Manipulation of EphB2 Regulatory Motifs and SH2 Binding Sites Switches MAPK Signaling and Biological Activity*

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Signaling by the Eph family of receptor tyrosine kinases (RTKs) is complex, because they can interact with a variety of intracellular targets, and can potentially induce distinct responses in different cell types. In NG108 neuronal cells, activated EphB2 recruits p120RasGAP, in a fashion that is associated with down-regulation of the Ras-Erk mitogen-activated kinase (MAPK) pathway and neurite retraction. To pursue the role of the Ras-MAPK pathway in EphB2-mediated growth cone collapse, and to explore the biochemical and biological functions of Eph receptors, we sought to re-engineer the signaling properties of EphB2 by manipulating its regulatory motifs and SH2 binding sites. An EphB2 mutant that retained juxtamembrane (JM) RasGAP binding sites but incorporated a Grb2 binding motif at an alternate RasGAP binding site within the kinase domain had little effect on basal Erk MAPK activation. In contrast, elimination of all RasGAP binding sites, accompanied by the addition of a Grb2 binding site within the kinase domain, led to an increase in phospho-Erk levels in NG108 cells following ephrin-B1 stimulation. Functional assays indicated a correlation between neurite retraction and the ability of the EphB2 mutants to down-regulate Ras-Erk MAPK signaling. These data suggest that EphB2 can be designed to repress, stabilize, or activate the Ras-Erk MAPK pathway by the manipulation of RasGAP and Grb2 SH2 domain binding sites and support the notion that Erk MAPK regulation plays a significant role in axon guidance. The behavior of EphB2 variants with mutations in the JM region and kinase domains suggests an intricate pattern of regulation and target recognition by Eph receptors.

The mechanisms by which signals are conveyed from receptor tyrosine kinases (RTKs)† at the plasma membrane to their intracellular targets in the cytoplasm and nucleus have been extensively explored (1–3). Ligand-induced autophosphorylation in the activation segment of the kinase domain, as well as phosphorylation of juxtamembrane (JM) tyrosines in RTKs such as Eph receptors, induce conformational changes that stimulate the activity of the kinase domain (4–6). Furthermore, receptor tyrosine phosphorylation, usually within non-catalytic sequences flanking the kinase domain, creates docking sites for proteins with SH2 or PTB domains, leading to the recruitment of cytoplasmic targets that regulate downstream signaling (3, 7).

This scheme is evident in the ability of RTKs to recruit negative or positive regulators of the Ras GTPase, such as the p120 Ras GTPase-activating protein (RasGAP) or Grb2 (8). RasGAP, which stimulates the hydrolysis of Ras-bound GTP, is a modular polypeptide with a C-terminal catalytic domain that down-regulates Ras, linked to two SH2 domains (surrounding an SH3 domain) at the N terminus (9, 10). In contrast, Grb2 is an adaptor comprised of a central SH2 domain flanked by two SH3 domains that bind to targets with proline-rich motifs (11–13). In particular, through recruitment of the Ras guanine nucleotide exchange factor Sos1, Grb2 directs phosphoryrosine-induced exchange of GDP for GTP on Ras, thereby stimulating the Erk MAPK pathway (3). RasGAP and Grb2 are therefore SH2-containing proteins with opposing effects on the state of Ras activation, and data from both invertebrate and mammalian systems suggest that RTKs potentially employ Grb2 and RasGAP in combination to achieve sophisticated regulation of the Ras signaling pathway (14, 15).

SH2 domains generally bind short phosphoryrosine-containing peptide sequences but differ in their ability to recognize residues C-terminal to the phosphoryrosine (7, 16, 17). For example, the Grb2 SH2 domain binds preferentially to pTyr-X-Asn motifs (17), whereas the RasGAP SH2 domains binds optimally to pTyr-X-X-Pro motifs (18). We have employed these observations as the basis to explore signaling by EphB2, a member of the predominant family of mammalian RTKs. Eph receptors are activated by their association with cell surface ligands, ephrins, and regulate biological events involving cell-cell interactions, including axon guidance, boundary formation, neural crest migration, angiogenesis, and synaptic function (19–23). In their cytoplasmic regions, Eph receptors have a JM sequence, followed by the kinase domain, a SAM domain, and a C-terminal PDZ domain binding motif. Activated receptors become phosphorylated on multiple tyrosine residues, including a tyrosine in the activation loop of the kinase domain and two conserved tyrosines in the JM region that regulate kinase activity (24–27). Phosphorylated tyrosines in the JM region and elsewhere can then directly engage cytoplasmic targets. In addition, the receptor can potentially phosphorylate docking proteins, such as p62Dok-1, with the ability to engage signaling
intricate regulation of EphB2 through the combined actions of phospho-Erk, and a block in the ability of EphB2 to induce neurite retraction in NG108 neuronal cells expressing EphB2.

In this study, we demonstrate that mutating the previously identified RasGAP binding sites in the EphB2 JM region is not sufficient to switch the EphB2 signal from down-regulation to up-regulation of the MAPK pathway. We identify a novel binding site for RasGAP and show that introduction of a Grb2 binding sequence in place of this second RasGAP binding site within the kinase domain stabilizes phospho-Erk levels in ephrin-B1-stimulated cells. Complete abrogation of RasGAP binding coupled with recruitment of Grb2 is sufficient to switch the EphB2 signal from down-regulation to up-regulation.

### EXPERIMENTAL PROCEDURES

**DNA Constructs, Mutagenesis, and Reagents**—Full-length murine EphB2 cDNA was cloned into the mammalian expression vector pcDNA3 (18). Mutagenesis of EphB2 was performed using the QuickChange system (Stratagene). Green fluorescent protein-tagged EphB2 (EphB2-GFP) was constructed by inserting the whole length of GFP cDNA into the C-terminal of EphB2 between Arg191 and Ala194. For glutathione S-transferase (GST) fusion constructs, cDNA sequences corresponding to the mouse Grb2 SH2 domain (residues 79-160) and human RasGAP SH2 domain (residues 169-470) were cloned into pGEX vector (Amersham Biosciences) and expressed as GST fusion proteins.

For immunoprecipitation and Western blotting, anti-EphB2 (18, 30) and anti-HA mouse monoclonal (12CA5) (31) antibodies have been described previously. Anti-Grb2 and anti-phospho-Erk1/2 antibodies were purchased from Transduction Laboratories and Cell Signaling Technologies, respectively. Other antibodies were purchased from Santa Cruz Biotechnology.

**Cell Culture, DNA Transfection, and EphB2 Stimulation**—NG108-15 (NG108) cells were routinely cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% hyoxanthine-aminopterin-thymidine (Invitrogen). Stable cell lines in NG108 cells were produced as previously described (18, 28). Briefly, parental NG108 cells were transfected with 20 μg of DNA using the calcium phosphate precipitation method (32). Cells were then grown in the presence of 400 μg/ml G418 (Invitrogen). Stable cell lines in NG108 cells were produced as previously described (18, 28). Briefly, parental NG108 cells were transfected with 20 μg of DNA using the calcium phosphate precipitation method (32). Cells were then grown in the presence of 400 μg/ml G418 (Invitrogen) to select stable transfectants. Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 20 μg of plasmid DNA was used to transfect 2 x 10^6 HEK-293 cells/100-mm plate by the calcium phosphate precipitation method (32). Control cells were treated in the same way but without DNA or with expression vector DNA only. Stimulation of EphB2-expressing cells was carried out as previously described (18, 28) with 2 μg/ml aggregated Ephrin-B1Fc. For Erk activation assays, EphB2 cells were seeded into six-well plates 24 h prior to stimulation at 3 x 10^5 cells/well.

**Immunoprecipitation, GST Fusion Protein Pull-down, and Western Blotting**—Unless otherwise indicated, NG108 cells were serum-starved overnight in DMEM containing 1% hyoxanthine-aminopterin-thymidine. For Erk and anti-phosphotyrosine (anti-pTyr) Western blots, cells were stimulated with Ephrin-B1Fc as indicated and lysed directly in 2 x SDS sample buffer. For immunoprecipitation experiments, cells were rinsed twice in ice-cold phosphate-buffered saline and routinely lysed in PLC lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM NaF, 100 mM NaF, 1 mM vanadate, and a mixture of protease inhibitors) as previous described (18, 30). Protein concentrations of cleared lysates were determined using a BCA assay (Pierce). Proteins were immunoprecipitated for 1-2 h at 4 °C, and beads were routinely washed three times in HNTG buffer (20 mM Hepes, pH 7.5, 10% glycerol, 0.1% Triton X-100, and 150 mM NaCl). Proteins were separated on SDS-PAGE gels and transferred to an Immobilon-P membrane (Millipore). Membranes were blocked in TBST containing 5% bovine serum albumin for anti-phosphotyrosine blots or 5% skimmed milk for other blots, and immunoblotted as per standard protocols. Primary antibodies were detected with anti-mouse- or protein-A-horseradish peroxidase followed by treatment with Enhanced Chemiluminescence (Pierce). For the GST fusion protein pull-down experiment, pre-bound GST fusion protein beads (around 6 μg of protein/ml) were used to pull down proteins from whole cell lysates in PLC lysis buffer at 4 °C for 1 h and washed three times in HNTG buffer.

**Peptide Spots Array Synthesis**—Peptide arrays were constructed according to the Spots-synthesis method as previously described (33). Acid-hardened cellulose membranes were pre-derivatized with polyethylene glycol (Abimed, Langfeld, Germany) were spotted with a grid of Fmoc β-alanine (Bachem) prior to peptide synthesis. Standard Fmoc chemistry was used throughout. Fmoc-protected and -activated amino acids were spotted in high density 24 x 18-spot arrays on 130 x 90-mm membranes using an Abimed ASP422 robot. All washing, Fmoc, and side-chain deprotection steps were done manually in propylene glycol containing. The amino acids were used at a concentration of 0.25 M and were twice spotted at a volume of 0.2 μl for each coupling reaction. The peptides covering all tyrosine residues in the EphB2 intracellular region were synthesized (see sequence in Fig. 3 below). Following peptide synthesis and side-chain deprotection, membranes were blocked overnight in 5% skim milk. Purified GST or GST fusion proteins were added at 0.1 μl in TBS and incubated for 1 h at 4 °C. Membranes were washed three times in TBS and incubated with horseradish peroxidase-conjugated anti-GST antibody for 1 h in TBS. Detection was by SuperSignal enhanced chemiluminescence (Pierce).

**Phosphopeptide Mapping by Two-dimensional Chromatography**—Wild-type and mutant (JX Grb2) forms of EphB2 were immunoprecipitated from transiently transfected NG108 cells, radiolabeled by in vitro kinase assay with [32P]ATP, and digested with trypsin. The digests were resolved in two dimensions on 100-μm x 20-cm x 20-cm thin-layer cellulose plates by electrophoresis followed by ascending chromatography. Electrophoresis was performed at pH 1.9 in 50:156:1794 88% formic acid/glacial acetic acid/water for 40 min at 1000 V on a Hunter HTLE-7000 thin layer electrophoresis system, and ascending chromatography was carried out in 750:500:150:600 n-butanol/pyridine/glacial acetic acid/water. The plates were exposed to x-ray film for indicated days at -70 °C.

**Luciferase Assay**—HEK-293 cells were transiently transfected with wild type EphB2, EphB2-EE, KIN<sub>667</sub>, KIN<sub>667</sub> or JX<sub>EE</sub>-KIN<sub>667</sub> by the addition of pFA-Eik1 and pFR-Luciferase reporter constructs (Stratagene PathDetect<sup>®</sup> system), and pSV2-β-galactosidase. At 48 h post-transfection, the cells were lysed with reporter lysis buffer (Promega Corp.). The luciferase activity in lysates was determined by a microplate luminometer LB 96V (EG&G Berthold, Germany) and Luciferin reagent (Promega Corp.). β-Galactosidase assays were performed as per the standard protocol (34).

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**FIG. 1.** Endogenous RasGAP is required for down-regulation of Erk MAPK phosphorylation in response to EphA2. MEF cells that are WT and null for RasGAP were stimulated with 4 μg/ml Ephrin-A1 for the indicated time points and lysed directly in sample buffer. Lysates were resolved by SDS-PAGE and probed for phosphorylated Erk 1/2 (upper panel) or total Erk (bottom panel).
Fig. 2. Response of EphB2 wild type (WT) and a mutant containing a tandem potential Grb2 SH2 binding motif in the JM region (JXGrb2) to ephrin stimulation. NG108 cells were stably transfected with pcDNA-EphB2 or pcDNA-EphB2-JXGrb2, and individual G418-resistant clones were isolated. A and B, parental, EphB2-, and JXGrb2-expressing NG108 cells were serum-starved overnight and challenged with 2 μg/ml clustered Ephrin-B1Fc for the indicated time points and lysed directly in 2× SDS-PAGE sample buffer. The lysates were electrophoresed, blotted and probed with antibodies against phosphorylated tyrosine (A, top panel) or phosphorylated Erk1/2 (B, top panel). The blots were stripped and reprobed for EphB2 receptor and total Erk1/2 (bottom panels, A and B, respectively). C, serum-starved parental, EphB2-, and JXGrb2-expressing NG108 cells were incubated in the presence or absence of 2 μg/ml aggregated ephrin-B1 for 20 min and lysed in PLC lysis buffer. EphB2 immunoprecipitates were separated by SDS-PAGE, the upper portion of the blot was probed with anti-pTyr antibody (upper panel) and reprobed with anti-EphB2 (second panel) and anti-p120-RasGAP (third panel) antibodies. The lower portion of the blot was probed with anti-Grb2 antibody (bottom panel). WCL, whole cell lysate.

**Neurite Retraction Assay**—NG108 cells were transiently transfected by using the calcium phosphate precipitation method with wild type or mutant EphB2-GFP. After 20 h, the transfected NG108 cells were differentiated by using 1 mM dibutyryl cyclic AMP (Sigma) for 5 h. Cells with neurite body length were stimulated with 2 μg/ml clustered ephrin-B1Fc and imaged every 1 min for 20 min on an inverted Leica DM IRB fluorescence microscope equipped with OpenLab software (Imaging Provision Co., UK).

**RESULTS**

**Endogenous RasGAP Is Required for Down-regulation of the Erk MAPK Pathway by Eph Receptor Signaling**—We have previously found that activation of ectopically expressed EphB2 in NG108 neuronal cells leads to a down-regulation of the Ras-MAPK pathway, in a fashion that is attenuated by a dominant negative RasGAP polypeptide (35). To explore whether this is a more general and physiological event, we have investigated the phosphorylation of Erk MAPK in wild type mouse embryo fibroblasts (MEFs) as well as MEFs derived from mouse embryos homozygous for a null mutation in the gene for RasGAP. We hypothesized that substitution of the RasGAP SH2 domain binding sites in the JM region with known Grb2 binding motifs, the sequence Y604VNVFTY610VNV (see Fig. 5A), might lead to recruitment of Grb2 to the JM region of the resulting JXGrb2 mutant EphB2 and induce up-regulation of MAPK activity, an effect opposite to that of wild type (WT) EphB2.

To study the Erk MAPK response to the EphB2 mutant JXGrb2, we derived an NG108-15 (NG108) cell line stably expressing this mutant. The parental NG108 cells do not express detectable EphB2 and do not respond to ephrin-B1 stimulation. In contrast, ephrin-B1 stimulation of NG-EphB2 cells, which stably express wild type EphB2 receptor, leads to an increase in the tyrosine phosphorylation of EphB2 and cellular proteins such as p62Dok-1 and induces neurite retraction (18) and down-regulation of MAPK activity (28). Similarly, ephrin-B1 stimulation of cells expressing JXGrb2 caused an increase in cellular tyrosine phosphorylation, although it was attenuated relative to WT EphB2 (Fig. 2A). We also observed ligand-dependent association of the mutant receptor with RasGAP in NG108 cells expressing JXGrb2 (Fig. 2C), albeit to a lesser extent than WT EphB2, and Erk1/2 dephosphorylation (Fig. 2B) in a manner similar to cells expressing WT receptor. Interestingly, we were unable to detect association of either the WT or JXGrb2 EphB2 with Grb2 following ephrin-B1 stimulation (Fig. 2C).

The observation that the JXGrb2 Eph receptor retains the ability to recruit RasGAP raises the possibility that tyrosine residues at the mutated Y604VNVFTY610VNV motif in JXGrb2 suppress Eph kinase activity (6, 24, 27); this inhibition is relieved by autophosphorylation of the JM motifs, which can subsequently serve as docking sites for SH2 domain containing proteins, including RasGAP (18). RasGAP recruitment to EphB2 is associated with the down-regulation of Erk MAPK activity and may play a significant role in the axon guidance function of EphB2 (28). To explore the regulation of MAPK activity, an effect opposite to that of wild type (WT) EphB2.

**Mutation of the EphB2 Juxtamembrane Region Reveals a Sequence Dependence for Receptor Regulation and Phosphorylation and a Secondary Binding Site for RasGAP**—To explore the determinants that regulate the coupling of Eph receptors to RasGAP, and the Ras-MAPK pathway, we turned to NG108 neuronal cells induced to express EphB2. Ephrin-B1 stimulation results in phosphorylation of EphB2 tyrosine residues, including two conserved JM tyrosines (Y19IDPFTY25EEDP) (18, 25). In their unphosphorylated form, these JM tyrosines
may still be able to preferentially recruit RasGAP instead of Grb2, or the mutant receptors could directly or indirectly associate with RasGAP at other sites. In addition, the JM tyrosine residues in the JXGrb2 mutant may not be phosphorylated upon ephrin-B1 stimulation, and therefore binding sites for the Grb2 SH2 domain may never be generated in the JXGrb2 mutant. To test the first hypothesis, arrays of tyrosine-phosphorylated and unphosphorylated peptides encompassing all tyrosine residues in the intracellular EphB2 region were synthesized directly on a membrane and probed with a GST-Grb2 SH2 domain fusion protein, followed by detection with horseradish peroxidase-anti-GST antibody. B, tryptic phosphopeptide maps of wild type (WT) and mutant JXGrb2 of EphB2. Wild type and mutant JXGrb2 receptors expressed in 293 cells were immunoprecipitated, autophosphorylated with \( \gamma \text{-}^32\text{P}}\)ATP, and digested with trypsin, and the resulting phosphopeptides were separated in two dimensions and exposed to film for 2 days at \(-70^\circ\text{C}\) for the WT and for 2 weeks at \(-70^\circ\text{C}\) for JXGrb2 EphB2 mutant.

**Introduction of a Grb2 Binding Site in the EphB2 Kinase Domain Stabilizes Erk Activity**—To identify other potential RasGAP binding sites in mutant EphB2, we screened a peptide array encompassing all the intracellular phosphopeptides of EphB2 for alternative sites of interaction with a GST fusion protein containing both RasGAP SH2 domains. This revealed another possible RasGAP SH2 binding site, Tyr<sup>667</sup>, in the EphB2 kinase domain (Fig. 3A). Tyr<sup>667</sup> has been previously identified as a phosphorylation site in vivo (25) and is on the surface of the kinase domain (6). We therefore considered the possibility that the Tyr<sup>667</sup> site might be a suitable candidate site for introducing an ectopic Grb2-binding motif in EphB2. To test whether such a mutant EphB2 (KIN<sub>Grb2</sub>) engineered to contain the Grb2-binding motif at this site (Y<sup>667</sup>VNV), can be phosphorylated, we expressed a GST fusion protein containing the intracellular domain of the KIN<sub>Grb2</sub> EphB2 mutant. The GST-KIN<sub>Grb2</sub> fusion protein became tyrosine-phosphorylated, as detected by an anti-phosphotyrosine antibody (data not shown). The GST-KIN<sub>Grb2</sub> protein was subsequently digested and analyzed by nanoelectrospray tandem mass spectrometry.
to map the phosphorylation sites. Ion peaks corresponding to the Tyr\(^{667}\)– containing phosphopeptide (SGpY\(^{667}\)VNVQR) were identified by sequencing and, thus, confirmed the specific phosphorylation of Tyr\(^{667}\) in mutant KIN\(_{Grb2}\) (data not shown). These results suggest that the KIN\(_{Grb2}\) binding site can be phosphorylated in vitro and may thus be able to recruit Grb2 in vivo.

To test whether the mutant KIN\(_{Grb2}\) receptor can bind to Grb2 and thereby influence Erk MAPK activity in vivo, we made an NG108 cell line stably expressing KIN\(_{Grb2}\). Stimulation of cells expressing WT or KIN\(_{Grb2}\) EphB2 caused an increase in cellular tyrosine phosphorylation in both cases; the KIN\(_{Grb2}\) receptor itself was inducibly tyrosine-phosphorylated, to a lesser extent than WT (Fig. 4, A and C). However, although we observed down-regulation of MAPK activity in WT EphB2 cells, cells expressing KIN\(_{Grb2}\) retained a constant level of phosphorylated Erk1/2 over time (Fig. 4D). Immunoprecipitation of EphB2 and blotting for either RasGAP or Grb2 revealed that, like WT EphB2, the KIN\(_{Grb2}\) mutant was able to associate with RasGAP, but in contrast to the WT receptor, KIN\(_{Grb2}\) also recruited endogenous Grb2 in a ligand-dependent manner (Fig. 4C). Thus, the mutant EphB2 receptor, KIN\(_{Grb2}\), is able to bind to Grb2 and to neutralize the down-regulation of MAPK activity observed in response to WT EphB2, suggesting that the positive effect of Grb2 may balance the inhibitory function of RasGAP. However, we could not immediately exclude the possibility that the stable MAPK activity in cells expressing the KIN\(_{Grb2}\) mutant after ephrin stimulation was due to a lower level of receptor activation, rather than Grb2 recruitment. To address this issue, we sought to engineer an EphB2 receptor that could up-regulate MAPK activity through Grb2 recruitment.

**Engineering an EphB2 Variant That Up-regulates Erk MAPK Activity in NG108 Cells**—We speculated that the inability of the KIN\(_{Grb2}\) mutant to stimulate Erk activation might be due to its association with RasGAP as well as Grb2 and, therefore, sought to engineer a receptor that lacks RasGAP binding sites but possesses a phosphorylated Grb2 binding motif. To this end, a series of mutations were introduced into EphB2 at tyrosine phosphorylation sites in the JM sequence, the kinase domain (Tyr\(^{667}\)), and the SAM domain (Tyr\(^{358}\)), either singly or in combination, as shown in Fig. 5A. Tyr\(^{358}\) in the EphB2 SAM domain is conserved across all Eph receptors except EphA3 and has been implicated in binding to the Grb10 adaptor protein (35). WT and mutant EphB2 receptors were transiently expressed in 293 cells and analyzed for tyrosine phosphorylation (Fig. 5B). EphB2 mutants became tyrosine-phosphorylated to varying degrees, with the exception of JX\(_{EE}\), in which the two JM tyrosines are changed to phenylalanine, thus locking the receptor in its autoinhibited state (6, 18). The phosphorylation of mutants with tandem Grb2 binding motifs in the JM region, and a YVVN site in either the kinase or SAM domain (JX\(_{Grb2}\), KIN\(_{Grb2}\) and JX\(_{Grb2}\)-SAM\(_{Grb2}\) was defective (Fig. 5B, upper panel), and indeed a GST-Grb2 SH2 fusion failed to bind JX\(_{Grb2}\)-KIN\(_{Grb2}\) or JX\(_{Grb2}\)-SAM\(_{Grb2}\) (Fig. 5C). We therefore sought to eliminate RasGAP binding in the JM region without significantly altering the surrounding amino acid sequence (24, 28). To this end, we replaced the JM tyrosines with glutamates, which preserve the ability of the receptor to be activated but do not provide SH2 binding sites (27), and introduced a Grb2 binding motif into the kinase domain at Tyr\(^{667}\). We anticipated that this mutant (JX\(_{EE}\)-KIN\(_{Grb2}\)) might retain kinase activity and bind selectively to Grb2 but not to RasGAP. Indeed, the JX\(_{EE}\)-KIN\(_{Grb2}\) receptor was tyrosine-phosphorylated in ephrin-B1-stimulated NG108 cells (Fig. 5B).

To test binding of the various EphB2 mutants to Grb2 or RasGAP, we used GST fusion proteins containing the SH2 domains of Grb2 or RasGAP in pull-down experiments with EphB2 mutants from whole-cell lysates (Fig. 5C). The GST-Grb2-SH2 fusion protein bound to KIN\(_{Grb2}\), but not the corresponding mutant with Tyr\(^{667}\) replaced with Phe (KIN\(_{F,Grb2}\)), indicating that the association of KIN\(_{Grb2}\) with Grb2 depends on Tyr\(^{667}\). As anticipated, both KIN\(_{Grb2}\) and KIN\(_{F,Grb2}\) bound the RasGAP SH2 domains, consistent with the presence of the JM RasGAP binding sites in these mutants. Significantly, the double-mutant JX\(_{EE}\)-KIN\(_{Grb2}\) bound the Grb2 SH2 domain but not the RasGAP SH2 domains (Fig. 5C). A similar Grb2 binding profile was observed when EphB2 was co-expressed with HA-tagged, full-length Grb2 (Fig. 5D), indicating that Grb2 can associate with both KIN\(_{Grb2}\) and JX\(_{EE}\)-KIN\(_{Grb2}\) in vivo.

To test the effect of the JX\(_{EE}\)-KIN\(_{Grb2}\) EphB2 on cellular signaling pathways, we constructed stable NG108 lines expressing this mutant receptor. Stimulation of cells expressing JX\(_{EE}\)-KIN\(_{Grb2}\) with ephrin-B1 induced tyrosine phosphorylation, albeit with slower kinetics than WT EphB2 (Fig. 6A), and association of the mutant receptor with Grb2 (Fig. 6C) but not RasGAP (data not shown). To determine the effect of JX\(_{EE}\)-KIN\(_{Grb2}\) on Erk activation, cells expressing WT and mutant EphB2 were stimulated with pre-clustered ephrin-B1, and lysates were probed with phospho-Erk antibodies. Stimulation of WT EphB2 led to attenuation of pErk levels, whereas stimulation of the JX\(_{EE}\)-KIN\(_{Grb2}\) variant produced a time-dependent,
biphasic increase in phospho-Erk (Fig. 6B), suggesting that this mutant can up-regulate signaling through the Erk MAPK pathway.

To pursue the effect of JX KIN Grb2 on MAPK signaling, HEK 293 cells were transiently transfected with WT EphB2, EphB2-EE, KIN667F, as well as JX KIN Grb2 mutants. Sepharose beads, containing 6 μg/ml of the GST-Grb2 SH2 domain (upper panel) or the GST-GAP SH2 domain (bottom panel) fusion proteins, were used to pull down WT or mutant EphB2 from transiently transfected HEK-293 cells. The pull-down products were electrophoresed and blotted with anti-pTyr antibody (top panel). Immunoblots were subsequently reprobed for EphB2 receptor (bottom panels). C, GST fusion protein pull-down assays of EphB2 mutants. Sepharose beads, containing 6 μg/ml of the GST-Grb2 SH2 domain (upper panel) or the GST-GAP SH2 domain (bottom panel) fusion proteins, were used to pull down WT or mutant EphB2 from transiently transfected HEK-293 cells. The pull-down products were electrophoresed and blotted with anti-pTyr antibody (top panel). Immunoblots were subsequently reprobed for EphB2 receptor (bottom panels).

The Effect of EphB2 Mutants on Neurite Retraction—To assess the biological properties of these EphB2 mutants, we established a neurite retraction assay measuring ephrin-induced changes of neurite length in NG108 cells transiently transfected with WT or mutant forms of EphB2, tagged at the C terminus with green fluorescent protein (GFP) to visualize expressing cells. Ephrin-B1 stimulation of differentiated, neurite-bearing NG-EphB2 cells leads to the reorganization of polymerized actin structures and to neurite retraction (18, 28). When transiently expressed in NG108 cells, WT EphB2-GFP was tyrosine-phosphorylated following ephrin-B1 stimulation and was bound by the RasGAP SH2 domains in vitro (data not shown), indicating that it has similar biochemical features as native EphB2. Transfected NG108 cells were therefore differentiated with cAMP, stimulated for 20 min with ephrin-B1 (Fig. 7A), and examined for neurite retraction in response to activation of WT or mutant EphB2-GFP receptors. Only the green fluorescent cells with neurite lengths more than two times their cell body length were included (Fig. 7B). After 20-min stimulation with 2 μg/ml ephrin-B1, the neurites of cells expressing WT EphB2-GFP showed a strong retraction to 23.1 ± 6.4% (n = 13) of their original length, whereas
neurites of cells expressing the constitutively autoinhibited receptor JX FF-GFP were not significantly retracted (91.5 ± 5.6% (n = 10) of the original neurite length). Cells expressing the KINGrb2-GFP (83.7 ± 6.6%, n = 14) or JXEE-KINGrb2-GFP (92.6 ± 3.7%, n = 10) mutants exhibited a lack of neurite retraction similar to the JX FF-GFP-expressing cells (Fig. 7B).

Thus, switching the binding and signaling properties of EphB2 from repressing Erk1/2 activation in neuronal cells, to stabilizing or stimulating this MAPK pathway, as in the case of KINGrb2 or JXEE-KINGrb2, also leads to a change in the receptor’s biological activity in NG108 neuronal cells. Together, these data suggest that EphB2 signaling can be altered by the rational design of mutant receptors with modified regulatory sites and SH2 binding motifs.

**DISCUSSION**

Activated RTKs can engage both positive regulators of the Ras-MAPK pathway, such as the Grb2/Sos1 complex, and Ras inhibitors such as RasGAP. Although the primary effect of most RTKs is to enhance Ras-GTP loading, stimulation of EphB2 or EphA2 down-regulates GTP-bound Ras and the level of phospho-Erk1/2 in at least some cells (28, 29). To probe EphB2 signaling, we have attempted to alter the binding properties of EphB2 to favor recruitment of Grb2 as compared with RasGAP. Surprisingly, YVNV Grb2 binding motifs incorporated in place of the JM YXXP RasGAP binding sites were apparently not phosphorylated, even though kinase activity of the mutant receptor was induced following ephrin-B1 stimulation, most likely due to autophosphorylation within the activation segment of the kinase domain (24, 25). These substitutions therefore appear to interfere with the inhibitory interaction of the JM sequence with the kinase domain and with recognition of the JM tyrosines by the kinase domain active site. These data suggest that the sequence, and possibly conformation, of the JM sequence is important not only for repression of kinase activity but also for the phosphorylation of the JM tyrosines induced by ephrin stimulation. Consistent with this interpretation, a JX-Grb2 mutant, in which the tyrosine residues in the JM YVNV motifs of JX Grb2 were changed to phenylalanine, behaved similarly to JX Grb2 (data not shown). Despite lacking the RasGAP binding sites in the JM region, the JXGrb2 mutant was effectively WT in activity, in the sense that ephrin-B1 stimulation of JXGrb2 NG108 cells still led to RasGAP recruitment (albeit less strongly than WT EphB2) and attenuation of Erk1/2 phosphorylation (Fig. 2).

We have previously found that an EphB2 mutant (EphB2-EE) in which the JM tyrosines are changed to glutamate also retained an ability to associate with RasGAP (28), raising the possibility of a secondary RasGAP binding site on EphB2. Peptide binding experiments demonstrated that the RasGAP SH2 domains were unable to associate with the JM peptides.
from either JXGrb2 or EphB2-EE but weakly associated with a peptide that includes phosphorylated Tyr667 in the kinase domain (Fig. 3A). In the crystal structure of the EphB2 kinase domain, Tyr667 is exposed on the upper surface of the N-terminal lobe, situated in the β3-αc linker (6), and is therefore potentially accessible for binding. Indeed we were able to abrogate RasGAP association with EphB2 only when both the JM motifs and the Tyr667 motif were mutated in concert, as in the JXEE-KINGrb2 mutant (Fig. 5). Single substitutions in the JM region as in EphB2-EE (28) or JXGrb2 (Fig. 2) or in the kinase domain such as KINGrb2 did not fully inhibit recruitment of RasGAP (Fig. 5), indicating EphB2 receptor may have multiple binding sites for RasGAP. These suggestions are further supported by luciferase assay results, which demonstrate that overexpression of EphB2 mutants lacking either of the RasGAP binding sites (EphB2-EE and KINGrb2) increased luciferase activity compared with WT and mutant EphB2 in response to ephrin-B1Fc. The data are expressed as a percentage of the original neurite length before the ligand stimulation. Each error bar represents the mean and S.E. of more than 10 cell experiments for each construct.

MAPK activation has provided an opportunity to engineer EphB2 variants in which biochemical signaling to the Ras-MAPK pathway is switched through the incorporation of a Grb2 binding site at Tyr667, such that ephrin stimulation induces Erk activation. The ability of this mutant (JXEE-KINGrb2) to up-regulate Erk phosphorylation in a ligand-inducible fashion was dependent on the ablation of RasGAP binding sites in the JM region (while at the same time suppressing the inhibitory effect of the JM sequence on kinase activity). Indeed a mutant with both Grb2 and RasGAP binding sites (KINGrb2) was neutral in its effects on Erk phosphorylation. These data suggest that an Eph receptor’s effects on the Erk MAPK pathway can be titrated from inhibitory to neutral to stimulatory by the relative incorporation of RasGAP and Grb2 binding sites. The switch in the biochemical output of the JXEE-KINGrb2 mutant is accompanied by a loss of the ephrin-B1-induced neurite retraction observed in NG108 cells expressing WT EphB2. These data are consistent with the possibility that down-regulation of the Ras-MAPK pathway is involved in the process of neurite retraction induced by EphB2 (28); indeed, these observations may have a more general significance, because neuronal outgrowth induced by netrin-1 signaling through the attractive DCC receptor appears dependent on the JXEE-KINGrb2 mutant. Although this scheme is generally applicable, it is apparent that Eph receptors are organized in a somewhat more complex fashion, in which the non-catalytic JM region can both regulate kinase activity and bind SH2-containing targets. The importance of the sequence and, likely, the conformation of the JM region in integrating Eph receptor signaling are revealed by the observation that altering the
sequence C-terminal to the JM tyrosines interferes with both its regulatory properties, as well as its ability to become phosphorylated and bind cytoplasmic polypeptides. Furthermore, we have identified a site within the kinase domain itself, Tyr^{667}, which has the potential to act as a docking site for SH2 proteins. Although the physiological importance and general applicability of this observation remains to be fully explored, it emphasizes that RTK targets may potentially interact with the kinase domain at sequences removed from the active site. In a similar fashion, the kinase domain of the serine/threonine-specific protein kinase glycogen synthase kinase 3\beta binds the phosphoserine/threonine residues of primed substrates through a positively charged pocket within the kinase domain (38). The complex regulation and signaling properties of Eph receptors likely reflect their sophisticated ability to control cell-cell interactions in both embryonic and adult tissues.

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