E-Cadherin Suppresses Cellular Transformation by Inhibiting β-Catenin Signaling in an Adhesion-independent Manner

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Abstract. E-cadherin is a tumor suppressor protein with a well-established role in cell–cell adhesion. Adhesion could contribute to tumor suppression either by physically joining cells or by facilitating other juxtacrine signaling events. Alternatively, E-cadherin tumor suppressor activity could result from binding and antagonizing the nuclear signaling function of β-catenin, a known proto-oncogene. To distinguish between an adhesion- versus a β-catenin signaling–dependent mechanism, chimeric cadherin constructs were expressed in the SW480 colorectal tumor cell line. Expression of wild-type E-cadherin significantly inhibits the growth of this cell line. Growth inhibitory activity is retained by all constructs that have the β-catenin binding region of the cytoplasmic domain but not by E-cadherin constructs that exhibit adhesive activity, but lack the β-catenin binding region. This growth suppression correlates with a reduction in β-catenin/T cell factor (TCF) reporter gene activity. Importantly, direct inhibition of β-catenin/TCF signaling inhibits the growth of SW480 cells, and the growth inhibitory activity of E-cadherin is rescued by constitutively activated forms of TCF. Thus, the growth suppressor activity of E-cadherin is adhesion independent and results from an inhibition of the β-catenin/TCF signaling pathway, suggesting that loss of E-cadherin expression can contribute to upregulation of this pathway in human cancers. E-cadherin–mediated growth suppression was not accompanied by overall depletion of β-catenin from the cytosol and nucleus. This appears to be due to the existence of a large pool of cytosolic β-catenin in SW480 cells that is refractory to both cadherin binding and TCF binding. Thus, a small pool of β-catenin that can bind TCF (i.e., the transcriptionally active pool) can be selectively depleted by E-cadherin expression. The existence of functionally distinct pools of cytosolic β-catenin suggests that there are mechanisms to regulate β-catenin signaling in addition to controlling its level of accumulation.

Key words: E-cadherin • tumor suppressor • adhesion • β-catenin • T cell factor

Introduction

E-cadherin is a calcium-dependent cell–cell adhesion molecule responsible for the major cell adhesion system in epithelia (Takeichi, 1995). Loss of E-cadherin expression or mutations in the E-cadherin gene have been found to be associated with several epithelial cancers and metastases (Birchmeier and Behrens, 1994). Indeed, E-cadherin has been identified as a bona fide tumor suppressor gene for diffuse gastric carcinomas, and loss of E-cadherin expression has been shown to play a causal role in tumor progression using a transgenic mouse model (Perl et al., 1998). Several different functions of E-cadherin could account for its tumor suppressor activity, but the molecular mechanism of tumor suppression has not yet been clearly established for any particular type of tumor.

E-cadherin is a transmembrane protein with an extracellular domain that mediates Ca^{2+}-dependent homophilic binding and a cytoplasmic domain that interacts with the catenin polypeptides α and β (or plakoglobin) and p120[ctn]. This multiprotein complex interacts with the actin cytoskeleton and physically links cells to each other (for review see Gottardi et al., 2001). Beyond forming the actual structure that joins neighboring cells, cadherin-mediated adhesion initiates formation and organization of functionally distinct cell junctions, such as tight and gap junctions, and desmosomes (Gumbiner et al., 1988; Musil et al., 1990; Watabe et al., 1994) and can facilitate juxtacrine or short range signaling, which depends on the close apposition of cells (for review see Fagotto and Gumbiner, 1996).

Although β-catenin is a critical component of the cadherin cell adhesion complex, it also has a well-established role as an essential mediator of the Wnt signal transduction pathway. Wnt/β-catenin signaling mediates many in-

ductive events in development, and upregulation of this pathway also plays a role in tumorigenesis (Wodarz and Nusse, 1998). In the final step in this pathway, β-catenin interacts with lymphocyte enhancer factor (LEF)[1]/T cell factor (TCF)–type transcription factors and activates transcription by providing a transactivation domain (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997; Hsu et al., 1998). The levels and activity of the signaling competent pool of β-catenin in the cytosol and nucleus is controlled by a complex of proteins that includes the adenomatous polyposis coli (APC) tumor suppressor gene product, axin, glycogen synthase kinase-3, and other polypeptides (Korinek et al., 1997; Behrens et al., 1998; Hart et al., 1998; Morin et al., 1997; for review see Seidensticker and Behrens, 2000). Constitutive upregulation of β-catenin levels and concomitant signaling, arising from either loss-of-function mutations in APC or axin or activating mutations in β-catenin, has been implicated in many human cancers.

The fact that β-catenin has two different functions, nuclear signaling and cadherin-mediated adhesion at the plasma membrane, raises the interesting possibility that cadherins could transduce a signal to the nucleus via β-catenin. Changes in cell–cell adhesion have not yet been found to alter β-catenin signaling or its translocation to the nucleus. However, several studies have shown that over-expression of cadherins in model systems can antagonize β-catenin signaling by binding and sequestering it from the nuclear signaling pool (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996). Conversely, experimental reduction of cadherin levels in *Drosophila* can lead to enhanced β-catenin (armadillo) signaling activity (Cox et al., 1996). It is not yet known whether naturally occurring changes in cadherin expression or function affect β-catenin nuclear signaling during normal development or tumor progression.

Elucidating the mechanism of tumor suppression by E-cadherin is complicated by the fact that E-cadherin has been implicated in both early and late stages of tumor progression. Initially it was thought that E-cadherin suppresses tumor progression by inhibiting local invasion and metastasis through increasing cell–cell adhesion because E-cadherin loss correlated with tumor invasiveness (Mareel et al., 1993; Takeichi, 1993), and reintroduction of E-cadherin into invasive cell lines significantly reduced their invasive properties (Frixen et al., 1991; Vleminckx et al., 1991). However, β-catenin can also regulate genes and processes that influence invasive behavior, such as fibronectin, matrilysin, or the epithelial–mesenchymal transition (Crawford et al., 1999; Gradl et al., 1999). More recent studies have revealed that E-cadherin loss may also contribute to early initiation stages of tumorigenesis. Somatic mutations of the E-cadherin gene have been detected in early noninvasive stages of lobular breast and stomach cancers (Vos et al., 1997), and germline mutations in E-cadherin have been found in families with a predisposition to a form of diffuse gastric carcinoma (Guilford et al., 1998). The role of E-cadherin in early stages implicates it in the control of cell growth or proliferation. In this re-

gard, reexpression of E-cadherin has been found to slow the growth rates of a few cell lines (Navarro et al., 1991; Miyaki et al., 1995; St. Croix et al., 1998). Since β-catenin has been also implicated in cell growth control and apoptosis (Ahmed et al., 1998; He et al., 1998; Orford et al., 1999; Tetsu and McCormick, 1999; Zhu and Watt, 1999), it might be expected to play a role in regulating growth by E-cadherin. However, an increase in cell adhesion resulting from expression of functional E-cadherin molecules could also control cell growth properties by altering overall cytoskeletal and junctional organization, or by facilitating juxtacrine signaling via other receptor systems. For example, junctional proteins such as discs large and scribbled have effects on cell proliferation (Woods and Bryant, 1991; Wodarz, 2000), and VE-cadherin has been found to facilitate signaling through VEGF receptors (Carmeliet et al., 1999). Thus, there are multiple ways that the cadherin could possibly mediate tumor suppression.

Therefore, we wished to determine the mechanism by which E-cadherin acts as a tumor suppressor using a colorectal cell line as a model system. This tumor cell type was chosen to analyze the relationship between cell adhesion and β-catenin signaling since the β-catenin signaling pathway is often upregulated in colon cancers. In this study, we asked whether the ability of E-cadherin to bind and antagonize the nuclear signaling activity of β-catenin or mediate cell–cell adhesion and close cell contact is most critical to its tumor suppressor function. We expressed wild-type E-cadherin or various E-cadherin chimeras lacking either adhesive function or β-catenin binding activity in a colorectal cell line expressing little or no endogenous E-cadherin. In vitro assays for cell growth and analysis of β-catenin/TCF signaling were used to assess tumor suppressor and signaling activities of the various constructs.

Materials and Methods

Plasmids

Partial cDNA for human E-cadherin was provided by D. Rimm (Yale University, New Haven, CT) and subcloned into the pcDNA3 mammalian expression vector (Invitrogen). Sequence analysis revealed that the 3′ end of the gene was missing after nucleotide 2644 (according to EMBL/GenBank/DDBJ under accession number L08599). This results in a truncation of the last 35 amino acids of the E-cadherin cytoplasmic domain and, as a result, does not contain the β-catenin binding region, as defined by Stappert and Kemler (1994). The COOH terminus of this truncation mutant (E-cadherin Δ β-catenin) ends at amino acid 844 (NH₃-ASLSSH); the frameshift adds a single histidine residue before a stop codon is introduced. The full-length human E-cadherin was reengineered using RNA from human A431 cells and the RT-PCR method (Primer A, 5′-TGA-CACCCGGGACAACGTTTATTA-3′, and Primer C, 5′-CTAGTCTA-GACCCCTAGTGGTCCTCG-3′) to generate a 425-bp fragment encoding the missing COOH-terminal residues. This fragment was subcloned into the truncated hEcad-Δ 35/pcDNA3 vector to generate full-length hEcad/pcDNA3. For the E-cadherin–α-catenin fusion construct that lacks the β-catenin binding domain in both E-cadherin and α-catenin, cDNA encoding the membrane proximal region of the cadherin cytoplasmic domain was amplified between nucleotides 1760 and 2530 using 5′ (5′-TGAGCACGTGAAGAACAGCACGTACAC-3′) and 3′ (5′-CCCTG-GCCATTTCCAATTTCATCGGGGA-3′) oligonucleotide primers, and the resultant 770-bp fragment was cloned into a *Xenopus* α-catenin cDNA such that amino acid 801 in E-cadherin was fused in frame with amino acid 201 in α-catenin (E-cad-DEIGN/GHRDQ-α-catenin). This fusion region was further subcloned into the E-cad/pcDNA3 plasmid to make the E-cadherin–α-catenin cDNA. For the IL2R/E-cadherin cytoplasmic chimera construct, the cDNA encoding the IL2R-1 α subunit was provided by Su-

san LaFlamme (pCMV-IL2R-1; Albany Medical College, Albany, NY). A PCR fragment encoding the entire cytoplasmic region of the cadherin was obtained using the 5' A and 3' C primers described above and subcloned into IL2R-1/pCMV expression vector. All constructs were verified by DNA sequencing. The human N-cadherin cDNA was provided by J. Hemperly (Becton Dickinson Technologies). The E-cadherin Δ p120^{ctn} cDNA was provided by A. Reynolds (Vanderbilt University, Nashville, TN) (Thoreson et al., 2000). The β-catenin–engrailed repressor fusion construct was provided by U. Mueller and Pierre McCrea (University of Texas, Houston, TX) (Montross et al., 2000). The constitutively activating LEF construct (LEFΔN-β CTA; where the COOH-terminal transactivation domain of β -catenin was fused to LEFΔN) was provided by K. Vleminckx (Vleminckx et al., 1999). The *Xenopus* TCF-3 cDNA was provided by H. Clevers; dominant-negative TCF (NH$_2$-terminal deletion of the β-catenin binding region of TCF-3) and activated TCF-3 (NH$_2$-terminal β-catenin binding region of TCF-3 replaced with the potent transactivation domain, VP16) are described elsewhere (Vonica et al., 2000).

Cell Culture and Stable Transfections

The SW480 human colon carcinoma cell line was obtained from American Type Culture Collection and grown in DME supplemented with 10% FBS, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). Cells were transfected with Lipofectamine reagent (GIBCO BRL), and colonies were selected in G418 (800 μg/ml). Stable clones were first isolated with cloning cylinders, expanded, and screened for protein expression by Western analysis. Positives were then subjected to one round of subcloning by limiting dilution and then examined by immunofluorescence to ensure homogeneous clonal expression. Three independent clones were selected per cadherin construct based solely on clonal expression characteristics.

Cell Growth Assays

Anchorage-independent Growth in Soft Agar. According to standard protocols, 10^4 cells from each cell line were suspended in 2.5 ml of 0.3% agarose medium containing DME + 5% FBS and layered onto a 2.5-ml bed of 0.6% agarose in a 35-mm dish with grids. Plates were incubated ~14 d, and the number of colonies >100 μm was counted.

Growth on Plastic Culture Dishes. 2 × 10^4 cells from each cell line were seeded in triplicate on six-well plates and cultured in DME supplemented with 2% FBS. At each time point, cells were trypsinized to single cell suspension and counted on a Coulter counter set >10-μm diameter.

Colony Formation Assays. For colony formation after transfection with drug selection, an equivalent number of SW480 cells (10^6–5 × 10^6) were transfected without DNA, 2 μg control or E-cadherin plasmid DNAs conferring neomycin drug resistance (pcDNA3neo or E-cadherin pcDNA3-neo), and 10–1000-fold dilutions of various LEF/TCF-expressing plasmids that confer no drug resistance (pCS2$^+$ vectors). The total amount of pcDNA3 and pCS2 vectors was held constant for each transfection by the addition of empty pCS2 vector. After transfection, cells were replated, selected in G418-containing media for ~3 weeks, and the resultant colonies were fixed and stained with crystal violet. Transfections were done in triplicate for each combination of plasmids. For colony formation of drug-selected stables, to confirm that colony formation reflects the anchorage-independent and -dependent growth properties of the stable cell lines characterized in Fig. 2, 500 cells from each cadherin construct–expressing stable cell line were seeded onto a 10-cm dish in DME supplemented with 10% FBS. After ~14 d, cells were fixed, stained, and quantified.

Adhesion Assay

Adhesion assays were performed as described essentially by Brieher et al. (1996), with the exception that a human E-cadherin–Fc-recombinant fusion protein was used as an adhesive substrate.

Glycoprotein Fractionation, Immunofluorescence, and SDS-PAGE/Western Analysis

Crude purification of glycoprotein-bound and -unbound fractions of β-catenin was performed as described essentially in Fagotto et al. (Fagotto et al., 1996). In brief, SW480 stable cell lines were extracted in a non-ionic detergent buffer (1% nonidet, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA) and equal amounts of total protein (20 μg) were incubated for 1 h with 50 μl (~300 μg concanavalin) concanavalin A (ConA)–Sepharose (Sigma-Aldrich). ConA-bound proteins were washed, and equivalent portions of the ConA-bound and -unbound proteins were subjected to standard SDS-PAGE and immunoblot analysis (Laemmli, 1970; Towbin et al., 1979). For experiments where the levels of cadherin constructs, β-catenin, or c-myc proteins were determined, cells were extracted in boiling RIPA buffer (same as above with addition of 2% SDS). All blots were incubated with either the anti–E-cadherin mAb (HECD-1; Zymed Laboratories), anti–N-cadherin mAb (3B9; Zymed Laboratories), anti–c-myc polyclonal antibody (C-19; Santa Cruz Biotechnology, Inc.), anti-myc epitope mAb (9E10), anti-β-catenin (rabbit polyclonal; McCrea et al., 1993), and HRP-conjugated anti–mouse and anti–rabbit secondary antibodies (Bio-Rad Laboratories). Labeled proteins were visualized with ECL (Amersham Pharmacia Biotech). To detect nuclear β-catenin, cells were fixed with methanol and processed according to standard immunofluorescence protocols.

Recombinant Fusion Protein Binding Experiments

To evaluate cadherin- or TCF-binding capacity of β-catenin in SW480 cells, a cell lysate (1% nonidet 40–containing buffer) or 100,000-g detergent-free supernatant (Reinacher-Schick and Gumbiner, 2001) was subjected to consecutive affinity precipitations (30' incubations × 3) with a glutathione S-transferase (GST) fusion protein containing the cytoplasmic domain of C-cadherin (Yap et al., 1998) or the β-catenin binding region of *Xenopus* TCF-3. β-catenin that was not depleted by these incubations was precipitated with TCA, and GST–cadherin or GST–TCF-bound and -unbound fractions were subjected to SDS-PAGE analysis.

β-Catenin/TCF Gene Reporter Assay

2.5–5 × 10^5 cells from each stable cell line were seeded in triplicate and transfected with 1 μg of the LEF/TCF luciferase reporter plasmid, TOP-FLASH (gift of H. Clevers, University of Utrecht, Netherlands), and 1 μg of a β-galactosidase reporter gene to control for transfection efficiency. After 36 h, luciferase activities were measured from cell lysates using a luciferase assay reagent (Promega). β-Galactosidase activities were determined by standard methods (Promega). Results are expressed as light units normalized to β-galactosidase activity.

Results

E-Cadherin Exhibits Growth Suppressor Activity in the SW480 Cell Line Via the Cytoplasmic Domain

The SW480 human colon carcinoma cell line expresses low, or virtually undetectable, amounts of E-cadherin. It also carries a mutation in the APC tumor suppressor gene product, resulting in sustained upregulation of the β-catenin/TCF signaling pathway (Korinek et al., 1997; Morin et al., 1997). The ability of cells to exhibit anchorage-independent growth is considered a hallmark of neoplastic transformation. Therefore, we examined the capacity of E-cadherin and the various constructs to inhibit growth in soft agar, a commonly used assay for anchorage-independent growth. Stable expression of a wild-type E-cadherin was able to inhibit the growth of this cell line in three independently derived clones (see Fig. 2, A and B). This growth suppression occurs at physiological levels of cadherin expression since the levels of E-cadherin in these clones were less than the levels detected in other colon-derived cell lines (i.e., HT29 and HCT116; data not shown). The role of E-cadherin in the suppression of cell invasiveness could not be evaluated because the SW480 cell line does not exhibit significant invasive activity using in vitro assays (de Both et al., 1999; data not shown).

Constructs were generated to evaluate the roles of adhesive function or β-catenin binding in growth suppression. Two E-cadherin constructs were used to restore intercellular adhesion without binding to β-catenin (Fig. 1): E-cadherin fused directly to α-catenin (E-cadherin–α-catenin)
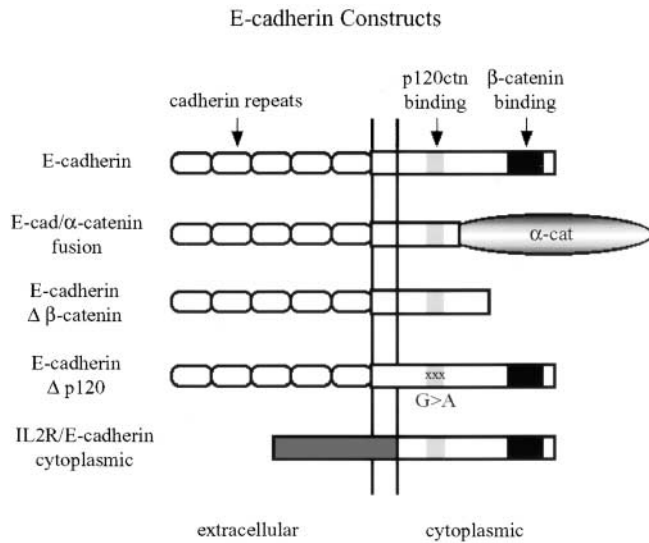
E-cadherin Constructs

Figure 1. Schematic diagram of E-cadherin constructs used in this study. Wild-type E-cadherin (top) is shown. The next two constructs (middle) were designed to mediate adhesion without interacting with β-catenin: E-cadherin–α-catenin fusion construct joins the extracellular and membrane proximal (p120[ctn] binding) region of E-cadherin directly with α-catenin; the E-cadherin Δ β-catenin construct is truncated before the β-catenin binding region. The E-cadherin Δ p120[ctn] construct contains 3 Gly > Ala point mutations and therefore cannot interact with p120[ctn]. The IL2R/E-cadherin cytoplasmic chimera fuses the extracellular and transmembrane domains of the interleukin-2 receptor α subunit to the cytoplasmic domain of E-cadherin. This construct can bind β-catenin but cannot engage in homophilic adhesive activity.

and an E-cadherin with a deletion of the β-catenin binding region (E-cadherin Δ β-catenin). The E-cadherin–α-catenin fusion links the membrane proximal region of the cadherin cytoplasmic domain directly to amino acid 201 of α-catenin, deleting the β-catenin binding regions of both proteins. A similar chimera was found to mediate strong adhesion when expressed in fibroblasts (Nagafuchi et al., 1994). Similarly, cadherin constructs that lack the β-catenin binding region have been shown to exhibit basic adhesive activity in several cell systems (Navarro et al., 1995; Yap et al., 1998). For a protein that binds β-catenin but does not mediate adhesion, the cytoplasmic domain of E-cadherin was fused to the extracellular domain of the interleukin-2 receptor α subunit (IL2R). In fact, similar protein constructs have been found to act as dominant-negative inhibitors of adhesion (Kintner, 1992; Broders and Thiery, 1995; Lee and Gumbiner, 1995).

cDNAs encoding the various constructs in Fig. 1 were transfected into the SW480 cell line, and cell clones were selected on the basis of homogenous expression throughout the population. Three independent clones were selected for each construct to ensure that any observed effects were not due to phenotypic variability inherent to genetically unstable human cancer cell lines. As stated previously, the wild-type cadherin significantly inhibited growth in soft agar (Fig. 2 A). This growth inhibitory activity maps largely to the cytoplasmic domain of the cadherin, since an IL2R/E-cadherin cytoplasmic domain chimera exhibited the strongest inhibition, whereas little or
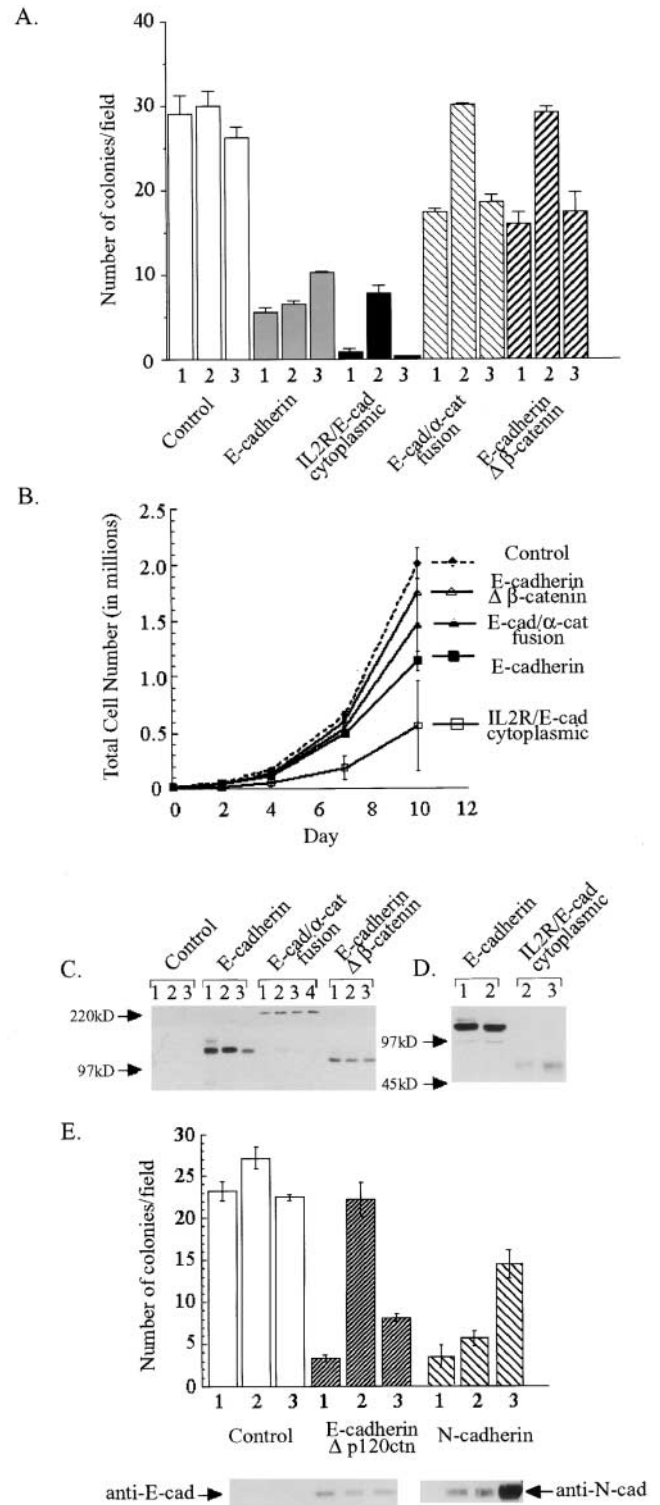

Figure 2. Growth properties of cadherin construct–expressing cell lines. Three independent clones per construct were analyzed. (A) Anchorage-independent growth. An equivalent number of cells from each stable cell line was seeded into soft agar, and colonies >100 μm diameter were counted after ~14 d in culture. Three independent clones per construct (columns 1, 2, and 3) were characterized (± SEM). (B) Growth properties of cell clones on plastic. Each data point represents the mean from the three independent cell lines. (C and D) Western blot analysis and relative expression levels of cadherin construct–expressing cell lines after immunoblotting with an mAb to the extracellular domain of E-cad-

no inhibition was observed for the cadherin constructs designed to rescue adhesion, E-cadherin–α-catenin fusion, and the E-cadherin Δ β-catenin. Similar results were observed when standard rates of log phase growth were measured for each cell line (Fig. 2 B). The differences in growth inhibition do not result from differences in levels of protein expression, as the IL2R-cytoplasmic construct is less well-expressed than either wild-type E-cadherin or the E-cadherin Δ β-catenin construct and about equally as well-expressed as the E-cadherin–α-catenin fusion (Fig. 2, C and D). Therefore, growth inhibition results primarily from the presence of the cadherin cytoplasmic domain but little if any from the adhesive function of E-cadherin.

The cytoplasmic domain of the cadherin can bind directly to p120[ctn] as well as β-catenin, and either protein could play a role in growth suppression. To determine whether p120[ctn] binding was involved in cadherin-mediated growth suppression, stable cell lines expressing mutant E-cadherin incapable of binding p120[ctn] were generated (Thoreson et al., 2000; Fig. 1). This E-cadherin Δ-p120[ctn] construct was still able to inhibit SW480 growth (Fig. 2 E). Together with the observation that deletion of the β-catenin binding region caused a loss of growth inhibitory activity of the cadherin (Fig. 2, A and B), these data suggest that β-catenin binding, but not p120[ctn] binding, is most critical for the growth suppressor activity of E-cadherin in SW480 cells. Furthermore, this growth-suppressing activity is not unique to E-cadherin since N-cadherin, which binds β-catenin strongly, also manifested this activity (Fig. 2 E).

The E-cadherin–α-catenin fusion chimera and E-cadherin Δ β-catenin were expected to mediate effective homophilic adhesion without being able to bind β-catenin. However, we wished to confirm that these proteins actually mediate adhesion in SW480 cells, especially since their levels were somewhat lower than the wild-type molecule (Fig. 2 C). We therefore measured relative adhesive activities of cells expressing these constructs using a flow assay to measure the strength of cell attachment to purified soluble E-cadherin ectodomain as described for C-cadherin (Brieher et al., 1996). All of the cadherin-expressing cell lines adhered more strongly than the mock-transfected E-cadherin–negative parental SW480 cells (Fig. 3). The E-cadherin–α-catenin fusion construct exhibited adhesive activity well over background and almost as good as the wild-type cadherin, even though it is less well expressed (Fig. 2 C). By contrast, the E-cadherin Δ β-catenin–expressing cell line exhibited even stronger adhesive activity than the wild-type cadherin–expressing cells, despite the fact that it is less well expressed (approximately threefold; Fig. 2 C). This finding is not really surprising in light of previous experiments showing that similar mutant C-cadherin
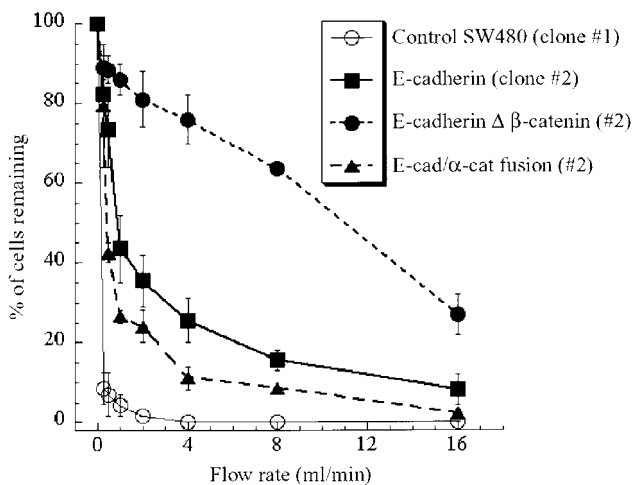

Figure 3. Adhesive properties of cadherin-expressing cell lines using a laminar flow cell attachment assay. Mock-transfected control SW480 cells and SW480 cells expressed wild-type E-cadherin, E-cadherin Δ β-catenin, or an E-cadherin–α-catenin fusion, remaining attached to glass capillary tubes coated with the extracellular domain of human E-cadherin after increasing flow rates (ml/min). Each curve represents adhesive activity of a given construct-expressing cell line. Adhesive activity shown above was completely calcium dependent (data not shown).

and VE-cadherin constructs have adhesive activities comparable to wild-type molecules when expressed in CHO cells, presumably due in some way to p120[ctn] binding (Navarro et al., 1995; Yap et al., 1998). Why this mutant cadherin has even higher adhesive activity than the wild type in SW480 cells is not yet clear. Nevertheless, since these two constructs failed to inhibit SW480 growth compared with the wild-type cadherin (Fig. 2, A and B), the adhesion activity of E-cadherin does not appear to be sufficient to mediate growth inhibition.

Growth-suppressing Activity of E-Cadherin Is Mediated through a Reduction in β-Catenin/TCF Signaling

The observation that the cytoplasmic domain of E-cadherin, and in particular the β-catenin binding region, contains the information to inhibit SW480 cell growth suggests that growth inhibition may be mediated through a β-catenin–dependent signaling pathway. We therefore asked whether expression of the different cadherin constructs could influence β-catenin signaling activity at target genes. A β-catenin/TCF–dependent reporter gene assay (TOPFLASH; Korinek et al., 1997) revealed that each of the wild-type E-cadherin and IL2R/E-cadherin cytoplasmic domain–expressing cell lines exhibit reduced target gene activation, but no consistent inhibition was observed for the other cell lines (Fig. 4 A). Furthermore, expression of an endogenous candidate target gene for the β-catenin/TCF signaling pathway in colon cells, the c-myc proto-oncogene (He et al., 1998), is reduced in the cadherin and IL2R/E-cadherin cytoplasmic domain–expressing cell lines (Fig. 4 B).

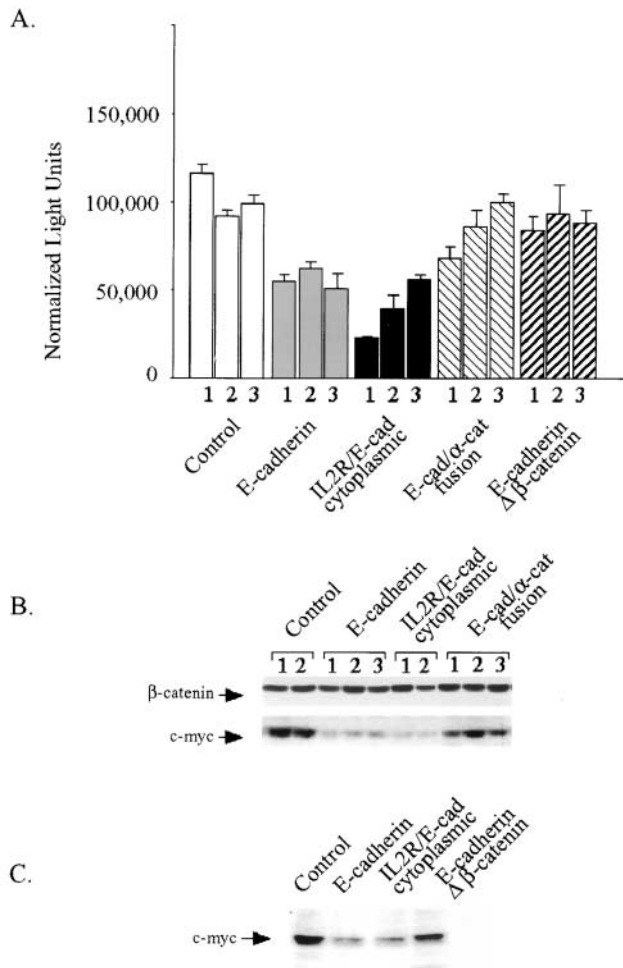To determine whether upregulation of the β-catenin signaling pathway contributes to the growth of SW480 cells,

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herin (HECD-1) (C), or with an antibody that recognizes the cytoplasmic domain of the cadherin (D) (Pep1 polyclonal antibody; Choi and Gumbiner, 1989). (E) Anchorage-independent growth properties and Western analysis of E-cadherin Δ p120[ctn] and N-cadherin. Mock-transfected (Control), E-cadherin Δ p120[ctn], and N-cadherin–expressing cell lines were seeded into soft agar, and the number of colonies was counted after ~15 d. Western analysis of E-cadherin Δ p120[ctn] and N-cadherin–expressing cell lines is shown.

A.

B.

C.

Figure 4. Activities of β-catenin/TCF target genes. E-cadherin or E-cadherin construct–expressing SW480 cell lines. (A) β-catenin/TCF-dependent reporter gene assay. Individual cell lines were transiently transfected with the β-catenin/TCF–dependent luciferase reporter gene (TOPFLASH) and a β-galactosidase reporter plasmid to control for transfection efficiency. Negligible activity was observed when the TCF binding sites were mutated (FOPFLASH; not shown). (B and C) Expression of product of endogenous β-catenin/TCF target gene, c-myc: control and cadherin construct–expressing cell lines were analyzed by SDS-PAGE and immunoblotting with β-catenin and c-myc specific antibodies. Individual cell lines were examined in B; cell lines were pooled in C.

we examined whether cell growth is affected by inhibition of β-catenin nuclear signaling at a downstream step. Either of two constructs known to inhibit β-catenin signaling at the level of target genes, β-catenin fused to the engrailed repressor domain, and a dominant-negative form of TCF were expressed in control SW480 cells (Molenaar et al., 1996; Montross et al., 2000). Stable expression of the β-catenin/engrailed chimera significantly inhibited anchorage-independent growth of SW480 cells compared with mock-transfected cells (Fig. 5 A). We failed to generate stable transfectants with readily detectable levels of the dominant-negative TCF and, therefore, turned to a colony-forming assay to measure growth potential. This assay appears to be a valid measure of anchorage-indepen-
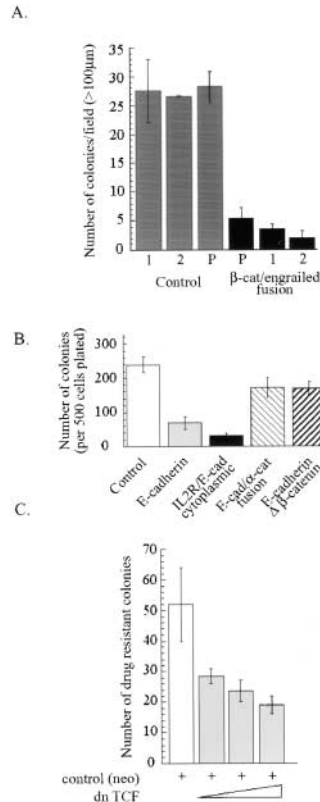
A.

B.

C.

Figure 5. Inhibition of β-catenin/TCF gene activation suppresses SW480 cell growth. (A) Anchorage-independent growth of β-catenin/engrailed repressor–expressing cell lines. (B) Colony-forming assay reflects the growth properties of stably transfected cadherin construct–expressing cell lines. The stable cell lines characterized for growth in soft agar and growth on plastic in Fig. 2 were assessed for their ability to inhibit colony formation when plated under dilute conditions (500 cells plated per dish). Each bar represents the mean of three independent cell lines evaluated for each cadherin construct. (C) Dominant-negative TCF inhibits growth of SW480 cells, as assessed with the colony-forming assay. Cells were transfected with a neomycin drug resistance plasmid (control [neo] +) with or without increasing amounts (0.002, 0.02, and 0.2 μg) of plasmid expressing a dominant negative (dn TCF). Results are from triplicate transfections (±SEM).

dent growth for SW480 cell lines because it mirrored the results of the soft agar assay (Fig. 5 B). Using this assay, a dominant-negative form of TCF potently inhibited the number of colonies formed compared with control-transfected cells (Fig. 5 C). This reduction in colony number is not due to simple toxicity from the TCF vector, as transfection with the β-catenin cDNA in the same vector caused no reduction in colony number (data not shown). Thus, specific inhibition of β-catenin nuclear signaling activity reduces SW480 cell growth, indicating that this pathway contributes to the high growth rate of these cells.

To determine whether the observed E-cadherin–mediated growth inhibition occurred through a reduction in the activity of a β-catenin/TCF signaling pathway, we asked whether the E-cadherin–mediated growth inhibition could be rescued at a downstream step with constitutively active forms of LEF or TCF. Transfection with E-cadherin–expressing plasmids alone reduced the number of drug-resistant colonies formed in the colony-forming assay, as expected (Fig. 6). Inhibition of colony formation by E-cadherin was reversed by cotransfecting with low amounts of plasmid encoding constitutively active forms of LEF or TCF. Specificity of the rescue for constitutively activated TCF was demonstrated by the lack of effect of either the wild-type or a dominant-negative form of TCF (Fig. 6 B). Titration of the LEF or TCF-expressing plasmids was required for optimal rescue effect, presumably because high levels of these transcription factors have inhibitory activity due to squelching or sequestration of transcription machinery components. The rescue of E-cadherin–mediated growth inhibition by
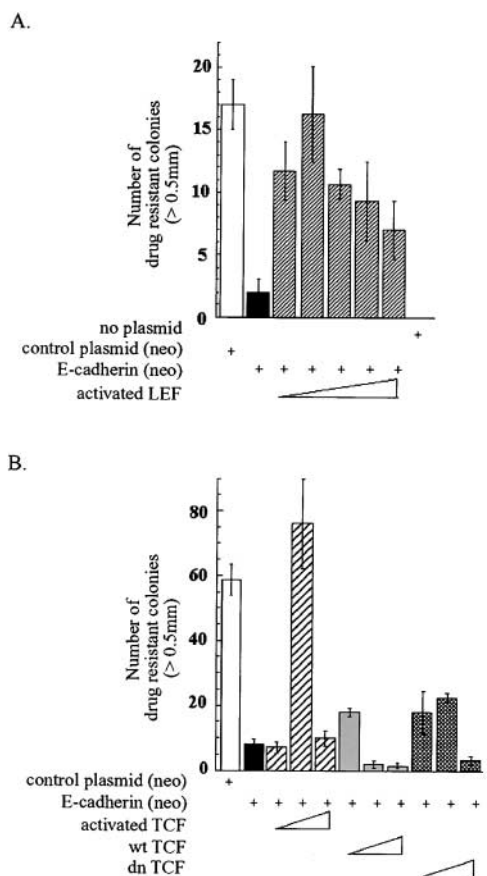
A.

B.

Figure 6. Constitutively activated forms of LEF and TCF rescue cadherin-mediated growth inhibition of SW480 cells. (A) Colony formation assay after transfection with E-cadherin or E-cadherin plus activated LEF (β-catenin COOH-terminal transactivation domain fused directly to LEF). Control plasmid (pcDNA3neo), E-cadherin/pcDNA3neo plasmid (E-cadherin [neo]) or activated LEF (0.0001–1.0 μg). (B) Colony–forming assay after transfection with E-cadherin or E-cadherin plus activated TCF (VP16 transactivation domain fused to TCF-3), and wild-type TCF (wt-TCF) or dominant-negative TCF (dnTCF) as controls. TCF-encoding plasmids were titrated for optimal effect (0.001–0.1 μg). Bars represent mean number of colonies from triplicate transfections (±SEM).

constitutively active forms of LEF or TCF provides compelling evidence that E-cadherin inhibits cell growth through a reduction in β-catenin/TCF signaling.

The Cadherin Cytoplasmic Domain Mediates Growth Inhibition Without Depleting Cytosolic or Nuclear β-Catenin

Previous studies with *Xenopus* embryos have shown that cadherins can inhibit β-catenin signaling activity through direct binding and recruitment to the plasma membrane, resulting in the depletion of the cytosolic and nuclear signaling pools of β-catenin (Heasman et al., 1994; Fagotto et al., 1996). Since both wild-type E-cadherin and the cytoplasmic domain of E-cadherin can interact with β-catenin, we wished to determine whether the reduction in β-catenin/TCF signaling and growth inhibition was due to sequestering β-catenin from the nuclear signaling pool. The sub-

cellular localization of β-catenin was determined by both cell fractionation and immunofluorescence microscopy techniques. The lectin ConA can be used to quantitatively separate the cadherin-bound pool of β-catenin from the unbound pool (see Materials and Methods; Fagotto et al., 1996). Although expression of wild-type cadherin or the IL2R/E-cadherin cytoplasmic chimera increased the amount of β-catenin recruited to the glycoprotein-associated fraction, the extremely large soluble pool of β-catenin in these cells is not significantly depleted (Fig. 7 A). Identical results were observed when cytosolic and membrane fractions were separated using a detergent-free/hypotonic lysis method (data not shown). The total levels of β-catenin were largely unaltered (Fig. 7 B), and no obvious difference in the nuclear accumulation of β-catenin was detected in any of the cadherin construct–expressing stable cell lines (Fig. 7 C). Similarly, no significant changes in the levels, sequestration, or nuclear localization of plakoglobin, p120[ctn], and α-catenin were detected between the various cell lines (data not shown). Thus, expression of E-cadherin or IL2R/E-cadherin did not significantly deplete the large cytosolic/nuclear pools of β-catenin in the SW480 cells, even though they inhibited β-catenin nuclear signaling.

Inhibition of β-catenin signaling without reducing cytosolic levels may appear at first glance to be inconsistent with the known mechanism of regulating β-catenin nuclear signaling (i.e., via reduction of its cytosolic levels). However, one possible explanation is that only a subfraction of the large cytosolic pool of β-catenin in SW480 cells is transcriptionally competent, and this competent active signaling pool is preferentially bound and depleted by E-cadherin in the cell. To determine whether SW480 cells contain distinct pools of β-catenin that differ in their competence to interact with E-cadherin, a cytosolic fraction (detergent-free 100,000 *g* supernatant) from SW480 cells was subjected to sequential affinity precipitations with a cadherin cytoplasmic domain–GST fusion protein. Only a very small amount of the cytosolic β-catenin could be precipitated by the cadherin–GST fusion protein (Fig. 8 A), showing that a sizable fraction of β-catenin in SW480 cells is refractory to cadherin binding. Similarly, using sequential precipitations with a TCF–GST fusion protein, only a small amount of the cytosolic β-catenin was capable of binding TCF (Fig. 8 B), suggesting that SW480 cells contain a large pool of transcriptionally incompetent or inactive β-catenin. The cadherin– and TCF–GST fusion proteins are able to bind β-catenin effectively since they could affinity precipitate β-catenin extracted from SW480 cells under more disruptive detergent lysis conditions (Fig. 8, C and D). The cadherin seems to be able to bind all of the β-catenin that is competent to interact with TCF because the cadherin depleted pool can no longer interact with TCF–GST (Fig. 8 C). Furthermore, the TCF–GST-depleted pool of β-catenin is not able to bind to the cadherin–GST protein (Fig. 8 D). Therefore, the cadherin-titratable pool of β-catenin appears to be the same as the transcriptionally competent active signaling pool that interacts with TCF. Moreover, the existence of a large cytosolic pool of β-catenin in SW480 cells that is refractory to both cadherin and TCF binding explains, at least in part, how E-cadherin expression can bind and inhibit the active signaling pool of β-catenin without significantly reducing its overall levels in the cytosol and nucleus.
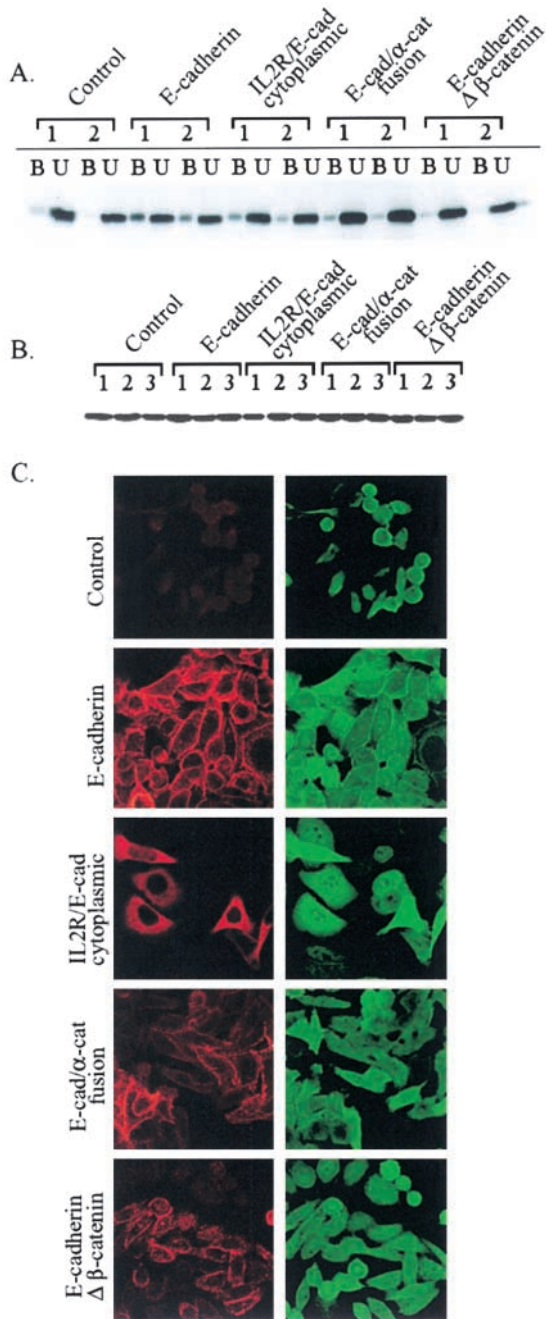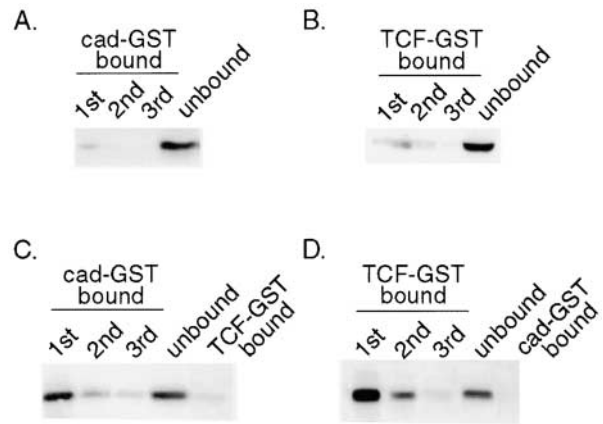
Figure 8. SW480 cells contain a large pool of β-catenin that does not interact with either cadherins or TCF. (A) Only a small fraction of cytosolic β–catenin is competent to interact with the cadherin cytoplasmic domain in vitro. Sequential depletion of β-catenin (1st, 2nd, and 3rd) from a detergent-free 100,000-*g* supernatant fraction of SW480 cells with cadherin–GST (cad-GST), and TCA precipitation of the unbound fraction. (B) Only a small fraction of cytosolic β-catenin is competent to interact with the TCF in vitro. Sequential depletion of β-catenin from the 100,000-*g* supernatant fraction of SW480 cells with TCF–GST, and TCA precipitation of the unbound fraction. (C) Cadherin-unbindable pool of β-catenin does not interact with TCF. Sequential depletion of β-catenin from an SW480 cell detergent lysate with cad-GST (1st, 2nd, and 3rd), and final depleted fraction subject to either TCA precipitation (unbound) or binding to TCF–GST. (D) TCF-unbindable pool of β-catenin does not interact with the cadherin cytoplasmic domain. Sequential depletion of β-catenin from an SW480 cell detergent lysate with TCF–GST (1st, 2nd, 3rd), and final depleted fraction subject to either TCA precipitation (unbound) or binding to cad-GST. Samples were separated by SDS-PAGE, and β-catenin was detected by immunoblotting.

Figure 7. Levels and subcellular distribution of β-catenin are not significantly altered in cadherin construct–expressing SW480 cell lines. (A) Fractionation of β-catenin into cadherin-associated and soluble pools using the lectin ConA. Detergent lysates were incubated with ConA-Sepharose (amount predetermined to deplete all of the cadherin) and equivalent proportions of the ConA-bound (B) and -unbound (U) fractions were analyzed by SDS-PAGE and immunoblotting. Two cell lines per constructs were analyzed. (B) Total levels of β-catenin: Western blot of SDS lysates of individual cell lines. (C) Indirect immunofluorescence microscopy of cadherin-constructs (CY-3) and β-catenin (FITC) in control and cadherin construct–expressing stable cell lines.

Discussion

We have examined the mechanism by which E-cadherin expression mediates tumor suppression in light of two known aspects of cadherin function: cell adhesion and the ability to bind β-catenin and influence its signaling and transcriptional activity in the nucleus. We find that E-cadherin suppresses the growth of the SW480 human colon carcinoma cell line and that this activity is mediated through an adhesion-independent, β-catenin/TCF–dependent signaling mechanism. Both wild-type E-cadherin and an IL2R/E-cadherin chimera that retains the cadherin cytoplasmic domain but lacks the extracellular homophilic adhesive domain potently inhibited anchorage-independent and log phase cell growth. Moreover, cadherin constructs that exhibit adhesive activity when expressed in SW480 cells, but do not bind β-catenin, failed to inhibit cell growth (e.g., the E-cadherin–α-catenin chimera and the E-cadherin Δ β-catenin mutant). Thus, the homophilic adhesive activity of E-cadherin is neither necessary nor sufficient to mediate strong growth inhibition in the SW480 cell line. Furthermore, using several different criteria, we show that cadherin-mediated growth inhibition acts through a β-catenin/TCF–dependent signaling mechanism. A construct that lacks the β-catenin binding region did not suppress growth. Growth inhibition of the cadherin correlated with a reduction in β-catenin transcriptional activity, as assessed with a β-catenin/TCF–responsive reporter assay. Moreover, β-catenin signaling clearly plays a physiological role in the growth properties of

SW480 cells, since direct inhibition of β-catenin target genes with either a dominant-negative form of TCF, or a β-catenin-engrailed repressor chimera, inhibited cell growth. E-cadherin–mediated growth inhibition was rescued with constitutively active forms of LEF or TCF that bypass the requirement for an upstream β-catenin signaling activity. Together, these results argue that the growth suppressor activity of E-cadherin in SW480 colorectal tumor cells is mediated through inhibiting the expression of β-catenin/TCF–dependent target genes.

A few previous studies have found that cadherin expression can lead to inhibition of cell growth (Miyaki et al., 1995; Hermiston et al., 1996; St. Croix et al., 1998). However, it had never been determined whether the effect on growth was mediated by the cadherin directly or indirectly through the establishment of close cell contacts, which invariably promote the formation of other junctional complexes (e.g., tight junctions or gap junctions) and/or juxtacrine signaling molecules with potentially independent growth suppressing functions. For example, cadherins have been shown to stimulate gap junction formation (Musil et al., 1990), and VE-cadherin has been found to regulate signaling through the VEGF receptor (Carmeliet et al., 1999), which could occur by a juxtacrine mechanism. Our finding that the growth inhibition of the cadherin is attributed to the cytoplasmic domain, but not to cell adhesion, demonstrates that the E-cadherin protein can directly transduce a growth inhibitory signal.

E-cadherin inhibits β-catenin/TCF signaling and SW480 tumor cell growth without noticeable changes in cytosolic/nuclear levels of β-catenin protein, the major mechanism thought to control β-catenin signaling (Polakis, 1999). However, several studies have shown that β-catenin signaling can be regulated, to some extent, independent of levels or stabilization (Young et al., 1998; Nelson and Gumbiner, 1999; Guger and Gumbiner, 2000). One possible interpretation, therefore, is that a large fraction of the β-catenin in this cell line may not be active in nuclear signaling and that sequestration of a minor active pool of β-catenin by E-cadherin could have significant effects on β-catenin signaling. We show that there are two distinct pools of β-catenin in SW480 cells, a pool that is competent to bind the cadherin and TCF, and a pool that binds neither. Thus, E-cadherin may be able to selectively sequester the transcriptionally competent pool of β-catenin without depleting overall cytosolic/nuclear levels of β-catenin. The biochemical basis of these two pools of β-catenin is not yet clear, but obvious candidates are phosphorylation or other types of modifications and/or binding proteins. For example, phosphorylation of a single tyrosine residue in β-catenin has been shown to alter cadherin binding (Roura et al., 1999), and two inhibitors of β-catenin signaling have been identified: a small nuclear/cytoplasmic–localizing polypeptide, ICAT, and a 120-kD nuclear protein, duplin (Sakamoto et al., 2000; Tago et al., 2000). Using two-dimensional electrophoresis or gel filtration analysis, we have not yet been able to distinguish these two distinct pools of β-catenin, but further work may reveal molecular differences.

Our findings demonstrate that one important mechanism by which E-cadherin mediates tumor suppression, and in particular growth suppression, is through an inhibition of β-catenin signaling. Since the SW480 colorectal

cells used in this study express mutant APC, and therefore significantly upregulate β-catenin signaling, these conclusions may be particularly relevant for tumors in which β-catenin signaling is implicated in cell transformation. Thus, these findings provide evidence that the same tumor cell can use two different mechanisms for negatively regulating β-catenin signaling: the APC/glycogen synthase kinase-3/axin–dependent destruction pathway and a cadherin-dependent pathway. Whether this adhesion-independent mechanism also explains E-cadherin tumor suppressor activity in cells that have not significantly upregulated the β-catenin signaling pathway is not known and will require further investigation.

In other tumor cell types, or different stages of the same tumor type, it is possible that different mechanisms may account for the tumor suppressor activity of E-cadherin. The fact that E-cadherin has been implicated in both early and late stages of tumor progression and has both growth suppressor and invasion suppressor activities raises the possibility of more than one mechanism. The role of E-cadherin in adhesion and epithelial junction formation may be more important for later stages of tumor progression and invasion. For example, adhesion-blocking E-cadherin antibodies increase the invasive behavior of cells (Behrens et al., 1989), and expression of the cadherin cytoplasmic domain, which is a dominant-negative for adhesion, actually promotes the adenoma-to-carcinoma transition in a pancreatic tumor model (Perl et al., 1998). Furthermore, E-cadherin and APC loss-of-function mutations tend to be associated with different cancer types (e.g., E-cadherin with breast, gastric, and prostate cancers; the APC pathway with colon, hepatic, and melanoma cancers). Therefore, it will also be important to determine how E-cadherin functions as either an invasion or tumor suppressor in other types of tumors, especially those that are not known to result from mutations in APC or β-catenin.

These findings also have broad implications for biological processes beyond tumorigenesis. Overexpression of cadherins in early *Xenopus* and *Drosophila* embryos has been found to antagonize β-catenin signaling. However, the relevance of these experimental perturbations to normal development has been uncertain. In this tumor suppressor model, normal levels of E-cadherin expression can regulate cell growth through β-catenin signaling, suggesting that there is a physiological relationship between cadherin expression (and/or function) and the β-catenin signaling pathway. Moreover, unlike the case of cadherin overexpression in *Xenopus*, the regulation of β-catenin signaling in tumor cells is not simply due to overall changes in cytosolic/nuclear levels, but rather to changes in subpools of β-catenin. Indeed, it would not be surprising if the physiological relationship between the two functions of β-catenin is not simply one of competition, but rather a more complex mechanism subject to additional layers of regulation. It will be interesting to determine whether the interactions between cadherins and β-catenin signaling are similarly regulated in developing tissues.

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