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Compartmentalization of hnRNP-K During Cell Cycle Progression and Its Interaction With Calponin in the Cytoplasm

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Abstract Coronary artery blockage, due to cardiovascular disease, is routinely treated by either balloon-angioplasty or bypass surgery. The limited success of these clinical interventions is due at least in part to smooth muscle cell (SMC) proliferation. Here we show that heterogeneous nuclear ribonucleoprotein complex K (hnRNP-K) protein levels increase in SMC with response to serum stimulation in vitro, in the aortas from an animal model of atherosclerosis, and in occluded human vein segments. hnRNP-K is a multi-functional protein that has been studied primarily in cancer cells and has been suggested to play a role in cell cycle progression. We show that in untransformed, cultured SMC, hnRNP-K protein sub-cellular localization modulates through the cell cycle in both the cytoplasm and nucleus. Using cycloheximide, we observed that cytoplasmic accumulation of hnRNP-K protein at later time points in the cell cycle occurred with a concomitant decrease in nuclear hnRNP-K protein, suggesting a translocation of nuclear hnRNP-K protein to the cytoplasm. Also, because we did not observe an increase in hnRNP-K protein at early time points in the cell cycle in the presence of cycloheximide, we propose that the early increase in cytoplasmic hnRNP-K protein following serum stimulation is due to new hnRNP-K protein synthesis. When present in the cytoplasm, hnRNP-K is part of a multi-protein complex that consists of at least two other proteins, calponin and ERK1/2. Our findings from this study are intriguing because they suggest that cytoplasmic hnRNP-K in SMC is part of a signaling complex that may be involved in growth-stimulated post-transcriptional regulation. J. Cell. Biochem. 95: 1042–1056, 2005.

Key words: smooth muscle; protein partners; K-protein; ERK1/2; 2D gel electrophoresis; cycloheximide; isoforms

hnRNP-K is a multi-functional protein that has been suggested to play a role in cell cycle progression, RNA transport, and transcriptional control (recently reviewed by Bomsztyk et al. [2004]). hnRNP-K results from one gene that can be alternatively spliced to generate four isoforms [Dejgaard et al., 1994]. There is a difference in the relative levels of the individual isoforms that is dependent on the quiescent or proliferative state of the cell, suggesting that hnRNP-K plays a role in cell cycle progression [Dejgaard et al., 1994].

hnRNP-K influences the translation of mRNAs by binding to the 3′-UTR and increasing mRNA stability. 15-Lipoxygenase (15LO) mRNA translation is inhibited in erythoid precursors by the binding of specific proteins, including hnRNP-K, to the 3′-UTR. In erythoid precursors, hnRNP-K regulates 15LO mRNA translation after activation by c-Src. Src tyrosine-phosphorylates hnRNP-K causing the release of the 15LO mRNA from hnRNP-K, thereby allowing translation of the message [Ostareck-Lederer et al., 2002]. Levels of plasma renin are controlled by transcriptional, post-transcriptional, and post-translational mechanisms. hnRNP-K has been shown to act post-transcriptionally to bind the human preprorenin mRNA 3′-UTR and regulate its stability [Persson et al., 2003; Skalweit et al., 2003]. Recent work demonstrates that hnRNP-K interacts with specific cellular, ribosomal and mitochondria RNA transcripts at specific

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binding sequences [a list can be found in Klimek-Tomczak, 2004 #435]. Interestingly, hnRNP-K binds mitochondrial COX-1 and COX-2 messages [Klimek-Tomczak et al., 2004].

While hnRNP-K was first identified as a RNA binding protein that binds cytidine-rich elements [Matunis et al., 1992; Dejgaard et al., 1994], other groups have characterized its DNA binding ability [Takimoto et al., 1993; Lee et al., 1996; Michelotti et al., 1996]. When bound to its DNA recognition site, hnRNP-K can act as a transcriptional activator and interact synergistically with the TATA-binding protein (TBP)-associated complex to activate transcription of a reporter gene [Takimoto et al., 1993; Michelotti et al., 1996]. Besides TBP, hnRNP-K has been reported to interact with other transcriptional regulation proteins, such as, transcription factors YB-1, SP1, and C/EBPβ [Du et al., 1998; Miau et al., 1998; Shnyreva et al., 2000], and Zik-1, a transcriptional repressor [Denisenko et al., 1996]. Lee et al., have also demonstrated the trans-activating potential of hnRNP-K [Lee et al., 1996].

hnRNP-K contains a shuttling domain that allows access to both nuclear and cytoplasmic compartments [Micheal et al., 1997]. This ability to transverse the nuclear envelope potentially facilitates interactions with proteins involved in transcriptional regulation and signal transduction. Post-translational modifications of hnRNP-K include methylation and phosphorylation. Two independent laboratories have reported that hnRNP-K is post-translationally methylated on arginines in cancer cells and lymphoblastoid cells [Huang et al., 2002; Wada et al., 2002]. Additionally, hnRNP-K is phosphorylated in vitro by tyrosine kinases Src and Lck [Ostrowski et al., 2000], as well as, in vivo and in vitro by inducible serine/threonine kinases [Bomsztyk et al., 1997, 2004; Ostrowski et al., 2000]. Interleukin-1-responsive kinase, protein kinase Cδ and casein kinase 2 have also been shown to phosphorylate hnRNP-K in vitro [Van Seuningen et al., 1995; Schullery et al., 1999; Wadd et al., 1999; Koffa et al., 2003]. Habelhah and coworkers reported that ERK and JNK phosphorylate hnRNP-K and that cytoplasmic localization is dependent upon phosphorylation [Habelhah et al., 2001a,b]. Interestingly, it has been shown that phosphorylated hnRNP-K interacts to a lesser extent with nucleic acid than non-phosphorylated [Dejgaard et al., 1994; Ostrowski et al., 2000]. Taken together, these reports and our initial investigation [Laury-Kleintop et al., 1999] suggested to us that regulation of hnRNP-K by pro-growth pathways may also occur in vascular SMC, not just in cancer models.

MATERIALS AND METHODS

Animals

After a 7 day acclimation period, three New Zealand White rabbits were placed on a cholesterol-enriched (0.5%) diet [Consigny et al., 1986]. After 8 weeks on the cholesterol-enriched diet the rabbits were euthanized and the thoracic aortas were fixed in 4% paraformaldehyde/PBS for immunofluorescence. Control New Zealand White rabbits were fed a normal chow diet and the thoracic aortas were either fixed in 4% paraformaldehyde/PBS for immunofluorescence and histology or processed for cell isolation by enzyme dispersion.

Cell Isolation and Maintenance

SMC were isolated as previously described [Laury-Kleintop et al., 1999]. For SMC isolation, the medial tissue was minced and incubated for 3–4 h in minimum essential medium (MEM) containing 1% FBS, 375 U/ml collagenase, 0.425 U/ml elastase, and 0.12% soybean trypsin inhibitor. Cells were seeded into wells/flasks in MEM containing 10% FBS. To maintain a smooth muscle phenotype, cells were used at passage 4 or less.

Immunohistochemistry

Human tissue was paraformaldehyde fixed (4%), decalcified, paraffin embedded, and sectioned onto ProbeOn Plus slides. After antigen retrieval, inhibiting endogenous peroxidase activity (with 3% H_2O_2 in 50% methanol) and blocking, sections were incubated with primary antibody (anti-SM α-actin, Sigma Chemicals, St. Louis, Missouri, or anti-hnRNP-K, 12G4-provided by Dr. Gideon Dreyfuss, Howard Hughes Medical Institute Research Laboratories, University of Pennsylvania School of Medicine). After washing with PBS, the sections were incubated with goat anti-mouse antibody conjugated to biotin. After Avidin–biotin–horseradish peroxidase amplification, (VECTASTAIN ABC Reagent), the sections were

incubated with filtered 3,3′-diaminobenzidine (DAB) until the desired stain intensity developed. After subsequent washing with PBS, the slides were counterstained with Hematoxylin.

Immunofluorescence

Tissue was fixed in 4% paraformaldehyde, embedded, and sectioned onto ProbeOn Plus slides. Tissue was then de-paraffined and rehydrated to PBS. Additionally, SMC grown on cover slips, were paraformaldehyde (4%) fixed and treated with 0.2% Triton X-100. Coverslips/slides were washed with PBS and incubated in 10% normal goat serum. Then, the coverslips/slides were incubated with the first primary antibody (monoclonal anti-mouse hnRNP-K antibody, 12G4 [Matunis et al., 1992]), washed, and incubated with the secondary antibody conjugated to FITC. Following a final wash, the coverslips/slides were mounted on slides with Vectastain containing DAPI, sealed, and analyzed on a Zeiss microscope fitted with an Axiocam digital camera and computer. For double-labeling, the coverslips were washed following the first secondary antibody and incubated with 10% normal mouse serum and then 10% mouse FAB fragment. Subsequently, the coverslips were incubated with the second primary antibody (monoclonal anti-calponin antibody, Sigma). After washing, the cover slips were incubated with the second secondary antibody conjugated to CY3. Following a final wash, the cover slips were mounted on slides with Vectastain containing DAPI, sealed, and assessed using a Zeiss Axiovert 220M microscope powered by Axiovision 4.0 software with multi-channel, Z-stack acquisition, 3D-deconvolution, and 4D rendering modules.

Flow Cytometry

SMC were collected at designated time points, washed in phosphate-buffered saline, and fixed in 70% ethanol [Vindelov et al., 1983; Francis et al., 1995]. The detergent-trypsin method as described by Vindelov [Vindelov et al., 1983] was used to prepare the cells and stain the DNA with propidium iodide for flow cytometry. Fluorescence intensity in the cell nucleus was measured by flow cytometry analysis on a FACScan (Beckon Dickenson) flow cytometer. Approximately 10,000 cells were acquired and analyzed for DNA content, cell cycle, and apoptosis analysis.

Cytoplasmic and Nuclear Extract Preparation

Cytoplasmic extracts were obtained in 10 mM HEPES, pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose & 0.5% Nonidet P-40 [Schreiber et al., 1989]. Nuclear extracts were obtained in 20 mM HEPES, pH 7.9, 0.75 mM $MgCl_2$, 210 mM NaCl, 50 mM KCl, 1 mM EDTA, 10% glycerol & 0.5 mM DTT [Schreiber et al., 1989]. Both extraction buffers contained protease and phosphatase inhibitors: 0.5 mM PMSF, 1 μg each leupeptin and aprotinin per ml, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4. The extracts were assayed for protein concentration using the Lowry protein determination method [Lowry et al., 1951].

SDS–PAGE and Western Blot Analysis

Protein extracts (45 or 15 μg/lane) were size fractionated on SDS–Polyacrylamide gels and transferred to nitrocellulose. After blocking in 5% milk in TEN (25 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA), blots were incubated with the primary antibody [anti-hnRNP-K, anti-Calponin (Sigma, Inc.), or anti-ERK1/2 (Upstate, Charlottesville, Virginia)] overnight at 4°C. After incubation with the Horseradish peroxidase (HRP)-conjugated secondary antibody and washing in 3% MILK in TEN, the blots were developed in chemiluminescense substrate (Western Lightening Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, Bostin, MA) and analyzed for light emission using autoradiographic film. Densitometry was performed with a Molecular Dynamics Scanning Densitometer equipped with ImagQuant software.

2D Gel Electrophoresis

Protein extracts (30 μg of cytoplasmic or 10 μg of nuclear) were separated in the first dimension according to isoelectric focusing (IEF) point and then in the second dimension according to size on SDS-Polyacrylamide gels [Gravel and Golaz, 1996]. IEF was performed using the Invitrogen, Carlsbad, California, Zoom IPG strip system where the protein extracts were incubated with the strip (pI 4–7) in the IEF sample rehydration buffer (8 M urea, 2% CHAPS, 0.5% (v/v) pH 4–7 carrier ampholytes, 20 mM dithiothreitol, 0.002% bromophenol blue). The proteins were focused as per manu-

facturer instructions (Invitrogen, Inc.). The second dimension was standard SDS–PAGE. The IEF strip was incubated in 1× NuPAGE sample buffer containing reducing agent (Invitrogen, Inc.) and then placed on a 4–20% SDS-PAGE gel (Invitrogen, Inc.). After SDS–PAGE electrophoresis the proteins were electrotransferred to nitrocellulose for Western Blot analysis.

Immunoprecipitation

Immunoprecipitations were performed as previously described [Matunis et al., 1992; Michelotti et al., 1996]. In brief, 100 μg of pre-cleared protein from total cell extracts was incubated with the precipitating antibody [anti-hnRNP-K, anti-Calponin (Sigma, Inc.), or anti-ERK1/2 (Upstate Biotech)] in extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% NP-40, & 0.5 mM DTT, 5 mM EDTA, 2 mM EGTA, 10% glycerol, 0.5 mM PMSF, 1 μg each leupeptin and aprotinin per ml, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) overnight at 4°C. After incubation, the antigen–antibody complex was bound to Protein A/G-Sepharose. The washed antigen–antibody–ProteinA/G complex was treated for SDS–PAGE, fractionated on 4–20% gradient Tris-Glycine gels (BioRad Laboratories, Hercules, CA),and analyzed by Western Blot.

RESULTS

hnRNP-K Protein Is Expressed in Rabbit Thoracic Aorta After Cholesterol Feeding and in Human Disease Vessels

In order to determine whether hnRNP-K is present in early atherosclerotic lesions that are similar to human fatty streak lesions, we immunostained the thoracic aorta from rabbits fed a 0.5% cholesterol enriched diet. Figure 1 (panel A) shows that hnRNP-K is present in normal aorta and in the media and lesion of cholesterol-fed rabbits (lower image). The Hematoxylin & Eosin stained serial section of the aorta isolated from the cholesterol-fed rabbit shows the developed fatty-streak lesion. We also investigated hnRNP-K protein expression in human tissue removed during coronary artery bypass graft surgery. Figure 1 (panel B) shows that hnRNP-K staining is present in occluded saphenous vein that was removed and replaced with normal saphenous vein during surgery. The staining pattern suggests that hnRNP-K is expressed in cells of the lesion and that the hnRNP-K staining appears to co-localized with SM α-actin. There was little co-localization staining with an endothelial-specific antibody and little co-localization with a macrophage-specific antibody (data not shown).

hnRNP-K Immunostaining Is Nuclear and Cytoplasmic in SMC After Growth Stimulation

Because hnRNP-K appears to play a role in cell growth [Dejgaard et al., 1994] and because we observed hnRNP-K in stenotic lesions, we investigated the protein expression of hnRNP-K in relation to cell growth in rabbit aorta SMC. Rabbit thoracic aorta SMC were isolated by enzymatic dispersion and grown for no more than four passages before use [Laury-Kleintop et al., 1999]. Using immunofluorescence we investigated the compartmentalization of hnRNP-K during cell growth. As shown in Figure 2, hnRNP-K staining is greatest in the nucleus and the amount of nuclear hnRNP-K appears to change with the cell cycle. These cells double by approximately 60 h and to avoid growth inhibition by cell/cell contact the coverslips used for immunostaining at middle and late time points were seeded with fewer cells. The hnRNP-K immunostaining corresponded to the DAPI staining; therefore, DAPI images are not shown. What is less discernable at lower magnification is the cytoplasmic staining pattern of hnRNP-K. Shown in Figure 3, at higher magnification, is an increase in the cytoplasmic localization of hnRNP-K with serum stimulation and its characteristic staining pattern. hnRNP-K immunostaining appears to cluster in the cytoplasm. These images in Figure 3 were taken at the same exposure that results in an over-exposure of the nuclear hnRNP-K staining.

Sub-Cellular Localization Changes of hnRNP-K With Cell Growth

In order to obtain a quantitative analysis of hnRNP-K changes in protein levels in the cytoplasmic and nuclear compartments, we isolated cellular extracts and performed western blotting analyses. Figure 4 shows hnRNP-K expression in the cytoplasm and nucleus of SMC following growth stimulation. Serum stimulation increased hnRNP-K protein expression in the cytoplasm of SMC with a concomitant increase in total Cyclin A protein and a decrease in total Rb protein (Cyclin A and Rb data not

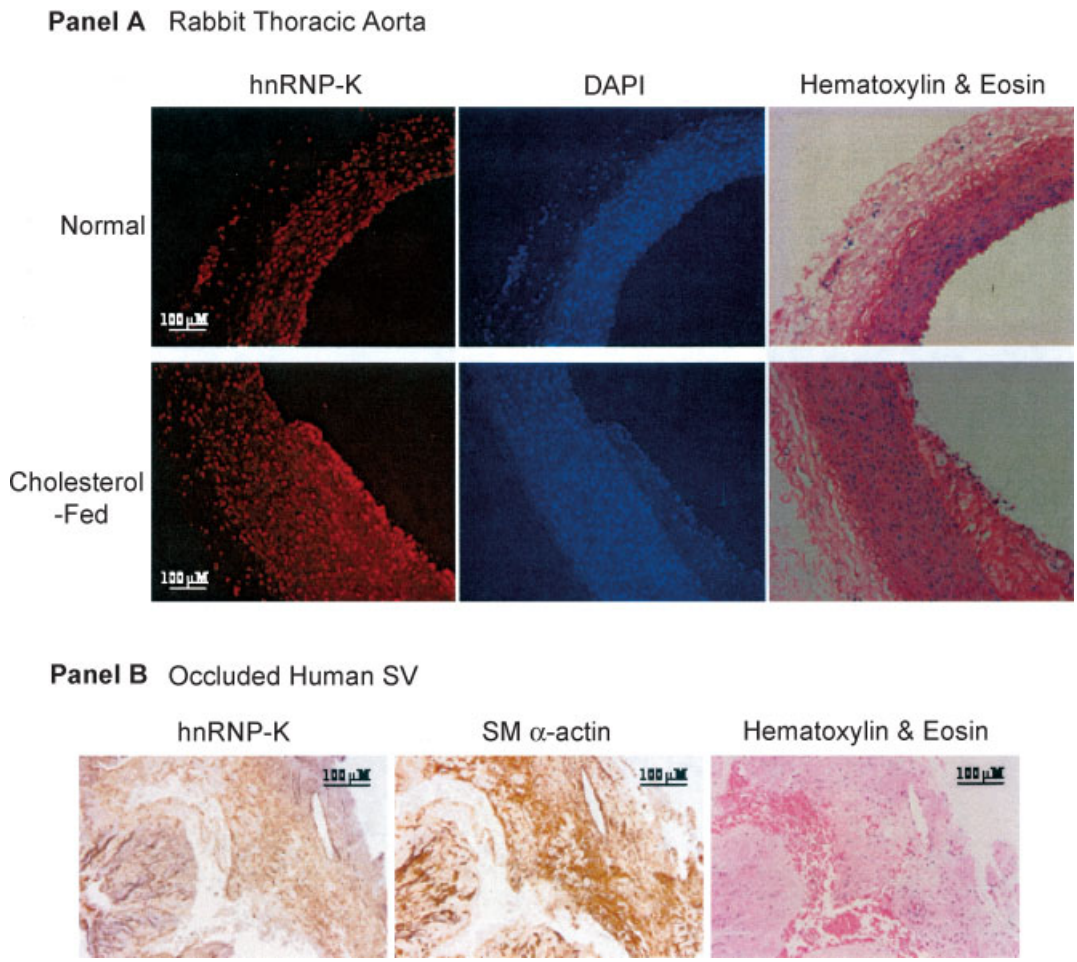

Fig. 1. hnRNP-K expression in rabbit thoracic aorta and human saphenous vein. The tissue was paraformaldehyde fixed, paraffin-embedded, and sectioned onto ProbeOn Plus slides. **Panel A**: Immunofluorescence was performed using anti-hnRNP-K monoclonal antibody and secondary CY3-conjugated antibody. The tissue from the cholesterol-fed animal shows fatty streak lesion formation and hnRNP-K staining cells in both the media and lesion areas. The Hematoxylin & Eosin staining provides histological detail. **Panel B**: Immunohistochemistry and histology of occluded SV. Tissue was fixed in 4% paraformaldehyde, decalcified, paraffin-embedded, and sectioned onto ProbeOn Plus slides. After antigen retrieval and blocking, sections were incubated with primary antibody (anti-SM α-actin, Sigma Chemicals or anti-hnRNP-K). The secondary antibody was goat anti-mouse conjugated to horseradish peroxidase and DAB was used to detect antigen. The slides were counterstained with Hematoxylin. Dark brown staining indicates a positive reaction between antigen and antibody.

shown). This together with FACS analysis shown in Figure 4 panel B, allowed us to determine that the increase in hnRNP-K in the cytoplasm was concomitant with S phase. The western and FACS data are consistent with the data presented in Figure 2 that suggested nuclear hnRNP-K levels change with the cell cycling. Data from the western analysis of the nuclear extracts indicates a rapid increase in hnRNP-K in the nucleus with a cell growth response. Experiments with cycloheximide (Fig. 5) show that the early increase of hnRNP-K in the cytoplasm is consistent with new protein synthesis and that the later presence of hnRNP-K in the cytoplasm is the result of translocation of hnRNP-K from the nucleus. We did observe a low level of hnRNP-K in the 48 h nuclear extract a longer exposure of the western blot.

Acidic hnRNP-K Isoforms in the Cytoplasm

Because the hnRNP-K protein present within cells can exist as multiple isoforms and these isoforms can exist in phosphorylated forms [Dejgaard et al., 1994], we investigated whether these multiple isoforms are present within SMC. Particularly, we were interested in determining whether SMC, a non-transformed cell-type, contains multiple isoforms as observed in transformed cells. Also, currently no information is available that relates the hnRNP-K

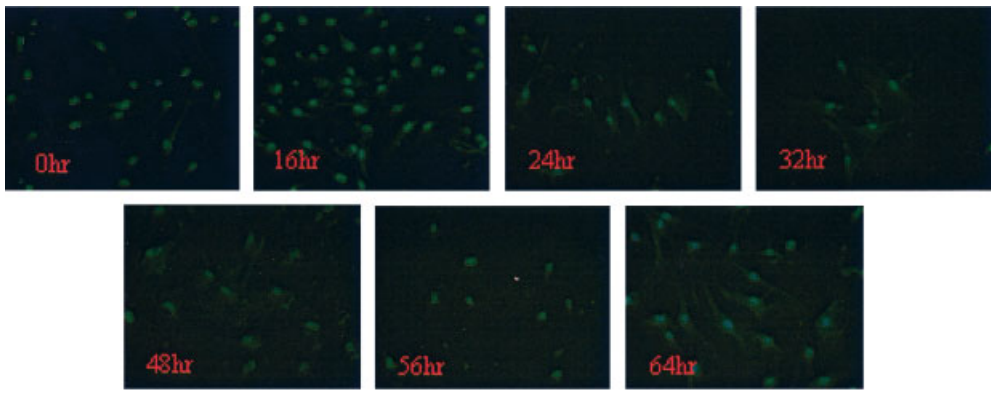

Fig. 2. hnRNP-K immunostaining of SMC. Rabbit SMC grown on coverslips were growth inhibited using serum free medium and stimulated with 10% fetal bovine serum over the course of 64 h. Cells were fixed with 4% paraformaldehyde, permeablized with Triton X-100, blocked, and then incubated with anti-hnRNP-K monoclonal antibody, washed with PBS, and finally, incubated with secondary antibody conjugated to FITC. Washed coverslips were mounted with DAPI containing mount and viewed on a Zeiss upright microscope.

isoform pattern to the cytoplasmic and nuclear compartments in either cancer cells or non-transformed cells. Using 2D gel electrophoresis, we determined that SMC do contain multiple hnRNP-K isoforms and importantly, that the more acidic isoforms are present in the cytoplasm (Fig. 6). The addition of cycloheximide did not alter the proportions of the isoforms in the cytoplasmic and nuclear compartments (data not shown), suggesting that the modifications do not rely on new protein synthesis. Dejgaard and coworkers suggest that the acidic isoforms are derived from the basic forms by multiple phosphorylations [Dejgaard et al., 1994].

Immunofluorescence of hnRNP-K Demonstrating Co-Localization With Calponin

Using double-labeling immunofluorescence we investigated the compartmentalization of K-protein after serum stimulation. Fortuitously, we were using the SM-specific protein calponin in our immunofluorescent experiments to monitor the SMC phenotype of our cultures. In SMC, calponin interacts with MAP kinases and in the absence of extracellular calcium, may play a role in smooth muscle cell (SMC) contraction [Menice et al., 1997]. We observed that the calponin staining pattern was

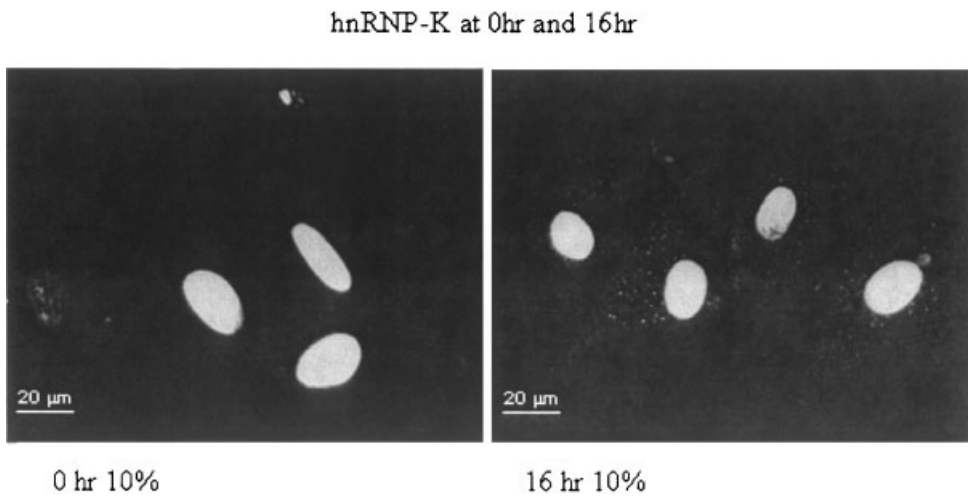

Fig. 3. hnRNP-K immunostaining of SMC at higher magnification. Rabbit SMC grown on coverslips were growth inhibited with serum free medium and stimulated with 10% fetal bovine serum for 16 h, as shown in Figure 2. Cells were stained as described in Figure 2.

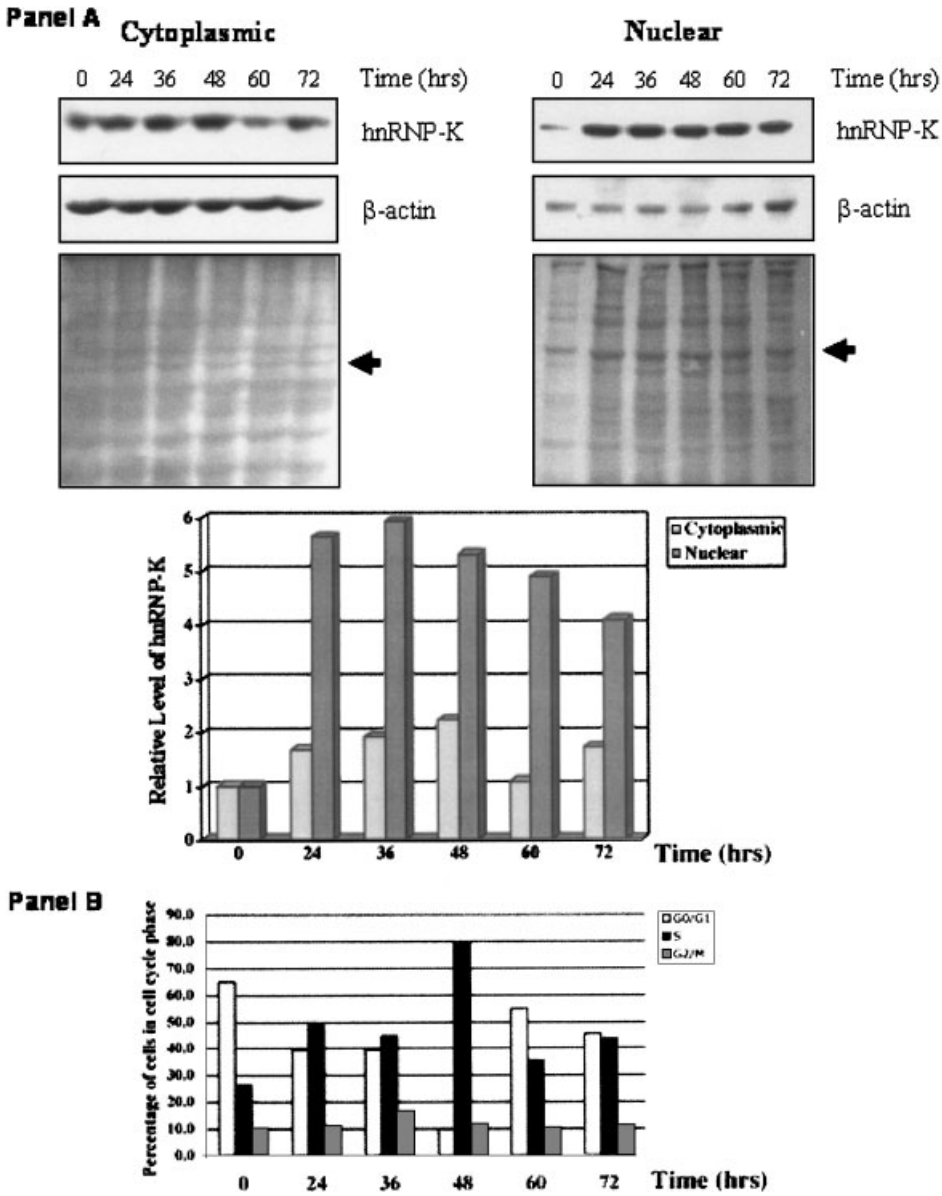

Fig. 4. hnRNP-K levels in cytoplasmic and nuclear extracts from SMC. **Panel A**: Western blotting analysis was performed on cytoplasmic and nuclear extracts from rabbit thoracic aorta SMC treated over a 72 h period with medium containing 10% FBS. Before treatment with10% FBS containing medium, SMC cultures at approx. 75% confluence were growth inhibited by stepping down to 1% FBS containing medium and then Serum-free Medium (SFM). SDS–PAGE was done using 45 μg of cytoplasmic extract and 15 μg of nuclear extract. Blots were probed with a monoclonal antibody generated against hnRNP-K (from Dr. Gideon Dreyfuss, Univ. of PA). These data are representative of three experiments from individual primary SMC isolates. The arrow next to the Ponceau S stained blot marks the band used to normalize hnRNP-K levels. **Panel B**: FACS analysis of SMC stimulated with medium containing 10% FBS over a 72 h period. These data are representative of three experiments from individual primary SMC isolates.

very similar to the hnRNP-K staining pattern in the cytoplasm. Therefore, we performed our double-labeling experiments with this SM-specific protein. Figure 7 shows the cytoplasmic co-localization of hnRNP-K with calponin in SMC. Calponin does not appear to associate with nuclear hnRNP-K. Appropriate isotype and secondary antibodies were used to control for non-specific staining. As a transporter of mRNAs [Matunis et al., 1992; Dejgaard et al., 1994; Ostareck-Lederer et al., 2002; Skalweit et al., 2003], hnRNP-K could be complexed in the cytoplasm with ribosomes or localized to mitochondria. hnRNP-K has been found to associate

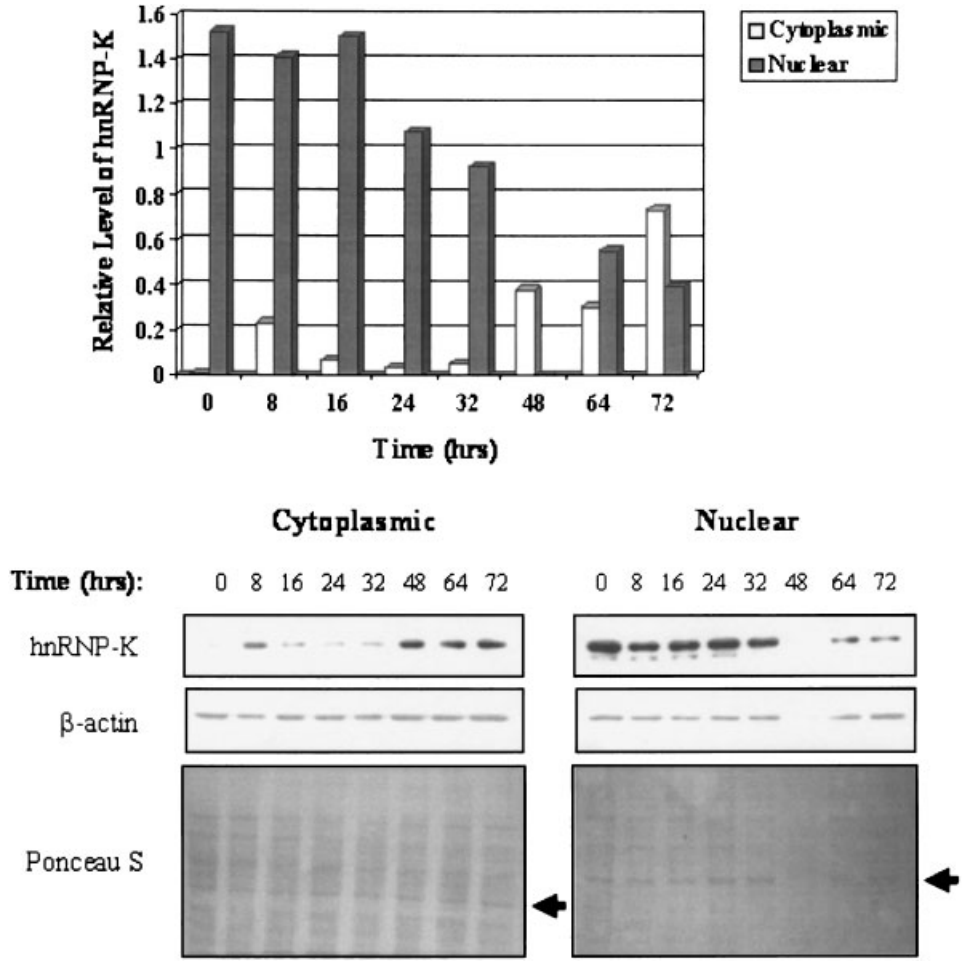

Fig. 5. hnRNP-K levels in cytoplasmic and nuclear extracts isolated from SMC after cycloheximide treatment. Western blotting analysis of cytoplasmic and nuclear extracts from hnRNP-K SMC treated over a 72 h period with medium containing10% FBS and 10 μg/ml (35.5 μM) cycloheximide before extraction of proteins. SDS–PAGE was done using 45 μg of cytoplasmic extract and 15 μg of nuclear extract. Blots were probed with the anti-hnRNP-K antibody. These data are representative of three experiments. The arrow next to the Ponceau S stained blot marks the band used to normalize hnRNP-K levels, the band at this position was used in all experiments.

with a number of mitochondrial transcripts, such as COX-1 and COX-2 [Ostrowski et al., 2002; Klimek-Tomczak et al., 2004]. We performed co-localization staining between hnRNP-K and ribosomal protein S6 or mitochondrial cytochrome c and did not observe that hnRNP-K co-localizes with these proteins (data not shown).

Immunoprecipitation of hnRNP-K and Calponin

To verify that hnRNP-K and calponin were associating together in SMC, we performed immunoprecipitations using protein specific antibodies. Also, because calponin has been demonstrated to complex with MAP kinases (ERK1/2) [Menice et al., 1997] and hnRNP-K is phosphorylated by ERK [Habelhah et al., 2001b], we used an antibody to ERK1/2 to determine if calponin and/or hnRNP-K associated with ERK in SMC. Using these antibodies against hnRNP-K, calponin, and ERK1/2, we observed that in total extracts from cells grown in serum for 24 or 48 h, hnRNP-K was pulled down with calponin and ERK1/2 (Fig. 8). More hnRNP-K was complexed with calponin than ERK1/2, as suggested by the lower intensity bands pulled down using the antiERK1/2 antibody. Also, more hnRNP-K was pulled down with the anti-calponin antibody than the hnRNP-K-specific antibody. Regardless of these differences in intensity, the results suggest that hnRNP-K interacts with calponin and/or ERK1/2. While it has been reported by

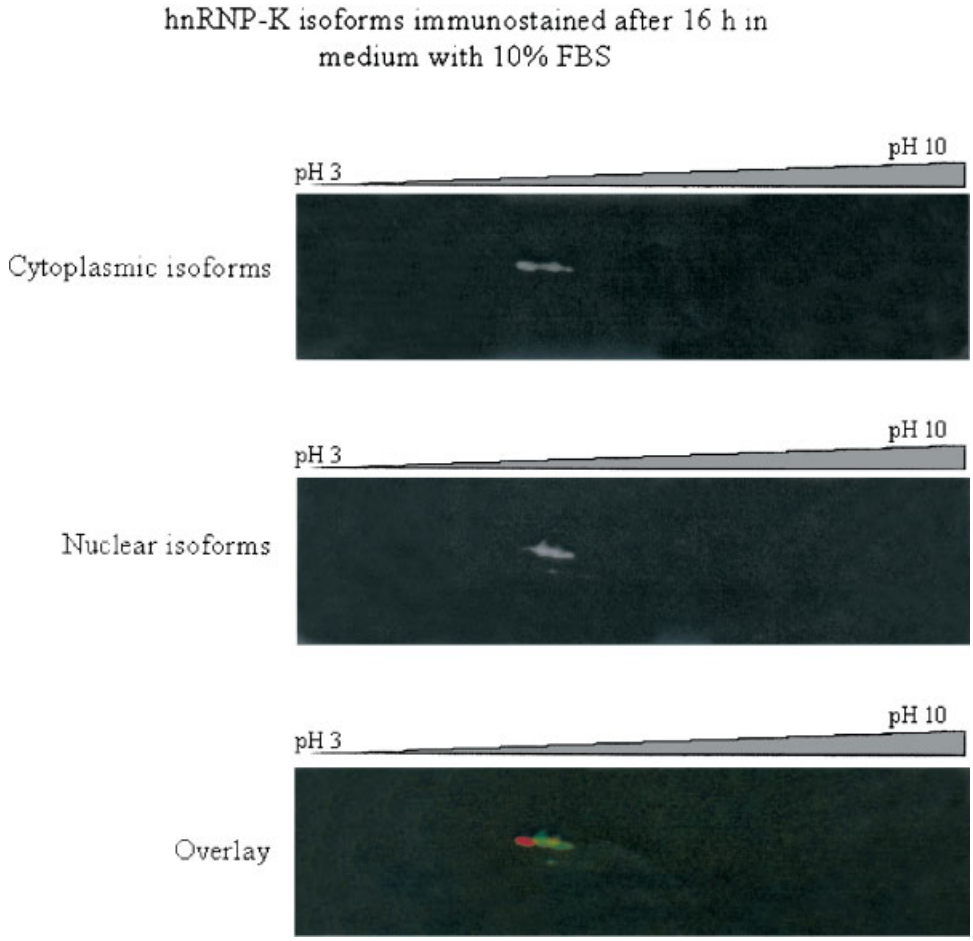

Fig. 6. hnRNP-K isoforms in the cytoplasm and nucleus. 2D gel western blots were generated and treated as described in Figures 4 and 5. Blots were first incubated with the anti-hnRNP-K monoclonal antibody and then a secondary antibody conjugated to HRP. These data are representative of three experiments from individual primary SMC isolates and 2D gel analyses. At 16 h after cell growth stimulation with 10% FBS extracts were prepared and separated by 2D gel analysis. Cytoplasmic hnRNP-K isoforms are shown at **top** (shown in red on the overlay); nuclear hnRNP-K isoforms are shown **middle** (shown in green on the overlay) and the overlay of the cytoplasmic and nuclear isoforms is shown in the **bottom panel**. The merged image was generated using Adobe Photoshop software.

others that ERK can phosphorylate hnRNP-K [Habelhah et al., 2001b], we extend that work to show that there is complex formation between hnRNP-K and ERK1/2 in SMC. Also, these protein associations occur in both the 24 and 48 h extracts, suggesting that the associations occur through out the course of the cell cycle. In extracts isolated at 0 h, there is very little association between these proteins (data not shown).

DISCUSSION

hnRNP-K is a member of a family with over 20 different proteins resulting from different genes. Their functions are primarily mRNA processing, transport, stability, and translation. Several hnRNPs have been identified as biomarkers of neoplasia and modulators of hypoxia, myogenesis, and gene transactivation [Hamilton et al., 1999; Shih and Claffey, 1999; Mulshine et al., 2000; Liu et al., 2001; Tolnay et al., 2002; Stone et al., 2003]. While the hnRNPs were first identified as RNA binding and pre-mRNA processing proteins, there is significant data in the literature that emphasizes the important regulatory roles of some members of this family. The nucleic acid binding activities, protein partners, and post-translational modifications of several of the hnRNP proteins regulate transcription and translation and, are themselves, regulated by signal transduction pathways. We highlight several members of this diverse family because of an emerging theme that we see developing.

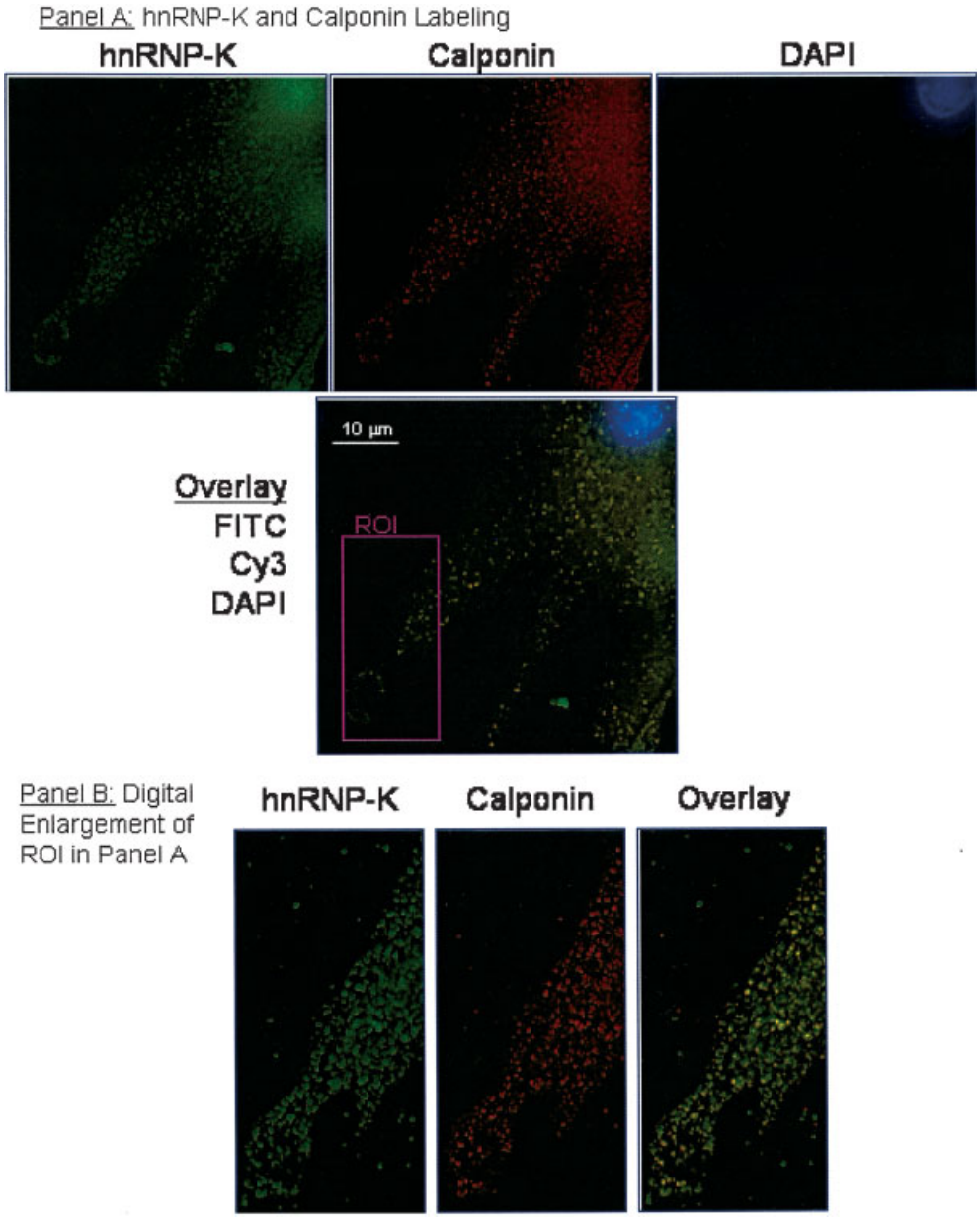

Fig. 7. hnRNP-K co-localization with calponin. Cells were grown on coverslips and fixed as described in Figure 2. Coverslips were first incubated with the anti-calponin monoclonal antibody and then a secondary antibody CY3-conjugated. After the first secondary antibody, the coverslips were re-blocked and then incubated with the anti-hnRNP-K antibody followed by a secondary conjugated to FITC. Washed coverslips were mounted with DAPI containing mount and viewed on a Zeiss upright microscope. **Panel A**: hnRNP-K/ FITC is shown left, calponin/ CY3, middle and DAPI stain is right. The 3D-deconvoluted and merged images were generated using Ziess Axiovision 4.0 software. **Panel B** is a digital enlargement of the region of interest (ROI) identified in panel A. hnRNP-K/ FITC is shown left, calponin/ CY3, middle and the merged image is shown at the right.

Several hnRNP proteins are playing important roles in regulating/modulating the growth and stress-responses of cells.

hnRNP-A2 is one of the better-characterized hnRNPs in relation to its mRNA transport function and post-translational modification. The increase of cytoplasmic hnRNP-A2 in bronchial epithelial cells is highly associated with the development of lung cancer [Mulshine et al., 2000]. Post-translational phosphorylation and methylation of hnRNP-A2 are affected by hypoxia and hypoglycemia, and these modifications affect the interaction of hnRNP-A2 with mRNA, thereby altering mRNA stability [Hamilton et al., 1999], as well as, sub-cellular localization [Nichols et al., 2000].

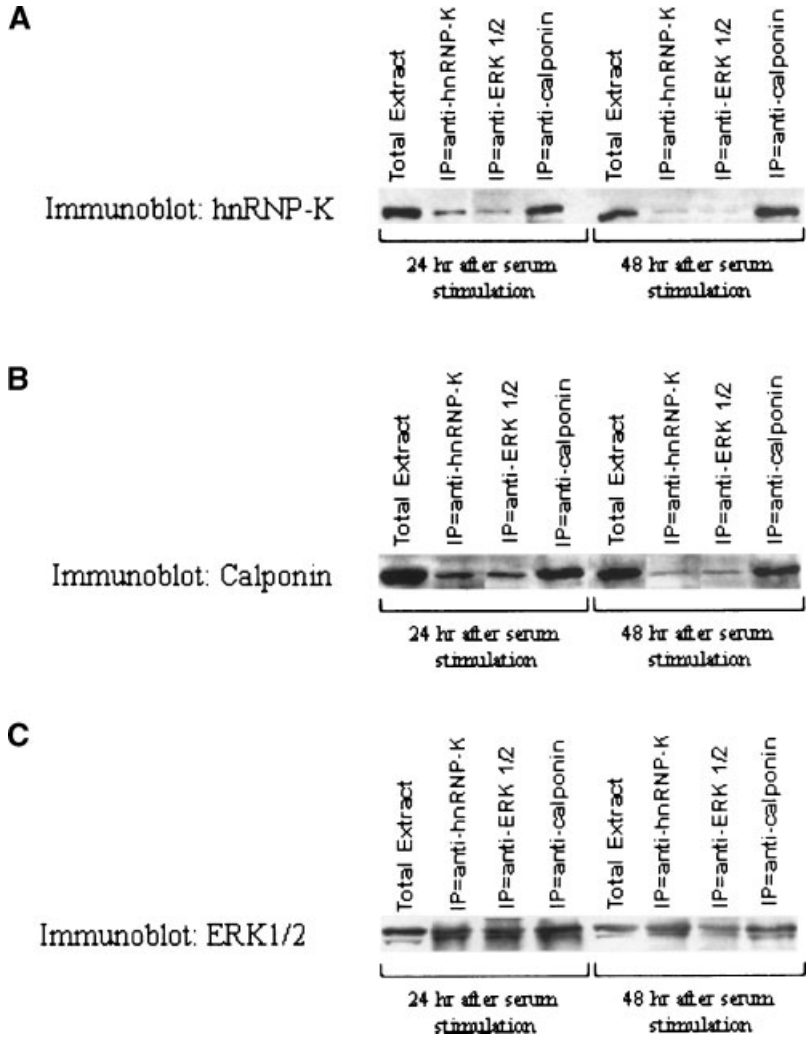

Fig. 8. Immunoprecipitation of hnRNP-K, calponin and ERK1/2. One hundred micrograms of extract generated from cells stimulated for 24 or 48 h was incubated with antibody against hnRNP-K, calponin, or ERK1/2. The immunocomplexes were pulled down with proteinA/G coupled to agarose and separated by SDS–PAGE and probed by Western analysis. The **top panel** shows the immunoblot probed with the hnRNP-K antibody. The **middle panel** shows the immunoblot using the calponin antibody and the **bottom panel** shows the immunoblot using the ERK1/2 antibody. These data are representative of three experiments from individual primary SMC isolates.

In concert with hnRNP-A2, hnRNP-L also interacts with the 3′UTR of VEGF mRNA regulating RNA stability [Hamilton et al., 1999; Shih and Claffey, 1999] in brain and cancer cells. hnRNP-L interacts with a sequence in the 3′UTR of VEGF mRNA that is distinct of hnRNP-A2 and post-transcriptionally provides stable regulation to VEGF mRNA under hypoxic stress [Shih and Claffey, 1999]. Hypoxia increases the cytoplasmic and nuclear levels of hnRNP-L and the interaction of hnRNP-L and VEGF mRNA [Shih and Claffey, 1999] in melanoma cells.

Endothelial cells treated with low concentrations of H_2O_2 show alterations in phosphorylation states and abundance of hnRNP-C [Stone and Collins, 2002; Stone et al., 2003]. Similar to hnRNP-K, hnRNP-C also has multiple splice forms that are then modified by phosphorylation, as shown by 2D gel electrophoresis [Stone and Collins, 2002]. The kinase acting on hnRNP-C in an H_2O_2-dependent fashion is protein kinase CK2 [Stone et al., 2003]. hnRNP-C may exert its affect on cell growth through its ability to bind c-myc mRNA at a specific heptameric U sequence and modulate the translation of c-myc message in a cell cycle dependent manner [Kim et al., 2003].

hnRNP-H has been reported to control cell-type specific alternative pre-mRNA splicing of β-tropomyosin in striated muscle cells, as well

as a number of other pre-mRNAs [Chen et al., 1999], whereas hnRNP-K has been shown to influence non-muscle β-tropomyosin splicing in cancer cells [Expert-Bezancon et al., 2002]. hnRNP-H is itself developmentally controlled. Liu and coworkers have demonstrated decreased mRNA and protein expression of hnRNP-H during SM differentiation [Liu et al., 2001]. By inhibiting hnRNP-H expression, the expression of SMC specific gene products, SM α-actin, desmin, and SM myosin are upregulated [Liu et al., 2001].

Like hnRNP-K, hnRNP-D has been shown to interact with RNA and DNA. In B lymphoblastoid cells, hnRNP-D binding to a sequence-specific site in the CR2 promoter, provides trans-activation activity that is phosphorylation dependent [Tolnay et al., 1999, 2002]. Phosphorylation of hnRNP-D by protein kinase A (PKA) and by glycogen synthase kinase 3β regulates its transactivation activity [Tolnay et al., 2002]. Also, as does hnRNP-K, hnRNP-D interacts with TATA-binding protein and p300 [Tolnay et al., 2002].

In this present study, we have begun to characterize hnRNP-K in a non-transformed cell type that can respond to a growth stimuli in the vasculature. The end organ damage, impaired vascular function, heart attack, and stroke, that results from atherosclerosis and restenosis are, in part, due to SMC proliferation. SMC proliferation during the genesis of atherosclerotic plaques has been speculated to occur as a result of mitogens released from accumulated macrophages [Ross et al., 1990] as well as from "injured" endothelial cells (EC) and SMC [Ross, 1986]. SMC growth is also a major problem in restenosis, the accelerated development of new plaques following revascularization techniques performed clinically to relieve under-perfusion due to the presence of atherosclerotic plaques [Goldman et al., 1997; Favaloro, 1998; Sriram and Patterson, 2001; Geng and Libby, 2002]. Using the rabbit animal model for early atherosclerotic lesions, the fatty streak, we observed hnRNP-K immunostaining in the cells of the thoracic aorta media and neointima. Also, we observed hnRNP-K immunostaining in SM α-actin positive regions of the lesions of failed human saphenous vein grafts. The occlusions found in vein grafts result from the vascular remodeling of these vessels, primarily orchestrated by SMC [Goldman et al., 1997; Favaloro, 1998].

Taken together, the in situ data suggested to us that hnRNP-K expression was increased in situations of SMC growth or vascular remodeling. We began investigations into the expression of hnRNP-K in isolated SMC in an effort to understand the role this multi-function protein is playing in SMC growth. Our data demonstrate that during the progression of the cell cycle, hnRNP-K is not only synthesized but also translocates from the nucleus to the cytoplasm in SMC. This translocation is presumably to traffic mRNAs necessary for cell growth to the cytoplasm for translation. Using cycloheximide we show that the increase in cytoplasmic hnRNP-K protein early in the cell cycle is presumably due to new protein synthesis, while the cytoplasmic increase later in the cell cycle is due to translocation from the nucleus to the cytoplasm. Ostrowski and Bomsztyk report a shift in hnRNP-K to the nucleus with cell growth and that nuclear hnRNP-K is greater in proliferating cells versus quiescent [Ostrowski and Bomsztyk, 2003]. Their observation is consistent with our data; however, our data extend their work by demonstrating two shifts in hnRNP-K movement, the first into the nucleus and then the second from the nucleus. Our data, in context with work of others, suggest that hnRNP-K is shuttling mRNA from the nucleus to the cytoplasm and then associating with proteins involved with signal transduction pathways to post-transcriptionally regulate mRNA translation in the cytoplasm [Micheal et al., 1997; Schullery et al., 1999; Wadd et al., 1999; Ostrowski et al., 2000; Habelhah et al., 2001a,b; Bomsztyk et al., 2004].

Our immunofluorescence data suggest that hnRNP-K is associated with a multimeric complex in the cytoplasm. Double-labeling experiments demonstrate that calponin is part of this multi-protein complex. Morgan and associates have shown that in SMC calponin complexes with ERK1/2 and PKCε [Menice et al., 1997; Leinweber et al., 1999; Morgan and Gangopadhyay, 2001]. As a consequence of this association calponin is post-translationally regulated by PKCε-directed phosphorylation events [Morgan and Gangopadhyay, 2001] and merges these signaling pathways. By complexing with calponin, hnRNP-K can also be in an association with ERK, which has been shown to phosphorylate hnRNP-K [Habelhah et al., 2001b]. We have used the ERK inhibitor U0126 and have not observed changes in hnRNP-K

translocation and compartmentalization (data not shown). Because calponin associates with PKCε [Morgan and Gangopadhyay, 2001], signaling through PKCε could also direct hnRNP-K function. Recent evidence by de Hoog and coworkers demonstrate that hnRNP-K is part of a multimeric complex at spreading initiation centers associated with focal adhesions [de Hoog et al., 2004]. Their studies suggest a role for RNA and RNA binding proteins at the initiation sites of cell spreading [de Hoog et al., 2004].

Interestingly, hnRNP-K has also been found to interact with a ubiquitous, cytoplasmic, calcium activated cysteine protease μ-Calpain [Kimura et al., 2003]. Kimura and coworkers demonstrated that a μ-Calpain and hnRNP-K complex is localized in the cytoplasm of COS-7 cells and that hnRNP-K is an in vitro and in vivo substrate for μ-Calpain [Kimura et al., 2003]. These data suggest a degradation pathway for cytoplasmic hnRNP-K that again is linked to a calcium-activated signaling mechanism.

Adding complexity to an already multifunctional protein, hnRNP-K exists as multiple isoforms. Our data suggest that the more acidic isoforms are prevalent in the cytoplasm. The regulation of these multiple hnRNP-K isoforms by post-translational modifications, compartmentalization and potentially by protein/protein interactions creates a powerful regulatory system that underscores the importance of hnRNP-K in cell growth.

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