The EphB6 Receptor Cooperates with c-Cbl to Regulate the Behavior of Breast Cancer Cells

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Abstract

Cancer invasiveness plays a major role in the mortality of patients with solid tumors, and deregulated cell adhesion and migration are suspected to drive invasive behavior. Since Eph receptor tyrosine kinases control both cell attachment and migration, they may act to define the level of cancer invasiveness. EphB6 is an unusual Eph receptor, lacking catalytic capacity due to alterations in its kinase domain. Interestingly, increased metastatic activity is associated with reduced EphB6 receptor expression in several tumor types, including breast cancer. This emphasizes the potential of EphB6 to act as a suppressor of cancer aggressiveness; however, the mechanism of its action is not well understood. We show that restoration of EphB6 expression in invasive breast cancer cells supports actin-dependent spreading and attachment and blocks invasiveness. EphB6 stimulation induces its tyrosine phosphorylation, which is crucial for its function and is mediated by the EphB4 receptor. This is accompanied by EphB6–c-Cbl interaction and phosphorylation of c-Cbl partner, the Abl kinase. Cbl silencing suppresses Abl phosphorylation, cell adhesion, and morphologic changes and blocks the ability of EphB6 to inhibit invasiveness, confirming its importance for EphB6 activity. Despite its crucial role in EphB6 responses, EphB4 also acts in an EphB6-independent manner to enhance invasive activity, suggesting that cancer invasiveness may be defined by the balance in the EphB6-EphB4 system. Overall, our observations suggest a new role for EphB6 in suppressing cancer invasiveness through c-Cbl–dependent signaling, morphologic changes, and cell attachment and indicate that EphB6 may represent a useful prognostic marker and a promising target for therapeutic approaches. Cancer Res; 70(3): 1141–53. ©2010 AACR.

Introduction

Accumulating observations suggest that Eph receptor tyrosine kinases may strongly influence cancer development and progression (1–3). Eph receptors form two groups: EphA receptors (EphA1–EphA10) and EphB receptors (EphB1–EphB6). EphA receptors predominantly interact with membrane-anchored ephrin-A ligands (ephrin-A1 to ephrin-A6), whereas EphBs are mostly activated by transmembrane ephrin-B proteins (ephrin-B1 to ephrin-B3). Through their signaling and cellular functions, Ephs play an important role in embryonic development (3, 4) and in homeostasis maintenance in adult organisms (5–8). Interestingly, both EphA and EphB groups possess kinase-deficient members, EphA10 (9) and EphB6 (10, 11), suggesting that these molecules may play an important role in the Eph receptor network and contribute to its complexity through the modulation of Eph receptor–initiated responses; however, the role of kinase-dead Eph receptors is only starting to be addressed.

Based on their ability to control cell attachment and migration, Ephs are thought to play an important role in cancer invasiveness (1, 2). Although the action of some EphB receptors in cancer often seems contradictory (12–19), reduced EphB6 expression consistently correlates with enhanced aggressiveness and invasiveness in melanoma (20), neuroblastoma (21), and non–small cell lung cancer (22). EphB6 is also known to be expressed in untransformed breast epithelial cells and in noninvasive breast cancer, whereas its expression is suppressed in invasive, metastatic breast cancer cells (12). Unusual EphB6 effect on cancer cells matches its unique signaling properties because it lacks catalytic activity due to sequence alterations in its kinase domain (10) and its phosphorylation is provided by EphB1 (23) or a Src family kinase (24) in Cos7 and HEK-293 cells.

All these observations indirectly suggest that EphB6 may play an important role in suppressing cancer aggressiveness, but little is known about the functions and the mechanism of action of this receptor.

We show here that EphB6 restoration suppresses the invasive activity of breast cancer cells. This is associated with EphB6-induced proadhesive responses and morphologic changes, including active cell spreading and formation of cell protrusions. Our observations suggest that the EphB4 receptor is responsible for EphB6 phosphorylation, whereas c-Cbl seems to play a central role in EphB6 signaling, allowing...
ligand-induced phosphorylation of the Abl kinase and supporting EphB6-mediated morphologic changes, adhesion, and invasion-suppressing activity. Whereas EphB6 signaling and anti-invasive action rely on EphB4-dependent phosphorylation, the EphB4 receptor also seems to support breast cancer invasiveness when operating without EphB6. This suggests that breast cancer invasiveness may be controlled by the relative activity of the EphB4 and EphB6 receptors.

Our work reveals some of the mechanisms used by EphB6 to control breast cancer cells and, because cancer cells typically express multiple EphB receptors, responding to all ephrin-B ligands in a promiscuous manner, it also supports an exciting model, where EphB6 may act as a molecular switch, redirecting ephrin-B–induced signaling and suppressing malignant activity.

Materials and Methods

**Antibodies, constructs, and chemicals.** Anti-phosphotyrosine antibody was obtained from Upstate Biotechnology, Inc. Extracellular signal-regulated kinase 2, Myc, Cbl, and Abl antibodies were from Santa Cruz Biotechnology, Inc. Anti-EphB6 and anti-EphB4 were from Sigma and Invitrogen. Myc-tagged EphB6 was described previously (23, 25). Wild-type EphB6 construct was a generous gift from Dr. C.M. Roifman ( Sick Children’s Hospital, Toronto, Ontario, Canada). A Myc-tagged tyrosine-deficient EphB6 mutant with all cytoplasmic tyrosines replaced by phenylalanines was generated using QuikChange Mutagenesis kit (Stratagene) according to the manufacturers’ instructions. EphB4 was purchased from OriGene Technologies, Inc. Ephrin-B2–Fc was from R&D Systems. PP2, PP3, and cytochalasin D were from Santa Cruz Biotechnology, Inc.

**Cell lines.** MDA-MB-231 cells [American Type Culture Collection (ATCC)] were transfected with pcDNA3 expression vector (Invitrogen), carrying cDNAs for EphB6, Myc-tagged EphB6, Myc-tagged tyrosine-deficient EphB6, or with empty pcDNA3 vector. Cells were electroporated with 20 μg DNA (70 μs, 140 V, ECM 830 electroporator; Harvard Apparatus Inc.). Cells were selected with G418 (1 mg/mL) for 30 d, and the resulting oligoclonal populations were screened by Western blotting. Transduction of Hs578T cells (ATCC) was performed as described in the Lentiviral transduction section.

**Cell stimulation.** Ephrin-B2–Fc was precomplexed with goat anti-human Fc for 30 min on ice (1 μg/mL of ephrin-B2–Fc with 1.5 μg/mL of anti-human Fc), and cells were stimulated at 37°C. Unstimulated cells were incubated at 37°C with precomplexed human IgG. Breast cancer cells were serum starved overnight before stimulation. In some experiments, cells were preincubated at 37°C with 8 μmol/L PP2 for 40 min (26, 27) or 3 μmol/L SU6656 for 1 h (28, 29). PP3- and DMSO-treated cells were used as controls for PP2 and SU6656, respectively.

**Adhesion assays.** Fibronectin (Upstate Biotechnology), ephrin-B2–Fc, or human IgG was immobilized onto 96-well plates overnight at 4°C. In some experiments, plates were additionally blocked for 1 h at 37°C with 10 mg/mL of bovine serum albumin (BSA) in DMEM. Cells (1 × 10^5 per well) were incubated at 37°C and 5% CO₂ in serum-free DMEM for 40 min. Nonadherent cells were removed by washing with PBS. Adherent cells were fixed with ice-cold methanol and stained with crystal violet, and absorbance was measured by a microplate reader at 550 nm. Error bars represent SD based on analysis of triplicates.

**Cell spreading assay.** Plates were precoated with ephrin-B2–Fc or human IgG overnight at 4°C and blocked with BSA. Cells (20 × 10^3 per well) were allowed to spread at 37°C and 5% CO₂ and imaged at ×400 magnification using a Nikon Diaphot inverted microscope and Nikon DS-L1 camera. Images were processed to uniform background brightness. Where indicated, cytochalasin D in DMSO (1 μmol/L) or equivalent volume of DMSO was added immediately before loading cells.

**Actin staining.** Glass coverslips were coated with 0.25 μg/mL ephrin-B2 overnight at 4°C. Coverslips were rinsed and blocked with 10 mg/mL BSA. Cells (1 × 10^5 per point) were allowed to attach at 37°C, fixed with 1% paraformaldehyde, permeabilized with ice-cold 0.1% Triton X-100 (Calbiochem), and stained with rhodamine-labeled phalloidin (Invitrogen). Coverslips were mounted with Pro-Long Antifade (Invitrogen). Images were captured with a Zeiss 510 Meta confocal microscope using LSM 5 version 3.2 software.

**Lentiviral transduction.** HEK-293 cells were cotransfected with psPAX2 (3 μg) and pMD2.G (1 μg) plasmids and 4 μg of a lentiviral construct encoding short hairpin RNA (shRNA; Open Biosystems) or EphB4 cDNA (GeneCo- poea) to produce lentiviral particles. Particles were collected 48 h after transfection by 0.44 μm filtration of conditioned medium. Virus-containing filtrate was mixed with serum-free medium containing 20 μg/mL polybrene (Sigma) in a 1:1 ratio, placed on cells overnight, and replaced with growth medium.

**Figure 1.** EphB6 supports attachment of breast carcinoma cells. A, EphB6-deficient MDA-MB-231 breast carcinoma cells (MDA) were electroporated with the pcDNA3 expression vector encoding EphB6 (MDA-B6), Myc-tagged EphB6 (MDA-B6-M), or empty pcDNA3 (MDA-pc3). Cells were selected for 30 d in the presence of 1 mg/mL G418 and analyzed by Western blotting. Erk2, extracellular signal-regulated kinase 2. B, MDA, MDA-pc3, MDA-B6, and MDA-B6-M cells were loaded into 96-well plates precoated with various concentrations of ephrin-B2–Fc (EFNB2-Fc) or human IgG (hIgG) and incubated for 40 min at 37°C. Unattached cells were removed by washing with PBS, and adherent cells were fixed with ice-cold methanol and stained with crystal violet. Cell adhesion was assessed at 550 nm using a microplate reader. Data represent the analysis of triplicates and are shown as a percent relative to untreated control. C, 96-well plates were precoated with various concentrations of fibronectin in the presence of ephrin-B2 or human IgG (0.15 μg/mL; top) or with fibronectin alone (bottom) and blocked with BSA (10 mg/mL). Adhesion of MDA-pc3 and MDA-B6-M cells was analyzed as in B and is shown relative to human IgG or BSA control. D, MDA cells were manipulated by lentiviral transduction of EphB4 cDNA (MDA-B4) or control EGFP cDNA (MDA-GFP). Cells were selected for 2 d with 10 μg/mL puromycin and analyzed by Western blotting. Adhesion of MDA-GFP and MDA-B4 to fibronectin was monitored as in C. Bars, SD. * P < 0.05, Student’s t test, for indicated points and their corresponding controls: MDA-B6 and MDA-B6-M are compared with corresponding IgG controls in B and C (top).
medium for 24 h. Cells were selected with 10 μg/mL puromycin (Sigma) for 2 d.

**Invasion assay.** The assay was performed in BD BioCoat Matrigel Invasion Chambers (BD Biosciences). Cells were loaded into the upper wells of invasion chambers in serum-free medium in the presence of 1.5 μg/mL of precomplexed ephrin-B2–Fc. Medium containing 8% serum was used as a chemoattractant in the lower wells. The cells were incubated for 21 h at 37°C, fixed with ice-cold methanol, and stained with Giemsa. Invading cells on the lower side of each membrane were counted in five randomly selected microscopic fields at ×400 magnification using a Nikon Diaphot inverted microscope with a Nikon DS-L1 camera, and the mean number of cells was displayed as a percent relative to appropriate control.

**Results**

**EphB6 supports attachment of breast cancer cells.** To analyze EphB6 functions, we restored EphB6 expression in highly invasive breast cancer cells, MDA-MB-231, which have been shown to lack this receptor (12), by transfection with wild-type EphB6 cDNA (MDA-B6) or DNA encoding Myc-tagged EphB6 (MDA-B6-M; Fig. 1A).

The invasiveness of cancer cells is known to strongly depend on their adhesive properties (30). To determine if EphB6 regulates attachment of breast cancer cells, we loaded EphB6-restored MDA-B6 and MDA-B6-M cells on immobilized EphB6 ligand, ephrin-B2 (31). Because an ephrin-B2–Fc fusion protein was used, human IgG was used as a specificity control. Immobilized ephrin-B2, but not human IgG, strongly enhanced adhesion of MDA-B6 and MDA-B6-M. This response was mediated by EphB6, as ephrin-B2 supported attachment of few MDA or mock-transfected MDA (MDA-pc3) cells (Fig. 1B).

To examine if EphB6 could also control interaction of breast cancer cells with the extracellular matrix, we loaded MDA-pc3 and MDA-B6-M onto fibronectin, immobilized at suboptimal concentrations in the presence of suboptimal concentrations of ephrin-B2. Although none of the proteins alone was efficient at supporting adhesion, their combination produced a synergistic effect and initiated a strong adhesive response in MDA-B6-M, but not MDA-pc3, cells. Moreover, EphB6 supported attachment to fibronectin even in the absence of ephrin-B2 (Fig. 1C). The effect was EphB6 specific because another breast cancer–related EphB receptor, EphB4 (32), although supporting adhesion to ephrin-B2 (Supplementary Fig. S1), was not efficient in supporting attachment to fibronectin even when overexpressed (Fig. 1D).

These observations suggested that EphB6 could play an active role in the regulation of breast cancer cell adhesion. **EphB6-induced morphologic changes are supported by actin cytoskeleton.** Cell attachment usually requires spreading, serving to increase cell-cell or cell-matrix contact areas, and enhancing adhesion (33). To assess EphB6 influence on breast cancer cell morphology, we monitored behavior of MDA-B6 and MDA-B6-M cells on immobilized ephrin-B2. MDA-B6 and MDA-B6-M attachment seemed to be supported by active spreading. In contrast, ephrin-B2–adherent MDA-MB-231 and MDA-pc3 cells failed to show any significant signs of spreading and strongly resembled cells nonspecifically interacting with IgG (Fig. 2A).

In agreement with EphB4 ability to support ephrin-B2–induced adhesion, EphB4-overexpressing MDA-B4 cells also spread on ephrin-B2 (Fig. 2B), suggesting that EphB6 may cooperate with other EphB receptors in some adhesive responses.

Cell spreading is typically supported by cytoskeletal modifications, including actin polymerization (34, 35). To examine the role of EphB6-mediated cytoskeletal rearrangements, we treated MDA-B6 and MDA-B6-M cells with cytochalasin D to prevent actin polymerization. Both MDA-B6 and MDA-B6-M failed to spread on ephrin-B2 when treated with cytochalasin D (Fig. 2C), highlighting the importance of EphB6-induced cytoskeleton-related signaling. To further assess cytoskeletal reassembling, we stained cells with rhodamine-labeled phalloidin and analyzed them by confocal microscopy. This revealed that attachment to ephrin-B2 was associated with actin rearrangements in EphB6-restored, but not EphB6-deficient, cells (Fig. 2D), supporting a role for EphB6 in cytoskeleton regulation.

**The EphB4 receptor is required for ligand-induced EphB6 phosphorylation.** Because cell spreading and cytoskeletal rearrangement are controlled through active signal transduction, our observations suggested that EphB6-induced responses relied on its ability to initiate cytoplasmic signaling. Eph receptor signaling is primarily initiated through autophosphorylation on tyrosine residues (36). However, EphB6 carries alterations in its kinase domain, rendering it catalytically inactive (10, 11), and its phosphorylation may be provided by EphB1 or a Src family kinase (23, 24). EphB6 phosphorylation could not be monitored in MDA-B6 cells due to the absence of an efficient EphB6-precipitating antibody, but ephrin-B2 stimulation of MDA-B6-M revealed a strong response (Fig. 3A).

Whereas EphB6 phosphorylation was blocked in cells pretreated with the Src inhibitor PP2, to our surprise, pretreatment with another broad spectrum Src inhibitor, SU6656, failed to affect EphB6 phosphorylation (Fig. 3B). This implied that PP2 may inhibit not only Src family members but also another tyrosine kinase that could be required for EphB6 phosphorylation.

EphB4 is strongly expressed in breast cancer cells, including MDA-MB-231 (32), and is efficiently inhibited by PP2 (37), suggesting that ligand-induced EphB6 phosphorylation could be provided by EphB4. Indeed, PP2, but not SU6656, strongly inhibited EphB4 phosphorylation (Fig. 3C, top left) and EphB6 coprecipitated with EphB4 in MDA-B6-M (Fig. 3C, top right).

Moreover, EphB4 coexpression resulted in strong phosphorylation of EphB6 in HEK-293 cells and this phosphorylation was enhanced in response to ephrin-B2 (Fig. 3C, bottom left). In line with the ability of PP2 to act as an EphB4 inhibitor (37) and to block EphB6 phosphorylation in MDA-B6-M, PP2 also suppressed EphB4-mediated EphB6 phosphorylation in HEK-293, whereas SU6656 had no inhibitory effect (Fig. 3C, bottom right). In further confirmation of the role of EphB4, silencing its expression in MDA-B6-M resulted in the pronounced inhibition of EphB6 phosphorylation (Fig. 3D).
Figure 2. EphB6 regulates actin-dependent cell spreading. A, plates were precoated with ephrin-B2 or human IgG, as indicated, and blocked with BSA. Cells were allowed to spread for 4 h at 37°C and imaged at x400 magnification. B, spreading of MDA-GFP and MDA-B4 cells was assessed and imaged as in A. No spreading was observed on human IgG control (data not shown). C, MDA-B6 and MDA-B6-M cells were treated with 1 μmol/L cytochalasin D in DMSO, or equal volume of DMSO, and cell spreading was followed as in A. D, cells were allowed to spread at 37°C on glass coverslips precoated with 0.25 μg/mL ephrin-B2. Cells were fixed in 1% paraformaldehyde and permeabilized with ice-cold 0.1% Triton X-100, and polymerized actin was visualized by staining with rhodamine-labeled phalloidin. Images were obtained with Zeiss 510 Meta confocal microscope. Scale bar, 10 μm.
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(A) MDA-pc3 vs MDA-B6-M
- EFN2B2-Fc (1 µg/mL)
  - 7 15
  - 7 15 (min)
  I.P. anti-Myc W.B. anti-PY
  EphB6-M

(B) MDA-B6-M
- PP3 vs PP2
- DMSO vs SU6656
- EFN2B2-Fc (1 µg/mL)
  - 7 15
  - 7 15 (min)
  I.P. anti-Myc W.B. anti-PY
  EphB6-M

(C) HEK-293-EphB4
- DMSO vs SU6656
- PP3 vs PP2

(D) MDA-B6-M vs MDA-B6-M
- B6-M-shEphB4 vs B6-M-NS-shRNA
- EFN2B2-Fc (1 µg/mL)
  - 7 10 12
  - 7 10 12 (min)
  I.P. anti-Myc W.B. anti-PY
  EphB6-M

W.B. anti-Erk2
These experiments strongly suggested that stimulation-induced EphB6 phosphorylation was mediated by the EphB4 receptor and that EphB4 represents an EphB6 signaling partner in breast cancer cells.

**c-Cbl plays a central role in EphB6 signaling and cellular responses.** EphB6 has previously been shown to constitutively interact with the c-Cbl ubiquitin ligase when cotransfected in Cos7 cells (23) and to control its phosphorylation in Jurkat cells (38), although the biological significance of EphB6–c-Cbl interaction remained unclear. Our experiments show that EphB6 also interacts with c-Cbl in MDA-MB-231, but, in contrast to the situation in Cos7, this interaction proved to be stimulation dependent (Fig. 4A).

c-Cbl is known to act as an adaptor, providing a link between cell surface receptors and cytoplasmic signaling proteins (39). Interestingly, tyrosine phosphorylation of one known c-Cbl–binding protein, the Abl kinase (40), was strongly enhanced by ephrin-B2 stimulation of MDA-B6 but not MDA-MB-231 or MDA-pc3 cells (Fig. 4B). This suggested that ligand-stimulated EphB6 supported Abl activation, potentially operating through c-Cbl. To determine whether EphB6 required c-Cbl to initiate Abl phosphorylation, we suppressed c-Cbl expression with c-Cbl–specific shRNA. When stimulated with ephrin-B2, EphB6 induced Abl phosphorylation in MDA-B6-M and MDA-B6-M transduced with nonsilencing shRNA but failed to do so in c-Cbl–silenced cells (Fig. 4C), supporting a crucial role for c-Cbl in the EphB6–Abl pathway.

Interestingly, a tyrosine-deficient EphB6 mutant with all cytoplasmic tyrosine residues converted to phenylalanines failed to interact with c-Cbl or trigger Abl phosphorylation (Fig. 4D), confirming the importance of EphB4-mediated phosphorylation.

Because Abl is an important regulator of cytoskeletal rearrangements, cell adhesion, and invasiveness (41–44), these findings implied that c-Cbl could operate downstream of EphB6 to support its proadhesive action. Indeed, ephrin-B2–induced adhesion was inhibited by c-Cbl silencing in MDA-B6-M (Fig. 5A), whereas control shRNA transduction did not result in any significant suppression (Fig. 5B). c-Cbl silencing also blocked attachment of MDA-B6-M to fibronectin, suggesting its overall importance for EphB6-mediated adhesion (Fig. 5C).

EphB6-mediated adhesion was associated with cell spreading, requiring actin polymerization, and we stained cells with rhodamine-labeled phalloidin to analyze c-Cbl involvement in this process. Phalloidin staining again showed that attachment to ephrin-B2 induced active cell spreading and this was inhibited by silencing c-Cbl expression (Fig. 5D).

In sum, these results suggested that c-Cbl played a central role in EphB6-mediated signal transduction and, consequently, EphB6-induced attachment and morphology of breast cancer cells.

**EphB6 requires c-Cbl to suppress the invasiveness of breast cancer cells.** Enhanced adhesion may potentially anchor breast cancer cells in their initial niche and suppress their invasiveness and metastatic activity (45). To analyze the role of EphB6, we assessed EphB6-deficient as well as MDA-B6 and MDA-B6-M cells in an invasion assay in the presence of precomplexed ephrin-B2. As predicted, MDA-MB-231 and MDA-pc3 cells showed a high level of invasiveness, whereas EphB6 restoration strongly reduced this activity (Fig. 6A), suggesting that EphB6 controls breast cancer cell morphology and adhesive behavior in a manner preventing invasion.

To determine the role of c-Cbl in mediating EphB6 effect, we assessed the behavior of c-Cbl–silenced MDA-B6-M. The silencing of c-Cbl compromised EphB6 ability to suppress invasion, thus strongly supporting the importance of c-Cbl for EphB6 action (Fig. 6A). The role of c-Cbl was further confirmed using an additional Cbl shRNA construct (Fig. 6B). In agreement with Cbl importance, Abl silencing and mutation of EphB6 tyrosine residues also inhibited EphB6 activity (Fig. 6B, top right and bottom left). EphB6 action also proved not to be restricted to MDA-MB-231 because its silencing enhanced invasion in Hs578t breast cancer cells (Fig. 6B, bottom right).

Surprisingly, although EphB4 silencing blocked EphB6–induced attachment (Supplementary Fig. S2), it did not suppress EphB6 anti-invasive action (Fig. 6C). This implied that, in addition to its role in EphB6 responses, EphB4 may act through EphB6-independent mechanisms to enhance breast cancer invasiveness. Indeed, introduction of EphB4–silencing shRNA inhibited invasiveness of MDA-MB-231, whereas EphB4 overexpression enhanced it in MDA-B6-M cells (Fig. 6D), suggesting that the balance in EphB6–EphB4 signaling could determine the invasive behavior.

Figure 3. Stimulation-induced EphB6 phosphorylation is EphB4 dependent. A, MDA-pc3 and MDA-B6-M cells were stimulated at 37°C for the indicated time periods with precomplexed ephrin-B2 (1 μg/mL). Unstimulated cells were treated for 15 min with precomplexed human IgG as a specificity control. EphB6 phosphorylation was analyzed by Western blotting of EphB6-Myc immunoprecipitates with anti-phosphotyrosine, and the phosphorylation was assessed as in A. B, MDA-B6-M cells were pretreated with Src kinase inhibitors PP2 or SU6656; PP3 and DMSO were used as respective controls. Cells were stimulated and EphB6 phosphorylation was monitored as in A. C, top left, EphB4-transfected HEK-293 cells were pretreated with Src inhibitors as in B and stimulated for 5 min with 1 μg/mL of precomplexed ephrin-B2 or human IgG. EphB4 was immunoprecipitated and its phosphorylation was assessed as in A. Top right, cells were stimulated with ephrin-B2 and EphB4/EphB6 interaction was analyzed by Western blotting of EphB6-Myc immunoprecipitates with anti-EphB4. No-antibody precipitation from ephrin-B2–stimulated cells was used as a specificity control (15). EphB6-Myc presence was monitored by blotting of cell lysates with anti-EphB6. Bottom left, HEK-293 were transfected with EphB6-M or cotransfected with EphB6-M and EphB4, pcDNA3 was used as a specificity control for EphB6 phosphorylation. EphB4 phosphorylation was monitored as in A. Bottom right, HEK-293 cells were cotransfected with EphB6-M and EphB4 and preincubated with PP2 or SU6656 as in B. Cells were stimulated and EphB6 phosphorylation was analyzed as in A. D, left, EphB4 expression was suppressed in MDA-B6-M by lentiviral transduction of EphB4–specific shRNA (EphB4-M-shEphB4). Cells were selected with puromycin and silencing was confirmed by Western blotting. Right, EphB4 silenced MDA-B6-M and MDA-B6-M cells transduced with nonsilencing shRNA (B6-M-NS-shRNA) were stimulated and EphB6 phosphorylation was analyzed as in A. Unstimulated cells were treated for 12 min with 1 μg/mL of precomplexed human IgG.
Figure 4. c-Cbl plays a central role in EphB6 signaling. A, MDA and MDA-B6-M cells were stimulated with precomplexed ephrin-B2. Unstimulated cells were treated for 7 min with precomplexed human IgG (1 μg/mL). EphB6–c-Cbl interaction was monitored by Western blotting of c-Cbl immunoprecipitates with anti-Myc. Sample loading was monitored by reblotting with anti-c-Cbl. The specificity of c-Cbl–EphB6 coprecipitation was confirmed in ephrin-B2–stimulated cells by no-antibody precipitation (15°) and immunoprecipitation with irrelevant matching antibody (I.A.). B, MDA, MDA-pc3, and MDA-B6 cells were stimulated and c-Abl phosphorylation was analyzed by Western blotting of c-Abl immunoprecipitates with anti-phosphotyrosine. C, left, MDA-B6-M cells were transduced with nonsilencing shRNA (B6-M-NS-shRNA) or c-Cbl–specific shRNA (B6-M-shCbl) and analyzed by Western blotting; right, MDA-B6-M, B6-M-shCbl, and B6-M-NS-shRNA cells were stimulated, and Abl phosphorylation was assessed as in B. D, top left, MDA-MB-231 cells were transfected with a Myc-tagged tyrosine-deficient EphB6 mutant (MDA-B6-Y→F) and selected with G418, and expression of EphB6-Y→F was confirmed by Western blotting. EphB6–c-Cbl interaction and c-Abl phosphorylation were analyzed in MDA-B6-Y→F cells as in A and B, respectively.
Figure 5. EphB6 controls breast cancer cells in a c-Cbl–dependent manner. A and B, the adhesion of B6-M-shCbl (A) and of control B6-M-NS-shRNA cells (B) was examined in ephrin-B2–precoated or IgG-precoated plates. Adhesion was measured as in Fig. 1B, analyzed in triplicate, and is presented for each ephrin-B2 concentration as a percent relative to matching IgG control. MDA-B6-M cells were used as a reference. C, adhesion of B6-M-NS-shRNA and B6-M-shCbl cells to fibronectin was assessed as in Fig. 1C (bottom). Bars, SD. *, \( P < 0.05 \), Student’s \( t \) test, for indicated points and corresponding controls. D, cells were allowed to attach for 7 h at 37°C to coverslips precoated with 0.25 \( \mu \)g/mL ephrin-B2. Cells were stained with rhodamine-labeled phalloidin and imaged as in Fig. 2D. Scale bar, 10 \( \mu \)m.
Discussion

The metastatic behavior and invasiveness of cancer cells relies on deregulated migration, cell-cell, and cell-matrix adhesion (30, 46–48). Eph receptors affect these responses in a very complex, cell type–specific, and often ligand concentration–dependent manner (4). This complexity is likely to partially reside in the ability of multiple members of the Eph family to be simultaneously activated by the same ligand, although potentially with different efficiencies. Different cell types express diverse combinations of Eph receptors, and each specific composition of Ephs could be expected to affect cell behavior in a very distinct manner. The reduced expression of a kinase-dead Eph family member, EphB6, has been consistently associated with increased aggressiveness and metastatic activity in various types of malignancies (12, 20–22), suggesting that this molecule may act as a suppressor of cancer metastasis. Our work shows that the reconstitution of EphB6 expression in highly invasive and metastatic EphB6-deficient breast cancer cells, MDA-MB-231, enhances their adhesive properties. This is associated with ephrin-B2–induced morphologic changes, including actin cytoskeleton rearrangements, cell spreading, and the formation of multiple cell protrusions, ultimately resulting in strong inhibition of the invasive behavior. EphB6 anti-invasive action is not restricted to MDA-MB-231 because its silencing strongly increases invasiveness of Hs578T breast cancer cells.

EphB6 expression has been shown to support signaling-independent tethering of HEK-293 cells to ephrin-B2 (24). In contrast, our observations in breast cancer cells suggest that EphB6-induced responses require active cytoskeletal rearrangements and rely on EphB6-specific signaling. Our investigation of the molecular mechanism of EphB6 action shows that, in response to ephrin-B2, EphB6 interacts with c-Cbl in breast cancer cells. Eph6–c-Cbl interaction has been reported previously in Cos7 (23) and in the T-cell line Jurkat (38); however, its biological significance has not been addressed. c-Cbl is a pluripotent signaling protein, and its best described function involves the ability to act as an E3 ubiquitin ligase and to target specific cell surface receptors and cytoplasmic proteins for degradation (49). c-Cbl is also known to operate in its much less well-understood capacity as an adaptor protein, interacting with multiple partners and controlling cytoskeletal rearrangements and cell morphology (39).

Interestingly, EphB6 not only interacted with c-Cbl but also supported tyrosine phosphorylation of the c-Cbl signaling partner and cytoskeletal regulator, Abl. Although we could not detect c-Cbl–Abl complexes in MDA-MB-231 cells, most probably due to the limited sensitivity of available antibodies, Abl phosphorylation proved to be mediated by c-Cbl because c-Cbl silencing blocked EphB6-induced phosphorylation of Abl. This suggested that c-Cbl could play a central role in EphB6-induced proadhesive and anti-invasive responses by allowing it to interact with cytoskeleton-regulating signaling molecules. This proved to be the case, as silencing c-Cbl in MDA-B6-M suppressed cell attachment and inhibited EphB6-dependent morphologic changes. In agreement with the importance of c-Cbl for EphB6-induced cell adhesion, c-Cbl silencing also blocked the ability of EphB6 to suppress breast cancer invasiveness. The negative effect of Abl silencing on EphB6 anti-invasive action provided an additional support for the importance of the EphB6-Cbl-Abl signaling pathway.

As EphB6 is kinase dead (11), its signaling was expected to be controlled through ligand-induced phosphorylation mediated by either EphB1 (23) or a Src family kinase (24). However, our experiments show that EphB4 interacts with EphB6 and can also mediate EphB6 phosphorylation, as inhibition of EphB4 blocks EphB6 phosphorylation in breast cancer cells. This supports a model, where EphB6 phosphorylation is assured by a catalytically active Eph receptor, although it does not exclude the possibility that in certain cell types this process may also rely on Src kinase activity. Because EphB6 phosphorylation is critical for its signaling and anti-invasive action, these observations are suggestive of a curious situation, where EphB6 operates as a partner of specific EphB receptors, switching their behavior to cancer suppressive. Indeed, our work shows that although EphB4 supports invasiveness, when acting in EphB6 absence or when overpowered EphB6, it is also indispensable for EphB6-induced adhesive responses. Therefore, the behavior of breast cancer cells is likely to be defined by the balance between EphB4 signaling and the activity of the EphB4–EphB6 complex.

Overall, our work describes a novel role for EphB6 and shows a new mechanism of EphB6 action in the regulation of breast cancer cell adhesion, morphology, and invasiveness. Combined with previous expression studies, showing that reduced EphB6 expression correlates with increased aggressiveness in multiple malignancies (12, 20–22), it also highlights EphB6 potential as a general suppressor of malignant behavior, which is likely to operate through the active modulation of cellular responses mediated by catalytically competent EphB receptors.

**Figure 6.** c-Cbl is required for EphB6-induced anti-invasive activity. A, breast carcinoma cells were examined in a Matrigel invasion assay. Cells were loaded into the upper wells of invasion chambers in serum-free medium containing 1.5 μg/mL of precomplexed ephrin-B2. Medium with 8% serum was used as a chemotactic attractant in the lower chambers. The cells were allowed to invade for 21 h at 37°C, fixed in ice-cold methanol, and Giemsa stained. Invading cells were counted on the lower sides of the membranes (three independent membranes per cell line) at ×400 magnification in five randomly selected microscopic fields for each membrane. Invasive activity is displayed as a percent relative to unmanipulated MDA-MB-231. B, invasive activity was assessed in MDA-B6-M cells transduced with additional c-Cbl shRNA (B6-M-shCbl 2; top left), c-Abl shRNA-transduced MDA-B6-M (B6-M-shAbl; top right), MDA-B6-Y-3F cells (bottom left), and Hs578T cells expressing EphB6-silencing (hs-shB6) or nonsilencing (hs-Ns-shRNA) shRNA (bottom right). Data are presented relative to appropriate controls. C, invasiveness of EphB4-silenced MDA-B6-M (B6-M-shEphB4) cells was examined as in A and displayed relative to nonsilencing control. D, invasiveness of MDA cells transduced with EphB4-silencing shRNA (MDA-shEphB4) and EphB4-overexpressing MDA-B6-M cells (B6-M-B4) was measured as in A and is shown relative to nonsilencing shRNA control (MDA-NS-shRNA) or EGF-transduced MDA-B6-M cells (B6-M-GFP), Bars, SD. n.s., not significant. *, P < 0.05, f test.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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