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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 NH_2-terminal head domain. Overexpression of p120, but not an NH_2-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 NH_2-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 NH_2-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;

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Abbreviations used in this paper: Arm, armadillo; JMD, juxtamembrane domain; KHC, kinesin heavy chain; KLC, kinesin light chain; MT, microtubule; p120, p120 catenin.

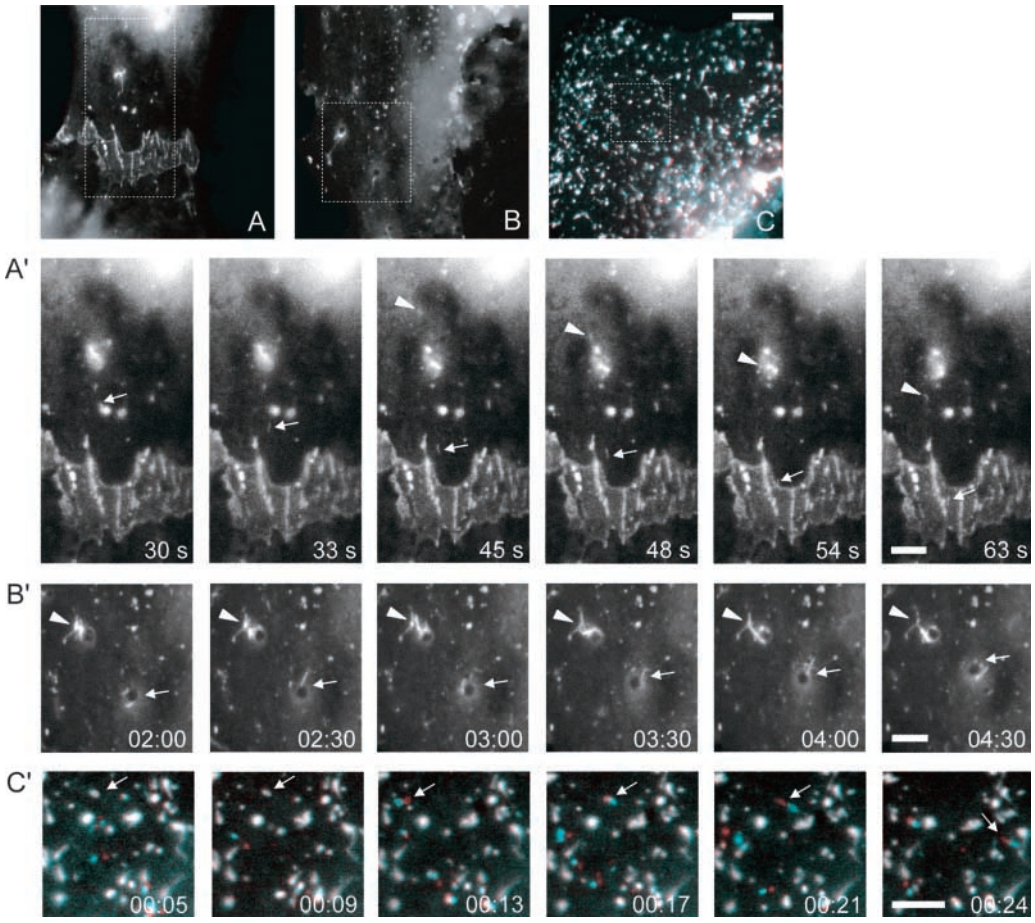

Figure 1. **Dynamics of p120-GFP in REF52 cells.** (A–C) The first frame of each movie in A′–C′ is shown. The boxed regions were enlarged and selected still frames of each movie are shown in A′–C′. (A′) p120-GFP particles move into existing cell–cell junctions (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). A region of two contacting cells is shown. The arrow indicates a p120-GFP particle that moved toward and eventually incorporated into an existing junction. The arrowhead shows another p120-GFP dot traveling in an anterograde direction toward the cell–cell contact area. (B′) p120-GFP shows dynamic structural changes characteristic of vesicles (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). Many p120-GFP particles appeared to associate with spherical structures of various sizes, and exhibited dynamic changes in their structure such as stretching and shortening. The arrow and arrowhead indicate two examples. (C′) p120-CFP and N-cad–YFP colocalize and move together (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). White dots represent colocalization of p120-CFP (blue) and N-cad–YFP (red). Due to the 3-s gap between the images taken from the CFP and YFP channel, moving complexes of p120-CFP and N-cad–YFP appeared as two adjacent blue and red dots, whereas static complexes remained white. The arrow indicates a complex of p120-CFP and N-cad–YFP particles moving together. In all the above experiments, REF52 cells were subjected to time-lapse imaging 48–72 h after transfection. Images were taken at 3-s intervals for 5–10 min. The time stamp in the bottom right-hand corner of each still image is displayed in min:sec, except in A′, which is displayed in seconds. Bars: 10 μm (A–C), 5 μm (A′–C′).

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.

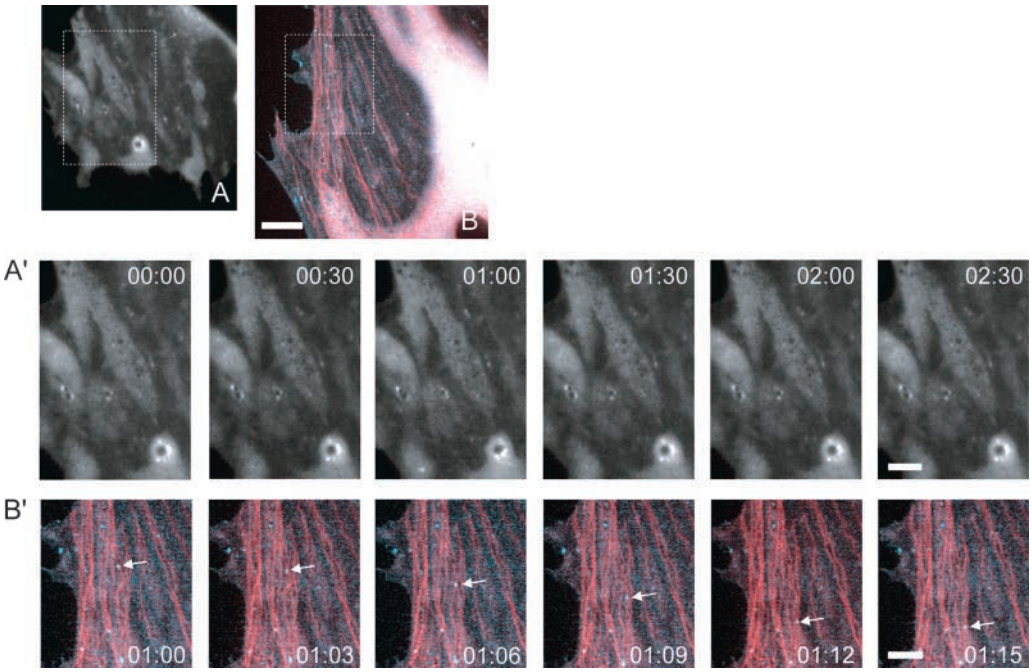

Figure 2. **Dependence of p120 dynamics on MTs.** (A and B) The first frame of each movie in A′ and B′ is shown. The boxed regions were enlarged and selected still frames of each movie are shown in A′ and B′. (A′) Unidirectional movement and dynamic structural changes of p120-GFP are MT-dependent (Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). REF52 cells transiently expressing p120-GFP were treated with nocodazole at 1 μg/ml for 30 min to disrupt MTs, followed by time-lapse imaging to observe p120-GFP dynamics. p120-GFP particles remained largely static with occasional random local jiggling. No unidirectional movements or structural changes of p120-GFP were observed. (B′) p120-CFP dots travel along MTs (Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). p120-CFP (blue) and YFP-α-tubulin (red) were cotransfected into REF52 cells. The arrow indicates one p120-CFP dot moving along MTs. In the above experiments, REF52 cells were subjected to time-lapse imaging 48–72 h after transfection. Images were taken at 3-s interval for 5–10 min. The time stamp in each still image is displayed in min:sec. Bars: 10 μm (A and B), 5 μm (A′, B′).

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-

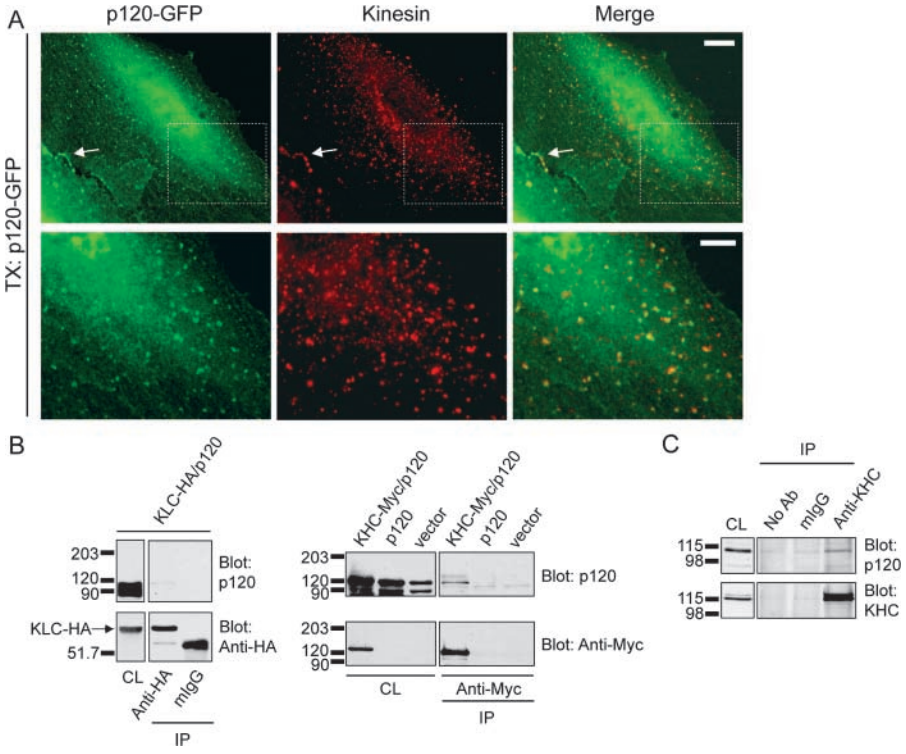

Figure 3. **p120 associates with kinesin.** (A) p120-GFP colocalizes with endogenous kinesin in the cytoplasm and at cell contacts. REF52 cells were transiently transfected with p120-GFP (green), followed by immunofluorescence using a monoclonal KHC antibody H1 (red). In some cells, cytoplasmic p120-GFP dots colocalized extensively with endogenous kinesin, which was also recruited to cell–cell contacts together with p120-GFP (arrow). Bottom panels are enlarged views of the boxed areas. (B) p120 associates with conventional KHC. Lysates of HEK293 cells transiently expressing p120 or in combination with HA-tagged KLC and Myc-tagged KHC as indicated were immunoprecipitated with monoclonal anti-HA antibody 3F10 or monoclonal anti-Myc antibody 9E10. The mouse IgG (mIgG) was used as a negative control. The band detected by the anti-HA antibody in the control immunoprecipitation is the mouse IgG heavy chain. (C) Detection of endogenous complexes of p120 and kinesin. An anti-KHC monoclonal antibody (anti-KHC) H2 was used to immunoprecipitate endogenous KHC and its associated proteins from a mouse brain high speed supernatant fraction. Beads alone (No Ab) or equal amount of nonspecific mouse IgG (mIgG) was used as a negative control. The immunocomplexes were analyzed by immunoblot using the rabbit anti-p120 antibody F1-SH and the anti-KHC peptide antibody αKHC13. TX, transfection; IP, immunoprecipitation; CL, cell lysate. Bars: 10 μm (A, top panels), 5 μm (A, bottom panels).

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–α-tubulin to visualize the MT network together with p120 dynamics. A subset of p120-CFP dots colocalized on MTs, but remained stationary during the movie, whereas others were seen traveling along MTs (Fig. 2 B′, arrow; Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1). Both plus- and minus-end movements of p120-CFP dots were observed (unpublished data). These data demonstrate that a fraction of cellular p120 is associated with intracellular vesicles that are translocated along MTs.

p120 associates with conventional kinesin heavy chain

To investigate the role of p120 in the delivery of cadherins to the cell surface, we examined the possibility that p120 might associate with endogenous kinesin in REF52 cells. A subset of p120-GFP dots colocalized with kinesin as discrete dots in the cytoplasm, and kinesin was also recruited to cell–cell contacts, where it colocalized with p120-GFP (Fig. 3 A). To test if p120 can physically associate with kinesin, we performed coimmunoprecipitation experiments from HEK293 cells cotransfected with p120 and conventional kinesin heavy chain (KHC) or kinesin light chain (KLC; Fig. 3 B). Virtually no p120 coimmunoprecipitated with KLC, whereas a small amount of p120 coimmunoprecipitated with KHC, indicating that p120 associates with kinesin through KHC.

To test if this association between p120 and KHC occurs in vivo, a high speed supernatant fraction from mouse brain was used for coimmunoprecipitation experiments with an anti-KHC antibody H2. Beads alone or equal amount of nonspecific mouse IgG (mIgG) was used as negative control. As shown in Fig. 3 C, immunoprecipitation (IP) of KHC resulted in the coprecipitation of endogenous p120 from mouse brain, demonstrating the existence of complexes between p120 and kinesin in vivo.

To further analyze the association of p120 with kinesin, a series of GFP-tagged p120 constructs were used in coimmunoprecipitation experiments to test their ability to associate with KHC. p120ΔN, but not p120ΔN2, lost the ability to associate with KHC (Fig. 4, A and B). The Arm repeats of p120 are required for cadherin binding, and p120ΔN2 has been shown previously to interact with cadherins (Reynolds et al., 1996). Coimmunoprecipitation experiments from cotransfected HEK293 cells confirmed that p120ΔN interacted with E-cadherin just as efficiently as full-length p120, but it no longer coimmunoprecipitated with KHC (Fig. 4 C). These data suggest that the association of p120 with kinesin is mediated by the p120 NH_2-terminal head domain, but not the Arm-repeat domain, which is involved in p120 binding to cadherins.

The ability of p120 to associate with kinesin raises the possibility that p120 might bind to cadherin and kinesin si-

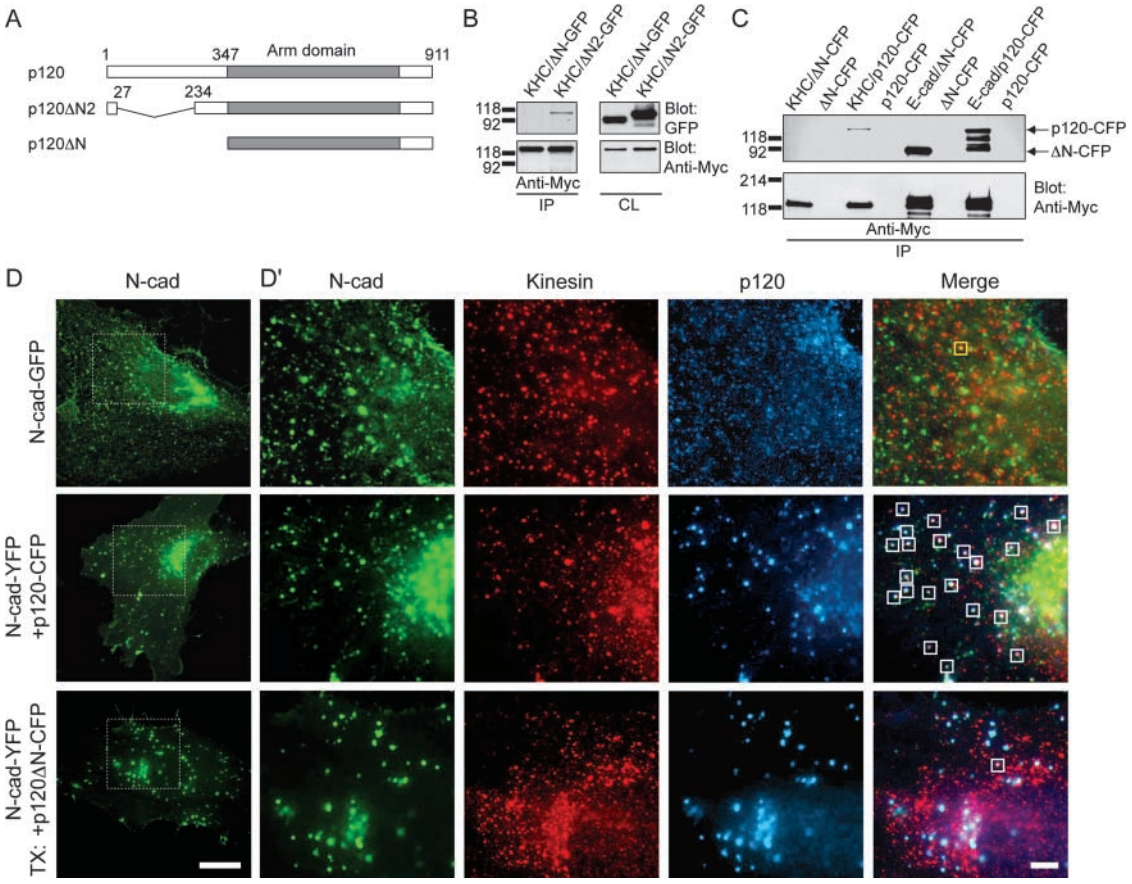

Figure 4. **p120 recruits endogenous kinesin to the cadherin–catenin complexes.** (A) Diagram of p120 and two of its NH_2-terminal deletion mutants. (B) p120ΔN2 (but not p120ΔN) associates with kinesin. p120ΔN2-GFP or p120ΔN-GFP was transiently transfected together with Myc-tagged KHC into HEK293 cells, followed by coimmunoprecipitation using the monoclonal anti-Myc antibody 9E10. (C) p120ΔN-CFP was coimmunoprecipitated with E-cad, but not with KHC. p120ΔN-CFP (or p120-CFP) was transiently transfected together with Myc-tagged KHC or Myc-tagged E-cad into HEK293 cells, followed by coimmunoprecipitation using the monoclonal anti-Myc antibody 9E10. The multiple bands of p120-CFP coprecipitated with E-cad detected in the top anti-GFP blot are likely breakdown products. (D and D′) p120, but not p120ΔN, recruits kinesin to N-cadherin. REF52 cells transiently expressing N-cad–GFP alone (top panels) were stained with the anti-KHC antibody H1 (red) and the anti-p120 monoclonal antibody (blue). REF52 cells transiently expressing N-cad–YFP (pseudocolored green) together with p120-CFP or p120ΔN-CFP (pseudocolored blue) were stained with the anti-KHC antibody H1 (red). Boxed regions in D were enlarged and shown for N-cad, kinesin, p120, and merged view in D′. In the absence of ectopic p120, very few N-cad–GFP dots colocalized with endogenous kinesin (D′, small yellow box in merged view). Introduction of ectopic p120 (but not p120ΔN) led to the colocalization of endogenous kinesin with the cadherin–catenin complexes, shown in the merged pictures as indicated by the small white box in D′ (colocalization of all three components appears as white, colocalization of N-cadherin and kinesin appears as yellow). TX. transfection; IP, immunoprecipitation; CL, cell lysate. Bars: 20 μm (D), 5 μm (D′).

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). Endogenous p120 was localized at cell borders and diffusely distributed in the cytoplasm (Fig. 4 D′; unpublished data). 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,

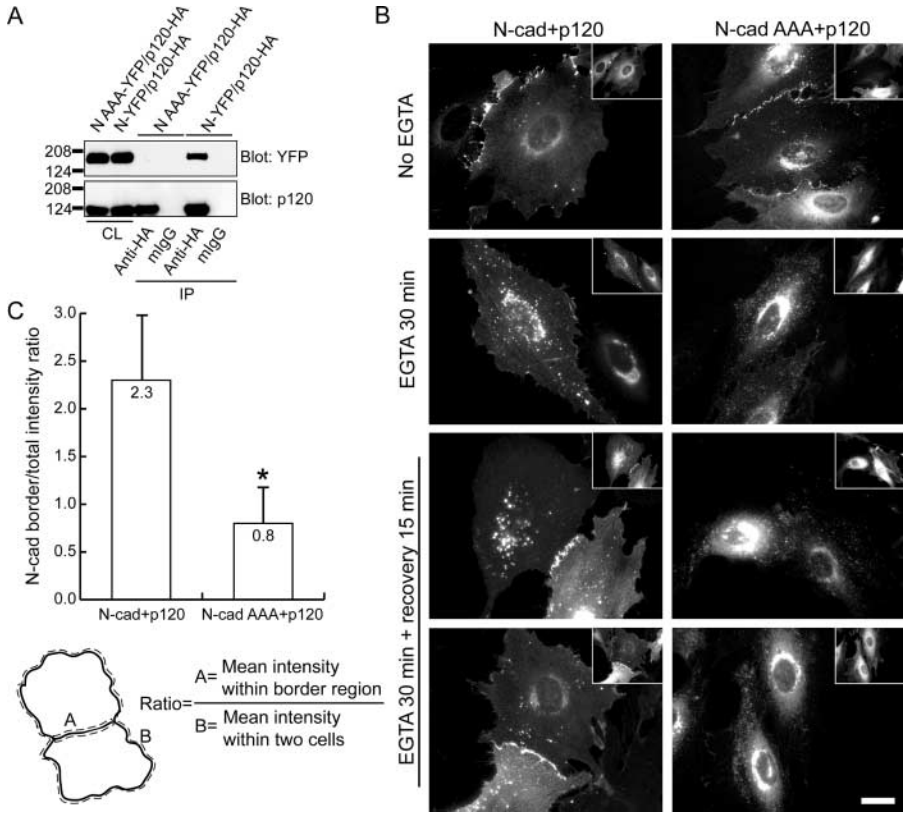

Figure 5. **Disruption of p120 binding to N-cadherin causes delayed accumulation of N-cadherin at cell–cell contacts in the presence of ectopic p120.** (A) Triple Ala mutation in the JMD domain of N-cadherin completely abolishes its ability to interact with p120. HEK293 cells transiently expressing N-cad–YFP (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 ($P = 3.8 \times 10^{-12} < 0.05$) by *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

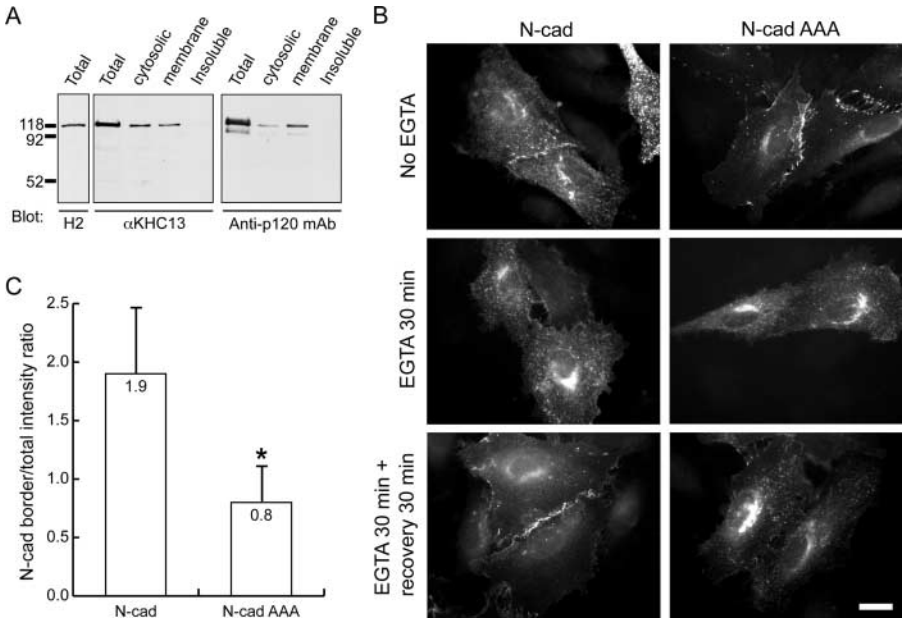

Figure 6. **Disruption of p120 binding to N-cadherin causes delayed accumulation of N-cadherin at cell–cell contacts in the presence of only endogenous p120.** (A) Sequential detergent extraction and immunoblot of endogenous kinesin and p120 in REF52 cells. A mouse anti-KHC antibody H2 and a rabbit anti-KHC antibody αKHC13 both detected a major band of KHC, which is mainly distributed in the saponin-soluble cytosolic fraction and the Triton X-100–soluble membrane fraction. Endogenous p120 is also primarily distributed within these two pools. (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 transfected 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 that both expressed N-cad were examined. After 30 min of calcium recovery, N-cad–YFP localized prominently at cell–cell borders, whereas most N-cad AAA-YFP still remained in the cytoplasm. (C) Quantification of N-cad–YFP or N-cad AAA-YFP accumulation at cell–cell contacts after 30 min of calcium recovery. Pairs of contacting cells expressing N-cad–YFP or N-cad AAA-YFP were randomly selected, and the cell border accumulation level 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 ($n = 25$) at cell–cell contacts is 58% less than that of N-cad–YFP ($n = 26$) 30 min after calcium recovery. Asterisk denotes significant difference from cells expressing N-cad–YFP ($P = 1.1 \times 10^{-10} < 0.05$) by *t* test. Bar, 20 μm.

cytosolic pool (Fig. 6 A). A major band of endogenous kinesin was recognized by two different anti-KHC antibodies. Both ectopic N-cad–YFP and N-cad AAA-YFP localized at cell borders and in the cytoplasm 20 h after transfection, and 30 min of EGTA treatment disrupted their accumulation at cell–cell contacts (Fig. 6 B). Within 30 min of calcium recovery, N-cad–YFP relocalized efficiently to cell–cell contact sites, whereas N-cad AAA-YFP still remained largely cytoplasmic with very little cell border accumulation. Consistent with a positive role for p120 in cadherin trafficking, the redistribution of N-cad–YFP to cell borders was slower in the absence of ectopic p120 because we could not detect strong border accumulation of N-cad–YFP after 15 min of calcium recovery without ectopic p120 (unpublished data). Quantification of adjacent pairs of transfected cells showed that the accumulation level of N-cad–YFP at cell borders was on average 2.4-fold greater than that of N-cad AAA-YFP after 30 min of calcium recovery (Fig. 6 C). Because no ectopic p120 was expressed in these cells, the binding of the endogenous pool of p120 to cadherins is sufficient to promote their delivery to the cell surface, and introducing ectopic p120 to elevate the p120 level further accelerates this process (Fig. 5). Together, these data strongly support a physiological role of p120 in facilitating the accumulation of cadherins at cell–cell contacts.

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. 7 A). 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. 7 B). 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 NH_2-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

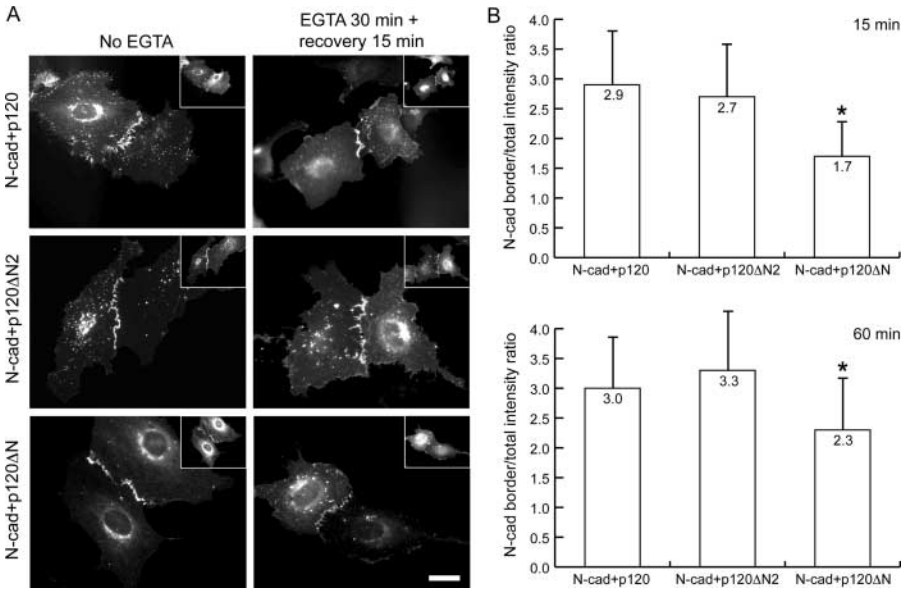

Figure 7. **Disruption of p120 binding to kinesin causes delayed accumulation of N-cadherin at cell–cell contacts during junction reassembly.** (A) N-cad–YFP is delayed in its accumulation at cell–cell contacts when coexpressed with p120ΔN-CFP compared with N-cad–YFP coexpressed with p120-CFP or p120ΔN2-CFP 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 ($P = 1.1 \times 10^{-7} < 0.05$ at 15 min; $P = 2.1 \times 10^{-3} < 0.05$ at 60 min) by *t* test. Bar, 20 μm.

cadherins to the cell surface through the association of a cadherin-bound catenin with MT-based motor proteins. Although cadherins are known to be transported to the cell surface along MTs in a kinesin-dependent manner (Mary et al., 2002), the involvement of the catenins in regulating this process had not previously been demonstrated. In this report, we show that p120 can associate with KHC through its head domain, thus enabling p120 to interact with cadherins simultaneously through its Arm-repeat domain and to recruit kinesin to the cadherins. By uncoupling the interaction between p120 and cadherin using a triple AAA mutant of N-cadherin, or by uncoupling the association between p120 and kinesin using a p120 NH_2-terminal deletion mutant p120ΔN, we demonstrate that a triple complex between cadherin and kinesin mediated by p120 is necessary for the rapid delivery of cadherins to the cell surface. Another p120 deletion mutant, p120ΔN2, which contains the first 27 aa and the last 114 aa of the NH_2-terminal head domain, still associates with kinesin and cadherins. p120ΔN2 is sufficient to support the rapid delivery of N-cadherin to the cell surface, further suggesting the importance of p120 and kinesin association in this process (Fig. 7). Because cadherins deficient in p120 binding are still able to localize to cell–cell borders, we propose that p120 promotes the trafficking of cadherins through the recruitment of kinesin to the cadherin–catenin-containing vesicles. This rapid delivery of junctional components facilitated by p120 would ensure a sufficient supply of cadherins at the cell surface for many processes such as neural crest cell and myoblastic precursor migration, tissue elongation in *Xenopus* embryo, and wound healing, during which cadherin-based contacts must be rapidly and dynamically regulated (Bronner-Fraser, 1993; Brieher and Gumbiner, 1994; Wilson and Gibson, 1997).

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.

Further insight into the in vivo functions of p120 came from several recent works examining the consequences of loss of p120 in *Drosophila* and *C. elegans* (Myster et al., 2003; Pettitt et al., 2003). In contrast to mammals, which express mul-

tiple 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 (Pacquelet 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 NH_2 terminus of mammalian p120 were not found in *Drosophila* and *C. elegans* p120. p120 associates with kinesin through its NH_2 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 NH_2-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-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 NH_2 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 NH_2 terminus immediately adjacent to the Arm-repeat domain of p120.

The relatively small amount of p120 coimmunoprecipitated 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 NH_2-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 NH_2 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

Constructs

The Rc-CMV-murine $p120^{ctn}$ 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 p120ΔN2-GFP were provided by Dr. A.D. Bershadsky (Weizmann Institute of Science, Rehovot, Israel; Grosheva et al., 2001). p120ΔN and p120ΔN2 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 *Eco*RI and KpnI and ligated into the pECFP-N1 vector (CLONTECH Laboratories, Inc.). N-cad–YFP was constructed by subcloning the N-cadherin cDNA fragment from N-cad–GFP into the AgeI and XhoI sites of pEYFP-N1 (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 transferring the human α1-tubulin cDNA from pEGFP-Tub (CLONTECH Laboratories, Inc.) into the XhoI and BamHI sites of pEYFP-C1 (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 DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech). REF52 cells were grown in DME/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

PBS, fixed in methanol for 2 min at −20°C, and incubated with appropriate primary and secondary antibodies for 30 min each at 37°C. Primary antibodies are as follows: p120 mouse monoclonal antibody used at 1:500 (Transduction Laboratories); anti-p120 rabbit polyclonal antibody F1-SH (provided by Dr. Al Reynolds, Vanderbilt University, Nashville, TN) used at 0.5 μg/ml; mouse monoclonal KHC antibody (H1) used at 1:100 (CHEMICON International). Alexa® Fluor–conjugated goat anti–mouse or goat anti–rabbit secondary antibodies were used at 1:300 (Molecular Probes, Inc.). Images were obtained on a microscope (DMR; Leitz) using a digital camera (Orca; Hamamatsu) and Openlab imaging software (Improvision).

Time-lapse fluorescence microscopy

REF52 cells growing on glass-bottom dishes (World Precision Instruments) were transiently transfected. 48–72 h later, the dish was mounted on an inverted microscope (Diaphot 300; Nikon) equipped with a Plan Fluor 100×, 1.30 NA oil objective and a slow-scan cooled CCD camera (model CH350; Photometrics). For GFP fusion protein imaging, fluorescent images were captured using a longpass filter set (Endow GFP; Chroma). For CFP/YFP double imaging, the lambda 10–2 optical filter changer (Sutter Instrument Company) was used with dual-band beamsplitters and single-band excitation/emission filters (Chroma Technology Corp.). Images were transferred to a computer workstation running MetaMorph® imaging software (Universal Imaging Corp.). Images were collected at 3-s intervals for 5 to 10 min. During live-cell imaging, temperature was kept at 33 ± 1°C by using flexible heaters (Omegalux) on the objective lens.

Coimmunoprecipitation, immunoblot, and sequential detergent extraction

Coimmunoprecipitation and immunoblot were performed as described in detail previously (Chen et al., 2002). Transfected HEK293 cells in 60-mm dishes were lysed with 500 μl ice-cold lysis buffer (1% Triton X-100, 145 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Lysates were vortexed for 30 s and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was transferred to a new tube, precleared with 40 μl GammaBind Plus Sepharose beads (Amersham Biosciences) for 1.5 h and centrifuged for 2 min to remove nonspecific complexes. Immunoprecipitation was performed using antibodies as follows: a mouse monoclonal anti-Myc antibody 9E10 (Sigma-Aldrich), a rat monoclonal anti-HA antibody 3F10 (Roche), and a rabbit polyclonal anti-GFP antibody that also recognizes YFP and CFP (CLONTECH Laboratories, Inc.). Immunoblots were performed using antibodies as follows: p120 mouse monoclonal antibody used at 1:1,000; anti-p120 rabbit polyclonal antibody F1-SH used at 1:3,440; mouse anti-KHC antibody (H2; CHEMICON International) used at 1:1,000; rabbit anti-KHC peptide antibody αKHC13 used at 1:2,500 (a gift from Dr. Kristen J. Verhey, University of Michigan Medical School, Ann Arbor, MI; Verhey et al., 1998); mouse monoclonal anti-Myc antibody 9E10 used at 1:1,000 (Sigma-Aldrich); rat monoclonal anti-HA antibody 3F10 (Roche) used at 1:1,000; mouse monoclonal Living Colors™ A.v. anti-GFP antibody JL-8 used at 1:4,000, and rabbit Living Colors™ A.v. anti-GFP peptide antibody used at 1:100 (CLONTECH Laboratories, Inc.). Sequential detergent extraction was performed as described previously (Palka and Green, 1997), except that cells were grown in 6-well dishes and the amount of buffer used at each step was adjusted so that the final volume of each pool was 400 μl.

Coimmunoprecipitation from mouse brain high speed supernatant fraction in the absence of detergent was performed as described in detail previously (Verhey et al., 2001). For immunoprecipitation, 1 mg high speed supernatant was used, and 90 μg mouse anti-KHC antibody (H2; CHEMICON International) or nonspecific mouse IgG was added to the supernatant and incubated for 4 h at 4°C. The immunocomplexes were isolated with 60 μl GammaBind Plus Sepharose beads and analyzed by SDS-PAGE and immunoblotting.

Calcium switch experiment and quantification

20–24 h after transfection, REF52 cells growing on glass coverslips were washed four times with PBS without calcium and magnesium and incubated in the growth medium containing 4 mM EGTA for 30 min at 37°C. Cells were then washed four times with the growth medium and incubated with it at 37°C for different period of time before being fixed in methanol. To quantify the N-cad–GFP or N-cad–YFP border/total intensity ratio in transfected REF52 cells, pairs of closely contacting cells expressing N-cad–GFP or both N-cad–YFP and p120-CFP were randomly selected. The region of the cell–cell contact with N-cad–GFP/YFP accumulation was selected and the average pixel intensity was measured for the selected region using the measurement tool of the Openlab imaging software (Improvision). 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 subtracted from the border intensity, and total intensity respectively before the border/total intensity ratio was calculated. Statistical analysis was performed using the *t* test.

Online supplemental material

Videos 1–5 correspond to still images shown in Fig. 1 and Fig. 2. For Videos 1, 2, and 4, REF52 cells were transfected with p120-GFP. For Video 3, REF52 cells were transfected with p120-CFP and N-cad–YFP. For Video 5, REF52 cells were transfected with p120-CFP and YFP–α-tubulin. 48–72 h after transfection, time-lapse movies of transfected cells were recorded. For Videos 1, 2, and 4, time-lapse images were taken at 3-s intervals and the movies are shown at 15 frames/s. For Videos 3 and 5 (double imaging), time-lapse images of the same fluorescent channel were taken at 6-s intervals, and the delay caused by switching to the second channel is 3 s. The adjacent CFP and YFP images were merged and the videos are shown at 7.5 frames/s. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200305137/DC1.

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