Human Fas-associated Factor 1 Interacts with Heat Shock Protein 70 and Negatively Regulates Chaperone Activity

Received for publication, June 7, 2004, and in revised form, November 27, 2004
Published, JBC Papers in Press, December 13, 2004, DOI 10.1074/jbc.M406297200

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We examined the cell death-inducing property of human Fas-associated factor 1 (hFAF1) in the heat shock signaling pathway. By employing co-immunoprecipitation and peptide mass fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, we found that hFAF1 binds to the 70-kDa heat shock protein family (Hsc70/Hsp70). Interaction mapping indicated that the 82–180 sequence of hFAF1 directly binds to the N-terminal region containing sequence 1–120 of Hsc70/Hsp70. This binding is very tight regardless of ATP and heat shock treatment. Hsc70/Hsp70 and hFAF1 co-localized in the cytosol and nucleus and concentrated to the perinuclear region by heat shock treatment. We examined how hFAF1 regulates Hsp70 function, and found that hFAF1 inhibited the Hsp70 chaperone activity of refolding denatured protein substrates, accelerated heat shock-induced SAPK/JNK activation, and raised heat shock-induced cell death in a binding dependent manner. These results suggest that hFAF1 prevents cells from recovery after stress by binding to and inhibiting the chaperone activity of Hsp70.

Fas-associated factor 1 (FAF1) was first identified as a binding protein to the Fas cytoplasmic region in the yeast two-hybrid screen in mouse. Transient overexpression of mouse FAF1 (mFAF1) enhances Fas-induced apoptosis in L cells (1). Unlike mFAF1, human FAF1 (hFAF1) initiates apoptosis in BOSC23 cells only by transient overexpression. hFAF1 binds to Fas through amino acid sequence 1–201 (2). Other than Fas, casein kinase 2 subunit (CK2) is the FAF1-binding molecule reported (3). hFAF1 is phosphorylated by CK2 on 289 and 291 serine residues, and the hFAF1-CK2 complex formation increases when apoptosis occurs (4, 5). Recently, hFAF1 was reported as a member of the Fas death-inducing signaling complex by interacting with Fas-associated via death domain and caspase-8 (6) and as a suppressor of NF-κB activity by cytoplasmic retention of NF-κB p65 via physical interaction (7).

Heat shock protein 70 (Hsp70) participates in the folding of newly synthesized proteins, translocation of intracellular proteins, assembly and disassembly of oligomeric protein structures, proteolytic degradation of denatured proteins, and in controlling the activity of regulatory proteins (8–10). As Hsp70 exerts its various roles through binding to various chaperone cofactors or co-chaperones, the fate of substrate proteins is determined by the nature of the co-chaperones. In Escherichia coli, the Hsp70 homologue, DnaK, is shown to be assisted by two co-chaperones, DnaJ, which yields the high substrate affinity form for substrate binding, and GrpE, which accelerates release of substrates (11, 12). In mammalian systems, where several co-chaperones have been identified, a homologue of DnaJ, Hsp40/Hdj-1, and Hsc70-interacting protein (Hip/p48) was shown to increase the affinity for substrate protein, thus preventing aggregation of denatured proteins (13, 14). The Hsc70-Hsp90-organizing protein (Hop/p60/Sti1) interacts with the C-terminal domain of Hsc70 and serves as an adaptor molecule that forms an Hsc70-Hop-Hsp90 complex, without affecting the chaperone activity of Hsc70 (15–17). BAG-1, which is known to bind to Bcl-2 and thus exerts anti-apoptotic activity, binds to the ATPase domain of Hsp70 and attenuates Hsc70 chaperone activity (9, 18, 19). The C terminus of Hsc70-interacting protein (CHIP) inhibits its ability to refold non-denatured proteins (20). Many co-chaperones were identified containing the Hsp70 binding domain and ubiquitin-like or U box domain, but even the functions of each domain were not well understood (21).

We found that hFAF1 is a new Hsc70/Hsp70-binding protein employing immunoprecipitation and identification of the bound proteins using MALDI-TOF MS. We examined whether hFAF1 regulates the chaperone activity through binding to chaperones in the heat shock-mediated signaling pathway because hFAF1 contains two ubiquitin-like domains and one ubiquitin-like module (UBX). Transient overexpression of hFAF1 inhibits chaperone activity of Hsp70, suggesting that hFAF1 is possibly a novel co-chaperone of Hsp70. We postulate that hFAF1 plays a role in the regulation of stress-induced cell death using Hsp70 as a binding partner.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length human hsp70 was subcloned into the pGEX-4T-1 vector or 3′-FLAG-CMV-7.1 vector pGEX-4T-1/Hsp70ΔABD (1–119, 429–640). The construct pGEX-4T-1/Hsp70N(1–119) was prepared by PCR using the sense primer 5′-GAAGAATTCTACGCGGCAA-AAGCCGGCGCAG-3′ and antisense primer 5′-GACATTCGAGGCGAT-ATCTCCTGGGTA-3′ and was subcloned into the pGEX-4T-1 vector.
pGEX-4T-1/Hsp70O(N-(120–640) was prepared by PCR using sense primer 5'-GGAAAAACTTGGTTATTTTGG-3' and antisense primer 5'-GCTGGTCAGGACAGGTTCTTT-3' and was subcloned into pGEX-4T-1 vector. pGEX-4T-1/Hsp70O(PBD-(1–435), 619–640) was prepared as described previously (22). The constructs pFLAG-CMV-2/HFAF1, pFLAG-CMV-2/HFAF1-(1–201), pFLAG-CMV-2/HFAF1-(1–345), pFLAG-CMV-2/HFAF1-(366–650) (hFAF1N), pFLAG-CMV-2/HFAF1-(181–381), and pCDINaHFAF1 were prepared as described previously (2).

RESULTS

Homozygous mutant Hck293T cells were transfected with expression plasmid using the calcium phosphate precipitation method. Cells were seeded in 10-cm plates a day before transfection at the density of 1.5 × 10^6 cells and transiently transfected with 6–12 µg of expression plasmid by the calcium phosphate method. The fresh medium was changed at 6 h after transfection and was cultured for an additional 18 h.

Materials and Immunoprecipitation—Cells were metabolically labeled with 2 µCi [35]S]methionine in methionine half-free medium for 18 h. Cells were disrupted with a buffer containing protease inhibitors (50 µM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 µg/ml aprotinin, 1 µg/ml leupeptin, 5 mM Na3VO4, 5 mM NaF) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 1 h, and the supernatant was incubated for 3 hours at 4 °C with monoclonal anti-FLAG M2 antibody purchased from Sigma. Monoclonal anti-Hsc70/Hsp70 antibody was obtained from StressGen. Polyclonal anti-hFAF1 antibody was generated and characterized in our group.

Microscopy—HEK293T cells were transfected with expression plasmid using the calcium phosphate precipitation method. Cells were seeded in 10-cm plates a day before transfection at the density of 1.5 × 10^6 cells and transiently transfected with 6–12 µg of expression plasmid by the calcium phosphate method. The fresh medium was changed at 6 h after transfection and was cultured for an additional 18 h.

Molecular and Immunoprecipitation—Cells were metabolically labeled with 2 µCi [35]S]methionine in methionine half-free medium for 18 h. Cells were disrupted with a buffer containing protease inhibitors (50 µM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 µg/ml aprotinin, 1 µg/ml leupeptin, 5 mM Na3VO4, 5 mM NaF) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 1 h, and the supernatant was incubated for 3 hours at 4 °C with monoclonal anti-FLAG M2 antibody purchased from Sigma. Monoclonal anti-Hsc70/Hsp70 antibody was obtained from StressGen. Polyclonal anti-hFAF1 antibody was generated and characterized in our group.

In Vitro Binding Assay—GST-Hsp70 deletion mutant expressed E. coli cytosolic GST was prepared with glutathione-Sepharose 4B beads in phosphate-buffered saline containing 0.1% Triton X-100 for 3 h at 4 °C. The beads were washed three times with phosphate-buffered saline containing 0.1% Triton X-100. Binding assays were performed with Hsp70 deletion mutants bound to glutathione-Sepharose 4B beads and hFAF1 cleaved by thrombin from GST-hFAF1 in binding buffer (50 mM NaCl with 1 mg of bovine serum albumin per ml) with rocking for 3 h at 4 °C. The beads were washed five times with phosphate-buffered saline containing 0.5% Nonidet P-40, resuspended in 2× gel sample buffer, resolved with SDS-PAGE, and Coomassie Blue-stained. After obtaining the gel image, the proteins in the gel were transferred to polyvinylidene difluoride membrane and immunologically probed with anti-hFAF1 antibody.

Confocal Microscopy—For morphological studies, cells were grown on coverslips, transiently transfected, and treated with heat shock. The cells were gently rinsed in HBSS and fixed with 4% paraformaldehyde in HBSS for 10 min at room temperature. After washing with HBSS, the cells were permeabilized by incubating with 0.1% Triton X-100 in HBSS for 10 min at room temperature before immunostaining. Non-specific protein binding was inhibited by incubation of the cells for 1 h in HBSS containing 3% bovine serum albumin, 0.2% Tween 20, and 0.2% gelatin. For Hsc70/Hsp70 staining, the cells were incubated with mouse monoclonal Hsc70/Hsp70 antibody (Stressgen) diluted at 1:150 in HBSS containing 1% sucrose and 1% bovine serum albumin for 2 h at 37 °C. After washing three times with HBSS, the cells were incubated with Texas Red-conjugated rabbit anti-mouse (Molecular Probes) diluted at 1:200 and subsequently washed with HBSS. FLAG-hFAF1 was stained with FITC-labeled monoclonal M2 anti-FLAG antibody diluted at 1:200 for 1 h at room temperature. Confocal microscopy was performed with a Triple photomicroscope (Zeiss) and a Multichannel Analyzer (Hamamatsu Photonics). Images were captured with a cooled charge-coupled device camera (CoolSnap-Digital Camera System; Roper Scientific) and processed using NIH Image software.
ern blot analysis of the NC membrane with monoclonal anti-
Hsc70/Hsp70 antibody (Fig. 1C). To confirm further the
binding, cell lysate from the FLAG-hFAF1-overexpressed cell
line was separated on gel filtration column chromatography,
and the binding complex of hFAF1 and Hsc70/Hsp70 was eluted at the 250–300-kDa fraction (data not shown).

To see if endogenous hFAF1 interacts with endogenous
Hsc70/Hsp70 in vivo, we performed immunoprecipitation studies
with polyclonal anti-hFAF1 antibody in HEK293T cells.
Fig. 2 shows that endogenous Hsc70/Hsp70 exists in the hFAF1
protein complex in vivo, and a small fraction of endogenous
Hsc70/Hsp70 could bind hFAF1. In addition, the recombinant
hFAF1 expressed in E. coli can bind to bacterial Hsp70, DnaK
(data not shown), suggesting that hFAF1 has intrinsic binding
property to Hsp70.

Hsc70/Hsp70 Interacts with the Amino Acids 82–180 of
hFAF1 in Vivo—To characterize the hFAF1 domains necessary
for interaction with Hsp70, we examined binding between en-
dogenous Hsc70/Hsp70 and a series of transiently transfected
FLAG-hFAF1 deletion mutants (Fig. 3A). HEK293T cells
were transfected with various FLAG-tagged hFAF1 deletion mu-
tants and immunoprecipitated by anti-FLAG M2-agarose
cross-linking affinity gel. The precipitates were analyzed by SDS-PAGE and Western
blot analysis using anti-FLAG antibody (Fig. 3B, upper panel)
and anti-hFAF1 polyclonal antibody (Fig. 3B, lower panel).

We examined whether the interaction between
Hsp70 and hFAF1—hFAF1 and Hsc70/Hsp70

Heat Shock Does Not Affect the Interaction between Hsp70
and hFAF1—We examined whether the interaction between
hFAF1 and Hsc70/Hsp70 in vivo could be regulated by heat
shock. HEK293T cells transiently transfected with pFLAG-CMV-2 vector or FLAG-tagged hFAF1 were heat-shocked at 45 °C for 45 min, recovered for various times at 37 °C, and immunoprecipitated with anti-FLAG M2-agarose cross-linking
affinity gel. Immunoprecipitates were analyzed by Western
blot analysis using anti-Hsc70/Hsp70 (Fig. 5, upper panel) and
anti-FLAG M2 antibody (Fig. 5, lower panel). When we quan-
titatively analyzed the ratio of Hsc70/Hsp70 to FLAG-hFAF1
in a linear range using LAS1000 (Fujifilm), Hsc70 interactions did
not change during recovery after heat shock, and even the
amino acids 82–180 of hFAF1 is necessary for binding with Hsc70/Hsp70. This
was confirmed in Fig. 3C. The amino acids 82–180 of hFAF1
bound to endogenous Hsc70/Hsp70. This region includes the
Fas death domain-binding domain (amino acid residues 1–201)
(23).

hFAF1 Binds Directly to the N-terminal Domain of Hsp70 in
Vitro—To examine whether hFAF1 might be associated di-
rectly or indirectly with Hsp70, in an accessory protein-medi-
ated process, we carried out in vitro pull down assay. We
prepared various truncated forms of GST-Hsp70 (Fig. 4A) as
follows: full Hsp70, 1–640; 1–120; 120–640; 1–119; 428–640;
1–435; and 616–640. Various recombinant GST-Hsp70s were
immobilized to glutathione-Sepharose beads and incubated
with purified hFAF1 in the presence of 10 mM ATP. We found
direct interaction between hFAF1 and GST-Hsp70-(1–120) by
immunoblotting with anti-hFAF1 antibody (Fig. 4B). In the
presence of various concentrations of ATP, the bindings be-
tween Hsp70 and hFAF1 were not affected (Fig. 4C). These
results suggest that hFAF1 directly interacts with the N ter-
minus of Hsp70 and that the binding was not affected by
ATP-induced conformational changes of Hsp70.

Subcellular Localization of hFAF1 and Hsc70/Hsp70 Was
Changed by Heat Shock—Because the interaction between
hFAF1 and Hsc70/Hsp70 was unaffected by ATP and stress,
the question arises whether the localization of the binding

**TABLE 1**

| Spot no. | Identified protein | NCBI GI no. | Mass (kDa) | pI | Peptide coverage |
|----------|-------------------|-------------|------------|----|-----------------|
| 1        | Hsc70             | 13273304    | 70.899     | 5.4 | 29              |
| 2        | Hsp70             | 386785      | 69.869     | 5.4 | 30              |

**FIG. 2. Endogenous hFAF1 interacts with endogenous Hsc70/Hsp70.** HEK293T cells were lysed and immunoprecipitated (IP) with mouse IgG as control (1st lane) and anti-hFAF1 polyclonal antibody (2nd lane). The precipitates were analyzed by SDS-PAGE and Western blot analysis by using anti-hFAF1 polyclonal antibody (upper panel) and anti-Hsc70/Hsp70 monoclonal antibody (lower panel). Cell lysates after immunoprecipitation were analyzed by Western blot analysis using anti-hFAF1 and anti-Hsc70/Hsp70 antibodies.
region, depending on heat shock and recovery conditions.

hFAF1 Inhibits Hsp70-mediated Reactivation of Heat-denatured Firefly Luciferase in vivo—Previous reports (24, 25) showed the activity of transiently expressed luciferase in cells can serve as a marker of chaperone activity of Hsp70 in vivo. To examine whether FLAG-tagged Hsp70 accelerates the reactivation of heat-denatured luciferase in vivo, we transiently transfected FLAG-tagged Hsp70 or Hsp70 ATP binding domain deletion mutant (Hsp70ΔABD-1{1-119} and 429-640) in HEK293T cells. We co-expressed firefly luciferase, whose enzymatic activity during recovery from thermal inactivation is highly dependent on chaperone activity. The overexpression of each protein was confirmed by Western blot analysis using anti-Hsp70 antibody (Fig. 7A), because we had problems detecting FLAG-Hsp70 with anti-FLAG antibody. The cells were heat-shocked at 45 °C for 15 min and recovered for the indicated times at 37 °C, harvested, and lysed, and luciferase assay was performed (Fig. 7B). Overexpression of Hsp70 alone is sufficient to enhance reactivation of luciferase activity during recovery at 37 °C. However, in Hsp70ΔABD-transfected cells, the reactivation of luciferase was slower than in Hsp70-expressed cells but faster than in control cells, suggesting that heat shock-inactivated luciferase was reactivated by the chaperone activity of Hsp70 and that ATP binding domain is not essential for chaperone activity in vivo.

To examine the effect of hFAF1 on the chaperone activity of endogenous Hsp70, HEK293T cells were transfected with various amounts of FLAG-tagged hFAF1. The overexpression of FLAG-tagged hFAF1 was confirmed by Western blot analysis using anti-FLAG antibody (Fig. 7C). The reactivation of luciferase activity was monitored during recovery at 37 °C after heat shock. Transfection of FLAG-CMV-2 vector plasmid alone caused basal level reactivation of heat-denatured cytoplasmic luciferase after 4 h at 37 °C, whereas in the presence of FLAG-hFAF1, luciferase activity was significantly reduced. Overexpression of hFAF1 significantly reduced the reactivation rate of luciferase activity in a dose-dependent manner, which indicates the inhibitory effect of hFAF1 on the chaperone activity of endogenous Hsp70 (Fig. 7D).

To confirm that the inhibitory effect of hFAF1 is through binding to endogenous Hsp70, HEK293T cells were transfected with FLAG-tagged hFAF1 or a deletion mutant, amino acid sequence 366–650 of FLAG-hFAF1 (FLAG-hFAF1ΔN), lacking the N-terminal domain required for Hsp70 interaction without Hsp70 overexpression. Expression of FLAG-hFAF1 and FLAG-hFAF1ΔN was monitored by Western blot analysis (Fig. 7E). In the presence of FLAG-hFAF1, luciferase activity was significantly reduced; however, expression of FLAG-hFAF1ΔN did not inhibit Hsp70 chaperone activity (Fig. 7F). To confirm the dominant negative-like effect of the overexpression of FLAG-hFAF1ΔN, hFAF1 lacking the N-terminal domain required for Hsp70 interaction in Fig. 7F, we transfected 1 and 2 μg of FLAG-hFAF1ΔN vector DNA in HEK293T cells. Transfection of FLAG-hFAF1ΔN increased the renaturation activity of Hsp70 in a dose-dependent manner (data not shown). To examine this effect on overexpressed Hsp70, we co-transfected a small amount of Hsp70 (0.3 μg DNA) with 1.0 μg of DNA of FLAG control, FLAG-hFAF1ΔN, or hFAF1 (Fig. 7G). Overexpression of Hsp70 alone increased the renaturation activity compared with FLAG control. Co-transfection of FLAG-hFAF1 with Hsp70 significantly reduced the higher basal luciferase activity; however, expression of FLAG-hFAF1ΔN did not inhibit Hsp70 chaperone activity (Fig. 7H). These studies establish that hFAF1 acts as an inhibitor of the chaperone activity of Hsp70 and that the N-terminal residues of hFAF1 play a role in this inhibition of Hsp70 chaperone activity.
Overexpression of hFAF1 Increases Cell Sensitivity to Heat Shock Stress—Activation of SAPK/JNK is required for stress-induced apoptosis, and Hsp70 overexpression prevents apoptosis by suppressing the activation of SAPK/JNK (26–28). We have shown that cells being tolerant to stress by expressing the elevated Hsp70 were less sensitive to stress, activated the

![Diagram A]

**FIG. 4.** hFAF1 directly interacts with the N-terminal domain of Hsp70 in vitro. A, diagram of GST-tagged Hsp70 fragments. B, purified recombinant hFAF1 was incubated with GST-Hsp70 deletion mutants immobilized on glutathione-Sepharose in the presence or absence of 10 mM ATP. Precipitates were detected by Coomassie Blue staining (B, upper panel) and immunoblotting using anti-hFAF1 polyclonal antibody (B, lower two panels). C, HEK293T cells were transiently transfected with FLAG-tagged hFAF1. Cells were lysed and incubated with monoclonal anti-FLAG M2 antibody and protein A-Sepharose 4B beads. Various concentrations of ATP were added to the immune complex and incubated for 1 h. Immunoprecipitates were analyzed by SDS-PAGE and Western blot analysis using anti-Hsc70/Hsp70 (upper panel) and anti-FLAG M2 (lower panel) monoclonal antibodies.

![Diagram B]

**FIG. 5.** Heat shock has no effect on the interaction between Hsc70/Hsp70 and hFAF1. HEK293T cells were transiently transfected with pFLAG-CMV-2 vector or FLAG-tagged hFAF1. Cells were heat-shocked at 45 °C for 45 min or untreated (C) and recovered for the indicated times. At each time point, cells were lysed and immunoprecipitated (IP) with monoclonal anti-FLAG M2 antibody. Precipitates were analyzed by Western blot analysis using anti-Hsc70/Hsp70 (upper panel) and anti-FLAG M2 (lower panel) monoclonal antibodies.
SAPK/JNK in response to a higher dose of stress, and had faster deactivation rate of SAPK/JNK in the same dose of stress. It would thus appear that kinetics of SAPK/JNK activation and deactivation can serve as a marker of cellular sensitivity to various stresses (29).

We then examined the effect of overexpression of hFAF1 on heat shock-induced cellular response, using SAPK/JNK activation as a marker. We transfected HEK293T cells with FLAG-CMV-2 vector, FLAG-tagged hFAF1, or FLAG-tagged hFAF1/H9004N. Transfected HEK293T cells were treated with heat shock at 45 °C for 45 min, and SAPK/JNK activities were measured (Fig. 8). The kinetics of SAPK/JNK activation in both control and FLAG-hFAF1/H9004N-expressing cells were similar; there was a 13-fold activation immediately after heat shock and deactivation to the basal level after a 7-h recovery. On the other hand, SAPK/JNK activity in FLAG-hFAF1-expressing cells increased 16-fold activation immediately after heat shock and lasted up to 2 h; deactivation was significantly delayed up to 7 h. This indicated that hFAF1-expressing cells were more sensitive to heat shock and were less able to recover. Also the hyper-activation of SAPK/JNK observed in heat-shocked FLAG-hFAF1-expressing cells was absent in heat-shocked FLAG-hFAF1ΔN-expressing cells. This suggests that overexpression of hFAF1 renders cells more sensitive to heat shock and more prone to apoptosis.

**hFAF1 Overexpression Increases Heat Shock-induced Cell Death**—To confirm the effect of hFAF1 on stress-induced cell death, we examined cell viability after heat shock. HEK293T cells transfected with FLAG-CMV-2 vector, FLAG-tagged hFAF1, FLAG-tagged hFAF1ΔN, or co-expression with Hsp70 were heat shocked at 45 °C for 45 min. After recovery at 37 °C for 24 h, the numbers of viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. FLAG-hFAF1-overexpressed HEK293T cells displayed reduced survival, compared with vector transfectants, in contrast to FLAG-hFAF1ΔN-transfected cells that showed enhanced survival (Fig. 9). This confirms that complexing with hFAF1 decreased the chaperone activity of Hsp70 and rendered the cells more sensitive to stress. FLAG-hFAF1ΔN-expressing cells, which lack of Hsp70 binding affinity, showed tolerance to heat shock. The same experiments were performed in the cells overexpressed with both Hsp70 and each hFAF1 in order to confirm the phenomena. Overexpression of Hsp70 slightly raised the resistance to stress, but the co-expression with hFAF1 increased the sensitivity to stress. FLAG-hFAF1ΔN co-expressing cells abolished the hFAF1 effect. This may be due to increased chaperone activity of Hsp70 or some unknown role of the C-terminal hFAF1. The results confirms that overexpression of hFAF1 renders cells more sensitive to heat shock and more prone to apoptosis by interacting with Hsp70.

**FAF1 Sequences Are Well Conserved in Vertebrates**—To determine whether FAF1 is well conserved through evolution, we aligned long and short forms of hFAF1s (23) with rat, mouse, quail, *Danio rerio*, *Caenorhabditis elegans*, and fly FAF1 as shown in the supplemental figure; the entire sequence of FAF1 is well conserved as well as the 82–180 amino acid region to which Hsp70 binds. It is 78–95% identical in vertebrates and 18–21% identical between vertebrates and invertebrates. This suggests that FAF1 plays an essential role throughout the vertebrates.
DISCUSSION

These studies demonstrate interaction between hFAF1 and Hsc70/Hsp70 and the functional significance of this interaction in heat shock-induced cell signaling. We previously identified the 1–201-amino acid region of hFAF1 as a Fas binding domain by using the yeast two-hybrid assay (23). The cellular function of hFAF1, a novel protein known to be an adaptor molecule of death receptor Fas, was identified as a member of the Fas death-inducing signaling complex (6) and as a suppressor of NF-κB activity by cytoplasmic retention of NF-κB p65 via physical interaction at the 181–381-amino acid region (7). In this study, by using two-dimensional gel electrophoresis and MALDI-TOF MS, gel filtration gel chromatography, and in vitro pull down assay, we have shown that the 82–180-amino acid region of hFAF1 directly interacts with the N terminus (amino acids 1–120) of Hsp70 in vivo. Hsp homologues
Hsp70 function. Our studies revealed that hFAF1 did not bind to the peptide binding domain, substrate-binding sites of Hsp70, but rather bound to N terminus of the ATPase domain of Hsp70, as co-chaperones Bag-1 and Hip bind to the ATPase domain. Also the expression level of hFAF1 was not varied in various stresses, indicating that hFAF1 is not a substrate of Hsp70. We therefore examined whether hFAF1 is involved in the regulation of Hsp70 activity. Transient overexpression of hFAF1 inhibited the chaperone activity of Hsp70, whereas transient expression of the N-terminally deleted hFAF1 mutant showed the opposite effect, indicating that hFAF1 may act as a co-chaperone and inhibit the chaperone activity of Hsp70. Recently, co-chaperones of Hsp70 such as Hsp40, Hip, Hop, Bag-1, CHIP, Chap1/PLIC-2, and Chap2/Bat3/Scythe have been identified. Hip and Hop facilitates refolding of unfolded proteins and Bag-1, CHIP, and Chap2/Bat3/Scythe attenuated the chaperone activity of Hsp70 (9, 14, 16, 17–20, 30). Because the latter proteins contain ubiquitin-like domains, which bind to proteasomes, they are thought to be involved in the degradation of Hsp70 substrates in proteasomes. Bag-1 contains a ubiquitin-like domain which binds to proteasome; CHIP has U box domain that has ubiquitin ligase activity, and Chap2/Bat3/Scythe also contains a ubiquitin-like domain. The whole sequence of FAF1 is 78–95% identical in vertebrates and 18–21% identical between vertebrates and invertebrates. It contains two ubiquitin-related domains, UAS and UBX (see the supplemental figure). This suggests that hFAF1 might play a role in protein degradation in combination with Hsp70. Our other data also suggest the involvement of hFAF1 in proteasome degradation.

To understand the biological function of hFAF1 reflected by its negative regulation of Hsp70 chaperone activity, we examined the effect of hFAF1 in heat shock responses. Hsp70 seems to play an important role in preventing programmed cell death in response to heat shock by suppressing the activation of SAPK/JNK (26, 28, 31). By using the kinetics of SAPK/JNK activation and deactivation as a marker of cellular sensitivity to stress, we found that the activation of SAPK/JNK in thermotolerant cells having elevated Hsp70 expression occurred at a higher dose of stress, and the deactivation in thermotolerant cells was much faster than in control (29). Recently we also reported that heat shock induced transient tyrosine phosphorylations of various proteins (32). As shown in Fig. 8, heat shock-induced SAPK/JNK activation was accelerated in cells overexpressing hFAF1, and the deactivation during recovery was significantly delayed compared with vector-transfected control cells. This effect was completely abolished by overexpression of the N-terminal truncated hFAF1, lacking affinity for Hsp70 binding. This indicates that overexpression of hFAF1 renders cells more sensitive to heat shock, probably due to inhibition of Hsp70 chaperone activity. It is possible that Hsp70 chaperone activity is related to the inhibition of SAPK/JNK activity induced by various stresses and to its anti-apoptotic effects. Volloch et al. (27) showed that ABD of Hsp70 is not necessary for the inhibition of SAPK/JNK. It seems that the chaperone activity of Hsp70, based on ATP hydrolysis as an energy source, does not affect SAPK/JNK activity. However, the relative roles of ABD and chaperone function of Hsp70 are not clear (33). In this study, the ABD deletion mutant of Hsp70 showed elevated chaperone activity, although lower than that of the full form of Hsp70, suggesting that ABD, having a smaller domain (amino acids 120–428) in this study than the conventional ABD (1–428), is not dispensable for the chap-

such as DnaK and DnaJ were co-purified during the purification of recombinant hFAF1 from the E. coli lysate.

Hsp70 plays an important protective role in stress-induced cell death. hFAF1 binding can affect this role of Hsp70 in two possible ways. One possibility is that hFAF1 may serve as a substrate and become refolded, degraded, or translocated by Hsp70. The second is that hFAF1 may act as modulator of SAPK/JNK activities were measured after the indicated recovery times.

**FIG. 8.** Overexpression of FLAG-hFAF1 accelerates heat shock-induced SAPK/JNK activation. HEK293T cells were transiently transfected with pFLAG-CMV-2 vector, pFLAG-CMV-2/hFAF1, or pFLAG-CMV-2/hFAF1ΔN and exposed to heat shock at 45 °C for 45 min. After recovery at 37 °C for 24 h, the percentage of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**FIG. 9.** Overexpression of FLAG-hFAF1 increases heat shock-induced cell death. HEK293T cells were transiently transfected with the pFLAG-CMV-2 vector, pFLAG-CMV-2/hFAF1, or pFLAG-CMV-2/hFAF1ΔN, co-expressed with 3FLAG-CMV-7.1/Hsp70 and each hFAF1, and exposed to heat shock at 45 °C for 45 min. After recovery at 37 °C for 24 h, the percentage of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

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2 E. J. Song, S. H. Yim, E. Kim, N. S. Kim, and K.-J. Lee, unpublished data.
hFAF1 as Negative Regulator of Hsp70

We then examined how the increased cellular sensitivity to heat shock in the hFAF1-overexpressed cell line is related to cell death. HEK293T cells with transient overexpression of FLAG-hFAF1 showed lower viability than vector-transfected cells following severe heat shock treatment. Also, the FLAG-hFAF1ΔN deletion mutant lacking the binding site to Hsp70 showed increased viability than vector-transfected cells, suggesting that hFAF1 renders cells more sensitive to heat shock by inhibiting the chaperone activity of Hsp70. The viability of FLAG-hFAF1ΔN-transfected cells increases possibly because the deletion mutant acts as a dominant negative mutant to the full form of hFAF1. It is possible that hFAF1 has multiple domains, and binding to the C terminus may regulate ubiquitination pathway. This hypothesis needs to be verified in future studies.

hFAF1 was originally identified as a Fas-associated protein. Amino acid sequence 1–201 is the binding domain for the Fas death domain (23). This region overlaps with amino acid residues 82–180, the Hsp70 interaction region. It is possible that hFAF1 binds to Fas or Hsp70, depending in the cellular conditions. In a previous finding, overexpression of Hsp70 did not protect cells from Fas-induced apoptosis (34). Further studies of the interaction between Fas and Hsp70 with hFAF1 are needed to explain this discrepancy.

The cellular function of hFAF1, as a novel protein, is still unclear. This study suggests that hFAF1 may play a role in the stress-induced signaling pathway through binding to Hsp70 and inhibition of the chaperone activity. Our study suggests that hFAF1 is a previously unrecognized new co-chaperone of Hsp70 having cell death-inducing potential. These functions of hFAF1 may possibly be regulated by post-translational modifications (4) and by changes in binding at multidomains of hFAF1.

Acknowledgments—we thank Dr. S. Subramani for providing cytoplasmic luciferase and Dr. R. I. Morimoto for providing Hsp70 clone.

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