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Caveolin-1 mediates the expression and localization of cathepsin B, pro-urokinase plasminogen activator and their cell-surface receptors in human colorectal carcinoma cells

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Summary
Cathepsin B and pro-urokinase plasminogen activator (pro-uPA) localize to the caveolae of HCT 116 human colorectal carcinoma cells, an association mediated by active K-RAS. In this study, we established a stable HCT 116 cell line with a gene encoding antisense caveolin-1 (AS-cav-1) to examine the effects of caveolin-1, the main structural protein of caveolae, on the expression and localization of cathepsin B and pro-uPA, and their cell-surface receptors p11 and uPA receptor (uPAR), respectively. AS-cav-1 HCT 116 cells secreted less procathepsin B than control (empty vector) cells as measured by immunoblotting and pepsin activation of the proenzyme. Expression and secretion of pro-uPA was also downregulated in AS-cav-1 HCT 116 cells. Localization of cathepsin B and pro-uPA to caveolae was reduced in AS-cav-1 HCT 116 cells, and these cells expressed less total and caveolae-associated p11 and uPAR compared with control cells. Previous studies have shown that uPAR forms a complex with caveolin-1 and β1-integrin, and we here show that downregulation of caveolin-1 also suppressed the localization of β1-integrin to caveolae of these cells. Finally, downregulation of caveolin-1 in HCT 116 cells inhibited degradation of the extracellular matrix protein collagen IV and the invasion of these cells through Matrigel. Based on these results, we hypothesize that caveolin-1 affects the expression and localization of cathepsin B and pro-uPA, and their receptors, thereby mediating cell-surface proteolytic events associated with invasion of colon cancer cells.

Key words: Colorectal carcinoma, Caveolin-1, Proteases, Proteolysis, Invasion

Introduction
Extracellular matrix (ECM) degradation by proteases occurs at many stages of malignant progression, including local invasion, angiogenesis and intravasation and extravasation of tumors during metastasis (Liotta et al., 1983; McCawley and Matrisian, 2001). Proteases of the plasminogen-activator system, matrix metalloproteinases (MMPs) and cysteine proteases such as cathepsin B have all been implicated in these invasive processes (Koblinski et al., 2000; Mignatti et al., 1986; Sloane et al., 2005). Localization of proteases to tumor-cell surfaces and secretion into the extracellular milieu gives these enzymes access to the ECM (Cavallo-Medved and Sloane, 2003; Koblinski et al., 2000). Evidence also shows the involvement of proteases in the intracellular degradation of ECM proteins (Everts et al., 1996; Kjoller et al., 2004; Sameni et al., 2000; Sameni et al., 2003).

In colorectal carcinomas, increased expression and activity of proteases contribute to degradation events during tumor invasion. Indeed, high levels of the lysosomal cysteine-protease cathepsin B in colon cancer patients are predictive of shorter overall survival (Campo et al., 1994). Furthermore, translocation of cathepsin B from the apical region of the cells to the basal plasma membranes parallels malignant progression of colon cancer, occurring in late adenomas and early carcinomas (Campo et al., 1994). The cathepsin B associated with the basal plasma membrane of human colon tumors is active (Hazen et al., 2000). Increases in expression of cathepsin B and translocation of active cathepsin B to the basal membrane are also observed during the progression of adenomas in Min mice (Marten et al., 2002), a model for familial adenomatous polyposis in humans (for a review, see Dove et al., 1995). Membrane-associated cathepsin B can be isolated from a range of cancer cells (Rozhin et al., 1987; Rozhin et al., 1994), and activities can be assayed on the surface of living cells in culture (Linebaugh et al., 1999), suggesting a functional role for this enzyme on tumor-cell surfaces.

Elevated levels of uPA (urokinase plasminogen activator) and its receptor, uPAR, have been associated with a negative prognosis (Ganesh et al., 1994) and distant metastases (Yang et al., 2000) of colorectal cancer. uPAR has also been localized to the invasive front of colon adenocarcinomas (Pyke et al., 1991). Antisense downregulation of uPAR expression both reduced receptor-bound uPA activities and ECM degradation
in vitro, and suppressed colon cancer metastasis in an in vivo nude mouse model (Wang et al., 2001). A functional role for uPA is to convert plasminogen to plasmin, a serine protease capable of degrading key ECM components and activating other zymogen proteases such as pro-MMP-9 and pro-MMP-3 (Davis et al., 2001; Hahn-Dantona et al., 1999; Ramos-DeSimone et al., 1999). Correlations have been made between expression of individual MMPs (e.g. MMP-1 and MMP-13) (Leeman et al., 2002; Murray et al., 1996) and poor prognosis in colorectal cancer (Leeman et al., 2003). In colorectal tumor tissues, the expression and activities of MMP-1, MMP-2, MMP-3 and MMP-9 are upregulated compared with normal mucosal tissue (Baker and Leaper, 2003). Moreover, the level of tissue inhibitor of metalloproteinase-2 (TIMP-2) was significantly decreased in these colorectal tumor tissues (Baker and Leaper, 2003). Hence, this suggests that, in colon cancer, the balance between proteases and inhibitors, and the complex interactions between protease systems are important to tumorigenesis.

Many proteases implicated in the progression and invasion of colorectal tumors have been found to associate with caveolae (Cavallo-Medved and Sloane, 2003), a region of the plasma membrane involved in endocytosis, cholesterol transport and cell signaling events (for reviews, see Anderson, 1998; Williams and Lisanti, 2004). Our laboratory recently reported the localization of cathepsin B to caveolae of HCT 116 human colorectal carcinoma cells (Cavallo-Medved et al., 2003). HCT 116 cells contain a mutant K-ras gene, disruption of which by homologous recombination (Shirasawa et al., 1993) led to a decrease in cathepsin-B trafficking to caveolae and a decrease in pericellular (membrane-associated and secreted) activity of the enzyme (Cavallo-Medved et al., 2003). Localization of active cathepsin C and p11 (the light chain of the annexin II tetramer (AIIt) and a cathepsin-B-binding protein (Mai et al., 2000)) to caveolae is intriguing because cathepsin B activates soluble and membrane-bound pro-uPA (Ikeda et al., 2000; Kobayashi et al., 1991) and p11 is involved in the binding and activation of plasminogen (Zhang et al., 2004). The loss of mutant K-ras in HCT 116 cells also suppressed the localization of uPA and uPAR to caveolae of these cells (Cavallo-Medved et al., 2003). Several MMPs, such as MMP-2 and membrane-type-1 MMP (MT1-MMP), have been localized to caveolae of glioblastomas, fibrosarcomas and endothelial cells, as have TIMP-2 and αvβ3 integrin, an integrin that acts as a receptor for MMP-2 (Annabi et al., 2001; Leeman et al., 2002; Murray et al., 1996) and poor prognosis in colorectal cancer. Hence, this suggests that, in colon cancer, the balance between proteases and inhibitors, and the complex interactions between protease systems are important to tumorigenesis.

Materials and Methods
Materials
Dulbecco’s minimal Eagle’s medium (DMEM), bovine serum albumin (BSA), antibiotics, DNA (salmon sperm), octylglucoside, 2-[[N-morpholino]ethanesulfonic acid (MES) and all other chemicals, unless otherwise stated, were from Sigma (St Louis, MO). Fetal bovine serum (FBS), fetal calf serum (FCS) and LipofectAmine were from Invitrogen Life Technologies. Polyclonal anti-caveolin-1 (610059) and monoclonal anti-p11 (mAb 148) antibodies were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-cathepsin-B antibodies (Moin et al., 1992) were produced and characterized in our laboratory. Polyclonal anti-uPA and polyclonal anti-uPAR antibodies were a kind gift from G. Hoyer-Hansen (The Finsen Center, Copenhagen, Denmark). Polyclonal anti-MMP-2 (AB19145), polyclonal anti-MMP-9 (AB16996), polyclonal anti-MT1-MMP (AB8345) and monoclonal anti-β1-integrin (MAB2247) antibodies were from Chemicon (Temecula, CA). Horseradish-peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG, and micro-bicinchoninic acid (BCA) reagents were from Pierce (Rockford, IL). Texas-Red-conjugated affinity-purified donkey anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Quenched fluorescent type-IV collagen (DQ-collagen IV), SYBR Green I nucleic-acid stain and SlowFade antifade reagent were from Molecular Probes (Eugene, OR). Matrigel was from BD Biosciences (Bedford, MA). Caveolin-encoding cDNA was from American Type Culture Collection (Rockville, MD). PCR primers were from Integrated DNA Technologies (Corvalde, IA). Vectors were from Stratagene (La Jolla, CA). Diff Quik stain set was from Dade Behring (Newark, DE). Acrylamide and nitrocellulose membranes were from BioRad (Hercules, CA). Prestained protein markers and chemiluminescent western-blotting detection kits were from Amersham Pharmacia Biotech (Piscataway, NJ). Benzoxycarbonyl-L-arginyl-L-arginine-4-methyl-7-coumarylamide (Z-Arg-Arg-CH2Cl) was from Molecular Probes (Eugene, OR). Centrifugal filters were from Millipore (Bedford, MA). Caveolin-encoding cDNA was from Transduction Laboratories (Lexington, KY). Polyclonal anti-uPA and polyclonal anti-uPAR antibodies were a kind gift from G. Hoyer-Hansen (The Finsen Center, Copenhagen, Denmark). Polyclonal anti-MMP-2 (AB19145), polyclonal anti-MMP-9 (AB16996), polyclonal anti-MT1-MMP (AB8345) and monoclonal anti-β1-integrin (MAB2247) antibodies were from Chemicon (Temecula, CA). Horseradish-peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG, and micro-bicinchoninic acid (BCA) reagents were from Pierce (Rockford, IL). Texas-Red-conjugated affinity-purified donkey anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Quenched fluorescent type-IV collagen (DQ-collagen IV), SYBR Green I nucleic-acid stain and SlowFade antifade reagent were from Molecular Probes (Eugene, OR). Matrigel was from BD Biosciences (Bedford, MA). Caveolin-encoding cDNA was from American Type Culture Collection (Rockville, MD). PCR primers were from Integrated DNA Technologies (Corvalde, IA). Vectors were from Stratagene (La Jolla, CA). Diff Quik stain set was from Dade Behring (Newark, DE). Acrylamide and nitrocellulose membranes were from BioRad (Hercules, CA). Prestained protein markers and chemiluminescent western-blotting detection kits were from Amersham Pharmacia Biotech (Piscataway, NJ). Benzoxycarbonyl-L-arginyl-L-arginine-4-methyl-7-coumarylamide (Z-Arg-Arg-CH2Cl) was from Molecular Probes (Eugene, OR). Centrifugal filters were from Millipore (Bedford, MA). Polycarbonate filters (polyvinylpyrrolidone, 12 μm pore, 13 mm diameter) were from Poretics (Livermore, CA). Findex grids were from Ted Pella (Redding, CA).

Cell culture
HCT 116 and NIH 3T3 cells were from the American Type Culture Collection (Rockville, MD) and grown in DMEM with glutamine, 4.5 g 1-1 glucose, antibiotics (penicillin and streptomycin) and 10% (v/v) FBS (for HCT 116 cells) or 5% (v/v) FCS (for NIH 3T3 cells) in 5% CO2 in a humidified atmosphere at 37°C.

Construction of caveolin-1 antisense vector
The cDNA fragment encoding human caveolin-1 was amplified by PCR using a human caveolin-1-encoding cDNA plasmid (ATCC, Manassas, VA) as a template. The primer pair used for the amplification was: 5′-GCCGCGGACGCTCATTTTCTTTCGCAAGTGA-3′ (containing the underlined SacI site); 3′ primer, 5′-GCCGCCCAGCCGATGCTTGGGCGAACATCGT-3′ (containing the underlined Smal site). The amplified cDNA fragment in the antisense orientation was subsequently cloned into the corresponding multiple cloning sites of the pcRES-hrGFP-1a vector to form the pAnti-caveolin-1-RES-hrGFP-1a vector. The pAnti-caveolin-1-RES-hrGFP-1a vector and the control vector pcRES-hrGFP-1a were then individually recombined with pEXchange-Puro.
recombinant vector according to manufacturer’s instructions. The identities and orientations of the resulting pAnti-caveolin-1-IREShGFP-1a-puro vector and pIREShGFP-1a-puro vector were confirmed by DNA sequencing.

Establishment of stable caveolin-1 antisense HCT 116 cell lines
Parental HCT 116 cells grown to 60% confluence were transfected with antisense caveolin-1 (AS-cav-1) or empty (control) pIREShGFP-1a recombinant vectors using LipofectAmine reagent according to the manufacturer’s instructions. Transfected cultures were selected with puromycin (0.5 µg ml⁻¹) for 15 days at 37°C and antibiotic-resistant colonies were picked and grown under selective conditions for further analysis.

Semiquantitative reverse-transcription PCR
For reverse-transcription PCR (RT-PCR), total RNA was isolated from cells transfected with mock or antisense vectors using a RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed. 1 µg total RNA was annealed with 0.5 µg oligo-dT₁₅ and reverse transcribed in a 20 µl volume containing 1× reverse-transcription buffer (Promega, Madison, WI), 0.1 mg ml⁻¹ BSA, 40 U RNasin (Promega, Madison, WI), 1 mM dNTPs and 200 U mouse Moloney leukemia virus reverse transcriptase (Promega, Madison, WI) at 37°C for 20 minutes. A 5 µl aliquot was used for subsequent PCR reactions using the PCR Master Mix from Promega (Madison, WI). For β₂-microglobulin (150-bp product), p11 (317-bp product) and caveolin-1 (557-bp product), the amplification conditions were as follows: 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by a final 10-minute extension at 72°C. PCR products were resolved on a 1.2% agarose gel stained with ethidium bromide. The primer sequences were as follows:

- β₂-microglobulin forward, 5′-TTAGCTGTGTCTGCGCTACTTCCTTC-3′;
- β₂-microglobulin reverse, 5′-GCCGCCGAGCTCTATTT-3′;
- p11 forward, 5′-AACCCAGACACA-3′;
- p11 reverse, 5′-CTTCTCAAATGGAACA-3′;
- caveolin-1 forward, 5′-AACCAGACACA-3′;
- caveolin-1 reverse, 5′-CTCTATTT-3′;
- cathepsin-B forward, 5′-GTCGGATGGATGA-GTCGGATGGATGA-3′;
- cathepsin-B reverse, 5′-ATTATTCGCGAGGTATCTTTCCTTGACTG-3′;
- caveolin-1 forward, 5′-GCCGCCCGGGATGATCTGGTG-3′;
- caveolin-1 reverse, 5′-AACCCAGACACA-3′.

Preparation of cellular extracts
Cells were washed twice with cold PBS and solubilized in lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octylglucoside) on ice. Cell extracts were then passed ten times through a syringe with a 20-gauge needle and centrifuged for 5 minutes at 10,000 g at 4°C. The cell homogenate supernatant was recovered and protein concentrations were quantified using micro-BCA reagents according to the manufacturer’s instructions. Equal protein aliquots of each sample were analysed by SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblot analysis
Samples were separated by SDS-PAGE (10%, 12% or 15% acrylamide) and transferred onto nitrocellulose membranes. Immunoblotting was performed with primary antibodies against cathepsin B (1:4000), caveolin (1:5000), p11 (1:5000), uPA (5 µg ml⁻¹), uPAR (5 µg ml⁻¹), β₁-integrin (1:1000), MMP-2 (1:1000), MMP-9 (1:1000) and MT1-MMP (1:1000), and secondary antibodies conjugated with horseradish peroxidase (1:10000) in TBS wash buffer (20 mM Tris, pH 7.5, 0.5 M NaCl) containing 0.5% Tween-20 and 5% (w/v) non-fat dried milk. After washing, specifically bound antibodies were detected by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Quantification and analysis of the bands were performed using a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm).

Caveolin-1 mediates protease localization
Preparation of caveola-enriched membrane fractions
Preparation of caveola-enriched membrane fractions was performed as previously described (Smart et al., 1995; Song et al., 1996). Briefly, cells from four 100 mm dishes were washed twice with cold (4°C) PBS and then scraped into 2 ml 500 mM sodium carbonate, pH 11.0. Cells were homogenized on ice by ten strokes in a loose-fitting Dounce homogenizer. The lysates were then sonicated on ice in a 50 W Ultrasonicator three times for 20 seconds each and adjusted to equal protein concentrations. The homogenates were adjusted to 45% sucrose by the addition of 2 ml 90% (w/v) sucrose prepared in MES-buffered saline (25 mM MES, pH 6.5, 0.15 mM NaCl) and plated on the bottom of a 12 ml ultracentrifuge tube. The homogenate was overlaid with a 5-35% discontinuous sucrose gradient by adding 4 ml 35% (w/v) sucrose (in MES-buffered saline containing 250 mM sodium carbonate) and 4 ml 5% (w/v) sucrose (in MES-buffered saline containing 250 mM sodium carbonate) and centrifuged at 185,000 g for 18-20 h in a SW41 rotor (Beckman Instruments) at 4°C. A light-scattering band was observed at the 5-35% sucrose interface. From the top of the gradient, 1 ml fractions were collected to yield a total of 12 fractions. Equal volume aliquots from each gradient fraction were analysed by SDS-PAGE and immunoblotting.

Immunocytochemical staining and confocal microscopy
Cells were grown on glass coverslips to 80% confluence and intracellular staining was carried out at room temperature using saponin-permeabilized cells as previously described (Sameni et al., 2000). Primary antibodies used were rabbit anti-human caveolin (5 µg ml⁻¹) and secondary antibodies used were Texas-Red-conjugated affinity-purified donkey anti-mouse IgG (20 mg ml⁻¹). Coverslips were mounted upside-down with Slow-Fade antifade reagent on glass slides and the cells were observed on a Zeiss LSM 310 microscope in confocal mode.

Cathepsin-B activity assay
Cathepsin-B activity assays on cells and media were performed as previously described (Koblinski et al., 2002; Linebaugh et al., 1999). Briefly, cells were serum starved overnight and media were collected, centrifuged to remove whole cells and concentrated using Amicon Ultra-4 10 K centrifugal filters. Cells were solubilized in lysis buffer (250 mM sucrose, 25 mM MES, pH 7.5, 1 mM EDTA, 0.1% Triton X-100) and sonicated on ice in a 50 W Ultrasonicator three times for 10 seconds each. To measure intracellular cathepsin-B activity, 50 µl cell lysates were incubated with 300 µl activator buffer (5 mM EDTA, 10 mM dithiothreitol, pH 5.2) for 15 minutes at 37°C. In a 96-well flat-bottomed black microtiter plate, 100 µl cell lysate/activator-buffer mixture was added to 200 µl assay buffer [Hank’s balanced salt solution lacking sodium bicarbonate and phenol red, and containing 0.6 mM CaCl₂, 0.6 mM MgCl₂, 25 mM piperazine-N,N’-bis-(2-ethanesulfonic acid) (disodium salt), pH 7.3] containing 150 µM Z-Arg-Arg-AMH-Mec substrate. Fluorescence was measured in triplicate at 1-minute intervals for 30 minutes using a Tecan SpectraFluor Plus plate reader with excitation at 360 nm and emission at 465 nm. To measure latent cathepsin-B activity in media samples, 50 µl concentrated medium was incubated for 15 minutes at 37°C in 0.1 M formate, pH 3.2, containing 0.4 mg ml⁻¹ pepsin. Then, 100 µl 20 mM dithiothreitol (prepared in 0.2 M sodium phosphate buffer, pH 6.8, containing 4 mM EDTA, 0.1% Triton X-100) was added to each sample and incubated at 37°C for 10 minutes. Finally, 200 µl 200 µM Arg-Arg-NHMec substrate was added to each sample and incubated at 37°C for 10 minutes. Finally, 200 µl 200 µM...
Z-Arg-Arg-NHMe substrate was added to each sample. 120 µl aliquots of the reaction mixture were added to a 96-well flat-bottomed black microtiter plate and fluorescence was read in triplicate at 1-minute intervals for 30 minutes with excitation at 360 nm and emission at 465 nm. DNA assays were performed on each sample and cathespin-B activity was recorded as pmoles min⁻¹ (µg DNA)⁻¹. Statistical significance was determined by a two-tailed Student’s t test with assumed equal variance. In the figures, asterisks represent a P value less than 0.05 and double asterisks represent a P value less than 0.01.

DNA assay
5 µl cell lysates were mixed with 195 µl DNA buffer (10 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM EDTA) containing 2 µl SYBR Green I nucleic-acid stain in a 96-well flat-bottomed black microtiter plate and incubated for 10 minutes at room temperature in the dark. Fluorescence was measured with excitation at 485 nm and emission at 535 nm, and sample DNA concentrations were calculated against a standard curve of salmon-sperm DNA.

Live-cell proteolysis assay
Proteolysis by live cells was assessed as previously described (Sameni et al., 2003). Briefly, glass coverslips were coated with 50 µl 12 mg ml⁻¹ Matrigel containing 25 µg ml⁻¹ quenched fluorescent substrate DQ-collagen IV (Molecular Probes) and incubated for 10 minutes at 37°C to solidify. Cells (2×10⁴) were seeded onto each coated coverslip and incubated for 60 minutes at 37°C. Complete medium (DMEM plus 10% FBS) was added to the cells and incubated for 48 hours at 37°C. After 48 hours, proteolysis of DQ-collagen IV (green fluorescence) by live cells was observed using a Zeiss LSM 310 confocal microscope with a 40× water-immersion objective. Levels of proteolysis were quantified using Metamorph Software (Universal Imaging).

Invasion assay
Invasion assays were performed using Boyden chambers as previously described (Demchik et al., 1999; Sameni et al., 2003). Briefly, polycarbonate filters (12 µm) were coated with 1% (w/v) gelatin and dried overnight. Matrigel (35 µg) was then added to each filter and dried for 4 hours. The coated filters were placed in Boyden chambers and conditioned medium from log-phase NIH 3T3 cells incubated overnight in DMEM supplemented with 4.5 g l⁻¹ glucose and 2% FCS was added to the lower compartment of the chambers as a chemoattractant. Cells (3×10⁴) in 200 µl DMEM with 4.5 g l⁻¹ glucose and 0.1% BSA were placed on top of the coated filters. Cells were incubated at 37°C for 24 hours and allowed to invade through the Matrigel-coated filters. After 24 hours, the filters were removed from the chambers and the cells were fixed and stained using the Diff-Quik stain set. Cells that did not invade through the Matrigel-coated filters were removed using a cotton swab and cells that did invade were counted three times by two independent counters on a Nikon TMS microscope with a 10× objective using finder grids.

Results
Downregulation of caveolin-1 in HCT 116 cells
HCT 116, the human colorectal cell line used for these studies, contains a point mutation that activates one of the K-ras alleles making them tumorigenic (Shirasawa et al., 1993). Our previous studies revealed that the mutant K-ras in HCT 116...
cells mediates localization of cathepsin B specifically to the caveolae on the surface of these cells (Cavallo-Medved et al., 2003). In the present study, we used HCT 116 cells to explore the effects of caveolin-1 expression, a main structural protein of caveolae, on caveolae-associated proteases. HCT 116 cells were transfected with either pIRES-hrGFP-1a expression vector containing caveolin-1-encoding cDNA in the antisense orientation or the empty vector alone. Clones were grown in vector containing caveolin-1-encoding cDNA in the antisense orientation or the empty vector alone. Clones were grown in selection medium containing 0.5 µg ml⁻¹ puromycin and each clone was tested by immunoblotting for the production of caveolin-1. In Fig. 1A, the production of caveolin-1 is illustrated for the parental HCT 116 cells, a selected HCT 116 clone transfected with the empty vector (control) and a selected HCT 116 clone transfected with the vector containing caveolin-1-encoding cDNA in the antisense orientation (AS-cav-1). Caveolin-1 expression in control cells was comparable to that observed in parental HCT 116 cells but caveolin-1 production in AS-cav-1 cells was downregulated ~80% compared with both the parental and control HCT 116 cells. Furthermore, we performed RT-PCR to show a downregulation of caveolin-1 RNA in AS-cav-1 HCT 116 cells compared with control cells (Fig. 1B).

To isolate caveolae and their associated proteins from HCT 116 colon-carcinoma cells, we used a well-established protocol (Song et al., 1996) based on the insolubility of caveolae to carbonate extraction and their specific buoyant density in equilibrium sucrose gradients. Fig. 1C illustrates the distribution pattern of caveolin-1 in fractions 2-11; fraction 1 is not included here or in subsequent figures because our previous studies revealed no detectable proteins in this fraction (Cavallo-Medved et al., 2003). Fractionation of parental, control and AS-cav-1 HCT 116 cells verified that the bulk of caveolin-1 (seen as α and β isoforms) was isolated in low-density fractions 4-6, hereafter designated as the caveolae fractions (Fig. 1C). There was a dramatic reduction in the expression of caveolin-1 in the caveolae fractions of AS-cav-1 cells compared with both the parental and the control HCT 116 cells. The levels of caveolin-1 in the caveolae fractions of control HCT 116 cells were comparable to those found in the parental HCT 116 cells and thus, hereafter, we chose to use control HCT 116 cells for comparison with the AS-cav-1 cells.

To illustrate further the difference in caveolin-1 expression between control and AS-cav-1 HCT 116 cells, we performed immunocytochemistry on these cells (Fig. 1D). Staining for caveolin-1 in saponin-permeabilized control cells showed discrete red rings adjacent to the cell membrane, a pattern similar to that previously observed in parental HCT 116 cells (Cavallo-Medved et al., 2003). Staining for caveolin-1 in AS-cav-1 cells was shown to be substantially less than that observed in control cells and no discrete rings were detected around the cell surface. The loss of caveolin-1 in AS-cav-1 cells might suggest a reduction in the number of caveolae formed at the surface of these cells, as previously reported by others (Cho et al., 2003; Griffoni et al., 2000).

Caveolin-1 expression alters cathepsin-B secretion

Previous studies from our laboratory have identified cathepsin B in the caveolae of HCT 116 cells (Cavallo-Medved et al., 2003). Furthermore, we established a positive correlation for active K-ras, the expression of caveolin-1 and the localization of cathepsin B in the caveolae of these cells. In the present study, we examined the expression levels of cathepsin-B RNA and protein in the cell lysates of control versus AS-cav-1 HCT 116 cells. RT-PCR results showed no differences in the RNA levels of cathepsin B between AS-cav-1 HCT 116 cells and controls (Fig. 2A). Immunoblotting revealed that, in both cell lines, intracellular cathepsin-B protein was found

![Fig. 2. Differences in the expression, localization and activity of cathepsin B in AS-cav-1 HCT 116 cells.](image)
Fig. 3. Expression and localization of p11 to caveolae are reduced in AS-cav-1 HCT 116 cells. (A) 1 μl total RNA isolated from control and AS-cav-1 HCT 116 cells was subjected to reverse transcription followed by PCR using primer sequences to p11 and β2-microglobulin (as a control). PCR products were resolved on a 1.2% agarose gel stained with ethidium bromide. (B) Control and AS-cav-1 HCT 116 cells were solubilized in lysis buffer containing 1% Triton X-100 and 60 mM octylglucoside. 25 μg protein were analysed by SDS-PAGE and immunoblotted with anti-human-p11 antibody. Immunoblots are shown in triplicate and are representative of at least three experiments. (C) Equal amounts of protein for control and AS-cav-1 HCT 116 cells were subjected to subcellular fractionation on a sucrose gradient after homogenization in sodium-carbonate buffer, pH 11.0. Fractions were collected from the top of the gradient and equal-volume aliquots of fractions 2-11 were analysed by SDS-PAGE and immunoblotted with anti-human-p11 antibodies. Immunoblots are representative of at least three experiments.

predominantly as the two active forms: the mature single chain (31 kDa) isoform and the heavy chain (25/26 kDa) of the two mature double-chain isoforms. Interestingly, the expression of 31 kDa cathepsin B was reduced by 50% in the AS-cav-1 cells compared with the control cells, and the smaller double chain isoform was not present in the control cells (Fig. 2B). We also compared the levels of cathepsin B secreted from these cells and found that AS-cav-1 cells secreted 50% less procathepsin B (the predominant isoform of cathepsin B secreted by tumor cells) than did control cells (Fig. 2B). Differences in the expression of cathepsin B isoforms suggest that trafficking of the enzyme is different in AS-cav-1 cells and control cells. Specifically, the expression level of caveolin-1 appears to affect secretion of cathepsin B from these colon cancer cells.

Trafficking of cathepsin B in HCT 116 cells was further investigated by comparing the intracellular and extracellular activity levels of cathepsin B in control and AS-cav-1 cells. In these assays, cathepsin-B activity was measured against the Z-Arg-Arg-NHMec substrate under conditions highly selective for cleavage by cathepsin B (Linebaugh et al., 1999); cleavage was completely inhibited by 10 μM CA074 (data not shown), a highly selective cathepsin-B inhibitor (Murata et al., 1991). The results show that there was a twofold increase in intracellular cathepsin-B activity in AS-cav-1 cells compared with the control cells (Fig. 2C), a trend that coincides with levels of production of cathepsin-B protein (Fig. 2B). To measure the activity of secreted procathepsin B, the medium samples were preincubated with pepsin to activate the proenzyme before conducting the activity assays. The data revealed that pepsin-activated procathepsin-B activity in the medium from AS-cav-1 cells was approximately half that measured in the medium from control cells (Fig. 2C), coinciding with the 50% decrease in procathepsin-B protein secreted from AS-cav-1 versus control cells (Fig. 2B). These results support the hypothesis that caveolin-1 expression regulates the trafficking and secretion of cathepsin B in HCT 116 cells.

Downregulation of caveolin-1 decreases the levels of caveolae-associated cathepsin B
To explore further the role of caveolin-1 in the localization of cathepsin B, we examined the distribution of cathepsin B to caveolae of control and AS-cav-1 cells. The data revealed that downregulation of caveolin-1 in AS-cav-1 cells reduced the association of cathepsin B with caveolae in these cells compared with control cells (Fig. 2D). Although our previous work showed that active K-RAS mediated caveolin-1 expression and cathepsin-B localization to caveolae (Cavallo-Medved et al., 2003), the present data suggest that the expression of caveolin-1 alone, independent of mutant K-ras, can also affect the localization of cathepsin B to caveolae of these cells. This is not surprising, because caveolin-1 is the main structural protein of caveolae and so downregulation of caveolin-1 would be expected to reduce the number of caveolae on the cell surface, as has been previously reported (Cho et al., 2003; Griffoni et al., 2000). A reduction in caveolae might contribute to the decrease in cathepsin B associating with these structures on the cell surface.

Caveolin-1 expression affects the expression and distribution of p11
The light chain of AIIt, p11, has previously been shown to bind procathepsin B on the surface of breast cancer and glioma cells (Mai et al., 2000). Several studies have identified AIIt as a caveolae-associated protein in various cell types (Harder and Gerke, 1994; Sargiacomo et al., 1993), including in HCT 116 cells (Cavallo-Medved and Sloane, 2003). Here, we examined the effects of caveolin-1 production on p11 RNA and protein. We detected no difference in the p11 RNA levels between control and AS-cav-1 HCT 116 cells (Fig. 3A) and only slightly less p11 protein expressed in AS-cav-1 compared with control cells (Fig. 3B). A more dramatic difference was observed in the distribution of p11 to caveolae in the two cell lines. In control cells, p11 was identified in both caveolae and non-caveolae fractions (Fig. 3C), a pattern also observed in parental HCT 116 cells (Cavallo-Medved et al., 2003). In AS-cav-1 cells, however, p11 was not detected in caveolae fractions and appears to be exclusively isolated in non-
caveolae fractions (Fig. 3C). A similar pattern was observed in HKh-2 cells (Cavallo-Medved et al., 2003), a daughter subline of HCT 116 cells in which the mutated K-ras allele has been deleted by homologous recombination (Shirasawa et al., 1993). HKh-2 cells, which are less tumorigenic than HCT 116 cells, were also shown to produce less caveolin-1 and p11 protein, and to contain less caveolae-associated cathepsin B and p11 than HCT 116 cells (Cavallo-Medved et al., 2003). Thus, caveolin-1 production appears to alter the distribution of p11 to caveolae, an effect that might contribute to the association of cathepsin B with caveolae and secretion of the protease from HCT 116 cells.

Downregulation of caveolin-1 decreases the expression and alters the distribution of uPA and uPAR
Active cathepsin B in caveolae may be involved in activation of pro-uPA (Guo, 2002; Ikeda et al., 2000; Kobayashi, 1993). Our previous study identified pro-uPA and its receptor uPAR in caveolae of HCT 116 cells, an association that is regulated by the expression of active K-RAS (Cavallo-Medved et al., 2003). In this study, we examined the effects of caveolin-1 production on the steady-state levels of uPA and uPAR, and their associations with caveolae in HCT 116 cells. Although the intracellular expression levels of mature uPA did appear to be slightly less in AS-cav-1 cells than in controls (Fig. 4A), we were unable to detect any mature uPA in the caveolae fractions of control or AS-cav-1 cells (Fig. 4B), a pattern consistent with the parental HCT 116 cells (Cavallo-Medved et al., 2003). Downregulation of caveolin-1 production reduced the levels of both intracellular and secreted pro-uPA (Fig. 4A). These data were also consistent with a reduction in the distribution of pro-uPA to caveolae of AS-cav-1 cells compared with control cells (Fig. 4B).

Steady-state levels of uPAR were dramatically decreased following downregulation of caveolin-1 (Fig. 5A). This finding was further complemented by a striking reduction in the amount of uPAR in caveolae fractions of AS-cav-1 cells compared with control cells (Fig. 5B). The downregulation in uPAR expression and localization to caveolae coincides with the reduction in the association of pro-uPA with caveolae and secretion of pro-uPA from AS-cav-1 cells (Fig. 4). In human articular chondrocytes (Schwab et al., 2001) and kidney 293 cells (Wei et al., 1999), uPAR has been shown to complex with β1-integrin and caveolin-1. Downregulation of uPAR in HCT 116 cells was shown to disrupt the uPAR/β1-integrin complex resulting in a fourfold suppression of pro-uPA secretion (Ahmed et al., 2003). We also examined the expression of β1-integrin in control versus AS-cav-1 cells and found the levels to be similar (Fig. 5D), a pattern that resembles that for uPAR (Fig. 5B). Thus, caveolin-1 expression appears to affect pro-uPA expression and secretion by mediating uPAR expression and its association with caveolae and β1-integrin.

Caveolin-1 expression affects the degradation of collagen IV
We used a well-established fluorescent proteolytic assay (Sameni et al., 2003) to measure the degradation of collagen-IV substrate by live colon cancer cells. In this assay, individual cells are plated on Matrigel containing DQ-collagen IV, a fluorescently quenched type-IV-collagen substrate, and, over 48 hours, the cells migrate to form spheroids. Degradation of DQ-collagen IV by the live colon cancer cells and the accumulation of green fluorescent products were visualized by confocal microscopy. Both control and AS-cav-1 cells exhibited intracellular (green punctate pattern within the spheroid) and pericellular (green ring around the perimeter of the spheroid) degradation of collagen IV, but the degradation was dramatically reduced in AS-cav-1 cells compared with controls (Fig. 6A). Quantification of DQ-collagen IV degradation was performed using the Metamorph analysis program and revealed that proteolysis of DQ-collagen IV in AS-cav-1 cells was approximately half that measured in control cells (Fig. 6B). Previous studies reported that the lower levels of uPAR in HKh-2 cells are responsible for these cells degrading laminin more slowly than HCT 116 cells (Allgayer et al., 1999). The lower level of uPAR expression in the AS-cav-1 cells here might similarly affect degradation of collagen-IV by these cells.
Downregulation of caveolin-1 reduces the invasive potential of colon cancer cells

Type-IV collagen, a component of the basement membrane, has been shown to be degraded at the invasive edges of tumors (Zeng et al., 1999). Here, we used Matrigel-invasion assays to measure the effects of caveolin-1 expression on the invasive potential of HCT 116 cells. We seeded equal numbers of control and AS-cav-1 HCT 116 cells on Matrigel-coated filters and counted the number of cells that had invaded through the Matrigel after 24 hours. The number of AS-cav-1 cells that invaded was expressed as a percentage of the number of control cells that invaded. The Matrigel-invasion assay demonstrated that the AS-cav-1 cells were ~80% less invasive than control cells. These data complement the results from the live-cell proteolysis assays (Fig. 6) and further support a functional role for caveolin-1, via caveolae-associated proteases and their receptors, in the invasion and degradation of ECM proteins by HCT 116 colon cancer cells.

Discussion

Upregulation of proteases and their trafficking to tumor cell surfaces have been shown to correlate with increased degradation of ECM proteins during tumor invasion. Many of the cell-surface proteases that degrade ECM have been localized to caveolae of cancer cells (Cavallo-Medved and Sloane, 2003). In the present study, we show that, in human colorectal carcinoma cells, expression of caveolin-1 (a structural protein of caveolae) influences the expression of pro-uPA and its receptor, uPAR, and the localization of cathepsin B and its receptor, p11. Moreover, our data show that downregulation of caveolin-1 expression reduces both the degradation of ECM by these cells and their invasiveness.

Expression and redistribution of active cathepsin B to the basal plasma membrane during the progression of colon cancer are associated with a shortened patient survival (Campo et al., 1994; Hazen et al., 2000). Although the mechanism of cathepsin-B distribution to the basal plasma membrane remains to be elucidated, it occurs in late adenomas (Campo et al., 1994) coincident with the activation of K-ras (Fearon and Vogelstein, 1990). We have shown in our previous studies using HCT 116 human colorectal-carcinoma cells that trafficking of cathepsin B to caveolae and its secretion is regulated by active K-ras (Cavallo-Medved et al., 2003). In the present study, we demonstrate a connection between caveolin-1 production and the secretion of cathepsin B from HCT 116 cells, suggesting a functional role for caveolin-1 in the distribution and activity of this enzyme. One mechanism by which caveolin-1 could affect cathepsin-B secretion is by mediating its association with caveolae. Downregulation of caveolin-1 expression in human umbilical-vein endothelial cells and human diploid fibroblasts has been shown to reduce the number of cell-surface caveolae (Cho et al., 2003; Griffoni et al., 2000). Therefore, a decrease in caveolae-associated cathepsin B in AS-cav-1 HCT 116 cells might be due to a reduction in the number of caveolae formed in these cells. Another potential reason for the reduction in caveolae-associated cathepsin B in AS-cav-1 HCT 116 cells is the absence of p11 from caveolae of these cells. These results are consistent with those observed in HKh-2 cells: in this HCT 116 daughter cell line, deletion of K-ras by homologous recombination results in loss of p11 from caveolae and a decrease in cathepsin B in the caveolae (Cavallo-Medved et al., 2003). The light chain subunit of the AII, p11, has been found to bind procathepsin B directly in breast cancer and glioma cells (Mai et al., 2000). In HCT 116 cells, p11 localizes with both caveolae-associated cathepsin B and caveolin-1 (Cavallo-
Caveolin-1 mediates protease localization

Medved et al., 2003). Furthermore, p11 has been identified in caveolae from a range of cells (Cavallo-Medved et al., 2003; Harder and Gerke, 1994; Sargiacomo et al., 1993).

Colocalization of cathepsin B and p11 to caveolae of colon cancer cells is intriguing because cathepsin B activates soluble and membrane-bound pro-uPA (Ikeda et al., 2000; Kobayashi et al., 1991) and p11 is involved in the binding and activation of plasminogen (Zhang et al., 2004), events associated with tumor invasion. Elevated levels of uPA and uPAR in colon cancer are associated with a negative prognosis (Ganesh et al., 1994). In colon tumors, overexpression of uPA and uPAR in colon cancer is associated with a negative prognosis (Ganesh et al., 1994). Consistent with these findings, in HKh-2 cells with disrupted mutant K-ras expression (Allgayer et al., 1999), localization of uPAR to caveolae is decreased (Cavallo-Medved et al., 2003). In our present study, we show that downregulation of caveolin-1 in HCT 116 cells decreases the expression of uPAR and the expression and secretion of pro-uPA. Consequently, the levels of caveolae-associated uPAR and pro-uPA were also reduced in these cells. The dramatic effects of caveolin-1 on the expression and secretion of pro-uPA, but not mature uPA, is not surprising because it is the proenzyme that binds uPAR on the cell surface. uPAR has been identified in caveolae of several cell types (Cavallo-Medved et al., 2003; Schwab et al., 2001; Stahl and Mueller, 1995; Wei et al., 1999). The clustering of uPA and uPAR in caveolae of human melanoma cells has been shown to enhance the activation of plasminogen at the cell surface (Stahl and Mueller, 1995). In human chondrocytes, uPAR forms a complex with caveolin-1 and β1-integrin (Schwab et al., 2001). Depletion of caveolin-1 in kidney 293 cells disrupted uPAR/β1-integrin complexes, resulting in a loss of uPAR-dependent adhesion and β1-integrin-induced signal transduction (Wei et al., 1999). A reduction of uPAR expression in HCT 116 cells has been shown to disrupt uPAR/β1-integrin complexes without the loss of β1-integrin expression (Ahmed et al., 2003). Similarly, there was no change in the overall expression of β1-integrin in AS-cav-1 HCT 116 cells. There was, however, a striking reduction in caveolae-associated β1-integrin in these cells. The decrease in caveolin-1 and uPAR expression in AS-cav-1 cells probably contributes to a loss in uPAR/β1-integrin complexes within caveolae, leading to the decrease in pro-uPA expression and secretion, ECM degradation and cell invasion.

uPA is involved in tumor-cell invasion by converting plasminogen to plasmin, which can either directly degrade ECM components or activate MMPs. Interestingly, several MMPs (including MMP-2 and MT1-MMP) have been localized to caveolae of glioblastomas, fibrosarcomas and endothelial cells (Annabi et al., 2001; Galvez et al., 2004; Puyraimond et al., 2001), consistent with the membrane region facilitating local invasion. Caveolae have also been identified as a novel pathway for the trafficking of MT1-MMP (MMP-14) during angiogenesis, regulating the localization, activity and function of the enzyme in migrating endothelial cells (Galvez et al., 2004). Nonetheless, we were unable to demonstrate an effect of caveolin-1 on the expression of MT1-MMP; MMP-2 or MMP-9 in the HCT 116 cells (data not shown). In MCF7 breast-carcinoma cells, it has been suggested that MT1-MMP translocated to caveolae of these cells is efficiently released from these lipid rafts, a process that is facilitated by the cytoplasmic tail of the protease (Rozanov et al., 2004). Perhaps the association of MMPs with caveolae of HCT 116 cells is also a short-term event, therefore explaining why we did not observe any differences in AS-cav-1 HCT 116 cells.

Few studies have investigated the link between caveolae/caveolin-1 and cell-surface proteolysis in cancer. We hypothesize that caveolae serve as organizing centers for these enzymes and their receptors in order to modulate proteolytic events that lead to ECM degradation and invasion. Proteolytic assays have shown that HCT 116 cells are capable of degrading type-IV collagen both intracellularly and pericellularly (Sameni et al., 2003). Moreover, inhibitors of MMPs, plasmin and cathepsin B reduce the degradation of type-IV collagen by HCT 116 cells (Sameni et al., 2003). Our results implicate caveolin-1 in both the intracellular and the pericellular degradation of type-IV collagen by HCT 116 cells, probably through regulation of caveolae-associated proteases. Furthermore, we show a reduction in invasion by AS-cav-1 HCT 116 cells, a pattern previously seen in HKh-2 cells, which express less caveolin-1 than their HCT 116 parental counterparts (Sameni et al., 2003).

Caveolin-1 expression has been studied in a wide range of cancers, although correlations between caveolin-1 and cancer
remain controversial. Studies in breast cancer have suggested that caveolin-1 possesses tumor-suppressor activities (for a review, see Williams and Lisanti, 2004), yet increased expression of caveolin-1 has been correlated with malignancy of primary and metastatic prostate (Yang et al., 1998; Yang et al., 1999), pancreatic (Suzuki et al., 2002), esophageal squamous cell (Kato et al., 2002), renal cell (Campbell et al., 2003) and oral (Hung et al., 2003) carcinomas. In colon adenocarcinomas, both increases and decreases in expression of caveolin-1 have been observed (Fine et al., 2001; Bender et al., 2000). Similar observations have been made in mammary and lung carcinomas (Lee et al., 1998; Racine et al., 1999; Yang et al., 1998; Ho et al., 2002). Perhaps caveolin-1 plays dual roles in tumor development by acting as a tumor suppressor in early cancers yet as a promoter of metastasis in late-stage cancers. During early stages of tumorigenesis, downregulation of caveolin-1 could promote cancer-cell proliferation and tumor growth, and, as tumors progress, caveolin-1 production might be induced or increased, thereby facilitating cell survival and metastasis. Our present studies using human colorectal-carcinoma cells suggest a regulatory role for caveolin-1 in the expression, activity and localization of proteases to the cell surfaces of these cells. These data are also significant in establishing a functional role for caveolae-associated proteases in the degradation of ECM proteins during cancer invasion and metastasis.

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References


