Association of Human Fas (CD95) with a Ubiquitin-conjugating Enzyme (UBC-FAP)*

(Received for publication, July 10, 1996)

Dowain A. Wright†§, Bruce Futcher, Propa Ghosh‡, and Raif S. Geha¶

From the ∆Department of Medicine, Division of Immunology, Children’s Hospital, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115 and ¶Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

A novel human ubiquitin conjugating enzyme (UBC) was found to associate with Fas (CD95). The mRNA for this UBC Fas-associated protein (FAP) was widely expressed in human tissues, and the protein was identified in several mammalian cell lines. UBC-FAP shows strong homology to two recently identified UBCs, Hus5 and Ubc9, which control yeast cell cycle progression. UBC-FAP, but not an active site mutant, complemented ubc9-1ts mutants. This suggests that UBC-FAP is a human homologue of Ubc9, possesses ubiquitin conjugating activity, and may play an important role in mammalian cell cycle regulation. A single amino acid substitution in the death domain of Fas that abolishes Fas-mediated apoptosis also abolished Fas association with UBC-FAP, suggesting that UBC-FAP may play a role in Fas signal transduction. The sequence of UBC-FAP is identical to that of HsUbc9, a UBC recently shown to interact with Rad51.

The Fas antigen (CD95) is a cell surface molecule which is a member of the tumor necrosis factor/nerve growth factor family of proteins (1). Binding of a monoclonal antibody (mAb) to Fas can cause apoptosis in cell lines expressing Fas (2). A defect in Fas gene expression has been identified as the cause of the lymphoproliferative syndrome and of the autoimmune disease resembling systemic lupus erythematosus in homozygous MRL-lpr mice (3) and in humans with autoimmune lymphoproliferative syndrome (4, 5). These observations suggest that Fas is involved in the removal of self-reactive T and B cells in the normal host and in the maintenance of self-tolerance by inducing the programmed cell death of self-reactive lymphocytes.

Mutational analysis of the human Fas antigen has defined a 68-amino acid signal-transducing cytoplasmic domain which is required for the transduction of an apoptotic signal and which is significantly conserved in both Fas and the TNF receptor (6). Recently, several novel proteins were isolated (MORT/FADD and RIP) that interact with the death domain of Fas, can independently cause apoptosis, and contain a sequence motif related to the Fas death domain (7–9). Immunoprecipitation studies have shown the association of four proteins called cytotoxicity-dependent APO-1-associated proteins (CAP) with aggregated CD95 (10). CAP 1/2 are phosphorylated forms of MORT/FADD and CAP 3/4 represent the recently cloned MORT/FADD interacting protein MACH/FLICE (11, 12). Finally, FAP1, a novel protein of unknown function, associates with the intracellular region of Fas and potentiates Fas-mediated killing (13). The role of these proteins in Fas killing remains to be established.

Although much is now known about the Fas signaling complex, the mechanisms that determine Fas sensitivity after ligand dissociation of the Fas receptor are not well understood. It is possible that additional proteins associate with the Fas complex to modulate Fas signaling. In an effort to investigate the mechanism of Fas signal transduction, we used the yeast two-hybrid system to probe a cDNA library from the Fas-sensitive KT3 cell line and cloned a novel intracellular protein which associated with the intracellular domain of Fas. This Fas-associated protein (FAP) is a human homologue of the recently identified ubiquitin conjugating enzyme (UBC), Ubc9, which controls cell cycle progression from G2 to M and has been shown to regulate the degradation of cyclins in Saccharomyces cerevisiae (14). A single amino acid substitution Val238→Asn in the death domain of the intracellular region of Fas that abolishes Fas-mediated apoptosis also abolished interaction of UBC-FAP with Fas, suggesting that UBC-FAP may play a role in Fas killing.

EXPERIMENTAL PROCEDURES

Cell Culture—The human T lymphoma cell line KT3 (kindly provided by S. Shimizu, Kanazawa Medical University) was maintained in RPMI 1640 media supplemented with 10% fetal calf serum and 0.2 to 2.0 ng/ml recombinant human IL-6 (R&D Systems). COS-7 cells (ATCC CRL-1651) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Construction of cDNA Library—Poly(A)+ mRNA was isolated from KT3 cells (Messenger RNA Isolation Kit, Stratagene) and unidirectional cDNAs were generated with the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer’s recommendations. The EcoRI to XhoI directional cDNAs were ligated into pGAD424 (Clontech) which was linearized with EcoRI andSalI and dephosphorylated. Approximately 1 × 106 independent clones were generated in Escherichia coli XL2-Blue MRF’ (Stratagene), and plasmid DNA was isolated directly without amplification.

Two-hybrid System—Human Fas cDNA in CDMS (15) was cleaved withPstI and partially digested with HphI to generate a 440-base pair Fas intracellular (IC) fragment which encoded the entire Fas intracellular region with the exception of the membrane-proximal 3 amino acids. A synthetic linker/adapter was synthesized with oligonucleotides 5′-AAATCAAGAAGG-3′ and 5′-TTTTGTTG-3′. The “bait” vector pGBT9 (Clontech) was digested with PstI and dephosphorylated, then cut with EcoRI. A trimolecular ligation with vector, linker/adapter, and FasIC generated a clone, pGBP1C, containing the Gal4 binding domain fused in-frame to the intracellular region of Fas from amino acids 174.
Fas Interacts with a Human UBC
to 319 and was confirmed by DNA sequence analysis. Two-hybrid strain YWO102a was created essentially by the manufacturer (Matchmaker Two-Hybrid System Protocol, Clontech).

For analysis of the self-association of Fas, the FAS_C cassette from pGBKFC was excised with EcoRI and PstI and cloned into pGAD424, which had been cleaved with EcoRI and PstI and dephosphorylated, generating pGADIC. For analysis of self-association of UBC-FAP, pFAP-1 was digested with AflIII (which cleaves at the initiating ATG, (A/C)ATGT), blunt-ended with Klenow polymerase, then cut with SpeI and gel-purified. The FAP cDNA was ligated into pGNT9 which has been dephosphorylated, generating pGFBAP.

**GST Fusion Protein Expression and in Vitro Binding Assay**—The intracellular regions of human Fas and CD40 and the entire UBC-FAP coding region were radiolabeled with 32P using heat muscle kinase and purified from E. coli (Pharmacia Biotech Inc.). Expression and purification of GST fusion proteins was performed essentially as described (16), typically yielding 3 to 5 mg of protein/ml of swollen glutathione-agarose beads.

GST fusion proteins containing UBC-FAP and the intracellular regions of Fas and CD40 were radiolabeled with 32P using heat muscle kinase and purified from E. coli (Pharmacia Biotech Inc.). Expression and purification of GST fusion proteins was performed essentially as described (16), typically yielding 3 to 5 mg of protein/ml of swollen glutathione-agarose beads.

GST fusion proteins containing UBC-FAP and the intracellular regions of Fas and CD40 were radiolabeled with 32P using heat muscle kinase and purified from E. coli (Pharmacia Biotech Inc.). Expression and purification of GST fusion proteins was performed essentially as described (16), typically yielding 3 to 5 mg of protein/ml of swollen glutathione-agarose beads.

GST fusion proteins containing UBC-FAP and the intracellular regions of Fas and CD40 were radiolabeled with 32P using heat muscle kinase and purified from E. coli (Pharmacia Biotech Inc.). Expression and purification of GST fusion proteins was performed essentially as described (16), typically yielding 3 to 5 mg of protein/ml of swollen glutathione-agarose beads.
The presence of 20 mM 3-aminotriazole, and a plus denotes growth both on the selective medium and β-galactosidase activity.

![Image](340x438 to 522x729)

**TABLE I**

Two-hybrid results for HF7c clones containing the Gal4 binding or activation domain vectors alone (None) or with the indicated fusion protein insert

| Gal4 activation domain inserts | UBC-FAP | Fas | SV40 T |
|-------------------------------|---------|-----|--------|
| None                          |         |     |        |
| Fas binding domain inserts    |         |     |        |
| Lamin 5′                      |         |     |        |
| p53                           |         |     |        |

The predominant band migrated at approximately 1.35 kilo-

Results

Identification of a Fas-associated Protein—We used the two-

hybrid system (17) to screen for cDNAs that encode for proteins which interact with the intracellular region of the Fas receptor (FasIC). We constructed a fusion protein bait vector containing the region from amino acids 175 to 319 of the Fas gene fused with the DNA binding domain of the yeast transcription factor Gal4 (amino acids 1–147). A human lymphocyte cDNA library constructed from the Fas-sensitive KT3 cell line, a T cell lymphoma with high levels of Fas expression and high sensitivity to Fas-mediated killing, was constructed and cloned into a vector containing the Gal4 activation domain (amino acids 768–881) to generate fusion proteins which can be screened for interaction with the FasIC gene product.

A total of 3 × 10^6 transformants were screened, and 47 large His⁺ colonies were obtained after 4–8 days at 30 °C. Eighteen colonies were positive in the β-galactosidase filter assay. Clones containing putative Fas-interacting cDNAs were characterized further by sequence analysis and retransformation into HF7c for verification of true positives. Of the 17 clones which could be sequenced, 13 formed four groups of cDNAs which encoded a single gene and were fused in the same reading frame at four different positions in the 5′-untranslated region. The longest cDNA clone was designated pFAP-1 and was utilized for further study. Table I summarizes the results of the two-hybrid screen. They demonstrate that the protein encoded by pFAP-1 interacts specifically with Fas and did not interact with the Fas region of human lamin, murine p53 (Table I), or the intracellular region of CD40 (data not shown). As previously reported (7), Fas was found to self-associate. pFAP-1 was also cloned into the Gal4 binding domain plasmid and was found to interact with itself suggesting that it has the capacity to dimerize or oligomerize.

Sequencing of pFAP-1 revealed a putative cDNA with a 5′-untranslated region of 136 base pairs to the first methionine and a coding region which consisted of 474 base pairs to the TAA stop codon corresponding to a 158-amino acid protein with a calculated molecular mass of 19.6 kDa. Data bank search of the protein sequence revealed a striking homology to two recently identified genes encoding a new family of E2 ubiquitin-conjugating enzymes (UBCs) which act as regulators of cell cycle progression in yeast (14, 18). Therefore, the protein encoded by pFAP-1 was termed UBC-FAP. The sequence of UBC-

Expression of the UBC-FAP in Human Tissues and Mammalian Cell Lines—Northern blot analysis showed the presence of the UBC-FAP mRNA in all human tissues examined (Fig. 1). The predominant band migrated at approximately 1.35 kilo-

bases which corresponded well with the size range of cDNAs isolated in the two-hybrid screen. UBC-FAP mRNA expression was variable, with the highest expression in thymus, testis, ovary, and heart, lower expression in spleen, prostate, small intestine, colon, peripheral blood lymphocytes, pancreas, and in skeletal muscle, and low but detectable expression in placenta, lung, liver, brain, and kidney. Some tissues showed hybridization to 3.5- and 7-kilobase mRNAs. These bands were also variably seen in Northern blots of RNA from lymphocyte cell lines (data not shown) and may represent spliced or alternatively spliced transcripts of UBC-FAP or result from cross-hybridization with RNA from homologous gene products as the probe contained the highly conserved active site of E2 proteins. When the Northern blots were stripped and reprobed with Fas cDNA, Fas mRNA was also found to be expressed in nearly all tissues examined. Fas mRNA was most abundant in ovary but could be readily detected in spleen, thymus, prostate, testis, small intestine, colon, peripheral blood lymphocyte, heart, placenta, lung, liver, and skeletal muscle. It was less abundant in kidney and pancreas and could not be detected in brain. Interestingly, this is different from the results published for murine Fas expression, which showed mRNA expression in thymus, liver, heart, lung, and ovary, but could not be detected in brain, spleen, bone marrow, kidney, testis, or uterus (3). The blots were stripped and reprobed with human actin (data not shown).

Rabbit polyclonal antiserum was generated by immunization with GST-FAP fusion proteins and the antiserum recognized a 20.5-kDa protein in lysates from multiple human cell lines including KT3, Jurkat T cells, and the B cell line BJAB, as well
with heart muscle kinase, then were cleaved from the with GSH. The recombinant fusion proteins were radiolabeled over glutathione (GSH)-Sepharose beads followed by elution of FLAG-tagged UBC-FAP fusion by the binding could be competed away by excess cold UBC-FAP but not CD40IC could specifically bind GST-Fas but not GST and the UBC-FAP could self-associate and co-migrated (Fig. 3). Fig. 3 also shows that radiolabeled FasIC bound specifically to immobilized GST-FAP but not to GST. This binding was specific because radiolabeled CD40IC did not bind to GST-FAP and because an excess of cold UBC-FAP but not CD40IC was specific because radiolabeled CD40IC did not bind to GST-FAP and because an excess of cold UBC-FAP but not CD40IC could inhibit the binding of radiolabeled FasIC to GST-FAP (Fig. 3B). Fig. 3 also shows that radiolabeled UBC-FAP binds to GST-FAP indicating that UBC-FAP can self-associate and confirming the results of the two-hybrid system. The reverse experiment was also performed and showed that radiolabeled UBC-FAP could specifically bind GST-Fas but not GST and the binding could be competed away by excess cold UBC-FAP but not CD40IC (data not shown).

Fig. 2. Western blot of COS-7 cell lysates which were transfected with FLAG (MOCK) or FLAG-tagged UBC-FAP fusion protein vectors (FLAG-FAP). The transfected cells were lysed in cytoplasmic lysis buffer (cytoplasmic), and the nuclei and debris were resuspended in total cell lysis buffer (Nuclear). The lysates were separated by SDS-PAGE and transferred to nitrocellulose. The filter was probed with rabbit anti-UBC-FAP polyclonal antiserum followed by horseradish peroxidase-conjugated goat anti-rabbit Ig and visualized by ECL (Amersham). UBC-FAP is indicated by the open arrow.

Fig. 4. Association of native Fas with GST-FAP. KT3 cell lysates were either cleared with mouse IgG, (lanes 1 and 2) or ZB4 anti-Fas mAb (lane 3) and adsorbed to GST (lane 1) or GST-FAP (lanes 2 and 3). Affinity-precipitated proteins were separated by SDS-PAGE along with ZB4, lane 4) and IgG, (lane 5) immunoprecipitates from KT3 lysates, transferred to nitrocellulose, probed with ZB4 mAb followed by biotinylated goat anti-mouse IgG and horseradish peroxidase-conjugated streptavidin, and developed by ECL (Amersham) for 2 h (lanes 1–3) or 15 min (lanes 4 and 5).

5-kDa band precipitated with GST-FAP but not with GST alone (lanes 1 and 2). In contrast, this band was not detected in lysates precleared with ZB4 mAb and adsorbed to GST-FAP (lane 3). Furthermore, the 45-kDa band comigrated with the band that was specifically revealed by the ZB4 mAb in Fas but not in isotype control immunoprecipitates from KT3 cells (lanes 4 and 5). These data demonstrate that native Fas protein is able to associate with recombinant UBC-FAP.

Mutational Analysis of the Interaction between Fas and UBC-FAP—A single amino acid substitution, Ile225 → Asn, in the intracellular domain of Fas underlies the deficiency in Fas-mediated killing in lpr/cg mice (3). The corresponding substitution in human Fas, Val238 → Asn, abolishes Fas killing in cells transfected with the mutant receptor (6). We therefore examined whether the Val238 → Asn mutation could affect the capacity of Fas to associate with UBC-FAP. The FasIC and UBC-FAP mutants were generated by a novel oligonucleotide-directed method called CMCR. This method utilizes a thermostable DNA polymerase to rapidly generate mutants in a single day with high fidelity and efficiencies up to 99%. The Fas 238 point mutation abolished association with UBC-FAP (Figs. 5 and 6A). Thus, the same amino acid substitution that blocks Fas killing also abolishes UBC-FAP association. Deletion of the carboxy-terminal region of Fas to amino acid 304, which deletes the putative negative regulatory region which was shown to bind to a Fas-associated phosphatase (20) did not affect association with UBC-FAP (Fig 6A). Further deletion to amino acid 209 in the death domain abolished association with UBC-FAP. These results suggest that UBC-FAP could be involved in...
the intracellular signal transduction leading to Fas-mediated apoptosis.

Mutational analysis of UBC-FAP showed that the aminoterminal 83 amino acids are required for association with Fas (Fig. 6B). A point mutation in the UBC active site (Cys93 → Ser) which has been shown to silence the enzymatic activity of other UBC enzymes did not affect association with Fas.

UBC-FAP Is a Human Homologue of Ubc9—It has been suggested that premature progression in the cell cycle may lead to apoptosis (21). A novel UBC from S. cerevisiae, Ubc9, which displays strong homology to UBC-FAP, has been shown to participate in the degradation of B-type cyclins, an event which is required for progression through the cell cycle (14). The temperature-sensitive ubc9-1 mutant strain when grown at the nonpermissive temperature, halts cell cycle progression at the G2 or early M phase. We asked whether UBC-FAP can rescue these mutants and if so whether this rescue requires the ubiquitin conjugating enzymatic activity of this UBC-FAP. UBC-FAP cDNA and a UBC-FAP Cys93 → Ser mutant, derived by a CMCR mutation of codon 93 changing the conserved Cys in the active site to Ser, were streaked with HF7c transformed with p53/pGADIC (A), pGBIF/pFAP-1 (B), pGBIFΔCG/pFAP-1 (C).

FIG. 5. Fas association with UBC-FAP is abolished in Fas"-like mutants. S. cerevisiae strain HF7c was sequentially transformed with pFAP-1 and the Gal4 binding domain fusion constructs containing native FasIC (pGBFIC) or the Val238 → Asn mutant (pGBFICΔCG). Association of murine p53 (pVA3) and the SV40 T antigen (pTD1) was used as a positive control. Two (SD) medium plates, one lacking Leu/Trp and one lacking Leu/Trp/His but containing 20 mM 3-aminotriazole, were streaked with HF7c transformed with p53/pGADIC (A), pGBFIC/pFAP-1 (B), pGBIFΔCG/pFAP-1 (C).

FIG. 6. Deletion mapping of UBC-FAP and FasIC to determine the minimal regions required for association in the two-hybrid system. Full-length UBC-FAP and mutants expressed as fusion proteins with the Gal4 AD were tested for the ability to associate with FasIC. Alternatively, FasIC and mutants expressed as fusion proteins with the Gal4 BD were tested for the ability to associate with UBC-FAP. A minus indicates no growth on Leu/Trp/His negative SD medium in the presence of 20 mM 3-aminotriazole, and a plus denotes both growth on the selective medium and β-galactosidase activity.

The temperature-sensitive ubc9-1 mutant strain when grown at the nonpermissive temperature, halts cell cycle progression at the G2 or early M phase. We asked whether UBC-FAP can rescue these mutants and if so whether this rescue requires the ubiquitin conjugating enzymatic activity of UBC-FAP. UBC-FAP cDNA and a UBC-FAP Cys93 → Ser mutant, derived by a CMCR mutation of codon 93 changing the conserved Cys in the active site to Ser, were placed in a yeast expression vector, and the clones were used to transform Ubc9 and ubc9-1 mutants. Fig. 7 shows that wild type Ubc9 grows well at either 23 or 36 °C, while the ubc9-1 mutants grew at 23 °C, but completely failed to grow at the nonpermissive temperature (36 °C). ubc9-1 mutants transformed with wild type UBC-FAP were able to grow at 36 °C at a rate 10% of that observed at 23 °C. This level of reconstitution is similar to that seen when the human homologue of the yeast UBC CDC34 was used to rescue temperature-sensitive CDC34 yeast mutants (22). In contrast, ubc9-1 mutants transformed with Cys93 → Ser-mutated UBC-FAP completely failed to grow at the nonpermissive temperature. This result strongly suggests that this UBC-FAP is a human homologue of Ubc9 and may play a role in the control of the cell cycle and that this role involves the ubiquitin conjugating enzymatic activity of this UBC-FAP.

DISCUSSION

Fas Associates with a Novel Human UBC—Programmed cell death is a common mechanism for regulation of development in multicellular organisms, with precise control of both cell num-
Fas Interacts with a Human UBC

bers and differentiation of specific lineages. Induction of programmed cell death by the Fas-Fas ligand axis (23) plays a critical role in normal lymphocyte development and function. Although much has been learned recently about the Fas signaling complex, the mechanisms for determining Fas sensitivity after ligation of the Fas receptor are not known. To address this question, we have utilized the two-hybrid system to identify putative signal-transducing proteins which interact with the intracellular region of human Fas. Using this method, we cloned a Fas-associated protein cDNA which encodes a novel human UBC E2 protein (UBC-FAP).

Ubiquitin-dependent proteolysis is a major pathway involved in the degradation of abnormal and short-lived proteins in eukaryotes (24). The hallmark of this pathway is the covalent attachment of ubiquitin to a target protein. Ubiquitin is a 76-amino acid polypeptide which is highly conserved among eukaryotic organisms. Ubiquitin conjugate formation requires the combined action of three classes of proteins. These are the ubiquitin activating enzyme (E1) (25), ubiquitin-conjugating enzymes (E2), and in some cases additional proteins (E3) referred to as ubiquitin-protein ligases, believed to play a role in the recognition of consensus instability sequences at the amino terminus of the substrate (26–29). Ubiquitin is first activated by E1 through an ATP-dependent formation of a thioester between the carboxyl-terminal Gly of ubiquitin and the active-site Cys residue in E1. The activated ubiquitin is then transferred to a Cys residue of an E2, preserving the high energy thioester bond. Finally, the E2 itself, or in concert with an E3 protein, catalyzes the formation of an isopeptide bond between the carboxyl-terminal Gly of ubiquitin and an ε-amino group of a lysine residue on the target protein. Ubiquitin can be linked to itself primarily through a lysine residue at position 46, resulting in mult ubiquitinated proteins that are recognized and degraded by the proteasome complex. For some precursor proteins, e.g. NF-κB1, ubiquitination serves to process the protein into a component that is degraded and an active component, e.g. NF-κB (30). Surface receptors can also be ubiquitinated after cross-linking, as in the T-cell receptor γ-chain (31) and FcεRI (32), which may target them for internalization, degradation, or activation, although the true physiologic significance of this remains unclear.

UBC-FAP Is a Functional Homologue of Ube9—UBC-FAP showed strong homology to S. cerevisiae Ubc9. Ubc9 has been shown to participate in degradation of the yeast S phase cyclin CLB5 and the M phase cyclin CLB2 in a cell cycle-specific manner. Mutants in Ubc9 function prevent yeast cell cycle progression at the G2 or early M phase, and disruption of the Ube9 gene was lethal (14). We have shown that UBC-FAP but not the Cys → Ser active site mutant can rescue ubc9-I mutants (Fig. 7). These data suggest that UBC-FAP is a functional UBC enzyme. It is possible that activation of UBC-FAP could lead to degradation of human homologues of CLB5 and CLB2 or other potential substrates and cause inappropriate initiation of mitosis leading to apoptosis.

Western blotting of cytoplasmic and nuclear fractions of COS-7 cells with rabbit anti-UBC-FAP suggested that UBC-FAP is predominantly present in the cytoplasm (Fig. 2), while Ubc9-β-galactosidase fusion proteins have been located in the nucleus (28). This suggests that UBC-FAP may have different substrate specificity or functions than Ubc9. Alternatively, UBC-FAP may be transported to the nucleus in a cell cycle-dependent manner or after activation during Fas ligation.

UBC-FAP Is Expressed and Associates with Fas in Human Cell Lysates—Northern blot analysis showed the presence of UBC-FAP mRNA in all human tissues examined (Fig. 1). UBC-FAP was expressed as a 20.5-kDa protein in mammalian cells including COS-7 cells (Fig. 2). The levels of UBC-FAP mRNA expression correlated in general with those of Fas. Interestingly, Fas expression is much more widespread in human than reported in mouse (33). The coexpression of Fas and UBC-FAP is consistent with UBC-FAP participating in Fas signal transduction.

Recombinant as well as native Fas from KT3 cells bound to recombinant UBC-FAP protein (Figs. 3 and 4). Only a relatively small fraction of Fas in KT3 lysates was affinity-purified by an excess of recombinant UBC-FAP. This may be due to a relatively low binding affinity or possibly represents an enzyme substrate interaction. Optimal binding may be contingent upon modification of either of these proteins following Fas ligation. A low binding affinity between Fas and UBC-FAP may explain our unsuccessful efforts to date to coprecipitate these proteins from cell lysates.

Support for a role of UBC-FAP in Fas signal transduction was obtained by generation of Fas mutants. A single amino acid substitution (Val228 → Asn) abolishes Fas killing in cells transfected with the mutant (6), and the corresponding substitution in murine Fas underlies the deficiency in Fas-mediated killing in lpr mice (33). This mutation destroys the association of other Fas-associated proteins found by two-hybrid screening (7–9, 13) This mutation also abolished association of Fas with UBC-FAP in the two-hybrid system. Carboxyl-terminal deletion into the death domain abolished association of Fas with UBC-FAP while deletion of the negative regulatory region did not (Fig. 6). This suggests that UBC-FAP plays an important role in Fas signal transduction possibly by ubiquitinating Fas or Fas-associated molecules. Attempts to detect ubiquitination of the intracellular region of Fas have not been successful to date.

It has been suggested previously that premature progression in the cell cycle can lead to apoptosis (21). Numerous genes that play a role in the regulation of cell proliferation are implicated in the control of apoptosis. These include p53, e-Myc, Rb-1, E1A, cyclins, and p34cdc2 kinase (34–36). Apoptosis induced by granzyme B as well as activation-induced T-cell death in synchronized T-cell hybridomas are associated with increased activity of cyclin-dependent kinases, in part p34cdc2 kinase (36, 37). Because activation-induced T-cell death is mediated by Fas-FasL interaction, it is possible that Fas ligation induces activation of p34cdc2 kinase. Thus, an alternate role for UBC-FAP in apoptosis is activation of a cyclin-dependent kinase, perhaps by increased degradation of an inhibitor or proteolytic processing of a proenzyme.

ICE and ICE-like proteases play a critical role in apoptosis (38). Recently, a novel member of the ICE family, MACH/FLICE has been shown to physically associate with Fas via interaction with FADD/MORT (11, 12). Following Fas ligation, MACH/FLICE is cleaved into an amino-terminal fragment which remains associated with the Fas complex. The carboxyl-terminal fragment of MACH/FLICE encodes a classical ICE-like protease and is released from the Fas complex into the cytoplasm. It has been shown that ubiquitination of NF-κB p105 precursor protein targets it for cleavage into an active p60 protein (30). This raises the possibility that ubiquitination of MACH/FLICE by UBC-FAP following Fas ligation may trigger its cleavage. Future experiments are aimed to determine whether FADD/MORT and/or MACH/FLICE are potential targets for UBC-FAP.

To date, transient overexpression of UBC-FAP in KT3 and Jurkat T cells did not cause cell death nor did it cause increased sensitivity to Fas-mediated killing and overexpression of a dominant negative UBC-FAP (with a mutation in the active site Cys227) did not interfere with Fas killing (data not shown).
However, we have been able to increase the level of UBC-FAP expression by only 2–3-fold. Thus, the role of UBC-FAP in Fas killing remains an open question.

Acknowledgments—We thank I. Stamenkovic and M. Clement for providing Fas cDNA.

REFERENCES

1. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991) Cell 66, 233–243
2. Yonehara, S., Ishii, A., and Yonehara, M. (1989) J. Exp. Med. 169, 1747
3. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992) Nature 356, 314–317
4. Fisher, G. H., Rosenberg, F. J., Straus, S. E., Dale, J. K., Millet, L. A., Lin, A. Y., Strober, W., Lenardo, M. J., and Puck, J. M. (1995) Cell 81, 935–946
5. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) Cell 85, 803–815
6. Steller, H. (1995) Science 267, 78–81
7. Paolilini, R., and Kinet, J.-P. (1993) EMBO J. 12, 779–786
8. Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995) Science 268, 411–415
9. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., McGahan, A., Mahboubi, A., and Green, D. R. (1994) J. Exp. Med. 180, 2413–2418
10. Shih, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W., and Greenberg, A. H. (1994) Science 263, 1143–1145
11. Fotedar, R., Flatt, J., Gupta, S., Margolis, R. L., Fitzgerald, P., Messier, H., and Fotedar, A. (1995) Mol. Cell. Biol. 15, 932–942
12. Miura, M., Zhu, H., Rotello, R., Hartweg, E. A., and Yuan, J. (1993) Cell 75, 653–660