

Caveolin-1 controls cell proliferation and cell death by suppressing expression of the inhibitor of apoptosis protein survivin

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Summary

Caveolin-1 is suggested to act as a tumor suppressor. We tested the hypothesis that caveolin-1 does so by repression of survivin, an inhibitor of apoptosis protein that regulates cell-cycle progression as well as apoptosis and is commonly overexpressed in human cancers. Ectopic expression of caveolin-1 in HEK293T and ZR75 cells or siRNA-mediated silencing of caveolin-1 in NIH3T3 cells caused downregulation or upregulation of survivin mRNA and protein, respectively. Survivin downregulation in HEK293T cells was paralleled by reduced cell proliferation, increases in G0-G1 and decreases in G2-M phase of the cell cycle. In addition, apoptosis was evident, as judged by several criteria. Importantly, expression of green fluorescent protein-survivin in caveolin-1-transfected HEK293T cells restored cell proliferation and viability. In

addition, expression of caveolin-1 inhibited transcriptional activity of a survivin promoter construct in a β -catenin-Tcf/Lef-dependent manner. Furthermore, in HEK293T cells caveolin-1 associated with β -catenin and inhibited Tcf/Lef-dependent transcription. Similar results were obtained upon caveolin-1 expression in DLD1 cells, where APC mutation leads to constitutive activation of β -catenin-Tcf/Lef-mediated transcription of survivin. Taken together, these results suggest that anti-proliferative and pro-apoptotic properties of caveolin-1 may be attributed to reduced survivin expression via a mechanism involving diminished β -catenin-Tcf/Lef-dependent transcription.

Key words: β -catenin, Caveolin-1, Cell death, Proliferation, Survivin

Introduction

Caveolins are a family of at least three different isoforms: caveolin-1, caveolin-2 and caveolin-3, which function in the organization as well as trafficking of caveolae and in signal transduction (Liu et al., 2002; Razani et al., 2002; van Deurs et al., 2003). In particular, caveolin-1 contains a so-called scaffolding domain that binds to and inhibits the activity of several signaling proteins in vitro and in situ, including the EGF and Neu receptors, Src-family kinases (Src/Fyn), PKCs, eNOS and the heterotrimeric G-proteins [for a detailed list see Razani et al. (Razani et al., 2002)].

A considerable body of evidence implicates caveolin-1 in preventing cell transformation (Engelman et al., 1997; Galbiati et al., 1998) and promoting cell-cycle arrest as well as senescence (Galbiati et al., 2001; Volonte et al., 2002). Indeed, decreased caveolin-1 expression in several human tumors combined with the effects observed upon caveolin-1 re-expression in human breast (Lee et al., 1998), lung (Racine et al., 1999), colorectal (Bender et al., 2002; Bender et al., 2000) and ovarian cancer cells (Wiechen et al., 2001a) as well as sarcomas (Wiechen et al., 2001b) support the above notion. However, although caveolin-1 knockout mice are viable and do not spontaneously develop tumors (Drab et al., 2001; Razani et al., 2001), they are more sensitive to carcinogen-induced

skin hyperplasia and tumor formation (Capozza et al., 2003). Taken together, these data support the hypothesis that caveolin-1 functions as a tumor suppressor protein in a large variety of cellular settings (Quest et al., 2004; Williams and Lisanti, 2005).

Despite the plethora of proteins that may potentially be regulated by caveolin-1 in vivo, changes in gene expression associated with the presence of caveolin-1 that explain its ability to function as a tumor suppressor remain scarce. An intriguing mechanism in this respect is via control of β -catenin-Tcf/Lef-dependent transcription (Galbiati et al., 2000; Lu et al., 2003; Lu and Hunter, 2004). β -catenin is a key component in the Wnt/ β -catenin/Tcf/Lef pathway and its protein levels are commonly elevated in several neoplasias (Logan and Nusse, 2004; Wong and Pignatelli, 2002). β -catenin is predominantly located at the plasma membrane complexed with E-cadherin and in the cytoplasm or in the nucleus associated with the Tcf/Lef transcriptional factors. The half-life of β -catenin is controlled by a cytosolic complex that includes the proteins adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK-3 β) and axin amongst others. In the absence of Wnt signaling, GSK-3 β phosphorylates β -catenin, thereby targeting it for degradation via the ubiquitin-proteasome system. Activation of the Wnt

pathway in cancer via mutations of APC or β -catenin, precludes efficient degradation of β -catenin, augments β -catenin in the cytosol and promotes translocation to the nucleus, where, in association with Tcf/Lef, target gene transcription is increased (reviewed by Logan and Nusse, 2004; Wong and Pignatelli, 2002).

Caveolin-1 is suggested to sequester β -catenin to the plasma membrane and, in doing so, prevents the transcription of genes such as cyclin D1, involved in cell-cycle progression (Galbiati et al., 2000; Hult et al., 2000). Additionally, caveolin-1 regulates cell-cycle progression via a p53/p21^{WAF1/Cip1}-dependent mechanism (Galbiati et al., 2001). However, caveolin-1 expression has also been found to promote apoptosis in ovarian cancer cells (Wiechen et al., 2001a) and T24 bladder carcinomas (Galbiati et al., 2001). Despite the fact that caveolin-1-promoted apoptosis has been established by morphological and biochemical criteria (Gargalovic and Dory, 2003; Liu et al., 2001; Ono et al., 2004), molecular mechanisms linking these events to the presence of caveolin-1 remain to be defined. Thus, depending on the working model, caveolin-1 may regulate cell proliferation by promoting cell-cycle arrest and/or by sensitizing cells to apoptosis.

In order to identify transcripts that change in response to caveolin-1 presence, mRNA from colon cancer cells expressing, or not expressing, caveolin-1 were compared by microarray analysis. Preliminary results identified survivin as one of the most strongly suppressed genes in the presence of caveolin-1 (Claudio Hetz, David Munroe and A.F.G.Q., unpublished data). Survivin is a 16.5 kDa member of the inhibitor of apoptosis (IAP) proteins, that is implicated in the regulation of both cell death and cell-cycle progression (Li et al., 1998; Reed, 2001). In cancer biology, this protein is of great interest, since it is commonly over-expressed in human cancers but absent in most normal tissues and is hence considered an excellent prognostic marker (reviewed by Altieri, 2003). Here, we investigated the effects of caveolin-1 on cell proliferation, viability and survivin expression using several approaches in HEK293T, ZR75 and NIH3T3 cells. Our results indicated that caveolin-1 presence reduced cell viability and modulated the cell cycle. Furthermore, caveolin-1-mediated control of survivin expression involved transcriptional regulation via the β -catenin/Tcf-Lef pathway. Importantly, re-expression of survivin was sufficient to overcome the limitations imposed by the presence of caveolin-1. Taken together, these results implicate loss of survivin as a crucial, to-date unappreciated, element in caveolin-1-mediated cell-cycle control and loss of cell viability.

Results

Caveolin-1 expression in HEK293T cells reduced cell proliferation, altered cell-cycle distribution and promoted cell death

Caveolin-1 is suggested both to facilitate cell death (Gargalovic and Dory, 2003; Liu et al., 2001; Ono et al., 2004) and to inhibit proliferation by inducing G₀-G₁ cell-cycle arrest (Galbiati et al., 2001). To investigate such possibilities and characterize potential targets down-stream of caveolin-1 involved in the observed responses, HEK293T cells were transiently transfected with pLacIOP, pLacIOP-caveolin-1, pEGFP-C1 or pEGFP-caveolin-1, and cell proliferation was assessed by the MTS[®] assay. Presence of either caveolin-1 or green fluorescent

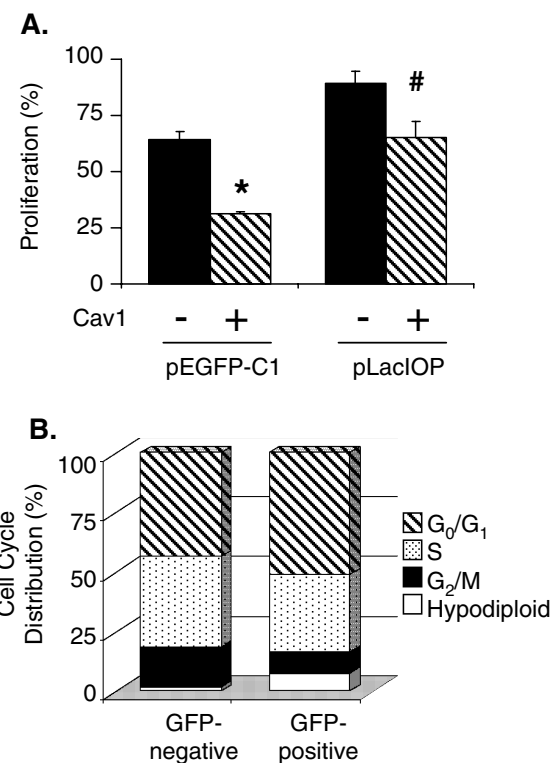


Fig. 1. Expression of caveolin-1 in HEK293T cells reduced proliferation and altered cell-cycle distribution. HEK293T cells were transfected with the plasmids pEGFP-C1, pEGFP-caveolin-1, pLacIOP or pLacIOP-caveolin-1 and plated 24 hours prior to measurements. Transfection efficiency in these experiments was roughly 50%. (A) Cell proliferation was measured by the MTS[®] assay. Values shown indicate the percentage of residual activity with respect to non-transfected controls. Black bars show viability of mock controls (pEGFP-C1 or pLacIOP) and hatched bars the viability of caveolin-1-transfected cells (pEGFP-caveolin-1 or pLacIOP-caveolin-1). Values were averaged from three independent experiments (mean \pm s.e.m.). *Comparison with pEGFP-C1 control ($P < 0.001$); #comparison with pLacIOP control ($P < 0.05$). (B) Post-transfection with pEGFP-caveolin-1, the cell-cycle distribution of GFP-positive and GFP-negative HEK293T cells was compared by flow cytometry. Data shown were averaged from three independent experiments (see Table 1).

protein (GFP)-caveolin-1 in these cells significantly decreased cell proliferation by 25 and 50%, respectively (Fig. 1A). The cell-cycle distribution of GFP-positive and GFP-negative cells was analyzed by flow cytometry following transfection with pEGFP-caveolin-1. Presence of GFP-caveolin-1 in HEK293T cells caused a significant decrease in the G₂-M phase of the cell-cycle, with a concomitant increment in cells with reduced DNA content (hypodiploid), when compared with GFP-negative, non-transfected cells (Fig. 1B, Table 1), suggesting that apoptosis was triggered by the presence of caveolin-1. Indeed, we observed that expression of caveolin-1 itself caused a 10-15% decrease in cell viability as assessed by the Trypan Blue exclusion assay (Fig. 2A), with a small increment in apoptosis, detected by a flow cytometry assay (Fig. 2B) (Hetz et al., 2002). Although the observed increase in apoptosis was not considered significant by statistical analysis ($P < 0.1$), induction of apoptosis by caveolin-1 was confirmed in a

Table 1. Cell-cycle distribution in HEK293T cells transfected with caveolin-1

	Hypodiploid	G ₀ /G ₁	S	G ₂ /M
GFP-negative (-caveolin-1)	1.9±1.0	43.6±0.8	38.3±1.5	16.5±1.4
GFP-positive (+caveolin-1)	7.6±4.3	51.4±3.3	32.8±4.3	8.7±1.4

Cell-cycle distribution was analyzed in HEK293T cells expressing GFP-caveolin-1 (GFP positive) and those that had not been transfected (GFP-negative), as indicated in Fig. 1B. All values are mean ± s.e.m. of three independent experiments.

standard DNA-laddering assay (Fig. 2C) and by confocal analysis of nuclear morphology (Fig. 2D). In the latter case, quantification revealed that nuclear fragmentation occurred in 25% of the caveolin-1-positive cells, whereas over 95% of the non-transfected cells had intact nuclei. Additionally, expression of caveolin-1 in HEK293T cells coincided with increased caspase-9 cleavage, and caveolin-1-triggered apoptosis was reduced both by caspase-3 and -9 inhibitors (data not shown). Taken together, these results suggest that the presence of caveolin-1 in HEK293T cells reduced cell proliferation and viability, while augmenting apoptosis.

Caveolin-1 expression in HEK293T cells reduced survivin mRNA and protein levels

Microarray results from our laboratory (Claudio Hetz, David Munroe and A.F.G.Q., unpublished data) and the fact that the IAP protein survivin is implicated in regulating events such as the ones presented here (Figs 1, 2) led us to investigate changes in the content of this protein upon caveolin-1 expression. Transfection of HEK293T cells with pLacIOP-caveolin-1 or pEGFP-caveolin-1 decreased survivin protein by 45-50% (Fig. 3A,B). No such changes were observed for other anti-apoptotic proteins, such as bcl-X1 or bcl-2 (data not shown). The results described so far were obtained in a transiently transfected population of cells, where efficiency was about 50%, as assessed by flow cytometry (data not shown). Using the same FACS experiment, endogenous survivin levels in the population of GFP-caveolin-1-positive and the GFP-negative cells were compared. In cells permeabilized and prepared for survivin detection using the appropriate antibodies (see Materials and Methods), two cell sub-populations (referred to as GFP-positive or -negative)

were distinguishable. Specifically, survivin was markedly decreased in GFP-positive as compared to GFP-negative HEK293T cells (Fig. 3B). Furthermore, survivin mRNA content was assessed in transfected cells. Expression of either GFP-caveolin-1 or caveolin-1 reduced survivin mRNA content by approximately 50% (Fig. 3C). Thus caveolin-1-mediated loss of survivin expression involved a transcriptional mechanism.

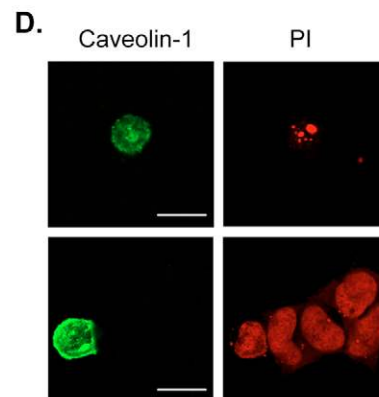
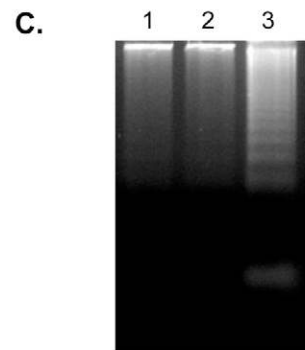
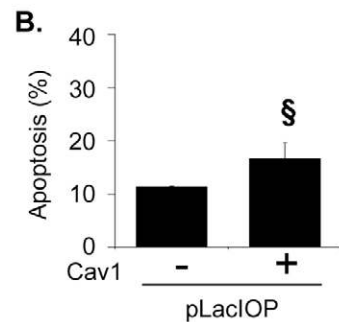
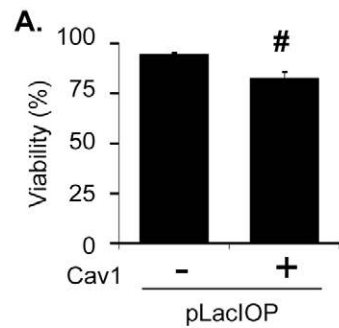


Fig. 2. Expression of caveolin-1 in HEK293T cells promoted cell death. HEK293T cells were transfected with pLacIOP or pLacIOP-caveolin-1 as indicated and analyzed after 24 hours. (A) Viability was determined by the Trypan Blue exclusion assay. (B) To determine apoptosis, cells were harvested, resuspended in PBS with 10 µg/ml propidium iodide (PI) and analyzed by flow cytometry. In A and B values were averaged from three independent experiments (mean ± s.e.m.). [#]comparison with pLacIOP control ($P < 0.05$), [§]comparison with pLacIOP control ($P < 0.1$). (C) Results from a standard DNA laddering assay. From left to right, samples from non-transfected (lane 1), pLacIOP-transfected (lane 2) and pLacIOP-caveolin-1-transfected (lane 3) cells are shown. (D) Nuclear fragmentation analysis. Caveolin-1 was detected by immunofluorescence (green) in cells transfected with pLacIOP-caveolin-1 and the integrity of the nuclei was assessed by PI staining (red). Samples were analyzed by confocal microscopy (Bar, 10 µm). In C and D a representative result from two independent experiments is shown.

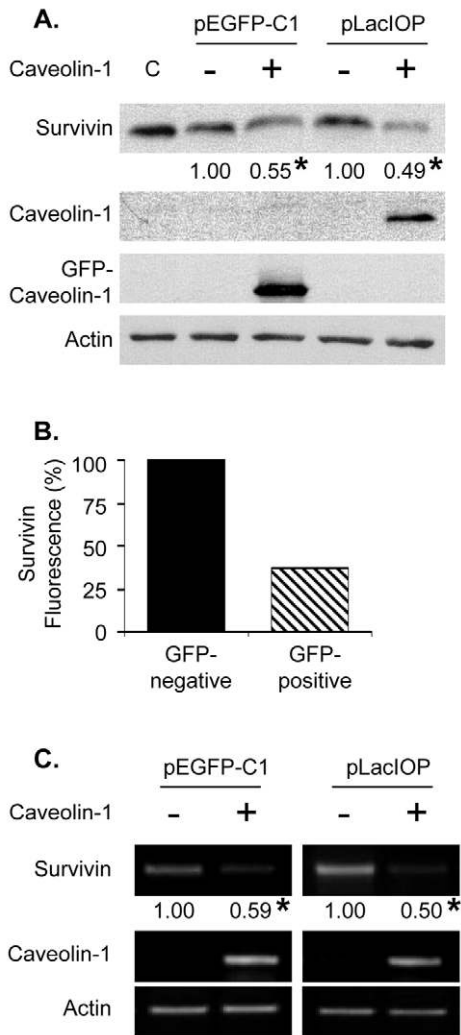


Fig. 3. Caveolin-1 expression in HEK293T cells reduced survivin mRNA and protein content. HEK293T cells were transfected with the plasmids pEGFP-C1, pEGFP-caveolin-1, pLacIOP or pLacIOP-caveolin-1 24 hours prior to measurements. (A) Cell extracts were analyzed by western blotting with anti-survivin, anti-caveolin-1 and anti-actin antibodies. From left to right: non-transfected cells (lane 1) and cells transfected with pEGFP-C1 (lane 2), pEGFP-caveolin-1 (lane 3), pLacIOP (lane 4) or pLacIOP-caveolin-1 (lane 5). Survivin levels were quantified by scanning densitometric analysis of western blots and normalized to actin. Residual survivin protein levels for pEGFP-caveolin-1 (0.55 ± 0.06) and pLacIOP-caveolin-1 (0.49 ± 0.05) transfected cells were compared to their respective mock controls. Numerical data represent the means \pm s.e.m. of results obtained in three independent experiments; *comparison with their respective mock controls, $P < 0.001$. (B) Alternatively, survivin was detected by immunofluorescence followed by FACS analysis in both green (GFP-positive) and non-green (GFP-negative) HEK293T cells, after transfection with the pEGFP-caveolin-1 vector. Data are representative of two experiments. (C) RT-PCR analysis of survivin. Actin was used as internal control. From left to right, samples from cells transfected with pEGFP-C1 (lane 1), pEGFP-caveolin-1 (lane 2), pLacIOP (lane 3) or pLacIOP-caveolin-1 (lane 4). Survivin mRNA levels for pEGFP-caveolin-1 (0.59 ± 0.07)- and pLacIOP-caveolin-1 (0.50 ± 0.07)-transfected cells were compared to their respective mock controls. Numerical data represent the means \pm s.e.m. of results obtained in three independent experiments; *comparison with their respective mock controls, $P < 0.05$.

Transcriptional regulation of survivin by caveolin-1 involved a β -catenin-Tcf/Lef-dependent mechanism

Survivin is a transcriptional target of β -catenin with Tcf/Lef-binding sites in the promoter region (Kim et al., 2003; Zhang et al., 2001a). In addition, caveolin-1 has been reported to interact with β -catenin and thereby inhibit Tcf/Lef-dependent transcription (Galbiati et al., 2000; Lu et al., 2003). Therefore, we analyzed whether loss of survivin expression observed in the presence of caveolin-1 was mediated by changes in β -catenin-Tcf/Lef-dependent transcription. In agreement with previous reports, caveolin-1 localized to the cell periphery and plasma membrane of HEK293T cells. Partial co-localization with β -catenin was apparent at the plasma membrane and was strongest at contact sites between adjacent cells (Fig. 4A). In addition, both proteins were associated in a protein complex isolated by immunoprecipitation (Fig. 4B). Interestingly, expression of caveolin-1 significantly inhibited transcriptional activity of β -catenin, as observed by the luciferase reporter assay (Fig. 4C). This effect was abrogated by the presence of lithium, a reported GSK3 β inhibitor and activator of Tcf/Lef-dependent transcription (Hedgepeth et al., 1997) that caused accumulation of β -catenin (data not shown). As expected, lithium reversed both caveolin-1-mediated downregulation of survivin and inhibition of β -catenin-dependent transcription in HEK293T cells (Fig. 4C).

The survivin promoter region contains three predicted Tcf/Lef-binding sites and luciferase reporter constructs containing either all three (pLuc-1710) or the two more proximal sites (pLuc-420) have been described (Kim et al., 2003). In addition, controls for pLuc-420 are available in which one of the two sites has been mutated (pLuc-420-2M, pLuc-420-3M). As expected, caveolin-1 expression reduced reporter activity detected with pLuc-1710 and pLuc-420 whereas no such regulation was apparent with the control constructs pLuc420-2M or pLuc-420-3M (Fig. 4E). Thus, caveolin-1 directly suppressed β -catenin-Tcf/Lef-mediated transcription within the survivin promoter region.

Survivin co-expression reversed caveolin-1-dependent loss of proliferation and changes in cell-cycle progression

To examine whether cytostatic and cytotoxic effects of caveolin-1 were linked to loss of survivin, HEK293T cells were co-transfected with pLacIOP-caveolin-1 and pEGFP-survivin. Fig. 5A shows that transfection with pLacIOP alone reduced cell proliferation by roughly 10%, but the expression of caveolin-1 reduced this value by an additional 25%. Interestingly, co-expression of increasing amounts of GFP-survivin with caveolin-1 reversed the inhibitory effect of caveolin-1 in a concentration-dependent manner and restored cell proliferation to levels comparable with those of mock-transfected cells. This effect of GFP-survivin was specific for survivin because expression of GFP alone did not reverse the reduction in cell proliferation caused by caveolin-1 (Fig. 5A). Increased cell proliferation in cells co-transfected with pEGFP-caveolin-1 and pEGFP-survivin was accompanied by recovery of the percentage of cells in G2-M and a concomitant decrease in the number of apoptotic cells compared with cells transfected with pEGFP-caveolin-1 alone (Fig. 5B). Importantly, no alteration in GFP-caveolin-1 expression was detected by western blotting when GFP-survivin was co-

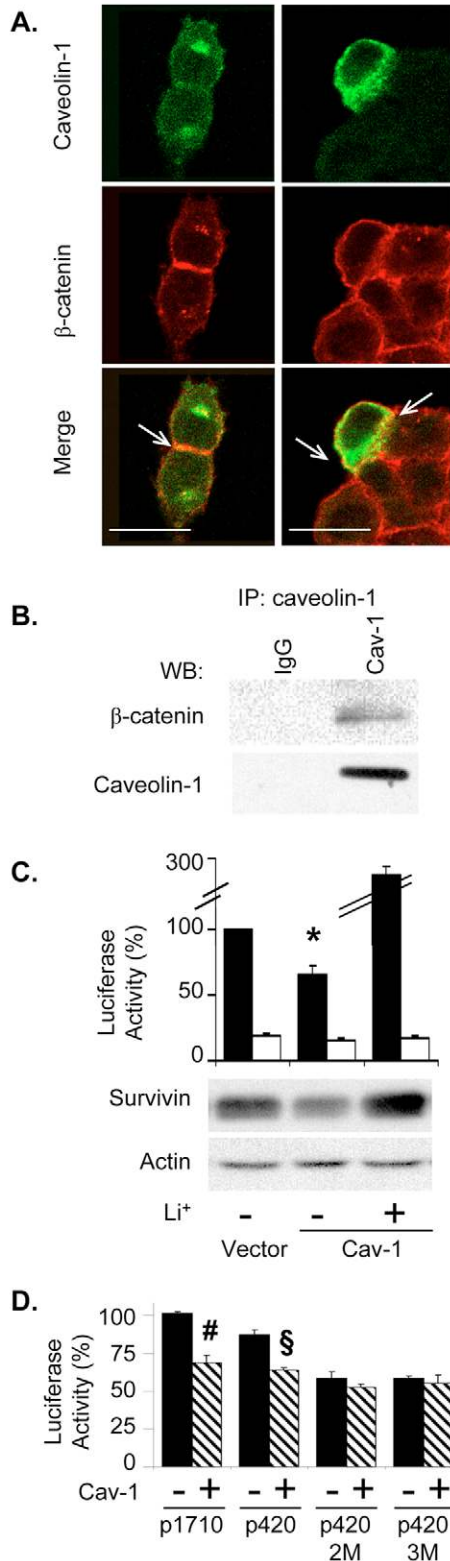


Fig. 4. Caveolin-1 suppressed β -catenin-Tcf/Lef-dependent transcription of survivin in HEK293T cells. HEK293T cells were transfected with the plasmids pLacIOP or pLacIOP-caveolin-1 and subjected to further analysis after 24 hours. (A) Cellular localization of caveolin-1 (upper panels) and β -catenin (middle panels) was detected by confocal microscopy; merged images (bottom panels). Bar, 10 μ m. Note that co-localization was predominantly detected at the plasma membrane cell-cell contact sites (white arrows). (B) Ectopically expressed caveolin-1 was immunoprecipitated from HEK293T cells and then caveolin-1 and β -catenin were detected by western blotting. A result representative of two independent experiments is shown. (C) HEK293T cells were co-transfected with the plasmids pLacIOP (vector) or pLacIOP-caveolin-1 (cav-1) and pTOP-FLASH (black bars) or pFOP-FLASH (white bars) in the presence or absence of 20 mM LiCl. After 24 hours, cell extracts were prepared and utilized for Tcf/Lef reporter assays (bar graph) and western blot analysis. Bar graph: luciferase activity (normalized to β -galactosidase) as a percentage of the values detected in vector-transfected cells (pLacIOP, defined as 100%). Data shown are mean \pm s.e.m. from three independent experiments; *comparison with pLacIOP control, $P < 0.01$. Western blot: determination of survivin and actin levels. From left to right: cells transfected with pLacIOP (vector) or pLacIOP-caveolin-1 (cav-1) in the absence (-) or presence (+) of LiCl (20 mM). (D) HEK293T cells were co-transfected with the plasmids pLacIOP (black bars) or pLacIOP-caveolin-1 (hatched bars) and the reporter constructs pLuc-1710, pLuc-420, pLuc-420-2M or pLuc-420-3M as indicated. After 24 hours, cell extracts were prepared and used to measure reporter activity. Data shown are mean \pm s.e.m. from three independent experiments; #comparison with pLuc-1710 in the presence of pLacIOP, $P < 0.001$; \$comparison with pLuc-420 in the presence of pLacIOP, $P < 0.01$.

caveolin-1 to levels comparable to these observed for mock-transfected cells, even in the presence of the drugs (Fig. 5C).

Caveolin-1 expression in breast cancer cells

To see whether caveolin-1-dependent modulation of survivin expression was a more general phenomenon, caveolin-1 was re-expressed in human breast cancer cell lines (ZR75 and MCF7). In breast cancer, caveolin-1 is downregulated and re-expression of caveolin-1 substantially reduces proliferation of MCF7 cells (Fiucci et al., 2002; Lee et al., 1998). In ZR75 breast cancer cells, caveolin-1 protein was essentially undetectable (Fig. 6A), whereas basal levels of caveolin-1 were higher in MCF7 cells (data not shown). Thus, the effects of caveolin-1 re-expression were mainly evaluated in ZR75 cells. In agreement with our previous results, survivin was downregulated by 40% in caveolin-1-transfected ZR75 cells, as compared to mock-transfected cells (Fig. 6A). Similar results were also obtained in MCF7 cells when caveolin-1 was re-expressed (data not shown). Furthermore, caveolin-1 presence in ZR75 cells was paralleled by a substantial reduction in endogenous survivin mRNA (Fig. 6B) and cell proliferation (Fig. 6C), as well as a modest twofold increase in apoptosis (Fig. 6D).

Downregulation of caveolin-1 in NIH3T3 cells using siRNA

To further increase the relevance of our findings, we used a complementary approach, where endogenous caveolin-1 was downregulated in NIH3T3 fibroblasts, using caveolin-1-specific siRNA. Transfection of NIH3T3 cells with a mixture

expressed (Fig. 5B). On the other hand, caveolin-1 was also found to sensitize HEK293T cells to the drugs taxol, doxorubicin and etoposide that were employed at cytostatic concentrations during a 24 hours incubation period (Fig. 5C). Under these conditions, co-expression of GFP-survivin with caveolin-1 restored proliferation of HEK293T cells expressing

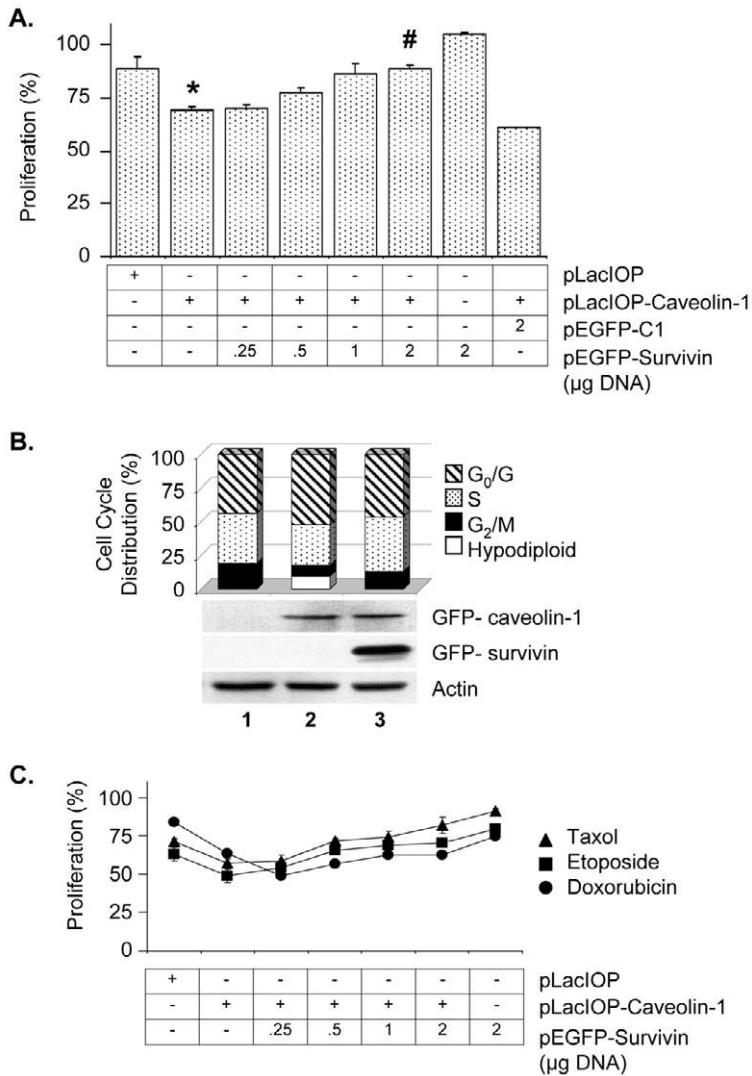


Fig. 5. Ectopic expression of GFP-survivin in HEK293T cells reversed the reduction in cell proliferation and changes in the cell cycle caused by caveolin-1. (A) HEK293T cells were transfected with pLacIOP alone (3 µg) or co-transfected with the vectors pLacIOP-caveolin-1 (3 µg) and increasing amounts of the vector pEGFP-survivin (up to 2 µg). Controls included transfections with pEGFP-C1 (2 µg) or pEGFP-survivin (2 µg). Cell proliferation was measured by the MTS[®] assay. Values detected following transfection are shown as a percentage of the MTS activity measured in non-transfected controls (100%). (B) HEK293T cells were either transfected with pEGFP-caveolin-1 alone (5 µg) or co-transfected with the vectors pEGFP-caveolin-1 (5 µg) and pEGFP-survivin (5 µg) and cell cycle was analyzed by flow cytometry 48 hours post-transfection by gating on GFP-negative (lane 1) and GFP-positive (lanes 2 and 3) subpopulations. GFP-caveolin-1 and GFP-survivin expression are shown in a representative western blot. A result representative of two independent experiments is shown. (C) HEK293T cells were transfected with the vector pLacIOP (3 µg) alone or co-transfected with the vectors pLacIOP-caveolin-1 (3 µg) and increasing amounts of pEGFP-survivin (up to 2 µg) and cultured post-transfection in the presence of either 250 nM doxorubicin, 25 µM etoposide or 250 nM taxol. Cell proliferation was evaluated by the MTS[®] assay. Values shown are equivalent to the percentage of residual activity as compared with non-transfected controls (100%). In A and C the data shown are mean ± s.e.m. from three independent experiments; *comparison with pLacIOP control, $P < 0.01$; #comparison with pLacIOP-caveolin-1, $P < 0.01$.

of siRNAs (see Materials and Methods section) decreased endogenous caveolin-1 content approximately threefold, as compared to siRNA control cells (Fig. 7A, black bar in B). A concomitant 2.8-fold increase in survivin protein was detected in siRNA-transfected cells (Fig. 7A,B), but no significant changes in caveolin-2 were observed (Fig. 7A, white bar in 7B). Silencing of caveolin-1 was accompanied by an increase in cell proliferation (Fig. 7C) and β -catenin-Tcf/Lef-dependent transcription (Fig. 7D).

Caveolin-1 decreased survivin expression in cells with enhanced or constitutive signaling via the Wnt pathway

To support our hypothesis that caveolin-1 downregulated survivin expression via a mechanism involving Tcf/Lef-dependent transcription, the effect of caveolin-1 was assessed in two different models where this transcriptional pathway is activated. First, Wnt3a-conditioned medium (see Materials and Methods) was employed to activate Tcf/Lef-dependent expression of survivin in HEK293T cells. As anticipated, Wnt3a-induced survivin expression was substantially reduced upon expression of caveolin-1 (Fig. 8A). Secondly, the effect of caveolin-1 on survivin expression was examined in a system where the β -catenin-Tcf/Lef-dependent transcription is constitutively activated. To this end, the colon adenocarcinoma cell line DLD1 was chosen for essentially two reasons: first, these cells are known to harbor an inactive-truncated form of the protein APC and thus β -catenin is endogenously stabilized (Polakis, 1997); and second, basal caveolin-1 levels are low in these cells and clones stably transfected with either pLacIOP (M1) or pLacIOP-caveolin-1 (C4) were available in our laboratory (Bender et al., 2000). Again, the presence of caveolin-1 led to a pronounced reduction in survivin protein and mRNA levels (Fig. 8B and C, respectively). Furthermore, as observed in HEK293T cells (Fig. 4), caveolin-1 co-localized and co-immunoprecipitated with β -catenin in DLD1 cells (data not shown). Taken together, these results are consistent with our current model whereby caveolin-1 suppresses survivin expression by sequestering β -catenin and precluding β -catenin-Tcf/Lef-dependent transcription.

Discussion

Effects of caveolin-1 on cell proliferation and cell death: involvement of survivin

An appreciable body of literature implicates caveolin-1 as a tumor suppressor gene in a variety of cellular settings (see Introduction) and links caveolin-1 presence to promotion of cell death and/or inhibition of cell proliferation. Caveolin-1 presence reportedly sensitizes cells to a variety of pro-apoptotic stimuli, such as exposure to ceramide (Zundel et al., 2000), staurosporine (Liu et al., 2001), arsenite (Shack et al., 2003) and TNF α (Ono et al., 2004). Our data in HEK293T cells are consistent with the notion that caveolin-1 alone is sufficient to promote apoptotic cell death with characteristics such as loss of plasma membrane

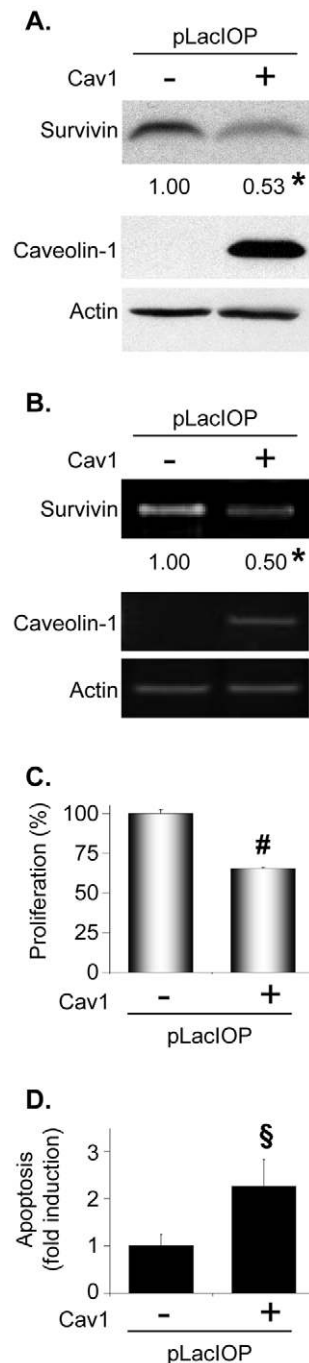


Fig. 6. Ectopic expression of caveolin-1 in ZR75 human breast cancer cells downregulated survivin and decreased cell proliferation. ZR75 cells were stably transfected with the plasmids pLacIOP (-) or pLacIOP-caveolin-1 (+). (A) Survivin and caveolin-1 expression was assessed by western blotting. A representative result is shown. Residual survivin protein levels were 0.53 ± 0.09 as determined in three independent experiments (* $P < 0.05$). (B) Survivin mRNA content was analyzed by RT-PCR. Residual survivin mRNA levels were 0.50 ± 0.06 as averaged from three independent experiments (* $P < 0.05$). (C) Cell proliferation was measured by the MTS[®] assay in the presence and absence of caveolin-1. (D) Apoptosis was determined as indicated above by PI staining followed by flow cytometry. Numerical results are mean \pm s.e.m. from three independent experiments; # and § comparisons with the mock control pLacIOP, # $P < 0.01$, § $P < 0.05$.

integrity (propidium iodide incorporation), DNA fragmentation, changes in nuclear morphology (Fig. 2) and caspase-9 activation (data not shown). However, caveolin-1 has been suggested to inhibit cell proliferation by arresting cells in the G₀-G₁ phase of the cell cycle (Galbiati et al., 2001). Our results in HEK293T and ZR75 cells indicate that both a reduction in proliferation and increase in cell death can be observed as a consequence of increased caveolin-1 expression (Figs 1, 2 and 6). Alternatively, however, increased proliferation was not accompanied by a reduction in cell death in siRNA-treated NIH3T3 cells (Fig. 7, data not shown). Thus, although cell death may occur in response to caveolin-1 expression, this effect alone cannot account for the observed changes in cell proliferation.

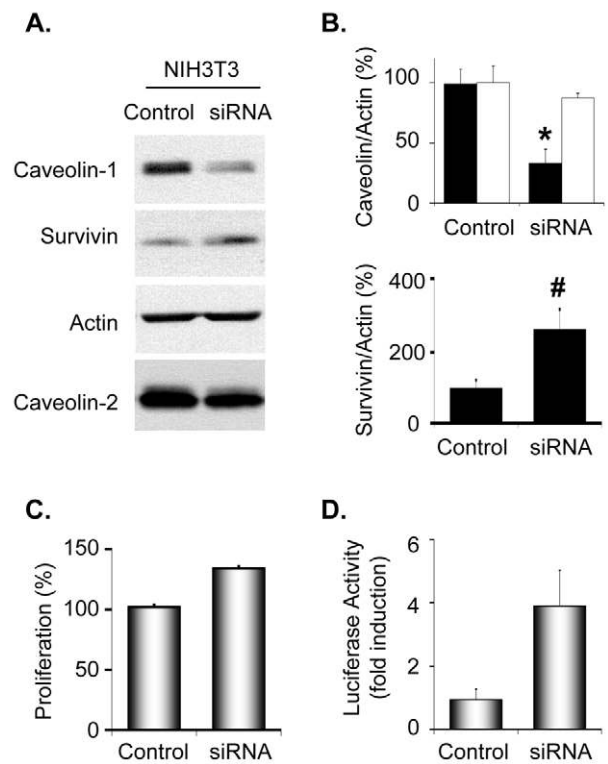


Fig. 7. Downregulation of caveolin-1 by si-RNA in NIH3T3 cells increased survivin expression. (A) NIH3T3 cells were transfected with caveolin-1-specific siRNA or control siRNA, as described. Expression of caveolin-1, survivin, actin and caveolin-2 was assessed by western blotting. A result representative of three independent experiments is shown. (B) Caveolin-1 and survivin levels were quantified by scanning densitometry. The ratios for caveolin-1 to actin (black bar), caveolin-2 to actin (white bar) and survivin to actin are shown as a percentage of the values obtained in control experiments. # and § comparisons with their respective controls, * $P < 0.01$, # $P < 0.05$. (C) Cell proliferation was measured by the MTS[®] assay. (D) Tcf/Lef reporter assay. NIH3T3 cells were transfected with caveolin-1-specific siRNA or the control siRNA and grown for 24 hours. Then, cells were additionally transfected with the plasmid pTOP-FLASH. After another 24 hours, luciferase activity was determined and normalized to β -galactosidase activity, as described above. All values are mean \pm s.e.m. from either three (B) or two (C,D) independent experiments.

Mechanisms by which caveolin-1 promotes cell death and inhibits cell proliferation are not completely understood and only a few transcriptional targets have been described downstream of caveolin-1. Here, we identified survivin as a target for caveolin-1-mediated effects on cell death and proliferation. Preliminary studies from our laboratory, using microarray analysis, compared mRNA levels of many genes from colon adenocarcinoma cells expressing or not expressing caveolin-1 (Bender et al., 2000). One of the most striking changes detected in response to caveolin-1 expression was suppression of the IAP protein survivin (Claudio Hetz, David Monroe and A.F.G.Q., unpublished data). The studies described here focused on first demonstrating that caveolin-1 indeed regulates survivin expression levels via a transcriptional mechanism in a variety of cellular settings and then linking such caveolin-1-mediated survivin suppression to changes in the cell cycle and

apoptosis. Our results showed that survivin was significantly downregulated at both the mRNA and protein levels in HEK293T and ZR75 breast cancer cells (Figs 3 and 6). Thus, the caveolin-1-survivin relationship was confirmed using siRNA-mediated caveolin-1 downregulation in NIH3T3 cells (Fig. 7), in HEK293T cells where survivin expression was augmented via stimulation of the β -catenin-Tcf/Lef pathway with Wnt3a (Fig. 8A) as well as in DLD1 colon adenocarcinoma cells where constitutive β -catenin-Tcf/Lef-dependent transcription results from APC mutation (Fig. 8B). Thus, caveolin-1-dependent regulation of survivin expression is a phenomenon that occurs in both non-tumoral and cancer cells.

Mechanisms by which survivin has been proposed to

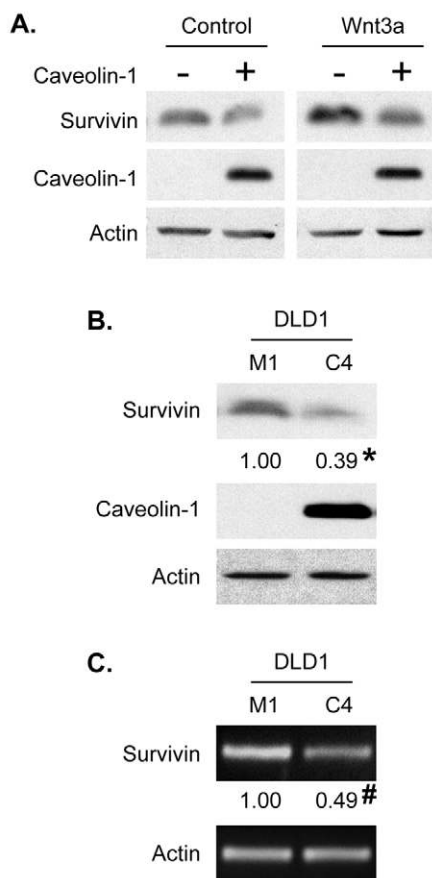


Fig. 8. Caveolin-1 downregulated survivin expression in cells with enhanced or constitutive activity via the Wnt pathway. (A) HEK293T cells were transfected with either pLacIOP (-) or pLacIOP-caveolin-1 (+) and then cultured either in normal medium (control) or conditioned medium containing Wnt3a for 24 hours (see Materials and Methods for details). Survivin, caveolin-1 and actin protein levels were assessed by western blotting. (B) Clones of DLD1 colon adenocarcinoma cells stably transfected with either pLacIOP (M1) or pLacIOP-caveolin-1 (C4) were described previously (Bender et al., 2000). Cell extracts were prepared post-induction with 1 mM IPTG for 24 hours. Survivin, caveolin-1 and actin expression were assessed by western blotting. Residual survivin level was 0.39 ± 0.10 (mean \pm s.e.m.; * $P < 0.05$), as determined in three independent experiments. (C) Survivin mRNA content in DLD1 clones was analyzed by RT-PCR. Residual survivin mRNA level was 0.49 ± 0.18 (mean \pm s.e.m.; # $P < 0.05$), as determined in three independent experiments.

regulate apoptosis include direct inhibition of caspases with a preference for caspase-9, sequestration of Smac/DIABLO, as well as stabilization of XIAP, another member of the IAP family (Altieri, 2003; Dohi et al., 2004; Reed, 2001; Song et al., 2003). Available evidence implicated caspase-9 as a downstream effector in the sequence of events triggered by caveolin-1 (data not shown). However, identification of the precise mechanisms and caspases by which caveolin-1-mediated decline in survivin promotes apoptosis requires further analysis. Here, it should also be mentioned that changes in survivin expression were not paralleled by alterations in other anti-apoptotic proteins such as Bcl-2 and Bcl-X1 (data not shown).

Cell-cycle changes associated with caveolin-1 expression

Our findings differed with respect to the cell-cycle changes that were previously reported to be associated with decreased proliferation due to caveolin-1 (Galbiati et al., 2001). When expressed in HEK293T cells, caveolin-1 increased the number of cells in G0-G1 and decreased the number in S and G2-M phases. However, percentages changes in G2-M were more prevalent than those in the G0-G1 and S phases. In addition, a hypodiploid (sub-G0-G1) apoptotic cell population was evident (Table 1). Importantly, caveolin-1-dependent downregulation of cyclin D1 was previously reported in NIH3T3 and CHO cell lines to occur via suppression of β -catenin-Tcf/Lef-mediated transcription (Hulit et al., 2000). As a consequence, pronounced accumulation of cells in G0-G1 was observed (Galbiati et al., 2001). Consistent with these findings, we observed, in NIH3T3 cells, that caveolin-1 downregulation was accompanied by increased cyclin D1 expression and changes in G0-G1 and S phases, whereas no changes in G2-M were observed (Fig. 7, data not shown). However, upon expression of caveolin-1 in HEK293T cells, where changes at G2-M were prevalent, cyclin D1 expression was not altered (data not shown). Alternatively, in ZR75 cells only minor changes in each phase of the cell cycle were observed, although cyclin D1 also remained unchanged upon caveolin-1 expression (data not shown). Thus, effects on the cell cycle and/or cell death observed in response to caveolin-1 expression were not confounded by simultaneous changes in cyclin D1 in these cells. In summary, changes in survivin expression in response to caveolin-1 presence were detectable in all cases characterized here, but the consequences observed were dependent on the cellular system under study.

The aforementioned findings indicated that caveolin-1 regulated both cell death and proliferation and these effects appeared to be, at least in HEK293T cells, independent of cyclin D1. Thus, we tested here the hypothesis that caveolin-1-mediated effects on cell cycle and cell death were associated with survivin modulation. Indeed, in HEK293T cells both viability- and cell-cycle-associated changes due to caveolin-1 presence were significantly reversed by ectopic expression of survivin (Fig. 5), suggesting that this protein is a crucial target for caveolin-1-mediated effects on cell fate.

Anti-apoptotic effects of survivin have been linked to the control of cell-cycle progression through G2-M. As previously reported (Li et al., 1998), we observed, particularly in HEK293T cells, accumulation of survivin protein in G2-M and downregulation in the G0-G1 phase (data not shown). Consistent

with this pattern of expression, significant changes in G2-M were detected when caveolin-1 was present (Fig. 1, Table 1). Interestingly, taxol treatment of A549 lung cancer cells has been reported to increase caveolin-1 expression and promote cell-cycle arrest in G2-M, as well as apoptosis (Roussel et al., 2004). These observations agree with our data in HEK293T cells, indicating that caveolin-1 controls passage through the G2-M checkpoint as well as apoptosis by modulating the presence of survivin.

Downregulation of survivin by caveolin-1 involved β -catenin-dependent transcriptional events

Some β -catenin-Tcf/Lef targets that control proliferation and/or viability include cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999), Myc (He et al., 1998), VEGF (Zhang et al., 2001b), COX-2 (Haertel-Wiesmann et al., 2000) and survivin (Kim et al., 2003; Zhang et al., 2001a). Cyclin D1 is transcriptionally controlled by the β -catenin-Tcf/Lef pathway (Shtutman et al., 1999; Tetsu and McCormick, 1999) and caveolin-1 reportedly reduces cyclin D1 expression through a mechanism involving the inhibition of this pathway (Galbiati et al., 2000; Hult et al., 2000). Given our results showing that caveolin-1 suppressed survivin expression and reported evidence indicating that survivin expression was transcriptionally controlled by the β -catenin-Tcf/Lef pathway (Kim et al., 2003; Zhang et al., 2001a), we hypothesized that downregulation of survivin by caveolin-1 may involve this pathway. In accordance with the model proposed by Lisanti and co-workers, whereby formation of a complex between caveolin-1 and β -catenin at the plasma membrane precludes formation of a transcriptionally active β -catenin/Tcf-Lef complex (Galbiati et al., 2000), we observed that ectopically expressed caveolin-1 and endogenous β -catenin co-immunoprecipitated and co-localized in HEK293T (Fig. 4), ZR75 and DLD1 cells (data not shown). In addition, caveolin-1 inhibited Tcf/Lef-dependent transcription in HEK293T cells and this effect was abrogated by lithium (an activator of this pathway). The same dependence on Tcf/Lef-mediated transcription was also observed in ZR75 and NIH3T3 cells (Fig. 7, data not shown). However, despite such intriguing convergence, these results need not exclude the existence of additional, transcriptional and post-transcriptional mechanisms downstream of caveolin-1 that control survivin expression. Indeed, survivin expression is known to be controlled at several levels by a variety of mechanisms (Altieri, 2003; Krysan et al., 2004). To what extent some of these other possibilities might be involved downstream of caveolin-1 is currently under investigation.

Since caveolin-1 presence has been shown to promote proteasome-mediated degradation of the inducible form of nitric oxide synthase (iNOS) (Felleo-Bosco et al., 2002; Felleo-Bosco et al., 2000), the possibility that caveolin-1 may affect β -catenin turnover in a similar manner was also tested. Our results indicated that caveolin-1 did not alter global β -catenin protein levels in HEK293T and ZR75 cells (data not shown). These observations favor the notion that caveolin-1 modulates subcellular distribution of β -catenin and, in doing so, limits the magnitude of the transcriptionally active pool of β -catenin rather than the rate of protein degradation.

Effects of caveolin-1 on survivin expression are independent of caveolin-2

In HEK293T, ZR75 and DLD1 cells, endogenous caveolin-2

protein levels were extremely low and were not augmented by ectopic expression of caveolin-1 (data not shown). NIH3T3 cells instead, express readily detectable levels of caveolin-2 protein, and decreasing caveolin-1 expression in these cells, from the use of siRNA did not modulate the level of caveolin-2 (Fig. 7). These observations are relevant, because caveolin-1 absence in knockout mice coincides with loss of caveolin-2, which is degraded via the proteasome pathway following accumulation in the Golgi (Drab et al., 2001; Razani et al., 2001). Thus, the results presented concerning changes in β -catenin-Tcf/Lef-mediated transcription and survivin expression cannot be attributed to fortuitous alterations in caveolin-2 protein levels.

Caveolin-1 in colon cancer

Rather intriguingly, increased cell proliferation observed in colonic crypts of caveolin-1 knock-out mice was recently attributed to augmented β -catenin-Tcf/Lef-dependent transcription (Li et al., 2005). In addition, accumulation of β -catenin in intestinal crypts in colorectal adenocarcinomas reportedly leads to survivin upregulation (Zhang et al., 2001a). Furthermore, our group has previously shown that caveolin-1 re-expression in colon adenocarcinoma cells reduces the tumor forming ability of these cells in nude mice (Bender et al., 2000). Thus, the mechanism proposed here linking increased caveolin-1 presence to decreased survivin levels and vice-versa offers an attractive possibility to connect all these observations and explain how caveolin-1 might function as a tumor suppressor. However, additional experimentation is required to substantiate this intriguing possibility.

Conclusions

In summary, our results show for the first time that survivin downregulation is an important event observed in response to augmented caveolin-1 expression. They also corroborate the notion that β -catenin-Tcf/Lef transcriptional activity is negatively regulated by caveolin-1 and identify survivin as an additional, to date, unappreciated target gene that is regulated by caveolin-1 via this pathway. Furthermore, we showed that limitations imposed on a cell by the presence of caveolin-1, such as reduced proliferation, increased apoptosis and changes in the cell cycle can be overcome by increased survivin expression. Finally, we propose that the mechanism described may provide a rationale to understanding the ability of caveolin-1 to function as a tumor suppressor protein in specific cellular settings.

Materials and Methods

Materials

Polyclonal anti-caveolin-1 (C13630), monoclonal anti-caveolin-2 (C57820) and monoclonal anti- β -catenin (C19220) antibodies were from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-human survivin (AF886) and anti-actin (A5060) antibodies were from RD Systems (Minneapolis, MN, USA) and Sigma (St Louis, MO, USA), respectively. Rabbit polyclonal anti-human cyclin D1 (AB1320) was from Chemicon (Temecula, CA, USA). Goat anti-rabbit IgG (#1706515) and goat anti-mouse IgG (A4416) antibodies coupled to horseradish peroxidase (HRPO) were from Bio-Rad Laboratories (Hercules, CA, USA) and Sigma, respectively. Cy3TM-conjugated goat anti-mouse IgG (#115-165-068) and FITC-conjugated goat anti-rabbit IgG (#111-0950144) antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The goat anti-rabbit IgG (A11010) antibody Alexa Fluor[®]546 was from Molecular Probes (Eugene, OR, USA). BCA and the Super Signal West Pico Chemiluminescent Substrate were from Pierce (Rockford, IL, USA). The Superfect Reagent (#301305) and Qiagen[®] Plasmid Midi Kit (#12143) were from Qiagen (Valencia, CA, USA). TriZOL[®] reagent was from Invitrogen, Life

Technologies (Carlsbad, CA, USA). Hygromycin was from Calbiochem (La Jolla, CA). Cell medium and antibiotics were from Gibco-BRL (Paisley, Scotland, UK). Fetal bovine serum (FBS) was from HyClone (Logan, Utah). The MTS[®] Proliferation Assay Kit was from Promega (Madison, WI). For reporter assays, luciferin and 2-nitrophenyl- β -D-galactopyranoside (ONPG) were purchased from Sigma and Boehringer Mannheim (Mannheim, Germany), respectively. 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 and NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (10,000 U/ml penicillin, 10 μ g/ml streptomycin) at 37°C, 5% CO₂. Human breast cancer cells ZR75 and MCF7 were cultured in DMEM-F12 with 10% FBS and antibiotics. Human colon adenocarcinoma cells DLD1 were cultured in RPMI with 10% FBS and antibiotics. For transfection experiments, the reagent Superfect[®] was used following instructions provided by the manufacturer.

Plasmids

The plasmids pLacIOP and pLacIOP-caveolin-1 were described previously (Bender et al., 2000) and the pEGFP-C1 vector was from Clontech (Palo Alto, CA). The Tcf/Lef-reporter plasmids pTOP-FLASH and pFOP-FLASH were described previously (van de Wetering et al., 1997). Survivin promoter constructs pLuc-1710, pLuc-420, pLuc420-2M and pLuc420-3M were kindly provided by Dario Altieri and have been described previously (Kim et al., 2003). The plasmid encoding β -galactosidase activity (pON) was provided by Sergio Lavandero (Universidad de Chile, Santiago, Chile). To generate pEGFP-caveolin-1, full-length caveolin-1 available in pGEX2T (Felley-Bosco et al., 2000) was subcloned in frame into pEGFP-C1 vector using the restriction sites *Bgl*III-*Kpn*I. In order to generate the plasmid pEGFP-survivin, a human cDNA encoding survivin was obtained by RT-PCR from mRNA of the human colorectal adenocarcinoma cell line HT29, using the sense (5'-GGATCCGGCGGCATGGGTGCCCGACGTTG-3'; *Bam*HI) and anti-sense (5'-GGTACCCTCAATCCATGGCAGCCAGCTGCT-3'; *Kpn*I) primers. The resulting cDNA was introduced into the pCR2.1-TOPO vector by TA-cloning (Invitrogen, Carlsbad, CA, USA), sequenced in both directions and thereafter subcloned in frame into pEGFP-C1 using the restriction sites *Bgl*III-*Kpn*I.

Western blotting

Cell extracts were prepared and analyzed by SDS-PAGE as described previously (Felley-Bosco et al., 2000). Blots were blocked with 5% milk in 0.1% Tween 20-PBS and then probed with anti-actin (1:5000), anti- β -catenin (1:1000), anti-caveolin-1 (1:5000), anti-caveolin-2 (1:1000), anti-cyclin D1 (1:4000) or anti-survivin (1:3000). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL 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'-CCGACGTTGCCCTCTGC-3'; anti-sense primer 5'-TCGATG-GCACGGCGCAC-3'), caveolin-1 (sense primer 5'-GGCAACATCTAGAAGC-CCAACAA-3'; anti-sense primer 5'-CTGATGCACTGAATCCAATCAGGAA-3') and actin (sense primer 5'-AAATCGTGCCTGACATTAAGC-3'; anti-sense primer 5'-CCGATCCACACGGAGTACTT-3') cDNAs were amplified by PCR. All reaction products were analyzed after 30 amplification cycles, each of which involved consecutive 1-minute steps at 94°C, 55°C and 72°C.

Immunoprecipitation

Cell extracts were prepared in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 60 mM octylglucoside. Supernatants obtained after centrifugation (13,000 g, 5 minutes, 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 on 12% acrylamide minigels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose. Blots were then probed as indicated above.

Immunofluorescence

HEK293T cells were transfected for 24 hours with the plasmids indicated and replated on glass coverslips for an additional 24 hours in normal culture medium. After rinsing with PBS, cells were fixed in PBS with 4% paraformaldehyde (30 minutes) and permeabilized with 0.1% Triton X-100 (10 minutes). Cells were incubated with either polyclonal anti-caveolin-1 IgG (1:200) or monoclonal anti- β -catenin IgG (1:50) first antibodies, followed by cy3-conjugated anti-mouse IgG (dilution 1:200) or FITC-conjugated anti-rabbit IgG (dilution 1:200) second antibodies. In some experiments, cells were further incubated with 1 μ g/ml propidium iodide (PI) for 10 minutes at room temperature before mounting them onto slides with Mowiol (Dabco). Samples were visualized with an LSM-400 Carl

Zeiss confocal microscope (Axiovert) following excitation at 488 and 543 nm (UACI, ICBM, Universidad de Chile).

Proliferation assay

HEK293T cells were transfected for 24 hours and subsequently re-plated on 96-well plates at a density of 1×10^4 cells per well. After an initial 24-hour period in culture, cells were treated with 250 nM doxorubicin, 25 μ M etoposide or 250 nM taxol and left for an additional 24 hours. Cell proliferation was evaluated by the MTS[®] assay, according to the manufacturer's (Promega, Madison, WI) instructions. Note that the MTS assay measures both cell proliferation and viability.

Analysis of survivin by fluorescence-activated cell sorting (FACS)

HEK293T cells were transfected with the plasmid pEGFP-caveolin-1 and grown for 24 hours. Thereafter, cells were harvested, permeabilized with methanol and blocked in 2% BSA-PBS for 30 minutes at room temperature. Cells were then incubated with the polyclonal anti-survivin first antibody (1:100) followed by an Alexa Fluor[®]546-coupled goat anti-rabbit IgG second antibody (1:200). Finally, cells were resuspended in PBS and analyzed by flow cytometry (FACSsort, Becton Dickinson, Mountain View, CA). Survivin content was determined in transfected and non-transfected cells by quantifying red fluorescence (survivin) in GFP-positive (green; caveolin-1) and GFP-negative (non-green; no caveolin-1) cells, respectively, using the Cell Quest program.

Viability assays

Cell viability was analyzed by flow cytometry following propidium iodide (PI) staining, essentially as described before (Hetz et al., 2002). HEK293T cells were transfected with the vectors pLacIOP, pLacIOP-caveolin-1, pEGFP-C1 or pEGFP-caveolin-1, as indicated above. Post-transfection (24 hours), 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 (Hetz et al., 2002). For DNA content analysis (cell-cycle distribution), cells were previously 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.

DNA laddering

Cells were harvested by brief centrifugation, resuspended in 100 μ l PBS and lysed by addition of 100 μ l phenol:chloroform:isoamylalcohol (25:24:1) followed by vortexing and centrifugation. Then, the aqueous phase was separated and incubated with RNase A for 30 minutes at 37°C and analyzed by electrophoresis on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. DNA laddering was visualized by exposure to UV light.

Luciferase reporter assays

For Tcf/Lef reporter assays, HEK293T cells were transfected with 2 μ g of the respective plasmids: pTOP-FLASH (Tcf/Lef reporter), pFOP-FLASH (mutated Tcf/Lef-binding sites), pON (β -galactosidase), pLacIOP-caveolin-1 or the vector alone (pLacIOP). Alternatively, experiments were done in the presence of 20 mM LiCl to block GSK3- β activity. For survivin promoter assays, HEK293T cells were transfected with 2 μ g of the respective plasmids: pLuc1710, pLuc420, pLuc420-2M, pLuc420-3M, pON, pLacIOP-caveolin-1 or pLacIOP. Post-transfection (24 hours), cells were lysed in a buffer containing 0.1 M KH₂PO₄ (pH 7.9) and 0.5% Triton X-100 and supernatants were either analyzed by SDS-PAGE or used to measure luciferase (80 μ l) and β -galactosidase (50 μ l) activities. The values reported for luciferase activity were standardized to β -galactosidase activity.

Survivin induction with Wnt3a-containing conditioned medium

Preparation of Wnt3a-containing medium has been described previously (Alvarez et al., 2004). Briefly, HEK293 cells stably transfected with the plasmid Wnt-3a-pUSEamp for constitutive expression and secretion of Wnt3a into the medium were grown to confluence in DMEM supplemented with 10% FBS and antibiotics. Culture medium was then replaced by DMEM supplemented with antibiotics only and cells were maintained for 7 days. The resulting cell supernatant, referred to as conditioned medium, was added to HEK293T cells for 24 hours to induce survivin expression after transfection with either pLacIOP or pLacIOP-caveolin-1. Cell extracts were then prepared as described.

Transfection of DLD1, ZR75 and MCF7 cells

Stably transfected clones of DLD1 cells with either the plasmid pLacIOP alone (M1) or pLacIOP-caveolin-1 (C4) were described previously (Bender et al., 2000). ZR75 and MCF7 cells were transfected with the plasmids pLacIOP and pLacIOP-caveolin-1 using the reagent Superfect[®], following instructions provided by the manufacturer (Qiagen, Valencia, CA). Post-transfection (48 hours), cells were grown in selection medium containing 750 μ g/ml hygromycin for 2-3 weeks. Mock (pLacIOP) and caveolin-1-expressing (pLacIOP-caveolin-1) cells (mixed, non-clonal populations) were obtained and characterized.

Silencing of caveolin-1 by siRNA in NIH3T3 cells

Synthesis of siRNA

siRNAs were designed to target sequences AAATACGTAGACTCGGAGGGA, AAGATTGACTTTGAAGATGTG, AAGATGTGATTGCAGAACCAG and AAAATATTCAGCAATGTCCGC, beginning at nucleotide positions 13, 193, 206 and 493, respectively, in the mouse caveolin-1 coding region (GenBankTM accession number NM 007616). Sense and anti-sense DNA oligonucleotides (MWG Biotech) were flanked at the 3' end by 5'-CCTGTCTC-3', a sequence that is complementary to the T7 promoter primer provided with the *Silencer* siRNA Construction Kit from Ambion (TX). Oligonucleotides were transcribed in vitro using the T7 promoter primer. For generation of siRNA duplexes, sense and antisense siRNA strands were mixed and were incubated at 37°C overnight. Duplex siRNAs corresponding to the four regions of caveolin-1 specified were mixed at equimolar concentration for experiments. NIH3T3 cells (2×10^5) were seeded on 6-well plates and grown for 24 hours. Subsequently, cells were transfected with the mixture of caveolin-1-specific siRNAs (final concentration 25 nM) using the reagent siPORT Amine (*Silencer*™ siRNA Transfection Kit, Ambion, TX) following instructions provided by the manufacturer. In control experiments, the same amount of *Silencer*® Negative Control #2 siRNA from Ambion was employed. Cell extracts were obtained 48 hours post-transfection for analysis of mRNA or protein levels. Alternatively, 24 hours post-siRNA transfection, cells were replated either to measure cell proliferation or for further transfection with the reporter plasmids pTOP-FLASH and/or pFOP-FLASH.

Statistical analysis

Where pertinent, results were compared using unpaired Student's *t*-tests of at least three independent experiments. $P < 0.05$ was considered significant.

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