CaSm-Mediated Cellular Transformation Is Associated with Altered Gene Expression and Messenger RNA Stability

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Abstract

CaSm (cancer-associated Sm-like) was originally identified based on elevated expression in pancreatic cancer and in several cancer-derived cell lines. CaSm encodes a 133 amino acid protein that contains two Sm motifs found in the common small nuclear RNA proteins and the LSm (like-Sm) family of proteins. Compared with normal human prostate tissue and primary prostate epithelial cells, some primary prostate tumors and prostate cancer–derived cell lines have elevated CaSm expression. Expression of antisense CaSm RNA in DU145 cells results in reduced CaSm protein levels and less transformed phenotype, measured by anchorage-independent growth in vitro and tumor formation in severe combined immunodeficient mice in vivo. Additional data shows that adenoviral delivery of antisense CaSm inhibits the growth of prostate cancer cell lines by altering cell cycle progression, and is associated with reduced expression of cyclin B1 and CDK1 proteins. Consistent with failure of antisense-treated cells to enter mitosis, microarray analysis identified altered expression of NEK2 and nucleophosmin/B23. Although the mechanisms by which CaSm contributes to neoplastic transformation and cellular proliferation are unknown, it has been shown that the yeast homologue (spb8/LSm1) of CaSm is required for 5′ to 3′ degradation of specific mRNAs. We provide data consistent with a similar role for CaSm in human cells, supporting the hypothesis that elevated CaSm expression observed in cancer leads to destabilization of multiple gene transcripts, contributing to the mutator phenotype of cancer cells. (Cancer Res 2005; 65(14); 6228-36)

Introduction

Prostate cancer is the most prevalent cancer in American males and the second leading cause of their cancer death (1). The development of many tumor types progresses from normal → dysplasia → carcinoma in situ → localized primary tumor → tumor with metastases. However, it is believed that the development of prostate tumors is a more stochastic process. The progression of prostate cancer varies considerably among patients. Indolent prostate cancers may remain localized for decades, whereas aggressive prostate cancers can metastasize rapidly to lymph nodes and/or by hematogenous routes. Human prostate cancer is heterogeneous in appearance and genetically unstable. Multiple genotypes from the same primary prostate tumor in phenotypically similar foci are frequently identified (2). A better understanding of the molecular controls that regulate prostate cancer growth and transformation is needed in order to develop more effective therapeutic approaches for aggressive prostate cancer. Furthermore, such understanding may allow insight into new markers for patient stratification, allowing those patients most likely to progress to clinical prostate cancer to be treated aggressively. Based on these observations, genetic and epigenetic studies of prostate cancer have been directed towards elucidation of possible mechanisms that could account for the rapid progression observed in some patients. We have hypothesized that alteration in transcriptional control and repair offer two mechanisms leading to rapid changes in the transcriptome of the prostate cancer cell. Specifically, we and others have shown that the ETS2 transcription factor is expressed at elevated levels in prostate cancer and is necessary for some of the transformed phenotypes of prostate cancer cells (3–5). In addition, we have shown that there are defects of mismatch repair genes in prostate cancer (6, 7), likely to contribute to genomic instability observed in prostate cancer (8, 9). In addition to transcriptional changes, alterations in mRNA stability would be expected to contribute to the mutator phenotype (10) in prostate cancer.

To characterize molecular alterations that lead to cancer, we previously employed subtractive hybridization cloning in order to identify and isolate genes whose expression pattern is associated with pancreatic cancer (11, 12). This approach led to the isolation and initial characterization of CaSm (for cancer-associated Sm-like) that was identified due to its elevated expression in pancreatic cancer. CaSm is overexpressed in a majority of pancreatic tumors and cancer-derived cell lines (12) as well as metastatic tumors (13). Furthermore, CaSm expression is required for the maintenance of transformed phenotype of pancreatic cancer cell lines (12). Elevated CaSm expression is not limited to pancreatic cancer, being overexpressed in lung, esophageal and bladder tumors, and pleural mesothelioma. Increased levels of CaSm mRNA are also observed in prostate, liver, lung, ovarian, rectal, bladder, and kidney cancer–derived cell lines. The current study provides evidence that CaSm is expressed at elevated levels in prostate cancer and uses antisense and small interfering RNA (siRNA) inhibition of CaSm expression to show that CaSm affects prostate cancer cell

Note: M.M. Fraser and P.M. Watson contributed equally to this work.

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6 Unpublished observations.
proliferation and tumor development. For the first time, we provide evidence that CaSm may exert its effect by altering message stability. These collective results support the importance of CaSm in the development and maintenance of cellular transformation.

**Materials and Methods**

**Cell lines and tissue culture.** DU145 and PC3 cell lines were purchased from the American Type Culture Collection (Manassas, VA), and were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies). The 267B-1 cell line (SV40 T-antigen immortalized neonatal prostate epithelium) was generously provided by Dr. J. Rhim (Bethesda Naval Hospital, Bethesda, MD) and cultured in P4-8 medium with 2% serum (Biological Research Facility and Faculty, Inc., Ijamsville, MD). Normal prostate epithelial cells were purchased and cultured in PrEGM medium (Clonetics, Rockland, ME) according to the manufacturer's instructions. All cell lines were propagated at 37°C in an atmosphere containing 5% CO2.

Transfection of DU145 cells was done in six-well plates using 1 μg of DNA, 6 μL LipofectAMINE PLUS reagent or 4 μL of LipofectAMINE reagent (Life Technologies). Stable cell lines were selected in 400 μg/mL of G418 (Calbiochem, La Jolla, CA). Cell lines were screened by Northern blot analysis and clones expressing antisense CaSm were selected for anchorage-dependent and -independent studies. Anchorage-dependent growth studies were done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assays. Parental and antisense expressing clone cells were plated at a density of 10,000 cells per well in 96-well plates. They were cultured for a period of 7 days and analyzed on a spectrophotometer on days 1, 3, 5, and 7. Anchorage-independent growth was assessed by soft agar colony formation. Parental and clone cells were plated at a density of 20,000 cells per well in six-well plates. Cells were fed and grown for a period of 4 weeks and colonies were counted. The bottom layer of agar was removed and the top layer and feeding layers were comprised of 0.4% agar. Both the MTT and soft agar assays were done in duplicate.

NIH-3T3 cells were kindly provided by Dr. Donald Blair (National Cancer Institute, Frederick, MD) and maintained in DMEM containing 8% calf serum at 37°C in an atmosphere containing 10% CO2. Cells were transfected with 1 μg of DNA using LipofectAMINE PLUS reagent in six-well plates at 40% to 50% confluence. Two days after transfection, cells were replated in 100 mm dishes, at a ratio of 1:2, and 0.25 μmol/L dexamethasone was added to the medium. Foci were counted on day 12 at 40× magnification on an inverted light microscope.

**RNA isolation and analysis.** RNA was purified from cultured cells or tissues using RNeasy (TEL-TEST) or Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Northern blot analysis was done on total RNA. The RNA was fractionated on a 1.2% agarose gel with 0.66 mol/L formaldehyde. Gels were transferred overnight to Duralon membrane (Stratagene, La Jolla, CA) in 0.1 mol/L sodium phosphate (pH 6.8). The Duralon membrane was UV cross-linked and hybridized with 32P-labeled probe (Stratagene prime II kit) in QuickHyb solution (Stratagene) according to the manufacturer's protocol. Quality of RNA and equivalent loading was verified by ethidium bromide staining of 18S and 28S RNA.

**Probes.** An 894 bp CaSm-specific insert was prepared by EcoRI digestion of pSGneoSK/CaSm (12). A p21/WAF1-specific insert was isolated by HindIII-AglI digestion of pC-WAF1-S (provided by Dr. Bert Vogelstein, Department of Oncology, Johns Hopkins University, Baltimore, MD). A B23 insert was obtained by EcoRI digestion of a nucleosamin/B23 fragment generated by RT-PCR (described below) and cloned into the pCRII-TA vector. Strand-specific probes were created by linear PCR using single primers (Table 1).

**RT-PCR.** DNA from prostate cell lines and normal tissue was used as a template for RT-PCR. cDNA was obtained using total RNA (5 μg) following the manufacturer's protocol (Superscript II, Invitrogen). cDNA was then used for PCR amplification using gene-specific primers (Table 1).

**Expression vectors and cloning.** Antisense CaSm in pSGneoSK was previously described (12). CaSm was amplified by PCR using sequences to provide restriction enzyme sites for directional cloning in FpcDNA3. FpcDNA3 is a modified pDNA3 vector that allows incorporation of an amino-terminal FLAG epitope (provided by Dr. Craig Hauser, The Burnham Institute, La Jolla, CA). Orientation and sequence was verified by analysis on ABI 373 automated sequencer (Applied Biosystems, Foster City, CA). Dr. D. Blair provided pM1, which contains the v-mos oncogene inserted into the EcoRI site of the pBR322 vector.

**Small interfering RNA.** CaSm siRNA sequences were identified using the criteria of Paddison and Hannon (14). Two sequences were identified that were unique to the CaSm gene and conformed to the consensus of 5'-AA19N-3'. These sequences were CaSm1-104-123, aacugguuuuggacgcgcu; and CaSm2-328-347, aauaggggauauuccgg. RNA in the sense and antisense direction was synthesized and annealed to form double-stranded RNA by Dharmacon Research, Inc. (Lafayette, CO). Transfection into prostate cancer cell lines was done using OligofectAMINE (Invitrogen) with dsRNA at a final concentration of 20 mmol/L. The transfection was repeated on days 3 and 5, and cellular protein and total RNA extracted on day 7.

**Western blot analysis.** Cells were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 8% protease inhibitors]. Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and incubated for 1 hour with one of the following antibodies: β-actin (Oncogene Research Products, Boston, MA), cyclin B1 (1:4541A, BD Biosciences/PharMingen, San Jose, CA), CDK1 (1:4391A, PharMingen), p21 (OP64-100UG, Oncogene Research Products), and Nek2 (SC-7440, Santa Cruz, Santa Cruz, CA). After washing, the

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membrane was incubated in horseradish peroxidase–labeled secondary antibody [HRP-goat-F(ab')2, anti-mouse-IgG (#11503507, Caltag, Burlingame, CA) or HRP-goat-F(ab')2 anti-rabbit IgG (#143007, Caltag)] for 20 minutes. The membrane was developed in enhanced chemiluminescence (#34080, Pierce, Rockford, IL) for 5 minutes and exposed to Kodak MR film.

The chicken anti-CaSm antibody was made using full-length bacterial expressed protein injected into laying hens (Aves Laboratory, Portland, OR). IgY was purified from eggs and affinity-purified with full-length protein covalently linked to agarose (Pierce). Rabbit anti-CaSm antibody was initially provided by the Lihrmann laboratory (Max-Planck Institute, Germany) and subsequently generated using a synthetic peptide corresponding to the 20 COOH-terminal amino acids of CaSm covalently coupled to bovine serum albumin (Rockland, Gilbertsville, PA). The antibody was affinity-purified as above. The specificity of this antibody was shown by the absence of immunoreactive proteins in Western blots of extracts prepared from cell lines that do not express CaSm mRNA. For Western blot, proteins were separated on a 12.5% acrylamide gel and transferred to nitrocellulose. After blocking, the membrane was incubated overnight in 2 µg/mL affinity-purified anti-CaSm IgY and for 1 hour in HRP-goat anti-IgY.

### Results

**CaSm expression is elevated in prostate cancer–derived cell lines.** To determine CaSm gene expression in prostate-derived cell lines, Northern blot analysis was done using a full-length CaSm probe. Expression was high in all prostate cancer–derived cell lines tested (LNCaP, DU145, and PC3; Fig. 1A). In contrast, CaSm expression is reduced in the immortalized fetal prostate epithelial cell line, 267B1.

RT-PCR analysis (Fig. 1B) was used to evaluate the expression of CaSm in prostate cancer cell lines compared with normal prostate tissue. High-level CaSm mRNA expression was detected in prostate tissue. The mRNA was isolated using Trizol (Invitrogen) and 1 µg of total RNA was used to isolate mRNA by Oligotex suspension (#70061, Quiagen, Valencia, CA) following the manufacturer's instructions. To identify genes that are differentially expressed between two samples, cDNAs were labeled with Cy3 or Cy5, and hybridized simultaneously to an identical array. The GEM V array (Genome Systems, Inc., St. Louis, MO) contained ~9,600 expressed sequence tags and cDNAs. The signals from each of 9,600 spots were measured in duplicate producing four data points per cDNA clone.
cancer cell lines; in contrast, relatively low expression levels were seen in normal prostate. Consistent with mRNA expression, elevated CaSm protein expression was also found in all prostate cell lines tested. Normal prostate epithelial cells express very low levels of CaSm compared with the cancer cell lines (Fig. 1C).

CaSm expression is necessary and sufficient for cellular transformation. To determine whether CaSm expression is necessary for the maintenance of the transformed phenotype, DU145 cells were transfected with a pSGneoSK plasmid vector expressing antisense CaSm. The effect of antisense expression on the ability of stable cell clones to grow independent of anchorage as colonies suspended in soft agar and form tumors in SCID mice was evaluated. The antisense-expressing cell clonelinesformed significantly fewer and smaller colonies than the parental cell line, demonstrating that CaSm expression is necessary for anchorage-independent growth (Fig. 2A). The parental DU145 cells formed >550 colonies, of which 65 were >280 μm in diameter. In contrast, the three antisense clones formed between 15 and 44 colonies each. Protein extracts were prepared from DU145 cells and these antisense clones and CaSm level assessed by Western blot. The antisense clones were found to consistently express less CaSm protein than parental DU145 cells (Fig. 2A, inset). We next examined the ability of DU145 clones expressing antisense CaSm to form tumors. The DU145 tumors grew to a size of 100 mm³ in an average of 31.6 days, whereas the antisense DU145 clone never grew to this size over the 60-day observation period. The Kaplan-Meier survival curves gave a log rank probability of 0.0018 using survival to a tumor volume of 100 mm³ as the y-axis (Fig. 2B).

Reduction of CaSm expression in human prostate cancer–derived cell lines inhibits their anchorage-independent growth and tumor formation, both hallmarks of transformation. To evaluate whether CaSm can function as an oncogene, NIH-3T3 cells were transfected with a vector containing the complete open reading frame of human CaSm and analyzed for in vitro foci formation. CaSm-transfected NIH-3T3 cells formed 80 foci of transformed cells (Fig. 2C) compared with 9 foci for untransfected NIH-3T3 cells. As a control for transformation, NIH-3T3 cells transfected with the v-mos oncogene formed 115 foci. Collectively, these studies show that CaSm expression is necessary and sufficient for cellular transformation.

Adenoviral expression of antisense CaSm inhibits cell proliferation. We have shown that multiple stable antisense clones fail to grow independent of anchorage. Adenoviral delivery of antisense CaSm allows examination of altered phenotypes of pools of infected cells rather than selected individual clones. We used a previously characterized adenoviral vector expressing antisense CaSm RNA (Ad-anti-CaSm; refs. 15, 16) to study the effect of reduced CaSm on the growth of prostate cancer. Northern blot analysis with a CaSm-specific sense ssDNA probe showed that Ad-anti-CaSm–infected cells expressed the appropriate antisense construct. This probe revealed a signal at 900 bp corresponding to expression of the CaSm antisense in prostate cancer lines treated with the virus (Fig. 3A, middle). To assess whether Ad-anti-CaSm expression affected the level of endogenous CaSm message, a CaSm-specific antisense ssDNA probe was used that selectively detected CaSm mRNA. CaSm mRNA level was significantly decreased in DU145 cells 48 hours after infection with Ad-anti-CaSm. In contrast, no change was observed in the uninfected and LacZ control infected cells (Fig. 3A, top).

To determine the effect of antisense CaSm on the growth of prostate cancer cells, DU145 and PC3 cells were infected with Ad-LacZ (MOI 100) or Ad-anti-CaSm (MOI 100) and assayed for anchorage-dependent cell proliferation by MIT assay. DU145 Ad-anti-CaSm–infected cells showed reduced cell numbers compared with untreated or Ad-LacZ–treated cells (Fig. 3B). There was no change in growth characteristics in the Ad-anti-CaSm–infected PC3 cells (data not shown).

To confirm the findings from the antisense CaSm experiments, we did complementary loss of function studies using siRNA (14, 18). Two siRNAs were designed to use in gene silencing experiments. Unique sequences were selected that are not homologous to other sequences in the human genome. Transfection of prostate cancer cells with either of these siRNAs significantly reduced expression of CaSm protein, whereas control sequences did not (Fig. 3C). In addition, cells treated with these siRNAs showed significant reductions in proliferation compared with control.

Figure 2. CaSm expression is associated with cellular transformation. A, parental cells and antisense-expressing clones were grown in soft agar for 4 weeks. The number of colonies greater than 140 μm in diameter was determined. The number of colonies for each transfected clone was compared with the parental cells using a two-sample t test. As three clones were analyzed, a Bonferroni adjustment was done, indicating a significant P value as <0.017. The P value for each antisense clone compared with parental cells was <0.001. A, Western blot of total extracts prepared from DU145 parental cells and stable antisense clones (19, 21, and 22) showing reduced protein expression of CaSm in antisense clones (inset). B, CaSm is necessary for tumor formation in vivo. Kaplan-Meier curves for DU145 cells and DU145 cells stably transfected with CaSm antisense. Cells were injected into SCID-Bg mice at day 0 and tumor growth was monitored weekly. The DU145 cells were injected into the right flank and the antisense cells were injected into the left flank. The data were plotted in Kaplan-Meier curves and analyzed using the log rank test and found to be significantly different (P = 0.0018). C, CaSm transfection of NIH-3T3 cells leads to loss of contact inhibition with foci formation of transformed cells. NIH-3T3 cells were transfected with a plasmid containing the full-length CaSm or the v-mos oncogene and scored for foci formation. The v-mos oncogene serves as a positive control for transformation. Number of foci for 3T3 parental cells and v-mos and CaSm transfected were compared using a two-sample t test. The P value for the v-mos oncogene was P = 3 × 10⁻⁶ and P = 3 × 10⁻⁶ for CaSm.

Figure 3. Loss of CaSm mRNA effects cell proliferation. A, ssDNA probe analysis confirms that Ad-antisense-CaSm is able to effectively reduce endogenous CaSm expression in human DU145 prostate cancer cells (top) and that antisense CaSm RNA is expressed in the infected cells. B, DU145 cells were maintained in RPMI or treated with Ad-LacZ or Ad-antisense-CaSm at an MOI of 100. Cells were treated for 90 minutes and cell number was assessed by MTT for 6 days. The Ad-antisense CaSm–treated cells show reduced growth compared with control and Ad-lacZ–treated cells. C, DU145 cells were transfected with each CaSm siRNA and a negative control scramble dsRNA or maintained in RPMI. Western blot analysis of DU145 cells treated with CaSm siRNA for 7 days show reduced CaSm protein levels compared with control and scramble siRNA. Loading control with actin staining (bottom). D, DU145 cells have a slower increase in cell number after treatment with siRNA. Cells were transfected as above at days 1, 2, and 5 (arrows) and cell number was assessed by MTT.

siRNAs have reduced proliferation, as observed after infection with Ad-antisense CaSm (Fig. 3D).

Ad-anti-CaSm treatment in vitro leads to a cytostatic inhibition of the cell cycle. Based on the observed differences in growth capabilities observed in anti-CaSm–infected cells, cell populations were further analyzed by flow cytometry. DU145 and PC3 prostate cancer–derived cell lines were infected at an MOI of 100 with Ad-anti-CaSm or Ad-LacZ and examined by flow cytometry. Treatment with the CaSm antisense virus alters the proportion of cells in each phase of the cell cycle, with a significant decrease in G0/G1 populations and an increase in the number of cells in the G2-M phase. Specifically, 41% of DU145 cells treated for 48 hours with Ad-anti-CaSm were arrested in the G2-M phase (30% in G1), compared with only 18% (62% G1) of control Ad-LacZ–infected cells. PC3 cells infected with Ad-anti-CaSm virus do not exhibit significant alterations in the cell cycle consistent with the MTT data.

Ad-anti-CaSm–infected cells display a phenotype similar to endoreduplication. DU145 cells treated with Ad-anti-CaSm exhibit a large percentage of cells with >4 N DNA content, compared with the untreated or Ad-LacZ–treated cells. Forty-eight hours after infection, 26% of Ad-anti-CaSm–treated DU145 cells had a >4 N DNA content, compared with 1% to 2% for untreated or Ad-lacZ–treated cells (Fig. 4A). A >4 N population is often referred to as “aggregate cells,” representing two or more cells stuck together. However, 4′,6-diamidino-2-phenylindole stained Ad-anti-CaSm–treated cells shows that these cells have enlarged nuclei (Fig. 4B). This data suggests that treatment of DU145 with Ad-anti-CaSm results in DNA synthesis in the absence of mitosis, a phenotype often associated with endoreduplication or endoreplication (19). It should be noted that despite the significant decreases in cell growth and the dramatic changes in the cell cycle, a substantial sub-G0 peak was not detected, suggesting that apoptosis is not occurring as a result of Ad-anti-CaSm treatment (Fig. 4A).

After infection with anti-CaSm–expressing virus, DU145 cells also exhibit a marked decrease in the number of cells undergoing mitosis (Fig. 4C). In every field of view at 400× magnification, two to four cells can be seen undergoing mitosis in both untreated and Ad-LacZ–treated cells. In contrast, Ad-anti-CaSm–treated cells rarely show any cells undergoing mitosis. Overall, we observe a 10-fold reduction of mitotic cells in Ad-anti-CaSm–treated DU145 cells relative to untreated or Ad-lacZ–treated cells.

Altered expression of G2-M regulatory genes as a consequence of antisense CaSm expression. Because cyclin B and CDK1 are essential for the G2-M transition, we examined whether their expression was altered following Ad-anti-CaSm infection. Cyclin B1 and CDK1 protein are reduced after infection with Ad-anti-CaSm (Fig. 4D), whereas these are not affected by Ad-LacZ.

Figure 4. Ad-anti-CaSm–treated cells have cells with an increased cell population with >4 N DNA and altered expression of G2-M regulatory genes. DU145 cells treated with Ad-anti-CaSm at an MOI of 100 have a larger population of aggregate cells than those left untreated or treated with adenoviral LacZ. A, DU145 cells were untreated or treated with adenoviral LacZ or antisense CaSm at an MOI of 100. At 48 hours, the cells were stained with propidium iodide and analyzed for DNA content by flow cytometry. B, cells were infected as in (A) and stained with 4′,6-diamidino-2-phenylindole and enlarged nuclei counted under 400× magnification. Fifty out of 124 Ad-antisense-CaSm–treated cells have enlarged nuclei, whereas untreated (control) or Ad-LacZ–treated cells have 5 of 63 and 0 of 69 enlarged nuclei, respectively. Number of enlarged nuclei were compared with parental cells using a two-sample t test (P < 0.001 Ad-LacZ versus Ad antisense CaSm). C, DU145 cells were left untreated or treated with Ad-LacZ or Ad-antisense-CaSm at a MOI of 100. Cells were fixed with 70% methanol in PBS. The cells were washed once with PBS and stained for 15 minutes at 37°C with 1 μg/mL 4′,6-diamidino-2-phenylindole and mitotic figures counted. DU145 cells untreated or treated with adenoviral LacZ showed at least 5 of 63 cells and 6 of 69 enlarged nuclei, respectively, in mitosis, whereas Ad-anti-CaSm–treated cells showed 1 mitotic cell out of 124 counted. The number of mitotic figures were compared with parental cells using a two-sample t test (P < 0.01 Ad-LacZ versus Ad-antisense CaSm). D, Western blot and RT-PCR analysis of Ad-antisense-CaSm–treated cells 48 hours post-infection compared with untreated or Ad-LacZ–treated DU145 cells for proteins involved in cell cycle regulation. Cyclin B and CDK1 protein levels are reduced in Ad-anti-CaSm–treated DU145 cells by Western blot consistent with a G2-M cell cycle block (top). Nek2 mRNA expression is increased in Ad-antisense-CaSm–treated DU145 cells by RT-PCR with S26 ribonucleoprotein as control (middle). Nucleophosmin/B23 RNA level is decreased in Ad-antisense-CaSm–treated DU145 by Northern blot (bottom).
CaSm Expression Is Required for Cellular Transformation

Virus. Microarray analysis was used to identify additional genes with altered expression as a consequence of reduced CaSm level. Polyadenylic acid (poly(A))-enriched RNA, isolated from DU145 parental cells and a stable cell line expressing antisense CaSm, was used to prepare cDNA probes for analysis of two different cDNA libraries. The Human UniGEMV (Genome Systems) array contained probes for 7,000 genes. The second microarray allowed assessment of >6,000 prostate-derived cDNAs (17, 20). The expression profiles identified differences between these cell lines and we selected two genes critical for cell cycle progression for validation. Nek2 [never in mitosis A (NIMA)-related kinase-2], a core component of the centrosome, peaks in S-G2 phase and is reduced when cells enter mitosis (21). Nek2 expression was increased in anti-CaSm expressing DU145 cells. Northern blot analysis confirmed Nek2 mRNA overexpression in stable antisense expressing cells compared with parental DU145 cells (data not shown). Differential expression was also seen in Ad-anti-CaSm–infected cell lines and confirmed that Nek2 mRNA level is elevated after infection with Ad-anti-CaSm virus (Fig. 4D, middle). Nucleophosmin/B23 associates with centrosomes and is required for DNA replication (22). Analysis of the prostate cDNA array indicated that nucleophosmin/B23 mRNA was reduced in RNA prepared from anti-CaSm expressing DU145 cells. Nucleophosmin/B23 mRNA expression is reduced in cells infected with the Ad-anti-CaSm virus (Fig. 4D, bottom), validating the microarray results. Collectively, these alterations, along with reduced cyclin B/CDK1, could contribute to the G2-M blockade in the adenovirus infection leads to cell cycle alterations in DU145 cells. Based on the importance of p21/Cip1 in cell cycle control, expression of p21/Cip1 mRNA was determined following Ad-anti-CaSm infection. The level of p21/Cip1 mRNA was compared between DU145, a DU145 clone with stable expression of anti-CaSm, and a pool of Ad-anti-CaSm or Ad-lacZ–infected DU145 cells (Fig. 5A). Northern analysis shows that cells expressing anti-CaSm have significantly higher levels of p21/Cip1 mRNA than the parental controls. To determine whether the increased message level resulted in increased p21 protein, total protein was extracted from untreated and Ad-LacZ or Ad-αCaSm–treated DU145 cells and subjected to Western blot analysis using a p21/Cip1-specific antibody. Unexpectedly, p21 protein levels were not significantly altered between untreated, Ad-LacZ and Ad-αCaSm–treated DU145 cells (Fig. 5B).

Based on the homology between CaSm and yeast Lsm1/spb8, a protein that contributes to mRNA stability, altered mRNA stability may contribute to the observed increase in p21/Cip1 mRNA. An actinomycin D blockade experiment was done to determine if antisense CaSm treatment led to changes in mRNA stability. Parental and antisense-expressing DU145 cell lines were treated with actinomycin D (5 μg/mL) and total RNA was isolated at 0, 2.5, 5, 7.5, 10, 15, 24, and 48 hours after the addition of the drug. Total RNA was resolved on a 1.2% agarose gel and transferred to a nitrocellulose membrane and hybridized using a32P-labeled p21/Cip1-specific probe. B, p21 protein levels are not changed by antisense CaSm expression. Western blot analysis was done on cell extracts from the indicated treated cells. C, DU145 cells and a stable antisense CaSm-expressing cell line were treated with actinomycin D (5 μg/mL) and total RNA was harvested at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 4, 5, and 6 hours after the addition of the drug. Total RNA was resolved on a 1.2% agarose gel, transferred to a nitrocellulose membrane and hybridized with a32P-labeled specific probe for p21. Relative mRNA abundance was determined using phosphoimaging and densitometry, comparing p21 mRNA to S26 mRNA as control. Lines were generated using an exponential equation. R2 values were 0.975 for DU145 and 0.901 for the antisense DU145 clone. D, p21 mRNA is deadenylated in antisense CaSm-expressing cells. Total and poly(A) RNA were isolated from DU145 and a clone stably expressing antisense CaSm RNA was resolved on a 1.2% agarose gel, transferred and hybridized using a32P-labeled p21/Cip1-specific probe.

The stabilized p21 mRNA was deadenylated, poly(A) selection was used to enrich for polyadenylated mRNA. Total RNA and poly(A)-containing mRNA from DU145 and stable antisense CaSm expressing cells were analyzed by Northern blot. Whereas an increased level of total p21 mRNA was observed in the antisense-expressing cells, no significant enrichment was observed in the poly(A) p21 message population (Fig. 5D). When compared with the parental cells, there was significantly less polyadenylated p21 mRNA in the antisense CaSm-expressing cells. From these data, we hypothesize that the stabilized p21 messages are deadenylated and thus, are inefficiently translated.

CaSm protein is elevated in prostate cancer tissue. Our data suggest that CaSm protein expression may be up-regulated during the progression of prostate cancer. To evaluate CaSm protein expression in prostate tissue, we conducted immunohistochemical staining of human prostate cancer sections. Only those sections containing both normal and tumor areas on the same slide were scored. As shown in Fig. 6, CaSm protein is found predominantly in the cytoplasm of normal and cancer epithelial cells. However, epithelial cells present in prostate carcinomas show increased protein expression in 40% of patients (4 of 10) examined (representative data, Fig. 6D). Elevated CaSm protein expression was not observed in all carcinoma specimens examined. The
remaining cases were divided between those with equivalent expression between tumor and normal regions (1 of 10) and those cases where CaSm protein level is higher in the normal tissue (5 of 10; Fig. 6B).

Discussion

Our current studies show that CaSm is required to maintain the transformed phenotype of prostate cancer cells and can function as an oncogene. Overexpression of this gene in NIH-3T3 cells leads to foci formation in vitro. Stable expression of antisense in DU145 cells results in reduced CaSm protein levels and reduced ability to grow independent of anchorage in vitro or as tumors in vivo. Additionally, siRNA-mediated reduction of CaSm protein also leads to altered cell growth. Ad-anti-CaSm treatment of DU145 cells has a cytostatic effect, with increased G2-M populations of cells along with a substantial increase in cells with nuclei containing more than the normal 4 N content of DNA. These phenotypic changes are associated with changes in the expression of proteins that regulate cell cycle progression through G2-M and mitosis, consistent with the model that down-regulation of the CaSm oncogene results in the blockage of the cell cycle prior to completion of mitosis.

The normal G1-S-G2-M progression of the cell cycle is controlled by a series of cyclins and cyclin-dependent kinases (23, 24). Cyclin B/CDK1 functions during G2 into M phase. A change in the levels of cyclin B or CDK proteins or CDK1 activity could explain the cell cycle changes observed after treatment with CaSm antisense. It has recently been shown that siRNA-mediated reduction of cyclin B1 inhibits cellular proliferation, with cells arrested in G2-M (25), a similar phenotype to that found following Ad-anti-CaSm treatment. Furthermore, reduced cyclin B1 level is also observed during the endomitotic cell cycle of megakaryocytic cells (26), and suggests that the reduced cyclin B level in anti-CaSm–treated cells may contribute to the accumulation of cells with >4 N DNA. Reduction in CDK1/cyclin B protein level by CaSm antisense may suggest that CaSm overexpression could have an inverse effect and increase CDK1 function in cancer cells. Thus, CaSm overexpressing cells may be able to bypass the G2 checkpoint and increase their rate of proliferation. Microarray analysis showed that anti-CaSm affects the expression of multiple genes. Two genes important for control of the G2-M transition and progression through mitosis, Nek2 and nucleophosmin/B23, were selected and altered expression initially detected by microarray analyses was validated. Nek2 is a NIMA-related kinase implicated in regulating centrosome structure at the G2-M transition (27, 28). Two Nek2 splice variants (Nek2A and Nek2B) have been identified, and Nek2A has been shown to be a target for APC/C and proteosome-mediated degradation during entry into mitosis (29), consistent with the observation that proteolysis of NIMA is required for completion of Aspergillus mitosis (30). Overexpression of Nek2 has been found to induce aneuploidy, chromosome instability, and supernumerary chromosomes, aborted mitosis or failed cytokinesis (31). Anti-CaSm expression results in increased Nek2 mRNA expression, consistent with altered spindle checkpoint signaling that could contribute to the failure to enter mitosis, contributing to G2-M arrest and/or >4 N DNA (endoreduplication). Nucleophosmin/B23 is a multifunctional protein implicated as a target for cyclin E/cyclin-dependent kinase 2 in modulating centrosome duplication and cell cycle control (22). Nucleophosmin/B23 has been considered to be a marker of cellular proliferation, is frequently overexpressed in a variety of human malignancies, and has been shown to transform NIH-3T3 cells (reviewed in ref. 22). Antisense-mediated down-regulation of nucleophosmin/B23 results in delayed entry into mitosis (32). Consistent with these properties, we find that nucleophosmin/B23 expression is reduced in proliferation-defective, anti-CaSm–expressing, cells.

A previous study reported that CaSm mRNA was down-regulated in metastatic prostate cancer compared with normal tissue and localized prostate cancer. They also reported that CaSm was not expressed in two prostate cancer–derived cell lines, PC3 and DU145 (33). In this study, we find that CaSm protein expression is low in primary normal prostate epithelial cells compared with prostate cancer–derived cell lines. Furthermore, the data presented here indicates that CaSm protein is expressed at higher levels in a subset of prostate cancer specimens compared with normal tissue areas. The current data also shows that CaSm mRNA and protein are both easily detectable in PC3 and DU145 cells, suggesting possible clonal variation in these cell lines.

Figure 6. Immunohistochemical staining of CaSm protein in human prostate cancer. Representative sections stained with rabbit anti-CaSm antibody using Vectastain Elite ABC Kit. Normal regions (arrows) and adjacent cancer regions (arrowheads). A, prostate tumor tissue that has elevated CaSm staining, compared with adjacent normal tissue. B, case where CaSm expression is not elevated in tumor tissue relative to normal. All of the original microscopic magnifications are 200×.
CaSm has homology to a family of Sm-containing proteins (34), first identified in yeast (35) and human (36) by database searching using core Sm sequences. Sm and LSm proteins form heptameric complexes involved in RNA metabolism. The LSm2-LSm8 complex functions during pre-mRNA splicing, binding to U6 small nuclear RNA and necessary for U4/U6 assembly (36, 37). LSm1 to LSm7 proteins are required for 5’ to 3’ mRNA degradation. Loss of function studies show that yeast CaSm homologue, spb8/LSm1, has a critical role in the decapping of mRNA (38). Consistent with such a role in mRNA stability, yeast LSm1-LSm7 has been shown to form a stable complex with the exonuclease Xrn1 as well as proteins that function in mRNA decapping, Dcp1/Dcp2 and Pat1 (39). Pat1 (Mrt1p) has also been shown to interact with spb8/LSm1 in vitro (40). Yeast LSm1, Pat1, Dcp1, Dcp2, and Xrn1 all localize to discrete cytoplasmic foci (P bodies; ref. 41). Together, these proteins control the stability of mRNA in yeast (34, 42). Although the function(s) of LSm1 (CaSm) has not been determined, previous publications show a correlation between LSm1 and mRNA degradation. Components of LSm1-LSm7 colocalize with human Dcp1/2 and Xrn1 in discrete cytoplasmic foci (43–45). Recently, additional factors linked to mRNA decay have been shown to be colocalized in these cytoplasmic foci (46). Collectively, the presence of similar complexes in yeast and human cells are consistent with the model that CaSm/LSm1 may have a role in message stability. Our finding that antisense-mediated reduction of CaSm is associated with increased 21/CIP mRNA stability provides the first direct evidence consistent with this model. It is generally accepted that gene expression is altered in cancer. Although many studies of gene expression examine the control of transcriptional initiation by specific transcription factors, it is evident that mRNA turnover provides an important mechanism for posttranscriptional control of gene expression. Thus, it is possible that alteration of decay rates of certain mRNAs (e.g., tumor suppressor and oncogenes) could play a role in cancer initiation and progression. The cyclin-dependent kinase inhibitor p21 is a critical regulator of cellular proliferation, mediating cell cycle arrest. Although the p21 transcript is stabilized in antisense CaSm expressing DU145 cell lines, the mRNA is deadenylated, and thus inefficiently translated. However, this data suggests that p21 decapping is a CaSm-associated process, and supports the model that CaSm overexpression leads to destabilization of p21 mRNA, leading to reduced p21 levels, allowing a bypass of a critical checkpoint in G1/S and G2-M transitions. Although it remains to be determined how CaSm function contributes to the control of message stability, CaSm’s impact on message stability represents an alternative mechanism for affecting the transcriptome in cancer cells.

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