Cell Death and Differentiation (2003) 10, 1137–1147

www.nature.com/cdd

Heat stress downregulates FLIP and sensitizes cells to Fas receptor-mediated apoptosis

SEF Tran[1,2], A Meinander[1,3], TH Holmström[1,7], A Rivero-Müller[1], KM Heiskanen[1,2,4], EK Linnau[1,8], MJ Courtney[5], DD Mosser[6], L Sistonen[1,2] and JE Eriksson*[,1,4]

[1] Turku Centre for Biotechnology, Åbo Akademi University and University of Turku, Turku, Finland
[2] Department of Biology, Åbo Akademi University, Turku, Finland
[3] Department of Biochemistry and Pharmacy, Åbo Akademi University, Turku, Finland
[4] Department of Biology, University of Turku, Turku, Finland
[5] AI Virtanen Institute for Molecular Sciences, University of Kuopio, Kuopio, Finland
[6] Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1
[7] Current address: Department of Cell Biology, Max-Planck-Institut for Biochemistry, D-82152 Martinsried, Germany
[8] Current address: Institute of Applied Microbiology, University of Agricultural Sciences, A-1190 Vienna, Austria
* Corresponding author: JE Eriksson, Department of Biology, University of Turku, FIN-20014 Turku, Finland Tel: +358 2 333 8036; Fax: +358 2 333 8000; E-mail: john.eriksson@utu.fi

Received 03.10.02; revised 08.4.03; accepted 22.4.03
Edited by E Alnemri

Abstract

The heat shock response and death receptor-mediated apoptosis are both key physiological determinants of cell survival. We found that exposure to a mild heat stress rapidly sensitized Jurkat and HeLa cells to Fas-mediated apoptosis. We further demonstrate that Hsp70 and the mitogen-activated protein kinases, critical molecules involved in both stress-associated and apoptotic responses, are not responsible for the sensitization. Instead, heat stress on its own induced downregulation of FLIP and promoted caspase-8 cleavage without triggering cell death, which might be the cause of the observed sensitization. Since caspase-9 and -3 were not cleaved after heat shock, caspase-8 seemed to be the initial caspase activated in the process. These findings could help understanding the regulation of death receptor signaling during stress, fever, or inflammation.

Cell Death and Differentiation (2003) **10,** 1137–1147. doi:10.1038/sj.cdd.4401278

Keywords: apoptosis; death receptor; Fas; CD95; stress; heat shock; FLIP; caspase

Abbreviations: DISC, death-inducing signaling complex; DR, death receptor; ERK1/2, extracellular-regulated kinase 1 and 2; HS, heat shock; HSE, heat shock element; HSF, heat shock factor; Hsp, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase

Introduction

Organisms have developed numerous strategies to protect cellular functions during periods of environmental stress. On the other hand, an equally important adaptation is the destruction and removal of irreparably damaged cells, when the protective mechanisms have been overwhelmed. Regulated cell death defends the organism against infected or mutated cells and thereby plays an important role in the maintenance of homeostasis. Stress-induced apoptosis deletes damaged cells through an intrinsic pathway that culminates in the activation of effector caspases.[1] The intrinsic mode of apoptotic induction is efficiently regulated by stress-induced protection mechanisms. Cells can also be eliminated under physiological conditions through an extrinsic pathway by activation of cell surface death receptors.[2] This extrinsic pathway is an essential homeostatic mechanism for controlling cell numbers, especially in the immune system. Therefore, it is important to determine whether the protective mechanisms that inhibit stress-induced apoptotic pathways could also influence the response of cells to death receptor-mediated cell killing.

Death receptors (DRs), such as the Fas-, TNF-, and TRAIL-receptors (FasR, TNF-R1, and TRAIL-R), are membrane proteins capable of inducing apoptosis, and belong to the TNF-R family.[2–4] Upon ligand binding, the DRs oligomerize and recruit a number of proteins to form a death-inducing signaling complex (DISC). Proteins in the DISC can either bind directly to the DR or with the aid of intermediate adaptor proteins, such as FADD, or, in the case of TNF-R1, through the combined binding of FADD and TRADD.[5,6] As an essential element in the apoptotic cascade of most DRs, FADD recruits caspase-8 to the DISC. After association to the complex, caspase-8 is activated and provokes the apoptotic signaling cascade (for review, see Cohen[7]). FasR signaling can either directly activate downstream effector caspases or trigger the mitochondrial apoptosis amplification loop. The latter involves caspase-8-mediated activation of the proapoptotic Bcl-2 family member, Bid, followed by release of cytochrome *c*, which binds to the apoptosome complex formed by Apaf-1 and caspase-9. In turn, caspase-9 cleaves more caspase-8 as well as downstream effector caspases. Other signaling modules such as RIP proteins,[8,9] Daxx,[10] FLIP,[11] or the whole NF-kB pathway[5,12] interact with the DISC. FLIP is especially interesting, as it acts as a physiological inhibitor of caspase-8.[11,13] Apart from proapoptotic signaling, there are also other proteins known to be activated by FasR stimulation, for example the mitogen-activated protein kinases (MAPKs) ERK1/2[14,15] and c-Jun N-terminal kinase (JNK)[16,17] are activated in many model systems after DR stimulation. Altogether, these signaling proteins can modulate the outcome of receptor activation.

Prolonged exposure to high temperatures is harmful for cells as it promotes protein damage. To cope with moderate and transient exposures to heat stress, the cells respond by

upregulating the expression of heat shock proteins (Hsps), which act as chaperones to prevent protein aggregation, help proper refolding of denatured proteins, and divert permanently damaged proteins to proteasome-mediated degradation (for review see Lindquist and Craig,[18] Hartl and Hayer-Hartl,[19] and Morimoto[20]).The heat shock response involves rapid and transient activation of the transcription factor heat shock factor 1 (HSF1), triggered by trimerization, phosphorylation, and subcellular relocalization (for review see Wu,[21] Pirkkala *et al*.,[22] and Holmberg *et al*.[23]). The activated HSF1 binds to heat shock elements (HSE) in the promoters of Hsp genes and induces expression of Hsps, the most abundant of which is Hsp70. Other signaling events take place during the heat shock response, including proapoptotic signaling, which is necessary to eliminate those cells that are damaged beyond repair. For instance, the stress-activated protein kinase JNK, which has been implicated in stress-induced apoptosis, is activated during heat shock (for review see Kyriakis and Arruch [24]).

Hsps, the survival factors generated by heat stress, have been well documented. Indeed, it has long been known that a mild heat shock can induce thermotolerance against a subsequent heat stress,[25] or crosstolerance to other apoptotic stimuli by Hsp-mediated inhibition of cell death.[25–27] The levels of Hsp70 expressed during the first stress remain elevated as the second stress occurs, permitting repair of the cell at the outset of the stress, and more specific inhibition of death pathways before their activation. Thus, Hsp70 has been shown to inhibit JNK, which is a mediator of the stress-induced death signals.[28–30] Hsp70 is also able to interfere more directly with the apoptotic machinery, as it has been shown to inhibit the apoptosome downstream of the mitochondria,[31,32] to prevent cytochrome *c* release from the mitochondria of stressed cells,[33] to stop the TNF-R1 death pathway downstream of caspase-3 activation,[34] and, more recently, to prevent Bid cleavage.[35] The interest in Hsp70 antiapoptotic properties has increased as they were shown to be constitutively expressed in a number of tumor cells, preventing their removal by apoptosis induction.[1,34,36,37]

Although it is well established that Hsp70 has an inhibitory effect on TNF-mediated apoptosis, the relations between stress and apoptosis induced by the other DRs have been poorly studied. Some reports describe a sensitizing effect of stress-related proteins on Fas-mediated apoptosis. Overexpression of Hsp70 has thus been suggested to sensitize Jurkat cells to Fas-induced apoptosis[38] and an active HSF1 mutant was observed to render cells more susceptible to Fas killing.[39] Furthermore, stress-activated protein kinases, such as JNK, which are also activated by DRs, have been implicated as especially important for induction of apoptosis.[40] In this study, we examined the possible interactions between the stress response and FasR signaling. We show that heat stress sensitizes Jurkat and HeLa cells to Fas-induced apoptosis and that this sensitization is not mediated by different members of the MAPK signaling protein family. We also observed that expression of Hsp70 provides neither protection nor sensitization to FasR-mediated apoptosis in this model system. Finally, we show that heat stress is able to rapidly downregulate FLIP and activate caspase-8 cleavage independently from activation of apoptosis, indicating that the sensitization to Fas is likely to be mediated by a facilitated caspase-8 activation in the absence of FLIP.

Results

Heat shock sensitizes cells to Fas-mediated apoptosis independently from Hsp70 expression

A mild heat shock can induce thermotolerance to a subsequent thermal stress. We wanted to determine whether heat shock would also protect against apoptosis induced by FasR in Jurkat cells. Surprisingly, exposure to a 30-min heat shock at 42°C prior to FasR stimulation did not inhibit, but rather increased the amount of apoptosis compared to cells subjected to Fas treatment alone. The effect was readily visible in phase contrast microscopy (Figure 1a) and obvious by quantification with flow cytometry after labeling of apoptotic cells with Annexin-V (Figure 1b). A stress as mild as 15 min at 42°C, followed by a 2-h Fas treatment, was sufficient to increase the amount of apoptotic cells (Figure 1b). This was not restricted to Jurkat cells or the use of Fas antibody. Indeed, we observed that HeLa cells subjected to heat shock were also sensitized to Fas-mediated cell death induced by treatment with Fas ligand (Figure 1c).

The heat shock response in Jurkat cells consisted of a rapid activation of HSF1 (Figure 2a), followed by upregulation of Hsp70 within 2 h (Figure 2b). FasR stimulation on its own did not induce HSF1 DNA-binding activity or Hsp70 expression (Figure 2a,b), thereby showing that the death receptor and heat stress signal through distinct pathways. Furthermore, FasR signaling did not affect the heat shock-induced HSF1 activation and Hsp70 expression (Figure 2a,b), suggesting that the sensitization does not occur through modulation of Hsp expression by FasR.

Hsps are known to protect against various apoptotic stimuli[41] (for a review, see Jäättelä[37]). Heat shock-induced expression of Hsps, however, did not seem to affect Fas-mediated killing. Indeed, the sensitization occurred, whether Fas was applied right after heat shock, before induction of Hsp70, or after 2-h recovery at 37°C, when the levels of Hsp70 were already high. To examine the protective effect of Hsp70 against Fas-induced cell death under nonstressful conditions, we used a Jurkat-based cell line expressing Hsp70 and GFP upon induction with doxycycline (Hsp70-GFP-tetON; Figure 3a). GFP-positive cells were not protected as both mitochondrial depolarization and nuclear fragmentation occurred (Figure 3b), showing that Hsp70 cannot rescue Jurkat cells from Fas-mediated cell death, which could explain why heat shock did not induce crosstolerance to another apoptotic stimulus. For further assessment, we quantified Fas-induced apoptosis in control cells and cells expressing Hsp70. However, FACS measurement of apoptosis did not reveal any significant protection by Hsp70 from Fas-induced apoptosis in Jurkat cells (Figure 3c). Additionally, this result also demonstrates that Hsp70 could not render the cells sensitive to Fas killing.

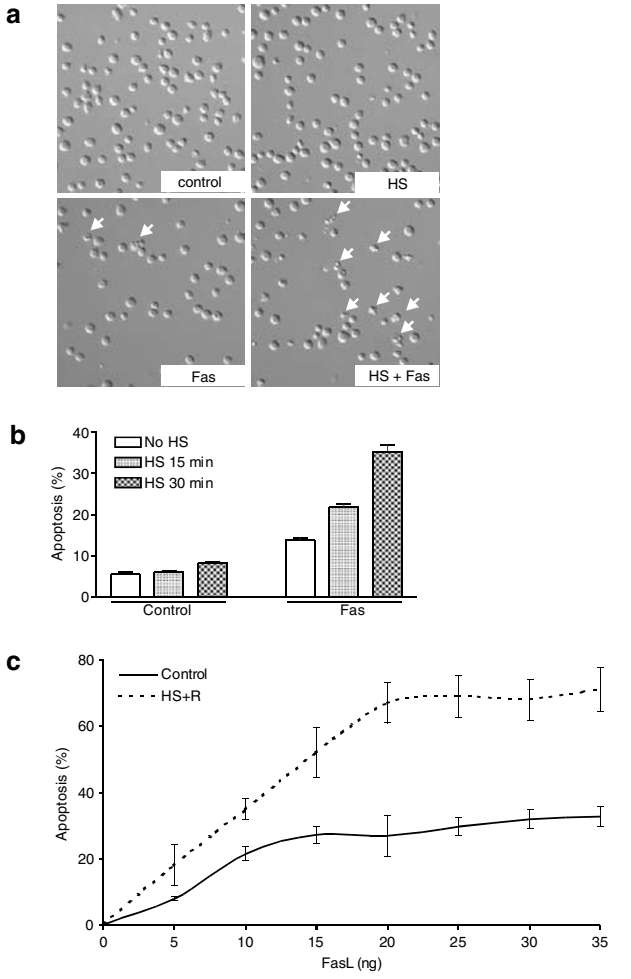

Figure 1 Heat shock sensitizes cells to Fas-mediated apoptosis. (**a**) Differential interference contrast pictures of Jurkat cells subjected to the indicated treatments: control, heat shock (HS, 30 min at 42°C followed by a 2-h recovery at 37°C), Fas (2-h treatment with 100 ng/ml anti-Fas), HS + Fas (30 min at 42°C followed by a 2-h anti-Fas treatment at 37°C). The arrows indicate the apoptotic cells, recognizable by their blebbing. (**b**) Jurkat cells were heat shocked for 15 or 30 min followed by a 2-h treatment with 200 ng/ml anti-Fas. Apoptotic cells were counted by FACScan after Annexin-V staining. The graph represents mean value (mean ± S.E.) of triplicates. (**c**) Control or heat-shocked HeLa cells (30 min 42°C and 2-h recovery prior to Fas stimulation) were treated with FasL in the indicated amounts. Cell death was measured by MTT assay.

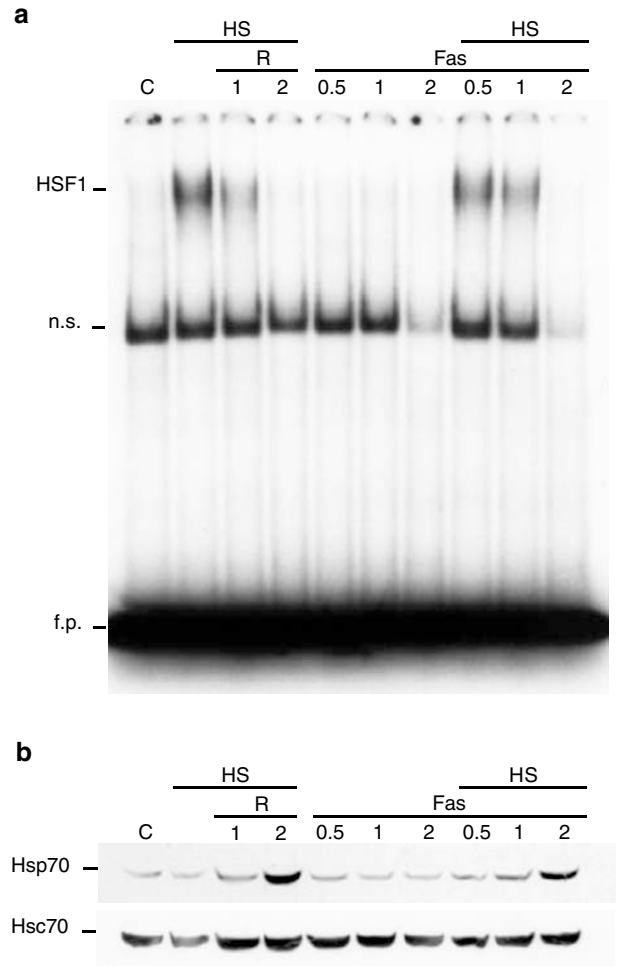

Figure 2 Fas does not activate the heat shock response. Jurkat cells were heat shocked (HS; 30 min at 42°C), before recovery at 37°C (R) with or without Fas treatment (200 ng/ml anti-Fas) for the indicated time periods. Cell extracts were subjected to electrophoretic mobility shift assay using an HSE probe to detect HSF1 DNA-binding activity (**a**) and Western blot with anti-Hsp70 antibody (**b**) Equal loading was shown using Hsc70 antibody. n.s., nonspecific binding, f.p., free probe.

Traditional stress-induced signaling pathways are not involved in the sensitization process

Members of the MAPK family are good candidates for modulating the fate of cells subjected to both stress and apoptotic stimuli. Especially, the JNK pathway is an essential signaling module involved in stress-induced apoptosis (for review, see Kyriakis and Arruch[24]). JNK is also activated by FasR stimulation in a number of cell lines, although it has been shown to be dispensable for Fas-mediated apoptosis.[17,42] However, a stress-induced activation of JNK preceding FasR stimulation could still result in increased apoptotic signaling. In Jurkat cells, JNK was activated both by heat shock and by FasR stimulation (Figure 4a). To disrupt the JNK pathway, we used a GFP-tagged dominant negative mutant (MKK4-DN) of the JNK activator MKK4, which has been shown to be necessary for heat shock-induced JNK activation.[43] Quantification of apoptosis among GFP-positive cells was carried out by fluorescence microscopy (Figure 4b), and flow cytometry. Surprisingly, expression of MKK4-DN did not prevent the heat shock-induced increase in apoptotic Jurkat cells after FasR stimulation (Figure 4c), indicating that JNK is not responsible for the sensitization. Furthermore, inhibition of the other MAPK family members, p38 and ERK1/2, using SB203580 and PD98059, respectively, resulted in higher sensitization rather than protection from heat shock- and Fas-mediated apoptosis (data not shown).

We have previously shown that in HeLa cells, FasR stimulation results in a rapid activation of ERK1/2 and subsequent protection of the cells from apoptosis.[14,15] Therefore, abrogation of ERK1/2 activity and survival signals could sensitize the cells. Heat shock, however, did not

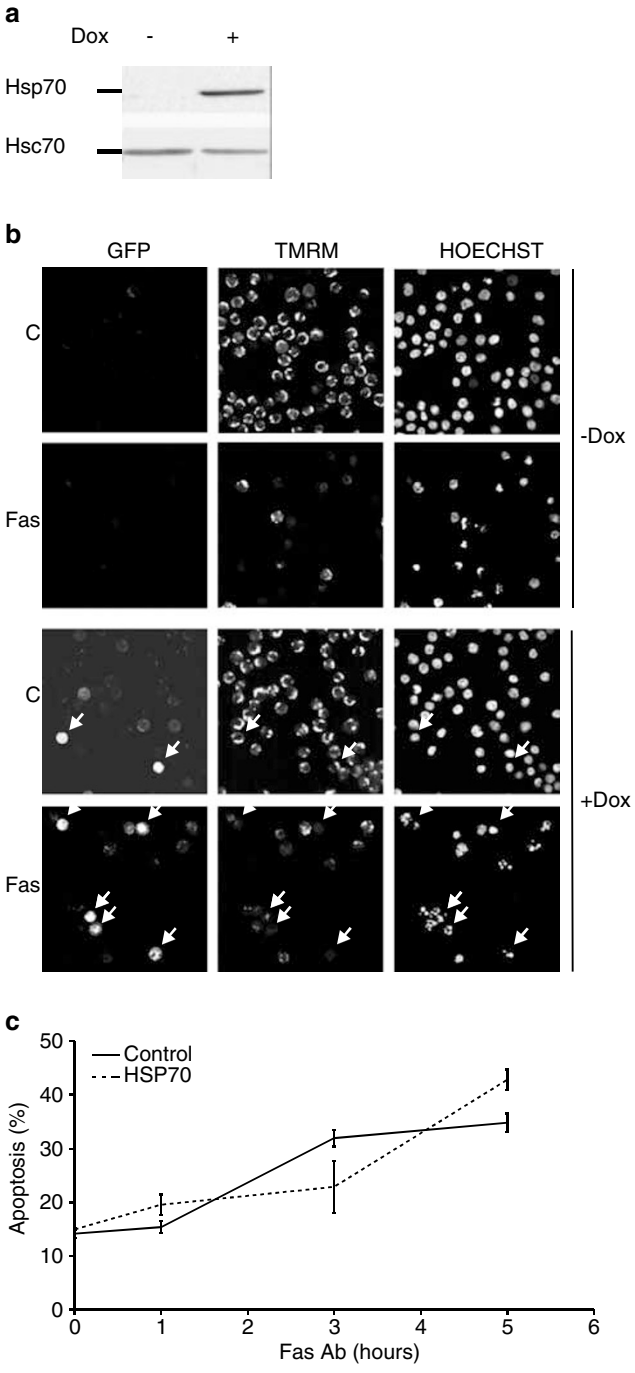

Figure 3 Inducible overexpression of Hsp70 does not affect the observed increase in apoptosis. (**a**) Control Western blot showing Hsp70 induction in Hsp70-GFP-tetON Jurkat cells after 24 h with 1 μg/ml doxycycline. (**b**) Micrographs of Hsp70-GFP-tetON Jurkat cells, with or without induction by 1 μg/ml doxycycline and 5 h of 200 ng/ml Fas treatment. The cells were loaded with TMRM for visualization of polarized mitochondria and the nuclei were stained with Hoechst. Arrows indicate examples of GFP-positive cells. (**c**) Apoptosis in Fas-treated Hsp70-GFP-tetON Jurkat cells with or without doxycycline was quantified by FACScan after Annexin-V staining. The graph represents mean value (mean $\pm$ S.E.) of three different experiments.

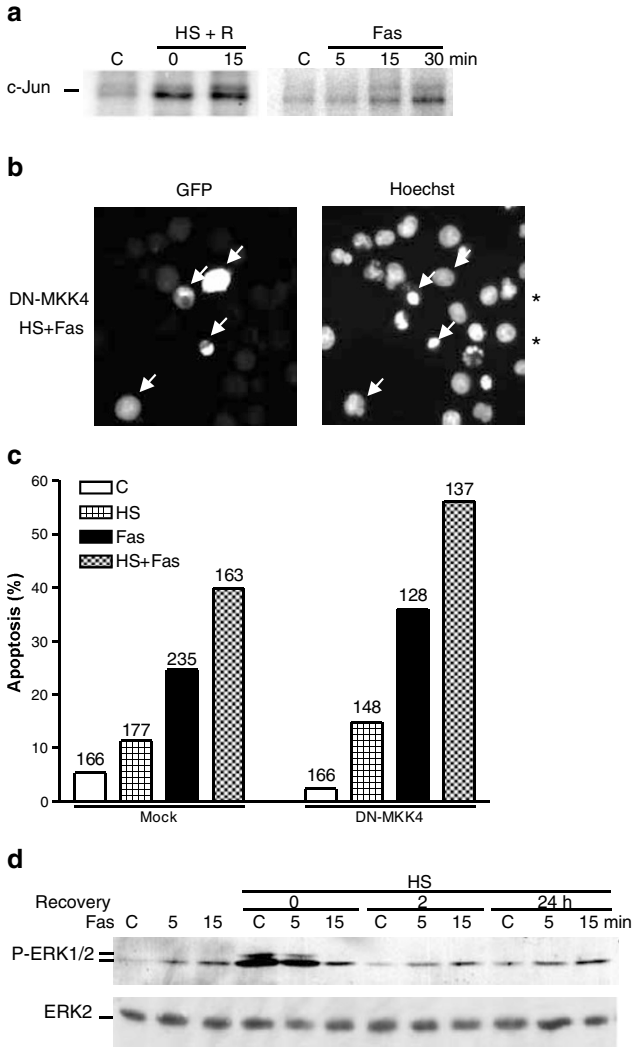

Figure 4 The sensitization is not due to antiapoptotic or proapoptotic MAPK signaling. (**a**) Jurkat cells were subjected to a heat shock (HS; 30 min at 42°C) followed by 0- or 15-min recovery (R), or to FasR stimulation for the indicated time periods. JNK assay was performed using c-Jun as a substrate. (**b**) Jurkat cells were mock transfected with vector-GFP, or transfected with dominant negative MKK4-GFP (DN-MKK4), and subjected to HS (30 min at 42°C) and Fas treatments (2 h, 200 ng/ml). Apoptotic cells were counted under the microscope. The HS + Fas micrographs are shown as an example: GFP-positive cells are indicated with arrows, and apoptotic cells with stars. (**c**) Graph representing the microscopic quantification. The number of cells counted is shown on top of the bars. Similar results were obtained with FACScan quantification (data not shown). (**d**) Western blot of phospho-ERK1/2. HeLa cells were subjected to heat shock prior to the indicated periods of recovery (0, 2, 24 h) followed by Fas treatment (200 ng/ml for 0, 5, or 15 min).

downregulate the activation of ERK1/2 by FasR in HeLa cells. Instead, heat shock strongly induced ERK1/2 phosphorylation, an activation state that disappeared rapidly when cells were left to recover at 37°C (Figure 4d). Furthermore, no significant ERK1/2 activity was detected in Jurkat cells after either heat shock or FasR stimulation (data not shown). Taken together, these results exclude the possibility of ERK1/2 being involved in the heat shock-induced sensitization to Fas-mediated apoptosis.

Stress-induced signaling has been previously shown to directly affect Fas-mediated cell death. Indeed, studies in *lpr* mice lymphoid cells have revealed that both γ-irradiation and

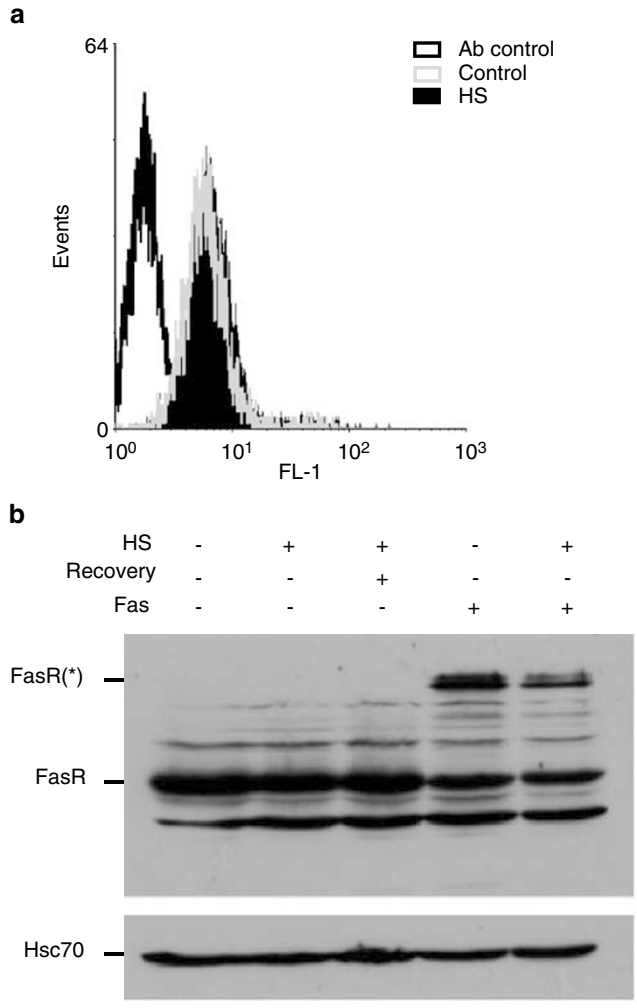

Figure 5 Heat shock does not affect FasR. (**a**) Flow cytometry histogram showing the expression of FasR on the surface of Jurkat cells in control cells, or cells heat shocked for 30 min followed by 2-h recovery. A negative control, with only secondary antibody is also shown (Ab control). (**b**) Jurkat cells were subjected to heat shock (30 min at 42°C), recovery (2-h), and Fas treatments (200 ng/ml) as described, and Western blot was performed against FasR. A typical SDS-stable, high molecular weight form of the FasR (*) appears in Fas-treated samples.

heat shock-induced apoptosis were partially dependent on FasR signaling, and γ-irradiation was able to upregulate FasR expression.[44] Furthermore, stress was also shown to increase cell death by upregulation of FasL.[45] However, heat shock did not provoke any increase in surface expression of FasR in Jurkat cells (Figure 5a), nor did it induce the SDS-stable high molecular form of FasR which appears in Western blots after FasR stimulation (Figure 5b). Furthermore, upregulation of FasL would be unlikely to have any effect, as we used saturating amounts of antibody, to engage all receptors (data not shown). This suggests that heat shock affects FasR signaling downstream of the receptor. Such signaling can be directed toward survival rather than cell death by activation of certain antiapoptotic mechanisms.

DISC components are affected by heat shock

As evidence for caspase-independent apoptosis is emerging,[46-50] we tested whether heat shock would activate a Fas-mediated, caspase-independent pathway. In this respect, we examined the induction of apoptosis in the absence or presence of the pan-caspase inhibitor z-VAD-fmk. A general caspase inhibition prevented equally well the induction of apoptosis by both Fas and heat shock followed by Fas, excluding the possibility that a caspase-independent pathway would be activated by heat shock (Figure 6a). A control Western blot of the same samples showed that caspase-8 cleavage into the intermediate p41/43 form and the p18 active form was effectively inhibited by z-VAD-fmk. Interestingly, we observed that heat shock by itself could induce cleavage of caspase-8 independently from activation of apoptosis (Figure 6b). The active form of caspase-8, which appeared within 2 h of recovery after heat shock, was not due to elevated levels of apoptosis in the heat shock samples, as shown in Figure 6a. In agreement with the heat stress-induced caspase-8 processing and sensitization to apoptosis, Fas treatment resulted in an augmentation of caspase-8 cleavage in heat-shocked cells compared to nonheat-shocked cells, which was clearly visible from the increase in p18 fragment (Figure 6b).

To assess whether the sensitization was due to general caspase activation by heat shock, we examined the cleavage of other caspases, namely caspase-9, which is another initiator caspase, functioning at the level of the mitochondrial amplification loop, and caspase-3, which is an effector caspase and a substrate for caspase-8 and -9. Heat shock did not promote caspase-3 or -9 cleavage (Figure 6c). Similarly, DEVDase activity, which includes caspase-3 activity, was detected in Fas-treated samples, but not after heat shock alone (Figure 6d). Furthermore, the specific caspase-8 inhibitor z-IETD-fmk completely prevented the heat shock-induced cleavage of caspase-8, indicating that autocatalytic processing was responsible for caspase-8 cleavage (Figure 7a). z-IETD-fmk also inhibited cleavage of caspase-3 and apoptosis induced by Fas with or without heat shock (Figure 7a,b). Taken together, our data suggest that a mild heat shock does not directly affect apoptosis, but is able to activate caspase-8, which is likely to sensitize the cells to the subsequent Fas-mediated apoptotic stimulus.

To further investigate the mechanism of heat shock-mediated caspase-8 cleavage, we examined the involvement of the DISC protein FLIP in this process, as FLIP has been characterized as an endogenous caspase-8 inhibitor.[11] It is expressed in two isoforms, FLIP long ($FLIP_L$) and FLIP short ($FLIP_S$), both of which can inhibit isoform-specific steps of caspase-8 activation.[51] As a consequence, elevated levels of FLIP protein in the cell often reduce the susceptibility of cells to undergo DR-mediated apoptosis.[13,52] We observed that a 30-min heat shock was sufficient to decrease the levels of both $FLIP_L$ and $FLIP_S$ in Jurkat cells (Figure 8a). FasR stimulation induced cleavage of only a fraction of total $FLIP_L$, whereas following heat shock-mediated downregulation of FLIP, FasR activation induced cleavage of the remaining $FLIP_L$ protein (Figure 8a). Although the FLIP proteins did not disappear completely, our results strongly suggest that the

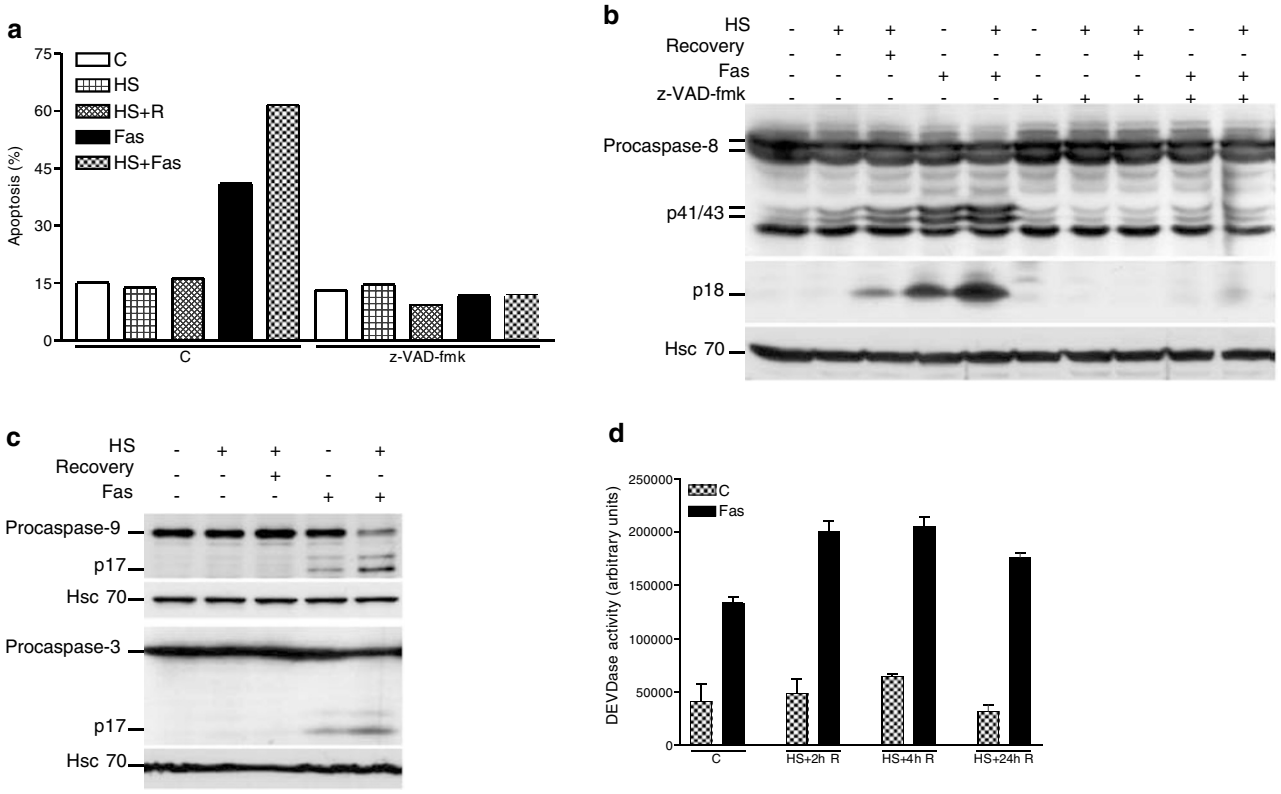

Figure 6 Heat shock induces caspase-8, but not caspase-9 or -3 cleavage. (**a**) Graph representing apoptosis levels with or without 20 μM z-VAD-fmk in control Jurkat cells, cells subjected to heat shock (30 min at 42°C) alone or followed by either 2 h recovery, or 2-h Fas treatment (200 ng/ml). (**b**) Processing of caspase-8 in the same samples as in panel a was determined by Western blot. The caspase-8 antibody recognizes procaspase-8, the intermediate fragments p43/44, and the active fragment p18. (**c**) Jurkat cells were subjected to heat shock (30 min at 42°C) alone or followed by either 2 h recovery, or 2 h Fas treatment (200 ng/ml), and Western blot was performed to detect caspase-3 and -9 cleavage products. The caspase-3 antibody recognizes the proform and the p17 fragment, the caspase-9 antibody detects the proform and p17. (**d**) DEVD cleavage activity was measured in Jurkat cells after heat shock (30 min at 42°C) followed by the indicated recovery periods before Fas treatment (200 ng/ml).

amount of FLIP has been reduced under a certain critical threshold, which could explain the sensitization by lack of protection. z-VAD-fmk or z-DEVD-fmk (inhibitor of caspase-3, -6, -7, -8, and -10) inhibited the caspase-8-mediated cleavage of FLIP, as expected, but not its downregulation (Figure 8a and data not shown), showing that the suppression of FLIP occurs in a caspase-independent manner. Other antiapoptotic proteins such as the mitochondrial proteins Bcl-X_L and Bcl-2 (reviewed in Gross *et al*.[53]) were not affected by heat shock, demonstrating the specificity of FLIP downregulation (Figure 8b).

Discussion

Heat shock-induced apoptotic signaling

The response of cells to hyperthermia is dependent upon the extent of temperature elevation and the duration of exposure. Moderate exposures to elevated temperature will induce synthesis of Hsps, which can be cytoprotective to cells that are subsequently exposed to more extreme temperature shifts, a phenomenon known as induced thermotolerance. When a cell's ability to repair heat-induced cellular damage is overwhelmed, an apoptotic signaling pathway is initiated, with the purpose of eliminating the damaged cell. It has been shown that induction of Hsps, especially Hsp70, prior to application of a severe stress can prevent the activation of this death pathway.[26,54] Furthermore, Hsp70 was shown to inhibit cell death induced by other apoptotic stimuli, for example by preventing cytochrome *c* release from mitochondria.[33] Therefore, it was surprising to observe that heat shock, with subsequent Hsp70 induction, did not have any protective effect against Fas-mediated apoptosis. In contrast to its protective role, constitutive overexpression of Hsp70 was previously suggested to increase the sensitivity to Fas-induced cell death in Jurkat cells.[38] However, in our Jurkat-based cell system, where expression of Hsp70 is inducible as it is in stress, Hsp70 induction did not affect Fas-mediated apoptosis, strongly suggesting that in Jurkat cells, Hsp70 neither protects from nor increases Fas-induced apoptosis. This is in accordance with the report that Hsp70, although able to inhibit the apoptosome, does not prevent Fas-mediated apoptosis.[32]

JNK has been implicated as a major factor in the stress-induced apoptotic signaling pathway (reviewed in Davis[40] and Tibbles and Woodgett[55]). Indeed, cells deficient in both JNK1 and JNK2 were resistant to UV or other stresses.[42] However, our data contradict the hypothesis that JNK would augment

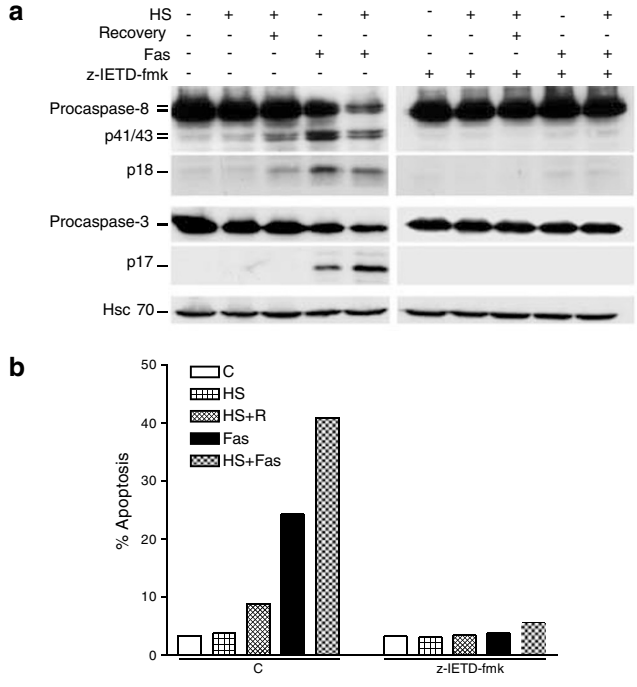

Figure 7 Cleavage of caspase-8 is specific. (**a**) Jurkat cells were subjected to heat shock (30 min at 42°C) alone or followed by either 2-h recovery, or 2-h Fas treatment (200 ng/ml), with or without 50 μM z-IETD-fmk. Western blot was performed against caspase-8 and 3. Note that the reduced levels of p18 in the heat-shocked and FasR-stimulated cells is likely to be due to postapoptotic proteolytic activity commonly observed in advanced states of apoptosis. (**b**) Similarly treated samples as in panel a were used to quantify apoptosis in the presence or absence of z-IETD-fmk.

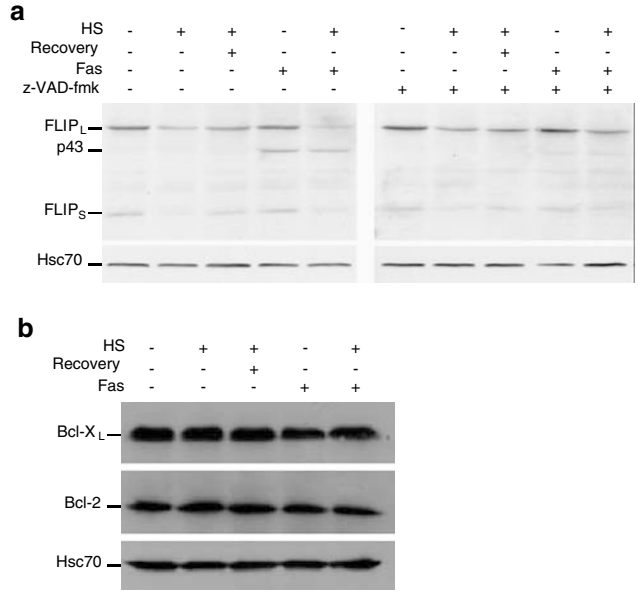

Figure 8 Heat shock downregulates FLIP but not other antiapoptotic proteins. (**a**) Jurkat cells were treated as in Figure 6a, and Western blot against FLIP$_L$ and FLIP$_S$ was performed. (**b**) Jurkat cells were treated as in Figure 6c and Western blot performed to detect Bcl-X$_L$ and Bcl-2.

Fas-mediated apoptosis following heat shock, as the sensitization was not inhibited by downregulation of JNK activity. In addition, our results also exclude the other MAPKs as possible factors involved in the sensitization. These data strongly suggest that the observed sensitization by heat shock to Fas-induced apoptosis is not a combination of stress-specific apoptotic signaling with that of the death receptor, but is a direct effect of the heat stress on one or more components of the Fas-induced apoptotic cascade.

Direct effect of heat shock on FasR-mediated signaling

Although there is evidence that certain stresses can directly sensitize cells to FasR-mediated apoptosis by upregulating either FasR or FasL,[44,45] heat shock does not seem to affect the death pathway at that level. Indeed, the sensitization still occurs when induction of apoptosis reaches a plateau after increasing concentration of FasL, clearly showing the saturation of FasR by its ligand (Figure 1c). Furthermore, we could not detect changes in surface expression or aggregation of FasR during heat shock, suggesting that heat stress affects FasR signaling downstream of the receptor.

FLIP proteins have been shown to protect cells from FasR-mediated apoptosis in many different models, and it has been reported that induced downregulation of FLIP could sensitize cells to apoptotic stimuli.[11,13] Although this remains true for FLIP$_S$, the function of FLIP$_L$ is more controversial. In fact, FLIP$_L$ has been found in some cases to sensitize cells to apoptosis.[56,57] It was recently suggested that the endogenous levels of FLIP$_L$ would activate caspase-8 and increase FasR-mediated apoptosis, while FLIP$_L$-mediated protection would occur only at higher levels of expression, which would explain the discrepancies in previous reports.[58] However, another model was subsequently proposed, in which FLIP$_L$ interaction with procaspase-8 triggers activation but restricts the caspase-8 activity to the DISC and promotes nonapoptotic signaling.[59] Our results indicate that the heat shock-mediated increase in Fas-induced apoptosis is likely to be due to the downregulation of at least one of the FLIP proteins. In the light of the above-described reports, there is the distinct possibility that downregulation of FLIP$_S$, rather than FLIP$_L$, would be the cause of the observed sensitization, since FLIP$_S$ is more strongly affected by the heat stress. However, we cannot exclude that downregulation of FLIP$_L$ also contributes to the increased Fas-mediated apoptosis by restoring release of active caspase-8. In any case, activation of FasR led to an incomplete FLIP cleavage, suggesting that FLIP would be present in excess compared to the cleaving activity. Thus, although the precise mechanism remains to be discovered, we can speculate that heat shock-promoted downregulation of FLIP proteins would prevent the inhibition of both caspase-8 cleavage and its cytosolic release and, as a consequence, would enhance the apoptotic activity of Fas. General inhibition of caspases prevented caspase-8 cleavage but not FLIP downregulation, demonstrating that caspase-8 activation is indeed a downstream event of FLIP downregulation. As the levels of FLIP decreased rapidly after heat shock, it is likely due to active degradation, rather than inhibition of protein synthesis.

As caspases are the main executioners of apoptosis, any sensitizing stimulus will affect the degree of activation of one or more caspases. However, the observation that caspase-8 is cleaved in the absence of apoptosis in heat-shocked samples indicates that caspase-8 cleavage can be the cause of the sensitization, rather than its consequence. This hypothesis is supported by the fact that heat shock specifically activates caspase-8 rather than caspase-3 or -9. Furthermore, caspase-8 inhibition prevented both its own cleavage in heat-shocked or Fas-treated cells, and cleavage of the effector caspase-3 in Fas-stimulated heat-shocked cells, indicating that caspase-8 activation is essential for induction of apoptosis by heat shock and Fas, and that other caspases are not necessary for its processing during heat shock. Thus, we can conclude that caspase-8 is the initial caspase in heat stress-mediated sensitization of cells to FasR-induced apoptosis.

Fever and the immune system

During an infection, the body's overall response involves elevation of its temperature. Although the role of fever has not been completely characterized, it is believed to have beneficial effects, for example by affecting viability of the pathogens. Hasday and Singh[60] have proposed several ways in which fever and the heat shock response interact, involving protection of the host cells, increased immunogenicity of the pathogen through Hsp production, and stimulation or inhibition of components of the immune response.[60] In accordance with the latter aspect, we can speculate that the heat shock response elicited during fever would enhance FasR-mediated destruction of infected cells by cytotoxic T lymphocytes. Furthermore, downregulation of the immune response is also dependent on FasR-induced cell death. Since we have shown that heat shock can sensitize a T cell model to FasR killing, we can speculate that fever could also be involved in regulating the destruction of T cells by activation-induced apoptosis.

It has long been known that significant numbers of spontaneous remission in cancer patients coincide with feverish infection.[61] Furthermore, hyperthermia has been shown to improve treatments in combination with radio- or chemotherapy in clinical trials (reviewed in Hildebrandt *et al*.[62]). As the mechanism for the regression of the tumors is not known, we can hypothesize that heat-induced down-regulation of FLIP could facilitate elimination of cancer cells. Indeed, constitutive FLIP expression has been implicated in tumor progression through escape from DR-induced cell death.[63,64] Recently, high-level expression of FLIP was detected in Fas-resistant Hodgkin's disease malignant cells, the expression of which was not affected by cycloheximide treatment.[65] In the light of these new data, it would be highly interesting to investigate how heat shock downregulates FLIP, and if this could assist in abrogating the enhanced resistance from DR-induced apoptosis seen in tumor cells.

Materials and Methods

Cell lines and plasmids

HeLa and Jurkat cells were obtained from ATCC, and cultured in Dulbecco modified Eagle's medium and RPMI (Sigma-Aldrich, St. Louis, MO, USA), respectively, supplemented with 10% inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, in a humidified incubator with 5% CO_2 in air at 37°C. A Jurkat cell line expressing the reverse tetracycline-controlled transactivator (rtTA) was generated by electroporation-mediated transfection with the plasmid pUHD17201neo.[66] A Jurkat-rtTA clone was then transfected with the tetracycline-regulated dicistronic Hsp70/GFP expression plasmid pTR5-DC/Hsp70-GFP*tk/hygro.[33] Stably transfected clones were selected and screened as described previously.[33,67] GFP-tagged kinase-dead mouse MKK4a was prepared by releasing the kinase-dead fragment from plasmid pEBG-SEK1(K129R)[68] (a kind gift from John Kyriakis, Diabetes Research Laboratory, Massachusetts General Hospital, Charlestown, MA, USA) with *Bam*HI, and ligating it in-frame with the C-terminus of GFP into EGFP-C1 cut with *Bgl*II and *Bam*HI and treated with Shrimp Alkaline Phosphatase (USB, Amersham Pharmacia). To validate dominant negative action, COS7 cells were transfected with JNK plasmid alone or in combination with GFP-MKK4KD, and dominant negative activity was observed as prevention of cotransfected JNK activation by a 40-min treatment with 10 μg/ml anisomycin. Jurkat cells were transfected with DN-MKK4 by electroporation 48 h before performing the experiment.

Reagents and treatments

Treatments were carried out for the time periods described in the figure legends, with an agonistic anti-human FasR immunoglobulin M antibody (100 or 200 ng/ml, MBL, Watertown, MA, USA), recombinant FasL (a kind gift from Jürg Tschopp, Institute of Biochemistry, University of Lausanne, Switzerland), 20 μM z-VAD-fmk, 50 μM z-IETD-fmk, or 50 μM z-DEVD-fmk (Sigma-Aldrich). Fas-induced apoptosis was measured in samples treated with a range of concentrations (1–100 μM) of caspase inhibitors to determine the concentration needed to inhibit all apoptosis. In all experiments, cells were incubated with caspase inhibitors 1 h prior to other treatments. For heat shock, culture dishes were sealed with parafilm and immersed in a water bath at 42°C for 30 min if not otherwise mentioned. Controls were left in the incubator at 37°C. Tests were made with sealed and unsealed controls to ensure that there was no difference. After heat shock, cells were either harvested or returned to the incubator for recovery and treatments.

Immunoblotting

For Western blot, cells were lysed in RIPA buffer (PBS pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM PMSF, DTT, complete protease inhibitor cocktail (Roche, Basel, Switzerland). A total of 20–50 μg of proteins was subjected to SDS-PAGE and transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), blocked in PBST (PBS, 0.1% Tween-20) with 5% nonfat milk, and incubated overnight with anti-caspase-8, anti-FLIP (C15 (Scaffidi *et al*.[69]) and NF6 (Scaffidi *et al*.[13]), respectively, were obtained from Peter Krammer, (German Cancer Research Center, Heidelberg, Germany), anti-phospho-p44/42-MAPK (New England Biolabs, Boston, MA, USA), anti-Hsp70 (4g4, Affinity Bioreagents, Golden, CO, USA) antibodies. After washes in PBST, the membranes were incubated for 1 h with the appropriate horseradish peroxidase-coupled secondary antibody (PIERCE, Amersham). Detection was performed by enhanced chemiluminescence reaction (ECL, Amersham). For loading controls, membranes were stripped in PBS with 1% NP-40, and probed as described with anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Hsc70 (StressGen, Victoria, Canada) antibodies.

Electrophoretic mobility shift assay

Cells were washed in cold PBS, lysed by freeze–thaw in buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES) containing PMSF and DTT (0.5 mM each), and supernatant was recovered by centrifugation at 4°C. Whole-cell extract (15 μg proteins) was incubated with a ^{32}P-labeled oligonucleotide probe corresponding to the consensus heat shock element.[70] The protein–DNA complexes were then resolved on 4% polyacrylamide native gel electrophoresis.

Kinase assay

ERK2 and JNK were immunoprecipitated from 400 μl RIPA lysates (6×10^6 cells/sample) by incubation with anti-ERK2 and anti-JNK antibody coupled to protein-A-Sepharose. Immunoprecipitates were washed three times in RIPA buffer. ERK2 immunoprecipitates were then washed three times in ERK assay buffer (10 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT). JNK immunoprecipitates were additionally washed three times with LiCl buffer (500 mM LiCl, 100 mM Tris pH 7.6, 0.1% Triton X-100, 1 mM DTT) and three times with JNK assay buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT). The kinase reactions were carried out in 120 μl kinase assay buffer containing 25 μM ATP, 2.5 μCi $^{32}P\gamma$ ATP and 1 mg/ml myelin basic protein or GST-c-Jun, respectively, as substrates, for 15 min at 37°C. Reactions were stopped by addition of 3 × Laemmli sample buffer and the samples were resolved by SDS-PAGE, analyzed on a phosphoimager (BioRad), and autoradiographed.

Quantification of apoptosis

Apoptotic Jurkat cells were detected by Annexin-V staining. Untransfected Jurkat cells were incubated with Annexin-V-FITC, and GFP-expressing cells were incubated with Annexin-V-PE, in medium and Annexin binding buffer for 15 min on ice. Samples were run on FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA). HeLa cell death was quantified by the MTT viability assay. After treatment of HeLa cells on 96-well plates, the medium was replaced with fresh medium containing 1 mM MTT (Sigma-Aldrich) and incubated for 2–4 h at 37°C before washing and solubilizing the formazan with 50 μl DMSO. Results were measured at 540 nm on a plate reader.

Caspase-3 assay was performed on RIPA cell extract without protease inhibitor, using the homogeneous time-resolved fluorescence quenching assay LANCE kit for caspase-3 (Perkin-Elmer Life Science, Turku, Finland), as described by the manufacturer. Results were measured on VICTOR (Perkin-Elmer Life Science).

Microscopy

Transfected cells were fixed with 3% paraformaldehyde in PBS for 30 min, printed on coverslips using a cytospin, and DNA-stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). After 3 washes, cells were mounted in Mowiol (Sigma-Aldrich) and visualized with a fluorescence microscope (Leica, Wetzlar, Germany). Tet-ON cell lines were treated with 1 μg/ml doxycycline (Sigma-Aldrich), resulting in a broad range of GFP induction, as detected by fluorescence microcopy. Hsp70 expression, assessed by cell staining with anti-Hsp70 antibody, correlated well with GFP-fluorescence intensity. To visualize cells retaining mitochondrial membrane potential, tetramethyl rhodamine ester (TMRM, 200 nM, Molecular Probes) was added to the medium, together with Hoechst 33342 (Molecular Probes). Pictures were taken by confocal microscopy (Leica TCS40) on the live cells at different time points after treatment with 200 ng/ml anti-Fas.

Acknowledgments

We thank Peter Krammer (German Cancer Research Center, Heidelberg, Germany) for caspase-8 and FLIP antibodies, Jürg Tschopp (Institute of Biochemistry, University of Lausanne, Switzerland) for Fas ligand, and John Kyriakis (Diabetes Research Laboratory, Massachusetts General Hospital, Charlestown, MA, USA) for the SEK1 plasmid. We also thank the other members of our laboratories for technical help and critical comments on the manuscript. Financial support from the Academy of Finland, Finnish Cancer Organizations, Sigrid Jusélius Foundation, Erna and Victor Hasselblad Foundation, Nordic Academy for Advanced Study (NorFA), Magnus Ehrnrooth Foundation, Åbo Akademi University, and University of Turku is gratefully acknowledged. SEFT was supported by the Turku Graduate School of Biomedical Sciences.

References

1. Jolly C and Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J. Natl. Cancer Inst. 92: 1564–1572.
2. Ashkenazi A and Dixit VM (1998) Death receptors: signaling and modulation. Science 281: 1305–1308.
3. Ashkenazi A and Dixit VM (1999) Apoptosis control by death and decoy receptors. Curr. Opin. Cell Biol. 11: 255–260.
4. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S and Peter M (1998) Apoptosis signaling by death receptors. Eur. J. Biochem. 254: 439–459.
5. Hsu H, Shu HB, Pan MG and Goeddel DV (1996) TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84: 299–308.
6. Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV and Mak TW (1998) FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279: 1954–1958.
7. Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem. J. 326: 1–16.
8. Sun X, Lee J, Navas T, Baldwin DT, Stewart TA and Dixit VM (1999) RIP3, a novel apoptosis-inducing kinase. J. Biol. Chem. 274: 16871–16875.
9. Stanger BZ, Leder P, Lee TH, Kim E and Seed B (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 81: 513–523.
10. Chang HY, Nishitoh H, Yang X, Ichijo H and Baltimore D (1998) Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. Science 281: 1860–1863.
11. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J, Schroter M, Burns B, Mattmann C, Rimoldi D, French LE and Tschopp J (1997) Inhibition of death receptor signals by cellular FLIP. Nature 388: 190–195.
12. Liu ZG, Hsu H, Goeddel DV and Karin M (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 87: 565–576.
13. Scaffidi C, Schmitz I, Krammer PH and Peter ME (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. J. Biol. Chem. 274: 1541–1548.
14. Tran SE, Holmström TH, Ahonen M, Kähäri VM and Eriksson JE (2001) MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. J. Biol. Chem. 276: 16484–16490.
15. Holmström TH, Tran SE, Johnson V, Ahn N, Chow SC and Eriksson J (1999) Inhibition of mitogen-activated kinase signaling sensitizes HeLa cells. Mol. Cell. Biol. 19: 5991–6002.
16. Chen YR, Wang X, Templeton D, Davis RJ and Tan TH (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma

radiation. Duration of JNK activation may determine cell death and proliferation. J. Biol. Chem. 271: 31929–31936.
17. He T, Stepulak A, Holmström TH, Omary MB and Eriksson JE (2002) The intermediate filament protein keratin 8 is a novel cytoplasmic substrate for c-Jun N-terminal kinase. J. Biol. Chem. 277: 10767–10774.
18. Lindquist S and Craig EA (1988) The heat-shock proteins. Annu. Rev. Genet. 22: 631–677.
19. Hartl FU and Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295: 1852–1858.
20. Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes. Dev. 12: 3788–3796.
21. Wu C (1995) Heat shock transcription factors: structure and regulation. Annu. Rev. Cell Dev. Biol. 11: 441–469.
22. Pirkkala L, Nykanen P and Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15: 1118–1131.
23. Holmberg CI, Tran SE, Eriksson JE and Sistonen L (2002) Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem. Sci. 27: 619–627.
24. Kyriakis JM and Avruch J (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. J. Biol. Chem. 271: 24313–24316.
25. Gerner EW and Schneider MJ (1975) Induced thermal resistance in HeLa cells. Nature 256: 500–502.
26. Mosser DD and Martin LH (1992) Induced thermotolerance to apoptosis in a human T lymphocyte cell line. J. Cell Physiol. 151: 561–570.
27. Samali A, Robertson JD, Peterson E, Manero F, van Zeijl L, Paul C, Cotgreave IA, Arrigo AP and Orrenius S (2001) Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. Cell Stress Chaperones 6: 49–58.
28. Gabai VL, Meriin AB, Mosser DD, Caron AW, Rits S, Shifrin VI and Sherman MY (1997) Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. J. Biol. Chem. 272: 18033–18037.
29. Mosser DD, Caron AW, Bourget L, Denis-Larose C and Massie B (1997) Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol. Cell. Biol. 17: 5317–5327.
30. Meriin AB, Yaglom JA, Gabai VL, Zon L, Ganiatsas S, Mosser DD and Sherman MY (1999) Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. Mol. Cell. Biol. 19: 2547–2555.
31. Saleh A, Srinivasula SM, Balkir L, Robbins PD and Alnemri ES (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat. Cell Biol. 2: 476–483.
32. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM and Green DR (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat. Cell Biol. 2: 469–475.
33. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI and Massie B (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol. Cell. Biol. 20: 7146–7159.
34. Jäättelä M, Wissing D, Kokholm K, Kallunki T and Egeblad M (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J. 17: 6124–6134.
35. Gabai VL, Mabuchi K, Mosser DD and Sherman MY (2002) Hsp72 and stress kinase c-jun N-terminal kinase regulate the bid-dependent pathway in tumor necrosis factor-induced apoptosis. Mol. Cell. Biol. 22: 3415–3424.
36. Jäättelä M (1993) Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J. Immunol. 151: 4286–4294.
37. Jäättelä M (1999) Escaping cell death: survival proteins in cancer. Exp. Cell Res. 248: 30–43.
38. Liossis SN, Ding XZ, Kiang JG and Tsokos GC (1997) Overexpression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat T cells. J. Immunol. 158: 5668–5675.
39. Xia W, Voellmy R and Spector NL (2000) Sensitization of tumor cells to fas killing through overexpression of heat-shock transcription factor 1. J. Cell. Physiol. 183: 425–431.
40. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103: 239–252.
41. Arrigo AP (1998) Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Biol. Chem. 379: 19–26.
42. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA and Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. Science 288: 870–874.
43. Yang D, Tournier C, Wysk M, Lu HT, Xu J, Davis RJ and Flavell RA (1997) Targeted disruption of the MKK4 gene causes embryonic death, inhibition of c-Jun NH2-terminal kinase activation, and defects in AP-1 transcriptional activity. Proc. Natl. Acad. Sci. USA 94: 3004–3009.
44. Reap EA, Roof K, Maynor K, Borrero M, Booker J and Cohen PL (1997) Radiation and stress-induced apoptosis: a role for Fas/Fas ligand interactions. Proc. Natl. Acad. Sci. USA 94: 5750–5755.
45. Faris M, Kokot N, Latinis K, Kasibhatla S, Green DR, Koretzky GA and Nel A (1998) The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. J. Immunol. 160: 134–144.
46. Carmody RJ and Cotter TG (2000) Oxidative stress induces caspase-independent retinal apoptosis *in vitro*. Cell Death Differ. 7: 282–291.
47. Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M and Jäättelä M (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J. Cell Biol. 153: 999–1010.
48. Leist M and Jäättelä M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat. Rev. Mol. Cell Biol. 2: 589–598.
49. Cande C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N and Kroemer G (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie. 84: 215–222.
50. Mathiasen IS and Jäättelä M (2002) Triggering caspase-independent cell death to combat cancer. Trends Mol. Med. 8: 212–220.
51. Krueger A, Schmitz I, Baumann S, Krammer PH and Kirchhoff S (2001) Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J. Biol. Chem. 276: 20633–20640.
52. Kataoka T, Ito M, Budd RC, Tschopp J and Nagai K (2002) Expression level of c-FLIP *versus* Fas determines susceptibility to Fas ligand-induced cell death in murine thymoma EL-4 cells. Exp. Cell Res. 273: 256–264.
53. Gross A, McDonnell J and Korsmeyer S (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13: 1899–1911.
54. Creagh EM, Carmody RJ and Cotter TG (2000) Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. Exp. Cell Res. 257: 58–66.
55. Tibbles LA and Woodgett JR (1999) The stress-activated protein kinase pathways. Cell. Mol. Life Sci. 55: 1230–1254.
56. Han DK, Chaudhary PM, Wright ME, Friedman C, Trask BJ, Riedel RT, Baskin DG, Schwartz SM and Hood L (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. Proc. Natl. Acad. Sci. USA 94: 11333–11338.
57. Shu HB, Halpin DR and Goeddel DV (1997) Casper is a FADD- and caspase-related inducer of apoptosis. Immunity 6: 751–763.
58. Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME and Yang X (2002) c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 21: 3704–3714.
59. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C and Grutter MG (2002) The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J. Biol. Chem. 277: 45162–45171.
60. Hasday JD and Singh IS (2000) Fever and the heat shock response: distinct, partially overlapping processes. Cell Stress Chaperones 5: 471–480.
61. Hobohm U (2001) Fever and cancer in perspective. Cancer Immunol. Immunother. 50: 391–396.
62. Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R and Riess H (2002) The cellular and molecular basis of hyperthermia. Crit. Rev. Oncol. Hematol. 43: 33–56.
63. Medema JP, de Jong J, van Hall T, Melief CJ and Offringa R (1999) Immune escape of tumors *in vivo* by expression of cellular FLICE-inhibitory protein. J. Exp. Med. 190: 1033–1038.
64. Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P and Grandien A (1999) The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J. Exp. Med. 190: 1025–1032.

65. Thomas RK, Kallenborn A, Wickenhauser C, Schultze JL, Draube A, Vockerodt M, Re D, Diehl V and Wolf J (2002) Constitutive expression of c-FLIP in Hodgkin and Reed-Sternberg cells. Am. J. Pathol. 160: 1521–1528.
66. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W and Bujard H (1995) Transcriptional activation by tetracyclines in mammalian cells. Science 268: 1766–1769.
67. Caron AW, Massie B and Mosser DD (2000) Use of a micromanipulator for high-efficiency cloning of cells co-expressing fluorescent proteins. Methods Cell Sci. 22: 137–145.
68. Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM and Zon LI (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 372: 794–798.
69. Scaffidi C, Medema JP, Krammer PH and Peter ME (1997) FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J. Biol. Chem. 272: 26953–26958.
70. Mosser DD, Theodorakis NG and Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol. Cell. Biol. 8: 4736–4744.