Vascular Biology, Atherosclerosis and Endothelium Biology

Protein Kinase CK1 α LS Promotes Vascular Cell Proliferation and Intimal Hyperplasia

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Protein kinase CK1 α regulates several fundamental cellular processes including proliferation and differentiation. Up to four forms of this kinase are expressed in vertebrates resulting from alternative splicing of exons; these exons encode either the L-insert located within the catalytic domain or the S-insert located at the C terminus of the protein. Whereas the L-insert is known to target the kinase to the nucleus, the functional significance of nuclear CK1 α LS has been unclear. Here we demonstrate that selective L-insert-targeted short hairpin small interfering RNA-mediated knockdown of CK1 α LS in human vascular endothelial cells and vascular smooth muscle cells impairs proliferation and abolishes hydrogen peroxide-stimulated proliferation of vascular smooth muscle cells, with the cells accumulating in G₀/G₁. In addition, selective knockdown of CK1 α LS in cultured human arteries inhibits vascular activation, preventing smooth muscle cell proliferation, intimal hyperplasia, and proteoglycan deposition. Knockdown of CK1 α LS results in the harmonious down-regulation of its target substrate heterogeneous nuclear ribonucleoprotein C and results in the altered expression or alternative splicing of key genes involved in cellular activation including CXCR4, MMP3, CSF2, and SMURF1. Our results indicate that the nuclear form of CK1 α in humans, CK1 α LS, plays a critical role in vascular cell proliferation, cellular activation, and hydrogen peroxide-mediated mitogenic signal transduction. (*Am J Pathol* 2010, 177:1562–1572; DOI: 10.2353/ajpath.2010.100327)

A key morphological distinction between vertebrates and invertebrates is the presence of a closed endothelial-lined vascular system in the vertebrates.¹ Activation of the cells comprising the vertebrate vasculature results in cellular proliferation, enhanced proteoglycan deposition, and secretion of growth factors and cytokines.^{2–4} Such vascular activation is an important process in both vascular development and in vascular diseases such as atherosclerosis and postangioplasty restenosis. Thus, an understanding of the vertebrate-specific signaling pathways regulating vascular cell activation is of high importance.

Protein kinase CK1 α regulates several fundamental cellular processes including proliferation and differentiation.⁵ Up to four different forms of the kinase exist owing to the alternative splicing of exons encoding either the L-insert located within the catalytic domain or the S-insert located at the C terminus of the protein.^{6–9} Protein kinase CK1 α itself is highly conserved among all metazoans. However, the exon encoding the nuclear localizing L-insert is restricted to vertebrates.¹⁰ Whereas vertebrates may contain up to four different splice forms of CK1 α , humans are thought to only express three forms: CK1 α , CK1 α S, and CK1 α LS, which are also referred to as CK1 α 1, CK1 α 2, and CK1 α 3, respectively. In the nucleus, CK1 α LS probably plays a role in pre-mRNA processing and alternative splicing based on its ability to phosphorylate the highly abundant vertebrate-specific pre-mRNA binding protein heterogeneous nuclear ribonucleoprotein C (hnRNP-C)^{10–12} and its localization to nuclear speckles,⁶ sites of accumulation of pre-mRNA processing factors.

Within the vessel wall, hydrogen peroxide (H₂O₂) plays important roles in mediating vascular activation resulting from diverse stimuli including altered flow, growth factors, cytokines, and vascular injury.^{13,14} In fact, vertebrate cells are known to proliferate in response to low concen-

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trations of H₂O₂.¹⁵ Low levels of H₂O₂ are generated by vertebrate cells in response to growth factor-mediated signaling, and this mitogenic H₂O₂ activates CK1 α LS, which then phosphorylates hnRNP-C.^{10,11} It is known that hnRNP-C modulates the expression of several genes regulating cell growth and survival, including platelet-derived growth factor B chain (PDGF-B),¹⁶ *c-myc*,¹⁷ p53,¹⁸ the X-chromosome-linked inhibitor of apoptosis,¹⁹ and the urokinase plasminogen activator receptor.²⁰ Thus, CK1 α LS phosphorylation of hnRNP-C may promote H₂O₂-stimulated vertebrate cell growth. However, in the cytoplasm, cytosolic forms of CK1 α (CK1 α and CK1 α S) play important roles inhibiting key proliferative signaling pathways involving both wnt/ β -catenin and the nuclear factor of activated T-cells.^{21,22} Thus, it has been unclear whether H₂O₂-activated CK1 α LS in the nucleus is promoting H₂O₂-stimulated growth or is, in fact, a compensatory counter-regulatory pathway. Here we demonstrate by selective and stable knockdown of CK1 α LS that the kinase is, in fact, an important positive regulator of vascular activation and H₂O₂ mitogenic signaling.

Materials and Methods

Expression Constructs

BamH1/EcoR1-prelinearized RNAi-Ready pSIREN-RetroQ vector (BD Biosciences, San Jose, CA) was ligated with double-stranded oligonucleotide encoding a short hairpin small interfering RNA (shRNA). Four 19-mer oligonucleotides targeting the L-insert of human CK1 α LS (exon 5 [937-1020], GenBank NM_001025105) were assessed: 5'-TCTCCAGTGGGGAAGAGGA-3' (946-964) for shRNA-S1, 5'-GGGAAGAGGAAAAGAAGCA-3' (955-973) for shRNA-S2, 5'-GAGGAAAAGAAGCATGACT-3' (960-978) for shRNA-S3, and 5'-GAAGCATGACTGTTAGTAC-3' (968-986) for shRNA-S4, using the hairpin loop sequence TTCAAGAGA. An shRNA-S3-resistant CK1 α LS rescue construct was created by introducing five silent mutations (A961C, G963A, A967C, C972T, and T978G) into the L-insert encoding sequence. The CK1 α LS rescue construct was subcloned into BamH1/NotI-prelinearized retroviral vector pCXbsr (Millipore Corporation, Billerica, MA) containing a blasticidin S resistance gene. Retroviral stocks were generated using 293T cells as described previously.²³

Cell Culture and Stable Transfection

Human umbilical vein endothelial cells (HUVECs) (VEC Technologies, Rensselaer, NY), human coronary artery smooth muscle cells (CASCs) (Lonza Walkersville, Inc., Walkersville, MD), and human coronary artery endothelial cells (HCAECs) (Lonza Walkersville, Inc.) were cultured in vascular cell growth medium (VCGM), consisting of medium M199 with 20% fetal bovine serum, 50 μ g/ml endothelial cell growth supplement, 100 μ g/ml heparin, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells (at 50 to 60% confluence) were infected in VCGM with threefold diluted retroviral stocks in the presence of 10 μ g/ml Polybrene.

After 48 hours, cells were washed, and stable transfectants were selected in VCGM containing 0.5 μ g/ml puromycin with or without 5 μ g/ml blasticidin S for 3 to 4 days. Stably transfected cells were maintained in VCGM with 0.5 μ g/ml puromycin with or without 5 μ g/ml blasticidin S for up to three passages.

Artery Culture

Segments of internal thoracic artery were obtained from patients undergoing coronary artery bypass surgery for atherosclerotic coronary artery disease. During the optimization of the artery culture model and execution of the experiments, arteries from 76 patients were used. The age of the patients ranged from 41 to 86 years (mean $\pm$ SD: 68 $\pm$ 11 years), with 64% being male, 91% having a history of hyperlipidemia and/or hypercholesterolemia, 91% having a history of hypertension, 67% having a history of smoking (current or previous), and 26% having a history of diabetes mellitus.

Before culture, adventitial tissue was removed. A cross section ring 4 to 6 mm in length was removed from the middle of the vessel and placed in 10% formalin solution or in TRIzol reagent (Invitrogen, Carlsbad, CA) or stored at -80°C. The remaining artery pieces were cut into 5-mm segments, opened longitudinally into two parts, placed endothelial side up into six-well tissue culture plates containing 3 ml of VCGM per well, and maintained at 37°C in a humidified atmosphere with 5% CO₂. On day 3, cultures were infected in the presence of 10 μ g/ml Polybrene with threefold diluted retrovirus containing CK1 α LS shRNA-S3 in RetroQ and/or the CK1 α LS rescue construct in pCXbsr and the corresponding empty vector(s). The culture medium with retroviruses was renewed every 7 days. After 3 to 4 weeks the artery segments were placed in 10% formalin solution or in TRIzol reagent or stored at -80°C. Formalin-fixed samples were processed and embedded in paraffin, followed by staining of 5- μ m sections with H&E or Movat stain. Immunohistochemical analysis was performed as described previously.²⁴

For each separate experiment, segments of artery from the same patient were treated with each of the experimental conditions, and each separate experiment used an artery from a different patient. During culture, acellular areas form primarily at the cut edges of the arteries, with intact arterial structure and endothelial lining within the middle portion of the arterial segments. Assessments were made using the intact portions of the arteries. Approximately 20% of the arteries did not survive the culture, and these were excluded from the analyses.

Cell Counting and Flow Cytometry

Cells were seeded into six-well plates at 5 $\times$ 10⁴ cells/well and cultured for 4 to 6 days (HUVECs or HCAECs) or 14 to 20 days (CASCs). Cells were then washed, detached with trypsin/EDTA, and centrifuged at 600 $\times$ g for 5 minutes. Cells were suspended in VCGM and counted in an hemacytometer. Cells were then centrifuged at 600 $\times$ g for 5 minutes. Cell pellets were fixed in ice-cold

Table 1. Primers Used for RT-PCR

mRNA	Accession no.	Strand	Sequence	Position
CK1 α LS	NM_001025105	Sense	5'-AGTACTTCTCAGGACCCA-3'	982-999
CK1 α LS	NM_001025105	Reverse	5'-TGCTTAGAAACCTTTCAT-3'	1564-1581
CK1 α S	NM_001892	Sense	5'-ATGGCGAGTAGCAGCGGCT-3'	481-499
CK1 α S	NM_001892	Reverse	5'-TGCTTAGAAACCTTTCAT-3'	1480-1497
MMP3	NM_002422	Sense	5'-ATGAAGAGTCTTCCAATC-3'	66-83
MMP3	NM_002422	Reverse	5'-TCAACAATTAAGCCAGCT-3'	1482-1499
CXCR4	NM_001008540	Sense	5'-ATGTCCATTCCCTTTCGCT-3'	305-322
CXCR4	NM_001008540	Reverse	5'-TTAGCTGGAGTGAAAAC-3'	1358-1375
CSF2	NM_000758	Sense	5'-ATGTGGCTGCAGAGCCTG-3'	33-50
CSF2	NM_000758	Reverse	5'-TCACTCCTGGACTGGCTC-3'	450-467
LCP1	NM_002298	Sense	5'-ATGGCCAGAGGATCAGTG-3'	238-256
LCP1	NM_002298	Reverse	5'-TCACACCCTCTTCATTCC-3'	2105-2122
VLDLR	NM_003383	Sense	5'-ATGGGCACGTCCGCGCTC-3'	398-415
VLDLR	NM_003383	Reverse	5'-TCAAGCTAGATCATCATC-3'	3002-3019
TSSK2	NM_053006	Sense	5'-ATGACGATGCCACAGTC-3'	593-610
TSSK2	NM_053006	Reverse	5'-TAGGTGCTTGCTTTCCC-3'	1652-1669
SMURF1	NM_020429	Sense	5'-CGGGAGATGTCGAACCCC-3'	315-332
SMURF1	NM_020429	Sense	5'-TCCCTCGGGGACCATTCC-3'	1127-1144
SMURF1	NM_020429	Reverse	5'-TGCTTTTCACTCCACAGC-3'	2583-2600
β -Actin	NM_001101	Sense	5'-TGGGCATGGGTCAGAAGG-3'	218-235
β -Actin	NM_001101	Reverse	5'-AGGAAGGAAGGCTGGAAG-3'	867-884
hnRNP-C	NM_031314	Sense	5'-ATGGCCAGCAACGTTACCA-3'	245-263
hnRNP-C	NM_031314	Reverse	5'-TGGCGAGGATGACTCTTAA-3'	1147-1165

70% ethanol for 24 hours, washed with PBS, and stained with 20 μ g/ml propidium iodide in the presence of 1 μ g/ml RNase (type IIA, Sigma-Aldrich, St. Louis, MO) in PBS. After 15 minutes of incubation, cells were collected on an LSR II cytometer using the 488-nm excitation line running DiVa acquisition software (BD, Franklin Lakes, NJ). Before analysis, instrument linearity was checked with propidium iodide-labeled chicken erythrocyte nuclei and BD DNA QC particles. Doublets were excluded with propidium iodide area versus width analysis. Cell cycle analysis was performed on list-mode data files with FlowJo cell cycle analysis software (Tree Star, Inc., Ashland, OR).

RT-PCR Analysis

Total RNA was extracted from cells and arteries with TRIzol and purified by an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (100 ng) was reverse-transcribed into cDNA in a final volume of 20 μ l using oligo(dT) primer with an SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed using the primers listed in Table 1 using a PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, MA). The resulting PCR products were electrophoresed through 0.9% agarose in 1 $\times$ TAE buffer and stained with ethidium bromide. Bands on gel images were quantitated using a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) and normalized to β -actin.

Western Blotting

Cells and arteries were washed with PBS. Cytosolic and nuclear extracts were prepared as described previously.²³ Extracts were mixed with 2 $\times$ SDS-polyacrylamide gel electrophoresis sample buffer containing 2-mercap-

toethanol and heated for 10 minutes at 100°C. Samples were electrophoresed on 12.5% Criterion Gels (Bio-Rad Laboratories) and electroblotted onto nitrocellulose. Membranes were blocked in 5% milk in Tris-buffered saline (10 mmol/L Tris.HCl [pH 7.4] and 150 mmol/L NaCl) + 0.05% Tween 20 (TBST) and probed sequentially with 1:1000 TBST-diluted L-insert-specific anti-CK1 α antibodies (Cell Signaling Technology, Danvers, MA), 1:1000 TBST-diluted S-insert-specific anti-CK1 α antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:1000 TBST-diluted anti-hnRNP C antibodies (Santa Cruz Biotechnology, Inc.), or 1:200 TBST-diluted anti-actin antibodies (Santa Cruz Biotechnology, Inc.) and then with 1:2000 TBST + 5% milk-diluted peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). Blots were imaged as described previously.¹¹

Exon Expression Arrays

Total RNA (1 μ g) was isolated using an RNeasy Mini Kit, and the sample integrity was verified using RNA 6000 Nano Assay RNA chips run in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Expression array analyses were performed at the Boston University Microarray Resource Facility as described in the GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual. Microarrays were scanned using a GeneArray Scanner 3000 7G Plus (Affymetrix, Santa Clara, CA). The resulting CEL files were summarized using an Affymetrix Expression Console (version 1.1). The robust multiarray analysis algorithm was used to generate gene-level expression estimates, and the iterPLIER algorithm was used to generate exon-level expression estimates using the hybridization intensities of the probes in the ~300,000 core exons, which consist of RefSeq-documented exons and

Figure 1. Knockdown of CK1 α LS inhibits vascular cell proliferation. Vascular cells were infected with retrovirus containing shRNA targeting the L-insert of CK1 α LS (S1 to S4) or vector control (V). **A, Top:** Assessment of mRNA by RT-PCR. **Bottom:** Immunoblots of CK1 α LS from nuclear extracts and CK1 α S from cytosolic extracts. Panels shown are for HUVECs; similar data were observed for CSMCs. **B:** By direct counting of cells, the four shRNA constructs were found to decrease the rate of proliferation of HUVECs, HCAECs, and CSMCs. Data represent 8 to 11 independent experiments for each cell type. * $P < 0.01$ versus vector by analysis of variance/Bonferroni. Error bars indicate SD. **C, Top:** HUVECs were infected with retrovirus containing shRNA-S3 directed against the L-insert of CK1 α LS and/or a CK1 α LS-rescue construct. Controls received the corresponding empty vectors. Cells were double-selected with blasticidin and puromycin, and proliferation was assessed by counting cells. $n = 3$ independent experiments. * $P < 0.001$ versus all other conditions by analysis of variance/Tukey. Error bars indicate SD. **Bottom:** Immunoblots for CK1 α LS in HUVEC nuclear extracts showing restoration of kinase expression with the rescue construct.

transcripts. The expression array data have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO Series accession number GSE19441, accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19441>).

Statistical Methods

For nonarray experiments, single comparisons were achieved by *t*-test, and multiple comparisons were made using analysis of variance with a Bonferroni or Tukey posttest. For exon arrays, differences in gene-level expression between groups were determined by paired *t*-test; none of the gene-level expression changes were significant after false discovery rate correction for multiple comparisons.

To assess for differences in exon usage, the ratio of the abundance of each exon in the presence of shRNA to that in the vector control was determined for three paired experimental groups. The abundance ratios for all exons within a given gene were then compared by analysis of variance with a posttest using the Bonferroni method to adjust for multiple comparisons. Exons without an expression value of at least 0.1 in all six samples were excluded, as were exons in genes that showed changes in gene-level expression. Exons for which the ratio of expression only differed significantly from less than half of the other exons in the gene were also excluded. In addition, exon level data were assessed using the software XRAY (Biotique Systems Inc., Reno, NV) with false discovery rate correction to adjust for multiple comparisons. For both approaches, $P < 0.001$ was considered significant.

Results

Knockdown of CK1 α LS Inhibits Vascular Cell Proliferation

To determine the role of CK1 α LS in vertebrate vascular cell activation, we designed four distinct shRNAs targeting the alternatively spliced exon encoding the L-insert. These shRNAs, labeled S1 to S4, were delivered by retrovirus in a RetroQ vector containing a puromycin resistance cassette to three distinct vertebrate vascular cell types: HUVECs, HCAECs, and CSMCs. Infected cells were selected by puromycin treatment. The four shRNA constructs were found to knock down the mRNA and protein for CK1 α LS but not the more abundant cytosolic splice form, CK1 α S (Figure 1A). The cells demonstrated stable knockdown of CK1 α LS for at least three passages. Knock down of CK1 α LS by the four shRNAs inhibited proliferation of HUVECs, HCAECs, and CSMCs to a degree correlating with the extent of CK1 α LS knockdown (Figure 1B). All subsequent experiments were performed using shRNA-S3, which was found to yield the most consistent knockdown of CK1 α LS. There was no evidence of enhanced cell death in shRNA-expressing cells (not shown).

To determine whether the inhibition of vascular cell proliferation was a specific result of CK1 α LS knockdown and not an "off-target" effect, an shRNA-resistant form of CK1 α LS was generated by placing silent mutations within the exon encoding the L-insert. This rescue construct was then introduced into HUVECs by retrovirus in vector pCXbsr, which also encodes a blasticidin resistance cassette. Cells were double-infected with L-insert shRNA in

Figure 2. Knockdown of CK1αLS inhibits H₂O₂-stimulated vascular cell proliferation. Human CASCs expressing either CK1αLS L-insert shRNA-S3 or control vector were plated at equal density and then treated with or without 1 μmol/L H₂O₂ every 24 hours for 14 days. **A, Top:** Cell proliferation was assessed by counting cells. The low concentration of H₂O₂ stimulated CASC proliferation, and this effect was inhibited by the knockdown of CK1αLS. ***P* < 0.00001 versus all other groups; **P* < 0.0002 versus shRNA-expressing cells by analysis of variance/Bonferroni. *n* = 3 experiments. Error bars indicate SD. **Bottom:** Immunoblot showing down-regulation of CK1αLS protein in CASCs in response to the shRNA both in the presence and absence of added H₂O₂. **B:** DNA content of the CASCs from above was assessed by flow cytometry. Shown are representative histograms with the data fit to three phases. Original data are in black, and fits are in blue. The S phase is shaded gray. **C:** Statistical analysis of the cell cycle analyses from three experiments. **P* < 0.05 and ***P* < 0.01 versus no shRNA for corresponding phase and H₂O₂ treatment by analysis of variance/Tukey. Error bars indicate SD.

RetroQ and/or CK1αLS-rescue in pCXbsr versus both empty vectors. Cells were then double selected with puromycin and blasticidin. Addition of the CK1αLS-rescue construct was found to completely reverse the proliferation defect caused by knocking down CK1αLS (Figure 1C).

CK1αLS Is Required for H₂O₂-Stimulated Vascular Cell Proliferation

To determine whether knockdown of CK1αLS affects H₂O₂-stimulated vascular cell proliferation, CASCs were infected with retrovirus containing either shRNA directed to the L-insert of CK1αLS or else vector control. After selection with puromycin, the cells in complete serum-containing media were treated with or without 1 μmol/L H₂O₂ every 24 hours for 14 days. Cell proliferation was assessed by counting cells. The low mitogenic levels of H₂O₂ stimulated the proliferation of the human CASCs as has been reported previously for other cell types.¹⁵ Remarkably, this mitogenic effect of H₂O₂ was abolished by knocking down CK1αLS (Figure 2A).

CK1αLS Knockdown Cells Accumulate in G₀/G₁

To more fully characterize the proliferation defect caused by knocking down CK1αLS, the CASCs above were analyzed for DNA content and cell cycle phase by flow cytometry. Knockdown of CK1αLS caused a significant reduction of cells in the S phase with accumulation of cells in G₀/G₁ (Figure 2, B and C). This effect was more prominent in the presence of H₂O₂. With H₂O₂ by itself, there was a trend for more cells in the S phase; however,

this effect was not statistically significant. These observations suggest a role for CK1αLS in G₀/G₁ and/or the G₁-S transition. By flow cytometry, there was no evidence of enhanced apoptosis in the setting of CK1αLS knockdown, which would appear as a shoulder or extra peaks to the left of the G₀/G₁ peak. Knockdown of CK1αLS did not result in the accumulation of cells in the M phase.

CK1αLS Mediates Cellular Activation in Human Arteries

Because human vascular cells in culture may not always recapitulate the biology of cells in an intact vessel wall, we have also assessed the role of CK1αLS in intact cultured human arteries. Vascular cells undergo dynamic time-dependent activation in cultured human arteries, indicated by increased medial smooth cell density, expansion of the inner intimal layer (intimal hyperplasia), and extensive proteoglycan deposition, as observed by a shift from red to blue-green on Movat staining (Figure 3). These arterial wall changes become prominent after 2 weeks in culture. This vascular activation is accompanied by significant up-regulation of both CK1αLS (Figure 4C) and hnRNP-C (Figures 3 and 4). This up-regulation of hnRNP-C mimics that which occurs in human arteries during the formation of intimal hyperplasia and atherosclerosis in people.²⁴ Remarkably, knocking down CK1αLS prevents vascular activation in the cultured arteries (Figure 4, A–D). Specifically, knocking down CK1αLS prevents smooth muscle cell proliferation in the medial layer and prevents the formation of intimal hyperplasia. Likewise knocking down CK1αLS prevents the deposition of proteoglycans in the artery wall and also prevents the up-regulation of CK1αLS and hnRNP-C.

Figure 3. Human arteries undergo activation in culture. Human arteries were cultured for up to 29 days. **Top:** H&E-stained sections. **Middle:** Movat-stained sections: red, normal smooth muscle cells; blue/green, proteoglycans; black, elastic fibers. **Bottom:** Immunohistochemical analysis for hnRNP-C. The **arrows** indicate the intima/media boundaries.

Only rare tunnel-positive apoptotic cells were observed in all of the arteries, and their presence was not affected by knocking down CK1 α LS (not shown). Both the vector-treated and shRNA-treated arteries maintained an endothelial-lined surface in culture (not shown).

To ensure that the inhibition of vascular activation in the cultured arteries was in fact due to knockdown of CK1 α LS and not an off-target effect, cultured arteries were double-infected with retroviruses containing shRNA-S3 and/or an shRNA-resistant CK1 α LS rescue construct. Expression of the rescue construct in the cultured arteries was found to reverse the inhibition of vascular activation caused by shRNA-S3, with recovery of intimal hyperplasia, proteoglycan deposition, and hnRNP-C up-regulation (Figure 5, A–C).

CK1 α LS Regulates Expression of hnRNP-C

The prevention of hnRNP-C up-regulation by knockdown of CK1 α LS in cultured human arteries was a surprising finding. To assess for the generality of this phenomenon, the role of CK1 α LS in regulating hnRNP-C expression was also assessed in HUVECs and CSMCs. Knockdown of CK1 α LS was observed to result in the down-regulation of hnRNP-C protein in both types of cultured cells, and coexpression of the CK1 α LS rescue construct restored hnRNP-C levels in HUVECs (Figure 4D). Interestingly, in the setting of CK1 α LS knockdown, hnRNP-C mRNA was not changed (Supplemental Figure S1, see

<http://ajp.amjpathol.org>), indicating that hnRNP-C expression is largely controlled at the post-transcriptional level by CK1 α LS.

CK1 α LS Regulates the Expression of Distinct Genes Involved in Vascular Activation

Because both CK1 α LS and hnRNP-C have been proposed to play a role in pre-mRNA processing, we hypothesized that knockdown of CK1 α LS results in the altered expression or splicing of key gene products involved in vertebrate cell activation. To identify CK1 α LS-dependent genes, which may be mediating vascular cell activation by the kinase, we have assessed for global changes in gene expression and mRNA splicing after CK1 α LS knockdown in CSMCs using the Affymetrix human GeneChip Exon ST Array system. Analysis of the array data revealed multiple candidate CK1 α LS-dependent genes that are in fact involved in cell proliferation or activation (Supplemental Tables S1 and S2, see <http://ajp.amjpathol.org>). For a subset of these genes, the expression changes resulting from CK1 α LS knockdown were confirmed by RT-PCR (Figure 6, A and B). These genes included genes involved in extracellular matrix remodeling (matrix metalloproteinase 3 [MMP3]), cytokine signaling (chemokine [C-X-C motif] receptor 4 [CXCR4]), and lipid metabolism (very-low-density lipoprotein receptor [VLDLR]). Because MMP3, CXCR4, and VLDLR all play

Figure 4. Knockdown of CK1αLS inhibits intimal hyperplasia and down-regulates expression of hnRNP-C. Human internal thoracic arteries were cultured *in vitro* and treated with retrovirus containing shRNA-S3 to CK1αLS or retrovirus containing vector control. **A, Top:** H&E-stained sections. **Middle:** Movat-stained sections: red, normal smooth muscle cells; blue/green, proteoglycans; black, elastic fibers. **Bottom:** Immunohistochemical analysis for hnRNP-C. The arrows indicate the intima/media boundaries. **B:** Statistical analyses of smooth cell density (**top**), intima/media ratio (**middle**), and fraction of cells expressing hnRNP-C (**bottom**) from three separate experiments using an artery from a different patient for each experiment. * $P < 0.03$ and ** $P = 0.01$ compared with the other conditions; analysis of variance/Tukey; Error bars indicate SD. **C, Top:** Immunoblots for CK1αLS protein and assessment of mRNA for CK1αLS and CK1αS by RT-PCR. **Bottom:** Statistical analysis of mRNA levels from seven separate experiments using an artery from a different patient for each experiment. ** $P < 0.01$ versus precultured arteries and < 0.05 versus shRNA-treated arteries; analysis of variance/Tukey. Error bars indicate SD. **D:** Immunoblots for hnRNP-C from HUVECs and CSMCs. CK1αLS was stably knocked down by retroviral delivery of shRNA-S3 with or without coexpression of the CK1αLS rescue construct. Control cells were treated with retrovirus with corresponding empty vectors.

important roles in vascular cell activation, the effect of CK1αLS knockdown on the expression of these genes was also assessed in HUVECs and in cultured human arteries. Knockdown of CK1αLS was found to down-regulate CXCR4 and VLDLR expression in both HUVECs and cultured human internal thoracic arteries. MMP3, which is not expressed in HUVECs, is also down-regulated on knockdown of CK1αLS in cultured arteries. Thus, the ability of CK1αLS to regulate expression of MMP3, CXCR4, and VLDLR is a general principle of human vascular cells.

CK1αLS Regulates Alternative Splicing

Because of its localization to nuclear speckles and its ability to regulate mRNA binding by hnRNP-C, CK1αLS has been proposed to play a role in pre-mRNA splicing/processing.^{6,11} To assess for altered pre-mRNA splicing in the setting of CK1αLS knockdown, we analyzed the exon level data from exon expression arrays with and without knockdown of CK1αLS. A subset of genes was identified as potentially undergoing altered splicing in the

setting of CK1αLS knockdown (Supplemental Table S3, see <http://ajp.amjpathol.org>). These potential alternative-splicing events included one known alternative splicing event involving SMURF1. Knockdown of CK1αLS results in a 5.4-fold increase in the use of exon 9, which codes for a portion of the substrate-binding domain in SMURF1 (Figure 7, A and B). This CK1αLS-dependent alternative splicing of SMURF1 was confirmed by RT-PCR (Figure 7C). Although nuclear CK1α has long been proposed to play a role in pre-mRNA processing, these observations represent the first evidence that nuclear CK1αLS deficiency is associated with altered mRNA splicing.

Discussion

Although it had been clear that the alternatively spliced L-insert in CK1αLS could drive nuclear localization of the kinase, the specific cellular functional significance of this kinase had been unclear. Here by selective stable knockdown, it is shown that, in fact, CK1αLS promotes multiple aspects of vascular cell activation including vascular cell

Figure 5. Expression of CK1 α LS restores vascular activation in arteries with CK1 α LS knockdown. Human internal thoracic arteries were cultured *in vitro* and treated with retrovirus containing shRNA-S3 to CK1 α LS, with or without expression of a CK1 α LS rescue construct or corresponding empty vectors. **A, Top:** H&E-stained sections. **Middle:** Movat-stained sections: red, normal smooth muscle cells; blue/green, proteoglycans; black, elastic fibers. **Bottom:** Immunohistochemical analysis for hnRNP-C. The **arrows** indicate the intima/media boundaries. **B:** Immunoblots for CK1 α LS protein show recovery of CK1 α LS expression in the arteries with the rescue construct. **C:** Statistical analyses of intima/media ratio from six separate experiments using an artery from a different patient for each experiment. * $P < 0.03$ compared with the no culture and knockdown conditions; analysis of variance/Tukey. Error bars indicate SD.

proliferation, H₂O₂-stimulated vascular cell proliferation, intimal hyperplasia, vascular proteoglycan deposition, and up-regulation of hnRNP-C. These observations indicate that nuclear CK1 α LS may be functioning antagonistically to its related cytosolic splice forms, CK1 α and CK1 α LS, which tend to negatively regulate pro-proliferative pathways.^{21,22}

Interestingly, knockdown of CK1 α LS caused a significant reduction of cells in the S phase with accumulation of cells in G₀/G₁, suggesting a role for CK1 α LS in G₀/G₁ and/or the G₁-S transition. Surprisingly, there was no accumulation of cells in the M phase on knockdown of CK1 α LS. Previously, CK1 α had been shown to play an important role in progression through the M phase,²⁵ and likewise it has recently been reported that knockdown of hnRNP-C results in the accumulation of cells in the M phase.²⁶ However, a recent phosphoproteomic study revealed evidence that the CK1 α LS phosphorylation sites on hnRNP-C are probably phosphorylated more frequently in G₁ than in the M phase,^{27,28} consistent with the findings here. In addition, the CK1 α , which is required for progression through the M phase, seems to be a cytosolic splice form, CK1 α or CK1 α LS, which translocates to the mitotic spindle during mitosis.¹⁰ Thus, the M phase functions of hnRNP-C may be independent of CK1 α LS.

Gene expression profiling revealed specific genes that are down-regulated in the setting of CK1 α LS knockdown

(Figure 6, Supplemental Table S1, see <http://ajp.amjpathol.org>), many of which are involved in cellular activation. MMP3 facilitates vascular smooth muscle cell proliferation and migration within arteries²⁹ and is down-regulated 3.5-fold on knockdown of CK1 α LS. CXCR4 is the receptor for the CXC chemokine stromal cell-derived factor 1 α (SDF-1 α) and is also down-regulated on knockdown of CK1 α LS. CXCR4 plays important roles in the formation of intimal hyperplasia after vascular injury and in angiogenesis.^{30–32} Previously, low concentrations of H₂O₂ were shown to result in the up-regulation of CXCR4,³³ consistent with the observations here that CXCR4 expression and H₂O₂ mitogenic signaling are both dependent on CK1 α LS. VLDLR is also down-regulated on CK1 α LS knockdown. VLDLR is a member of the low-density lipoprotein receptor gene family that is up-regulated in atherosclerosis, binds apolipoprotein-E containing lipoproteins, and regulates cell growth.^{34,35} Two additional genes down-regulated were L-plastin and colony-stimulating factor 2 (CSF2). L-plastin/lymphocyte cytosolic protein 1 (LCP1) is an actin-binding protein that positively regulates integrin activation and cell proliferation and migration.³⁶ CSF2, also known as granulocyte-macrophage colony-stimulating factor is released by activated vascular smooth muscle cells to elicit activation of monocytes, endothelial cells, and progenitor cells.^{37,38} Recently CSF2 has also been shown to play a key role

Figure 6. Knockdown of CK1 α LS results in the altered expression of distinct genes. **A:** For a subset of the genes whose expression was suggested to be altered by the knockdown of CK1 α LS, mRNA levels were assessed by RT-PCR. For these genes, RT-PCR analysis confirmed the gene expression changes identified by expression array. Shown are the *P* values (without false discovery rate correction) and fold expression changes from the array along with the corresponding PCR products (1.5-fold = 50% change in expression). **B:** For three genes, CXCR4, VLDLR, and MMP3, the effect of CK1 α LS knockdown on their mRNA levels was assessed in cultured arteries (Ar) and HUVECs (En) in addition to CASCs (Sm). *n* = 3 experiments and/or patients. **P* < 0.05 and ***P* < 0.005 compared with vector control. Error bars indicate SD.

in stimulating proliferation of intimal dendritic cells in the low-density lipoprotein receptor-deficient mouse model.³⁹

Thus, in general, knockdown of CK1 α LS results in the down-regulation of several genes that positively regulate cellular activation. In contrast, a smaller number of candidate genes for up-regulation in the setting of CK1 α LS knockdown were also identified (Supplemental Table S2, see <http://ajp.amjpathol.org>). In contrast to the down-regulated genes, the up-regulated genes are not, in general, positive regulators of cellular activation. For example, one of the up-regulated genes encodes the Ser/Thr kinase TSSK2 (Figure 6). TSSK2 is a relatively widely expressed protein kinase.⁴⁰ In the testis TSSK2 is important for spermiogenesis,⁴¹ but the role of TSSK2 in the vasculature is unclear.

Figure 7. Knockdown of CK1 α LS results in the altered splicing of SMURF1. **A:** Cartoon diagram of the domains of SMURF1 depicting the location of the alternatively spliced exon 9. **B:** The expression level of each exon in SMURF1 in CASCs was obtained by exon expression arrays. For each exon in SMURF1, the ratio of the expression level in the setting of CK1 α LS knockdown to that present in the vector control condition is plotted as the mean $\pm$ SE for three experiments. **C:** mRNA levels for total SMURF1 and specifically for SMURF1 isoform 1, which contains exon 9, were determined by RT-PCR.

Because of its localization to nuclear speckles and its ability to regulate mRNA binding by hnRNP-C, CK1 α LS has been proposed to play a role in pre-mRNA splicing/processing,^{6,11} but the specific gene products whose processing may be regulated by the kinase have been unclear. Analysis of exon-level data from the exon expression array revealed a subset of genes that potentially undergo altered splicing in the setting of CK1 α LS knockdown (Supplemental Table S3, see <http://ajp.amjpathol.org>). These potential alternative-splicing events included one known alternative splicing event involving SMURF1, an E3 ubiquitin protein ligase that regulates transforming growth factor- β /SMAD signaling and RhoA protein levels.^{42–44} Knockdown of CK1 α LS results in a 5.4-fold increase in the use of exon 9, which codes for a portion of the substrate-binding domain in SMURF1 (Figure 7). Thus, CK1 α LS may be regulating the substrate specificity of SMURF1 through alternative splicing.

Although once viewed as a highly abundant constitutive nuclear protein, hnRNP-C is now recognized to play important roles in the expression of specific genes regulating cell growth and survival. Correspondingly, although hnRNP-C protein expression is typically constitutively high in cultured cells, its expression is dynamically regulated in diseased vertebrate tissues. Specifically, hnRNP-C is up-regulated in adult human arteries during development of vascular diseases, particularly intimal hyperplasia and atherosclerosis.²⁴ Likewise, hnRNP-C is up-regulated in brain tissue after transient ischemia in a murine stroke model.⁴⁵ Recently hnRNP-C has been shown to be up-regulated in human cervical tissue after human papillomavirus infection and neoplastic transformation.⁴⁶ The regulation of hnRNP-C protein levels by

CK1 α LS represents novel insight into how vertebrate cells regulate the expression of this key nuclear pre-mRNA binding protein.

Interestingly, the gene expression changes identified above suggest that regulation of hnRNP-C protein levels may be a key mechanism by which CK1 α LS regulates vascular cell activation. For a few genes, hnRNP-C has been shown to directly regulate translation of the mRNA. These hnRNP-C-dependent gene products include platelet-derived growth factor B chain¹⁶ and c-Myc¹⁷. It is possible that the expression of many of the CK1 α LS-dependent genes such as CXCR4, MMP3, CSF2, and VLDLR is in fact regulated by these hnRNP-C-dependent gene products. For example, c-Myc is known to positively regulate expression of CXCR4 mRNA by direct inhibition of Ying Yang 1 (YY1), a transcriptional repressor of CXCR4.^{47,48} In addition, platelet-derived growth factor has been shown to positively regulate MMP3 mRNA expression via c-fos,^{49,50} and c-fos is known to positively regulate CSF2 expression, which itself has been shown to positively regulate expression of VLDLR.^{51,52} Thus, hnRNP-C and the hnRNP-C-dependent gene products may in fact be mediating vascular activation by CK1 α LS.

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