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A Mouse Fas-Associated Protein with Homology to the Human Mort1/FADD Protein Is Essential for Fas-Induced Apoptosis

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The Fas cell surface receptor belongs to the tumor necrosis factor receptor family and can initiate apoptosis in a variety of cell types. Using the Fas cytoplasmic domain as bait in a yeast two-hybrid screening, we isolated a mouse cDNA encoding a 205-amino-acid protein. Its predicted protein sequence shows 68% identity and 80% similarity with the sequence of recently described human Mort1/FADD. This protein, most likely the mouse homolog of human FADD, associates with Fas in vivo only upon the induction of cell death. A fraction of this protein is highly phosphorylated at serine/threonine residues, with both phosphorylated and unphosphorylated forms being capable of binding to Fas. Stable expression of a truncated form of the Mort1/FADD protein protects cells from Fas-mediated apoptosis by interfering with the wild-type protein–Fas interaction. Thus, mouse Mort1/FADD is an essential downstream component that mediates Fas-induced apoptosis.

Programmed cell death or apoptosis is a general phenomenon that occurs in a wide variety of cell types in many organisms and plays an important role during development. This process is usually characterized by chromatin condensation, membrane blebbing, and the generation of nucleosome-sized DNA ladders (24). In the immune system, apoptosis is a mechanism for the elimination of self-reactive immature and mature lymphocytes as well as for the destruction of target cells by cytotoxic T cells (for a review, see references 10, 12, 17, 26 to 28, 34, 41, 51, 52, and 54). Apoptosis of immature lymphocytes can be initiated by signaling through the T-cell receptor or the B-cell receptor (immunoglobulin). It can also be induced by irradiation, a glucocorticoid, or a depletion of growth factors. Prolonged engagement of the T-cell receptor in mature T cells also results in apoptosis by signaling of the Fas/APO1 (CD95) cell surface receptor (27, 34).

The Fas/Apo-1 (CD95) cell surface receptor is a member of the tumor necrosis factor (TNF) receptor family. The Fas ligand possesses structural homology to the TNF family (for a review, see references 27, 34, and 43). Both Fas and the Fas ligand are expressed in many cell types, and engagement of the pair can lead to apoptosis. Fas-mediated apoptosis plays an important physiological role, which is indicated by the phenotypes of two mutant mouse strains. The *lpr*/*lpr* strain of mice contains mutations at the Fas receptor, whereas the Fas ligand is mutated in the *gld*/*gld* strain of mice (46, 53). Both strains of mice have similar phenotypes: accumulation of $CD4^-$ $CD8^-$ T cells in spleen and lymph nodes, severe autoimmune diseases, and lymphadenopathy (11). Similarly, recent work also showed a correlation between Fas gene mutation and the development of human lymphoproliferative syndrome and autoimmunity (16, 38). Experiments by several groups have shown that the *lpr* mutation results in a defective apoptotic process in mature but not immature T cells, underscoring the importance of Fas in peripheral tolerance (33, 39, 42). In addition, Fas-mediated apoptosis may also play a role in immune privilege (2, 18).

In a manner similar to that of Fas, the type I TNF receptor (p55) can also deliver a death signal (reviewed in references 34 and 43). Both Fas and TNF receptor p55 contain a homologous cytoplasmic domain termed the death domain (22, 47). An amino acid substitution within the Fas death domain in the lpr^{cg} strain of mice results in a nonfunctional protein and leads to defective Fas-mediated apoptosis (53). The downstream events initiated by the Fas receptor are beginning to be understood. These events include the activation of a sphingomyelinase (8, 19, 48) and the involvement of an ICE-like protease (1, 15, 29, 49, 50). Recently, two different death domain-containing molecules, RIP and Mort1/FADD, were cloned in a yeast two-hybrid screening with the human Fas cytoplasmic domain being used as a bait (3, 7, 9, 45). Both proteins can initiate Fas-independent apoptosis when overexpressed. The FADD death domain appears to be crucial for its ability to interact with the Fas cytoplasmic domain but not for the initiation of apoptosis (3, 7). In addition to the two death domain-containing proteins, FAP-1/PTP-BAS phosphatase was shown to bind to the C-terminal portion of the Fas protein outside the death domain in vitro (40). Only the human Mort1/FADD protein, however, was shown to associate endogenously with the Fas receptor upon the induction of apoptosis (25). Furthermore, as most studies so far were done in an overexpression system, the physiological role of the proteins described above in Fas-mediated signaling is yet to be clearly defined.

During our own search for the downstream factors involved in Fas signaling in which the mouse Fas cytoplasmic domain was used as bait in a yeast two-hybrid screen, we isolated a mouse cDNA encoding a protein with homology to human Mort1/FADD. This protein, which exists in both phosphorylated and nonphosphorylated forms, interacts with Fas only under death-inducing conditions. Expression of an N-terminally deleted protein which serves as a dominant negative mutant protected a B-cell line from Fas-initiated apoptosis. Thus, we have identified mouse Mort1 and defined its essential role in Fas-mediated signaling.

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MATERIALS AND METHODS

Cell culture and apoptosis assay. A20 murine B lymphoma cells (obtained from the American Type Culture Collection) and DO11.10 T-cell hybridomas (provided by P. Marrack, University of Colorado, Denver) were maintained in RPMI 1640 (Gibco-BRL) medium plus 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Cell viability was assayed by propidium iodide uptake on a Coulter EPICS XL machine as described previously (31). For the induction of cell death, A20 parental or stably transfected cells were treated with anti-Fas monoclonal antibody Jo2 (Pharmingen) or RK8 (Kamiya Inc.) in the presence of 30 μg of cycloheximide per ml as described previously (35, 36).

Cloning and sequencing. The DNA sequence (nucleotides 608 to 1033) encoding the mouse Fas intracellular domain (53) was amplified by PCR with two primers (CG**GGATCC**GAAAGTACCGGAAAAGAAAG and CG**GGATCC**TC ACTCCAGACATTGTCCTTC) containing *Bam*HI sites (in boldface). The Gal4-Fas fusion bait was then generated by inserting this fragment into the *Bam*HI site of pAS1-CYH1 (a gift from S. Elledge). Two-hybrid screening was performed with a mouse peripheral blood T-cell library in pACT (a generous gift from S. Elledge) as described previously (14).

The *mort1* genomic clones were isolated by screening a mouse 129Sv liver genomic cosmid library (provided by M. Koshland), with the 1.4-kb cDNA fragment of *mort1* being used as a probe. The 4.7-kb *Eco*RI fragment of cos16A and 9.6-kb *Eco*RI fragment from cos2A were cloned into pSP72 (Promega) to make the pMortE4.7 and pMortE9.6 plasmids, respectively. For mapping of the 3′ flanking region, a 9-kb *Xho*I fragment of cos16A was cloned into the *Sal*I site of pSP72 to generate the pMortX9.0 plasmid. These plasmids were then used for the mapping of exon and intron structures by restriction mapping and sequencing with the oligonucleotide primers listed below: oligonucleotide 240 (CGAGCGC CTGGACGACTTTG [positions 240 to 258]), oligonucleotide 398 (ATCCCAT CCATCTTGGCC [positions 398 to 381]), oligonucleotide 550 (GCCCAGGA ATCTGTGAGC [positions 550 to 567]), oligonucleotide 1096 (GGTGCTACC CTTTAAACC [positions 1096 to 1079]), and oligonucleotide 1372 (CCTCACA ACTAGCACCCAG [positions 1372 to 1354]).

Plasmids and establishment of stable clones. For the expression of truncated Mort1, nucleotide sequence 250 to 1372 was PCR amplified. The 5′ primer (CG **GGATCC**TCTTTGGCAAGCGCCTTGTAGAAGCGCGTATGGCTGACGA CTTCGAGGCGGGG) contains the herpes simplex virus thymidine kinase translation initiation codon and sequence from pEVRF2 (32) as well as a *Bam*HI site (in boldface). The 3′ primer is CG**GGATCC**TCACAACTAGCGCCCAG, containing a *Bam*HI site (in boldface). After digestion, the amplified fragment was inserted into the *Bam*HI site of either pHβAPr-1-neo (20) or pcDNA3 (Invitrogen). Stable clones expressing Mort1 truncation were established in A20 cells by electroporation with a Gene Pulser (Bio-Rad) and then by selection in medium containing 1 and 2 mg of Geneticin (Gibco-BRL) per ml.

Antibody production, Western blot (immunoblot), immunoprecipitation analysis, and in vitro dephosphorylation analysis. Full-length Mort1 was fused to glutathione *S*-transferase by cloning a 1.4-kb *Bam*HI-*Bgl*II fragment from two-hybrid clone pACT-Mort1 17B into the *Bam*HI site of pGEX1. Glutathione *S*-transferase–Mort1 fusion protein was purified as described previously (44). Rabbit polyclonal anti-Mort1 antibodies were prepared and purified on a glutathione *S*-transferase–Mort1–Affi-Gel A10 (Bio-Rad) column as described previously (6).

For Western blots, whole-cell extracts were prepared by lysing the cells in lysis-immunoprecipitation buffer (see below). After centrifugation, 40 to 80 μg of the lysates was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were blotted to nitrocellulose Protran (Schleicher & Schuell). The filters were then treated with 5% nonfat milk in TBST (10 mM Tris-HCl [pH 8.0], 200 mM NaCl, 0.1% Tween 20) overnight at 4°C. Primary antibody interaction was carried out by a 1- to 2-h incubation of the filters in 10 to 15 ml of 5% nonfat milk in TBST plus 1 to 2 μg of anti-Mort1 antibody. After the binding of goat anti-rabbit immunoglobulin G–horseradish peroxidase (Caltag) for 30 to 45 min in the same buffer as that described above, the filters were washed in TBST and developed with Renaissance (Du Pont) per the manufacturer's instructions.

For immunoprecipitation, 2×10^7 A20 cells were lysed by rotating them at 4°C for 30 to 60 min in 1.4 ml of lysis-immunoprecipitation buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1 mM NaF, 0.5 or 1% Nonidet P-40 (Calbiochem), 0.2 mM phenylmethylsulfonyl fluoride, 1 mg of pepstatin (Sigma) per ml, and 1 mg of antipain (Sigma) per ml. After centrifugation, Mort1 in the supernatant was immunoprecipitated with 2 μg of anti-Mort1 antibody and 30 μl of 50% protein A–agarose beads (Pierce) by rotation at 4°C for 3 to 4 h. Fas was immunoprecipitated with 2 to 5 μg of monoclonal anti-Fas antibody Jo2 (Pharmingen) or RK8 (Kamiya) and protein G-agarose (Pierce) as described above. The beads were then washed four times with 1 ml of ice-cold lysis-immunoprecipitation buffer. The beads with the immunocomplex were boiled in sample buffer (0.125 mM Tris-HCl [pH 6.8], 2% SDS, 10% β-mercaptoenthanol), and the proteins were separated by SDS–12% PAGE. For the detection of the Fas signaling-specific interaction between Mort1 and Fas, A20 cells were incubated with anti-Fas antibody (1 μg per 10^6 cells) in medium for the times indicated in Results. DO11.10 cells were treated with phorbol myristate acetate (10 ng/ml) and ionomycin (A23187; 0.5 mM) for 6 h. Immunoprecipitation was carried out with anti-Fas antibody, and coimmunoprecipitation of Mort1 was detected with anti-Mort1 antiserum and Western blots as described above.

For in vitro dephosphorylation, Mort1 in the anti-Mort1 or anti-Fas immunoprecipitates was treated with 20 U of calf intestinal phosphatase (New England Biolabs) for 1.5 h at 37°C. The proteins were then resolved by SDS–12% PAGE and blotted onto a nitrocellulose filter. Mort1 was detected by Western analysis as described above.

Phosphoamino acid and phosphopeptide analysis. A20 cells were starved for 40 to 60 min in phosphate-free RPMI 1640 medium (Gibco-BRL) supplemented with dialyzed 10% fetal calf serum and labeled with $^{32}P_i$ (Du Pont) for 4 h. For apoptosis induction, anti-Fas RK8 or Jo2 was added for the time periods indicated in Results. Immunoprecipitation was carried out with anti-Mort1 antibody or anti-Fas antibody as described above. Phosphorylated Mort1 was resolved by SDS–12% PAGE and blotted onto polyvinylidene difluoride Polyscreen (Du Pont) or nitrocellulose. Phosphoamino acid and phosphopeptide analysis was performed as described before (4, 30).

Nucleotide sequence accession number. The cDNA sequence has been submitted to GenBank under accession number U50406.

RESULTS

Cloning of a mouse cDNA encoding a protein that interacts with Fas and is homologous to human Mort1/FADD. We have employed the yeast two-hybrid system (14) to identify the downstream components involved in Fas-mediated apoptosis. The cytoplasmic domain (amino acids 166 to 306) of the mouse Fas protein was fused to the *Saccharomyces cerevisiae* GAL4 DNA binding domain and used as bait to screen a mouse peripheral T-lymphocyte cDNA library constructed as fusions with the yeast GAL4 activation domain. During the initial screening of 10^6 yeast transformants, twelve *his*$^+$ clones were isolated and shown to form blue colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-containing plates. Complete sequencing of a 1.4-kb insert in one of the clones, 17B, revealed an open reading frame encoding a novel mouse protein with a size of 205 amino acids in frame with the Gal4 protein. This protein contains a region with homology to the death domain found in Fas and TNF receptor I. As a control, we constructed a Fas bait harboring the *lpr*^{cg} mutation. There was barely any interaction between the 17B clone and the *lpr* mutant bait detected in the yeast cells (data not shown). The deduced amino acid sequence of the 17B cDNA clone shows extensive homology, with 68% identity and 80% overall similarity, to that of human Mort1/FADD (3, 7). It is likely that we have isolated the mouse counterpart of human Mort1; therefore, this novel mouse protein is designated mouse Mort1/FADD. After the submission of this paper, a paper describing the cloning of mouse FADD by interaction with TRADD in yeast cells was published (21).

The mouse *mort1* gene contains two exons. The *mort1* gene locus was cloned by screening a mouse 129Sv cosmid genomic library with the 17B cDNA. The screening of 2×10^6 colonies yielded 15 different clones which overlap in sequence, indicating that there is likely only one copy of the gene per haploid genome. Clones cos2A, cos5A, cos10A, and cos16A encompass approximately 64 kb of DNA (Fig. 1). This section of DNA includes the entire *mort1* coding sequence, which is within a 3-kb region, as well as 35 and 26 kb of the 5′ and 3′ flanking regions, respectively. To delineate the *mort1* gene exon-intron structure, several oligonucleotides corresponding to the mouse *mort1* cDNA sequence were used to sequence the genomic DNA. Sequencing data showed that the mouse *mort1* gene appears to contain two exons, with the first exon encoding amino acids 1 to 95 and the second one encoding the rest of the polypeptide and the 3′ untranslated region (Fig. 1).

In vivo cell death-specific association between mouse Mort1 and Fas. The yeast two-hybrid screening frequently picks up proteins that nonspecifically interact with the bait. To see if the mouse Mort1-Fas association also occurs in vivo, we first gen-

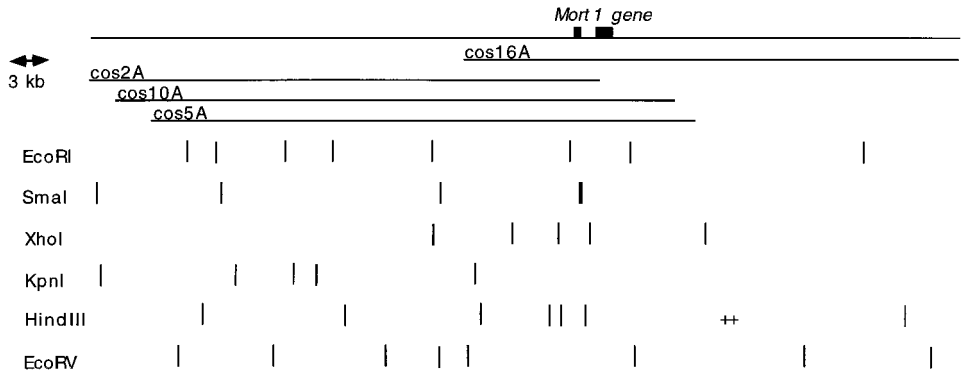

FIG. 1. Genomic structure of the mouse *mort1* gene. The restriction map of four cosmid clones encompassing 64 kb of the *mort1* gene locus is shown. The *mort1* exons further defined by sequencing (Materials and Methods) are depicted as black boxes. ++, additional restriction sites that are not completely mapped.

erated anti-Mort1 antisera. Mort1 protein was prepared in a large quantity with a glutathione *S*-transferase fusion-expression and purification system. Anti-Mort1 sera were then raised in rabbits and purified to high specificity with a Mort1 affinity column. In a Western blot analysis, these antibodies recognize specifically two closely migrating bands with sizes of about 30 kDa from mouse B-cell line A20 (Fig. 2, WCE lanes). Two protein bands with the same molecular weights were also immunoprecipitated with these purified antibodies from ^{35}S-labeled A20 B cells or DO11.10 T-cell hybridomas (55).

To detect the association between Fas and Mort1, we first used a monoclonal anti-mouse Fas antibody (RK8 or Jo2 [35, 37]) to immunoprecipitate Fas and its associated proteins. The immunocomplex was then resolved by SDS-PAGE, which was followed by a Western blot analysis using the purified Mort1 antibodies. As can be seen from Fig. 2, anti-Fas immunoprecipitate from untreated A20 cell extract does not contain the 30-kDa Mort1 protein (Fig. 2, Non-ind. lane). After cells were treated with monoclonal anti-Fas antibody to initiate apoptosis, however, two conspicuous Mort1 protein bands with sizes of around 30 kDa were coimmunoprecipitated with Fas. In addition, a third smaller band was also seen to coimmunoprecipitate with anti-Fas antibody and was recognized by the anti-Mort1 antibodies (see Fig. 3B). This result indicates that the endogenous Mort1-Fas association occurs in vivo only after the triggering of cell death. Similar results were also obtained with

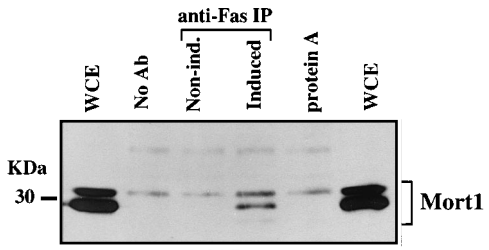

FIG. 2. The mouse Mort1 protein exists as two bands with sizes of about 30 kDa and coimmunoprecipitates with the Fas receptor upon stimulation. Whole-cell extracts were made from A20 cells (3×10^7) treated with 10 μg of anti-Fas RK8 for 2 h (Induced) or from those left untreated (Non-ind.). Immunoprecipitation was performed in lysis buffer with monoclonal anti-Fas RK8–protein A–agarose. Proteins were resolved by SDS–12% PAGE and subjected to Western blot analysis with purified anti-Mort1 polyclonal antibodies. In lanes WCE, whole-cell extract (100 μg of total protein) from untreated A20 cells was loaded directly. In lane protein A, 30 μl of 50% protein A–agarose was boiled and resolved, serving as a control. In lane No Ab, as a mock control, no antibody RK8 was used in the immunoprecipitation of untreated cell extracts. Rainbow marker (220 to 14.3 kDa; Amersham) was used as a size reference.

DO11.10 T-cell hybridomas (data not shown) treated with phorbol ester and ionomycin, which trigger T-cell receptor signaling, leading to Fas-mediated apoptosis (5, 13, 23).

Mort1 protein exists in both phosphorylated and nonphosphorylated forms. The inducibility of Fas-Mort1 interaction suggests that Fas trimerization after stimulation may recruit Mort1 to its cytoplasmic tail, leading to the initiation of downstream events. In many systems, receptor-mediated protein phosphorylation and/or dephosphorylation is the mechanism, for signal transduction. To explore these possibilities, A20 B cells were labeled in vivo with $^{32}P_i$ and Mort1 was immunoprecipitated with the purified Mort1 antisera. As can be seen from Fig. 3A (anti-Mort1 lanes), a fraction of the endogenous Mort1 protein is highly phosphorylated before and after the onset of apoptosis. Interestingly, the heavily phosphorylated species corresponds only to the upper Mort1 band detected by Western blotting (Fig. 3A). A faint phosphoprotein band which runs immediately below the major phosphorylated protein does not comigrate with the lower, unphosphorylated band detected on the Western blot. It could be a partially phosphorylated Mort1 protein. Phosphatase treatment of a Mort1 immunoprecipitate reduces the two Mort1 bands detected by the Western blot analysis to the faster migrating one (Fig. 3B). This result confirms that the mouse Mort1 protein consists of both phosphorylated (upper band) and nonphosphorylated (lower band) forms.

We also analyzed the phosphorylation status of the Fas-associated Mort1 protein. Surprisingly, no ^{32}P-labeled protein corresponding to the 30-kDa Mort protein could be detected in the coimmunoprecipitate of the anti-Fas antibody (Fig. 3A, anti-Fas lane), even at a much longer exposure (data not shown). As a control, the same filter was probed with anti-Mort1 antibodies, and both bands of the Mort1 protein were present in the anti-Fas coimmunoprecipitate after the initiation of apoptosis, as was expected (Fig. 3). It appears that both phosphorylated and nonphosphorylated Mort1 is recruited to Fas upon induction. Thus, the failure to detect ^{32}P-labeled Mort1 associated with Fas is most probably due to the poor sensitivity of the labeling experiments. Interestingly, a third-fastest-migrating band that is recognized by the anti-Mort1 antibodies was present in the anti-Fas coimmunoprecipitates (Fig. 3B). This protein can only be seen in the Fas-associated complex; it cannot be detected in the whole-cell extract, even after anti-Fas treatment. It might correspond to a proteolytically processed Mort1 (see Discussion).

Phosphoamino acid analysis of the labeled Mort1 protein showed that it is phosphorylated at both serine and threonine

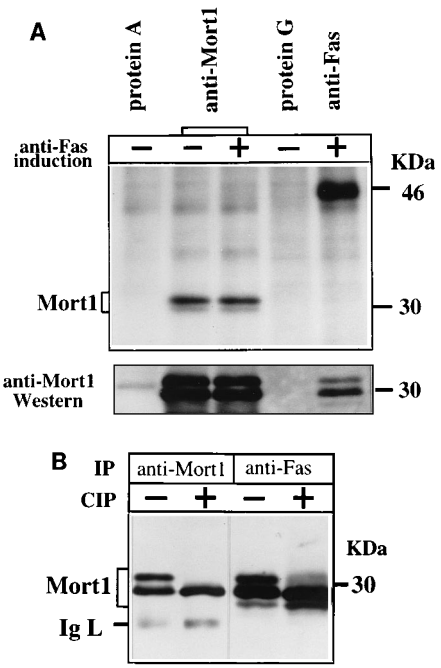

FIG. 3. Mouse Mort1 exists as both phosphorylated and unphosphorylated proteins. (A) A20 cells were metabolically labeled with $^{32}P_i$. For the initiation of Fas signaling, the cells were treated with anti-Fas Jo2 for 30 min (the lanes labeled with plus signs). Immunoprecipitation was performed with anti-Mort1 antibody (the anti-Mort1 lanes). A mock immunoprecipitation of untreated cell extract with protein A-agarose or protein G-agarose but without antibodies served as a control (the protein A and G lanes). To immunoprecipitate Fas and its associated proteins, protein G-agarose was added to extracts from anti-Fas-treated cells (the anti-Fas lane). The proteins were resolved by SDS–12% PAGE, blotted to a polyvinylidene difluoride membrane, and exposed to X-ray film for 4.5 h (upper panel). To show that Mort1 is coimmunoprecipitated with Fas, the same filter was probed with anti-Mort1 antibody and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (lower panel). Note, as was discussed in Results, that the lower, faint, ^{32}P-labeled band in the upper panel (the anti-Mort1 lanes) has a slightly lower mobility than, and therefore is not identical to, the lower band of Mort1 on the Western blot (lower panel). The 45-kDa phosphoprotein in the anti-Fas lane matches the size of Fas and is also present in the nontreated cells (data not shown). Molecular masses (in kilodaltons) are indicated on the right. (B) In vitro dephosphorylation of Mort1. A20 cells were treated with anti-Fas Jo2, and the cell lysates were immunoprecipitated (IP) first with anti-Fas Jo2 (the anti-Fas lanes) and then with anti-Mort1 antibodies (the anti-Mort1 lanes). Half of the immunoprecipitates were treated with calf intestinal phosphotase (CIP; the lanes labeled with plus signs). IgL, the rabbit immunoglobulin light chain of the anti-Mort1 antibody which is recognized by the secondary antibody.

residues, with no changes being detected before and after anti-Fas treatment (Fig. 4A). To see if the phosphorylation pattern of the total cellular Mort1 protein might change during apoptosis, tryptic phosphopeptide analysis was performed on a two-dimensional gel. Tryptic digestion of phospho-Mort1 yields two prominent spots on the two-dimensional gel (Fig. 4B). Several other weaker spots were also visible after a longer exposure. No major changes for Mort1 proteins from noninduced and anti-Fas-induced cells could be detected in the profile of tryptic phosphopeptides.

A truncated Mort1 protein protects cells from Fas-mediated apoptosis and interferes with endogenous Mort1-Fas interaction. To examine the specific role of Mort1 in Fas-mediated cell death, we generated mutant forms of Mort1 and tested their effects on Fas signaling after being stably expressed in the B-cell lymphoma A20 line. Since the death domain appears to be the site of interaction between Fas and human Mort1 (3, 7), we first tried to overexpress a truncated Mort1 containing residues 111 to 175 (the death domain) in A20 cells. However, we failed to detect the mutant protein after stable transfection, possibly because of its instability. Transfections were then carried out with vectors expressing a truncated form of Mort1 lacking the first 80 amino acids (81 to 205), as a similar truncated human Mort1 protein was previously shown not to initiate apoptosis after being overexpressed in a transient transfection experiment (7). Two different expression vectors were used: pHβAPr-1-neo with the actin promoter and pcDNA3

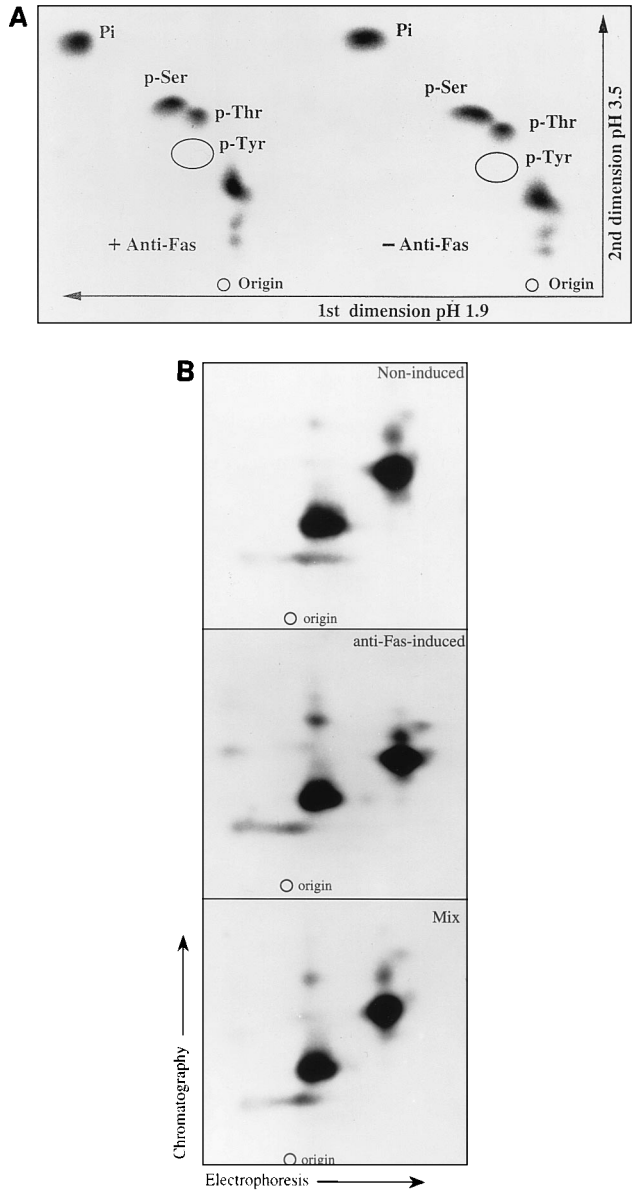

FIG. 4. Phosphoamino acids and phosphopeptide mapping of Mort1. Mort1 was immunoprecipitated from ^{32}P-labeled, anti-Fas RK8-treated and -untreated A20 cell lysates and resolved by SDS–12% PAGE. (A) For phosphoamino acid analysis, proteins were blotted onto polyvinylidene difluoride membrane and partially hydrolyzed with HCl. The phosphoamino acids were resolved by two-dimensional electrophoresis. (B) Part of the labeled proteins were blotted onto nitrocellulose and digested with trypsin. The peptides were separated by electrophoresis in one dimension and chromatography in the other. For mobility control, one-third of the peptides from either Fas-treated or -untreated cells were mixed and resolved in the same way (bottom panel). The plates were exposed to X-ray film for 30 days.

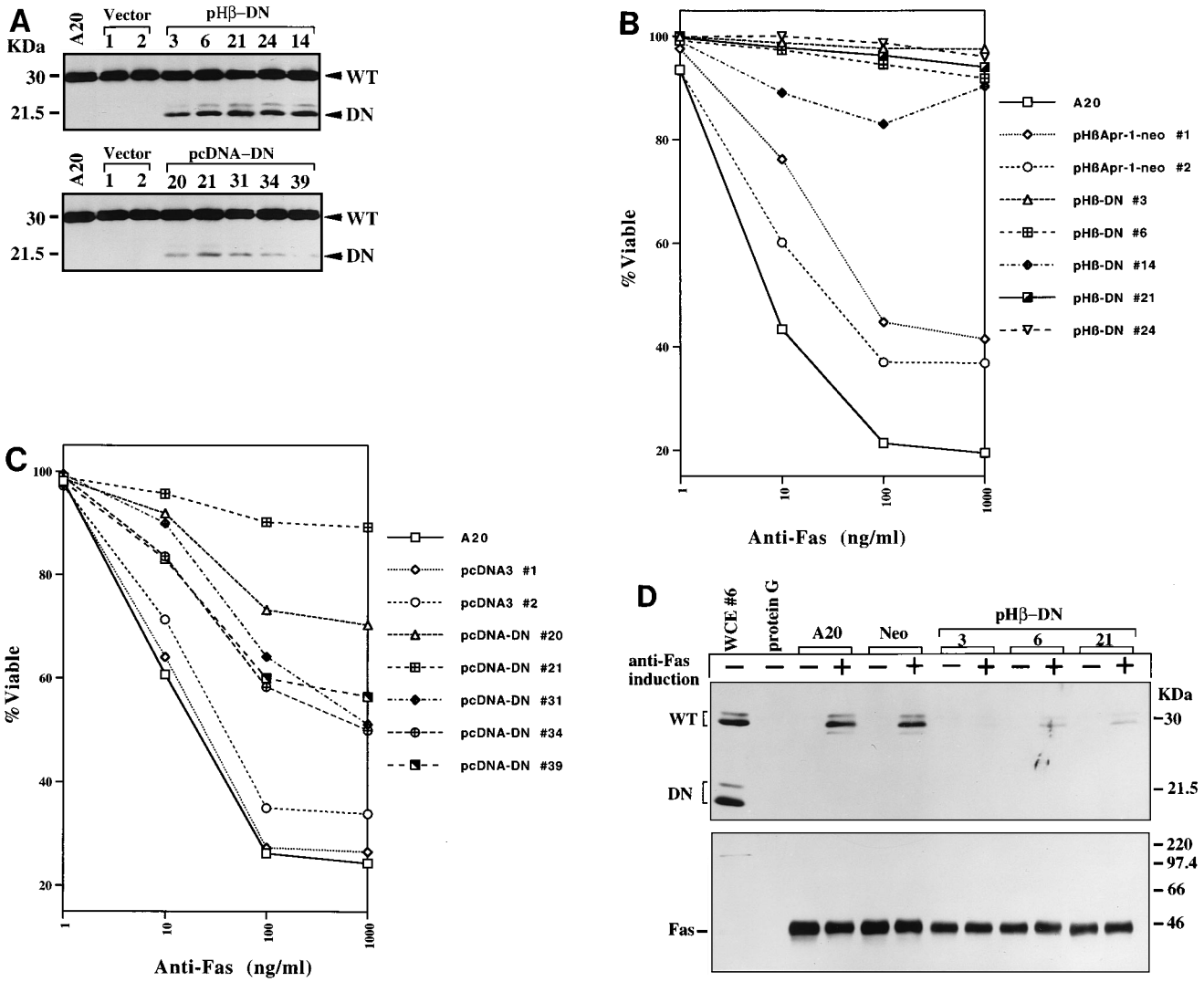

FIG. 5. A truncated Mort1 function as a dominant negative protein protects cells from Fas-mediated apoptosis because of its interference with the endogenous protein interaction. (A) A truncated Mort1 protein lacking the first 80 amino acids was expressed in A20 cells by using two different expression vectors. The mutant protein expression was confirmed by detecting the presence of the protein in cell lysates with anti-Mort1 antibody and Western blots. The wild-type (WT) and the mutant (DN) proteins are indicated on the right. A20 is the parental cell. Two stable clones (Vectors 1 and 2) containing the cloning vector from each were included as controls. pHβ-DN (3, 6, 21, 24, and 14) and pcDNA-DN (20, 21, 31, 34, and 39) are each five stable clones expressing the mutant protein from the pHβApr-1-neo (top panel) and pcDNA3 (bottom panel) vectors, respectively. (B) Parental A20 cells, vector control clones (pHβApr-1-neo 1 and 2), and five stable clones (pHβ-DN 3, 6, 14, 21, and 24) expressing the Mort1 dominant negative protein were tested for their sensitivities to Fas-mediated killing. Cells were incubated with various concentrations of anti-Fas RK8 in medium for 17 h, and the viability of the cells was assayed as described in Materials and Methods. (C) Parental A20 cells, vector control stable clones (pcDNA3 1 and 2), and stable clones expressing Mort1 dominant negative protein (pcDNA-DN 20, 21, 31, 34, and 39) were assayed for their sensitivities to Fas-mediated killing as described above. (D) Fas-Mort1 interaction was analyzed by immunoprecipitation with anti-Fas and then by Western blotting with anti-Mort1 antibody (upper panel) as described in the legend to Fig. 3. The same filter was stripped of the antibodies and reprobed with polyclonal anti-Fas antibody (lower panel). WCE, whole-cell extract of pHβ-DN clone 6; protein G, mock immunoprecipitation control with protein G-agarose and nontreated A20 cell lysates; Neo, pHβApr-Neo clone 2; WT, wild-type protein; DN, mutant protein.

with the cytomegaloviral promoter. Five different stable clones from each transfection experiment were chosen for analysis (pHβ-DN 3, 6, 21, 24, and 14 and pcDNA-DN 20, 21, 31, 34, and 39). As a control, we also generated stable clones by using the corresponding expression plasmids (pHβAPr-1-neo 1 and 2 and pcDNA3 1 and 2). Whole-cell extracts were made from these clones and the parental A20 cells and were analyzed by Western blots, with the Mort1 rabbit polyclonal antisera being used. All of the clones express an additional Mort1 antiserum-reactive protein with a size of around 21 kDa, which most likely corresponds to the size of the truncated Mort1 protein (Fig. 5A). As expected, this protein species is not present in the cell extracts from either the parental A20 cells or the four vector control stable cell lines. Expression of the truncated Mort1 protein varies among clones, with pcDNA-DN 20, 31, 34, and 39 expressing low levels of the truncated protein and pHβ-DN clones expressing higher amounts of the truncated protein (Fig. 5A).

To see if expression of the truncated Mort1 protein (amino acids 81 to 205) affects the process of Fas-mediated apoptosis, all the stably transfected clones were tested for their sensitivities to the anti-Fas antibodies. Fifteen hours later, the extent of cell death was measured by propidium iodide dye exclusion and quantitated on a fluorescence-activated cell sorter ma-

chine. As can be seen from Fig. 5B and C, the A20 parental cell line and the four vector control clones were killed efficiently by the anti-Fas antibody. In contrast, all 10 stable clones expressing the truncated Mort1 protein exhibited various degrees of protection from Fas-mediated killing (Fig. 5B and C). The extent of protection correlated largely with the expression levels of the truncated Mort1. An almost complete protection was seen for the pHβ-DN clones and pcDNA-DN 21, whereas the resistance to Fas killing is incomplete for the other four pcDNA-DN clones (20, 31, 34, and 39) that express lower levels of the truncated Mort1 protein. The levels of the Fas cell surface receptor for the different stable clones were similar to that for the parental A20 cells, except for clone pHβAPr-1-neo 1, which expresses Fas at 85% of the A20 level (data not shown).

To see if the dominant negative protein interferes with the Fas-Mort1 association, we analyzed the amount of Mort1 that coimmunoprecipitated with the anti-Fas antibody. The same amounts of Mort1 protein coimmunoprecipitated with Fas in cell extracts from the control neomycin stable cell lines and the parental A20 cells (Fig. 5D). In contrast, little wild-type Mort1 can be seen to coimmunoprecipitate with Fas in three of the dominant negative clones analyzed (Fig. 5D). The same filter was probed with the polyclonal anti-Fas antibodies (Santa Cruz), which showed similar amounts of Fas immunoprecipitated in all lanes (Fig. 5D). These data indicate that the Mort1 dominant negative protein interferes with the Fas-Mort1 association, hence protecting the cells from delivering the apoptotic signals.

DISCUSSION

Fas-mediated apoptosis is a rapid process that is independent of any new RNA-protein synthesis. The process involves activation of a protease, which most likely acts in the downstream pathway common to all apoptotic processes (1, 15, 29, 49, 50), and stimulation of a sphingomyelinase (8, 19, 48). The immediate signaling components for Fas are just beginning to be characterized. Four proteins that constitute the major components of the human Fas-signaling complex were identified (25). Two of these (CAP1 and CAP2) were shown to be the human Mort1/FADD protein, which was previously cloned by using a yeast two-hybrid screen (3, 7). Using a similar technique and the mouse Fas cytoplasmic tail as a bait, we have identified a mouse cDNA (17B) encoding a protein with significant homology to the human Mort1/FADD protein. In the Western blot analysis with the rabbit polyclonal antibodies specific for clone 17B, this protein appears as two closely migrating bands with sizes of 29 to 30 kDa. Transfection of the 17B cDNA in mammalian expression vector pCI into A20 cells resulted in the overproduction of the 29- to 30-kDa protein (data not shown), indicating that clone 17B encodes a full-length cDNA. In addition, we also showed that this protein coimmunoprecipitates with the mouse Fas receptor upon stimulation with anti-Fas antibodies, indicating that it interacts specifically with Fas in vivo. Thus, clone 17B represents mouse Mort1.

A fraction of the mouse Mort1 protein is heavily phosphorylated in vivo. Engagement of the Fas receptor, however, does not lead to any significant phosphorylation changes in the total Mort1 protein. Mort1 proteins with two slightly different apparent molecular weights were detected in the total cell extracts. They correspond to the phosphorylated and nonphosphorylated proteins. Although both forms of Mort1 protein were recruited to the Fas complex, as was indicated by Western blot analysis, a ^{32}P-labeling experiment failed to detect obvious phosphorylation. The most likely explanation is that the Western blot analysis is more sensitive than the ^{32}P-labeling experiment in this case. In the human system, Fas-associated proteins CAP1 and CAP2, identified as the human Mort1 proteins, were also phosphorylated (25). In the total cell extracts, we also detected two phosphorylated mouse Mort1 proteins, with one being a minor species. Interestingly, in addition to the two forms of Mort1 proteins, a faster-migrating Mort1 species was detected in the Fas complex. This protein species was not found in the cytoplasm and is not due to a lack of phosphorylation, as phosphatase treatment of the bound Mort1 proteins only reduced the slowest-migrating protein to the mid-range species. This 28-kDa Mort1 species was not detected in the cytoplasm, even after a prolonged exposure of the Western blot. The most likely explanation for this observation is the activation of a protease which digests the Mort1 protein in the Fas complex to a smaller molecule. The mechanism and physiological significance of this possible processing of Fas-bound Mort1 are currently under investigation.

In the ^{32}P-labeling experiment, we also noticed a phosphoprotein with the same molecular weight as the Fas protein in the immunoprecipitate of anti-Fas antibodies (Fig. 3A). Thus, it appears that Fas itself is phosphorylated, but the phosphorylation status does not change before or after stimulation with anti-Fas antibodies (unpublished data). In addition, it is also interesting that the SDS-stable, high-molecular-weight human Fas aggregates that are formed immediately upon anti-Fas treatment (25) were not seen for the mouse Fas protein in B cells (Fig. 5D).

Human Mort1 has been previously shown to associate with the human Fas protein; its role in the death-inducing signals by Fas, however, had not been addressed. Overexpression of human Mort1/FADD was shown to lead to apoptosis in a variety of cell lines (3, 7). It is not clear how these effects are related to the normal function of Mort1 in vivo. The overexpression studies showed that truncation of the 41-amino-acid N-terminal peptide of human Mort1 inactivates its ability to initiate cell death whereas the C terminus of Mort1 serves as a protein-protein interaction domain with Fas (3, 7). Thus, an N-terminally deleted Mort1 protein may serve as a dominant negative protein to interfere with Fas-mediated killing (7). We tested this idea by expressing an N-terminally deleted mouse Mort1 protein in a B-cell lymphoma to see if it can suppress Fas-mediated apoptosis. As was predicted from the overexpression studies, stably transfected cells expressing the N terminal-truncated Mort1 protein with the death domain (amino acids 81 to 205) did not undergo apoptosis and were actually protected from Fas-mediated cell death. The extents of protection correlate with the expression levels of the truncated Mort1. Introduction of this dominant negative protein blocks cell death by interfering with the ability of the wild-type protein to associate with Fas. Surprisingly, however, the dominant negative protein does not bind to Fas under apoptotic or nonapoptotic conditions. Although a truncated Mort1 protein containing the death domain was previously shown to be able to interact with Fas (3, 7), these studies were carried out with yeast cells. The Mort1-Fas association in yeast cells is clearly nonphysiological, as the endogenous Mort1-Fas association in mammalian cells cannot be detected under nonapoptotic conditions. Similarly, the results obtained from overexpression experiments in mammalian cells can also be misleading, as was indicated by the ability of an overexpressed Mort1 protein to associate with Fas under nonapoptotic conditions (3, 7). We have repeated the experiments reported on in Fig. 5D several times, and it is clear that the blockage of wild-type Mort1-Fas association is not due to competition between wild-type and mutant Mort1 proteins

for binding to the Fas cytoplasmic tail. It is known that Fas needs to be trimerized to initiate apoptosis (34). Thus, it is likely that Mort1 also needs to be trimerized to bind to Fas. Previous studies have shown that human Mort1 can associate with itself (3). It is possible that association of the dominant negative and wild-type Mort1 proteins results in a complex that cannot bind to Fas. Consistent with this hypothesis is the almost complete effect of the dominant negative in blocking apoptosis, despite its lower than wild-type level of expression. Further studies on the Mort1 protein should lead to an elucidation of how Fas signaling is mediated and how the apoptotic machinery operates in mammalian cells.

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