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Recruitment of Phosphoinositide 3-Kinase Defines a Positive Contribution of Tyrosine Kinase Signaling to E-cadherin Function*

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Classical cadherin adhesion molecules can function as adhesion-activated cell-signaling receptors. One key target for cadherin signaling is the lipid kinase phosphoinositide (PI) 3-kinase, which is recruited to cell-cell contacts and activated by E-cadherin. In this study, we sought to identify upstream factors necessary for E-cadherin to activate PI 3-kinase signaling. We found that inhibition of tyrosine kinase signaling blocked recruitment of PI 3-kinase to E-cadherin contacts and abolished the ability of E-cadherin to activate PI 3-kinase signaling. Tyrosine kinase inhibitors further perturbed several parameters of cadherin function, including cell adhesion and the ability of cells to productively extend nascent cadherin-adhesive contacts. Notably, the functional effects of tyrosine kinase blockade were rescued by expression of a constitutively active form of PI 3-kinase that restores PI 3-kinase signaling. Finally, using dominant negative Src mutants and Src-null cells, we identified Src as one key upstream kinase in the E-cadherin/PI 3-kinase-signaling pathway. Taken together, our findings indicate that tyrosine kinase activity, notably Src signaling, can contribute positively to cadherin function by supporting E-cadherin signaling to PI 3-kinase.

Classical cadherin adhesion receptors mediate cell-cell recognition and patterning in all solid tissues of the body. In cooperation with the actin cytoskeleton, cadherins regulate cell shape, cell-cell cohesion, and intercellular locomotility, processes that collaborate to orchestrate tissue morphogenesis during development and post-embryonic life (1, 2). It is increasingly apparent that cell-signaling events play critical roles in cadherin-dependent morphogenesis, particularly in coordinating surface adhesion and cytoskeletal activity (3, 4). Indeed, one emerging hypothesis is that cadherins exert their morphogenetic effects, at least in part, by acting as adhesion-activated cell-signaling receptors. In this model, productive adhesive ligation of the cadherin ectodomain activates membrane-local cell-signaling pathways capable of regulating cytoskeletal activity at the contacts (5). Because the cadherin cytoplasmic tail possesses no known catalytic activity, it is implied that membrane-associated signaling molecules must be recruited to the cadherin-catenin complex for signaling to occur. Ultimately, such recruitment is likely to involve coordinators of membrane signaling, such as ancillary adaptor proteins, scaffolds, and membrane microdomains.

One such cadherin-activated signaling pathway involves the lipid kinase, phosphoinositide (PI)[1] 3-kinase. Signaling by Class 1 PI 3-kinases is activated in response to a wide variety of extracellular stimuli, including growth factors and hemopoietic cytokines (6, 7). Paradigmatically, such signaling pathways involve the initial recruitment of PI 3-kinase to ligand-activated cell surface receptors (8, 9). In the case of the Class IA PI 3-kinases, membrane recruitment is mediated by the p85 adaptor subunit (6). Subsequently, the p110 catalytic subunit catalyzes the local production of 3′-phosphoinositides (9), notably $PI_{(3,4,5)}P_3$ (PIP_3), which trigger signaling cascades by recruiting proteins that contain pleckstrin homology domains, including the serine/threonine kinase Akt (Protein kinase B) and exchange factors for a range of Rho family GTPases (6).

Cadherin-activated PI 3-kinase signaling appears to follow this basic paradigm. Thus, Class 1A PI 3-kinase is recruited to cadherin-based adhesive contacts, as epithelial cells form contacts with one another (10). Indeed, cadherin homophilic ligation alone is sufficient to recruit PI 3-kinase to adhesive contacts, where it appears to preferentially accumulate in newly forming adhesive contacts (11). Furthermore, cadherin-induced recruitment stimulates PI 3-kinase signaling, as reflected in the membrane recruitment and phosphorylation of Akt and the generation of PIP_3 at cadherin contacts (10–12). Such cadherin-activated PI 3-kinase signaling has a clear impact on cadherin function, being necessary both for adhesive strengthening and for the efficient assembly of cadherin-based adhesive contacts (11, 13). These functional consequences are likely to be mediated by activation of the Rac GTPase (11), which can regulate cooperation between cadherins and the actin cytoskeleton in cell-cell contacts (5, 14).

As with many other models of PI 3-kinase signaling, recruitment of PI 3-kinase to the plasma membrane is a key step in the cadherin-activated signaling pathway. This may entail binding of the p85 adaptor subunit to cadherin-associated proteins, such as β-catenin (15–17) and potentially also hDlg (18). In many instances of receptor-activated PI 3-kinase signaling, binding of the p85 adaptor subunit is, itself, a response to

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[1] The abbreviations used are: PI, phosphoinositide; CHO, Chinese hamster ovary; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ConA, concanavalin A; mRFP, monomeric red fluorescent protein.

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upstream tyrosine kinase activity (7). Recruitment commonly entails high affinity interactions between Src homology 2 domains and tyrosine-phosphorylated sequences in components of the receptor complex (6, 8). Such upstream tyrosine phosphorylation may be due to either receptor tyrosine kinases or nonreceptor tyrosine kinases. The observation that recruitment of PI 3-kinase to E-cadherin appears to be transient and preferentially confined to newly forming adhesive contacts suggested that this, too, might depend on upstream signaling events. Accordingly, in this study, we sought to examine the potential contribution of tyrosine kinase activity to cadherin-activated PI 3-kinase signaling. We report that upstream tyrosine kinase signaling is necessary to recruit PI 3-kinase to E-cadherin-adhesive contacts, providing a mechanism for tyrosine kinase activity to positively regulate cadherin function. We further identify c-Src as a potentially key intermediary kinase in the E-cadherin/PI 3-kinase-signaling pathway.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Reagents—Parental CHO cells, CHO cells stably expressing full-length human E-cadherin (hE-CHO cells), and MCF-7 cells were cultured as reported previously (11, 19). For the experiments, the cells were plated at subconfluent density on glass coverslips or dishes and maintained for 1–2 days before being used.

The plasmids used were: pcDNA3.1_{zeo}-GFP-p85 (a gift of Dr. W. Gullick; Imperial College School of Medicine, London, UK); Myc-tagged pSG5p110^{CAAX} (kindly provided by Dr. J. Downward; Imperial Cancer Research Fund); and CA10Src-mf (K295M, Y527F) (20). To generate a plasmid that expressed both Src-mf (K295M, Y527F) and GFP, the coding sequence for the mutant Src was excised as a BamHI/SalI fragment from CA10Src-mf and subcloned into pIRES2EGFP via BglII/SalI. To generate an mRFP-tagged version of p85, the coding sequence for p85 was amplified by PCR from pcDNA3.1_{zeo}-GFP-p85 and cloned as a SalI/BamHI fragment into pEXFP-C3 (a kind gift of Dr. Roger Tsien) to result in a cytomegalovirus promoter-driven expression of an N-terminal mRFP fusion protein. Transient transfections were performed with Lipofectamine™ (Invitrogen) according to the manufacturer's instructions. Genistein and herbimycin were purchased from Calbiochem.

Antibodies—The primary antibodies were: 1) mouse mAb directed against the conserved cytoplasmic tail of human E-cadherin (Transduction Laboratories) for immunofluorescent staining, 2) mouse mAb SHE 78–8 (Zymed Laboratories Inc.) raised against the ectodomain of human E-cadherin for immunoprecipitation, 3) a rabbit pAb raised against the extracellular domain of human E-cadherin (21), 4) mouse mAb against GFP (Roche Applied Science), 5) mouse mAb PY20 against phosphotyrosine (Cell Signaling), and 6) mouse mAbs against Akt or phospho-Akt (Cell Signaling Technologies). Alexa-488 and Texas Red-conjugated phalloidin were used to stain F-actin (Molecular Probes, Inc.). Secondary antibodies were species-specific antibodies conjugated with Alexa-488 or Texas Red (Molecular Probes, Inc.).

hE/Fc Assays and Quantitation—hE/Fc protein was purified from conditioned media as described previously (11) and used at 100 μg/ml to coat latex beads or planar substrata. Bead assays were performed as described previously (22) with the addition of tyrosine kinase inhibitors during the 90-min incubation as indicated. Accumulation of GFP-p85 or phosphotyrosine was quantified from digital images using Image J (version 1.30). The area of fluorescence that accumulated around each bead was identified using the region of interest tool in Image J (version 1.30). Accumulation was expressed as the ratio of the mean pixel intensity in the region around the bead divided by the background pixel intensity in the cell away from the beads. Planar spreading assays (11), hE/Fc adhesion assays (23), and calcium manipulation assays (21) were performed as described previously. Image J was used to measure the surface area of cells adherent to hE/Fc in planar adhesion assays. Briefly, the perimeters of phalloidin-stained cells were marked using the region of interest tool and the number of pixels within the area measured. Reformation of cell-cell contacts following the manipulation of extracellular calcium was quantitated by measuring the lengths of individual cadherin contacts using Image J as described previously (21).

Immunofluorescence Microscopy—Cells were fixed on ice for 30 min in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS at room temperature, washed with PBS, and blocked overnight with 5% (w/v) nonfat dried milk in PBS. The cells were then incubated in primary antibodies diluted in 5% (w/v) nonfat dried milk in PBS or 1% (w/v) gelatin in TBSP buffer (10 mM Tris, pH7.5, 100 mM NaCl, 0.1% Tween 20) for detection of phosphotyrosine and visualized with Alexa-488- or Alexa-594-conjugated secondary antibodies (Molecular Probes). For detection of F-actin, the specimens were stained with Alexa-488- or Texas Red-conjugated phalloidin in 5% (w/v) nonfat dried milk in PBS. Immunolabeled cells were mounted in 1% *N*-propyl-gallate in 50% glycerol:PBS for immunofluorescence microscopy.

Fixed specimens were examined by immunofluorescence microscopy using an Olympus IX81 microscope equipped with 60× 1.40 numerical aperture objectives. Images were captured with Hamamatsu Orca I or Orca I-ER cameras operated by MetaMorph software version 5.0 (Universal Imaging Corp.). The figures were processed and assembled for presentation in Adobe Photoshop (version 7).

Immunoprecipitation and Immunoblotting—Cells were lysed in cold lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaF, 0.1 mM sodium vanadate, and Complete Protease Inhibitors (Roche Applied Science, Mannheim, Germany). Lysates were sheared through a 30-gauss needle (30 times) and then further solubilized on ice for 30 min. Post-nuclear supernatants were obtained by centrifugation at 3000 rpm for 3 min.

For co-immunoprecipitations, cell extracts were incubated with mouse E-cadherin mAb SHE 78–7 raised against the ectodomain of human E-cadherin for 2 h at 4 °C followed by incubation with protein G-agarose for 30 min at 4 °C. Precipitates were recovered by centrifugation and then washed several times with cold lysis buffer. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting for either E-cadherin or p85.

To determine Akt activation, cells were serum-starved by overnight incubation in media containing 0.2% fetal calf serum. The cells were then allowed to adhere for 90 min to either 0.5% poly-L-lysine- or hE/Fc-coated 10-cm bacteria dishes in the presence or absence of genistein at 37 °C. The post-nuclei supernatants were centrifuged at 50,000 rpm at 4 °C for 1 h and separated into cytosolic (S100) and membrane-enriched (P100) fractions. The P100 fractions were then resuspended in 500 μl of lysis buffer. Akt was immunoprecipitated with an Akt antibody for 1 h at 4 °C, and immune complexes were separated by SDS-PAGE and probed with Akt or activation-specific phospho-Akt antibodies.

RESULTS

Tyrosine Kinase Activity Is Necessary for PI 3-Kinase to Recruit to E-cadherin-adhesive Contacts—As a first step, we compared the localization of tyrosine-phosphorylated proteins and PI 3-kinase at E-cadherin-adhesive contacts (Fig. 1). For this study, we used a recombinant cadherin adhesive ligand (hE/Fc) consisting of the complete E-cadherin ectodomain expressed as an Fc fusion protein. This well validated reagent (11, 21–24) provided the opportunity to specifically examine E-cadherin-activated signaling pathways independent of other juxtacrine signals that come into play when native cell surfaces are brought into contact with one another.

We used latex beads coated with hE/Fc to provide spatially defined cadherin-adhesive signals to cells. As previously reported (21, 22, 24), cellular E-cadherin rapidly accumulated at sites of adhesion between hE/Fc-coated beads and the dorsal surfaces of CHO cells stably expressing human E-cadherin (hE-CHO cells) (Fig. 1*A*). Type 1A PI 3-kinase also accumulated at these sites of adhesion, as identified using either transiently expressed GFP-p85 (Fig. 1, *A* and *C*) or by staining for endogenous p85 (not shown). GFP-p85 did not accumulate at contacts with ConA-coated beads (Fig. 1, *A* and *C*), indicating that the recruitment of PI 3-kinase was relatively specific for E-cadherin adhesion; nor did GFP alone accumulate at sites of contact with hE/Fc-coated beads (Fig. 1, *A* and *C*).

Immunostaining with anti-phosphotyrosine antibodies demonstrated that tyrosine-phosphorylated proteins co-accumulated with GFP-p85 at cadherin-adhesive contacts (Fig. 1*A*). This indicated that cadherin-specific adhesions are potential sites of tyrosine kinase activity, consistent with the well documented observation that phosphotyrosine accumulates at adherens junctions (25–27). To test the potential functional significance of this activity, we treated cells with broad spectrum inhibitors of tyrosine kinase signaling. Neither herbimycin (10

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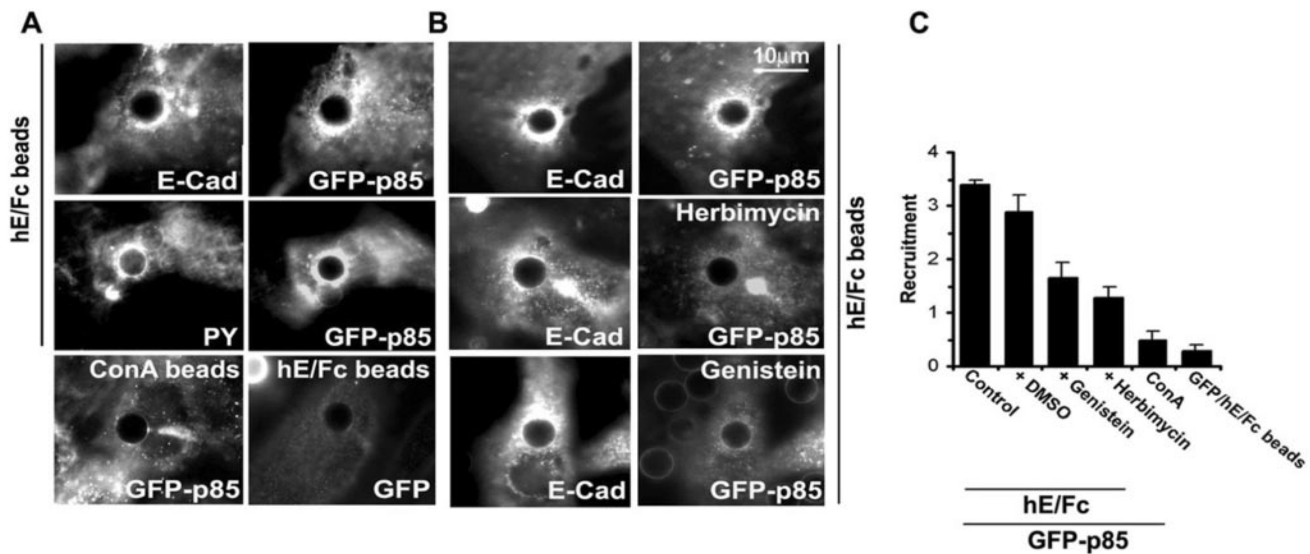

FIG. 1. **Tyrosine kinase activity is necessary for PI 3-kinase to recruit to cadherin-adhesive contacts.** E-cadherin homophilic contacts were generated by allowing latex beads coated with either hE/Fc or ConA to adhere for 90 min to the dorsal surfaces of CHO cells stably expressing human E-cadherin (hE-CHO cells). *A*, co-accumulation of PI 3-kinase and phosphotyrosine at cadherin-adhesive contacts. Representative images are shown of cellular E-cadherin (*E-Cad*), transiently expressed GFP-p85 (*GFP-p85*), or phosphotyrosine (*PY*) at adhesive contacts with beads. *Left* and *right images* of hE/Fc beads are of simultaneous dual color images. As controls, we assessed recruitment of GFP-p85 recruitment to ConA beads to test the specificity of recruitment for cadherin adhesion and recruitment of GFP alone to hE/Fc beads to exclude potential edge artifacts. *B*, effect of tyrosine kinase inhibitors on GFP-p85 recruitment to cadherin contacts. hE-CHO cells transiently expressing GFP-p85 were incubated with either herbimycin (10 μM) or genistein (100 μM) for 90 min prior to bead adhesion assays; drugs were maintained for the duration of the bead assays. *Left* and *right panels* are from simultaneous dual color images. *C*, GFP-p85 recruitment to beads was quantitated by digital image analysis as described under "Materials and Methods." Cells were transfected with either GFP-p85 or GFP alone and allowed to bind beads coated with either hE/Fc or ConA. Where shown, the cells were incubated with herbimycin (10 μM), genistein (100 μM), or Me_2SO (*DMSO*) alone (0.01% w/v) for 90 min before and during the adhesion assays. Data are means ± S.E., $n = 29$.

μM) nor genistein (100 μM) materially affected the recruitment of cellular E-cadherin to hE/Fc beads (Fig. 1*B*). However, both drugs markedly inhibited the accumulation of GFP-p85 (Fig. 1, *B* and *C*) and phosphotyrosine (not shown) at hE/Fc beads. These data thus suggested that tyrosine kinase signaling was necessary to recruit PI 3-kinase to E-cadherin adhesions.

Tyrosine Kinase Activity Is Necessary for PI 3-Kinase to Complex with E-cadherin—PI 3-kinase appears to be recruited to cadherin adhesions via indirect biochemical interactions that may involve β-catenin (17) and hDlg (18). Accordingly, we sought to test whether tyrosine kinase signaling affects the ability of PI 3-kinase to biochemically associate with the E-cadherin molecular complex. As shown in Fig. 2*A*, endogenous p85 co-immunoprecipitated with cellular E-cadherin in cells adherent to hE/Fc-coated substrata but not in cells adherent to the nonspecific adhesive ligand poly-L-lysine. This is consistent with our earlier observation that cadherin homophilic adhesion alone induces the formation of a biochemical complex between E-cadherin and PI 3-kinase (11). However, inhibition of tyrosine kinase signaling with genistein substantially reduced the amount of p85 that complexed with E-cadherin (Fig. 1*A*).

We then extended these observations to study the biochemical interaction between these two proteins as cells formed native cell-cell contacts with one another. For these experiments, we used MCF-7 breast epithelial cells, which contain endogenous E-cadherin and form adherens junctions with one another (Fig. 2*B*). Cell-cell contacts were first disrupted by chelating extracellular calcium and then induced to reassemble by replacing extracellular calcium. As reported previously (10, 11), p85 progressively associated with E-cadherin as cells reassembled cell-cell contacts. Genistein reduced the amount of p85 that co-immunoprecipitated with E-cadherin in these assays. The effect of genistein was most prominent early in the process of reassembling contacts; with time, the amount of p85 that co-precipitated with E-cadherin increased toward control levels. Taken together, these data complement our fluorescence recruitment assays to demonstrate that tyrosine kinase activity is necessary for E-cadherin to recruit PI 3-kinase.

Tyrosine Kinase Activity Is Necessary for E-cadherin-activated PI 3-Kinase Signaling—Activation of downstream signaling pathways accompanies recruitment of PI 3-kinase by E-cadherin (10–12). We therefore used activation-specific phospho-Akt antibodies to test whether tyrosine kinases affected the activation of PI 3-kinase signaling by E-cadherin adhesion (Fig. 2*C*). Serum-deprived hE-CHO cells were allowed to adhere to hE/Fc- or poly-L-lysine-coated substrata. After 60 min of adhesion, the cells were lysed and fractionated, and Akt was immunoprecipitated from the plasma membrane-enriched (P100) fraction. As we have observed previously (11), adhesion to hE/Fc, but not to poly-L-lysine, increased phospho-Akt levels in the plasma membrane fraction. This was inhibited by wortmannin, confirming that activation of Akt occurred in response to PI 3-kinase signaling. Treatment of cells with genistein also reduced the amount of pAkt that accumulated in membranes in response to E-cadherin to levels similar to those seen upon attachment to poly-L-lysine (Fig. 2*C*). This indicated that cadherin-activated PI 3-kinase signaling, as well as its recruitment to cadherin contacts, required tyrosine kinase activity.

Tyrosine Kinase-dependent PI 3-Kinase Signaling Contributes to Cadherin Contact Formation and Adhesion—E-cadherin-activated PI 3-kinase signaling regulates cellular responses to E-cadherin homophilic ligation, including adhesive strengthening and cell contact formation (11, 18). This suggested that tyrosine kinase activity might contribute to E-cadherin activity through its effect on cadherin-activated PI 3-kinase signaling. To assess this, we began by investigating the impact of tyrosine kinase inhibitors on cadherin-based cell contact formation, a process that requires punctate initial cell-cell contacts to be actively extended into broad stable zones of cadherin-based adhesion (14, 28). Such contact zone extension

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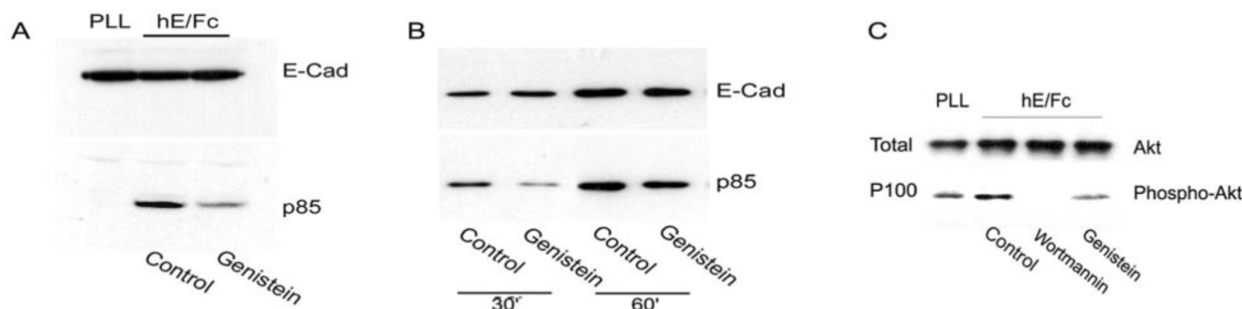

FIG. 2. **Tyrosine kinase activity is necessary for the E-cadherin complex to interact with PI 3-kinase and activate Akt.** *A* and *B*, effect of tyrosine kinase inhibitors on the biochemical association between E-cadherin and PI 3-kinase. The ability of p85 to associate with the cadherin-catenin complex was tested in hE-CHO cells after 90 min of adhesion to either hE/Fc- or PLL-coated substrata (*A*) or in MCF-7 cells (*B*), where contacts were allowed to reassemble for 30–60 min after chelation of extracellular Ca^{2+}. E-cadherin immunoprecipitates from cell lysates were separated by SDS-PAGE and Western blots probed for both p85 and E-cadherin (*E-Cad*). Where shown, the cells were incubated with genistein (100 μM). *C*, tyrosine kinase activity is necessary for E-cadherin ligation to activate PI 3-kinase signaling. hE-CHO cells were allowed to attach to hE/Fc- or poly-L-lysine-coated dishes in the presence of either wortmannin (60 nM) or genistein (100 μM). Akt was immunoprecipitated from a plasma membrane-enriched (P100) fraction, and immune complexes were separated by SDS-PAGE. Western blots were probed for phospho-Akt to measure activation of PI 3-kinase-dependent signaling. Total cellular Akt levels were assessed by immunoblotting total cell lysates (*Total*).

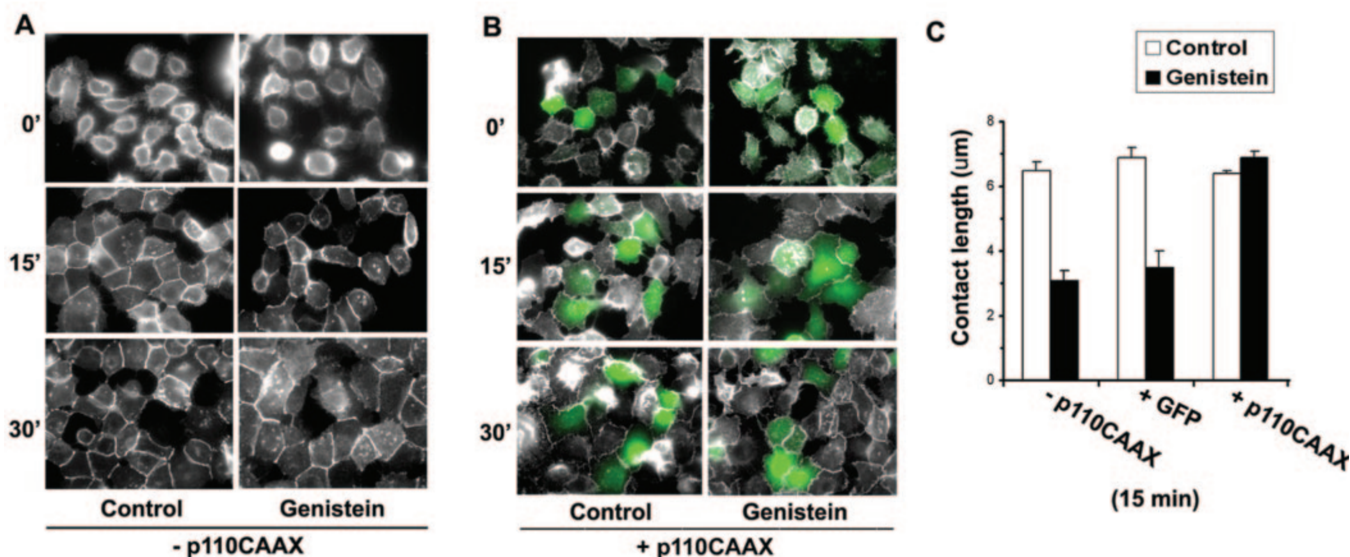

FIG. 3. **Tyrosine kinase inhibitors perturb efficient assembly of cell-cell contacts in a PI 3-kinase-dependent fashion.** Cell-cell contacts between MCF-7 cells were disrupted by chelation of extracellular Ca^{2+} (0′) and then monitored as they reassembled after replacement of Ca^{2+} (15–30′). Where indicated, untransfected MCF-7 cell monolayers (*−p110CAAX*) (*A*) and MCF-7 cell monolayers transiently transfected with constitutively active $p110^{CAAX}$ (+p110CAAX) (*B*) were treated with genistein (100 mM) (*Genistein*) for 90 min prior to and during the experiments. Transfected cells were identified by co-expression of enhanced GFP (*green*). *C*, the ability of cells to reassemble contacts 15 min after replacement of extracellular Ca^{2+} was quantitated by measuring the lengths of individual contacts made by untransfected cells (*−p110CAAX*), cells transfected with GFP alone (*+GFP*), and cells transfected with $p110^{CAAX}$ (*+p110CAAX*). Data are means ± S.E., *n* = 20–30.

appears to entail cell-signaling pathways that are activated by cadherin homophilic ligation to regulate cytoskeletal activity in newly assembling cell-cell contacts, including PI 3-kinase (5).

We measured the efficiency with which MCF-7 monolayers reassembled cell-cell contacts following chelation of extracellular calcium, conditions where genistein inhibited recruitment of PI 3-kinase to E-cadherin (Fig. 2). MCF-7 cells rapidly reassembled cell-cell contacts following replacement of extracellular calcium; contacts broken after chelation of extracellular calcium had largely reformed within 15 min (Fig. 3, *A* and *C*). Treatment with genistein, however, significantly retarded the ability of cells to reassemble contacts (Fig. 3, *A* and *C*). After 15 min, genistein reduced the lengths of contacts between cells by ~50% compared with untreated cultures (Fig. 3*C*), and contacts did not fully reform until after ~30–45 min (Fig. 3*A*).

To test whether this inhibition of contact formation involved a PI 3-kinase-dependent pathway, we transiently expressed $p110^{CAAX}$-Myc, a well characterized membrane-tethered form of the PI 3-kinase catalytic unit that provides constitutively active PI 3-kinase signaling (29). Contact formation was quantitated by measuring the length of individual cell-cell contacts made by cells expressing the transgene, identified by staining for the Myc epitope tag. Expression of $p110^{CAAX}$ alone did not affect the ability of cells to reform contacts with one another, but it largely restored the efficiency with which genistein-treated cells reassembled cell-cell contacts (Fig. 3, *B* and *C*). Expression of GFP alone, as a transfection control, did not affect contact formation (Fig. 3*C*).

It was possible, however, that changes in cell-substrate interactions also delayed reassembly of cell-cell contacts when tyrosine kinase signaling was inhibited. To isolate the potential impact of tyrosine kinase signaling on the cadherin-activated pathways that mediate contact formation, we examined the ability of cells to extend contacts on hE/Fc-coated substrata (Fig. 4). This assay has proven to be a useful strategy to identify cadherin-specific cellular mechanisms that support

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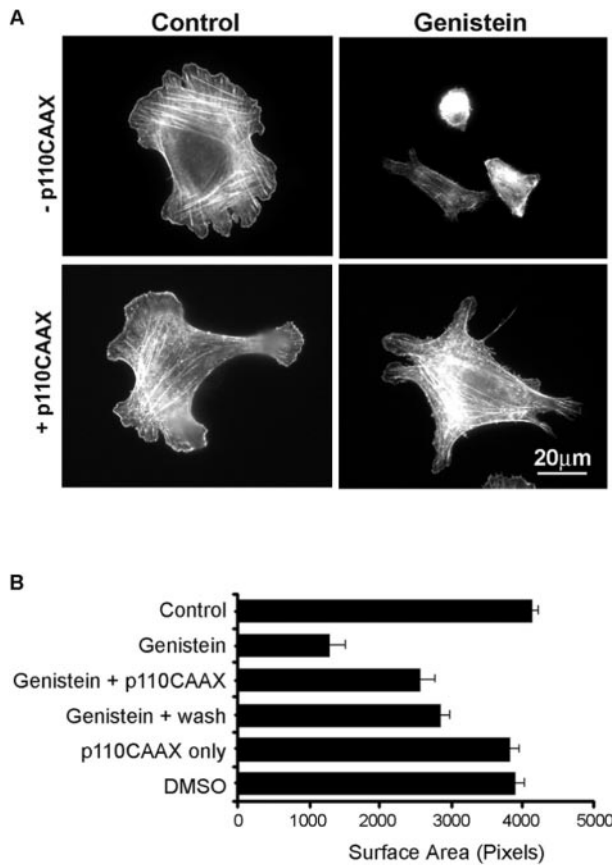

FIG. 4. **Tyrosine kinase-dependent PI 3-kinase signaling is necessary for efficient extension of cadherin-specific adhesive contact zones.** Untransfected hE-CHO cells and hE-CHO cells transiently transfected with constitutively active $p110^{CAAX}$ were plated onto hE/FC-coated substrata in the presence or absence of genistein (100 μM) for 90 min. As a control for nonspecific irreversible toxicity, genistein-treated hE-CHO cells were washed into drug-free medium for a further 60 min (*Genistein + wash*). Carrier controls were incubated with Me_2SO (*DMSO*) (0.1% w/v) for 90 min. *A*, representative images of adherent hE-CHO cells stained with Alexa-488 phalloidin to identify the full extent of contact zones. *B*, contact zone extension was quantitated by measuring the average surface area (expressed in pixels) of adherent cells. Data are means ± S.E., $n = 20$.

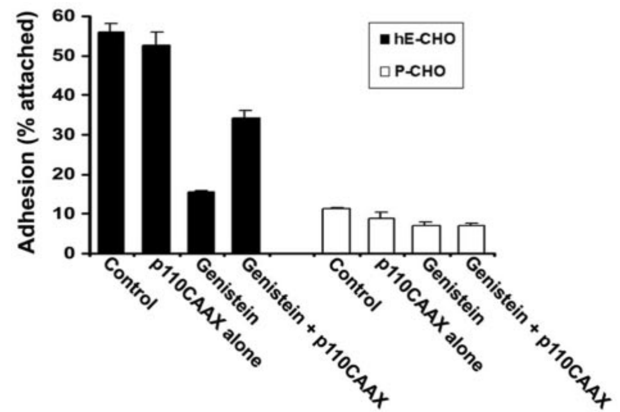

FIG. 5. **Effect of tyrosine kinase inhibition on cadherin-specific cell adhesion.** Control cells and cells transiently transfected with constitutively active $p110^{CAAX}$ were allowed to attach for 90 min to hE/Fc-coated substrata in the presence or absence of genistein (100 μM). Adhesion was measured by the resistance of cells to detachment, as described under "Materials and Methods." Control hE-CHO cells (*hE-CHO*) displayed significantly greater adhesive strength than cadherin-deficient parental CHO (*P-CHO*) cells.

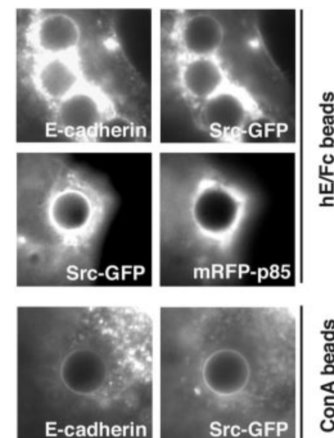

FIG. 6. **Src co-accumulates with PI 3-kinase in cadherin-adhesive contacts.** hE-CHO cells transiently expressing Src-GFP alone or co-expressing Src-GFP and mRFP-p85 were exposed to either hE/Fc-coated beads or ConA-coated beads. The samples were then fixed and E-cadherin was identified by indirect immunofluorescence microscopy and the transgenes by their fluorescent tags. *Right* and *left panels* represent simultaneous dual color fluorescent images.

contact zone extension (13, 21, 23). Consistent with our experience assaying cell-cell contacts (Fig. 3), we found that inhibiting tyrosine kinase signaling profoundly reduced the ability of hE-CHO cells to extend adhesive contacts on hE/Fc-coated substrata (Fig. 4). In these planar adhesion assays, contact zone extension is driven by the formation of broad cadherin-based lamellipodia. Preincubation with genistein profoundly inhibited the ability of cells to form lamellipodia, as reflected in a ~4-fold reduction in the adhesive surface area formed by the cells (Fig. 4). This was reversed when the drug was removed, indicating that it was not because of cell toxicity. Again, expression of $p110^{CAAX}$ alone did not affect the degree to which cells spread on hE/Fc but largely restored the ability of genistein-treated cells to extend adhesive contacts (Fig. 4*B*).

We then tested the impact of tyrosine kinase-dependent PI 3-kinase signaling on cadherin adhesion, as measured by the resistance of cells to detachment from hE/Fc-coated substrata (23) (Fig. 5). As described previously (11, 23), hE-CHO cells, but not cadherin-deficient parental CHO cells, effectively adhered to hE/Fc. Consistent with its effects on contact formation, genistein significantly reduced the adhesiveness of hE-CHO cells but had no effect on parental CHO cells. Expression of $p110^{CAAX}$ alone did not affect cadherin-based adhesion, but $p110^{CAAX}$ substantially restored the adhesiveness of cells treated with genistein. Note that, in contrast to our assays of cell contact formation, which identify individual transfected cells, our adhesion assays sampled populations of transiently transfected cells. It is therefore likely that these data underestimate the degree to which $p110^{CAAX}$ rescued adhesion, as our transfection efficiency was ~50–60%.

Src Kinase Is Required for the Recruitment of PI 3-Kinase to E-cadherin Adhesion—Finally, we sought to identify candidate kinases that might regulate the recruitment of PI 3-kinase to cadherin contacts. We focused our attention on Src, which has been identified at cell-cell contacts (25) and is known to act upstream in other signaling pathways where PI 3-kinase is activated (30, 31). As a first step, we tested whether Src might co-accumulate with PI 3-kinase at cadherin-adhesive contacts in our assay systems (Fig. 6). The localization of Src was identified in hE-CHO cells by transient expression of a novel GFP-tagged Src construct that faithfully reproduces the regulation and subcellular targeting of endogenous c-Src (32). PI 3-kinase was identified by either staining for endogenous p85 (not shown) or by co-expression of an mRFP-tagged p85 construct. We found that Src-GFP co-accumulated with mRFP-p85 where cells made

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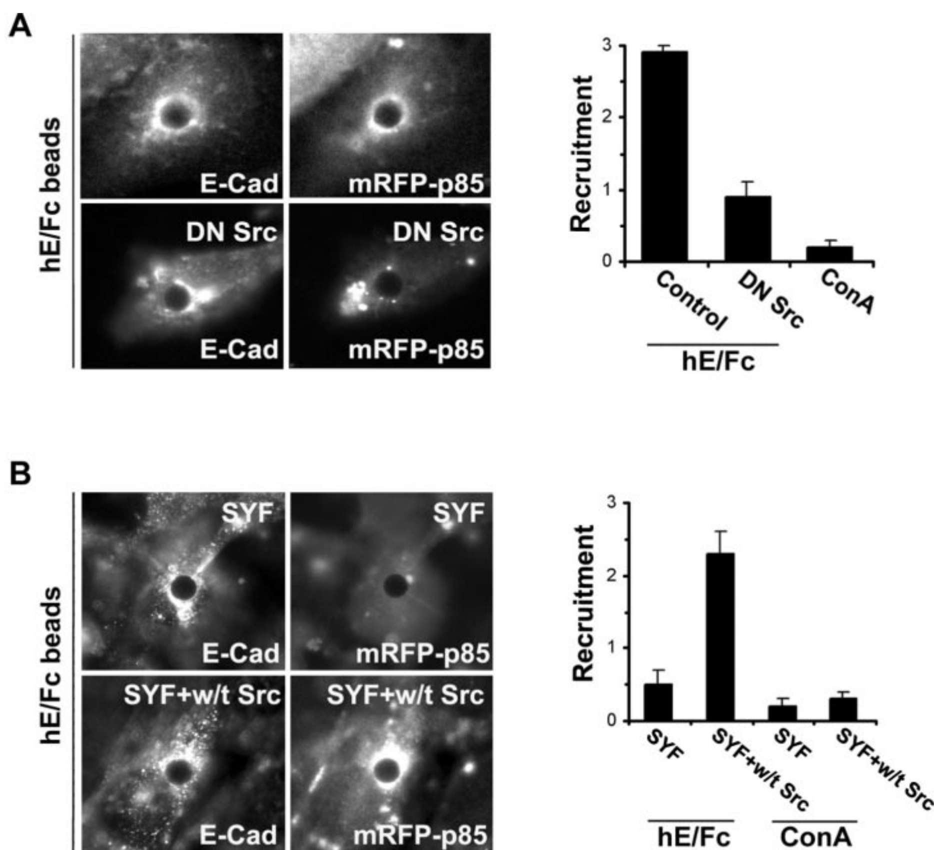

FIG. 7. **c-Src is necessary and sufficient to support E-cadherin recruitment of PI 3-kinase.** *A*, hE-CHO cells were transiently co-transfected with mRFP-p85 and either pIRES2-Src-mf-EGFP or enhanced GFP alone. Recruitment of mRFP-p85 to hE/Fc-coated beads was assessed by immunofluorescence microscopy and quantitated by digital image analysis. Data are means ± S.E., $n = 20$ beads. *B*, SYF-null fibroblasts or SYF cells stably expressing c-Src (*SYF+w/t Src*) cells were transiently co-transfected with human E-cadherin and mRFP-p85. Recruitment of mRFP-p85 to hE/Fc-coated beads or to ConA-coated beads was assessed by immunofluorescence microscopy and quantitated by digital image analysis. Data are means ± S.E., $n = 20$ beads.

contacts with hE/Fc beads but not at contacts made by ConA beads (Fig. 6). Therefore, Src was appropriately located to participate in recruiting PI 3-kinase to E-cadherin adhesions.

To test whether the activity of Src family kinases was necessary for E-cadherin to recruit PI 3-kinase, we transiently expressed dominant negative Src-mf (K295M, Y527F) (20) in an internal ribosomal entry site (IRES)-containing vector that allowed us to identify transfected cells by co-expression of GFP. PI 3-kinase was identified using mRFP-tagged p85 in double transfection studies. As shown in Fig. 7*A*, Src-mf did not appreciably affect the ability of cellular E-cadherin to accumulate at adhesions with hE/Fc-coated beads. However, the co-accumulation of mRFP-p85 was substantially reduced in Src-mf-transfected cells compared with control cells expressing GFP alone, suggesting that Src family kinase activity was necessary for recruitment of PI 3-kinase.

To confirm this, we assessed the ability of E-cadherin to recruit PI 3-kinase in fibroblasts derived from mice deficient in the ubiquitous Src family kinase members, Src, Fyn, and Yes (SYF-null fibroblasts) (Fig. 7*B*). E-cadherin was transiently co-expressed with mRFP-p85 in either SYF-null fibroblasts or SYF cells stably complemented with wild-type c-Src alone (SYF-Src). Using hE/Fc- coated beads to engage cellular cadherins, we found that E-cadherin recruited to hE/Fc-coated beads in both SYF-null and SYF-Src cells. In contrast, mRFP-p85 failed to recruit to cadherin contacts in SYF-null cells, but recruitment was restored in SYF-Src cells. These observations confirm that SKF activity is necessary for E-cadherin to recruit PI 3-kinase and, in extension to this, demonstrate that c-Src is sufficient to support PI 3-kinase recruitment.

DISCUSSION

We are exploring the concept that classical cadherin adhesion molecules can influence cellular behavior by acting as adhesion-activated signaling receptors. In this model, adhesive ligation activates a range of cell signals that determine functional responses, such as adhesive strengthening and contact formation. As many of these cellular responses require active cooperation of the actin cytoskeleton, membrane-local signals (such as Rho family GTPases) provide an attractive mechanism to coordinate surface adhesion with actin activity.

One key signaling molecule is PI 3-kinase, which is activated by homophilic cadherin ligation to participate in adhesive strengthening and contact formation (11). Accordingly, in the present study, we sought to find potential upstream elements responsible for activation of PI 3-kinase by E-cadherin. Our data identify a crucial role for tyrosine kinase signaling in this process. First, we found that tyrosine kinase activity was necessary for E-cadherin to couple to a PI 3-kinase-signaling pathway. Second, tyrosine kinase inhibitors perturbed cadherin adhesion and adhesive contact formation in a PI 3-kinase-dependent fashion. Third, we identified c-Src as one key potential mediator of this upstream tyrosine kinase activity. Hence, we conclude that tyrosine kinase activity, notably Src signaling, exerts a positive effect on cadherin function by regulating the ability of E-cadherin to recruit and activate PI 3-kinase.

In many contexts, intermediary tyrosine phosphorylation events are necessary for PI 3-kinase signaling to be activated by cell-signaling receptors. In this regard, E-cadherin-activated PI 3-kinase signaling closely resembles the paradigms established for growth factor and cytokine-activated PI 3-kinase pathways (6, 7). Thus, broad spectrum tyrosine kinase inhibitors blocked the recruitment of PI 3-kinase to E-cadherin, as assessed both by accumulation of p85 at localized sites of cadherin homophilic ligation and by the ligation-dependent assembly of a molecular complex containing both E-cadherin and p85. Further, these changes in p85 recruitment were accompanied by reduction in the ability of cadherin homophilic ligation to activate Akt, an index of signaling downstream of PI 3-kinase.

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Similarly, in well characterized models of growth factor and cytokine signaling, phosphorylated tyrosines on receptors or adaptor proteins provide docking sites for the Src homology 2 domain of p85, thereby recruiting PI 3-kinase to the plasma membrane. Interestingly, both β-catenin (15–17) and hDlg (18), which have been implicated in cadherin recruitment of PI 3-kinase, are potential targets for tyrosine phosphorylation. Although translocation to the plasma membrane alone may activate PI 3-kinase signaling (7), binding of phosphotyrosine sequences to p85 can also alleviate inhibition of the p110 catalytic subunit by p85 (6). Either or both mechanism(s) might therefore underlie the contribution of tyrosine kinase activity to cadherin-activated PI 3-kinase signaling.

It has long been appreciated that cadherin-based adherens junctions are major sites for tyrosine kinase signaling, being enriched in tyrosine-phosphorylated proteins and a range of receptor and nonreceptor tyrosine kinases (25–27, 33, 34). However, the relationship between tyrosine kinase signaling and cadherin function is not yet well understood and is likely to be complex. Tyrosine phosphorylation has commonly been reported to have a negative impact on cadherin adhesion (reviewed in Refs. 35 and 36). However, our current data clearly demonstrate that tyrosine kinase signaling can also contribute positively to cadherin function. Thus inhibition of tyrosine kinase activity blocked several functional consequences of cadherin ligation, namely the efficient assembly of contacts between epithelial cells, contact zone extension on cadherin substrata, and cadherin-based cell adhesion. Therefore, some tyrosine kinase signaling pathway(s) are necessary to activate these cellular responses to cadherin-adhesive ligation.

Several earlier reports also suggested that tyrosine kinases might contribute positively to cadherin function (37). Notably, Calautti *et al.* (33) reported that tyrosine kinase signaling was necessary for differentiating keratinocytes to assemble cadherin-based cell-cell contacts. Genistein further blocked the stable association of α-catenin and p120-ctn with E-cadherin, leading to the suggestion that tyrosine kinases might support cadherin function by promoting the assembly of the cadherin molecular complex (33). Certainly, *in vitro* studies indicate that tyrosine phosphorylation affects the ability of both p120-ctn and β-catenin to bind the cadherin cytoplasmic tail (38). Our current data extend these observations to identify the recruitment and signaling of PI 3-kinase as another key mechanism for tyrosine kinases to support cadherin function. Previously, we showed that inhibition of PI 3-kinase signaling perturbs contact formation and cadherin adhesion (11), effects identical to those seen with tyrosine kinase inhibitors in the current study. Importantly, in our current experiments, we found that the inhibition of cadherin function by genistein or herbimycin was effectively rescued using a constitutively active form of the p110 catalytic subunit that restores PI 3-kinase signaling. This suggests that, in addition to possibly affecting the assembly of the cadherin-catenin complex, tyrosine kinase signaling also promotes cadherin function by allowing this adhesion receptor to signal to PI 3-kinase. We envisage that this allows the adhesion receptor to trigger signaling cascades that coordinate adhesion and the actin cytoskeleton, thereby driving processes such as adhesive strengthening and contact zone extension.

Many tyrosine kinases have been reported to concentrate at cadherin-adhesive contacts. Our data implicate c-Src as a major contributor to the cadherin/PI 3-kinase-signaling pathway. Thus we found that c-Src co-recruits to cadherin homophilic contacts made with hE/Fc-coated beads, consistent with earlier reports that Src family kinases accumulate in adherens junctions between epithelial cells (25, 33, 34). Importantly, two independent lines of evidence showed that Src signaling was necessary to recruit PI 3-kinase to cadherin contacts. First, expression of a well characterized inhibitory Src mutant blocked the ability of hE/Fc beads to recruit PI 3-kinase. Second, cadherin recruitment of PI 3-kinase was also abolished in SYF-null cells lacking the ubiquitous Src family members, and recruitment was restored by expression of c-Src alone. This positive contribution of Src to cadherin signaling is consistent with the earlier demonstrations that Fyn was necessary for the efficient assembly of cell-cell junctions in keratinocytes (33), and Src inhibitors reduced N-cadherin adhesiveness (37). Furthermore, in other experimental systems, Src is often required for PI 3-kinase to interact with growth factor and cytokine receptors (6, 7). Src may generate phosphotyrosine-based binding sites to recruit the p85 subunit, as well as directly regulate PI 3-kinase activity itself. Thus, although these data do not exclude potential contributions from other tyrosine kinases, they identify Src as critical for recruitment of PI 3-kinase in E-cadherin signaling.

How then can we reconcile this positive contribution of tyrosine kinase signaling, and Src specifically, to cadherin function with other reports that clearly demonstrate the potential negative influence of tyrosine kinase signaling (35, 36)? Some of the discrepancies may reflect differences in cell types and assay systems. More importantly, it is likely that very tight regulation of tyrosine kinase signaling is critical for cadherin biology. Many studies that document the negative impact of tyrosine kinase activity have utilized constitutively active kinases or cells that overexpress growth factor receptors, situations that likely reflect one extreme in the spectrum of Src or tyrosine kinase activity. Thus, although endogenous Fyn was necessary for the assembly of cell-cell contacts and maturation of the cadherin-catenin complex in keratinocytes, expression of constitutively active Src in the same cells perturbed adhesion and contact formation (33). Similarly, v-Src can reduce the number and size of integrin-based focal adhesions, whereas c-Src participates in focal adhesion assembly (39). Therefore, the dynamic regulation of tyrosine kinase activity may be critical to understanding its pleiotropic impact on cadherin function.

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Recruitment of Phosphoinositide 3-Kinase Defines a Positive Contribution of Tyrosine Kinase Signaling to E-cadherin Function

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