Arp2/3 Activity Is Necessary for Efficient Formation of E-cadherin Adhesive Contacts*†

Suzie Verma‡‡, Annette M. Shewan‡, Jeannie A. Scott‡, Falak M. Helwani‡, Nicole R. den Elzen††, Hiroaki Miki‡‡‡, Tadaomi Takenawa§§§§, and Alpha S. Yap†††

From the †Institute for Molecular Bioscience, ‡School for Biomedical Science, The University of Queensland, St. Lucia, Queensland 4072, Australia. Tel.: 61-7-3346-2103; Fax: 61-7-3346-2101; E-mail: a.yap@imb.uq.edu.au.

Classical cadherin adhesion molecules are fundamental determinants of cell-cell recognition that function in cooperation with the actin cytoskeleton. Productive cadherin-based cell recognition is accompanied by a distinct morphological process of contact zone extension, where limited initial points of adhesion are progressively expanded into broad zones of contact. We recently demonstrated that E-cadherin ligation recruits the Arp2/3 actin nucleator complex to the plasma membrane in regions where cell contacts are undergoing protrusion and extension. This suggested that Arp2/3 might generate the protrusive forces necessary for cell surfaces to extend upon one another during contact assembly. We tested this hypothesis in mammalian cells by exogenously expressing the CA region of N-WASP. This fragment, which potently inhibits Arp2/3-mediated actin assembly in vitro, also effectively reduced actin assembly at cadherin adhesive contacts. Blocking Arp2/3 activity by this strategy profoundly reduced the ability of cells to extend cadherin adhesive contacts but did not affect cell adhesiveness. These findings demonstrate that Arp2/3 activity is necessary for cells to efficiently extend and assemble cadherin-based adhesive contacts.

This work was supported by the Australian Research Council and Human Frontiers Science Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The on-line version of this article (available at http://www.jbc.org) contains Movie 1.
† Peter Doherty Fellow of the National Health and Medical Research Council of Australia.
‡‡ International Senior Medical Research Fellow of the Wellcome Trust (UK); To whom correspondence should be addressed: Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia. Tel.: 61-7-3346-2103; Fax: 61-7-3346-2101; E-mail: a.yap@imb.uq.edu.au.
MATERIALS AND METHODS

**Plasmids**—To generate pIRESpuro-hE-Cad, hEcad/pcDNA3 (a kind gift from C. Gottiardi and Barry Gumbiner) (16) was digested with HindIII and XbaI and the coding sequence fragment for human E-cadherin subcloned into pIRESpuro. pEGFP-CA was derived from FLAGtagged N-WASP-CA (17), with an N-terminal EGFP tag substituted for the FLAG tag. pEGFP-CA was digested with Nhel and XbaI, and the coding sequence fragment was subcloned into pUHD10-3-hygro (a kind gift from Dr. C. Gottiardi) digested with XbaI to yield pUHD10-3 Hygro/EGFP-CA.

**Cell Culture and Protein Purification**—To generate CHO cell lines containing both E-cadherin and the Tet-OFF transcriptional regulator, CHO-AA8 cells stably expressing the Tet-OFF transcriptional regulator (Clontech) were transfected with pIRESpuro-hE-Cad using Lipofectamine (Invitrogen). Individual clones (hE-CHO/Tet cells) were picked and expanded after incubation with puromycin (10 μg/ml). Lines were chosen that showed E-cadherin expression at levels (by Western blotting) similar to those observed in MCF-7 cells (not shown) as well as CHO-Tk lines stably expressing human E-cadherin (18).

hE-CHO/Tet cells were then co-transfected by lipofection with pUHD10-3 Hygro/EGFP-CA. Initial selection was conducted in the presence of doxycycline (1 μg/ml) and hygromycin (200 μg/ml). Hygromycin-resistant clones were picked and expanded in the absence of doxycycline to induce expression of EGFP-CA. EGFP-expressing populations were then collected by fluorescence-activated cell sorting and subcloned. MDCR cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. hE-FC was prepared and purified as described previously (14, 18).

**Antibodies**—The primary antibodies used are as follows: 1) mouse mAb HEC1D-1 against human E-cadherin (a kind gift from Dr. Peggy Wheelock with the permission of Dr. Masatoshi Takeichi); 2) rabbit pAb against human E-cadherin, raised against hE-Fc (19); 3) mouse mAb directed against the conserved cytoplasmic tail of human E-cadherin (Transduction Laboratories); 4) rabbit pAb against β-catenin; 5) rabbit pAb against α-catenin (both kind gifts from Dr. Barry Gumbiner, University of Virginia); 6) mouse mAb pp120 against p120-catenin (Transduction Laboratories); 7) mouse mAb against GFP (Roche Applied Science); 8) polyclonal antibody against α14 was raised in rabbits immunized with the diphertheria toxoid-conjugated peptide EKKEMKTITITGKTF (based on amino acids 285–298) (20). Species-specific secondary antibodies were obtained from Molecular Probes. F-actin was identified with TRITC-phalloidin (Sigma).

**Adhesion Assays**—Luminar flow detachment adhesion assays were performed as described previously (21). Longer term detachment assays were performed as described previously (21) with modifications. In brief, nitrocellulose-coated 6-well plates were incubated with hE-FC (in Hanks’ balanced salt solution, HBSS, containing 5 mM CaCl2) overnight at 4°C, and then blocked with bovine serum albumin (10 mg/ml, 2 h, 4°C). Cells were isolated by incubation for 10 min in 0.01% (w/v) trypsin (Sigma) in HBSS containing 5 mM CaCl2. Freshly isolated cells were allowed to attach to the substrata for 90 min at 37°C in a CO2 incubator and were then subjected to detachment by systematic pipetting. For this, five regions in each well (the four quadrants and center) were washed twice with 200 μl of HBSS/CaCl2 delivered using a stand-mounted pipette. Cells remaining adherent to the wells were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10 mg/ml) dissolved in Me2SO and read at A595 in a microplate reader. Cellular content in wells after pipetting was compared with the cellular content of wells prepared under identical conditions but was not subjected to pipetting (yielding the total number of cells plated in each well). Cell aggregation assays (22) and hE-FC-coated bead assays (21) were performed as described previously.

To quantitate cell extension on hE/FC-coated substrata, phalloidin stained images were analyzed using Metamorph (version 5.0; Universal Imaging). The outer margins of cadherin-based lamellipodia were identified by their characteristic broad pattern of phalloidin staining. For each cell the lengths of each outer margin were measured and summed, and the total number of the outer margins was expressed as a percentage of the perimeter of that cell (lamellipodial index). Cell contact formation following manipulation of extracellular calcium was quantitated in E-cadherin-stained specimens as described previously (19).

**Actin Accumulation Assay**—Actin accumulation at cadherin-coated or ConA-coated beads was measured by quantifying fluorescence intensity of phalloidin-stained specimens using Image J (version 1.30). Images of phalloidin staining were taken in the plane of adhesion of the beads, and care was taken to ensure that all pixel values were within the linear range of the camera. For each cell the region of phalloidin staining immediately adjacent to, and extending from, the beads was manually identified using the region of interest function in Image J. To correct for background actin staining, independent of adhesive binding, the average pixel intensity in a region of the surface clearly separate from the beads was measured, and this was then subtracted from each pixel value in the sample. The mean corrected pixel intensity and number of pixels within the region of interest was then measured, and the product of these was used as an index of phalloidin accumulation.

**G-actin Incorporation Assay**—Incorporation of Alexa 568-labeled G-actin (Molecular Probes) into saponin-permeabilized cells was performed as described previously (14). Quantification of G-actin incorporation at cell-cell contacts of MDCK cells was performed with Metamorph (Universal Imaging), using epi-illumination images taken with identical exposures and camera settings. Fluorescence intensity at cell-cell contacts was assessed by measuring the mean intensity in 12 × 12 pixel boxed regions placed at cell-cell contacts. To correct for inter-sample variation and photobleaching, fluorescence intensity at contacts between microinjected test cells was normalized to the fluorescence intensity in contacts between uninjected cells within the same coverslip.

**Microscopy**—Epi-illumination fluorescence microscopy of fixed specimens was performed using Olympus AX70 or IX81 microscopes equipped ×100, 1.40 NA objectives. Images were acquired with Hamamatsu Orca I or Orca I-ER cameras driven by Metamorph software version 5.0 (Universal Imaging). Total internal reflection fluorescence microscopy was performed using an Olympus IX81 microscope and ×60, 1.45 NA objective illuminated with a 10-milliwatt argon laser (Melles-Griot). For live cell microscopy, cells were maintained in an individual isolated incubation chamber at 37°C. Background correction and contrast adjustment of raw images were performed in either Adobe Photoshop (version 7) or ImageJ (version 1.30). Panels were assembled in Adobe Photoshop.

RESULTS

**Generation of Cells Inducibly Expressing the CA Domain of N-WASP**—To inhibit specifically Arp2/3 activity in cells, we utilized an EGFP fusion protein bearing the C-terminal domains of N-WASP (EGFP-CA), a fragment that potently inhibited Arp2/3 activity in vitro (15) blocked Rac-induced ruffling in vivo (17). CHO-AA8 cells stably co-expressing the Tet-OFF transregulator and human E-cadherin (hE-CHO/Tet cells) were transfected with pTRE2-EGFP-CA, where the EGFP-CA coding region is placed under control of the Tet promoter. Selection-resistant clonal lines expressing EGFP-CA (hE-CHO/Tet cells) were picked by fluorescence-activated cell sorting and subcloned. Low levels of EGFP-CA were detectable by immunoblotting (but not by immunofluorescence) in uninduced cells, but expression increased considerably after doxycycline was removed from the media (Fig. 1A), typically peaking after ~3 days.

Induction of EGFP-CA did not affect the total cellular levels of E-cadherin, β-, α-, or p120-catenin compared with either uninduced cells or with hE-CHO/Tet cells not transfected with EGFP-CA (Fig. 1B). The surface level of E-cadherin was also not affected by EGFP-CA as assessed by the proportion of cellular E-cadherin susceptible to surface trypsination (Fig. 1C). Immunofluorescence microscopy of cell monolayers demonstrated that E-cadherin localized with β-catenin and F-actin to cell-cell contacts in uninduced hE-CHO/Tet-CA cells (Fig. 2). Some E-cadherin and β-catenin staining was also seen where cells induced to express EGFP-CA formed contacts with each other. However, the contacts between induced cells appeared less extensive than in uninduced cells, typically being more punctate and ragged. Similarly, relatively little phalloidin

1 The abbreviations used are: EGFP, enhanced green fluorescent protein; MDCK, Madin-Darby canine kidney cells; mAb, monoclonal and polyclonal antibody; GFP, green fluorescent protein; HBSS, Hanks’ balanced salt solution; TRITC, tetramethylrhodamine isothiocyanate; CHO, Chinese hamster ovary cell; ConA, concanavalin A; TIRF, total internal reflection fluorescence.
staining was observed at the cell contacts in EGFP-CA-induced cells compared with the more intense F-actin seen in contacts between uninduced cells. These observations suggested that expression of EGFP-CA might affect the ability of cells to establish contacts with one another, although the cellular levels of E-cadherin and catenins were unaffected.

Expression of EGFP-CA Inhibits Cadherin-directed Actin Assembly—Although the CA fragment of N-WASP has been shown to be a potent inhibitor of Arp2/3-mediated actin assembly in vitro (15), it was important for us to test whether inducible expression of this fragment could inhibit cadherin-directed actin assembly in cells. Phalloidin staining in cell monolayers suggested that this might be the case, because F-actin bundles and actin-rich structures were less prominent in cells induced to express EGFP-CA than in uninduced cells (Fig. 2). To address this more directly, however, we wished to determine whether EGFP-CA affected the ability of cadherin adhesion alone to induce actin assembly at adhesive contacts. For this purpose we first used latex beads coated with recombinant cadherin ligands to present spatially confined homophilic adhesive signals that can recruit Arp2/3 and induce actin assembly (13, 14). In these experiments we employed a dimeric ligand consisting of the complete ectodomain of human E-cadherin fused to the Fc region of IgG (hE/Fc). This and similar recombinant proteins (13, 23, 24) support cadherin-specific cell adhesion without detectable contribution of integrins (18, 25), and directly activate cell signaling via Rho-GTPases and phosphatidylinositol 3-kinase (18, 26).

As reported previously (14), phalloidin staining of uninduced cells (Fig. 3A) and cells not transfected with EGFP-CA (not shown) revealed prominent circumferential accumulation of F-actin and E-cadherin at the sites where cadherin-coated beads bound. Characteristically, phalloidin stained densely in the immediate vicinity of the beads and extended outward on the dorsal surfaces of the cells. No accumulation of either F-actin or E-cadherin was observed at sites of adhesion to ConA-coated beads (Fig. 3A). Induction of EGFP-CA profoundly inhibited the accumulation of actin at cadherin bead contacts. By comparison with uninduced cells, much less phalloidin staining was observed at these sites of adhesion, although cellular cadherin was recruited to the beads. This difference was confirmed when the extent of phalloidin accumulation around the beads was quantitated (Fig. 3B). This indicated that expression of EGFP-CA in cells indeed inhibited local cellular F-actin accumulation in response to cadherin homophilic signals.

Arp2/3-dependent Actin Assembly Occurs Preferentially in Nascent Adhesive Contacts—Planar substrata coated with hE/Fc provide reductionist models to identify molecular mechanisms capable of influencing contact zone extension (14, 18, 27). Upon attachment in this assay, cadherin-containing cells progressively extend broad lamellipodia to expand their adhesive contacts. The outer margins of these lamellipodia thereby
participate in contact zone extension, analogous to the pattern observed as MDCK cells extend nascent contacts upon one another (5).

We had observed earlier (14) that the Arp2/3 complex characteristically accumulated at the outer margins of adhesive contacts as cells attached to cadherin-coated substrata. To better define the dynamics of Arp2/3 accumulation during contact zone extension, we used total internal reflection fluorescence (TIRF) microscopy to image molecular events that occur within ~100 nm of the adhesive interface between cells and hE/Fc-coated substrata (28). We found that GFP-Arp3 clearly localized at the very outer margins as adhesive contacts were extending (Fig. 4A and supplemental Movie 1) but was often lost from those regions undergoing transient retraction. This extended our earlier observations to indicate that Arp2/3 concentrates at the cell cortex in regions of adhesion where contacts are actively extending. Furthermore, TIRF imaging of fixed specimens revealed that although the p34 subunit of the Arp2/3 complex stained prominently at the very outer margins of cadherin-based lamellipodia, cellular E-cadherin was found in clusters throughout the adhesive interface (not shown). Therefore, the preferential accumulation of GFP-Arp3 at the outer margin was not because of differences in proximity between the cell surface and the glass substrate.

Taken together, these observations reinforced our earlier inference that the Arp2/3 complex is selectively recruited to those regions of the cell margin where contact zones were undergoing active extension. This suggested that Arp2/3-mediated actin assembly might occur preferentially at these sites. To test this, we examined the pattern of fluorescent G-actin incorporation in hE-CHO cells adherent to hE/Fc (Fig. 4B). Uninduced hE-CHO/Tet-CA cells displayed fluorescent actin incorporation at the outer margins of cadherin-based lamellipodia (Fig. 4B, arrows) as well as in actin bundles. In contrast, induction of EGFP-CA largely abolished G-actin incorporation at the outer margins (Fig. 4B, arrowheads), although incorporation was seen elsewhere in the cells (Fig. 4B). In both conditions, cellular E-cadherin stained in clusters and streaks at the cell-substrate interface. This suggested that, indeed, Arp2/3 preferentially supported actin assembly in the extending regions of cadherin-based cell contacts.

EGFP-CA Inhibits Cadherin-dependent Contact Zone Extension—To test the potential contribution of Arp2/3 activity to cadherin contact zone extension, we first compared the ability of induced and uninduced cells to form native cell-cell contacts. To test this, we examined the pattern of fluorescent G-actin incorporation in hE-CHO cells adherent to hE/Fc (Fig. 4B). Uninduced hE-CHO/Tet-CA cells displayed fluorescent actin incorporation at the outer margins of cadherin-based lamellipodia (Fig. 4B, arrows) as well as in actin bundles. In contrast, induction of EGFP-CA largely abolished G-actin incorporation at the outer margins (Fig. 4B, arrowheads), although incorporation was seen elsewhere in the cells (Fig. 4B). In both conditions, cellular E-cadherin stained in clusters and streaks at the cell-substrate interface. This suggested that, indeed, Arp2/3 preferentially supported actin assembly in the extending regions of cadherin-based cell contacts.

EGFP-CA preferentially inhibits G-actin incorporation in nascent contacts as cells adhere to cadherin-coated substrata. A, TIRF imaging reveals that Arp2/3 localizes at the outer margins of extending cadherin contact zones. CHO cells stably expressing human E-cadherin (hE-CHO) cells were transiently transfected with GFP-Arp3, allowed to adhere to substrate coated with hE/Fc, and visualized by TIRF. Sequential frames from a representative TIRF movie (supplemental Movie 1) show regions of cadherin-based lamellipodia undergoing dynamic extension. Note that GFP-Arp3 transiently recruits to regions of lamellipodia undergoing extension (A, arrows, asterisks) and filopodia undergoing dynamic extension (A, arrowheads). B, expression of EGFP-CA inhibits G-actin incorporation at the outer margins of cells adherent to hE/Fc-coated substrata. hE-CHO/Tet-CA cells were grown in the presence (–CA) or absence (+CA) of doxycycline (1 µg/ml), isolated, and allowed to adhere to hE/Fc-coated substrata for 90 min. Live cells were then permeabilized and incubated with Alexa 568-G-actin, before fixation, processing, and co-labeling for cellular E-cadherin. Representative dual label fluorescence images of E-cadherin and G-actin incorporation (G-actin) are shown. Arrows and arrowheads identify the outer margins of contact zones in Uninduced and Induced cells, respectively.
Depletion of extracellular calcium was used to break cadherin-mediated contacts in hE-CHO/Tet-CA cells, and the ability of cells to reform contacts was monitored after restoration of calcium (Fig. 5). Cells rapidly separated from one another upon chelation of extracellular calcium, associated with redistribution of E-cadherin away from the free cell margins (Fig. 5A, −Ca²⁺). Cells were then washed into fresh medium containing CaCl₂ (2 mM) to allow contacts to reform. At various times (15’ to 120’) after replenishment of calcium, cells were fixed and stained for E-cadherin or with phalloidin (F-actin). A, representative fields showing immunostaining for E-cadherin and F-actin at various stages of calcium manipulation. B, reassembly of contacts was quantitated by measuring the lengths of individual cell-cell contacts (data are means ± S.E., n = 40–60; *, p < 0.0001, Student’s t test). As Arp2/3-mediated actin assembly is implicated in cell motility (12), it was possible that defects in the ability of cells to move on substrate toward one another contributed to the delay in reforming cell-cell contacts. To test directly the contribution of Arp2/3 activity to cadherin-based contact formation, independent of any possible effects on substrate-based motility, we then assayed the ability of cells to extend adhesive contacts in hE/Fc planar adhesion assays. As noted previously, in this system cells extend adhesive contact zones via protrusion of cadherin-based lamellipodia. Lamellipodial extension was quantitated by summing the length of the outer margins of all lamellipodia in individual cells, expressed as a proportion of the total perimeter for each cell (lamellipodial index). This gave a measure of lamellipodial extension that was independent of cell size and surface area. As shown in Fig. 6, induction of EGFP-CA profoundly reduced lamellipodial extension in cells allowed to adhere to hE/Fc for 90 min (p < 0.001, Student’s t test). In contrast, lamellipodial extension in uninduced cells was comparable with that in control cells transiently expressing EGFP alone.
Inhibition of Arp2/3 Activity Has Minimal Effect on Cell Surface Adhesion—We then asked whether these dramatic changes in contact zone extension were accompanied by changes in cadherin adhesive strength (Fig. 7). We first tested the effects of expressing EGFP-CA on adhesion measured by the resistance of cells to detachment from cadherin-coated substrata. By using a sensitive laminar flow assay (23, 24) to detect changes in adhesive strength after short periods (20 min) of attachment to hE/Fe (Fig. 7A), we found that both hE-CHO/Tet cells and uninduced hE-CHO/Tet-CA cells showed significantly greater adhesion compared with cadherin-negative CHO cells, consistent with previous observations (23). Surprisingly, however, induction of EGFP-CA did not appreciably affect cell adhesion in this short term assay.

In case the attachment times were too short to allow differences in adhesion to become apparent, we modified the assays to measure adhesion after 90 min (i.e. at times identical to those used in the planar spreading assays) (Fig. 7B). For these assays, freshly isolated cells were allowed to attach to hE/Fe-coated substrata in a CO2 incubator and then detached by pipetting (21). As seen with the short term flow detachment assay, cadherin-negative parental CHO cells detached readily from hE/Fe-coated substrata, whereas hE-CHO cells not transfected with EGFP-CA significantly resisted detachment. Uninduced hE-CHO/Tet-CA cells resisted detachment to a similar degree as in hE-CHO cells not transfected with EGFP-CA. Induction of EGFP-CA did not, however, significantly affect the strength of attachment to hE/Fe-coated substrata, although adhesion was reduced by latrunculin A (1 μM).

To pursue these surprising results, we then tested the ability of cells to aggregate in suspension culture. After 60 min of incubation, parental CHO cells remained as individual cells, whereas both hE-CHO/Tet and uninduced hE-CHO/Tet-CA cells formed clearly defined aggregates that were apparent both by microscopy (not shown) and as reflected in the fall in number of particles present in suspension (Fig. 7C). Consistent with the results of the hE/Fe-based adhesion assays, hE-CHO/Tet-CA cells induced to express GFP-CA aggregated to the same degree as both control cadherin-expressing cell lines. Taken together, these data indicate that expression of GFP-CA did not measurably affect E-cadherin adhesiveness in these cells.

Arp2/3 Participates in Actin Assembly at Cadherin Contacts in MDCK Cells—We then sought to assess the potential activity of Arp2/3 at cell-cell contacts in polarized epithelial cells. As a first step, we raised an antibody against the p34 subunit in order to immunolocalize the endogenous Arp2/3 complex in cells. This pAb identified a single polypeptide band in a range of cultured cell lines, including MDCK cells, a classic polarized epithelial model (Fig. 8A). Indirect immunofluorescence showed clear p34 staining which co-localized with E-cadherin at cell-cell contacts in confluent MDCK monolayers (Fig. 8B). Moreover, semi-permeabilized MDCK cells readily incorporated fluorescently labeled G-actin into cell-cell contacts, where the label co-localized with E-cadherin (Fig. 8C). To test whether Arp2/3 participates in this junctional actin assembly, we transiently expressed GFP-CA in MDCK cells by microinjection, thereby allowing us to reliably express GFP-CA in clusters of epithelial cells. Cells were examined at 5 and 16 h post-injection with identical results. Staining for E-cadherin at contacts between microinjected cells was not appreciably affected (Fig. 8C). In contrast, G-actin incorporation was less prominent at contacts between cells expressing GFP-CA than in contacts either between uninjected cells (Fig. 8, C and D) or cells expressing GFP alone (Fig. 8D). This was confirmed by quantitation of fluorescence intensity which revealed that G-actin was reduced by ~40% in contacts between microinjected cells compared with those formed between control cells (Fig. 8D). These data then indicate that Arp2/3 supports ongoing actin assembly in established contacts within epithelial monolayers.

DISCUSSION

In this study we sought to test the potential contribution of Arp2/3 activity to E-cadherin function. Prompted by our recent discovery that cadherin homophilic ligation marks sites that recruit Arp2/3 to the cell surface (14), we hypothesized that Arp2/3 might provide a force-generating mechanism to drive cell surface protrusion during adhesive recognition. Our current TIRF movies reinforce this possibility, by showing that Arp2/3 localized preferentially and with fidelity to the outer margins of cadherin-based lamellae as adhesive contacts were being extended in planar adhesion assays. Thus Arp2/3 was well placed, both spatially and temporally, to contribute to contact zone extension.
also treated with latrunculin A (1 μM; Lat).

**FIG. 7.** Effect of EGFP-CA on cadherin-based cell adhesion. Adhesion assays compared hE-CHO/Tet-CA cells incubated with doxycycline (1 μg/ml; +Dox), hE-CHO/Tet-CA cells 3 days after removal of doxycycline (−Dox), hE-CHO/Tet cells not transfected with EGFP-CA (hE-CHO/Tet), and parental CHO cells (p-CHO). A and B, cells were allowed to adhere to substra coated with hE/Fc, and adhesion was measured as the proportion of cells remaining adherent despite detachment force (Adhesion, % attached). A, in short term laminar-flow adhesion assays, cells were allowed to adhere to hE/Fc-coated capillary tubes for 20 min and then exposed to progressively increasing flow rates of buffer. Data are means ± S.E. (n = 4). B, in longer term detachment assays cells were allowed to attach for 90 min and then detached by systematic pipetting. In the longer term assays, uninduced cells were also treated with latrunculin A (1 μM; Lat). Box and whisker plots represent medians, 25th centiles (boxes), and ranges (outer whiskers) (n = 6). C, for aggregation assays, freshly isolated cells were incubated in agarose-coated wells rotated at 45 rpm for 60 min before being counted. Aggregation was measured by counting the number of particles remaining after 60 min, expressed as a percentage of the number present at the beginning of the assay (Nend/N0 × 100).

To test this we used a fragment of N-WASP that potently inhibited Arp2/3-mediated actin assembly in pyrene nucleation assays (15). Several observations indicate that expression of EGFP-CA in cells similarly inhibited actin assembly at cadherin contacts. First, F-actin staining at cell-cell contacts was reduced upon expression of EGFP-CA, both in established monolayer cultures and when cell-cell contacts reassembled in calcium manipulation experiments. Second, EGFP-CA profoundly reduced the ability of homophilic cadherin ligation to accumulate F-actin, measured at cellular sites of adhesion to hE/Fc-coated beads. Finally, expression of EGFP-CA inhibited the incorporation of fluorescently labeled G-actin into adhesive contacts in permeabilized cells, a direct test of actin assembly. Taken with the in vitro data (15), these findings suggested that EGFP-CA provided the opportunity to reasonably and specifically target Arp2/3 activity in cells.

We found that that expression of EGFP-CA profoundly inhibited the ability of cells to extend cadherin adhesive contacts. The rate at which cell-cell contacts reassembled following manipulation of extracellular calcium was significantly retarded upon induction of EGFP-CA. This strongly suggested that Arp2/3 activity was necessary for contact extension in native cell-cell contacts and was consistent with our observation that cells expressing EGFP-CA formed less extensive, more ragged, contacts with one another when grown in monolayer culture. We considered the possibility that failure of cells expressing EGFP-CA to efficiently re-establish contacts might also be due to a requirement for Arp2/3 activity to drive cell movement on substrate (12), thereby delaying the ability of cells to spread and migrate toward one another. However, the observation that contact extension on cadherin-coated substrata was also inhibited by EGFP-CA indicates that Arp2/3 activity is necessary for homophilic ligation to support adhesive contact extension, independent of any contribution to integrin-based motility. Furthermore, although actin binding may participate in stabilizing the cadherin-catenin complex at the cell surface (29), the expression of EGFP-CA was not accompanied by any detectable changes in cadherin or catenin levels. This suggests that EGFP-CA affected contact zone extension by disrupting the cytoskeletal response to homophilic ligation, rather than by indirect changes in the pool of cadherins or catenins available within cells.

Our work taken with other recent studies, notably from Fuchs and co-workers (7, 30), establishes cadherin adhesive contacts as sites for active actin assembly. Already two distinct molecular mechanisms have been identified that can support such actin assembly. In addition to the role for Arp2/3 that we have identified, formin-1 was recently shown to associate with α-catenin in adherens junctions of keratinocytes (30). Like other formin homology proteins, formin-1 likely mediates assembly in actin bundles, in contrast to the branched meshworks characteristically thought to result from Arp2/3 activity. Such distinct mechanisms are likely to support functionally distinct modes of cadherin-actin cooperativity. Although our present data indicate that Arp2/3 participates in the extension of nascent contacts during early cell-cell recognition, formin-1 appears to be critical for the later stages of junctional maturation that couple epithelia into mechanically coherent sheets (30). Indeed, these distinct processes may co-exist. Thus we identified Arp2/3 at cell-cell contacts in confluent MDCK monolayers, and expression of EGFP-CA significantly reduced G-actin incorporation into those contacts. This indicated that even in polarized epithelia, cadherin contacts are sites for ongoing actin assembly, to which Arp2/3 is a major contributor. Furthermore, within the individual contacts that cells formed in planar spreading assays, Arp2/3 appears to be principally responsible for actin assembly in the actively extending outer margins, suggesting that other mechanisms may co-exist to support actin assembly elsewhere in the contacts.

Despite its profound effect on contact extension, expression of EGFP-CA had surprisingly little discernible effect on cell adhesion, measured either by cellular resistance to detachment from cadherin-coated substrata or by the ability of cells to aggregate in suspension. Consistent with this, we observed in planar spread-
ing assays that lateral cadherin clustering, which can strengthen adhesion (25), was unaffected by the presence or absence of EGFP-CA. In contrast, earlier studies clearly demonstrated that drug inhibitors of actin assembly potently inhibit cadherin-based adhesion, as we confirmed in our present experiments (31, 32). There are, however, significant differences in the potential effects of drug inhibitors on cells compared with those of EGFP-CA. First, multiple distinct molecular mechanisms exist to support actin assembly in cells (33, 34). Whereas EGFP-CA is predicted to target Arp2/3-mediated actin assembly, drugs, such as cytochalasins and latrunculin, that cap the barbed ends of filaments or sequester G-actin, respectively (35), likely inhibit all these processes indiscriminately. Drug inhibitor studies therefore establish the general requirement for actin integrity to support cadherin function but cannot define specific molecular mechanisms. Second, by virtue of their ability to ultimately perturb actin filament integrity, drug inhibitors may affect other processes that require actin filament integrity to contribute to cell adhesion, such as myosin-mediated contractility, but which are not necessarily immediate consequences of Arp2/3-mediated actin assembly. Thus we believe that our findings build on the general requirement for actin integrity, to identify a specific requirement for Arp2/3 in the morphological process of cadherin contact extension.

The discrepant effects of EGFP-CA on contact formation and surface adhesiveness further imply that these two processes are mechanistically distinguishable. Adhesive contact formation is commonly envisaged to reflect changes in surface adhesion, a concept often based on earlier demonstrations that experimental manipulation of cell surface adhesive properties influence contact formation in cell spreading assays (36). However, our data suggest that although surface adhesion is clearly essential to provide traction for cell surfaces to move upon one another as contacts assemble, the degree to which cells extend contacts is also affected by the ability of Arp2/3 to support surface protrusion. This implies that contact formation is not solely determined by changes in the adhesive properties of cadherins at the cell surface, but rather arises from functional cooperation between the surface binding activity of cadherins and the force-generating capacity of the actin cytoskeleton. Thus, given a minimally necessary level of surface adhesiveness, Arp2/3-mediated actin assembly acts as a distinct, rate-limiting factor to drive cell surfaces upon one another to form contacts. Because the ability to form contacts was not altogether abolished by expression of EGFP-CA, Arp2/3 may principally determine the kinetics or efficiency of contact formation. Such efficient contact formation is likely to be particularly germane during processes such as gastrulation (37), dorsal closure, and wound repair (38), where rapid contact formation must occur for tissue remodeling to occur.

Finally, this study suggests that interactions between cadherin molecules and different functional states of the actin cytoskeleton molecules and different functional states of the actin cytoskeleton support different aspects of cadherin function. The spatially restricted recruitment of Arp2/3 to the outer margins of adhesive contact zones that we have observed is likely to principally provide the local protrusive forces needed to extend those contacts. Other distinct cadherin-actin interactions may collaborate to support other molecular mechanisms necessary for efficient contact formation. For example, passive scaffolding onto subcortical actin filaments can restrict lateral movements of cadherin molecules (10), whereas association with actin bundles is likely to couple surface adhesion and contractility (6). The functional and biochemical cooperativity between classical cadherins and the actin cytoskeleton is thus unlikely to be a single process, but rather an ensemble of molecular processes that are subject to strict spatio-temporal regulation in cells.

Acknowledgments—We thank all our aforementioned colleagues for their generous gifts of reagents, Teresa Munchow for assistance with tissue culture, and all the members of our lab for their advice, support, and encouragement.

REFERENCES

1. Takeichi, M. (1995) Curr. Opin. Cell Biol. 7, 619–627
2. Tepass, U., Treeng, K., Godt, D., Ikura, M., and Peifer, M. (2000) Nat. Rev. Mol. Cell Biol. 1, 91–100
3. Birchmeier, W., and Behrens, J. (1994) Biochim. Biophys. Acta 1198, 11–26
4. Adams, C. L., Chen, Y.-T., Smith, S. J., and Nelson, W. J. (1998) J. Cell Biol. 1105–1119
5. Ehrelih, J. S., Hansen, M. D. H., and Nelson, W. J. (2002) Dev. Cell 3, 259–270
6. Vaes, A., Bauer, C., Vasioukhin, V., and Fuchs, E. (2002) Dev. Cell 3, 367

* A. M. Shewan, et al., unpublished observations.
7. Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000) Cell 100, 209–219
8. Yap, A. S., and Kovacs, E. M. (2003) J. Cell Biol. 160, 11–16
9. Adams, C. L., and Nelsen, W. J. (1998) Curr. Opin. Cell Biol. 10, 572–577
10. Sakai, Y., Nagafuchi, A., Tsukita, S., Takeichi, M., and Kusumi, A. (1998) J. Cell Biol. 140, 1227–1240
11. Bear, J. E., Krause, M., and Gertler, F. B. (2001) Curr. Opin. Cell Biol. 13, 158–166
12. Pollard, T. D., and Borisy, G. G. (2003) Cell 112, 453–465
13. Lambert, M., Choquet, D., and Mege, R. M. (2002) J. Cell Biol. 157, 469–479
14. Kovacs, E. M., Goodwin, M., Ali, R. G., Paterson, A. D., and Yap, A. S. (2002) Curr. Opin. Cell Biol. 14, 379–382
15. Rohtagi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takeiwa, T., and Kirschner, M. W. (1999) Cell 97, 221–231
16. Gotardi, C. J., Wong, E., and Gumbiner, B. M. (2001) J. Biol. Chem. 276, 20533–20539
17. Sasaki, N., Miki, H., and Takeiwa, T. (2000) Biochem. Biophys. Res. Commun. 272, 386–390
18. Kovacs, E. M., Ali, R. G., McCormack, A. J., and Yap, A. S. (2002) J. Biol. Chem. 277, 6708–6718
19. Helwani, F. M., Kovacs, E. M., Paterson, A. D., Verma, S., Ali, R. G., Fanning, A. S., Weed, S. A., and Yap, A. S. (2004) J. Cell Biol. 164, 899–910
20. Bally, M., Ichetokvin, I., Grant, W., Zebda, N., Macheky, L. M., Segall, J. E., and Condeelis, J. (2001) Curr. Biol. 11, 620–625
21. Goodwin, M., Kovacs, E. M., Thoresen, M. A., Reynolds, A. B., and Yap, A. S. (2003) J. Biol. Chem. 278, 20533–20539
22. Yap, A. S., Niessen, C., and Gumbiner, B. M. (1998) J. Cell Biol. 141, 779–789
23. Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996) J. Cell Biol. 135, 487–489
24. Niessen, C. M., and Gumbiner, B. M. (2000) J. Cell Biol. 156, 389–400
25. Yap, A. S., Brieher, W. M., Pruschy, M., and Gumbiner, B. M. (1997) Curr. Biol. 7, 308–315
26. Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001) J. Biol. Chem. 276, 33305–33308
27. Gavard, J., Lambert, M., Grosheva, I., Marthiensa, V., Irinopoulou, T., Riou, J.-F., Bershadsky, A., and Mege, R. M. (2004) J. Cell Sci. 117, 257–270
28. Steyer, J. A., and Almers, W. (2001) Nat Rev. Mol. Cell. Biol. 2, 268–275
29. Troxell, M. L., Chen, Y. T., Cobb, N., Nelson, W. J., and Mars, J. A. (1999) Am. J. Physiol. 276, C404–C418
30. Kohselak, A., Psalvi, H. A., and Fuchs, E. (2004) Nat. Cell Biol. 6, 21–30
31. Angres, B., Barth, A., and Nelson, W. J. (1996) J. Cell Biol. 134, 549–557
32. Jaffe, S. H., Friedlander, D. R., Matsuoka, F., Crossin, K. L., Cunningham, B. A., and Edelman, G. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3589–3593
33. Evangelista, M., Pruyne, D., Amberg, D. C., Boone, C., and Bretscher, A. (2001) Nat. Cell Biol. 4, 260–269
34. Li, F., and Higgs, H. N. (2000) Curr. Biol. 13, 1335–1340
35. Ayscough, K. (1998) Methods Enzymol. 298, 18–25
36. Grinnell, F. (1978) Int. Rev. Cytol. 53, 65–144
37. Gumbiner, B. M. (1992) Cell 69, 385–397
38. Jacinto, A., Martinez-Arias, A., and Martin, P. (2001) Nat. Cell Biol. 3, E117–E123
Arp2/3 Activity Is Necessary for Efficient Formation of E-cadherin Adhesive Contacts

Suzie Verma, Annette M. Shewan, Jeanie A. Scott, Falak M. Helwani, Nicole R. den Elzen, Hiroaki Miki, Tadaomi Takenawa and Alpha S. Yap

J. Biol. Chem. 2004, 279:34062-34070.
doi: 10.1074/jbc.M404814200 originally published online May 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404814200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/07/02/M404814200.DC1

This article cites 35 references, 14 of which can be accessed free at http://www.jbc.org/content/279/32/34062.full.html#ref-list-1