β-Catenin Associates with the Actin-bundling Protein Fascin in a Noncadherin Complex

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Abstract. Catenins were first characterized as linking the cytoplasmic domains of cadherin cell–cell adhesion molecules to the cortical actin cytoskeleton. In addition to their essential role in modulating cadherin adhesivity, catenins have more recently been indicated to participate in cell and developmental signaling pathways. β-Catenin, for example, associates directly with at least two receptor tyrosine kinases and transduces developmental signals within the Wnt pathway. Catenins also complex with the tumor suppressor protein adenomatous polyposis coli (APC), which appears to have a role in regulating cell proliferation.

We have used the yeast two-hybrid method to reveal that fascin, a bundler of actin filaments, binds to β-catenin's central Armadillo repeat domain. Western blotting of immunoprecipitates from cell line and mouse and rat brain extracts indicate that this interaction exists in vivo. Fascin and β-catenin's association was further substantiated in vitro using purified proteins isolated from recombinant bacterial and baculoviral sources. Immunoprecipitation analysis indicates that fascin additionally binds to plakoglobin, which is highly homologous to β-catenin but not to p120^{cas}, a newly described catenin which contains a more divergent Armadillo-repeat domain. Immunoprecipitation, in vitro competition, and domain-mapping experiments demonstrate that fascin and E-cadherin utilize a similar binding site within β-catenin, such that they form mutually exclusive complexes with β-catenin. Immunofluorescence microscopy reveals that fascin and β-catenin colocalize at cell–cell borders and dynamic cell leading edges of epithelial and endothelial cells. In addition to cell–cell borders, cadherins were unexpectedly observed to colocalize with fascin and β-catenin at cell leading edges. It is conceivable that β-catenin participates in modulating cytoskeletal dynamics in association with the microfilament-bundling protein fascin, perhaps in a coordinate manner with its functions in cadherin and APC complexes.

How cell–cell interactions are regulated in developing and adult organisms to enable coordinate adhesive interactions and dynamic cell movements is not well established. Such coordination is, however, essential during processes such as wound repair and embryonic gastrulation. Correspondingly, the misregulation of adhesive and motility functions is a hallmark of certain disease processes, including cancers (for review see references 3, 71).

The cadherin family of transmembrane cell–cell adhesion proteins and their intracellularly associated catenin proteins form complexes of central importance to the sorting and morphogenic processes of virtually all developing animal tissues and in maintaining the integrity and identity of adult tissues (for reviews see references 35, 70). With the exception of T-cadherin (56), which lacks a cytoplasmic tail, the catenin proteins have been shown to link nondesmosomal cadherins to cortical actin (26, 37, 46, 51), intracellular and receptor tyrosine kinases and phosphatases (5, 31, 34), and additional proteins. The loss of cadherin (for reviews see references 32, 71) or of catenin function (29, 50) is well correlated with dedifferentiated and invasive cell phenotypes, suggesting that the function of the cadherin–catenin complex profoundly influences cell identity and motility.

Although the mechanisms by which cell motility is effected are multifactorial, one likely regulatory point resides in the assembly/disassembly of microfilament bundles. Bundled actin filaments, for example, are essential in extending lamellopodia and microspikes (filopodia in neurons) from cell leading edges, an early and obligate step in cell migration. Conversely, the disassembly of actin bundles and filaments in these structures is thought to result in leading edge retraction, which in response to various stimuli may be transient or result in further cell redirection (for reviews see references 12, 66).

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Proteins that are capable of bundling actin filaments are likely to be numerous. Examples of known actin bundlers include fimbrin, α-actinin, α-catenin, and fascin (59; for reviews see references 15, 48). α-Actinin bundles filaments as an antiparalell dimer, while fimbrin and fascin appear to possess two actin-binding domains within a single molecule, permitting tight filament packing. While functional redundancies are known to occur within families of actin-associating proteins, little is known concerning the degree of in vivo functional overlap amongst bundling proteins, including, for example, the degree to which different bundling proteins exist within the same cell.

Fascin associates with filamentous actin, often within highly motile and dynamic subcellular structures including stress fibers, lamellopodia, and filopodia (75, 76). In marine invertebrates such as echinoderms (sea urchins), fascin appears to have diverse roles, including the rapid and highly ordered (7) bundling of actin filaments in egg microvilli, sperm acrosomal processes, and the filopodia of phagocytic coelomocytes (primitive immune surveillance cells) (for reviews see references 6, 15). That fascin function is crucial in certain cell types was indicated in recent experiments in which rat hippocampal neurons were exposed to fascin antisense oligonucleotides, leading to a dramatic collapse in neuronal growth cones (15). Within the mouse, brain, spleen, and uterus tissues are enriched in fascin mRNA and protein (15), while within *Xenopus*, testis and oocytes express the highest levels of fascin mRNA (30). While the levels vary, surveys suggest that most cells express fascin mRNA and protein (14). Fascin contains one MARCKS consensus sequence near its NH_2 terminus (43) but otherwise is not homologous to any known protein (8).

Drosophila possesses a fascin homolog named Singed, and mutants of the *singed* locus evidence a number of embryonic defects, including aberrant mechanosensory bristle and oocyte formation. Microscopy indicates that the morphological phenotypes of affected cells correlate well with the disorganization or absence of microfilament bundles at the molecular level (10, 49, 73). That all embryonic tissues were not grossly defective in mutant *singed* alleles may indicate the presence of residual Singed protein functionality even within apparently strong *singed* alleles or, more likely, may indicate the presence of functional redundancies in some *Drosophila* tissues (10, 73).

As indicated above, the cytoplasmic domain of cadherins are joined via catenins to a meshwork of cortical actin-containing microfilament bundles. In epithelia, this large and incompletely characterized complex is distributed continuously along cell–cell borders within a region known as the *zonula adherens*, which is distinct from the tight junction and desmosomal cell–cell adhesion structures. Integrin-containing cell substrate adhesion complexes, or focal contacts, likewise associate with bundled microfilaments known as stress fibers, but these appear as larger and more singular entities that extend away (or loop out) from the plasma membrane. Given that the functions of cell adhesion and motility complexes are interdependent, it is important to seek and examine shared protein components that may facilitate their coordinate activities.

Through use of the yeast two-hybrid system, we have identified a novel interaction of the actin-bundling protein fascin with β-catenin. We have then authenticated the existence of this complex using a number of different in vivo and in vitro biochemical assays, mapped β-catenin's interaction domain, and examined the colocalization of fascin, β-catenin, and cadherins using immunofluorescent microscopy.

Materials and Methods

Yeast Two-Hybrid Screen

The yeast two-hybrid system of Vojtek et al. (74) was generously provided by Stan Hollenberg (University of Oregon Health Sciences Center, Portland, OR) and used to identify novel components that interact with a "bait" cDNA construct expressing most of the Armadillo repeat region of *Xenopus* β-catenin (repeats ~0.5–11 > amino acids 167–583) (40) fused to the lexA DNA-binding domain. The embryonic mouse cDNA library consisted of 300–700-base pair cDNA inserts fused to the VP16 activation domain (74). 1.1×10^6 colonies were screened, and only clones meeting all standard two-hybrid specificity tests were considered as positive (2). These tests included robust colony growth in His^- media containing 25 mM 3-amino-triazole, the absence of an interaction between prey constructs and a lamin negative-control construct, and the inability of colonies containing the prey construct alone to pass His^- (survival) and β-galactosidase (blue) assays.

Antibody Production and Cell Lines

All polyclonal and monoclonal antibodies were raised, respectively, in rabbits and mice. Antifascin polyclonal antibodies were raised against mouse fascin protein that was affinity purified after expression from bacteria as a thioredoxin fusion protein (thioredoxin domain removed by enterokinase treatment) (InVitrogen, San Diego, CA) (13). Anti–β-catenin polyclonal antibodies were raised and affinity purified as indicated (41). Antiplakoglobin polyclonal antibodies were raised against a mixture of three human plakoglobin peptides (amino acids 1–22, 49–72, and 690–710) (19), and two *Xenopus* plakoglobin peptides (amino acids 173–196 and 223–239) (18). Anti–α-catenin polyclonal antibodies were raised as indicated (20). Anti–mouse E-cadherin and $p120^{cas}$ (mouse) monoclonal antibodies were obtained from Transduction Labs (Lexington, KY), and normal rabbit IgG was obtained from Sigma Immunochemicals (St. Louis, MO). Anti–N-cadherin monoclonal antibodies (clone 13A9) were generously provided by M. Wheelock (University of Toledo, Toledo, OH) (37).

A-431 cells were derived from an epidermoid human carcinoma by Giard et al. (21), and grown as indicated (CTL 1555; American Type Culture Collection, Rockville, MD) (23). HeLa cells were grown in D-MEM-F12 with 10% fetal calf serum. Primary normal human lung microvascular endothelial cells (HMVEC-L) were kindly provided by A. Jacobs (D. Carson Lab, University of Texas M.D. Anderson Cancer Center, Houston, TX) and grown in EGM-MV Bullet Kit Media (cells and media from Clonetics, San Diego, CA).

Tissue Extraction, Immunoprecipitations, SDS-PAGE, and Western Blotting

Fresh mouse or rat brain tissues were extracted in 0.75% NP-40 in G buffer (20 mM Tris-HCl, pH 7.6, 150 mM KCl, 0.6 mM CaCl, 2.0 mM $MgCl_2$, 0.5 mM ATP, 0.05% Triton X-100). Cell line extractions occurred in G buffer. After debris removal via centrifugation (14,000 rpm microfuge, 30 min), the resulting supernatants were subjected to immunoprecipitation using affinity-purified polyclonal anti–β-catenin antibodies, antifascin antibodies, or normal rabbit IgG antibodies (negative control), directly coupled to cyanogen bromide Sepharose-4B beads following the manufacturer's protocol (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Alternatively, uncoupled primary antibodies were precipitated using protein G coupled to Sepharose-4B beads as previously indicated (40). Immunoprecipitates were washed three times in G buffer, once in 0.5 M NaCl in G buffer without NP-40, and once in PBS^- (136 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) before their reduction (20 mM DTT, 5 min, 95°C) in 50–100 μl of SDS-PAGE sample buffer (3.3% SDS). Samples destined for fascin Western blotting were not reduced to prevent the comigration of antibody heavy chains (~55 kD). Proteins were resolved upon 7% SDS-PAGE gels, electrotransfered to nitrocellulose (40),

and detected by enhanced chemiluminescence (ECL)[1] (Amersham Corp., Arlington Heights, IL). Primary antibodies included affinity-purified anti-β-catenin and fascin polyclonal antibodies (0.5–2.0 μg/ml), immune sera (1,000–5,000-fold dilution), and hybridoma supernatants (ascites, 500–2,000-fold dilution) and were incubated with blots in minimal volumes for 2–16 h at room temperature or 4°C. The ECL Western blot development protocol was used to visualize proteins of interest (Amersham Corp.).

In Vitro Fascin-β-Catenin Binding

Recombinant full-length β-catenin was purified from baculovirus Hi-5 cells (PharMingen, San Diego, CA), infected with AcMNPV phage expressing $(His)_6$-tagged *Xenopus* β-catenin (kindly provided by Robert Kypta, University of California, San Francisco/HHMI) and affinity purified using NTA (nickel)-agarose chromatography according to the manufacturer (Quiagen, Chatsworth, CA). The Armadillo repeat region of β-catenin (amino acid residues 167–583) was expressed as a glutathione-*S*-transferase (GST)-fusion protein in *Escherichia coli* BL-21 and affinity purified by GST-column absorption from bacterial extracts according to the manufacturer's protocol (Pharmacia LKB Biotechnology, Inc.); briefly, bacterial pellets were washed and resuspended in $\frac{1}{50}$ of their culture volume in PBS^- containing protease inhibitors, probe sonicated, brought to 1% in Triton X-100, and supernatant fraction retained after centrifugation (20 min at 15,000 *g*). Full-length recombinant fascin was expressed as a thioredoxin fusion protein in *E. coli* BL-21 and affinity purified by column absorption (13). Protein solutions were precleared by centrifugation immediately before use in in vitro assays (20–30 min, 15,000 *g*). For binding assays, 1.5 μg of fascin protein was added to either 2.0 μg of full-length β-catenin or 2.0 μg of β-catenin Armadillo repeat fusion protein in 600 μl of G buffer containing 0.75% NP-40, 0.5 mg/ml BSA, and 0.5 mg/ml *E. coli* BL-21 whole-protein extract obtained using the same protocol as noted above for isolation of GST-fusion proteins (Pharmacia LKB Biotechnology, Inc.). After a 20-min incubation at room temperature, the mixture was equally separated into three tubes, brought to a buffer volume to 800 μl, and subjected to anti-β-catenin (β), antifascin (F), and normal rabbit IgG (negative control) immunoprecipitation. Immunoprecipitates were blotted with affinity-purified anti-β-catenin or antifascin antibodies.

Yeast Two-Hybrid Mapping of Fascin's Binding Site within β-Catenin

cDNA fragments coding for the indicated (see Fig. 4) Armadillo repeats of β-catenin were obtained by PCR and cloned in-frame into the yeast two-hybrid "bait" vector pBTM116 using directional polylinker sites EcoRI and SalI (74). The initiation and termination of each construct approximated that of the *Xenopus* β-catenin Armadillo repeats as follows (repeat No. > amino acid): 1 > 141, 2 > 175, 3 > 218, 4 >259, 5 > 301, 6 > 344, 7 > 384, 8 > 423, 9 > 468, 10 > 513, 11 > 577, 12 > 618, and 13 > 657 (terminus 695) (40). The 5′ and 3′ cDNA ends of each construct were sequenced to confirm the inserts' orientation and identity. After transformation into yeast, the ability of the β-catenin fusion constructs to interact with fascin was assessed by two criteria (20 clones tested per construct) (17): (*a*) ability of yeast expressing both constructs to transactivate the His reporter and confer growth in the absence of His and (*b*) ability to transactivate the lacZ reporter construct, assessed by β-galactosidase filter assay. " −" indicates clones that did not grow on His− medium and/or failed the β-galactosidase filter assay. " +" indicates clones which both grew on His− medium and passed the β-galactosidase color assay ("+", "++", "+++," and "++++" indicates the clone's relative size and color density). Viable ("+") clones passed standard authenticity tests (Materials and Methods) (2).

E-Cadherin/Fascin Competition for β-Catenin

Affinity-purified full-length fascin and β-catenin were obtained as noted in the above Materials and Methods. A GST–E-cadherin fusion protein was constructed to contain the cytoplasmic domain (residues 734–884) of mouse E-cadherin and affinity purified (E-cadherin cDNA a kind gift of M. Takeichi, Kyoto University [45].). A 3.4:1 molar ratio of E-cadherin/β-catenin (2.7 nmol [12.5 μg]/0.8 nmol [7.5 μg]) was preincubated for 30 min at room temperature (reaction in 0.6 ml G buffer containing 0.75% NP-40, 1 mg/ml BSA, 0.5 mg/ml *E. coli* protein extract, and protease inhibitors), and the mixture was equally divided into five tubes containing fascin at increasing molar ratios of fascin/β-catenin. After an additional incubation (30 min, room temperature), the reaction was immunoprecipitated for E-cadherin, the washed precipitates subjected to SDS-PAGE, and coprecipitating β-catenin protein resolved by Western blotting (ECL; Amersham Corp.)

Immunofluorescence Microscopy

A-431 cells, HeLa cells (data not shown), and human endothelial cells were grown for 18–48 h on coverslips to 50–80% confluence, fixed for 15 min at room temperature using 3.75% formaldehyde/PBS, and permeablized with 0.1% Triton X-100 in PBS. Cells were blocked using 0.2% BSA and 5% (vol/vol) normal donkey serum (Jackson ImmunoResearch Labs, Inc., West Grove PA) and double-stained using our affinity-purified antifascin polyclonal antibody, anti-β-catenin monoclonal antibody (Transduction Labs), anti-E-cadherin monoclonal antibody (Transduction Labs) or anti-P-cadherin monoclonal antibody (Transduction Labs). Negative controls used normal rabbit IgG or normal mouse IgG. Alternatively, the specificity of immunofluorescent staining was established by competing anti-E-cadherin antibodies with excess affinity-purified GST–E-cadherin cytoplasmic domain fusion protein or by competing anti-β-catenin antibodies with excess affinity (his tag)-purified recombinant baculovirus-expressed β-catenin. Primary antibodies were detected by fluorescein-conjugated donkey anti-rabbit IgG antibodies and rhodamine (TRITC)-conjugated donkey anti-mouse IgG antibodies (Jackson ImmunoResearch Labs, Inc.). Immunofluorescence microscopy was performed on a Leitz Dialux microscope using a Leitz 60 × NPL Fluorar objective (Rockleigh, NJ).

Results

The Yeast Two-Hybrid Systems Resolves Known and Novel β-Catenin Interactions

We used the yeast two-hybrid system of Vojtek et al. (74) to identify novel components that interact with β-catenin. A "bait" cDNA construct expressing the repeat region of *Xenopus* β-catenin (40) fused to the lexA DNA-binding domain was used to screen for "prey" clones in an embryonic mouse cDNA library composed of 300–800-base pair cDNA inserts fused to the VP16 activation domain (74). The 97% amino acid identity between the *Xenopus* and mouse β-catenin proteins (9) indicated to us that cross species screening was feasible.

Full-length and NH_2- and COOH-terminal regions of β-catenin were not used in similar screens as these constructs "autoactivated," that is, they survived selection conditions in the absence of an interaction with prey library constructs.

19 of our positive clones met all standard two-hybrid specificity tests (2). 18 of the positive prey clones were authentic based upon sequence comparisons; mouse OB-cadherin (47), N-cadherin (42), adenomatous polyposis coli (APC) (67), and a homologue of human vascular endothelial (VE)-cadherin (cadherin-5) (38, 69, 72). As cadherins and APC are known to bind the β-catenin repeats via interaction domains (33, 44, 51, 61) that were present in our clones (data not shown), these results provided a strong indication that our system was working.

That no artifactural interactions were resolved may have been a consequence of the particular two-hybrid system employed, which requires that each interaction complex must possess sufficient avidity to survive cotransport into the nucleus, as only the prey (not both the prey and bait) fusion protein possesses a nuclear localization se-

1. *Abbreviations used in this paper*: APC, adenomatous polyposis coli; ECL, enhanced chemiluminescence, GST; glutathione-*S*-transferase.

quence. The accounting of our clones indicated, however, that our screen was incomplete, as additional cadherins and perhaps the EGF receptor (31) would have been isolated under saturating conditions.

One positive clone identified an unknown interaction between β-catenin's repeat domain and the actin-bundling protein fascin (16). The cDNA of mouse fascin was recently isolated and sequenced; its deduced amino acid sequence is 32 and 36% identical, respectively, to echinoderm and *Drosophila* fascin (Singed). Yeast harboring the fascin clone grew as well in selective media as the cadherin and APC fusions (even in the presence of 3-amido triazole) and developed an intense blue color within 30 min after β-galactosidase staining. Fascin thus arose as a strong candidate in our search for novel binding partners of β-catenin, especially given that our remaining clones represented known partners of β-catenin in vivo.

Immunoprecipitation of the Fascin–β-Catenin Complex In Vivo

To test the fascin–β-catenin interaction more rigorously, we conducted both fascin and β-catenin immunoprecipitations from HeLa, mouse, and rat brain detergent extracts (Fig. 1), which contain easily detectable levels of each protein. Western blots of washed immunocomplexes indicate that fascin specifically coprecipitates with β-catenin (Fig. 1 *A*), and β-catenin specifically coprecipitates with fascin (Fig. 1, *B* and *E*). Plakoglobin (γ-catenin) and p120^{cas} possess a central Armadillo repeat domain similar to that in β-catenin, and each interacts with cadherins (1, 36, 54, 58, 62, 65). Fig. 1 *C* shows that plakoglobin is likewise present in fascin immunoprecipitates, although the Western blot (ECL) signal is weaker and more variable than that of β-catenin. This is characteristic of the weaker but authentic association of plakoglobin with cadherins and APC (39, 61). In contrast to β-catenin and plakoglobin, p120^{cas} appeared to be absent from the antifascin immunoprecipitates (Fig. 1 *D*). p120^{cas} is less homologous to β-catenin than is plakoglobin, even within the Armadillo repeat domain (57), and apparently is unable to associate with fascin, or too weakly to be detected via coimmunoprecipitation.

The Fascin-β-Catenin Interaction Does Not Require Cadherins

The cadherin–catenin complex contains β-catenin and interacts with cortical actin. Thus, we next tested if fascin is a component of the E-cadherin complex. To our surprise, α-catenin and N-cadherin are not detectable in antifascin immunoprecipitates (Fig. 1, *F* and *G*, respectively), and fascin is correspondingly absent in E- and N-cadherin and α-catenin immunoprecipitates (data not shown). Various positive controls indicated that the primary precipitations were successful in coprecipitating known protein partners (Fig. 1). The absence of α-catenin in fascin–β-catenin complexes is unexpected given that a cadherin or APC molecule may bind to β-catenin's Armadillo repeat domain while permitting the subsequent binding of α-catenin to its NH_2-terminal domain (1, 33, 39, 61). Steric, conformational, or phosphorylation/acetylation constraints may preclude the simultaneous association of β-catenin with fascin and α-catenin, or a weak association of α-catenin with the complex may lessen its resolution under coimmunoprecipitation conditions.

Given that E- and N-cadherin, α-catenin, and p120^{cas} are absent from fascin immunoprecipitates (and vice versa), we conclude that the fascin–β-catenin complex is not an integral subcomponent of cadherin complexes. This interpretation is consistent with data presented below that the fascin and cadherin binding sites within β-catenin are overlapping and that fascin and cadherins compete in vitro for binding to β-catenin.

Fascin Binds to β-Catenin in In Vitro Immunoprecipitation Assays

An in vitro assay, using affinity-purified protein components, further supports the authenticity of the fascin–β-catenin interaction. Two recombinant β-catenin constructs were used giving similar results, the first a His-tagged and baculovirus-expressed full-length construct and the second a bacterially expressed GST fusion protein containing the Armadillo repeat region of β-catenin. Recombinant full-length fascin was bacterially expressed as a fusion protein with thioredoxin. As shown in Fig. 2 and consistent with their direct physical interaction, β-catenin immunoprecipitates specifically contain fascin, and conversely, fascin immunoprecipitates specifically contain β-catenin. Consistent with our yeast two-hybrid and in vivo immunoprecipitation results, the in vitro coimmunoprecipitation data indicate that fascin interacts directly with β-catenin.

Mapping the Fascin-binding Domain of β-Catenin

Given that the Armadillo repeat domain of β-catenin is composed of 13 related but nonidentical repeats (each of ~42 amino acids), it was of interest to test which repeats were necessary for binding fascin. Using the yeast two-hybrid system, we determined that none of β-catenin's chosen overlapping triplets of repeats (1–3, 3–5, 5–7, 7–9, 9–11, and 11–13) is capable of supporting a fascin–β-catenin interaction (Fig. 3). Overlapping septets of repeats were then tested (1–5, 5–9, and 9–13), and only the most COOH-terminal construct (9–13) generated a positive interaction. Two longer stretches of seven repeats were tested (1–7 and 7–13), and again, the more COOH-terminal repeats (7–13) demonstrated the stronger interaction with fascin. As the entire β-catenin Armadillo domain, composed of repeats 1–13, generated an interaction that was stronger than that of the repeats 7–13, and of repeats 0.5–11 and 3–11, an extended portion of β-catenin's Armadillo domain appears to be necessary for full associative activity of β-catenin with fascin.

Fascin and the Cytoplasmic Domain of E-cadherin Compete for the Binding of β-Catenin

It has been shown by others that β-catenin's Armadillo repeats 4–13 interact with the conserved cytoplasmic domain of cadherins (33). Because this repeat region is similar to that mapped for fascin's association with β-catenin, we tested if E-cadherin's cytoplasmic domain would compete with fascin for binding to β-catenin. Increasing molar ratios of fascin to E-cadherin were incubated with β-catenin before immunoprecipitation. As shown in Fig. 4, the in

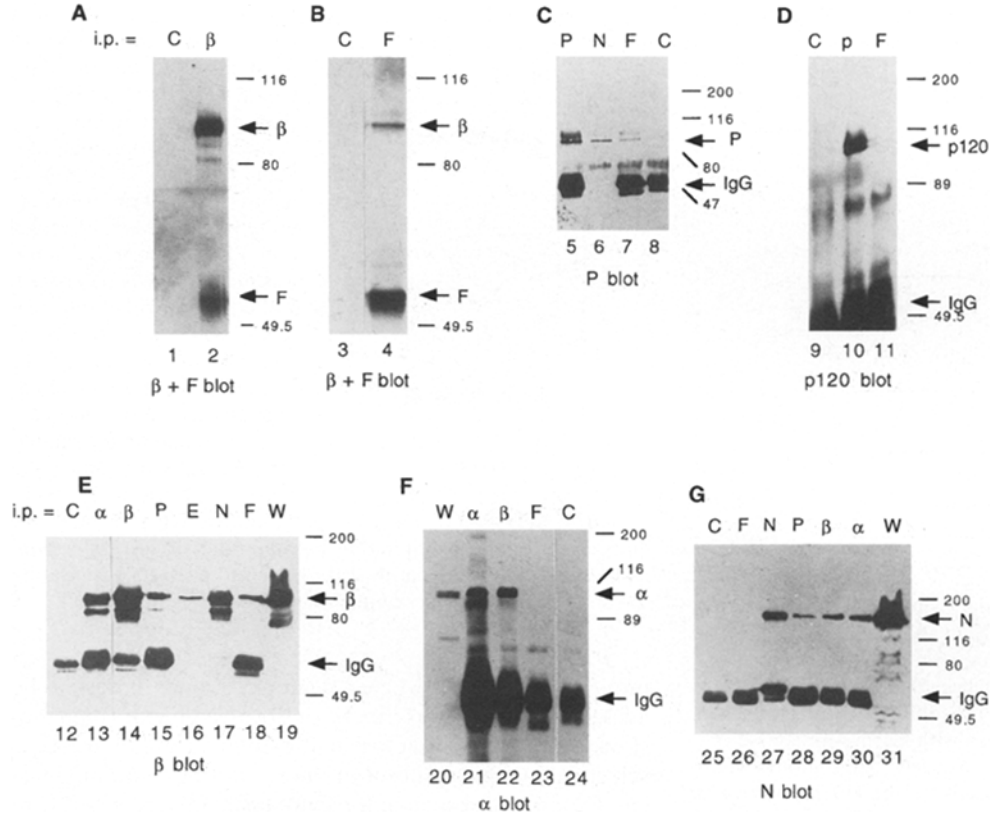

Figure 1. Coimmunoprecipitation of fascin with β-catenin and plakoglobin but not with cadherins, α-catenin, or p120^{cas}. (*A* and *B*) Coimmunoprecipitation of β-catenin and fascin from HeLa cells. As indicated above each lane, cell lysates were subjected to immunoprecipitation using affinity-purified polyclonal anti-β-catenin antibodies (*β*), antifascin antibodies (*F*), or normal rabbit IgG antibodies (*C*, control lanes), cyanogen-bromide conjugated to Sepharose-4B beads (Pharmacia LKB Biotechnology, Inc.). Proteins within the washed immunoprecipitated complexes were resolved by SDS-PAGE gels and Western blotted using anti-β-catenin (upper half) and fascin (lower half) antibodies. Samples destined for fascin Western blotting were not reduced to prevent the comigration of antibody heavy chains (~55 kD). Rat (*C*, *E*, and *G*) or mouse brain tissue (*D* and *F*) was lysed as for (*A* and *B*) and immunoprecipitated with the indicated antibodies against plakoglobin (*P*), p120^{cas} (*p*), α-catenin (*α*), β-catenin (*β*), E-cadherin (*E*), N-cadherin (*N*), fascin (*F*), and normal rabbit IgG (*C*, control lanes). Protein G–conjugated Sepharose-4B was then used to precipitate the immune complexes. Whole-cell extracts (*W*) and immunoprecipitates were Western blotted with antiplakoglobin (*C*), anti-p120^{cas} (*D*), anti–β-catenin (*E*), anti–α-catenin (*F*), and anti–N-cadherin (*G*) antibodies. The electrophoretic mobilities of prestained molecular markers are indicated for each panel. (β-Catenin = 92 kD, fascin = 55 kD, p120 = 110–120 kD, α-catenin = 102 kD, and N-cadherin = 120 kD.)

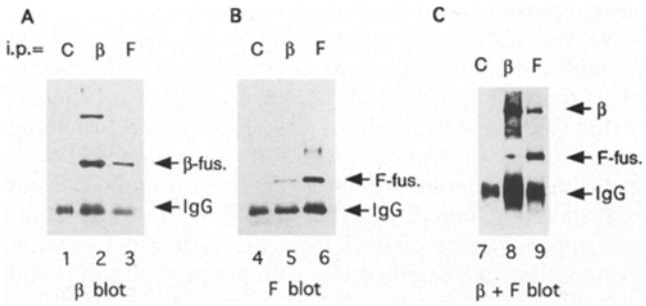

Figure 2. Association of affinity-purified recombinant β-catenin and fascin in vitro. Fascin protein was added to full-length β-catenin (*C*) or to a GST fusion protein containing β-catenin's central Armadillo repeat domain (*A* and *B*). After incubation, the mixtures were equally separated into three tubes and subjected to anti–β-catenin (*β*), antifascin (*F*), and normal rabbit IgG (*C*, control lanes) immunoprecipitation as indicated above each lane. Immunoprecipitates were blotted with anti–β-catenin (*A*) or antifascin (*B*) antibodies. (Higher molecular weight bands in positive-control lanes *2* and *6* are likely precipitated oligomers of β-catenin and fascin, respectively.) In *C*, which used full-length β-catenin, the upper half of the nitrocellulose filter was Western blotted using affinity-purified anti–β-catenin antibodies, and the lower half using affinity-purified antifascin antibodies. (Full-length β-catenin = 92 kD, GST-β-catenin-Armadillo repeat fusion protein = 80 kD, thioredoxin-fascin fusion protein = 67 kD.)

vitro coimmunoprecipitation of β-catenin with E-cadherin is reduced in the presence of increasing molar ratios of fascin, suggesting indeed that E-cadherin and fascin require overlapping sites within β-catenin. These results are consistent with the immunoprecipitation data presented above, which indicated that cadherins are not found in fascin–β-catenin complexes formed in vivo.

These results are further reminiscent of the mutually exclusive binding of cadherin and APC proteins to β-catenin (33, 61), and suggests that fascin, β-catenin, and APC may each use overlapping sites within β-catenin. Conversely, preliminary data of fascin's β-catenin binding domain is indicated within our initial yeast two-hybrid fascin clone, which contains mouse fascin amino acids 326–453. This region is localized within the COOH-terminal half of fascin (493 amino acids total in mouse) and contains one of fascin's two putative actin-binding domains. This region also contains the conserved amino acid sequence motif GKYW, present in all known fascin sequences (16), that could participate in binding β-catenin.

Immunofluorescent Colocalization of Fascin and β-Catenin

We tested for the colocalization of β-catenin and fascin using indirect immunofluorescence in the human epider-

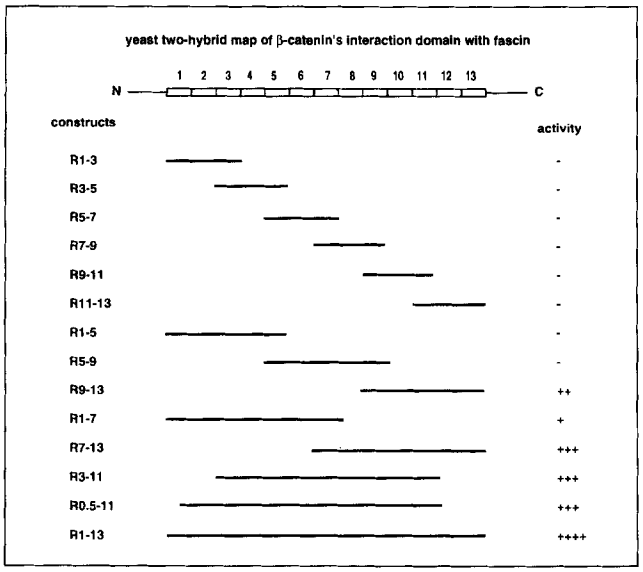

Figure 3. Mapping of fascin's binding site within β-catenin by yeast two-hybrid analysis. cDNA fragments coding for the indicated Armadillo repeats of β-catenin were obtained by PCR and cloned in-frame into the yeast two-hybrid "bait" vector pBTM116. After transformation into yeast, the ability of the indicated β-catenin fusion constructs to interact with fascin was assessed by two criteria (20 clones tested per construct): (*a*) ability of yeast expressing both constructs to transactivate the His reporter and confer growth in the absence of His and (*b*) ability to transactivate the lacZ reporter construct, assessed by β-galactosidase filter assay. −, clones that did not grow on His− medium and/or failed the β-galactosidase filter assay. +, clones which both grew on His− medium and passed the β-galactosidase color assay (+, ++, +++, and ++++ indicate the clones' relative size and color density). Viable (+) clones passed standard authenticity tests (Materials and Methods) (2).

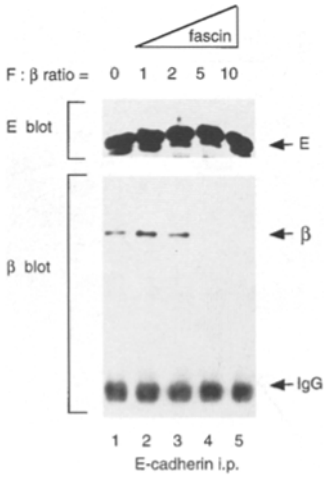

Figure 4. E-cadherin and fascin bind competitively to β-catenin. Recombinant full-length fascin and a GST fusion protein containing the E-cadherin cytoplasmic domain (residues 734–884) of mouse E-cadherin were affinity purified. A 3.4:1 molar ratio of E-cadherin/β-catenin was mixed, briefly incubated, and equally divided into five tubes containing fascin at the indicated molar ratios of fascin/β-catenin. After an additional incubation, the reaction was immunoprecipitated for E-cadherin and the washed precipitates subjected to SDS-PAGE and Western blotting. Western blots are presented for the precipitating E-cadherin protein (*upper blot*, anti–E-cadherin [*E*] antibody), and for the coprecipitating β-catenin protein (*lower blot*, anti–β-catenin [*β*] antibody).

moid A-431 cell line and in human lung endothelial cells (Fig. 5). β-catenin and fascin (detected using rhodamine- and fluorescein-conjugated anti–rabbit or anti–mouse antibodies respectively) were colocalized within identical optical fields to the cell–cell borders of highly confluent A-431 cells (*D* and *H*). Cadherins display a similar cell–cell border localization in many cell types, including A-431 (for review see [70]). In common with β-catenin's staining pattern in confluent cells, β-catenin was also present along the cell–cell borders of small groups of subconfluent A-431 and human endothelial cells (*small arrows* in *A* and *B*). In contrast, fascin staining was considerably less apparent along the cell–cell borders of subconfluent relative to confluent cells (*small arrows* in *E* and *F* relative to *H*). Thus, while fascin is likely present and functioning at immature cell–cell borders, it is most easily observed at mature cell–cell borders and cell leading edges, likely reflecting its recruitment to β-catenin and/or bundled-actin complexes (see also Fig. 6).

HeLa cells, mouse embryonal carcinoma p19 cells, and primary rat hippocampal neurons also displayed clear β-catenin and fascin costaining at cell leading edges, as did the recovering leading cell edges of wounded A-431 cell monolayers (data not shown). Fascin was also localized to actin stress fibers, which terminate at focal contacts containing integrins. However, β-catenin did not appear to be associated with stress fibers or focal contacts (data not shown), indicating that fascin–β-catenin interactions occur selectively within a subset of the structures containing fascin. Fascin is also seen in the cytoplasm, in agreement with work upon *Drosophila* fascin (Singed) (10).

We were most interested to observe that β-catenin and fascin colocalize to cell leading edges that are not in contact with other cells (Fig. 5, *large arrows*). Cell leading edges are regions where actin bundles are known to play an active role in multiple processes including cell motility, and where fascin is known to function (for reviews see references 15, 66). β-Catenin, however, has not been previously reported at cell leading edges.

We were further very interested to observe specific E- and P-cadherin immunofluorescence staining at cell leading edges that colocalized with fascin, in addition to the expected staining at cell–cell borders (Fig. 6). The costaining of both β-catenin and cadherins were each highly colocalized with fascin (compare fine structures within Figs. 5 and 6). It should be noted that while the fascin–β-catenin complex appears to be distinct from the cadherin–β-catenin complex as evaluated using immunoprecipitation and other above-noted assays, the protein components of the two complexes colocalize along cell–cell borders and cell leading edges and may conceivably share in higher-order interactions within cells.

Negative controls indicated that the noted β-catenin, fascin, and cadherin immunofluorescent staining was specific in the experimental samples of all used cell types. Controls included the use of anti–β-catenin, antifascin, or anti–E-cadherin antibodies that had briefly been preincubated with a 5–10-fold molar excess of their corresponding protein or fusion protein (data not shown) (see Materials and Methods), and the use of purified (whole sera-derived) nonspecific mouse or rabbit IgG as the primary antibody (see Fig. 6). Additionally, the observed temporal nature of fascin's cell–cell border staining in subconfluent versus confluent cells provides a reasonable internal con-

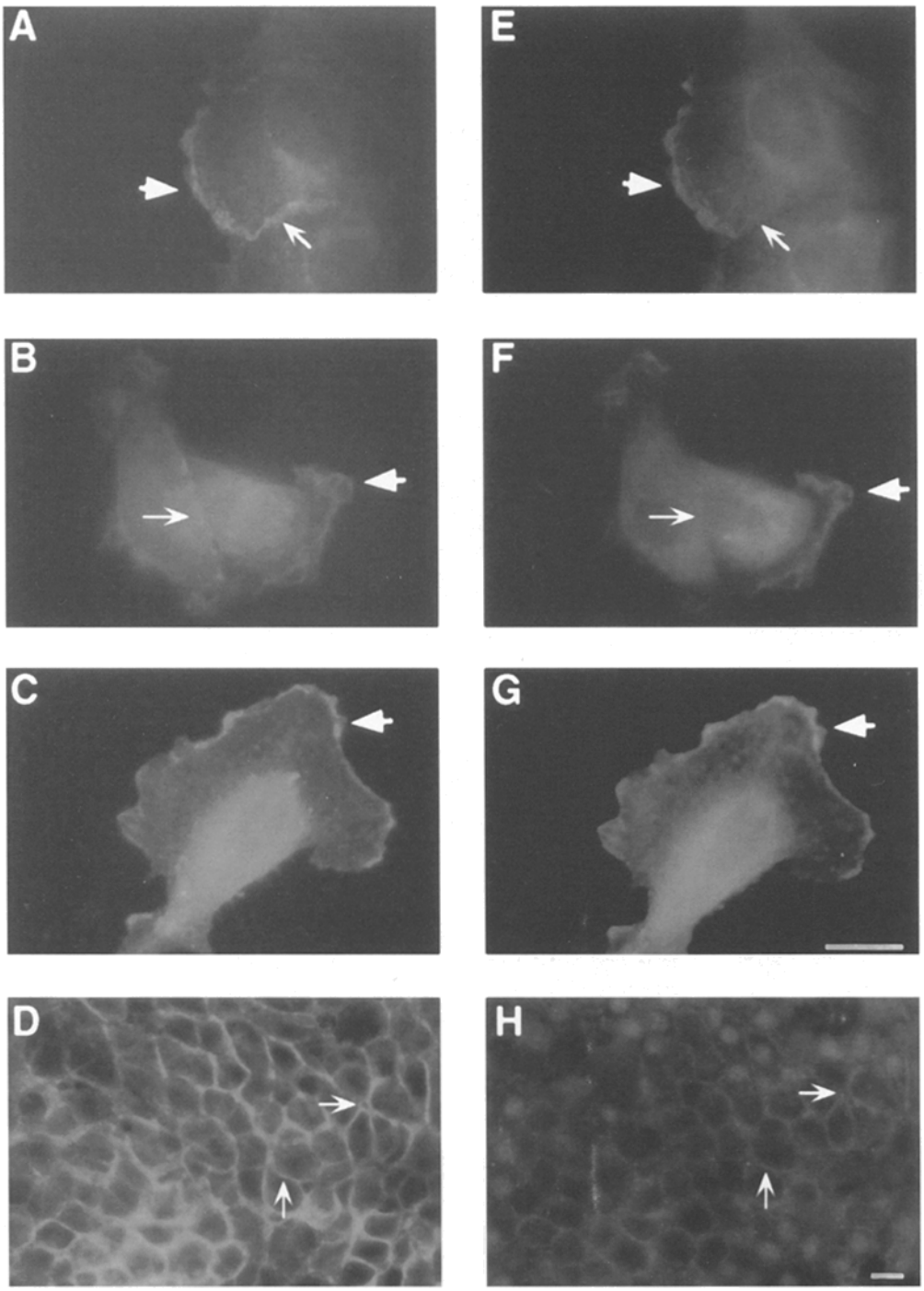

Figure 5. Immunofluorescence colocalization of β-catenin and fascin in A-431 cells (pairs *A* and *E*, *B* and *F*, and *D* and *H*), and human lung endothelial cells (pair *C* and *G*). Cells were grown, fixed, and extracted as described in Materials and Methods. After blocking, cells were double-stained using affinity-purified anti–β-catenin monoclonal antibodies (*A–D*), and an antifascin polyclonal antibody (*E–H*). Primary antibodies were detected using Rhodamine (TRITC)-conjugated donkey anti–mouse IgG antibodies (β-catenin; *A–D*), or fluorescein-conjugated donkey anti–rabbit IgG antibodies (fascin; *E–F*). Large arrowheads in the upper three panel pairs (*A* and *E*, *B* and *F*, and *C* and *G*) and small arrowheads in the bottom panel pair (*D* and *H*), indicate the colocalization of β-catenin with fascin at the leading edges of subconfluent cells and at the cell–cell borders of highly confluent cells, respectively. Small arrowheads in the upper two panel pairs (*A* and *E* and *B* and *F*) indicate the presence of β-catenin, but less so of fascin, at cell–cell borders adjoining the edges of subconfluent cell islands (see also Fig. 6). HeLa cells demonstrated equally striking colocalization of β-catenin with fascin at cell leading edges (data not shown). (*D* and *H* objective magnification 16; all others 100.) Bars, 10 μm.

trol, suggesting that the observed fascin-derived immunofluorescent staining is specific.

Discussion

Various observations, in addition to the consideration of "simple" cellular mechanics, suggest that cell adhesion and motility functions must be regulated coordinately during the execution of many cell behaviors, including embryonic cell migration and wound repair. Such behaviors may in some cases be modeled in cell culture, as is evidenced in the reduction of cadherin-mediated cell–cell adhesion and concurrent increase in cell motility upon addition of hepatocyte growth factor (HGF/scatter factor) to columnar epithelial cells (63). While significant scientific inroads have been made in our understanding of adhesion and motility events, our knowledge of the molecular mechanisms that balance their interplay is not yet well established.

We have used the yeast two-hybrid system to identify a novel interaction of the actin-bundling protein fascin with β-catenin. This association was shown to exist in vivo and in vitro as assayed by specific coimmunoprecipitation of fascin with β-catenin. Yeast two-hybrid mapping indicates that β-catenin associates with fascin via an extended region that traverses at least five or its thirteen Armadillo repeats, with larger constructs displaying yet stronger in-

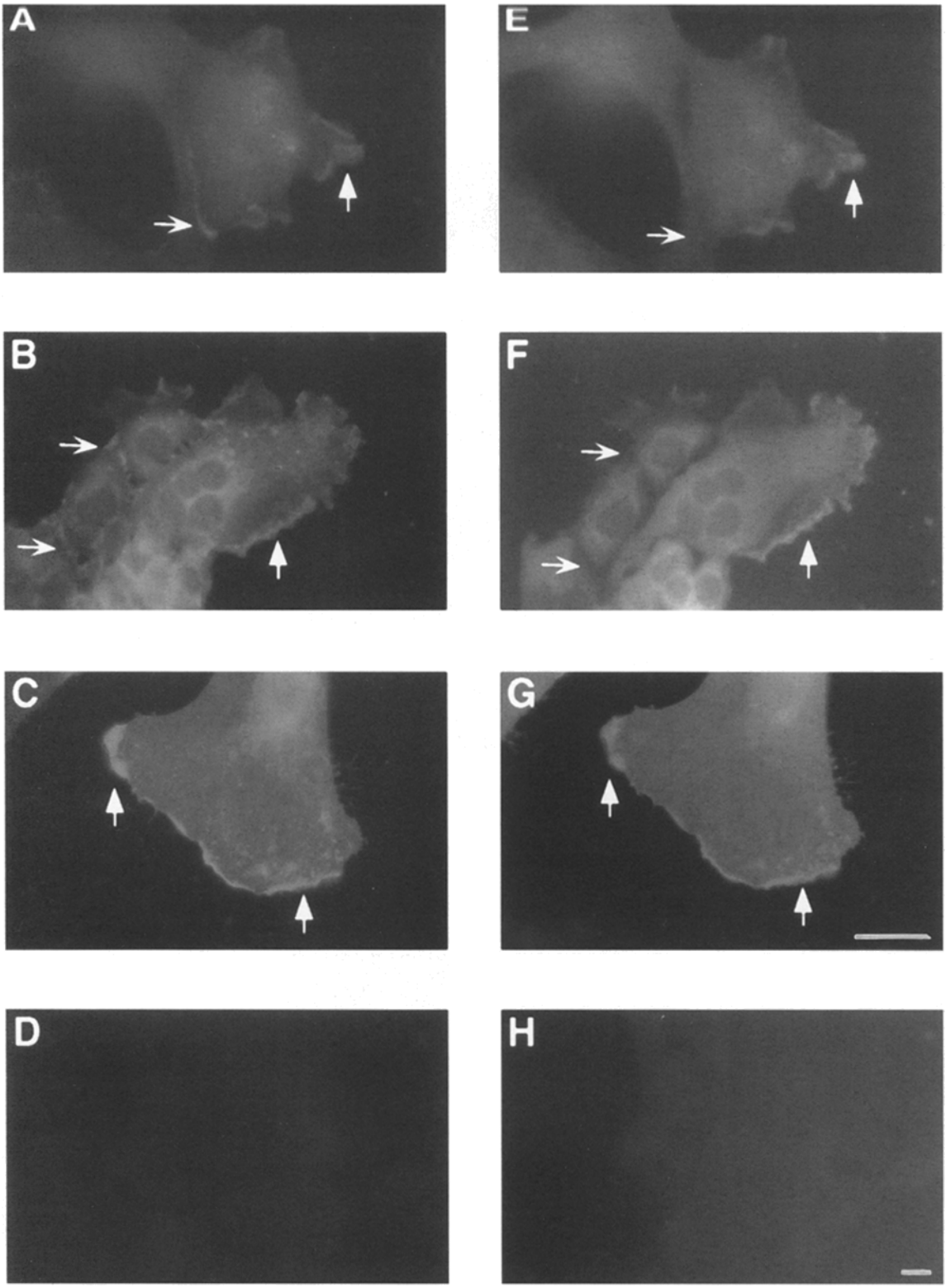

Figure 6. Immunofluorescence colocalization of E-cadherin with fascin (pairs *A* and *E* and *C* and *G*) and of P-cadherin with fascin (pair *B* and *F*). Cells were grown, fixed, and extracted as described in Materials and Methods. Cadherins were detected using mouse monoclonal primary antibodies followed by rhodamine (TRITC)-conjugated donkey anti–mouse secondary antibodies (*A–C*), while fascin was detected using rabbit polyclonal primary antibodies followed by fluoresceine-conjugated donkey anti-rabbit secondary antibodies (*E–G*). A-431 cells were employed in panel pairs *A* and *E*, *C* and *G*, and *D* and *H*. Human lung endothelial cells were utilized in panel pair *B* and *F*. Large vertical arrowheads indicate the colocalization of E-cadherin or P-cadherin with fascin at cell leading edges. Small horizontal arrowheads indicate the presence of E- or P-cadherin, but not of fascin, at cell–cell borders adjoining the edges of subconfluent cell islands (see also Fig. 5). Purified mouse and rabbit IgG were respectively used as negative-control primary antibodies in *D* and *H*. Cadherin and fascin immunofluorescence staining of cell leading edges and cell–cell borders was likewise absent when anticadherin or fascin primary antibodies were correspondingly preincubated with excess cadherin or fascin protein (data not shown). (*D* and *H* objective magnification 40×; all others 100.) Bars, 10 μm.

teractions. Those yeast two-hybrid constructs displaying the strongest fascin interactions included Armadillo repeats that were COOH-terminal within the domain. This extended but somewhat COOH-terminal region is also known to be necessary for β-catenin's interaction with E-cadherin and APC (33, 61).

Indeed, our work indicates that fascin and the cytoplasmic domain of E-cadherin compete for association with β-catenin in vitro. We expect likewise that fascin may compete with APC for binding to β-catenin; in this regard, we have not observed APC in fascin immunoprecipitates nor fascin in APC immunoprecipitates (data not shown).

Finally, the immunofluorescence colocalization of fascin and β-catenin to leading cell edges and cell–cell borders is, respectively, consistent with the complex's conceivable role in modulating or effecting the functions of cell motility and adhesion. Interestingly, we also observe that cadherins colocalize with fascin at cell leading edges, which raises the further possibility that the cadherin–catenin complex may function in coordination with the fascin–β-catenin complex, perhaps via a higher-order physical complex that we could not detect using standard coimmunoprecipitation assays.

β-Catenin has been shown previously to bind via its Armadillo repeat domain to a number of distinct proteins (see Introduction and below). The binding of at least two of these associated proteins, namely cadherins and the APC tumor supressor protein, is "competitive" and mutually exclusive (33, 61). Our in vitro work indicates that fascin likewise competes with cadherins, and likely also APC, for binding to β-catenin.

While such mutually exclusive β-catenin interactions appear to occur in vivo and in vitro, it is not yet clear if the cellular levels of β-catenin are limiting such that a competition occurs for its association. It has been suggested previously that β-catenin's (or plakoglobin's) stoichiometry within cell–cell (cadherin) and APC complexes may influence their respective functionalities (4, 25, 28, 33, 60, 61, 68). Thus, for example, a stoichiometric increase of β-catenin within the cadherin complex following its loss from the APC complex (or vice versa) would effectively couple the biological activity of these two distinct complexes. While the veracity of this model awaits further testing, it might now be extended to fascin, and it would predict that the biological processes of cell–cell adhesion (cadherin), cell proliferation-death (APC), and filament-bundling/cell motility (fascin) are coordinately regulated via the competitive titration of β-catenin.

It is known with greater certainty that β-catenin (*Drosophila* Armadillo) transduces signals within the *Wnt* developmental signaling pathway (4, 20, 28, 41; for review see reference 53), which is initiated by the binding of the Wnt protein to a cell surface receptor yet to be identified (reviewed in 52). Within this pathway, β-catenin's activity is likely determined by the immediately upstream glycogen synthase kinase (*Drosophila* Shaggy), a serine/threonine kinase (27, 55, 64). Within the context of the cadherin complex, β-catenin is likewise known to associate directly with receptor tyrosine kinases, including the EGF receptor and c-erb-2 (31, 34). While β-catenin's functions within the Wnt pathway may be separable from those within the cadherin complex (22, 24), it is thought likely that the phosphorylation of β-catenin is functionally significant within both contexts (less is known concerning APC) (53, 63).

Whatever the biological role of the fascin–β-catenin complex in vivo, its activity is likely to be specific to some but not all fascin-containing bundles, for while both fascin and β-catenin are present at bundle-rich leading cell edges and cell–cell borders and coassociate with filaments in vitro, β-catenin was not observed in fascin-containing stress fibers emanating from cell substrate (focal) contacts. Further, fascin itself does not appear to be required for the function of all bundled filaments. For example, *Drosophila* fascin (Singed) mutants evidence a variety of apparently normal cell activities even in embryos harboring strong *singed* alleles, leaving open the possibilities that nonaffected cells are permissive to reduced fascin functionality, or more likely that other bundling proteins may effectively assume fascin's activities (10, 73). Fascin is, however, clearly essential in various filament-bundling capacities in all organisms studied including *Drosophila* (see Introduction). Most recently, work upon rat hippocampal neurons has indicated that the antisense-directed loss of fascin function leads to the dramatic collapse of protrusive processes at growth cone leading edges, consistent with fascin's role in assembling dynamic microfilament-based structures (15).

In summary, the presence of β-catenin within both "motility" (fascin–β-catenin) and adhesion (cadherin–catenin) complexes may conceivably facilitate their coordinate regulation. Plakoglobin, which is highly homologous to β-catenin, may also coordinately (but distinctly?) modulate the function of motility and adhesion complexes, as it also associates with fascin (but more weakly) and is a known component of cadherin complexes present at both *zonula adherens* and desmosomal junctions (11). Receptor tyrosine kinase and *src* signaling pathways might be conjectured to influence motility and adhesion processes via the direct or indirect modulation of β-catenin's (and plakoglobin's?) functions in both fascin and cadherin complexes. Such functional regulation is likely to require phosphorylation and/or small G protein coupled events. In the future, these conceptual possibilities must be experimentally tested.

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