done at least three times. Measurements were done in triplicate.

To measure three-dimensional movement, the Transwell migration assay was used as described previously (27). Transwell filters (B-D Falcon, Bedford, MA) with 8-µm pores were coated on the undersurface with 10 µg/ml laminin. The following day, they were washed three times with PBS and blocked for 1 h with 1% fatty acid-free bovine serum albumin. In addition to providing a necessary substrate for migration, the laminin undercoating filled the 8-µm pores of the Transwell, creating a liquid diffusion barrier effectively dividing the well into two

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Chambers, upper and lower, to which factor and/or inhibitors were added. Subconfluent U251 cells in 10-cm dishes were serum starved overnight. On the day of the experiment, they were lifted from the plate using 0.5 mM EDTA in PBS, pelleted, and resuspended in MAB to attain a concentration of 100,000 cells/ml. Equal numbers of cells (40,000/ filter) were aliquoted onto the top surface of the Transwell filter and allowed to adhere for 30 min prior to the addition of any drugs. Inhibitors Tyrphostin AG 825, PP2, and PP3 were added for 30 min prior to the addition of NRG-1β. The cells were returned to the incubator for 4–6 h, rinsed with PBS, fixed, and stained with an ethanol-based crystal violet solution. Cells on the upper surface of the filter were removed using a cotton-tipped applicator. Cells on the lower surface of the filter were visualized and imaged as above for immunostaining. Experiments were repeated at least four times.

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NRG-1β Increases Motility of U251 Human Glioma Cells—Because erbB2 and erbB3 receptors localized in regions of the cell important for movement, we set out to determine whether their activation could modulate motility, defined herein as movement in a two-dimensional plane. This was accomplished by using a scratch motility assay in which the repopulation of cells into a cell-free region (scar) could be examined quantitatively. U251 cells were maintained until they reached confluence. Subsequently, a scar was created in the monolayer, and the cells were washed and placed in MAB without or with 1 ng/ml NRG-1β. We have demonstrated previously that exogenous NRG-1β can activate erbB2 and erbB3 receptors in U251 glioma cells as measured by induction of tyrosine phosphorylation (29). After 24 h, the surface area of the scarred region was quantified using imaging software (see “Experimental Procedures”) and compared with the surface area of the scarred region at the initiation of the experiment. Movement of cells into the scarred region resulted in a decrease in the surface area of the scar. Fig. 2A is a digital image of the scarred region before (t = 0) and after the 24-h incubation period (t = 24) from a typical experiment. The same experiment is quantified in Fig. 2B. The overall change in the surface area of the scar for control, untreated samples was 31.83% ± 0.23%, whereas the change in the surface area of the scar for samples treated with 1 ng/ml NRG-1β was 52.48% ± 0.88%, an increase of about 20% (p < 0.001).

NRG-1β Increases Transwell Migration of U251 Human Glioma Cells—Our results from the scratch motility assay indicated that NRG-1β could modulate glioma cell movement in a two-dimensional plane. In the brain, such movement is not likely. Instead, glioma cells must be able to migrate and invade through the three-dimensional spatial constraints of the surrounding brain tissue. To replicate this type of environment more closely, we employed a Transwell migration assay (Fig. 3). To induce glioma cell migration, it was necessary to coat the undersurface of the filter with an extracellular matrix (ECM) protein (data not shown). A similar dependence for ECM proteins is thought to occur with glioma cells in vivo (30). We performed our migration assays using laminin, a potent inductor of U251 cell migration (27). Serum-starved U251 glioma cells were plated onto the top of the Transwell filter and allowed to migrate for 4–6 h before they were fixed, stained, and counted. Fig. 3, A and B, shows representative digital images of cells on the undersurface of a filter which migrated in response to laminin alone (control) or in response to laminin plus 1 ng/ml NRG-1β added to the lower chamber. These images show that...
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U251 cells migrated in response to laminin but that the number of migrating cells increased markedly in the presence of NRG-1β. Similar results were seen with two other human glioma cell lines, U87-MG and U118-MG (data not shown), suggesting that this was not a cell type-specific phenomenon. These experiments were repeated with the U251 glioma cell line using a concentration range of NRG-1β from 0.1 to 100 ng/ml. The resulting dose-response relationship is shown in Fig. 3C and Table I. To determine whether NRG-1β effects on migration were permissive (chemokinetic) or instructive (chemotactic), we designed a series of Transwell experiments with 1) NRG-1β in the lower chamber, 2) NRG-1β in the upper chamber, or 3) NRG-1β in both the lower and upper chambers. The results for these studies are summarized in Fig. 3D and Table I. In each experimental configuration, NRG-1β significantly increased the number of cells successfully migrating through to the under-surface of the filter, implying that NRG-1β acted in a permissive manner. However, although migration was enhanced in each configuration, more cells were induced to migrate when NRG-1β was present in the bottom of the chamber (2.36 ± 0.02-fold increase) versus in the top (1.42 ± 0.07-fold increase), suggesting an instructive role (see “Discussion”).

Although our data clearly indicated that NRG-1β could enhance the Transwell migration of glioma cells, a role for erbB2 in the effect was not conclusive. Therefore, we examined NRG-1β effects in the presence of the erbB2-specific pharmacological inhibitor, AG 825 (31–33). For these experiments, 1 ng/ml NRG-1β and increasing concentrations of AG 825 were included in both the upper and lower chambers. As demonstrated (Fig. 3E) AG 825 dose-dependently inhibited the NRG-1β-induced stimulation of Transwell migration with an apparent IC₅₀ of 12.28 μM. Results for the migration experiments are summarized in Table I.

Because the ability of cells to migrate depends critically on their interactions with the ECM, we next examined the potential effect of NRG-1β on the adhesion of U251 cells to laminin. Serum-starved cells were plated onto a laminin-coated 96-well plate and allowed to adhere for 30 min to 1 h. Subsequently, the cells were washed, fixed, and stained. The number of adherent cells was quantified using an enzyme-linked immunosorbent assay plate reader. As indicated (Fig. 3F), the ability of the U251 cells to adhere to laminin was not altered by exposing the cells to a range of NRG-1β concentrations, or to 50 μM AG 825.

ErbB2 Receptor Immunoreactivity Localizes to the Invadipodia of Migrating Cells and Colocalizes with the Focal Complex Proteins β₁-Integrin and FAK—Thus far, our results indicated that stimulation of glioma cells by NRG-1β could enhance motility and migration. Our initial immunostaining (Fig. 1) demonstrated that the erbB2 and erbB3 receptors localized in regions of the plasma membrane called lamellipodia. We hypothesized that this localization might help facilitate the ability of the receptors to modulate migration. To investigate further, we performed erbB2 immunostaining in actively migrating cells. For these studies, we used the same Transwell migration assays; however, instead of fixing and staining the cells for cell counting, we processed them for immunostaining further, we performed erbB2 immunostaining in actively migrating cells. For these studies, we used the same Transwell migration assays; however, instead of fixing and staining the cells for cell counting, we processed them for immunostaining of the erbB2 receptor. As with the migration assays (see “Experimental Procedures”), cells on the surface of the filter were removed, leaving only the cells and the invading processes on the undersurface. The invading processes of the migrating cells, called invadipodia (34), contain the components necessary for migration into the pore. The digital image displayed in Fig. 4 is a composite image made by combining sample images taken from the undersurface of one Transwell filter. For this experiment, the cells were exposed to 1 ng/ml NRG-1β in both the upper and lower chambers. It is clear from this composite that the erbB2 receptor localized in the invadipodia. The morphology of the invadipodia varied depending on the temporal status of the cells in the migration process. We saw no significant difference in the erbB2 staining pattern from cells on filters that were unstimulated, exposed to NRG-1β in the lower chamber only, or exposed to NRG-1β plus AG 825 (data not shown), suggesting the constitutive and invariant expression of these receptors on glioma cells.

In light of our immunostaining results localizing erbB2 in invadipodia and based on evidence from the literature indicating the presence of focal complex proteins in these processes (34), we decided to examine whether erbB2 could colocalize with other molecules implicated in the migration process. Specifically, we examined the localization of erbB2 with F-actin, the polymerized form of actin which is present in these cellular extensions (35); β₁-integrin, an ECM receptor that mediates...
engagement with the laminins (36); and FAK, a nonreceptor protein-tyrosine kinase that has been implicated in mediating both integrin- and growth factor-induced cellular migration (37). In addition, we examined whether the erbB3 receptor also colocalized with erbB2 in these invading processes. These experiments were performed as described above, by immunostaining the fixed processes of U251 cells actively migrating through a Transwell filter in the absence or presence of NRG-1β. The image is a composite made of several images taken from the underside of one filter. Cells were exposed to 1 ng/ml NRG-1β on both sides of the filter. The inset is an enlargement of one invadopodia, and the arrow points to an 8-μm pore without an invadopodia. Similar results were seen on numerous occasions. The scale bar is 24 μm.

Fig. 4. ErbB2 immunostaining of invadopodia. U251 glioma cells actively migrating through a Transwell filter were fixed and stained for erbB2. The image is a composite made of several images taken from the underside of one filter. Cells were exposed to 1 ng/ml NRG-1β on both sides of the filter. The inset is an enlargement of one invadopodia, and the arrow points to an 8-μm pore without an invadopodia. Similar results were seen on numerous occasions. The scale bar is 24 μm.

| Table 1 | Summarized data from migration experiments displayed in Fig. 3 |
|-----------------|-----------------|-----------------|-----------------|
| Factor/Drug     | Upper concentration | Lower concentration | Fold change relative to control |
| None            |                   |                  |                          |
| NRG-1β          | 0.1 ng/ml         | 0.1 ng/ml        | 1 ± 0.02                |
| NRG-1β          | 1 ng/ml           | 1 ng/ml          | 1.60 ± 0.05**           |
| NRG-1β          | 10 ng/ml          | 10 ng/ml         | 2.36 ± 0.02**           |
| NRG-1β          | 100 ng/ml         | 100 ng/ml        | 1.95 ± 0.04**           |
| NRG-1β          | 1 ng/ml           | 1 ng/ml          | 1.60 ± 0.03**           |
| NRG-1β (1 ng/ml) + AG 825 | 6.25 μM | 6.25 μM | 1.42 ± 0.07** |
| NRG-1β (1 ng/ml) + AG 825 | 12.5 μM | 12.5 μM | 1.94 ± 0.06** |
| NRG-1β (1 ng/ml) + AG 825 | 25.0 μM | 25.0 μM | 1.64 ± 0.12** |
| NRG-1β (1 ng/ml) + AG 825 | 50.0 μM | 50.0 μM | 1.26 ± 0.04** |
| NRG-1β (1 ng/ml) + AG 825 | 100 ng/ml | 100 ng/ml | 0.41 ± 0.04** |

NRG-1β Activates FAK—Our results described in Fig. 6 allowed us to formulate the hypothesis that erbB receptor activation by NRG-1β led to the stimulation of FAK. Such modulation of FAK has been described downstream from NRG-1...
treatment in human breast cancer cells (12) and in cultured Schwann cells (38). Therefore, we examined, in more detail, whether erbB2 could modulate the activation status of FAK in human glioma cells. This was accomplished by measuring the level of tyrosine-phosphorylated FAK in U251 cells that had been treated with increasing concentrations of NRG-1β or NRG-1α plus increasing concentrations of AG 825 (Fig. 7A). Lysates were immunoprecipitated with agarose-conjugated phosphotyrosine antibodies then immunoblotted with antibodies specific for FAK. As demonstrated, NRG-1β dose dependently increased the level of tyrosine phosphorylation of FAK, and AG 825 (in the presence of NRG-1β) dose dependently decreased the level of tyrosine phosphorylation of FAK.

FAK contains at least six tyrosine residues that can be phosphorylated resulting in kinase activation (39). Tyr-397 is the major phosphorylation (activation) site on FAK (40). Phosphorylation of this residue recruits another nonreceptor protein-tyrosine kinase, Src, to FAK. Subsequently, Src is believed to activate FAK completely through additional tyrosine phosphorylation events (41). In this way, the FAK-Src complex is thought to activate other key proteins involved in migration such as p130Cas (42, 43). Therefore, we took our analysis one step further by investigating whether a Src-specific inhibitor (PP2) could decrease the effects of NRG-1β on glioma cell migration. Indeed, our results (Fig. 7B) indicated that PP2 but not PP3 (an inactive analog of PP2) could inhibit the stimulatory effects of NRG-1β on U251 glioma cell migration. Specifically, NRG-1β increased migration 1.95 ± 0.11-fold, relative to control. PP3 treatment in the presence of NRG-1β did not significantly alter cell migration (1.76 ± 0.08-fold increase), whereas PP2 treatment markedly reduced NRG-1β effects (0.51 ± 0.05-fold decrease, p < 0.001).

**DISCUSSION**

The experiments described in this report demonstrate the enhancement of human glioma cell motility and migration by NRG-1β. We began our study by indicating the subcellular localization of erbB2 and erbB3 receptors in glioma cells in areas of the plasma membrane involved in movement, namely the lamellipodia. Next, we specifically activated these receptors in the U251 human glioma cell line using NRG-1α. Our results showed that NRG-1α could increase movement of human glioma cells in both the two-dimensional and three-dimensional planes and that an erbB2-specific inhibitor, AG 825, could attenuate the effect. We demonstrated that erbB2 and erbB3 receptors localized in the invadipodia. Importantly, we found that erbB2 colocalized with and coimmunoprecipitated with members of the focal complex. Finally, we provided evidence that suggested erbB2 functionally modulated a key member of the complex, FAK. Taken together, these data support the hypothesis that NRG-1 activation of erbB receptors stimulates glioma cell movement. In this way, the NRG-1/erbB receptor network may contribute to the aggressive migratory phenotype exhibited by human glioma cells.

**Evidence That ErbB2 Is Localized in Regions of the Cell Involved in Motility and Migration—Lamellipodia in motile cells and invadipodia in migrating or invading cells are dynamic processes that contain members of the focal complex (44). Growth factor receptors have been reported to associate with components of this complex. In particular, the erbB2 receptor coimmunoprecipitated with integrins β1, β3, and β4 (45–47) and with FAK (12, 39). In our study, we demonstrated the localization of erbB2 receptors in lamellipodia and invadipodia. Importantly, we evidenced the coimmunostaining of erbB2 with members of the focal complex. Our immunostaining results demonstrated similar localization of components in cells treated without or with NRG-1β or NRG-1α plus AG 825. This suggested that the activation status of erbB2 did not alter its subcellular localization or the localization of actin, β1-integrin, vinculin, or FAK. Instead, it appeared that the colocalization between these components was constitutive and represented a co-clustering (48) in the same region of the plasma membrane. Indeed, coimmunoprecipitation experiments between erbB2 and β1-integrin confirmed a level of association which did not change after stimulation or inhibition of erbB2.

Interestingly, our coimmunoprecipitation experiments between erbB2 and FAK demonstrated an association status that was dependent upon the activation of erbB2. In this case, we...
believe that FAK, already localized in the same region of the cell as erbB2, was stimulated to complex either directly or indirectly with erbB2 in a manner that was dependent upon the activation status of erbB2. Such an association between FAK and activated platelet-derived growth factor receptors has recently been demonstrated in transiently transfected human 293T cells (49). These studies place the erbB receptors, specifically erbB2, in regions of the membrane which are important for cellular motility and may facilitate their ability to modulate movement.

Activation of ErbB2 and ErbB3 Receptors by a Specific Ligand, NRG-1-β, Can Modulate Motility and Migration—ErbB2 and erbB3 receptors were selectively activated using the specific ligand NRG-1-β (29). This recombinant form of NRG-1 contains only the active epidermal growth factor-like domain. The endogenous NRG-1 protein is synthesized as a transmembrane-bound proprotein by both neurons and glia (18–20) and by glioma cells themselves (22). It is believed that this membrane-bound form of NRG-1 can stimulate erbB receptors on the same (autocrine) or surrounding cells (juxtacrine). For example, membrane-bound NRG-1 expressed by radial glial cells has been shown to induce the migration of cortical neurons (50).

In addition to producing NRG-1 as a membrane-bound form, cells of the central nervous system and glioma cells (data not shown) can proteolytically cleave and release the functional portion of the NRG-1 protein. Included in the domains of this released form of NRG-1 is an Ig-like domain that allows it to adhere to the surrounding ECM (21). In this way, released NRG-1 appears to function in a paracrine manner to stimulate erbB receptors on the same (autocrine) or surrounding cells (juxtacrine). For example, membrane-bound NRG-1 expressed by radial glial cells has been shown to induce the migration of cortical neurons (50).

In our cellular movement experiments we demonstrated that the motility and migration of glioma cells could be enhanced by exogenous application of NRG-1-β. Notably, we saw that glioma cells could migrate in the absence of NRG-1-β but that the magnitude of movement under a variety of experimental conditions could be increased in response to NRG-1-β. This meant that activation of erbB2 and erbB3 by NRG-1-β could increase a process that was already activated in the glioma cells. More specifically, in our migration experiments, we saw that laminin could induce migration; however, the effect was enhanced in the presence of NRG-1-β. Indeed, NRG-1-β appeared to function in both a chemoattractant and manner.

Laminin induces migration of glioma cells in a manner that is dependent upon the activation of integrin receptors and subsequently, the activation of FAK (52). We believe that the enhancement seen with NRG-1-β was caused by the concomitant activation of FAK by signals generated downstream from both integrins and erbB2 receptors. Such a convergence onto FAK has been demonstrated in the coactivation of integrins and the epidermal growth factor receptor (erbB1) (49).

 Signals from ErbB2 and Integrins Converge on FAK—FAK is a nonreceptor protein-tyrosine kinase that can localize in the focal complex through a direct (53) or indirect (54) association with the β1-integrin cytoplasmic tail. Upon β1-integrin engagement with an ECM such as laminin, FAK becomes autophosphorylated and initiates signaling cascades that ultimately lead to a variety of effects including cytoskeletal reorganization (39). ErbB2 has been shown to associate with and modulate FAK after stimulation with NRG-1. As discussed previously, we observed colocalization between erbB2 and FAK in human glioma cells. Additionally, we were able to communoprecipitate erbB2 and FAK in a manner that appeared to be dependent upon the activation status of the erbB2 receptor. In subsequent experiments we were able to demonstrate that NRG-1-β dramatically increased the amount of tyrosine-phosphorylated FAK in U251 glioma cells. This stimulation was dependent upon the erbB2 receptor as demonstrated by a pharmacological inhibitor, AG 222. It is possible that FAK is a substrate for erbB2 kinase, implying a direct association between the two proteins. Alternatively, erbB2 has been shown to associate and/or activate a number of proteins such as Src and Grb2 (16), which also associate with FAK. Therefore, erbB2 may indirectly modulate FAK by stimulating a kinase, which subsequently modulates its activity.

As a first step to elucidate the downstream effects of NRG-1-β stimulation of FAK, we demonstrated that the Src-specific inhibitor PP2, but not its inactive analog (PP3), could abrogate NRG-1-β effects on migration. At least two possible scenarios could explain the PP2 effects: 1) Src is upstream from FAK activation by erbB2, and its inhibition prevents FAK stimulation; or 2) Src is downstream from FAK activation, and its inhibition prevents the formation of the FAK-Src complex. The exact mechanisms mediating erbB2 activation of FAK and Src inhibition of migration remain to be clarified and will be the subject of future studies.

In conclusion, our results demonstrate that the activation of erbB receptors by NRG-1-β enhances glioma cell migration. Such a function for the NRG-1/erbB receptor cascade has not been demonstrated previously in human brain malignancies. These findings are intriguing, however, in light of NRG-1 effects on the development of the brain. In fact, NRG-1 is believed to serve a number of important functions related to cellular migration in the nervous system. For example, during embryonic development, GGF2 (an isoform of NRG-1) promotes the migration of mammalian neural crest cells (55). In the developing central nervous system, GGF/NRG-1 expression on radial glial cells was documented to be necessary for cortical neuronal migration (50), and NRG-1 expression on migrating granule cells is necessary for radial glia formation in the developing cerebellum (56). In the peripheral nervous system, NRG-1 has been shown to enhance the motility of Schwann cells (57). One study reported that this function was dependent upon the activation of the mitogen-activated protein kinase cascade (58).

In addition, Vartanian et al. (38) demonstrated that NRG-1 could induce the rapid association of FAK with erbB2 and erbB3 in cultured Schwann cells. Our report, demonstrating a similar effect of NRG-1 on glioma cells, may provide additional support to the theory that gliomas recapitulate many features of cells of the developing brain (59, 60). Indeed, it is still unclear whether gliomas arise from differentiated glial cells or from their progenitors. Regardless of their origin, it is apparent from our report that glioma cells respond to NRG-1 in a manner that is similar to immature neural cells migrating through the brain. A better understanding of the mechanisms involved in this effect would not only contribute to our knowledge of the events that lead to the spread of human malignant gliomas but would also apply to the way brain cells, in general, migrate within the developing brain.

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