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

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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)[1] 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 nonnative 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′-GAAGAATTCATGGCCA-AAGCCGCGGCAG-3′ and antisense primer 5′-GCATCTCGAGCGAG-ATCTCCTCGGGGTA-3′ and was subcloned into the pGEX-4T-1 vector.

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[1] The abbreviations used are: FAF1, Fas-associated factor 1; hFAF1, human Fas-associated factor 1; mFAF1, mouse FAF1; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; GST, glutathione *S*-transferase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; HBSS, Hanks' buffered saline solution; FITC, fluorescein isothiocyanate; Hsp70, heat shock protein 70; ABD, ATP-binding domain.

pGEX-4T-1/Hsp70ΔN-(120–640) was prepared by PCR using sense primer 5′-CGAGGAATTCATGTCCATGGTGCTGACC-3 and antisense primer 5′-GGCCTCGAGCTAATCTACCTCCTCAATG-3′ and was subcloned into the pGEX-4T-1 vector. pGEX-4T-1/Hsp70ΔPBD-(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-(82–650), pFLAG-CMV-2/hFAF1-(366–650) (hFAF1ΔN), pFLAG-CMV-2/hFAF1-(181–381), and pCDNA/hFAF1 were prepared as described previously (2). pCytluc (pRSVLL/V) encoding cytoplasmic luciferase was provided by S. Subramani (University of California, San Diego).

Cells and Reagents—Human embryonic kidney epithelial (HEK293T) cells were grown and maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Monoclonal anti-FLAG antibody M2 was purchased from Sigma. Monoclonal anti-Hsc70/Hsp70 antibody was obtained from StressGen. Polyclonal anti-hFAF1 antibody was generated and characterized in our group.

Transient Transfection—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.

Metabolic Labeling and Immunoprecipitation—Cells were metabolically labeled with 2 μCi/ml [^{35}S]methionine in methionine half-free medium for 18 h. Cells were disrupted with a buffer containing protease inhibitors (50 mM 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 Na_3VO_4, 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 h at 4 °C with monoclonal anti-FLAG M2-affinity cross-linking agarose beads or incubated for 2 h with anti-FLAG antibody and for an additional 1 h with protein A beads at 4 °C. The beads were washed more than three times with 1 ml of lysis buffer.

Two-dimensional Gel Electrophoresis—The protein samples were mixed with a buffer containing 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM EDTA, 10 mM Na_3VO_4, 10 mM NaF for 30 min at room temperature and electrofocused in 7-cm Immobiline DryStrips (pH 4–7) with IPGphor (Amersham Biosciences). The following focusing protocol was used: 50 μA per strip at 20 °C; 1) rehydration for 16 h; 2) 500 V for 1 h (step and hold); 3) 1000 V for 1 h (step and hold); 4) 8000 V for 3 h (step and hold). After electrofocusing, the strips were shaken for 15 min with equilibration buffer (1.5 M Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml dithiothreitol) and loaded onto mini two-dimensional SDS-polyacrylamide gel (Bio-Rad).

In Vitro Binding Assay—GST-Hsp70 deletion mutant expressed *E. coli* cytosol was incubated 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 immunostained with polyclonal anti-hFAF1 antibody.

Confocal Microscopy—For morphological studies, cells were grown on coated 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. Nonspecific protein absorption 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 for 1 h 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 Multiphoton Radiance 2000 (Bio-Rad) connected to a microscope (Nikon Eclipse TE 300).

In-gel Digestion and Mass Spectrometric Analysis—The cellular proteins were separated on two-dimensional gel electrophoresis and stained with Coomassie Blue or Silver. The gel spots were excised with a scalpel, crushed, and destained by washing with 25 mM NH_4HCO_3, 50% acetonitrile. The silver-stained gel was destained by washing with 15 mM K_4FeCN_6, 50 mM sodium thiosulfate. After crushing, the gels were dehydrated by addition of acetonitrile, rehydrated by adding 10–20 μl of 25 mM ammonium bicarbonate with 10 ng/μl of sequencing grade trypsin (Promega), and incubated at 37 °C for 12–15 h. Peptides were extracted by adding 30 μl of solution containing 60% acetonitrile/0.1% trifluoroacetic acid. The extraction was repeated three times and completed by adding 20 μl of acetonitrile. The extracted solutions were pooled and evaporated to dryness in a SpeedVac vacuum centrifuge. Samples were reconstituted in 10 μl of 0.1% trifluoroacetic acid and treated with ZipTips containing C18 resin (Millipore) according to the manufacturer's instructions. The washed peptides were eluted with saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.1% trifluoroacetic acid). Peptide mixtures were analyzed with MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Voyager-DE STR; Applied Biosystems, Inc.). External calibration was carried out using sEQUAZYME peptide mass standard kit (PerSeptive Biosystems) and internal calibration, by using the autolytic peaks of trypsin. This procedure typically results in mass accuracies of 50 ppm. For interpretation of the mass spectra, we used the Ms-Fit program available on the World Wide Web site of the University of California at San Francisco (prospector.ucsf.edu/). This result were confirmed by peptide sequencing using liquid chromatography-electrospray ionization-Q-TOF tandem MS.

Luciferase Reactivation Assay—Cells were transiently transfected with pCytluc and FLAG-tagged Hsp70 or hFAF1. Twenty-four hours after transfection, the cells were transferred into tissue culture dishes in medium with 20 μg/ml cycloheximide for 30 min at 37 °C. Luciferase was inactivated by heating the cells at 45 °C for 15 min and recovered at 37 °C for various times. Luciferase activities of the harvested cells were measured using luciferase assay kit (Promega).

SAPK/JNK Assay—SAPK/JNK was assayed using GST-c-Jun-(1–89)-peptide (provided by Dr. J. R. Woodgett) as a substrate after immunoprecipitation with anti-JNK antibody (Santa Cruz Biotechnology). Cell pellets were homogenized with buffer A containing 20 mM HEPES, pH 7.4, 50 mM β-glycerophosphate, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 400 μM phenylmethylsulfonyl fluoride, and 10 units/ml aprotinin. The supernatant containing 1 mg of protein was incubated with antibodies against mammalian SAPK/JNK and then with protein A-Sepharose 4B suspension. The immunocomplexes were washed three times and then incubated with a kinase buffer (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100) supplemented with GST-c-Jun and [γ-^{32}P]ATP for 20 min at 37 °C. To stop the reaction, 2× gel sample buffer was added. Samples were subjected to 12% SDS-PAGE, and the gels were stained, destained, and dried. Phosphorylation of c-Jun was measured by BAS2500 (Fuji).

RESULTS

Identification of Hsc70 and Hsp70 as hFAF1-interacting Protein in Vivo—To identify the proteins interacting with hFAF1, we performed co-immunoprecipitation experiments. FLAG-tagged hFAF1 was transiently overexpressed in HEK293T cells, and cellular proteins were metabolically labeled with 2 μCi/ml [^{35}S]methionine. hFAF1-interacting proteins were divided into two fractions and analyzed by two-dimensional gel electrophoresis. One fraction was transferred to NC membrane for autoradiogram and Western blot analysis, and the other fraction was silver-stained for visualization (Fig. 1). Protein spots detected in the autoradiogram were cut out from the corresponding silver-stained gel and subjected to in-gel digestion with trypsin and mass analyses using MALDI-TOF MS and electrospray ionization-Q-TOF tandem MS. We identified spot numbers 1 and 2 as heat shock protein 70 cognate (Hsc70) and heat shock protein 70 inducible (Hsp70), respectively (Table I). Binding to Hsc70 and Hsp70 was reconfirmed by West-

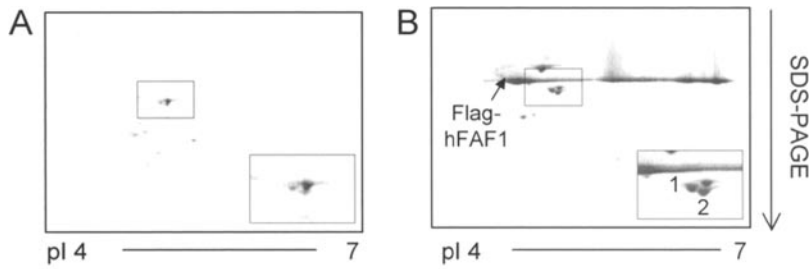

FIG. 1. **FLAG-hFAF1 interacts with Hsc70 and Hsp70.** HEK293T cells were transfected with pFLAG-CMV-2 vector (*A*) and FLAG-tagged hFAF1 (*B*) and labeled with 2 μCi/ml [^{35}S]methionine. Cells were lysed and immunoprecipitated with anti-FLAG M2-agarose cross-linking affinity gel. Precipitates were analyzed by two-dimensional gel electrophoresis and autoradiographed using BAS2500. Protein spots detected on the autoradiograph were cut out from the corresponding silver-stained gel and subjected to in-gel digestion with trypsin, and mass peptide fingerprint analyses were conducted. Enlarged figure of the *boxed section* is shown at the *right bottom* of each gel. *C,* Hsc70/Hsp70 were confirmed by Western blot analysis.

TABLE I
FLAG-hFAF1-binding proteins identified by two-dimensional gel electrophoresis and MALDI-TOF MS

Spot no.	Identified protein	NCBI GI no.	Mass	pI	Peptide coverage
			Da		
1	Hsc70	13273304	70,899	5.4	29
2	Hsp70	386785	69,869	5.4	30

ern blot analysis of the NC membrane with monoclonal anti-Hsc70/Hsp70 antibody (Fig. 1*C*). 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 co-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 endogenous Hsc70/Hsp70 and a series of transiently transfected FLAG-hFAF1 deletion mutants (Fig. 3*A*). HEK293T cells were transfected with various FLAG-tagged hFAF1 deletion mutants and immunoprecipitated by anti-FLAG M2-agarose cross-linking affinity gel. The precipitates were analyzed by Western blot analysis using anti-FLAG antibody (Fig. 3*B, upper panel*) and anti-Hsc70/Hsp70 antibody (Fig. 3*B, lower panel*). Hsc70/Hsp70 did not bind to anti-FLAG M2-agarose cross-linking affinity gel of vector-transfected cells, bound only negligibly to amino acid sequences of 366–650 and 181–381 of FLAG-tagged hFAF1, but bound well to N-terminal hFAF1 fragments containing amino acid sequences, 1–201, 1–345, and 82–650. These data showed that the amino acid sequence 82–180 of hFAF1 is necessary for binding with Hsc70/Hsp70. This was confirmed in Fig. 3*C*. 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 directly or indirectly with Hsp70, in an accessory protein-mediated process, we carried out *in vitro* pull down assay. We prepared various truncated forms of GST-Hsp70 (Fig. 4*A*) as

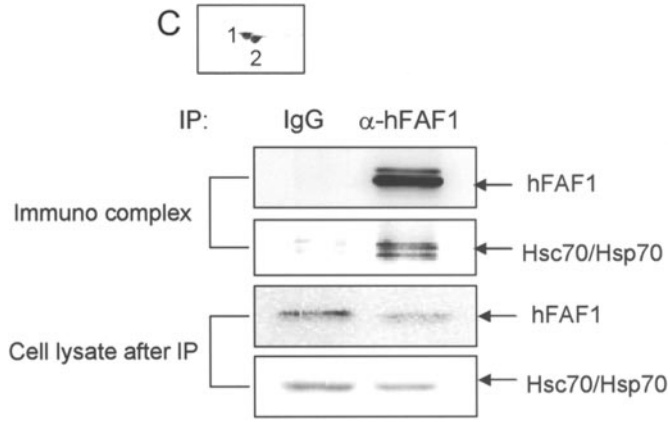

FIG. 2. **Endogenous hFAF1 interacts with endogenous Hsc70/Hsp70.** HEK293T cells were lysed and immunoprecipitated (*IP*) with mouse IgG as control (*1st lane*) and anti-hFAF1 polyclonal antibody (*2nd lane*). The precipitates were analyzed by SDS-PAGE and Western blot analysis by using anti-hFAF1 polyclonal 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.

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. 4*B*). In the presence of various concentrations of ATP, the bindings between Hsp70 and hFAF1 were not affected (Fig. 4*C*). These results suggest that hFAF1 directly interacts with the N terminus of Hsp70 and that the binding was not affected by ATP-induced conformational changes of 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 quantitatively analyzed the ratio of Hsc70/Hsp70 to FLAG-hFAF1 in a linear range using LAS1000 (Fuji), Hsc70 interactions did not change during recovery after heat shock, and even the amount of Hsp70 was slightly increased in cell lysates. This suggests that the interaction between hFAF1 and Hsc70/Hsp70 is homeostatic and is not affected by heat shock treatment.

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

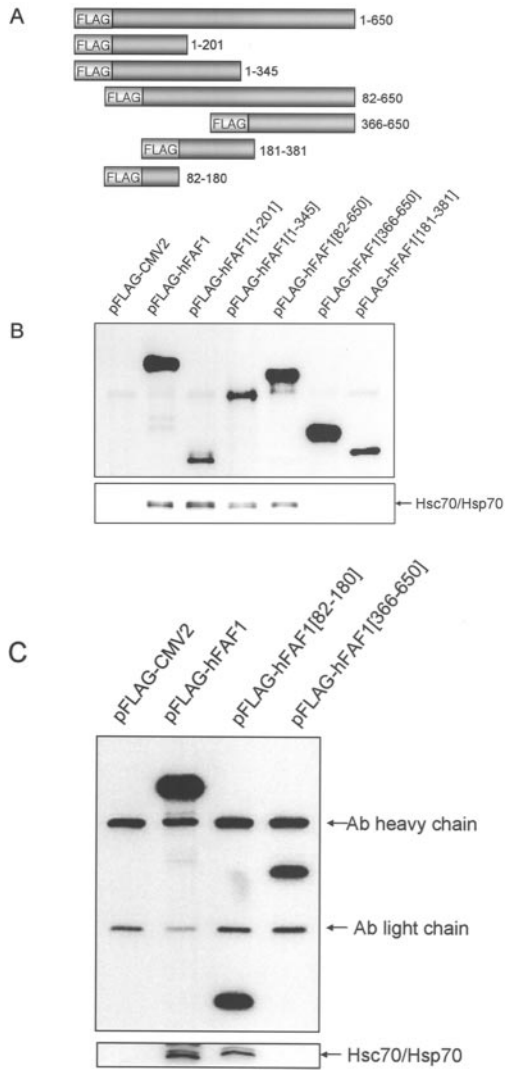

FIG. 3. **Hsc70 and Hsp70 interact with amino acids 82–180 of hFAF1 *in vivo*.** *A,* diagram of FLAG-tagged hFAF1 fragments. HEK293T cells were transfected with pFLAG-CMV-2 vector or pFLAG-CMV/hFAF1 fragments. Cells were lysed and immunoprecipitated with anti-FLAG M2-affinity cross-linking agarose beads (*B* and *C*) or with monoclonal anti-FLAG antibody (*Ab*) and with protein A beads. Precipitates were analyzed by Western blot analysis using anti-FLAG M2 monoclonal antibody (*upper panels* of *B* and *C*) and anti-Hsc70/Hsp70 antibody (*lower panels* of *B* and *C*).

complex is specific and is changed by heat shock. FLAG-hFAF1 transiently overexpressed in HeLa cells was treated with heat shock at 45 °C for 30 min and recovered at 37 °C after 24 h. Hsp70/Hsc70 proteins were stained with monoclonal anti-Hsc70/Hsp70 antibody, and secondary antibody was labeled with Texas Red and FLAG-hFAF1 with FITC-labeled anti-FLAG M2 monoclonal antibody. hFAF1 and Hsp70 normally appeared in the cytosol and nucleus (Fig. 6, *A* and *B*). When two images were merged, the cytosolic and nuclear regions showed bright yellow color, suggesting co-localization of two proteins (Fig. 6*C*). When the cells were treated with heat shock at 45 °C for 30 min, immediately after heat shock, hFAF1 and Hsp70 co-localized in perinuclear region, and speckles were bright yellow in the cytosol (Fig. 6*F*). After 24 h of recovery at 37 °C, hFAF1 relocalized mainly to the nucleus and cytosol, and Hsp70 showed enrichment in the nucleus when compared with the control cells (Fig. 6, *G* and *H*). Again the two proteins co-localized in the perinuclear region (Fig. 6*I*). These results show that the sites of co-localization of hFAF1 and Hsp70 vary between the cytosolic and nuclear region and the perinuclear 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-(1–119 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. 7*A*), 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. 7*B*). 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. 7*C*). 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. 7*D*).

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. 7*E*). In the presence of FLAG-hFAF1, luciferase activity was significantly reduced; however, expression of FLAG-hFAF1ΔN did not inhibit Hsp70 chaperone activity (Fig. 7*F*). 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. 7*F*, 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. 7*G*). 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. 7*H*). 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.

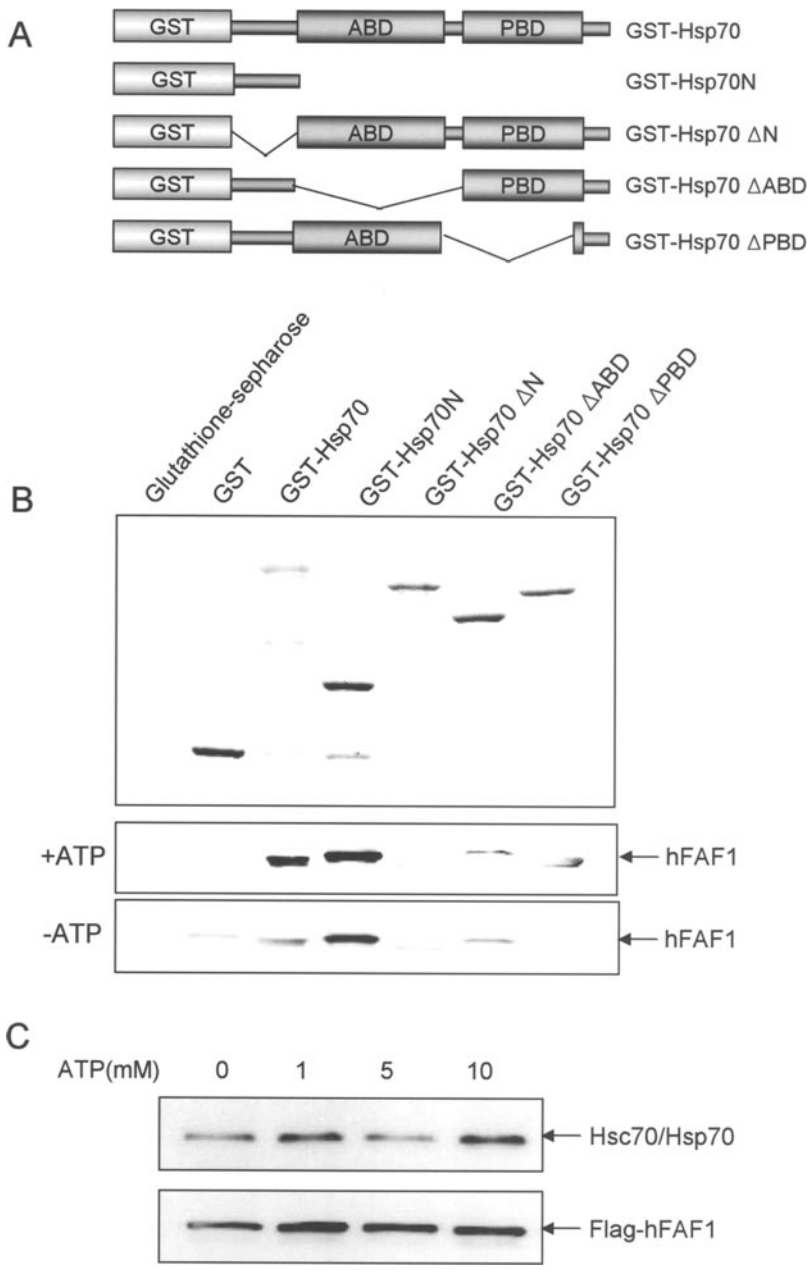

FIG. 4. **hFAF1 directly interacts with the N-terminal domain of Hsp70 *in vitro*.** *A*, diagram of GST-tagged Hsp70 fragments. *B*, purified recombinant hFAF1 was incubated with GST-Hsp70 deletion mutants immobilized on glutathione-Sepharose in the presence or absence of 10 mM ATP. Precipitates were 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.

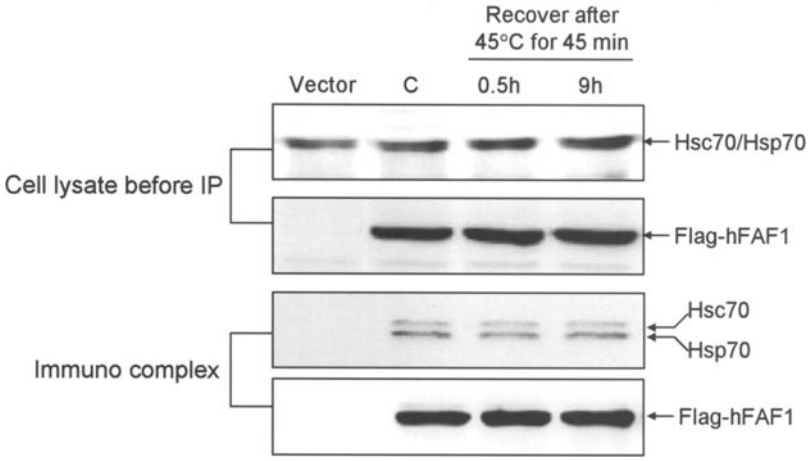

FIG. 5. **Heat shock has no effect on the interaction between Hsc70/Hsp70 and hFAF1.** HEK293T cells were transiently transfected with pFLAG-CMV-2 vector or FLAG-tagged hFAF1. Cells were heat-shocked at 45 °C for 45 min or untreated (*C*) and recovered for the indicated times. At each time point, cells 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.

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

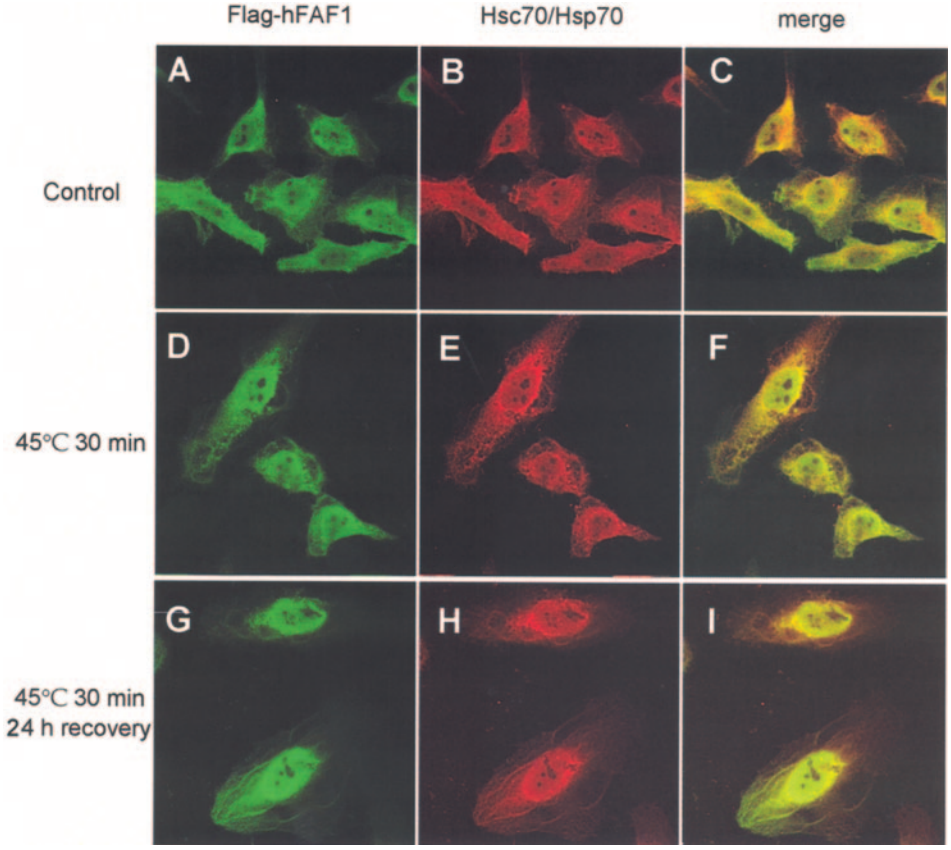

FIG. 6. **Immunofluorescence analysis of FLAG-hFAF1 and Hsp70 localization.** HeLa cells were transiently transfected with pFLAG-CMV-2 vector or pFLAG-CMV-2/hFAF1. Cells were immunostained without heat shock (*A–C*), after heat shock at 45 °C for 30 min (*D–F*), or after a recovery period of 24 h (*G–I*). All cells were stained with a monoclonal antibody to the Hsc70/Hsp70 followed by incubation with Texas Red-labeled secondary antibody (*red*). The cells were then stained with anti-FLAG M2 monoclonal antibody labeled with FITC (*green*). *Yellow* in the merged figures (*C, F,* and *I*) means co-localization of *red* and *green* fluorescence. The images were made by confocal microscopy.

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 hFAF1ΔN. 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-hFAF1ΔN-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.

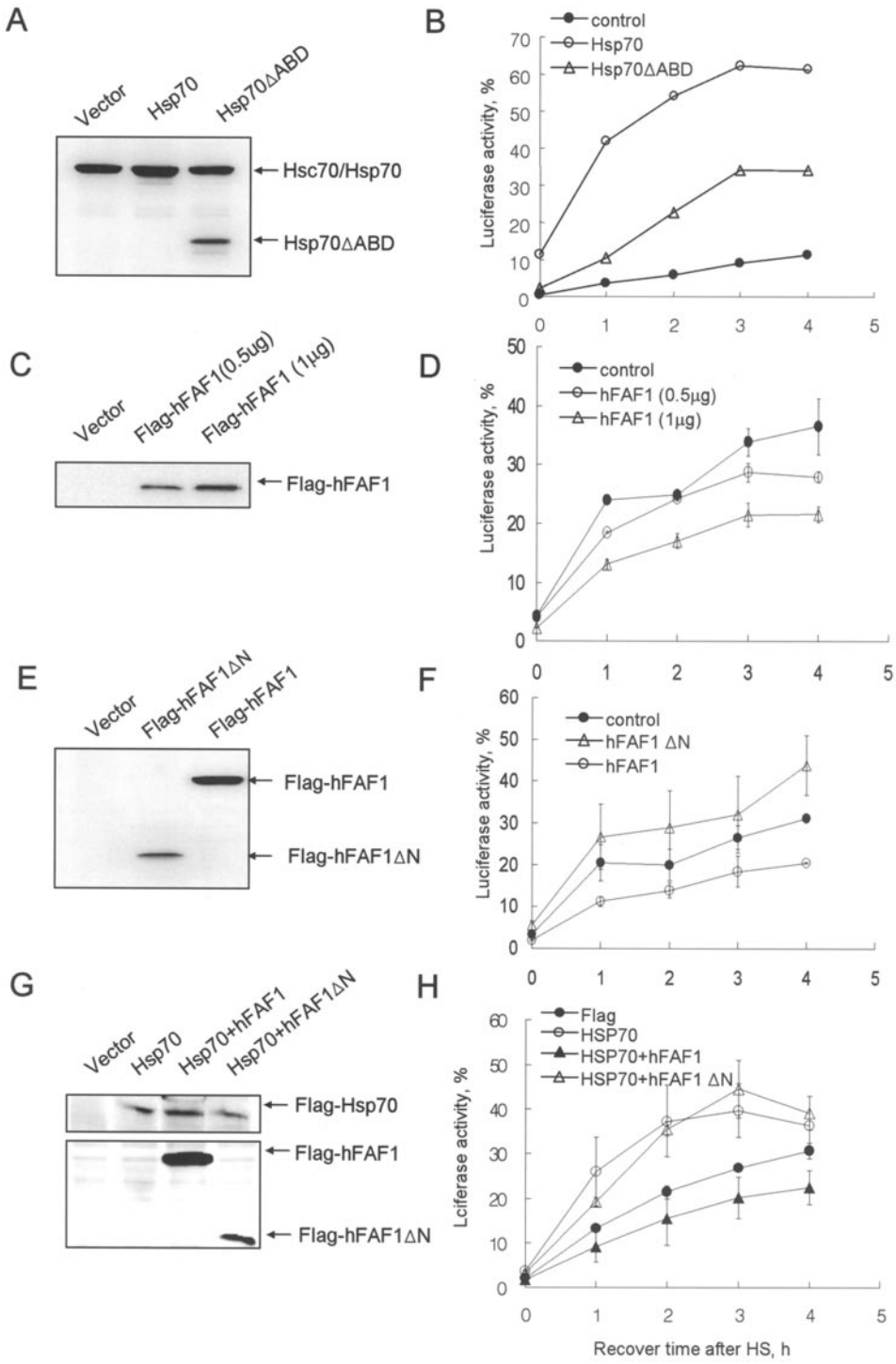

FIG. 7. **Overexpression of hFAF1 inhibits Hsp70-mediated reactivation of heat-denatured firefly luciferase.** HEK293T cells were transiently transfected with pCytluc (encoding cytosolic luciferase) together with pFLAG-CMV-2 vector, pFLAG-CMV-2/Hsp70, or pFLAG-CMV-2/Hsp70 ΔABD, pFLAG-CMV-2/hFAF1 (*A, C,* and *E*), or pFLAG-CMV-2/hFAF1ΔN 3FLAG-CMV-7.1/Hsp70 as indicated (*G*), and were detected with Western blot analysis by using anti-Hsp70 antibody for FLAG-tagged Hsc70/Hsp70, Hsp70ΔABD, and anti-FLAG or anti-FAF1 antibody for hFAF1, and hFAF1ΔN expression (*A, C, E,* and *G*) and anti-FLAG antibody for 3FLAG-Hsp70 (*G*). After pretreatment with cycloheximide (20 μg/ml) for 30 min, luciferase was inactivated by heating the cells at 45 °C for 15 min. During a subsequent recovery period at 37 °C, luciferase activity (*B, D, F,* and *H*) at each time point was measured.

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* by 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

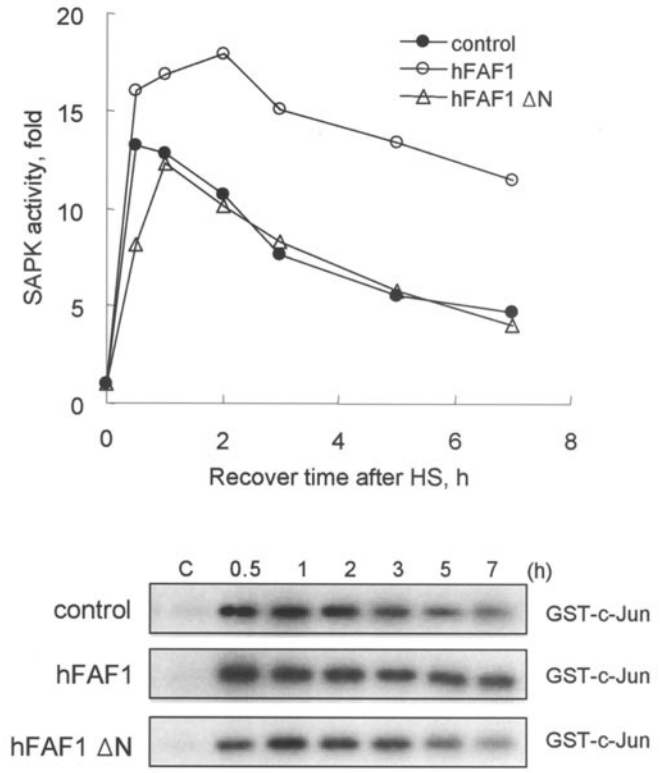

FIG. 8. **Overexpression of FLAG-hFAF1 accelerates heat shock-induced SAPK/JNK activation.** HEK293T cells were transiently transfected with pFLAG-CMV-2 vector, pFLAG-CMV-2/hFAF1, or pFLAG-CMV-2/hFAF1ΔN and exposed to heat shock at 45 °C for 45 min. SAPK/JNK activities were measured after the indicated recovery times. *C*, control without heat shock treatment.

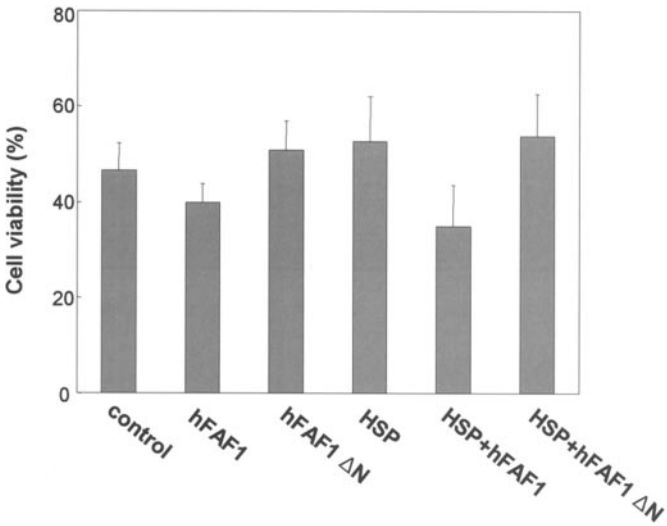

FIG. 9. **Overexpression of FLAG-hFAF1 increases heat shock-induced cell death.** HEK293T cells were transiently transfected with the pFLAG-CMV-2 vector, pFLAG-CMV-2/hFAF1, or pFLAG-CMV-2/hFAF1ΔN, co-expressed with 3FLAG-CMV-7.1/Hsp70 and each hFAF1, and exposed to heat shock at 45 °C for 45 min. After 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.

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 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[2] 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 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 indispensable for the chap-

[2] E. J. Song, S. H. Yim, E. Kim, N. S. Kim, and K.-J. Lee, unpublished data.

erone activity. Thus, the possibility that chaperone activity of Hsp70 can affect SAPK/JNK activation should not be excluded.

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.

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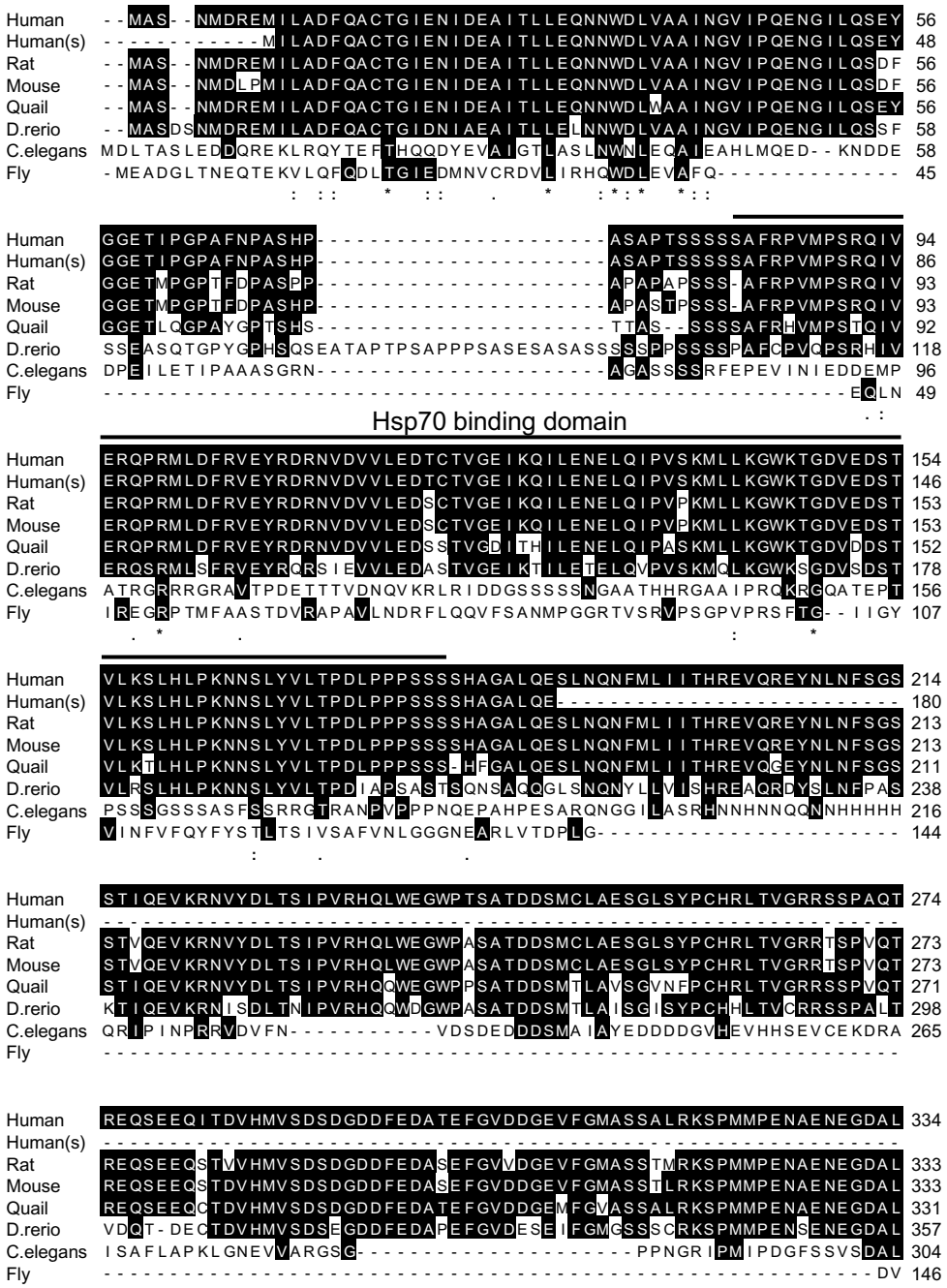

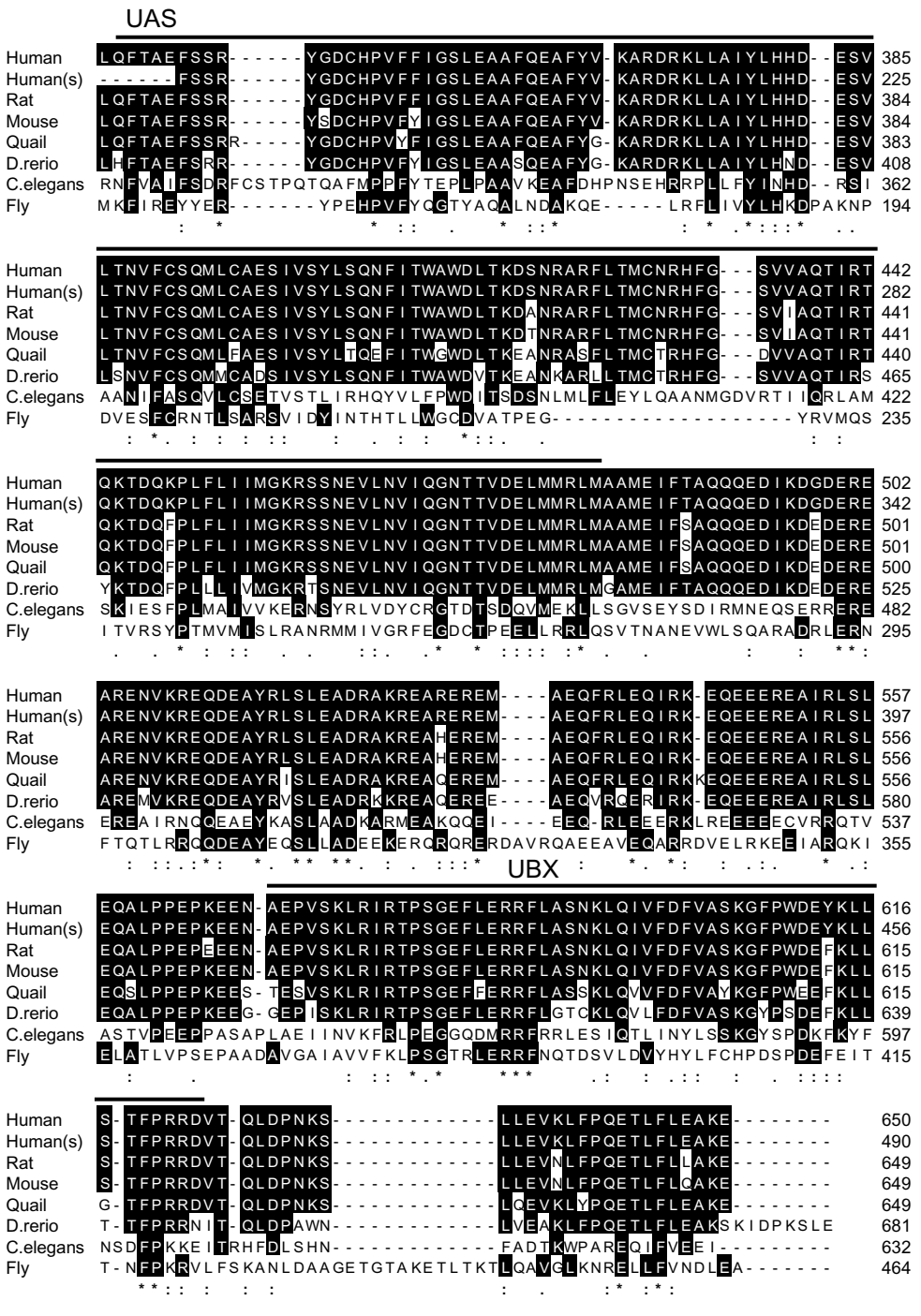
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Human Fas-associated Factor 1 Interacts with Heat Shock Protein 70 and Negatively Regulates Chaperone Activity

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