ErbB4 Expression in Neural Progenitor Cells (ST14A) Is Necessary to Mediate Neuregulin-1β1-induced Migration*

Received for publication, July 23, 2004, and in revised form, August 24, 2004 Published, JBC Papers in Press, September 8, 2004, DOI 10.1074/jbc.M408374200


Giovanna Gambardella‡‡, Donatella Garzotto‡‡, Erika Destro‡‡, Beatrice Mautino‡, Costanza Giampietro‡, Santina Cutrupi, Claudio Dati‡, Elena Cattaneo**, Aldo Fasolo‡, and Isabelle Perrotte‡†

From the ‡Department of Human and Animal Biology, University of Torino, Torino 10123, Italy, the °Department of Medical Sciences, University of Piemonte Orientale, Novara 28100, Italy, and the **Department of Pharmacological Sciences, University of Milan, Milan 20133, Italy

Activation of the receptor tyrosine kinase ErbB4 leads to various cellular responses such as proliferation, survival, differentiation, and chemotaxis. Two pairs of naturally occurring ErbB4 isoforms differing in their juxtamembrane (JMa/JMb) and C termini (cyt1/cyt2) have been described. To examine the role of ErbB4 in neuron migration, we cloned and stably transfected each of the four ErbB4 isoforms in ST14A cells (a neural progenitor cell line derived from the striatum of embryonic day 14 rats) endogenously expressing the other members of the ErbB family: ErbB1, ErbB2, and ErbB3. Using immunoprecipitation assays, we showed that the neuregulin-1β1 (NRG1β1) stimulus induced ErbB4 tyrosine phosphorylation and phosphatidylinositol 3-kinase (PI3K) recruitment and activation (as demonstrated by Akt phosphorylation) either directly (ErbB4 cyt1 isoform) or indirectly (ErbB4 cyt2 isoform). We examined the ability of the four ErbB4 isoforms to induce chemotaxis and cell proliferation in response to NRG1β1 stimulation. Using migration assays, we observed that only ErbB4-expressing cells stimulated with NRG1β1 showed a significant increase in migration, whereas the growth rate remained unchanged. Additional assays showed that inhibition of PI3K (but not of phospholipase Cγ) dramatically reduced migratory activity. Our data show that ErbB4 signaling via PI3K activation plays a fundamental role in controlling NRG1β1-induced migration.

The ErbB receptor family consists of four receptor tyrosine kinases named the epidermal growth factor (EGF) receptor, ErbB2, ErbB3, and ErbB4 (reviewed in Ref. 1). Ligand-dependent activation of ErbB receptors results in homo- or heterodimerization, which stimulates receptor trans-phosphorylation on cytoplasmic tyrosine residues, creating binding sites for adaptor or enzymatic proteins. EGF receptor and ErbB homodimers are active kinases in the absence of coreceptors, whereas ErbB3 (which has little or no intrinsic tyrosine kinase activity) and ErbB2 (for which no ligand has been identified) necessitate coreceptor interaction for signal transduction (2). Thus, whereas ErbB2 and ErbB3 are limited to heterodimerization, the EGF receptor and ErbB4 can be activated by either homo- or heterodimerization.

An interesting ErbB4 feature (recently reviewed in Ref. 3) is the existence of isoforms generated by alternative splicing (4). One isoform pair (5) is characterized by alternative splicing of exons located in the extracellular juxtamembrane region conferring (JMa), or not (JMb), susceptibility to proteolytic cleavage (6) by a member of the ADAM (a disintegrin and metallo-protease) family, the tumor necrosis factor-α-converting enzyme (7). ErbB4 proteolytic cleavage produces a membrane-associated 80-kDa fragment that can be degraded by proteasome activity following polyubiquitination (8) or that can be the substrate for subsequent γ-secretase cleavage, which releases the cytoplasmic domain from the membrane and allows, intriguingly, nuclear translocation of a fragment (9, 10) that can act as cotranscriptional activator of the Yes-associated protein (11, 12).

The ErbB4 isoform pair (13) is characterized by the presence (cyt1) or absence (cyt2) of a cytoplasmic exon containing a docking site for phosphatidylinositol 3-kinase (PI3K). The existence of these isoforms suggests that potentially four isoforms may exist and evoke functional differences. Actually, in stable NIH3T3 transfectants, it has been shown that the ErbB4 isoform that does not activate PI3K (cyt2) mediates proliferation, but not survival or chemotaxis, as does cyt1 (13).

ErbB4 ligands belong to two groups: the neuregulins (NRG1–4), also termed heregulins, and some members of the EGF family (betacellulin, epiregulin, and HB-EGF). NRG1 and NRG2 exist in a number of splicing isoforms and recognize also ErbB3 as a binding site, whereas NRG3 and NRG4 interact, with lower affinity, only with ErbB4 (1). In most assays, NRG1 with a β-type EGF-like domain is 10–100 times more potent than NRG1 with an α-type EGF-like domain. Moreover, experiments performed with mice with targeted mutations showed that NRG1β is involved in nervous system and cardiac development, whereas NRG1α is involved in breast development (reviewed in Ref. 14).

In the central nervous system, NRG1 is expressed by both neurons and glia (15–17), and ErbB4 is expressed in various part of the brain and nervous system (18–22), including the...
ErbB4 Mediates NRG1β-induced Migration in ST14A Cells

ErbB4 Mediates NRG1β-induced Migration in ST14A Cells

The ST14A cell line was derived from primary cells dissociated from embryonic day 14 rat striatal primordia and conditionally immortalized by retroviral transduction of the temperature-sensitive variant tsA58/U19 of the SV40 large T antigen (34). Cells were cultured on dishes (BD Biosciences) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM l-glutamine, and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). Stable transfectants were grown in medium containing 5 μg/ml puromycin (Sigma). Cells were grown as monolayers at the permissive temperature of 33 °C in a 5% CO2 atmosphere saturated with H2O. Cells were allowed to grow to near confluence, and adherent cells were harvested by the trypsin/EDTA method.

The EGFR-like domain of mouse NRG1β was produced in our laboratory as a His-tag fusion protein in Escherichia coli (41). The PI3K inhibitor LY294002 was dissolved in dimethyl sulfoxide (20 mM stock solution) and stored at −20 °C. The phospholipase Cγ inhibitor U73122 was dissolved in chloroform according to the manufacturer's instructions to a final concentration of 2 mM, divided in aliquots, lyophilized, stored at −20 °C, and resuspended in dimethyl sulfoxide just before use to obtain a 2 mM stock solution. In all experiments, control samples received the same concentration of dimethyl sulfoxide or other vehicle.

Polyclonal anti-ErbB4 (C-18, sc-283) and monoclonal anti-phosphotyrosine (PY99) primary antibodies were from Santa Cruz Biotechnology. Polyclonal anti-PI3K p85 antibody was from Upstate Biotechnology, Inc. Polyclonal anti-phospho-Ser737 Akt and anti-Akt antibodies were from Cell Signaling. Horseradish peroxidase-linked donkey anti-rabbit and sheep anti-mouse secondary antibodies were from Amersham Biosciences.

Proliferation Assay—The proliferation assay was performed according to the protocol described by Kueng et al. (42). Briefly, cells were plated in 200 μl of DMEM containing 10% FBS at a density of 1000 cells/well in 96-well plates. The following day (t = 0), the medium was replaced with 2% FBS-containing DMEM with or without 5 ng NRG1β (35). For each plate, an 8-well line without cells was treated as the entire plate and used as a blank in the following microplate reader analysis. At t = 0, a 96-well plate was fixed and used as a growth starting point. Cell growth was subsequently calculated as a function of treatments. Briefly, the medium was removed, and cells were fixed by addition of 100 μl of 2% glutaraldehyde in phosphate-buffered saline (PBS). After being shaken (200 cycles/min) for 20 min at room temperature, plates were washed five times by submersion in deionized water and air-dried for at least 24 h. Plates were then stained by addition of a solution (100 μl/well) containing 0.1% crystal violet dissolved in freshly prepared 200 mM boric acid (pH 9.0). After being shaken (200 cycles/min) for 20 min at room temperature, plates were washed five times by submersion in deionized water and air-dried for at least 24 h. Plates were solubilized (addition of 100 μl/well) and 5 min of shaking at room temperature. The absorbance of dye extracts was measured directly in plates using a Microplate Reader (Bio-Rad) at a wavelength of 590 nm.

Reverse Transcription (RT)-PCR and Cloning of Rat ErbB4 cDNAs—Total RNA was prepared from olfactory bulbs obtained from one adult Wistar rat (Charles River Laboratories) using the Trizol reagent (Gibco) according to the manufacturer’s instructions. Total RNA (1 μg) was subsequently reverse-transcribed to cDNA in a total volume of 25 μl using 200 units of reverse transcriptase (U. S. Biochemical Corp.) in 1× buffer (U. S. Biochemical Corp.) with 7.5 μM random exonucleotide primers (Amersham Biosciences), 0.05% Triton X-100, 0.5 mM dNTPs (Amersham Biosciences), 0.1 μg/μl acetylated bovine serum albumin (Amersham Biosciences), and 30 Amersham Biosciences). As a control, an enzyme-less reaction was performed for each sample. PCRs were carried out in a total volume of 50 μl containing 5 μl of cDNA, enzyme-less reaction sample, or water (negative controls), 250 nM each 5′- and 3′-primers (see below), 2.5 units of PfuTurbo® DNA polymerase (Stratagene), 100 μM dNTPs, and 5% glycerol. The primers used (produced by Sigma) were designed according to the GenBank®/EJB rat ErbB4 sequence (accession number NM_021687; corresponding to the JmA-cyt1 isoform, herein numbered considering the start site as +1: primer ErbB4-11 (5′-TGCTAGCCAA-AATGGAAGCTGCGG-3′, with the artificial Nhel site underlined and the ATG codon in boldface) and primer ErbB4-4 (5′-GGTACCCAGATTCC-3′) to obtain the full-length ErbB4 cDNAs, the previously obtained amplicons (nucleotides 4025. Samples were amplified using a simplified touchdown PCR protocol: denaturation at 95 °C for 5 min; followed by 10 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 30 s, and extension at 72 °C for 3 min; followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 30 s, and extension at 72 °C for 3 min. The extension step of the last cycle was increased to 30 min. PCR products were separated by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and visualized with UV light. A 1-kb DNA ladder (Invitrogen) or BenchTop pGem DNA markers (Promega) was used as a size marker. PCR products were purified from agarose gel using the GenElute™ gel purification kit (Sigma). To obtain the full-length ErbB4 cDNAs, the previously obtained amplicons (nucleotides 7 to 2...
ErbB4 Mediates NRG1-induced Migration in ST14A Cells

+2228 and nucleotides +2105 to +4025) were used as templates in the presence of primers ErbB4-11 and ErbB4-6 in recombinant PCR according to the following protocol: denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 8 min. The extension step of the last cycle was increased to 30 min. PCR products were separated by electrophoresis on a 0.8% agarose gel, purified from agarose gel using the GenElute™ gel purification kit, and cloned into the pCR®-BluntII-TOPO® vector (Invitrogen) using the Zero Blunt®-TOPO® PCR cloning kit (Invitrogen) following the manufacturer’s instructions.

The DNA of 18 single colonies was obtained using the GenElute™ plasmid miniprep kit (Sigma). For each clone, the orientation of the cDNA in the vector (sense or antisense) was detected by restriction enzyme digestions using EcoRI and BamHI (Amersham Biosciences). To identify the different ErbB4 isoforms (cyt1/cyt2 and JMa/JMb), individual sense clones were analyzed by PCR; the juxtamembrane domain (fragment +1773 to +2019) was amplified with primer ErbB4-4-12 (5′-GAAGAATTCACAGGGTCTACGG-3′) and primer ErbB4-13 (5′-AAGGGCAATACAGGCAGCTG-3′), and the cytoplastin domain (fragment +3054 to +3267) was amplified with primer ErbB4-14 (5′-T-GCTGAAAGATATTGTCGAGGCTGCA-GACG-3′) and primer ErbB4-2 (5′-TCTGGTATGACTTGGCTCGTTGCTGCT-3′). PCRs were carried out in a total volume of 50 µl containing 5 µl of DNA or water (negative controls), 250 mM KCl, 1 unit of RNaseH, 100 µM dNTPs, and 10% glycerol. Samples were amplified by denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C (JMa/JMb) or 60 °C (cyt1/cyt2) for 30 s, and extension at 72 °C for 1 min. The extension step of the last cycle was increased to 20 min. To amplify the glyceraldehyde-3-phosphate dehydrogenase cDNA (5′-GTTAGGAGGATCCCTGGACTGAGGCTGCGGAC-3′), primers used for sequencing were designed according to the GenBank™/EBI rat ErbB4 sequence (accession number NM_021687).

Western blotting as described below.

Immunoprecipitation and Western Blotting—For immunoprecipitation experiments, confluent cells grown on 10-cm dishes were serum-starved overnight. The following day, 1 mM sodium orthovanadate was added to both the untreated and treated cells for at least 30 min prior to addition of 5 mM NRG1. After 10 min of NRG1 stimulation, cells were rinsed twice with ice-cold PBS containing 1 mM sodium orthovanadate and lysed for 20 min on ice with 500 µl of cold extraction buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1% Triton X-100, 100 µM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Roche Applied Science)). For immunoprecipitations to be assayed for PI3K activity, cells were lysed with 500 µl of cold lysis buffer (25 mM Hepes (pH 8), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 2 mM EGTA, 1% Triton X-100, 100 µM sodium orthovanadate, and a mixture of protease inhibitors) (45). Lysates were collected with a cell scraper, placed in microcentrifuge tubes, rocked at 4 °C for 10 min, and spun at 4 °C for 20 min at 11,000 × g to discard cell debris. Protein concentration was determined using the BCA kit for protein determination, and lysates (1.5–2 µg) were diluted to equal concentrations using 1% Agarose (50 µl; Santa Cruz Biotechnology) pre-washed three times with extraction buffer were precubicated for 45 min with primary antibody (1 µg/ml of protein), and lysates were added and incubated overnight with constant rotation at 4 °C. As a control, representative samples (NRG1-treated or not) were incubated with protein A beads without primary antibody. The next day, immunoprecipitates were spun at 4 °C for 20 min. Protein concentrations were adjusted to 1 µg/ml, and 900 µl of cold extraction buffer was added. To release immunoprecipitates from the beads, 2× sample buffer (62.5 mM Tris (pH 6.8); 4% SDS, 480 µM 2-mercaptoethanol, and 40% glycerol) was added, and the proteins were denatured at 100 °C for 5 min. Proteins were resolved by 8% SDS-PAGE, transferred to a Hybond™ C Extra membrane (Amersham Biosciences) following the manufacturer’s instruction, and blocked for 1 h at 37 °C in 1× TBST (10 mM Tris-HCl (pH 7.4); 0.1% Tween) plus 5% nonfat milk. The membranes were incubated overnight at 4 °C in primary antibodies diluted 1:1500 in TBST plus 1% nonfat milk. The next day, they were rinsed four times with TBST for 5 min each at room temperature and incubated for 1 h at room temperature with horseradish peroxidase-linked anti-rabbit (1:3000) or anti-mouse (1:10,000) secondary antibodies (Amersham Biosciences) in blocking solution (100 mM glycine buffer (pH 7.5); 1% Tween; 10% nonfat milk) for 1 h at room temperature, and specific binding was detected by the enhanced chemiluminescence ECL system (Amersham Biosciences) using Hyperfilm™ (Amersham Biosciences). In some cases, the membrane was stripped for 30 min at 50 °C in 0.05 µM phosphatase buffer (pH 6.5), 10 µl urea, and 0.1 M 2-mercaptoethanol, blocked, tested with secondary antibody to verify accurate cleaning, and repotted with the indicated primary antibodies for further analysis.

The molecular masses of proteins were estimated relative to the electrophoretic mobility of cotransfected prestained molecular mass markers (Invitrogen and Bio-Rad).

Total Protein Extraction—Total proteins were extracted by lysing cells with extraction buffer as described above or by solubilizing cells in boiling Laemmli buffer (2.5% SDS and 0.125% Triton-X114 (pH 6.8)), followed by 5 min of denaturation at 100 °C in 240 µM 2-mercaptoethanol and 18% glycerol. Protein concentration was determined by the BCA method, and equal amounts of proteins were loaded onto each lane, separated by SDS-PAGE, transferred to a Hybond™ C Extra membrane, and analyzed as described above. Bands were quantified by densitometry using the Gel Doc image documentation system (Bio-Rad) with the Quantity One software (Bio-Rad). Conclusions were drawn after experiments were repeated a minimum of three times.

PI3K Assays—PI3K activity was assayed in the ErbB4 immunoprecipitates in the presence of [γ-32P]ATP (Amersham Biosciences) and phosphoinositides (Sigma) as described previously (45, 46).

Motility Assay—The wound healing motility assay was used to measure two-dimensional movement. Cells were grown to confluence in 12-well plates and serum-starved for 24 h, and then a cross-shaped
wound was made on the monolayers using a sterile 200-μl pipette tip. The cells were rinsed three times with PBS and placed in serum-free DMEM with or without 5 nM NRG1β. To obtain good reproducibility, in all experiments, the left arm of the cross was photographed. Photos were taken at the initiation of the experiment (t = 0) and 24 h later (t = 24 h) using an Olympus IX50 inverted microscope equipped with a Cool SNAP-Pro CCD camera (Media Cybernetics, Silver Spring, MD). Images were edited with Image Pro-Plus software (Media Cybernetics). The areas were measured using the measurement program included. The two-dimensional movement of the cells was quantified by measuring the surface area of the wound at t = 0 and comparing it with the surface area at t = 24 h; healing percentage = (1 − A(t)/A(0)) × 100.

Migration Assay—The Transwell migration assay was used to measure three-dimensional movement. Cells (10^5) resuspended in 200 μl of DMEM containing 2% FBS were seeded in the upper chamber of a Transwell (cell culture insert, no. 353907, BD Biosciences) on a porous transparent polycarbonate membrane (0.0-μm pore size, 1 × 10^6 pores/cm^2). The lower chamber (a 24-well plate well) was filled with DMEM containing 2% FBS with or without 5 nM recombinant NRG1β. The 24-well plates containing cell culture inserts were incubated at 33 °C in a 5% CO₂ atmosphere saturated with H₂O. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde in PBS for 15 min at room temperature, washed five times with water, stained with 0.1% crystal violet and 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using an Olympus IX50 inverted microscope equipped with a Cool SNAP-Pro CCD camera; images were edited with Image Pro-Plus software. Migrated cells (2 mm^2 of microscope field/sample) were counted using the enclosed measurement program. Inhibitors (LY294002, U73122, or the same concentration of dimethyl sulfoxide as a control) were added to both the upper and lower chambers 30 min prior to addition of NRG1β. In each case, the final concentration represented a 1:200 dilution of stock solutions in Me₂SO, and control cultures were treated with Me₂SO at a 1:200 dilution. We observed that they can be divided into two groups: a lower rate group, including wild-type ST14A cells and cells expressing ErbB4, and a higher rate group. Statistical Analysis—Results from different experiments (performed at least three times in duplicate) were averaged and expressed as means ± S.D. One-way analysis of variance was used to compare data as indicated in the figure legends. Data were graphed using Microsoft Excel.

RESULTS

ErbB Expression Patterns in ST14A Cells—Previous data (13) describe functional studies on chemotaxis mediated by ErbB4 following stable expression of two isoforms (cyt1/cyt2) of this receptor in fibroblasts (NIH3T3) that endogenously express only moderate levels of the ErbB2 receptor (47). Being interested in the mechanisms by which ErbB4 intervenes in neural cell migration, we expressed the ErbB4 cDNAs in neural progenitor cells previously derived and conditionally immortalized from embryonic striatum (34). We verified in these cells the expression of ErbB1, ErbB2, and ErbB3 and the absence of ErbB4 by RT-PCR (data not shown) and Western blotting (Fig. 1). The three bands corresponding to apparent molecular masses of 80, 60, and 50 kDa were nonspecific, being recognized by anti-ErbB4 primary antibody in whole cell protein extracts from ST14A cells, which do not express ErbB4, as assessed by RT-PCR performed on parent ST14A cells (data not shown) or ST14A cells transfected with the pIRESpuro2 vector alone (see Fig. 3B, vector lane).

Expression of the Four ErbB4 Isoforms in ST14A Cells—The cDNAs corresponding to the various ErbB4 isoforms produced by alternative splicing, JMa-cyt1, JMa-cyt2, JMb-cyt1, and JMb-cyt2 (clones a1, a2, b1, and b2, respectively) (Fig. 2), were obtained by RT-PCR performed on ratolfactory bulb RNA, sequenced, and subcloned into the pIRESpuro2 vector, an internal ribosome entry site-containing vector for bicistronic expression of ErbB4 and puromycin resistance (see “Experimental Procedures”). ST14A cells were stably transfected with the expression vector incorporating the cDNAs corresponding to the four ErbB4 isoforms and with the parent vector as a control. Several puromycin-resistant subclones were isolated, and the presence of exogenous protein was confirmed by Western blot analysis (data not shown). For further analysis, two positive clones for each isoform and one control were selected (Fig. 3A). To investigate for possible transcriptional trans-regulation between different ErbB4 isoforms, each positive clone was analyzed by RT-PCR to detect the known alternative splicing isoforms (Fig. 3B). Our data show that each subclone expressed only the isoform we transfected, ruling out trans-regulation events.

Transfected cells do not show significant morphology variations. To better characterize the different ErbB4-expressing clones, growth rate analysis was performed in low serum for comparison in the presence and absence of NRG1β (data not shown). At t = 18 h, the time used for motility and migration assays, we did not observe significant differences between different clones and between treated and untreated cells; however, comparing all clones at t₂₄ = 48 h and t₄₈ = 96 h, we observed that they can be divided into two groups: a lower rate group, including wild-type ST14A cells and cells expressing ErbB4; and a higher rate group, including wild-type ST14A cells and cells expressing ErbB4/ERB4 (data not shown).
whether the different isoforms can be activated by treatment
with ErbB4 JMb-cyt1 isoforms (b1-7 and b1-15), and a higher rate
of PI3K associating with ErbB4 increased
when the receptors were stimulated by NRG1
1. Immunoprecipitation analysis con-
mirmed that, whereas ErbB4 cyt1 could bind PI3K independently
of ErbB3, ErbB4 cyt2 needed wild-type ErbB3 to co-
immunoprecipitate with PI3K (Fig. 5).

All ErbB4 Isoforms Activate PI3K and Akt—By co-immuno-
precipitation analysis, we demonstrated that ErbB4 recruits PI3K upon NRG1β stimulation. To prove PI3K activation, as
a preliminary test, PI3K activity was analyzed in ErbB4 im-
munoprecipitates by in vitro kinase assays in which the phos-
phatidylinositol 3-phosphate product was resolved by thin
layer chromatography (data not shown). PI3K activity co-
immunoprecipitated with both the ErbB4 cyt1 and cyt2 isoforms,
although with lower intensity with the cyt2 isoform.

Phosphorylation of Akt provides an excellent indication of
PI3K activation in the cell; phosphorylation at Thr308 and
Ser473 is critical for activation of the protein Ser/Thr kinase
activity of Akt (53). Therefore, to confirm PI3K activation, we
investigated Akt phosphorylation at Ser473 in ST14A cells
expressing either the ErbB4 cyt1 isoform (clones b1-15 and a1-2)
or the ErbB4 cyt2 isoform (clones b2-16 and a2-6) following
NRG1β stimulation (Fig. 6). We observed a very strong NRG1β-
dependent increase in phosphorylation of Akt at Ser473 in cells
expressing ErbB4 cyt1 and cyt2. The very slight phosphoryla-
tion observed in control cells (vector) could be the consequence
of ErbB2-ErbB3 heterodimer activation (44) and demonstrates
that the strong phosphorylation observed in our samples can be
ascribed to ErbB4 activation.

Two-dimensional Motility Assay—Finally, being interested
in studying migration mediated by ErbB4, we performed a first
round of experiments to determine whether NRG1β treatment
can modulate motility, defined here as two-dimensional
movement or chemokinesis. We selected as a model the wound
healing assay, in which the repopulation of cells in a cell-free
region (wound) can be examined quantitatively. Stable ErbB4
clones and control cells were grown until they progressively
reached confluence and then serum-starved for 24 h. A cross-
shaped wound was created in the monolayers, and cells were
placed in medium with or without NRG1β and photographed.
Twenty-four hours later, the cross-shaped wound was photo-
graphed in the same region, and the healing percentage was
quantified comparing the wound area at \( t = 0 \) and \( t = 24 \) h. Fig.
7 (upper) shows representative digital images of the wounded
region before and after the incubation period; the healing per-
percentage was quantified and is displayed graphically (Fig. 7, lower). Our results show that NRG1β1 treatment induced a statistically significant motility increase only in clones expressing ErbB4-JMb-cyt1 and JMb-cyt2. We suppose that the wound (and the consequent absence of contact inhibition) stimulates motility and proliferation so strongly that the differences between clones and between treated and untreated samples cannot be appreciated significantly. Only clone a2-6 showed a significant increase in motility compared with control cells; however, it displayed high basal activity observed also in the Transwell assays discussed below.

**ErbB4 Activation Stimulates Cell Invasion in Three-dimensional Migration Assays**—To evaluate the ability of stable transfectants to migrate in a three-dimensional environment, hence more similar to brain tissues, we assayed Transwell migration. A preliminary experiment was carried out with clone b2-16 using NRG1β1 at 10 pm to 10 nm. Cells were plated in the upper chamber of a Transwell filter and allowed to migrate for 18 h in response to NRG1β1 added to the lower chamber before being fixed, stained, and counted. Fig. 8 shows that the number of migrating cells was augmented significantly in the presence of increasing amounts of NRG1β1 from 1 nm, with a peak at 5 nm.

Representative clones for each ErbB4 isoform and a negative control (cells transfected with empty vector) were plated in the upper chamber of a Transwell filter and allowed to migrate for 18 h with or without 5 nm NRG1β1 added to the lower chamber. Fig. 9 shows that, for each ErbB4 isoform, NRG1β1 treatment significantly increased the number of cells that migrated through the filter for each treated clone compared with the respective untreated clone and with the treated negative control (empty vector).

**ErbB4-mediated Migration Requires PI3K (but Not Phospholipase C) Activity**—Data highlighting the need for PI3K in ErbB4 (cyt1 isoform)-mediated migration (13) and our results concerning ErbB4 and PI3K co-immunoprecipitation prompted us to investigate a possible role of this signal transduction protein in cell migration. We performed Transwell assays in the presence of 5 nm NRG1β1 and increasing amounts of the PI3K inhibitor LY294002 (1–100 μM) (Fig. 10). To exclude a
ErbB4 Mediates NRG1β1-induced Migration in ST14A Cells

FIG. 7. Motility of ErbB4-expressing cells. A cross-shaped wound was made on serum-starved confluent monolayers of cell clones representative of each ErbB4 isoform and the control vector. The cells were rinsed and placed in medium with (+) or without (−) 5 nM NRG1β1. Upper, images were taken at the start of the experiment (t = 0) and 12 and 24 h later. Clone b1-15 was chosen as a representative. Wound healing was evaluated as the decrease in the surface area of the wound at t = 24 h. Lower, the percent wound healing was quantified and is displayed, where healing percentage = \((1 - t_3/t_1) \times 100\). Bars represent means ± S.D. Experiments were performed three times in duplicate. *, p < 0.05.

FIG. 8. NRG1β1 stimulates migration of ErbB4-expressing cells. The ability of NRG1β1 to stimulate chemotaxis of a representative clone expressing ErbB4 (b2-16) was analyzed by Transwell migration assay. Cells (10⁵) were plated in the upper chamber of a Transwell filter and allowed to migrate for 18 h in response to NRG1β1 added to the lower chamber (using NRG1β1 at 10 pM to 10 nM, corresponding to 0.1–100 ng/ml) before being fixed and stained. Cells (2 mm² of microscopic field/sample) were photographed, and migrated cells were counted. Data represent means ± S.D. *, p < 0.05 (stimulated samples compared with the untreated sample).

This is the first work demonstrating that, in immortalized neural progenitor cells, ErbB4 expression is necessary to mediate NRG1β1-induced migration and that this phenomenon occurs via a PI3K-mediated mechanism. We began our research prompted by several intriguing studies suggesting that ErbB4 may be involved in cell migration and, specially, in neural cell migration. In fact, interneurons tangentially migrating from the ganglionic eminences express ErbB4 (29). When the glial ErbB4 receptor is blocked, neurons fail to induce radial glial formation, and their migration along radial glial fibers is impaired (33). ErbB4 knockout mice (genetically rescued from embryonic lethality) display defects in cranial neural crest migration and increased numbers of large interneurons within the cerebellum (32).

In our laboratory, long-distance migration and differentiation of neuronal precursors in adult mammalian brain have been extensively investigated in the paradigm represented by the subependymal layer-olfactory bulb system (reviewed in Ref. 54). The olfactory bulb, together with the dentate gyrus of the hippocampus, belongs to those few specific regions of the brain undergoing constant neurogenesis during adulthood (55). Newly formed cells of the olfactory bulb are generated from multipotent stem cells that reside in the subependymal layer of the lateral ventricles (see Ref. 56 for a review). The progeny of these stem cells undergo long-distance tangential migration along the rostral extension (rostral migratory stream) of the subependymal layer, reaching the main (57) and accessory (58) olfactory bulb, where they differentiate into the two principal classes of interneurons, the granule and periglomerular cells. Intriguingly, cells coming out of the rostral migratory stream of the subependymal layer, as well as isolated cells in the granule cell layer, possibly migrating cells, strongly express ErbB4, and ErbB4 immunoreactivity is found in all the periglomerular and mitral/tufted cells of the olfactory bulb (26, 28).

Many efforts have been made to solve the ErbB1 signal transduction mechanism, whereas little is known about ErbB4 receptor signaling. Neuregulin-stimulated ErbB4 receptor (cyt1 isoform) has been shown to interact with the adaptor protein Shc (Src homology and collagen domain) and with the p85 subunit of PI3K (59). In addition, it has been demonstrated that, in NIH3T3 cells overexpressing ErbB4 (cyt1 isoform), HB-EGF induces association of PI3K activity with ErbB4 and is a potent chemotactic factor (60). Moreover, chemotaxis is inhibited by wortmannin, a PI3K inhibitor, suggesting a possible role for PI3K in mediating HB-EGF-stimulated chemotaxis.

A number of studies in several types of cell lines (reviewed in Ref. 61) showed that, in response to external stimuli, PI3K activates protein kinase B/Akt, which phosphorylates Raf-1 at Ser²⁵⁹ inhibiting Raf-1 and the subsequent extracellular signal-regulated kinase activity (62, 63). This pathway has been recently confirmed for neuregulin-induced ErbB4 signal transduction (64).

Previous studies suggest that neuregulin (and other growth factors)-activated PI3K may be involved in actin reorganization, formation of lamellipodia, membrane ruffling, and cell migration (65–68). PI3K cooperates with the small GTPases Rac and Cdc42 and with p21-activated kinase-1 in the formation of actin-rich extensions via stimulation of actin polymerization (66, 69–71). There is also evidence for the association of PI3K with microtubules (72) and for the involvement of the microtubule system in the outgrowth of cell processes induced by PI3K (73, 74).

The aim of our research was to study the role of ErbB4 in neural cell migration. We began our analysis by seeking a
ErbB4 Mediates NRG1β1-induced Migration in ST14A Cells

suitable in vitro model, and we identified an embryonic neuroepithelial striatal immortalized cell line, ST14A (34), which lacks ErbB4, but expresses all other members of the ErbB family. Starting from data describing two different isoform pairs (5, 13), we cloned the full-length cDNAs encoding the four ErbB family. Starting from data describing two different isoform pairs (5, 13), we cloned the full-length cDNAs encoding the four ErbB isoforms (47) and stably transfected with an ErbB4 isoform were studied, whereas we studied a neural progenitor cell line endogenously expressing ErbB1, ErbB2, and ErbB3 and one transfected ErbB4 isoform. Therefore, in NIH3T3 fibroblasts, ErbB4 can homodimerize or heterodimerize only with ErbB2. ErbB2 has been described to associate with Src (75, 76), a potential mediator of PI3K activation (77). Nevertheless, in NIH3T3 cells, it has been shown that cyt2, the ErbB4 isoform that does not activate PI3K, mediates proliferation, but not survival and chemotaxis (13), which are mediated by PI3K, suggesting that ErbB2 cannot account for the PI3K recruitment mediated by NRG1β1-stimulated ErbB4.

Intriguingly, there are six PI3K-binding motifs in the C-terminal domain of ErbB3 (50–52), and one simple explanation may include direct recruitment of PI3K by the activated ErbB3 receptor. We tested this hypothesis by expressing ErbB4 cyt2 together with wild-type ErbB3 (43) or ErbB3 mutated at the six tyrosines included in the PI3K-binding motifs (44), and we demonstrated that the ErbB4 cyt2 isoform could co-immunoprecipitate with PI3K only when coexpressed with the wild-type ErbB3 receptor.

Finally, to test our preliminary hypothesis, we performed migration assays and showed that ErbB4 confers to ST14A cells the ability to respond to NRG1β1 through migration. This ligand is not ErbB4-specific and could activate the ErbB3 receptor; nevertheless, control cells expressing ErbB3, but lacking ErbB4, failed to migrate, indicating that, in our cell model, ErbB4 is necessary for this activity. Our data show that every ErbB4 isoform (endowed or not with the PI3K-binding motif) mediates NRG1β1-induced migration, although with different intensity, and we have demonstrated that PI3K activation is necessary for ErbB4-mediated migration either directly or indirectly through ErbB3 heterodimerization.

Acknowledgments—We thank Dr. John G. Koland for the generous gift of the ErbB3 and ErbB3-6F mammalian expression vectors. We gratefully acknowledge Dr. Andrea Graziani for fruitful discussions, Drs. Daniela Gramaglia and Andrea Rasola for helpful suggestions, and Dr. Tiziana Merlo for technical assistance.

REFERENCES
