Distinct regions of the cadherin cytoplasmic domain are essential for functional interaction with Go12 and β-catenin*

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Running title: Go12 and β-catenin bind distinct regions of cadherin
SUMMARY

Heterotrimeric G proteins of the G12 subfamily mediate cellular signals leading to events such as cytoskeletal rearrangements, cell proliferation, and oncogenic transformation.

Several recent studies have revealed direct effector proteins through which G12 proteins may transmit signals leading to various cellular responses. Our laboratory recently demonstrated that G12 proteins specifically interact with the cytoplasmic domains of several members of the cadherin family of cell adhesion molecules (Meigs, T. E., Fields, T. A., McKee, D. D., and Casey, P. J. (2001) Proc. Natl. Acad. Sci. USA 98, 519-524).

This interaction causes β-catenin to release from cadherin and relocalize to the cytoplasm and nucleus, where it participates in transcriptional activation. Here we report that two distinct regions of the epithelial cadherin (E-cadherin) tail are required for interaction with β-catenin and Gα12, respectively. Deletion of an acidic, 19 amino acid region of E-cadherin abolishes its ability to bind β-catenin in vitro, to inhibit β-catenin-mediated transactivation or to stabilize β-catenin, causes subcellular mislocalization of β-catenin, and disrupts cadherin-mediated cell adhesion. On the other hand, deletion of a distinct 11 amino acid region of E-cadherin dramatically attenuates interaction with Gα12; furthermore, Gα12 is ineffective in stimulating β-catenin release from an E-cadherin cytoplasmic domain lacking this putative G12-binding region. These findings indicate that Gα12 and β-catenin do not compete for the same binding site on cadherin, and
provide molecular targets for selectively disrupting the interaction of these proteins with cadherin.

INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G proteins) regulate cellular physiology by transducing extracellular signals to intracellular effector molecules (1). G proteins are composed of two functional signaling units, a nucleotide-binding \( \alpha \) subunit and a tightly coupled \( \beta\gamma \) subunit dimer. Based on the primary sequence of the \( \alpha \) subunits, G proteins have been classified into four subfamilies: Gs, Gi, Gq, and G12 (2). The G12 subfamily, consisting of G\( \alpha \)12 and G\( \alpha \)13, has been reported to mediate a variety of cellular processes, including regulation of Rho-dependent cytoskeletal rearrangements (3) and Na\(^+\)/H\(^+\) antiporter activity (4), activation of c-Jun N-terminal kinase (5), stimulation of phospholipase D activity (6), regulation of membrane depolarization (7), and conformational activation of radixin (8). Additionally, G12 proteins have been implicated in pathways controlling cell proliferation and early developmental events, as well as oncogenesis (9-12). While Rho-dependent responses to G12 signaling have been shown to involve specific guanine nucleotide exchange factors that directly interact with G12 proteins (13-16), signaling pathways involved in other G12-mediated responses, particularly cellular transformation, are not well understood.
Recently, our laboratory reported a specific interaction between G12 subfamily proteins and the cytoplasmic domain of several cadherins (17), which are cell surface proteins involved in calcium-dependent cell-cell adhesion (18). The G12/cadherin interaction was shown to cause β-catenin, a multifunctional protein involved in both cell adhesion and transcriptional activation (19), to release from its cadherin-bound state and relocalize to the cytoplasm and nucleus. Furthermore, in cells deficient in β-catenin degradation, expression of Gα12 or Gα13 was found to up-regulate β-catenin-mediated transcriptional activation (17). These findings have provided a mechanism that may explain the role of G12 proteins in developmental processes as well as malignant transformation.

Identifying the regions of the cadherin tail necessary for interaction with both Gα12 and β-catenin is critical for understanding the mechanism of Gα12-triggered release of β-catenin, and for developing reagents to dissect the biological importance of the G12/cadherin interaction. In the present study, we undertook a systematic analysis to identify the regions of E-cadherin that are required for interaction with G12, and also expanded upon earlier studies by other researchers in which the region of cadherin critical for interaction with β-catenin was mapped. Our work demonstrates that distinct regions of the cadherin tail are required for interaction with G12 and with β-catenin, and
provides experimental systems that should allow dissection of the roles of these two key regulatory molecules in cadherin-mediated biological processes.

**EXPERIMENTAL PROCEDURES**

*Miscellaneous Materials and Methods* - The cDNAs for Go12Q229L and GozQ205L were a gift of Henry Bourne (University of California, San Francisco). The plasmid pXEH2, encoding the full-length cDNA for human E-cadherin, was a gift of Yutaka Shimoyama (National Okura Hospital, Okura, Setagaya-ku, Tokyo, Japan). The Go12 baculovirus construct was provided by Alfred Gilman (University of Texas Southwestern Medical Center, Dallas). Baculovirus stock used for production of His-tagged β-catenin was from Barry Gumbiner (Memorial Sloan Kettering Cancer Center). Anti-Go12 antibody was purchased from Santa Cruz Biotechnology, anti-Goz antibody has been previously described (20), anti-E-cadherin and anti-β-catenin antibodies were purchased from Zymed (S. San Francisco, CA), and anti-green fluorescent protein (GFP) antibody was purchased from Clontech (Palo Alto, CA). K562 human chronic myelogenous leukemia cells and SW480 human colorectal carcinoma cells were from American Type Culture Collection (Manassas, VA). L mouse fibroblast cells were provided by Karl Willert (Stanford University). Protein concentrations were determined by the method of Bradford (21) or by staining with Coomassie blue using bovine serum albumin standards.
Production of E-cadherin Mutants – cDNA corresponding to the 98 C-terminal amino acids of human E-cadherin was amplified by polymerase chain reaction (PCR). Deletion mutants within the E-cadherin cytoplasmic domain were generated by a two-step PCR procedure using primers corresponding to the N- and C-terminal ends of the domain, that introduced BamHI and EcoRI restriction sites, respectively. For each mutant, these primers were used, along with internal primers flanking the region to be deleted, in two separate PCR reactions to amplify the N- and C-terminal regions of cDNA; products from the two reactions were then mixed and subjected to a second round of PCR using only the end primers. This reaction effectively “sewed” the N- and C-terminal regions together, producing constructs encoding the C-terminal domain of E-cadherin with the desired regions deleted. Constructs were then subcloned into pGEX-2T (Amersham Pharmacia, Piscataway, NJ) for production as glutathione-S-transferase (GST) fusion proteins. Selected constructs were also subcloned into pEGFP-C (Clontech, Palo Alto, CA) for generation of fusions to GFP.

Full-length E-cadΔβ-cat (full-length E-cadherin lacking residues 819-837) was produced by first subcloning the full open reading frame of E-cadherin from pXEH2 into pcDNA1.1/CAT (which lacks a BspHI site), producing the plasmid E-cad-pcDNA. The region of E-cadΔβ-cat containing the deleted residues was then excised from the corresponding GST construct (described above) via digestion with Xho I and BspHI, and subcloned into E-cad-pcDNA that had been digested in the identical manner. Finally, the
open reading frame encoding the full-length version of E-cadΔβ-cat was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) using the XbaI and EcoRV sites present in both the pcDNA1.1/CAT and pcDNA3.1 (+) vectors. Full-length wild-type E-cadherin was subcloned into pcDNA3.1(+) in a similar manner. All constructs were verified by sequencing.

*Production of Gα12 and β-catenin Protein* – Recombinant Gα12 protein was produced in Sf9 cells as previously described (22). His-tagged β-catenin, produced in Sf9 cells and purified as previously described (23), was a generous gift from Tim Fields (this institution).

*In Vitro Protein-Binding Studies* - GST-fusion proteins were produced in *Escherichia coli* strain BL21-DE3 and purified from cell lysates using glutathione Sepharose 4B (Amersham Pharmacia). Bound GST-fusion protein was washed with buffer containing 10 mM HEPES (pH 8.0), 1 mM DTT, and 150 mM NaCl and then stored in the same buffer.

Binding of Gα12 to GST-E-cadherin or GST-E-cadherin mutants was evaluated as described (17). Briefly, 350 ng purified Gα12 was incubated in buffer containing 10 µM GTPγS for 2h at 30 °C, the reaction was divided and then incubated with approximately 1 µg of each glutathione sepharose-bound GST-fusion protein. Reactions were incubated with gentle agitation for 2 h at 4 °C, followed by pelleting of glutathione sepharose and
extensive washing. Bound Gα12 was eluted by heating for 10 min at 70 °C in Laemmli sample buffer, subjected to SDS-PAGE, and subsequently analyzed by immunoblotting with 200 ng/ml Gα12 primary antibody followed by 200 ng/ml of horseradish peroxidase-conjugated anti-rabbit secondary antibody. Binding of β-catenin (1 µg per reaction) to GST-fusion proteins was evaluated in the same manner, except that the nucleotide loading step was omitted. β-catenin primary antibody was used at a concentration of 250 ng/ml followed by horseradish peroxidase-conjugated anti-mouse secondary antibody at a concentration of 200 ng/ml.

Cell Culture – SW480 cells were grown in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (FBS). L cells, stably expressing full-length E-cadherin or the corresponding E-cadΔβ-cat, were selected in the same media supplemented with 1 mg/ml Geneticin® (Invitrogen), and clonal populations were isolated and maintained in the same medium containing 500 µg/ml Geneticin®. K562 cells were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% FBS and 10 mM HEPES (pH 7.5). Transfections were performed using Lipofectamine™ (Invitrogen) according to the manufacturer’s protocol unless otherwise noted. For stable transfection of K562 cells, approximately 10 x 10⁶ cells were washed in RPMI 1640 medium to remove serum, combined with 30 µg DNA encoding either full-length E-cadherin or the corresponding E-cadΔβ-cat, and subjected
to electroporation (340 V, 10 msec, 1 pulse, using a T820 square-wave electroporator [BTX, San Diego, CA]), and then plated in fresh medium. After 48 h, cells were seeded at varying dilutions into 96-well plates in the presence of 1 mg/ml Geneticin®. After two weeks, plates with growth in <20% of wells were chosen, and wells were expanded and maintained in 500 µg/ml Geneticin®.

Transcriptional Activation Assays – SW480 cells were transfected with the T cell factor (TCF) reporter construct TOPFLASH (Upstate Biotechnology, Lake Placid, NY), the control reporter construct pRL-TK (Promega, Madison, WI), and additional plasmids as indicated. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS), lysed in Passive Lysis Buffer (Promega), cleared by centrifugation, and assayed for protein. Lysates were analyzed using the Dual-Luciferase® Reporter Assay System (Promega), first for firefly (TOPFLASH) luciferase activity, followed by quenching and immediate measurement of Renilla (pRL-TK) luciferase activity. Luminometry was performed using either a Lumat LB-9501 (Berthold, Nashua, NH) or a TD-20/20 (Turner Designs, Sunnyvale, CA) luminometer.

Immunofluorescence Microscopy – L cells stably expressing full-length or mutant E-cadherin were grown on glass cover slips, washed with PBS, fixed in 4% paraformaldehyde, and incubated in blocking buffer (PBS containing 10% FBS and 0.2% saponin). Cover slips were incubated with 2.5 – 5 µg/ml primary antibody in blocking buffer, followed by three washes in PBS containing 10% FBS and subsequent incubation
with 7 – 14 µg/ml of fluorescein isothiocyanate-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) in blocking buffer. β-Catenin localization was observed using an LSM-410 laser scanning confocal microscope with a 63X oil-immersion objective (Zeiss, Thornwood, NY).

RESULTS

Identification of Regions of the Cadherin Cytoplasmic Domain that Interact with Gα12 and β-catenin – A series of eight deletion mutants within the E-cadherin cytoplasmic domain, with each deleted region being defined by the primary sequence as proline-rich, serine-rich, acidic, basic, or mixed-charged (Fig. 1), were constructed as fusions to GST. The fusion proteins were produced in a bacterial expression system and the purified proteins evaluated in a cell-free system for their ability to interact with purified Gα12 and with purified β-catenin (Fig. 2). The wild-type E-cadherin cytoplasmic tail interacted with activated (GTPγS-loaded) Gα12 and with β-catenin (Fig. 2C), as did 6 of the 8 deletion mutants (data not shown). Deletion F, which lacks a highly charged region of 11 amino acids (Fig. 2A), interacted poorly with Gα12, yet retained the ability to bind β-catenin (Fig. 2C). Based on these results, Deletion F will subsequently be referred to as E-cadΔG12 (Fig. 2A). Conversely, Deletion D, which lacks a 19 amino acid stretch containing 5 acidic residues (Fig. 2A), was completely
unable to interact with β-catenin, but maintained effective binding to Gα12 (Fig. 2C).

Thus, Deletion D will subsequently be referred to as E-cadΔβ-cat (Fig. 2A).

Unexpectedly, Deletion E, which lacks a serine-rich region (Fig. 1) previously implicated as essential for β-catenin binding (24, 25), bound to both β-catenin and Gα12 (Fig. 2C); this finding will be discussed further below. The ability of each deletion mutant to bind at least one of the two proteins (Gα12 or β-catenin) diminishes the likelihood that these mutations produce misfolded proteins with a global loss of protein binding capacity. These data demonstrate that distinct regions of the cadherin cytoplasmic domain are required for interaction with Gα12 and β-catenin.

_E-cadΔβ-cat is Unable to Mediate Biological Functions of Cadherin that Require Interaction with β-catenin_ — Expression of the cytoplasmic domain of cadherins has been shown to attenuate β-catenin-mediated transcriptional activation, presumably by sequestering β-catenin and preventing its interaction with target transcription factors such as TCF or lymphoid enhancer factor (26). To determine whether the lack of interaction between β-catenin and E-cadΔβ-cat translates to functional changes within a cellular context, we fused the E-cadΔβ-cat construct to GFP, expressed this fusion protein in SW480 cells along with the TOPFLASH reporter plasmid, and measured β-catenin-mediated activation of the reporter gene. SW480 cells express a truncated form of the adenomatous polyposis coli protein that is unable to promote β-catenin degradation; this
results in high levels of β-catenin and a robust β-catenin-mediated transactivation
response in these cells. Consistent with previous results (17, 26), we found that
expression of a fusion between GFP and the wild-type E-cadherin tail significantly
attenuated β-catenin-mediated transcriptional activation (Fig. 3A). However, the GFP-E-
cadΔβ-cat fusion protein was without effect (Fig. 3A), indicating that deletion of the
acidic region of the E-cadherin tail abolishes its ability to bind β-catenin in a cellular
context.

Formation of β-catenin/cadherin complexes has been demonstrated to stabilize β-
catenin in cells by preventing its interaction with components required for its degradation
(27-29). Thus, we examined the ability of both wild-type E-cadherin and E-cadΔβ-cat to
protect β-catenin from degradation. Full-length E-cadherin or full-length E-cadΔβ-cat
was stably expressed in K562 cells, which lack endogenous cadherins (30), and lysates
from these cells were analyzed by immunoblotting for E-cadherin and β-catenin. While
mock-transfected K562 cells had very low levels of β-catenin (Fig. 3B), cells stably
expressing wild-type E-cadherin exhibited a dramatic stabilization of endogenous β-
catenin (Fig. 3B). In contrast, essentially no stabilization of β-catenin was observed in
cells stably expressing full-length E-cadΔβ-cat (Fig. 3B).

In most cell types expressing cadherins, β-catenin is localized primarily at the cell
periphery, while in cells lacking cadherins, β-catenin is generally cytoplasmic or nuclear
(31, 32). We reasoned that a form of cadherin unable to bind β-catenin, such as E-cadΔβ-cat, should fail to promote peripheral localization of β-catenin. To test this hypothesis, we first examined the localization of β-catenin in L cells, which like K562 cells, lack endogenous cadherins (30). L cells are adherent and were used in place of K562 suspension cells to better facilitate growth on cover slips. Indirect immunofluorescence performed on these cells revealed a primarily cytoplasmic and nuclear staining pattern for endogenous β-catenin (Fig. 4A). As expected, in L cells stably expressing wild-type E-cadherin, β-catenin was now found predominantly at the cell periphery (Fig. 4B). However, no relocalization of β-catenin was observed in L cells stably expressing E-cadΔβ-cat (Fig. 4C); the staining pattern instead was similar to that observed in control L cells. Pre-incubation of primary antibody with purified β-catenin ablated the immunofluorescence signal, while pre-incubation with the irrelevant protein farnesyltransferase did not (data not shown).

Having demonstrated that E-cadΔβ-cat is unable to functionally interact with β-catenin in vitro or in cells, we next evaluated the impact of loss of β-catenin binding on cadherin function. A primary biological function of cadherins is to mediate cell-cell adhesion through homophilic interaction between cadherins on adjacent cells. Although the extracellular domain of cadherins is directly involved in adhesion, cadherins lacking the cytoplasmic domain are unable to promote cell-cell interaction (33). To evaluate the
importance of the region of E-cadherin we defined as essential for β-catenin binding for cell adhesion, we utilized K562 cells, which do not normally exhibit cell-cell adhesion (30). Stable expression of wild-type E-cadherin in these cells resulted in the formation of large aggregates of cells (Fig. 5B). However, cells stably expressing equivalent levels (see Fig. 5D) of E-cadΔβ-cat showed no such aggregation (Fig. 5C), and were indistinguishable from control K562 cells (see Fig. 5A). Taken together, these data clearly demonstrate that the 19 amino acid region deleted in E-cadΔβ-cat is essential for both β-catenin binding and for cadherin function. The absence of this domain abolishes the ability of the cadherin tail to inhibit β-catenin-mediated transactivation, eliminates cadherin’s ability to localize and stabilize β-catenin, and disrupts cadherin-mediated cell adhesion.

_E-cadΔG12 is Impaired in Gα12-mediated Release of β-catenin_ – The inhibition of β-catenin-mediated transactivation resulting from expression of the GFP-E-cadherin tail (see Fig. 3A) can be attenuated by co-expression of mutationally-activated Gα12; this effect is thought to be due to Gα12 disrupting the interaction between β-catenin and the E-cadherin tail and thus freeing β-catenin to function as a transcriptional co-activator (17). Hence, we hypothesized that the G12 binding region of cadherin identified by deletion analysis (see Fig. 2) should be required for Gα12 to elicit dissociation of β-catenin from the E-cadherin tail. To test this hypothesis, the E-cadΔG12 cytoplasmic tail
was fused to GFP and this construct was transfected into SW480 cells along with the
TOPFLASH reporter plasmid and different mutationally-activated G proteins. Consistent
with our previous study (17), expression of activated G\(\alpha\)12 resulted in a significant
increase in TOPFLASH reporter activity in cells expressing a GFP-wild-type E-cadherin
tail fusion protein (Fig. 6). However, activated G\(\alpha\)12 was much less effective in
stimulating \(\beta\)-catenin-mediated transactivation in cells expressing GFP-E-cad\(\Delta\)G12 (Fig.
6). Expression of activated G\(\alpha\)z did not stimulate \(\beta\)-catenin-mediated reporter activation
in the presence of either form of cadherin (Fig. 6). Our findings indicate that G\(\alpha\)12
indeed requires the 11 amino acid region deleted in the E-cad\(\Delta\)G12 construct in order to
effectively trigger \(\beta\)-catenin dissociation from cadherin.

**DISCUSSION**

Inappropriate accumulation of \(\beta\)-catenin is believed to be a key event in the
development of most colorectal tumors (34), as well as a variety of other human cancers
(35). Additionally, the majority of epithelial tumors lose E-cadherin function as they
progress to a malignant phenotype (36). Our recent finding that G12 family proteins
interact with the cytoplasmic domain of cadherins in a fashion that stimulates the release
of \(\beta\)-catenin suggests that G12 proteins may be involved in \(\beta\)-catenin-mediated
tumorigenesis (17). In the present study, we have demonstrated that distinct domains of
the E-cadherin tail are required for interaction with Gα12 and with β-catenin. The mutant E-cadherin molecules generated in this study should be of great utility for dissecting the roles of Gα12 and β-catenin in cadherin-mediated functions.

Since the discovery that the cytoplasmic domain of E-cadherin is required for cell-cell adhesion (37) and the identification of β-catenin as a protein associated with the cadherin cytoplasmic domain (38), numerous studies have analyzed the cadherin cytoplasmic domain to identify the region to which β-catenin binds. Early studies demonstrated that deletion of the 72 C-terminal residues of E-cadherin abolished interaction with β-catenin (37-40). Stappert and Kemler later demonstrated that the mutation of eight serines to alanines between residues 838 and 853 of E-cadherin abolished interaction with β-catenin, as determined by co-immunoprecipitation (24). Conversely, a study by Jou et al. presented data indicating that deletion of a 25 residue acidic region, corresponding to amino acids 815-839 of human E-cadherin, eliminated the ability of β-catenin to interact with E-cadherin in a yeast two-hybrid system (41). Near the completion of our study, the crystal structure of the murine β-catenin/E-cadherin complex was reported (42). The crystal structure indicates that E-cadherin binds to β-catenin as an extended polypeptide with as many as 100 residues of the E-cadherin tail interacting with β-catenin (42). However, the authors concluded that 18 residues of the cadherin cytoplasmic tail form a “core” binding region that is essential for interaction with β-catenin, while the other
residues involved in the extended interface serve to modulate the affinity of the interaction between the two proteins. The core domain identified in the crystal structure corresponds to amino acids 821-838 of human E-cadherin, which is nearly identical to the 19 residue acidic region (amino acids 819-837) we identified as essential for interaction with β-catenin in the current study. Thus, our results support the conclusion from the structural study that the core β-catenin-binding region of cadherin is essential for the biologically functional interaction between cadherin and β-catenin.

The β-catenin/E-cadherin crystal structure was solved with both unphosphorylated and phosphorylated E-cadherin. The authors of the study found that the serine-rich region of the E-cadherin tail, corresponding to residues 838-853 of human E-cadherin, interacted with β-catenin only when phosphorylated. This finding provides an explanation for earlier reports demonstrating that phosphorylation of serine residues in this region of cadherin increases the affinity of its interaction with β-catenin (25, 43). The structural data suggests that, while the serine-rich region is involved in modulating the affinity of the interaction between the two proteins, it is not essential for the interaction to occur. Our finding that E-cadherin missing this precise serine-rich region (Deletion E, see Fig. 1) still binds effectively to β-catenin (Fig. 2C) supports this conclusion.

Another very recent study reported identification of a minimal region of the Drosophila E-cadherin cytoplasmic tail that was able to interact with β-catenin (44).
This analysis found that a polypeptide encoding as few as 23 residues of the *Drosophila*
E-cadherin tail could bind to β-catenin in a yeast two-hybrid system; this 23 residue
region corresponds to amino acids 828-850 of the human E-cadherin tail. This region
overlaps substantially with the domains identified as essential for β-catenin binding in
both the present study and the structural study noted above, although only about half the
residues in this region are conserved from fly to mammals.

The present study validates the G12-cadherin interaction as a key event leading to
destabilization of the cadherin/β-catenin complex in cells. Identification of residues 854-864 of human E-cadherin as critical for its interaction with Gα12 allowed testing of the
hypothesis that this interaction leads to release of β-catenin from the E-cadherin tail (see
Fig. 6). In the β-catenin/E-cadherin crystal structure, these residues of E-cadherin are
seen to form part of a cap that protects hydrophobic residues of the first armadillo repeat
region of β-catenin from solvent exposure (42). Earlier studies have reported that
deletion of the cap region reduces interaction of E-cadherin with β-catenin (45, 24).
However, since robust binding to β-catenin was still observed when the N- or C-terminal
half of the E-cadherin cap region was deleted (see “Results”), it is unlikely that disruption
of the interaction between the hydrophobic cap of cadherin and β-catenin could account
for the ability of Gα12 to stimulate β-catenin release from cadherin. Rather, we would
speculate that binding of Gα12 to cadherin disrupts additional regions involved in
formation of the β-catenin/cadherin complex, likely including interactions involving the acidic core region of cadherin that is essential for β-catenin binding.

An intriguing study that may shed light on additional mechanisms by which G12 proteins can affect β-catenin signaling was recently reported by Fujino et al (46). They demonstrated that stimulation of FPβ prostanoid receptors, which are G protein-coupled receptors thought to signal at least in part through G12 and G13 proteins (47), leads to activation of a β-catenin/TCF signaling pathway, thereby providing additional evidence of a link between heterotrimeric G proteins and β-catenin signaling.

The specific mechanism by which Gα12 stimulates β-catenin release is not yet apparent, but our data clearly demonstrate that these two proteins bind to distinct regions of the cadherin tail. Therefore, a simple competition model cannot adequately explain Gα12-stimulated β-catenin release, and the possibility exists that additional proteins may be involved in this process. We are currently attempting to identify such proteins to investigate whether G12 may serve to recruit them to the cadherin/β-catenin complex. These and other studies noted above should result in elucidation of the complex interplay between the G12 and β-catenin signaling pathways.
REFERENCES

1. Hamm, H. E. (1998) *J. Biol. Chem.* **273**, 669-672

2. Strathmann, M. P. and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5582-5586

3. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) *J. Biol. Chem.* **270**, 24631-24634

4. Hooley, R., Yu, C.-Y., Symons, M., and Barber, D. L. (1996) *J. Biol. Chem.* **271**, 6152-6158

5. Vara Prasad, M. V. V. S., Dermott, J. M., Heasley, L. E., and Johnson, G. L. (1995) *J. Biol. Chem.* **270**, 18655-18659

6. Plonk, S. G., Park, S. K., and Exton, J. H. (1998) *J. Biol. Chem.* **273**, 4823-4826

7. Postma, F. R., Jalink, K., Hengeveld, T., Offermanns, S., and Moolenaar, W. H. (2001) *Curr. Biol.* **11**, 121-124

8. Vaiskunaite, R., Adarichev, V., Furthmayr, H., Kozasa, T., Gudkov, A., and Voyno-Yasenetskaya, T. A. (2000) *J. Biol. Chem.* **275**, 26206-26212

9. Offermanns, S., Mancino, V., Revel, J.-P., and Simon, M. I. (1997) *Science* **275**, 533-536

10. Chan, A. M., Fleming, T. P., McGovern, E.S., Chedid, M., Miki, T., and Aaronson, S. A. (1993) *Mol. Cell. Biol.* **13**, 762-768

11. Jiang, H., Wu, D., and Simon, M. I. (1993) *FEBS Lett.* **3**, 319-322

12. Jones, T. L. and Gutkind, J. S. (1998) *Biochemistry* **37**, 3196-3202
13. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G.,
Sternweis, P. C., and Bollag, G. (1998) *Science* **280**, 2112-2114

14. Fukuhara, S., Murga, C., Zohar, M., Igishi, T., and Gutkind, J. S. (1999) *J. Biol. Chem.* **274**, 5868-5879

15. Seasholtz, T. M., Majumdar, M., and Brown, J. H. (1999) *Mol. Pharmacol.* **55**, 949-956

16. Ross, E. M. and Wilkie, T. M. (2000) *Annu. Rev. Biochem.* **69**, 795-827

17. Meigs, T. E., Fields, T. A., McKee, D. D., and Casey, P. J. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 519-524

18. Pötter, E., Bergwitz, C., and Brabant, G. (1999) *Endocr. Rev.* **20**, 207-239

19. Provost, E. and Rimm, D. L. (1999) *Curr. Opin. Cell Biol.* **11**, 567-572

20. Casey, P. J., Fong, H. K., Simon, M. I., and Gilman, A. G. (1990) *J. Biol. Chem.* **265**, 2383-2390

21. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254

22. Kozasa, T. and Gilman, A. G. (1995) *J. Biol. Chem.* **270**, 1734-1741

23. Fagotto, F., Glück, U., and Gumbiner, B. M. (1998) *Curr. Biol.* **8**, 181-190

24. Stappert, J. and Kemler, R. (1994) *Cell Adhes. Comm.* **2**, 319-327

25. Lickert, H., Bauer, A., Kemler, R., and Stappert, J. (2000) *J. Biol. Chem.* **275**, 5090-5095
26. Sadot, E., Simcha, I., Shtutman, M., Ben-Ze’ev, A. and Geiger, B. (1998) Proc. Natl. Acad. Sci. USA 95, 15339-15344

27. Papkoff, J. (1997) J. Biol. Chem. 272, 4536-4543

28. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996) Mol. Cell. Biol. 16, 2128-2134

29. Salomon, D., Sacco, P. A., Roy, S. G., Simcha, I., Johnson, K. R., Wheelock, M. J., and Ben-Ze’ev, A. (1997) J. Cell Biol. 139, 1325-1335

30. Ozawa, M., and Kemler, R. (1998) J. Biol. Chem. 273, 6166-6170

31. Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E., and Gumbiner, B. M. (1992) J. Cell Biol. 118, 681-691

32. Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J., and Nelson, W. J. (1994) J. Cell Biol. 125, 1341-1352

33. Kintner, C. (1992) Cell 69, 225-236

34. Polakis, P. (1999) Curr. Opin. Genet. Dev. 9, 15-21

35. Polakis, P. (2000) Genes Dev. 14, 1837-1851

36. Christofori, G. and Semb, H. (1999) Trends Biochem. Sci. 24, 73-76

37. Nagafuchi, A. and Takeichi, M. (1988) EMBO J. 7, 3679-3684

38. Ozawa, M., Baribault, H., and Kemler, R. (1989) EMBO J. 8, 1711-1717

39. Nagafuchi, A. and Takeichi, M. (1989) Cell Regul. 1, 37-44
40. Ozawa, M., Ringwald, M., and Kemler, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4246-4250

41. Jou, T.-Z., Stewart, D. B., Stappert, J., Nelson, W. J., and Marrs, J. A. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5067-5071

42. Huber, A. H. and Weis, W. I. (2001) *Cell* **105**, 392-402

43. Serres, M., Filhol, O., Lickert, H., Grangeasse, C., Chambaz, E. M., Stappert, J., Vincent, C., and Schmitt, D. (2000) *Exp. Cell Res.* **257**, 255-264

44. Simcha, I., Kirkpatrick, C., Sadot, E., Shtutman, M., Polevoy, G., Geiger, B., Peifer, M., and Ben-Ze’ev, A. (2001) *Mol. Biol. Cell* **12**, 1177-1188

45. Finnemann, S., Mitrik, I., Hess, M., Otto, G., and Wedlich, D. (1997) *J. Biol. Chem.* **272**, 11856-11862

46. Fujino, H. and Regan, J. W. (2001) *J. Biol. Chem.* **276**, 12489-12492

47. Pierce, K. L., Fujino, H., Srinivasan, D., and Regan, J. W. (1999) *J. Biol. Chem.* **274**, 35944-35949

**FOOTNOTES**

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FIGURE LEGENDS

FIG. 1. **Deletion mutants of the E-cadherin C-terminal cytoplasmic region.**

Individual deletion constructs of the human E-cadherin tail were generated as GST-fusions as described in “Experimental Procedures”. The eight constructs prepared for evaluation are designated Deletion A through Deletion H and the residues deleted in each are indicated.

FIG. 2. **G\(\alpha\)12 and \(\beta\)-catenin bind to distinct regions of the E-cadherin cytoplasmic domain.**

_A_. Diagram highlighting regions of E-cadherin cytoplasmic domain absent in Deletions (Del.) D-F. Amino acid sequence is shown for regions deleted in Del. D (E-cad\(\Delta\beta\)-cat) and Del. F (E-cad\(\Delta\)G12). _B_. SDS-PAGE analysis of purified GST-fusion proteins of the human E-cadherin cytoplasmic domain and specified deletion mutants (see “Experimental Procedures”). Bacterially-produced proteins were visualized by Coomassie blue staining; molecular mass standards (in kDa) are indicated at left. Approximately 500 ng of protein was loaded in each lane. _C_. Analysis of the interaction of the E-cadherin cytoplasmic domain and select deletion mutants with G\(\alpha\)12 and with \(\beta\)-catenin. GST-E-Cad fusion proteins were incubated with purified G\(\alpha\)12 (loaded with GTP\(\gamma\)S) or \(\beta\)-catenin protein (see “Experimental Procedures”). Bound proteins were separated by SDS-PAGE and identified by immunoblot analysis using anti-G\(\alpha\)12 or anti-
β-catenin antibody as indicated. Data are representative of six independent experiments.

IB, immunoblot.

FIG. 3. **E-cadΔβ-cat does not inhibit β-catenin-mediated transactivation** or protect β-catenin from degradation.  

A, Effect of deleting the β-catenin interacting region on the ability of E-cadherin fusion proteins to suppress β-catenin-mediated transcriptional activation. SW480 cells were transfected with TOPFLASH, pRL-TK, and the indicated E-cadherin tail constructs fused to GFP (see “Experimental Procedures”). Cell lysates were analyzed for firefly luciferase (TOPFLASH) activity and *Renilla* luciferase (pRL-TK) activity; the latter was used as an internal standard for transfection efficiency. TOPFLASH activity was normalized for pRL-TK activity in each sample and is shown here as a percent of the TOPFLASH activity in cells expressing GFP alone.

Data represent mean ± standard error of the mean of four independent experiments, each performed in duplicate. *Inset*, anti-GFP immunoblot from SW480 cell lysates.  

B, Stabilization of endogenous β-catenin in K562 cells expressing full-length E-cadherin and the variant lacking the putative β-catenin binding region (E-cadΔβ-cat). Stable cell lines were obtained as described in “Experimental Procedures”. Cells were lysed and equivalent amounts of lysate from each separated by SDS-PAGE and subject to immunoblot analysis using either anti-E-cadherin or anti-β-catenin antibody as indicated.
Data are representative of three independent experiments, each performed in duplicate.

IB, immunoblot.

**FIG. 4.** **E-cadΔβ-cat does not bind β-catenin at the cell periphery.** A-C, subcellular localization of endogenous β-catenin, in either control L cells or L cells stably expressing the indicated full-length E-cadherin construct, was analyzed by indirect immunofluorescence for β-catenin (see “Experimental Procedures”). β-Catenin staining was visualized using laser scanning confocal microscopy. D, anti-E-cadherin immunoblot of lysates from stable L cell lines indicating level of E-cadherin or E-cadΔβ-cat expression. Data shown are representative of that obtained in four separate experiments. Scale bar represents 10 µm.

**FIG. 5.** **E-cadΔβ-cat is unable to promote cell-cell adhesion.** K562 cells were stably transfected with either wild-type E-cadherin or E-cadΔβ-cat, and clonal populations were isolated as described in “Experimental Procedures.” A-C, cells were visualized under phase-contrast microscopy using a light microscope with 10X objective and images were captured using a digital camera. D, cells were analyzed for expression of E-cadherin or E-cadΔβ-cat by immunoblotting. Clonal lines that expressed approximately equal amounts of the wild-type and mutant E-cadherin were selected for analysis. Data shown are representative of that obtained in three separate experiments.
FIG. 6. **G12 is ineffective in eliciting β-catenin release from E-cadΔG12.** β-Catenin release from GFP fusions of the E-cadherin or E-cadΔG12 tail was evaluated by measuring β-catenin-mediated reporter activation. SW480 cells were transfected with TOPFLASH, pRL-TK, and the indicated combinations of GFP-E-cadherin tail fusions and mutationally-activated (QL) G protein α subunits. TOPFLASH activity from cell lysates was normalized for pRL-TK activity in each sample and is shown here as percent increase over cells expressing the corresponding GFP-E-cadherin fusion alone. Data represent mean ± standard error of the mean of four independent experiments, each performed in duplicate. These errors for basal luciferase activity of cells expressing only GFP-E-cadherin or GFP-E-cadΔG12 were 11.6% and 5.4%, respectively.

**ABBREVIATIONS**

The abbreviations used are: E-cadherin or E-cad, epithelial cadherin; β-cat, β-catenin; wt, wild-type; TCF, T cell factor; Gα12QL, constitutively-active Q229L variant of G12 protein α-subunit; GαzQL, constitutively-active Q205L variant of Gz protein α-subunit; GST, glutathione-S-transferase; GFP, green fluorescent protein; FBS, fetal bovine serum.
A

GFP adduct:  none  E-Cad  E-CadΔβ-Cat

Luciferase activity (% of control)

B

Cadherin Expressed

none  E-Cad  E-CadΔβ-Cat

IB: E-cad

IB: β-cad
Kaplan et al. Fig. 4

A. Vector

B. E-cad

C. E-cadΔβ-cat

D. E-cadherin Immunoblot

- Vector
- E-cad
- E-cad-Δβ-cat
Kaplan et al. Fig. 5

A: Untransfected
B: E-cad
C: E-cadΔβ-cat
D: E-cadherin Immunoblot

Untransfected  E-cad  E-cad-Δβ-cat
Luciferase activity (% increase over basal)

GFP adduct:
- E-Cad
- E-Cad
- E-CadΔG12
- E-CadΔG12

α-subunit:
- 12QL
- ZQL
- 12QL
- ZQL
FIG. S1. Immunoblot for β-catenin from whole lysate of L cells stably-expressing full-length E-cadherin.
**FIG S2.** Indirect immunofluorescence for β-catenin in L cells stably-expressing full-length E-cadherin. 

**A.** No primary antibody was used. 

**B.** β-Catenin primary antibody was used as described in “Experimental Procedures”. 

**C.** β-Catenin primary antibody was pre-incubated with 100-fold molar excess of recombinant β-catenin. 

**D.** β-Catenin primary antibody was pre-incubated with a 100-fold molar excess of farnesyltransferase. Images were obtained using a light microscope with a fluorescence filter.
**Fig S3. Load reactions.** Immunoblot analysis as shown in Fig. 2C with load reactions added. The load reactions represent 10% of the amount of protein used for each pull down.
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