Overexpression of EPHA2 receptor destabilizes adherens junctions via a RhoA-dependent mechanism

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Summary
EPHA2 receptor tyrosine kinase is overexpressed in several human cancer types and promotes malignancy. However, the mechanisms by which EPHA2 promotes tumor progression are not completely understood. Here we report that overexpression of a wild-type EPHA2, but not a signaling-defective cytoplasmic truncation mutant (ΔC), in human mammary epithelial cells weakens E-cadherin-mediated cell-cell adhesion. Interestingly, the total level of cadherins and the composition of the adherens junction complexes were not affected, nor was the tyrosine phosphorylation of the cadherin complex components changed. By contrast, RhoA GTPase activity was significantly affected by modulating the EPHA2 activity in MCF-10A cells. Treatment with a ROCK kinase inhibitor rescued cell-cell adhesion defects in EPHA2-overexpressing cells, whereas expression of constitutively activated Rho disrupted adherens junctions in ΔC-expressing cells. EPHA2-dependent Rho activation and destabilization of adherens junctions appeared to be regulated via a signaling pathway involving Src kinase, low molecular weight phosphotyrosine phosphatase (LMW-PTP) and p190 RhoGAP. EPHA2 interacted with both Src and LMW-PTP, and the interactions increased in EPHA2-overexpressing cells. In addition, LMW-PTP phosphatase activity was elevated, and this elevation was accompanied by a decrease in tyrosine phosphorylation of p190 RhoGAP and destabilization of cell-cell adhesion. Expression of either a dominant negative LMW-PTP mutant, C12S, or a wild-type p190 RhoGAP rescued adhesion defects in EPHA2-overexpressing cells. Together, these data suggest that EPHA2 promotes tumor malignancy through a mechanism involving RhoA-dependent destabilization of adherens junctions.

Key words: EPHA2, RhoA, Adherens junction

Introduction
Protein tyrosine phosphorylation is a powerful signal that regulates cell proliferation, cell invasion, and cell migration. Mutation, gene amplification or aberrant regulation of protein tyrosine kinase has been linked to tumor initiation and progression. Specific tyrosine kinase inhibitors, such as inhibitors of the Her-2/Neu receptor and EGFR, have been developed for cancer therapies with varying degrees of success. Although exciting in their clinical effectiveness, these inhibitors are only suitable for treating a small subset of cancer types. The hope remains that other receptor tyrosine kinases will be identified and that their inhibition will have broader efficacy in cancer treatment.

A new family of receptor tyrosine kinases, the Eph family, plays a critical role in cancer. Originally discovered as modulators of axonal guidance and embryonic patterning during development, subsequent studies have shown that many Eph receptors are overexpressed in a large number of cancers (Brantley-Sieders et al., 2004b; Pasquale, 2005). One family member in particular, the EPHA2 receptor, has been linked to breast cancer, prostate cancer, lung cancer, ovarian and cervical cancer, esophageal and colorectal cancer, as well as malignant melanoma (Ireton and Chen, 2005). Furthermore, the level of EPHA2 receptor expressed on tumor cells correlates with the degree of tumor malignancy (Kinch and Carles-Kinch, 2003). Overexpression of the EPHA2 receptor in MCF-10A cells is associated with increased cell growth in soft agar and increased tumor growth when these cells were implanted into nude mice (Zelinski et al., 2001). However, despite the strong correlation of EPHA2 receptor expression with malignant phenotypes, the mechanisms by which EPHA2 contributes to tumor cell malignancy are not completely understood.

One hallmark of malignancy in tumors is the loss of cell-cell adhesion. In non-malignant circumstances, cell-cell adhesion connects epithelial cells in their normal polarized position. In mammals, adhesion between epithelial cells is generally mediated by three types of junctions: tight junctions, adherens junctions and desmosomes. Adherens junctions are cadherin-dependent adhesive structures that are intimately linked to the cytoskeleton. The extracellular domain of classical cadherins mediates Ca2+-dependent homophilic interactions, whereas the intracellular domain interacts with several catenins. In general, the cadherin-bound catenins function to either anchor the adhesion complex to the actin cytoskeleton (β- and α-catenin) or regulate the cadherin stability at the junction (p120 catenin). Cadherins and their associating catenins are collectively known as the cadherin complex. The stability of the epithelial cadherin E-cadherin and its associated complex dictates both the polarity and the motility of epithelial cells (Perez-Moreno et al., 2003). Several studies have shown that the loss of E-cadherin downregulates EPHA2 receptor levels and induces EPHA2 mislocalization in tumor cells and mouse embryonic stem cells (Orsulic and Kemler, 2000; Zantek et al., 1999). Likewise, VE-cadherin, another type of cadherin, appears to regulate EPHA2 localization in melanoma cells that undergo vascular mimicry (Hess et al., 2006). However, the effect of EPHA2 overexpression on adherens junctions remains unclear.

Cell-cell adhesion is regulated by multiple mechanisms. The Rho family of GTPases have been increasingly recognized as key...
players in regulation of adherens junction (reviewed by Fukata and Kaibuchi, 2001; Lozano et al., 2003; Perez- Moreno et al., 2003). Rho proteins are small GTPases that cycle between an active, GTP-bound, conformation and an inactive, GDP-bound, conformation. In response to extracellular cues through cell surface receptors, Rho proteins can be activated by guanine nucleotide exchange factor (GEF) or inactivated by GTPase activating protein (GAP). Multiple Eph receptors have been shown to modulate Rho family GTPase activity (reviewed by Noren and Pasquale, 2004). EPHA2 receptor activation leads to elevation of GTP-Rac1 via Vav GEFs in vascular endothelial cells to regulate angiogenesis (Brantley-Sieders et al., 2004a; Hunter et al., 2006). In epithelial and tumor cells, stimulation of EPHA2 receptor induces activation of RhoA GTPase and affects cell migration (Fang et al., 2005; Miao et al., 2003).

In addition to activation of Rho family GTPases, EphA receptors have been shown to both regulate low molecular weight phosphotyrosine phosphatase (LMW-PTP) activity and serve as substrates for the same phosphatase. The EphA8 receptor phosphorylates and activates LMW-PTP in vitro (Park, 2003). Conversely, LMW-PTP negatively regulates EphA receptors by dephosphorylation of EPHA2 and EphA8 receptors (Kikawa et al., 2002; Park, 2003; Parri et al., 2005). Another substrate of the LMW-PTP is the p190RhoGAP. In fibroblasts, LMW-PTP regulates adherens junction stability by modulating phosphorylation levels of p190RhoGAP (Nimmual et al., 2003). However, the role of LMW-PTP in regulating receptor tyrosine kinases and/or cell-cell adhesion remains to be determined in epithelial cells.

To determine whether the EPHA2 receptor can regulate adherens junctions, we expressed wild-type and mutant EPHA2 in the immortalized, non-transformed MCF-10A breast epithelial cell line. We found that overexpression of wild-type EPHA2 destabilizes adherens junctions. Interestingly, EPHA2 overexpression does not affect overall levels or phosphorylation status of E-cadherin, p120 catenin, β-catenin and α-catenin, but appears to weaken the adherens junction by upregulating RhoA GTPase activity via p190 RhoGAP and LMW-PTP. Thus, in addition to regulating tumor cell motility (Fang et al., 2005), the increased EPHA2 receptor levels in tumors also promote destabilization of cell-cell adhesion by regulating RhoA GTPase activity.

Results

Overexpression of EPHA2 receptor weakens cell-cell adhesion

Previous studies showed that loss of E-cadherin downregulates EPHA2 receptor levels and induces mislocalization of EPHA2 in both tumor cells and mouse embryonic stem cells (Hess et al., 2006; Orsulic and Kemler, 2000; Zantek et al., 1999). However, the reciprocal effect of EPHA2 on adherens junction components remains unclear. Because EPHA2 levels are often elevated in tumor cells (reviewed by Ireton and Chen, 2005), we studied the effects of overexpression of EPHA2 receptor tyrosine kinase in the immortalized nontransformed MCF-10A breast epithelial cell line. MCF-10A cells were infected with LZRS retrovirus overexpressing moderate levels of either full-length wild-type EPHA2-IRES-GFP or an EPHA2 cytoplasmic truncation mutant, ΔC-IRES-GFP (Fang et al., 2005). Pools of transduced cells were sorted by fluorescence-activated cell sorting (FACS) for comparable levels of EPHA2 receptor expression and subjected to assays that measure adhesion strength and stability.

To study the impact of increased EPHA2 receptor levels on cell-cell adhesion, we first performed a hanging drop aggregation assay – an assay that was designed to assess the strength of cell-cell adhesion without the influence of cellular adhesion to the plate. As shown in Fig. 1A, A431 cells expressing wild-type E-cadherin induced tightly compacted cell aggregates that could not be dissociated by shear force. By contrast, the control A431D cells, which do not express E-cadherin, exhibited loose association and immediate separation of cells when subjected to shear force. MCF-10A cells grown in hanging drop suspension culture do not form compact cellular masses as A431 cells, but they do form a cell spheroid structure that was resistant to shear force separation. However, stimulation of ephrin-A1 resulted in cell-cell dissociation of MCF-10A cells. (B) MCF-10A-EPHA2, but not MCF-10A-ΔC cells, exhibited decreased cell-cell adhesion. (C) Western blot analysis of EPHA2 and Myc-tagged mutant ΔC protein in MCF-10A cells.
suggest that EPHA2 receptor signaling is required for regulating the strength of cell-cell adhesion.

**EPHA2 overexpression does not affect the expression and phosphorylation of cadherin or catenin nor the composition of adherens junction complexes**

To dissect the mechanisms by which overexpression of EPHA2 promotes dissociation of cell-cell contacts, we examined whether the altered cell-cell adhesion mediated by EPHA2 receptor activation involved modulation of the adherens junction complex. We observed no alteration in E-cadherin protein levels in stable junctions from cells cultured to confluence, as judged either by western blot analysis (Fig. 2A) or immunofluorescence (Fig. 2C-F). Since EPHA2 activation does not result in cadherin loss, we evaluated whether it could compromise cell adhesion by modulating the composition of adherens junctional complexes. Adherens junctions consist of E-cadherin molecules, the cytoplasmic domains of which interact directly with p120 catenin and β-catenin. α-catenin interacts with E-cadherin indirectly through binding with β-catenin and links the adherens junction to the actin cytoskeleton. Thus, any member of the catenin family could impact the adhesive capacity and association with the cytoskeleton (Ireton et al., 2002; Perez-Moreno et al., 2003). However, the levels of p120 catenin, β-catenin and α-catenin were not altered in MCF-10A/WT-EPHA2 cells compared with the control cell lines (Fig. 2A). When E-cadherin was immunoprecipitated from MCF-10A/WT-EPHA2 cells, the levels of associated catenins were likewise unaltered in these cells compared with the immunoprecipitations from the control cell lines (Fig. 2B). Nor were tyrosine phosphorylation levels of cadherin or catenin protein significantly changed among parental MCF-10A, MCF-10A/EPHA2 or MCF-10A/ΔC cells (Fig. 2G). Together, these data suggest that EPHA2 receptor activation alone is unable to initiate the disruption of adherens junctions. However, as shown in Fig. 1, overexpression of EPHA2 weakens the strength of cell-cell adhesion and may accelerate dissociation of cells in a dynamic environment.

**Overexpression of EPHA2 accelerates adherens junction disruption by decreasing the localization of E-cadherin at the adherens junction**

To test whether EPHA2 can accelerate the dissociation of cell-cell adhesion, we performed a Ca²⁺ depletion assay. Cells were cultured in low Ca²⁺ medium and disappearance of cellular junctions was monitored by immunofluorescence with anti-E-cadherin. The rate of junction dissociation was compared in cells expressing either wild-type or mutant EPHA2 receptor. As shown in Fig. 3E, 8 hours after Ca²⁺ depletion, E-cadherin localization was substantially reduced at the junction in cells expressing wild-type EPHA2
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Although RhoA activity is often regulated by Rac (Nimnual et al., 2003), we did not observe significant changes in the level of activated Rac1 between cells expressing WT-EPHA2 and cells expressing the ΔC-EPHA2 mutant.

To test the functional role of EPHA2-activated RhoA GTPase in cell-cell adhesion, we assessed the effect of inhibition of RhoA activity on cell adhesion. Rho proteins can regulate adherens junctions by signaling through ROCK kinase (Sahai and Marshall, 2002). Therefore, we performed a Ca\(^{2+}\) depletion assay in the presence of the ROCK inhibitor Y-27632 to specifically block the actin-myosin contraction pathway. As shown in Fig. 4B and D, 8 hours after Ca\(^{2+}\) depletion, E-cadherin was substantially reduced in cells expressing wild-type EPHA2 receptor (Fig. 4D) relative to parental MCF-10A (Fig. 4B). However, inhibition of ROCK activity in EPHA2-overexpressing cells (Fig. 4E) partially restored the expression of E-cadherin to a level similar to the expression in parental MCF-10A cells (Fig. 4B) and cells expressing the ΔC mutation (Fig. 4C). These data suggest that RhoA activity is required for EPHA2-mediated destabilization of cell-cell adhesion.

To investigate whether increased RhoA activation is sufficient to disrupt cell-cell adhesion in MCF-10A and MCF-10AΔC cells, these cells were transduced with control Ad-LacZ or a constitutively activated Rho mutant Ad-Rho (Q63L) (Lee et al., 2004; Mayer et al., 1999). As shown in Fig. 4F and H, the MCF-10A and MCF-10A-ΔC cells infected with control viruses exhibited stable adherens junctions. By contrast, expression of activated Rho in the MCF-10A and MCF-10A-ΔC cells destabilized adherens junctions, consistent with the notion that activation of RhoA GTPase is sufficient to regulate the stability of cell-cell adhesion (reviewed by Fukata and Kaibuchi, 2001; Lozano et al., 2003; Perez-Moreno et al., 2003).

EPHA2-dependent activation of RhoA is regulated by p190 RhoGAP

Rho GTPases cycle between an inactive GDP-bound conformation and an active GTP-bound conformation. The Rho proteins can exchange nucleotide and hydrolyse GTP at slow rates in vitro, and these reactions are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Recent studies showed that p190 RhoGAP binds to p120-catenin and regulates cell-cell adhesion via inhibition of Rho activity (Wildenberg et al., 2006). To investigate whether EPHA2-dependent activation of RhoA is regulated by GAP proteins, we measured the phosphorylation state of p190RhoGAP. As shown in Fig. 5A, tyrosine phosphorylation of p190RhoGAP in MCF-10A cells overexpressing the EPHA2 ΔC mutant was significantly elevated, compared with cells overexpressing wild-type EPHA2.

To assess the functional role of p190 RhoGAP in EPHA2-dependent destabilization of adherens junctions, MCF-10A and MCF-10A-ΔC cells were transduced with LZRS retrovirus expressing wild-type p190 or p190/30-1, a p190 mutant that is defective in GTPase-activating enzymatic activity (Brouns et al., 2001; Wildenberg et al., 2006). As expected, in the MCF-10A and MCF-10A-ΔC cells, E-cadherin remains localized to cell-cell contacts even after Ca\(^{2+}\) depletion for 8 hours (Fig. 5B,E). Expression of wild-type p190 did not affect the localization of E-cadherin, whereas expression of p190/30-1 restored the expression of E-cadherin to levels similar to those observed in parental cells (Fig. 5G and H). These data suggest that p190 RhoGAP is required for EPHA2-mediated destabilization of cell-cell adhesion.

RhoA activity is required for EPHA2-mediated destabilization of cell-cell adhesion

Adherens junctions are regulated by multiple mechanisms. In addition to regulation of cadherin/catenin protein levels, phosphorylation state and composition of adherens junction complex, Rho family GTPases are known to modulate adherens junction stability. As Eph family RTKs are often capable of activating Rho family GTPases (Noren and Pasquale, 2004), we investigated whether EPHA2-induced destabilization of cell-cell adhesion is mediated by Rho proteins. We have previously shown that ephrin-A1 stimulation of 4T1 mammary tumor cells induced activation of RhoA GTPase (Fang et al., 2005). Accordingly, we used a Rhotekin pull-down assay to test whether RhoA GTPase is also activated by EPHA2 receptor signaling in MCF-10A cells. As shown in Fig. 4A, ephrin-A1 stimulation of MCF-10A cells induced RhoA activation within 5 minutes. The basal level of RhoA activity was elevated in cells expressing WT-EPHA2, but was diminished in cells expressing the ΔC-EPHA2 mutation.

Fig. 3. EPHA2 overexpression destabilizes adherens junctions. MCF-10A cells were cultured in normal (A,C) or low Ca\(^{2+}\) (B) medium for 24 hours. Cells were subsequently fixed and stained for anti-E-cadherin (A,B) or secondary antibody alone (C). MCF-10A cells carrying was diminished in cells expressing the WT-EPHA2, but was diminished in cells expressing the ΔC-EPHA2 mutant. RhoA activity was elevated in cells expressing WT-EPHA2, but was diminished in cells expressing the ΔC-EPHA2 mutant.
cadherin (Fig. 5C,F). However, although overexpression of EPHA2 resulted in loss of E-cadherin expression at cell-cell contacts (Fig. 5H), expression of wild-type p190 in these cells completely restored E-cadherin expression (Fig. 5I). Conversely, expression of the p190/30-1 in the MCF-10A and MCF-10A-ΔC cells resulted in a destabilization of the adherens junctions, as shown by loss of E-cadherin localization at the cell-cell contacts (Fig. 5D,G). These data suggest that EPHA2 destabilizes the adherens junction by regulating p190 RhoGAP activity.

LMW-PTP acts downstream of the EPHA2 receptor to regulate p190 RhoGAP

Next, we wanted to determine how p190 RhoGAP is regulated by EPHA2. A plausible candidate is the ubiquitously expressed low molecular weight protein tyrosine phosphatase (LMW-PTP), because p190Rho-GAP has been shown to be a target of LMW-PTP (Chiarugi et al., 2000; Nimnual et al., 2003). In addition, Eph receptors are known to recruit LMW-PTP upon receptor activation (Stein, 1998; Zantek et al., 1999). To investigate whether LMW-PTP function can be regulated by the EPHA2 receptor, we first measured LMW-PTP phosphatase activity in MCF-10A-EPHA2 and MCF-10A-ΔC cells using the synthetic substrate DiFMUP (Marlo and Desai, 2006; Montalibet et al., 2005; Welte et al., 2005).

As shown in Fig. 6A and B, LMW-PTP activity was significantly higher in MCF-10A-EPHA2 cells than that in MCF-10A-ΔC cells.

To determine the mechanisms by which EPHA2 regulates LMW-PTP activity, we investigated whether EPHA2 affects LMW-PTP protein levels, phosphorylation state or recruitment of LMW-PTP to the EPHA2 receptor. Neither the total levels of LMW-PTP protein nor the tyrosine phosphorylation state was affected by overexpression of EPHA2 or inhibition of EPHA2 signaling (data not shown). However, higher levels of the interaction between EPHA2 and LMW-PTP were detected in MCF-10A-EPHA2 cells (Fig. 6C), suggesting that EPHA2 receptor recruits LMW-PTP. We reasoned that if EPHA2 is signaling through LMW-PTP to destabilize the adherens junctions, this phenotype should be rescued by overexpression of C12S, the phosphatase inactive mutant of LMW-PTP (Chiarugi et al., 1997). To test this possibility, MCF-10A-EPHA2 cells were transduced with retrovirus expressing C12S and assayed for tyrosine phosphorylation of p190 RhoGAP and stability of the adherens junction. Consistent with data shown in Fig. 5A, p190 RhoGAP tyrosine phosphorylation is decreased in cells overexpressing EPHA2, and expression of the dominant negative C12S mutant restores RhoGAP tyrosine phosphorylation levels (Fig. 6D). In addition, although MCF-10A-EPHA2 cells transduced with control virus exhibited reduced E-cadherin staining at sites of cell-cell contact (Fig. 6E), expression of the LMW-PTP-C12S mutant stabilized the adherens junctions (Fig. 6F). Taken together, these data suggest that LMW-PTP provides a molecular link between EPHA2 receptor activation and the inhibition of p190 RhoGAP, leading to activation of Rho GTPase and destabilization of the adherens junctions.

**Fig. 4.** EPHA2 regulates adherens junction stability via modulation of RhoA activity. (A)Activated Rho and Rac GTPases in MCF-10A, MCF-10A-EPHA2, or MCF-10A-ΔC cells in response to ephrin-A1 stimulation were measured by GST-Rhotekin binding domain and GST-Pak binding domain pull-down assays, respectively. Total levels of Rho and Rac proteins were assay by western blot analysis. *P<0.05. (B-E) MCF-10A, MCF-10A-ΔC, MCF-10A-EPHA2 or MCF-10A-EPHA2 treated with ROCK kinase inhibitor, Y27632, were subjected to Ca²⁺ depletion for 8 hours, followed by detection of E-cadherin. (F-I) MCF-10A and MCF-10A-ΔC cells expressing a constitutively active Rho (Q63L) or control β-galactosidase (LacZ) were assayed for adherens junction stability by Ca²⁺-depletion assay. (J) The expression and activity of Q63L in control and MCF-10A-ΔC cells were confirmed by Rhotekin pull-down assays and western blot analysis.
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Src kinase interacts with EPHA2 to alter adherens junction stability

In addition to LMW-PTP, Src kinase was shown to regulate p190 RhoGAP function (Haskell et al., 2001; Roof et al., 2000; Roof et al., 1998). Moreover, several EphA receptors were found to interact with Src family kinases (Knoll and Drescher, 2004; Parri et al., 2007). To determine whether Src is involved in EPHA2-mediated destabilization of the adherens junctions, total Src and tyrosine phosphorylated Src levels, as well as Src association with EPHA2 receptor were assayed in cells overexpressing EPHA2 or MCF-10A-ΔC cells expressing p190 or the dominant negative p190 mutant 30-1 were assayed for adherens junction stability by Ca²⁺-depletion assay. Expression of wild-type p190 rescued the adherens junctions in MCF-10A-EPHA2 cells (I), whereas expression of the 30-1 mutant destabilized cell-cell adhesion in parental MCF-10A cells and MCF-10A-ΔC cells (D,G).

Fig. 5. EPHA2 regulates adherens junction stability through p190 RhoGAP. (A) p190 RhoGAP was immunoprecipitated from MCF-10A, MCF-10A-ΔC, or MCF-10A-EPHA2 cell lysates and blotted for tyrosine phosphorylation. Tyrosine phosphorylation of p190 was decreased in MCF-10A-EPHA2 cells. (B-I) MCF-10A, MCF-10A-EPHA2 and MCF-10A-ΔC cells expressing p190 or the dominant negative p190 mutant 30-1 were assayed for adherens junction stability by Ca²⁺-depletion assay. Expression of wild-type p190 rescued the adherens junctions in MCF-10A-EPHA2 cells (I), whereas expression of the 30-1 mutant destabilized cell-cell adhesion in parental MCF-10A cells and MCF-10A-ΔC cells (D,G).

Cells than those in MCF-10A or MCF-10A-ΔC cells (Fig. 7A,B). Differential recruitment of Src kinase by EPHA2 receptor in these cells suggests a possibility of Src-kinase-dependent destabilization of cell-cell adhesion. To investigate this possibility, MCF-10A cells overexpressing EPHA2 were treated with the Src kinase inhibitor, PP2. MCF-10A-EPHA2 cells treated with vehicle control lost E-cadherin expression after Ca²⁺ depletion for 8 hours (Fig. 7C). By contrast, treatment of cells with PP2 stabilized the adherens junction (Fig. 7D), as determined by the increase E-cadherin levels at cell-cell contacts.

To test specifically whether association of EPHA2 with Src is required for mediating the destabilization of the adherens junctions,
we generated a series of tyrosine (Y) to phenylalanine (F) mutants in the juxtamembrane and kinase domains of EPHA2 receptor. These mutants, or control wild-type EPHA2, were co-transfected with Src into COS7 cells and the ability of Src to interact with these mutants was assessed by co-immunoprecipitation and western blot analysis. Although the majority of EPHA2 mutants were capable of interacting with Src, two kinase domain mutants, Y812F and Y816F, exhibited significantly decreased association with Src kinase (Fig. 7E). To determine the requirement of EPHA2 interaction with Src in mediating the destabilization of the adherens junction, we first examined the EPHA2 expression levels in MCF-10A/Y812F and MCF-10A-Y816F cells.

MCF-10A/Y816F cells were transduced with retrovirus expressing LMW-PTP and assayed for E-cadherin localization at cell-cell contact in low Ca²⁺ medium. As shown in Fig. 7I-L, expression of LMW-PTP in either MCF-10-A/Y812F or MCF-10-A/Y816F cells destabilizes adherens junction, suggesting that Src kinase functions upstream of LMW-PTP (see diagram in Fig. 9).

High levels of EPHA2 expression correlate with accelerated destabilization of the adherens junction.

EPHA2 receptor tyrosine kinase overexpression is common in malignant tumor cells and the level of EPHA2 receptor expressed on tumor cells correlates with the degree of tumor malignancy (Kinch and Carles-Kinch, 2003). To determine how the level of EPHA2 expression affects cell-cell adhesion, we first examined the EPHA2 expression levels in MCF-10A, MCF-10A/LZRS-EPHA2, MCF-10A/Ad-EPHA2 and two commonly used human breast cancer cell lines, BT-549 and MDA-MB-231. As shown in Fig. 8, EPHA2 is expressed at low levels in MCF-10A, a nontransformed breast epithelial cell line. Expression of exogenous EPHA2 receptor via retroviral transduction increased EPHA2 expression modestly above endogenous level, but the EPHA2 levels in these cells are still considerably lower that those observed in two tumor cell lines or cells infected with adenoviruses (Fig. 8A). Interestingly, although levels of EPHA2 were significantly higher in MDA-MB-231 or BT-549 cells, the phosphorylation state of the receptor was dramatically lower in the tumor cells, possibly reflecting the fact that the tumor cells have lost cell-cell contact and were unable to interact with ephrin ligands on adjacent cells.

To determine the effect of EPHA2 expression on cell-cell adhesion, we compared E-cadherin expression in low Ca²⁺ medium between MCF-10A, MCF-10A/LZRS-EPHA2, and MCF-10A/Ad-EPHA2. Compared with MCF-10A or MCF-10A/LZRS-EPHA2, MCF-10A/Ad-EPHA2 cells depleted of Ca²⁺ exhibited a dramatic increase in the rate at which the adherens junctions were disrupted (Fig. 8B). Within 2 hours of Ca²⁺ depletion, overexpression of EPHA2 in MCF-10A/Ad-EPHA2 cells resulted in a complete loss
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By contrast, destabilization of the adherens junctions was only detected after 8 hours of Ca2+ depletion in cells expressing moderate amounts of EPHA2 (MCF-10A/LZRS-EPHA2). Taken together, these data suggest that EPHA2 overexpression can destabilize the adherens junctions and the rate of destabilization of the adherens junctions is dependent upon EPHA2 levels.

Discussion

Despite significant evidence supporting a role for EPHA2 receptor tyrosine kinase in tumorigenesis, the mechanisms by which EPHA2 overexpression contributes to tumor progression are not completely understood. Adherens junctions play a fundamental role in embryonic development and in the maintenance of tissue architecture in adults. Loss of cadherin function has been associated with migratory behavior in vitro and is a hallmark of invasive carcinoma in vivo. Previous studies showed that loss of E-cadherin inhibits the expression of Eph receptors in embryonic stem cells (Orsulic and Kemler, 2000), suggesting EPHA2 expression is regulated by E-cadherin. However, many malignant tumor cells that have lost E-cadherin expression exhibited elevated EPHA2 receptor levels (Ireton and Chen, 2005; Zantek et al., 1999), raising the question of whether EPHA2 levels affect the adherens junctions. In this report, we studied how cell-cell adhesion is affected by EPHA2 overexpression. We found that overexpression of EPHA2 does not induce epithelial to mesenchymal transition directly; however, elevated EPHA2 expression destabilizes adherens junctions and accelerates dissociation of cell-cell contact. The rate of destabilization of the adherens junctions is dependent upon EPHA2 levels; higher EPHA2 expression leads to more rapid downregulation of E-cadherin localization at cell-cell contacts. The fact that overexpression of EPHA2 also affects E-cadherin suggests that a reciprocal regulation exists between E-cadherin and EPHA2. A delicate balance of relative levels of E-cadherin and EPHA2 receptor appears to ensure normal cellular architecture and function.

Several cellular mechanisms have been proposed to perturb adherens junctions. These include cleavage of the cadherin extracellular domain, phosphorylation of the cadherin complexes, increased turnover of cadherin receptors and regulation of cytoskeletal attachment to cadherin complexes (Daniel and Reynolds, 1997; Fukata and Kaibuchi, 2001; Lozano et al., 2003). Activation of receptor tyrosine kinases (RTKs) has been shown to destabilize adherens junctions through phosphorylation of cadherin or catenin junctional proteins (Daniel and Reynolds, 1997) and ephrin-A1 stimulation is reported to induce β-catenin phosphorylation in HT-29 colon carcinoma cells (Potla et al., 2002). However, we did not observe changes in tyrosine phosphorylation of E-cadherin, p120, and β- or γ-catenin in MCF-10A, MCF-10A/EPHA2 or MCF-10A/ΔC cells—a result that is consistent with the data reported by Orsulic et al. (Orsulic and Kemler, 2000). Nor did we detect any alternations of cadherin expression or composition of adherens junction complex (Fig. 2). In addition, no significant changes in cortical actin cytoskeleton were observed, suggesting that the effects of EPHA2 on adhesive strength are probably not due to gross disruption of the cytoskeleton.

A growing number of studies have linked Rho family small GTPases with cadherin-dependent cell-cell contacts (reviewed by Fukata and Kaibuchi, 2001; Lozano et al., 2003; Perez-Moreno et al., 2003). Rho family GTPases can regulate cell-cell adhesion by acting on the cadherin-catenin complex or on the actin cytoskeleton and other components. Rac1 and Cdc42 have been shown to directly affect the cadherin-catenin complex by modulating the interaction of IQGAP1, a GTPase-activating protein, with β-catenin (Fukata et al., 1999). By contrast, activation of RhoA has been implicated in regulating E-cadherin-mediated adhesive activity through the actin cytoskeleton (Fukata and Kaibuchi, 2001; Nagafuchi et al., 1994). Here we show that EPHA2 signaling activates RhoA GTPase. As the ROCK kinase inhibitor suppresses EPHA2-induced destabilization of cell-cell adhesion, these data suggest a critical role of EPHA2-induced RhoA activation in regulating the strength of adherens junctions.
In fibroblasts, Rho activity can be regulated by Rac via a signaling pathway involving ROS, LMW-PTP and p190 RhoGAP (Nimmul et al., 2003). More recently, Wildenberg et al. showed that a p120 catenin-p190 RhoGAP interaction is required for Rac inhibition of Rho in the stabilization of adherens junctions (Wildenberg et al., 2006). Although EPHA2 receptor activation in vascular endothelial cells upregulates Rac1 activity (Brantley-Sieders et al., 2004a), EPHA2 receptor overexpression in mammary epithelial cells does not appear to affect Rac-GTP levels (Fig. 4A). Enhanced RhoA activity in MCF-10A-EPHA2 cells is apparently regulated by enhanced LMW-PTP phosphatase activity and inhibition of tyrosine phosphorylation of p190 RhoGAP, ultimately leading to the destabilization of cell-cell adhesion (see diagram in Fig. 9).

It is interesting to note that although LMW-PTP can be activated by EphA receptors [this report; (Park, 2003)], EPHA2 receptor has also been shown to be a substrate for the same phosphatase (Kikawa et al., 2002; Parri et al., 2005). However, in our system, phosphorylation of EPHA2 does not appear to be affected by LMW-PTP activity. Although the general PTP inhibitor pervanadate increased EPHA2 phosphorylation, overexpression of wild-type or a C12S mutant of LMW-PTP did not change the EPHA2 phosphorylation level significantly (data not shown), suggesting that EPHA2 may not be a major substrate of LMW-PTP in MCF-10A cells.

How does EphA receptor activation enhance LMW-PTP activity? In the case of EphA8, the kinase activity of the receptor directly phosphorylates LMW-PTP (Park, 2003). However, direct phosphorylation of LMW-PTP by EPHA2 was not observed in these cells, indicating that EPHA2 regulates LMW-PTP activity by alternative mechanism(s). One possible mechanism is through Src kinase. Although Src can phosphorylate both LMW-PTP and p190 RhoGAP (Bucciantini et al., 1999) (Haskell et al., 2001; Roof et al., 2000; Roof et al., 1998), the phenotype of MCF-10A-EPHA2 cells suggest that p190 RhoGAP is most likely not a candidate substrate for Src. As EPHA2 can physically associate with both Src kinase and LMW-PTP, Src could possibly regulate cell-cell adhesion through modulating LMW-PTP activity. Alternatively, Src could also affect adherens junctions by upregulating Rho GEF activity. Further experiments are needed to dissect these possibilities.

In addition to adherens junctions, tight junctions also contribute to intercellular junctional complexes (Perez-Moreno et al., 2003). Members of the Eph family have also been implicated in regulating tight junctions. Ephrin-B1 binds to Claudin-1 and Claudin-4, which are components of tight junction complexes. In addition, ephrin-B1 is phosphorylated in a Src-kinase-dependent manner upon cell-cell contact (Tanaka et al., 2005b). Furthermore, Tanaka et al. reported that EPHA2 phosphorylates the cytoplasmic tail of Claudin-4 and mediates paracellular permeability in MDCK cells (Tanaka et al., 2005a). Whether or not EPHA2 overexpression in MCF-10A cells also affects tight junctions in addition to regulation of adherens junctions remains to be determined. Furthermore, it will be interesting to determine whether other members of the Eph RTK family also play a role in regulating adherens junctions.

In summary, our results support a role of the EPHA2 receptor in the regulation of cell-cell adhesion. EPHA2 overexpression probably promotes destabilization of the adherens junction through a signaling pathway of recruitment of Src kinase, enhanced LMW-PTP activity, inhibition of p190 RhoGAP and activation of RhoA GTPase. As EPHA2 level is linked to tumor malignancy, these studies provide a foundation for investigating EPHA2 as a potential target for therapeutic intervention.

Materials and Methods

Antibodies and reagent
Antibodies used for immunoblotting include anti-EPHA2 (1:1000, Upstate Biotechnology), anti-phosphotyrosine (1:250, Santa Cruz Biotechnology), anti-p190 RhoGAP (1:1000, Transduction Laboratory), anti-E-cadherin (0.1 μg/ml, Transduction Laboratory), anti-p120 (1:0 μg/ml, Transduction Laboratory), anti-β-catenin (1:5000, Sigma), anti-α-catenin (1:5000, Sigma), anti-tubulin (1:1000, Sigma), anti-phospho-Src (Y416) (1:1000, Cell Signaling Technology), anti-Src (1:1000, Cell Signaling Technology), and anti-RhoA (1:200, Santa Cruz Biotechnology). Anti-LMW-PTP (1:2000) was a kind gift from Takamune Takahashi (Vanderbilt University, TN). Recombinant ephrinA1-Fc proteins were purchased from R&D Systems (Minneapolis, MN). ROCK inhibitor, Y27632, and Src inhibitor PP2 were purchased from Calbiochem.

Plasmids and viruses
EPHA2 mutations were generated by PCR amplification using EPHA2-specific primers containing tyrosine to phenylalanine mutations. Retroviral plasmids, LZRS-EPHA2, LZRS-ΔC, LZRS-Y812F, LZRS-Y816F, were generated by subcloning from existing plasmids (Fang et al., 2005). LZRS-p190RhoGAP and LZRS-30-1 mutant were described previously (Brouns et al., 2001; Wildenberg et al., 2006). Wild-type (pBabe-LMW-PTP) and mutant LMW-PTP (pBabe-C12S) retroviruses were generated from plasmids as described (Chiarugi et al., 1997). Adenovirus expressing constitutively active RhoA (Q63LA) was purchased from Cell Bioslabs (San Diego, CA) (Lee et al., 2004; Mayer et al., 1999). Adenoviruses expressing EPHA2 or control β-galactosidase were described previously (Brantley-Sieders et al., 2004a; Cheng and Chen, 2001).

Cell culture and retroviral infection
MCF-10A, a spontaneously immortalized but non-transformed human mammary epithelial cell line, was obtained from the American Type Culture Collection (ATCC) and cultured in 5% horse serum (Hyclone), 20 mg/ml EGF (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml Cholera Toxin (Calbiochem), 10 μg/ml insulin (Sigma), and 1% penicillin-streptomycin (Life Technologies) in 10% FBS, 0.023 IU/ml insulin (Sigma), and 1% penicillin-streptomycin (Life Technologies). BT-549 cells were cultured in RPMI media with 10% FBS, 0.023 IU/ml insulin (Sigma), and 1% penicillin-streptomycin.

Hanging drop aggregation assay
MCF-10A cells were trypsinized and resuspended at a concentration of 500,000 cells/ml growth medium. Approximately 15,000 cells (30 μl cell suspension) were plated in 96-well plates with 30 μl per well. After 7 days, the hanging drops were fixed and stained with rhodamin-phalloidin and Hoechst 33342.
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