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A Paradigm Shift in EPH Receptor Interaction: Biological Relevance of EPHB6 Interaction with EPHA2 and EPHB2 in Breast Carcinoma Cell Lines

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Abstract. *EPH receptors are the largest known family of receptor tyrosine kinases characterized in humans. These proteins are involved in axon guidance, tissue organization, synaptic plasticity, vascular development and the progression of various diseases including cancer. The varied biological effects of EPH receptors are mediated in part by the expression of these proteins and their intracellular binding proteins. The ability of EPH molecules to form heterodimers within their own class has been suggested, although not exhaustively characterized. We have clarified this phenomenon by showing that EPHB6, a kinase-deficient receptor, can interact with EPHB2 in mammalian cells, and more significantly EPHB6 interacts with EPHA2. However, EPHB6 does not interact with another kinase-deficient receptor, EPHA10. The interaction between EPHB6 and EPHA2 is the first demonstration of an A-type receptor interacting with a B-type receptor. Furthermore, we correlated relative expression of EPHB6, EPHB2 and EPHA2 with non-invasive and invasive phenotypes of breast tumor cell lines. Our results indicate that tumor invasiveness-suppressing activity of EPHB6 is mediated by its ability to sequester other kinase-sufficient and oncogenic EPH receptors. These observations suggest that cellular phenotypes may, in part, be attributed to a combinatorial expression of EPH receptors and heteromeric interactions among the same class, as well as between two classes, of EPH receptors. Our results also suggest that EPHA10 may transduce signals by interacting with other kinase-sufficient receptors in a similar manner.*

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The erythropoietin producing hepatocellular carcinoma (EPH) family of receptors is the largest known family of receptor tyrosine kinases (RTK) with 14 members identified to date in humans. These EPH receptors are divided into A and B classes based on their homology to one another as well their affinity for their ephrin ligands. The ephrins are similarly divided into two classes based on their homology and binding affinities for their respective receptors. Generally, the EPHA receptors (EPHA1 to EPHA8 and EPHA10) bind to the membrane-anchored ephrin A ligands (ephrin A1 to ephrin A5) and the EphB receptors (EPHB1 to EPHB4 and EPHB6) bind to the transmembrane ephrin B ligands (ephrin B1to ephrin B3) (1). There is, however, some evidence of cross-talk between receptor and ligand families. The most notable of these exceptions include the activation of EPHB2 by ephrin A5 ligand and the binding of EPHA4 receptor with ephrin B2 and ephrin B3 (2, 3). Although both of these cross-family interactions take place at lower affinities than with their cognate ligands, there is evidence that they are physiologically relevant. For instance, EPHA4-ephrin B interactions are essential for proper axon guidance and synaptic plasticity in the brain (4). In addition, the binding of EPHB2 with ephrin A5 results in the collapse of growth cones and neurite retraction (2). The ability of these low-affinity interactions to occur *in vivo* is likely the result of the clustering of EPH and ephrins on adjacent cells and tissues.

In addition to their involvement in axon guidance, tissue organization, synaptic plasticity, and vascular development (3, 4), EPH receptors are also known to play significant roles in cancer progression and pathological angiogenesis (5-7). The complementary expression of EPH receptors and ephrin ligands on adjacent cells leads to adhesion, repulsion and migration of cells consequent to integrin activation and actin cytoskeletal rearrangements (4, 8-12).

Interactions between EPH and ephrin molecules have been elucidated in a variety of ways. For instance, biochemical and X-ray crystallographic analyses of the interaction between EPHB2 and ephrin B2 indicate that these molecules form a ring-like structure consisting of two

EPHB2 receptors and two ephrin B2 ligands (13). EPH-ephrin tetramers aggregate further and may cluster into lipid raft microdomains on the surface of cells (4, 13-14). Importantly, it is known that additional EPH receptors can be recruited into these signaling clusters even when not bound to an ephrin molecule (15). The microdomain regions described above with high concentrations of EPH receptors and the proteins associated with them serve as signaling centers mediating a variety of biological activities regulated by these molecules.

It warrants mention that EPH receptors within a class are known to heterodimerize (4). However, the binding affinities of two receptor monomers with each other in a heterodimer are expected to be much different from the affinity of monomers in a homodimer. Such redundancy of dimerization makes it difficult to decipher the function of individual EPH receptors in the progression of cancer. We have shown that the expression pattern of EPH receptors in breast carcinoma cell lines varies depending on the phenotype of the cell, and the expression of EPHB6 is an indicator of invasiveness (16, 17). The abundance of EPHB6 protein is also known to be an indicator of tumor stage in melanoma, non-small cell lung carcinoma, and neuroblastoma (18-22). EPHB6, although kinase-deficient, is a target for phosphorylation by EPHB1 following its activation by ephrin B1 or ephrin B2 (23-25). However, no evidence exists to suggest that EPHA and EPHB receptors are capable of heterodimerizing. We describe here such an interaction between EPHA2 and EPHB6 and discuss the possible implications of these interactions in cancer progression.

Materials and Methods

Cell lines and growth media. MCF-10A, a cell line established from normal breast epithelium, and seven breast carcinoma cell lines (MCF-7, BT-20, MDA-MB-231, MDA-MB-435, MDA-MB-468 and BT549) were used in these investigations (American Type Culture Collection, Manassas, VA, USA). All cells were cultured at 37˚C in the presence of 7% CO_2. MCF-10A cells were grown in 1:1 ratio of DMEM and F12 media (Gibco, Carlsbad, CA, USA) with 5% horse serum (Gibco), 20 mM HEPES (Gibco), 10 ng/ml EGF (Invitrogen), 10 units/ml penicillin (Gibco), 10 μg/ml streptomycin (Gibco), 2.0 mM L-glutamine (Gibco), 10 μg/ml insulin (Invitrogen), 0.1 μg/ml cholera toxin (Sigma, St Louis, MO, USA) and 500 ng/ml hydrocortisone (Sigma). All other cell lines were grown in DMEM (Gibco) with 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 2.0 mM L-glutamine (Gibco), 1.0 mM sodium pyruvate (Gibco), 25 units/ml penicillin (Gibco), and 25 μg/ml streptomycin (Gibco) unless indicated otherwise.

RNA isolation. RNA was isolated from cells by using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) with slight modifications of the recommended protocol. Briefly, cells were grown in a 10 cm^2 dish and lysed by adding 1.0 ml of TRI reagent. The lysate was mixed with 200 μl of chloroform and the mixture centrifuged in a microfuge at 16,000 ×*g* for 15 minutes at 4˚C to separate the solution into aqueous and organic phases. RNA was precipitated from the aqueous phase by adding 250 μl of isopropanol and centrifuging at 16,000 ×*g* at 4˚C for 20 minutes. The pellet was then washed sequentially with 80% and 100% ethanol, resuspended in diethyl pyrocarbonate-treated H_2O and stored in aliquots at –80˚C. The quality of RNA was evaluated by electrophoresis on 1.2% agarose gel made in 246 mM formaldehyde and 3-*N*-morpholino propanesulfonic acid (MOPS) buffer, pH 7.0. The amount of RNA was determined by measuring the absorbance at 260 nm and the purity was confirmed by calculating the ratio of absorbance values at 260 nm and 280 nm.

DNase treatment of total RNA. RNA (20 μg) was treated with 500 ng DNaseI, 80 units RNasin (Promega, Madison, WI, USA) and 1.0 mM $MgCl_2$ in 40 mM Tris buffer (pH 8) in a total volume of 50 μl. The reaction was carried out at 37˚C for one hour and then terminated by incubation at 65˚C for 30 minutes. The elimination of genomic DNA in RNA samples was confirmed in the following manner. First, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for 38 cycles on an aliquot of RNA using primers spanning a small intron of the ephrin A4 gene. The absence of an amplified product corresponding to the intron indicated the absence of genomic DNA. The effectiveness of the DNase treatment was further evaluated by PCR amplification for 38 cycles using either ephrin A4- or EPHB6-specific primers. The absence of a product indicated that genomic DNA had been successfully removed from the mixture.

Semi-quantitative RT-PCR. SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen) was used for all reactions. Equal amounts of RNA, as confirmed by amplification of actin transcript, from each cell line were used in sets of parallel reactions. Except for the number of cycles, which was optimized for each primer set in order to compare relative abundance of these transcripts, the conditions were as follows. The reaction mixtures (20 μl) containing 20 ng DNase-treated RNA, 0.6 pmol each of forward and reverse primers, 0.8 μl of a mixture of reverse transcriptase and *Taq*DNA polymerase and 1× supplied reaction buffer were subjected to RT-PCR. The reverse transcription reaction was performed at 53˚C for 30 minutes followed by incubation at 94˚C for 2 minutes. Each PCR cycle consisted of the following incubations: 94˚C for 15 seconds, 63˚C for 30 seconds, and 68˚C for 1 minute. The primer sequences as well as the number of cycles used for each primer set are listed in Table I.

Amplification of EPHA2, EPHB2, and EPHB6 for cloning. Each transcript was amplified using the TripleMasterTaqPCR kit (Eppendorf, Hauppauge, New York, USA) in 50 μl according to the manufacturer's recommendations. After an initial incubation for 2 minutes at 95˚C, the amplification conditions consisted of 35 cycles of a three temperature PCR: 94˚C for 30 seconds, 58˚C for 30 seconds and 68˚C for 3.5 minutes. Following the 35th cycle, a final extension was performed by incubating the samples at 68˚C for 10 minutes.

Generation of fusion protein constructs. Full-length EPHA2 and EPHB2 were cloned into pCDNA4 TOmycHis A vector (Invitrogen), and full-length EPHB6 was cloned into pCDNA3.1V5His vector (Invitrogen). The cloning sites, primers and templates used to

Table I. *Primer sequences used for transcript amplification.*

Transcript name	Forward primer sequence	Reverse primer sequence	Size of RT-PCR product	Number of cycles
Actin	CTGACTGACTACCTCATGAAG	ATCCACATCTGCTGGAAGGTG	497 bp	18, 20
EPHA2	TCAGCAGCAGCGACTTCGAGGCA	CAGTGGCCAGGGAAGGTGCA	221 bp	27
EPHA10	CCAAGTGTGCCCTGACTACCTGTC	GTTCAGCCAAAGAGATGCCTAGGCTCAC	219 bp	35
EPHB2	ATGGCGCCCCTCTCCTCTGGCATCA	GTTCAGCCAAAGAGATGCCTAGGCTCAC	416 bp	33
EPHB6	GTTCTGGACGACCAGCGACG	GACGTTCAGTTGCAGTCCAG	408 bp	35

Table II. *Cloning strategy for generating full-length constructs into tagged vectors. Gene for EPHB6 was cloned into pCDNAV5His A, and EPHA2 and EPHB2 were cloned into pCDNA4TOmycHisA.*

Gene name (accession no.)	Restriction sites	Forward primer	Reverse primer	Template
EPHA2 (NM_004431)	*Kpn*I/*Xho*I	GATTCAGGTACCCCACC ATGGAGCTCCAGGCAGCC	GGTTACCTCGAGGATGG GGATCCCCACAGTGTTC	Human leukocyte, Marathon-Ready cDNA
EPHB2 (NM_017449)	*Eco*RI	GATTCAGAATTCCCACCAT GGCTCTGCGGAGGCTGG	GGTTACGAATTCAACCTC CACAGACTGAATCTGG	Human brain, Marathon-Ready cDNA
EPHB6 (NM_004445)	*Eco*RI/*Xho*I	CTCAGTTGAATTCCCACCATG GTGTGTAGCCTATGGGTGC	CTCAGTTCTCGAGGACCTC CACTGAGCCCTGCTGC	EPHB6 cDNA in pCDNA3.1

amplify the corresponding transcripts are listed in Table II. Each clone was sequenced to confirm that the protein coding sequences were in frame with the *myc*, or *V5* tag present on the vector.

Transfection and western blotting. EPHA2, EPHB2, and EPHB6 constructs were transfected into HEK293T or MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Thirty hours after transfection, culture medium was removed and the plates were washed with ice-cold PBS. An aliquot (500 µl) of cell lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing 1 mM phenylmethylsulphonyl fluoride (Sigma) was added to each plate and the plates were kept on ice for 5 minutes. The cell lysate was collected in a 1.5 ml tube. The lysates were sonicated 4 times for 5 seconds each using a Misonix 3000 sonicator at a setting of 2.0 and the homogenate was then centrifuged at 16,000 ×*g* in a microfuge at 4˚C for 10 minutes. The supernatant was collected in a fresh tube and used for analysis. Approximately 50 µg of protein (in a volume of 20 µl), as determined by Bradford reagent (Sigma), was combined with 10 µl of 3× loading buffer (Cell Signaling Technology) supplemented with 125 mM dithiothreitol. The samples were loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoresed at 60 mA for approximately 1.5 hours. The proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA) and processed as described below for the detection of EPHB6 or the *myc* epitope.

Detection of EPHB6. First, the membrane was incubated for one hour at room temperature in Tris-buffered saline (TBS) containing 5% nonfat dry milk. The membrane was subsequently incubated with 5 µg of goat anti-mouse EPHB6 polyclonal antibody (R&D systems, Minneapolis, MN, USA) in 15 ml of TBS containing 0.1% Tween® 20 (TBS-T) and 5% bovine serum albumin (BSA) at 4˚C overnight. The blot was then washed 3 times for 5 minutes each with TBS-T at room temperature and incubated with a horse radish peroxidase (HRP)-conjugated donkey anti-goat-antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100,000 in TBS-T for one hour at room temperature. The membrane was washed 3 times for 5 minutes each with TBS-T and incubated with HRP substrate (Millipore, Billerica, MA, USA). The chemiluminescent signal was detected by exposing the membrane to an X-ray film, and the intensity of the signal was used to determine the relative abundance of EPHB6 protein.

Detection of the myc epitope. The membrane was incubated for one hour at room temperature in TBS containing 5% nonfat dry milk. It was subsequently incubated overnight at 4˚C with 15 ml of blocking buffer containing 7.5 µl of an alkaline phosphatase (AP)-conjugated anti-myc monoclonal antibody generated in mouse (Invitrogen). The blot was then washed 3 times for 5 minutes each with TBS-T at room temperature, and incubated with 1-Step™ NBT/BCIP reagent (Millipore), as recommended by the manufacturer.

Results

Expression of kinase-deficient receptors EPHB6 and EPHA10 in breast carcinoma cell lines. We have previously correlated the expression profiles of the EPH family of transcripts to the phenotype of breast carcinoma cell lines (16) and confirmed the importance of EPHB6 protein as a suppressor of invasiveness (17). The mechanism of action of this protein in this process has yet to be elucidated. We therefore investigated a comparison of *EPHB6* transcript

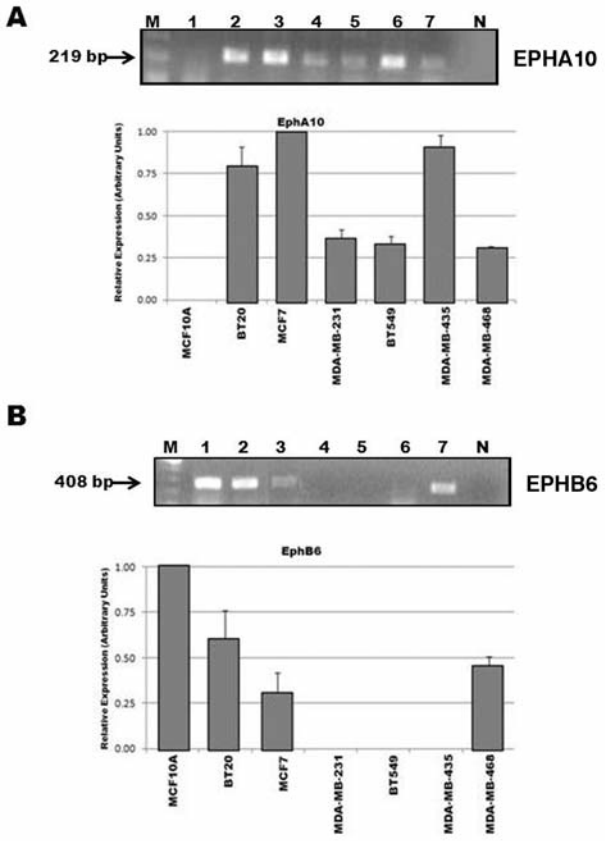

Figure 1. *Relative abundance of EPHA10 and EPHB6 receptor transcripts in breast carcinoma cell lines by RT-PCR. A: DNase-treated RNA templates (20 ng) from MCF-10A (lane 1), BT-20 (lane 2), MCF-7 (lane 3), MDA-MB-231 (lane 4), BT549 (lane 5), MDA-MB-435 (lane 6) and MDA-MB-468 (lane 7) were amplified with gene-specific primers as described in the Materials and Methods section and Table I. "M" designates DNA size marker and "N" refers to a no-template control. The products were separated on 1.5% agarose gels and photographed using Gel Doc (Bio-Rad) imaging software. The results shown are representative of three trials for each transcript. The bar diagram shows quantification of EPHA10 transcript. The relative levels of the EPHA10 were determined using SigmaGel™ gel analysis software (SPSS Science) and normalized to β-actin. B: EPHB6 transcript levels in various cell lines were determined by using gene specific primers and relative expression was quantified as above. The lanes and bars corresponding to various cell lines are identical to those described for panel A.*

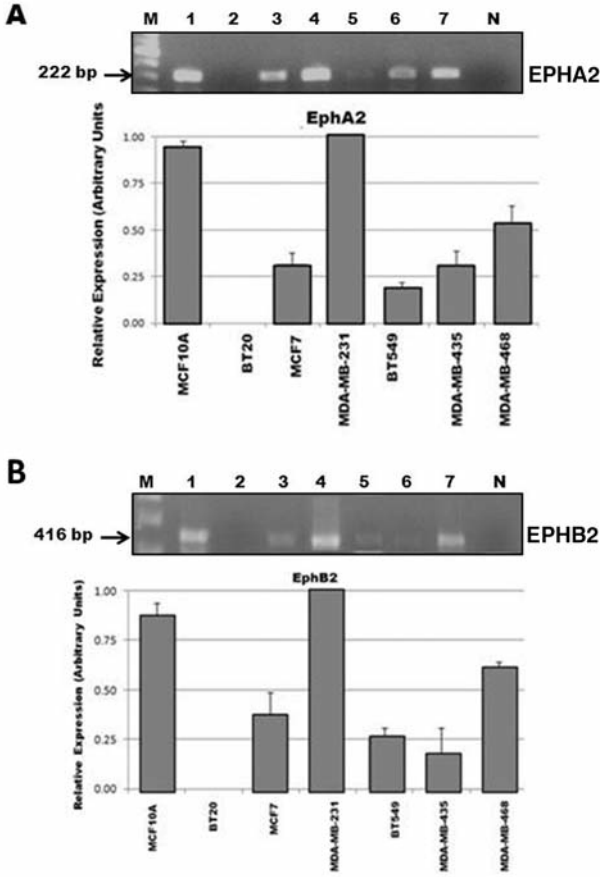

Figure 2. *Relative abundance of EPHA2 and EPHB2 receptor transcripts in breast carcinoma cell lines. A: DNase-treated RNA templates (20 ng) from MCF-10A (lane 1), BT-20 (lane 2), MCF-7 (lane 3), MDA-MB-231 (lane 4), BT549 (lane 5), MDA-MB-435 (lane 6) and MDA-MB-468 (lane 7) were amplified with gene-specific primers as described in the Materials and Methods section and Table I. "M" designates DNA size marker and "N" refers to a no-template control. The products were separated on 1.5% agarose gels and photographed using Gel Doc (Bio-Rad) imaging software. The results shown are representative of three trials for each transcript. The bar diagram shows quantification of EPHA2 transcript. The relative levels of EPHA2 transcript were determined using SigmaGel™ gel analysis software (SPSS Science) and normalized to β-actin. B: EphB2 transcript levels in various cell lines were determined by using gene specific primers and relative expression was quantified as above. The lanes and bars corresponding to various cell lines are identical to those described for panel A.*

with that of *EPHA10*, another receptor known to be kinase-deficient. We used RNA isolated from: i) a cell line derived from normal breast epithelial tissue (MCF-10A), ii) two tumorigenic/non-invasive cell lines (BT-20 and MCF-7), and iii) four invasive cell lines (MDA-MB-231, BT-549, MDA-MB-435 and MDA-MB-468). As shown in Figure 1, *EPHA10* is expressed at various levels in all but the normal cell line. The expression of *EPHB6* transcript, on the other hand, confirms our earlier observation that its levels are high in normal cells and lower in tumor cells, according to the aggressiveness of the phenotype. These results suggest that the mechanisms underlying the actions of kinase-deficient receptors EPHB6 and EPHA10 may be quite different.

Semi-quantitative expression of kinase-sufficient receptors EPHA2 and EPHB2 in breast carcinoma cell lines. As described above for *EPHB6* and *EPHA10*, similar profiling was performed to relate the expression of *EPHA2* and

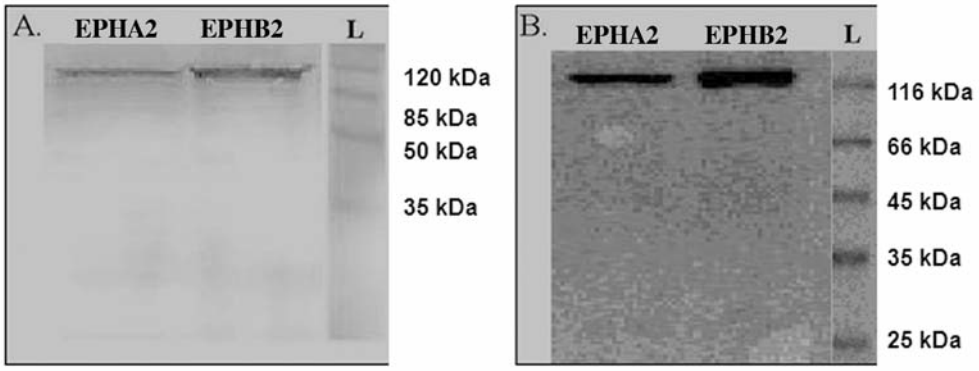

Figure 3. *EPHB6 interacts with EPHA2 and EPHB2 in HEK293T cells. A: The indicated fusion constructs (along with an EPHB6 fusion construct) were transfected into HEK293T followed by detection of myc epitope as described in the Materials and Methods section. B: Aliquots of the lysates from transfected samples described in panel A were immunoprecipitated with an anti-EPHB6 antibody as described in the Materials and Methods section and subjected to Western blotting with an anti-myc antibody.*

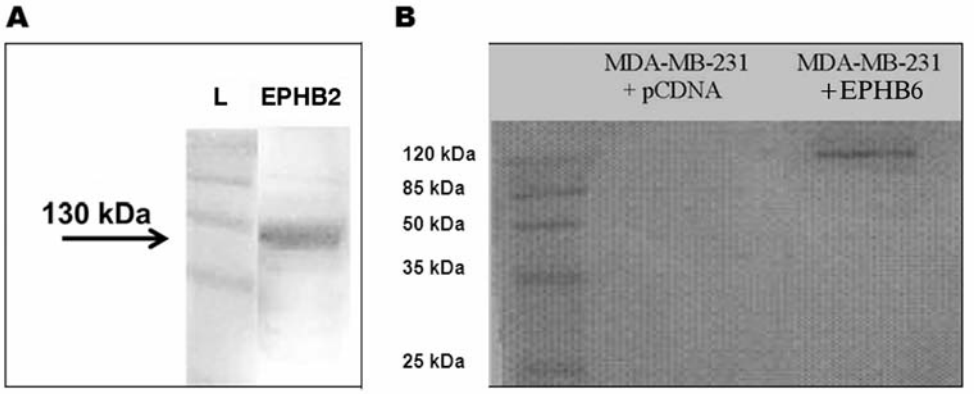

Figure 4. *EphB2 interacts with EPHB6 in MDA-MB-231 cells stably transfected with EPHB6. A: EPHB2 fusion construct was transfected into stable MDA-MB-231 clones and cell lysates were Western blotted and probed with anti-myc antibody as described in the Materials and Methods section. B: EPHB6 expressing stable clone of MDA-MB-231 cell line was transfected with EPHB2 and subjected to co-immunoprecipitation with anti-EphB6 antibody as described in the Materials and Methods section. The precipitate was electrophoresed and blots probed with anti-myc antibody as described in the Materials and Methods section.*

EPHB2 with phenotypes of breast carcinoma cell lines. The patterns of expression for *EPHA2* and *EPHB2* in various cell lines were remarkably similar. Specifically, these transcripts were expressed in MCF-10A, non-invasive cell line MCF7 and all four invasive cell lines (Figure 2).

EPHA2 and EPHB2 are capable of interacting with EPHB6 in mammalian cells. Given the lack of kinase activity in EPHB6 and EPHA10 receptors and similar patterns of expression for *EPHA2* and *EPHB2* in various cell lines, we reasoned that cell line-specific phenotypes might, in part, be explained after investigating interactions between receptors. We therefore investigated potential interactions of EPHB6 with EPHA2, EPHA10 and EPHB2 in mammalian cells. There is direct experimental evidence of heterodimerization of EPH receptors within the same class (26), but nothing is known about the interactions between two classes of receptors. Thus, EPHA2 and EPHA10 were chosen for investigation of their interactions with EPHB6.

To perform the above analyses, EPHA2, EPHA10 and EPHB2 cDNAs were cloned into pCDNA4TOmycHis (Invitrogen) containing *His* and *myc* epitopes. Similarly, EPHB6 was cloned into pCDNA3.1V5His (Invitrogen) containing *His* and V5 epitopes. The *EPHA2* and *EPHB2* constructs were transfected separately into HEK293T cells along with the *EPHB6 V5-His* construct, as described in the Materials and Methods section. The lysates from the transfected cells were electrophoresed and the proteins were

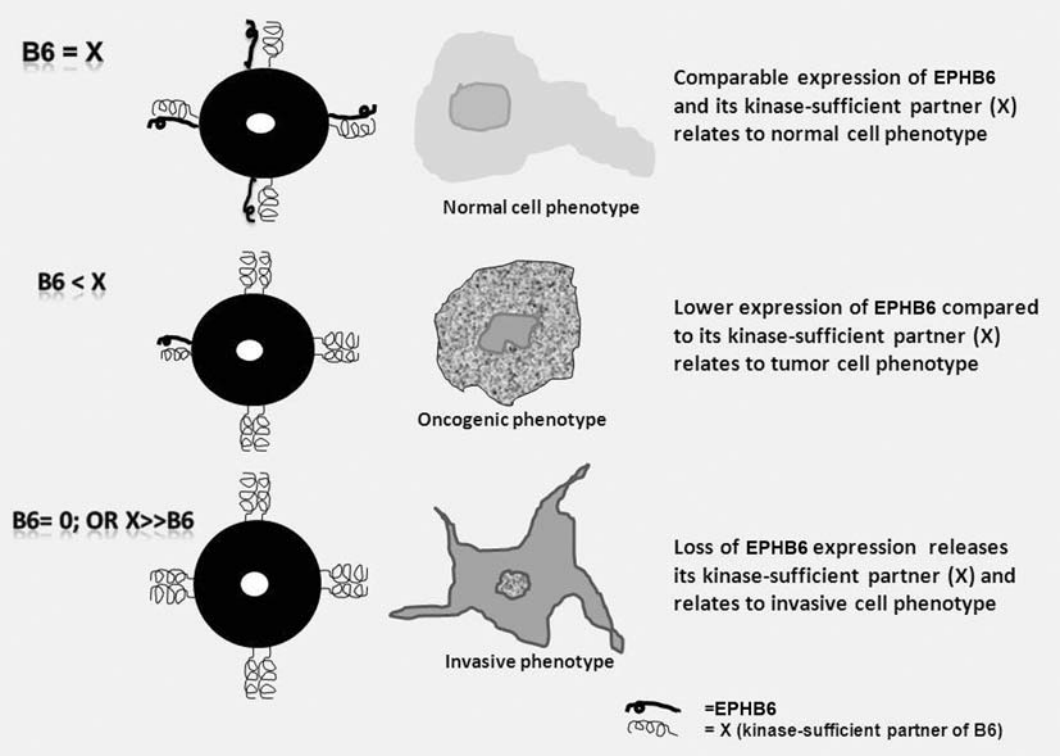

Figure 5. *A model to correlate receptor interactions with cellular phenotypes. Differential expression of EPHB6 modulates the levels of its interacting partner and influences downstream signaling pathways.*

transferred to a membrane. The incubation of the membrane with anti-myc antibody confirmed the presence of fusion proteins in transfected cells based on their expected molecular weights (Figure 3A). Subsequently, 200 μl aliquot (1 μg protein/μl) of cell lysate from each transfection was subjected to immunoprecipitation using an anti-EPHB6 antibody (R&D Systems) and analyzed for the presence of the *myc* fusion protein. As seen in Figure 3B, both EPHA2 and EPHB2 fusion proteins were present in the immunoprecipitated samples, suggesting that they interact with the EPHB6 fusion protein. No fusion proteins were detected in control immunoprecipitation reactions performed without antibody or with whole goat IgG antibodies. Together, these results demonstrate that the fusion proteins interact with the EPHB6 protein as opposed to the anti-EPHB6 antibody or agarose beads used for precipitation. Furthermore, an additional set of experiments indicated that EPHB6 is incapable of interacting with the protein of *EPHA10* transfected into EPHB6-expressing cells.

After recognizing that EPHB2 and EPHA2 interact with EPHB6 in HEK293T cells, EPHB2 was tested for its ability to interact with EPHB6 in stable transfectants of MDA-MB-231 cells. We have previously shown that there is no detectable endogenous EPHB6 protein expression in MDA-MB-231 cells (16). For these studies, two clones were used: i) MDA-MB-231 + pCDNA (control) and MDA-MB-231 + EPHB6 (a clone previously shown to express the EPHB6 protein). Figure 4A indicates that EPHB2 is detectable in MDA-MB-231 cells transfected with the EPHB2 construct. A co-immunoprecipitation experiment performed with anti-EPHB6 antibody indicated that EPHB2 is detectable in the immunoprecipitate under these conditions (Figure 4B). However, EPHB2 was not detectable in control immunoprecipitations. It warrants mention that the green fluorescence protein transfections indicated a significantly higher efficiency of transfection in HEK293T cells as compared to MDA-MB-231 cells. Together these results demonstrate that EPHB2 is capable of interacting with EPHB6 in MDA-MB-231 cells stably transfected with *EPHB6*. Furthermore, EPHA2 and EPHB2 are capable of interacting with EPHB6 in the mammalian cell line HEK293T.

Model for EPHB6 interactions and prediction of cellular phenotype. We have proposed a model (Figure 5) to explain the results described here. As shown in the figure, comparable levels of kinase-deficient and kinase-sufficient

EPH receptors lead to the formation of heteromeric dimers of these two receptors that transduce signals required to maintain normal cell phenotypes. A decrease in the level of the kinase-deficient receptor, or an increase in the level of its cognate kinase-sufficient receptor results in sufficient excess of the kinase-sufficient receptor such that it homodimerizes to mediate signaling pathways specific to tumor cells. In the absence of the kinase-deficient receptor, the kinase-sufficient receptor cannot be sequestered and thus forms abundant amounts of homodimers that mediate tumorigenic as well as invasive pathways.

Discussion

We have previously used the yeast two-hybrid system to show that the cytoplasmic domain of EPHB6 interacts with a variety of intracellular proteins (27). However, this assay was not adequate to address interaction between transmembrane proteins. Thus, interactions between specific EPH receptors required a targeted investigation, and co-immunoprecipitation was considered a useful method to investigate such interactions.

It is known that EPH receptors within the same family are capable of heterodimerization (4). Such heteromeric interactions of EPHB6 with other members of the EPHB family would likely lead to a change in their binding affinities for ephrin ligands, as well as for intracellular docking and/or signaling proteins (28). While comprehensive analyses have been performed to determine the affinities of EPH receptors for ephrin ligands, these studies have not explicitly considered the effect that heterodimerization may have on the binding of these two types of proteins. Previously, it was shown that EPHB6 is phosphorylated upon dimerization with EPHB1 (23), and the activated receptor can transduce signals in a phosphorylation-dependent manner (23, 29, 30). In support of these observations, we have demonstrated that EPHB6 can also interact with EPHB2 and EPHA2. Thus, the expression profile of other EPH molecules is likely to alter the nature and magnitude of signaling through EPHB6 by allowing EPHB6 to sequester oncogenic and/or invasion-promoting/-suppressing molecules. This phenomenon is likely not unique to EPHB6 and therefore suggests that a better understanding of the nature of EPH receptor heterodimerization and its effects on downstream signaling are required. The inability of two kinase-deficient receptors such as EPHB6 and EPHA10 to interact suggests that phosphorylation may be required for maintaining a stable interaction. Alternatively, if phosphorylation is not a pre-requisite for downstream signaling then kinase-deficient receptor may damp signal transduction by sequestering a kinase-sufficient receptor. Based on these observations, we propose a model for the interaction of EPHB6 with EPHA2 and EPHB2 (Figure 5). Such a model predicts a similar mechanism for the interaction of kinase-deficient EPHA10 receptor with other EPH receptors.

Given the important roles EPHB6 and EPHA2 play in tumor progression (5, 17, 20, 22, 31-34), the possible interaction between EPHB6 and EPHA2 becomes biologically relevant. Overexpression of EPHA2 is associated with an increase in the growth of MCF-10A cells in soft agar and their ability to invade through matrigel (33). Furthermore, it has been shown that high levels of EPHA2 are found in more aggressive stages of melanomas and prostate, breast, colon, lung, and esophageal carcinomas (5). On the other hand, the loss of EPHB6 expression is correlated with the aggressiveness of breast carcinoma and neuroblastoma cells (16-17, 20). It should be noted that although EPHA2 is overexpressed in 40% of human carcinomas, its expression alone is not a perfect predictor of a tumor's phenotype (35, 36). Furthermore, the localization and phosphorylation status of this protein may, in part, determine the cellular phenotype (34, 37, 38). While overexpression of EPHA2 has been correlated to tumor phenotype (5), the interaction of EPHB6 with EPHB2 assumes significance for the following reasons. EPHB2, a tumor suppressor, is required to prevent the progression of colorectal carcinoma (39). Furthermore, overexpression of EPHB2 in a mouse xenograft model was shown to reduce tumor growth (40). Thus, an interaction between two tumor suppressor molecules may possibly counteract other oncogenic activities in the cell. The mechanisms of downstream effects of such interactions remain to be elucidated.

We propose that EPHB6 can mediate the localization and signaling through EPHA2 to reduce the invasiveness or aggressiveness of cancer cells (Figure 5). This hypothesis is supported by the following observations. The overexpression of EPHA2 and underexpression of EPHB6 are both indicators of more advanced phenotypes in several types of cancer. Specifically, elevated levels of *EPHA2* and reduced levels of *EPHB6* transcript were observed in a panel of breast carcinoma cell lines (16). We have also shown that re-expression of EPHB6 in MDA-MB-231 cells can alter the phenotype of these cells and significantly reduces their invasiveness without impacting the expression levels of EPHA2 (17). In view of these observations, we believe that EPHB6 suppresses invasiveness by dampening the oncogenic effects of EPHA2 as illustrated in Figure 5. The heterodimeric interactions between EPH receptors may thus be used to designate tumor suppressor–oncogene pairs of receptors and to develop appropriate high-affinity peptides for impairing the oncogenic activities of specific receptors.

The crystal structure of EPHA4 receptor provides an explanation for its binding to a B-class ephrin based on two distinct conformations of the receptor in a ligand-free state (38, 41, 42). It is thus likely that native structural domains in EPH receptors may facilitate heterodimerization of A-type

receptors with B-type receptors. Such cross-class heterodimerization presents a unique opportunity to target either one or both of these heterodimerizing partners for therapeutic interventions. In particular, binding of small peptides to high-affinity sites (43-45) on these receptors can impair receptor dimerization and may likely prevent progression towards an invasive cancer cell phenotype.

In conclusion, we have shown that EPHB6 is capable of interacting with EPHB2 in HEK293T and MDA-MB-231 cells. More importantly, we have demonstrated that EPHA2 is capable of interacting with EPHB6 in HEK293T cells. The interaction between A- and B-type receptors has not been reported in the literature. The observation that EPHA2 interacts with EPHB6 is the first such report, and it has significant implications for the description and interpretation of EPH receptor-mediated signal transduction. These interactions also offer explanations for specific profiles of EPH receptor expression and their relationship to cellular phenotypes.

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