ANKYRIN-G IS A MOLECULAR PARTNER OF E-CADHERIN IN EPITHELIAL CELLS AND EARLY EMBRYOS

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E-cadherin is a ubiquitous component of lateral membranes in epithelial tissues, and is required to form the first lateral membrane domains in development. Here, we identify ankyrin-G as a molecular partner of E-cadherin, and demonstrate that ankyrin-G and beta-2 spectrin are required for accumulation of E-cadherin at the lateral membrane in both epithelial cells and early embryos. Ankyrin-G binds to the cytoplasmic domain of E-cadherin at a conserved site distinct from that of beta-catenin. Ankyrin-G also recruits beta-2-spectrin to E-cadherin/beta-catenin complexes, thus providing a direct connection between E-cadherin and the spectrin/actin skeleton. In addition to restricting membrane mobility of E-cadherin, ankyrin-G and beta-2-spectrin also are required for exit of E-cadherin from the trans-Golgi network in a microtubule-dependent pathway. Ankyrin-G and beta-2 spectrin co-localize with E-cadherin in pre-implantation mouse embryos. Moreover, knockdown of either ankyrin-G or beta-2 spectrin in one cell of a two-cell embryo blocks accumulation of E-cadherin at sites of cell-cell contact. E-cadherin thus requires both ankyrin-G and beta-2-spectrin for its cellular localization in early embryos as well as cultured epithelial cells. We have recently reported that ankyrin-G and beta-2 spectrin collaborate in biogenesis of the lateral membrane (Kizhatil et al., J. Biol. Chem. 282: 2029-37, 2007). Together with the current findings, these data suggest a ankyrin/spectrin-based mechanism for coordinating membrane assembly with extracellular interactions of E-cadherin at sites of cell-cell contact.

INTRODUCTION

The lateral membrane domain of epithelial cells is of considerable physiological importance due to its roles in salt and water homeostasis and protection of epithelial tissues from mechanical stress. Moreover, loss of this specialized domain is a hallmark of metastatic cancer cells (1). Recent studies employing siRNA have revealed that ankyrin-G and beta-2 spectrin collaborate in formation of the lateral membrane of bronchial epithelial cells (2,3). Cells depleted of either protein maintain polarity, but are converted from columnar to a squamous morphology with minimal lateral membrane and expanded apical and basal membranes. The loss of lateral membrane in ankyrin-G- and beta-2 spectrin-depleted cells is accompanied by a failure in de novo membrane biogenesis during mitosis (2,3). Ankyrin-G and beta-2 spectrin thus are required for bulk delivery of proteins and phospholipids to the lateral membrane.

The cell adhesion molecule E-cadherin is a prototypic component of lateral membranes in epithelial cells and is required for the first formation of lateral domains in development during compaction in 8-cell embryos (4-6). E-
cadherin has been proposed to recruit cytoskeletal proteins including spectrin (7,8). According to these models, spectrin in turn recruits ankyrin, which directly interacts with resident membrane-spanning proteins such as the Na/K ATPase (9-11).

E-cadherin forms a complex and co-immunoprecipitates with ankyrin and spectrin from extracts of cultured epithelial cells (12). This provocative result raises the question of whether E-cadherin associates directly with either ankyrin or spectrin. We report here that E-cadherin binds directly to ankyrin-G, and present evidence that this interaction is required for exit of E-cadherin from the trans-Golgi network and for maintenance at the lateral membrane of cultured epithelial cells. We also show that ankyrin-G and beta-2 spectrin are required in early mouse embryos for compaction and accumulation of E-cadherin at cell-cell contacts.

EXPERIMENTAL PROCEDURES

cDNA constructs: cDNAs encoding human E-cadherin fused at the C-terminus to either GFP or three copies of the HA tag were constructed using standard molecular biology techniques. cDNAs encoding E-cadherin cytoplasmic domain and beta-catenin were inserted into a pGEX6P1-7 His bacterial expression vector (13) for protein expression and purification. Four E-cadherin cytoplasmic domain polypeptides with progressive C-terminal deletions were generated by terminating the coding sequence at amino acid 772, 813, 831, or 862, where amino acids are numbered based on the E-cadherin propeptide amino acid sequence. cDNA encoding repeats 14-16 of beta-2-spectrin containing the ankyrin binding site (amino acids 1669-1991) was introduced into the pET15b vector.

Mutagenesis was performed using the Quickchange mutagenesis kit (Stratagene) and was confirmed by DNA sequencing. shRNA plasmids: The pSuper plasmids encoding human ankyrin-G and beta-2-spectrin shRNA have been described (2,3). A pFIV H1 (SBI systems) vector was used to express shRNA against mouse ankyrin-G and mouse beta-2-spectrin in experiments with early embryos. The following sequences were targeted: ankyrin-G (NM 170728) GGATTTGCTCCGCTCCATC; beta-2 spectrin (NM 175836) - TGGTTAAAGCATCAAGCA. Corresponding control siRNAs were generated by introducing mutations into the siRNA sequence at three wobble nucleotide positions (underlined) to make them non-functional: ankyrin-G: GGGATCGCTTCCGCTCCATC; beta-2-spectrin: TGGTTAAAGCTTCCGAGCA.

In vitro interaction assays with purified proteins: Histidine-tagged human 220 kDa ankyrin-B was expressed using the BacPak baculovirus protein expression system (Clontech) and purified as described (14). Histidine-tagged rat 190 kDa ankyrin-G was expressed and isolated in a similar fashion to ankyrin-B. Recombinant beta-catenin was expressed in BL21 bacteria as a fusion protein with a glutathione-S-transferase (GST) tag at the N-terminus and a hepta-histidine tag at the C-terminus. The beta-catenin fusion protein was first isolated on NiNTA-sepharose followed by purification on glutathione-sepharose beads. This two-step protocol ensures the purification of the full length beta-catenin polypeptide. The GST tag fused to beta-catenin was removed by Precision protease treatment and the beta-catenin polypeptide was further purified using anion exchange chromatography. Histidine-tagged beta-2-spectrin (repeats 14-16 including the ankyrin binding site) was isolated on NiNTA-sepharose, and then purified by anion exchange chromatography followed by gel filtration chromatography. E-cadherin cytoplasmic domain polypeptides (wild type, truncations, and point mutants) were expressed as fusion proteins with the GST tag at the N-terminus and a hepta-histidine tag at the C-terminus in BL21 E.Coli cells. The cytoplasmic domain polypeptides were first isolated on NiNTA-sepharose. Then the isolated cytoplasmic polypeptides fused to GST (GST-EC-CD) were immobilized on glutathione-sepharose for binding studies.

In vitro protein interaction studies were performed as described (14). Briefly, either GST-EC-CD or GST immobilized on glutathione-agarose beads were incubated with a candidate binding partner in 20mM HEPES,
pH 7.3, either 50 or 100 mM NaCl, 1 mM EDTA, 1 mM NaN₃, 5 mg/ml bovine serum albumin. Following incubation, the glutathione beads were pelleted (at 5000Xg for 15 min) and proteins bound to the beads were resolved by SDS-PAGE followed by Coomassie blue-staining. Relative levels of ankyrin-G and EC-CD in individual complexes were determined as follows: Stained protein bands were cut from SDS-PAGE gels and the Coomassie blue dye was eluted using pyridine (25%). Relative levels of dye were measured based on absorbance at 650 nm. Binding stoichiometries expressed as moles of ankyrin bound per mole of GST-EC-CD were determined in each sample from the ratio of Coomassie blue associated with each polypeptide, and were calculated based on relative molecular weights of ankyrins and GST-EC-CD, with the assumption that these proteins bind equivalent amounts of dye per microgram. Ankyrin-G was used at 0.5 µM in experiments designed to determine binding to mutant EC-CD. In all other experiments ankyrin-G, beta-catenin and beta-spectrin were used at 1 µM.

Cellular assays: The HEK 293-based plasma membrane recruitment assay used to study the interaction of ankyrin-G and E-cadherin in cells has been described (15). Briefly, HEK 293 cells (80,000 cells) were plated on the 1.4 mm diameter coverslips of a Matek plate and then co-transfected with 150 ng of wild type or mutant E-cadherin-3HA and 100 ng of 190 kDa ankyrin-G-GFP using Effectene (6.25 µg in 200 µl; Qiagen). Cells were fixed with 2% paraformaldehyde at room temperature and processed for immunofluorescence with an anti-HA (1 µg/ml) antibody and anti-GFP antibody (1 µg/ml) 18-20 h following transfection.

Human bronchial epithelial cells (HBE) were cultured as described (2). Lateral membrane heights of cells grown for various time points were determined after fixation using E-cadherin as a marker for the lateral membrane. Cells grown on Matek plates were depleted of ankyrin-G or beta-2-spectrin by transfecting HBE cells with 1 µg of the respective siRNA plasmids with Lipofectamine-2000 as described (2)(see supplementary method for more details). E-cadherin-3HA plasmid DNA (150 ng; wild type or mutant) was transfected into HBE cells using Lipofectamine 2000 (450 ng in 200 µl).

Embryo injections: One-cell embryos were isolated from super-ovulated B6SJL/F1/J mice and allowed to develop to the 2-cell stage at 37°C in KSOM media (Speciality media) under 5% CO₂. 2 µl of a 3:1 mixture of shRNA plasmid and pCAGGS-GFP at a final concentration of 10 ng/µl was injected into one cell of the 2-cell embryo using a fire polished glass needle attached to a TL1-100 Micromanipulator (Harvard Apparatus). The injected embryos were cultured for another 40 h at 37°C under 5% CO₂. Embryos were screened by phase contrast light microscopy, and those that were dead following the 40 h incubation were removed.

Immunofluorescence microscopy and three-dimensional rendering: Immunofluorescence and laser scanning confocal microscopy were performed as described (2,3) on a Zeiss LSM 510 microscope using a 100 X objective with a NA of 1.45. Three-dimensional rendering of confocal Z-stacks collected at 0.2 µm intervals was performed using the Volocity 4.0 software (Improvision) with the 3D rendering option set to opacity. Overlap of voxels representing organelle markers with voxels representing intracellular E-cadherin was calculated from three-dimensional renderings of confocal stacks of cells labeled for E-cadherin and marker protein using the co-localization function.

Rabbit polyclonal affinity purified antibodies against ankyrin-G, beta-2-spectrin and GFP have been described (2,3). Rabbit anti-Rab7 was a gift of Dr. Ignacio Sandoval. The following antibodies were obtained commercially; rat anti E-cadherin (Sigma), mouse anti-golgin-97 (Invitrogen), mouse anti-giantin (EMD biosciences), mouse anti-EEA1 (BD biosciences), and chicken anti-HA (Aves laboratory). Dextran uptake in cells was performed as described before (16). Briefly, cells transfected with siRNA were washed with serum free-DMEM with 10 mM HEPES, pH 7.4, 0.05% bovine serum albumin 18 h after transfection and then incubated with 1 mg/ml FITC-dextran (Mini emerald, Mr = 10000; Molecular Probes) for 30 min at 37°C.
Cells were washed further then fixed and processed for immunofluorescence as described before (2,3). Dextran uptake in cells after nocodazole treatment for 6 h was performed in a similar manner. 

**Fluorescence recovery after photobleaching (FRAP):** HBE cells grown on Matek plates were transfected with 600 ng of either wt or RD748DR E-cadherin pEGFPN1 with Lipofectamine 2000 (1.8 µg in 200 µl). 18-20 hours after transfection the cells form a monolayer where the transfected E-cadherin localize to cell-cell junctions. Cells were visualized using an Apochromat 100 X NA 1.40 objective on a LSM 5 live duo scan confocal system. Images of cells were collected at five time points before photobleaching using a 488 nm laser. Images were obtained every second following bleach for 3 min. Fluorescence intensities from the bleached region of the cell, the whole cell and background region were obtained for each time point using the LSM 4.0 software. Fluorescence intensities over time following bleach were normalized using Phair’s double normalization technique. In this method the ratio of background subtracted fluorescence intensities during recovery and background subtracted average pre-bleach fluorescence intensity is corrected for acquisition bleaching. The normalized fluorescence intensities were plotted against time to obtain a FRAP recovery curve. The curve was then fitted using the single exponential equation \( y = y_0 + Ae^{-bx} \). The mobile fraction was calculated according the equation \( Mob = -A / (1 - (y_0 + A)) \). The half time was calculated using \(-\ln 0.5/b\).

**RESULTS**

**Ankyrin-G recruits beta-2 spectrin to an E-cadherin-beta-catenin complex in vitro —** Based on the report that E-cadherin co-immunoprecipitates with ankyrin and spectrin (12), as well as functional experiments (see below), we directly evaluated interaction of ankyrin-G with the cytoplasmic domain of E-cadherin using purified recombinant proteins. Ankyrin-G associated with GST-E-cadherin cytoplasmic domain that was immobilized on glutathione-agarose beads, while ankyrin-B exhibited only minimal binding under identical conditions (Fig. 1A). Increasing concentrations of ankyrin-G bound to the E-cadherin cytoplasmic domain in a saturable manner at close to 1:1 molar stoichiometry, with half-maximal binding at 0.5 µM (Fig. 1B). We also evaluated binding of E-cadherin to ankyrins in a cell-based assay where GFP-tagged proteins are recruited to the plasma membrane when co-expressed with membrane protein binding partners (Fig. 1C) (15). Ankyrin-G-GFP in 293 cells was present in the cytoplasm when expressed alone, but was confined to the plasma membrane when co-expressed with E-cadherin (Fig. 1C). In contrast, ankyrin-B-GFP remained cytoplasmic in the presence of E-cadherin (Fig. 1C). The fact that ankyrin-G is markedly more active than ankyrin-B in binding to E-cadherin, even though these proteins are closely related in sequence and isoelectric point, indicates that the interaction is highly specific.

We next determined the site in the E-cadherin cytoplasmic domain required for binding of ankyrin-G. Ankyrin-G associated with N-cadherin equivalently to E-cadherin (data not shown), indicating that ankyrin-binding may be a conserved feature of type 1 classical cadherins. We found, using a series of truncations, that the juxtamembrane 41 amino acids (aa 732-773) of the E-cadherin cytoplasmic tail were sufficient for ankyrin-binding (supplemental Fig. 1). We performed alanine-scanning mutagenesis within the minimal ankyrin-G binding region of the complete E-cadherin cytoplasmic domain, and focused on clusters of charged residues conserved between E-cadherin and N-cadherin (Fig. 2A). These mutations resulted in loss of binding to ankyrin-G, ranging from 30-40% for residues 738-750 to 60% for residues 756-764. These results, considered with the intrinsically unstructured fold of the E-cadherin cytoplasmic domain (17), indicate an extended ankyrin-binding site distributed along at least 26 residues (Fig. 2B).
We next evaluated interaction of ankyrin-G and E-cadherin in the context of physiological binding partners for each protein. Beta-catenin associates with the C-terminal 100 residues of the cytoplasmic domain of E-cadherin, which are distinct from the juxtamembrane binding site for ankyrin-G (18-20). We determined directly whether ankyrin-G and beta-catenin could simultaneously associate with E-cadherin as expected from their separate binding sites (Fig. 2A). Ankyrin-G associated with the E-cadherin cytoplasmic tail complexed with beta-catenin in a 1:1 molar stoichiometry, the same as with E-cadherin alone, although the affinity was reduced about 1.5-fold (Fig. 3A). We next asked if ankyrin-G could bind simultaneously to beta-2 spectrin and the E-cadherin/beta-catenin complex. Again, ankyrin-G associated equivalently to E-cadherin/beta-catenin in the presence and absence of recombinant beta-2 spectrin (repeats 14-16 containing the ankyrin-binding site). Beta-2 spectrin was present in sufficient concentrations to form a 1:1 complex with ankyrin-G (Fig. 3B). Beta-2 spectrin exhibited no binding to E-cadherin/beta-catenin in the absence of ankyrin-G (Fig. 3B). A small amount of bovine serum albumin (BSA) (present in the assay at 5 mg/ml to reduce nonspecific binding) remains in all samples, including those with GST alone (Fig. 3B). These results demonstrate that ankyrin-G can recruit beta-2 spectrin to an E-cadherin/beta-catenin complex. Ankyrin-G's ability to form a ternary complex is in contrast to alpha-catenin, which can associate either with beta-catenin or actin but not both proteins at the same time (21).

Impaired cellular localization of E-cadherin with reduced ankyrin-G-binding — We determined the cellular consequences of mutations of E-cadherin that reduce its ankyrin-G-binding activity. Binding sites of p120-catenin and ankyrin-G overlap in residues 756-764 (Fig. 2A) (22). However, the RD748AA mutation that reduces ankyrin-G-binding is N-terminal to the binding site for p120-catenin (22). We further increased the specificity of this mutation by performing a charge reversal of RD to DR, thus preserving the isoelectric point. Interestingly, RD residues at this site are conserved in nematode and Drosophila as well as other vertebrate type 1 cadherins (Fig. 2A). RD748DR and RD748AA mutations of E-cadherin exhibited a similar reduction in binding to ankyrin-G (Fig. 2B). We determined the fate of HA-tagged RD748DR E-cadherin and RD748AA E-cadherin expressed in human bronchial epithelial cells. RD748DR E-cadherin accumulated primarily in a sub-apical intracellular compartment, and only to a limited extent in the lateral membrane (Fig. 4A). Representative cells are shown in XY and XZ planes. RD748DR E-cadherin exhibited marked intracellular localization in 200 out of 200 cells (not shown). Identical results were obtained with the RD748AA mutant E-cadherin (Supplementary information, Fig. S2). In contrast, HA-tagged wildtype E-cadherin expressed under identical conditions was entirely confined to the lateral membrane in over 95 percent of cells (Fig. 4A). Intracellular RD748DR E-cadherin partially co-localized in three-dimensions with structures that co-labeled with a marker for the trans-Golgi network (TGN) (Fig. 4A).

We next evaluated the effect of the RD748DR mutation on the dynamic behaviour of GFP-tagged E-cadherin expressed on the lateral membrane in confluent HBE cells. This was achieved by measuring fluorescence recovery of GFP-tagged E-cadherin after photobleaching (Fig. 4B). RD748DR E-cadherin-GFP compared to wildtype E-cadherin-GFP exhibited a 2.2-fold increase in the mobile fraction from 0.35 +/- 0.1 to 0.79 +/- 0.14. A 1.2-fold decrease in the time for half-maximal recovery from 60 +/- 1.6 sec for wild type E-cadherin to 50 +/- 3 sec for the mutant E-cadherin (n= 4) was also determined. A representative curve of recovery of fluorescence is shown in Fig. 4B. Together, these experiments demonstrate that RD748DR E-cadherin exhibits both impaired exit from the TGN and increased mobility on the lateral membrane. Given that the E-cadherin cytoplasmic domain is intrinsically
unstructured (17), the RD748DR mutation is unlikely to affect folding. Therefore, altered behavior of RD748DR E-cadherin may result from loss of ankyrin-G-binding.

E-cadherin contains an atypical dileucine motif within the ankyrin-binding site in the juxtamembrane domain, which has been proposed to function as a basolateral targeting signal (23). We found that LL/AA mutation of this motif had no effect on binding to ankyrin-G, either with pure proteins or in the cell recruitment assay (Fig. 5A and B). However, we also found that the LL/AA mutant E-cadherin was targeted to the lateral membrane when expressed at physiological levels and only mis-localized to the apical surface when highly expressed (Fig. 5C). Similar findings have been reported for LL/AA mutated E-cadherin in MDCK cells, and were interpreted as evidence for a role of the dileucine motif in endocytosis (24). E-cadherin apparently can independently associate with either ankyrin-G or the endocytosis machinery through an overlapping interaction surface provided by its intrinsically unstructured cytoplasmic domain.

**Exit of E-cadherin from the TGN requires ankyrin-G and beta-2 spectrin**—We wanted to further evaluate roles of ankyrin-G and its partner beta-2 spectrin in determining cellular behavior of E-cadherin. Conventional wisdom would predict that the principal function of an ankyrin-spectrin skeleton is to immobilize E-cadherin and prevent its recycling through endocytosis (7). In support of a retention function for ankyrin-G, RD748DR E-cadherin-GFP, which has reduced ankyrin-G-binding activity, also has increased mobility in the lateral membrane (Fig. 4A).

In order to directly evaluate roles of ankyrin-G and its partner beta-2 spectrin in stabilizing E-cadherin at the lateral membrane, we knocked down ankyrin-G and beta-2 spectrin in human bronchial epithelial cells using plasmids encoding siRNAs for these proteins, as previously described (2,3). Controls in these experiments include use of siRNA mutated at three positions, as well as rescue of depleted cells with ankyrin-G or beta-2 spectrin resistant to siRNA (2,3). We measured the height of the lateral membrane, as marked by E-cadherin staining, as a function of time following transfection after 12 hours in culture. Cells depleted of either ankyrin-G or beta-2 spectrin by siRNA exhibited loss of lateral membrane height from 4 microns at the time of transfection, to less than 2 microns at 26 hours (Fig. 6, bottom right panels). Control cells, in contrast, extended their lateral membrane height from 4 microns at 12 hours to 10 microns at 24 hours (Fig. 6). These results indicate that ankyrin-G and beta-2 spectrin are both required to preserve existing membrane, as expected from a scaffolding model.

In addition to preserving existing membrane, ankyrin-G and beta-2 spectrin also are required for de novo biogenesis of lateral membrane between anaphase and telophase in dividing cells (2,3). The dramatic loss of the entire lateral membrane in cells depleted of either ankyrin-G or beta-2 spectrin (Fig. 6) (2,3) thus is likely to reflect an essential role for these proteins in bulk assembly of the lateral membrane as well as to their function in stabilizing the membrane.

In support of a direct role of ankyrin-G in delivery of E-cadherin to the lateral membrane, RD748DR E-cadherin with reduced ankyrin-binding activity accumulates in the TGN (Fig. 4 A). This suggests that ankyrin-G is required for exit of E-cadherin from the TGN. We therefore determined the fate of E-cadherin in cells depleted of either ankyrin-G or beta-2 spectrin (Fig. 7). We observed a 5-fold increase in E-cadherin mis-localized from the lateral membrane in cells depleted of either ankyrin-G or beta-2-spectrin (Fig. 7). We determined the compartment containing mis-localized E-cadherin using fluorescent dextran as a marker for plasma membrane internalized by endocytosis, and antibodies against EEA1 and rab 7 to label early and late endosomes, respectively. We also evaluated antibodies against ERP57 to label ER, giantin to label cisternal Golgi, and golgin 97 to mark the trans-Golgi network (TGN). Co-localization between E-cadherin and these markers was evaluated in three-dimensions (voxel overlap) to reduce false overlap of objects occupying the same XY
coordinates but separated in the Z-dimension. Images were obtained by confocal microscopy using a high-resolution 100X, NA 1.45 objective, and were rendered in 3-dimensions from Z-stacks of 0.2 micron sections. This approach of determining localization in three-dimensions with high resolution was required due the relatively flat geometry of cells following knockdown of ankyrin-G or beta-2 spectrin. However, with these methods we readily resolved localization at intracellular sites from apical and basal surfaces (Fig. 7B).

Strikingly, in cells depleted of either ankyrin-G or beta-2-spectrin, E-cadherin accumulated only in an intracellular compartment that partially overlapped with the TGN (Fig. 7C, D). E-cadherin exhibited approximately 55 percent overlap with golgin 97. In contrast E-cadherin had less than 5 percent co-localization with endosomes, cisternal Golgi, or ER (Fig. 7C). These results provide strong evidence that E-cadherin requires ankyrin-G as well as beta-2 spectrin for exit from the TGN. Given that ankyrin-G requires beta-2-spectrin for biogenesis of the lateral membrane (3), E-cadherin, ankyrin-G, and beta-2-spectrin may all cooperate in lateral membrane assembly.

We next explored a possible role of ankyrin-G and beta-2 spectrin in post-Golgi trafficking of E-cadherin. Spectrin has been reported to link intracellular membranes to microtubule-based motors either via the dynactin complex (25-27) or through KIF3A kinesin (28). Moreover, E-cadherin is transported from the TGN in tubulovesicular carriers via microtubules (29). In support of an essential role of microtubules in post-Golgi transport, E-cadherin accumulated in the TGN in the absence of microtubules in cells treated with nocodazole (Fig. 7). We hypothesized that ankyrin-G couples E-cadherin to spectrin in microtubule-dependent post-Golgi carriers. Only minimal levels of intracellular E-cadherin are detectable in fully polarized cells (Fig. 4; Fig. 7). However, intracellular E-cadherin was abundant in nocodazole-treated cells, where exit of newly synthesized E-cadherin from the TGN was blocked due to the absence of microtubules (Fig. 7). Recovery of microtubules following removal of nocodazole resulted in rapid re-growth of the lateral membrane (Fig. 8A and B). We therefore examined localization of E-cadherin with ankyrin-G or beta-2 spectrin under these conditions of synchronized membrane assembly immediately following recovery from nocodazole. Neither ankyrin-G nor beta-2 spectrin (green, Fig. 8C and D) localized with intracellular E-cadherin (red, Fig. 8C and D) in microtubule-depleted cells. In fact, beta-2 spectrin is substantially reduced in nocadazole-treated cells (note lack of green in Fig. 8D). However, 30 min following removal of nocodazole, intracellular E-cadherin (red) co-localized in three-dimension with both ankyrin-G (42% voxel overlap, green, Fig. 8C) and beta-2 spectrin (51% voxel overlap, green, Fig. 8D). The areas within the boxes in the upper panel after 30 min of recovery are shown magnified in the lower panels (Fig. 8C and D). These E-cadherin/ankyrin-G/beta-2 spectrin-staining structures are 200-500 nm in diameter, and are comparable in size to post-Golgi carriers containing E-cadherin that were visualized in HeLa cells (29). Intracellular E-cadherin largely disappeared by two hours following removal of nocodazole (Fig. 8C and D). The intracellular E-cadherin/ankyrin-G/beta-2 spectrin structures therefore are transient, and we hypothesize that they are trafficking intermediates derived from the trans-Golgi network following restoration of microtubules. These results are consistent with an active role for ankyrin-G and beta-2 spectrin in trafficking E-cadherin to a post-Golgi destination.

**E-cadherin requires ankyrin-G and beta-2 spectrin in early embryos** — Having demonstrated that ankyrin-G and beta-2 spectrin are obligatory partners for E-cadherin in cultured epithelial cells, we next asked if these proteins collaborate with E-cadherin in vivo in early vertebrate development. E-cadherin is required for formation of the first lateral membrane domains as cells become polarized during development of pre-implantation mouse embryos (30). Moreover, spectrin has been previously reported to co-localize with E-cadherin at sites of cell-cell contact in 8-cell mouse embryos (31,32). We detected 190 kDa ankyrin-G and 270 kDa
beta-2-spectrin in immunoblots of early mouse embryos using affinity-purified polyclonal antibodies (Supplementary information, Fig. S3 A). We also observed that both proteins co-localize with E-cadherin in 2-cell embryos and at the newly formed sites of cell-cell contact following compaction in 8-16 cell embryos (Fig. 9A). We depleted either ankyrin-G or beta-2-spectrin in one cell of a 2-cell embryo by co-injection of two plasmids, one encoding siRNA, and the other GFP, into a single blastomere (Fig. 9B). The progeny of the injected cell in these experiments were traced by GFP fluorescence. The majority of the embryos injected with either ankyrin-G siRNA (35 of 42) or beta-2-spectrin siRNA (37 of 52) (Supplementary information, Fig. S3) formed aberrant morula-like structures, but did not progress to blastocysts. In contrast, most of the embryos injected with either a control plasmid or mutated ankyrin-G or beta-2-spectrin siRNA plasmids inactivated by changes at 3 sites (see Experimental procedures) developed normally to form blastocysts in 2 independent experiments (Supplementary information, Fig. S3). 40 hr after injection of siRNA, GFP-expressing cells show an overall reduction of either ankyrin-G or beta-2-spectrin staining at sites of cell-cell contact (compare arrowheads to arrows, Fig. 9 B). Strikingly, embryonic cells depleted of beta-2-spectrin or ankyrin-G also exhibited impaired recruitment of E-cadherin to sites of cell-cell contact and did not undergo compaction. Compaction is essential for normal development, and is a process where blastomeres flatten on to each other and the cell adhesion molecule E-cadherin is recruited to sites of cell-cell contact and did not undergo compaction. Compaction is essential for normal development, and is a process where blastomeres flatten on to each other and the cell adhesion molecule E-cadherin is recruited to sites of cell-cell contact (30,33). Injected cells were viable based on their expression of both GFP and E-cadherin, and ability to undergo at least one cycle of cell division. Ankyrin-G and beta-2-spectrin thus are required for accumulation of E-cadherin at sites of cell-cell contact in early embryos as well as cultured epithelial cells.

**DISCUSSION**

It has long been recognized that function of E-cadherin requires its cytoplasmic domain (20,34). Here, we identify ankyrin-G as a new binding partner for the E-cadherin cytoplasmic domain, and demonstrate that ankyrin-G can recruit beta-2 spectrin to E-cadherin/beta-catenin complexes. Interestingly, these findings were foreshadowed 17 years ago by work of Nelson and colleagues who reported that E-cadherin forms a complex with ankyrin and spectrin in epithelial cells (12). We also present evidence that ankyrin-G, beta-2-spectrin, and E-cadherin cooperate in formation as well as maintenance of the lateral membrane. Moreover, we show that ankyrin-G and beta-2 spectrin are both required for concentration of E-cadherin at sites of cell-cell contact in early mouse embryos, suggesting that these proteins are functional partners of E-cadherin in vivo as well as in cultured epithelial cells.

Our discovery of a new link between E-cadherin and the actin cytoskeleton is particularly timely given uncertainties regarding the commonly accepted molecular pathway linking cadherins to actin filaments (21,35). According to textbook models, alpha-catenin binds to both beta-catenin/E-cadherin and F-actin (35). However, alpha-catenin could not couple E-cadherin-beta-catenin directly to actin in assays with pure proteins (21). Nevertheless, an E-cadherin-alpha-catenin fusion protein lacking a binding site for beta-catenin but retaining the ankyrin-binding site is still active in cell adhesion (36,37). Thus an indirect linkage of alpha-catenin to actin through alpha catenin-binding proteins such as spectrin, alpha-actinin, or ZO-1 could still occur. The affinity of ankyrin-G for E-cadherin is relatively low, and may be enhanced by accessory proteins in vivo. For example, it is conceivable that stable complexes of E-cadherin could arise from the interaction of alpha-catenin with the N-terminal region of beta-2 spectrin (8), in combination with ankyrin-G-dependent coupling of beta-2-spectrin to E-cadherin. It will be important to determine directly whether alpha-catenin and spectrin can engage in such a multimeric complex.
The E-cadherin cytoplasmic domain is intrinsically unstructured in solution (17), which is a fold shared by ankyrin-binding sites in other proteins including L1 CAMs (15), and the anion exchanger (38,39). Moreover, the ankyrin-binding sites of voltage-gated Na channels (40,41), and the RhBG ammonium transporters (42) are predicted to be unstructured (http://iupred.enzim.hu/index.html). It is of interest that the atomic structure of the ankyrin membrane-binding domain contains a groove capable of accommodating extended polypeptides (43). Together these considerations suggest that requirements for ankyrin recognition are simple: a stretch of residues (26 in the case of E-cadherin) containing a mixture of charged and hydrophobic residues. A potential advantage of an unstructured fold is that multiple proteins can bind to the same or overlapping sites. In the case of E-cadherin, p120-catenin and components of the endocytosis machinery may share an interaction surface with ankyrin.

An important conclusion of this study is that ankyrin-G and beta-2 spectrin directly participate in delivery of E-cadherin to the lateral membrane in addition to their expected role in stabilizing pre-formed membrane. A working model for how these proteins cooperate in formation of membranes is shown schematically in Figure 10. According to this scheme, newly synthesized E-cadherin/beta catenin complexes bind directly to ankyrin-G in the trans-Golgi network. Ankyrin-G in turn couples these complexes to beta-2 spectrin. Beta-2 spectrin binds to phosphatidylinositol and phosphatidylserine as well as microtubule-based motors (26,28), and thus can promote transport of ankyrin-G-associated proteins together with bulk phospholipid.

Transcellular interactions between E-cadherin molecules in adjacent cells are likely to provide the spatial cues that direct the ankyrin-spectrin machinery (44). Coupling of E-cadherin to a versatile adaptor protein such as ankyrin-G could promote co-recruitment of diverse proteins to sites of cell-cell contact. For example, ankyrin-G associates with other lateral membrane proteins including the Na/K ATPase (10,11,45) and the RhBG ammonium transporter (42). Ankyrin-G also binds to N-cadherin (not shown) and likely other type 1 classical cadherins. Ankyrin-G and cadherin partners thus could direct formation of a variety of specialized membrane domains at sites of cell-cell contact, ranging from synapses in the nervous system to intercalated discs in cardiomyocytes.
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FOOTNOTES

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Abbreviations- The abbreviations used are: HBE, Human bronchial epithelial; GFP, green fluorescent protein; siRNA, small interfering RNA; HA, hemaglutinin; TGN, Trans-golgi network
Figure Legends

**Figure 1.** Ankyrin-G recruits beta-2-spectrin to E-cadherin/beta-catenin complexes through direct interaction with the E-cadherin cytoplasmic domain. A and B, E-cadherin cytoplasmic domain (EC-CD) binds selectively to ankyrin-G and not ankyrin-B. A. Coomassie blue-stained SDS PAGE gel profile of a binding assay between GST-E-cadherin cytoplasmic domain (GST-EC-CD) and either ankyrin-G (AnkG) or ankyrin-B (AnkB). 1, AnkG; 2, AnkB; 3, GST, and 4, GST-EC-CD. B. Stoichiometric binding of ankG (closed circle) to GST-EC-CD (Methods). Note weak association of ankB (open circles). C. HEK 293 based plasma membrane recruitment assay shows that E-cadherin (EC) recruits ankyrin-G-GFP(AnkG) to the plasma membrane but not ankyrin-B-GFP (AnkB). Scale, 5 µm

**Figure 2.** Ankyrin-G binds to a distributed site in the juxta-membrane region of the E-cadherin cytoplasmic domain. A. The minimal ankyrin-G binding site (amino acid sequence expanded), the beta-catenin binding site (grey) and the p120 catenin binding site (blue) are identified in the E-cadherin cytoplasmic domain. The amino acid sequences of the minimal ankyrin-G binding site of E-cadherin (EC) and the homologous regions in N-cadherin (NC), P-cadherin (PC), R-cadherin (RC) VE-cadherin (VEC), the Drosophila shotgun, Drosophila N-cadherin (D-NC) and the C.elegans cadherin (HMR-1) are shown. Amino acids within EC-CD mutated to alanine and also conserved in the other cadherins are shown in red. B. Bar graphs show ankyrin-G binding to mutant GST-EC-CD polypeptides as a percentage of ankyrin-G binding to wild type GST-EC-CD (right). RD748DR GST-EC-CD and RD748AA GST-EC-CD show a similar reduction in ankyrin-G binding (left).

**Figure 3.** Ankyrin-G links an E-cadherin/beta-catenin complex to beta-spectrin. A. GSTEC-CD binds simultaneously to both ankyrin-G and beta-catenin. Stoichiometric binding of ankyrin-G to GST-EC-CD in either the presence (open circle, AnkG+ beta-catenin) or absence (closed circle, AnkG) of 1µM of beta-catenin. The Coomassie blue-stained SDS PAGE gel shows the results of binding between 1µM of ankyrin-G and EC-CD in the presence or absence of saturating amounts of beta-catenin: 1-GST + AnkG; 2-GST-EC-CD + AnkG; 3-GST + AnkG + beta-catenin; 4-GST-EC-CD + AnkG + beta-catenin. B. Ankyrin-G links an EC-CD/beta-catenin complex to beta-2-spectrin. The Coomassie blue-stained gel shows the results of binding between EC-CD, ankyrin-G, beta-catenin and beta-2-spectrin repeats (14-16) including the ankyrin binding site: 1-GST; 2-GST-EC-CD; 3-beta-catenin; 4- beta-2-spectrin; and 5- ankyrin-G.

**Figure 4.** Mutant E-cadherin with reduced binding to ankyrin-G accumulates in the TGN, and has increased mobility at the lateral membrane of polarized epithelial cells. A. RD748DR E-cadherin accumulates in the TGN of HBE cells. Panels (left) show XY (top) and XZ (bottom) confocal sections of cells expressing wild type E-cadherin-3HA (EC, WT) or RD748 DR E-cadherin-3HA (EC, RD748DR). The RD748 DR E-cadherin shows strong intracellular accumulation. Note lack of intracellular accumulation of WT E-cadherin. The intracellular RDDR E-cadherin partially overlaps with the TGN (right panels) identified by golgin-97 immunofluorescence (red). Scale, 5 µm. B. Increased mobile fraction of RD748DR E-cadherin in the lateral membrane of HBE cells. Images of HBE cells expressing wild type (WT, top) or RD748DR (bottom) E-cadherin-GFP (EC-GFP) cells before bleaching (prebleach), at the point of bleach (bleach, arrow) and during recovery from bleaching (post bleach). Representative graph showing normalized fluorescence intensities showing recovery after photobleaching of wild type E-cadherin-GFP (black) or RD748DR (red) E-cadherin-GFP. Scale, 2 µm

**Figure 5.** LL740AA E-cadherin binds ankyrin-G and mis-localizes in HBE cells only at high levels of expression. A. GST-LL740AA EC-cytoplasmic domain (EC-CD) and wildtype EC-CD bind equivalently to ankyrin-G in solution (Methods). B. LL740AA E-cadherin can recruit ankyrin-G to the plasma membrane of HEK 293 cells. Ankyrin-G-GFP is recruited to the membrane in the presence of wild type (WT) or LL740AA E-cadherin (EC). Scale, 5 µm. C. Exogenous wild type (WT) and LL740AA E-cadherin (EC) localize to the lateral membrane in HBE when expressed at low levels (right panels). In contrast, LL740AA E-cadherin, unlike WT
E-cadherin, localizes to both the lateral membrane and apical membrane when expressed at high levels. Cells were stained with anti-HA antibody (green) and anti-golgin-97 antibody (red). Scale, 5 µm

**Figure 6. Time course of loss of E-cadherin and lateral membrane upon depletion of ankyrin-G or beta-2-spectrin by siRNA.** Height of the lateral membrane marked by E-cadherin staining is shown as a function of time in control HBE cells and cells transfected 12 h after plating with ankyrin-G siRNA or beta-2-spectrin siRNA. Control cells (black line), AnkG, ankyrin-G siRNA (red line) and β-2-sp, beta-2-spectrin siRNA (blue line). Comparison of XZ confocal sections of control HBE cells at 12 h and 24 h post plating show the dramatic growth of the lateral membrane marked with E-cadherin (green, top and middle). The localization of ankyrin-G (left) and beta-2-spectrin (right) in the same cells are shown. The bottom panels show the loss of E-cadherin accompanying loss of either ankyrin-G (left) or beta-2-spectrin (right). Arrowheads point to the apical tip of the lateral membrane.

**Figure 7. Exit of E-cadherin from the TGN of HBE cells requires ankyrin-G, beta-2-spectrin and microtubules.** A. Intracellular accumulation of E-cadherin results from depletion of either ankyrin-G or beta-2-spectrin by siRNA, or depletion of microtubules with nocodazole. Arrows indicate the sites of intracellular accumulation of E-cadherin. Scale 20 µm. B, C and D. E-cadherin accumulates in the TGN in cells depleted of either ankyrin-G, beta-2-spectrin or microtubules. B. XZ confocal sections shows a dramatic increase in intracellular accumulation of E-cadherin (red) in either cells depleted of ankyrin-G or beta-2-spectrin using siRNA or microtubule depleted cells compared to control cells. There is minimal intracellular E-cadherin in control cells. The TGN is identified by the golgin-97 immunofluorescence (green, left panels) and the cis /medial Golgi is identified by the localization of giantin (green, right panels). The intracellular E-cadherin (red) in cells resulting from depletion of ankyrin-G, beta-2-spectrin or microtubules overlaps partially with the TGN marker golgin-97 (yellow, left panels). In contrast, intracellular E-cadherin (red) is distinct from the cis/medial golgi marked by giantin (green). C and D. Three-dimensional renderings of depleted cells in B show that the intracellular E-cadherin (red) co-localizes with the TGN identified by golgin-97 localization (green, top) but not with cis /medial Golgi identified by giantin localization (green, bottom). E-cadherin localizes predominantly to the plasma membrane in control cells. Overlap of voxels representing golgin-97 with voxels representing intracellular E-cadherin (% overlap) is shown in the bar graph. Note the minimal overlap of E-cadherin with markers for the cis/medial golgi (giantin), ER (ERP57) and endosomes (dextran, EEA1 and Rab7). Scale, 5 µm

**Figure 8. E-cadherin transiently co-localizes with intracellular ankyrin-G and beta-2-spectrin during synchronized lateral membrane biogenesis.** A and B. Microtubule depletion in HBE cells results in a reversible loss of lateral membranes. A. Time course of loss of lateral membrane heights (black line) in cells upon treatment with nocodazole (arrow, nocodazole) and re-growth of lateral membranes after removal of the drug (arrow, Wash). Results are means and s.d. B, Images at 0, 6, and 18 hours of XZ confocal sections of cells in (a) stained for E-cadherin EC, E-cadherin. C and D. Intracellular complexes containing E-cadherin and both ankyrin-G and beta-2-spectrin appear transiently after nocodazole washout in HBE cells. Three-dimensional renderings of cells treated with nocodazole show that intracellular E-cadherin (red) does not co-localize with either ankyrin-G (green, top left, C) or beta-2- spectrin (green, bottom left, D) six hours after nocodazole treatment. In contrast, in cells that have recovered from the nocodazole treatment for 0.5 h (middle), E-cadherin (red) colocalizes with ankyrin-G (green, C) or beta-2-spectrin (green, D). A higher magnification of the area within the box at the 0.5 h time point is shown in the second row of panels in c and d. The intracellular structures are not present after 2 h of recovery from nocodazole treatment. Scale, 4µm (1st row panels) and 2µm (2nd row panels).
Figure 9. E-cadherin localization to sites of cell-cell contact in early mouse embryos requires both ankyrin-G and beta-2-spectrin.
A. E-cadherin co-localizes with ankyrin-G or beta-2-spectrin at the sites of cell-cell contact in early mouse embryos. Confocal images of a 2-cell stage mouse embryo (top), and a morula stage embryo (bottom, ~60 h post fertilization). B, C. E-cadherin, ankG, ankyrin-G. B, E-cadherin at the plasma membrane of mouse embryos is reduced upon depletion of either ankyrin-G or beta-2-spectrin. Missense siRNA injected embryos (GFP positive) develops normally into a blastocyst. In contrast, ankyrin-G or beta-2-spectrin and E-cadherin are reduced at the sites of cell-cell contact between the progeny cells of ankyrin-G or beta-2-spectrin siRNA injected blastomere (arrowhead, GFP positive). The plasma membrane between the progeny cells of the uninjected blastomere (arrow) labels robustly with ankyrin-G or beta-2-spectrin and E-cadherin. Ankyrin-G or beta-2-spectrin depleted embryo fail to compact, and undergo only two rounds of cell division (compare number of GFP labeled cells in missense siRNA injected embryo and siRNA injected embryo). Scale, 20 µm

Figure 10. A schematic model showing the collaboration of ankyrin-G, beta-2-spectrin and E-cadherin in membrane domain formation. In this model E-cadherin/beta-catenin complexes linked to ankyrin-G are transported to the plasma membrane along microtubules by motor proteins. Beta-spectrin couples the microtubule motors to ankyrin-G / E-cadherin/beta-catenin complexes. Bulk lipid transport is also achieved by interaction of beta-spectrin with phospholipids. Transcellular interactions between E-cadherin at the plasma membrane likely targets the complexes to sites of cell-cell contact. Other ankyrin-G binding membrane proteins (NaKATPase (NKA) and yet to be identified membrane proteins X) are co-recruited along with E-cadherin to the sites of cell-cell contact. The membrane proteins are retained at the plasma membrane through an interaction between ankyrin-G and spectrin actin-skeleton.
Kizhatil Fig 9

A

AnkG  EC  Overlap

β-2-spectrin  EC  Overlap

B

DIC  GFP  AnkG  EC

siRNA

Missense  AnkG

β-2-spectrin  EC

siRNA

Missense  β-2-spectrin
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