p120 catenin associates with kinesin and facilitates the transport of cadherin–catenin complexes to intercellular junctions

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p120 catenin (p120) is a component of adherens junctions and has been implicated in regulating cadherin-based cell adhesion as well as the activity of Rho small GTPases, but its exact roles in cell–cell adhesion are unclear. Using time-lapse imaging, we show that p120-GFP associates with vesicles and exhibits unidirectional movements along microtubules. Furthermore, p120 forms a complex with kinesin heavy chain through the p120 NH2-terminal head domain. Overexpression of p120, but not an NH2-terminal deletion mutant deficient in kinesin binding, recruits endogenous kinesin to N-cadherin. Disruption of the interaction between N-cadherin and p120, or the interaction between p120 and kinesin, leads to a delayed accumulation of N-cadherin at cell–cell contacts during calcium-initiated junction reassembly. Our analyses identify a novel role of p120 in promoting cell surface trafficking of cadherins via association and recruitment of kinesin.

Introduction

Cadherin-mediated cell–cell adhesion plays critical roles during embryonic development and tissue morphogenesis (Takeichi, 1995; Gumbiner, 1996), and is often down-regulated during tumor progression (Yap, 1998; Nollet et al., 1999). In adherens junctions, the transmembrane cadherins mediate intercellular adhesion through calcium-dependent homophilic interaction of their extracellular domains. The intracellular domains of cadherins associate with several cytoplasmic catenin proteins, including β-catenin (or its homologue plakoglobin), which interacts directly with the cadherin cytoplasmic tail, and α-catenin, which binds to β-catenin and links the complex to the actin cytoskeleton (Aberle et al., 1994; Hulsken et al., 1994; Jou et al., 1995). A fourth catenin, p120 catenin (p120), binds directly to the juxtamembrane domain (JMD) of the cadherin tail, but not to α-catenin (Daniel and Reynolds, 1995; Yap et al., 1998).

p120 is a member of the armadillo (Arm) supergene family (Reynolds et al., 1992; Peifer et al., 1994), and it was originally discovered as a substrate for Src (Reynolds et al., 1992) and various other tyrosine kinases (Downing and Reynolds, 1991; Kanner et al., 1991). p120 is composed of an NH2-terminal head domain, an ARM domain containing 10 Arm repeats and a short COOH-terminal tail (Anastasiadis and Reynolds, 2000). The Arm repeats are involved in the direct interaction of p120 to classical cadherins (Reynolds et al., 1996), whereas the functions of the NH2-terminal head domain and the COOH-terminal tail are not known.

The function of p120 in cell–cell adhesion has remained controversial, as previous reports suggest that p120 can both positively and negatively regulate cadherin-mediated adhesion, likely depending on cellular context and the activity of specific signaling pathways. Several reports suggested that p120 might be required for clustering of cadherins and strong cell–cell adhesion (Yap et al., 1998; Thoreson et al., 2000). In contrast, another paper indicated a role of p120 in the inhibition of cell–cell adhesion in Colo205 cells (Aono et al., 1999). In human cancer cells deficient in p120 expression, p120 binding to cadherins stabilizes cadherins and restores their accumulation at cell borders (Ireton et al., 2002), which is consistent with genetic experiments in Drosophila and Caenorhabditis elegans supporting a positive regulatory role of p120 in cadherin function (Myster et al., 2003;...
Pettitt et al., 2003). Moreover, p120 has been shown to regulate the activity of Rho small GTPases (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001) and thus influence cadherin-mediated cell adhesion and cell migration, but the exact mechanisms by which p120 regulates cadherin function and cell–cell adhesion are still unclear.

The pivotal roles of cadherin-based junctions during development and tissue morphogenesis require the dynamic regulation of their assembly and function (Gumbiner, 2000). Several mechanisms may contribute to the regulation of cadherin-based adhesion (Gumbiner, 2000), including the cell surface delivery of junctional complexes and their turnover (Hinck et al., 1994; Le et al., 1999; Palacios et al., 2002). In neural crest cells, formation of stable intercellular adhesion results from the recruitment of N-cadherin from an intracellular pool rather than from a redistribution of surface-bound N-cadherins (Monier-Gavelle and Duband, 1995, 1997). Using GFP-tagged N-cadherin and time-lapse imaging, Mary et al. (2002) showed that N-cadherin transport to cell surface in fibroblasts is dependent on the formation of cell–cell contact and requires the microtubule (MT) network and the MT-associated motor protein kinesin. However, it is not known how cadherin trafficking to the cell surface is regulated or which cadherin-associated proteins might be involved in this process.

Here, we provide evidence that p120 promotes the trafficking of cadherins to the cell surface via association and recruitment of kinesin. Our data reveal a novel role of p120 in the trafficking of cadherins, and suggest a mechanism by which the delivery of cadherins to the cell surface is specifically regulated by a catenin protein.
Results

**p120 associates with vesicles and travels along MTs**

To analyze the potential involvement of p120 in the trafficking of cadherins, we investigated the dynamics of p120 in living cells using time-lapse imaging of GFP-tagged proteins. REF52 cells were used because they form adherens junctions that accumulate endogenous β-catenin (Mary et al., 2002) and p120 (unpublished data). In addition, detailed analysis of the intracellular transport of N-cadherin in REF52 cells has been documented (Mary et al., 2002). Transfected REF52 cells expressing low levels of p120-GFP were selected for time-lapse imaging to minimize potential complications from overexpression of proteins. p120-GFP often appeared as moving dots in the cytoplasm and frequently traveled unidirectionally at 0.5–1.0 μm/s (Fig. 1 A’, arrow and arrowhead; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1), a velocity comparable to that exhibited by vesicles undergoing MT-dependent transport. p120-GFP particles frequently associated with spherical structures of various sizes, presumably membrane-bound organelles such as vesicles or vacuoles. p120-GFP itself exhibited dynamic structural changes characteristic of membrane vesicles, being stretched into long tubular forms or shortened (Fig. 1 B’, arrow and arrowhead; Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). Sometimes these p120-GFP structures detached from the spherical organelles and translocated across the cytoplasm unidirectionally. Some p120-GFP structures moved and merged into existing cell–cell junctions containing p120-GFP (Fig. 1 A’, arrow), indicating that p120, and possibly its associated cadherins, can be delivered to cell–cell contacts along MTs.

To begin to address whether the observed p120 dynamics are related to the transport of cadherins, REF52 cells were cotransfected with p120-CFP and N-cad–YFP and analyzed by time-lapse imaging. Most cytoplasmic particles of p120-CFP and N-cad–YFP colocalized perfectly, as expected from their ability to interact with each other (Fig. 1 C’, white dots; see also Fig. 4). Time-lapse imaging showed that some of these complexes of cadherin–p120 remained static (Fig. 1 C’, white dots; Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1), but other complexes exhibited fast and unidirectional translocation as described in the previous paragraph for p120-GFP (Fig. 1 C’, arrow). Because of the 3-s delay between images from the CFP and YFP channels, moving complexes appeared as two adjacent blue (p120-CFP) and red (N-cad–YFP) particles. N-cadherin transport has been shown to be a microtubule- and kinesin-dependent process (Mary et al., 2002). Our data demonstrate that the majority of p120 is associated with N-cadherin, and they are transported together in the same complexes.

To further examine whether the dynamics of p120-GFP particles were dependent on MTs, REF52 cells expressing p120-GFP were examined after treatment with nocodazole to disrupt the MT networks. Both the unidirectional trans-
location and structural changes were abolished by this treatment (Fig. 2 A'; Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). Some local random movements of p120-GFP were observed in the presence of nocodazole, which lacked the directionality of MT-dependent transport of vesicles, suggesting that they were caused by Brownian motion or MT-independent mechanisms.

To confirm that the characteristic movements of p120-GFP occurred along MTs, REF52 cells were cotransfected with p120-CFP and YFP–GFP occurred along MTs, REF52 cells were cotransfected by Brownian motion or MT-independent mechanisms.

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multaneously and recruit kinesin to cadherins. To test this idea, we examined the colocalization of N-cad–GFP/YFP with endogenous kinesin in REF52 cells (Fig. 4, D and D’). N-cad–GFP localized in the cytoplasm and the perinuclear Golgi region, as well as at cell–cell contacts as described before (unpublished data; Mary et al., 2002). Many discrete dots of endogenous kinesin were distributed in the cytoplasm, but very few kinesin dots colocalized with N-cad–GFP (Fig. 4 D’, small yellow box). In contrast, the introduction of excess p120-CFP together with N-cad–YFP not only led to extensive colocalization of p120-CFP with N-cad–YFP, but many of these cadherin–catenin complexes also contained endogenous kinesin (Fig. 4 D’, small white box). The codistribution of kinesin with cadherin–catenin complexes was rarely observed when N-cad–YFP was coexpressed with p120ΔN-CFP, indicating that p120 binding to kinesin is responsible for the accumulation of kinesin to cadherins. These findings suggest that p120 may function as a linker between the cadherin tail and kinesin, and recruit kinesin to cadherin–catenin complexes.

Disruption of p120 binding to N-cadherin delays accumulation of N-cadherin at cell–cell contacts during junction reassembly

An E-cadherin mutant deficient in p120 binding can still accumulate at cell borders (Thoreson et al., 2000; Ireton et al., 2002), indicating that p120 binding to cadherin tail is not absolutely required for the cell surface delivery of cadherins. However, our observations that p120 associates with kinesin and recruits it to cadherins suggest a positive role of p120 in facilitating the transport of cadherins to the cell surface. To investigate the involvement of p120 in cadherin trafficking,
Figure 5. Disruption of p120 binding to N-cadherin causes delayed accumulation of N-cadherin at cell–cell contacts in the presence of ectopic p120. (A) Triple Ala mutation in the JMD domain of N-cadherin completely abolishes its ability to interact with p120. HEK293 cells transiently expressing N-cad–YFP (N-YFP) or N-cad AAA–YFP (N AAA–YFP) together with HA-tagged p120 were subjected to coimmunoprecipitation using a monoclonal anti-HA antibody or mouse IgG (mIgG) as control. (B) N-cad AAA–YFP is delayed in its accumulation at cell–cell contacts compared with N-cad–YFP during calcium switch. REF52 cells were transiently cotransfected with p120–CFP (insets) together with N-cad–YFP or N-cad AAA–YFP, 20 h after transfection, cells were incubated with growth medium containing 4 mM EGTA for 30 min, followed by incubation in complete growth medium (recovery). Only adjacent cells that were in close contact and expressed both N-cad and p120 were examined. (C) Quantification of N-cad–YFP or N-cad AAA–YFP accumulation at cell–cell contacts after 15 min of calcium recovery. Pairs of contacting cells expressing p120–CFP with either N-cad–YFP or N-cad AAA–YFP were randomly selected, and the level of cell border accumulation of N-cad–YFP or N-cad AAA–YFP is expressed as ratios of the average YFP fluorescent intensity at cell–cell contacts over the average total YFP fluorescent intensity within the two contacting cells. The number in each bar represents the mean value. The average accumulation of N-cad AAA–YFP (n = 23) at cell–cell contacts is 66% less than that of N-cad–YFP (n = 27) 15 min after calcium recovery. Asterisk denotes significant difference from cells coexpressing N-cad–YFP and p120–CFP (P = 3.8 × 10^{-12} < 0.05) by Student’s t test. IP, immunoprecipitation; CL, cell lysate. Bar, 20 µm.

We generated N-cad–YFP and N-cad AAA–YFP with a triple Ala mutation in its JMD domain. This triple Ala mutation in the cadherin JMD domain has been shown to specifically disrupt its interaction with p120 (Thoreson et al., 2000), and our coimmunoprecipitation experiments confirmed the loss of interaction between N-cad AAA–YFP and p120 (Fig. 5 A). Coexpression of N-cad–YFP and p120–CFP in REF52 cells resulted in their extensive colocalization as cytoplasmic dots and at cell borders, whereas p120–CFP was mostly diffuse in the cytoplasm when coexpressed with N-cad AAA–YFP (unpublished data).

To examine a potential role of p120 in the efficient delivery of cadherins to the cell surface, we used a calcium switch procedure in which junction assembly is rapidly initiated by the restoration of normal calcium concentration in the medium. REF52 cells transiently expressing N-cad–YFP or N-cad AAA–YFP together with p120–CFP were treated with EGTA containing medium for 30 min to disrupt cell–cell junctions, and then were incubated with normal medium for different periods of time (calcium recovery; Fig. 5 B). 20 h after transfection, both N-cad–YFP and N-cad AAA–YFP distributed at cell borders as well as in the cytoplasm, and 30 min of EGTA treatment led to their disappearance from cell–cell contacts. 15 min after calcium recovery, there was already strong accumulation of N-cad–YFP at cell–cell contacts where it colocalized with p120–CFP. In contrast, N-cad AAA–YFP appeared to have a much slower rate of accumulation at cell–cell contact sites, such that many of the close-contacting cells lacked apparent border localization of N-cad AAA–YFP 15 min after calcium recovery. Most of the N-cad AAA–YFP was distributed in the cytoplasm and around the perinuclear Golgi region. To normalize the level of border accumulation of N-cad–YFP or N-cad AAA–YFP against their total expression level, we measured the average YFP fluorescent intensity at cell–cell contacts and the average total YFP fluorescent intensity within the two contacting cells normalized by the area of the selected regions, and expressed the accumulation of N-cad–YFP or N-cad AAA–YFP at cell–cell contacts as a ratio between the border and total YFP intensity (Fig. 5 C). The level of accumulation of N-cad–YFP at cell borders was on average 2.9-fold greater than that of N-cad AAA–YFP after 15 min of calcium recovery. The accumulation of N-cad AAA–YFP at borders was less efficient compared with N-cad–YFP even after 60 min of calcium recovery (unpublished data). These data suggest that binding of p120 to cadherins facilitates the rapid redistribution of cadherin–catenin complexes to the cell surface.

To further evaluate the physiological role of p120 in promoting the delivery of cadherins to the cell surface, we performed similar calcium switch experiments with REF52 cells transiently expressing only ectopic N-cad–YFP or N-cad AAA–YFP, but not ectopic p120. Sequential detergent extraction and immunoblot showed that REF52 cells express endogenous kinesin and p120, and they are mainly distributed in the Triton-soluble membrane pool and the saponin-soluble
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of N-cad–YFP to cell borders (Fig. 7A). Before EGTA treatment the effect of p120

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The accumulation of N-cadherin at cell–cell contacts during junction reassembly is delayed by a p120 deletion mutant that does not bind to kinesin

Next, we performed the calcium switch experiment to examine the effect of p120ΔN or p120ΔN2 on the redistribution of N-cad–YFP to cell borders (Fig. 7A). Before EGTA treatment, N-cad–YFP accumulated at cell borders in the presence of either full-length p120 or its deletion mutants. 30 min of EGTA treatment resulted in the disappearance of N-cad–YFP from cell borders (unpublished data). After just 15 min of calcium recovery, prominent cell border accumulation of N-cad–YFP coexpressed with p120 or p120ΔN2 was already detected between contacting cells. In contrast, many of the contacting cells expressing N-cad–YFP and p120ΔN exhibited only weak accumulation of N-cad–YFP at cell–cell borders, which usually appeared discontinuous and thinner in width. Quantification of the N-cad–YFP border to total intensity ratio demonstrated that there was an average 1.7-fold higher accumulation of N-cad–YFP at cell–cell contacts when coexpressed with p120-CFP compared with p120ΔN-CFP 15 min after calcium recovery (Fig. 7B). After 60 min of calcium recovery, the border accumulation level of N-cad–YFP was still lower in cells cotransfected with p120ΔN-CFP than with p120-CFP or p120ΔN2-CFP. Importantly, the accumulation of N-cad–YFP was not delayed when coexpressed with p120ΔN2-CFP, which can still associate with kinesin despite deletion of most of the NH2-terminal head domain. These results further support that p120 binding to kinesin facilitates the delivery of cadherin–catenin complexes to the cell surface.

Discussion

The role of p120 in regulating cell–cell adhesion has been controversial and has remained elusive. Here, we demonstrate an association between p120 and the KHC, and identify a novel mechanism that regulates the delivery of
Disruption of p120 binding to kinesin causes delayed accumulation of N-cadherin at cell–cell contacts during junction reassembly. (A) N-cad–YFP is delayed in its accumulation at cell–cell contacts when coexpressed with p120ΔN-CFP compared with N-cad–YFP coexpressed with p120-CFP or p120ΔN2-CFP during calcium switch. REF52 cells were transiently co-transfected with p120-CFP, p120ΔN2-CFP, or p120ΔN-CFP (insets) together with N-cad–YFP. 20 h after transfection, cells were incubated with growth medium containing 4 mM EGTA for 30 min, followed by incubation in complete growth medium (recovery). Only adjacent cells that were in close contact and expressed both N-cad and p120 were examined. (B) Quantification of N-cad–YFP accumulation at cell–cell contacts after 15 and 60 min of calcium recovery. Pairs of contacting cells expressing N-cad–YFP with either p120-CFP, p120ΔN2-CFP, or p120ΔN-CFP were randomly selected, and the level of cell border accumulation of N-cad–YFP was measured. The number in each bar represents the mean value. The accumulation of N-cad–YFP at cell–cell contacts when coexpressed with p120ΔN-CFP (n = 33 at 15 min; n = 27 at 60 min) is 42% less than that of N-cad–YFP coexpressed with p120-CFP (n = 32 at 15 min; n = 29 at 60 min) 15 min after calcium recovery, and is 24% less after 60 min of calcium recovery. There is no significant difference between the border accumulation of N-cad–YFP when coexpressed with p120-CFP or p120ΔN2-CFP (P = 0.32 > 0.05 at 15 min; P = 0.22 > 0.05 at 60 min). Asterisk denotes significant difference from cells coexpressing N-cad–YFP and p120-CFP (P = 1.1 × 10⁻⁷ < 0.05 at 15 min; P = 2.1 × 10⁻³ < 0.05 at 60 min) by t test. Bar, 20 μm.

Experiments in MDCK cells reported that shortly after its synthesis, E-cadherin forms a complex with β-catenin and is then transported to the cell surface, where α-catenin associates with the complex (Hinck et al., 1994). A more recent report demonstrated that β-catenin, α-catenin, and p120 were all found in a complex with proN-cadherin in HeLa cells, but only p120 was associated with the earliest form of N-cadherin (Wahl et al., 2003), suggesting that proN-cadherin associates immediately with p120 after synthesis. This is consistent with our observation that some p120 colocalized with N-cadherin in the perinuclear Golgi region (Fig. 5). Together with our result showing the coincidence of p120 and N-cadherin dynamics (Fig. 1), these observations raise the possibility that p120 is involved throughout the process of transporting newly synthesized cadherins from the Golgi to the cell surface. It remains to be determined if p120 also regulates the exit of cadherins from the Golgi, or if it is only involved in the transport of cadherins along MTs. In the calcium switch model, EGTA treatment induces the disassembly of cell–cell junctions and the endocytosis of cadherins, some of which are recycled back to the cell surface upon calcium restoration (Kartenbeck et al., 1982, 1991; Le et al., 1999; Mary et al., 2002). Therefore, it is possible that both the delivery of newly synthesized cadherins and the recycling of endocytosed cadherins contribute to the reaccumulation of cadherins at the cell surface after calcium recovery. p120 might facilitate both of these processes by recruiting kinesin to different subsets of cadherin-containing vesicles, and future experiments will be needed to address these questions.
ple isoforms of p120 and several p120-related proteins, Drosophila and C. elegans each have only a single p120 homologue. Loss of p120 function by genetic mutation or RNA interference revealed that p120 is not an essential component of adherens junctions in either organism, but loss of p120 greatly enhances the phenotypes caused by mutations in cadherins, β-catenin, and α-catenin. These results suggest that p120 plays a positive role in modulating cadherin functions, and its absence or reduced level leads to increased sensitivity toward disruption of cadherin–catenin functions. The nonessential role of p120 in cadherin function in flies and worms is further supported by an experiment in which a Drosophila E-cadherin AAA mutant defective in p120 binding completely substituted for the activity of endogenous E-cadherin in a variety of cadherin-dependent processes (Pacquet et al., 2003). However, these reports do not necessarily indicate that the role of mammalian p120 is also nonessential in cadherin functions. It is likely that the increased complexity of tissue organization and morphogenetic events has enabled p120 to evolve into a more important regulator of cell–cell adhesion than its counterparts in flies and worms. This might be achieved through additional protein interactions and regulatory domains, as suggested by sequence comparisons of Drosophila and C. elegans p120 with mammalian p120 (Myster et al., 2003; Pettitt et al., 2003), which showed that they share little sequence homology outside of the Arm-repeat domain. Importantly, several putative protein–protein interaction motifs and the phosphorylation domain in the NH2 terminus of mammalian p120 were not found in Drosophila and C. elegans p120. p120 associates with kinesin through its NH2 terminus, which shows great sequence diversity among different species; therefore, it will be very interesting to test if this is one of the conserved functions of p120 throughout evolution.

One of the most poorly understood aspects of MT-based trafficking is the identity of the cargo protein for each motor and the nature of the motor–cargo interaction. Conventional kinesin is a heterotetramer composed of two KHCs and two KLCs. KHC contains three domains; an NH2-terminal motor domain, a central coiled-coil stalk region involved in dimerization, and a COOH-terminal globular tail domain (Vale and Fletterick, 1997; Diefenbach et al., 1998; Verhey et al., 1998). The tail region of the kinesin molecule, including the KHC COOH terminus and KLC, is most likely to be involved in cargo binding. Several transmembrane and cytoplasmic–COOH-binding partners of KHC and KLC have been reported (Karcher et al., 2002). Here, we identify p120 as a potential novel binding partner for KHC and a linker between kinesin and the transmembrane cadherin molecules. Whether this association between p120 and KHC is a direct interaction awaits further investigation, but the p120 NH2 terminus deletion mutant (p120ΔN) loses its ability to associate with KHC while still being able to bind cadherins, suggesting that the association between p120 and KHC is not mediated by cadherins. Another recent report demonstrated a direct interaction between dynein and β-catenin (Ligon et al., 2001), but we did not detect p120 associating with dynein by either coimmunoprecipitation or immunofluorescence (unpublished data). The ability of p120ΔN2 to associate with KHC implies that the binding site for KHC might lie within the first 27 aa, or the last 114 aa in the NH2 terminus immediately adjacent to the Arm-repeat domain of p120.

The relatively small amount of p120 communoprecipitated with KHC from cotransfected cells suggests that this association must be a tightly regulated event that responds to proper positional and temporal signals. Several mechanisms might be involved in regulating the association between p120 and KHC, including association of KLC or other proteins with KHC, binding of p120 to cadherins, or post-translational modification of KHC or p120. p120 is a prominent Src substrate (Reynolds et al., 1992), and is tyrosine phosphorylated in response to activation of many receptor tyrosine kinases (Daniel and Reynolds, 1997). Interestingly, the NH2-terminal region immediately adjacent to the Arm-repeat domain of p120 encompasses a 100-aa phosphorylation domain that contains the majority of the tyrosine phosphorylation sites on p120 (Mariner et al., 2001), raising the possibility that tyrosine phosphorylation of p120 in the NH2 terminus regulates its association with kinesin. The importance of p120 function is emphasized by a number of recent reports showing the loss of p120 expression in many types of tumors, and a correlation with poor prognosis in many cases (Thoreson and Reynolds, 2002). The identification of a new role of p120 in cadherin trafficking provides mechanistic insight into the functions of p120 in regulating junctional complex assembly, and is an important step toward understanding the dynamic nature of cell–cell adhesion during cell migration and metastasis.

Materials and methods

Construcnts

The Rc-CMV-murine p120CTN 1A was a gift from Dr. Al Reynolds (Vanderbilt University, Nashville, TN; Reynolds et al., 1996). The p120-GFP construct was a gift from Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC; Noren et al., 2000). p120ΔN-GFP and p120Δ2N-GFP were provided by Dr. A.D. Bershadsky (Weizmann Institute of Science, Rehovot, Israel; Groshova et al., 2001). p120ΔN and p120Δ2N have a deletion of aa 1–346 and aa 28–233, respectively. Murine N-cad–GFP was provided by Dr. Cécile Gauthier-Rouvière (Centre National de la Recherche Scientifique, Montpellier, France; Mary et al., 2002). To construct CFP-tagged p120, the coding sequences were excised from p120-GFP with EcoRI and KpnI and ligated into the pECFP-N1 vector (CLONTECH Laboratories, Inc.). N-cad–YFP was mutagenized using the QuikChange® site-directed mutagenesis kit (Stratagene) to create N-cad AAA–YFP in which Glu–Glu–Asp (aa 780–782) residues of N-cadherin cDNA fragment from N-cad–GFP into the Agel and Xhol sites of pET-3d (CLONTECH Laboratories, Inc.). N-cad–YFP was mutagenized using the QuikChange® site-directed mutagenesis kit (Stratagene) to create N-cad AAA–YFP in which Glu–Glu–Asp (aa 780–782) residues of N-cadherin were substituted with Ala–Ala–Ala. YFP–α-tubulin was generated by transfecting the human α1-tubulin cDNA from pEGFP-Tub (CLONTECH Laboratories, Inc.) into the Xhol and BamHI sites of pET-3d (CLONTECH Laboratories, Inc.). Plasmids encoding Myc-tagged rat KHC and HA-tagged rat KLC were provided by Dr. Kristen J. Verhey (University of Michigan Medical School, Ann Arbor, MI; Verhey et al., 1998).

Cell culture and transfections

HEK293 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech). REF52 cells were grown in DMEM/Ham’s F12 (50/50 mix) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech). For transient transfection of HEK293 cells, calcium phosphate transfection was performed as described previously (Stappenbeck and Green, 1992). REF52 cells were transfected using FuGENE™ 6 reagent (Roche) according to the manufacturer’s protocol and assayed 20–24 h later.

Immunofluorescence

Immunofluorescence procedures have been previously described in detail (Chen et al., 2002). In brief, 20–24 h after transfection, cells were washed in
Images were obtained on a microscope (DMR; Leitz) using a digital camera (provided by Dr. Al Reynolds, Vanderbilt University, Nashville, TN) used at Transduction Laboratories; anti-p120 rabbit polyclonal antibody F1-SH bodies are as follows: p120 mouse monoclonal antibody used at 1:500 20–24 h after transfection, REF52 cells growing on glass coverslips were Calcium switch experiment and quantification were grown in 6-well dishes and the amount of buffer used at each step formed as described previously (Palka and Green, 1997), except that cells and rabbit Living Colors™ A.v. anti-GFP peptide antibody used at 1:100; mouse monoclonal anti-HA antibody 3F10 (Roche) used at 1:1,000; mouse anti-KHC antibody (H2; p120 mouse monoclonal antibody used at 1:1,000; anti-p120 rabbit poly-

A new lab developed with 10 μl of GaMP32 positive beads (Amersham Biosciences) for labelled N-cadherin (1:500) and p120-CFP were randomly selected. The ratio was calculated. Statistical analysis was performed using the measurement tool of the Openlab imaging software. The average total N-cad–GFP/YFP intensity was measured by selecting the area covering both of the two contacting cells. Background intensity was measured by selecting an empty area and subtracting the background intensity. The mean fluorescence intensity was calculated and the average pixel intensity was measured for the selected region using the measurement tool of the Openlab imaging software. The average total N-cad–GFP/YFP intensity was measured by selecting the area covering both of the two contacting cells. Background intensity was measured by selecting an empty area and subtracting the background intensity. The mean fluorescence intensity was calculated.
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