The $MKK_{3/6}$-p38–signaling Cascade Alters the Subcellular Distribution of hnRNP A1 and Modulates Alternative Splicing Regulation

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Abstract. Individual members of the serine-arginine (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) A/B families of proteins have antagonistic effects in regulating alternative splicing. Although hnRNP A1 accumulates predominantly in the nucleus, it shuttles continuously between the nucleus and the cytoplasm. Some but not all SR proteins also undergo nucleo-cytoplasmic shuttling, which is affected by phosphorylation of their serine/arginine (RS)–rich domain. The signaling mechanisms that control the subcellular localization of these proteins are unknown. We show that exposure of NIH-3T3 and SV-40 transformed green monkey kidney (COS) cells to stress stimuli such as osmotic shock or UVC irradiation, but not to mitogenic activators such as PDGF or EGF, results in a marked cytoplasmic accumulation of hnRNP A1, concomitant with an increase in its phosphorylation. These effects are mediated by the $MKK_{3/6}$-p38 pathway, and moreover, p38 activation is necessary and sufficient for the induction of hnRNP A1 cytoplasmic accumulation. The stress-induced increase in the cytoplasmic levels of hnRNP A/B proteins and the concomitant decrease in their nuclear abundance are paralleled by changes in the alternative splicing pattern of an adenovirus E1A pre-mRNA splicing reporter. These results suggest the intriguing possibility that signaling mechanisms regulate pre-mRNA splicing in vivo by influencing the subcellular distribution of splicing factors.

Key words: alternative splicing • hnRNP A1 • signal transduction • p38 kinase • stress signaling.

Introduction

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP)[1] is an abundant nuclear RNA-binding protein involved in the processing of pre-mRNA (Dreyfuss et al., 1993). hnRNP A1 binds to most RNA polymerase II transcripts, displaying high affinity for sequences that resemble splice sites (Burd and Dreyfuss, 1994), and it can regulate alternative splicing in vitro and in vivo by antagonizing the serine-arginine (SR) family of proteins (Mayeda and Krainer, 1992; Cáceres et al., 1994; Yang et al., 1994; reviewed in Cáceres and Krainer, 1997).

Although hnRNP A1 localizes predominantly in the nucleus, it shuttles continuously between the nucleus and the cytoplasm, and treatment with the transcriptional inhibitor actinomycin D results in its cytoplasmic accumulation (Piñol-Roma and Dreyfuss, 1992). A subset of human SR proteins also shuttles rapidly and continuously between the nucleus and the cytoplasm (Cáceres et al., 1998). The ability of hnRNP and SR proteins to shuttle may be essential for additional roles played by these proteins in nuclear export of mature mRNAs (Izaurralde et al., 1997), translation, and/or posttranslational events. For example, hnRNP A1 can render translation cap-dependent in vitro, which suggests a role for hnRNP A1 in the regulation of protein translation (Svitkin et al., 1996). Other shuttling proteins, such as hnRNP K and hnRNP E1, regulate 15-lipoxygenase translation by binding to specific sequences in the 3′ untranslated region of the mRNA (Ostareck et al., 1997).

Transient overexpression of Clk/Sty kinase affects the subcellular distribution of the SR protein SF2/ASF, causing its cytoplasmic accumulation (Cáceres et al., 1998).

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[1]*Abbreviations used in this paper*: ActD, actinomycin D; CBC, nuclear cap-binding complex; COS, SV-40 transformed green monkey kidney; ERK, extracellular signal regulated kinase; hnRNP, heterogeneous nuclear ribonucleoprotein; JNK, jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK-1 kinase; MKK, MAP-kinase kinase; OSM, osmotic shock; PKA, protein kinase A; RS, arginine/serine; SAPK, stress-activated protein kinase; SR, serine-arginine.

This effect is most likely due to phosphorylation of the RS domain of SF2/ASF. However, it is not clear whether these changes in cellular localization are due to activation of nuclear export or inhibition of nuclear import of SF2/ASF. This observation suggests that signaling mechanisms that control the subcellular distribution of splicing regulators can affect splicing regulation by altering the ratios of antagonistic factors in the nucleus. Indeed, transient transfection of Clk/Sty kinase affects the splicing pattern of transcripts from a cotransfected E1A splicing reporter (Duncan et al., 1997).

The signaling pathways that link membrane events with cytoplasmic/nuclear effects have recently been grouped into kinase cascades activated by either mitogenic or stress stimuli (Davis, 1993, 1994). The mitogenic signals converge into the MEK/MAP kinase module, whereas the stress signals lead to the activation of two kinase cascades that are similar to the mitogenic MAP kinase (also known as ERK; Canman and Kastan, 1996). Osmotic shock, inflammatory cytokines, as well as UVC-irradiation and ceramide, stimulate these two stress-response kinase cascades that involve the activation of SAPK (also known as JNK) by SEK-1, and that of p38 kinase by $MKK_{3/6}$ (Kyriakis and Avruch, 1996, Karin 1998). Although the events upstream of SEK-1 and $MKK_{3/6}$ are still poorly defined, the activation of SAPK and/or p38 are critical steps in the control of the stress response. All three MAP kinases culminate in the phosphorylation and activation of important transcription factors such as elk-1, c-Jun, or ATF-2 (Davis, 1993, 1994; Kyriakis and Avruch, 1996). However, other substrates exist for these kinases, such as cPLA2 and $p90^{rsk}$ (also known as MAPKAP kinase-1) in the case of ERK kinase, or MAPKAP kinase-2 in the case of p38 (Davis, 1994; Kyriakis and Avruch, 1996).

The signaling mechanisms that control hnRNP A1 shuttling, if any, are unknown. The existence of a link between these signaling routes and hnRNP A1 shuttling would clarify the mechanism whereby signal transduction pathways regulate not only gene transcription, but also the correct processing of mRNA. We demonstrate here that the subcellular localization of hnRNP A1 can be modulated by the $MKK_{3/6}$-p38 pathway in response to stress, but not to mitogenic stimulation. The stress-induced increase in the cytoplasmic levels of hnRNP A1 and the concomitant decrease in nuclear hnRNP A1 abundance are reflected in changes in alternative splicing activity.

We propose that the subcellular distribution of splicing factors is a regulated process, which in the case of hnRNP A1 appears to be an early response to stress, involving the p38 kinase cascade.

Materials and Methods

Plasmids

Plasmids expressing HA-tagged versions of ERK, SAPK, p38 and ζPKC, and their respective dominant-negative mutants were described previously (Berra et al., 1995; Raingeaud et al., 1996) and were generously provided by J. Pouyssegur, J. Woodget, and R. Davis, respectively. The myc-tagged $MKK_{3/6}$ wild-type and constitutively active mutant ($MKK_{3/6}^{DD}$) were a generous gift from A. Nebreda (EMBL; Cuenda et al., 1996). The E1A gene plasmid pMTE1A used in the alternative splicing assays was described previously (Zerler et al., 1986; Cáceres et al., 1994) and was kindly provided by B. Moran.

Cell Culture and Transfections

NIH 3T3 cells and arginine/serine (RS)–rich SV-40 transformed green monkey kidney (COS) cells were cultured in DME supplemented with 10% FCS. Cells were treated with the different agonists as described in the figure legends. Sorbitol and actinomycin D were purchased from Sigma Chemicals. Transfections were carried out by the calcium phosphate method. In brief, subconfluent cultures of COS cells grown on coverslips in 60-mm dishes were transfected with 5 μg of plasmids expressing MAPK, SAPK, p38, $MKK_{3/6}$, or their respective mutants. Plasmid DNA was removed 12 h later, and DME containing 10% FCS was added. At 24 h after transfection, cells were either left untreated or were exposed to different stimuli and fixed for indirect immunofluorescence analysis.

Immunofluorescence Microscopy

Cells were fixed with 4% *p*-formaldehyde in PBS for 15–30 min at room temperature, followed by incubation for 10 min in 0.2% Triton X-100. Endogenous hnRNP A1, hnRNP U and hnRNP C1/C2 were visualized with a 1:1,000 dilution of 4B10, 3G6 or 4F4 monoclonal antibodies, respectively, which were kindly provided by Dr. Gideon Dreyfuss (Dreyfuss et al., 1984; Piñol-Roma et al., 1988). Endogenous hnRNP B1 was visualized using a 1:1,000 dilution of 2B2 monoclonal antibody (Kamma et al., 1999). For localization of endogenous SF2/ASF protein, a monoclonal antibody against SF2/ASF (mAb 103) was used at a 1:500 dilution (Cáceres et al., 1997). For localization of endogenous SC35 protein, a monoclonal antibody against SC35 was used as ascites at a 1:1,000 dilution (Fu and Maniatis, 1990). The cells were then washed three times with PBS and incubated at room temperature with a 1:200 dilution of fluorescein-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). After washing three times, the coverslips were inverted and mounted on glass microscope slides. Double immunofluorescence analysis of endogenous hnRNP A1 and HA- or myc-tagged proteins was carried out using monoclonal anti-hnRNP A1 4B10 and rabbit polyclonal anti-HA or anti-myc antibodies (Santa Cruz Biotechnology, Inc.), followed by a mix of fluorescein-conjugated anti–mouse and rhodamine-conjugated anti–rabbit antibodies (Jackson ImmunoResearch Laboratories). Samples were observed on a Zeiss Axioskop microscope and images acquired with a Photometrics CH250 cooled CCD camera using Digital Scientific Smartcapture extensions within IP Lab Spectrum software.

In Vivo Phosphorylation of hnRNP A1 and Western Blot Analysis

Metabolically ^{32}P-labeled NIH-3T3 cells were either left untreated or were exposed to high-osmolarity medium (OSM) for different times. Subsequently, cell lysates were made with RIPA buffer containing 10 μg/ml of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM orthovanadate, 20 mM β-glycerophosphate and 10 mM sodium fluoride. Endogenous hnRNP A1 was immunoprecipitated with the monoclonal antibody 4B10 and its phosphorylation level determined by autoradiography following SDS-PAGE.

For Western blot analysis, cell lysates were made with RIPA buffer as described above. The electrophoretically separated proteins were transferred onto Hybond *P* membranes (Amersham Pharmacia Biotech). Nonspecific binding was blocked by incubating the blot with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.6, 137 mM NaCl and 0.1% Tween 20). Proteins were detected by subsequent incubation with the primary antibody in TBST. The following primary antibodies were used: anti–phospho-p38 kinase rabbit polyclonal antibody at a 1:1,000 dilution (9211; New England Biolabs), anti–phospho-SAPK rabbit polyclonal antibody at a 1:1,000 dilution (9251; New England Biolabs), anti-cdc2 (PSTAIRE) rabbit polyclonal antibody at a 1:500 dilution (sc-53; Santa Cruz Biotechnology, Inc.) and the anti-hnRNP A1 mouse monoclonal antibody 4B10 at a dilution of 1:1,000. After extensive rinsing with TBST, the blots were incubated with secondary antibodies (either goat anti–rabbit IgG or goat anti–mouse IgG; sc-2004 or 2005, respectively; Santa Cruz Biotechnology, Inc.) conjugated to horseradish peroxidase at a 1:7,000 dilution. After further rinsing in TBST, the blots were developed using ECL.

E1A Alternative Splicing

The experiments involving E1A alternative splicing were performed as

described (Cáceres et al., 1994). In brief, COS cells grown on 90-mm dishes were transfected with 6 μg of pMTE1A alone or in combination with 7 μg of expression plasmids encoding myc-tagged versions of $MKK_{3/6}$ or its permanently active mutant ($MKK_{3/6}^{DD}$), in conjunction with 7 μg of HA-tagged versions of wild-type p38 kinase or its dominant-negative mutant ($p38^{DN}$). Plasmid DNA was removed 12 h later, and DME containing 10% FCS was added. After 24 h, cells were either left untreated or exposed to 600 mM sorbitol for 4 h. RNA was extracted using the Total RNA Isolation Reagent (Advanced Biotechnologies LTD). Total RNA (5 μg) was analyzed by RT-PCR with Superscript II reverse transcriptase (Life Technologies) and AmpliTaq DNA polymerase (Perkin Elmer). E1A mRNA detection was carried out with the exon 1 forward primer 5′-GTTTTCTCCTCCGAGCCGCTCCGA-3′ and the 5′ end-labeled exon 2 reverse primer 5′-CTCAGGCTCAGGTTCAGACACAGG-3′.

Results

Cytoplasmic Accumulation of hnRNP A1 in Stress-activated Cells

Exposure of NIH-3T3 cells to osmotic shock (OSM; DME + 400 mM sorbitol) induced a detectable accumulation of hnRNP A1 in the cytoplasm within 30 min of cell stimulation, and this response peaked after 2 h (Fig. 1 A). This effect was reversible and by 5 h of osmotic shock, hnRNP A1 protein was nuclear in most cells (Fig. 1 A, upper panel; Table I). Whereas ~65–80% of NIH-3T3 cells reversibly accumulated hnRNP A1 in the cytoplasm when exposed to 400 mM sorbitol; (Fig. 1 A and Table I), ~100% of the cells displayed irreversible cytoplasmic accumulation of hnRNP A1 when the concentration of sorbitol was increased to 600 mM (Fig. 1 A, middle panel). As a control, staining of the nonshuttling hnRNP U protein with the appropriate antibody (Dreyfuss et al., 1984) demonstrated that this effect was specific and not due to gener-

Table I. Percentage of Cells Showing Cytoplasmic Staining for Endogenous hnRNP A1, SF2 and hnRNP B1 after Treatment with OSM and UV

		3T3 cells		COS cells		
		hnRNP A1	SF2	hnRNP A1	SF2	hnRNP B1
		%			%	
Control		<2	<2	<2	<2	<2
0.4 M OSM	2 h	65–80	5–10	35–45	5	3–5
	5 h	25–40	ND	30–35	ND	2–3
0.6 M OSM	2 h	>98	15–20	95–100	ND	95–100
	5 h	>98	ND	90–95	ND	80–90
UV	5 h	15–20	<2	3–6	<2	10–15

NIH-3T3 and COS cells grown on coverslips were either left untreated or exposed to the indicated stress stimuli, and subsequently fixed and incubated with either the 4B10 antibody to detect endogenous hnRNP A1, the 103 antibody to detect endogenous SF2, or the 2B2 antibody to detect endogenous hnRNP B1.

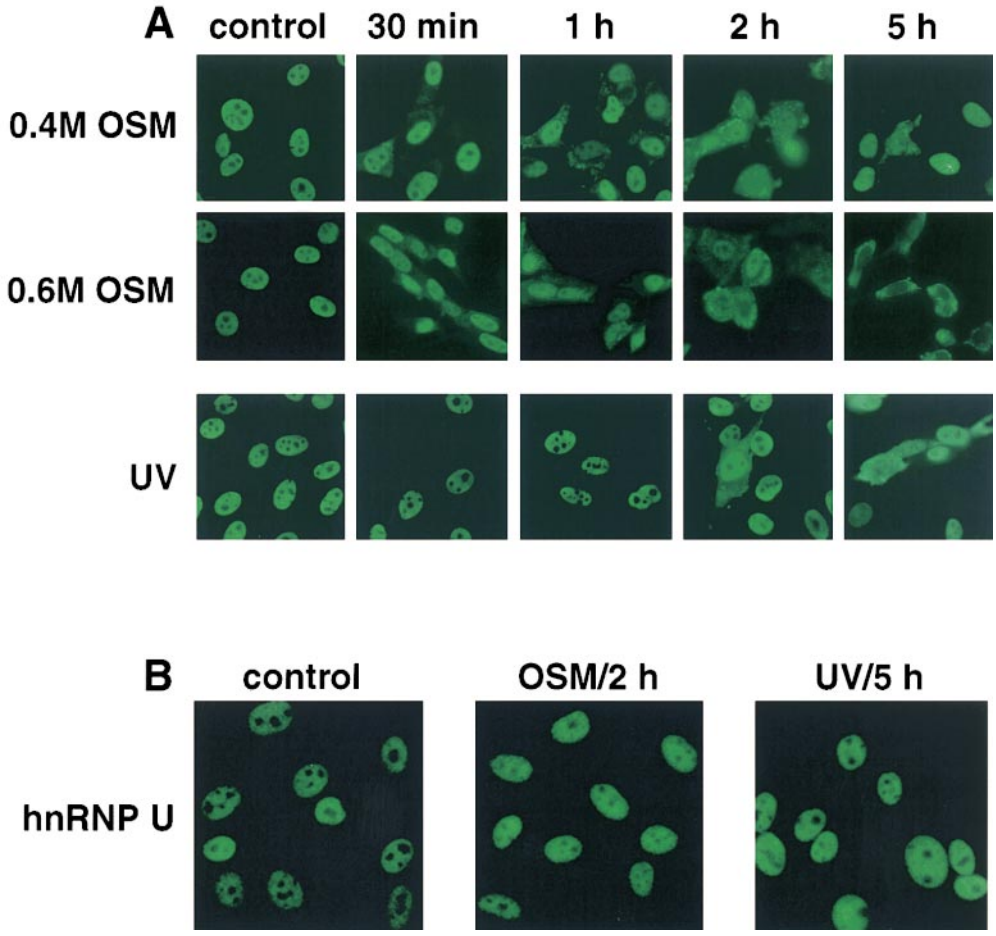

Figure 1. hnRNP A1 accumulates in the cytoplasm in stress-activated cells. (A) NIH-3T3 cells cultured on glass coverslips were left untreated or were exposed to either high-osmolarity medium (OSM; DME + 400 mM sorbitol, upper panel; DME + 600 mM sorbitol, middle panel) for the indicated times, or to UV-C light (UV; 180 J/m^2, lower panel) and were then further cultured for the indicated times. The cells were then fixed and immunostained with the anti-hnRNP A1 monoclonal antibody 4B10 to detect endogenous hnRNP A1 protein, as described in Materials and Methods. 100 mM Z-VAD, a potent caspase inhibitor, was included in the 5-h incubation time point (lower panel). (B) As a control, cells exposed to either 400 mM sorbitol (OSM) for 2 h or to UV-C light (UV) and further incubated for 5 h, were processed as above but stained with the monoclonal antibody 3G6 to detect endogenous hnRNP U protein, a nonshuttling hnRNP protein. Essentially identical results were obtained in several independent experiments.

alized nuclear leakage, since no accumulation of hnRNP U protein in the cytoplasm was observed (Fig. 1 B).

When cells were irradiated with UV-C light, hnRNP A1 accumulated in the cytoplasm, but with slower kinetics than observed after osmotic stress. In UV-C–irradiated cells, hnRNP A1 cytoplasmic accumulation was detectable by 2 h and was maximal at 5 h (Fig. 1 A, lower panel). UV-C light induces cells to undergo apoptosis; however, hnRNP A1 cytoplasmic accumulation was independent of this process, since the 5-h time point was obtained in the presence of a potent Caspase inhibitor (Z-VAD). As observed with OSM, UV-C irradiation provoked no changes in the subcellular distribution of hnRNP U (Fig. 1 B).

In contrast to the effects of OSM and UV-C light, stimulation of serum-starved quiescent 3T3 cells with different growth factors such as PDGF or EGF, did not alter the cellular distribution of hnRNP A1 (not shown). Significantly, pretreatment of NIH-3T3 cells with 100 μM cycloheximide to block protein synthesis 4 h before the stimulus had no effect on the ability of the different stress stimuli to promote the cytoplasmic accumulation of hnRNP A1 (not shown). This observation demonstrates that the accumulation of hnRNP A1 in the cytoplasm does not represent newly synthesized protein in conjunction with a blockage of nuclear import.

To test whether the cytoplasmic accumulation of hnRNP A1 in response to stress is cell specific, we exposed different cell lines to OSM and/or UV-C irradiation and analyzed the subcellular distribution of a panel of hnRNP and SR proteins. Exposure of COS cells to OSM (DME + 400 mM sorbitol) for 2 h induced cytoplasmic accumulation of hnRNP A1 in 35–45% of the cells (Fig. 2 and Table I). No detectable changes in the subcellular distribution of the nonshuttling hnRNP U and hnRNP C proteins were observed. The kinetics of hnRNP A1 cytoplasmic accumulation differed between NIH-3T3 and COS cells, since in the latter hnRNP A1 remained cytoplasmic after a 5-h incubation, and reversion to the nuclear localization pattern was observed only after a 10-h incubation (data not shown). The response of SR proteins to OSM in COS cells varied. In the case of SF2/ASF, only a small fraction of cells accumulated endogenous SF2/ASF in the cytoplasm (5%), which is similar to what has been observed in 3T3 cells (see Table I). In contrast, SC35 remained nuclear (Fig. 2). In accordance to what has been observed in 3T3 cells, UV-C irradiation of COS cells induced cytoplasmic accumulation of hnRNP A1 in a low number of cells (3–6%, compared with 15–20% in 3T3 cells, Table I), whereas SF2/ASF remained nuclear (Table I). Similar results were obtained with two human cell lines, HeLa and 293 (data not shown).

We analyzed the distribution of another shuttling hnRNP protein, hnRNP B1, which is closely related in sequence to hnRNP A1 and also antagonizes SR proteins in splicing regulation (Biamonti et al., 1994; Mayeda et al., 1994). Interestingly, we found that 400 mM sorbitol did not significantly change the nuclear localization of hnRNP B1 in COS cells, in contrast to what has been observed for hnRNP A1 (Table I). On the other hand, 600 mM sorbitol induced cytoplasmic accumulation of hnRNP B1 in ~100% of the cells, similar to what has been observed for hnRNP A1. Exposure to UV-C light resulted in cytoplasmic relocalization of hnRNP B1 in 10–15% of the cells (Table I). These experiments strongly suggest that the stress-induced cytoplasmic accumulation of hnRNP and SR proteins is not a general feature of shuttling versus nonshuttling proteins, but rather a unique response of individual proteins to stress signaling (Figs. 1 and 2 and Table I). A summary of the localization of different hnRNP and SR proteins in cells treated with OSM and UV is presented in Table I.

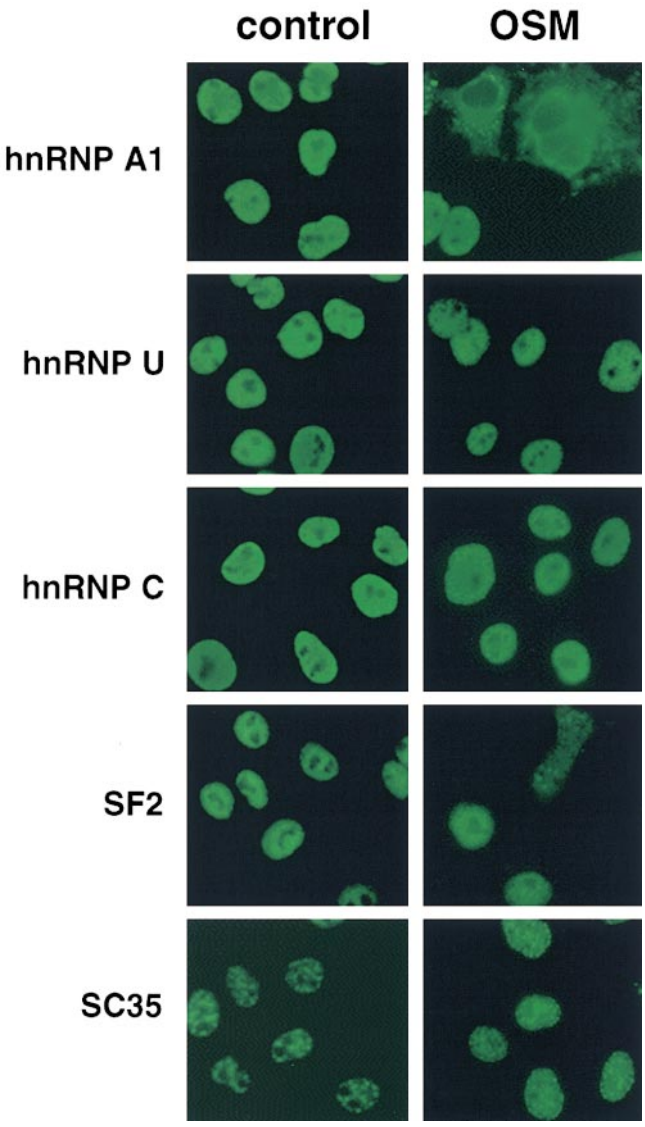

Figure 2. Subcellular distribution of several SR/hnRNP proteins in COS cells subjected to OSM. COS cells cultured on glass coverslips were left untreated or were exposed to 400 mM sorbitol (OSM) for 2 h. The cells were then fixed and stained with monoclonal antibodies specific for hnRNP A1 (4B10), hnRNP U (3G6), hnRNP C (4F4), SF2/ASF (103), or SC35 (anti-SC35).

This set of experiments demonstrates that changes in the subcellular distribution of hnRNP A1 following stress signaling are not a cell type-specific phenomenon. The fact that hnRNP U (Fig. 1 B), hnRNP C and SC35 (Fig. 2) remained exclusively nuclear in cells exposed to different stress stimuli also indicates that the OSM and UV treat-

ments do not affect general cell integrity or nuclear envelope morphology. In addition, these controls demonstrate that general cellular protein import and export are not affected following stress signaling.

OSM Induces hnRNP A1 Phosphorylation

We did not detect phosphorylation of hnRNP A1 by activated p38 kinase in vitro (not shown). However, phosphorylation of hnRNP A1 increased dramatically following the exposure of NIH-3T3 cells to OSM. The time course of hnRNP A1 phosphorylation at 600 mM sorbitol correlated well with the kinetics of its cytoplasmic accumulation (Fig. 3 B). hnRNP A1 phosphorylation increased gradually throughout the time course and under these experimental conditions, ~100% of the cells accumulated hnRNP A1 in the cytoplasm in an irreversible manner (Fig. 1 A and Table I). When NIH-3T3 cells were exposed to 400 mM sorbitol, hnRNP A1 also became phosphorylated although to a much lesser extent. HnRNP A1 phosphorylation reaches a plateau at 2–5 h after stimulation, however, reversion to a nuclear localization was observed at 5 h when hnRNP A1 was still phosphorylated. The inability of hnRNP A1 to return to the nucleus after exposure to 600 mM sorbitol may be a consequence of the hyperphosphorylated state of the protein under these conditions. We also observed a comparable phosphorylation pattern of hnRNP A1 in COS cells exposed to OSM (not shown). Collectively, these results indicate that although hnRNP A1 may not be a direct substrate of p38 kinase in vitro, it becomes phosphorylated in response to stress, and the kinetics of phosphorylation and of cytoplasmic accumulation are similar.

Role of p38 Kinase in the Cytoplasmic Accumulation of hnRNP A1

The signaling pathways activated by stress have been extensively studied (Canman and Kastan, 1996; Hannun 1996; Kyriakis and Avruch, 1996; Karin, 1998). Different stress stimuli, such as osmotic shock, UV-C irradiation and ceramide, activate two stress-response kinase cascades which are critical steps in the control of the stress response: SAPK (also known as JNK) by SEK-1, and p38 kinase by $MKK_{3/6}$ (Canman and Kastan, 1996; Kyriakis and Avruch, 1996, Karin, 1998). We therefore analyzed the potential role of the SAPK and p38 kinase pathways in the control of hnRNP A1 subcellular redistribution after stress signaling. COS cells were transfected with expression plasmids encoding HA-tagged versions of wild-type or dominant-negative mutants of MAPK/ERK, SAPK, or p38 kinase (Berra et al., 1995; Raingeaud et al., 1996). The transfected cells were exposed to OSM, and the subcellular localization of endogenous hnRNP A1 was determined by double immunofluorescence staining (Fig. 4 a). Expression of the dominant-negative mutant of p38 abrogated the effect of OSM by blocking the accumulation of hnRNP A1 in the cytoplasm (Fig. 4, a and b), whereas expression of wild-type p38 had no effect (Fig. 4 b). Expression of dominant-negative ERK or SAPK (Fig. 4, a and b) and of wild-type ERK or SAPK had no effect either (Fig. 4 b). Thus, blocking the p38 pathway by a dominant-negative form of p38 kinase is sufficient to prevent the cytoplasmic accumulation of hnRNP A1 after OSM, strongly suggesting that this signaling pathway is responsible for its altered subcellular distribution.

hnRNP A1 is directly phosphorylated in vitro by protein kinase A (PKA) and ζPKC (Cobianchi et al., 1993; Municio et al., 1995). Although expression of a constitutively active mutant of ζPKC promotes the cytoplasmic accumulation of hnRNP A1 in proliferating NIH-3T3 cells (Municio et al., 1995), transfection of a kinase-inactive dominant-negative mutant of ζPKC had no effect on the cytoplasmic accumulation of hnRNP A1 in response to OSM (Fig. 4 b). Therefore, ζPKC does not appear to play a role in the regulation of hnRNP A1 shuttling in response to stress.

The nucleo-cytoplasmic shuttling properties of hnRNP A1 were originally discovered by exposure of cells to actinomycin D (Piñol-Roma and Dreyfuss, 1992). These experiments suggested that the accumulation of hnRNP A1 in the cytoplasm is a direct consequence of the inhibition of RNA polymerase II transcription. The experiment in Fig. 4 c clearly demonstrates that transfection of a dominant-negative form of p38 kinase does not interfere with the ability of actinomycin D to promote the cytoplasmic accumulation of hnRNP A1, indicating that the effect of actinomycin D on hnRNP A1 shuttling does not involve the p38 pathway. Moreover, stress signals do not cause a general inhibition of transcription; on the contrary, transcription of several genes, such as the aldose reductase gene, is activated as a cellular response to hyperosmolarity (Ferraris et al., 1996; Ruepp et al., 1996). In addition, several of the downstream targets of both stress pathways, such as hsp27, ATF-2, and c-jun are transcription factors, which upon activation by SAPK and p38 kinase will in turn activate the transcription of downstream genes. We conclude that the cytoplasmic accumulation of hnRNP A1 in response to stress is not due to a general inhibition of transcription.

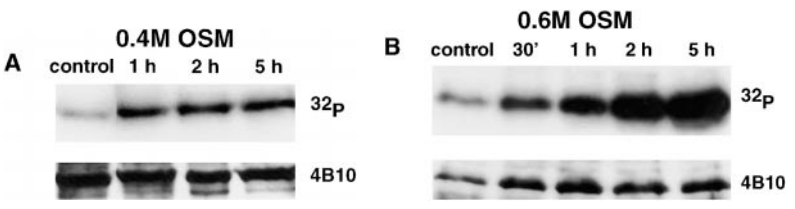

Figure 3. OSM induces hnRNP A1 phosphorylation. Metabolically ^{32}P-labeled NIH-3T3 cells were either left untreated or were exposed to OSM (DME + 400 mM sorbitol (A) or DME + 600 mM sorbitol (B)) for the indicated times. Total protein was isolated, endogenous hnRNP A1 was immunoprecipitated with the monoclonal antibody 4B10, and its phosphorylation level was determined by autoradiography following SDS-PAGE. Western blotting analysis using the 4B10 antibody showed comparable amounts of hnRNP A1 protein present in all lanes (lower panel in A and B). Exposure of NIH-3T3 cells to 600 mM sorbitol resulted in cytoplasmic accumulation of hnRNP A1 in ~100% of the cells after 2 h, and hnRNP A1 cytoplasmic localization was not reversible after 5 h.

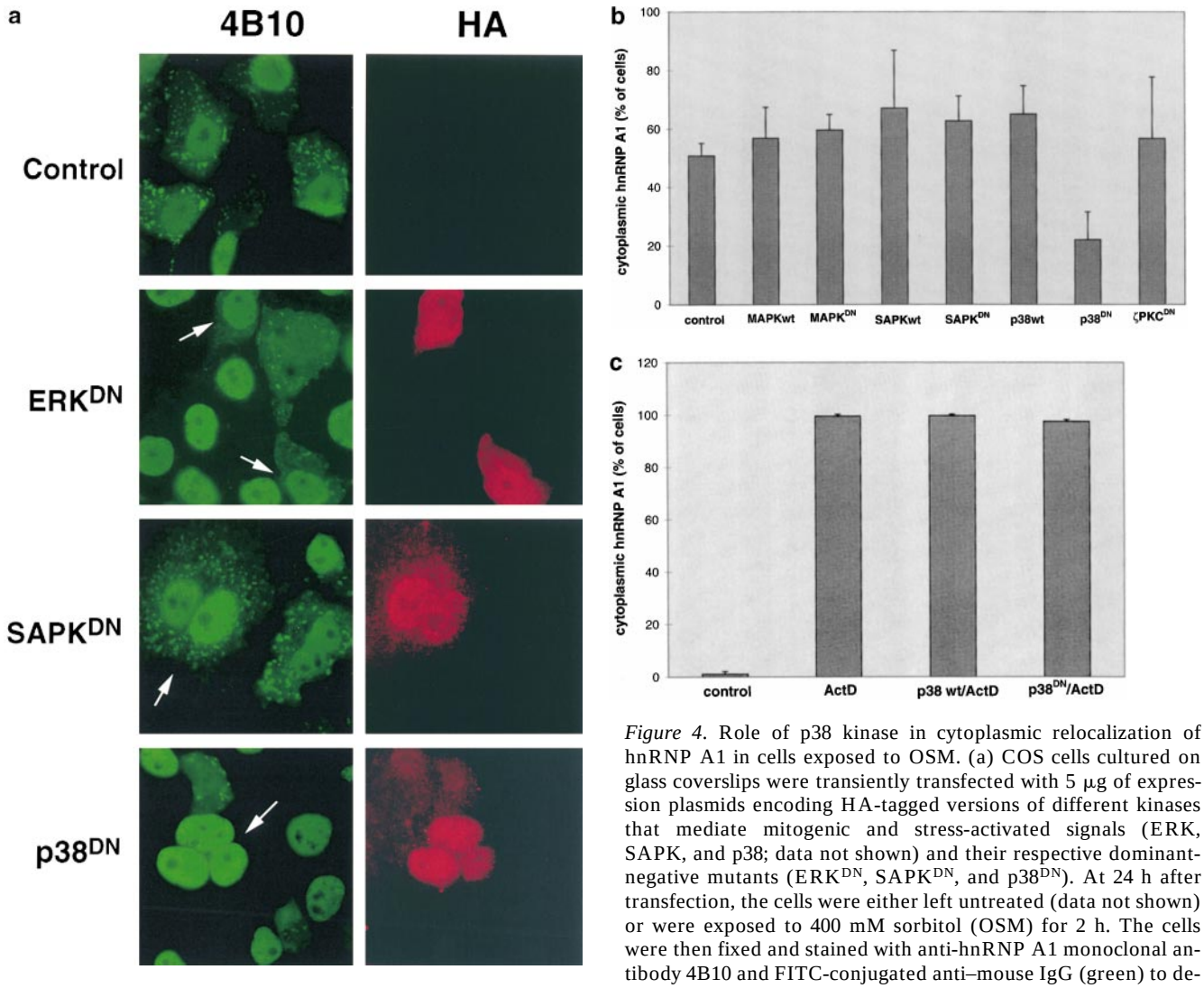

Figure 4. Role of p38 kinase in cytoplasmic relocalization of hnRNP A1 in cells exposed to OSM. (a) COS cells cultured on glass coverslips were transiently transfected with 5 μg of expression plasmids encoding HA-tagged versions of different kinases that mediate mitogenic and stress-activated signals (ERK, SAPK, and p38; data not shown) and their respective dominant-negative mutants (ERK^{DN}, $SAPK^{DN}$, and $p38^{DN}$). At 24 h after transfection, the cells were either left untreated (data not shown) or were exposed to 400 mM sorbitol (OSM) for 2 h. The cells were then fixed and stained with anti-hnRNP A1 monoclonal antibody 4B10 and FITC-conjugated anti–mouse IgG (green) to detect endogenous hnRNP A1 protein, and with rabbit polyclonal anti-HA antibody and rhodamine-conjugated anti–rabbit IgG (red) to detect transiently expressed kinases. Essentially identical results were obtained in several independent experiments. (b) Histogram showing the percentage of cells displaying cytoplasmic hnRNP A1 after transfection of the respective HA-tagged kinase followed by exposure to OSM. (c) Histogram showing the percentage of cells displaying cytoplasmic accumulation of hnRNP A1 after transfection with 5 μg of the respective HA-tagged kinase followed by actinomycin D treatment (5 μg/ml) for 3 h.

Activation of p38 Kinase and SAPK by OSM and UV

Although OSM and UV induce the cytoplasmic accumulation of hnRNP A1, these stimuli may trigger distinct pathways. We analyzed the activation of both p38 kinase and SAPK in cells exposed to different stress stimuli by Western blots using specific antibodies that recognize the phosphorylated, i.e., the activated form of these protein kinases. We compared the activation of p38 kinase and SAPK in cells exposed to either 400 mM sorbitol, 600 mM sorbitol or UV-C. In agreement with previous results (reviewed in Canman and Kastan, 1996; Kyriakis and Avruch, 1996, Karin, 1998), we found that both p38 kinase and SAPK get activated by OSM and UV-C irradiation, as seen by the appearance of their respective phosphorylated forms following different stress stimuli (Fig. 5). However, maximum activation of p38 kinase was obtained with 600 mM sorbitol, which also has the strongest relocalization effect on hnRNP A1. At the same time, 600 mM sorbitol also induces the highest activation of SAPK. However, the fact that a dominant-negative form of p38 kinase, but not a dominant-negative form of SAPK, blocks the cytoplasmic accumulation of hnRNP A1 induced by OSM, clearly demonstrates that the effect observed is exerted through the p38 kinase pathway (Fig. 4). It remains, however, possible that the cytoplasmic accumulation of hnRNP A1 induced by UV-C may involve the SAPK pathway.

Activation of the $MKK_{3/6}$-p38 Pathway Is Sufficient to Promote the Cytoplasmic Accumulation of hnRNP A1

Collectively, the above results suggest that the cytoplasmic accumulation of hnRNP A1 provoked by cell stress requires a functional p38 kinase. To test whether p38 activation is not only required, but also sufficient to promote hnRNP A1 cytoplasmic accumulation, COS cells were

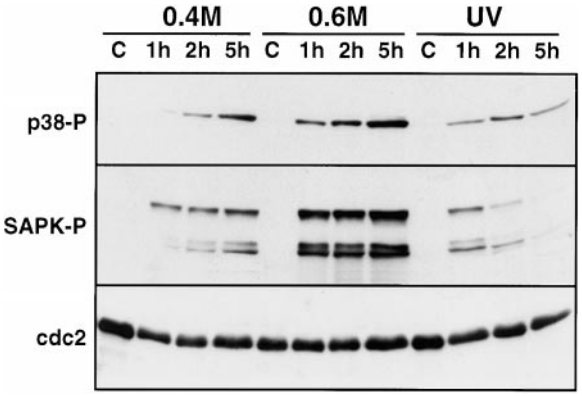

Figure 5. OSM and UV irradiation induce p38 kinase activation. NIH-3T3 cells were either left untreated or exposed to 0.4 M, 0.6 M sorbitol or UV-C light (UV) for the indicated times. Total protein extracts were analyzed by Western blot using an anti–phospho-p38 kinase antibody. The same blot was subsequently incubated with an anti phospho-SAPK antibody, followed by a cdc2 antibody as loading control.

transfected with a myc-tagged version of a constitutively active mutant of $MKK_{3/6}$ ($MKK_{3/6}^{DD}$, Cuenda et al., 1996) either alone or in combination with plasmids encoding HA-tagged versions of wild-type or dominant-negative mutants of p38 kinase. Subsequently, the localization of endogenous hnRNP A1 in the absence of any other stimulus was determined by double-label immunofluorescence. Transfection conditions were optimized to achieve simultaneous expression of the myc and HA epitope-tagged kinases in 75–85% of the transfected cells as judged by immunofluorescence microscopy (not shown). Expression of the constitutively active mutant $MKK_{3/6}^{DD}$ alone was not sufficient to induce cytoplasmic accumulation of endogenous hnRNP A1. However, cotransfection of $MKK_{3/6}^{DD}$ with p38 wild-type resulted in cytoplasmic accumulation of endogenous hnRNP A1 in >80% of the cells that expressed the tagged $MKK_{3/6}^{DD}$ (Fig. 6, a and b). This cytoplasmic accumulation was not observed when $MKK_{3/6}^{DD}$ was cotransfected with the dominant-negative mutant of the p38 kinase. In addition, a wild-type version of $MKK_{3/6}$ in combination with the wild-type p38 kinase induced cytoplasmic hnRNP A1 in <15% of the transfected cells, and hnRNP A1 was not observed in the cytoplasm of cells transfected with a wild-type version of $MKK_{3/6}$ alone (Fig. 6 b). Moreover, this effect was specific for hnRNP A1, since no changes in the subcellular distribution of either hnRNP U or hnRNP C were observed (not shown). Because of the technical difficulty in simultaneously detecting endogenous hnRNP A1 together with both cotransfected kinases (myc-tagged $MKK_{3/6}$ and HA-tagged p38), we also analyzed the localization of endogenous hnRNP A1 and of the HA-tagged p38 in parallel experiments. Virtually identical results were obtained (not shown), confirming that both tagged kinase constructs are expressed in the same cells.

We conclude from these experiments that activation of the $MKK_{3/6}$-p38 pathway is necessary and sufficient to induce the cytoplasmic accumulation of hnRNP A1 in response to cellular stress.

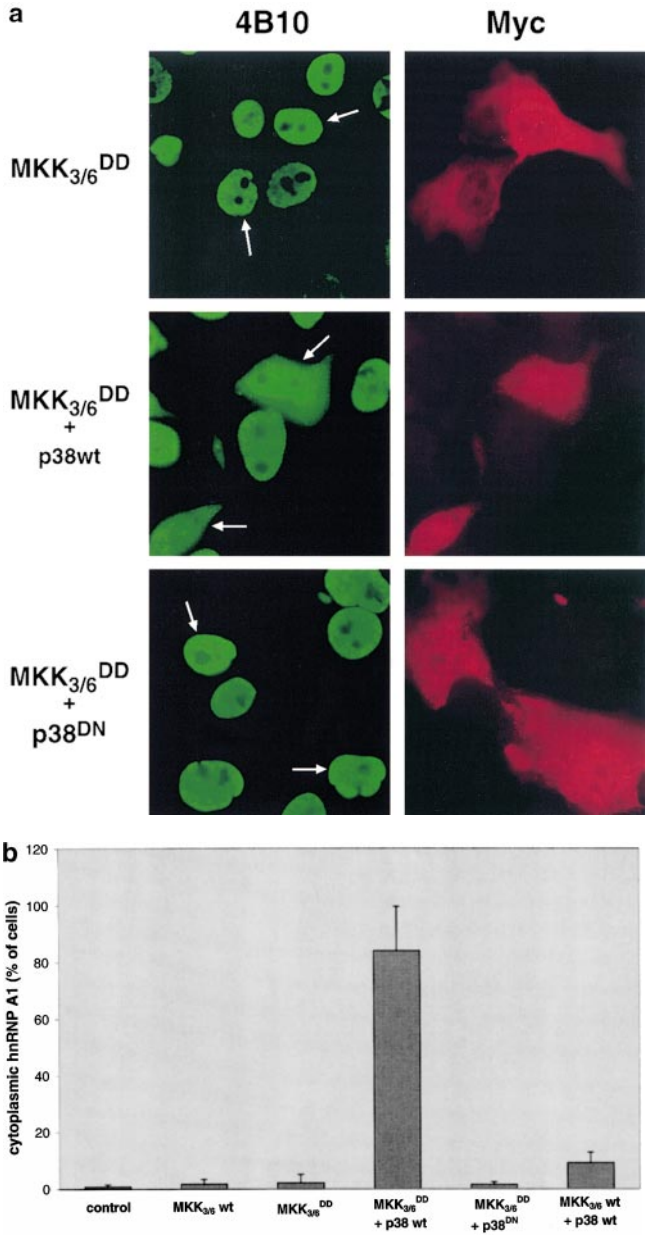

Figure 6. Activation of the $MKK_{3/6}$-p38 pathway is sufficient to promote cytoplasmic accumulation of hnRNP A1. (a) COS cells cultured on glass coverslips were transiently transfected with 5 μg of expression plasmids encoding myc-tagged versions of the $MKK_{3/6}$ kinase (data not shown) or its permanently active mutant ($MKK_{3/6}^{DD}$), in conjunction with 5 μg of expression plasmids encoding HA-tagged versions of wild-type p38 kinase or its dominant-negative mutant ($p38^{DN}$). The cells were then fixed and stained with the mouse monoclonal antibody 4B10 and FITC-conjugated anti–mouse IgG (green) to detect endogenous hnRNP A1 protein, and with the rabbit polyclonal anti-myc antibody and rhodamine-conjugated anti–rabbit IgG (red) to detect the transiently expressed $MKK_{3/6}^{DD}$. (b) Histogram showing the percentage of cells displaying cytoplasmic hnRNP A1 accumulation after transfection as described in a.

Changes in Alternative Splicing Regulation after Activation of the $MKK_{3/6}$-p38 Pathway

Alternative splicing is a widespread mechanism for the

spatial or temporal control of gene expression. hnRNP A1 and other closely related hnRNP A/B proteins have a concentration-dependent effect on the selection of alternative 5′ splice sites both in vitro and in vivo (Mayeda and Krainer, 1992; Cáceres et al., 1994; Yang et al., 1994). The amount of hnRNP A1 relative to that of the splicing factor SF2/ASF determines the use of alternative 5′ splice sites, such that when hnRNP A1 is in excess, distal 5′ splice sites are usually selected, and when SF2/ASF is in excess, proximal 5′ splice sites are favored (Mayeda and Krainer, 1992; Cáceres et al., 1994; Yang et al., 1994). Because SF2/ASF cytoplasmic levels did not increase appreciably in stress-activated cells (Fig. 2 and Table I), we reasoned that the stress-induced increase in the cytoplasmic levels of hnRNP A1, and the concomitant decrease in nuclear hnRNP A1 abundance, should favor the selection of proximal 5′ splice sites. To test this hypothesis, COS cells were transfected with a reporter adenovirus E1A gene and the cells were either left untreated or exposed to OSM (DME + 600 mM sorbitol) for 4 h. In cells exposed to 0.6 M sorbitol, the use of the most distal 5′ splice site (which gives rise to the 9S isoform), was reproducibly inhibited (as shown by a reduction of 9S from 45% in the control to 24% after OSM; Fig. 7). Similar results have been obtained in 3T3 cells (data not shown).

We have shown that cotransfection of $MKK_{3/6}^{DD}$ and wild-type p38 elicits the biological response, i.e., hnRNP A1 cytoplasmic accumulation, in the absence of an upstream signal (i.e., OSM, Fig. 6). To further confirm that the observed change in the alternative splicing pattern of the E1A reporter after OSM was mediated by the activation of the $MKK_{3/6}$-p38 kinase cascade, COS cells were transfected with expression plasmids encoding myc-tagged versions of the $MKK_{3/6}$ kinase or its permanently active mutant ($MKK_{3/6}^{DD}$), in conjunction with HA-tagged versions of wild-type p38 kinase or its dominant-negative mutant ($p38^{DN}$). Indeed, the use of the most distal E1A alternative 5′ splice site (9S isoform) was reproducibly inhibited in cells cotransfected with $MKK_{3/6}^{DD}$ and wild-type p38, but not in cells transfected with either kinase alone (Fig. 7). In contrast, distal splice site inhibition was not observed when the E1A splicing reporter was cotransfected with $MKK_{3/6}^{DD}$ and $p38^{DN}$, or with wild-type $MKK_{3/6}$ either alone or in combination with wild-type p38 (Fig. 7). Expression of p38 wild-type or $MKK_{3/6}^{DD}$ alone resulted in an increased level of the 9S transcript, probably due to nonspecific events. Thus, there is a strong correlation between the activation of the $MKK_{3/6}$-p38 kinase cascade (which induces cytoplasmic accumulation of hnRNP A1), and inhibition of the 9S distal splice site of E1A. Although the decreased levels of 9S mRNA upon decrease of nuclear hnRNP A1 are consistent with the known concentration-dependent effects of hnRNP A1 on alternative 5′ splice-site selection, we cannot rule out the possibility that the accumulation of hnRNP A1 in the cytoplasm leads to differential stabilization of the different alternatively spliced isoforms.

In conclusion, these experiments strongly suggest that stress stimuli trigger changes in the alternative splicing of cellular genes by decreasing the nuclear ratio of hnRNP A1 to SF2/ASF through the modulation of hnRNP A1 nucleo-cytoplasmic transport.

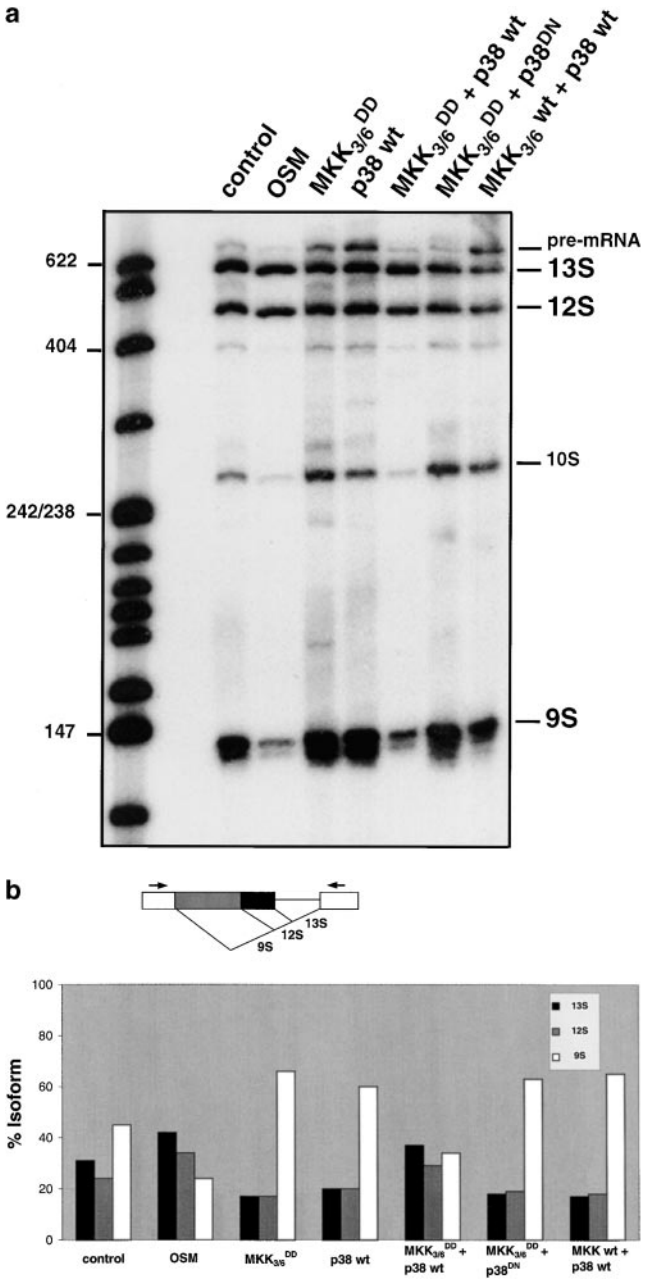

Figure 7. Activation of the $MKK_{3/6}$-p38 pathway and the concomitant reduction of the level of nuclear hnRNP A1 correlates with changes in alternative splicing. COS cells were transiently transfected with 6 μg of plasmid encoding the E1A splicing reporter minigene alone or together with 7 μg of expression plasmids encoding myc-tagged versions of the $MKK_{3/6}$ kinase, or its permanently active mutant ($MKK_{3/6}^{DD}$), in conjunction with 7 μg of expression plasmids encoding HA-tagged versions of wild-type p38 kinase or its dominant-negative mutant ($p38^{DN}$). At 24 h after transfection cells were either left untreated or exposed to 600 mM sorbitol for 4 h. Total RNA was then isolated and the alternative splicing pattern of the E1A transcripts was determined by RT-PCR. The relative levels of 13S, 12S, and 9S mRNAs were quantitated as described (Cáceres et al., 1994; Screaton et al., 1995). The E1A transcript isoforms are shown schematically. Essentially identical results were obtained in three independent experiments.

Discussion

We show here for the first time that a signal transduction pathway can control alternative splice site selection in vivo by regulating the cellular localization of pre-mRNA splicing factors. We have shown that the bidirectional transport of hnRNP A1 between the nucleus and the cytoplasm is not a constitutive process. Instead, it is subject to modulation by extracellular signals, which trigger rapid changes in the distribution of the protein between these cellular compartments. The relocalization of hnRNP A1 to the cytoplasm in stress-activated cells is mediated by the $MKK_{3/6}$-p38 pathway, which activation is both required and sufficient for hnRNP A1 cytoplasmic accumulation. At the same time, OSM leads to phosphorylation of hnRNP A1 with kinetics that parallel the localization of hnRNP A1. Although the role of protein phosphorylation in splicing is unclear, recent observations suggest that phosphorylation modulates protein–protein interactions within the spliceosome and controls the subnuclear distribution of SR proteins in interphase and mitosis (Misteli, 1999). In addition, phosphorylation of splicing regulators may be a way of regulating the subcellular distribution of antagonistic factors, and the resulting changes in the ratios of these factors in the nucleus may in turn affect splicing activity. This proposed mechanism represents a novel way of regulating alternative splice-site selection.

hnRNP A1 is directly phosphorylated in vitro by PKA, casein kinase II (CKII) and ζPKC (Cobianchi et al., 1993; Municio et al., 1995), but is not a direct target of p38 kinase (not shown). Phosphorylation by PKA significantly alters the properties of hnRNP A1, suppressing its strand annealing ability with no effect on its nucleic acid binding capacity (Cobianchi et al., 1993). We have shown that stress signals induced the cytoplasmic accumulation of hnRNP A1, concomitant with an increase in its phosphorylation. Although the expression of a permanently active mutant of ζPKC promotes the cytoplasmic accumulation of hnRNP A1 in proliferating cells (Municio et al., 1995), transfection of a kinase-inactive dominant-negative mutant of ζPKC had no effect on the cytoplasmic accumulation of hnRNP A1 in response to stress (not shown). Regarding the potential role of PKA in this pathway, exposure of cells to dibutyryl cAMP or cholera toxin, which potently activate PKA in vivo, did not induce the cytoplasmic accumulation of hnRNP A1 (not shown). Therefore, neither ζPKC nor PKA kinases appear to play a role during the stress-induced subcellular redistribution of hnRNP A1.

The mechanism whereby p38 kinase regulates hnRNP A1 cytoplasmic accumulation is presently unknown. The large increase in phosphorylation of hnRNP A1 upon osmotic shock may be part of the signal transduction pathway that leads to its altered partitioning between the nucleus and the cytoplasm. Alternatively, phosphorylation of hnRNP A1 may be a consequence of its accumulation and the cytoplasm, where it may be exposed to a kinase with which it does not normally interact. Although hnRNP A1 it is not a direct substrate for p38, it is known that p38 kinase activates other kinases, such as MAPKAP kinase-2 and -3 (McLaughlin et al., 1996), which in turn may directly phosphorylate hnRNP A1, altering its shuttling properties. MAPKAP kinase-2 and p38 form a complex in the nucleus of unstimulated cells, where p38 kinase is inactive. In stimulated cells, phosphorylation of MAPKAP kinase-2 by p38 leads to a conformational change in MAPKAP kinase-2, which results in the masking of a nuclear localization signal (and perhaps the exposure of a nuclear export signal) that causes the relocalization of the p38-MAPKAP kinase-2 complex to the cytoplasm. Thus, MAPKAP kinase-2 acts both as an effector of p38 by phosphorylating substrates and also as a determinant of the cellular localization of p38 (Ben-Levy et al., 1998; Engel et al., 1998). This cytoplasmic translocation event may allow p38 and MAPKAP kinase-2 to phosphorylate cytoplasmic targets.

Alternatively, the effect of p38 on hnRNP A1 cytoplasmic accumulation could be indirect, through modulation of the activity of proteins involved in the nucleo-cytoplasmic transport of hnRNP A1. In this regard, a novel receptor-mediated nuclear import pathway, which is distinct from the classical importin-mediated NLS pathway, has been described. This new pathway involves a novel 90-kD protein, transportin, which physically interacts with, and serves to facilitate the nuclear import of, hnRNP A1 (Pollard et al., 1996). An attractive possibility is that the p38 kinase cascade, through the phosphorylation of hnRNP A1, modulates its interaction with transportin, resulting in a decreased rate of nuclear import. However, it is not clear whether the altered subcellular distribution of hnRNP A1 following stress signaling is the result of inhibition of nuclear import or of an increased rate of nuclear export.

For the majority of nuclear events regulated by extracellular stimuli, the targets of the signaling pathways are transcription factors. However, other nuclear activities, such as mRNA processing, are also potential targets for these signaling pathways. One example of a growth factor–regulated splicing event is the processing of the PTP-1B pre-mRNA, which gives rise to a unique mRNA isoform via alternative splicing upon stimulation of quiescent cells with a variety of growth factors (Shiffrin and Neel, 1993). The nuclear cap-binding complex (CBC) has also been identified as a target for growth factor receptor-coupled signal transduction (Wilson et al., 1999). The ability of growth factors to stimulate the capped RNA-binding activity correlates with growth factor stimulation of splicing activity. The CBC receives inputs from multiple pathways, since it is activated by the Ras-Raf-MEK pathway and also by stress-activated signaling pathways. However, the subcellular localization of both CBC protein subunits (CBP80 and CBP20) is not affected by growth factor stimulation (Wilson et al., 1999).

The results reported here establish a new link between signaling cascades that are central to the control of important cell functions, such as the stress response, and the control of gene expression by mRNA processing. We showed that stress signals induce the cytoplasmic accumulation of hnRNP A1, concomitantly with changes in the alternative splicing of a cotransfected reporter. The cytoplasmic accumulation of hnRNP A1 after OSM presumably causes an altered ratio of the antagonistic alternative splicing factors SF2/ASF and hnRNP A1 in the cell nucleus. Indeed, the alternative splicing pattern of the E1A reporter mini-gene shows a decrease of the relative level of 9S transcripts in cells exposed to stress signals compared with untreated cells, as would be expected for splic-

ing in the presence of reduced levels of hnRNP A1. In addition to the effects attributable to hnRNP A1, it is possible that other splicing factors are also altered by osmotic stress and p38 activation, contributing to the overall effect on alternative splicing. For example, we have shown that hnRNP B1 also accumulates in the cytoplasm under these conditions (Table I), and this protein is structurally and functionally closely related to hnRNP A1 (Mayeda et al., 1994).

The present findings demonstrate that changes mediated by signaling can modulate alternative splicing by altering the localization of known splicing regulators. This regulatory mechanism may turn out to be a general one, by which different signaling pathways may affect the nuclear ratio of specific antagonistic splicing regulators, thereby modulating alternative splicing regulation of particular pre-mRNAs.

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