EPHA3 FUNCTION IS REGULATED BY MULTIPLE PHOSPHOTYROSINE RESIDUES

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EphA3, a member of the Eph family of tyrosine kinase receptors, has been implicated in a variety of tumors including melanoma, glioblastoma, pancreatic carcinoma, lung and colon cancers. The loss of EphA3 function has been associated with colorectal cancer. To identify potential roles in tumor formation, we examined effects of EphA3 expression and activation on melanoma cell migration, and identified key signal transducer docking sites of the receptor. This analysis showed that the human melanoma cell line WM115 lacks EphA3 expression, while two other melanoma cell lines, WM239A and C8161, both retain expression of the receptor. Activation of EphA3 with ephrin-A5 leads to the inhibition of cell migration of WM239A and C8161, but not WM115 cells. Transfection of EphA3 into WM115 cells restores ephrin-A5-induced inhibition. Furthermore, mutation of tyrosine 602 and 779 each partially reduces the inhibition activity in EphA3-transfected 293A cells. Double mutants completely abolished the inhibition, although the receptor kinase is fully active. Interestingly, the Y602 and 779 mutations are associated with the loss of Erk inhibition and RhoA activation, respectively. These observations suggest that EphA3 receptor acts as inhibitors to the migration of melanoma cells. In addition, the inhibition of cell migration requires both Y602 and Y779, and different EphA3 phosphotyrosine docking sites regulate distinct signal transduction pathways.

The Eph family tyrosine kinase receptors have been associated with many different types of cancers (reviewed in 1 - 3). EphA2 has been shown to facilitate angiogenesis-dependent metastatic spread upon ephrin-A1 stimulation in animal models (4). Clinically, EphA2 overexpression in esophageal squamous cell carcinoma was significantly correlated with regional lymph node metastasis (5). Overexpression of ephrin-B2, a ligand for EphB receptors, in B16 melanoma cells enhanced integrin-mediated ECM-attachment and migration (6). EphB2 positively regulates glioma cell adhesion, growth, and invasion via R-Ras signaling (7). In contrast to promote tumor metastasis and cancer cell line migration, Eph receptors may also regulate the cell motility negatively. Hafner and coworkers compared EphB6 mRNA level in benign nevi, primary melanomas, and metastatic melanomas, and found a progressive and significant reduction of EphB6 mRNA expression in more advanced tumors (8). Similarly, Fox and coworkers found that EphA3 along with EphB3 and ephrin-A3 were over-expressed in normal prostate, while EphA3 was not detectable in the primary prostate cancer and only in one out of four metastatic prostate cancer cell lines assayed (9). These data implicate complex roles of Eph receptors in tumorigenesis instead of simply promoting or inhibiting cancer progression.

EphA3 was first identified as a novel tumor-associated receptor-type tyrosine kinase (Hek) from an acute pre-B cell lymphoblastic leukemia cell line (LK63) in 1992 (10, 11). EphA3 has been shown to regulate several developmental functions including the formation of axon pathways and the maturation of synapses (12, 13), heart development (14), and leukocyte adhesion of endothelia (15). EphA3 is overexpressed in clinical cancer samples and cell lines (9, 16). Particularly, 75% - 80% of melanoma samples had positive expression of EphA3, while it was scarcely detectable in normal adult melanocytes (17). In addition to melanoma, EphA3 expression was also reported in various other cancer cell lines such as small cell lung cancer, non-small cell lung cancer, renal cell carcinomas, prostate carcinoma, neuroblastoma, and lymphoblastic leukemia (3, 9, 17). Accumulating evidence shows that EphA3

may have multiple functions in melanoma and other cancerous development (9).

It remains unclear what roles EphA3 may play and what are the signal transduction pathways that mediate the functions. The present study explores the roles of EphA3 in regulating cell migration in melanoma cell lines and transfected 293A cells, using site-directed mutagenesis of tyrosine residues of the receptor. Our results are the first to show that ephrin-A5 inhibits melanoma cell migration through interaction with EphA3. Our results also demonstrated that the phosphorylation of juxtamembrane conserved tyrosine residues Y596 and Y602 as well as catalytic domain conserved tyrosine Y779 is required for the suppression of cell migration. These results suggest an interesting model for EphA3 function in which different signaling pathways docked at different phsophorylated tyrosine residues collaborate to regulate cell migration.

Materials and Methods

Site-directed mutagenesis
Mouse EphA3 cDNA was a gift from Dr. Elena B. Pasquale. Desired mutations were generated using a Site-Directed Mutagenesis kit (BD Bioscience, Palo Alto, CA) following the instructions of the manufacturer, and confirmed by DNA sequence analysis. The primers used in the mutagenesis are listed in Table 1.

Cell culture and transfection
Melanoma tumor cells (WM115, WM239A, and C8161) were cultured in RPMI-1640 medium plus 10% fetal bovine serum at 37 °C. HEK 293A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum at 37 °C. DNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions.

Ephrin-A5 preparation and treatment
Ephrin-A5-Fc fusion protein was purchased from R&D Systems (Minneapolis, MN). To form clustered ephrin-A5, ephrin-A5-Fc was cross-linked with anti-human Fc IgG (Jackson Immuno-Research, West Grove, PA) at 1/5 ratio in ugs for 2 hours at 37 °C as described in our previous studies (18). Ephrin-A5 treatment was carried out at 37°C for 15 minutes.

Immunoprecipitation and in vitro kinase assay
Transfected HEK 293A cells were lyzed with the cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM sodium vanadate, and protease inhibitors). Cell lysates were then centrifuged to clear off cell debris for 10 minutes at 16,000g at 4°C. The supernatants were incubated with a rabbit polyclonal anti-EphA3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 hours and then with protein A agarose (Millipore, Billerica, MA) for 1 hour at 4°C. The beads were collected and washed three times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100) and then twice with kinase buffer (25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM sodium vanadate). Samples were incubated in kinase buffer containing 10 μg of acid denatured Enolase (Sigma-Aldrich, St. Louis, MO) and 50 μM ATP at 30 °C for 30 minutes. The reaction was stopped by adding SDS sample loading buffer and boiling. The reaction products were analyzed using Western blot technique.

Western Blot Analysis
Proteins were separated by 10% SDS-polyacrylamide gel and were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% BSA in Tris-buffered saline containing 0.05% Tween-20 (TBST), and then incubated with the primary antibody. The primary antibodies were detected with horseradish peroxidase-conjugated second antibodies (Sigma-Aldrich, St. Louis, MO). The antigen-antibody complex was visualized using a chemiluminescence detection kit (Roche, Indianapolis, IN). The density of each protein band was scanned and the data were subjected to statistical analysis. Anti-phosphotyrosine, anti-phospho-Erk/MAPK, and anti-total Erk/MAPK antibodies were purchased from Cell Signaling (Danvers, MA). Anti-EphA3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell migration assay

For wound-healing assay, 5×10^5 melanoma or transfected 293A cells were seeded on fibronectin-coated dishes and cultured for 1 day. The cell monolayer was scratched with micropipette tips and images were captured at 0 and indicated hours after wounding using a Nikon microscope. To quantify the results, cells expressing GFP with or without EphA3 that migrated into the gap were counted. For Transwell migration assay, 2×10^4 cells were re-plated onto the upper chamber of a Transwell filter with 8 μm pores (Corning Inc.- Life Sciences, Wilkes-Barre, PA) coated with 10 μg/ml fibronectin. After 16 hours, cells were fixed with 4% paraformaldehyde in PBS. Non-migrated cells on the upper side of the filter were wiped off with a cotton swab. Melanoma cells were stained with 4% crystal violet. Transfected WM115 and 293A cells were analyzed under a Nikon fluorescence microscope. Same amount of cells was plated to fibronectin-coated plates in parallel to confirm cell counting. Relative cell migration was determined by the number of the migrated cells normalized to the total number of the cells adhering to fibronectin-coated plates. For transfected cultures, only GFP-expressing cells were counted. For each experiment, the number of cells in five random fields on the underside of the filter was counted and two to six independent assays were performed.

RhoA Activation Assay

RhoA activity was analyzed using Active Rho Pull-Down and Detection Kit from Pierce Biotechnology, Inc. (Rockford, IL) following manufacturer's instructions. Briefly, transfected 293A cells were treated with or without cross-linked ephrin-A5 for 10 minutes. After the treatment, the cells were lyzed with Lysis/Binding/Washing Buffer provided with the kit. The cell lysate containing 1 mg of total protein was incubated with 400 μg of GST-Rhotekin-RBD and glutathione agarose beads at 4 °C for 1 hour with agitation. The beads were washed three times with the same lysis buffer and boiled at 100 °C for 5 minutes in 2x SDS PAGE sample buffer. The precipitates were analyzed by Western Blot analysis using the anti-RhoA antibody provided with the kit.

Statistical Analysis

For comparison of multiple groups, One-Way ANOVA was used and followed by Bonferroni's post hoc test of selected pairs. The α level (0.05) used in post hoc analyses was adjusted by the number of comparisons (Bonferroni correction).

Results

EphA3 expression and activation inhibits melanoma cell migration. To examine roles of Eph receptors and ephrins in the regulation of tumor cell migration, we analyzed effects of ephrin-A5 on the migration of three different melanoma cell lines, using transwell and wound-healing cell migration assays. Melanoma cell line WM115 was derived from a vertical growth phase (VGP) melanoma and WM239A cells were originated from metastasized lymph-nodes from a melanoma patient (19). C8161 is an amelanotic human melanoma cell line characterized with wide metastasis after inoculation into experimental animals (20). Ephrin-A5 treatment resulted in substantial reduction of transwell cell migration in two cell lines, WM239A and C8161, but not in WM115 cells (Fig. 1A). Consistent with this observation, Ephrin-A5 treatment also resulted in a 70% and 80% decreases in the migration of WM293A and C8161 cells, respectively, in wound healing assay (Fig. 1B). WM115 cells grow with many micro-clumps instead of an even monolayer, which interferes with cell counting in wound healing assay. Thus, WM115 was not included in the wound healing assay.

EphA receptor expression has been shown to associate with melanoma (9). To examine whether the differential effects of ephrin-A5 on melanoma cell migration relate to EphA3 expression and activation, the melanoma cell lines were treated with cross-linked ephrin-A5 for 15 min. Cell lysates were analyzed by means of Western Blot using an anti-phosphotyrosine antibody to identify Eph receptor phosphorylation. This analysis showed that ephrin-A5 induced phosphorylation of Eph receptor(s) in WM239A and C8161, but not in WM115 cells (Fig. 1C). The presence of activated Eph receptor phosphorylation is correlated with positive expression of EphA3 in WM239A and C8161 cells (Fig. 1C). WM115 cells showed no expression of EphA3, and no phosphorylation of any Eph receptors, suggesting the lack of response to ephrin-A5 is due to the lack

of ephrin-A5 sensitive Eph receptors. To critically test the idea that EphA3 expression was responsible for ephrin-A5-induced inhibition of cell migration in melanoma cells, we transfected EphA3 into WM115 cells and tested the effects on migration (Fig. 2). Treatment of EphA3-expressing WM115 cells with ephrin-A5 led to a 40% reduction of cell migration (Fig. 2B). To test whether EphA3 kinase activity was required, lysine 653 was mutated to arginine. Substitution of this conserved lysine residue has been shown to inactivate Eph receptor kinase activity (21). Transfection of the kinase-dead EphA3 mutant (K653R) showed no effect on WM115 cell migration, indicating that EphA3 is responsible for the inhibition of cell migration by ephrin-A5, and that the kinase activity is required.

Specific tyrosine residues of EphA3 are required for ephrin-A5-induced inhibition of cell migration. To reveal underlying mechanisms by which EphA3 receptor relays ephrin-A5 signals, we mutagenized tyrosine residues in the cytoplasmic domain of EphA3. There are a total of 15 tyrosine residues in this domain, and each was mutated to either phenylalanine (F) or glutamic acid (E) (Table 1). Mutation of tyrosine into phenylalanine prevents phosphorylation of the residue, while into glutamic acid adds a negative charge that mimics phosphorylated tyrosine (22). The mutants were transfected into human embryonic kidney (HEK) 293A cells, and assayed for their effects on cell migration in the presence of ephrin-A5 using would-healing and transwell assays (Fig. 3). Transfection of wild type EphA3 resulted in efficient inhibition of cell migration by ephrin-A5 in both assays (Fig. 3). In contrast, ephrin-A5 had no effects on the migration of cells transfected with the kinase-dead mutant K653R (Fig. 3). Furthermore, our analyses showed that substitution of juxtamembrane tyrosine 596 with phenylalanine completely abolished effects on cell migration, while two other mutations, Y602F and Y779F, resulted in partial loss of the function in both assays (Fig. 3B, C). Mutations in other tyrosine residues did not have any obvious effects (Fig. 3B, C). These results indicate that both the tyrosine kinase activity and the phosphorylation of 602 and 779 are crucial for the inhibition of cell migration.

Y596 regulates EphA3 kinase activity, and Y602 and Y779 are major phosphorylation sites. To identify the mechanisms by which the mutations disrupt EphA3 activity, we examined effects of mutations on the levels of receptor tyrosine phosphorylation and on its kinase activity. The EphA3 mutants were transfected into 293A cells and two days later, the transfected cells were lysed and EphA3 was immunoprecipitated with a rabbit anti-EphA3 polyclonal antibody. The precipitated EphA3 was then analyzed for tyrosine phosphorylation using Western blot technique with an anti-phophotyrosine-specific antibody. This analysis showed that Y596F mutant exhibited a total loss of autophosphorylation, and so did the kinase-dead mutant K653R (Fig. 4), suggesting a loss of kinase activity in these two mutants. Several other mutants including Y602F, Y736F, Y742F, Y779F, Y798F, Y825F, Y841F, and Y937F, all showed reduction in autophosphorylation levels (Fig. 4). However, Y602F and Y779F showed more substantial decreases than the other mutants except Y596F and K653R (Fig. 4B). These observations indicate that multiple tyrosines are phosphorylated and tyrosine 596 may regulate EphA3 kinase activity.

The decrease of tyrosine phosphorylation of EphA3 mutants could be due either to the loss of kinase activity or phosphorylation sites. To differentiate between these two possibilities, we assayed the kinase activity using enolase as a substrate. This analysis showed that Y596F, just like the kinase-dead mutant K653R, did not have any detectable kinase activity, while all other tyrosine mutants have normal enzymatic activity (Fig. 5A). Phosphorylation of the juxtamembrane domain tyrosine residues has been shown to play key roles in releasing intramolecular inhibition of the Eph receptor kinases (22). To study whether Y596 phosphorylation is required for EphA3 activation, we replaced this tyrosine residue with glutamic acid and assayed for the effects on the kinase activity. The negative charge of glutamic acid residue in the juxtamembrane region has been shown to mimic that of phosphorylated tyrosine residues (22). Replacement of Y596 with a glutamic acid residue completely restored EphA3 kinase activity (Fig. 5B), suggesting that the negative charge on Y596 is crucial to activate EphA3. In contrast, phosphorylation of Y602 and 779 is not required for EphA3 kinase activity,

since Y602F, Y779F, the double mutants Y596E/602F and Y602/779F, as well as the triple mutant Y596E/602/779F all possessed normal kinase activity (Fig. 5B). Although Y602 and 779 were not required for kinase activation, Y602F and Y779F mutations had significantly reduced levels of receptor tyrosine phosphorylation (Figs. 4 and 5C), indicating that these two tyrosine residues were major EphA3 phosphorylation sites. In addition, although Y596E mutant was fully active in phosphorylating enolase, the receptor tyrosine phosphorylation was also significantly reduced (Figs. 5B and C), suggesting that Y596 was another major phosphorylation site. Consistent with these conclusions, tyrosine phosphorylation signals of the double mutants Y596E/602F and Y602/779F and the triple mutant Y596E/602/779F were barely detectable (Fig. 5C). Taken together these studies provide evidence that Y596 is required for EphA3 activation, and together with Y602 and 779, these three tyrosine residues are major phosphorylation sites.

Cooperative regulation of cell migration by Y602 and Y779. Since Y602F and Y779F mutants each reduced EphA3-mediated inhibition of cell migration to about half of the wild type (Figs. 3B and C), we asked whether these two tyrosine residues cooperatively regulate cell migration. Indeed, double EphA3 mutant Y602/779F lost all activity in mediating inhibition of cell migration in both the wound-healing and transwell assays (Fig. 6). Similarly, triple mutant Y596E/602/779F also lost all activity in mediating inhibition of cell migration. In contrast, double mutant Y596E/602F had similar activity as that of Y602F single mutant (Fig. 3 and Fig. 6), which was consistent with the idea that phosphorylation of Y596 merely serves to activate the receptor kinase. These observations support the notion that Y602 and 779 together provide docking sites for downstream signal transduction pathways to regulate cell migration.

Tyrosine 602 is required for the inhibition of Erk phosphorylation by ephrin-A5. To explore downstream pathways docked onto these critical phosphorylated tyrosine residues of activated EphA3, we examined effects of tyrosine mutations on Erk phosphorylation. We observed that in the presence of ephrin-A5, Erk phosphorylation was significantly ($\alpha < 0.05$) reduced in the 293A cells expressing wild type EphA3 (Fig. 7). Analysis of the tyrosine mutants showed that the two kinase-inactive mutants, Y596F and K653R, completely lacked Erk inhibitory activity (Fig. 7B). Interestingly, Y602F also failed to inhibit Erk activity. Mutations of all other tyrosine residues including Y779 showed no defects in inhibiting Erk phosphorylation (Fig. 7B). These observations indicate that only Y602 is required for transmitting signals for Erk inhibition.

Tyrosine 779 mediates the activation of the small GTPase RhoA. Eph receptor activation has been shown to activate the small GTPase protein RhoA (21, 23 - 25). We therefore examined effects of the mutations on RhoA activation. Stimulation of wild type EphA3 with ephrin-A5 led to a 38% increase of activated RhoA (Fig. 8). Similarly, Y602 mutant showed similar RhoA activation as the wild type, indicating that this tyrosine residue is not critical in the activation of RhoA. Intriguingly, Y779F abolished the ability of EphA3 to activate RhoA (Fig. 8). These results indicate that Y779 phosphorylation is crucial for RhoA activation, while Y602 is not.

Discussion

Activation of several Eph receptors has been shown to regulate cell migration both *in vivo* and *in vitro* (reviewed in 26). However, the molecular mechanisms underlying this function have not been well characterized. In this study, we made the observation that EphA3 activation regulates cell migration, and mapped critical tyrosine residues that control kinase activation or serve as docking sites for downstream signaling pathways. These studies identified Y596 phosphorylation as necessary for EphA3 kinase activation and Y602 and 779 important to regulate cell migration co-operatively. Our studies here, along with previous studies from other laboratories, show that activation of Eph receptors regulates multiple signal transduction pathways, which are mediated by distinct tyrosine residues, and these different pathways may regulate the same biological function co-operatively or regulate distinct processes.

EphA3 regulates melanoma cell migration. In the developing vertebrate hindbrain and somites, interaction between Eph receptors and ligands has been shown to maintain boundaries between different tissue segments by restricting cell migration and mingling. In addition, levels of Eph receptor and ligand expression may underlie the direction of cell migration and the positioning of specific types of cells (27). *In vitro*, ephrin-A1 inhibits migration of Madin-Darby canin kidney (MDCK) cells induced by hepatocyte growth factor (HGF) (28). In addition, ephrin-B1 also inhibited migration of colorectal epithelial cells (29). Ephrin-A1 was reported to inhibit CD 4+ T-cell migration when using immobilized ligand (30), but promote migration when used in soluble form (31). Here we extended these previous observations to show that ephrin-A5 inhibits migration of melanoma cells expressing EphA3 receptor. Mutations of EphA3 have been detected in melanoma, lung cancer and colon cancer (32 - 34), and the protein is overexpressed in several tumors including melanoma, lung, kidney, and prostate cancer (17, 35). A previous study by Lawrenson et al. (35) showed that stimulation of melanoma cell expressing EphA3 resulted in retraction of cellular protrusions, membrane blebbing and cell detachment, through activation of RhoA. It is possible the same biochemical processes induced by EphA3 activation also mediate inhibition of cell migration as shown in the present study, since loss of RhoA activation is associated with the loss of phosphorylation in tyrosine 779 in Y779F mutant (Fig. 3; Fig. 8). The ability of ephrin-A5 to regulate both cell morphology and migration of melanoma cells suggests that EphA3 may contribute to regulate tumor progression and metastasis. This notion is supported by a previous study showing that EphA3 is a major tumor antigen targeted by lytic CD4 T-cells (17).

Tyrosine residues regulate Eph receptor activity. Our analyses show that the phosphorylation of the juxtamembrane tyrosine 596 is necessary for EphA3 activation. Replacing Y596 with phenylalanine inactivates EphA3. Both autophosphorylation assay and kinase activity assay using the exogenous substrate enolase yielded consistent results that support this conclusion. However, the kinase activity is fully restored by a glutamic acid replacement in the tyrosine 596 mutant Y596E (Fig. 5), which mimics the negative charge of phosphorylated tyrosine at this position (36). The Y596E mutant displayed a relatively weaker tyrosine phosphorylation on EphA3 receptor than the wild type EphA3, suggesting that in the wild type EphA3, Y596 is one of the major phosphorylation sites (Fig. 5). However, a study by Lawrenson and colleagues (35) reported no loss of kinase activity when tyrosine 596 is mutated to phenylalanine in human EphA3 as measured by autophosphorylation. The cause of this difference is not known at present, although it could be due to differences between mouse EphA3 receptor (this study) and human EphA3 receptor (35). A critical role of the juxtamembrane domain tyrosine residues in regulating the kinase activity is supported by studies of other Eph receptors (36). Mutagenesis of the corresponding tyrosine at position 605 of EphB2 resulted in a reduction in both the kinase activity and autophosphorylation levels (36), consistent with our observations reported here. Interestingly, mutation of a second juxtamembrane region tyrosine (residue 611) of EphB2 also reduces the kinase activity. Mutation of both residues led to complete inactivation of EphB2 kinase activity (36). In contrast to EphB2, mutation of the Y602 in EphA3 corresponding to tyrosine 611 in EphB2 does not affect tyrosine kinase activity (Fig. 4). Thus, although some variation exists among different Eph receptors, phosphorylation of the juxtamembrane tyrosine residues is necessary for the activation of the Eph receptors. Chrystallographic studies indicate that the juxtamembrane domain of Eph receptors interacts with the kinase domain to inhibit kinase activation. Phosphorylation of juxtamembrane tyrosines results in the release of binding to the kinase domain and allows kinase activation (22). Our findings here are consistent with this model of Eph receptor activation, since phosphorylation of Y596 may serve to activate the kinase activity. This is further supported by the observation that substituting Y596 with glutamic acid which mimics the negative charge of phosphotyrosine, restores the ability of EphA3 to be activated.

Different phosphotyrosine residues collaborate in the regulation of cell migration. The current study identified two cytoplasmic tyrosine residues of

EphA3 that together regulate cell migration, Y602 and Y779. Mutation of any of these two residues alone leads to a partial reduction in EphA3 biological activity in inhibiting cell migration (Fig. 3; Fig. 6). Mutation of both residues leads to a complete loss of biological activity, although the kinase activity of the receptor remains intact (Fig. 5). This observation indicates that Y602 and 779 co-operate to regulate cell migration. Phosphorylated tyrosine residues have been known to serve as docking sites for SH (Src Homology) 2 domain-containing adaptor proteins to allow transduction of signals downstream (35, 37). Substitution of EphA3 Y602 and 779 with phenylalanine maintained the kinase activity, but prevents docking of down-stream adaptor proteins (Fig. 4; Fig. 5), supporting their roles in linking EphA3 receptor with critical downstream signaling pathways that regulate cell migration.

Several different adaptor proteins containing the SH2 domain have been shown to bind to the corresponding juxtamembrane domain tyrosine residues on Eph receptors including RasGAP, Src, Abl, Nck and Vav (38 - 42). Among these proteins, RasGAP has been shown to inhibit R-ras activity and regulate cell migration and growth cone collapse (43 - 45). In addition, Src family kinases are also required for growth cone collapse (46). It is likely that Eph receptors use similar biochemical pathways in the regulation of cell migration, and the inhibitory effects mediated by EphA3 Y602 may be due to interaction with either RasGAP, Src family kinases, or both. The phosphorylated Y779 has been shown to bind to the SH2 domain-containing Crk adaptor protein (35). Interaction of Crk with Y779 is required for cell rounding effects induced by EphA3 activation (35). Activation of the small GTPase RhoA is a downtream event required by both cell rounding and growth cone collapse induced by ephrin-A5 through the activation of EphA receptors, and RhoA activiation depends on Crk activity (23, 25, 35). We show here that Y779 mutation blocks RhoA activation while the Y602 mutation does not, suggesting that the loss of Crk-mediated RhoA activation is responsible for the decrease of EphA3 activity in regulating cell migration. Thus, it is likely that both the R-Ras and RhoA pathways contribute to the regulation of cell migration by EphA3.

In light of the notion that RasGAP links Eph receptor to inhibition of Erk, we analyzed the activation of the Erk under EphA3 activation. Our results have shown that EphA3 activation down regulates Erk activity. The effects of Eph receptor activation on Erk activity have been controversial (47 - 49). Our results are consistent with several earlier studies which demonstrated that Eph receptor activation leads to inhibition of Erk activity (7, 18, 38, 47, 48, 50). However, a number of reports also showed that Erk was activated by Eph receptor activation (49, 51 - 53). Whether Eph receptors activate or inhibit Erk activity may depend on the presence of different downstream adaptor proteins. Erk inhibition has been shown to be mediated by recruitment of RasGAP to the activated Eph receptor (38) while Erk activation was associated with Grb2 (38, 49). RasGAP inactivates while Grb2 stimulates the Ras-Erk pathway, and differential recruitment of these different proteins to Eph receptors may result in opposite effects on Erk activity. Consistent with this view, artificial switching of the RasGap binding site of EphB2 to a Grb2 binding site indeed leads to a switch of effects on Erk activity (38).

The involvement of Erk in cell motility and cancer metastasis has been noticed (54, reviewed in 55). In the present study, although loss of Erk inhibition by EphA3 mutant Y602F associates with the loss of inhibition of cell migration (Fig. 3B and C; Fig. 7B), Erk inhibition is insufficient for inhibiting cell migration. Furthermore, constitutive Erk activation failed to block ephrin-A5-induced axon growth cone collapse, suggesting that the down-regulation of Erk activity is either not required or not sufficient for mediating eprhin-A5 activity (18, 44). Thus, the failure of Y602F and Y779F mutants in regulating cell migration is more likely due to the loss of RasGAP binding and consequently the failure of R-Ras inhibition (44), and the failure of RhoA activation. Roles of Erk inhibition by Eph receptor activation remain to be further elucidated, which should be an interesting area for future studies.

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Figure Legends

Figure 1. Effects of ephrin-A5 on melanoma cell migration. (A) Transwell migration assay. The migration of WM239A and C8161, but not WM115, cells was significantly reduced after treatment with cross-linked ephrin-A5 as compared with the untreated controls. (B) Wound healing assay. The cells were seeded on fibronectin-coated dishes. After one day of culture, cell monolayer was scratched with a micropipette tip and images were captured at 0 and 18 hours in WM239A, and 0 and 6 hours in C8161. (C) Expression of EphA3 in melanoma cells. Melanoma cells were treated with pre-clustered ephrin-A5 or human IgG as control. Cell lysates were fractionated with 10% SDS-polyacrylamide gel, and analyzed for the expression and activation of EphA3 using Western blot technique with anti-EphA3 and anti-phosphotyrosine antibodies. The same blot was reprobed for actin expression to control protein loading. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies.

Figure 2. EphA3 expression confers response to ephrin-A5 in WM115 cells. (A) Western blot analysis of transfected WM115 cells. Only the wild type EphA3 showed tyrosine kinase activation after transfection. (B) Transwell migration assay of transfected WM115 cells. Bars represent means of cell counts from 10 different fields in two independent experiments. Error bars indicate the standard deviation (SD). * indicates $\alpha < 0.05$ vs vector control (one-way ANOVA followed with Bonferroni test for selected pairs).

Figure 3. Effects of tyrosine mutations on EphA3 function. (A) Inhibition of cell migration by EphA3 is kinase-dependent. 293A cells were transfected with wild type EphA3, kinase-dead mutant K653R, or pCMV vector only, and assayed for effects on transwell cell migration. Images were taken at 0 and 24 hours after wounding. (B) Effects of EphA3 tyrosine mutants on cell migration in the wound-healing assay. (C) Effects of EphA3 tyrosine mutations on cell migration in the Transwell assay. In both assays, cell counts were normalized to that of the vector controls. Bars represent the means of six independent experiments. Error bars indicate standard deviation (SD). * indicates $\alpha < 0.05$ vs wild type (WT) (one-way ANOVA followed with Bonferroni test for selected pairs).

Figure 4. Effects of tyrosine mutations on EphA3 autophosphorylation. (A) Reduction of EphA3 autophosphorylation in several tyrosine mutants. EphA3 clones were transfected into 293A cells, treated with clustered ephrin-A5 for 15 min, and immunoprecipited 2 days later with an anti-EphA2 antibody. The precipitated EphA2 protein was examined for tyrosine phosphorylation using an anti-phosphotyrosine antibody Western blot analysis. Top panel: Western blot anlaysis with anti-phosphotyrosine antibody; Bottom panel: the same membrane was reprobed with anti-EphA3 antibody to control protein loading. (B) Quantification of the relative intensities of receptor autophosphorylation with optical intensity scanning. Data were normalized to cells harboring wild type EphA3 (WT). Bars represent the means of three independent experiments; error bars indicate SD. Asterisk * indicates $\alpha < 0.05$ vs wild type (WT) (one-way ANOVA followed with Bonferroni test for selected pairs).

Figure 5. Kinase activity of various EphA3 mutants. EphA3 were immunoprecipitated from transfected 293A cells and incubated with 10 μg of acid denatured Enolase. The reaction was stopped by adding SDS sample loading buffer and boiling. The reaction products were analyzed by immunoblotting with anti-phosphotyrosine antibody (upper panel) and anti-EphA3 antibody (lower panel), respectively. (A) Effects of the tyrosine to phenylalanine mutations on the kinase activity. (B) Tyrosine kinase activity of the double and triple EphA3 mutants. (C) Receptor autophophorylation levels in the double and triple EphA3 mutants.

Figure 6. Effects of the double and triple EphA3 mutants on cell migration. (A) Wound healing assay. (B) Transwell assay. Cell counts are normalized to that of the vector controls (Vector). Bars represent means

from six independent experiments; error bars represent SD. * indicates $\alpha < 0.05$ vs vector control (one-way ANOVA followed with Bonferroni test for selected pairs).

Figure 7. Effects of EphA3 mutants on Erk activity. (A) Wild type EphA3 inhibits Erk activity induced by EGF stimulation. 293A cells transfected with EphA3 were stimulated with EGF alone or EGF in combination with ephrin-A5. The stimulated cells were lysed and the cell lysates analyzed using Western blot analysis with anti-phosphorylated Erk and anti-total Erk antibodies. (B) Quantification of the relative Erk signal intensity. Data were normalized to that of the 239A cells containing the empty vector and without ephrin-A5 treatment (empty box of "Con"). Bars represent the means of at least three independent experiments; error bars indicate SD. * indicates $\alpha < 0.05$, EGF treatment (empty bar) vs the corresponding group treated with EGF plus ephrin-A5 (filled bar) (one-way ANOVA followed with Bonferroni test for the each paired samples).

Figure 8. EphA3 Y779F mutant fails to activate RhoA. (A) 293A cells transfected with EphA3 or controls were treated with or without ephrin-A5, and then lysed. Activated RhoA protein was precipitated using GST-Rhotekin-RBD and glutathione agarose beads. The precipitates were analyzed with Western blot analysis using anti-RhoA antibody. (B) Quantitative analysis of active RhoA signals.

Table 1. Mutagenic primers used in the site-directed mutagenesis

Mutations	**Mutagenic Primers** (Mutated nucleotides are underlined)
Y569F	5'-GGAGGTTTTGTGGCTTTCACAAGTCAAAACACAG-3'
Y596F	5'-CTCAGGACCTTTGTTGATCCAC-3'
Y602F	5'-GATCCACATACATTTGAAGACCCTACTC-3'
Y659F	5'-CCCTGAAAGTAGGGTTTACCGAGAAACAGAGG-3'
Y701F	5'-GTCACGGAATTCATGGAGAATGGC-3'
Y736F	5'-GCATCAGGTATGAAATTCCTCTCAGATATGGG-3'
Y742F	5'-GATATGGGCTTCGTCCACCGG-3'
Y779F	5'-GAAGCTGCTTTCACAACCAGG-3'
Y798F	5'-GCAATTGCCTTCCGCAAGTTC-3'
Y810F	5'-CCAGCGATGTATGGAGTTTCGGGATTGTTC-3'
Y820F	5'-GGGAAGTGATGTCTTTCGGAGAAAGGCC-3'
Y825F	5'-GAAAGGCCATTCTGGGAGATG-3'
Y841F	5'-GGATGAGCGGTTTCGCCTGCCACC-3'
Y854F	5'-CCAGCTGCCTTGTTTCAGTTGATGTTGG-3'
Y937F	5'-GGCGTCGAATTCAGCTCCTGTG-3'
Y596E	5'-GGTCTCAGGACCGAGGTTGATCCACATAC-3'
Y602E	5'-GATCCACATACAGAGGAAGACCCTACTC-3'
Y779E	5'-AGAAGCTGCTGAGACAACCAGGGG-3'
K653R	5'-GGTGGCCATAAGGACCCTGAAAG-3'

Figure 1

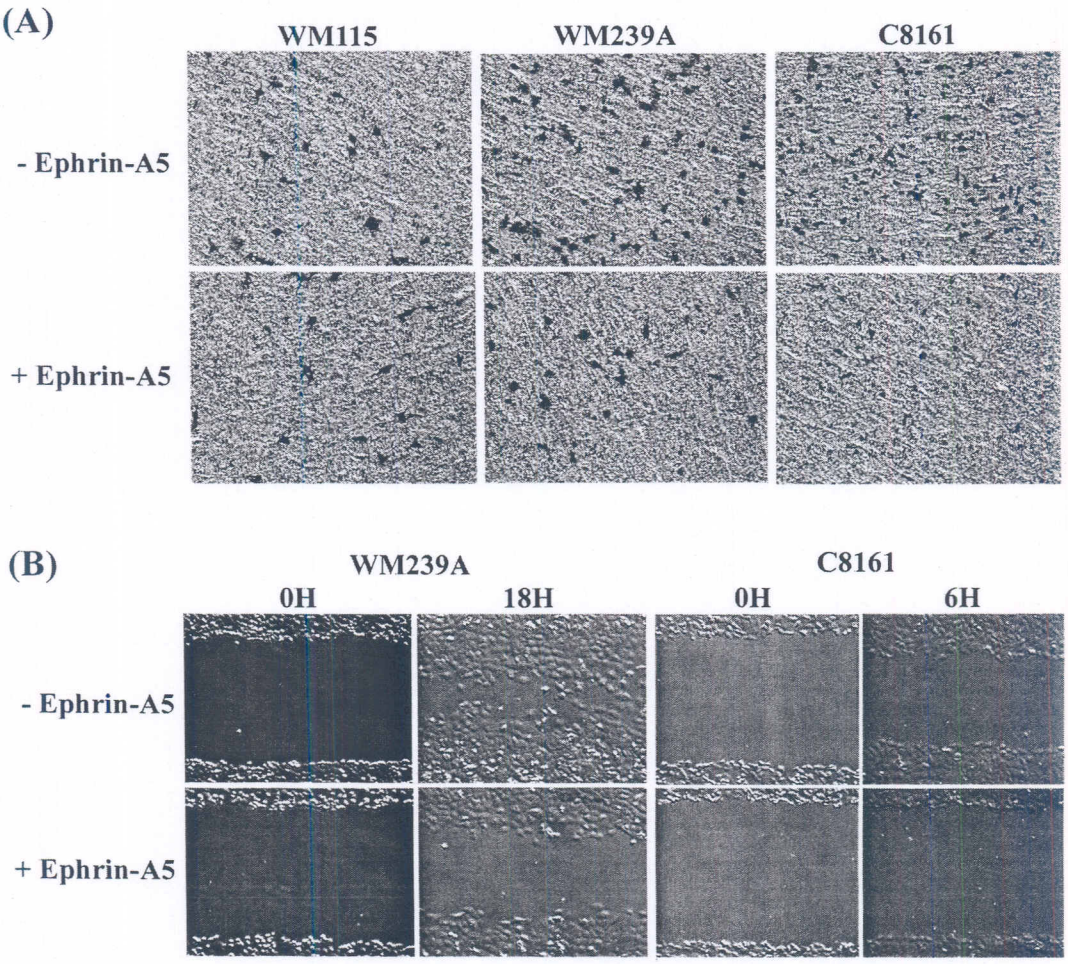

(A)
WM115
WM239A
C8161
- Ephrin-A5
+ Ephrin-A5
(B)
WM239A
C8161
0H
18H
0H
6H
- Ephrin-A5
+ Ephrin-A5
(C)
WM239A
C8161
WM115
Ephrin-A5
- + - + - +
anti-p-Y
anti-EphA3
anti-Actin

Figure 2

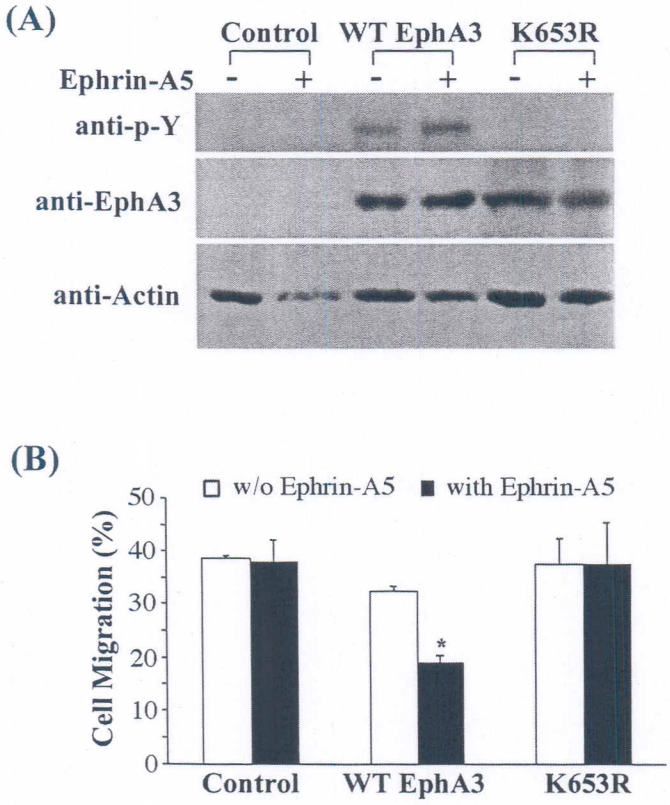

(A)
Control
WT EphA3
K653R
Ephrin-A5
- + - + - +
anti-p-Y
anti-EphA3
anti-Actin
(B)
w/o Ephrin-A5
with Ephrin-A5
Cell Migration (%)
50
40
30
20
10
0
*
Control
WT EphA3
K653R

Figure 3

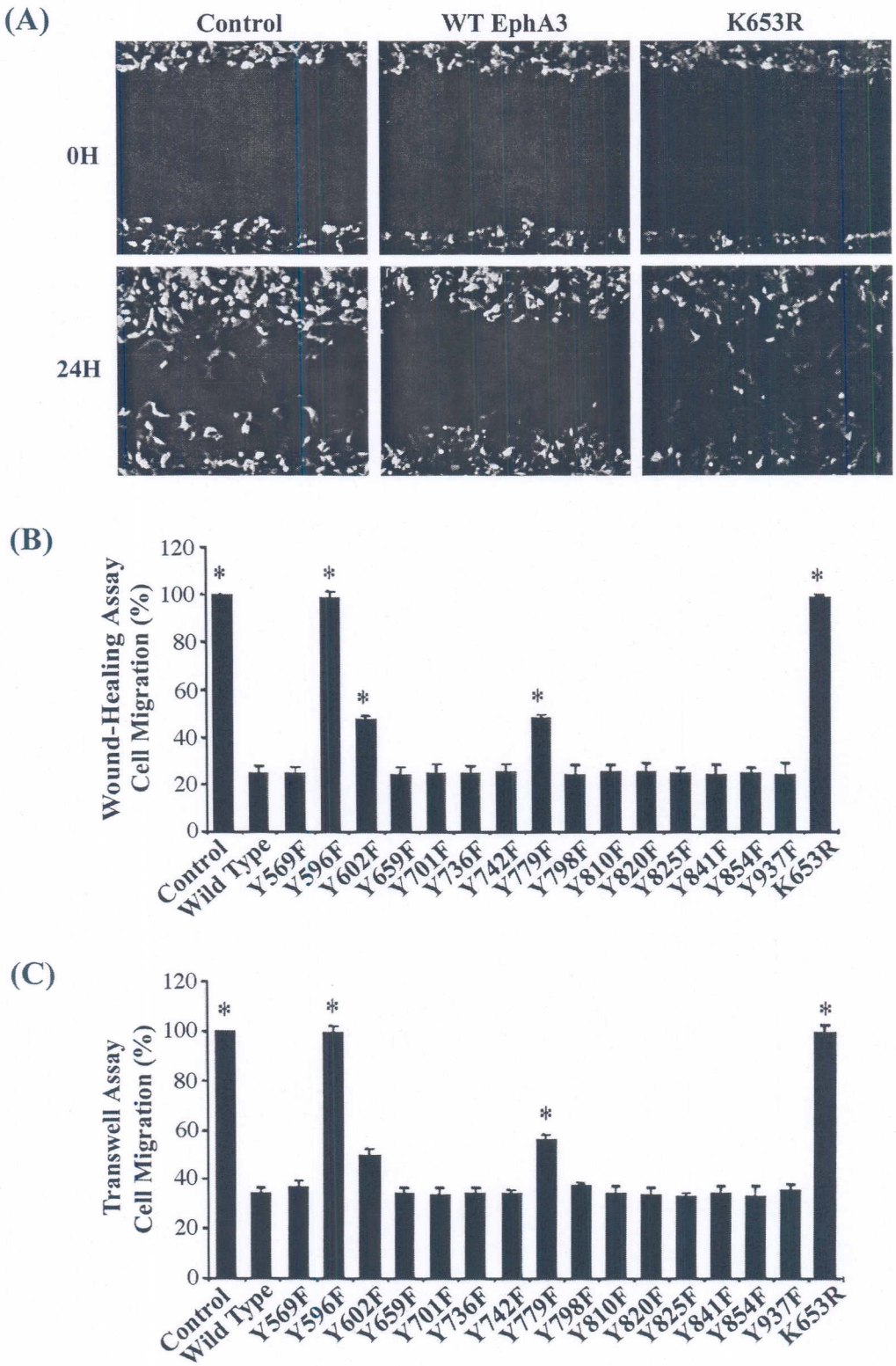

(A)
Control
WT EphA3
K653R
0H
24H
(B)
Wound-Healing Assay
Cell Migration (%)
120
100
80
60
40
20
0
Control
Wild Type
Y569F
Y596F
Y602F
Y659F
Y701F
Y736F
Y742F
Y779F
Y798F
Y810F
Y820F
Y825F
Y841F
Y854F
Y937F
K653R
(C)
Transwell Assay
Cell Migration (%)
120
100
80
60
40
20
0
Control
Wild Type
Y569F
Y596F
Y602F
Y659F
Y701F
Y736F
Y742F
Y779F
Y798F
Y810F
Y820F
Y825F
Y841F
Y854F
Y937F
K653R

Figure 4

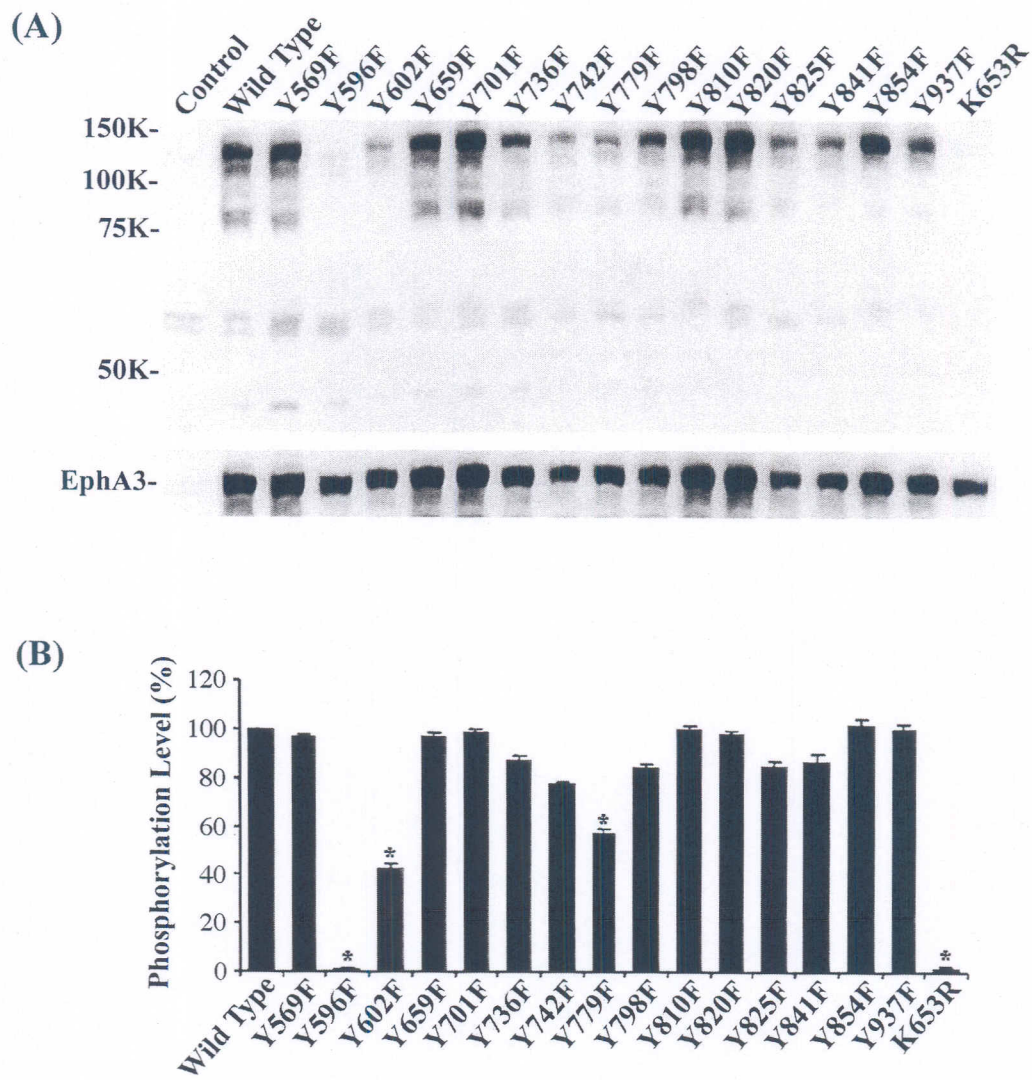

(A)
Control
Wild Type
Y569F
Y596F
Y602F
Y659F
Y701F
Y736F
Y742F
Y779F
Y798F
Y810F
Y820F
Y825F
Y841F
Y854F
Y937F
K653R
150K-
100K-
75K-
50K-
EphA3-
(B)
Phosphorylation Level (%)
120
100
80
60
40
20
0
Wild Type
Y569F
Y596F
Y602F
Y659F
Y701F
Y736F
Y742F
Y779F
Y798F
Y810F
Y820F
Y825F
Y841F
Y854F
Y937F
K653R

Figure 5

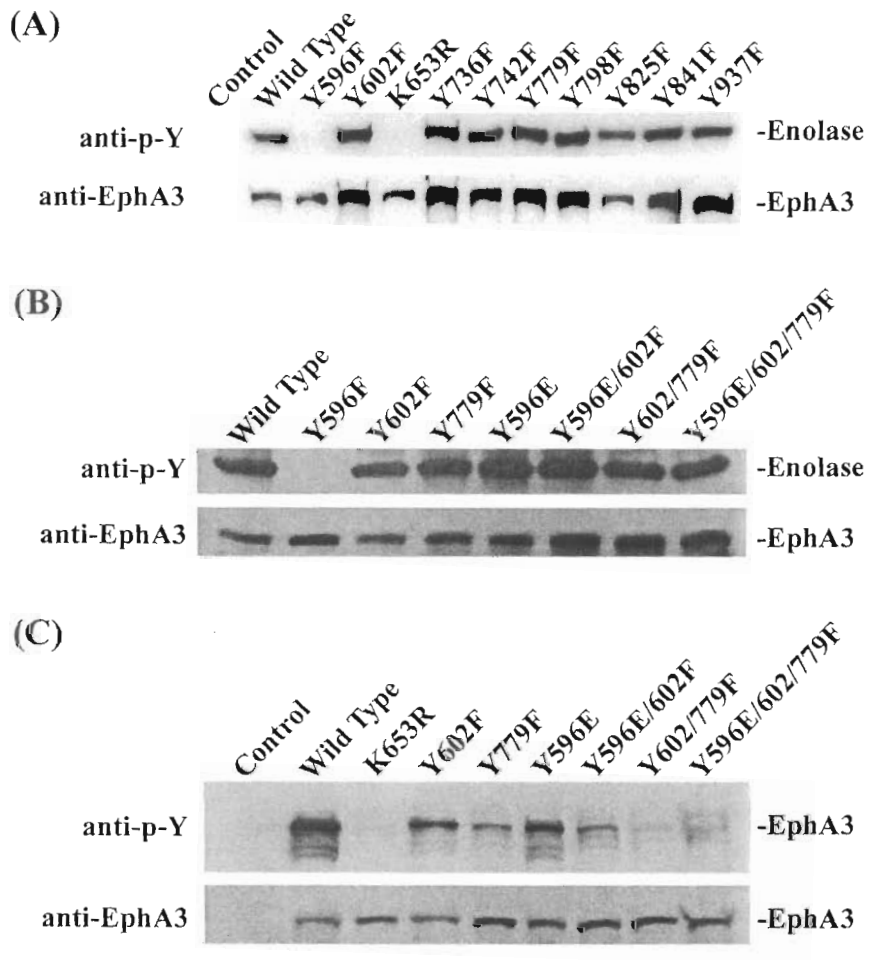

(A)
Control
Wild Type
Y596F
Y602F
K653R
Y736F
Y742F
Y779F
Y798F
Y825F
Y841F
Y937F
anti-p-Y
-Enolase
anti-EphA3
-EphA3
(B)
Wild Type
Y596F
Y602F
Y779F
Y596E
Y596E/602F
Y602/779F
Y596E/602/779F
anti-p-Y
-Enolase
anti-EphA3
-EphA3
(C)
Control
Wild Type
K653R
Y602F
Y779F
Y596E
Y596E/602F
Y602/779F
Y596E/602/779F
anti-p-Y
-EphA3
anti-EphA3
-EphA3

Figure 6

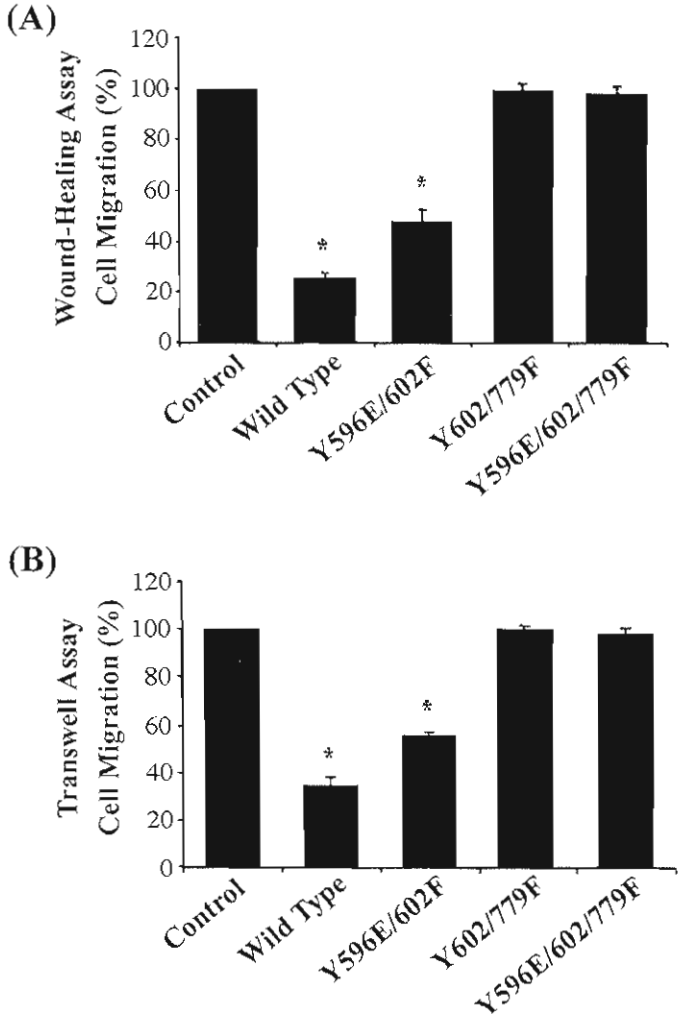

(A)
Wound-Healing Assay
Cell Migration (%)
120
100
80
60
40
20
0
*
*
Control
Wild Type
Y596E/602F
Y602/779F
Y596E/602/779F
(B)
Transwell Assay
Cell Migration (%)
120
100
80
60
40
20
0
*
*
Control
Wild Type
Y596E/602F
Y602/779F
Y596E/602/779F

Figure 7

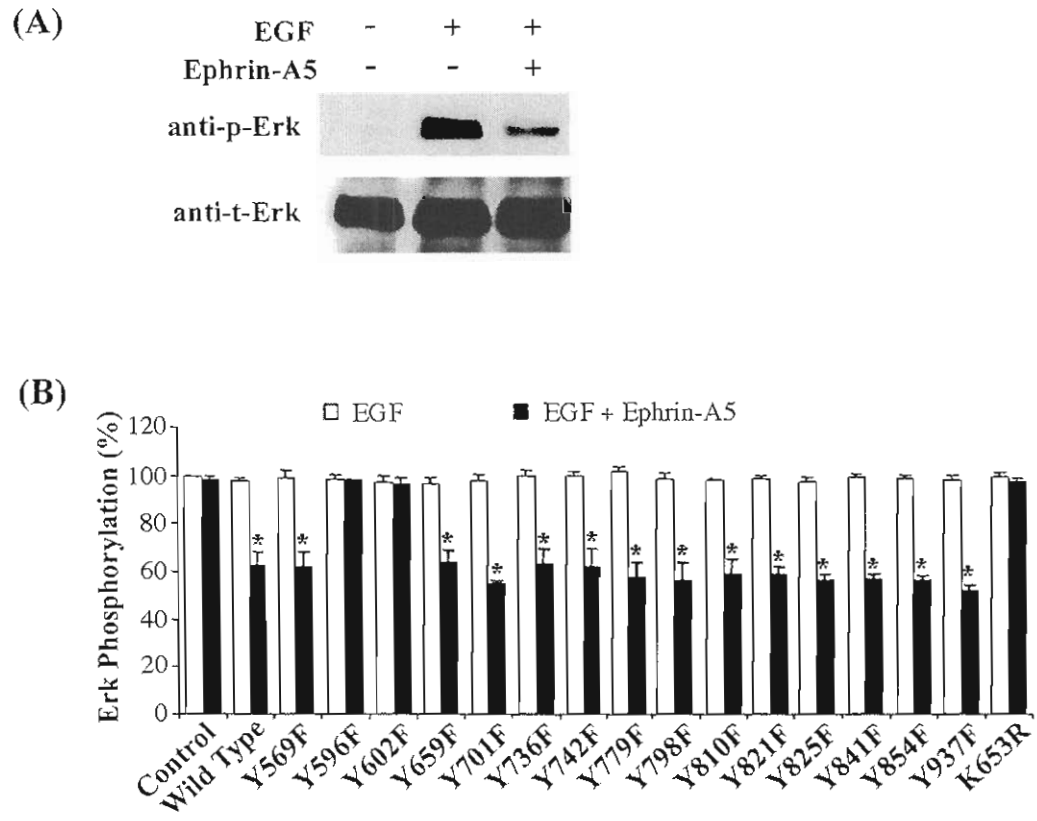
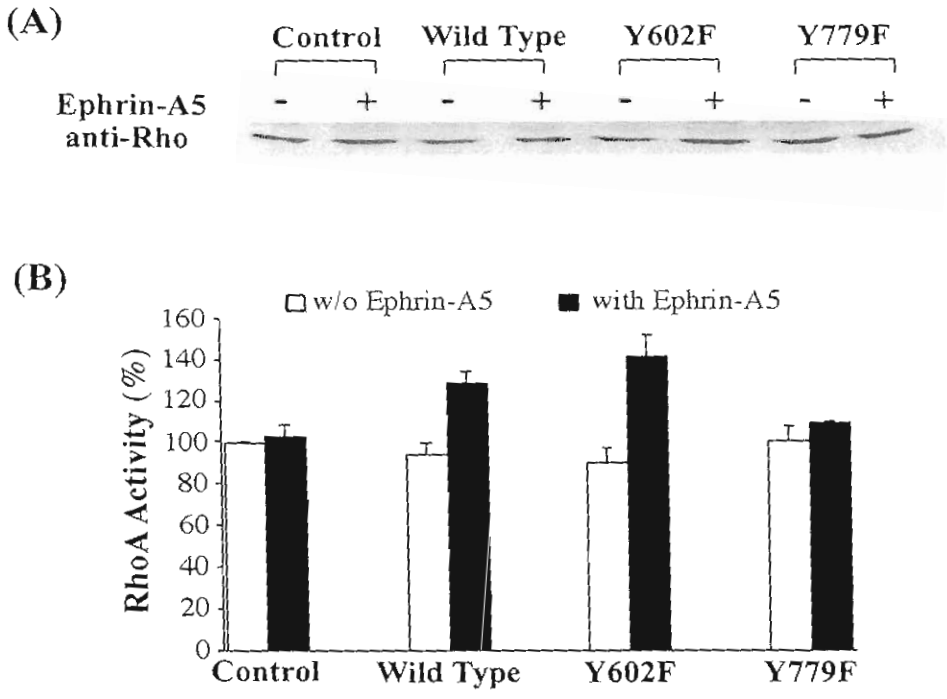

(A)
EGF - + +
Ephrin-A5 - - +
anti-p-Erk
anti-t-Erk
(B)
□ EGF
■ EGF + Ephrin-A5
Erk Phosphorylation (%)
120
100
80
60
40
20
0
Control
Wild Type
Y569F
Y596F
Y602F
Y659F
Y701F
Y736F
Y742F
Y779F
Y798F
Y810F
Y821F
Y825F
Y841F
Y854F
Y937F
K653R

Figure 8


(A)
Control
Wild Type
Y602F
Y779F
Ephrin-A5
anti-Rho
- + - + - + - +
(B)
w/o Ephrin-A5
with Ephrin-A5
RhoA Activity (%)
160
140
120
100
80
60
40
20
0
Control
Wild Type
Y602F
Y779F