Several studies have reported the up-regulation of EphB receptor-tyrosine kinases and ephrin-B ligands in a variety of tumors, suggesting a functional relation between EphB/ephrin-B signaling and tumor progression. The ability of the EphB receptors to regulate cell migration and promote angiogenesis likely contributes to tumor progression and metastasis. Here we show that EphB receptors, and especially EphB4, regulate the migration of murine melanoma cells. Highly malignant melanoma cells express the highest levels of EphB4 receptor and migrate faster than less malignant melanoma cells. Furthermore, inhibition of EphB receptor forward signaling by overexpression of a form of EphB4 lacking the cytoplasmic portion or by treatment with competitively acting soluble EphB2-Fc results in slower melanoma cell migration. In contrast, overexpression of active EphB4 significantly enhances cell migration. The effects of EphB4 receptor on cell migration and cell morphology require its kinase activity because the inhibition of EphB4 kinase activity by overexpression of kinase dead EphB4 inhibits cell migration and affects the organization of actin cytoskeleton. Activation of EphB4 receptor with its ligand ephrin-B2-Fc enhances the migratory ability of melanoma cells and increases RhoA activity, whereas inhibiting EphB receptor forward signaling decreases RhoA activity. Moreover, expression of dominant negative RhoA blocks the effects of active EphB4 on cell migration and actin organization. These data suggest that EphB4 forward signaling contributes to the high migratory ability of invasive melanoma cells by influencing RhoA-mediated actin cytoskeleton reorganization.

In recent years significant progress has been achieved in the prevention and early detection of melanoma. Nevertheless, melanoma remains the most deadly type of skin cancer because of the lack of effective therapeutic treatments. Melanoma progression is a multistep process that begins with changes in melanocyte morphology and an increased migratory ability (1). Both genetic and environmental factors have been implicated in melanoma progression. However, the molecular mechanisms that contribute to the progression of melanoma are not well understood. An increasing body of evidence suggests that Eph receptor-tyrosine kinases and their ligands, the ephrins, may play an important role in the molecular events responsible for melanoma progression.

There are numerous examples of misregulated expression of Eph receptors in tumors, including melanomas (2, 3). The EphA2 receptor, for example, is highly expressed in more than 90% of melanoma cell lines, with especially high levels in more malignant melanomas (4–8). EphA receptors have also been reported to be up-regulated during the development of breast and prostate carcinomas (9–11). Besides the EphA/ephrin-A system, several studies have also reported the expression of B class Eph receptors and ephrins in different tumors, including melanomas, and suggested a functional relation between EphB/ephrin-B expression and tumor progression (12–21).

The Eph receptors are the largest family of receptor-tyrosine kinases. The family is divided into two classes, A and B, on the basis of their sequence similarity and ability to bind ephrin-A or ephrin-B ligands, although some binding interactions between the two groups have also been reported (22, 23). The A class ephrins are glycosylphosphatidylinositol-anchored molecules, whereas the B class ephrins are transmembrane proteins. The kinase activity of the Eph receptors is stimulated by binding to ephrin ligands. Eph receptor activation triggers forward signaling through interactions with cytoplasmic signaling proteins and tyrosine phosphorylation of target proteins. Eph receptor-ephrin interactions also generate reverse signaling through the ephrin.

The downstream signaling pathways that link Eph receptors to cell migration are beginning to be elucidated. Some signaling pathways target Rho GTPases leading to the reorganization of the actin cytoskeleton that accompanies cell migration (24). Recent work has also highlighted the ability of Eph receptors and ephrins to affect cell–extracellular matrix adhesion by modulating integrin activity, which also plays an important role in cell migration (21, 25–29). Moreover, Eph receptors and ephrins can promote angiogenesis through their effects on endothelial cell migration as well as proliferation (30–35). The EphA2 receptor has been reported to promote angiogenesis in a variety of tumor types and has also been implicated in vascular mimicry in aggressive melanoma tumors (36–38). The ectodomain of the EphB4 receptor, on the other hand, promotes breast tumor growth by stimulating angiogenesis through
reverse ephrin-B2 signaling (20). The ability of EphB receptors to regulate cell migration and promote tumor angiogenesis might both contribute to melanoma progression.

Here we examine the role of B class Eph receptors and ephrins in melanoma cell migration. Using a panel of murine melanoma cells with well defined metastatic properties in vivo and migratory abilities in vitro, we show that the EphB receptors enhance the migration of melanoma cells. Highly malignant melanoma cells that migrate rapidly express the highest levels of EphB receptors, including EphB2, EphB3, and EphB4. Our data reveal the involvement of EphB4 receptor forward signaling because EphB4 lacking the cytoplasmic domain slows melanoma cell migration, whereas overexpression of active EphB4 significantly enhances cell migration. Furthermore, the effects of EphB4 on melanoma cell migration and morphology depend on its kinase activity because expression of kinase-inactive EphB4 slows cell migration and also alters cell morphology and actin organization. Finally, our data suggest that the EphB4 receptor regulates the migration and morphology of melanoma cells by influencing Rho-mediated actin cytoskeleton reorganization.

**EXPERIMENTAL PROCEDURES**

*Antibodies—*The primary antibodies were: goat anti-EphB1 (Santa Cruz Biotechnology, Inc.; 2 μg/10^6 cells for flow cytometry); goat anti-EphB2, EphB3, EphB4, ephrin-B1, ephrin-B2, and ephrin-B3 (R&D Systems; 2 μg/10^6 cells for flow cytometry and 0.2 μg/ml for Western blot); rabbit anti-EphB2 (0.2 μg/ml for Western blot (39)); rabbit anti-Src (BIOSOURCE International, Inc.; 1 μg/ml for Western blot); horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY20; BD Transduction Laboratories, 0.1 μg/ml for Western blot).

The secondary antibodies were: horseradish peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, 0.08 μg/ml for Western blot); horseradish peroxidase-conjugated donkey anti-goat (Jackson ImmunoResearch Laboratories, 0.008 μg/ml for Western blot); fluorescein isothiocyanate-conjugated donkey anti-goat (Molecular Probes, 0.5 μg/10^6 cells for flow cytometry).

*Cell Culture and Transfection—*Murine melanoma cell lines K1735 SW1, M2, P, C19, and C23 were cultured in Dulbecco’s modified Eagle’s high glucose medium with 2 mM l-glutamine (Irvine Scientific), 0.1 mM nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 1× vitamin solution (Invitrogen), 1% penicillin-streptomycin, and 10% fetal bovine serum (Irvine Scientific) under 5% CO2 at 37 °C. Cell transfections were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol with modifications. Briefly, cell cultures with 80% confluency grown on 35-mm plates were incubated with transfection solution containing 0.5–1.5 μg of plasmid DNA for 2–3 h. The transfection solution was then replaced with fresh Dulbecco’s modified Eagle’s high glucose medium.

*Expression Vectors—*The DNA constructs used were: pEGFP-N1 (Clontech); pEFGP-F (Clontech); pEGFP-actin (Clontech); hemagglutinin-tagged dnRhoA N19 (a kind gift of Dr. Kathryn DeFea); EphB4ΔC-GFP (consisting of a region of human EphB4 cDNA encoding amino acids 1–584 in pEGFP-N2 vector (Clontech)); full-length EphB4 cDNA in pcDNA3 plasmid (20); kinase-dead EphB4 (kdEphB4, which contains a K647R mutation in the kinase domain); EphB4FF with double mutations of Tyr-596 and -602 in the juxtamembrane domain of EphB4 into phenylalanine; EphB4EE with double mutations of Tyr-596 and -602 into glutamate. All single and double amino acid mutations in cytoplasmic domain of EphB4 receptor were generated by site-directed mutagenesis using the QuikChange kit (Stratagene). All mutations were confirmed by nucleotide sequencing.

*Ligand Induction of EphB Receptors—*To activate EphB receptors in melanoma cells, we used 2 μg/ml ephrin-B2-Fc (R&D Systems) or control human Fc (Jackson ImmunoResearch Laboratories) preclustered with anti-human Fc antibody (Jackson ImmunoResearch Laboratories) before the application. For preclustering, ephrin-B2-Fc or Fc was incubated with anti-human Fc antibody at a 1:2 ratio on ice for 1 h before application to the cultured cells. To inhibit endogenous ephrin-B/EphB interaction, we used 4 μg/ml EphB2-Fc (R&D Systems).

**Immunoprecipitation and Western Blot Analysis—*Melanoma cells were lysed in lysis buffer (25 mM Tris, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, protease inhibitors (Sigma), and 50 mM sodium pervanadate). Cell lysates were collected after centrifugation at 13,400 rpm. The protein concentrations were measured by BCA kit (Pierce), and equal amounts of proteins were resolved on the 8–16% Tris-glycine gels and transferred to nitrocellulose membranes. The proteins of interest were probed with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were visualized by enhanced chemiluminescence substrate (Amersham Biosciences).

For immunoprecipitation, melanoma cells were treated with 2 μg/ml ephrin-B2-Fc, 4 μg/ml EphB2-Fc, or control human Fc for 15 or 30 min, and cell lysates were collected as described above. The lysates were then incubated with rabbit anti-EphB2 or goat anti-EphB4 and protein A-Sepharose beads (Sigma) for 4 h at 4 °C. The beads were washed three times with lysis buffer, and the proteins bound to beads were resolved as previously described (40). For quantification purpose, the densitometry values of tyrosine-phosphorylated proteins were normalized to the values of the corresponding total protein bands using Adobe Photoshop.

**RhoA Activity Assay—*To assess RhoA activity, preclustered ephrin-B2-Fc (2 μg/ml, 15 min), EphB2-Fc (4 μg/ml, 30 min), or control human Fc was applied to melanoma cells as described above. After treatment, the cells were lysed in lysis buffer (50 mM Tris, pH 7.4, containing 150 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 5 mM EDTA, protease inhibitors (Sigma), and 50 mM sodium pervanadate) and collected as described above. The lysates of SW1 melanoma cells transiently transfected with GFP, EphB4ΔC-GFP, or kdEphB4 were also

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2 The abbreviations used are: GFP, green fluorescent protein; ANOVA, analysis of variance; RhoGEF, Rho GTPase exchange factor; dn, dominant negative; kd, kinase dead.
assessed for RhoA activity. A portion of the lysates was retained for use in the assessment of total RhoA, and GTP-RhoA (active RhoA) was precipitated from the remainder of the lysates using GST-RBD beads (Upstate Cell Signaling Solutions). After precipitation, the beads were washed three times with lysis buffer, the bound material was eluted with Laemmli buffer, and Western immunoblot analysis was performed. The membranes were probed with anti-Rho antibody (Santa Cruz Biotechnologies). The level of GTP-RhoA was quantified by densitometry and normalized to total RhoA levels in the lysates.

**Immunostaining**—Melanoma cells cultured on glass coverslips were fixed in 4% paraformaldehyde and then treated with 0.1% Triton X-100 and blocked with 1% bovine serum albumin. The transfected cells were identified by GFP fluorescence. F-actin was labeled by incubation with rhodamine-coupled phalloidin (0.165 μM, Molecular Probes). Immunostaining was analyzed under a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) or a Nikon TE2000 inverted fluorescent microscope with 20× air objective and recorded by a Hamamatsu ORCA-AG 12-bit CCD camera using Image-Pro Software.

**Live Imaging**—We monitored the actin rearrangements in GFP-actin-expressing melanoma cells plated on fibronectin-coated glass coverslips. Time-lapse imaging was performed under an inverted fluorescent microscope (model TE2000; Nikon) with a 40× air Fluor objective and monitored by a 12-bit CCD camera (model ORCA-AG; Hamamatsu) using Image-Pro software (Media Cybernetics). During imaging the melanoma cells were maintained in medium at 37 °C and 5% CO₂, and images were captured at 2-min intervals for 1 h.

**Flow Cytometry**—Cells were harvested by spinning down and were washed once in binding buffer (20 mM HEPES, pH 7.4, 2% fetal bovine serum, 2 μg/ml mouse IgG, and 0.02% sodium azide). The cells were resuspended in binding buffer at 10⁶ cells/ml and divided into 100-μl aliquots with 2 μg of primary antibody (anti-EphB1, -B2, -B3, and -B4 and ephrin-B1, -B2, -B3 and goat IgG as negative control) added to each aliquot. The reactions were incubated at 4 °C for 30 min in the dark. Cells were washed twice with binding buffer and incubated with 0.5 μg secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-goat; Molecular Probes) for 30 min at 4 °C in the dark. Cells were washed twice with binding buffer and assayed on the FACSScan cytometer (Becton Dickinson). The values were calculated as F1-F2/F2, where F1 is the experimental value (immunofluorescent labeling with specific antibodies), and F2 is the control value (negative immunofluorescent control with unspecific goat IgG). Three independent experiments were performed for each condition. The statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

**Real-time PCR**—mRNA samples were extracted from five murine melanoma cell lines, K1735 SW1, M2, P, C19, and C23, using TRizol (Invitrogen) as described by the manufacturer. mRNA concentrations were measured, and reverse transcription was performed. The concentrations of cDNA products were then measured, and equal amounts of cDNA samples from five melanoma cell lines (K1735 SW1, M2, P, C19, and C23) were used for real-time PCR against specific primers designed for EphB1, B2, B3, B4, B6, ephrin-B1, B2, B3, and actin. Three reactions were performed for each group. Actin was used as an internal standard. Three independent experiments were performed for each condition. The statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

**Scratch Assay**—After melanoma cells were plated on fibronectin (BD Science)-coated dishes for 12 h (overnight), a thin strip of cells (~1.5 mm²) was removed from the dish by scratching with a blue Eppendorf tip. Detached cells were removed, and cell migration into scratched area was monitored after 0, 3, and 6 h. The phase-contrast images were taken under a Nikon TE2000 inverted fluorescent microscope with a 4× air objective and a Hamamatsu ORCA-AG 12-bit CCD camera using Image-Pro Software. Fifty scratches (10/plate) were analyzed in each experimental group. The number of cells migrated into the scratched area was counted, and the area of the scratch was measured. The number of migrated cells/1 mm² of scratch area was calculated. In experiments with transfected cells, the number of transfected migrating cells was normalized for transfection efficiency. Three independent experiments were performed for each condition (total 150 scratches/group). Experimental values represent the mean ± S.D. Statistical analysis was performed using Microsoft (Redmond, WA) Excel. The statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

**Cell Viability Assay**—To assess cell viability in the scratch assay, live cells were labeled with calcein AM (Molecular Probes) for 10 min at 37 °C and dead cells with propidium iodide for 5 min at room temperature (Molecular Probes). The images were taken as described above. The number of calcein-positive cells (live cells) and propidium iodide-labeled cells (dead cells) were counted. The percentage of live cells was calculated for each cell line. Three independent experiments were performed. The statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

**Single Cell Motility Assay**—The single cell motility assay was performed with Cell Motility Hitkit (Cellomics). Briefly, the blue fluorescent beads were resuspended in washing buffer M and plated on fibronectin-coated 96-well plates. Then GFP-transfected melanoma cells were plated on the plates coated with the beads and incubated for 5 or 8 h at 37 °C in Dulbecco’s modified Eagle’s medium. The M2 and P melanoma cells were treated with 2 μg/ml ephrin-B2-Fc (R&D Systems) or control human Fc (Jackson ImmunoResearch Laboratories) for 6 h. After the incubation, the melanoma cells were fixed in form/aldehyde. The images were taken under a Nikon TE2000 inverted fluorescent microscope with 20× air objective and a Hamamatsu ORCA-AG 12-bit CCD camera using Image-Pro Software. As cells migrate, they push aside the beads, clearing tracks behind them. Approximately 500 cells were randomly selected and analyzed in each experimental group. The number of migrating cells and the track areas of migrating cells (migration area) were calculated to assess cell migration. Three independent experiments were performed for each condition (total 1500 cells/group). Experimental values represent the mean ± S.D. The results show low variability in number of migrating...
cells per group. The statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

RESULTS

Expression of the EphB2, EphB3, and EphB4 Receptors Is High in Fast Migrating Melanoma Cells—To investigate whether melanoma cells acquire increased expression of EphB receptors and ephrin-B ligands during malignant progression, we examined five murine melanoma cell lines with well defined metastatic properties: SW1, M2, P, C19, and C23. Four of these cell lines represent distinct clones (derived by in vitro cloning or in vivo selection) originating from a parental cell line induced by UV irradiation and croton oil promotion (41). The parental cell line, designated K1735 P and hereafter referred to as P, shows low metastatic properties overall but has been shown to contain subpopulations of highly metastatic cells as well as essentially non-metastatic cells. The disparate biologic behavior of these sub-populations is represented in the four clones designated SW1 and M2, which are highly metastatic to the lung, and C19 and C23, which are essentially non-metastatic (42, 43). We have analyzed the migratory ability of these murine melanoma cell clones in vitro using two different migration assays, the scratch assay and the single cell motility assay (Figs. 1 and 2). The migratory ability of the melanoma cells was found to correlate with their metastatic properties (44). The highly metastatic SW1 melanoma cells migrated faster, whereas the least aggressive clones C19 and C23 migrated slower (Figs. 1 and 2). Analysis of cell viability by labeling live cells with calcein AM and dead cells with propidium iodide showed no significant difference between the different clones in scratch assay (supplemental Fig. 1).

To evaluate whether the migratory ability of the different melanoma clones correlates with the levels of EphB receptors and their ephrin-B ligands, we examined cell surface expression of these molecules by flow cytometry. The results show that the cell surface levels of EphB2, EphB3, and EphB4 correlate with the migratory ability of the melanoma cells, whereas EphB1 is
ings demonstrate an association between the migratory ability of melanoma cells and the expression of the EphB4 receptor and its ligand ephrin-B2.

**The EphB4 Receptor Is Active in the Fast Migrating SW1 Melanoma Cells**—To examine whether the EphB4 receptor expressed in the SW1 cells is activated given the high expression of the ephrin-B2 ligand, we examined receptor tyrosine phosphorylation. EphB4 immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibody revealed that EphB4 is phosphorylated on tyrosine in SW1 cells (Fig. 4B), suggesting endogenous ephrin-B2/EphB4 signaling in these cells. In contrast, we did not detect EphB4 tyrosine phosphorylation in the slow migrating C23 melanoma cells (not shown). Signaling of the EphB4 receptor in SW1 cells was confirmed by the association of EphB4 with its downstream signaling target, the Src non-receptor-tyrosine kinase (Fig. 4B). EphB4 phosphorylation and association with Src were further enhanced by treatment of the SW1 melanoma cells with a soluble form of the ligand, ephrin-B2-Fc, and were inhibited by treatment with competitively acting soluble EphB2-Fc (Fig. 4B). These data show that the EphB4 receptor-tyrosine kinase is actively engaged in signaling in the fast migrating SW1 melanoma cells.

**Inhibition of EphB4 Activation Decreases the Migratory Ability of SW1 Melanoma Cells**—To examine the role of EphB4 signaling in melanoma cell migration, we used a loss-of-function approach. The highly invasive SW1 melanoma cells were transiently transfected with a truncated form of EphB4 that lacks the cytoplasmic portion (EphB4ΔC-GFP) and inhibit
EphB4 receptor forward signaling in a dominant-negative manner. The inhibiting effect of EphB4ΔC-GFP on endogenous EphB4 receptor signaling was confirmed by the decreased level of EphB4 tyrosine phosphorylation (Fig. 5B). SW1 cells transfected with EphB4ΔC-GFP had altered morphology, exhibiting flattened shapes and multiple processes compared with the less spread GFP-transfected cells (Fig. 5A). Furthermore, SW1 cells transfected with EphB4ΔC-GFP migrated significantly slower than control GFP-transfected cells in both a scratch assay and a single cell migration assay (Fig. 5, C and D). In contrast, overexpression of full-length EphB4 slightly enhanced SW1 cell migration (Fig. 5, C and D). Treatment of confluent SW1 mel-
EphB Receptors in Melanomas

The Effects of EphB4 on Melanoma Cell Migration Require an Active Kinase Domain—Having shown that deletion of the EphB4 cytoplasmic portion inhibits cell migration, we next examined whether the receptor kinase domain and the phosphorylated juxtamembrane motifs that recruit downstream signaling molecules are important for the effects of EphB4 on cell migration. For this, we used a kinase dead mutant that contains a single amino acid mutation (K647R) in the kinase domain (kdEphB4). We also generated EphB4FF and EphB4EE mutants in which the conserved tyrosines Tyr-596 and Tyr-602 in the juxtamembrane domain are mutated to phenylalanine or glutamic acid (E, Fig. 7A). These EphB4 mutants cannot interact with the SH2 domains of downstream signaling molecules that bind to the Tyr-596 and Tyr-602 phosphorylated motifs, such as Src. It has also been shown that the corresponding FF mutants of other Eph receptors have low kinase activity, whereas the EE mutants have constitutive kinase activity (45).

Overexpression of the kinase inactive kdEphB4 or EphB4FF mutants in SW1 cells decreased both the level of EphB4 tyrosine phosphorylation (Fig. 7B) and migration in the single cell migration assay (Fig. 7C, supplemental Table 1). In contrast, overexpression of the constitutively active EphB4EE mutant slightly enhanced cell migration, similar to full-length EphB4. These results reveal that EphB4 kinase activity is necessary for its promoting effects on melanoma cell migration.

EphB Kinase Activity Controls Cell Morphology and Actin Organization in Melanoma Cells—Inhibition of EphB4 kinase activity and forward signaling also affected cell morphology. Cell contours were visualized by GFP fluorescence and actin polymerization by rhodamine-coupled phalloidin (Fig. 8). Although most of the control GFP-expressing SW1 cells as well as P cells and some M2 cells had a polarized morphology with accumulation of F-actin at the leading edge (arrowheads in Fig. 8), many cells expressing EphB4ΔC-GFP or kdEphB4 exhibited more spread, flattened shapes with multiple processes and more symmetric distribution of F-actin around the cell rim (see the arrows in Fig. 8). The SW1 cells expressing EphB4ΔC-GFP or kdEphB4 acquired the morphology similar to that of the slower migrating C19 and C23 cells, whereas overexpression of full-length EphB4 or EphB4EE in SW1 cells did not result in obvious changes in cell morphology (Fig. 8). The EphB4ΔC-GFP or kdEphB4-transfected SW1 melanoma cells also showed many long filopodia or lamellipodia-like extensions, suggesting control of Rho family GTPases by EphB receptors in melanoma cells. Interestingly, the activation of EphB4 receptor with pre-clustered ephrin-B2-Fc in cultures of M2 melanoma cells, which contain both polarized cells and cells with more spread and flattened shapes, increased the proportion of fast migrating polarized cells with the morphology similar to the SW1 cells.
These data suggest that EphB receptor signaling might contribute to the high migratory ability of melanoma cells by influencing actin cytoskeleton organization.

**EphB4 Positively Regulates RhoA Activity, and Dominant Negative RhoA Blocks the Effects of EphB4 on Melanoma Cell Migration**—Increasing evidence indicates that EphB receptors regulate actin dynamics through Rho GTPases by recruitment and activation of different Rho GTPase exchange factors (Rho-GEFs). Members of the Rho family of small GTPases (RhoA, Rac, and Cdc42) are known to control cell migration through reorganization of actin cytoskeleton (46). Rac and Cdc42 regulate actin organization at the leading edge through formation of protrusive structures, lamellipodia, and filopodia, whereas RhoA induces cell migration through myosin-mediated contractility that promotes locomotion of cell body at the rear end. In addition, the cancer cells might use either Rac- or RhoA-dependent migration mode or convert between these types of migration under certain conditions. The rounded cancer cells usually show motility that is promoted by RhoA and ROCK activity, whereas the elongated cells use mesenchymal migration mode that depends on Rac activity (46). Inhibition of RhoA or ROCK suppresses migration and invasion of rounded A375m2 melanoma and LS174T colon carcinoma cells but not elongated SW962 squamous carcinoma cells, suggesting that RhoA signaling promotes rounded cell motility (47).

The fast migrating SW1 melanoma cells also exhibit strikingly rounded morphology and use a RhoA-dependent motility mode because the inhibition of RhoA or ROCK suppresses migration and invasion of SW1 melanoma cells (Fig. 9, C and D). To determine whether the EphB4 receptor induces migration of melanoma cells through RhoA-mediated control of the actin cytoskeleton, we examined the effects of the EphB4 receptor activation or inhibition on level of RhoA activity. The level of active RhoA (GTP-RhoA) was significantly increased in response to EphB4 receptor activation with its ligand ephrin-B2-Fc (Fig. 9, A and B). In contrast, inhibiting EphB4 forward signaling with competitively acting soluble EphB2-Fc or by overexpressing dnEphB4 or kdEphB4 mutant decreases RhoA activity in SW1 cells (Fig. 9, A and B). Importantly, the inhibition of RhoA blocked EphB4-induced cell migration. Although the overexpression of full-length EphB4 slightly enhanced the migratory ability of SW1 melanoma cells, the co-expression of dnRhoAN19 significantly enhanced the migratory ability of SW1 melanoma cells (Fig. 9, C and D). Thus, the EphB4 promotes melanoma cell migration through RhoA-mediated actin reorganization.
FIGURE 8. The EphB4 forward signaling controls cell morphology and actin organization of murine melanoma cells. Murine melanoma cells were transfected with GFP (A), EphB4ΔC-GFP (B), GFP and kdEphB4 (C), or GFP and full-length EphB4 (D) or GFP and EphB4EE (E). Cell contours were visualized by GFP fluorescence (green) and F-actin with rhodamine-coupled phallolidin (red). Many cells with polarized cell morphology are seen among GFP-expressing SW1 (A) and P cells (F), some in M2 (G) cell cultures (indicated by arrowheads). Most of the GFP-expressing C23 (H) and C19 (I) cells as well as SW1 cells expressing EphB4ΔC-GFP (B) or kdEphB4 (C) are not polarized and have spread cell bodies and multiple dendrites (indicated by arrows). M2 cells treated with preclustered ephrin-B2-Fc (K) show increased proportion of round, polarized cells as compared with control Fc-treated cells (J). Scale bars, 40 μm. L. Quantification of the percentage of spread cells with multiple dendrites (flat) and round or elongated polarized cells (round) M2 cells treated with preclustered ephrin-B2-Fc (eB2-Fc) or control Fc. The histogram shows average values from three independent experiments. Error bars indicate S.D. (n = 1500 cells/group). ***, p < 0.001.
Using a panel of murine melanoma cells with well defined metastatic behavior in vivo, we demonstrate a role for EphB4 receptor forward signaling in melanoma cell migration. Highly malignant melanoma cells that migrate fast express the highest levels of EphB4. The receptor is phosphorylated on tyrosine and associated with Src, suggesting that it is activated and engaged in forward signaling. Furthermore, increasing the level of activated EphB4 by treating melanoma cells with ephrin-B2-Fc or by expressing full-length EphB4 in slow migrating melanoma cells significantly enhanced their migratory ability. Conversely, inhibition of EphB receptor kinase activity and forward signaling in highly migratory melanoma cells resulted in increased cell spreading and slower migration. Several different approaches were used to inhibit EphB receptor forward signaling, including (i) treatment with competitively acting soluble EphB2-Fc, (ii) overexpression of a truncated form of EphB4 that lacks the cytoplasmic portion of the receptor and inhibits endogenous EphB4 receptor signaling in a dominant-negative manner, and (iii) overexpression of two inactive mutants of EphB4 (kdEphB4 and EphB4FF), which also act in a dominant negative manner. All of these manipulations reduced the migratory ability of the highly invasive SW1 melanoma cells.

Ephrin-B2 is also present in the melanoma cells, and it will be important to determine whether reverse signaling mediated by ephrin-B2 plays a role in the migratory and invasive properties of these cells (21). Our data suggest that the effects of EphB4 forward signaling may predominate because overexpression of full-length EphB4 has opposite effects on melanoma cell migration as compared with overexpression of dominant negative EphB4 mutants or EphB2-Fc treatment, even though all of these approaches should promote ephrin-B2 reverse signaling. Although it is clear that forward signaling through EphB4 receptor contributes to its effects on cell migration, the role of reverse signaling in melanoma cells remains to be established.

Consistent with our data, there is increasing evidence that EphB receptor forward signaling is a key regulator of the migration of certain cell types. For example, EphB4 has been shown to

**DISCUSSION**

Using a panel of murine melanoma cells with well defined metastatic behavior in vivo, we demonstrate a role for EphB4 receptor forward signaling in melanoma cell migration. Highly malignant melanoma cells that migrate fast express the highest

**FIGURE 9.** The effects of EphB4 on cell migration are mediated through RhoA-dependent actin cytoskeleton reorganization. A, Western blot analysis of GST-RBD pulldowns for active RhoA (GTP-RhoA, upper panels) and lysates for total RhoA (lower panels) in P cells after treatment with Fc or ephrin-B2-Fc (eB2-Fc, left panels) or SW1 cells after treatment with Fc or EphB2-Fc (middle panels) and SW1 transfected with GFP, EphB4ΔC-GFP, or kdEphB4 (right panels). B, quantification of the levels of total and GTP-bound active RhoA by densitometry show a significant increase in the level of active RhoA (GTP-RhoA) in response to EphB4 receptor activation with its ligand ephrin-B2-Fc in P cells while inhibiting EphB4 forward signaling with competitively acting soluble EphB2-Fc or by overexpressing dominant negative EphB4 or kdEphB4 mutant decreases RhoA activity in SW1 cells. C and D, quantification of the migratory ability of melanoma SW1 cells expressing GFP, EphB4ΔC-GFP, or GFP with EphB4, dnRhoAN19, or EphB4 plus dnRhoAN19 in a single cell migration assay. The track areas of migrating cells (migration area) and the percentage of transfected cells that migrate (migrating cells) were calculated to assess cell migration. The histogram shows average values from three independent experiments. Error bars indicate S.D. (n = 1500 cells per group). Statistical differences were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests (supplemental Table 1) *, p < 0.05; **, p < 0.01; ***, p < 0.001. E-G, fluorescence images of SW1 cells overexpressing GFP (E), kdEphB4 (F), or dnRhoAN19 (G). Cell contours were visualized by GFP fluorescence (green) and F-actin with rhodamine-coupled phalloidin (red). Although many cells with polarized cell morphology are seen among GFP-expressing SW1 cells (E, indicated by arrows), most of the cells expressing kdEphB4 (F) or dnRhoAN19 (G) are not polarized and have spread cell bodies with multiple dendrites (indicated by arrows). Scale bars, 40 μm.
promote the migration of microvascular endothelial cells through the Akt pathway (48) and EphB1 to promote the migration of endothelial and other cell types (49). The migration and invasion of human glioma cells is promoted by activation of the EphB2 receptor and inhibited by blocking EphB2 (50). On the other hand, EphB receptors have also been reported to inhibit cell migration. For example, EphB4 inhibits migration and invasion of breast cancer cells in response to ephrin-B2-Fc stimulation (51), and EphB/ephrin-B repulsive signaling has been implicated in the positioning of epithelial cells in the intestinal cript (52).

Several downstream signaling pathways may link Eph receptors to melanoma cell migration and invasion. EphB receptors have been shown to promote cell migration through recruitment of Src, Shc, and components of the focal adhesion complex (26, 49, 53, 54). The inhibiting or activating effects of EphB receptors on cell migration have also been attributed to their ability to phosphorylate and activate the cytoskeletal protein paxillin (54) or to inhibit the ability of small GTPase R-Ras to support integrin-mediated adhesion (25). Our results show that the effects of EphB4 on melanoma cell migration require its kinase activity because inhibition of EphB4 kinase activity by overexpression of kinase dead EphB4 with a single K647R mutation in its kinase domain resulted in slower cell migration and significantly altered cell morphology and actin cytoskeleton organization.

The ability of EphB receptors to control cell migration seems to involve remodeling of the actin cytoskeleton. Our results show that inhibition of EphB4 receptor kinase activity in highly malignant SW1 cells resulted in pronounced effects on cell morphology and actin organization. Actin cytoskeleton remodeling and cell migration are known to be controlled by small GTPases of the Rho family. We indeed found that that inhibition of EphB4 kinase activity in SW1 melanoma cells results in increased cell spreading and decreased cell contractility, suggesting a role for EphB4 in the regulation of RhoA. Consistent with this hypothesis, EphB4 activation with ephrin-B2-Fc in melanoma cells up-regulated RhoA activity, whereas EphB4 inhibition with competitively acting soluble Ephrin-B2-Fc or by overexpressing a dominant negative EphB4 mutant decreased the levels of active RhoA. Moreover, overexpression of dominant negative RhoA mimicked the effects of kinase dead EphB4 and blocked the effects of EphB4 activation on cell migration and actin organization, supporting a model in which the effects of EphB4 on melanoma cell migration are mediated through RhoA-dependent actin remodeling.

Several studies have demonstrated the ability of Eph receptors to activate different Rho-GTPases (20, 55, 56). In melanoma cells, ephrin-A5 has been shown to induce RhoA activation (57), and in colon carcinoma cells, treatment with clustered ephrin-B1 decreased Rac activity (52). The effects of Eph receptors on RhoGTPases can be mediated through recruitment and/or activation of RhoGEFs, which promote exchange of GDP to GTP and activate RhoGTPases. For example, stimulation of EphA receptors in retinal ganglion cells with ephrin-A5 induces growth cone collapse in a RhoA-dependent manner through recruitment and activation of RhoGEF Ephexin (58). Another RhoGEF, Vms-RhoGEF, constitutively binds to the EphA4 receptor and mediates RhoA activation in rat vascular smooth muscle cells stimulated with ephrinA1-Fc (59). Moreover, EphB2 has been shown to control actin rearrangements in dendritic spines of hippocampal neurons through regulation of Rac and Cdc42 by recruiting Kalirin and Intersectin, two exchange factors for Rac1 and Cdc42, respectively (60, 61). Recent reports also show that EphB receptors induce RhoA activation, although how EphB receptors mediate RhoA activity remains to be established (29, 62, 63, 65). EphB receptors might regulate RhoA activity through focal adhesion kinase-dependent activation of p190RhoGEF (62), recruitment of the scaffolding protein Daam1 (65), or indirectly through inhibiting activity of R-Ras (64).

Here we show that the EphB4 promotes melanoma cell migration through RhoA-mediated actin reorganization. Cancer cells with rounded morphology usually show RhoA-dependent motility, whereas well spread cancer cells use a mesenchymal migration mode that depends on Rac activity (46). Consistent with this, inhibition of RhoB pathways has been shown to suppress the migration and invasion of the rounded A375m2 melanoma and LS174T colon carcinoma cells but not of the elongated SW962 squamous carcinoma cells (47). We found that the fast migrating SW1 cells also exhibit a rounded morphology and use a RhoA-dependent motility mode. In contrast, breast cancer cells in which EphB4 signaling inhibits migration and invasion appear to use a mesenchymal Rac-dependent migratory mode (51). Thus, Eph receptors may differentially control the migratory and invasive ability of different cancer cell types through activating RhoA or inhibiting Rac. In conclusion, our findings strongly suggest that EphB receptor signaling plays an important role in melanoma progression by promoting the migratory ability of melanoma cells.

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REFERENCES

1. Bogenrieder, T., and Herlyn, M. (2002) Crit. Rev. Oncol. Hematol. 44, 1–15
2. Dodelet, V. C., and Pasquale, E. B. (2000) Oncogene 19, 5614–5619
3. Lamorte, L., and Park, M. (2001) Surg. Oncol. Clin. N Am. 10, 271–288
4. Easty, D. J., Ganz, S. E., Farr, C. J., Lai, C., Herlyn, M., and Bennett, D. C. (1993) J. Investig. Dermatol. 101, 679–684
5. Easty, D. J., Herlyn, M., and Bennett, D. C. (1995) Int. J. Cancer 60, 129–136
6. Easty, D. J., Guthrie, B. A., Maung, K., Farr, C. J., Lindberg, R. A., Toso, R. J., Herlyn, M., and Bennett, D. C. (1995) Cancer Res. 55, 2528–2532
7. Easty, D. J., Hill, S. P., Hsu, M. Y., Fallowfield, M. E., Flores, V. A., Herlyn, M., and Bennett, D. C. (1999) Int. J. Cancer 84, 494–501
8. Easty, D. J., and Bennett, D. C. (2000) Melanoma Res. 10, 401–411
9. Walker-Daniels, J., Coffman, K., Azimi, M., Rhim, J. S., Bostwick, D. G., Snyder, P., Kerns, B. J., Waters, D. J., and Kinch, M. S. (1999) Prostate 41, 275–280
10. Zelinski, D. P., Zantek, N. D., Stewart, J. C., Irizarry, A. R., and Kinch, M. S. (2001) Cancer Res. 61, 2301–2306
11. Nakamoto, M., and Bergemann, A. D. (2002) Microsc. Res. Tech. 59, 58–67
12. Nikolova, Z., Djonov, V., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1998) J. Cell Sci. 111, 2741–2751

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13. Vogt, T., Stolz, W., Welsh, J., Jung, B., Kerbel, R. S., Kobayashi, H., Landthaler, M., and McClelland, M. (1998) Clin. Cancer Res. 4, 791–797
14. Stephenson, S. A., Slomka, S., Douglas, E. L., Hewett, P. J., and Harding-Jam, J. E. (2001) BMC Mol. Biol. 2, 1–9
15. Takai, N., Miyazaki, T., Fujisawa, K., Nasu, K., and Miyakawa, I. (2001) Oncol. Rep. 8, 567–573
16. Liu, W., Ahmad, S. A., Jung, Y. D., Reinmuth, N., Fan, F., Bucana, C. D., and Ellis, I. M. (2002) Cancer 94, 934–939
17. Liu, W., Jung, Y. D., Ahmad, S. A., McCarty, M. F., Stoeltzing, O., Reinmuth, N., Fan, F., and Ellis, I. M. (2004) Br. J. Cancer 90, 1620–1626
18. Martiny-Baron, G., Stoner, J., Schaffner, F., Esser, N., Eggstein, S., Marme, D., and Augustin, H. G. (2004) Neoplasia 6, 248–257
19. Wu, Q., Suo, Z., Risberg, B., Karlsson, M. G., Villman, K., and Nesland, M. J. (2005) J. Biol. Chem. 280, 1587–1598
20. Cowan, C. W., Shao, Y. R., Sahin, M., Shamah, S. M., Greer, P. L., Gao, S., Griffith, E. C., Brugge, J. S., and Greenberg, M. E. (2005) Neuron 46, 191–204
21. Cowan, C. W., Shao, Y. R., Sahin, M., Shamah, S. M., Lin, M. Z., Greer, P. L., Gao, S., Griffith, E. C., Brugge, J. S., and Greenberg, M. E. (2005) Neuron 46, 205–217
22. Lawrenson, I. D., Wimmer-Kleikamp, S. H., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F., and Lackmann, M. (2002) J. Cell Sci. 115, 233–244
23. Ogita, H., Kunimoto, S., Kamioka, Y., Sawa, H., Masuda, M., and Mochizuki, N. (2003) Cancer Res. 63, 51–60
24. Penzes, P., Beeser, A., Chernoff, J., Schiller, M. R., Epper, B. A., Mains, R. E., and Huganir, R. L. (2003) Neuron 39, 623–274
25. Irie, F., and Yamaguchi, Y. (2002) Nat. Neurosci. 5, 1117–1118
26. Moeller, M. L., Shi, Y., Reichardt, L. F., and Eshel, I. M. (2006) J. Biol. Chem. 281, 1587–1598
27. Ogawa, K., Wada, H., Okada, N., Harada, I., Nakajima, T., Pasquali, E. B., and Tsuwayama, S. (2006) J. Cell Sci. 119, 559–570
28. Dall, M., Richter, M., Gadement, P., and Pasquali, E. B. (2006) J. Cell Sci. 119, 1244–1254
29. Pande, A., Shao, H., Marks, R. M., Pulverini, P. J., and Dritschel, E. M. (1995) Science 268, 567–569
30. Sahin, M., Niska, J. A., Miyamori, H., McDonough, W. S., Wu, J., Sato, H., and Berens, M. E. (2004) Cancer Res. 64, 3179–3185
31. Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O’Connell, S., Cowan, C. W., Hult, L., Goldberg, J. L., Debatant, A., Corfas, G., krull, C. E., and Greenberg, M. E. (2005) Neuron 46, 191–204
32. O’Toole, T., Teli, C., Crotti, D. P., Turner, C. E., and Huynh-Do, U. (2004) J. Biol. Chem. 279, 27965–27970
33. Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O’Connell, S., Cowan, C. W., Hult, L., Goldberg, J. L., Debatant, A., Corfas, G., Krull, C. E., and Greenberg, M. E. (2005) Neuron 46, 205–217
34. Sahin, M., Niska, J. A., Miyamori, H., McDonough, W. S., Wu, J., Sato, H., and Berens, M. E. (2004) Cancer Res. 64, 3179–3185
35. Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O’Connell, S., Cowan, C. W., Hult, L., Goldberg, J. L., Debatant, A., Corfas, G., Krull, C. E., and Greenberg, M. E. (2005) Neuron 46, 191–204
36. O’Toole, T., Teli, C., Crotti, D. P., Turner, C. E., and Huynh-Do, U. (2004) J. Biol. Chem. 279, 27965–27970