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Death-associated Protein 4 Binds MST1 and Augments MST1-induced Apoptosis*

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The protein kinase MST1 is proapoptotic when overexpressed in an active form, however, its physiologic regulation and cellular targets are unknown. An overexpressed inactive MST1 mutant associates in COS-7 cells with an endogenous 761-amino acid polypeptide known as "death-associated protein 4" (DAP4). The DAPs are a functionally heterogeneous array of polypeptides previously isolated by Kimchi and colleagues (Kimchi, A. (1998) *Biochim. Biophys. Acta* 1377, F13–F33 in a screen for elements involved in the interferon γ-induced apoptosis of HeLa cells. DAP4, which is encoded by a member of a vertebrate-only gene family, contains no identifiable domains, but is identical over its amino-terminal 488 amino acids to p52^{rIPK}, a putative modulator of protein kinase R. DAP4 is a widely expressed, constitutively nuclear polypeptide that homodimerizes through its amino terminus and binds MST1 through its carboxyl-terminal segment. MST1 is predominantly cytoplasmic, but cycles continuously through the nucleus, as evidenced by its rapid accumulation in the nucleus after addition of the Crm1 inhibitor, leptomycin B. Overexpression of DAP4 does not cause apoptosis, however, coexpression of DAP4 with a submaximal amount of MST1 enhances MST1-induced apoptosis in a dose-dependent fashion. DAP4 is not significantly phosphorylated by MST1 nor does it alter MST1 kinase activity *in vivo* or *in vitro*. MST1-induced apoptosis is suppressed by a dominant interfering mutant of p53. MST1 is unable to directly phosphorylate p53, however, DAP4 binds endogenous and recombinant p53. DAP4 may promote MST1-induced apoptosis by enabling colocalization of MST with p53.

MST1 (1) (also known as Krs-2 (2)) is a 487-aa[1] mammalian protein kinase best classified as a group II GC kinase (3, 4). MST1 (and its close homolog MST2/Krs-1) contains a Ste20-related kinase catalytic domain in the amino-terminal segment (aa 30–270) followed by a noncatalytic tail that contains successively an autoinhibitory domain (aa 331–394), a dimerization domain (after aa 431), and a nuclear localization signal at the COOH terminus (1, 2, 5). Although initially identified as a kinase activated late after transformation by v-Src, MST kinase activity can also be activated by certain severe stresses, such as 0.25 M sodium arsenite or heat shock at 55 °C, as well as by protein phosphatase inhibitors such as okadaic acid (2). Nevertheless, the physiologic regulation of MST1 and -2 remains obscure. MST1 contains a caspase 3 cleavage site at DMED326, just amino-terminal to the autoinhibitory domain, and cleavage of MST1 occurs during apoptosis initiated by a variety of stimuli, which yields a 36-kDa catalytic fragment whose specific activity is increased about 10-fold over the parent MST1 (6–8). In addition, overexpression of either wild-type MST1 or a carboxyl-terminal truncated mutant (but not a kinase inactive MST) is itself sufficient to initiate apoptosis. Reciprocally, overexpression of a kinase-inactive mutant of MST1 is able to partially suppress the apoptosis induced by the anti-tumor agents cytotrienin A (9), MT-21 (10), or staurosporine, suggesting that the recruitment of MST plays a significant role in the apoptosis induced by these agents. The mechanism of MST-induced apoptosis is not well defined; overexpression of MST1 is accompanied by activation of SAPK/JNK (6, 9, 11), and coexpression with a dominant inhibitory SAPK mutant partially suppresses MST1-induced apoptosis (12). Moreover, whereas wild-type MST1 is a cytoplasmic protein, caspase-cleaved MST enters the nucleus and induces chromatin condensation followed by internucleosomal DNA fragmentation (13, 14), suggesting that nuclear entry of MST may be important to the expression of its proapoptotic action.

In an effort to gain further insight into the mechanism of MST1 cellular actions, we transiently overexpressed a kinase-inactive, caspase-resistant mutant of MST1 and analyzed by mass spectrometry the polypeptides recovered in association with MST1. Herein we characterize the physical and functional interactions of MST1 with one candidate partner identified thereby, death-associated protein 4 (DAP4).

MATERIALS AND METHODS

Identification of DAP4 as a MST1-associated Protein—In an effort to identify proteins that associate with MST1 in intact cells with sufficient avidity to survive extraction and washing, a FLAG-tagged MST1 was overexpressed transiently in COS-7 cells and recovered by affinity purification on anti-FLAG-agarose. The MST1 was rendered kinase inactive by mutation at the ATP binding site (K59R) so as to avoid the induction of apoptosis, and the caspase 3 cleavage site was also mutated (D326N). Cells transfected in parallel with empty vector were processed in tandem. Forty-eight hours after transfection, the cells were extracted into buffer containing 1% Triton X-100 A with protease inhibitors. The supernatant of a 13,500 × *g*, 15-min centrifugation was adsorbed onto FLAG-agarose, washed extensively in the lysis buffer with and without

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[1] The abbreviations used are: aa, amino acid(s); SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; DAP, death associated protein; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

1 M NaCl, eluted at pH 2.5, concentrated, and subjected to SDS-PAGE. After staining with Coomassie Blue, the lane containing the sample prepared from cells transfected with empty vector showed only a faint band of light chain; the lane containing FLAG-MST1 exhibited a major band at 61 kDa and 10 minor bands at about 1–10% abundance of the 61-kDa FLAG-MST1. Each of the latter was excised, subjected to tryptic digestion *in situ,* and analyzed by tandem mass spectrometry, using the nanospray MS-MS approach (15). Among the seven bands at M_r < 61,000, five were fragments of MST1. Among the three bands at M_r > 61,000, two were heat shock proteins (HSP 90 and 70). A third band, at M_r 88,000, contained 5 tryptic peptide fragments, two of which could be assigned to $p52^{rIPK}$ (regulator of the inhibitor of protein kinae R), a 492-amino acid polypeptide modulator of the PKR inhibitor, $p58^{IPK}$ (16), and three other tryptic peptides that could be identified within the human expressed sequence tag polypeptide GI1616050 (AA076164). Alignment of overlapping human, mouse, and rat expressed sequence tags enabled the assembly of a sequence that extended amino-terminal from GI1616050 to produce an identical overlap with amino acids 389–488 of $p52^{rIPK}$. Combining the sequence of $p52^{rIPK}$ and the overlapping human, mouse, and rat expressed sequence tags yielded a polypeptide of 761 amino acids. Blast of this combined sequence against human genomic sequences yielded several matches exceeding 90% on different chromosomes. Shortly thereafter a 761-amino acid sequence was deposited in the nonredundant data bank (accession number AF081567) by Barzilay and Kimchi under the name "death-associated protein 4," which differed at five amino acids from our assembled sequence. The sequence of DAP4 is shown in Fig. 1*A*; it is identical with $p52^{rIPK}$ over the NH_2-terminal 488 amino acids.

DNA Constructs and Manipulations—DAP4 was cloned from human skeletal muscle cell cDNA library using PCR 5′ primer, GCGGGATCCATGCCGAACTTCTGCGCTGCC, and 3′ primer, GGCGGCCGCTTAGGTATTTTCCACAGTTTC. Mouse MST1 cDNA was kindly provided by Leonard Zon (Children's Hospital, Boston, MA). Constructs encoding wild-type p53 and a mutant p53 miniprotein (p53 aa 302–393) (17, 18) were generous gifts from Dr. Moshe Oren. DNA manipulations were performed using the standard techniques (19). Full-length, truncated, and point mutations of constructs were subcloned into different plasmids including pCMV5 FLAG, pEBG GST, pEGFP-C1, pEGFP-N1, and pdsRed-N1. All the constructs were verified by DNA sequencing.

Cell Cultures and Transfection—COS-7, HEK 293, and HeLa cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 0.1 mg/ml streptomycin in 10% CO_2 atmosphere at 37 °C. NIH 3T3 were cultivated as above except 10% bovine calf serum (Invitrogen) was used. Cells were replaced at a density of 3–5 × 10^6 per 10-cm dish and transfected 5 h later with a total of 8 μg of DNA and 30 μl of LipofectAMINE per dish following the manufacturer's instruction (Invitrogen).

Cell Lysate, Immunoprecipitation, and Immunoblot Assay—The binding assays *in vitro* employed extracts from HEK 293 cells expressing recombinant FLAG or GST fusion proteins individually. At 48 h after transfection, cells were snap frozen and stored at −70 °C until use. One 10-cm dish of frozen cells was scraped into 1 ml of lysis buffer (50 mM Tris base, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 nM calyculin A, 0.5% Triton X-100, 1 tablet/50 ml of protease inhibitor (Roche Molecular Biochemicals)). Lysates were centrifuged at 13,500 rpm for 10 min. Aliquots of the supernatants containing equal amounts of protein, measured by Bradford assay (Bio-Rad), were added to 15 μl of settled GSH-agarose beads (Amersham Biosciences) and incubated at 4 °C for 3 h. Beads were washed three times with 1 ml of lysis buffer, once with 1 ml of lysis buffer containing 0.5 M LiCl, and again with 1 ml of lysis buffer. Thereafter, aliquots of the lysate from the FLAG fusion protein-transfected cells matched for proteins were added to the beads and incubated at 4 °C for another 3 h. The beads were then washed extensively and adsorbed proteins were eluted in 15 μl of 2× SDS sample buffer, heated at 95 °C for 10 min, separated by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and probed with antibodies as indicated. Blots were visualized using horseradish peroxide-conjugated secondary antibody followed by chemiluminescence as outlined by the manufacturer (Pierce).

The studies of binding *in vivo*, mapping of the sites of DAP4/MST1 association, DAP4/p53 association, and DAP4 dimerization were carried out after cotransfection of the constructs indicated into COS-7 or HEK 293 cells. The procedures of cell lysis and immunoaffinity purification were as described above.

Immunocytochemistry and Fluorescence Microscopy of Cultured Cells—HEK 293, HeLa, and NIH 3T3 cells were cultured in 60-mm plates and transfected with 3–4 μg of GFP/red fluorescent protein-tagged cDNA constructs using 15 μl of LipofectAMINE. In some cases, 6 μl of FuGENE (Roche Molecular Biochemicals) or 15 μl of LipofectAMINE 2000 (Invitrogen) were used. At 24 h after transfection, cells were incubated with 0.2 μg/ml Hoechst 33342 for 30 min at 37 °C and observed directly by fluorescence microscopy. The same plates were also observed after 48 and 72 h transfection. To examine the effect of leptomycin B on cellular localization, 10 ng/ml leptomycin B or carrier was added to the cells at 24 h after transfection, and images were taken from 20 min to 1 h after addition of leptomycin B.

When used for immunocytochemistry, cells were seeded onto glass coverslips 24 h before the transfection. At the times indicated after transfection, cells were washed with PBS, fixed in methanol for 10 min, and blocked in 2% bovine serum albumin in PBS for 10 min. Anti-FLAG mAb (10 μg/ml) was added for 30 min followed by washing with PBS. The secondary antibody, fluorescein isothiocyanate-conjugated anti-mouse antibody (Cappel), was used at a 1:400 dilution for 30 min then washed with PBS. Cells were incubated with 0.2 μg/ml Hoechst 33342 for an additional 30 min and washed again in PBS. The coverslips were then mounted on a glass slide using Fluoromount-G (Southern Biotechnology). Images were collected using a Zeiss Axiovert S100M microscope (Carl Zeiss) connected to a CCD camera. The figures were prepared using MetaMorph Imaging software (Universal Imaging).

Cell Death Assay—The method used for measuring cell death was modified from Rabizadeh *et al.* (20) and Sperandio *et al.* (21). Briefly, HEK 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transient transfections were performed using LipofectAMINE 2000 according to the manufacturer's instructions. A total of 1 × 10^6 cells were seeded in 60-mm plates and 15 μl of LipofectAMINE 2000 was used. Twenty-four to forty-eight hours after transfection, the pro-apoptotic agent tamoxifen was added at a final concentration of 20–25 μM to increase the rate of apoptosis. Floating cells were collected at 20–24 h after the addition of tamoxifen and cell death was assessed by trypan blue exclusion method.

MST1 Kinase Assay—For the experiment shown in Fig. 7, HEK 293 cells were transfected with FLAG-tagged MST1 and DAP4 (total 8 μg/plate in a 60-mm plate) using LipofectAMINE; some cells were incubated with zVAD-fmk at 50 μM (BioMol) and other cells were transfected with plasmid encoding BV p35 (22) along with the DAP4 and MST1. At 72 h after transfection, cells were rinsed, frozen, and harvested into 0.6 ml of lysis buffer, followed by centrifugation at 13,500 × *g* for 10 min. Supernatant were incubated for 3 h at 4 °C with anti-FLAG antibody prebound to protein G beads. The beads were then washed 4 times with 1 ml of lysis buffer and 3 times with 1 ml of kinase buffer (40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerophosphate). The kinase assay was performed in 30 μl of reaction buffer (40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.8 μg/μl histone 2B, 100 μM ATP, and 2 μCi/tube) for 15 min at 30 °C on a thermomixer. The reaction was stopped by addition of SDS sample buffer to 1× concentration followed by boiling for 5 min. An aliquot of the sample was separated by SDS-PAGE on a 12% SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane and staining with Coomassie Blue, the ^{32}P incorporation into DAP4, MST1, and H2B was quantified by phosphorimaging. The same membrane was used for immunoblot determination of polypeptide abundance after decay of the ^{32}P signal.

In the experiments shown in Figs. 8, *A* and *B,* and 9*C*, the kinase assay was performed as above except using purified, soluble proteins. To obtain the proteins, individual FLAG-tagged constructs were transfected into HEK 293 cells. At 48 h after transfection, the clarified supernatant of a concentrated cell lysate was incubated with 15 μl of FLAG-agarose beads (Sigma) for 3 h at 4 °C. The beads were washed 4 times with 1 ml of lysis buffer, 4 times with 1 ml of lysis buffer containing 0.5 M LiCl, and the FLAG-tagged proteins were then eluted by three successive incubations in lysis buffer containing 0.1 mg/ml FLAG peptide (Sigma) for 10 min on ice. The elutes were combined and centrifuged for 5 min at 5000 × *g* through Spin-X centrifuge tube filters (Corning).

Statistical Analysis—Data are presented as mean ± S.E. Treatment effects were evaluated using a two-tailed Student's *t* test. A *p* value <0.05 was considered to be statistically significant.

RESULTS

Characterization of the Human DAP4 Gene Family—DAP4 was identified as a polypeptide that was co-isolated with recombinant, kinase-inactive MST1 (see "Materials and Methods"). In view of the prior recovery of DAP4 in a functional

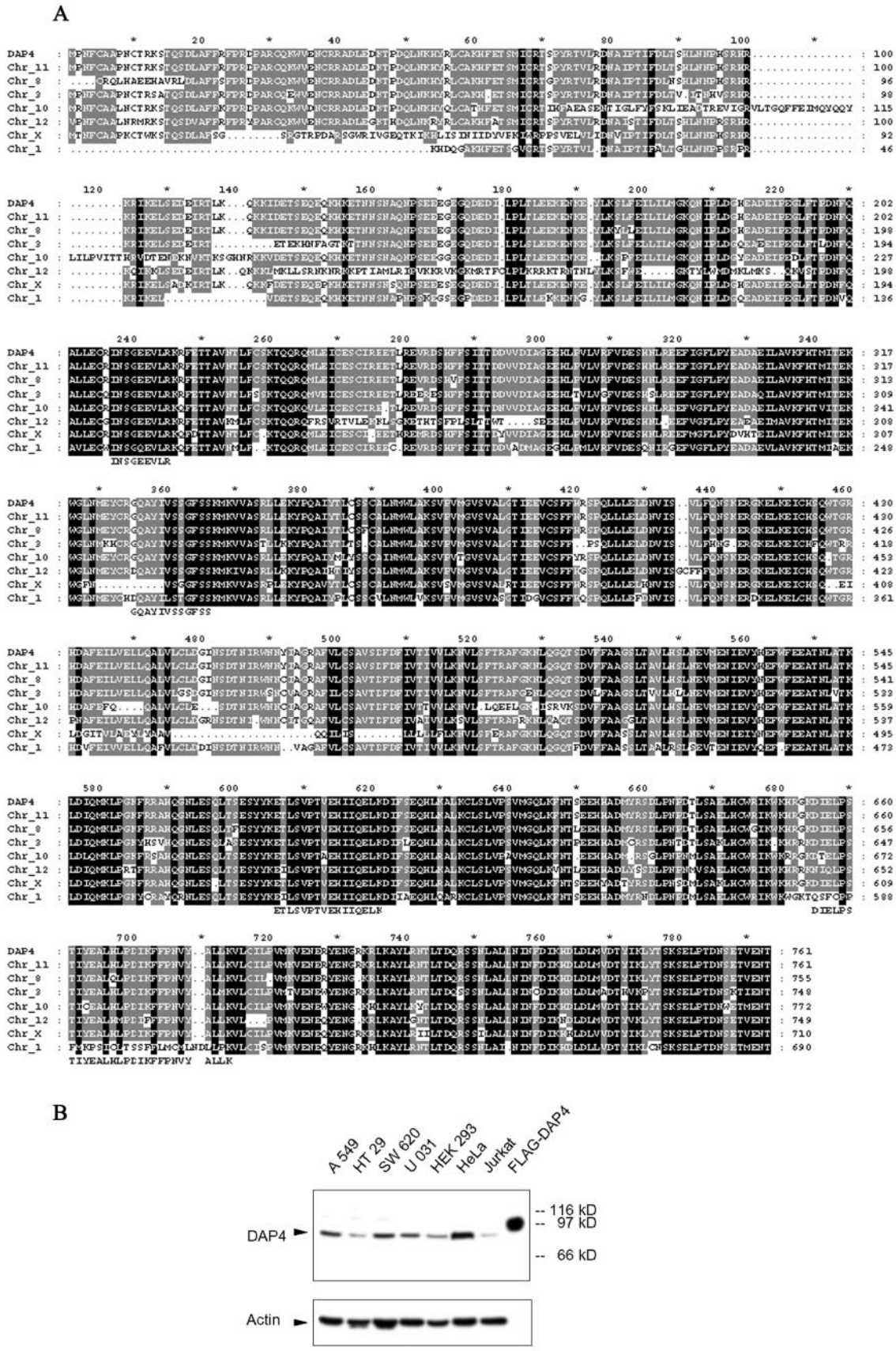

FIG. 1. *A*, alignment of DAP4 with polypeptide sequences derived from the human genomic DNA data base (tBlastn). Identical/conserved residues are indicated in the *black* background. Highly conserved residues are indicated in the *dark gray* background. The identities relative to the deposited DAP4 cDNA are: DAP4A, 100%; DAP4B, 95%; DAP4C, 87%; DAP4D, 82% DAP4E, 78% DAP4F, 79% DAP4G, 75%. The five tryptic peptide sequences identified by MS-MS in the digest of the 88-kDa MST1-associated polypeptide are shown *below* the aligned sequences. *B*, immunoblot of DAP4-related polypeptide expression in human cell lines.

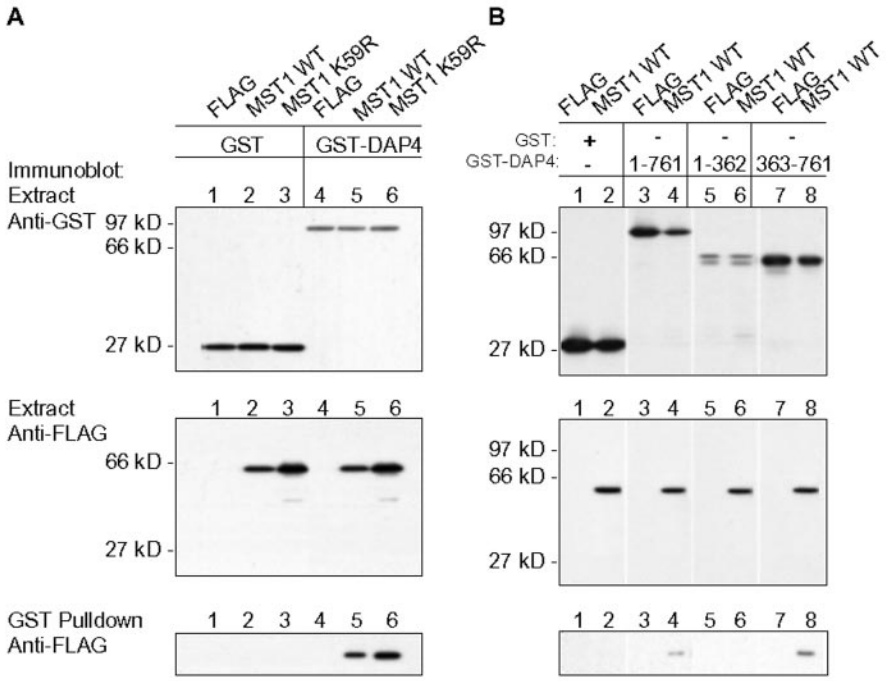

FIG. 2. **The binding of DAP4 to MST1.** *A*, HEK 293 cells were transfected with vectors encoding GST, GST-DAP4, or FLAG-MST1 (wild-type or the inactive mutant K59R). The GST proteins were immobilized on GSH-agarose, washed, and lysates from mock transfected or MST1-transfected cells were added to the GSH-agarose beads as indicated. After further washing, the bound proteins were subjected to SDS-PAGE and blotted with anti-FLAG mAb immunoblot. *B*, recombinant GST or a GST-DAP4 fusion protein (wild-type, aa 1–362 and 363–761) were each coexpressed in HEK 293 cells with empty FLAG vector or FLAG-MST1. The FLAG blot of the GSH-agarose isolate is shown in the *bottom panel*. The blot is representative of one experiment repeated 3 times.

screen for elements involved in interferon-induced apoptosis, we attempted to determine whether DAP4 participated in MST-induced apoptosis.

Blast of the DAP4 polypeptide sequence against the human genome sequence data base yields seven polypeptide sequences (shown in Fig. 1*A*) that exhibit >75% identity in amino sequence with DAP4; we have arbitrarily named these polypeptides as DAP4A (Chr11) through DAP4G(Chr1) in order of decreasing identity. In addition, other homologous sequences can be identified at a second site on Chr11 (65% identity to DAP4), on Chr13 (63% identity), Chr4 (52% identity), Chr6 (45% identity), and Chr15 (47% identity), with probable additional homologues on Chr6, -13, -1, and -12, and a third homologous gene on Chr11; altogether 17 human *DAP4*-related genes were identifiable. The *DAP4A* DNA sequence on Chr11 is identical to the deposited *DAP4* cDNA, and certainly encodes the gene corresponding this cDNA polypeptide. The *DAP4A* gene contains at least three introns, and the *DAP4C* sequence on Chr3 contains at least one intron, however, the *DAP4B* sequence on chromosome 8 lacks introns, and may represent a processed *psuedo*gene. Among these polypeptides only the genes on Chr11 (*DAP4A*) and Chr3 (*DAP4C*) encode sequences identical to the five peptides identified by MS analysis of the 88-kDa MST1-associated polypeptide (indicated in Fig. 1), and the sequence on Chr8 (*DAP4B*) differs by 1 aa of the aggregate 66 aa within these peptides. Based on available data we conclude that the polypeptide recovered in association with MST1 is either DAP4A (=DAP4) or DAP4C; the latter is 87% identical in amino acid sequence to DAP4A. The DAP4 polypeptides contain no domain motifs identifiable within the Prosite, Pfam, or Interpro algorithrims.

DAP4 Polypeptide Expression—A polyclonal antibody was raised against the synthetic peptide Cys-KSELPTDNSETVENT, coupled through its amino terminus to KLH. This sequence corresponds to the carboxyl-terminal 15 amino acids of DAP4A, and is highly conserved among the DAP4-related gene products described in Fig. 1*A*. Immunoblot of extracts prepared from a variety of human cell lines, using affinity purified anti-DAP4A(CT peptide) IgG (Fig. 1*B*) showed the ubiquitous presence of a single major polypeptide band at ~88 kDa, which we presume to be DAP4A, and perhaps one or more of the closely related gene products indicated in Fig. 1, many of which differ in length from DAP4A by less than 15 amino acids. Some extracts exhibit a second, faint band at ~103 kDa; the identity of this polypeptide is unknown. Thus, DAP4-related polypeptides are commonly expressed in human cell lines.

MST Binds to DAP4 in Vitro and in Vivo through the DAP4-(AA 363–761)—We next employed PCR to isolate, from a human skeletal muscle library, a cDNA corresponding exactly in sequence to the human DAP4A, and proceeded to characterize its properties and its interactions with MST1. Plasmids encoding GST or a GST-DAP4 fusion protein were expressed separately in HEK 293 cells and each recombinant GST polypeptide was immobilized at comparable concentrations by adsorption to GSH-agarose. Extracts of cells transfected with FLAG-MST1, FLAG-MST (K59R), or empty vector were matched for protein content and incubated with immobilized GST proteins; after washing, the adsorbed proteins were eluted, subjected to SDS-PAGE, and probed with anti-FLAG antibody. As seen in Fig. 2*A*, both wild-type and kinase-inactive MST1 polypeptides bind specifically to GST-DAP4. We next examined the ability of MST1 and DAP4 to associate during transient coexpression. Both FLAG-MST1 wild-type (Fig. 2*A*) and K59R (not shown) bind specifically to GST-DAP4. The binding site on DAP4 for MST1 is located carboxyl-terminal to aa 362, and predominantly after aa 489; thus MST1 associates with a segment in the carboxyl-terminal half of DAP4 that is mostly distal to the $p52^{rIPK}$ splice site.

DAP4 Dimerizes through Its Amino Terminus and Localizes to the Nucleus—Recombinant DAP4 is capable of homodimerization during transient expression, as demonstrated by the specific recovery of coexpressed FLAG-DAP4 with GST-DAP4; DAP4 homodimerization is mediated by the DAP4 amino-terminal segment aa 1–488 (Fig. 3*A*); self-association increases progressively as the amino-terminal fragment is lengthened from 60 to 300 amino acids, mediating that the self-association surface involves most of the amino-terminal half of DAP4 (Fig. 3*B*). These results also indicate that DAP4 is as likely to dimerize with the $p52^{rIPK}$ splice variant as with the full-length DAP4 polypeptide. We next ascertained the cellular localization of DAP4 by examining the distribution of recombinant FLAG-DAP4 or DAP4 fused in-frame with eGFP, either at the DAP4

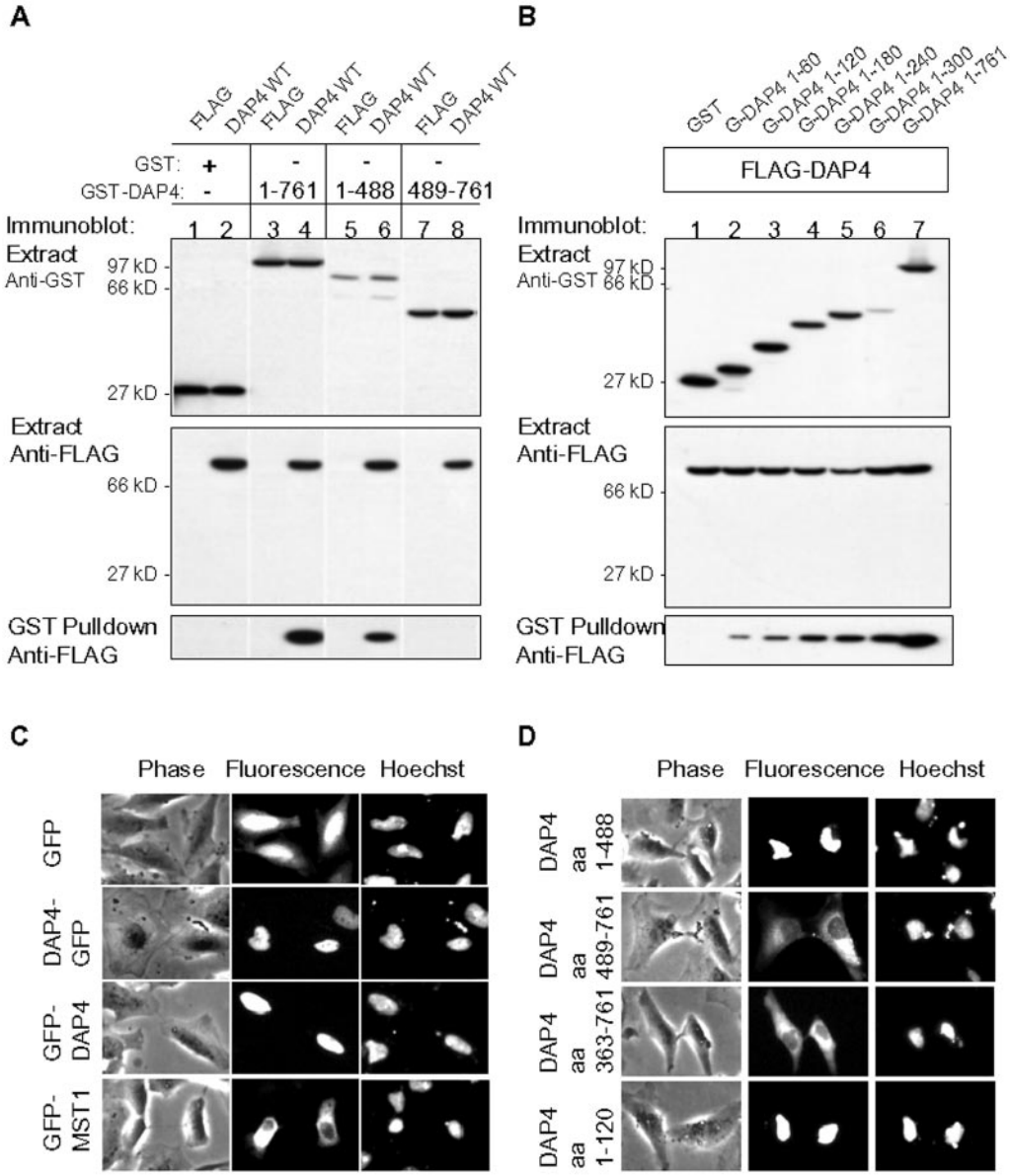

FIG. 3. **Dimerization and localization of DAP4.** *A*, HEK 293 cells were co-transfected with FLAG-DAP4 wild-type and GST or GST-DAP4 wild-type or GST-DAP4 aa 1–488 or GST-DAP4 aa 489–761. *B*, HEK 293 cells were co-transfected with FLAG-DAP4 wild-type and GST or GST-DAP4 fusion proteins (aa 1–60, 1–120, 1–180, 1–240, 1–300 and wild-type). Cells were lysed and supernatants were adsorbed to GSH-agarose. After washing, retained polypeptides were separated on an 8% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and immunoblotted with anti-FLAG antibody. The FLAG blot of the GSH-agarose isolate is shown in the *bottom panel*. *C*, HeLa cells were transfected with GFP, DAP4-GFP, GFP-DAP4, or GFP-MST1. *D*, HeLa cells were transfected with GFP or GFP fused to fragments of DAP4 (aa 1–488, 489–761, 1–120, and 363–761). Twenty-four hours after transfection the cells were incubated with 0.2 μg/ml Hoechst 33342 to stain nuclei, and imaged by fluorescence microscopy.

NH_2 or COOH terminus; all three DAP4 polypeptides gave indistinguishable results. DAP4 localizes exclusively in the nucleus, whether examined in HeLa (Fig. 3*C*), HEK 293, or NIH 3T3 cells (not shown). DAP4 nuclear localization is determined by sequences near the DAP4 amino terminus, between aa 1 and 120 (Fig. 3*D*). In contrast to DAP4, GFP-MST1 is localized predominantly in the cytoplasm (Fig. 3*C*), at least prior to the onset of apoptosis. The finding that endogenous DAP4 can be recovered from the cell in association with MST1, together with the presence of a classical bipartite nuclear localization signal at the MST1 carboxyl terminus (aa 469–487) led us to inquire whether MST1 enters and exits the nucleus; we therefore coexpressed GFP-MST1 with red fluorescent protein-DAP4 in several cell types, and followed the localization of these polypeptides over time; the localization of these two polypeptides remained distinct (Fig. 4*A*) until the onset of apoptosis and nuclear membrane breakdown, whereupon substantial overlap ensued (not shown). If, however, NIH 3T3 cells expressing GFP-MST1 were treated with leptomycin B, an inhibitor of CrmA-mediated nuclear export (23), substantial MST1-GFP fluorescence became evident in the nucleus within 20 min (Fig. 4*B*); identical results were seen in the HeLa cells. Thus MST1 is transiting rapidly through the nucleus, with its export mediated by the leptomycin B-sensitive CrmA nuclear export apparatus. We next sought to define the MST1 NES signals; the MST1 carboxyl-terminal tail contains two candidate leucine-rich NES motifs, at aa 361–370 and 444–451 (Fig. 5*A*). Each was mutated separately as shown in Fig. 5*B*, resulting in the partial localization of FLAG-MST to the nucleus. Concurrent mutation of both motifs resulted in the exclusive

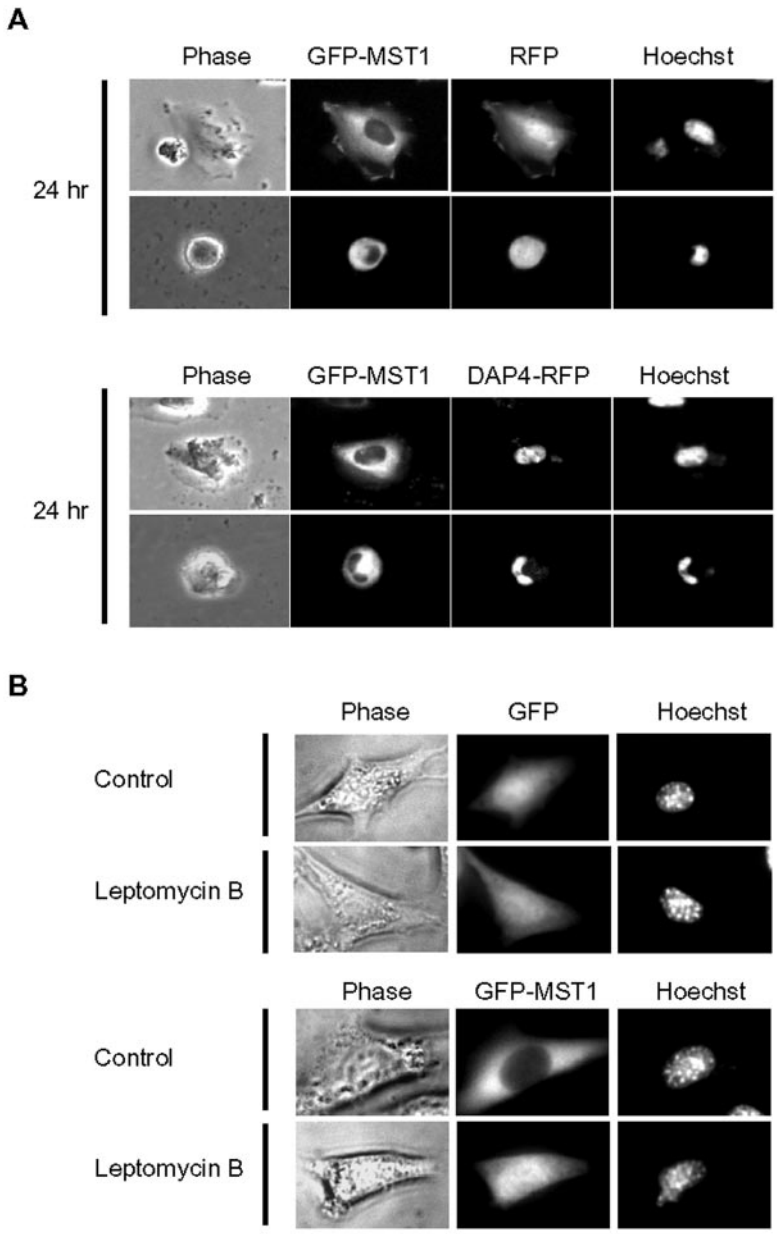

FIG. 4. **MST1 cycles between cytoplasm and nucleus.** *A*, HeLa cells were transfected with GFP-MST1 and DAP4-red fluorescent protein using FuGENE according to the manufacturer's instruction. Twenty-four hours after transfection, cells were stained with Hoechst 33342 for 30 min and were observed under a microscope; representative non-apoptotic and early apoptotic cells were shown. *B*, leptomycin B (10 ng/ml) was added to NIH 3T3 cells expressing GFP or GFP-MST1 at 24 h after transfection. The images shown were obtained 20 min after the addition of leptomycin B.

localization of MST1 to the nucleus. Similar findings were reported by Ura *et al.* (13). In summary, DAP4 is a constitutively nuclear protein that can bind directly the protein kinase MST1; the latter in turn cycles in and of the nucleus.

DAP4 Augments MST1-induced Apoptosis—As shown previously, transient expression of MST1 results in a dose-dependent apoptosis (6–14), *e.g.* as observed in HEK 293 cells (Fig. 6*A*). DAP4 expressed alone does not induce cell death in HEK 293 (Fig. 6*B*) or HeLa cells (not shown), however, coexpression of DAP4 with a low dose of MST1 substantially increases the extent of MST1-induced apoptosis. The ability of MST1 to induce apoptosis requires an active kinase function, and expression of MST1 (K59R) with or without DAP4 does not lead to increased cell death (Fig. 6*C*).

The ability of DAP4 to augment MST1-induced apoptosis is not associated with any change in the expression of the recombinant MST1 polypeptide (Fig. 6, *B* and *C*). We therefore examined whether DAP4 alters FLAG-MST1 kinase activity (Fig. 7), measured by the FLAG-MST1-catalyzed phosphorylation of histone 2B *in vitro*. Coexpression of increasing amounts of DAP4 with FLAG-MST1 gave an apparent 2-fold increase in FLAG-MST1 kinase activity measured *in vitro* (Fig. 7, *lanes 2–4*). A significant caveat, however, is that the coexpression of DAP4 with MST1 augments MST1-induced apoptosis, one consequence of which is the partial cleavage of MST1 itself, so as to generate a catalytic fragment with increased specific activity. The autophosphorylation of this fragment is evident in Fig. 7 (in the *third panel* from the *top*). We therefore also carried out this experiment by adding the caspase inhibitor zVAD to the cells during the entire period (Fig. 7, *lanes 5–7*) as well as by coexpression of a plasmid encoding the baculoviral general caspase inhibitor, p35 (Fig. 7, *lanes 8–10*). Whereas zVAD mildly inhibited basal and DAP4-stimulated MST1 kinase activity, p35 inhibited basal MST1 activity by 70% and essentially eliminated any stimulation of MST1 kinase by coexpressed DAP4. Based on these results we conclude that DAP4 does not activate the kinase activity of the full-length MST1 polypeptide, however, by augmenting MST1-induced apoptosis, DAP4 promotes the caspase-catalyzed cleavage of MST1 and the generation of an activated MST catalytic fragment. The

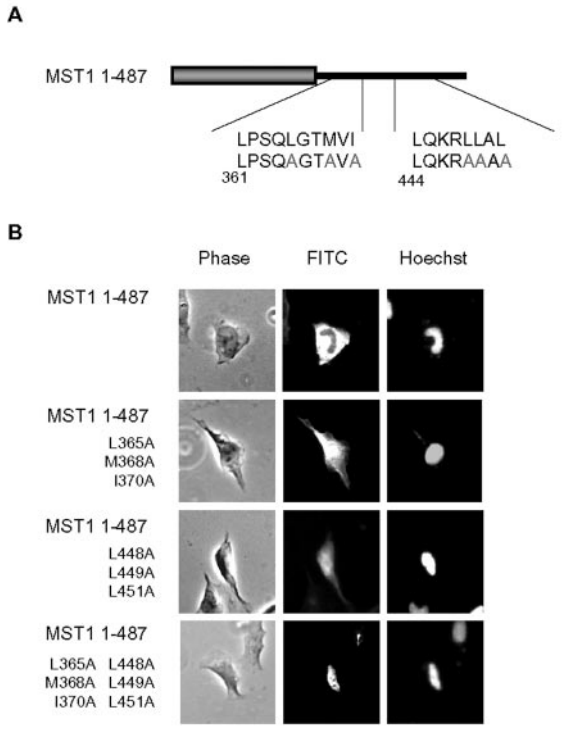

FIG. 5. **MST1 bears two nuclear export signals in its COOH-terminal noncatalytic tail.** *A*, two putative nuclear export signals and the mutations introduced are indicated in the *cartoon*. Within the two segments, the Leu^{365}/Met^{368}/Iso^{370} in the aa 361–370 and Leu^{448}/Leu^{449}/Leu^{451} in the aa 444–451 were all mutated to Ala. *B*, HeLa cells were transfected with FLAG-MST1 wild type (*upper panels*) or FLAG-MST1 bearing mutations in one or both of the putative NES. After fixation and blocking, cells were incubated with anti-FLAG mAb followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Cells were then incubated with Hoechst 33342 for 30 min and imaged under the fluorescence microscope.

ability of p35 to suppress the generation of this fragment is evident in Fig. 7 (*third panel* from the *top*), wherein the autophosphorylation of the 36-kDa fragment is seen to be suppressed by the presence of p35 to an extent far greater than that of the full-length MST1 polypeptide.

We next examined the ability of DAP4 to alter MST1 kinase activity *in vitro*. The addition of increasing amounts of recombinant DAP4 to purified recombinant FLAG MST1 does not significantly alter the rate of MST1-catalyzed H2B phosphorylation (Fig. 8*A*). Moreover, DAP4 is itself not significantly phosphorylated *in vitro* by recombinant MST1, either in the presence (Fig. 8*A*) or absence (Fig. 8*B*) of histone 2B. In summary, DAP4 augments MST1-induced apoptosis without altering MST1 abundance or intrinsic specific activity, and without serving as a substrate for MST1.

We next sought to gain insight into the mechanism by which DAP4 augments MST-induced apoptosis. DAP4s exclusive nuclear localization suggested that it might interact with a nuclear-localized proapoptotic effector of MST1. Perhaps the best characterized proapoptotic nuclear protein is p53 (24). A role for p53 (17, 18) in MST1-induced apoptosis is indicated by the ability of a dominant inhibitory mutant of p53 to suppress MST1-induced cell death by about 50% (Fig. 9*A*). The ability of the p53 miniprotein to inhibit MST-induced apoptosis is similar in magnitude to its ability to inhibit apoptosis in the interleukin-3-dependent DA-1 cell line induced by interleukin-3 withdrawal or UV (18). MST1 neither binds (Fig. 9*B, left panel*) nor phosphorylates p53 directly *in vitro* (Fig. 9*C*), however, recombinant DAP4 is capable of binding endogenous (Fig. 9*B, right panel*) or recombinant p53 (Fig. 9*B, middle panel*), both

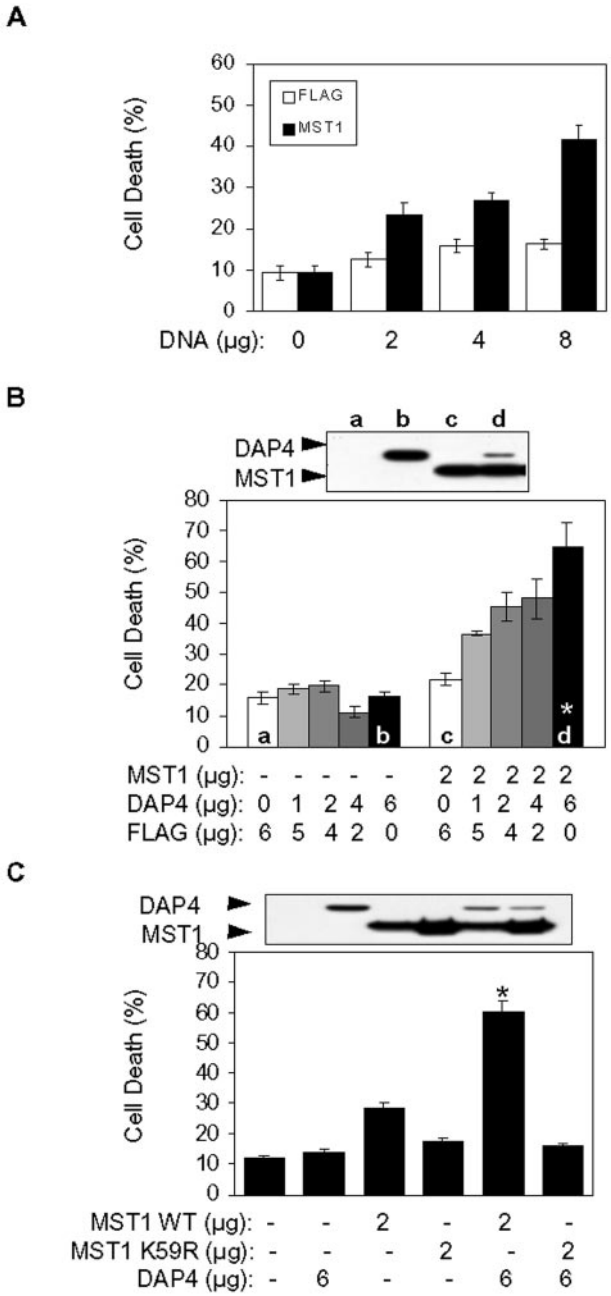

FIG. 6. **Effect of DAP4 on MST1-induced cell death.** HEK 293 cells were transfected with plasmids encoding DAP4 and/or MST1 in the amounts indicated. At 72 h after transfection, cells were collected and analyzed for apoptosis as described (20, 21). *A*, the effect of different amounts of MST1 on cell death. *B*, cell death in response to different amounts of DAP4 alone, or in the presence of 2 μg of MST1 and increasing amounts of DAP4; the *inset* is an immunoblot of DAP4-MST1 expression in the conditions indicated. *C*, effect of DAP4 on cell death induced by MST1 wild type (WT) or mutant, inactive MST1 (K59R); the *inset* is an immunoblot of DAP4-MST1 expression. The result is representative of one experiment repeated at least 3 times. Data are shown as mean ± S.E. ($n = 3$). *, $p < 0.05$ compared with the absence of DAP4.

during transient expression or directly *in vitro* (Fig. 9*B, left panel*). We propose that the ability of DAP4 to bind p53 may underlie, at least in part, the ability of DAP4 to promote MST1-induced cell death.

DISCUSSION

MST1 is a protein kinase whose overexpression results in apoptosis in many cell backgrounds. Seeking insight into the mechanism of MST-induced apoptosis we overexpressed a ki-

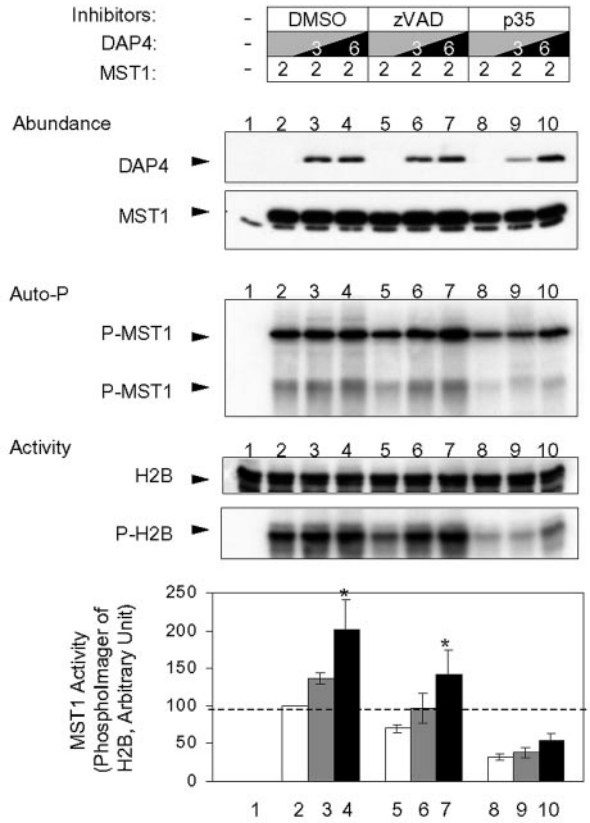

FIG. 7. **The effect of DAP4 on MST1 activity *in vivo*.** HEK 293 cells were co-transfected with a constant amount of MST1 and increasing amounts of DAP4. Some cells (*lanes 5–7*) were incubated with 50 μM zVAD throughout, whereas other cells (*lanes 8–10*) received 4 μg of p35 plasmid DNA together with the DAP4-MST1 plasmid cDNAs. After 72 h, cells were lysed and FLAG-tagged proteins were immunoprecipitated using anti-FLAG mAb prebound to protein G. MST1 kinase activity was measured by addition of exogenous substrate H2B. The PhosphorImager value of H2B phosphorylation by MST1 (transfected singly, *lane 2*) was set as 100%. The blot is representative of one experiment repeated 3 times. Data were mean ± S.E. ($n = 3$). *, $p < 0.05$. Compared with the absence of DAP4.

nase-inactive, caspase-resistant mutant of the MST1 in COS-7 cells and analyzed the copurifying endogenous polypeptides using mass spectroscopy of tryptic digests prepared from excised gel bands. We identified five tryptic peptides from an MST1-associated 88-kDa polypeptide that were all encompassed within a 761-aa polypeptide previously cloned by Barzilay and Kimchi, and named DAP4. Analysis of the *DAP4* gene family *in silico* revealed that the human genome data base contains 17 distinct genes encoding polypeptides homologous to DAP4. At least seven of these genes, each on a different chromosome, encode polypeptides with ≥75% sequence identity to DAP4; these are shown in Fig. 1. Some of these genes, such as *DAP4B* on Chr8, are likely to be processed *psuedo*genes, as DAP4B lacks introns and may have a frameshift near the translational start site. In addition to the seven sequences shown in Fig. 1, another 10 loci can be identified that encode clearly homologous polypeptides. Six of these 17 loci are situated nearby to another family member with three distinct homologues identifiable on Chr11. Thus the size of this newly discovered human gene family is substantial; moreover it is clear from the prior description of $p52^{rIPK}$ (16), a 492-aa polypeptide that is identical to DAP4A over their amino-terminal 488 amino acids, that the expression of these genes is further diversified by alternative mRNA splicing. The DAP4 polypeptides contain no previously recognized domain motifs. Moreover, whereas several zebrafish expressed tag sequences encoding closely related sequences are deposited in the data base, analysis of the completed *Caenorhabditis elegans* on *Drosophila melanogaster* genomes fails to reveal polypeptide sequences or domains with homology to the *DAP4* polypeptide family. We therefore infer that the *DAP4* genes arose early in vertebrate evolution (although an earlier origin in the chordates is possible) and underwent a rapid expansion by gene duplication, transposition, and mutation (25–28). It is well appreciated that a substantial increase in gene number occurred early in vertebrate evolution, and a substantial minority of human genes and/or domains lack an orthologue in invertebrates (29). This has been specifically noted for a variety of polypeptides broadly involved in apoptosis, particularly the extracellular components, adaptors, Bcl2 family members, and the NACHT family of NTPases (30). Although the first molecular insight into apoptosis emerged from *C. elegans*, elements such as the pyrins (and the pyrin domain), SMAC/Diablo, calpain inhibitors, interleukin-1-like molecules, and others have no counterparts at all nematodes or arthropods (30). The disproportionate expansion in the repertoire of apoptotic genes in vertebrates may reflect their greater developmental complexity or perhaps more narrowly, the requirements of acquired immunity. Thus, the elaboration of the *DAP4* gene family during vertebrate evolution is consistent with the participation of these polypeptides as apoptotic effectors.

The death-associated proteins represent a functionally heterogeneous array of polypeptides isolated by Kimchi and colleagues (31, 32) in a screen of an antisense cDNA library for inserts that could block interferon γ-induced apoptosis in HeLa cells. Seven sets of DNA fragments with confirmed antiapoptotic activity were recovered; two encoded fragments of the known proteins, cathespin D and thioredoxin. Five cDNAs encoded fragments of novel polypeptides, among which the most fully characterized at present are DAP2, also known as DAP kinase, and DAP5. The latter is a novel 97-kDa homolog of p220 eIF-4G that lacks the NH_2-terminal eIF-4E-binding domain (33). It is suggested that DAP5 may promote translation of mRNAs whose 5′-untranslated region segments contain IRES; many such mRNAs encode proteins involved in the control of apoptosis, whose translation is up-regulated during stress (34). DAP2 or DAP kinase is a calmodulin-binding protein kinase with a carboxyl-terminal death-effector domain, and bears no structural similarities to MST1/2 (35). DAP kinase overexpression promotes apoptosis at least in part by up-regulation of $p19^{ARF}$ and p53 (36). Moreover, DAP kinase expression is frequently lost in human tumors (37). DAP1 is a 15-kDa proline-rich phosphoprotein, whereas DAP3 is a ubiquitously expressed 46-kDa polypeptide; overexpression of each results in marked apoptosis of HeLa cells (31). The initial identification of DAP4 by Kimchi (31) as a possible participant in interferon γ-induced apoptosis in HeLa cells (31), together with the present finding that DAP4 binds to the proapoptotic protein kinase MST1, raises the question of whether MST1 is a participant in interferon γ-induced apoptosis in HeLa cells. In preliminary experiments we have not detected any activation of endogenous MST1 in response to interferon γ, and further experiments are ongoing.

The current report is to our knowledge, the first description of the properties of the DAP4 polypeptide, however, some properties of $p52^{rIPK}$, a carboxyl-terminal shortened splice variant of DAP4, have been previously described (16). The $p52^{rIPK}$ polypeptide was identified in a two-hybrid screen using as bait $p58^{IPK}$, a cellular inhibitor of PKR (38, 39). $p58^{IPK}$ contains nine tandem TPR motifs and a carboxyl-terminal DnαJ-like domain (13, 14), and binds to PKR through one TPR motif. $p58^{IPK}$ becomes available as a PKR inhibitor upon influenza

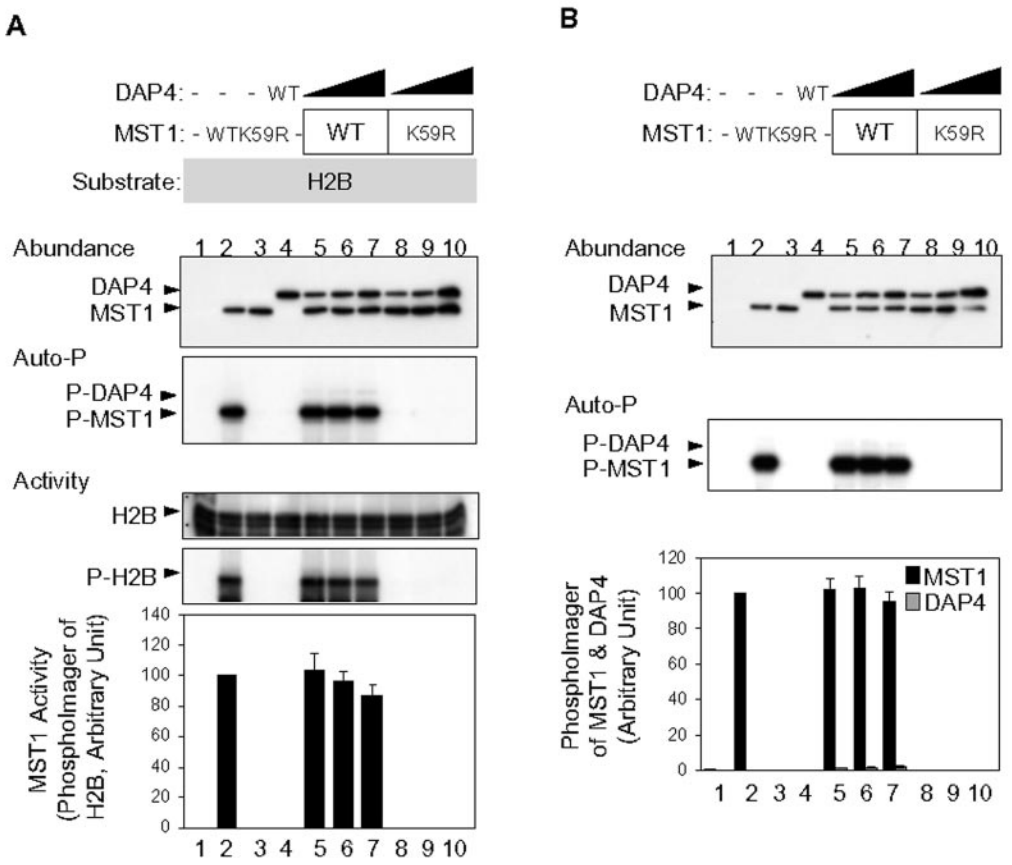

FIG. 8. **DAP4 is neither a regulator nor substrate of MST1 *in vitro*.** FLAG-MST1, FLAG-MST1 (K59R), and FLAG-DAP4 were each expressed individually and purified using FLAG-agarose. After elution of the purified FLAG-tagged polypeptides, a kinase assay was performed as described under "Materials and Methods." *A*, H2B serves as substrate; *B*, DAP4 is substrate. In *A* the PhosphorImager value of H2B phosphorylation by MST1 was set to 100%; *B,* MST1 autophosphorylation was set as 100%. The blot is representative of one experiment repeated 4 times. Data are mean ± S.E. ($n = 4$).

viral infection (40) or heat shock (41) and by binding to PKR, blocks its dimerization and activation (42). $p58^{IPK}$ also inhibits apoptosis in NIH 3T3 induced by double-stranded RNA as well as by tumor necrosis factor-α (43). The availability of $p58^{IPK}$ as a PKR inhibitor appears to be negatively regulated primarily through its association with HSP40 (44); $p58^{IPK}$ also binds to HSP70 (41). Based on these structural and functional properties, $p58^{IPK}$ has been suggested to be a co-chaperone (41). The $p52^{rIPK}$ polypeptide can bind $p58^{IPK}$ and abrogate its ability to inhibit PKR, at least when reconstituted in a yeast system. Moreover, Katze and co-workers (41) point to amino acids 86–200 of $p52^{rIPK}$/DAP4 as a segment bearing 24% identity to the charged domain of HSP90 (aa 170–300), a region implicated in regulatory interactions with the glucocorticoid receptor. They therefore suggest that $p52^{rIPK}$ (and thus DAP4) may be related to HSP90 in function (16). Inasmuch as DAP4 encompasses 488 of the 492 amino acids of $p52^{rIPK}$ it is very likely that DAP4 can also bind $p58^{IPK}$ (unless this is abrogated by the unique DAP4 carboxyl-terminal segment). DAP4 binding of $p58^{IPK}$ would occur through the DAP4 amino-terminal segment, distinct from the site of MST1 binding, pointing to the possible assembly of a heterotrimeric complex. Thus if DAP4 does bind $p58^{IPK}$, the latter polypeptide may act as a modulator of MST1 kinase activity as well as of PKR. In fact the ability of MST1 overexpression to initiate apoptosis suggests strongly that endogenous MST1 activity is restrained by a cellular inhibitor; moreover the activation of endogenous MST1 by severe cellular stress is consistent with the idea that heat shock proteins participate in the negative regulation of MST1 activity. Thus, further examination of the functional interactions and subcellular localizations of $p58^{IPK}$, DAP4, and MST1 is clearly warranted.

It appears that MST1 can promote apoptosis through several, possibly independent pathways. Ura *et al.* (12) reported that MST1 induction of caspase 3 activation, nucleosomal DNA fragmentation, membrane blebbing, and cell rounding can be partially inhibited by a dominant negative mutant of JNK1/SAPKγ (TPY to APF). We have observed that MST1 can activate coexpressed p54 JNK2/SAPKα (but not p38α); and coexpression of MST1 with a catalytically inactive mutant of SEK1 (Lys to Arg) inhibits MST1-induced apoptosis by 70% (data not shown). Thus, activation of SAPK plays a significant role in MST1-induced apoptosis. The present finding that inhibition of p53 suppresses MST1-induced apoptosis (Fig. 9*A*) unveils another pathway for MST1 promotion of cell death. Although JNK/SAPK has been reported to phosphorylate p53 Thr^{81}, and this modification is claimed to be necessary for stress-induced p53 activation (45), we have been unable to detect phosphorylation of p53 Thr^{81} *in vivo* by immunoblot (using antibody provided by Ronai, Ruttenberg Cancer Center) in the course of MST-induced apoptosis. Moreover, MST1 does not itself directly phosphorylate p53 *in vitro* (Fig. 9*B*). It is possible that MST1 and p53 contribute to apoptosis in an additive but largely independent manner. Nevertheless, the partial dependence of MST1-induced apoptosis on p53 and the ability of DAP4 to augment MST1-induced apoptosis and to bind both MST1 and p53 raises the possibility that DAP4 may promote MST1 action by enabling the apposition of MST1 to a p53-associated protein that acts to enhance the proapoptotic efficacy of p53, such as JMY or ASPP (46, 47).

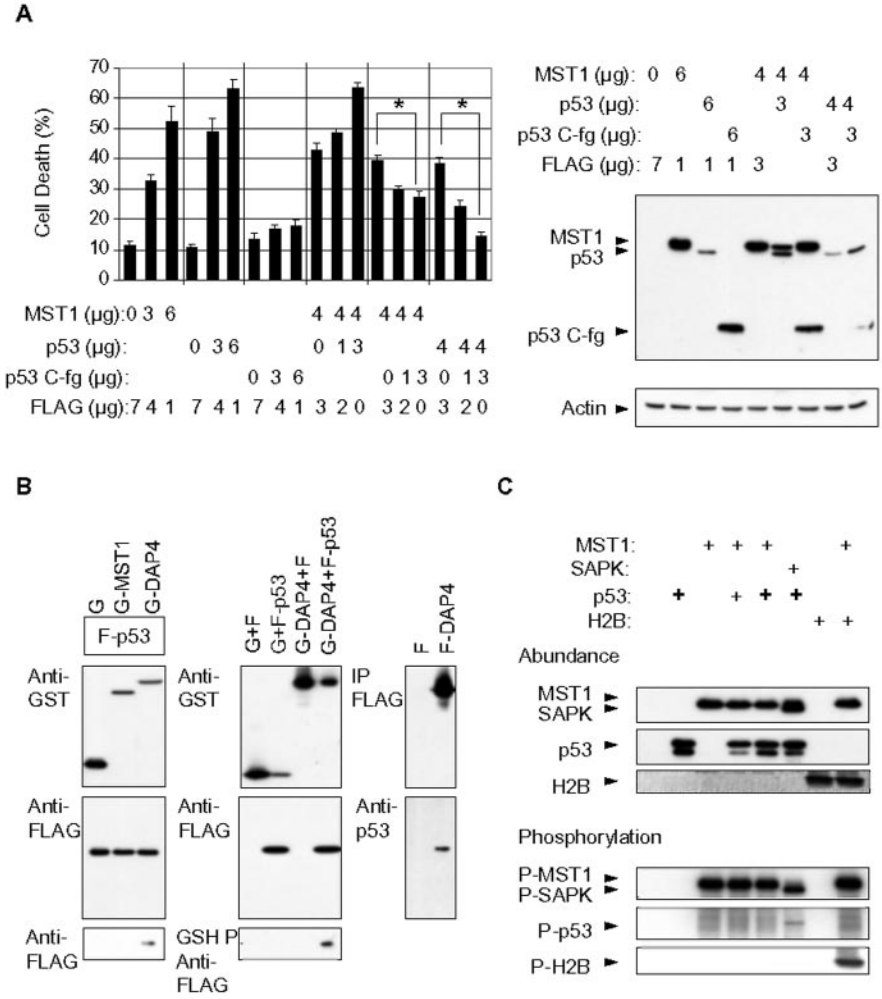

FIG. 9. **p53 participates in MST1-induced cell death.** *A*, the effect of dominant inhibitory p53 miniprotein (p53 aa 302–393, p53 C-fg, Refs 17 and 18) on MST1-induced cell death. HEK 293 cells were transfected with equal amounts of total DNA as indicated. Cell death induced by p53 or MST in the absence and presence of the p53 miniprotein was measured. A representative immunoblot of polypeptide expression in selected conditions is shown; an actin blot was used as a control. *B*, DAP4 binds p53. *Left panel*, binding of p53 to DAP4 or MST1 *in vitro*: HEK 293 cells were transfected with vectors encoding GST (*G*), GST-MST1 (*G-MST1*), GST-DAP4 (*G-DAP4*), or FLAG-p53 (*F-p53*). The GST proteins were immobilized on GSH-agarose, washed, and then the lysates from FLAG-p53-transfected cells were added to the GSH-agarose beads. After washing, the bound proteins were subjected to SDS-PAGE and blotted with anti-FLAG mAb. *Middle panel*, association of coexpressed DAP4 and p53. Plasmids encoding GST (*G*) or a GST-DAP4 fusion protein (*G-DAP4*) were each coexpressed in HEK 293 cells with empty FLAG vector or FLAG-p53 (*F-p53*). The FLAG blot of the GSH-agarose isolate is shown in the *bottom*. *Right panel*, recombinant DAP4 binds endogenous p53. HEK 293 cells were transfected with FLAG (*F*) or FLAG-DAP4 (*F-DAP4*). FLAG-tagged proteins were immunoprecipitated using anti-FLAG mAb prebound to protein G. The bead was subjected to intensive washed and run on 10% SDS-PAGE. The endogenous p53 blotting is shown in the *bottom*. *C*, SAPK, but not MST1 phosphorylates p53 *in vitro*. FLAG-p53 were expressed transiently in HEK 293 cells, purified, and eluted from FLAG-agarose. A phosphorylation reaction was carried out *in vitro* using purified recombinant MST1 or SAPK as described under "Materials and Methods." H2B was included as a positive control. All the blots are representative of one experiment repeated 2–3 times. Data are mean ± S.E. ($n = 3$). *, $p < 0.05$.

In summary, we identified DAP4 as an MST1-associated protein. DAP4, which is encoded by a member of a gene family apparently found only in vertebrates, is a constitutively nuclear protein. Overexpression of DAP4 does not initiate apoptosis, but substantially augments the extent of apoptosis caused by overexpression of MST1. MST1-induced apoptosis is dependent, in part on p53; DAP4 binds p53 as well as MST1, and is likely to act by promoting the p53-dependent component of MST1-induced apoptosis.

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Death-associated Protein 4 Binds MST1 and Augments MST1-induced Apoptosis

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