Association of Lbc Rho Guanine Nucleotide Exchange Factor with α-Catenin-related Protein, α-Catenulin/CTNNAL1, Supports Serum Response Factor Activation*

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The Rho GTPase signaling pathway is required for actin cytoskeletal organization and serum response factor-dependent gene transcription. Lbc is a Rho-specific guanine nucleotide exchange factor that contains a modulatory C-terminal region. To elucidate Lbc regulatory mechanism(s), a yeast two-hybrid screen for proteins that interact with the Lbc C-terminal region was carried out, resulting in multiple isolation of cDNAs encoding the same 794-amino acid Lbc interacting protein. The Lbc interacting protein has homology with the α-catenin cell adhesion component and is identical to the α-catenin-like α-catenulin protein of unknown function. The human α-catenulin gene (CTNNAL1) maps to 9q31-32. Here we identify the predicted endogenous α-catenulin product, document α-catenulin and Lbc co-expression in multiple human cell lines, and show α-catenulin and Lbc subcellular co-fractionation and intracellular localization. The required regions for Lbc and α-catenulin interaction were mapped, and complex formation between Lbc and α-catenulin in mammalian cells was detected. Functionally, α-catenulin co-expression leads to increased Lbc-induced serum response factor activation in vivo as measured by a transcriptional reporter assay. Furthermore, α-catenulin co-expression enhances Lbc-induced GTP-Rho formation in vivo. These results support the concept that the recently identified α-catenulin protein may modulate Rho pathway signaling in vivo by providing a scaffold for the Lbc Rho guanine nucleotide exchange factor.

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The ubiquitous Rho small GTPase is required for several key cellular responses including contractility, adhesion, migration, and growth. Rho controls a distinct signaling pathway that regulates serum-induced actin filament assembly (1) and gene transcription via serum response factor (SRF) (2). Furthermore, evidence increasingly implicates involvement of the Rho pathway in disorders such as cancer, metastasis, and cardiovascular disease (3-5). However, regulation of this pathway is not well understood. Guanine nucleotide exchange factors (GEFs) for the Rho family GTPases, Rho, Rac, and Cdc42, induce formation of the active, GTP-bound GTPase capable of transducing signals (6) and play a key role, because they transduce signal inputs from activated cell surface receptors to Rho members (7). All Rho family GEFs contain the common Dbl oncogene homology (DH) domain, which confers GEF activity, and a tandem pleckstrin homology (PH) domain that has several functions (6, 8). In addition, Rho GEFs contain unique N- or C-terminal regions that likely represent regulatory regions and often contain consens signaling domains/motifs (6, 8). Although the functional role of Rho GEFs is now apparent, Rho GEF regulation is in many cases poorly understood.

The DH domain-containing Lbc oncogene product (9) is a specific GEF for Rho (10) and is a member of the Rho-specific GEF family that includes p115 Rho GEF (11), Lsc (12, 13), p190 RhoGAP (14, 15), PDZ Rho GEF (16), and LARG GEF (17, 18). Previous comparison of onco-Lbc with the normal Lbc homolog isolated from human muscle revealed that although both forms contain intact DH and PH domains, proto-Lbc or wild type Lbc (wt-Lbc) cDNA encodes an extended C-terminal region of 453 residues that is deleted from onco-Lbc (ΔCT-Lbc) (19). Subsequent reports of normally occurring Lbc splice variants with longer N termini has recently highlighted the multi-functional-ity of the Lbc Rho GEF. For example, Brx is an Lbc variant enriched in testes that is implicated in nuclear hormone modulation (20). In addition, AKAP-Lbc, which is enriched in the heart, functions as a protein kinase A-anchoring protein, because it contains an additional N-terminal protein kinase A-binding domain (21, 22). However, all normally occurring Lbc Rho GEF forms described to date contain a common C-terminal region following the DH/PH GEF domain cassette. The C-terminal region contains a unique sequence the notable feature of which is a −110-residue region of predicted α-helical
structure that contains a consensus leucine zipper motif (19). Deletional analysis of the wt-Lbc C-terminal region indicated that this region contributes to regulation of Lbc transforming activity and subcellular localization (19).

To investigate the mechanism of wt-Lbc modulation by the C-terminal region, we focused on identifying proteins that interact with this region using the yeast two-hybrid system, because many may yield clues to Lbc regulatory mechanisms. Here we describe isolation and characterization of the Lbc-associated protein-1, α-catenin, which interacts specifically with the Lbc C-terminal region. α-Catenin, which has no previously ascribed function, has homology with the α-catenin adhesion component. We identify the endogenous α-catenin protein and document α-catenin and Lbc co-expression and co-fractionation in multiple human cell lines. The required regions for Lbc and α-catenin interaction were defined; furthermore, Lbc and α-catenin complex formation in mammalian cells was detected. Moreover, the functional effect of α-catenin on Lbc activity was determined in vivo by using a Rho-dependent SRF transcriptional reporter assay and by measuring changes in GTP-Rho formation. These results for the first time characterize the endogenous α-catenin protein and indicate a role for α-catenin in modulating the Rho signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Lines—The Cos-1, HeLa, HT29, T84, HCT116, HEK293T, MCF-7, and Caco-2 cell lines are from ATCC.

Plasmids—Lbc C-terminal region cDNA (418–893) (19) was used as template to generate the LbcAT (418–780), LbcID (418–468), LbcAID (669–893), and LbcT (780–893) deletion mutants in the pGIBT yeast expression vector by PCR. Following this double selection procedure, 10^7 total clones were screened, representing a stringent conditions. Northern blot hybridization was carried out according to the manufacturer’s recommendation (Clontech). The filters were washed under stringent conditions.

Fluorescence in Situ Hybridization—Metaphase spreads were prepared from human peripheral blood lymphocytes cultured for 72 h in the presence of phytohemagglutinin. α-Catenin cDNA was labeled with [γ-^32P]ATP (Roche Molecular Biochemicals) by nick translation. Fluorescence in situ hybridization and detection of immunofluorescence were performed as previously described (23). Chromosomes were counterstained with diamidino-2-phenylindole and observed with a Zeiss epifluorescence microscope equipped with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) operated by a Macintosh computer work station. Digitized images of diamidino-2-phenylindole staining and α-CATULIN/CNN1 Lbc fluorescent signals were captured and merged using Oncor Image version 1.6 software.

GST Fusion Protein Expression and Purification—wt-Lbc C-terminal region (Lbc residues 416–893; Ref. 19) and α-catenin N-terminal region cDNAs (residues 1–537) were each subcloned into the pGEXX vector (Pharmacia Corp.) for expression in E. coli DH5α. Optimal growth conditions for soluble GST:cDNA expression were 30 °C to 0.5Amax for 1; for GST-LbcCTerm, conditions were 37 °C to 0.5Amax. Two soluble fusion proteins were affinity-purified with glutathione-Sepharose 4B bead conjugate (Pharmacia Corp.) and eluted following standard procedures (22).

In Vitro Binding Assays—An equimolar amount of GST:Lbc CTerm was incubated with either GST or α-catenin for 1 h at room temperature and then immunoprecipitated with 5 μl of bead-coupled M2 anti-FLAG antibody for 1 h at room temperature in TBS with 1% BSA. Reciprocally, GST:α-catenin was incubated with either GST or GST:LbcTerm and then immunoprecipitated with 5 μl of bead-coupled 9E10 anti-Myc antibody. BSA-coated beads were preincubated with 1% BSA to reduce nonspecific binding. Following four washes in TBS, 1% Triton, 1% octylglucoside, the α-catenin-imunoprecipitated material was resuspended in sample buffer with dithiothreitol, boiled, and analyzed by immunoblot.

Cell Transfection—100-mm dishes at 70% confluence were transfected with plasmids using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s recommendation for 5 h. After transfection the cells were grown 1–2 days and harvested.

Subcellular Fractionation—HEK293T cells were fractionated into the S-100 soluble fraction and the P-100 particulate fraction as previously described (19).

Immunofluorescence—HEK293T cells expressing Mycα-catenin and FLAG:Lbc were grown on glass coverslips and washed twice with Dulbecco’s modified Eagle’s medium followed by two washes with PBS. The cells were fixed in 4% paraformaldehyde, permeabilized to 5 μg/ml aprotinin. As primary antibodies, 1:150 dilution of rabbit anti-Myc A-14 and 1:200 dilution of mouse anti-FLAG M2 were used for α-catenin and Lbc detection, respectively. After three washes in PBS, the cells were incubated with TRITC-conjugated anti-rabbit IgG (Pierce) at 1:300 or fluoresein isothiocyanate-conjugated anti-mouse IgG (Fierce) at 1:200. After two washes in PBS, the coverslips were mounted and viewed on a Zeiss Axiovert 10 microscope. The images were then captured using a Hamamatsu digital camera using Metamorph software.

Immunoprecipitation—Insoluble lysate material was removed by centrifugation at 10,000 × g for 10 min. For immunoprecipitation of epitope-tagged products, agarose-bead conjugated anti-Myc 9E10 antibody or affinity gel-conjugated anti-FLAG M2 antibody was used. Bead-coupled antibodies were preincubated with 1% BSA overnight. The images were then captured using a Hamamatsu digital camera using Metamorph software.

Immunoblotting—Cellular material in sample buffer with dithiothreitol was heated at 94 °C for 5 min. After transfer onto nitrocellulose, the filters were blocked overnight at 4 °C in 5% nonfat dry milk in TBS and then washed twice in TBS. The filters were probed for 1 h with either anti-FLAG M2 antibody at 10 μg/ml, anti-Myc 9E10 antibody at 1 μg/ml, or 1:1500 dilution of anti-Lbc 9E10 antibody at 1 μg/ml, or 1:1500 dilution of anti-Lbc 9E10 antibody at 1 μg/ml.
or anti-catulin LP-1 antisemirum in TBS with 5% nonfat milk. The filters were washed three times each for 15 min in TBS with 0.05% Tween. After incubation for 1 h with anti-mouse or anti-rabbit horseradish peroxidase in TBS with 5% nonfat milk, the filters were washed three times each for 15 min. In TBS with 0.05% Tween, followed by two additional washes in TBS alone. For GST fusion protein detection, the filters were probed for 1 h with anti-GST-HRP antibody at 1:3000 dilution in TBS, followed by three washes for 15 min in TBS. ECL was performed using Renaissance Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

**Dual Luciferase Reporter Assay**—The SRE.L luciferase reporter plasmid, which encodes a mutant SRE that contains SRF binding sites but eliminates the ternary complex factor-binding site and is potently activated by Rho signals (2), was used. All of the samples were transfected with 10 ng of *Renilla* luciferase plasmid as an internal control, and the total plasmid amount transfected was equalized by the addition of vector. 24 h following transfection of HEK293T cells in 6-well dishes, luciferase assays were performed using a dual luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s recommendation, and the luciferase activity was measured by luminometer. The results were internally normalized by sequential measurement of control *Renilla* and experimental firefly luciferase reporter plasmid activities. Each point was performed in triplicate.

**RESULTS**

**Characterization of Lbc-interacting cDNAs**—18 cDNA clones were confirmed to positively interact with the GAL4 BD:Lbc C terminus fusion bait following a yeast two-hybrid screen of a GAL4 activation domain (AD):human HeLa cDNA fusion library. Sequencing revealed that all of the positives were one of two cDNAs of 2.3 or 2.45 kb, representing the same mRNA. The longest Lbc-interacting cDNA contained a 5’/H11032 Kozak consensus sequence for translation initiation (26), followed by a 2.2-kb coding sequence in-frame with the GAL4 AD fusion sequence, a translation stop site, a 3’/H11032-untranslated sequence, and a poly(A) tail. Analysis of the deduced 734-residue polypeptide of predicted 82-kDa size indicates that it is hydrophilic, and analysis with the PROSITE data base (27) for known protein motifs reveals a consensus tyrosine kinase phosphorylation site at residue 536, and multiple consensus protein kinase C phosphorylation sites.

BLAST (28) search of the deduced Lbc-interacting peptide sequence against GenBank™ entries revealed most similarity to -catenin. In addition, the search revealed that the sequence is identical to two recently described cDNAs alternately called CTNNAL1 (AF030233; Ref. 29) and -catulin (30) reported to have similarity to -catenin. On this basis, we adopted the use of -catulin/CTNNAL1 names to designate the Lbc-interacting peptide and gene, respectively. As shown in Fig. 1, BESTFIT similarity alignment of -catulin with its closest human homologue, -catenin-related protein CAP-R, CTNNA2 gene; A45011; Ref. 31). The identity is 29%, overall homology is 50%. Alignment was generated using the BESTFIT program. The dots above each line represent 10 amino acids. The vertical lines indicate identity, conservative substitutions of high or low similarity are indicated by a colon or a single dot, respectively.
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P26232) (31), shows 29% identity and 50% overall homology. α-Catenin also has similar overall homology (~28% identity and 40% homology) to human αE-catenin (epithelial) (CTNNAL1 gene; P35221) (32), in addition to murine αN-catenin and Dro sophila and Lytechinus variegatus (sea urchin) α-catenin (not shown). Fig. 1 indicates that α-catenin contains an extra 16 N-terminal residues not found in mammalian α-catenins. The homology between α-catenin and α-catenin lies within two blocks; the first homology block is a nearly contiguous sequence between α-catenin residues 18-524 and αN-catenin residues 2-504. This is followed by a region of ~110 residues present in α-catenin that is missing from α-catenin. The second homology block spans α-catenin residues 525 until the final residue 734 and αN-catenin residues 615-897. This region contains four limited gaps in homology in which α-catenin lacks 68 residues present in αN-catenin. After this, αN-catenin contains 48 additional C-terminal residues. Comparison of α-catenin and human αE-catenin sequences generates a highly similar alignment to that shown in Fig. 1, although αE-catenin is a shorter product (907 residues) than αN-catenin (945 residues). Overall, in comparison with the original report describing CTNNAL1 (29), our analysis and the report by Janssens et al. (30) reveals a greater similarity between CTNNAL1/α-catenin and αN-catenin based on the detection of the second homology block in α-catenin residues 525-734. α-Catenin is a member of the vinculin superfamily (32, 33), and α-catenin also shows substantial but lower homology (21% identity) to vinculin (30).

α-CATULIN/CTNNAL1 Gene Chromosomal Localization—

An α-catenin cDNA probe was used to map the chromosomal location of the CTNNAL1 gene by fluorescence in situ hybridization analysis. The α-catenin probe hybridized to chromosome 9 in 20 of 20 (100%) metaphase spreads from a normal male donor. As indicated by Fig. 2, altogether, 43 of 64 fluorescence signals (67%) were located specifically at 9q31–32.

α-Catenin Expression and Subcellular Localization—Fig. 3A shows the presence of a single major ~2.8-kb α-CATULIN mRNA transcript in several human tissues such as thymus, prostate, testes, ovary, small intestine, and colon. α-CATULIN mRNA expression is also reported in skeletal muscle, lung, heart, and placenta (30), indicating that it is widely expressed. We previously reported high levels of Lbc mRNA co-expression in these tissues (19).

To identify the endogenous α-catenin protein, rabbit anti-catenin LP-1 antiserum was generated. Fig. 3B (left panel) shows that LP-1 immune serum detects endogenous α-catenin (lane 2) of predicted ~82-kDa size, in addition to the larger epitope-tagged form (lane 1), in HEK293T cells. In contrast, as shown in the right panel of Fig. 3B, the preimmune serum did not detect these bands. Next, the LP-1 antiserum was used to detect α-catenin protein by immunoblot analysis of lysates of human epithelial cell lines. Fig. 3C shows expression of the predicted endogenous α-catenin product of ~82 kDa in all human cell lines tested of different origins, such as cervical (HeLa), colon (HT29, T84, HCT116, CaCo2), breast (MCF-7), and human embryonic kidney (HEK293T). These data indicate that α-catenin is widely expressed. In Fig. 3C shows that when the same filters were immunoblotted with anti-Lbc antiserum, the 102–105-kDa wt-Lbc Rho GEF form is present in these cell lines.

To investigate subcellular localization, untransfected HEK293T cells were subjected to high speed fractionation into soluble (cytosolic) and pellet (membrane-rich) fractions. The fractions were resolved by SDS/PAGE and immunoblotted for endogenous RhoA, Lbc, α-catenin, and α-catenin. Fig. 3D shows that consistent with previous reports (34), RhoA is present mainly in the soluble fraction, with a minor proportion in the pellet, verifying the fractionation procedure used. In contrast, substantial portions of endogenous Lbc and α-catenin localized to both the membrane-rich (pellet) fraction and the soluble (cytosolic) fraction. Finally, α-catenin was found to localize almost exclusively to the membrane-rich fraction. Thus, the overall fractionation patterns of α-catenin and α-catenin are distinct.

Intracellular localization was further analyzed by immunofluorescence. The cells were transfected with FLAG epitope-tagged Lbc (pSR FLAG:wt-Lbc) and Myc-tagged α-catenin (pcDNA Myc:α-catenin) either singly or in combination, and indirect immunofluorescence was performed. Fig. 4 (A and B) shows that when expressed alone, α-catenin and Lbc, respectively, are present in the cytosol and at plasma membrane-proximal sites. Nuclear localization was not observed. In addition, when co-expressed (Fig. 4, C and D), α-catenin and Lbc continue to show a similar distribution in the cytosol and at plasma membrane-proximal sites. These results are consistent with the fractionation results.

Mapping of Lbc and α-Catenin Interaction Sites—The sites of interaction within the wt-Lbc C-terminal region and α-catenin were initially mapped by yeast two-hybrid assay. As indicated in Fig. 5A, the first 250 residues of the Lbc C-terminal region, Lbc CTerm, is designated the interdomain region (IDR). This is followed by a ~110-residue α-helical region designated α-Hel and finally the extreme C-terminal 115 residues designated Lbc-T. As shown in Fig. 5A, deletion mutants of each of these domains were generated as yeast GAL4 BD fusion proteins; these fusion proteins were not toxic in yeast and did not induce autonomous activation in yeast reporter strains (results not shown). Following co-transformation of these BD:Lbc fusion mutants with the GAL4 AD: α-catenin fusion plasmid, co-transformants were assayed for interaction by testing for growth on triple drop-out medium and by β-galactosidase positivity. Fig. 5A summarizes the results obtained, which showed that the Lbc CTerm, Lbc-T, and Lbc IDR constructs strongly interacted with α-catenin, whereas the Lbc-ΔIDR and Lbc-T constructs were negative. These results indicate that the IDR within the Lbc C-terminal region is sufficient for the interaction and that the α-helical region and extreme C terminus are not required.

Next, the required α-catenin interaction region was investigated. As indicated in Fig. 5B, two separate positively interacting α-catenin cDNAs were originally isolated from the cDNA library screen: full-length α-catenin (shown in Fig. 1) and...
residues of α-catenin are sufficient for interaction.

To independently verify the defined interaction sites, recombinant GST fusion proteins containing the interaction regions were generated. To allow separate immunoprecipitation, FLAG epitope-tagged GST:LbcIDR (53 kDa) and Myc epitope-tagged GST:NH-Catulin (82 kDa) fusion proteins were expressed in bacteria, and purified proteins were tested for direct interaction in vitro. As indicated in Fig. 5C (first and second lanes), GST:LbcIDR-FLAG was incubated with either GST:NHCatu-Myc or GST as control at equimolar amounts and then immunoprecipitated with anti-FLAG antibody. Immunoblotting of immunoprecipitates with anti-GST antibody showed that GST: NHCatu-Myc, but not GST, co-immunoprecipitates with GST:LbcIDR-FLAG (first and second lanes). Reciprocally, GST:NHCatu-Myc was incubated with either GST or GST:LbcIDR-FLAG (third and fourth lanes). Following immunoprecipitation of NHCatu-Myc with anti-Myc antibody, immunoblotting showed that the precipitate contains GST:LbcIDR but not GST (third and fourth lanes). These data, summarized in Fig. 5D, provide independent evidence that recombinant LbcIDR and NH-Catulin domains are sufficient for direct interaction.

Complex Formation by Epitope-tagged Lbc and α-Catenin in Mammalian Cells—To investigate whether Lbc and α-catenin associate in mammalian cells, immunoprecipitation was carried out to detect complex formation. For this purpose, Myc-tagged α-catenin (pcDNA-Myc:Catu) and FLAG-tagged wt-Lbc (pSR-FLAG:wt-Lbc) plasmids were transiently transfected either together or with vector at submaximal levels to yield transiently expressed proteins in COS-1 cells. As shown in Fig. 6A, anti-FLAG antibody was used to immunoprecipitate FLAG: wt-Lbc. Subsequent immunoblotting of this precipitate with anti-Myc antibody readily showed co-immunoprecipitation of the 82-kDa α-catenin in the group co-transfected with α-catenin plasmid but not with vector. Reciprocally, Fig. 6B shows that following α-catenin immunoprecipitation with anti-Myc antibody, an immunoblot of this material with anti-FLAG antibody
endogenous partner. For this purpose, FLAG-tagged wt-Lbc was transiently expressed in HEK293T cells, and cell lysates were incubated either with M2 anti-FLAG antibody or with beads conjugated to mouse Ig. After immunoprecipitation, the material was immunoblotted with anti-catulin antiserum. Fig. 7A shows the presence of the co-precipitated endogenous 82-kDa α-catulin product in the anti-FLAG antibody precipitate (+M2) but not in the control group (+Ig). In contrast, Fig. 7B shows that endogenous α-catulin was not present in FLAG:ΔCT-Lbc immunoprecipitates (+M2). Reciprocally, Myc-tagged α-catulin was transiently expressed in HEK293T cells, and the cell lysates were incubated with either anti-Myc antibody or Ig. Fig. 7C shows that the −102-kDa Lbc form was detected in the Myc-Catulin immunoprecipitate (+Myc) but not in the +Ig group. These results demonstrate complex formation of endogenous α-catulin and wt-Lbc with the corresponding exogenously expressed partner in mammalian cells and indicate the specificity of the association with wt-Lbc.

α-Catulin Effect on Lbc-induced SRE Reporter Activation in Vivo—Based on the physical association observed, we next investigated whether α-catulin can modulate wt-Lbc signaling in vivo using a SRF-dependent transcriptional reporter that encodes a mutant form of the serum response element (SRE), designated SRE.L (2). SRF activity requires Rho function (2) and is a commonly used measure of Rho-dependent signals in vivo. The same epitope-tagged wt-Lbc and/or α-catulin cDNAs used above were transiently expressed with the SRE.L luciferase reporter plasmid in HEK293 cells. As shown in the first bar in the graph of Fig. 8A, transfection of the pSR and pcDNA vectors with the reporter plasmid causes minimal luciferase activity, thus providing a low background. As we previously reported (35), expression of wt-Lbc at submaximal levels induced substantial SRF-dependent luciferase reporter activity. Next, α-catulin was co-expressed at increasing doses with a constant submaximal amount of Lbc to test for any effects.
Interestingly, increasing doses of α-catenin caused an increase in Lbc-induced SRF reporter activation up to a doubling of luciferase levels; inhibition was not observed. In contrast, α-catenin expression at the same doses in the absence of Lbc had no significant effect on SRE reporter activity. Immunoblots of total cell lysates showed that Lbc expression levels remained the same in the presence of α-catenin, indicating that increased reporter activity was not due to increased Lbc expression. These results indicate that α-catenin cooperates with Lbc to stimulate the SRE-L reporter but has no activity on its own.

To investigate the cooperative response, a plasmid encoding the Clostridium botulinum C3 transferase, which inactivates Rho (36), was used. As expected, Fig. 8B (graph, bars 3 and 4) shows that C3 expression completely blocked wt-Lbc-induced SRE reporter activation. In addition, C3 expression effectively blocked the combined effect of α-catenin and Lbc expression (bars 5 and 6), indicating that the cooperative effect of α-catenin is Rho dependent. Next, α-catenin and Lbc mutants were used to investigate the domains involved. Expression of a plasmid encoding the CT-Catenin mutant, which lacks the required binding site (Fig. 5), at levels comparable with full-length α-catenin, had no effect (bar 7). Furthermore, CT-Catenin was unable to cooperate with wt-Lbc in SRE induction (bar 8). Reciprocally, ΔCT-Lbc was expressed at a submaximal dose to induce SRF activity at a level comparable with that of wt-Lbc (bar 9). In contrast to co-expression with wt-Lbc, α-catenin was unable to cooperate with ΔCT-Lbc in SRE reporter activation (bar 10), either at the ratio used or at other ratios tested. Immunoblots of cell lysates showed that the Lbc expression level was not altered by α-catenin co-expression, indicating that increased SRE reporter activity was not due to increased Lbc expression. These results indicate that the in vivo cooperative response requires the α-catenin N-terminal and the Lbc C-terminal regions, consistent with the definition of these regions as being the regions of interaction.

α-Catenin Effect on Lbc-induced GTP-Rho Formation in Vivo—To determine whether α-catenin co-expression affects in vivo Rho activation, GTP-Rho pull-down assays were performed to evaluate changes in GTP-Rho levels in vivo. α-Catu-
The cells were transfected with the designated plasmids, and GTP-RhoA pull-down assays using Rhotekin RBD were carried out on a portion of the lysates as described under “Experimental Procedures.” In addition, a portion of the lysate was directly immunobotted for RhoA. The upper panel shows the levels of RBD affinity-purified GTP-RhoA. The lower panel shows the amount of total RhoA present in the cell lysate.

DISCUSSION

Here we report direct association of the Lbc Rho GEF with the α-catenin-related protein α-catenin by three independent means: yeast two-hybrid interaction, direct binding in vitro, and complex formation in mammalian cells. The yeast two-hybrid screen used here to isolate α-catenin did not detect any other interacting cDNAs, in contrast to the report by Rubin et al. (20), who found that the Lbc C-terminal region can bind to certain nuclear hormone receptors by yeast two-hybrid assay. This difference may reflect the different expression levels of these proteins represented in the different cDNA libraries screened.

Our analysis indicates that the α-catenin peptide sequence has the most homology with human αN-catenin at 50% overall homology, closely followed by αE-catenin at 40% overall homology. α-Catenin is a key component of the cadherin-catenin complex that mediates cell-cell adhesion in many vertebrate tissues (37). Adhesion by the transmembrane cadherin receptor is dependent on complex formation of the cadherin cytoplasmic tail with intracellular catenins (α, β, and γ) (37). Although β- and γ-catenin share sequence similarity, α-catenin is a distinct protein. Within this complex, β- and γ-catenin directly bind to the cadherin cytoplasmic tail in a mutually exclusive fashion, and α-catenin is part of this complex through binding to β- or γ-catenin (37). α-Catenin is essential for cadherin adhesive activity and links the adhesion complex to the actin cytoskeleton (37). This occurs via α-catenin binding with several actin-binding proteins including α-actinin (38), vinculin (39), and ZO-1 (40); additionally, α-catenin can itself directly bind to actin filaments (41). Furthermore, α-catenin links to growth signaling pathways (42). α-Catenin expression down-regulates Wnt-induced differentiation (43) and inhibits β-catenin-dependent transcriptional activation (44). In addition, α-catenin acts as a tumor growth suppressor (45) and regulates skin cell proliferation through a mitogen-activated protein kinase-dependent pathway (46). Moreover, the cadherin-catenin complex links to Rho family GTPase signaling pathways. For example, Rho, Rac, and Cdc42 are required for cadherin-mediated adhesion (47, 48). In addition, the Rac/Cdc42 effector IQGAP binds to β-catenin (49), and p120 catenin, a β-catenin family member, inhibits Rho function (50). Thus, our findings linking Lbc Rho GEF with α-catenin are consistent with an emerging theme of links between Rho GTPase pathways and catenins. Nevertheless, our results are novel, because they describe a link between a Rho family pathway and an α-catenin relative rather than the β-catenin family members reported to date.

Both our data as well as the other two published reports on CTNNAL1/α-catenin (29, 30) have identified the same 5’ transcriptional start site. Because an upstream stop codon was not previously detected, our lab attempted to obtain additional α-catenin 5’ cDNA sequence by cDNA library screening and reverse transcription-PCR approaches. Although these approaches did not identify an upstream stop codon in the open reading frame shown in Fig. 1, they did detect α-catenin cDNAs with shorter, alternative 5’ open reading frame sequence encoding an upstream stop codon (results not shown; Toksoz lab), although the functional significance of these cDNA forms remains to be determined. Thus, whereas a more 5’ transcriptional start site cannot be ruled out at this point, the length of the open reading frame shown is closely compatible with the predicted protein size as detected by immunoblotting as shown here.

The α-catenin N-terminal region contains extensive homology with the α-catenin N-terminal region, the latter of which contains binding sites for β-catenin, α-actinin, and vinculin. This raises the possibility that α-catenin may also bind the same or related partners and thus link to signaling/adhesion and/or cytoskeleton complexes. Following the N-terminal region, α-catenin lacks a central core of −110 residues contained in α-catenin; although the precise function of this region in α-catenin is not clear, it may modulate the strength of cell adhesion (51). This is followed by the C-terminal homology block corresponding to α-catenin residues 631–906, which includes the binding sites for the ZO-1 cytoskeletal linker (52), and to actin (41). The corresponding level of homology in this region is less than the N-terminal region, which may reflect a functional divergence. Thus, overall the predicted 734-residue α-catenin polypeptide is smaller than αN-catenin and αE-catenin, because of the absence of the central −110-residue region and the shorter C terminus. The differences in peptide sequence may indicate functional differences between α-catenin and α-catenin. This is supported by our preliminary finding that the Lbc C-terminal region does not interact with αE-catenin as determined by yeast two-hybrid assay (results not shown).

Like α-catenin, α-catenin is a member of the vinculin superfamily (33). Vinculin is present both in cadherin-catenin adhesion complexes and in focal adhesion complexes that mediate cell–extracellular matrix adhesion (52, 53). Both α-catenin and vinculin function as linkers to the actin cytoskeleton. The overall similarity of α-catenin to α-catenin and vinculin suggests that α-catenin likely acts as a cytoskeletal linker protein, although our prediction would be that its precise role is distinct from its relatives. Interestingly, α-CATULIN mRNA appears to be differentially regulated following growth arrest of cells, sug-
suggesting that it may play a role in growth regulation (29), although no function was ascribed. On the basis of these combined findings, it will be important to determine the functional similarities and differences between α-catenin, α-catenin, and vinculin.

The chromosomal mapping of the α-CATULIN/CTNNAL1 gene to 9q31–32 described here is consistent with the previous localization to 9q31.2 (29). Thus, the CTNNAL1 locus is distinct from both αN-catenin (CTNNB2) and αE-catenin (CTNNB1) genes, which have been mapped to human chromosomes 2 and 5, respectively (31, 54). The 9q31–32 region is a frequent site of allelic loss, a hallmark of tumor suppressor genes, in a variety of human carcinomas (discussed in Ref. 29). Co-localization of the CTNNAL1 gene to this region raises the possibility that α-catenin protein may be a putative tumor suppressor implicated in human cancer. Such a possibility would be in keeping with the demonstrated function of its relative, αE-catenin, as a tumor growth suppressor (45) and tumor invasion suppressor (55). Janssens et al. (30) compare the genomic structure of α-CATULIN/CTNNAL1 with that of α-catenin and vinculin genes. They conclude that the α-catenin gene CTNNAL1 has a closer evolutionary relationship with the α-catenin gene CTNNB1 but not at all to the vinculin gene, suggesting a more closely related function to the former. Thus, it will be of interest to determine whether α-catenin, like α-catenin, is implicated in human malignancy.

Our results identify the predicted ~82-kDa α-catenin protein and determine α-catenin expression in all human epithelial cell lines tested. The cadherin-catenin complex acts as an invasion suppressor, and loss of cadherin and/or α-catenin expression correlates with increased malignancy in carcinomas (55). Although several invasive human carcinoma cell lines show loss of E-cadherin and/or α-catenin (56, 57), none of the cell lines tested here are reported to lack cadherin and α-catenin, consistent with the report that the CaCo-2, HCT116, and HT29 colorectal carcinoma and MCF-7 breast carcinoma cell lines tested are noninvasive (56). Thus, although none of these noninvasive cell lines exhibit defective α-catenin protein expression, it is conceivable that a wider screen of human carcinomas/cell lines, which includes metastatic and invasive samples, may reveal altered α-catenin expression.

In keeping with being physiologic binding partners, endogenous wt-Lbc and α-catenin are co-expressed in all cell lines tested; furthermore, they co-fractionate to both a membrane-rich fraction and a soluble fraction. The presence of wt-Lbc in these two fractions may reflect a membrane-proximal pool that is available for activation and/or already active and a reserve, cytosolic pool, in keeping with its substrate Rho (34). Comparison of α-catenin with α-catenin reveals a similar, although not identical, subcellular localization; α-catenin is exclusively in the membrane-rich fraction, consistent with its being part of the membrane-proximal cadherin-catenin complex (37). In contrast, a soluble α-catenin fraction is detected, which may indicate that α-catenin is part of a distinct complex. These findings are also compatible with the intracellular localization of α-catenin as detected by indirect immunofluorescence.

The required site of interaction within the Lbc C-terminal region maps to the ~253-residue IDR, which contains unique sequence between the PH domain and the ensuing α-helical region. This finding is consistent with our previous report that the Lbc IDR plays a role in regulating Lbc activity (19), although no precise function was previously ascribed. Furthermore, we determined that the required α-catenin interaction site lies within N-terminal residues 34–524. As discussed above, this region has contiguous homology with the α-catenin N terminus, which contains the binding sites for several key partners such as β-catenin and α-actinin. Whether interaction of Lbc with the α-catenin N-terminal region competes with binding of other α-catenin binding partner(s) remains to be determined.

Our results show Lbc and α-catenin complex formation in mammalian cells under conditions where only one partner or both are exogenously expressed; furthermore, this association requires the Lbc C-terminal region. Whereas the complex formation observed in vivo is modest and less than that observed in vitro with recombinant protein domains, this is not unexpected, because the regulated, transient associations formed by signaling proteins in vivo are frequently inefficiently detected by the available methods as used here. Thus, the in vivo Lbc-α-catenin interaction may be regulated by other binding partners for α-catenin, similar to α-catenin, which could potentially limit access to Lbc, and future studies will investigate these and other potential regulatory events.

Investigation of the functional consequence of Lbc and α-catenin interaction in vivo by SRE reporter assay showed that although α-catenin expression alone has no intrinsic activity, co-expression of α-catenin with wt-Lbc causes increased Lbc-induced SRE reporter activation. Because the combined response is effectively blocked by C3 transferase expression, the α-catenin effect is Rho-dependent, thus ruling out any potential Rho-independent effects on SRE by α-catenin. In addition, the lack of cooperation of the negative CTCoat mutant or the ΔCT-Lbc mutant with its respective full-length partner in this assay indicates the requirement for the α-catenin N-terminal and Lbc C-terminal regions for the observed response. The finding that α-catenin and wt-Lbc co-expression causes increased GTP-Rho formation in vivo is consistent with the cooperative response observed in the SRE reporter assay and presents an independent measure of the cooperative effect. Moreover, these data imply that α-catenin lies upstream of Rho.

One possible basis for the observed cooperative effects in vivo may be that α-catenin acts as a scaffold protein for Lbc Rho GEF and facilitates Lbc-induced Rho signals. As discussed above, α-catenin likely has other binding partners and thus may serve to localize Lbc in a multimolecular signaling complex linked to the cytoskeleton. Such a role would be analogous to its relative, α-catenin, which acts as a scaffold for a signaling and adhesion protein complex that is linked to the actin cytoskeleton (58). Analogously, Dbl, a Cdc42/Rho GEF, interacts with the radixin adhesion component, which may serve to localize Dbl at the actin cytoskeleton-plasma membrane interface (59). On this basis, α-catenin binding may facilitate Lbc to link with additional components that may regulate Lbc in vivo. For example, our findings and other reports indicate that Lbc may physically associate with and receive signal inputs from activated heterotrimeric Gaq (21, 35, 60) and Gαq (35) subunits, and it is conceivable that α-catenin may act as a scaffold for such a signaling complex. This model would be analogous to regulation of other Rho family GEFs; for example, Cdc42, a yeast Cdc42 GEF, forms a protein complex with the scaffolding protein Far1 and Gβγ subunits (61). In addition, it is also conceivable that α-catenin binding may contribute to stimulation of intrinsic Lbc GEF activity. Furthermore, α-catenin may serve to localize an Lbc-Rho complex in proximity to particular downstream Rho effectors. Examples of this include p190 RhoGEF, which binds to the 14-3-3 scaffold protein (62), and to the c-Jun N-terminal kinase-interacting protein-1, Jip-1 (63), each of which likely serve to interface varying functions of p190 Rho GEF. Similarly, binding of the TIAM-1 and Ras GRF Rac GEFs to the Jip-2 scaffold modulates Rac effector specificity (64). In summary, our findings reveal that the α-catenin relative α-catenin, of previously unknown function, can associ-
ate with the Lbc Rho GEF and enhance Rho signaling in vivo. Elucidation of the functional relationship of α-catenin to its relatives and its potential role in cytoskeletal responses, cell adhesion, and malignancy will further define this new α-catenin-related protein.

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