Lamellipodium extension and cadherin adhesion: two cell responses to cadherin activation relying on distinct signalling pathways

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Summary

Cell adhesion molecules of the cadherin family contribute to the regulation of cell shape and fate by mediating strong intercellular adhesion through Ca^{2+}-dependent interaction of their ectodomain and association of their cytoplasmic tail to actin. However, the mechanisms co-ordinating cadherin-mediated adhesion with the reorganization of the actin cytoskeleton remain elusive. Here, the formation of de novo contacts was dissected by spreading cells on a highly active N-cadherin homophilic ligand. Cells responded to N-cadherin activation by extending lamellipodium and organizing cadherin-catenin complexes and actin filaments in cadherin adhesions. Lamellipodium protrusion, associated with actin polymerization at the leading edge sustained the extension of cadherin contacts through a phosphoinositide 3-kinase (PI 3-kinase)-Rac1 pathway. Cadherin adhesions were formed by PI 3-kinase-independent, Rac1-dependent co-recruitment of adhesion complexes and actin filaments. The expression and localization of p120 at the plasma membrane, associated with an increase in membrane-associated Rac1 was required for both cell responses, consistent with a major role of p120 in signalling pathways initiated by cadherin activation and contributing to Rac1-dependent contact extension and maturation. These results provide additional information on the mechanisms by which cadherin co-ordinates adhesion with dynamic changes in the cytoskeleton to control cell shape and intercellular junction organization.

Supplemental data available online

Key words: N-cadherin, Catenin, PI 3-kinase, Rac1, p120

Introduction

Interactions of cells with the extracellular matrix (ECM) and their neighbours play a crucial role in embryonic development and in the regulation of tissue architecture and homeostasis (Takeichi, 1995; Hynes, 1999). Cell adhesion molecules of the cadherin family, as well as ECM receptors of the integrin family, participate to the regulation of cell shape, behaviour and fate. Integrin-mediated adhesion has been shown to involve several interdependent steps, starting with the establishment of initial cell-substrate contacts, extension of these adhesions and finally formation of focal adhesions and spreading on the matrix with concomitant reorganization of the cell cytoskeleton and the matrix itself (Geiger et al., 2001). During this process, integrins make transmembrane connections to the cytoskeleton and activate intracellular signalling pathways that are now largely described (Hynes, 2002). By comparison, the molecular mechanisms involved in cadherin-mediated contact formation and in the initiation of signalling cascades upon cadherin binding remain poorly understood.

Cadherins contribute to cell aggregation, segregation and migration through dynamic cell-cell contact remodelling (Friedlander et al., 1989; Tepass et al., 2000). They further specify differentiated cell-cell contacts by inducing the recruitment of junctional complexes during mesenchymal-epithelial transition (Mège et al., 1988; Gumbiner, 1996), cardiomyocyte assembly (Radice et al., 1997) and synaptogenesis (Uchida et al., 1996). Cadherins mediate cell adhesion through Ca^{2+}-dependent homophilic interactions of their ectodomain and association of their conserved cytoplasmic domain to catenins (β-, α- and p120) that link cadherin tails to actin filaments (Yap et al., 1997a). The integrity of the cadherin-catenin complex, as well as its correct association with the actin cytoskeleton, is required for cell migration, cell aggregation and intercellular junction formation. Both aspects of the developmental role of cadherins imply a tight co-ordination between cell adhesion and dynamic changes in cytoskeleton organization.

Several studies suggest that the establishment of these contacts involves both the recruitment of catenins and the anchoring of cadherin to the actin cytoskeleton (Yap et al., 1997b; Adams and Nelson, 1998; Lambert et al., 2000). Moreover, dynamic changes in cadherin-cytoskeleton interactions take place during cell-cell contact formation. Sako and collaborators identified two populations of E-cadherin at the cell surface: one fraction that

was highly mobile in the plasma membrane and the other one that was tethered to actin filaments (Sako et al., 1998). We further showed that, during the very first steps of cadherin-mediated contact formation, the homophilic interaction of N-cadherin induces its rapid and strong anchoring to actin filaments (Lambert et al., 2002). Furthermore, the establishment of cadherin-mediated contacts involves complex changes in actin organization and membrane dynamics in the two adjacent cells (Ehrlich et al., 2002). Complementary approaches designed to investigate cadherin-activated cell signalling demonstrated that the activity of the small GTPase Rac1 is stimulated in response to homophilic liganding of E-cadherin (Noren et al., 2001; Kovacs et al., 2002a). Conversely, Rac1 appears to be essential for regulating the changes in actin cytoskeleton dynamics associated with cadherin-mediated contact formation (Braga et al., 1997; Ehrlich et al., 2002; Lambert et al., 2002).

The association of β- and α-catenin with cadherin is crucial for the mechanical link of adhesion complexes to the actin cytoskeleton. The p120 catenin, interacting with the juxtamembrane domain (JMD) of cadherins, remains a controversial modulator of cadherin activity (Anastasiadis and Reynolds, 2001). When overexpressed in a cell, p120 has been reported as a potent regulator of Rho-like GTPases, activating Rac1 and cdc42 and inhibiting RhoA (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). The deletion or mutation of the E-cadherin JMD combined with the expression of exogenous p120 suggested a putative function of p120 in cadherin adhesiveness, with either positive or negative effects depending on the cell backgrounds (Yap et al., 1998; Ozawa and Kemler, 1998; Ohkubo and Ozawa, 1999; Aono et al., 1999; Thoreson et al., 2000).

To study further the molecular and cellular mechanisms regulating the formation and maturation of cadherin-based contacts, we analysed the behaviour of single cell membranes in the interaction with N-cadherin planar surfaces. Cells specifically responded to N-cadherin activation by: (1) extending large lamellipodium; and (2) reorganizing their actin cytoskeleton, cadherin-catenin complexes and cadherin-associated proteins in highly patterned radial structures named cadherin adhesions. We provide evidence that p120 plays a pivotal role in signalling cascades initiated by cadherin activation. p120 is absolutely required for both PI 3-kinase-Rac1-dependent contact extension associated with lamellipodium protrusion and PI 3-kinase-independent, Rac1-dependent reorganization of cadherin-catenin complexes and actin filaments in cadherin adhesions.

Materials and Methods

Cell culture

The mouse myogenic C2 cell line (Yaffe and Saxel, 1977) and the chicken N-cadherin-expressing S180 cell line, N-cad S180 (Matsuzaki et al., 1990), were grown in Dulbecco's modified Eagle's medium (DMEM) plus Glutamax supplemented with 10% fetal calf serum under 7.5% CO_2 at 37°C. The human adenocarcinoma SW48 epithelial cell line (from ATCC) was grown in Leibovitz's medium (L-15) plus L-Glutamine 2.0 mM and 10% fetal calf serum in the absence of CO_2 at 37°C.

Electroporation and plasmid constructs

For experiments requiring the expression of exogenous proteins, 5×10^6 C2 or SW48 cells were electroporated (Easyject Plus, Equibio, Ashford, UK) with 35 μg of expression vectors under 260 V, 1500 μF in 400 μL DMEM plus 15 mM Hepes pH 7.2, or under 260 V, 750 μF in 600 μL L-15 medium plus 15 mM Hepes pH 7.2, respectively. The transfection efficiency was around 60% in all the cases and transfected cells were easily identified by green fluorescent protein (GFP) expression.

To obtain the Ncad-GFP construct, the chicken N-cadherin cDNA (nucleotides 1-2736, GenBank accession # X07277, ID GGNCAD_2) was inserted in a pEGFP-N2 vector (Clontech, Palo Alto, CA), placing the N-cadherin sequence in frame with a sequence encoding GFP at the C-terminus. (Torso)-JMDC-cad-(myc)6 (abbreviated to JMD-cad) was constructed by replacing the sequence encoding the intracellular domain of a constitutive fibroblast growth factor receptor (FGFR) 4 (Sp64T Torso-FGFR-4) (Umbhauer et al., 2000) by the sequence of the JMD of C-cadherin (aa 731-824). The chimaeric protein comprising the extracellular and transmembrane domains of the dimerizing *Drosophila* protein Torso, fused to the juxtamembrane p120-binding domain of C-cadherin (JMD) was designed to trap and address p120 to the plasma membrane. This was confirmed by checking that p120-GFP co-immunoprecipitates with JMD-cad (data not shown). Vectors containing the dominant-active (V12 Rac1, V12 cdc42 and V14 RhoA) and dominant-negative (N17 Rac1, N17 cdc42 and N19 RhoA) forms of the small GTPase cDNAs tagged with GFP were provided by C. Gauthier-Rouvière. Actin-GFP was a gift from D. Choquet. The full-length p120-GFP expression vector has been described before (Grosheva et al., 2001).

For p120 silencing, a 64 bp oligonucleotide, containing a p120-specific sequence in both sense and antisense orientations was cloned in the pSUPER vector (I.G., M. Shtutman, S. Boguslavsky and A.B., unpublished). C2 cells were electroporated with empty-pSUPER (Brummelkamp et al., 2002) or p120-pSUPER vectors 16 hours before cell spreading assays. p120 silencing evaluated by immunoblot analysis of p120 cellular content was already optimum at 16 hours post-transfection and was not further increased until 72 hours (data not shown).

Drug treatments

All drugs were purchased from Sigma except indicated otherwise and were kept during the cell spreading assays. Cytochalasin D, Jasplakinolide (Molecular Probes, Eugene, OR) or Nocodazole were added before cell spreading at 2 μM for 5 minutes, 0.5 μM for 5 minutes and 10 μM for 30 minutes, respectively. C2 cells were pre-treated with 1 μM bafilomycin and 20 μM brefeldin A alone or in combination for 1 hour. Alternatively, cells were pre-treated with 0.05 μM wortmannin for 30 minutes, 10 μM Taxol for 30 minutes, 270 μM H7 for 1 hour, 5 μM lysophosphatidic acid (LPA; BIOMOL Research Laboratories, Plymouth Meeting, PA) for 5 hours. 3 mM EDTA and 25 mM 2,3-butanedione monoxime (BDM) were added when cells were seeded on adhesion substrates.

Preparation of adhesion substrates and cell spreading assays

Glass coverslips were treated with 20% nitric acid, washed in methanol-acetone and coated with organopolysiloxane (Sigmacote, Sigma). Air-dried silanized coverslips were loaded with goat anti-mouse, anti-rabbit or anti-human Fcγ fragment antibodies (Jackson ImmunoResearch, West Grove, PA) at 1 $\mu g/cm^2$ in 0.1 M borate buffer pH 8.0 for at least 5 hours. Coverslips were then incubated for 2 hours with a concentration of 5-10 $\mu g/cm^2$ of purified Ncad-Fc chimera (extracellular domain of the chicken N-cadherin fused to the mouse IgG2b Fcγ fragment) (Lambert et al., 2000). Alternatively, coverslips were coated with human Ecad-Fc chimera (R&D systems, Minneapolis, MN), laminin (prepared from Engelbreth-Holm-Swarm mouse tumours, a gift from N. de Carvalho and M. Vigny), human fibronectin (PAA Lab, GmbH, Linz, Austria), monoclonal anti-

chicken N-cadherin antibody (clone GC4, Sigma) or polyclonal anti-mouse N-cadherin antibody (Charrasse et al., 2002). Surfaces were then saturated with 1.5% purified BSA (Sigma) in borate buffer for 5 minutes at room temperature. To preserve cell-surface cadherins, C2 cells were mechanically dissociated in trypsin-free conditions with PBS, 5 mM EDTA, 2% BSA on ice; this treatment should not exceed 5 minutes (Takeichi, 1977; Stevenson et al., 1992). Cells were then plated on the different adhesion substrates in serum-free conditions and at very low cell density (5×10^2 to 5×10^3 cells/cm^2) for 2 hours.

Immunostainings

Cells were fixed in 3% PBS-formaldehyde at 37°C for 15 minutes, washed in 0.1 M PBS-glycine and permeabilized in 0.5% PBS-Triton for 5 minutes. Coverslips were then incubated with primary polyclonal antibodies in 3% PBS-BSA for 1 hour, washed and incubated with goat FITC-, TRITC- or Cy5-conjugated anti-rabbit antibodies (1/500 dilution; Jackson ImmunoResearch). The following rabbit polyclonal antibodies were used: anti-β-catenin (1/500 dilution, Sigma), anti-α-catenin (1/200 dilution, Sigma), anti-p120 F1αSH (1/500 dilution) (Wu et al., 1998). Alternatively, cells were stained with monoclonal anti-Myc (1/500, clone 9E10; Santa Cruz) or anti-tubulin (1/1000 dilution, clone E7; Hybridoma Bank) antibodies, revealed with biotin-conjugated goat anti-mouse κ chain (1/200 dilution; Southern Biotechnologies, Birmimgham, AL), then with rhodamine-conjugated streptavidin (1/200 dilution, Molecular Probes). Alexa488- or 546-conjugated phalloidin (1/1500 dilution, Molecular Probes) were used to visualize F-actin. Samples mounted in Citifluor (UKC Chem. Lab., Canterbury, UK) were observed by conventional fluorescence microscopy (Provis, Olympus Optical Co., Tokyo, Japan) and images were captured with a Micromax CCD camera driven with the Metamorph software (Roper Scientific, Trenton, NJ). Alternatively, preparations were analysed with a TCS SP2 confocal microscope (Leica, Mannheim, Germany) set on sequential mode.

Subcellular fractionation

Transfected C2 cells were rinsed three times in cold PBS and incubated in 200 μL hypotonic buffer without detergent [10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 μM NaVO4, 25 mM NaF, plus protease inhibitor cocktail (Complete; Roche, Mannheim, Germany)] during 30 minutes on ice. Cells were scraped and homogenized by twenty passings through a 23-gauge needle (cell lysis was checked in phase contrast and stopped when more than 75% of nuclei were free of ghost cell membranes). Whole cell lysates were spun at 750 ***g***, 10 minutes, at 4°C to exclude nuclei. Post-nuclear extracts, equivalent to 200 μg total protein, estimated by micro BCA (Pierce, Rockford, IL) were submitted to ultracentrifugation (100 000 ***g***, 30 minutes, 4°C in a TLA 100.1 rotor; Optima Beckman). The pellets were rinsed twice in PBS, then resuspended in 100 μL hypotonic buffer. Equal amounts of proteins from each fraction (membrane-containing pellet and cytosol-containing supernatant) were further analysed by western blot.

Western blot

Subcellular fractions or whole cell lysates were analysed by 7% or 13% SDS-PAGE and blotted on 0.2 μm nitrocellulose membranes. Blots were incubated with the following mouse antibodies: anti-p120 (1/2000 dilution; Transduction Laboratories, Lexington, KY), anti-β-catenin (1/2000 dilution; Transduction Laboratories), anti-GFP (1/2000 dilution; Roche), anti-Rac1 (1/3000 dilution; Upstate Biotechnologies) or anti-tubulin (1/20000, clone E7). After extensive washes, the membranes were incubated with goat anti-mouse HRP-conjugated antibody (1/10000 dilution; Dako, Denmark). Blots were then developed for electrochemiluminescence (ECL) using ECL western blotting reagents (Amersham biosciences, Uppsala, Sweden).

Results

Specific cell adhesion and spreading triggered by homophilic liganding of N-cadherin

Myogenic C2 cells in culture, like Madin-Darby canine kidney (MDCK) cells (Ehrlich et al., 2002) or keratinocytes (Vasioukhin et al., 2000), establish cadherin-dependent cell-cell contacts in an opportunistic fashion. The newly formed intercellular contacts are complex and associated with lamellipodium of adjacent cells growing on top of each other (Fig. 1A). β-catenin staining was mostly restricted to these contact regions and oriented perpendicularly to the cell-cell contacts, whereas actin fibres were organized in a complex network at the contact regions. However, in such conditions, it was difficult to discriminate between intracellular signalling triggered by adhesion per se and a more global response of the cell to this modification of its neighbourhood, both contributing to cell-cell contact formation and maturation.

In order to characterize the molecular and cellular factors involved in the establishment of cadherin-mediated contacts, we decided to increase artificially the contacting area by spreading cells directly onto a cadherin-mimicking substrate. C2 cells expressing endogenous N-cadherin were allowed to attach at very low density on an immobilized Ncad-Fc surface in the absence of serum (Figs 1B, 2). These conditions avoided any contribution from other adhesion receptors and growth factors, allowing the analysis of N-cadherin-induced signalling, thanks to the homophilic properties of the Ncad-Fc chimera (Lambert et al., 2000). C2 cells attached in less than 15 minutes to this planar substrate, but did not on BSA or anti-Fc-antibody-coated surfaces (data not shown). S180 cells that do not express N-cadherin or C2 cells depleted of their membrane cadherins by trypsinization did not bind to Ncad-Fc, demonstrating a specific interaction between cellular N-cadherin and the Ncad-Fc substrate. Because of its strict dependence upon cadherin homophilic liganding (Brieher et al., 1996; Lambert et al., 2000; Yap and Kovacs, 2003), this approach was further used as a convenient model for the establishment of cadherin-mediated contacts.

Cells responded to this uniform Ncad-Fc substrate with extensive spreading by 30 minutes. By 2 hours, they exhibited a typical fried egg morphology characterized by a large circular lamellipodium (Fig. 1B). These non-polarized cells represented nearly 50% of attached cells (Fig. 3B). In addition, 20% of attached cells displayed a more elongated shape with multiple lamellipodium protrusions, whereas cell attachment by electrostatic interactions on poly-ornithine did not show any cell spreading (Fig. 3B). Moreover, C2 cells grown on laminin (or fibronectin, data not shown) showed the expected elongated cell shape characterized by more limited, polarized, lamellipodium extensions (Fig. 1A, Fig. 3A-C). The fried egg shape induced by N-cadherin homophilic liganding suggested a specific architecture of the cytoskeleton. The actin cytoskeleton was characterized by a circular network of filaments surrounding the nucleus together with radial cables directed towards and ending in the lamellipodium (Fig. 1B). No stress fibres were detected in these non-polarized cells, whereas C2 cells grown on laminin presented typical stress fibres (Fig. 3A). Microtubules were also symmetrically distributed in the circular lamellipodium, confirming the non-polarized character of cells spread on Ncad-Fc (Fig. 1B). Thus,

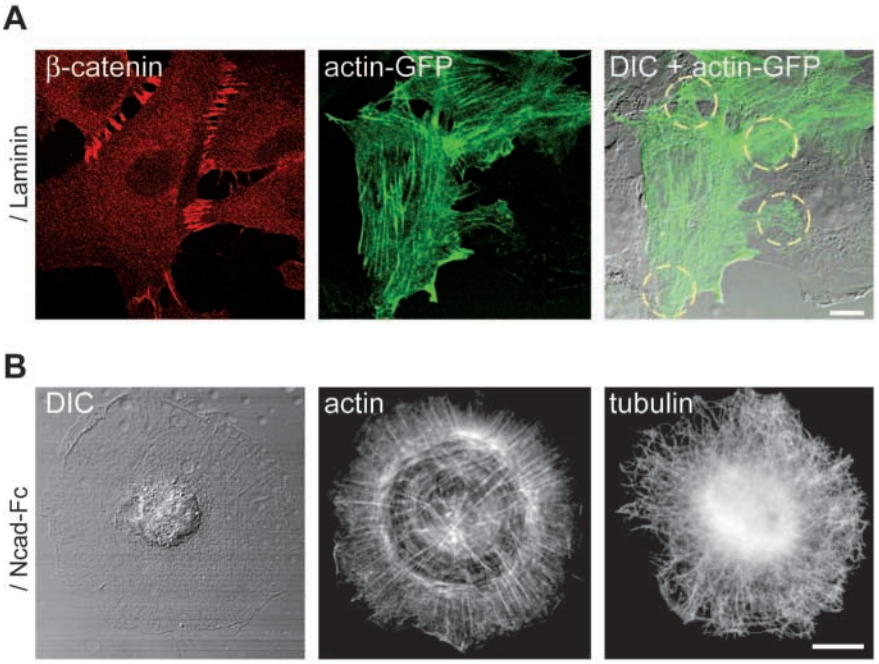

Fig. 1. N-cadherin adhesion, typical cell spreading and cytoskeleton organization on the Ncad-Fc substrate. (A) Myogenic mouse C2 cells expressing endogenous N-cadherin were spread on laminin (/Laminin), stained for β-catenin and analysed by confocal microscopy. Alternatively, actin-GFP-transfected cells were spread on laminin and analysed by confocal microscopy in combination with differential interference contrast (DIC) imaging. Note the radial distribution of β-catenin and the more complex actin network (circles) at cell-cell contacts. (B) C2 cells dissociated in trypsin-free conditions were seeded in serum-free medium on silanized glass coverslips coated by immunoadsorption with purified Ncad-Fc (/Ncad-Fc). Cells spread for 2 hours exhibited typical fried egg morphology (DIC). Cytoskeleton organization was assessed by labelling actin filaments with phalloidin and microtubules with anti-tubulin antibodies. Bar, 10 μm.

the radial distribution of actin fibres represents a specific response to N-cadherin liganding, establishing here the ability of cadherin to control the cytoskeleton organization, and therefore the shape of the cell.

N-cadherin homophilic liganding specifically induces the co-organization of cadherin-catenin complexes and actin cytoskeleton in cadherin adhesions

As catenins link cadherin to the actin network, we determined the localization of these proteins by immunofluorescent staining in cells contacting the Ncad-Fc surface. β-catenin was recruited but not uniformly distributed at the cell-substratum interface. Anti-β-catenin antibodies strongly labelled radial structures within the lamellipodium (Fig. 2A). Confocal microscopy showed that these highly patterned structures were located at the ventral face of the cells, whereas the intense β-catenin staining observed in the centre of the cell might correspond to the labelling of intracellular vesicle-like structures. The three catenins, α-catenin, β-catenin and p120, as well as N-cadherin-GFP, displayed the same patterned distribution in the lamellipodium. More than 75% of cells attached on Ncad-Fc showed these radial organizations of cadherin-catenin complexes, reminiscent of the actin fibre distribution. Double staining of actin and β-catenin showed that radially oriented actin cables were directed towards and ended in β-catenin-positive radial structures (Fig. 2B). These structures, which reflect a higher order organization of adhesion complexes and actin filaments, were named cadherin adhesions. The actin-binding proteins vinculin and α-actinin, as well as signalling molecules known to be accumulated at intercellular junctions, were also recruited in cadherin adhesions (data not shown). By contrast, proteins specific of ECM-cell contacts such as laminin, activated β1-integrin, talin and phospho-focal adhesion kinase were not co-recruited in these structures (data not shown), indicating that integrin-ECM interactions do not contribute to cadherin adhesions. Altogether, these data indicate that the molecular composition of cadherin adhesions carries the hallmark of cadherin-dependent cell-cell contacts. In addition, electron microscopy examination indicated the cell membrane adjoined Ncad-Fc-coated surface with a regular spacing of 20-30 nm, in agreement with the intermembrane spacing at adherens junctions (data not shown).

Cadherin adhesion formation was a specific response to cadherin ectodomain mobilization, since cells plated on fibronectin (data not shown) or laminin (Fig. 3A) never presented such radial organizations of cadherin-associated adhesion complexes and actin filaments. The cell response to attachment on Ncad-Fc could result from either agonistic cadherin liganding or cadherin crosslinking. C2 cells grown on surfaces coated with anti-N-cadherin did not present the typical fried egg shape assumed on the Ncad-Fc substrate (Fig. 3A,B). Cells spread on anti-N-cadherin antibodies displayed diffuse β-catenin immunostaining at their ventral surface and no reorganization of their actin cytoskeleton in radial structures (Fig. 3A). S180 cells expressing chicken N-cadherin (N-cad S180) were further used to compare the cell response to substrates coated with Ncad-Fc and a monoclonal anti-N-cadherin antibody at comparable loading density (Fig. 3C). These cells failed to organize β-catenin in radial structures on the monoclonal anti-chicken N-cadherin antibody, whereas they organized cadherin-catenin complexes and actin filaments in radial structures on Ncad-Fc. These observations further reflect that antibody-induced N-cadherin crosslinking by itself was not sufficient to promote cadherin adhesion formation. Thus, the formation of cadherin adhesions appears to require cadherin homophilic liganding. Although less organized than in C2 cells, cadherin adhesions were observed for various cell types, including primary myoblasts, as well as S180, HeLa or MDCK cells transfected with N-cadherin (Fig. S1; http://jcs.biologists.org/supplemental/), indicating that this cell response was not cell-type specific.

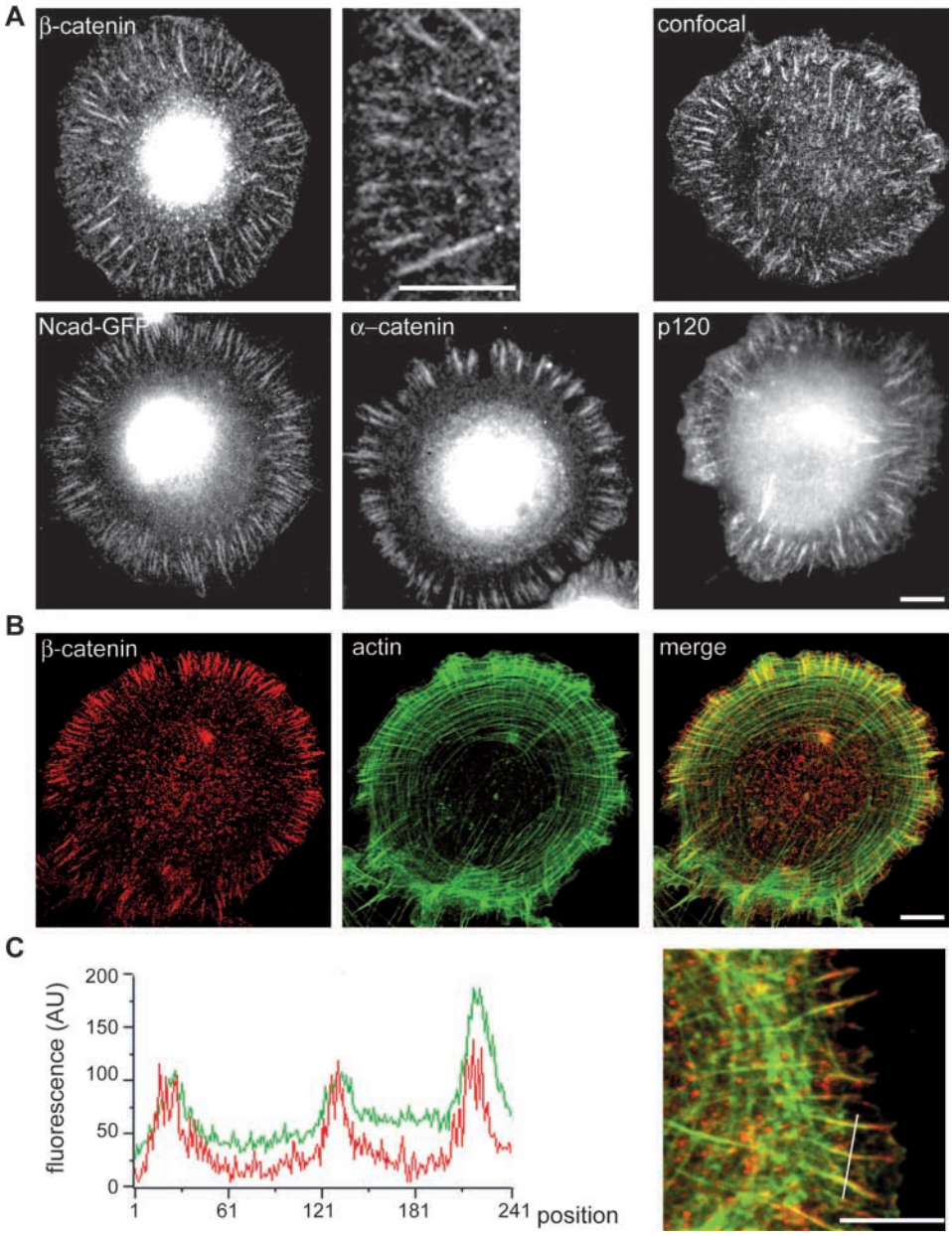

Fig. 2. Cell spreading on Ncad-Fc induces the formation of cadherin adhesions. (A) C2 cells spread for 2 hours on Ncad-Fc were immunolabelled for β-catenin, α-catenin and p120 or directly observed for the localization of transfected N-cadherin-GFP. The three catenins as well as N-cadherin presented the same radial distribution in the lamellipodium. The radial distribution of β-catenin was specifically detected on a 500 nm thin confocal section taken at the ventral side of fried-egg-shaped cells (confocal). The strong vesicle-like immunostaining in the central area of the cells was not detected in this section. (B) Double staining for β-catenin (red) and F-actin (green) was performed on cells spread on Ncad-Fc and analysed by confocal microscopy. (C) Co-localization of the red (β-catenin) and green (F-actin) signals was analysed along the line indicated on the overlay image by line scan (Metamorph software). An optical section taken close to the cell-substratum interface revealed a zone of coincidence of β-catenin-positive structures and F-actin labelling within the lamellipodium. Bars, 10 μm.

The formation of cadherin adhesions does not need cadherin trafficking and solely depends on the actin cytoskeleton

The redistribution of cadherin receptors in radial structures may involve different mechanisms, among which is the export of molecules from intracellular pools to the cell surface (Mary et al., 2002). The removal of N-cadherin from the cell surface by trypsin in the presence of EDTA totally prevented cell binding (Table 1). The microtubule-destabilizing drugs Nocodazole and Taxol had no effect on cell spreading and radial structure formation, suggesting that cadherin adhesions did not depend on microtubule-dependent vesicle transport. Finally, treatment of cells with the vesicle-trafficking blockers brefeldin A and bafilomycin had no effect on cell spreading and cadherin adhesion formation (Table 1). These data indicate that neither initial homophilic adhesion nor cadherin adhesions require the export to the membrane or recycling of N-cadherin intracellular pools and solely involve the engagement of pre-existing cell-surface pools.

To assess further the role of actin dynamics in cadherin adhesion biogenesis, we studied the effects of actin cytoskeleton destabilizing drugs (Table 1). Cytochalasin D and Jasplakinolide totally inhibited cell spreading and radial structure formation, but they had no effect on the attachment of cells on the Ncad-Fc substrate. Interestingly, if added after cell spreading, these drugs led to lamellipodium shrinking and to the disappearance of radial structures. As expected, the actin cytoskeleton integrity was absolutely required for cadherin redistribution in radial structures. In order to characterize how actin contributes to both processes, we assessed where actin polymerization takes place in cells spread on Ncad-Fc. G-actin incorporation in living cells spread on Ncad-Fc indicated that actin polymerization occurred at the tip of the lamellipodium (data not shown). Furthermore, GFP-Arp3, a protein belonging to the actin nucleation complex, was present at the tip of the lamellipodium but absent from the cadherin adhesions (data not shown). Thus, actin polymerization occurred only at the tip of the lamellipodium, probably sustaining cell spreading, but was not associated with cadherin adhesions.

Rac1 and PI 3-kinase are differentially involved in cell spreading and in cadherin adhesion formation

In order to get insight into the mechanisms underlying

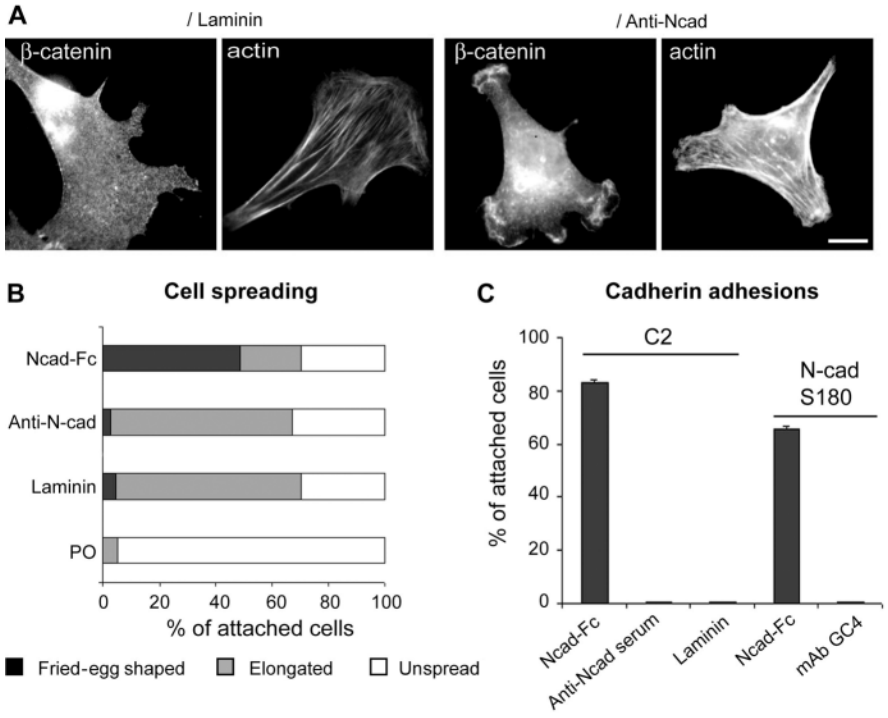

Fig. 3. Laminin or anti-N-cadherin antibodies failed to induce cadherin adhesions. (A) C2 cells were seeded on laminin (/Laminin) or anti-N-cadherin antibodies (/Anti-Ncad) and immunostained for β-catenin or F-actin. The usual elongated shape of C2 cells grown on laminin was also observed on the anti-N-cadherin substrate. In both conditions, β-catenin immunostaining was uniformly distributed at the cell surface and no radial distribution of β-catenin and actin fibres was detected. Bar, 10 μm. (B) Quantitative analysis of cell shape achieved after 2 hours of spreading on the Ncad-Fc, laminin, anti-N-cad and poly-ornithine (PO) substrates; the number of counted cells was superior to 250 for each condition and repeated in three independent experiments. (C) Quantification of mouse C2 cells or S180 chicken N-cad cells forming cadherin adhesions when seeded on Ncad-Fc, polyclonal anti-mouse N-cadherin serum, laminin or monoclonal GC4 anti-chicken N-cadherin antibody, respectively (at least 250 cells counted in two independent experiments).

cadherin-mediated contact formation, we searched for signalling proteins potentially regulating their genesis, taking advantage of the spatial dissociation between the sites of nascent contacts at the tip of the lamellipodium and mature cadherin adhesions. Several reports implicate Rac1 in cadherin-based contact genesis (Braga et al., 1997; Ehrlich et al., 2002; Kovacs et al., 2002a; Lambert et al., 2002). Furthermore, the phenotype of C2 cells spread on Ncad-Fc by itself is a potential indication of Rac1 activation (Nobes and Hall, 1995). Endogenous Rac1 was detected in the whole lamellipodium of C2 cells spread on Ncad-Fc, with stronger accumulations at the leading edge and partial colocalization with β-catenin in radial structures (Fig. S2; http://jcs.biologists.org/supplemental). We never detected PI 3-kinase, a downstream and upstream regulator of Rac1 activity, in cadherin adhesions (Fig. S2; http://jcs.biologists.org/supplemental). The PI 3-kinase immunostaining was rather uniformly distributed in the lamellipodium, with some accumulation at the tip of the lamellipodium. These results suggest that cadherin homophilic liganding induces a differential recruitment of signalling proteins at nascent contacts or in cadherin adhesions.

Table 1. Actin cytoskeleton but not cadherin transport was crucial for cadherin adhesions

	Cell responses		
Cell treatment	Binding	Spreading	Radial structures
Trypsin + EDTA*	–		
EDTA	–		
Bafilomycin + Brefeldin A	+	+	+
Cytochalasin D	+	–	–
Jasplakinolide	+	–	–
Nocodazole	+	+	+
Taxol	+	+	+
BDM	+/–	+/–	+
H7	+	+	+
LPA	+/–	+/–	–

The drugs were applied on C2 cells prior to their dissociation in trypsin-free conditions and their addition on Ncad-Fc substrate, as described in Materials and Methods. Their effect on: (1) cell attachment to the substrate; (2) cell spreading; and (3) cadherin adhesion formation after 2 hours of plating was qualitatively analysed in three independent experiments and compared with the effect of DMSO alone.

*Alternatively, cells were treated with trypsin+EDTA before the assay.

We then investigated the involvement of Rac1 on cell spreading and radial structure formation induced by N-cadherin homophilic interactions, thanks to the transient expression in C2 cells of dominant-negative (DN) and dominant-active (DA) Rac1 mutants. We did not detect any obvious effect of the expression of DA Rac1 on the spreading and cadherin adhesion formation (Fig. 4A, D). By contrast, the spreading of DN Rac1-expressing cells was strongly impaired since fewer than 5% of attached cells achieved a fried egg shape (Fig. 4A, D). The radial distribution of β-catenin staining was altered, even in the few cells exhibiting membrane protrusions. Thus, both cell spreading and cadherin adhesions were dependent on Rac1 activity. Strikingly, the expression of DA Rac1 was able to increase cell spreading on anti-N-cadherin surfaces (Fig. 4B) in such a way that cells assumed morphology very similar to the one induced by Ncad-Fc. Increased Rac1 activity was not sufficient to promote the formation of cadherin adhesions on the anti-N-cadherin substrate.

Neither the DA nor the DN forms of cdc42 had an effect on cell spreading and radial structure formation (Fig. 4E). Cell spreading was not impaired by the expression of RhoA mutants, whereas the DA form of RhoA specifically inhibited the organization of cadherin adhesions (Fig. 4E). Furthermore, the activation of Rho-dependent cell contractility by LPA altered cell spreading and prevented the

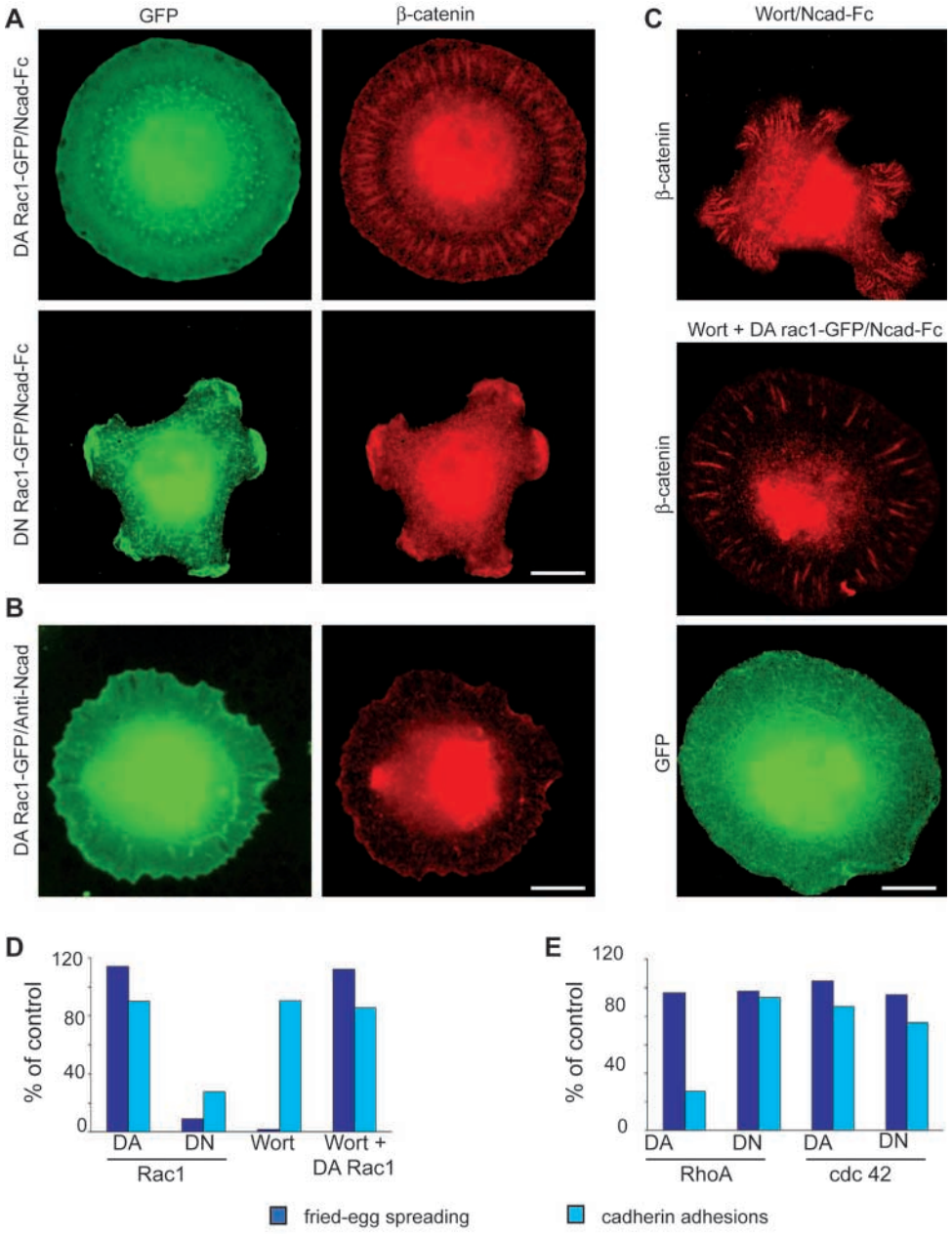

Fig. 4. Rac1 and PI 3-kinase are differentially involved in cell spreading and cadherin adhesion formation. (A) C2 cells transiently transfected with GFP-tagged DA (V12) or DN (N17) forms of Rac1 were plated for 2 hours on Ncad-Fc (/Ncad-Fc), fixed and stained for β-catenin. (B) DA Rac1-transfected cells were seeded on anti-N-cadherin antibodies (/Anti-Ncad) and stained for β-catenin. (C) Cells were treated with wortmannin (Wort, 50 nM) before plating for 2 hours on Ncad-Fc and labelled for β-catenin. Alternatively, the same experiment was performed with DA Rac1-transfected cells. Bars, 10 μm. (D,E) The histograms present the quantification of cell spreading and cadherin adhesions in the various conditions, expressed as a percent of the control conditions. At least 300 cells were counted in three independent experiments (except two for the cdc42 mutants).

formation of cadherin adhesions (Table 1). We did not detect any effect on cadherin-induced phenotypes by the broad-spectrum inhibitors of actomyosin contractility BDM or H7 (Table 1). Thus, the formation of cadherin adhesions is dependent on Rac1 activity and is inhibited by increased RhoA activity or contractility.

The PI 3-kinase inhibitors wortmannin (Fig. 4C,D) and LY294002 (data not shown) drastically inhibited cell spreading on Ncad-Fc and only 1% of attached cells exhibited a fried egg shape. The radial accumulations of β-catenin were formed normally at the remaining cell-to-substrate contact sites. Interestingly, the expression of DA Rac1 restored the spreading of wortmannin-treated cells (Fig. 4D). Thus, the lamellipodium extension induced by cadherin activation was controlled by a PI 3-kinase-Rac1 pathway. By contrast, the formation of cadherin adhesions was under the control of a Rac1-dependent, PI 3-kinase-independent pathway, further supporting the notion that the extension of nascent cadherin contacts and the maturation of cadherin adhesions are differentially regulated. Altogether, these results provided strong evidence for a two-step contribution of Rac1 in the formation and maturation of cadherin-mediated contacts.

p120 expression is necessary for the co-organization of cadherin complexes and actin cytoskeleton in cadherin adhesions

We assessed whether p120 could contribute to the PI 3-kinase-independent regulation of Rac1 activity in cadherin adhesion genesis. In order to address this question, we performed p120 silencing thanks to the production in the cells of short interfering (si)RNAs. C2 cells were transiently transfected with a mammalian expression vector (pSUPER) (Brummelkamp et al., 2002) containing a mouse p120-specific sequence in both

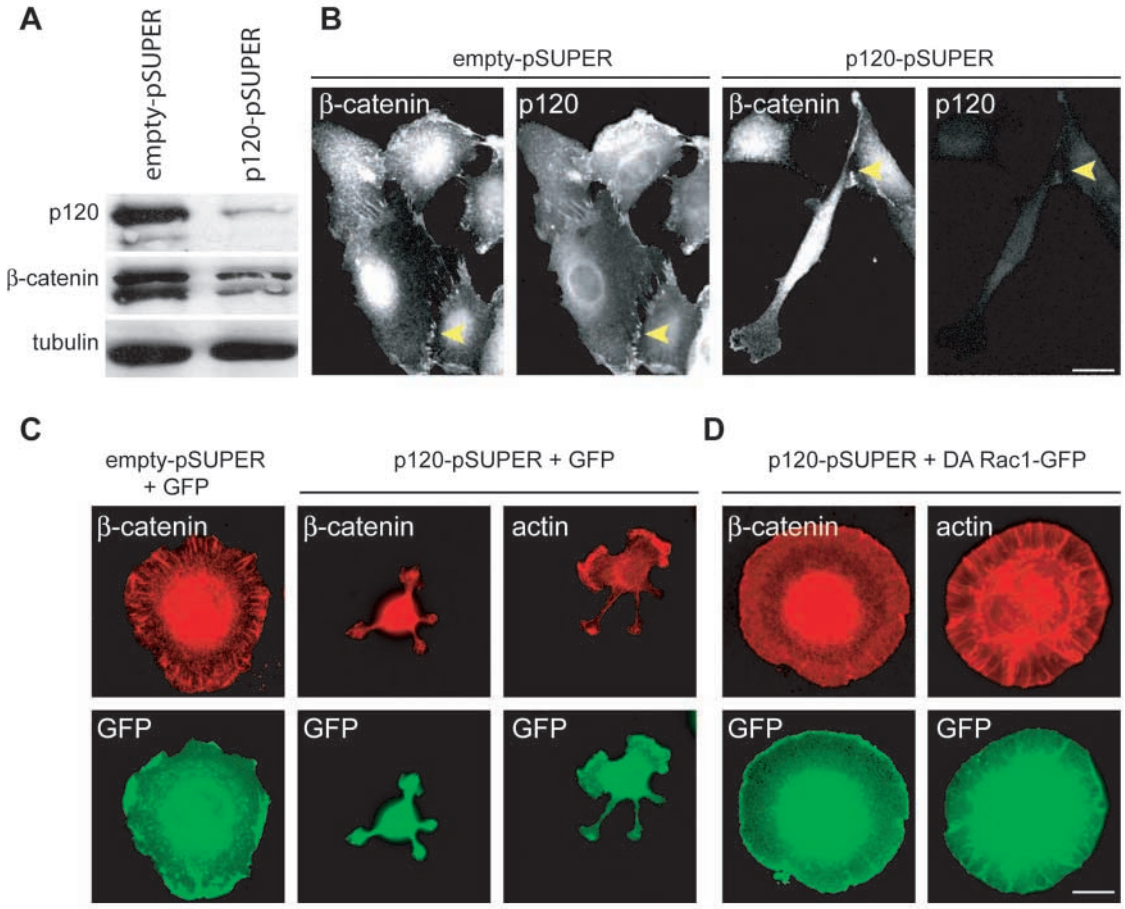

Fig. 5. p120 is necessary for both cell spreading on Ncad-Fc substrate and cadherin adhesion formation. (A) C2 cells were transfected either with siRNA p120-pSUPER or the empty pSUPER vectors, 16 hours prior to protein extraction. Twenty μg of protein extracts were separated on 7% SDS-PAGE and analysed by immunoblotting with anti-p120 and anti-β-catenin antibodies. Tubulin was used as a loading control. (B) p120-pSUPER-transfected cells and mock transfectants were fixed and immunostained for p120 and β-catenin. Note the very low level of p120 immunostaining in p120-pSUPER-transfected cells and the remaining accumulation of β-catenin at cell-cell contacts (arrows). (C) C2 cells double transfected with pEGFP and either p120-pSUPER or empty-pSUPER were plated on Ncad-Fc substrate for 2 hours and labelled with anti-β-catenin antibodies or Alexa-conjugated phalloidin. Note the absence of spreading of p120-pSUPER-transfected cells, contrasting with the normal spreading and cadherin adhesion formation of mock transfected cells. (D) C2 cells double transfected with the p120-pSUPER and DA Rac1-GFP were plated on Ncad-Fc and stained for β-catenin or actin. Notice the restored spreading of the cells. Bars, 10 μm.

sense and antisense orientations (I.G., M. Shtutman, S. Boguslavsky and A.B., unpublished). The ability of siRNA to switch off p120 expression in C2 cells was assessed by western blotting 16 hours post-transfection (Fig. 5A). Only a faint p120 immunoreactive band was detected in the extracts of transfected cells. The decrease in p120 expression was at least of 80% in these transiently transfected cultures. Interestingly, we also noticed a decrease of the level of β-catenin in protein extracts of p120 siRNA-expressing cells (Fig. 5A). β-catenin normally accumulated at cell-cell contacts, despite the highly reduced p120 immunostaining (Fig. 5B). When plated on Ncad-Fc, siRNA and GFP co-transfected C2 cells attached normally, but more than 90% of transfected cells failed to spread (Fig. 5C). Neither β-catenin nor actin were organized in radial structures, whereas these structures were typically observed in mock transfected cells, indicating that p120 is necessary for both cell spreading and cadherin adhesion formation.

Since the phenotype of siRNA-treated cells was reminiscent of that of DN Rac1-expressing cells, we wanted to know whether Rac1 could rescue the absence of p120. To this aim, we combined p120 RNA interference (RNAi) with DA Rac1 expression. The fried egg morphology was restored in the cells lacking p120 but expressing a constitutively active form of Rac1, as around 60% of double-transfected cells displayed a fried egg shape (Fig. 5D). Moreover, the actin cytoskeleton was organized in radial cables, resembling the one formed in control cells on Ncad-Fc. Despite the rescue of cell spreading and actin cytoskeleton organization, β-catenin was not radially structured in the lamellipodium, but instead remained diffuse in the zone where cadherin adhesions were normally formed (Fig. 5D). Thus, DA Rac1 could partially rescue for the depletion of p120 in the process of cell spreading and actin reorganization induced by N-cadherin activation. The presence of p120 itself is absolutely needed for the redistribution of cadherin-catenin complexes in cadherin adhesions.

To demonstrate further the crucial role of p120 in cadherin adhesion genesis, we took advantage of the properties of the human SW48 colon carcinoma cell line endogenously expressing E-cadherin, and recently characterized for its absence of functional p120 (Ireton et al., 2002). These cells

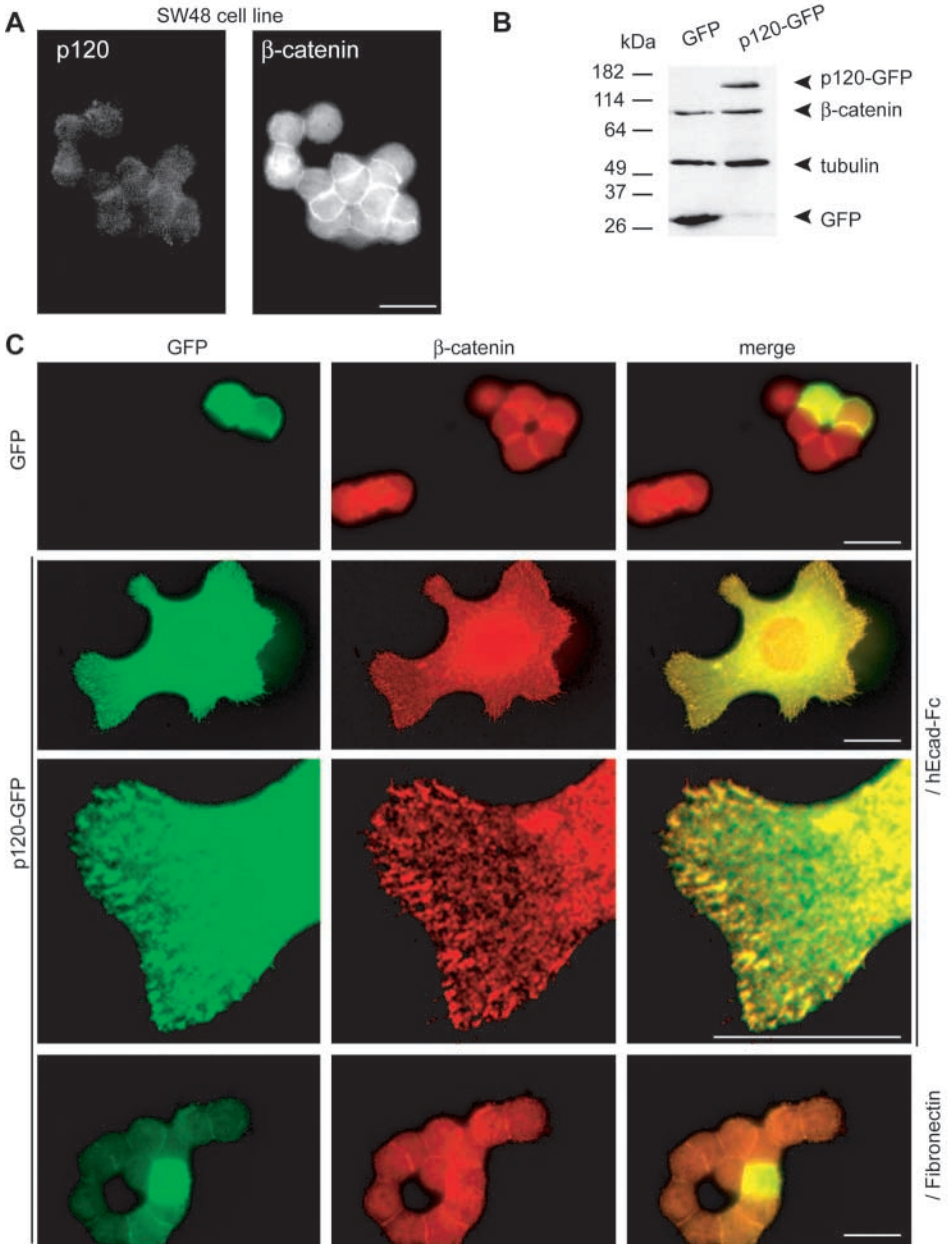

Fig. 6. The expression of p120 in epithelial SW48 cells is sufficient to induce cell spreading on hEcad-Fc and cadherin adhesions. (A) E-cadherin-expressing SW48 cells grown in standard conditions and immunostained for p120 and β-catenin. (B) Equal quantities (10 μg) of proteins extracted from SW48 cells transfected with GFP (lane 1) or p120-GFP (lane 2) were separated on 7% SDS-PAGE and immunoblotted with anti-p120, anti-GFP, anti-β-catenin antibodies, then with anti-tubulin antibodies as a loading control. (C) GFP- and p120-GFP-expressing SW48 cells were plated on hEcad-Fc (/hEcad-Fc) for 2 hours and immunostained for β-catenin. While GFP-expressing cells behave exactly as GFP-negative cells (top row), p120-GFP SW48 cells spread extensively on the hEcad-Fc substrate (second row). Concomitantly, all these spread cells organized β-catenin-positive radial structures in their lamellipodium (enlargement, third row). By contrast, p120-transfected SW48 cells did not spread better than untransfected cells on fibronectin (/Fibronectin). Bar, 10 μm.

did not spread on fibronectin, exhibited a compact shape without any membrane protrusion and only formed chains or small colonies of round cells that could however form cell-cell contacts and accumulate β-catenin at the cell-cell junctions (Fig. 6A). Interestingly, SW48 cells bound but did not spread on hEcad-Fc substrate. In order to evaluate the importance of the p120 defect in this phenotype, SW48 were transiently transfected with p120-GFP (Fig. 6B). p120-GFP-transfected SW48 cells spread extensively on hEcad-Fc, whereas SW48 cells expressing GFP alone remained unspread (Fig. 6C). The p120-induced lamellipodium extensions increased considerably the surface of the cell. Moreover, p120-GFP and β-catenin accumulated in small dots in the lamellipodium (Fig. 6C), reminiscent of radial structures observed in C2 cells. This indicated that the re-expression of p120 compensated for the defect in cell spreading and cadherin adhesion formation observed in SW48 cells. Altogether, our data show that p120 expression is both necessary and sufficient to rescue the extension of cadherin contacts and the dynamic reorganization of cadherin-catenin complexes and the actin cytoskeleton.

p120 membrane recruitment positively regulates Rac1-dependent cell spreading and cadherin adhesion formation

SW48 cells expressing p120-GFP at higher levels did not spread on hEcad-Fc (data not shown), indicating that p120 overexpression may alter cell spreading and cadherin adhesion formation. We further asked whether p120 overexpression might have a similar effect on the cell response to cadherin activation in the C2 cell model. The overexpression of p120-GFP in C2 cells induced the typical branching phenotype on fibronectin (data not shown) observed previously in other cell types (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). The spreading on Ncad-Fc of C2 cells overexpressing p120 was strongly inhibited (Fig. 7A) and fewer than 25% of the cells presented a typical fried egg shape. p120 overexpression also prevented the formation of cadherin adhesions, having similar inhibitory effects to the silencing of p120. In order to assess whether the expression of DA Rac1 could also compensate for the deleterious effects of p120 overexpression, C2 cells were double transfected with p120-GFP and DA Rac1 (Fig. 7A,C). Both cell spreading and

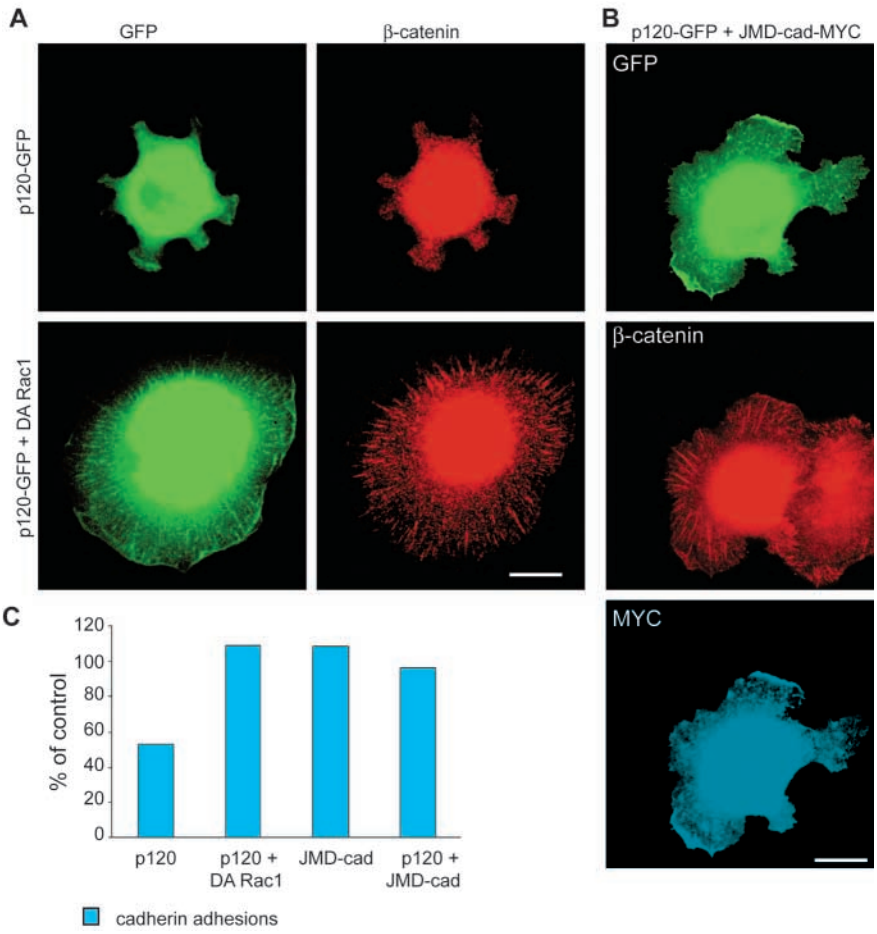

Fig. 7. Cell spreading and cadherin adhesions are inhibited by p120 overexpression in C2 cells. (A) C2 cells expressing p120-GFP alone or together with DA Rac1 were plated on Ncad-Fc for 2 hours before labelling with anti-β-catenin antibodies. (B) Cells were double transfected with p120-GFP and the Myc-tagged JMD-cad construct, then seeded on Ncad-Fc for 2 hours and labelled with anti-β-catenin and anti-Myc antibodies. Bar, 10 μm. (C) The histogram presents the quantification of cadherin adhesions in the various conditions, expressed as a percent of the control conditions. At least 200 cells were counted in two independent experiments.

cadherin adhesions were rescued in these cells, suggesting that increased levels of p120 might act negatively on the Rac1 pathways needed for cadherin-induced cell spreading and cadherin adhesion formation.

Since both the silencing of p120 and its overexpression altered cadherin contact extension and maturation, we hypothesized that the regulation of the cell response by p120 could instead be associated with its subcellular localization. Thus, we played more precisely on the balance of the cytoplasm versus membrane pools of p120. C2 cells were double transfected with p120-GFP and a chimera comprising the extracellular and transmembrane domains of the *Drosophila* Torso protein fused to the juxtamembrane p120-binding domain of C-cadherin (JMD-cad) designed to trap p120 at the cell membrane (Fig. 7B). The expression of JMD-cad alone had no obvious effect on β-catenin-containing radial structures (Fig. 7C), indicating that this chimera did not perturb cadherin contact formation. Cells co-expressing p120 and JMD-cad spread and formed typical radial structures, indicating that the phenotype induced by p120 overexpression was partially restored by membrane trapping of p120 molecules. Thus, both cadherin-induced cell spreading and cadherin adhesions might be affected by a modification of the relative amount of p120 at the plasma membrane versus cytosol.

To check directly for p120 recruitment at the membrane, we performed subcellular fractionation and immunoblotting on C2 cells transiently transfected with either p120-GFP, JMD-cad or both (Fig. 8A). p120-GFP-transfected cells presented levels of exogenous p120 in their cytosol comparable with endogenous p120 levels, indicating that total p120 cytosolic content was approximately doubled in the cells. The expression of JMD-cad strongly increased the quantity of both endogenous and exogenous p120 in membrane fractions (Fig. 8A, membrane, lanes 2, 3). As expected, only a very faint β-catenin band was detected in C2 cell cytosolic fractions, whereas β-catenin was massively recovered in membrane fractions. JMD-cad expression led to a dramatic increase in the level of β-catenin in membrane fractions. These results clearly show that, related to its ability to rescue for the deleterious effect of p120 overexpression, the expression of JMD-cad induces the recruitment of p120 in membrane pools. Moreover, its expression also leads to β-catenin stabilization, which might reflect an increased stability of cadherin-mediated contacts.

In order to get insight into the link between p120 and Rac1, we searched for a co-translocation of Rac1 at the plasma membrane. It was shown that the targeting of Rac1 to the membrane is a crucial process for the regulation of its activity (Fleming et al., 1996; Michaelson et al., 2001). We thus investigated changes in the level of membrane-associated Rac1 (Fig. 8B). When JMD-cad was expressed in C2 cells alone or with p120-GFP, the level of total Rac1 detected in membrane fractions was highly augmented and directly followed the recruitment of p120 at the membrane. Conversely, the overexpression of p120-GFP did not support increased Rac1 accumulation at the membrane (Fig. 8B). These data indicate that the accumulation of p120 at the plasma membrane, which favours lamellipodium extension and cadherin adhesion formation, is associated with an increase in membrane-associated Rac1. Taken together, these findings indicate that the cadherin-dependent recruitment of p120 at the plasma membrane plays an essential role in cadherin contact formation and maturation, probably through the local mobilization of Rac1. Alternatively, the partition of p120 between membrane and cytoplasmic pools could be involved in the regulation of such processes.

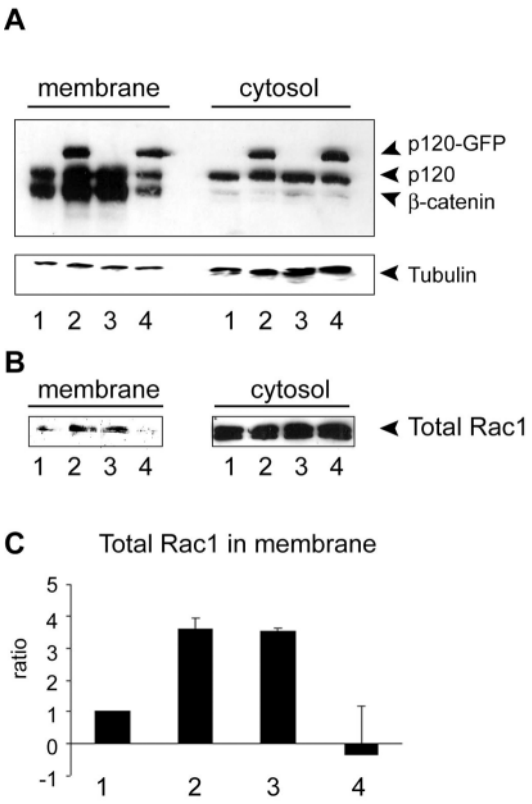

Fig. 8. Co-enrichment of p120 and Rac1 in membrane fractions upon JMD-cad expression. C2 cells were transiently transfected with GFP (lane 1), p120-GFP plus JMD-cad (lane 2), JMD-cad (lane 3) or p120-GFP (lane 4), then cultured for 16 hours, harvested and submitted to subcellular fractionation. (A) Membrane-associated and cytosolic proteins (10 μg and 20 μg, respectively) were separated on 7% SDS-PAGE and blotted with anti-p120, anti-β-catenin antibodies, then with anti-tubulin antibodies as a loading control. The results are representative of two independent experiments. (B) The same extracts were separated on 13% SDS-PAGE and immunoblotted against Rac1. (C) The JMD-cad expression induced a threefold increase in Rac1 recovered in membrane fractions. The densitometry analysis was performed with the NIH Image 2.0 software on two independent experiments.

Discussion

Cadherins are major constituents of intercellular junctions mediating cell adhesion through homophilic interactions of their extracellular domain and anchoring of their cytoplasmic domain to the actin cytoskeleton. Here, the mechanisms linking these two aspects of cadherin function were dissected through the formation of contacts between N-cadherin-expressing cells and an N-cadherin homophilic substrate. The cell response to cadherin activation was characterized by two striking features that could be uncoupled and that rely on distinct signalling pathways: (1) the induction of lamellipodium protrusions; and (2) the reorganization of cadherin-catenin complexes and actin filaments in cadherin adhesions. We further showed that the recruitment of p120 at the membrane is absolutely required for these two cell responses, contributing to Rac1-dependent extension and maturation of cadherin-mediated contacts.

The lamellipodium protrusion: a cadherin-triggered p120-PI 3-kinase-Rac1 pathway and a way to extend cadherin contacts

One of the responses to N-cadherin liganding was a strong lamellipodium induction, extending a previous report on E-cadherin-expressing CHO cells spread on hEcad-Fc (Kovacs et al., 2002a). Lamellipodium spreading and nascent contacts appear intimately associated and imply a crosstalk between cadherin liganding and actin cytoskeleton dynamics. The adhesive engagement of new cadherins takes place at the tip of the lamellipodium protruding on the Ncad-Fc substrate, which can be considered as a preferential site of new contact formation. The extension of cadherin contacts is associated with actin nucleation as previously reported and new actin assembly [our observations and Kovacs et al. (Kovacs et al., 2002b)], which might provide the driving force for lamellipodium protrusion. We further observed that N-cadherin-triggered lamellipodium extension was dependent on both PI 3-kinase and Rac1. In this regard, lamellipodium extension sustained by cadherin liganding appears to rely on a very general signal mechanism that also sustains chemoattractant-, growth factor- or integrin-induced lamellipodium extensions (Small et al., 2002). Nevertheless, our own data and previous data (Kovacs et al., 2002a) show that cadherin engagement is the trigger of the PI 3-kinase-, Rac1-dependent lamellipodium protrusion. Lamellipodium extension was also described as a crucial event that takes place at newly formed cell-cell contacts in MDCK cells (Ehrlich et al., 2002). Interestingly, this cell response is not induced by antibody-mediated receptor crosslinking, but requires cadherin homophilic liganding.

The p85 catalytic subunit of PI 3-kinase interacts with β-catenin (Pece et al., 1999; Woodfield et al., 2001) and is recruited independently of Rac1 and activated by E-cadherin liganding (Kovacs et al., 2002a). We show here that PI 3-kinase inhibitors prevent C2 cell spreading without affecting cadherin adhesions, both processes being dependent on Rac1. Spreading was restored in wortmannin-treated cells by the expression of dominant-active Rac1, indicating that PI 3-kinase is involved upstream of Rac1 in cell spreading. We further show that cadherin-induced lamellipodium extension also requires the expression and recruitment of p120 at the membrane, probably to control the local regulation of Rac1 activity. Very recently, minimum mutation of the JMD domain pointed towards a role of p120-E-cadherin interactions in E-cadherin-triggered lamellipodium spreading and the associated Rac1 signalling pathway, independently of PI 3-kinase (Goodwin et al., 2003). Thus, in addition to the PI 3-kinase-Rac1 signalling initiated by cadherin activation, which may represent a common pathway in different systems to activate the actin nucleation machinery, the extension of cadherin contacts also requires p120, contributing cooperatively to Rac1 activity. Altogether, these data show that cadherin homophilic liganding initiates a p120, PI 3-kinase-Rac1-dependent lamellipodium induction that promotes active extension of nascent contacts in tight association with actin polymerization.

Cadherin adhesions: a p120-Rac1-dependent co-organization of adhesion complexes and actin cytoskeleton

One of our major findings is that myogenic C2 cells spread on

Ncad-Fc substrate reorganize N-cadherin, catenins, actin filaments and signalling proteins in highly patterned radial structures: the cadherin adhesions. Cadherin adhesions were observed with a variety of cell types. However, they were nicely observed in cells with a very flat morphology and a well-organized actin network like C2 or HeLa cells. By contrast, they were smaller in cells that displayed a round morphology and less organized phalloidin labelling of F-actin, like S180 or SW48 cells. Despite these morphological differences, cadherin adhesion formation appeared to constitute a general response to cadherin activation.

It is overwhelming that, at cell-ECM contacts, integrins through bidirectional transduction of mechanical and chemical signals organize adhesion complexes, actin cytoskeleton and signalling complexes in adhesive structures known as matrix adhesions (Zamir et al., 2000). We describe here amazingly similar co-organization of cadherin-associated complexes and actin cytoskeleton. Indeed, cadherin adhesions present the same radial distribution as newly formed matrix adhesions (Geiger et al., 2001). Whereas matrix adhesions evolve in focal adhesions associated with the formation of Rho-dependent stress fibres after a primary Rac1-dependent phase (Chrzanowska-Wodnicka and Burridge, 1996), cadherin adhesions conserved their architecture for hours, suggesting different mechanisms of regulation. This correlates well with the fact that the expression of dominant-active RhoA or an increase in cell contractility, known to stimulate focal adhesions, blocked cadherin adhesion formation. Altogether, our data suggest that cadherin adhesions are primarily dependent on Rac1 activity.

For cadherin adhesions to occur, N-cadherin molecules that initially bind to the unpatterned Ncad-Fc substrate at the tip of the lamellipodium should redistribute to form patterned structures, spatially distinct from sites of nascent contacts. This clustering of adhesion complexes requires a tight co-ordination with actin cytoskeleton, but cadherin adhesions did not represent active sites of actin nucleation. PI 3-kinase is not involved either in this reorganization of cadherin-catenin complexes, which should occur independently of lamellipodium protrusion. Rac1 activity was nevertheless required for the recruitment of cadherin-catenin complexes. We showed previously that cadherin liganding triggers their anchoring and transport by the actin cytoskeleton tread-milling (Lambert et al., 2002). This process might play an essential role in the gathering of cadherins in cadherin adhesions. Cadherin adhesion formation also requires p120. Dominant-active Rac1 rescued the defects in cell spreading and actin cytoskeleton organization induced by the depletion in p120, but was unable to restore the recruitment of cadherin-catenin complexes in radial structures. These data suggest that p120 is not only necessary to regulate Rac1 activity, but should also act independently on cadherin clustering (Yap et al., 1998). Moreover, neither anti-N-cadherin antibody itself nor in combination with the activated form of Rac1 were sufficient to promote cadherin reorganization. This unexpected result may rely on the high stability of antigen-antibody interactions preventing a fast dissociation-reassociation of N-cadherin from its immobilized ligand. Thus, the recruitment of adhesion complexes in cadherin adhesions may need intracellularly the Rac1-dependent anchoring of cadherins to the actin cytoskeleton (Lambert et al., 2002) and extracellularly the fast dissociation-reassociation of cadherin-cadherin adhesive dimers (Perret et al., 2002).

Regulation of cadherin-mediated cell contact extension and maturation by p120

One puzzling question is how cadherin liganding may signal to activate these two independent cell responses. We show here that the silencing of p120 totally inhibits lamellipodium extension and cadherin adhesion formation. This essential role of p120 was further confirmed in SW48 cells bearing a nonsense mutation in the p120 gene (Ireton et al., 2002). In contrast to MDCK cells, these E-cadherin-expressing cells are unable to spread on hEcad-Fc. The expression of p120 in these cells promotes E-cadherin-dependent spreading and cadherin-catenin complex reorganization, in agreement with the specific requirement of p120 for cadherin-mediated contact extension and maturation. We show here by p120 interference or re-expression that this catenin was required at the plasma membrane for the Rac1-dependent extension and the maturation of cadherin-mediated contacts. Independently, Goodwin et al. showed that mutation of the p120-binding domain of E-cadherin prevents the ability of E-cadherin to induce Rac1 activation (Goodwin et al., 2003). The depletion of p120 in C2 cells leads to a decrease in β-catenin and conversely the membrane trapping of p120 stabilizes β-catenin at the membrane, indicating that, at the global cell level, p120 favours the stabilization of cadherin-catenin complexes, further supporting a role in contact maturation.

The overexpression of p120 inhibits both cell spreading and cadherin adhesion formation, suggesting that these cell responses are tightly dependent on the level of p120 expression. p120 has been shown to shuttle from the cytosol to the plasma membrane upon cadherin expression or activation affecting the regulation of small GTPases (Grosheva et al., 2001). The alteration of the cellular responses by p120 overexpression may thus depend on the relative amount of p120 recruited at the plasma membrane versus the cytosol. The trapping of p120 at the membrane that counteracts the deleterious effect of its overexpression increases the level of Rac1 at the plasma membrane. We hypothesized that p120 recruitment at the membrane may signal directly or indirectly the local activation of Rac1, either by compartmentalization of its activity or by dissociation from cytoplasmic Rho-guanine dissociation inhibitor (GDI) (Fleming et al., 1996; Michaelson et al., 2001). Cadherin-dependent recruitment of p120 at the plasma membrane, through local Rac1 activation, may thus initiate the signalling pathways leading to lamellipodium protrusion and cadherin adhesion formation. The overexpression of p120 would have a negative effect on the cell response by preventing or competing with the cadherin-dependent recruitment of Rac1 at the cell membrane. Cytosolic p120 may act through scavenging specific guanine nucleotide exchange factors (GEFs) in the cytoplasm, or participating itself in a Rho-GDI complex. Such a modulation of the membrane/cytosolic balance of p120 may have a physiological significance in the normal regulation of cadherin function.

In conclusion, upon activation, cadherins act as nucleation centres to organize scaffolding molecules, actin cytoskeleton and cell-signalling machinery implicated in the control of cell shape, cell migration and cell fate, in which the catenin p120

plays a major role. The molecular mechanisms underlying both the initiation of cadherin-mediated contact, their extension and maturation in higher order organizations described here are likely to play a crucial role in the formation of actual cell-cell contacts.

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References

Adams, C. L. and Nelson, W. J. (1998). Cytomechanics of cadherin-mediated cell-cell adhesion. *Curr. Opin. Cell Biol.* **10**, 572-577.

Anastasiadis, P. Z., Moon, S. Y., Thoreson, M. A., Mariner, D. J., Crawford, H. C., Zheng, Y. and Reynolds, A. B. (2000). Inhibition of RhoA by p120 catenin. *Nat. Cell Biol.* **2**, 637-644.

Anastasiadis, P. Z. and Reynolds, A. B. (2001). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J. Cell Sci.* **113**, 1319-1334.

Aono, S., Nakagawa, S., Reynolds, A. B. and Takeichi, M. (1999). $p120^{ctn}$ acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J. Cell Biol.* **145**, 551-562.

Braga, V. M. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.

Brieher, W. M., Yap, A. S. and Gumbiner, B. M. (1996). Lateral dimerization is required for homophilic binding activity of C-cadherin. *J. Cell Biol.* **135**, 487-496.

Brummelkamp, T. R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553.

Charrasse, S., Meriane, M., Communale, F., Blangy, A. and Gauthier-Rouvière, C. (2002). N-cadherin-dependent cell-cell contact regulates Rho GTPases and β-catenin localization in mouse C2C12 myoblasts. *J. Cell Biol.* **158**, 953-965.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403-1415.

Ehrlich, J. S., Hansen, M. D. and Nelson, W. J. (2002). Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. *Dev. Cell* **3**, 259-270.

Fleming, I. N., Elliott, C. M. and Exton, J. H. (1996). Differential translocation of Rho family GTPases by lysophoshatidic acid, endothelin-1, and platelet-derived growth factor. *J. Biol. Chem.* **271**, 33067-33073.

Friedlander, D. R., Mège, R. M., Cunningham, B. A. and Edelman, G. M. (1989). Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces. *Proc. Natl. Acad. Sci. USA* **86**, 7043-7047.

Geiger, B., Bershadsky, A. D., Pankov, R. and Yamada, K. M. (2001). Transmembrane extracellular matrix-cytoskeleton crosstalk. *Nat. Rev. Mol. Cell. Biol.* **2**, 793-805.

Goodwin, M., Kovacs, E. M., Thoreson, M. A., Reynolds, A. B., and Yap, A. S. (2003). Minimal mutation of the cytoplasmic tail inhibits the ability of E-cadherin to activate rac but not PI3-kinase. Direct evidence of a role for cadherin-activated Rac signaling in adhesion and contact formation. *J. Biol. Chem.* **278**, 20533-20539.

Grosheva, I., Shtutman, M., Elbaum, M. and Bershadsky, A. D. (2001). p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link between cell-cell contact formation and regulation of cell locomotion. *J. Cell Sci.* **114**, 695-707.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.

Hansen, M. D., Ehrlich, J. S. and Nelson, W. J. (2002). Molecular mechanism for orienting membrane and actin dynamics to nascent cell-cell contacts in epithelial cells. *J. Biol. Chem.* **277**, 45371-45376.

Hynes, R. O. (1999). Cell adhesion: old and new questions. *Trends Cell Biol.* **9**, 33-37.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Ireton, R. C., Davis, M. A., van Hengel, J., Mariner, D. J., Barnes, K., Thoreson, M. A., Anastasiadis, P. Z., Matrisian, L., Bundy, L. M., Sealy, L. et al. (2002). A novel role for p120 catenin in E-cadherin function. *J. Cell Biol.* **159**, 465-476.

Kovacs, E. M., Ali, R. G., McCormack, A. J. and Yap, A. S. (2002a). E-cadherin homophilic ligation directly signals through Rac and Phosphatidyl 3-kinase to regulate adhesive contacts. *J. Biol. Chem.* **277**, 6708-6718.

Kovacs, E. M., Goodwin, M., Ali, R. G., Paterson, A. D. and Yap, A. S. (2002b). Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct assembly in nascent adhesive contacts. *Curr. Biol.* **12**, 379-382.

Lambert, M., Padilla, F. and Mège, R. M. (2000). Immobilized dimers of N-cadherin-Fc chimera mimic cadherin-mediated cell contact formation: Contribution of both inside-out and outside in signals. *J. Cell Sci.* **113**, 2207-2219.

Lambert, M., Choquet, D. and Mège, R. M. (2002). Dynamics of ligand induced, Rac1-dependent anchoring of cadherins to the actin cytoskeleton. *J. Cell Biol.* **157**, 469-479.

Mary, S., Charrasse, S., Meriane, M., Comunale, F., Travo, P., Blangy, A. and Gauthier-Rouvière, C. (2002). Biogenesis of N-cadherin-dependent cell-cell contacts in living fibroblasts is a microtubule-dependent kinesin-driven mechanisms. *Mol. Biol. Cell* **13**, 285-301.

Matsuzaki, F., Mège, R. M., Jaffe, S. H., Friedlander, D. R., Gallin, W. J., Goldberg, J. I., Cunningham, B. A. and Edelman, G. M. (1990). cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in transfected S180 cells. *J. Cell Biol.* **110**, 1239-1252.

Mège, R. M., Matsuzaki, F., Gallin, W. J., Goldberg, J. I., Cunningham, B. A. and Edelman, G. M. (1988). Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* **85**, 7274-7278.

Michaelson, D., Silletti, J., Murphy, G., D'Eustachio, P., Rush, M. and Philips, M. R. (2001). Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *J. Cell Biol.* **152**, 111-126.

Nobes, C. D. and Hall, A. (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. *Cell* **14**, 1583-1589.

Noren, N. K., Liu, B. P., Burridge, K. and Kreft, B. (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J. Cell Biol.* **150**, 567-579.

Noren, N. K., Niessen, C. M., Gumbiner, B. M. and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. *J. Biol. Chem.* **276**, 33305-33308.

Ohkubo, T. and Ozawa, M. (1999). $p120^{ctn}$ binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. *J. Biol. Chem.* **274**, 21409-21415.

Ozawa, M. and Kemler, R. (1998). The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. *J. Cell Biol.* **142**, 1605-1613.

Pece, S., Chiariello, M., Murga, C. and Gutkind, S. (1999). Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. *J. Biol. Chem.* **274**, 19347-19351.

Perret, E., Benoliel, A. M., Nassoy, P., Pierres, A., Delmas, V., Thiery, J.-P., Bongrand, P. and Feracci, H. (2002). Fast dissociation kinetics between individual E-cadherin fragments revealed by flow chamber analysis. *EMBO. J.* **21**, 2537-2546.

Radice, G. L., Rayburn, H., Matsunami, H., Knudsen, K. A., Takeichi, M. and Hynes, R. O. (1997). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* **181**, 64-78.

Sako, Y., Nagafuchi, A., Tsukita, S., Takeichi, M. and Kusumi, A. (1998).

Cytoplasmic regulation of the movement of E-cadherin on the free cell surface was studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. *J. Cell Biol.* **140**, 1227-1240.

Small, V. J., Stradal, T., Vignal, E. and Rottner, K. (2002). The lamellipodium: where motility begins. *Trends Cell Biol.* **12**, 112-120.

Stevenson, B. R., Gallin, W. J. and Paul, D. L. (1992). *Cell-cell interactions: A practical approach. The practical approach series* (ed. D. Rickwood and B. D. Hames), Oxford: Oxford University Press.

Takeichi, M. (1977). Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* **75**, 464-474.

Takeichi, M. (1995). Morphogenic roles of classic cadherins. *Curr. Opin. Cell Biol*. **7**, 619-627.

Tepass, U., Truong, K., Godt, D., Ikura, M. and Peifer, M. (2000). Cadherins in embryonic and neural morphogenesis. *Nat. Rev. Mol. Cell. Biol.* **1**, 91-100.

Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. H. and Reynolds, A. B. (2000). Selective uncoupling of p120^{ctn} from E-cadherin disrupts strong adhesion. *J. Cell Biol.* **148**, 189-201.

Uchida, N., Honjo, Y., Johnson, K. R., Wheelock, M. J. and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. *J. Cell Biol.* **135**, 767-779.

Umbhauer, M., Penzo-Mendez, A., Clavilier, L., Boucaut, J. and Riou, J. F. (2000). Signaling specificities of fibroblast growth factor receptors in early *Xenopus* embryo. *J. Cell Sci.* **113**, 2865-2875.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209-219.

Woodfield, R. J., Hodgkin, M. N., Akhtar, N., Morse, M. A., Fuller, K. J., Saqib, K., Thompson, N. T. and Wakelam, M. J. (2001). The p85 subunit of phosphoinositide 3-kinase is associated with beta-catenin in the cadherin-based adhesion complex. *Biochem. J.* **360**, 335-344.

Wu, J., Mariner, D. J., Thoreson, M. A. and Reynolds, A. B. (1998). Production and characterization of monoclonal antibodies to the catenin p120^{ctn}. *Hybridoma* **17**, 175-183.

Yaffe, D. and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**, 725-727.

Yap, A. S., Brieher, W. M. and Gumbiner, B. M. (1997a). Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev. Cell Dev. Biol.* **13**, 119-146.

Yap, A. S., Brieher, W. M., Pruschy, M. and Gumbiner, B. M. (1997b). Lateral clustering of the adhesive ectodomain: a fundamental determinant of cadherin function. *Curr. Biol.* **7**, 308-315.

Yap, A. S., Niessen, C. M. and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherincytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120^{ctn}. *J. Cell Biol.* **141**, 779-789.

Yap, A. S. and Kovacs, E. M. (2003). Direct cadherin-activated cell signaling: a view from the plasma membrane. *J. Cell Biol.* **160**, 11-16.

Zamir, E., Katz, M., Posen, Y., Erez, N., Yamada, K. M., Katz, M., Lin, S., Lin, D. C., Bershadsky, A. D. and Geiger, B. (2000). Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat. Cell Biol.* **2**, 191-196.