

# Hsp27 Enhances Recovery of Splicing as well as Rephosphorylation of SRp38 after Heat Shock

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A heat stress causes a rapid inhibition of splicing. Exogenous expression of Hsp27 did not prevent that inhibition but enhanced the recovery of splicing afterward. Another small heat shock protein,  $\alpha$ B-crystallin, had no effect. Hsp27, but not  $\alpha$ B-crystallin, also hastened rephosphorylation of SRp38—dephosphorylated a potent inhibitor of splicing—after a heat shock, although it did not prevent dephosphorylation by a heat shock. The effect of Hsp27 on rephosphorylation of SRp38 required phosphorylatable Hsp27. A Hsp90 client protein was required for the effect of Hsp27 on recovery of splicing and on rephosphorylation of SRp38. Raising the Hsp70 level by either a pre-heat shock or by exogenous expression had no effect on either dephosphorylation of SRp38 during heat shock or rephosphorylation after heat shock. The phosphatase inhibitor calyculin A prevented dephosphorylation of SRp38 during a heat shock and caused complete rephosphorylation of SRp38 after a heat shock, indicating that cells recovering from a heat shock are not deficient in kinase activity. Together our data show that the activity of Hsp27 in restoring splicing is not due to a general thermoprotective effect of Hsp27, but that Hsp27 is an active participant in the (de)phosphorylation cascade controlling the activity of the splicing regulator SRp38.

## INTRODUCTION

In response to an increase in ambient temperature cells shift to the production of protective proteins, among which are the small heat shock proteins (sHsps). sHsps are characterized by a conserved C-terminal  $\alpha$ -crystallin domain and a variable N-terminal domain. The human sHsp family has 10 members (Kappé *et al.*, 2003) of which Hsp27 and  $\alpha$ B-crystallin are the best known (Van Montfort *et al.*, 2001; Arrigo and Muller, 2002). Hsp27 and  $\alpha$ B-crystallin are constitutively expressed in a number of tissues but also up-regulated upon heat shock. High levels of these two proteins are often found in degenerative diseases and in tumors (Krueger-Naug *et al.*, 2002). The *in vivo* function of the sHsps is largely unknown but is commonly thought to be based on chaperoning. sHsps can associate with the cytoskeleton and might protect this structure from stress (Quinlan, 2002). Hsp27 has also been implicated in the ubiquitin-proteasome pathway (Parcellier *et al.*, 2003), in Akt kinase signaling (Konishi *et al.*, 1997; Rane *et al.*, 2001; Rane *et al.*, 2003), and in apoptosis (reviewed in Garrido *et al.*, 2001). The importance of the sHsps is highlighted by their recently discovered role in ageing: the transcription of sHsps genes is increased in long-lived *Caenorhabditis elegans* and *Drosophila* mutants and RNAi against sHsps mRNAs results in a decreased lifespan

of these animals (Hsu *et al.*, 2003; Murphy *et al.*, 2003; Morley and Morimoto, 2004).

A heat stress causes inhibition of macromolecular synthesis: transcription of non-Hsp genes, splicing, and translation initiation is blocked. The repression of splicing is due to the rapid dephosphorylation of SRp38, which then sequesters U1 small nuclear ribonucleoprotein (snRNP; Shin *et al.*, 2004). In addition, the U4/U5/U6 snRNP complex dissociates (Bond, 1988; Utans *et al.*, 1992; Bracken and Bond, 1999; Bond and James, 2000). Finally, splicing factors such as SF2/ASF are sequestered in nuclear stress bodies (Chiodi *et al.*, 2004; Metz *et al.*, 2004). The block in splicing is not complete as for example the Hsp90 $\alpha$  and Hsp90 $\beta$  transcripts are spliced in heat-shocked human fibroblasts (Jolly *et al.*, 1999). Similarly, in heat-shocked HeLa cells the splicing of the Hsp27 mRNA is only partially inhibited (Bond, 1988).

Cells that have accumulated Hsps as a result of stress become thermotolerant, *i.e.*, more resistant to subsequent stress. Splicing is less inhibited by a heat stress in thermotolerant cells (Yost and Lindquist, 1986; Bond, 1988; Yost and Lindquist, 1991; Corell and Gross, 1992; Bracken and Bond, 1999). The exact mechanism of the splicing thermotolerance is unclear. In yeast, Hsp104 and Hsp70 act to repair splicing after heat shock both *in vitro* and *in vivo* (Yost and Lindquist, 1991; Bracken and Bond, 1999), although surprisingly these proteins do not appear to be involved in establishing thermotolerance of splicing (Bracken and Bond, 1999). In yeast Hsp70 associates with the U4/U5/U6 snRNPs in thermotolerant cells (Bracken and Bond, 1999), whereas in mammalian cells a “heat reversal factor” has been identified (Delannoy and Caruthers, 1991). Because this fraction contained proteins in the 70-kDa range, it was suggested, although not proven, that Hsp70 is involved. Later experiments showed a novel 65-kDa protein to be associated

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with snRNPs in thermotolerant HeLa cells (Bond and James, 2000).

Because exogenous expression  $\alpha$ B-crystallin and Hsp27 can also confer thermotolerance (Aoyama *et al.*, 1993; Lavoie *et al.*, 1993; Carper *et al.*, 1997), we have investigated whether either one or both of these two sHsps prevent the inactivation of splicing by a heat shock or hasten the recovery of splicing after a heat shock. We show here that expression of Hsp27, but not of  $\alpha$ B-crystallin, does indeed increase the extent of splicing during recovery from a heat shock. We also show that in the presence of Hsp27 the splicing inhibitor SRp38 is rephosphorylated, and thus inactivated, more rapidly during recovery from a heat shock. Furthermore, we demonstrate that the ability of Hsp27 to affect the phosphorylation state of SRp38 depends on its own phosphorylation state. We also show that pretreatment with geldanamycin (GA), an inhibitor of Hsp90, blocks the recovery of splicing as well as the rephosphorylation of SRp38 after heat shock, indicating that an Hsp90 client protein is required in this process. Finally, we show that exogenous expression of Hsp70 does not affect the phosphorylation state of SRp38. Together these results show that Hsp27 is an active player in the kinase cascade that regulates the splicing activity after a heat shock.

## MATERIALS AND METHODS

### Cell Culture

T-REx  $\alpha$ B-crystallin HeLa cells, T-REx Hsp27 HeLa cells, Flp-In T-REx HEK-293 Hsp27 or YFP-Hsp70 HEK-293 cells (HeLa cells or HEK-293 cells stably transfected with the tetracycline repressor [Invitrogen, Breda, The Netherlands] and expression constructs for human  $\alpha$ B-crystallin, hamster Hsp27, Hsp27 S15A, S90A, and S15A/S90A mutants or YFP-Hsp70 under the control of the tetracycline repressor) were cultured in minimum Eagle's medium (EMEM; BioWhittaker, Verviers, Belgium) with glutamax (Invitrogen), 10% fetal calf serum, penicillin, and streptomycin. Blasticydin (Invitrogen) was added during maintenance of the cell lines.

### Transfection, Heat Shock, and Induction of sHsp Expression

Twenty-four hours before transfection,  $2.5 \times 10^5$  T-REx cells were plated per well in a six-well plate. Cells were transfected with 1  $\mu$ g of DNA using 6  $\mu$ l of DDAB: DOPE liposomes (0.5:1 M ratio) prepared as described (Campbell, 1995). As a control for the transfection efficiency, 0.1  $\mu$ g of a CMV- $\beta$ -galactosidase construct was cotransfected. Where indicated, the expression of  $\alpha$ B-crystallin, (mutant) Hsp27, or YFP-Hsp70 was induced by adding 1  $\mu$ g/ml doxycycline 24 h after transfection. Forty-eight hours after transfection, cells were heat shocked for 1 h at 45°C. Cells were harvested during recovery at 37°C at the times indicated.

### RNA Isolation and Real Time Quantitative RT-PCR

RNA isolation, DNase treatment of the RNA, and the reverse transcriptase reaction were performed as described (Doerwald *et al.*, 2003). cDNA, 2.5  $\mu$ l, was used per SYBR green PCR (Eurogentec, Luik, Belgium). Parallel PCR reactions were performed with 0.125  $\mu$ g of the DNase-treated RNA to test for DNA contamination. The PCRs were performed on a Gene Amp 5700 Sequence Detection System instrument (PE Applied Biosystems, Foster City, CA) using the thermal cycling conditions of 10 min at 95°C, followed by 40 cycles of amplification of 15 s at 95°C and 1 min at 60°C.

Primers used for the PCR were as follows:  $\gamma$ D-crystallin primers across second intron: 5'-CCGACCACCAGCAGTGGAT-3' and 5'-GCCTCTGTAGTCTCCTCTCA-3';  $\gamma$ D-crystallin primers located in exon 2 (total mRNA from pHsp- $\gamma$ D-crystallin): 5'-CCTACTTGAGCCGCTGCAAC-3' and 5'-ACTGCTGGTGGTCGGCATAG-3';  $\beta$ -globin primers across second intron: 5'-TGCACGTGGATCTGAGAAGT-3' and 5'-TTTCTGATAGGCAGCCTGCAC-3';  $\beta$ -globin primers within exon 2 (total mRNA from pHsp- $\beta$ -globin): 5'-CCTTAGTGATGGCCTGGCTCAC-3' and 5'-CCTGAAGTCTCAGGATCCACG-3'; and human  $\beta$ -actin mRNA: 5'-CCATCATGAAGTGTGACGTGG-3' and 5'-TCTGCATCTGTGCGCAAT-3'.

All primers were designed with the Primer Express program (PE Applied Biosystems). Gene Amp 5700 SDS software (PE Applied Biosystems) was used to quantify the signals. The melting curve of the PCR products indicated a single product in all cases. The melting curves obtained for the  $\beta$ -globin and  $\gamma$ D-crystallin products were identical to those obtained with cloned DNA. Linearity of the QT-PCR was confirmed by using serial dilutions of cDNA as

substrate. The efficiency of the primers was calibrated using DNA as well as RNA isolated from cells transfected with CMV- $\beta$ -globin or SV40- $\gamma$ D-crystallin genes cultured at 37°C, making the assumption that under those conditions essentially all of the RNA is spliced. The signals obtained from the  $\gamma$ D-crystallin or  $\beta$ -globin transcripts were normalized to the signal obtained from the  $\beta$ -actin mRNA and corrected for primer efficiency as well as transfection efficiency. The latter was determined from the  $\beta$ -galactosidase activity, which was measured as previously described (Doerwald *et al.*, 2003). In no case was significant DNA contamination of the RNA detected.

### SDS-PAGE and Western Blot Analysis

Cells were harvested by adding 50  $\mu$ l of reporter lysis mix (25 mM bicine, pH 7.5, 0.05% Tween-20, and 0.05% Tween-80) per well and scraping. The material from two wells was then pooled. Then 30  $\mu$ l of SDS sample buffer (20% glycerol, 4% SDS, 200 mM dithiothreitol, 200 mM Tris-HCl, pH 6.8, and bromophenol blue) was added to each sample. Ten microliters of each sample was separated by PAGE and Western blotted. The Western blots were stained for SRp38 (polyclonal anti-SRp38 at a dilution of 1:10,000), Hsp70 (monoclonal anti-Hsp70 at dilution of 1:1000; Stressgen, Sanbio BV, Uden, The Netherlands), hamster Hsp27 (polyclonal anti-hamster Hsp27 at dilution of 1:5000),  $\alpha$ B-crystallin (monoclonal anti- $\alpha$ B-crystallin at dilution 1:500), or human Hsp27 (monoclonal anti-human Hsp27 at dilution of 1:500; Stressgen, Sanbio BV, Uden, The Netherlands) using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Perbio Science Nederland BV, Etten-Leur, The Netherlands). The density of the bands was quantified using LabWorks image acquisition and analysis software (Ultra-Violet Products, Cambridge, United Kingdom).

### Inhibitors

Calyculin A was used at 0.1  $\mu$ M and added 1 h before harvesting. 2-Aminopurine was used at a concentration of 10 mM. GA was added to the cells to a final concentration of 10  $\mu$ M. An equivalent amount of the solvent was added to control cells in all cases. All inhibitors were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands).

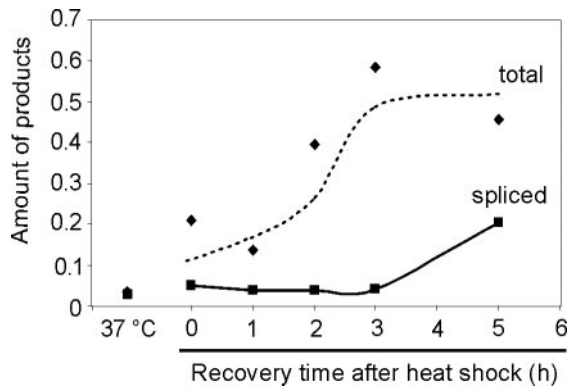
### Constructs

For pHsp- $\gamma$ D-crystallin and pSV- $\gamma$ D-crystallin, the genomic human  $\gamma$ D fragment was excised from pSV- $\gamma$ D (Brakenhoff *et al.*, 1994) with *Nco*I and *Eco*RI (blunted) and inserted *Nco*I/*Hpa*I in pHsp-Cap-Luc (Doerwald *et al.*, 2003) or pGL3 control (Promega, Leiden, The Netherlands), thus replacing the luciferase coding region by the  $\gamma$ D-crystallin gene. For pHsp- $\beta$ -globin, the  $\beta$ -globin gene was excised *Hind*III/*Xba*I from the pcDNA3-wt $\beta$  clone (Lykke-Andersen *et al.*, 2000; kindly donated by Dr. Steitz) and inserted *Hind*III/*Xba*I in pHsp-Cap-Luc, again replacing the luciferase coding region.

## RESULTS

### Hsp27, but Not $\alpha$ B-Crystallin, Enhances the Recovery of Splicing after a Heat Shock

To monitor splicing efficiency, we used constructs based on the human  $\gamma$ D-crystallin gene. The transcripts from this gene accumulate to a very high level in lens fiber cells and are presumably efficiently spliced. Indeed, when splicing of the (2166 nt long) second intron of the pSV40- $\gamma$ D-crystallin transcript was assayed at 37°C, no unspliced products were detected (unpublished data). To measure splicing efficiency of this intron during and after a heat shock, we used the Hsp70 heat shock promoter to drive transcription (pHsp- $\gamma$ D-crystallin). Using this promoter ensures that the transcription unit is actively transcribed during and after the heat shock. Furthermore, this promoter is poorly active before heat shock, and most of the spliced products detected have thus been processed during or after heat shock. Measuring the efficiency of splicing of transcripts of non-heat shock genes is difficult because of the high background of pre-existing spliced transcripts. The transcripts of pHsp- $\gamma$ D-crystallin showed a 10–20-fold increase in level after heat shock and reached a steady state level after ~3 h recovery. A marked increase in the number of spliced transcripts was seen between 3 and 5 h recovery (Figure 1). The time at which splicing recovers in our experiments is longer than previously reported (see for example, Corell and Gross, 1992; Bond and James, 2000). This could be due to a more severe heat stress. In addition, the measured rate of recovery



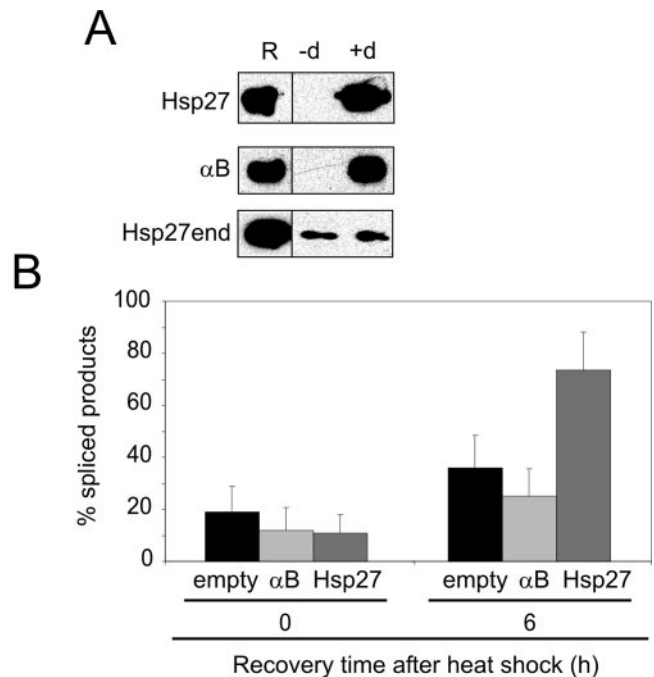
**Figure 1.** Effect of a heat shock on splicing efficiency. The accumulation of total (♦) and spliced (■) transcripts from pHsp- $\gamma$ D-crystallin was determined by real time quantitative RT-PCR before heat shock (37°C) and during recovery from a heat shock (pHsp- $\gamma$ D-crystallin), as described in *Materials and Methods*. The accumulation is expressed in arbitrary units.

is also dependent on the intron of which the splicing is assayed. We have also checked the efficiency of splicing of the large intron of the  $\beta$ -globin gene under our conditions and found only ~1% spliced transcripts (see also Figure 3). In contrast, ~65% of the small  $\gamma$ D-crystallin first intron was spliced out, and splicing of this intron is thus more efficient than that of the large second intron during recovery from a heat shock (unpublished data). Other studies have shown that splicing of at least some introns continues during and directly after heat shock (Bond, 1988; Jolly *et al.*, 1999).

The splicing of the second intron of the pHsp- $\gamma$ D-crystallin transcript after a heat shock was also monitored in cells overexpressing human  $\alpha$ B-crystallin or hamster Hsp27. For these experiments we used T-REx HeLa cells stably expressing these proteins from the CMV promoter placed under control of the Tet repressor (see Figure 2A; note that the antibody used to detect hamster Hsp27 does not cross-react with human Hsp27 and vice versa). The induced level of exogenous Hsp27 is about 10 times that of the endogenous Hsp27 level in control T-REx HeLa cell and about twice the endogenous level found in cells after 18 h of recovery from heat shock. The level of exogenous Hsp27 thus mimics that in thermotolerant cells. The sHsps did not affect the level of accumulation of the transcript from pHsp- $\gamma$ D-crystallin (unpublished data). Immediately after heat shock, the splicing efficiency was also not affected by the presence of either one of these two sHsps (Figure 2B). However, when cells were left to recover for 6 h at 37°C, more spliced products were present in cells expressing Hsp27 than in cells that contained  $\alpha$ B-crystallin or in control cells (Figure 2B). Similarly, the splicing of the second intron of the  $\beta$ -globin gene was enhanced about fourfold in the presence of Hsp27 (Figure 3). The extent of splicing of the small  $\gamma$ D-crystallin intron, which is already efficient in the absence of Hsp27, was not significantly increased by Hsp27 (unpublished data).

#### *The Effect of Hsp27 and $\alpha$ B-Crystallin on the Phosphorylation State of SRp38*

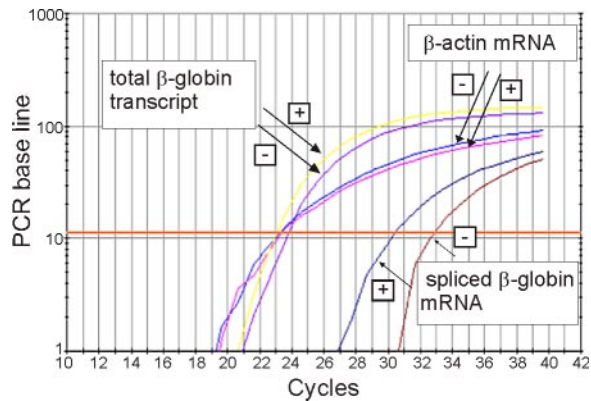
SRp38 plays a key role in the impairment of splicing during heat shock (Shin *et al.*, 2004): it is rapidly dephosphorylated during a heat shock and the dephosphorylated form (dSRp38) then sequesters U1 snRNP. At 37°C SRp38 is mainly found in its fully phosphorylated form (Figure 4A, 37°C) and the presence of Hsp27 had no effect on the phos-



**Figure 2.** Hsp27 enhances splicing of the second  $\gamma$ D-crystallin intron after heat shock. (A) Expression levels of Hsp27 or  $\alpha$ B-crystallin in T-REx HeLa cells before or after induction. T-REx HeLa expressing hamster Hsp27 or  $\alpha$ B-crystallin under the regulation of the Tet repressor (see *Materials and Methods*) were cultured without (-d) or for 24 h with doxycycline (+d). The lysate of  $\sim 3 \times 10^4$  cells was loaded on gel. For comparison, 25 ng of purified recombinant protein (lane marked R) was loaded on the same gel. Levels of hamster Hsp27 (Hsp27), endogenous Hsp27 (Hsp27end) or  $\alpha$ B-crystallin were determined by Western blotting. Note that the antibodies against Hsp27 are rodent or primate specific. (B) Splicing in heat-shocked cells expressing Hsp27 or  $\alpha$ B-crystallin. The relative amount of spliced transcripts of pHsp- $\gamma$ D-crystallin was measured directly after heat shock or after 6 h of recovery from a heat shock in T-REx HeLa cells expressing Hsp27 (dark gray columns) or  $\alpha$ B-crystallin (light gray columns) or in cells cultured without doxycycline (black columns). The bar indicates the SD of several independent experiments.

phorylation state of SRp38. Immediately after heat shock, SRp38 is mostly dephosphorylated as previously reported (Figure 4; Shin *et al.*, 2004). Hsp27 did not prevent this dephosphorylation of SRp38. During recovery from the heat shock, phosphorylation of SRp38 was only slowly restored. In the absence of exogenous Hsp27 little fully phosphorylated SRp38 was detected even 24 h after the heat shock. When exogenous Hsp27 was present, phosphorylation of SRp38 was restored more rapidly (Figure 4A). Quantitation of high-resolution gels such as shown in Figure 6 showed that ~30% of SRp38 is rephosphorylated after 6 h of recovery in the presence of exogenous Hsp27 against ~10% in its absence (note that the anti-SRp38 antibody recognizes dSRp38 better than SRp38, which leads to a visual underestimate of the level of rephosphorylation).

The effect of Hsp27 on the phosphorylation level of SRp38 could just be a manifestation of a general thermotolerance caused by the expression of a sHsp. Therefore, the same experiment was performed using cells expressing  $\alpha$ B-crystallin, which also confers thermotolerance (Aoyama *et al.*, 1993). As shown in Figure 4B, the state of phosphorylation of SRp38 after heat shock was not affected by the expression of  $\alpha$ B-crystallin: the same level of dephosphorylated SRp38

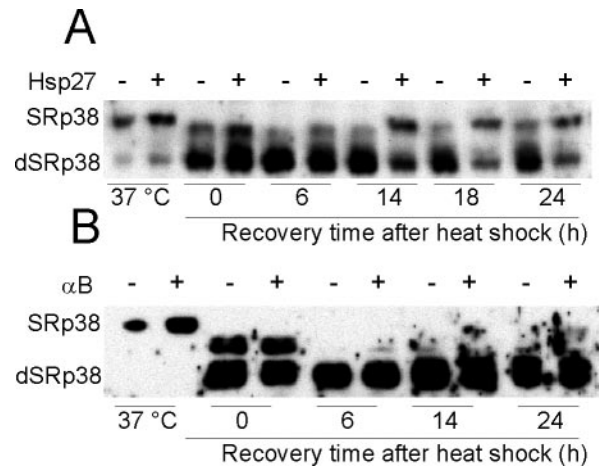


**Figure 3.** Hsp27 also enhances splicing of the second  $\beta$ -globin intron after heat shock. The results of the real time quantitative PCR experiments as displayed by the MyiQ System Software are shown. T-REx-Hsp27 HeLa cells were transfected with the pHsp- $\beta$ -globin construct, 24 h later the expression of Hsp27 was induced (+ Hsp27) or not (- Hsp27) and 48 h after transfection cells were heat shocked for 1 h at 45°C and allowed to recover for 6 h at 37°C. RNA was isolated, reversed transcribed and quantitated by QT-PCR as described in *Material and Methods*. The graph shows the amount of fluorescence obtained during the amplification cycle for reactions performed on an aliquot of the cDNA of RNA isolated from cells with or without exogenous Hsp27, as indicated by “-” or “+”, using primers directed against  $\beta$ -actin messenger, total  $\beta$ -globin transcript or spliced  $\beta$ -globin messenger, as indicated. Note that the amount of  $\beta$ -actin RNA, used to normalize the QT-PCR data (see *Materials and Methods*), and that of the total  $\beta$ -globin transcripts is the same irrespective of the presence of Hsp27, as expected. Only the amount of spliced  $\beta$ -globin RNA differs. Note also that to calculate the relative amount of spliced  $\beta$ -globin RNA the threefold lower efficiency of the primers for the spliced RNA needs to be taken into account (see also *Materials and Methods*). The calculated % spliced  $\beta$ -globin RNA in the absence of Hsp27 based on the data shown in 0.65%; in the presence of Hsp27 it is 2.23%. The threshold cycle ( $C_t$ ) is shown by the thicker orange horizontal line.

was found during recovery after a heat shock in the presence or absence of  $\alpha$ B-crystallin. Thus the effect of Hsp27 on the phosphorylation state of SRp38 is not due to a general thermotolerance conferred by Hsp27.

#### The Effect of the Phosphorylation State of Hsp27

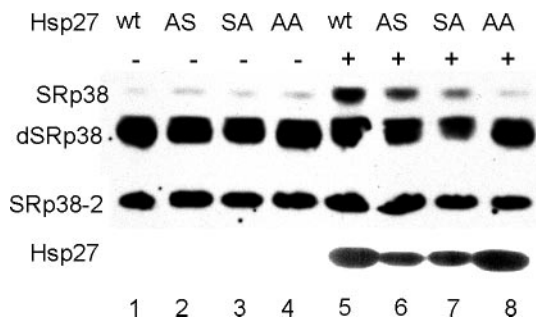
Hsp27 is phosphorylated upon stress (for review, see Gaestel, 2002; Kato *et al.*, 2002) and this phosphorylation is necessary for thermotolerance (Gabai and Sherman, 2002; Geum *et al.*, 2002). To determine whether the phosphorylation state of Hsp27 affects the rate of rephosphorylation of SRp38 during recovery after a heat shock, stable transfected cell lines with inducible expression of mutants mimicking non-phosphorylated forms of Hsp27 (Hsp27 S15A, S90A, and S15A/S90A) were used. Mutants which retain a single phosphorylation site (S15A and S90A) still enhanced the rephosphorylation of SRp38, although the amount of rephosphorylated SRp38 in the presence of Hsp27 S15A and S90A was less than in the presence of wild-type Hsp27 (Figure 5, lanes 5–7). However, the Hsp27 mutant in which both phosphorylation sites have been mutated (Hsp27 S15A/S90A) did not enhance the amount of phosphorylated SRp38 after 6 h of recovery from a heat shock (Figure 5, lane 8), suggesting that phosphorylation of Hsp27 is required for a faster rephosphorylation rate of SRp38 after heat shock.



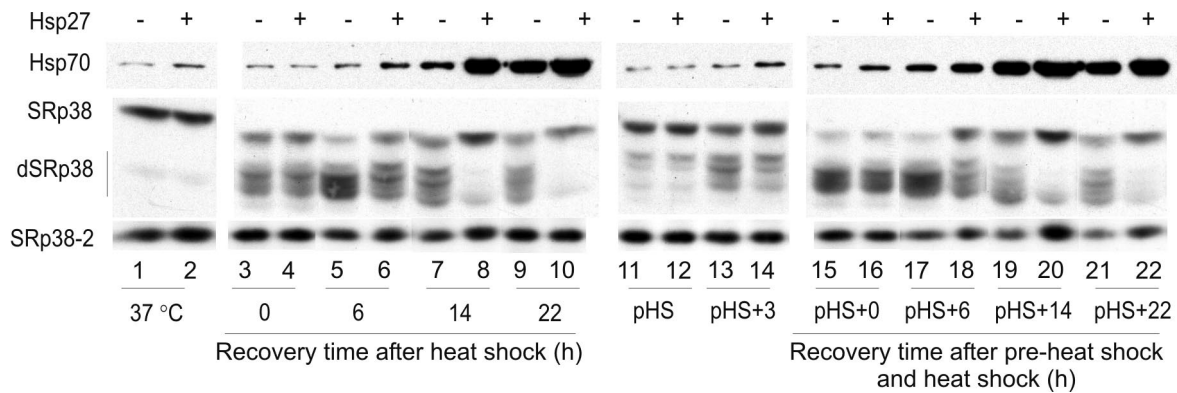
**Figure 4.** Effect of Hsp27 and  $\alpha$ B-crystallin on the rephosphorylation of SRp38 after a heat shock. (A) Western blot showing the different forms of SRp38 found after heat shock in the presence or absence of Hsp27. T-REx HeLa Hsp27 cells were cultured with (+ Hsp27) or without doxycycline (- Hsp27) and harvested either after culture at 37°C (lanes 37°C) or after a heat shock and recovery at the time indicated. SRp38 indicates the fully phosphorylated form and dSRp38 the (partially) dephosphorylated form. (B) Western blot showing the different forms of SRp38 found after heat shock and recovery in the presence or absence of  $\alpha$ B-crystallin. The experimental procedure was as described above except that T-REx HeLa  $\alpha$ B-crystallin cells were used. The various forms of SRp38 are indicated as above.

#### The Effect of Hsp27 on the Rephosphorylation of SRp38 Is Not Affected by the Presence of the Heat-Shock Proteins Made during a Mild Pre-Heat Shock, and the Phosphorylation State of SRp38 Is Not Influenced by Hsp70

The rephosphorylation of SRp38 after a heat shock is relatively slow, and during the recovery the full complement of heat shock proteins will be synthesized. Hence it is possible that Hsp27 just keeps cells in a competent state but that the



**Figure 5.** Effect of the phosphorylation state of Hsp27 on rephosphorylation of SRp38 after heat shock. T-REx HeLa cell lines expressing different mutants mimicking partially unphosphorylated Hsp27 were cultured with (+ Hsp27 or its mutants) or without (- Hsp27 or its mutants) doxycycline, heat shocked, and harvested 6 h after recovery at 37°C. Lanes 1 and 5: Hsp27 wild-type (Wt) cell line; lanes 2 and 6: Hsp27 S15A (AS) cell line; lanes 3 and 7: Hsp27 S90A (SA) cell line; lanes 4 and 8: Hsp27 S15/90A (AA) cell line. SRp38 indicates the fully phosphorylated form, dSRp38 the (partially) dephosphorylated form, whereas the bottom panel shows the alternative spliced form of SRp38 (SRp38-2). The bottom panel shows the induced expression level of wild-type or mutant hamster Hsp27.



**Figure 6.** A pre-heat shock does not alter the Hsp27 effect on SRp38 rephosphorylation. Western blots showing the Hsp70 levels and the distinct forms of SRp38 found in naïve and pre-heat-shocked cells before and after a heat shock. T-REx HeLa Hsp27 wild-type cells were either kept at 37°C (lanes 1 and 2), or pre-heat shocked at 45°C for 30 min (lanes 11 and 12) or pre-heat shocked, left to recover for 3 h (lanes 13 and 14) or pre-heat shocked, left to recover for 3 h, and then heat shocked at 45°C for 60 min. Where indicated, Hsp27 expression was induced 24 h before the heat shock. After the heat shock cells were either harvested immediately (lanes 15 and 16), or 6, 14, or 22 h after recovery (lanes 17–22). The same heat shock and recovery times were used for cells that were not pre-heat shocked (lanes 3–10). The various forms of SRp38 are indicated as in the legend to Figure 4. Note that in this experiment the samples were separated on a large gel, yielding better separation of the partially phosphorylated forms of SRp38.

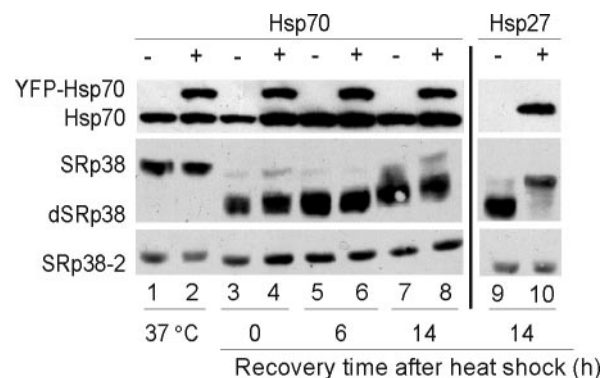
increase in rephosphorylation requires another, newly synthesized, heat shock protein. To test this, cells were subjected to a mild pre-heat shock and allowed to recover for 3 h, after which the standard heat shock was applied. As shown in Figure 6A (compare lanes 3 and 4 with lanes 11 and 12), SRp38 was as extensively dephosphorylated by a heat shock in pre-heat-shocked cells as it was in naïve cells. Furthermore, exogenous expression of Hsp27 still promoted rephosphorylation of SRp38.

We did note that in cells overexpressing Hsp27, the level of Hsp70 tended to be higher and we therefore tested the effect of overexpressing Hsp70 directly. To that end we used HEK-293 cells stably transfected with tetracycline-inducible YFP-Hsp70. The N-terminal YFP-Hsp70 fusion protein has been shown to have the same *in vivo* protective properties as the wild-type Hsp70 (Zeng *et al.*, 2004; H. H. Kampinga, personal communication). Induction of exogenous expression of Hsp70 protected splicing to the same extent as induction of exogenous Hsp27 expression in HEK-293 cells (unpublished data). However, upon exogenous expression of Hsp70, there was no change in the phosphorylation state of SRp38 after heat shock compared with noninduced cells. SRp38 was still fully dephosphorylated after heat shock and only a trace of rephosphorylated SRp38 was seen after 14 h of recovery in the presence of Hsp70 (Figure 7, lanes 7 and 8). In comparison, exogenous expression of Hsp27 in HEK-293 cells had the same effect as exogenous expression of Hsp27 in HeLa cells: 14 h after recovery from the heat shock, all SRp38 is at least partially rephosphorylated (Figure 7, lanes 9 and 10). These results indicate that the enhanced rephosphorylation of SRp38 after a heat shock is a specific effect of Hsp27 and not a consequence of the increase in Hsp70 levels in response to the heat shock.

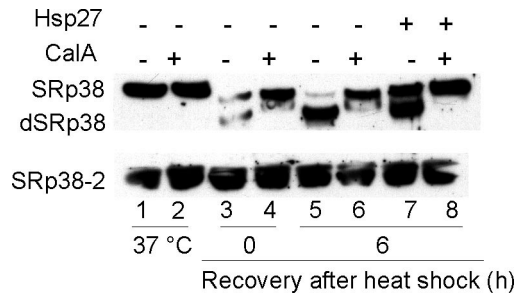
#### *The Dephosphorylation of SRp38 Is Sensitive to the Phosphatase Inhibitor Calyculin A*

The steady state SRp38 phosphorylation level is the outcome of the balance between the activity of kinases and phosphatases. Hsp27 changes that balance in favor of phosphorylation but could do so by activating a kinase or by inhibiting a phosphatase. If Hsp27 inhibits a phosphatase, then inhibition of dephosphorylation by other means should abolish

the effect of Hsp27. When cells were treated with calyculin A during the heat shock, only the partial dephosphorylated form of SRp38 was seen after the heat shock (Figure 8, lanes 3 and 4). Treatment with calyculin A between 5 and 6 h after recovery from heat shock resulted in a shift in SRp38 toward the phosphorylated form. Apparently there is sufficient kinase activity in cells recovering from a heat shock to rephosphorylate SRp38 and it would thus suffice for Hsp27 to inhibit a phosphatase (but see also *Discussion*).



**Figure 7.** Overexpression of Hsp70 does not enhance SRp38 rephosphorylation after heat shock. Western blot showing Hsp70 levels and the different forms of SRp38 found after heat shock and recovery in the presence or absence of exogenous Hsp70. HEK-293 cells expressing YFP-Hsp70 were cultured with (+) or without (–) doxycycline, and either heat shocked and harvested at the indicated time points (lanes 3–8) or cultured at 37°C (lanes 1 and 2). The top panel shows the staining for the induced Hsp70 (YFP-Hsp70) and the endogenous Hsp70 (Hsp70). SRp38 indicates the fully phosphorylated form and dSRp38 the (partially) dephosphorylated form. For comparison the Western blots showing Hsp27 levels and the different forms of SRp38 found in HEK-293 cells after heat shock and 14 h of recovery in the absence or presence of exogenous Hsp27 are also shown (lanes 9 and 10).

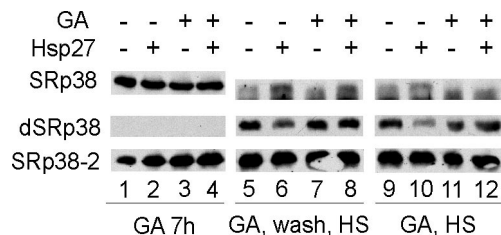


**Figure 8.** Effect of the phosphatase inhibitor calyculin A on SRp38 phosphorylation. T-REx HeLa Hsp27 cells were either kept at 37°C (lanes 1 and 2) or heat shocked at 45°C for 60 min (lanes 3–8) and harvested immediately after heat shock (lanes 3 and 4) or 6 h after recovery (lanes 5–8). Where indicated (CalA +), cells were cultured for 1 h before harvesting with 0.1  $\mu$ M calyculin A. Where indicated (Hsp27 +), Hsp27 expression was induced 24 h before the heat shock.

### The Rephosphorylation of SRp38 during Recovery from a Heat Shock Requires a Hsp90 Client Protein

Many proteins involved in signaling, including kinases, are client proteins of Hsp90. We have therefore tested whether the activity of Hsp90 is required for the rephosphorylation of SRp38 after a heat shock. Treatment with GA, an inhibitor of Hsp90, at 37°C for 7 h (Figure 9, lanes 1–4) did not result in a change in the phosphorylation state of SRp38. However, when GA was added to the cells for 1 h and was either washed away before the heat shock (Figure 9, lanes 5–8) or remained present during heat shock and subsequent recovery (Figure 9, lanes 9–12) the rephosphorylation of SRp38 was inhibited, irrespective of the presence of Hsp27 (note the loss of dSRp38 in Figure 9, lanes 6 and 10, compared with lanes 8 and 12).

If the extent of rephosphorylation of SRp38 is causally related to the recovery of splicing, then GA should also



**Figure 9.** The Hsp90 inhibitor geldanamycin (GA) inhibits SRp38 phosphorylation in T-REx HeLa Hsp27 cells. Lanes 1–4: cells were cultured at 37°C with (Hsp27 +; lanes 2, 4) or without (Hsp27 –; lanes 1 and 3) induction of expression of Hsp27. Where indicated (GA +, lanes 3 and 4) cells were treated with GA for 7 h at 37°C. Lanes 5–8: cells were treated with GA (GA +) for 1 h before the heat shock. To control cells (GA –) an equivalent amount of dimethyl sulfoxide (DMSO) was added. GA or DMSO was then washed away, cells were heat shocked for 1 h at 45°C and left to recover for 6 h at 37°C. Where indicated (Hsp27 +, lanes 6 and 8), the expression of Hsp27 had been induced 24 h before the heat shock. Lanes 9–12: GA (GA +; lanes 11 and 12) or an equivalent amount of DMSO (GA –; lanes 9 and 10) was added to the cells just before the heat shock and remained present during the 6 h of recovery. Where indicated (Hsp27 +; lanes 10 and 12), Hsp27 expression was induced by adding doxycycline 24 h before the heat shock. Note that for the dSRp38 a shorter exposure of the same blot is shown than for SRp38.

block recovery of splicing irrespective of the presence of Hsp27. We therefore measured the recovery of splicing in the presence of GA and found that it blocked the effect of Hsp27: the level of spliced products in the presence of Hsp27 and GA was equal to that seen in the absence of Hsp27 (unpublished data). Hence an Hsp90 client protein is required both for rephosphorylation of SRp38 and for the recovery of splicing.

## DISCUSSION

Under normal conditions, transcription, RNA processing, and RNA export are integrated events, where each step is monitored by quality control systems (Hirose and Manley, 2000; Jensen *et al.*, 2003; Dimaano and Ullman, 2004; Fasken and Corbett, 2005). Properly processed mRNAs are marked as such and efficiently recruited by the translation machinery. During a heat shock the coordination between these steps is lost. The phosphorylation pattern of the CTD of RNA polymerase II, which plays an essential role in integration of transcription and processing, is changed (Egyhazi *et al.*, 1998; Palancade and Bensaude, 2003), with as yet unknown consequences for the link between transcription and processing. The exosome is still recruited to the site of transcription (Andrulis *et al.*, 2002), but apparently fails to recognize improperly processed transcripts as unspliced RNAs are exported to the cytoplasm and translated (Yost and Lindquist, 1988; unpublished results). During recovery from a heat shock, the coordination between RNA synthesis, processing and quality control must be restored. If the quality control is less temperature sensitive than splicing, then, once splicing starts to recover, partially spliced transcripts will be recognized and removed. Our experiments are at least suggestive that this occurs. The level of total  $\gamma$ D-crystallin RNA reaches a steady state after  $\sim$ 3 h of recovery from a heat shock, yet the level of spliced mRNA only starts to increase at this time (Figure 1). Hence, unspliced or partially spliced RNA is being preferentially removed. Hsp27 does not appear to affect the turnover rates of either unspliced or spliced mRNAs, as Hsp27 would then also have an effect on the total transcript level, which it does not. However, it does mean that we are overestimating the actual level of splicing both in the presence and absence of Hsp27, making it difficult to correlate the level of splicing and the extent of rephosphorylation of SRp38 directly. A further complication in measuring the level of splicing after heat shock is that there is quite a bit of difference in the efficiency with which the scarce snRNPs are recruited to various introns. The splicing of some introns has been reported not to be inhibited by a heat shock (Bond, 1988; Jolly *et al.*, 1999). We found that the small  $\gamma$ -crystallin first intron is efficiently spliced out in  $\sim$ 65% of the transcripts directly after heat shock, whereas only  $\sim$ 15% of the transcripts lack the large second intron; the second intron of the  $\beta$ -globin transcript was spliced even less efficiently. This suggests that the splicing efficiency after a heat shock is determined not only by the availability of the general splicing factors but also by exonic and intronic splicing enhancers and silencers as well as the SR proteins and hnRNP complexes that interact with these transcript-specific splicing regulators (for recent review, see Matlin *et al.*, 2005). However, because Hsp27 stimulates the splicing of the  $\gamma$ D-crystallin as well as the  $\beta$ -globin introns, its action is more likely on a general than on a specific splicing component. We have shown here that Hsp27 stimulates the recovery of splicing and the rephosphorylation of SRp38. The effect of Hsp27 on the rephosphorylation of SRp38 appears to be delayed and more modest relative to its effect on splicing.

However, as we have argued above, the extent of recovery of splicing is probably overestimated in our experiments because we cannot correct for degradation of partially spliced transcripts. Furthermore, in heat-shocked cells transcription from non-heat shock promoters decreases sharply, giving transcripts from the heat shock promoters full access to the few snRNPs that escape from the general inhibition. Finally, with  $\sim 3 \times 10^6$  molecules per cell, SRp38 is only in about a twofold excess to U1 snRNP (Shin and Manley, 2002). Thus even a 30% rephosphorylation of SRp38 in the presence of Hsp27 could result in a relatively large increase in free U1 snRNP.

The requirement for heat shock proteins in the recovery of splicing after a heat shock has been best studied in yeast, using mutants lacking Hsp104 and/or members of the Hsp70 family (Yost and Lindquist, 1986, 1991; Bracken and Bond, 1999). Hsp104 and Hsp70 were found to enhance the recovery of splicing in a synergistic manner, suggesting that the splicing pathway is inhibited by a heat shock at at least two sites and that the release of inhibition at those sites has a different requirement for Hsps. Unfortunately, the effect of yeast Hsp26 has not been studied. This would be of interest as it has been reported that the establishment of splicing thermotolerance in yeast by a mild heat shock does not require either Hsp104 or Hsp70 (Bracken and Bond, 1999) and in the light of the recent finding that Hsp26 cooperates with Hsp104 in yeast (Cashikar *et al.*, 2005). To what extent the yeast data can be extrapolated to the mammalian system is not clear. A heat sensitivity of the U4/U5/U6 snRNP complex is found in both yeast and HeLa cells and in both cell types a pre-heat shock lessens the sensitivity of the trisRNP assembly to a heat shock (Utans *et al.*, 1992; Bracken and Bond, 1999; Bond and James, 2000). A major difference between the regulation of splicing in heat-stressed yeast and mammalian cells is, however, the lack of SR proteins and thus of the SRp38 splicing regulator in yeast (Shin *et al.*, 2004). The SR proteins in mammalian cells impose an additional layer of regulation on splicing, and, as we have shown here, it is this layer that is influenced by the presence of Hsp27 but not by that of Hsp70. Given the evolutionary conservation of both the splicing machinery and Hsp70, it is obvious to suggest that Hsp70 acts in mammalian cells to protect the U4/U5/U6 snRNP complex as it does in yeast (Bracken and Bond, 1999). However, in mammalian cells a direct association of Hsp70 with the U4/U5/U6 snRNP complex could not be demonstrated (Bond and James, 2000) and the site of action of Hsp70 thus remains unclear. Our data do show that Hsp70 does not affect the phosphorylation state of SRp38 after heat shock and its action thus differs from that of Hsp27. Our data do not address the question as to whether Hsp70 is required for the recovery of splicing after heat shock when exogenous Hsp27 is present and vice versa: as the challenge heat shock induces the heat shock system, the full complement of endogenous heat shock proteins is present in the cells recovering from that heat shock.

Of the two sHsps tested in this study, only Hsp27, not  $\alpha$ B-crystallin, affected the recovery of splicing after a heat shock. This difference is rather surprising because both sHsps are *in vitro* chaperones (for review, see Haslbeck and Buchner, 2002), because both colocalize with SC35 (van den IJssel *et al.*, 2003; van Rijk *et al.*, 2003; den Engelsman *et al.*, 2004), a marker of the nuclear speckles that are thought to be transit or storage sites for splicing components (Phair and Misteli, 2000; note that SRp38 does not colocalize with SC35; Shin *et al.*, 2005), and because both traffic to the nucleus in normal as well as stressed cells (Arrigo *et al.*, 1988; Loktionova *et al.*, 1996; van den IJssel *et al.*, 2003; van Rijk *et al.*,

2003). The nuclear localization of Hsp27 is phosphorylation dependent (Geum *et al.*, 2002), which could explain the lack of activity of the Hsp27 Ser $\rightarrow$ Ala mutants.

Small Hsps cannot refold proteins, they can merely transfer substrates for refolding to Hsp70 (Ehrnsperger *et al.*, 1997; Lee *et al.*, 1997; Wang and Spector, 2000). Hence, if the general chaperoning capacity of Hsp27 is vital for the restoration of the phosphorylation of SRp38, then one would expect the level of phosphorylated SRp38 to be enhanced by an increase in Hsp70. However, raising the Hsp70 level by either pre-heat shocking the cells or by exogenous expression of Hsp70 had no effect. Our data thus suggest that a specific interaction of Hsp27, perhaps stabilization of a single client protein, is required to enhance SRp38 phosphorylation. Unfortunately it is difficult to trace back the phosphorylation cascade to the site of action of Hsp27. The data show that inhibition of a phosphatase would suffice to restore SRp38 phosphorylation, but it is not known which phosphatase is responsible for the dephosphorylation of SRp38 and the spectrum of inhibition by calyculin A is too broad to allow an informed guess. Furthermore, we cannot rule out the possibility that Hsp27 directly or indirectly activates a kinase and thereby shifts the balance toward phosphorylation. SR proteins are known to be phosphorylated by SRPK and Clk/Sty kinases but the regulation of activity of these kinases by extrinsic factors remains to be elucidated (Mermoud *et al.*, 1994; Shin and Manley, 2004). The only known interaction of Hsp27 with a kinase is that with Akt kinase (Konishi *et al.*, 1997; Rane *et al.*, 2001; Rane *et al.*, 2003). In neutrophils Akt was found in a complex with Hsp27, p38 MAPK, and MAPKAPK-2 and the Hsp27-Akt interaction was required for activation of Akt (Rane *et al.*, 2003). This interaction was disrupted by phosphorylation of Hsp27. If a similar interaction takes place in the cells used here, then dissociation of the Hsp27-Akt complex is apparently required for the rephosphorylation of SRp38 as the nonphosphorylatable Hsp27 mutant could not enhance the rephosphorylation.

A role of Akt in the recovery of splicing after a heat shock is consistent with the finding that treatment of the cells with GA, an inhibitor of Hsp90, before heat shock inhibited the recovery of splicing and the rephosphorylation of SRp38, even in the presence of Hsp27. Akt is a client protein of Hsp90 and disruption of Hsp90 function by GA causes proteasome-dependent Akt degradation (Basso *et al.*, 2002). Possibly Hsp27 controls the level and/or duration of the activation of Akt after a heat shock. Clearly the interplay between Hsp90, Hsp27, and Akt needs further investigation.

Expression of Hsp27 is not only stress-induced; some tissues contain constitutively high levels of Hsp27. Our results suggest that it would be of interest to investigate whether Hsp27 contributes to the regulation of alternative splicing in those tissues through an effect on the phosphorylation state of SRp38 and whether the anti-apoptotic effect of Hsp27 is in part mediated by preventing the alternative splicing of transcripts of genes in the apoptotic pathway. Finally, it has recently been shown that SR proteins, including SRp38, are also involved in translation (Sanford *et al.*, 2004; Huang and Steitz, 2005; Liu and Harland, 2005). Because Hsp27 also confers translational thermotolerance through an as yet unknown mechanism, the link between Hsp27 and SRp38 in translation deserves further study.

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