E-Cadherin Is Required for Caveolin-1-Mediated Down-Regulation of the Inhibitor of Apoptosis Protein Survivin via Reduced β -Catenin-Tcf/Lef-Dependent Transcription^{∇‡}

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Received 24 October 2006/Returned for modification 5 January 2007/Accepted 16 August 2007

Caveolin-1 reportedly acts as a tumor suppressor and promotes events associated with tumor progression, including metastasis. The molecular mechanisms underlying such radical differences in function are not understood. Recently, we showed that caveolin-1 inhibits expression of the inhibitor of apoptosis protein survivin via a transcriptional mechanism involving the β -catenin-Tcf/Lef pathway. Surprisingly, while caveolin-1 expression decreased survivin mRNA and protein levels in HT29(ATCC) human colon cancer cells, this was not the case in metastatic HT29(US) cells. Survivin down-regulation was paralleled by coimmunoprecipitation and colocalization of caveolin-1 with β -catenin in HT29(ATCC) but not HT29(US) cells. Unlike HT29(ATCC) cells, HT29(US) cells expressed small amounts of E-cadherin that accumulated in intracellular patches rather than at the cell surface. Re-expression of E-cadherin in HT29(US) cells restored the ability of caveolin-1 to down-regulate β -catenin-Tcf/Lef-dependent transcription and survivin expression, as seen in HT29(ATCC) cells. In addition, coimmunoprecipitation and colocalization between caveolin-1 and β -catenin increased upon E-cadherin expression in HT29(US) cells. In human embryonic kidney HEK293T and HT29(US) cells, caveolin-1 and E-cadherin cooperated in suppressing β -catenin-Tcf/Lef-dependent transcription as well as survivin expression. Finally, mouse melanoma B16-F10 cells, another metastatic cell model with low endogenous caveolin-1 and E-cadherin levels, were characterized. In these cells, caveolin-1-mediated down-regulation of survivin in the presence of E-cadherin coincided with increased apoptosis. Thus, the absence of E-cadherin severely compromises the ability of caveolin-1 to develop activities potentially relevant to its role as a tumor suppressor.

Caveolin-1 is a 21- to 24-kDa protein involved in caveola formation, cell trafficking, cholesterol homeostasis, and signal transduction (7, 45). Observations linking caveolin-1 presence to inhibition of a variety of proteins whose activity is associated with cell proliferation and survival, including protein kinase C, Src, epidermal growth factor receptor, and endothelial nitric oxide synthase, led to the proposal that caveolin-1 may function as a tumor suppressor (42). This view has been corroborated in several studies showing that caveolin-1 is down-regulated in a variety of human tumors, including those of the lung (46), breast (29), and ovary (59), as well as in colon adenocarcinomas (2, 3) and sarcomas (60). In addition, caveolin-1 expression is reduced in many human cancer cell lines. Furthermore, caveolin-1 re-expression decreases cell proliferation, anchorage-independent growth, and tumorigenicity of such cell lines. Mechanistically, caveolin-1 has been shown to inhibit cell proliferation by promoting cell cycle arrest (15) as well as apoptotic cell death (35, 56, 59).

Although a large body of data in the literature implicates

caveolin-1 as a tumor suppressor, some evidence favors an alternative view, arguing that caveolin-1 promotes tumor metastasis and multidrug resistance (reviewed in reference 45). In prostate cancer, in particular, caveolin-1 presence in tumors is associated with elevated metastatic potential and poorer patient prognosis (32, 53, 64). This could be considered a peculiarity of prostate tissue, since caveolin-1 is normally not expressed there (64). However, work with cancer cells derived from other tissues where caveolin-1 down-regulation is associated with the process of tumor formation, such as breast, colon, and lung cancers, indicates that caveolin-1 suppression is not irreversible and that reappearance of caveolin-1 may be associated with multidrug resistance and/or elevated metastatic potential (3, 21, 27, 28), although this is not always the case (66). Clearly, therefore, effects associated with the presence of caveolin-1 depend very much on the experimental model employed. To date, however, essentially nothing is known at the molecular level about how caveolin-1 function is defined by the cellular context.

Colon cancer is commonly characterized by mutations in the protein APC, which leads to up-regulation of β -catenin-dependent transcription (37, 61). β -Catenin, a key element in the Wnt- β -catenin-Tcf/Lef pathway, is found predominantly in three locations: at the plasma membrane in a complex with E-cadherin; in the nucleus, where it promotes transcription of target genes together with the Tcf/Lef DNA binding factors; and in the cytoplasm associated with a multiprotein complex

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[‡] Supplemental material for this article may be found at <http://mcb.asm.org/>.

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[∇] Published ahead of print on 4 September 2007.

formed by GSK3 β , axin, and APC, among other proteins. β -Catenin stability is tightly controlled by this cytoplasmic complex, via phosphorylation and subsequent degradation via the proteasome pathway (reviewed in references 37 and 61). In addition, both total β -catenin levels and localization throughout the cell are controlled by E-cadherin, a protein that mediates cell-cell adhesion through calcium-dependent homophilic interactions of the extracellular domain. E-cadherin binds β -catenin or plakoglobin through its cytoplasmic tail. The last two associate with α -catenin, which in turn links these complexes to the actin cytoskeleton (43, 58). Loss of E-cadherin expression is intimately related to tumor progression and metastasis. In colon cancer, both mutations in APC and E-cadherin down-regulation are linked to enhanced β -catenin-mediated transcriptional activity (6, 37).

The expression of a large number of genes, including those for cyclin D1, *c-myc*, vascular endothelial growth factor, and survivin, is controlled via the β -catenin-Tcf/Lef pathway (24, 61, 65). Of these genes, several modulate cell cycle progression and/or apoptosis, and increases in their expression are associated with tumor development or progression. In this respect, survivin, a member of the IAP (inhibitor of apoptosis protein) family, has attracted a great deal of interest, since expression of survivin is dramatically up-regulated in most human tumors and is required for tumor survival (reviewed in reference 1). The precise mechanisms underlying survivin function remain controversial, but they include inhibition of apoptosis and control of progression through the G₂/M checkpoint of the cell cycle (10, 30, 49, 50, 54).

Recent work from this laboratory demonstrated that caveolin-1 controls cell proliferation and viability by inhibiting expression of survivin via a transcriptional mechanism involving the β -catenin-Tcf/Lef pathway in several cellular settings (56). Caveolin-1 recruits β -catenin to a Triton X-100-insoluble protein complex, presumably at the plasma membrane, thereby precluding Tcf/Lef-dependent transcription (14, 38, 39, 56). While this mechanism was functional in a variety of different cell lines available, we did notice that there were exceptions. In particular, expression of caveolin-1 in a subline derived from the human adenocarcinoma cell line HT29, termed HT29(US), which was obtained by selection for higher metastatic potential, did not alter cell proliferation, apoptosis, or survivin expression. Given that loss of E-cadherin is often associated with the process of metastasis, we wondered whether such loss might account for the inability of caveolin-1 to regulate survivin. Indeed, we observed that E-cadherin levels were dramatically reduced in HT29(US) cells, compared to those in HT29 cells obtained from the American Type Culture Collection (ATCC), referred to in this study as HT29(ATCC) cells. Furthermore, re-expression of E-cadherin was sufficient to restore the ability of caveolin-1 in HT29(US) cells to regulate survivin expression and control cell proliferation. Likewise, results obtained in HEK293T and metastatic murine melanoma B16-F10 cells indicated that E-cadherin facilitates caveolin-1-mediated effects. Taken together, these results identify E-cadherin as an important permissive element in defining functions of caveolin-1 potentially relevant to its role as a tumor suppressor. In doing so, they also shed light on how the consequences of caveolin-1 presence may vary in a cell context-dependent fashion.

(Results were presented in preliminary form as meeting abstracts at the XX Annual Meeting of the Chilean Cell Biology Society [October 2006, Pucon, Chile] and the 46th Annual ASCB Meeting [December 2006, San Diego, CA].)

MATERIALS AND METHODS

Materials. Polyclonal anti-caveolin-1, monoclonal anti-E-cadherin, and monoclonal anti- β -catenin antibodies were from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-human survivin and antiactin antibodies were from RD Systems (Minneapolis, MN) and Sigma (St. Louis, MO), respectively. Rabbit polyclonal anti-cyclin D1 antibody was from Chemicon International (Temecula, CA). Goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG antibodies coupled to horseradish peroxidase were from Bio-Rad Laboratories (Hercules, CA) and Sigma, respectively. Cy3-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Bicinchoninic acid was from Pierce (Rockford, IL). The EZ-ECL chemiluminescent substrate was from Biological Industries (Kibbutz Beit Haemek, Israel). The Superfect reagent and plasmid midi kit were from QIAGEN (Valencia, CA). TRIzol reagent was from Invitrogen, Life Technologies (Carlsbad, CA). Hygromycin was from Calbiochem (La Jolla, CA). Cell medium and antibiotics were from Gibco-BRL (Paisley, Scotland, United Kingdom). Fetal bovine serum (FBS) was from HyClone (Logan, UT). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonylphenyl)-2H tetrazolium inner salt (MTS) proliferation assay kit was from Promega (Madison, WI). Luciferin for reporter assays was from Sigma. Protein A-Sepharose used in immunoprecipitation assays and all other reagents used for immunofluorescence and Western blots were from Sigma or of the highest grade available.

Cell culture. HEK293T, MDCK, human colon adenocarcinoma HT29(ATCC), and metastasis-derived HT29(US) cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 10 μ g/ml streptomycin) at 37°C and 5% CO₂. Metastatic murine melanoma cells B16-F10 (provided by Laurence Zitvogel, Institut Gustav Roussy, Villejuif, France) were cultured in RPMI with 10% FBS and antibiotics. For stable transfection of HT29(ATCC) and HT29(US) cells and transient-transfection experiments in HEK293T cells, the reagent Superfect was used following instructions provided by the manufacturer. B16-F10 cells were transfected by electroporation (see below).

HT29(US) cells were selected from HT29(ATCC) cells for higher metastatic potential by repeated passages in nude mice whereby cells were injected dorsally and then recovered from lung metastases (kindly provided by Bernard Sordat, ISREC, Epalinges, Switzerland).

Plasmids. The plasmids pLacIOP and pLacIOP-caveolin-1 were previously described (3, 12). The reporter plasmids pTOP-FLASH (containing wild-type Tcf/Lef-binding sites fused to the luciferase reporter gene) and pFOP-FLASH (containing mutated Tcf/Lef-binding sites), described previously (57), were kindly provided by Hans Clevers (Hubrecht Laboratory, Uppsalalaan, The Netherlands). The plasmid pBATEM2, encoding murine E-cadherin, was provided by Amparo Cano (Universidad Autónoma de Madrid, Madrid, Spain).

Caveolin-1-expressing colon cancer cells. HT29(US) and DLD-1 cells transfected stably with either pLacIOP alone (mock) or pLacIOP-caveolin-1 (clones C14 and C4, respectively) were described previously (3).

Transfection of HT29(ATCC) and HT29(US) cells. HT29(ATCC) cells were transfected with the plasmids pLacIOP and pLacIOP-caveolin-1 using the reagent Superfect, following instructions provided by the manufacturer. After transfection (48 h), cells were grown in selection medium containing 750 μ g/ml hygromycin for 2 to 3 weeks. Mock (pLacIOP) and caveolin-1-expressing (pLacIOP-caveolin-1) cells (mixed, nonclonal populations) were obtained and characterized. For some experiments (see Fig. 5), clonal populations of caveolin-1-expressing HT29(ATCC) cells were obtained using the cloning ring procedure. Similarly, HT29(US) cells were cotransfected with either pBATEM2 and pLacIOP or pBATEM2 and pLacIOP-caveolin-1 using Superfect. Since pBATEM2 does not contain a resistance marker, stably transfected cells (mixed, nonclonal populations) were obtained in cotransfection experiments with either pLacIOP or pLacIOP-caveolin-1 by exposure to 750 μ g/ml hygromycin for 2 to 3 weeks.

Transfection of B16-F10 melanoma cells. B16-F10 cells were transfected with the pLacIOP and pLacIOP-caveolin-1 by electroporation (see below). After transfection (48 h), cells were grown in selection medium containing 100 μ g/ml hygromycin for 2 to 3 weeks. Mock-transfected (pLacIOP) and caveolin-1-expressing (pLacIOP-caveolin-1) cells were obtained and characterized.

Electroporation conditions. For ectopic expression of E-cadherin, B16-F10 melanoma cells stably transfected with either pLacIOP or pLacIOP-caveolin-1 were grown to 60 to 80% confluence in 10-cm plates. Cells were electroporated with 25 μ g of plasmid pBATEM2 in 1 ml of RPMI medium without serum using a pulse of 270 V and 1,600 μ F in a cell Porator (GibcoBRL, Life Technologies, Bethesda, MD). After electroporation, cells were plated in complete RPMI medium containing 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Protein and mRNA levels were analyzed 48 h later.

Western blotting. Cell extracts were prepared and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide minigels (Bio-Rad), loading 50 μ g total protein per lane and transferred to nitrocellulose as described previously (3). Blots were blocked with 5% milk in 0.1% Tween-phosphate-buffered saline (PBS) and then probed with antiactin (dilution, 1:5,000), anti-E-cadherin (1:3,000), anti- β -catenin (1:1,000), anti-caveolin-1 (1:5,000), or antisuvinin (1:3,000) antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence system.

Analysis of mRNA levels by RT-PCR. Total RNA was isolated with the reagent TRIzol following instructions provided by the manufacturer. RNA samples, characterized by electrophoresis in 1% agarose gels (quality control), were employed as templates to generate cDNA. Survivin (sense primer, 5'-CCGACGTTGCCCTGC-3'; antisense primer, 5'-TCGATGGACGCGCAC-3'), caveolin-1 (sense primer, 5'-GGGCAACATCTAGAAGCCCAACA-3'; antisense primer, 5'-CTGATGCACTGAATTCCAATCAGGAA-3') and actin (sense primer, 5'-AAATCGTGCGTGACATTAAGC-3'; antisense primer, 5'-CCGATCCACGAGTACTT-3') cDNAs were amplified by PCR. All reaction products were analyzed after 30 amplification cycles, each of which involved consecutive 1-min steps at 94°C, 55°C and 72°C. Survivin mRNA levels were normalized to actin RNA levels in this case.

The results obtained by semiquantitative PCR were confirmed by quantitative real-time PCR (QPCR) with brilliant SYBR green quantitative PCR (Stratagene, La Jolla, CA). The PCRs were carried out in a Chromo4 real-time PCR detection system (Bio-Rad Laboratories) using thermal cycle conditions following suggestions by the manufacturer and according to primer design. The relative gene expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method (36). Survivin levels were normalized to RNA of the 18S rRNA housekeeping gene. All data were expressed relative to values obtained for mock-transfected cells (100%). The assays were performed at least in triplicate.

Immunoprecipitation. Cell extracts were prepared in a buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100. Supernatants obtained after centrifugation (13,000 $\times$ g, 5 min, 4°C) were used for immunoprecipitation assays (500 μ g total protein per assay) with protein A-Sepharose-immobilized antibodies. Immunoprecipitated samples were solubilized in sample buffer, separated by SDS-PAGE, and analyzed by Western blotting as described above.

Immunofluorescence. Cells were cultured for 24 h in the absence of IPTG followed by 24 h in the presence of 1 mM IPTG in normal culture medium. After rinsing with PBS, cells were fixed in PBS-4% paraformaldehyde (30 min) and permeabilized with 0.1% Triton X-100 (10 min) at 4°C. Cells were then incubated with either polyclonal anti-caveolin-1 IgG (dilution, 1:100), monoclonal anti- β -catenin IgG (1:100), or monoclonal anti-E-cadherin IgG (1:100) primary antibodies, followed by Cy3-conjugated anti-mouse IgG (1:200) or FITC-conjugated anti-rabbit IgG (1:200) secondary antibodies. Samples were then mounted onto slides with 10% Mowiol-2.5% 1,4-diazobicyclo[2,2,2]octane (DABCO) and visualized with a Carl Zeiss Axiovert-135 M confocal microscope (LSM Microsystems) following excitation at 488 or 543 nm. Optical sections obtained for colocalization studies and acquired-image z stacks for three-dimensional visualization were processed with Imaris software (Bitplane AG, Zurich, Switzerland) as specified in individual figure legends.

Subcellular fractionation. Subcellular fractions of HT29(US) and HT29(ATCC) cells were prepared using the commercially available ProteoExtract subcellular proteome extraction kit (Calbiochem), which permits differential extraction of proteins with specific extraction buffers according to subcellular localization. Following a sequential extraction procedure, four fractions are isolated, corresponding to cytosol, membranes and organelles, nucleus, and cytoskeleton. The identity of the nuclear fraction was corroborated by the presence of characteristic low-molecular-weight histone bands visible upon Ponceau red staining following protein transfer to nitrocellulose (data not shown) and detection of cyclin D1 using specific antibodies.

Proliferation assay. Transfected HT29(ATCC), HT29(US), and B16-F10 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h in the absence of IPTG, followed by 24 h in either the absence or presence of 1 mM IPTG in complete medium. Cell proliferation was evaluated

by the MTS assay, according to the manufacturer's instructions. Note that the MTS assay measures both cell proliferation and viability.

Viability assays. Apoptosis was assessed by flow cytometry following propidium iodide (PI) staining, essentially as described before (20). B16-F10 cells were transfected as described above. After transfection (24 h), cells were harvested, resuspended in PBS containing RNase A and 10 μ g/ml of PI, and analyzed by flow cytometry. The extent of apoptosis was determined by plotting PI fluorescence versus the forward scatter parameter, using the Cell Quest program. For DNA content analysis (cell cycle distribution), cells were permeabilized in methanol and resuspended in PBS containing RNase A and 10 μ g/ml of PI. Samples containing roughly 2×10^4 cells were analyzed using the Cell Quest program.

Luciferase reporter assays. For Tcf/Lef reporter assays, HEK293T, HT29 (ATCC), and HT29(US) cells were transfected with 2 μ g of each plasmid: pTOP-FLASH (Tcf/Lef reporter), pFOP-FLASH (mutated Tcf/Lef binding sites), pLacIOP-caveolin-1, the vector alone (pLacIOP), and/or pBATEM2 (E-cadherin). After transfection (24 h), cells were lysed in a buffer containing 0.1 M KH_2PO_4 (pH 7.9) and 0.5% Triton X-100, and supernatants were used to measure luciferase (50 μ l) activities. Luciferase activity was detected using a luminescence counter (Topcount; Perkin Elmer, MA). Luciferase activity data were standardized for each condition by calculating the pTOP-FLASH/pFOP-FLASH (TOP/FOP) activity ratios. Values shown are means $\pm$ standard errors of the means (SEM) of activity measurements in at least three independent experiments.

Analysis of E-cadherin surface expression by fluorescence-activated cell sorting. HT29(US) wild-type cells, cells expressing caveolin-1 (clone C14), and cells cotransfected with the plasmids pBATEM2 (E-cadherin) and pLacIOP-caveolin-1 were harvested and incubated in 2% bovine serum albumin-PBS for 30 min at room temperature. Samples were then treated with the monoclonal anti-E-cadherin first antibody (1:100), followed by a FITC-coupled goat anti-mouse second antibody (1:200). Finally, cells were resuspended in PBS and analyzed by flow cytometry (FACSCanto; Becton Dickinson, Mountain View, CA). E-cadherin content in transfected and nontransfected cells was determined by quantifying green fluorescence using FACSDiva software.

Statistical analysis. Where pertinent, results were compared by using unpaired or paired *t* tests of two or more independent experiments in triplicate. A *P* value of <0.05 was considered significant.

RESULTS

Cell line-dependent down-regulation of survivin by caveolin-1. HT29(ATCC) cells were stably transfected with either the empty vector (pLacIOP) or the plasmid permitting IPTG-inducible caveolin-1 expression (pLacIOP-caveolin-1). Basal levels of caveolin-1 were augmented in HT29(ATCC) cells stably transfected with pLacIOP-caveolin-1. Furthermore, addition of IPTG increased expression of caveolin-1 in these cells but not in those transfected with pLacIOP alone (Fig. 1A). The presence of caveolin-1 in HT29(ATCC) cells coincided with a reduction in survivin levels that was generally more prevalent in the presence of IPTG (Fig. 1A). Survivin mRNA levels were also reduced by the presence of caveolin-1 in HT29(ATCC) cells, as assessed by semiquantitative PCR (Fig. 1B) and QPCR (Fig. 1C) (see also Table S1 in the supplemental material). In contrast, survivin protein (Fig. 1D) and mRNA levels (Fig. 1E and F) observed in caveolin-1-expressing HT29(US) cells were essentially identical to those in cells transfected with pLacIOP only (Fig. 1D to F). Although changes in mRNA levels observed with these two methods of quantification were not identical, they both suggested that reduced transcription of the survivin gene may account for caveolin-1-induced alterations in survivin protein expression in HT29(ATCC) cells. These changes at the molecular level were accompanied by a significant reduction in cell proliferation due to caveolin-1 presence in HT29(ATCC) but not HT29(US) cells [proliferation in transfected cells compared with mock-

FIG. 1. Ectopic caveolin-1 expression reduced survivin mRNA and protein levels in HT29(ATCC) but not HT29(US) human colon cancer cells. Results obtained with HT29(ATCC) cells (A, B, and C) and HT29(US) cells (D, E, and F) stably transfected with the plasmids pLacIOP and pLacIOP-caveolin-1 are shown. Cells were grown for 24 h in the absence (–) or presence (+) of 1 mM IPTG. (A and D) Total protein extracts from HT29(ATCC) or HT29(US) cells were separated by SDS-PAGE (50 μ g total protein per lane) and analyzed by Western blotting with anti-caveolin-1, antisurvivin, and antiactin antibodies. Survivin levels were quantified by scanning densitometric analysis of Western blots and normalized to actin. Survivin levels in cells with IPTG, pLacIOP-caveolin-1, or both were compared to those in the pLacIOP no-IPTG control. Data are means $\pm$ SEM for three independent experiments. (B and E) Survivin mRNA levels were assessed by semiquantitative RT-PCR analysis in cells grown in the absence (–) or presence (+) of 1 mM IPTG. Actin was used as an internal control. Data are means $\pm$ SEM for three independent experiments. Survivin mRNA content in cells with IPTG, pLacIOP-caveolin-1, or both were compared to the pLacIOP no-IPTG control. (C and F) Survivin mRNA levels were determined by QPCR analysis in cells grown as indicated for panels B and E. 18S rRNA was used as an internal control. Data are means $\pm$ SEM for three independent experiments. Statistically significant differences compared with the pLacIOP no-IPTG control are indicated (*, $P < 0.01$; #, $P < 0.05$).

transfected cells: HT29(ATCC), 67.4% $\pm$ 2.1% ($P < 0.05$), and HT29(US), 95.6% $\pm$ 3.3%]. Thus, caveolin-1 down-regulated survivin and reduced cell proliferation in HT29(ATCC) but not HT29(US) cells.

Expression of β -catenin, E-cadherin, and caveolin-1 in colon adenocarcinoma cells. As expected based on previous results (3), caveolin-1 levels were very low in both HT29(ATCC) and HT29(US) cells, compared to MDCK cells (Fig. 2A). Given our working model indicating that the ability of caveolin-1 to regulate certain genes, including that for survivin, may depend on its capacity to sequester β -catenin (56), we wondered whether β -catenin localization differed in the two cell lines. Indeed, in HT29(ATCC) cells, β -catenin was located at the cell surface, while in HT29(US) cells, β -catenin accumu-

lated predominantly in the nucleus (Fig. 2B). Since E-cadherin is a key regulator of β -catenin localization, and loss of the protein is known to be associated with metastasis, expression and distribution of E-cadherin were also assessed in both cell lines. Indeed, E-cadherin was abundantly expressed (Fig. 2A) and prevalent at the surface of HT29(ATCC) cells (Fig. 2B), while in HT29(US) cells E-cadherin was expressed at low levels (Fig. 2A) and distributed diffusely throughout the cytosol in punctate structures (Fig. 2B).

Caveolin-1 associated with β -catenin in HT29(ATCC) but not in HT29(US) cells. Caveolin-1 recruits β -catenin to a protein complex in detergent-insoluble microdomains (14, 38, 56), thereby precluding transcription of genes, such as those for cyclin D1 (22) and survivin (56). Hence, the hypothesis that

FIG. 2. Expression and localization of β -catenin and E-cadherin in HT29(ATCC) and HT29(US) colon cancer cells. (A) HT29(ATCC), HT29(US), and MDCK cells were cultured for 48 h and then harvested. Protein extracts were prepared, separated by SDS-PAGE (50 μ g total protein per lane), and analyzed by Western blotting with anti-E-cadherin, anti-caveolin-1, and antiactin antibodies. Bands corresponding to caveolin-1 α and -1 β are indicated. (B) HT29(ATCC) and HT29(US) cells were grown for 48 h on glass coverslips, and samples were analyzed by indirect immunofluorescence and confocal microscopy. Cellular localization of β -catenin (β -cat) and E-cadherin (E-cad) are shown in green, while the nucleus was stained with PI. Results are representative of two independent experiments. White arrows indicate areas corresponding to insets showing merged images at a higher magnification. Bar, 10 μ m.

caveolin-1 may not modulate survivin expression in HT29(US) cells because association between caveolin-1 and β -catenin is impaired was tested, initially by comparing the cellular localization of caveolin-1 and β -catenin in HT29(ATCC) and HT29(US) cells. Here, MDCK cells served as a well-established control in which E-cadherin, β -catenin, and caveolin-1 are all known to colocalize at the cell surface (14).

In HT29(ATCC) cells, ectopically expressed caveolin-1 accumulated near the cell surface in a manner similar to that of β -catenin, and an appreciable degree of colocalization was apparent in cells expressing high levels of caveolin-1, as was also the case for MDCK cells (Fig. 3A). In HT29(US) cells, by contrast, β -catenin accumulated predominantly in the nucleus, although some cytoplasmic staining was also detectable. Caveolin-1 was mostly cytoplasmic, with a modest degree of accumulation at the cell surface in or near the plasma membrane (Fig. 3A). As a consequence, colocalization between caveolin-1 and β -catenin was evident in HT29(ATCC) but not in HT29(US) cells (Fig. 3A, merge). Importantly, total β -catenin and E-cadherin levels determined by Western blotting were essentially unaltered in the presence or absence of caveolin-1 in both HT29(ATCC) and HT29(US) cells (Fig. 3B and C, respectively).

Expression of caveolin-1 reduced the nuclear presence of β -catenin and β -catenin-Tcf/Lef reporter activity in HT29(ATCC) cells. Given that nuclear β -catenin is considered the pool essential for β -catenin-Tcf/Lef-dependent transcriptional activity, we also evaluated whether changes were apparent in this respect. First, nuclear β -catenin levels were compared by confocal mi-

croscopy in HT29(ATCC) cells expressing or not expressing caveolin-1. Nuclear β -catenin was clearly more prevalent in cells transfected with pLacIOP (Fig. 4A and C) than in cells transfected with pLacIOP-caveolin-1 (Fig. 4B and D). As a second approach, different subcellular fractions of HT29(ATCC) and HT29(US) cells were prepared using a commercially available kit that relies on sequential extraction steps to obtain cytosolic, membrane/organelle, nucleus, and cytoskeleton fractions. As anticipated, substantially smaller amounts of β -catenin were detectable in the nuclear fraction of HT29(ATCC) cells expressing caveolin-1 than in the same cells without caveolin-1 (Fig. 4E). Alternatively, this was not observed for HT29(US) cells expressing caveolin-1. In all experiments, cyclin D1 was employed as an independent marker for the nuclear fraction.

The results obtained so far linked reduced survivin expression observed upon caveolin-1 expression in HT29(ATCC) cells to more efficient sequestration of β -catenin, possibly within an E-cadherin/caveolin-1 complex at the cell surface, and reduced nuclear presence of β -catenin as a consequence. This being the case, reduced β -catenin-Tcf/Lef reporter activity was expected. Indeed, caveolin-1 presence in HT29(ATCC) cells coincided with substantially reduced reporter activity; the changes were slightly more pronounced when ectopic caveolin-1 expression was enhanced upon induction with IPTG (Fig. 4F).

Survivin regulation in clonal HT29(ATCC) cells expressing caveolin-1. In the experiments described so far, the effects of caveolin-1 expression in HT29(ATCC) and HT29(US) cells

FIG. 3. Localization of β -catenin and caveolin-1 in HT29(ATCC) and HT29(US) cells expressing caveolin-1. (A) HT29(ATCC), HT29(US), and MDCK cells were grown on glass coverslips and incubated for 24 h in the presence of 1 mM IPTG. Samples were analyzed by indirect immunofluorescence and confocal microscopy. Cellular localization of caveolin-1 (cav-1, green) and β -catenin (β -cat, red) is shown. White arrows indicate areas corresponding to insets (bottom row), shown at a higher magnification. Merged images were generated with Imaris software, whereby a third pseudocolor channel (white) was used to show colocalization of red and green pixels. The software provides an automated selection of colocalization based on previously described algorithms (8). Bar, 10 μ m. (B and C) Proteins (50 μ g per lane) from HT29(ATCC) cells (B) and HT29(US) cells (C) transfected with pLacIOP or pLacIOP-caveolin-1 were separated by SDS-PAGE, and the presence of E-cadherin, β -catenin, caveolin-1, and actin was assessed by Western blotting.

were compared, and differences were potentially linked to the presence of E-cadherin in HT29(ATCC) cells. However, an important additional difference that we needed to consider was that HT29(US) cells expressing caveolin-1 were clones, while HT29(ATCC) cells were mixed, nonclonal populations. Thus, to exclude the possibility that these differences (clonal versus nonclonal) might account for the observed alterations in survivin regulation downstream of caveolin-1, individual hygromycin-resistant clones of caveolin-1-expressing HT29(ATCC) cells were isolated. As was apparent by Western blot analysis, caveolin-1 expression was inducible in the two lines isolated (clones 1 and 2), and survivin expression decreased in both cases, particularly in the presence of IPTG (Fig. 5A). Note also that β -catenin and E-cadherin essentially did not change in these two clonal populations compared to pLacIOP- or

pLacIOP-caveolin-1-transfected cell populations (Fig. 5A, batch). In the presence of IPTG, survivin mRNA levels (Fig. 5B; see also Table S3 in the supplemental material) and β -catenin-Tcf/Lef reporter activity (Fig. 5C) were reduced to a similar extent in the two clonal lines and batch-transfected HT29(ATCC) cells. Hence, in terms of survivin regulation downstream of caveolin-1, no significant differences were detectable between the clonal and mixed nonclonal HT29(ATCC) populations. The latter, on the other hand, represented the most appropriate comparison for HT29(US) cells that were cotransfected with plasmids permitting expression of caveolin-1 and E-cadherin (see Fig. 8, 9, and 10).

Coimmunoprecipitation of β -catenin with caveolin-1 was elevated in cells expressing E-cadherin. Caveolin-1 reportedly coimmunoprecipitates with β -catenin and E-cadherin in sev-

FIG. 4. Ectopic caveolin-1 expression reduced the nuclear presence of β -catenin in HT29(ATCC) cells. HT29(ATCC) cells transfected with pLacIOP (A and C) or pLacIOP-caveolin-1 (B and D) were stained with anti- β -catenin antibodies (green) and PI (red). A confocal optical section through the center of the cell is shown for cells lacking (A) or expressing (B) caveolin-1. Bar, 7 μ m. Images in panels C and D correspond to those in A and B, respectively, after processing with Imaris software. The laser confocal image z stack was visualized three-dimensionally using the IsoSurface processing mode, which creates an artificial real-volume object from available voxels. The z axis of the group of cells was rotated to the position indicated (yellow line), and a virtual section through the images was generated at the center of the cells so that the green label inside the nucleus could be clearly identified. (E) HT29(ATCC) and HT29(US) cells transfected with pLacIOP or pLacIOP-caveolin-1 were processed with a proteomic extraction system kit following the manufacturer's instructions to yield subcellular fractions corresponding to cytosol (1), plasma membrane/organelles (2), nucleus (3), and cytoskeleton (4). Proteins from equal volumes of each fraction were concentrated, separated by SDS-PAGE, and analyzed by Western blotting with anti- β -catenin and anti-cyclin D1 antibodies. Cyclin D1 was utilized here as an internal control for the nuclear fraction. (F) HT29(ATCC) cells were cotransfected with 2 μ g of pTOP-FLASH, pFOP-FLASH, pLacIOP, or pLacIOP-caveolin-1 and cultured for 24 h in the absence (-) or presence (+) of 1 mM IPTG. Then, cell extracts were prepared and luciferase activity was determined as described in Materials and Methods. Values for luciferase activity were standardized by calculating the TOP/FOP activity ratio for each condition. Data are means $\pm$ SEM for two independent experiments in triplicate. Statistically significant differences compared with pLacIOP vector alone are indicated (*, $P < 0.01$).

FIG. 5. Characterization of clonal HT29(ATCC) cell populations expressing caveolin-1. (A) Clonal HT29(ATCC) cell populations stably transfected with pLacIOP-caveolin-1 were isolated as described in Materials and Methods from the parental HT29(ATCC) pLacIOP-caveolin-1-transfected population (batch). The pLacIOP- and pLacIOP-caveolin-1 batch-transfected cells, as well as clonal cell populations (clones 1 and 2), were cultured for 24 h in the presence of 1 mM IPTG. Total protein extracts (50 μ g per lane) were separated by SDS-PAGE and analyzed by Western blotting for the presence of E-cadherin (E-cad), β -catenin (β -cat), caveolin-1 (cav-1), survivin, and actin using specific antibodies. (B) Survivin mRNA levels were determined by QPCR in the stably transfected HT29(ATCC) cell populations used for panel A and expressed as a percentage of the value for nontransfected cells. 18S rRNA was used as an internal control. Statistically significant differences compared with the pLacIOP vector are indicated (#, $P < 0.05$). (C) HT29(ATCC) cell populations analyzed for panel A were also transfected with pTOP-FLASH and pFOP-FLASH (2 μ g each). After 24 h, cell extracts were obtained and luciferase activity was determined. Values for luciferase activity were standardized by calculating the TOP/FOP activity ratio for each condition. The data are means $\pm$ SEM from two independent experiments in triplicate. Statistically significant differences compared with pLacIOP vector alone are indicated (*, $P < 0.01$).

eral cell lines (14). Given the immunolocalization and fractionation results, as well as reporter assay data for HT29(ATCC) cells expressing caveolin-1, we next examined the extent to which the three proteins coimmunoprecipitated in cell lines

FIG. 6. Caveolin-1 associated with β -catenin and E-cadherin in several cell lines. (A) HT29(US), DLD1, and HT29(ATCC) cells stably transfected with pLacIOP-caveolin-1 were cultured in presence of 1 mM IPTG for 24 h. MDCK cells at confluence after 48 h in culture were included as a control for endogenous caveolin-1. HEK293T cells were transiently transfected with pLacIOP-caveolin-1 and analyzed 24 h posttransfection in the presence of 1 mM IPTG. Endogenous or ectopically expressed caveolin-1 was immunoprecipitated from cell extracts (500 μ g total proteins). Results obtained with an unrelated antibody (IgG) were included as a negative control. Then caveolin-1 (cav-1), β -catenin (β -cat), and E-cadherin (E-cad) presence was detected by Western blotting. Numerical values shown below individual blots indicate the percentage of total β -catenin averaged from two independent experiments in the respective cell extracts that coimmunoprecipitated with caveolin-1 corrected in each case for efficiency of caveolin-1 immunoprecipitation (percent recovery). (B) HT29(ATCC), HT29(US), and DLD1 cells transfected with pLacIOP vector were cultured in the presence of 1 mM IPTG, and protein extracts were subsequently immunoprecipitated using the conditions described above. Immunoprecipitation of an extract of DLD1 cells stably transfected with pLacIOP-caveolin-1 and cultured in the presence of IPTG was utilized as a positive control.

expressing caveolin-1 either endogenously (MDCK) or after transient (HEK293T) or stable [HT29(ATCC), HT29(US), and DLD1] transfection with pLacIOP-caveolin-1. Readily detectable amounts of β -catenin coimmunoprecipitated with caveolin-1 from transfected HEK293T, DLD1, and HT29(ATCC) cells, as well as MDCK cells (Fig. 6A). However, β -catenin coimmunoprecipitated only poorly with caveolin-1 from HT29(US) cells ectopically expressing the protein (Fig. 6A). Numerical values refer to the fraction of the total β -catenin pool that coimmunoprecipitated with caveolin-1 (β -catenin recovery), after correcting for caveolin-1 immunoprecipitation efficiency in each case. By this criterion, the fraction of total β -catenin that appeared to be sensitive to caveolin-1, as reflected in coimmunoprecipitation with caveolin-1, was reduced 10-fold or more in HT29(US) cells compared with the other E-cadherin-expressing cells. Furthermore, caveolin-1 was abundantly detected in β -catenin immunoprecipitates from MDCK but not HT29(US) cells (data

FIG. 7. Caveolin-1 and E-cadherin cooperated to inhibit β -catenin-Tcf/Lef-dependent transcription. (A) HEK293T cells were either transfected with pLacIOP-caveolin-1 alone (2 μ g) or cotransfected with pLacIOP-caveolin-1 (2 μ g) and pBATEM2 (4 μ g). After 24 h in the presence of 1 mM IPTG, caveolin-1 (cav-1), β -catenin (β -cat), and E-cadherin (E-cad) protein levels were assessed in caveolin-1 immunoprecipitates by Western blotting. (B) HEK293T cells were cotransfected with pTOP-FLASH and pFOP-FLASH and with pLacIOP or pLacIOP-caveolin-1 and in the absence or presence of pBATEM2, coding for E-cadherin. For transfection experiments, 2 μ g of each plasmid was employed. After 24 h in the presence of 1 mM IPTG, cell extracts were obtained and luciferase activity was determined. Values for luciferase activity were standardized by calculating TOP/FOP activity ratios for each condition. The data are means $\pm$ SEM from two independent experiments in triplicate. Statistically significant differences are indicated ([#], $P < 0.05$).

not shown). Finally, E-cadherin was also readily detectable in caveolin-1 immunoprecipitates obtained from HEK293T, MDCK, DLD1, and HT29(ATCC) cells but not those from HT29(US) cells (Fig. 6A). Note that the appearance of β -catenin and E-cadherin in immunoprecipitates requires the expression of caveolin-1, since the corresponding bands were not detected in nontransfected colon adenocarcinoma cells used as controls (Fig. 6B).

Augmented E-cadherin expression in HEK293T cells promoted caveolin-1- β -catenin interaction and caveolin-1-mediated loss of β -catenin-Tcf/Lef-dependent transcriptional activity. To see whether E-cadherin promoted association between caveolin-1 and β -catenin, basal E-cadherin levels were increased in HEK293T cells. Transiently expressed caveolin-1 was immunoprecipitated from cells either cotransfected with pBATEM2 for E-cadherin expression or not. In HEK293T cells with augmented E-cadherin levels, coimmunoprecipitation of E-cadherin and β -catenin with caveolin-1 increased (Fig. 7A). Since E-cadherin promoted association between caveolin-1 and β -catenin in HEK293T cells (Fig. 7A) and caveolin-1 was previously shown to inhibit Tcf/Lef-dependent transcription (14, 38, 56), the effect of caveolin-1 on Tcf/Lef reporter activity in HEK293T cells with increased E-cadherin levels was assessed. The presence of ectopically expressed caveolin-1 or E-cadherin reduced β -catenin-Tcf/Lef-depen-

FIG. 8. Coexpression of E-cadherin and caveolin-1 in HT29(US) cells. HT29(US) cells were cotransfected with pBATEM2 and either pLacIOP or pLacIOP-caveolin-1. Stably transfected cells were selected in the presence of hygromycin as described in the text. These cells were compared to HT29(US) cells transfected with pLacIOP or pLacIOP-caveolin-1. (A) Cell extracts were obtained from each cell line 48 h after plating. Proteins were separated by SDS-PAGE (50 μ g total protein per lane) and analyzed by Western blotting using antibodies specific for E-cadherin (E-cad), caveolin-1 (cav-1), and actin. (B) Cells were grown for 48 h in the presence of 1 mM IPTG, harvested, fixed, and prepared for detection of E-cadherin using a monoclonal antibody and a FITC-coupled second antibody. E-cadherin-specific cell surface staining was visualized by flow cytometry for cells expressing caveolin-1 alone (not shown), E-cadherin (gray line), or caveolin-1 and E-cadherin (black area). Results for a negative control (cells without first antibody) are also shown; the fluorescence was similar to that for caveolin-1 expressed alone (not shown). A representative result from two independent experiments is shown in each panel.

dent reporter activity individually. However, inhibition was more substantial when expression of both proteins was simultaneously increased by cotransfection (Fig. 7B). Taken together, these results in HEK293T cells suggest that E-cadherin promoted association between caveolin-1 and β -catenin and, in doing so, caveolin-1-mediated inhibition of β -catenin-Tcf/Lef-dependent transcription.

E-cadherin expression in HT29(US) promoted association between caveolin-1 and β -catenin. To further substantiate the role of E-cadherin in promoting association between caveolin-1 and β -catenin, E-cadherin was re-expressed in HT29(US) cells. These cells were stably cotransfected (Fig. 8A) with pBATEM2 and either pLacIOP or pLacIOP-caveolin-1. In pBATEM2-transfected HT29(US) cells, E-cadherin expression increased five- to sevenfold with respect to endogenous protein levels. In cells cotransfected with pLacIOP-caveolin-1, expression of caveolin-1 was also increased substantially in the presence of IPTG (Fig. 8A). Importantly, ectopically expressed E-cadherin was detected by flow cytometry at the surface of nonpermeabilized cells following transfection with pBATEM2 alone or upon cotransfection with pBATEM2 and pLacIOP-caveolin-1 (Fig. 8B).

Subsequently, we examined the association between caveo-

lin-1 and β -catenin in transfected cells. As in previous experiments (Fig. 6), minor amounts of β -catenin were detected in caveolin-1 immunoprecipitates from HT29(US) cells expressing caveolin-1 alone. However, in HT29(US) cells expressing both caveolin-1 and E-cadherin, the extent of β -catenin coimmunoprecipitation with caveolin-1 was dramatically increased (Fig. 9A). Importantly, E-cadherin was essentially detected only in immunoprecipitates from HT29(US) cells transfected with pBATEM2 (Fig. 9A). In immunofluorescence studies, ectopically expressed E-cadherin was detected as punctate structures within the cell and at the cell surface, where it colocalized with caveolin-1 (Fig. 9B, left and middle columns). Furthermore, in E-cadherin-expressing cells, endogenous β -catenin accumulated within the nucleus and at the cell surface, where it colocalized with caveolin-1 (Fig. 9B, right column). These observations indicate that E-cadherin facilitates and is required to promote, at least partially, the association between caveolin-1 and β -catenin in HT29(US) cells.

Survivin was down-regulated by caveolin-1 in an E-cadherin-dependent manner. Since re-expression of E-cadherin in HT29(US) cells restored caveolin-1- β -catenin colocalization and association, we next examined whether transcriptional events linked to control of survivin expression became responsive to caveolin-1 when E-cadherin was available. Indeed, the presence of caveolin-1 in E-cadherin expressing HT29(US) cells reduced survivin levels by 40%, and the loss of survivin increased to 62% in cells where caveolin-1 expression was induced with IPTG (Fig. 10A; see also Table S2 in the supplemental material). Similarly, survivin mRNA levels were substantially reduced by caveolin-1 in HT29(US) cells expressing E-cadherin (Fig. 10B). Since caveolin-1 expression was shown to reduce cell proliferation in HT29(ATCC) cells (see above) and other cells (56), we also asked whether E-cadherin sensitized HT29(US) cells to caveolin-1 presence in this respect. Indeed, the presence of caveolin-1 alone had no effect on proliferation of HT29(US) cells. However, proliferation was substantially reduced beyond the effect of E-cadherin alone when caveolin-1 and E-cadherin were coexpressed (Fig. 10C). Furthermore, β -catenin-Tcf/Lef reporter activity was significantly reduced in HT29(US) expressing both caveolin-1 and E-cadherin (Fig. 10D). Taken together, these observations suggest that expression of E-cadherin is required for caveolin-1-dependent transcriptional control of survivin expression and cell proliferation in HT29(US) cells.

Survivin down-regulation and apoptosis upon caveolin-1 expression in B16-F10 melanoma cells. To extend the relevance of our findings, we wished to investigate the effects of caveolin-1 in an additional cancer cell model. Interestingly, high levels of β -catenin have been observed in malignant melanomas. Furthermore, activating mutations in the β -catenin gene (CTNNB1), as well as genetic and epigenetic alterations of the APC gene, occur in malignant melanomas and are associated with melanoma progression (26, 48, 62). Thus, as in colon cancer, aberrant Wnt signaling is relevant to the genesis and progression of melanomas. Here, the highly metastatic murine melanoma cell line B16-F10 was chosen, since endogenous expression of both caveolin-1 and E-cadherin was low in these cells (Fig. 11A). Unlike for HT29(US) cells, where cotransfection and subsequent selection using hygromycin yielded mixed, nonclonal cell populations with significant increases in both

FIG. 9. E-cadherin promoted the association of caveolin-1 with β -catenin in HT29(US) cells. HT29(US) cells transfected with pLacIOP-caveolin-1 (cav-1) or HT29(US) cells cotransfected with pLacIOP-caveolin-1 and pBATEM2 (cav-1 + E-cad) were cultured for 24 h in the presence of 1 mM IPTG. (A) Cell extracts were obtained and caveolin-1 was immunoprecipitated. Thereafter, E-cadherin (E-cad) and β -catenin (β -cat) levels were assessed in caveolin-1 (cav-1) immunoprecipitates by Western blotting. (B) Caveolin-1, β -catenin, and E-cadherin localization was assessed by indirect immunofluorescence and confocal microscopy. White arrows indicate areas shown in insets at higher magnifications. Merged images were generated with Imaris software as described for Fig. 3. Bar, 10 μ m.

caveolin-1 and E-cadherin (Fig. 8), this approach did not work well for B16-F10 cells. Thus, an alternative approach was utilized. B16-F10 cells were first transfected with either pLacIOP or pLacIOP-caveolin-1, and stably transfected populations were obtained by selection in hygromycin. Thereafter, pLacIOP or pLacIOP-caveolin-1 cells were transiently transfected with the plasmid pBATEM2 as described in Materials and Methods. Efficiency of transfection was again 40 to 50%, as detected by flow cytometry, following transfection with green fluorescent protein-caveolin-1 as a marker (data not shown). As expected, caveolin-1 alone had no or little effect on endogenous survivin. However, when coexpressed with E-cadherin, caveolin-1 sub-

stantially reduced survivin protein levels (Fig. 11A) and mRNA levels (data not shown). Also, caveolin-1 presence reduced proliferation of B16-F10 cells in an E-cadherin-dependent manner (Fig. 11B), as seen in HT29(US) cells (Fig. 10C). Thus, the ability of E-cadherin to promote caveolin-1- β -catenin association and to facilitate caveolin-1-dependent transcriptional regulation of effector genes like those for survivin and control of cell proliferation appears to represent a generally valid phenomenon. In addition, cell cycle distribution was also assessed here as previously described (56). We found that expression of either caveolin-1 or E-cadherin caused slight alterations in cell cycle distribution, but changes in the S and

FIG. 10. Re-expression of E-cadherin in HT29(US) cells restored caveolin-1-mediated inhibition of survivin expression and cell proliferation. (A) HT29(US) cells stably cotransfected with pBATEM2 and pLacIOP or pLacIOP-caveolin-1 were grown for 24 h in the absence (–) or presence (+) of 1 mM IPTG. Then, cell extracts were obtained for analysis. Following protein separation by SDS-PAGE (50 μ g total protein per lane), E-cadherin (E-cad), caveolin-1 (cav-1), survivin, and actin levels were assessed by Western blotting. Survivin levels were quantified by scanning densitometric analysis of Western blots and normalized to actin. Survivin levels in cells with IPTG, pLacIOP-caveolin-1, or both were compared to those in the pLacIOP no-IPTG control. Data are means $\pm$ SEM for three independent experiments. Statistically significant differences compared with the control are indicated (*, $P < 0.01$). (B) Survivin mRNA levels were assessed by QPCR analysis in HT29(US) cells stably cotransfected as indicated above. 18S rRNA was used as an internal control. Statistically significant differences compared with the control are indicated (#, $P < 0.05$). (C) Cell proliferation was measured by the MTS assay in HT29(US) cells stably transfected with pBATEM2 (E-cad) and either pLacIOP (–cav1) or pLacIOP-caveolin-1 (+cav1). Here, IPTG was present at 1 mM in all four conditions evaluated. Data are means $\pm$ SEM from two independent experiments in triplicate. Statistically significant differences are indicated (#, $P < 0.05$). (D) HT29(US) cells were transfected with the plasmids used for panel C together with pTOP-FLASH and pFOP-FLASH (2 μ g each). After 24 h, cell extracts were obtained and luciferase activity was determined. Values for luciferase activity were standardized by calculating the TOP/FOP activity ratio for each condition. Data are means $\pm$ SEM from two independent experiments in triplicate. Statistically significant differences are indicated (*, $P < 0.01$).

G₂/M phases of the cell cycle were more pronounced upon simultaneous expression of both proteins. Furthermore, an increase in the hypodiploid sub-G₀/G₁ population, indicative of apoptosis, was detected (Fig. 11C). Apoptosis, as assessed by an alternative method (20), was greatest in cells expressing caveolin-1 together with E-cadherin (Fig. 11D). Thus, in melanomas the coexpression of caveolin-1 together with E-cadherin resulted in a loss of survivin expression, reduced proliferation, and changes in the cell cycle highly reminiscent of those previously reported for HEK293T cells upon expression of caveolin-1 alone (56).

DISCUSSION

Caveolin-1 reportedly functions as a tumor suppressor, possibly as a consequence of its inhibitory role in cell signaling (see the introduction). Specifically, caveolin-1 mRNA and protein levels are down-regulated in several human tumors and cancer

cell lines. Furthermore, reexpression of caveolin-1 in cancer cells or oncogenically transformed cell lines inhibits tumor cell growth and reduces tumorigenicity (3, 29, 46, 59, 60). In this respect, prostate cancer is a notable exception. Caveolin-1 is not expressed in normal prostate tissue but increases during tumor development, where its presence favors metastatic activity. Moreover, expression of caveolin-1 in prostate tumors correlates with increased metastatic potential and poor patient prognosis (32, 63, 64). Interestingly, even in epithelial cells from tissues where loss of caveolin-1 expression, generally via transcriptional silencing, is associated with tumor development, reexpression at later stages can occur. Frequently, caveolin-1 reappearance is then associated with enhanced cell survival and metastasis. For instance, increases in caveolin-1 expression have been reported in multidrug-resistant HT29 colon and MCF7 breast cancer cells (3, 27, 28), as well as in colon and lung cancer cells with elevated metastatic potential (3, 21). Increased caveolin-1 expression has also been reported

FIG. 11. Expression of E-cadherin in the metastatic melanoma B16-F10 cell line facilitated caveolin-1-mediated down-regulation of survivin and cell cycle control. B16-F10 murine melanoma cells stably transfected with either pLacIOP or pLacIOP-caveolin-1 were grown for 24 h in complete medium and then transfected with the plasmid pBATEM2 by electroporation, as described in Materials and Methods. Thereafter, cells were grown in the presence of 1 mM IPTG for 48 h and then subjected to analysis. (A) Cell extracts were obtained and analyzed by Western blotting. A result representative of three independent experiments is shown. Residual survivin levels in cells transfected with pLacIOP-caveolin-1, pLacIOP/pBATEM2, or pLacIOP-caveolin-1/pBATEM2 were compared to levels in cells transfected with pLacIOP alone (control). Data are means $\pm$ SEM for three independent experiments. Statistically significant differences compared with the pLacIOP control are indicated (*, $P < 0.01$). (B) Cell proliferation was measured by the MTS assay. Data were averaged from three independent experiments (means $\pm$ SEM; #, $P < 0.05$). (C) Cell cycle distribution was assessed by flow cytometry. Data are representative of three independent experiments. (D) To determine apoptosis, cells were harvested, resuspended in PBS with 10 μ g/ml PI, and analyzed by flow cytometry. Data are means $\pm$ SEM from three independent experiments. Statistically significant differences detected with an unpaired (#, $P < 0.05$) or paired (&, $P < 0.01$) t test are indicated.

for infiltrating ductal human breast cancer specimens (63). Here again, the data are contradictory, since caveolin-1 expression in MTLn3 breast cancer cells blocks traits associated with elevated metastatic potential (66). Alternatively, in MCF7 breast cancer cells, caveolin-1 promotes anchorage-independent growth but reduces matrix metalloproteinase secretion associated with metastasis (13). Also, enhanced activity of metalloproteinases 2 and 9 observed in breast epithelial cells from caveolin-1 knockout mice is suppressed by caveolin-1 reexpression (52).

Taken together, at least two conclusions may be drawn from these data. First, the role that caveolin-1 plays in cancer is tissue specific; second, the impact of expression of caveolin-1 at the onset of cancer and at later stages of the disease may differ substantially even in cells of the same epithelial origin (45). The mechanisms underlying these radical changes in caveolin-1 function have not been defined to date. Our results indicate that expression of E-cadherin is critical in this respect. Loss of E-cadherin, frequently observed in epithelial tumors, is considered a crucial event that favors metastasis (6). In our study, the ability of caveolin-1 to inhibit cell proliferation and reduce survivin expression was drastically reduced in two metastatic cell models essentially devoid of E-cadherin expression. Thus, characteristics potentially relevant to caveolin-1 function

as a tumor suppressor are compromised in E-cadherin-deficient cells.

Caveolin-1 typically inhibits the activity of a large number of signaling proteins via an interaction involving the scaffolding domain of caveolin-1 and a scaffolding domain-binding domain motif in the respective target proteins (42, 47). However, alternative modes of caveolin-1 action exist, such as inhibition of Src via phosphorylation of caveolin-1 in tyrosine 14 (5). Also, caveolin-1 association with inducible nitric oxide synthase via a putative WW domain element is suggested to promote degradation of inducible nitric oxide synthase via the proteasome pathway in detergent-resistant membrane microdomains (11, 12). In summary, caveolin-1 often restricts signaling via pathways that promote cell proliferation and survival by direct physical interaction with and inhibition of target protein function.

Evidence from in vitro and in situ assays also indicates that caveolin-1 associates with β -catenin and recruits the protein to caveolar microdomains (14). Furthermore, inhibition of β -catenin-Tcf/Lef-dependent transcription by caveolin-1, due to association between these proteins (14, 38, 56), leads to inhibition of the expression of genes, such as those for cyclin D1 and survivin, involved in the regulation of cell proliferation and apoptosis (22, 56). Despite this well-established link be-

tween caveolin-1 and β -catenin, no results demonstrating direct binding between these two proteins are available. Our results indicate that direct caveolin-1- β -catenin association either does not exist or is of such low affinity in HT29(US) cells that it cannot be detected under the experimental conditions employed, while this was not the case in HT29(ATCC) cells.

Consistent with the possibility that E-cadherin presence may promote or even be required for caveolin-1- β -catenin complex formation, all cells with readily detectable E-cadherin levels displayed significantly elevated amounts of β -catenin associated with caveolin-1 compared with caveolin-1-transfected HT29(US) cells in coimmunoprecipitation experiments (Fig. 6). Furthermore, coimmunolocalization of caveolin-1 and β -catenin was also substantially enhanced in cells expressing E-cadherin (Fig. 3A). Quite remarkably, coexpression of E-cadherin in HT29(US) cells with caveolin-1 was sufficient to augment coimmunoprecipitation efficiency as well as colocalization of β -catenin with caveolin-1 (Fig. 9). An intriguing interpretation of these results, therefore, is that E-cadherin, a protein that typically binds directly to β -catenin at the plasma membrane, is required for caveolin-1- β -catenin complex formation.

Although endogenous β -catenin was localized at the surface of HT29(US) cells coexpressing E-cadherin and caveolin-1, a substantial amount of β -catenin remained transcriptionally active within the nucleus (Fig. 9 and 10). One possible interpretation is that the nuclear β -catenin pool is heterogeneous and composed of different " β -catenin populations," only some of which are sensitive to the presence of caveolin-1. The existence of different subpopulations within compartmentalized β -catenin pools was suggested previously (18). Furthermore, β -catenin is known to be regulated by several serine/threonine kinases that modulate subcellular distribution and stability (9, 16, 25, 34, 55). Also, tyrosine phosphorylation by Fer, cMet, Abl, and Src kinases reportedly precludes association of β -catenin with cadherin complexes at the plasma membrane (4, 17, 19, 33, 44). Such modifications may also be expected to prevent β -catenin sequestration at the plasma membrane by the caveolin-1-E-cadherin complex. Clearly, there are a number of possible explanations for such variations. The fact that transcriptional reporter activity was never completely suppressed in any of the cells tested (Fig. 4, 5, 7, and 10) suggests that such restraints exist in a variety of experimental models. In each case, however, the mechanism responsible may be distinct. Thus, an interesting goal for future research will be to define factors that modulate the extent to which the caveolin-1-E-cadherin complex is able to sequester β -catenin at the membrane.

The existence of an intricate relationship between caveolin-1 and E-cadherin was established when caveolin-1 was shown to promote E-cadherin expression by reducing β -catenin-Tcf/Lef-dependent transcription of the transcriptional repressor Snail (38). However, we did not detect significant changes in E-cadherin levels upon ectopic expression of caveolin-1 in either HT29(ATCC) or HT29(US) cells (Fig. 3B, 3C, and 5A). In HT29(US) cells, residual E-cadherin expression was apparent at intracellular sites rather than the cell surface (Fig. 2). Indeed, the distribution of E-cadherin in HT29(US) cells was to some extent reminiscent of that reported for E-cadherin in breast epithelial cells from caveolin-1 knockout mice, where

absence of caveolin-1 contributes to hyperproliferation (52). Our observations suggest that, in addition to regulating the expression of E-cadherin (38), caveolin-1 may also contribute to defining the subcellular distribution of E-cadherin (Fig. 9B). Consistent with this interpretation, spontaneous epithelial-to-mesenchymal transition with reduced cell-cell attachment and E-cadherin redistribution from plasma membrane to intracellular compartments is observed in mammary epithelial cells from caveolin-1-null mice (52). Furthermore, caveolin-1 promotes membrane association of E-cadherin and thereby favors cell-cell adhesion as well as stabilizes adherent junctions by a mechanism involving inhibition of Src family kinases (41).

Rather intriguingly, the caveolin-1-E-cadherin connection appears to be reciprocal, since ectopically expressed caveolin-1 was detectable preferentially at intracellular sites with only little accumulation at the cell surface in HT29(US) cells lacking E-cadherin (Fig. 3A). Coexpression of caveolin-1 with E-cadherin clearly augmented the presence of caveolin-1 at the cell surface in these cells. This was most obvious at sites of cell-cell contact, as was the colocalization with β -catenin (Fig. 9). Thus, E-cadherin promotes caveolin-1 colocalization at the cell surface as well as complex formation with β -catenin and in doing so is likely to favor caveolin-1-mediated inhibition of β -catenin-Tcf/Lef-dependent transcription.

Caveolin-1 inhibits β -catenin-dependent transcription and thereby controls specific aspects of the cell cycle in a cell-specific fashion (22, 56). Here, the role of E-cadherin in promoting such functions of caveolin-1 was evaluated. In HT29(US) cells, re-expression of E-cadherin was sufficient to restore the ability of caveolin-1 to promote down-regulation of survivin mRNA and protein levels, suppress β -catenin-Tcf/Lef-dependent transcription, and decrease proliferation, as seen in HT29(ATCC) cells (compare Fig. 1, 4F, and 10). Likewise, in HEK293T cells, which express low levels of E-cadherin, ectopic expression of this protein together with caveolin-1 augmented the efficiency with which caveolin-1 suppressed β -catenin-Tcf/Lef-dependent transcription (Fig. 7). Finally, in B16-F10 melanoma cells, another model system with very low E-cadherin levels, the ability of caveolin-1 to control survivin expression and proliferation was greatly enhanced by coexpression of E-cadherin (Fig. 11). These results are consistent with the interpretation that E-cadherin promotes characteristics of caveolin-1 associated with its ability to regulate at the transcriptional level the expression of survivin and, in doing so, cell proliferation. VE-cadherin- and E-cadherin-dependent regulation of survivin expression was noted previously (23). Our results extend these observations by identifying caveolin-1 as a protein that requires E-cadherin presence to display a number of characteristics potentially relevant to its ability to function as a tumor suppressor, including the suppression of survivin expression.

Metastasis requires reduced susceptibility to apoptosis, particularly upon loss of cell-cell or cell-matrix interactions (40). Increased cell death as a consequence of caveolin-1 expression has been reported, although the underlying mechanisms were not defined (35, 59). Interestingly, hyperactivation of β -catenin-Tcf/Lef-dependent transcription is associated with aberrant cell proliferation, hyperplasia, and impaired cell-cell adhesion in caveolin-1 knockout mice (31, 51). Data from this laboratory linked caveolin-1-mediated cell death to transcrip-

tional suppression of survivin, particularly in HEK293T cells (56). Clearly, the ability of caveolin-1 to reduce proliferation is impaired in metastatic HT29(US) (Fig. 10 and data not shown) and B16-F10 (Fig. 11) cells deficient in E-cadherin. Forced expression of E-cadherin alone had essentially no significant effect on survivin expression in the absence of caveolin-1 in HT29(US) and B16-F10 cells (Fig. 10A and 11A, respectively). However, while the substantially increased loss of survivin upon caveolin-1 and E-cadherin coexpression was paralleled by reduced proliferation in both cases (Fig. 10C and 11B), significantly augmented apoptosis was detectable only in B16-F10 cells (Fig. 11C and 11D), not in HT29(US) cells (data not shown). Thus, although the results presented here show that caveolin-1 requires E-cadherin to suppress survivin expression and reduce cell proliferation, they should not be taken to imply that loss of survivin alone accounts entirely for the changes in proliferation or that the consequences of such loss are similar in all cases. Given the plethora of genes that are regulated by β -catenin-Tcf/Lef-dependent transcription (see references in the introduction and <http://www.stanford.edu/%7Eernusse/wntwindow.html>), such variations between cell types are not surprising.

In the experiments described here, E-cadherin was introduced into cells using pBATEM2. This vector does not contain a marker for selection in transfected eukaryotic cells. Thus, expression of E-cadherin was ensured in our experiments by cotransfection with pLacIOP or pLacIOP-caveolin-1 and subsequent selection with hygromycin, the resistance marker of pLacIOP. We did notice, however, that initially observed changes in HT29(US) cells, including E-cadherin expression, were lost after a limited number of passages following the initial selection procedure. This outcome was to some extent predictable, since cells expressing both E-cadherin and caveolin-1 proliferated less rapidly than cells expressing caveolin-1 alone. Thus, to ensure that the effect of caveolin-1 on transcriptional regulation of survivin endured over time, HT29(ATCC) clones expressing caveolin-1 were isolated and characterized. Indeed, caveolin-1-expressing HT29(ATCC) clones behaved similarly to the mixed, nonclonal population (Fig. 1 to 4) with respect to down-regulation of survivin mRNA and protein levels and β -catenin-Tcf/Lef reporter activity. Somewhat surprisingly, however, the ability of caveolin-1 expression to reduce cell proliferation was lost in these clonal populations (data not shown). Thus, while caveolin-1-mediated down-regulation of survivin was maintained upon cloning, the ability to reduce proliferation was not. This can be taken to indicate that the cloning procedure selected for cells in the nonclonal population with mechanisms in place that are sufficient to bypass limitations imposed by caveolin-1 presence and reduced expression of survivin.

In summary, we show here (i) that the ability of caveolin-1 to regulate survivin expression and cell proliferation is severely impaired in metastatic cancer cells lacking E-cadherin, (ii) that these caveolin-1 traits are restored in the presence of E-cadherin, and (iii) that E-cadherin promotes colocalization and coimmunoprecipitation of caveolin-1 with β -catenin, as well as inhibition of β -catenin-Tcf/Lef dependent transcription. Loss of E-cadherin is frequently observed in human epithelial tumors (6). Thus, combined removal of caveolin-1 and E-cadherin from epithelial cells is likely to accelerate loss of cell-cell contacts, epithelial-mesenchymal transition, and transformation. Moreover, and

perhaps most importantly, loss of E-cadherin contributes to creating a permissive cellular environment where re-expression of caveolin-1 at a later stage in tumor progression need not be linked to detrimental effects for tumor growth. Instead, traits of this protein associated with malignant tumor progression are more likely to prevail.

ACKNOWLEDGMENTS

Amparo Cano and Hans Clevers are gratefully acknowledged for providing the pBATEM2 plasmid and TOP-FLASH/FOP-FLASH reporter constructs, respectively. We thank Freddy Meza and Stefan Hartel for assistance with the evaluation of colocalization experiments, as well as David Mears for careful reading of the manuscript.

This work was supported by FONDAP 15010006 (to A.F.G.Q.), FONDECYT 3050037 (to J.C.T.), FONDECYT 1070699 (to L.L.), CONICYT Ph.D. fellowships (to A.L. and V.A.T.), a MECESUP Ph.D. fellowship (to D.A.R.), and an ICBM Ph.D. fellowship (to C.A.).

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