Ligand Binding Up-Regulates EphA2 Messenger RNA Through the Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Pathway

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Abstract
The EphA2 receptor tyrosine kinase is overexpressed in aggressive cancer cells, where it critically influences many aspects of malignant character. Although high levels of EphA2 have been documented in many different cancers, relatively little is known of the mechanisms that govern EphA2 gene expression in normal or malignant cells. Our present studies demonstrate that EphA2 influences the regulation of its own gene expression. Specifically, ligand-mediated phosphorylation of EphA2 transmits signals to the nucleus via extracellular signal-regulated kinase kinases to up-regulate de novo EphA2 gene expression and synthesis. This mechanism governs EphA2 expression in normal and malignant cells. In normal cells, EphA2 protein expression is balanced by ligand-mediated induction of EphA2 gene expression countered by EphA2 protein turnover. These findings suggest that EphA2 expression and ligand binding are intimately linked in epithelial cells. Increased understanding of this mechanism could have important implications for understanding the causes of EphA2 overexpression and for developing new strategies for therapeutic intervention in the many cancers that overexpress EphA2.

Introduction
The EphA2 receptor tyrosine kinase is frequently elevated and functionally altered in cancer cells. High levels of EphA2 have been documented in many cell models and clinical specimens of human cancer, including metastatic melanoma and breast, prostate, lung, renal cell, colon, and esophageal carcinomas (1–6). Despite the prevalence of EphA2 in cancer, less is known of the mechanisms that govern EphA2 overexpression. What is known suggests that EphA2 gene expression is negatively regulated by ERα (7) and wild-type p53 (8), which is notable because both effectors are functionally altered or misexpressed in highly aggressive tumors (9, 10). Increasing evidence also suggests a potential linkage between EphA2 overexpression and signaling by Ras oncogenes. For example, high levels of EphA2 have been observed in Ras-transformed cell lines and in the mammary tumors of transgenic mice that express mutant Ras in the mammary gland (11–14). Other signaling pathways that have been linked with Ras have also been shown to modulate EphA2 gene expression, including interleukin-1β, basic fibroblast growth factor, interleukin-2, epidermal growth factor, and transforming growth factor-β (15). These observations are nonetheless correlative, and the specific mechanisms that might allow Ras to regulate EphA2 levels have yet to be identified.

EphA2 is also functionally altered in malignant cells. Normal epithelial cells generally have highly stable cell-cell contacts (16). Because EphA2 binds ligands that are anchored to the cell membrane (17, 13). This outcome is important because tyrosine-phosphorylated EphA2 negatively regulates cell growth and invasion (2, 13, 18, 19). A very different situation arises in malignant cells. Cancer cells frequently have unstable intercellular contacts (16, 20), which decreases the duration and magnitude of EphA2-ligand binding (13). Consequently, the EphA2 in malignant cells is generally not tyrosine phosphorylated. Notably, this EphA2 retains enzymatic activity, but the unphosphorylated EphA2 promotes (rather than inhibits) tumor cell growth, invasion, and survival (2). These observations have generated much recent interest in developing strategies that mimic or restore ligand binding using monoclonal antibodies or artificial ligands (21, 22).

Because EphA2 has been linked with important biological outcomes, there has also been a recent emphasis on understanding how EphA2 mediates these events (18, 19, 23–27). Mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinases are among the intracellular pathways that have been linked with EphA2 (18, 19, 28). In the present study, we have shown that EphA2 signaling to MAP/ERK kinases contributes to EphA2 overexpression in malignant cells. Ligand binding increases EphA2 gene expression and also alters a fine balance in EphA2 gene expression, synthesis, and protein degradation. These results suggest that unbalanced synthesis versus degradation contributes to EphA2 overexpression in cancer. These findings provide new insight about mechanisms that govern the expression of a powerful oncogene and demonstrate a novel means by which EphA2 expression and ligand binding are linked in normal cells and dysregulated in cancer cells.
Results

Ligand Up-Regulates EphA2 Messenger RNA Expression

High levels of EphA2 protein often preside in cells that have unstable EphA2-ligand binding (2, 13). Thus, we asked if ligand binding might regulate EphA2 gene expression. Our initial studies used MDA-MB-231, an aggressive human breast cancer cell line, where EphA2 is overexpressed and largely unphosphorylated. Ligand stimulation of EphA2 could be induced using artificial ligands or antibodies (13, 21). Incubation of MDA-MB-231 cells with a fusion of ephrinA1 and Fc immunoglobulin (EA1-Fc) was sufficient to increase EphA2 mRNA product. Quantitative real-time PCR (RT-PCR) analyses revealed that incubation with 1 μg/ml EA1-Fc increased EphA2 mRNA levels by at least 4- or 2-fold normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) compared with matched controls (P < 0.003; Fig. 1A). The increased EphA2 mRNA levels were detected within 1 h and persisted for at least 2 h (data not shown). Western blot analysis of immunoprecipitated EphA2 with phosphotyrosine-specific antibody (P-Tyr) confirmed that ligand binding had increased EphA2 phosphorylation. Parallel studies with cell lysates evaluated EphA2 protein levels. β-catenin served as a loading control (Fig. 1B). These studies demonstrated a slight decrease in EphA2 protein, which is consistent with recent evidence for ligand-mediated EphA2 protein degradation (29). To avoid complications caused by protein degradation, a relatively short (1 h) time point was used for all studies below. Multiple cell systems including MDA-MB-231 and MDA-MB-435 yielded comparable results, indicating that ligand-mediated up-regulation of EphA2 was not unique to any particular cell system (data not shown).

Additional controls confirmed the specificity and relevance of ligand-mediated up-regulation of EphA2 mRNA. First, ligand binding did not significantly increase EphA2 gene expression in cells where EphA2 already interacts with its endogenous ligands. For example, EA1-Fc treatment of nontransformed MCF-10A breast epithelial cells did not significantly increase EphA2 gene or protein levels relative to matched controls (Fig. 2A, left). Consistent with this, the phosphotyrosine content and protein levels of EphA2 did not change following incubation with EA1-Fc (Fig. 2A, right). To ask if increased EphA2 expression might have resulted from the soluble nature of EA1-Fc, this ligand was immobilized onto protein A-Sepharose (EA1-PAS). Incubation of MDA-MB-231 cells with EA1-PAS increased EphA2 mRNA levels by at least 2-fold relative to matched control (P < 0.003; Fig. 2B, left). The magnitude of the increase in EphA2 gene expression was lower in studies using immobilized ligand, but this related to decreased EphA2 stimulation (phosphotyrosine content) and likely reflected steric hindrances that limit immobilized EA1-Fc binding to EphA2. Equal sample loading was verified with cell lysates probed with β-catenin antibody (Fig. 2B, right).

An analogous mechanism regulates EphA2 in nontransformed epithelial cells. The studies above modeled EphA2-ligand binding by using malignant cells treated with an engineered ligand. To preclude potential artifacts that could arise from the use of an artificial system, the inverse experiment was performed by disrupting EphA2-ligand binding in nontransformed cell systems. For this, MCF-10A epithelial cells were used because previous studies have shown that these cells demonstrate stable EphA2-ligand binding and that inhibitors of cell-cell adhesion, such as EGTA, prevent EphA2 activation by endogenous ligands (13). EGTA treatment decreased EphA2 mRNA levels by 65% relative to GAPDH and the matched control (P < 0.0011; Fig. 3A, left). Consistent with this finding, EGTA treatment also decreased EphA2 phosphorylation and increased EphA2 protein levels that reflect a reduction in ligand-mediated EphA2 degradation (Fig. 3A, right). While performing these studies, we considered that decreased
extracellular calcium might have induced nonspecific effects. To obviate this potential concern, MCF-10A cells were treated with EphA2-Fc, a fusion protein that functions as a dominant negative inhibitor of EphA2-ligand binding. Quantitative RT-PCR revealed that a 2-h treatment with EphA2-Fc reduced EphA2 mRNA levels by 63% relative to matched controls ($P < 0.00002$; Fig. 3B, left). EphA2-Fc treatment significantly decreased phosphotyrosine content of EphA2 revealed by Western blot of EphA2 immunoprecipitations (Fig. 3B, right). Moreover, EphA2-Fc modestly increased EphA2 protein levels (because of decreased protein turnover).

**EphA2 Gene Expression Requires ERK Signaling**

EphA2 phosphorylation increased on binding of EA1-Fc, which suggests that certain downstream signals likely allow EphA2 to regulate its own gene expression. Based on the recent demonstration that ligand stimulation of EphA2 transiently activates ERK kinases, a postulate was developed to ask if ERK signaling might regulate EphA2 gene expression (17). To test this hypothesis, MDA-MB-231 cells were incubated with PD98059, a specific inhibitor of MAP kinase kinase 1 (MEK1) signaling. Two-hour treatment with PD98059 decreased EphA2 mRNA levels ($P < 3 \times 10^{-8}$) and prevented ligand (EA1-Fc)-mediated induction of EphA2 gene expression ($P < 0.003$; Fig. 4A). Western blot of corresponding cell lysate with phospho-ERK (P-ERK) antibody confirmed the effectiveness of PD98059 (Fig. 4B). Ligand binding caused high EphA2 protein degradation alone, regardless of the presence of PD98059 (data not shown). Chemical blockade of ERK similarly decreased EphA2 gene expression in nontransformed MCF-10A cells (data not shown). Consistent data in these cell systems confirmed that ligand-mediated induction of EphA2 gene expression requires ERK signaling.

**Coordinated Regulation of EphA2 Gene Expression and Protein Degradation**

EA1-Fc causes a significant but transient decrease in EphA2 protein due to proteasomal degradation (29). Over time (~3 h), the levels of EphA2 are replenished by newly synthesized material. Thus, the net levels of cellular EphA2

![FIGURE 2](image-url)  

**FIGURE 2.** EphA2 expression is regulated by ephrinA1/EphA2 binding. RT-PCR results from (A) MCF-10A cells treated with EA1-Fc or (B) MDA-MB-231 cells incubated with EA1-PAS for 1 h at 37°C. A, 1 μg/ml soluble EA1-Fc treatment for 1 h of MCF-10A did not further increase EphA2 expression as compared with matched controls (C; defined as 1.0). Consistent with this, analysis of phosphotyrosine levels of immunoprecipitated EphA2 demonstrates that EphA2 is already stimulated by endogenous ligands and that EA1-Fc does not further stimulate EphA2 (P-Tyr; top right). Western blot analysis illustrated a modest change in the level of EphA2 protein (middle right). Equal loading was verified using β-catenin antibody (bottom right). B, EA1-PAS induced EphA2 gene expression by at least 2-fold ($P < 0.003$) as compared with matched controls (C; defined as 1.0). The EA1-PAS-mediated induction of EphA2 mRNA was consistent with the results recorded with EA1-Fc (see Fig. 1 for comparison) and was related to the magnitude of EphA2 stimulation (phosphotyrosine content; P-Tyr) indicated by Western blot analysis of immunoprecipitated EphA2 (top right). Western blot analysis also confirmed a modest decrease in the level of EphA2 protein (middle right). Equal loading was verified using β-catenin antibody (bottom right). Averages of at least four independent experiments.
protein are balanced by de novo synthesis and turnover. To investigate further the role of EphA2 protein degradation, MDA-MB-231 cells were pretreated for 2 h with MG-132, a chemical inhibitor of proteasome activity, or a matched vehicle control. The samples were then incubated with EA1-Fc for 1 h to stimulate EphA2. The levels of EphA2 protein were then measured using immunoprecipitated EphA2 probed with EphA2-specific antibody, D7. Western blot analyses revealed that MG-132 did not significantly change EphA2 protein levels. This outcome was consistent with evidence that EphA2 is stable in malignant cells (29). In contrast, EA1-Fc treatment induced efficient degradation of EphA2 within 2 h. Notably, the combination of EA1-Fc and MG-132 prevented EphA2 degradation and caused a dramatic increase in EphA2 protein levels. This outcome suggests that ligand mediates EphA2 translation and EphA2 gene expression and provides further evidence that the net levels of EphA2 are differentially balanced in normal and malignant cells by a combination of de novo synthesis and protein degradation (Fig. 5).

**Discussion**

The major findings of our present study are that ligand-mediated stimulation of EphA2 up-regulates EphA2 mRNA expression and that this induction of EphA2 gene expression requires ERK signaling. Ligand binding of EphA2 can trigger de novo EphA2 gene expression as well as EphA2 degradation. The net levels of EphA2 in nontransformed epithelial cells result from a balance between these two outcomes. Moreover, evidence is provided that changes in the balance between EphA2 production and degradation contribute to the high levels of EphA2 that have been observed in many cancer cells.

The present findings are unique, in part, because they offer a new perspective about mechanisms that govern EphA2 levels. EphA2 is expressed at relatively low levels in normal cells (1, 5, 6, 30). Stable cell-cell contacts cause the EphA2 protein to be constitutively tyrosine phosphorylated, which is notable because tyrosine-phosphorylated EphA2 is rapidly turned over via internalization and degradation (29). These results suggest that stable ligand binding maintains low levels of EphA2 by
balancing de novo EphA2 synthesis and turnover. This hypothesis predicts that changes in ligand binding could cause dramatic changes in EphA2 levels. These changes could arise from decreased cell-cell adhesions (as a result of changes in E-cadherin), impaired or occluded ligand binding, increased dephosphorylation of EphA2 (by associated phosphatases), or insufficiencies in critical signaling pathways (i.e., ERK). Frequently, ERK kinases are dysregulated in cancer (31–33), and the mechanism akin to those described herein could allow other stimuli to similarly up-regulate EphA2 expression.

Based on the aforementioned model, malignant cells likely circumvent normal controls that govern EphA2 gene expression and protein degradation. Malignant cells generally have unstable cell-cell interactions (34–36). Consequently, the EphA2 in malignant cells could transiently bind ligand sufficiently long enough to transmit signals through ERK but brief enough to preclude EphA2 internalization. Thus, high levels of EphA2 could arise at the levels of both gene expression and protein stability. It is also possible that additional stimuli that increase MAP/ERK activity could similarly promote EphA2 overexpression.

This new model has interesting implications for understanding how imbalances in cancer cell signaling could contribute to EphA2 overexpression. For example, high levels of EphA2 have been observed in cell lines and in the tumors of transgenic mice that express oncogenic Ras (11, 13, 14). It is also intriguing that high levels of EphA2 have been observed in clinical specimens and cell models of pancreatic cancer, which frequently have elevated Ras activity (M. S. Kinch, unpublished observations). Such observations are nonetheless correlative, and further investigation (e.g., covariate analyses) should be performed to ask if Ras signaling results in EphA2 overexpression.

Recent reports have also demonstrated that ephrin receptors can down-regulate ERK and Ras signaling (18, 19, 37, 38). For example, inhibition of ligand/kinase binding resulted in elevated Ras activity and aberrant EphB2 regulation (37). One mechanism appears to involve induction of p120 GTPase-activating protein (37, 38). Because MDA-MB-231 cells express an activated form of Ras (39), it is unlikely that this effect has been modeled in our present studies. However, future studies could address the potential complexity of the interactions between EphA2 and Ras signaling.

Mechanisms that alter the stability of cell-cell adhesions could similarly alter a fine balance of EphA2 synthesis and turnover, albeit in an indirect manner. For example, E-cadherin is the primary mediator of cell-cell adhesions (40) and E-cadherin expression inversely relates to EphA2 in breast cancer cell lines (13). Consistent with this, ectopic restoration of E-cadherin expression in aggressive MDA-MB-231 cells increases EphA2 mRNA levels in combination with decreased EphA2 protein levels (R. L. Pratt, unpublished observations).

A recent report showed that antisense targeting of ephrinA1 decreases the growth of HT-29 colon tumor cells (41).
Supportive of this, high levels of ephrins have been reported in cancer tissues (4, 42, 43). At first glance, these results seem inconsistent with evidence that EphA2 agonists (monoclonal antibodies, ligand-bodies, and peptides) negatively regulate tumor cell growth by favoring EphA2 protein degradation (2, 13, 18, 21, 22). In light of the present findings, we conjecture that high levels of endogenous ligands in cancer cells could up-regulate EphA2 gene expression without favoring protein turnover. High levels of endogenous ephrinA1 could favor EphA2 overexpression if unstable cancer cell-cell contacts cause transient EphA2-ligand binding in a manner that transmits signals for EphA2 synthesis but which does not promote EphA2 turnover.

Materials and Methods

Cell Culture and Antibodies

MDA-MB-231 cells were propagated in RPMI, 10% fetal bovine serum, Pen/Strep (100 units/ml penicillin, 100 µg/ml streptomycin), and 2 mM L-glutamine. MCF-10A cells were grown in DMEM/F12, 5% horse serum, 20 ng/ml epidermal growth factor (Upstate Biotechnology, Inc., Lake Placid, NY), 10.0 µg/ml insulin (Sigma Chemical Co., St. Louis, MO), 0.5 µg/ml hydrocortisone (Sigma), Pen/Strep (100 units/ml penicillin, 100 µg/ml streptomycin), 0.25 µg/ml Fungizone, and 2 mM L-glutamine. MDA-MB-435 cells were cultured in DMEM, 10% fetal bovine serum, Pen/Strep (100 units/ml penicillin, 100 µg/ml streptomycin), and 2 mM L-glutamine. Monoclonal antibodies specific for EphA2 (clones D7 and B2D6) were generated in our laboratory or purchased from Upstate Biologicals, Inc. (Lake Placid, NY). Antibodies specific for P-ERK, ERK1, and β-catenin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories (Lexington, KY). Antibodies specific for P-Tyr (4G10) were purchased from Upstate Biologicals. MEK1 inhibitor, PD98059 (used at 4 µM/ml), was purchased from Cell Signaling Technology, Inc. (Beverly, MA) and dissolved in DMSO for a stock concentration of 20 µM. Proteosome degradation inhibitor, MG-132 (used at 10 µM/ml), was purchased from Calbiochem (San Diego, CA) and dissolved in sterile water.

EphA2 Stimulation

Aggregation of EphA2 receptors was performed as described previously (2). Unless noted otherwise, all stimulations of EphA2 were with 1 µg/ml of the soluble ligand EA1-Fc, for 0 min or 1 h at 37°C. A 1-h time point was specifically chosen to avoid complications from ligand-mediated degradation of EphA2 that arise at later time points (2 h). For experiments in combination with MG-132 or PD98059, cells were incubated for 2 h at 37°C. These inhibitors remained in the conditioned media during the additional ligand treatment.

Bead-Bound EA1-PAS Experiments

EphrinA1 was conjugated to washed protein A-Sepharose beads as recommended by the manufacturer (Sigma). Duplicate samples (for RT-PCR and protein analysis) of MDA-MB-231 cells were cultured at 70–80% confluence at 37°C before treatment with 1 µg/ml of soluble EA1-PAS for 1 h at 37°C.

EGTA and EphA2-Fc Treatments

Duplicate tissue culture plates (for RT-PCR and protein analysis) of MCF-10A cells were grown to 70–80% confluence at 37°C. EGTA was used at a final concentration of 4 mM for the indicated times at 37°C. EphA2-Fc was added to a final concentration of 1.45 µg/ml. For experiments in combination with EA1-Fc, EGTA and EphA2-Fc remained in the conditioned media during the additional ligand treatment.

Western Blot Analysis

Western blot analyses were performed on normalized cell lysates as described previously (2) and antibody binding was detected by enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL) and autoradiography (Kodak X-OMAT; Kodak, Rochester, NY).

Total RNA and cDNA and Quantitative RT-PCR

Total RNA and cDNA were created as described (44). Primers for EphA2 and GAPDH, the control gene, were as follows: EphA2 P1 = 5’-ATGGAGGCCTCAAGGACCGGC-3’, EphA2 P2 = 5’-GGCATACGGGTTGTTGAGCCAGC-3’, GAPDH P1 = 5’-CAGTGGTGGACCCTGACC TGGCGTCT-3’, and GAPDH P2 = 5’-CTCAGTGTAACGCCACG-GAC TGCCTTGGAG-3’. RT-PCR was carried out using the SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, CA). RT-PCR was performed using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) RT-PCR resulted in a 150-bp product for EphA2 and a 104-bp product for GAPDH. Note that RT-PCR data represented in the figures are relevant to GAPDH. Analyses of RT-PCR data required that all control samples be set at a value of 1 (or 100%) after loading was equalized against GAPDH; therefore, no error bars appear on the controls.

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References

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