Abstract. Erythropoietin-producing hepatocellular carcinoma cell (EPH) receptors comprise the most abundant receptor tyrosine kinase family characterized to date in mammals including humans. These proteins are involved in axon guidance, tissue organization, vascular development and the intricate process of various diseases including cancer. These diverse functions of EPH receptors are attributed, in part, to their abilities for heterodimerization. While the interacting partners of kinase-deficient EPHB6 receptor have been characterized, the interaction of the kinase-dead EPHA10 with any other receptor has not been identified. By using co-immunoprecipitation, we demonstrated physical interaction between kinase-deficient EPHA10 with kinase-sufficient EPHA7 receptor. Immunocytochemical analyses have revealed that these two receptors co-localize on the cell surface, and soluble portions of the receptors exist as a complex in the cytoplasm as well as the nuclei. While EPHA7 and EPHA10 co-localize similarly on the membrane in MCF10A and MCF7 cells, they were differentially co-localized in MDA-MB-231 cells stably transfected with empty pcDNA vector (MDA-MB-231-PC) or an expression construct of EPHB6 (MDA-MB-231-B6). The full-length isoforms of these receptors were co-localized on the cell surface, and the soluble forms were present as a complex in the cytoplasm as well as the nucleus in MDA-MB-231-PC cells. MDA-MB-231-B6 cells, on the other hand, were distinguished by the absence of any signal in the nuclei. Our results represent the first demonstration of physical interaction between EPHA10 and EPHA7 and their cellular co-localization. Furthermore, these observations also suggest gene-regulatory functions of the complex of the soluble forms of these receptors in breast carcinoma cells of differential invasiveness.

Breast cancer is the leading cause of cancer mortality in women worldwide (1). In the United States, breast cancer accounts for 29% of new cancer cases and 14% of deaths among women (2). The prognosis is positive when breast cancer is localized and diagnosed in early stages of the disease. Cancer cells from aggressive tumors, however, invade adjacent tissues, enter the circulation and form secondary tumors at distant sites in a process known as metastasis. This metastatic progression of tumor cells is responsible for a majority of cancer deaths.

A variety of genes have been implicated in transformation of a normal cell into a cancer cell, and cell surface receptors have emerged as an important class of genes to influence cellular phenotypes. Erythropoietin-producing hepatocellular carcinoma cell (EPH) receptor family with 14 distinct receptor tyrosine kinases (RTK) constitutes an important class of cell surface proteins. Among these, EPHA10 and EPHB6 are two kinase-deficient proteins (3). Upon binding to cognate ephrin ligands, which are also cell surface proteins, EPH receptors are auto- and cross-phosphorylated. The extracellular domain of EPH receptors contains the N-terminal ephrin-binding region, a cysteine-rich domain and two fibronectin type-III repeats. The cytoplasmic region of the receptor includes a juxtamembrane segment, the tyrosine activation domain, the sterile-alpha motif, and a post-synaptic density protein-Drosophila disc large tumor suppressor-zonula occludens-1 (PDZ) domain binding sequence at the C-terminus (4). Interestingly, there are a variety of alternate transcripts of these receptors that reside in the cytoplasm (5-7). Some shorter isoforms of EPH receptors found in the cytoplasm also arise by proteolytic cleavage (8, 9), and these cleavage products, as well as the alternatively-spliced isoforms, are implicated in various cellular functions (6).
Some pre-clinical and laboratory studies have established the correlation of EPH RTKs in tumor growth, metastasis, and the formation of functional microvascular networks in cancer (10-12). The investigations of breast cancer cell lines with differing phenotypes have illustrated the involvement of a variety of cell surface proteins in the progression of breast carcinoma (13, 14). While EPHA7 was recently correlated with poor prognosis and metastasis (15,16), higher expression levels of EPHA10 were found in invasive breast carcinoma cells (13). Given its lack of kinase activity, the biochemical mechanisms underlying EPHA10 activation have, thus, become relevant to explaining its involvement in breast tumorigenesis.

In a phylogenetic analysis of the EPH receptors, EPHA7 and EPHA10 were found to be the most closely related, with 65% identity in the extracellular region, for which they share compatible ephrin ligands (7, 17, 18). However, an increase in both these receptors is correlated with poor prognosis and metastasis in breast cancer respectively (13, 16). It has been shown that EPHA7 reduces ERK phosphorylation and causes cellular dedifferentiation (6). EPHA10, on the other hand, increases phosphorylation of p38 and DNA synthesis (17).

In light of the structural and sequence similarities between EPHA7 and EPHA10 receptors and their abundance in breast cancer cells, our studies aimed at determining the expression and cellular localization of these two receptors and possible physical interaction between them. We reasoned that physical interaction between EPHA7 and EPHA10 would demonstrate the mechanism of activation of kinase-deficient EPHA10 and biological relevance of EPHA7-EPHA10 complexes in influencing phenotypes of breast cancer cells.

Materials and Methods

Cell culture. MCF-10A cells were grown in 1:1Dulbecco’s Modified Eagle Medium (DMEM):F12 media from Gibco-Thermo Fisher Scientific (Grand Island, NY, USA) supplemented with 5% horse serum and 0.1 μg/ml cholera toxin, and 500 ng/ml hydrocortisone from Sigma-Aldrich (St. Louis, MO, USA), 10 ng/ml epidermal growth factor and 10 μg/ml of insulin from Invitrogen (Thermo Fisher Scientific). MCF-7, MDA-MB-231, and MDA-MB-231-B6, a cell line stably transfected with a mammalian expression construct of full-length EPHB6 cDNA, were all maintained in DMEM from Gibco (Thermo Fisher Scientific) supplemented with 10% horse serum from Sigma-Aldrich. All cell lines were supplemented with 5000 U/ml of penicillin/streptomycin from Gibco and grown in a humidified chamber with 5% CO2 at 37°C. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231-PC and MDA-MB-231-B6 were generated by stably transfecting MDA-MB-231 cells either with an empty pCDNA (PC) vector or an expression construct of EPHB6 (B6), respectively (19).

Co-immunoprecipitation. All four cell lines were grown to 90-95% confluency. Briefly, cells were washed with phosphate-buffered saline (PBS) and suspended in 1 ml of immunoprecipitation lysis buffer. Cells were passed through a hypodermic needle attached to a 1 ml syringe for complete lysis, and the lysate centrifuged for 15 minutes at 16,000 g at 4°C to clear cell debris. One hundred micrograms of lysate was incubated with 50 μl of A/G agarose beads from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for 1 hour. The lysate was transferred to a fresh tube with 10 μl of primary antibody and rotated overnight at 4°C. The following day, A/G agarose beads were added to the tubes and rotated for 1 hour. At 4°C, cells were washed with immunoprecipitation buffer four times and all washes were collected. The beads were boiled in 50 μl of sodium dodecyl sulfate sample buffer and prepared for western blot.

Immunocytochemistry and co-localization. The cell lines were grown on Falcon two-chamber tissue culture slides from Becton-Dickinson (Franklin Lakes, NJ, USA) and fixed to the slides with ice-cold methanol. Briefly, fixed cells were rinsed in PBS and permeabilized with 0.25% Triton-X-100 for 5 minutes. After rinsing with PBS twice, cells were treated with 10% bovine serum albumin (BSA) for 30 min and then incubated with 1:20 dilution of EPHA7 or EPHA10 antibody purchased from Santa Cruz Biotechnology for 1 h. Control chambers were incubated in 3% BSA without antibody. Cells were then washed in 3% BSA four times for 5 minutes each and incubated with 1:1000 dilutions of Alexa-Fluor 488 or Alexa-Fluor 647-conjugated fluorescent secondary antibodies from Invitrogen for 30 min in the dark. After washing four times for 5 minutes, the process was repeated in the dark for the second primary antibody. Cells were then rinsed with water, stained with 4',6'-diamidino-2-phenylindole (DAPI) using mounting medium from Vector Laboratories (Burlingame, CA, USA) and stored in the dark at −20°C. The slides were visualized with a Nikon Eclipse Ti-S microscope (Nikon, Irvine, CA, USA) and analyzed with Nikon’s NIS-Elements-AR software.

Western blotting. Cells were grown in a 75 cm2 tissue culture flask to 85-90% confluency, washed with PBS and then lysed with RIPA buffer. The lysate was centrifuged at 16000 g for 15 minutes at 4°C, dissolved in sample buffer and an aliquot of the lysate was electrophoresed on a 12% polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride membrane overnight. The membrane was blocked with 10% non-fat dry milk in a mixture of Tris-buffered saline and Tween 20 (TBST) for 1 h and incubated with either EPHA7 or EPHA10 primary antibody from Santa Cruz Biotechnology overnight. Blots were washed with TBST three times for 15 min and then incubated in secondary antibody for 1 h at room temperature. The non-specifically bound antibody was removed by washing the blots three times in TBST for 15 min and antibodies bound to target proteins were visualized on VersaDoc Imaging System from Bio Rad (Hercules, CA, USA).

Results

EPHA7 and EPHA10 protein isoforms are detectable in normal breast cells and breast carcinoma cell lines. To investigate biological significance of kinase-deficient EPHA10 via its interaction with kinase-sufficient EPHA7, we first addressed the presence of these proteins in different cell lines established from normal breast and breast carcinomas. These cells correspond to normal breast (MCF10A), non-invasive breast tumor cells (MCF7), highly
invasive MDA-MB-231 breast carcinoma cells transfected with an empty pCDNA vector and MDA-MB-231 cells transfected with an expression construct of EPHB6. All these cells had detectable levels of both EPHA7 and EPHA10. It is noteworthy that several isoforms of these proteins have been reported in mammalian cells. While the relative amounts of protein isoforms in these cell lines were variable, three isoforms each of EPHA7 and EPHA10 were detected in all cell lines used here. EPHA7 receptor isoforms were 56 kDa, 93 kDa, and 112 kDa, whereas EPHA10 appeared to exist as 48 kDa, 50 kDa and 86 kDa proteins in various cell lines (Figure 1). The confirmation of the presence of EPHA7 and EPHA10 complexes in cell lysates was confirmed, and the results described below demonstrate physical interaction between these two proteins.

Cell-line model system for interaction between EPHA7 and EPHA10. We previously showed that EPHA10 is expressed in the invasive breast carcinoma cell line MDA-MB-231 but not in noninvasive breast carcinoma cells (13). While low levels of EPHA7 are associated with metastasis in esophageal squamous and lung carcinoma (15), higher expression of EPHA7 is seen in breast cancer cells (16). In light of the changes seen in the levels of kinase-deficient EPHA10 and some preliminary observations in our laboratory, we considered investigating the physical interaction between EPHA10 and EPHA7.

To facilitate functional studies, we used a cell-line model system consisting of normal breast cells (MCF 10A), noninvasive breast carcinoma cells (MCF-7), invasive breast carcinoma cells (MDA-MB-231-PC) and experimentally engineered less-invasive breast carcinoma cells (MDA-MB-231-B6). The presence of EPHA7 and EPHA10 complexes in cell lysates was confirmed, and the results described below demonstrate physical interaction between these two receptors in all four cell lines.

**EPHA7 and EPHA10 interaction in MCF10A and MCF7 cells.** The cell lysates were immunoprecipitated with antibody against EPHA7 and the complex was then analyzed on a western blot by probing with antibody against EPHA10. To confirm the interaction, the antibodies used for immunoprecipitation and western blot analyses were switched in a reciprocal experiment. MCF10A and MCF7 cells express EPHA10 isoforms of 48 kDa, 86 kDa and 109 kDa. However, co-immunoprecipitation with EPHA7 specifically pulled down the 48 kDa fragment only. Nearly all of the 48-kDa protein was found to be complexed with EPHA7, and the results were similar for both MCF10A and MCF7 cells (Figure 2, panels A and C).

A complementary finding emerged when the antibodies used from immunoprecipitation and western blot analyses
were switched. In these experiments, the lysate was precipitated with EPHA10, the precipitate was electrophoresed, and the blot probed with an EPHA7 antibody. As shown in Figure 2 (panels B and D), the antibody recognized proteins of approximately 56 kDa, 93 kDa, and 112 kDa in both MCF10A and MCF7 cells. The relative amounts of these proteins were variable, with 56 kDa protein being the most abundant. Immunoprecipitation of MCF10A lysate by EPHA10 antibody, however, primarily pulled-down the 56 kDa protein fragment, and
levels of 93-kDa and 112-kDa proteins were barely detectable by western blotting. It warrants mention that the larger fragments were not abundant in the input lysate. Interestingly, nearly 75% of the 56-kDa protein in MCF10A lysate was pulled-down by EPHA10 antibody compared to 10% of the protein in MCF7 cell lysate. The abundance of 93 kDa and 112-kDa proteins, however, was reversed in the two cell lines. The fraction of these larger proteins pulled-down by EPHA10 in MCF10A cell lysate was relatively smaller than that in MCF7 cells.

Figure 3. Co-immunoprecipitation (IP) of proteins associating/interacting with Erythropoietin-producing hepatocellular carcinoma cell receptor A7 (EPHA7) and EPHA10 in MDA-MB-231 cells transfected with an empty pCDNA (PC) vector (MDA-MB-231-PC) and MDA-MB-231 cells transfected with an expression construct of EPHB6 (MDA-MB-231-B6). The experiments were carried out with 100 μg of lysate protein from MDA-MB-231-PC and MDA-MB-231-B6 cells. The lysates were precipitated with EPHA7 antibody, electrophoresed, and probed with EPHA10 antibody (A and C). In other experiments, immunoprecipitation of lysates was carried out with EPHA10 antibody and western analysis was performed with EPHA7 antibody (B and D). Lanes 1, 2 and 3 represent input protein, immunoprecipitated proteins and proteins in the washes, respectively. The sizes of proteins are indicated. All experiments were performed in triplicates.
Formation of EPHA7 and EPHA10 complex in MDA-MB-231 and MDA-MB-231-B6 cells. We previously showed that native MDA-MB-231 cells and clones of this cell line stably transfected with an empty pCDNA vector (MDA-MB-231-PC) have comparable in vitro invasiveness (19). However, stable expression of EPHB6 in these cells (MDA-MB-231-B6) significantly reduces their invasive characteristics (19). We therefore investigated the interaction between EPHA7 and EPHA10 in this cell-line pair to reveal biological significance of these receptors in the context of cellular phenotypes.

MDA-MB-231-PC and MDA-MB-231-B6 cells displayed quantitative differences in the abundance of EPHA10 isoforms. Although MDA-MB-231-PC cell line had an abundant amount of 48-kDa isoform, it also had detectable amounts of 86-kDa and 109-kDa proteins. When cell lysates were immunoprecipitated with EPHA7 antibody and the precipitate was probed with EPHA10 antibody, all of the 48-kDa isoform as well as the higher-size isoforms of EPHA10 were pulled-down by EPHA7 antibody (Figure 3, panels A and C).

The antibodies for immunoprecipitation and western analyses were switched in a reciprocal experiment to confirm the results presented above. In these experiments, the lysate was precipitated with EPHA10 antibody and western blot probed with EPHA7. As shown in Figure 3B, EPHA7 isoforms of 56 kDa, 93 kDa and 112 kDa were pulled down nearly completely by EPHA10 antibody from lysates of MDA-MB-231-PC cells. MDA-MB-231-B6 cells, on the other hand, predominantly had the 56 kDa isoform of EPHA7 and all of the isoform was pulled down by EPHA10 antibody (Figure 3D).

The preceding results clearly demonstrate differential manifestation of physical interactions of various isoforms of EPHA7 and EPHA10 in normal breast cells, non-invasive breast carcinoma cells, and invasive breast carcinoma cells stably transfected with either an empty pCDNA vector or an expression construct of EPHB6.

Although EPHA7 was readily detectable in cell lysates of MCF10A, these cells did not appear to be positive for EPHA7 signal. EPHA10, on the other hand, was intensely stained in the cytoplasm as well as the cell surface. The signal was quite intense for EPHA7 as well as EPHA10 in MCF7 cells. Furthermore, the majority of staining was restricted to the cell periphery, indicating their localization on the cell membrane. The merged images suggest that EPHA7 and EPHA10 co-localize on the membrane, which support the physical interaction between these proteins demonstrated by co-immunoprecipitation (Figure 4).

EPHA7 and EPHA10 co-localize differentially MDA-MB-231 cells transfected with an empty vector or an expression construct of EPHB6. Based on our earlier observation of differential invasiveness of MDA-MB-231-PC and MDA-MB-231-B6, we investigated the co-localization of EPHA7 and EPHA10 in these two cell lines by fluorescent microscopy. MDA-MB-231-PC cells were intensely stained with both EPHA7 and EPHA10, and the staining appeared to be dispersed throughout the cell (Figure 5, panels A and B). A unique pattern of receptor localization emerged when the EPHA7 and EPHA10 signals were merged with the image of the DAPI stained nuclei. In large part, EPHA7 and EPHA10 were co-localized in the membrane, cytoplasm and the nucleus. However, speckles of isolated EPHA7 and EPHA10 were also visible in some parts of the cytoplasm. MDA-MB-231-B6 cells, however, displayed qualitative as well as quantitative differences. The abundance of EPHA7 and EPHA10 in MDA-MB-231-B6 was lower than MDA-MB-231-PC cells (Figure 5 E and F). The merged image indicate co-localization of EPHA7 and EPHA10, both in the membrane and the cytoplasm. However, the presence of EPHA7 and EPHA10 was not detected in the nuclei of these cells (Figure 5H).

Discussion

The roles of EPH receptors and ephrin ligands have been described in normal development (20-22), and aberrant patterns of their expression have been linked to a variety of human cancer types (23-25). These EPH receptors are phosphorylated upon activation by their cognate ephrin ligands, and the phosphorylated receptors then activate proteins of various signaling pathways (26-29). While the initial activation events and hetero-dimerization for kinase-deficient EPHB6 receptor have been described to some extent (30), the activation of kinase-dead EPHA10 receptor is unclear (31). Based on amino acid sequence identity of protein interaction domains in EPHA10 and EPHA7, we hypothesized that activated EPHA7 receptor could be a candidate receptor for dimerization with kinase-dead EPHA10. The correlation of EPHA10 expression with invasive breast carcinoma and low expression of EPHA7 in lymph node metastasis warranted exploration of these receptors for their heteromeric interactions and biological significance.

Our studies on transcript profiling indicated that the levels of EPHA7 and EPHA10 transcripts are barely detectable in normal breast cells compared to MDA-MB-231 cells (13). Furthermore, the abundance of EPHA7 transcript was relatively lower than EPHA10 in MDA-MB-231 cells. These observations suggested that cancer cell phenotypes are likely to be determined, in part, by relative expression levels of these two receptors, and the invasiveness of cancer cells may be modulated by altering the combinatorial abundance of EPHA7 and EPHA10 in a phenotype-specific manner.
EPHA7 binds to and is activated by all A-class ephrins, and the activated receptor is involved in development (5, 6, 11), ERK phosphorylation, proliferation and apoptosis (18, 32, 33). EPHA7 is also known to interact with PDZ domain-containing non-EPH receptor proteins (34-37). The secreted form of EPHA7, which is expressed in lymphoma and lung...
cancer, interacts with EPHA2 (6, 38, 39). EPHA10 is also activated by all A-class ephrin ligands, but the affinity of the receptor is highest for ephrin A5 (7).

We have shown that three isoforms of approximately 56 kDa, 93 kDa and 112 kDa of EPHA7 are present in breast cells, and likewise three isoforms of EPHA10 corresponding to approximate sizes of 35 kDa, 50 kDa and 86 kDa are also detectable. The interaction of EPHA7 with EPHA2 has been described (40, 41), but the mechanisms for EPHA10 interaction and its subsequent activation are not clear. Our co-immunoprecipitation and western blotting experiments revealed physical interaction between EPHA7 and EPHA10. Although these interactions were observed in all cell lines, some cell lines displayed unique interactions. While EPHA7 specifically interacted with the 48-kDa isoform of EPHA10 in MCF10A and MCF7 cell lines, EPHA10 antibody pulled down all three isoforms of EPHA7 in both these cell lines. The major EPHA7 isoform, however, was of 56 kDa molecular weight. The interaction between these proteins in MDA-MB-231 and MDA-MB-231-B6 were distinguished by the lack of higher-size isoforms in the precipitated complex. Although the higher-size isoforms of EPHA10 and EPHA7 were pulled-down to some extent by EPHA7 and EPHA10 antibodies, the smallest isoforms were most abundant in the complex precipitated by antibodies. In light of the sequence identity between EPHA7 and EPHA10 (7), it is not surprising that these two receptors interact, and we speculate that the kinase activity of EPHA7 cross-phosphorylates EPHA10. The interactions between the smaller isoforms signify their biological relevance for the following reason. The secreted version of the smaller EPHA7 isoform has been shown to trigger cellular reprogramming by inducing markers of pluripotency (6). Such reprogramming has important consequences for proliferation, invasion, and the tumor microenvironment. Thus, our observations of EPHA7 and EPHA10 interaction suggest the mechanistic aspects of EPHA10 activation and underscore the implications of combinatorial pattern of EPH receptor expression.

The physical interactions demonstrated by co-immunoprecipitation and western blotting were further confirmed by immunohistochemically co-localizing EPHA7 and EPHA10. The physical territories of both receptors overlapped in MCF7, MDA-MB-231-PC and MDA-MB-231-B6 cells. While the majority of the merged signal was restricted to the cell periphery, some co-localized spots were also observed in the cytoplasm. The presence of nuclear staining in MDA-MB-231-PC cells, however, provided some clues to the invasiveness of this cell line. It is noteworthy that MDA-MB-231 cells transfected with EPHB6 did not display any nuclear staining. EPHB6 has been shown to be a tumor suppressor and metastasis regulator (19, 42, 43). Our results indicate that EPHA7 and EPHA10 physically interact, and the complex of these receptors can migrate to the nucleus. However, it is unclear whether the nuclear complexes represent the full-length receptor, cleaved receptor or soluble receptor. Our data are insufficient to distinguish among these three possibilities. The smaller cytoplasmic isoforms of EPHA7 and EPHA10 may also exist in a complex, that could migrate into the nucleus upon sensing specific signals.

Although the involvement of EPH receptors in modulating cytoskeletal changes via Rho signaling has been described (44-46), the mechanisms of transcription activation by translocation of EPH receptors into the nucleus are largely unknown. The transcriptional changes mediated by signals transduced by EPH receptors are relayed to the nucleus via signal transducer and activator of transcription (STAT3), SRC and ERK. However, it is interesting to note that activation of EPHB2 by ephrin-B2 leads to secretase cleavage of intracellular domain of EPHB2 and its subsequent migration into the nucleus (8, 47-49). These observations support the ability of EPHA7 and EPHA10 to regulate gene transcription, and the presence of EPHA7-EPHA10 complex in the nucleus suggests them as putative factors associated with the transcriptional apparatus. The localization of EPHA7 and EPHA10 in breast cells is supported by the demonstration that EPHB4 protein exists in the nucleus of prostate cancer cells (50). It, however, remains to be confirmed whether our observations on nuclear presence of EPHA7 and EPHA10 represent a transcriptional regulation of some target genes or an indication of a receptor recycling-mediated mechanism for regulating receptor activation (51).

We have demonstrated the physical association and cellular co-localization of EPHA7 and EPHA10 in breast carcinoma cells. The nuclear co-localization of these two receptors in invasive MDA-MB-231 cells suggests their involvement in transcriptional activation of genes involved in invasiveness. The absence of such nuclear localization in EPHB6 transfected MDA-MB-231 cells further supports the metastasis-suppressor role of EPHB6. Our observations set the basis for confirming the role of specific EPH receptor fragments/isoforms in gene transcription.

References


