IQ-domain GTPase-activating Protein 1 Regulates β-Catenin at Membrane Ruffles and Its Role in Macropinocytosis of N-cadherin and Adenomatous Polyposis Coli*

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β-Catenin is an integral component of E-cadherin dependent cell-cell junctions. Here we show that β-catenin co-localizes with IQ-domain GTPase-activating protein 1 (IQGAP1), adenomatous polyposis coli (APC), and N-cadherin at actin-positive membrane ruffles in NIH 3T3 fibroblasts. We used deletion mapping to identify the membrane ruffle-targeting region of β-catenin, localizing it to amino acids 47–217, which overlap the IQGAP1 binding site. Knockdown by small interference RNA (siRNA) revealed IQGAP1-dependent membrane targeting of β-catenin, APC, and N-cadherin. Transient overexpression of IQGAP1 or N-cadherin increased β-catenin at membrane ruffles. IQGAP1/APC regulates cell migration, and using a wound healing assay we demonstrate that siRNA-mediated loss of β-catenin also caused a modest reduction in the rate of cell migration. More significantly, we discovered that β-catenin is internalized by Arf6-dependent macropinocytosis near sites of membrane ruffling. The β-catenin macropinosomes co-stained for APC, N-cadherin, and to a lesser extent IQGAP1, and internalization of each binding partner was abrogated by siRNA-dependent knockdown of β-catenin. In addition, β-catenin macropinosomes co-localized with the lysosomal marker, lysosome associated membrane protein 1, consistent with their recycling by the late endosomal machinery. Our findings expand on current knowledge of β-catenin function. We propose that in motile cells β-catenin is recruited by IQGAP1 and N-cadherin to active membrane ruffles, wherein β-catenin mediates the internalization and possible recycling of the membrane-associated proteins N-cadherin and APC.

β-Catenin is a multifunctional protein with roles in cell-cell adhesion and as a mediator of nuclear signaling in the Wnt pathway and in various types of cancer (1). β-Catenin is involved in cell adhesion through its interaction with E-cadherin at cell-cell junctions (2), and as a transcriptional activator it binds/activates the lymphoid enhancing factor-1/T-cell factor transcription factors to induce genes involved in cell transformation (3). Cytoplasmic β-catenin is normally subject to strict regulation by the APC/axin/glycogen synthase kinase-3β degradation complex, which promotes β-catenin turnover through the proteasome (1). Disruption of β-catenin degradation results in increased β-catenin protein levels and an altered distribution of β-catenin, which shifts from membrane and cytoplasm to the nucleus, culminating in changes typical of the transformed cellular phenotype including enhanced cell migration and increased proliferation. Despite an increase in our knowledge concerning β-catenin subcellular distribution, nuclear transport, and turnover (3, 4), our understanding of how membrane-associated β-catenin is internalized and recycled is rather poor.

Membrane-localized β-catenin is mostly bound to E-cadherin at adherens junctions, structures that mediate cell-cell adhesion and maintain epithelial cell contact, polarity, and communication (5). The adherens junctions are positioned at the basolateral membrane, and their formation is dependent on the homophilic binding of the transmembrane protein E-cadherin. β-Catenin together with α-catenin tethers adherens junctions to the actin cytoskeleton (2, 6). The formation and disassembly of adherens junctions is a dynamic process linked to tissue remodeling and tumor cell invasion (7). The loss of adherens junctions can result in disruption of apical-basal polarity and loss of the epithelial phenotype and is a contributing factor to the so-called epithelial-to-mesenchymal transition, a shift in cellular phenotype associated with the progression of epithelial-derived cancers (8). The loss of β-catenin and E-cadherin from the cell surface is emerging as a key event in this process, especially in breast and colon carcinomas where E-cadherin down-regulation occurs through multiple mechanisms (7–9). In addition to the reduced anchorage of cells caused by loss of E-cadherin, they can also acquire a more migratory phenotype through the release of β-catenin from the membrane and its translocation to the nucleus, where it activates genes involved in cell transformation and migration (1, 3).

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1—S6.

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2 The abbreviations used are: APC, adenomatous polyposis coli; ASEF, APC-stimulated exchange factor; GFP, green fluorescent protein; IQGAP1, IQ-domain GTPase-activating protein 1; LAMP1, lysosome-associated membrane protein 1; PBS, phosphate-buffered saline; siRNA, small interference RNA; ca, constitutively active; wt, wild type; ct, C terminus; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate.
More recently, a second membrane localization of cellular \(\beta\)-catenin was reported. Etienne-Manneville et al. (10) showed that \(\beta\)-catenin was detectable at the leading edge of migrating rat astrocytes; however, this localization pattern has not yet been confirmed or characterized. Although it is possible this atypical membrane staining pattern reflected artifactual antibody staining, it is equally plausible that \(\beta\)-catenin does localize to lamellipodia and active ruffling membrane in mesenchymal cells or fibroblasts. The appearance of the \(\beta\)-catenin binding partner, APC, was recently reported at such dynamic membrane structures (11), and the membrane association of APC was functionally linked to cell migration. In this study we performed a series of experiments that provide compelling evidence for the localization of \(\beta\)-catenin at membrane ruffles. We further show that membrane-associated \(\beta\)-catenin correlates with a modest contribution to cell migration and that this unexpected localization pattern is important for the internalization of \(\beta\)-catenin and specific binding partners through the process of macropinocytosis. We identified IQGAP1, an important regulator of actin dynamics and cell migration (11, 12), as a key regulator of \(\beta\)-catenin at the membrane.

### MATERIALS AND METHODS

**Cell Culture, Reagents, and Transfection**—The following cell lines were used: non-tumor derived epithelial cell lines (canine kidney epithelial cells), HEK-293T (immortalized human embryonic kidney cells), and MCF10A (breast epithelial cell lines). Many other cell lines were used: non-tumor derived epithelial cell lines (canine kidney epithelial cells), breast tumor epithelial cell lines T47D, MCF-7, and HEK-293T (immortalized human embryonic kidney epithelial cells), and MCF10A (breast epithelial cell lines). HEK-293T (immortalized human embryonic kidney epithelial cells), breast tumor epithelial cell lines T47D, MCF-7, and MCF10A (breast epithelial cell lines) were used: non-tumor derived epithelial cell lines (canine kidney epithelial cells), breast tumor epithelial cell lines T47D, MCF-7, and MDA.MB.231; mouse fibroblast cell line NIH 3T3. All cell lines were confirmed mycoplasma-free and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) at 37 °C in 5% CO2 humidified atmosphere. For transfection cells were grown on glass coverslips in 8-well dishes (Nunc) and 12 h post-seeding were transfected with 2 μg of DNA or 3 μg of siRNA per ml of media using Lipofectamine 2000 (Invitrogen); the lipid-DNA mix was left on cells for 6 h before replacing the medium and processing cells 40 h later. Cells were processed for microscopy or wound healing assay. The effect of ARF6 was studied by treating the cells with 30 μM Myr-Arf6 peptide (Calbiochem) for 1 h.

**Plasmids**—The following plasmids were transfected into NIH 3T3 cells: pEGFP-Rac1 and pEGFP-RhoA constructs were gifts from Dr. Mark Philips (13); human pEGFP-E-cadherin was a gift from Dr. Alpha Yap; murine pEGFP-N-cadherin was a gift from Dr. C. Gauthier-Rouviere; human pASEF-wt and pASEF-ca were gifts from Prof. Tetsu Akiyama (14, 15); human pEGFP-IQGAP-wt and -ct (amino acids 1503–1657) were gifts from Dr. Kozo Kaibuchi (11). Human \(\beta\)-catenin-wt-FLAG plasmid and \(\beta\)-catenin-Δ218–467-FLAG plasmid were gifts from Dr. Eric Fearon (16) and were used as templates to construct a set of \(\beta\)-catenin-GFP vectors in the expression plasmid pEGFP-N1 (Clontech). The \(\beta\)-catenin cDNA insert sequences were PCR-amplified using forward and reverse primers (see Table 1) containing KpnI site (in forward primer) and BamHI (in reverse primer) restriction sites and cloned in-frame into pEGFP-N1. \(\beta\)-Catenin-wt-FLAG plasmid was used as template for all constructs except \(\beta\)-catenin-Δ218–467-FLAG, where \(\beta\)-catenin-Δ218–467-FLAG was used as the template. All clones were confirmed by sequencing.

**Immunofluorescence Microscopy, Confocal Microscopy, and Antibodies**—Cells were washed with PBS and fixed with 3.7% formalin, PBS for 20 min followed by permeabilization with 0.2% Triton-X-100, PBS for 10 min. Cells were blocked with 3% bovine serum albumin, PBS for 45 min, incubated with 150 μl of primary antibody (diluted in blocking solution; see below for dilutions), and washed 3 times with PBS. Cells were subsequently incubated with secondary antibody. All incubations were carried out at room temperature. After extensive washes in PBS, slides were mounted with Vectashield (Vector Labs) and visualized by fluorescence microscopy. The following antibodies were used for immunofluorescence labeling and Western blotting: \(\beta\)-catenin polyclonal antibody (1:100, Santa Cruz Biotechnology, Inc., #H102), \(\beta\)-catenin monoclonal antibody (1:100, Transduction Laboratories #610153), M-APC rabbit polyclonal antibody (a gift from Dr. Inke Nathke), N-cadherin monoclonal antibody (1:100, Zymed Laboratories Inc. #33–3900), IQGAP1 polyclonal antibody (1:100, Santa Cruz, H109), IQGAP1 monoclonal antibody (1:100, Transduction Laboratories, #610612), clathrin monoclonal antibody (1:100, Transduction Laboratories #610499), anti-HA (1:200, Santa Cruz, clone Y-11), Arf6 monoclonal antibody (1:100, Santa Cruz, clone 3A-1), \(\beta\)-actin monoclonal antibody (1:5000, Sigma clone AC-74), Rab3A polyclonal antibody (1:100, Santa Cruz, clone K-15), Rab5b (1:100, Transduction Laboratories #610281), EEA1 polyclonal antibody (1:100, Santa Cruz clone C-15), LAMP1 monoclonal antibody (1:200, Transduction Laboratories #611042), Rab11 monoclonal antibody (1:100, Transduction Labs #610656), TGN38 (1:200, Transduction Laboratories, #610898), β-COP (1:2000, Affinity Bioreagents #PA1–061), TRITC- or FITC-conjugated phalloidin (0.5 μg/ml, Sigma P1951 or P5282, respectively). Secondary antibodies used were anti-mouse or anti-rabbit antibody conjugated to biotin (1:500, 1:1000, respectively) and visualized by fluorescence microscopy.
DAKO), Texas Red conjugated to avidin (1:800, Vecta Laboratories), anti-mouse conjugated to FITC (1:100, Santa Cruz), or anti-rabbit conjugated to FITC (1:100, Santa Cruz). Confocal microscope images of labeled cells were taken at ×600 magnification using the Leica TCS SP2 confocal system attached to a Leica upright fluorescence microscope (DMRE) and using Leica Confocal Software Version 2.00. Other images were taken using an Olympus BL51 fluorescence microscope at ×400 magnification. A SPOT 32 camera and SPOT Advanced software was used for general image capture. The images were modified in Adobe Photoshop 7.0.

**Deconvolution Analysis of Microscope Images**—For high resolution imaging of cells, an inverted Zeiss Axiovert 200M microscope was used to capture 21 sections of cell samples, each section of 0.35 μm, a z axis range that spanned the apical to basal part of the cell. Images were resolved by iterative deconvolution using the Zeiss software inverse filter algorithm. The deconvolved images selected for presentation in this study correspond to sections 10–15 and represent a mid-plane section of the cell.

**Dextran Labeling Experiment**—For uptake of dextran-FITC (71 kDa; Sigma) or dextran-TRITC (76 kDa; Sigma), cells were transfected with ASEP-ca plasmid. After 30 h cells were incubated with 4 mg/ml dextran for 15 min to 1 h at 37 °C in serum-free media followed by 3 washes with fresh media and PBS (17). Cells were then fixed with 3.7% formalin, stained with β-catenin monoclonal antibody or M-APC polyclonal antibody, and analyzed by Zeiss deconvolution microscopy as described above.

**Western Blot Analysis**—Cells were resuspended in protein extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, supplemented with protease inhibitor mix (Roche Diagnostics) and shock-frozen in liquid nitrogen. After a quick thaw at 37 °C, cells were refrozen in liquid nitrogen, thawed on ice for 20 min, and cleared of insoluble components by centrifugation at 13,000 rpm at 4 °C for 15 min. The supernatant containing total protein was quantified using the Bio-Rad protein assay. Cell extracts were then denatured at 95 °C for 5 min in sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol, 5% SDS), and 40 μg of proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Millipore). Membranes were treated in blocking solution (5% dry milk in PBS containing 0.2% Tween 20) and incubated with primary antibody at room temperature for 2 h followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000; Sigma) for 1 h at room temperature. Proteins were visualized by ECL (Amersham Biosciences). Prestained broad range molecular weight marker (Bio-Rad) was used as the molecular size standard.

**RNA Interference**—Double-stranded 21-mer RNA oligonucleotides homologous to sequences in mouse IQGAP1 or β-catenin (see Table 2) were purchased as purified duplexes (Qiagen-Xeragon Inc). Cells at medium density were transfected with 3 μg of RNA duplexes in 1 ml of Dulbecco’s modified Eagle’s medium using Lipofectamine for 6 h and either harvested 48 h post-transfection for image analysis or analyzed by time-lapse microscopy.

### Table 2

| siRNA sequences used in this study | Target | Sequence |
|-----------------------------------|--------|----------|
| Control siRNA                     |        | 5'-AATPCTCGCAACGTGTCAGCAGT-3' |
| β-Catenin siRNA 1                 |        | 5’-CAGGGTGCTATTGGCCAGCTA-3’ |
| β-Catenin siRNA 2                 |        | 5’-CAGTAGAAGATGGCCGAGTA-3’ |
| IQGAP1 siRNA                      |        | 5’-CGACATGATGATGATGATAAACAA-3’ |

**Wound Healing Assay**—NIH 3T3 cells were grown on 4-well chamber slides (Lab-Tek, #155383). After 72 h, the confluent cells were wounded using a Gilson P1000 pipette tip. Cells were imaged live along the wound at 8 different regions per well for 15 h using a Zeiss Axiovert 200M inverted microscope. For each region, the distance migrated by cells was measured at three different points. Thus, for each well the distance migrated was recorded as an average of 24 points every hour. At the end of the time-lapse experiment cells were fixed and stained with β-catenin and Texas Red-conjugated secondary antibody and FITC-conjugated phalloidin to confirm knock-down of the β-catenin. The assay was repeated twice.

**RESULTS**

**β-Catenin Is Localized at Membrane Ruffles in Migrating Cells**—Our laboratory has an ongoing interest in the subcellular localization and intracellular trafficking of β-catenin (18–20). The steady-state localization of β-catenin in the nucleus and at cell-cell junctions is well documented (1). β-Catenin has been reported at cell membrane lamellipodia (10), and we have detected β-catenin at lamellipodia, filopodia, and short membrane ruffles; however, such localization patterns remain poorly characterized. We tested for non-adherens junction membrane staining of β-catenin in a panel of cell lines using the wound healing assay, which permits analysis of actively migrating cells. Confluent monolayers of different epithelial cell lines were scratched and allowed to heal for 6 h then immunostained with antibody to β-catenin and with fluorescent-tagged phalloidin to detect actin (Fig. 1A and supplemental Fig. S1). In canine kidney epithelial cells, visible β-catenin was primarily restricted to cell-cell junctions, consistent with the fact that these cells form extremely tight and stable cell-cell junctions and are not highly motile. In the breast cancer epithelial cell lines T47D and MCF-7, β-catenin was detected both at cell-cell junctions and also at membrane ruffles in migrating cells at the wound edge. The staining of β-catenin was more biased toward actin-positive membrane ruffles in NIH 3T3 fibroblasts (Fig. 1B). To confirm specificity of the membrane staining of β-catenin, we treated NIH 3T3 cells with siRNA to silence β-catenin and observed a consequent loss of staining from cell-cell junctions, the nucleus and cytoplasm, and also from the membrane ruffles. In contrast, a control siRNA had no effect on β-catenin staining patterns, and the actin staining pattern was unaffected, indicating that membrane ruffles are a genuine site of β-catenin accumulation.

To further test the localization of β-catenin at membrane ruffles, we transfected NIH 3T3 cells with plasmids encoding different factors known to modulate actin dynamics and induce membrane ruffling; a constitutively active form of Rac1 GTPase (13, 21), the Rac1 GTPase exchange factor, ASEC (14) and a constitutively active form of ASEC (15), and stained cells for
actin and β-catenin. Both Rac1 and ASEF increased the proportion of transfected cells with actin-positive membrane ruffles by 1.5–2.5-fold, and a corresponding increase in cellular β-catenin was detected at these membrane structures (Fig. 1C). The strongest effect was elicited by constitutively active ASEF (ASEF-ca), which induced a 3-fold increase in actin-positive ruffles and a 2.5-fold increase in β-catenin-positive ruffles. Thus, the localization of β-catenin at membrane ruffles is associated with induction of these migratory structures.

**β-Catenin Co-localizes with IQGAP1, APC, and N-cadherin at Membrane Ruffles**—NIH 3T3 cells were transfected with ASEF-ca plasmid to induce membrane ruffles then stained for β-catenin and three partner proteins previously reported to locate at membrane ruffles (Fig. 2A). Cells positive for β-catenin were compared for the extent to which β-catenin co-localized with each partner at ruffles (Fig. 2A and supplemental Fig. S2). IQGAP1 is a known marker protein of membrane ruffles (22). We detected co-localization of IQGAP1 or actin in ~97% of NIH 3T3 cells with β-catenin-positive ruffles. Watanabe et al. (11) showed that IQGAP1 recruits APC to membrane ruffles. We, therefore, stained for APC protein (using M-APC antibody) and found that 62% of cells with β-catenin-positive membrane ruffles co-stained with APC.

We next tested β-catenin-positive membrane ruffles for the presence of N-cadherin, a transmembrane protein and binding partner of β-catenin previously observed at free membrane and implicated in cell migration and invasion (23). A high degree of co-localization was observed between these two proteins at membrane ruffles (95% of cells analyzed). Other membrane proteins such as E-cadherin and Zo-1 displayed poor co-staining with β-catenin at ruffles (see supplemental Fig. S3). We conclude that β-catenin-positive membrane ruffles co-localize with actin, IQGAP1, APC, and N-cadherin.

**Transiently Expressed N-cadherin and IQGAP1 Increase β-Catenin at Membrane Ruffles**—In contrast to APC, which regulates β-catenin turnover (1, 3), N-cadherin and IQGAP1 independently locate at free membrane and are potential regulators of β-catenin targeting to membrane ruffles. Indeed, when transiently expressed in cells, GFP-tagged forms of both N-cadherin and IQGAP1 stimulated
The IQGAP1 Binding Region (Amino Acids 47–217) Is Required for β-Catenin Localization to Membrane Ruffles—We next investigated the sequences that are necessary to target β-catenin to membrane ruffles. To address this, NIH 3T3 cells were transfected with a series of GFP-tagged β-catenin deletion constructs (Fig. 3A) and co-expressed with pASEF-ca to elicit membrane ruffling. We scored transfected cells for the localization of ectopic ASEF-ca and β-catenin-GFP. Wild-type β-catenin localized to ASEF-positive membrane ruffles in 25% of transfected cells, displaying ~80% co-localization with ASEF at ruffles (Fig. 3, B and C). Deletion of amino acids 1–47, the primary phosphorylated sequence of β-catenin, did not change its localization at membrane ruffles, suggesting that phosphorylation per se is not required for membrane localization. Subsequent deletion of residues 1–132 reduced β-catenin localization at membrane ruffles by ~40%, whereas deletion of amino acids 1–217 reduced this staining pattern by 75%.
Interestingly, an internally deleted of the arm domain (amino acids 218–467) that prevents binding to APC and N-cadherin did not impair β-catenin localization at ruffles. Thus, although N-cadherin may boost β-catenin at the free membrane, it is not essential. These results indicate that residues 47–217 are critical for localization of β-catenin at membrane ruffles. This sequence contains the binding site for IQGAP1 (amino acids 123–183; Ref. 24), implicating IQGAP1 as a primary anchor or regulator of β-catenin at membrane ruffles.

Silencing IQGAP1 Expression Reduces β-Catenin, APC, and N-cadherin at Membrane Ruffles—We examined the effect of silencing IQGAP1 expression in NIH 3T3 cells. Relative to cells treated with a control siRNA, endogenous IQGAP1 was reduced ~80% by an IQGAP1-specific siRNA as shown by Western blot (Fig. 4A). NIH 3T3 cells were transfected with control or IQGAP1 siRNA, and 48 h later cells were immunostained with antibodies against IQGAP1 and partner proteins. More than 200 cells were scored in each case, comparing cells that were either positive for IQGAP1 membrane ruffles (control siRNA group) or cells that were negative for IQGAP1-stained ruffles (IQGAP1 siRNA) as shown in Fig. 4B. Cells were subconfluent, and all displayed free membrane with the potential to form ruffles. We found that ~55% of cells that stained positive for IQGAP1 at membrane ruffles also stained positive for β-catenin at these structures. In contrast, those cells negative for IQGAP1 at ruffles showed only ~20% staining of β-catenin at ruffles. The loss of IQGAP1 did not result in a net loss of ruffle formation as revealed by staining for actin-positive ruffles, which was unaffected by IQGAP1 knock-down (data not shown). Moreover, siRNA-dependent silencing of APC, although less efficient in NIH 3T3 cells (50% of cells with loss of APC ruffles), caused an increase in β-catenin nuclear staining but had no significant affect on β-catenin at membrane ruffles (data not shown). We, therefore, conclude that IQGAP1 contributes to recruitment of β-catenin at ruffling membrane. A similar conclusion was proposed by Watanabe et al. (11) for the effect of IQGAP1 on APC in human Vero fibroblasts. Indeed, we confirmed the ability of IQGAP1 siRNA to facilitate a similar loss of APC in NIH 3T3 cells. Furthermore, we observed an unexpected and novel inhibitory effect of IQGAP1 siRNA on N-cadherin at membrane ruffles (Fig. 4C). These data indicate a role for IQGAP1 in recruiting not only APC but also β-catenin and N-cadherin to membrane ruffles.

Silencing of β-Catenin Reduces APC and N-cadherin, but Not IQGAP1, at Membrane Ruffles—NIH 3T3 cells were treated with β-catenin siRNA, resulting in 90% specific loss of β-catenin protein (Fig. 5A). These cells were then stained and assessed for protein localization at membrane ruffles as described above for the IQGAP1 knockdown (Fig. 4). We found that β-catenin silencing had no effect on IQGAP1 at membrane ruffles, whereas it abolished staining of APC and N-cadherin in 40–50% of cells (Fig. 5B). These data indicate that IQGAP1 acts upstream of β-catenin. The knockdown of either IQGAP1 or β-catenin reduced APC and N-cadherin at ruffles, suggesting that IQGAP1 may co-operate with β-catenin to enlist membrane recruitment of specific partners.

The Loss of β-Catenin Retards Cell Migration in a Wound Healing Assay—We investigated the possibility that β-catenin contributes to cell migration in a wound healing assay. NIH 3T3 cells were transfected with control or β-catenin siRNA, and 72 h post-transfection cells were wounded, and cells at the wound edge were imaged by time-lapse microscopy. A Zeiss Axiovert 200M live cell imaging system was used to track 8 different wound regions every hour over a 15-h period. For each region three points were selected for post-image analysis, and the distance migrated by cells every hour was measured using
Zeiss Axiovision software. After the wound-heal experiment, cells were fixed and stained for β-catenin and actin to confirm the siRNA-mediated loss of β-catenin. Using this approach we observed a slight retardation of wound healing in β-catenin siRNA-transfected cells relative to control siRNA-treated cells (Fig. 6). The most consistent effect was observed within the first 6 h, suggesting that loss of β-catenin slows the response to wounding and the initiation of cell movement. It has previously been suggested that β-catenin might contribute to cell migration or invasion through its nuclear transcriptional activity; however, a transcription-independent link between β-catenin and cell migration has also been suggested (23). We note that membrane ruffle-associated β-catenin forms a significant fraction of the β-catenin pool in migrating 3T3 cells, leading us to propose that the membrane-associated fraction of β-catenin may contribute to cell migration in this assay. However, because the change in cell migration activity was relatively modest, it was evident that β-catenin might in fact be recruited to membrane ruffles for another reason.

The Localization of β-Catenin at Membrane Ruffles Triggers Its Internalization through Macropinocytosis—Membrane ruffles are associated not only with cell motility but also with the process of fluid phase protein uptake through macropinocytosis (25, 26). During our study we observed frequent staining of β-catenin in large cytoplasmic particles, not only in NIH 3T3 cells, but also in breast cancer cell lines such as T47D and MCF-7 (see Fig. 7A and supplemental Fig. S5). We induced membrane ruffling in NIH 3T3 cells and noticed that the appearance of β-catenin particles increased in proportion to the extent of membrane ruffling (see Fig. 7B). Indeed the proportion of cells with β-catenin particles was 4-fold higher in cells that displayed membrane ruffles.

To determine whether the particles we observed are macropinosomes, we transfected NIH 3T3 cells with pASEF-ca and 48 h later incubated these cells with the fluid phase pinocytic marker, TRITC-conjugated dextran, then fixed the cells after 1 h and immunostained for β-catenin. Deconvolution analysis of microscopy cell images was used to show that surface-labeled dextran was internalized from the membrane into the cytoplasm of the cells and that the internalized dextran co-localized with β-catenin in the cytoplasmic particles (Fig. 7C). This is strong evidence that the β-catenin is indeed being internalized by macropinocytosis at the site of membrane ruffles. We also observed co-localization between dextran and APC in macropinosome particles (Fig. 7C).

We further tested some breast tumor epithelial cell lines for the presence of β-catenin macropinosomes. β-Catenin was frequently observed at membrane ruffles and in macropinosomes. β-Catenin macropinosomes were seen in 11–13% of cells in the cell lines T47D, MCF7, and MDA.MB.2321 (Fig. 7A and supplemental Fig. S5).
β-Catenin Localization at Membrane Ruffles

β-Catenin Macropinocytosis Is Regulated by Arf6 GTase—The Arf6 GTase has been implicated in endocytosis and actin remodeling (27), E-cadherin internalization (28, 29), and macropinocytosis (17). Arf6 must cycle between GTP and GDP-bound forms to function properly, and the overexpression of either constitutively active or dominant negative mutants can inhibit its activity by preventing its GTP/GDP binding cycle (27). Palacois et al. (28) reported that GTP-bound Arf6 can induce disassembly of adherens junctions and the internalization/endocytosis of E-cadherin. They also observed Arf6-mediated ruffling of the lateral membrane, which is implicated in its role in facilitating macropinocytosis (30). We hypothesized that a block in the Arf6 GTase cycle might affect macropinocytosis of β-catenin in NIH 3T3 cells. To address this, we tested a myristoylated synthetic peptide corresponding to the N-terminal region of Arf6, which was previously reported to block Arf6 activities in various cellular processes including the aluminum fluoride induced activation of phospholipase D and release of β-arrestin protein (31, 32). Cells were transfected with the ASEF-ca plasmid to induce ruffling, and 30 h later cells were treated with the myrArf6 (2–13) peptide and stained with antibodies against Arf6 and β-catenin (Fig. 7D). Microscopic analysis revealed that Arf6 peptide treatment caused a 67% reduction in β-catenin staining at membrane ruffles and in β-catenin positive macropinosomes relative to vehicle-treated cells. A similar result was observed for human T47D breast cancer cells (see supplemental Fig. S6). We conclude that macropinocytosis of β-catenin from membrane ruffles is regulated by Arf6 GTase.

β-Catenin Co-localizes with Actin, APC, N-cadherin, and IQGAP1 at Macropinosomes—The macropinocytosis of β-catenin involves involution of membrane at sites of ruffling and might represent an avenue for internalization of those β-catenin binding partners found to co-localize with β-catenin at membrane ruffles (Fig. 2). To test this we transfected NIH 3T3 cells with pASEF-ca and stained cells for β-catenin and the different partner proteins. A strong co-localization with β-catenin (≥90%) was observed for actin and N-cadherin at macropinosomes (Fig. 8). Actin itself is known to coat macropinosomes early during internal transit (33), whereas the identification of N-cadherin is novel. Nearly 60% of β-catenin macropinosomes co-localized with APC, whereas only 28% stained positive for IQGAP1. ASEF-ca protein provided a valuable internal control, and although it was present at membrane ruffles with β-catenin, only 8% of β-catenin macropinosomes displayed detectable ASEF protein. We propose that actin, APC, N-cadherin, and IQGAP1 are to varying degrees internalized from the membrane in macropinosomes along with β-catenin.

IQGAP1 Regulates β-Catenin Macropinocytosis—We scored for the presence of β-catenin macropinosomes after knockdown of IQGAP1. An ~70% reduction in cells with β-catenin-positive macropinosomes was observed after IQGAP1 siRNA treatment compared with cells treated with control siRNA (Fig. 9A). This is consistent with the idea that loss of IQGAP1 reduces targeting of β-catenin to membrane ruffles, indirectly affecting its ability to be internalized through macropinocytosis. To further show that IQGAP1 regulates β-catenin internalization, we transiently expressed different GFP-fusion proteins in NIH 3T3 cells. Overexpressed GFP-IQGAP1 increased the formation of β-catenin-positive macropinosomes, whereas expression of the C-terminal dominant negative sequence of IQGAP1 (IQGAP1-ct) reduced β-catenin macropinocytosis (Fig. 9B). The overexpression of GFP-N-cadherin had little effect on β-catenin macropinosomes.

β-Catenin Regulates Macropinocytosis of APC, IQGAP1, and N-cadherin—Does β-catenin itself contribute to macropinocytosis? To address this question, NIH 3T3 cells were treated with control siRNA or β-catenin siRNA to assess the impact of β-catenin silencing on macropinosome formation. The β-catenin siRNA treatment reduced visible β-catenin-positive macropinosomes in cells by 84% compared with cells treated with...
It is interesting to note that this knockdown impacted modestly on the overall macropinocytosis process, as staining for actin-positive macropinosome particles was reduced by ~28% compared with control. More striking was the specific effect on the β-catenin binding partners APC, N-cadherin, and IQGAP1; cells with visible staining of these partners at macropinosomes were reduced by 80–90% after β-catenin siRNA treatment (Fig. 9C). In contrast, knockdown of β-catenin had little effect on IQGAP1 staining at membrane ruffles (Fig. 5). These data imply a possible chaperone/scaffold-

FIGURE 8. β-Catenin co-localizes with actin, APC, IQGAP1, and N-cadherin at macropinosomes. NIH 3T3 cells were transfected with ASEF-ca and stained with antibodies to β-catenin and the different partner proteins. Co-staining at macropinosomes was scored (see arrows in the cell images) and quantified. Values shown in graph are mean ± S.E. from two experiments.

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β-Catenin Macropinosomes Co-localize with the Lysosomal Marker, LAMP1, Identifying a Route for Protein Recycling—E-cadherin is internalized by different endocytosis pathways and was subsequently degraded or recycled back to the membrane, but much less is known about the internalization of β-catenin (34–36). Internalized E-cadherin vesicles have been reported to co-localize with Rab5- and EEA1-positive early endosomes (34, 36, 37). Macropinosomes do not usually co-stain with markers of early or late endosomes, although the constituents can be recycled through these pathways after the macropinosome breaks down (17, 27). To confirm that the macropinosomes detected here are not an unusual form of vesicle, we stained for various endosomal markers but observed no co-localization with TGN38 (Golgi marker), EEA1, Rab3 and Rab5 (early endosome proteins), or Rab11 (recycling endosomes) using both standard fluorescence microscopy and deconvolution microscopy. All of the antibodies tested are well characterized and have been published for clear detection of their respective targets (see “Materials and Methods”), and all gave the expected vesicular or granular staining patterns as published. The only antibody tested that produced a pattern of co-localization with β-catenin macropinosomes was the lysosomal marker, LAMP1 (Fig. 10A). Using a Zeiss Axiovert 200M microscope and deconvolution software, we found that 38% of β-catenin macropinosomes co-localized with LAMP1 antibody (Fig. 10A). This finding is consistent with the ultimate merging of macropinosomes with the late endocytic machinery and suggests that β-catenin-positive macropinosomes are eventually targeted for lysosome-mediated degradation, with the constituents presumably degraded or recycled for trafficking to other parts of the cell.

DISCUSSION

β-Catenin is a mediator of the wnt signaling pathway and is generally regarded to have two major sites of action in the cell; that is, at membrane adherens junctions and in the nucleus (1, 38). In this study we show that β-catenin also accumulates at membrane ruffles in actively migrating cells and identified the β-catenin binding partner, IQGAP1, as a key regulator of this process. IQGAP1 is known to modulate cell migration (11, 12), and the specific silencing of β-catenin likewise displayed a reproducible, albeit modest, influence on the rate of cell migration. The main consequence of β-catenin recruitment to membrane ruffles was its rapid internalization through ruffle-induced macropinocytosis. This macropinocytic pathway was regulated by Arf6 and IQGAP1. We propose, based on data reported in this study, that β-catenin may act as a linker or scaffold for the internalization of membrane-associated proteins including APC and N-cadherin (our findings are summarized in Fig. 10B). The concept of β-catenin acting as a chaperone or scaffold for the internalization of membrane-associated proteins provides a new context for its targeting to these dynamic membrane regions.

β-Catenin at Membrane Ruffles—β-Catenin is usually described as an integral component of cell adherens junctions and plays a key role in the nucleus as a transcriptional regulator and activator of cell transformation in several cancers (3, 38). In

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addition to cell-cell membrane junctions, β-catenin has been detected at other membrane locations including microtubule-dependent membrane protrusions (39) and actin-dependent membrane ruffles (10). We recently demonstrated a role for β-catenin in regulating APC localization at membrane protrusions (20). Here we addressed the question of why β-catenin localized to membrane ruffles. First, we tested its involvement in cell migration by imaging live cells after wounding and tracking the rate of wound closure in NIH 3T3 fibroblasts transfected with control or β-catenin siRNA, then stained for β-catenin and partner proteins and processed for microscopy. Cells were scored randomly for the presence of β-catenin or partner at macropinosomes. The data shown are the mean ± S.E. from three experiments. The loss of β-catenin reduced staining of partners at macropinosomes.

Increased β-catenin at membrane ruffles. IQGAP1 is an important regulator of cell migration (11, 12) and adhesion (22, 42), and its binding to β-catenin may modulate these processes. In this regard it was proposed that direct binding of IQGAP1 to β-catenin-E-cadherin complexes at epithelial cell adherens junctions may displace α-catenin and actin, leading to weakening of the adherens junction and a consequent increase in cell movement (42). On the other hand, our data with fibroblasts implicates an association between β-catenin and IQGAP1 at membrane ruffles that might also favor cell migration, a view supported by the negative influence on migration by siRNA-mediated knockdown of IQGAP1 (11, 12), APC (11), and β-catenin (this study).

IQGAP1-dependent Macropinocytosis of β-Catenin—Membrane ruffling is also important for solute uptake by macropinocytosis. A key finding of this study is that IQGAP1-dependent recruitment of β-catenin to membrane ruffles facilitates the macropinocytosis of β-catenin and specific binding partners. Ruffle-mediated macropinocytosis provides a mechanism for internalizing large sections of lateral plasma membrane in an actin-dependent but clathrin-independent manner (25, 26, 43). We stimulated membrane ruffle formation by overexpressing

FIGURE 9. IQGAP1 and β-catenin regulate macropinocytosis of partner proteins. A, NIH 3T3 cells were transfected with control or IQGAP1 siRNA, and >200 cells were scored for the presence of β-catenin or IQGAP1 at macropinosomes. B, cells were transfected with plasmids encoding GFP-fusions of N-cadherin or IQGAP1 (wild-type or ct mutant) and co-stained for β-catenin. The proportion of transfected cells with β-catenin-positive macropinosomes was scored and graphed. Data are the mean ± S.E. from two experiments. C, cells were transfected with control or β-catenin siRNA, then stained for β-catenin and partner proteins and processed for microscopy. Cells were scored randomly for the presence of β-catenin or partner at macropinosomes. The data shown are the mean ± S.E. from three experiments. The loss of β-catenin reduced staining of partners at macropinosomes.
that is, the regulated internalization of membrane ruffle-associated APC and N-cadherin. Once internalized, these proteins may subsequently follow the endocytic pathway for membrane proteins where they are targeted for recycling or degradation (44). This is suggested by our observation that some β-catenin macropinosomes co-stained with the lysosome marker LAMP1, indicating that they are targeted for lysosome-mediated degradation.

The cadherins are a family of transmembrane proteins that display variable cellular distribution and function in different cell types. E-cadherin is highly expressed in epithelial cells and promotes cell-cell adhesion (45, 46), whereas N-cadherin is implicated in promoting cell motility and invasion (23, 47, 48). Paterson et al. (29) previously speculated that E-cadherin might be internalized by active macropinocytosis in MCF-7 breast cancer cells, but this was not experimentally verified. In this study N-cadherin co-localized strongly with β-catenin in NIH 3T3 cells at both adhering and ruffling membrane, in the cytoplasm in macropinosomes, and to a lesser degree in the nucleus (data not shown). It is possible that the two form an intracellular complex important for regulating their cellular targeting, at least for their co-location at membrane ruffles and their internalization by macropinocytosis as demonstrated here. Indeed, the overexpression of N-cadherin contributed to β-catenin recruitment at membrane ruffles, and knockdown of β-catenin reduced N-cadherin at ruffles, predicting that the two proteins may form a mutual complex with IQGAP1 at actin filaments just below the membrane surface. Our data implicate β-catenin as an intermediate in the IQGAP1-regulated internalization of APC and N-cadherin by macropinocytosis.

Although Rac1 is essential for membrane ruffle formation (49), other small GTPases such as Rah/Rab34 have been implicated in the generation of macropinosomes from ruffled membrane (33). We detected actin in most, but not all β-catenin-positive macropinosomes, and this is consistent with the notion that actin coats the macropinosome but comes off as the vesicles move toward the center of the cell (33). There is very little known in regard to internalization and recycling of IQGAP1, N-cadherin, or APC, and it is tempting to speculate that one reason β-catenin moves to this dynamic membrane region is to help facilitate the internalization of these factors. It is yet to be determined if β-catenin moves to ruffles from a defined pool, although its targeting does not seem to be regulated by phosphorylation (Fig. 3 and data not shown). Further study of these questions should help elucidate new roles for membrane-associated β-catenin.

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