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PRINCIPAL INVESTIGATOR: Junn Yanagisawa, Ph.D.
Shinichi Kitada, Ph.D.
John Reed, Ph.D.

CONTRACTING ORGANIZATION: La Jolla Cancer Research Foundation
La Jolla, California 92037

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The cytosolic domain of Fas (APO-1; CD95), a member of the TNF-receptor family, was used as a bait for screening yeast two-hybrid cDNA libraries. cDNAs encoding a protein tyrosine phosphatase (PTPase) were thus obtained. This Fas-associated phosphatase (FAP-1) was shown to bind specifically to Fas, but not to other members of the TNF-receptor family. The region within the Fas cytosolic tail responsible for interactions with FAP-1 was identified and coincided with a negative regulatory domain that has been reported to inhibit Fas-induced signals leading to apoptosis. The endogenous levels of FAP-1 mRNA inversely correlated with sensitivity of various tumor cells to Fas-induced apoptosis. Ectopic expression of FAP-1 in a leukemia cell line that normally fails to express FAP-1 increased its resistance to Fas-mediated cytotoxicity. Taken together, the results indicate that this PTPase is a negative regulator of Fas signaling pathways, implying an opposing role for a protein tyrosine kinase in Fas-induced apoptosis. The expression of FAP-1 by tumors may represent a mechanism for avoidance of immune surveillance mechanisms.

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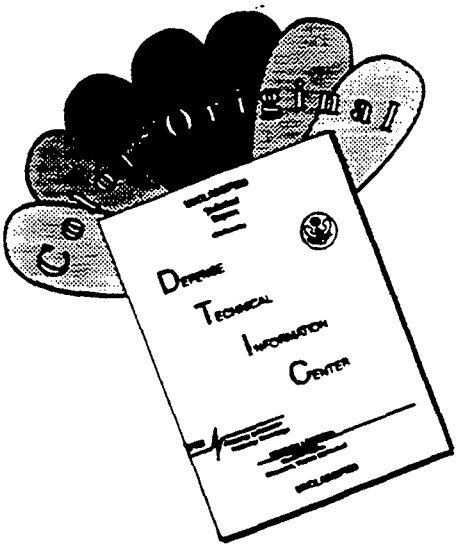

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INTRODUCTION:

Cell-mediated immunity represents one of the chief natural defense mechanisms against tumors. Cytolytic T-cells have two principal mechanisms for killing neoplastic cells: (a) secretion of perforins and granzymes and (b) expression of the cytotoxic cytokine Fas-ligand (reviewed in [1]). Fas, also known as APO-1 and CD95, is a member of the Tumor Necrosis Factor (TNF) receptor family (reviewed in [2]). Fas is widely expressed on the surface of various types of cells in the body and provides a conduit through which Cytolytic T-cells (CTLs) and Natural Killer (NK) cells can trigger apoptosis of the tumor target cells.

The ability of Fas to mediated cell death in human tumors however varies widely, and is unrelated to the relative levels of surface expression of this receptor protein. Some tumors, for example, are exquisitely sensitive to Fas-based cytotoxicity, whereas others are entirely resistant despite expressing similar levels of Fas [3]. This observation suggests that other factors modulate the relative efficiency with which Fas transduces signals leading to apoptosis.

At the time this study was initiated, nothing was known about the mechanisms by which Fas-transduces signals for cell death. The cytosolic domain of the receptor, for example, contains no sequences resembling kinases or other enzymes that might suggest a function. By analogy to many other cytokine receptors, however, we hypothesized that the cytosolic domain of Fas would bind to intracellular proteins that participate in Fas-mediated signaling. To identify such proteins, therefore, we employed yeast two hybrid cDNA library screening methods in an effort to identify proteins that specifically bind to the cytosolic tail of Fas.

BODY:

All of the proposed goals of the proposal were accomplished. Using the cytosolic domain of Fas as a bait for yeast two-hybrid screening of cDNA libraries, 2 cDNA clones were obtained from a screen of over 10 milllion clones which encoded proteins that specifically bound to Fas. These two cDNAs represented overlapping clones derived from the same gene, and both encoding a ~100 amino-acid domain within the PTPase, FAP-1 (also known as PTP-Bas, PTP-L1, and PTP-1E). This domain is known as a GLGF-repeat,

and is believed to represent a type of protein-protein interaction domain that is found in a variety of signal transducing proteins, including some guanylate kinases and nitric oxide synthetases [4-6].

Using the initial partial cDNAs obtained through yeast two-hybrid screening, a human breast cancer cDNA library was screened and longer cDNAs were obtained. Using these cDNA and RT-PCR methods, a full-length FAP-1 cDNA was derived that encoded the full-length PTPase.

In vitro binding studies using bacterial produced GST-Fas(cyto) fusion protein confirmed the authenticity of the binding of FAP-1 which was seen initially in the yeast two-hybrid assays. Moreover, using both two-hybrid methods and in vitro binding assays,the region in the cytosolic domain of Fas that is required for binding to FAP-1 was mapped. The last 15 amino-acids of Fas were found to be both necessary and sufficient for binding in vitro and in yeast two-hybrid assays to FAP-1 as well as to the GLGF-repeat domain of FAP-1. Interestingly, an analogous situtation has recently been discovered for another GLGF-repeat domain-containing protein PSD-90 which binds to the C-terminal 6 amino-acids of the NMDA receptors [6]. Thus, proteins that contain GLGF-repeat domains may have a tendency to bind to the C-termini of some types of receptors.

The C-terminal 15 amino-acids of Fas has been shown previously to represent a negative regulatory domain [7]. Deletion of this stretch of 15 amino-acids from Fas enhances its ability to promote apoptosis. The binding of FAP-1 to this site therefore suggested that rather than mediating signals for cell death, the FAP-1 protein was likely to function as a repressor of Fas-induced apoptosis. Consistent with this idea, the levels of FAP-1 mRNA in various tumor cell lines were found to correlate inversely with the sensitivity of these cells to anti-Fas antibody induced apoptosis.

To further test the functional relevance of FAP-1 to the regulation of Fas-controlled pathways that induce apoptosis, mammalian expression plasmids were prepared that encoded either the full-length FAP-1 or a deletion mutant of FAP-1 missing the catalytic domain of this PTPase. These proteins were then expressed in Jurkat T-cells, a leukemia cell line which is exquisitely sensitive to Fas and that has been employed extensively as a model for studying Fas-induced apoptosis. Based on Northern blot analysis using FAP-1 DNA probes, we determined that Jurkat T-cells do not express FAP-1. Our goal therefore was to ectopically express the full-length FAP-1 protein and the catalytically inactive mutant

FAP-1 in Jurkat through gene transfer methods and then determine the consequences as far as Fas-induced apoptosis is concerned. These experiments showed that expression of the full-length FAP-1 protein in Jurkat cells resulted in increased resistance to Fas-induced apoptosis. In contrast, the mutant of FAP-1 lacking the catalytic domain had no effect on Fas-induced apoptosis when expressed at comparable levels in Jurkat cells.

These studies describing the initial cloning of FAP-1, its binding to the negative regulatory domain of Fas, and its ability to impair Fas-induced apoptosis were published in *SCIENCE* (see Bibliography).

In addition, the human FAP-1 gene was cloned from a cosmid library and genomic clones were used to determine the chromosomal location of FAP-1 by in situ fluorescence hybridization and somatic cell hybrid analysis. The human FAP-1 gene thus was determined to reside on chromosome 4 at band q21.3. This region has been reported to become deleted in some types of tumors, but not in breast cancers. These results were published in *GENOMICS*.

Finally, monoclonal antibodies were raised against a GST-FAP-1 fusion protein that was produced in bacteria and affinity purified on glutathione-Sepharose. 10 independent hybridomas were obtained which specifically bind to FAP-1 (unpublished data). These antibodies are being employed now for more detailed biochemical investigations of FAP-1.

CONCLUSIONS:

In summary, the first Fas-binding protein FAP-1 was discovered by screening cDNA libraries using a yeast two-hybrid method that employed the cytosolic domain of Fas as a bait. Unlike other Fas-binding proteins that were subsequently described by others, FAP-1 does not bind to the signal transducing "death domain" of Fas but rather interacts with a negative regulatory domain that resides in the last 15 amino-acids of this receptor. Gene transfer experiments indicate that FAP-1 opposes Fas-induced apoptosis. The catalytic domain of this PTPase is necessary for inhibition of Fas-based cytotoxicity, implying that FAP-1 opposes the actions of a Fas-regulated protein tyrosine kinase (PTK). Recent data from another group suggest that the PTK, Fyn, is associated with Fas and is required for Fas-induced apoptosis [8]. Current efforts therefore should be directed toward delineating the interactions between FAP-1 and Fyn within the context of Fas-mediated signaling.

Enhanced understanding of the molecular mechanisms by which Fas-mediates apoptosis in tumor cells and improved knowledge about the mechanisms employed by tumors for developing resistance to Fas could ultimately lead to novel immunotherapies for the treatment of breast cancer and other types of malignancies.

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Inazawa I, Ariyama T, Abe T, Druck T, Ohta M, Huebner K, Yanagisawa J, Reed JC, Sato T: PTPN13, a Fas-associated protein tyrosine phosphatase, is located on the long arm of chromosome 4 at band q21.3. Genomics 31:240-242, 1996.

PERSONNEL:

1. Takaaki Sato, PhD
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3. Shinichi Kitada, MD, PhD

IMMUNOBLOT ANALYSIS OF ANTI-FAP-1 MONOCLONAL ANTIBODY

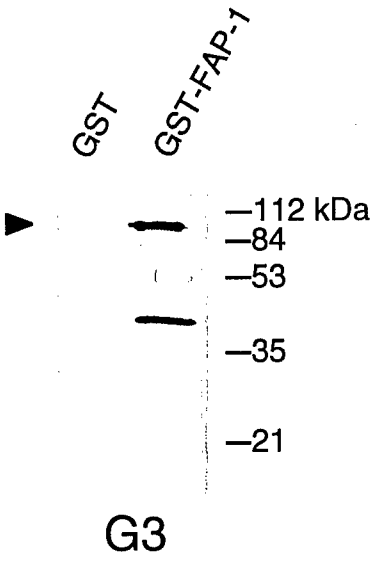

Figure 1

Cells Expressing FAP-1 are Resistant to Fas- but not Ceramide-Induced Apoptosis

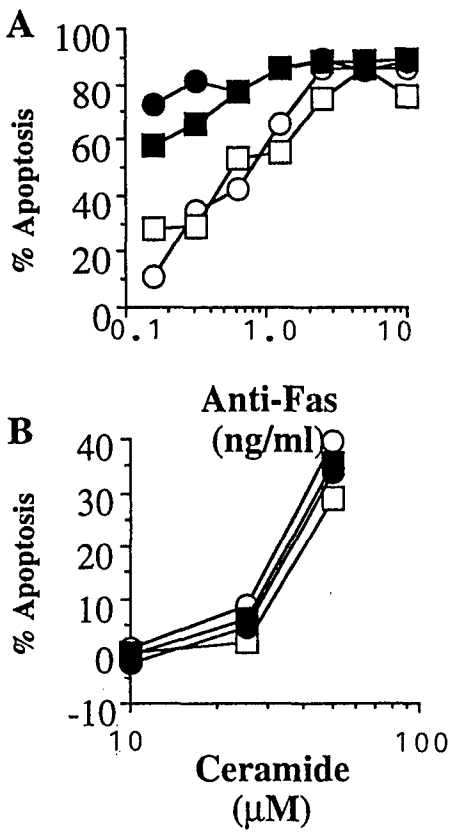

Figure 2

SHORT COMMUNICATION

PTPN13, a Fas-Associated Protein Tyrosine Phosphatase, Is Located on the Long Arm of Chromosome 4 at Band q21.3

JOHJI INAZAWA,* TAKESHI ARIYAMA,* TATSUO ABE,* TERESA DRUCK,† MASATAKA OHTA,† KAY HUEBNER,† JUNN YANAGISAWA,‡ JOHN C. REED,§ AND TAKAAKI SATO‡,[1]

*Department of Hygiene, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602, Japan; †Jefferson Medical College, Thomas Jefferson University, 233 S. 10th Street, Philadelphia, Pennsylvania 19107; §La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, California 92037; and ‡Division of Molecular Oncology, Department of Otolaryngology/Head & Neck Surgery and Pathology, Columbia University, 630 West 168th Street, New York, New York 10032

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PTPN13 is a protein tyrosine phosphatase that associates with the C-terminal negative regulatory domain in the Fas (APO-1/CD95) receptor. The PTPN13 protein contains six GLGF repeats that have been found in the rat postsynaptic density protein (PSD-95) and the *Drosophila* tumor suppressor protein, lethal-(1)-disc-large-1 (*dlg-1*). The localization of the PTPN13 gene to human chromosome 4q21.3 was determined by both FISH and PCR analysis of somatic cell hybrids. This 4q21.3 chromosomal region contains a gene for autosomal dominant polycystic kidney disease as well as the region frequently deleted in liver and ovarian cancers, suggesting that PTPN13 is a candidate for one of the putative tumor suppressor genes on the long arm of chromosome 4.

PTPN13, Fas-associated protein tyrosine phosphatase-1 (FAP-1), binds to a negative regulatory domain in Fas (APO-1/CD95) that inhibits Fas-induced apoptosis (16). PTPN13 has several alternative spliced forms that are identical to PTP-BAS/hPTP1E/PTPL1 (1, 11, 15) and that were originally cloned by a reverse transcriptase–polymerase chain reaction (RT-PCR) method that utilized degenerate primers in the conserved catalytic domain of PTPases. PTPN13 lacks a transmembrane domain, but contains a membrane-binding region similar to that found in the cytoskeleton-associated proteins, ezrin (5), radixin (4), moesin (10), neurofibromatosis type II gene product (14), and protein 4.1 (3), as well as in the PTPases PTPH1 (20), PTP-MEG (6) and PTPD1 (18). In addition to a catalytic domain located near its carboxy terminus, PTPN13 contains six GLGF repeats. These structures are thought to mediate intra- and intermolecular interactions among protein domains and may play an important role in targeting proteins to the submembranous cytoskeleton or in regulating enzyme activity (11). GLGF repeats have been found previously in guanylate kinases as well as in the rat postsynaptic density protein (PSD-95), which is a homolog of the *Drosophila* tumor suppressor protein, lethal-(1)-disc-large-1 [*dlg-1*] (2, 19). Furthermore, the PTPN13 protein contains a leucine zipper motif ($LX_6LX_6LX_6MX_6L$), suggesting that the PTPN13 leucine zipper may mediate homo- and heterodimerization, which potentially could influence PTPase activity, binding to Fas, or interactions with other signal transduction proteins.

To investigate the chromosomal localization of the PTPN13 gene, both FISH (fluorescence *in situ* hybridization) and PCR analysis of somatic cell hybrid panels were used to map the location of the PTPN13 gene in the human genome. A full-length PTPN13 cDNA (pSKII-FAP-1, 7.5-kb insert) was labeled with biotin-16–dUTP by a nick-translation method. Human (pro)metaphase chromosomes were prepared from normal female lymphocytes by using a thymidine synchronization/BrdU release technique (8). Hybridization signals were revealed by FITC-conjugated avidin and amplified with additional layers of a biotinylated goat anti-avidin antibody, as described (13). The chromosomes were then counterstained with propidium iodide (1 μg/ml) in an anti-fade solution containing 1% DABCO [1,4-diazabicyclo(2,2,2)octane] (Sigma). To identify the sublocalization of these signals, microscopy was performed with a Nikon epifluorescent microscope. Hybridization signals were detected on (pro)metaphase chromosomes through a B-2A filter (Nikon), and G-banding patterns of the same metaphase chromosomes were sequentially visualized through UV-2A filters (Nikon). A total of 40 metaphase cells exhibiting hybridization signals were examined. Among them, 11 showed double-spot signals on both homologous chromosomes 4 at q21.1–q21.3 (Fig. 1A), and 14 showed either single or double spots on both homologous chromosomes 4q21.1–q21.3. Such specific signals could not be observed on the other chromosomes. To localize the PTPN13 gene, more precisely, 6 of the elongated prometaphase chromosomes 4 bearing twin-spot signals

[1] To whom correspondence should be addressed. Telephone: (212) 305-5820. Fax: (212) 305-2249. E-mail: TS174@columbia.edu.

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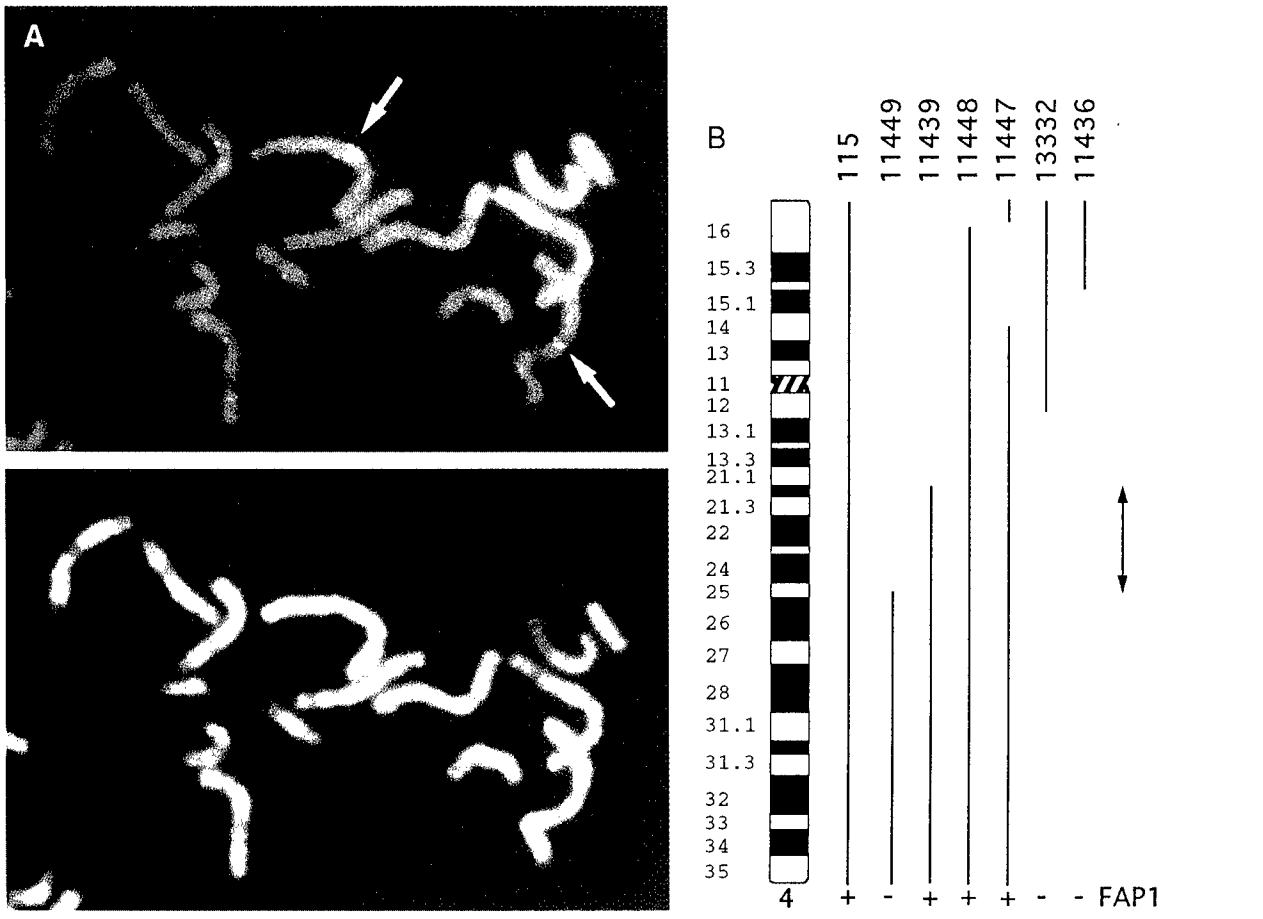

FIG. 1. (**A**) Fluorescence *in situ* hybridization of the human PTPN13 gene. (**Top**) Partial metaphase showing the FITC signals (arrows) on both chromatids of chromosome 4. (**Bottom**) The G-banding pattern of the same chromosome delineated though a UV filter, indicating the signals at 4q21.3. (**B**) Mapping of the PTPN13 gene to chromosome region 4q21 to 4q25 by PCR analysis of rodent–human hybrids. To localize the PTPN13 gene regionally, a second panel of hybrids retaining various portions of chromosome 4 was tested. Only hybrids retaining 4q21 to 4q25 exhibited the expected PCR product.

were examined, and the hybridization signals were localized on band 4q21.3. Thus, we sublocalized the PTPN13 gene to chromosome 4q21.3.

To confirm the FISH-based chromosomal localization of the PTPN13 gene, genomic DNA derived from a rodent–human cell hybrid panel [obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) or as previously described (7)] was analyzed for the presence or absence of the PTPN13 gene by PCR. Briefly, PCR amplification was performed in a final volume of 12.5 μl with 100 ng of DNA template, 10 m*M* Tris–HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% w/v gelatin, 200 m*M* dNTPs, 20 ng each primer, and 1 unit *Taq* polymerase. The amplifications were performed in a Perkin–Elmer Cetus thermal cycler for 30 cycles, consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The PCR products were visualized in ethidium bromide-stained gels. The product was sequenced to confirm identity using a *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (ABI) and a 373 DNA Sequencer (ABI). The forward and reverse primers used were PTPN13F 5′-CAGAGGGATGGAAAGAAGAA-3′ at nucleotide position 1910 bp (relative to the translation initiation site) and PTPN13R 5′-GTAATACTGATG-ACACGTCAG-3′ at 2020 bp, respectively, which were derived from the human PTP-BAS3 cDNA sequence (GenBank Accession No. D21211). PTPN13 primers generated an unique ~400-bp PCR product from genomic templates. Only hybrids retaining chromosome 4 were positive for this PCR product. To localize the PTPN13 gene regionally, a second panel of hybrids retaining various portions of chromosome 4 was tested by PCR. Only hybrids retaining 4q21 to 4q25 exhibited the expected PCR product, as illustrated in Fig. 1B.

We have mapped the PTPN13 gene to the long arm of chromosome 4q at band 21.3 by fluorescence *in situ* hybridization and have confirmed its location to the 4q21–q25 region by PCR analysis of hybrid cell panels. Our results strongly suggested that the mapping results reported previously for the hPTP1E gene, which was assigned to chromosome 11, are in error (1). The 4q21.3 chromosomal region is believed to contain a gene for autosomal dominant polycystic kidney disease (9, 12), as well as putative tumor suppressor genes of relevance to several types of cancers, includ-

ing liver and ovarian cancers (17, 21). It remains to be determined whether the PTPN13 protein has functions beyond its role as an inhibitor of Fas-induced apoptosis that would make it a candidate for one of the postulated tumor suppressor genes on the long arm of chromosome 4.

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FAP-1: A Protein Tyrosine Phosphatase That Associates with Fas

Takaaki Sato, Shinji Irie, Shinichi Kitada, and John C. Reed*

FAP-1: A Protein Tyrosine Phosphatase That Associates with Fas

Takaaki Sato, Shinji Irie, Shinichi Kitada, John C. Reed*

Fas is a cell surface receptor that controls a poorly understood signal transduction pathway that leads to cell death by means of apoptosis. A protein tyrosine phosphatase, FAP-1, capable of interacting with the cytosolic domain of Fas, was identified. The carboxyl terminal 15 amino acids of Fas are necessary and sufficient for interaction with FAP-1. FAP-1 expression is highest in tissues and cell lines that are relatively resistant to Fas-mediated cytotoxicity. Gene transfer–mediated elevations in FAP-1 partially abolished Fas-induced apoptosis in a T cell line. These findings are consistent with an inhibitory effect of FAP-1 on Fas signal transduction.

Fas (also known as APO-1 and CD95) is a cell surface receptor that is expressed on a variety of normal and neoplastic cells. It shares significant amino acid sequence homology with several members of the tumor necrosis factor receptor (TNFR) family, including p55-TNFR, CD40, and the p75–nerve growth factor receptor (NGFR), which have been shown to act as either inhibitors or inducers of cell death (*1*). The ligand for Fas is expressed predominantly on cytolytic T cells (*2*), suggesting that Fas plays a role in the effector branch of cellular immune responses. Mutations in the genes encoding Fas or its ligand have been associated with lymphoproliferative and autoimmune disorders in mice (*3*). Furthermore, alterations in Fas production have been associated with autoimmune disease in humans and susceptibility to induction of apoptosis of T cells in human immunodeficiency virus–infected persons (*4*).

Monoclonal antibodies specific against Fas induce apoptosis in many types of cells (*5*). However, in some cases antibodies to Fas stimulate cell proliferation (*6*, *7*), suggesting that the intracellular signal transduction pathways used by this receptor are subject to regulation. The cytosolic domain of Fas contains no similarity to known kinases or other enzymes that might transduce signals into cells. Deletion mapping analysis has identified a domain that is required for induction of apoptosis, which is called the "death domain." This domain shares homology with sequences located in the cytosolic domains of p55-TNFR1, CD40, and p75-NGFR (*8*). For some cells, apoptosis induced by antibodies to Fas is dependent on protein synthesis inhibitors such as cycloheximide (*8*). This implies either the existence of a labile protein that suppresses Fas-generated signals leading to cell death or the induction of proteins that inhibit the ability of Fas to trigger apoptosis. A negative regulatory domain has been mapped to the COOH-terminal 15 amino acids of Fas that is not homologous to other TNFR-like proteins; deletion of this domain can abrogate the dependence on protein synthesis inhibitors for apoptosis induced by antibody to Fas (*8*).

To identify complementary DNAs (cDNAs) encoding proteins that can potentially modulate the activity of Fas, we used a yeast two-hybrid system for cDNA library screening. We used the cytosolic domain of human Fas fused to a LexA DNA binding domain (*9*, *10*) and random cDNAs fused in frame with a VP16 transactivation domain. Using a His synthetase gene (*HIS3*) under the control of LexA operators as a reporter, we identified 395 His^+ colonies from an initial screen of 3×10^8 transformants. Of these, 84 were also positive when a *lacZ* gene (β-galactosidase) under the control of a LexA operator was used as an alternative reporter. Mating tests were then performed (*11*); only 2 of the 84 candidate clones (numbers 31 and 43) reacted with the cytosolic domain of Fas (Ta-

La Jolla Cancer Research Foundation, Oncogene and Tumor Suppressor Gene Program, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed.

ble 1). These mouse cDNAs represented overlapping independent clones with insert sizes of 381 base pairs (bp) (clone 31) and 351 bp (clone 43); these clones share >95% homology with a human cDNA sequence encoding a protein tyrosine phosphatase (PTP) termed PTP-BAS (*12*).

PTP-BAS was originally cloned from human basophils by a reverse transcriptase–polymerase chain reaction (RT-PCR) method that used degenerate primers targeted against conserved sequences found in PTPs. Three isoforms arising from alternative splicing have been identified by cDNA cloning, the longest of which is predicted to encode a 2485–amino acid protein (*12*). PTP-BAS lacks a transmembrane domain but contains a membrane-binding domain similar to that found in the cytoskeleton-associated proteins ezrin, radixin, moesin, and protein 4.1, as well as in the PTPs PTPH1, PTP-MEG, and PTPD1 (*12*, *13*). In addition to a catalytic domain located near its COOH-terminus, PTP-BAS contains six repeats (Gly-Leu-Gly-Phe; GLGF) (Fig. 1). These structures are thought to mediate intra- and intermolecular interactions among protein domains and may play a role in targeting proteins to the submembranous cytoskeleton or in regulating enzyme activity (*12*). Both Fas-interacting clones 31 and 43 correspond to the third GLGF repeat in PTP-BAS (Fig. 1), suggesting that this domain mediates specific interactions with the cytosolic domain of Fas and implying that PTP-BAS is a Fas-associated phos-

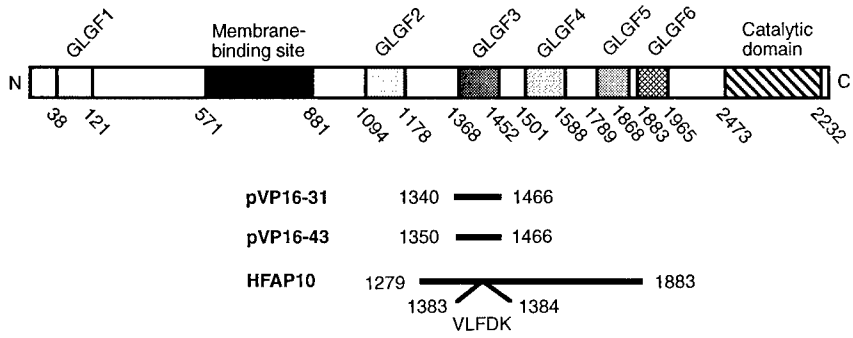

Fig. 1. Diagram of FAP-1 protein and cDNA clones. (**Top**) The structure of the 2485–amino acid human FAP-1 protein (also known as PTP-BAS type-1) (*12*), showing the locations of the catalytic, membrane-binding, and GLGF repeat domains (*12*, *13*). (**Bottom**) The cDNAs identified by two-hybrid screening of a mouse embryo cDNA library: pVP16-31 and pVP16-43 with encoded amino acids relative to the human FAP-1 protein. A human FAP-1 partial cDNA (encoding amino acids 1279 through 1883 of PTP-BAS type-1) from brain was cloned and found to contain a five–amino acid insert in the GLGF3 domain, as compared with the published sequence (*12*). V, Val; L, Leu; F, Phe; D, Asp; and K, Lys. GenBank accession numbers of pVP16-31, pVP16-43, and HFAP10 are L34581, L34582, and L34583, respectively. C, COOH-terminal; N, NH_2-terminal.

Table 1. Specific interaction of FAP-1 with the Fas cytosolic domain. L40 strain cells with pVP16-31 or pVP16-43 were cured of plasmid pBTM116-Fas and then mated with NA87-11A cells transformed with various pBTM116 plasmids as described (*24*). Growth was measured on His-deficient media, and color was measured by a β-galactosidase colorimetric filter assay (*22*).

Proteins	pVP16	Growth	Color
Fas(191–335)	31 and 43	+	Blue
Ras(V12)	31 and 43	–	White
Ras(L35R37)	31 and 43	–	White
CD40(216–277)	31 and 43	–	White
CD40(225–269)	31 and 43	–	White
Bcl-2(83–218)	31 and 43	–	White
Lamin	31 and 43	–	White
Ras(V12)	c-Raf	+	Blue
Lamin	c-Raf	–	White

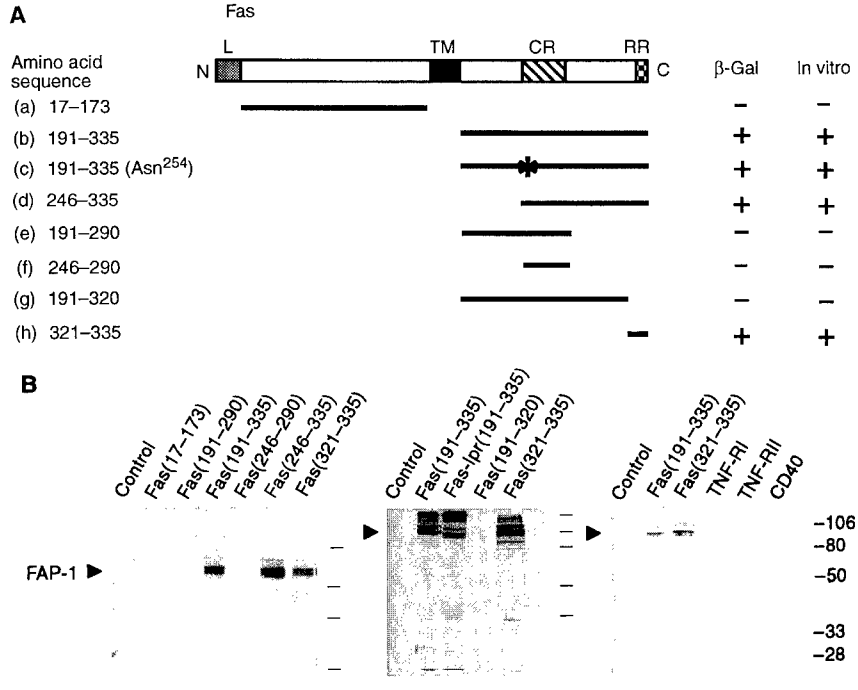

Fig. 2. Mapping of the site on Fas involved in binding to the GLGF3 domain of FAP-1. (**A**) The structure of the Fas protein is depicted with the relative locations of its leader sequence (L), transmembrane domain (TM), conserved cytosolic regions (CR), and unique regulatory region (RR) indicated. A series of Fas deletion mutants were generated (*14*) that contained (a) the extracellular domain of Fas minus its leader sequence (amino acids 17 through 173); (b) the complete cytosolic domain of Fas (191 through 335); (c) a cytosolic domain mutant containing a Val→Asn^{254} mutation (indicated by an asterisk), analogous to an identified mutant allele of Fas in the cg-strain of *lpr* autoimmune mice, which has been shown to be deficient in Fas-mediated induction of apoptosis (*8*); (d) a deletion mutant (246 through 335) lacking the sequences between the TM of Fas and the CR that are required for the induction of apoptosis (*8*); (e) a COOH-terminal deletion mutant (191 through 290) that contains sequences from the TM to the end of the CR; (f) a mutant containing only the CR of Fas (246 through 290); (g) a truncation mutant lacking the COOH-terminal 15 amino acids (191 through 320) that have been shown to constitute a negative RR in Fas (*8*); and (h) a mutant containing only the COOH-terminal 15 amino acids of Fas (321 through 335). They were expressed in L40 strain yeast cells as LexA DNA binding domain fusion proteins with VP16–FAP-1 (clone 31). Protein-protein interactions were detected through the use of a *lacZ* reporter gene under the control of LexA operators with β-galactosidase plate and filter assays (*22*) and scored as positive (blue, +) or negative (white, –). Essentially identical results were obtained in EGY48 strain yeast (*25*). (**B**) These same Fas cDNAs were also subcloned in frame into either pGEX–4T-1 or pGEX–2T-1 and expressed as GST fusion proteins in *E. coli* (*15*). The indicated affinity-purified GST-Fas fusion proteins immobilized on glutathione-Sepharose were incubated with ^{35}S-labeled human FAP-1 protein fragment (1323 through 1883), and specifically bound proteins were detected by SDS-PAGE analysis (*16*) and scored as either positive for binding (+) or negative (–) in (A). Examples of autoradiography results from an SDS-PAGE of samples from the in vitro binding assays are shown. GST nonfusion protein was used as a control. Molecular size markers are shown at right in kilodaltons.

phatase. We therefore called it FAP-1.

To determine the region in the cytosolic domain of Fas that is required for binding to the GLGF3 domain of FAP-1, we prepared a series of Fas deletion mutants that were expressed in yeast as fusion proteins with the LexA DNA binding domain (Fig. 2A) (*14*). When tested in the two-hybrid system, the polypeptide encoded by the longer of the FAP-1 cDNAs (pVP16-31) mediated interactions with LexA fusion proteins containing only the last 15 amino acids of Fas (amino acids 321 through 335) but not with Fas mutants lacking the COOH-terminal 15 amino acids. Thus, the 15–amino acid COOH-terminus of Fas that functionally represents a negative regulatory domain appears to be both necessary and sufficient for interactions with the GLGF3 domain of Fas.

To confirm these two-hybrid results, we performed in vitro binding assays. The Fas deletion mutants were expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins, affinity-purified on glutathione-Sepharose (*15*), and tested for binding to a ^{35}S-L-Met–labeled fragment of the human FAP-1 protein (amino acids 1323 through 1883) prepared by translation in vitro (*16*). The human FAP-1 partial cDNA used for preparation of in vitro–translated protein was obtained by hybridization screening of a human fetal brain cDNA library (*17*). It contained a five–amino acid insert in the GLGF3 domain, relative to the published PTP-BAS sequence, and presumably therefore represents a different isoform of this PTP that is expressed in brain (Fig. 1). Using this in vitro binding assay approach, we obtained results identical to those with the two-hybrid system: The last 15 amino acids of Fas were found to be both necessary and sufficient for binding to the FAP-1 fragment (Fig. 2B). Moreover, no interaction was detected with GST fusion proteins containing the cytosolic domains of p55-TNFRI, p75-TNFRII, or CD40 (*18*).

The finding that FAP-1 interacts with

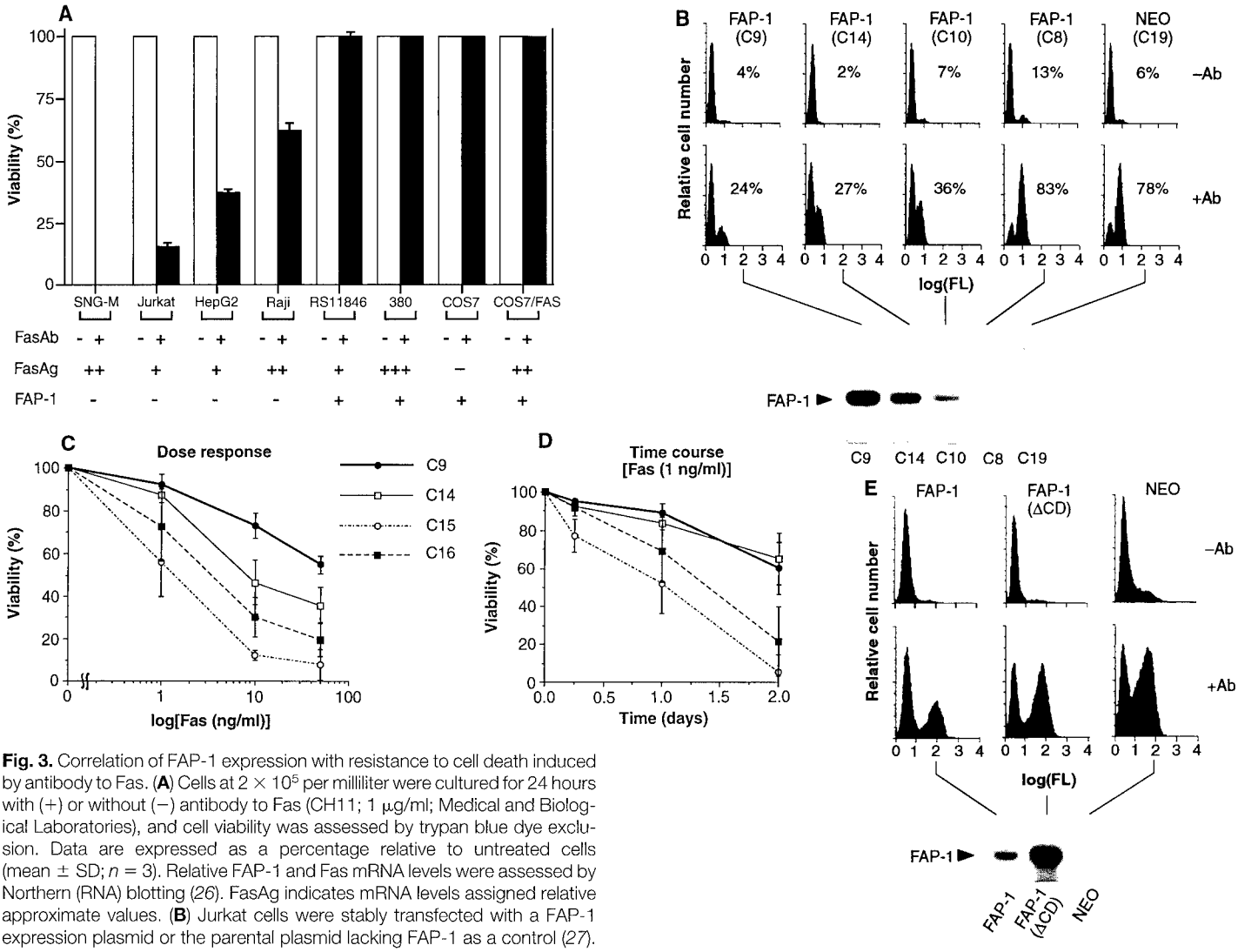

Fig. 3. Correlation of FAP-1 expression with resistance to cell death induced by antibody to Fas. (**A**) Cells at 2×10^5 per milliliter were cultured for 24 hours with (+) or without (−) antibody to Fas (CH11; 1 μg/ml; Medical and Biological Laboratories), and cell viability was assessed by trypan blue dye exclusion. Data are expressed as a percentage relative to untreated cells (mean ± SD; $n = 3$). Relative FAP-1 and Fas mRNA levels were assessed by Northern (RNA) blotting (*26*). FasAg indicates mRNA levels assigned relative approximate values. (**B**) Jurkat cells were stably transfected with a FAP-1 expression plasmid or the parental plasmid lacking FAP-1 as a control (*27*). The relative levels of FAP-1 expression in G418-resistant subclones were then determined by a RT-PCR assay (bottom) (*28*). Data shown are derived from a single exposure of the same blot (lanes reordered for clarity of presentation). Transfected clones were incubated with or without (Ab) CH11 (50 ng/ml), and the TUNEL assay was performed 4 hours later (*19*). Representative histograms are shown (top). Clones C8, C9, C10, and C14 received the FAP-1 plasmid pRc/CMV–FAP-1, whereas clone C19 was transfected with the parental pRc/CMV plasmid (NEO). Data are representative of multiple clones. A control immunoglobin M (IgM) antibody (MOPC-104E; Cappel) did not induce DNA fragmentation and apoptosis in Jurkat cells (*23*). FL, fluorescence. (**C**) FAP-1 transfectant clones C9 and C14 were compared with Neo-transfected control clones C15 and C16 with regards to survival [determined by trypan blue dye exclusion (mean ± SD; $n = 3$)] when cultured for ~1 day with various concentrations of CH11 antibody (zero equals no antibody) (C) or for various times with CH11 antibody (1 ng/ml) (**D**). Cell viability was >95% for cells treated with an IgM control antibody (*23*). (**E**) TUNEL assays and RT-PCR analyses were performed as described in (B) with untransfected Jurkat cells and bulk transfectants that expressed either full-length FAP-1 or a truncated FAP lacking the catalytic domain (ΔCD) (*27*).

the COOH-terminal negative regulatory domain of Fas suggests that this PTP may somehow inhibit Fas-generated signals that lead to apoptosis. To explore this possibility, we correlated the presence or absence of FAP-1 expression with relative sensitivity to apoptosis induced by antibody to Fas in a variety of cell lines that express Fas. Four of four tumor cell lines (SNG-M, Jurkat, HepG2, and Raji) that lacked FAP-1 mRNA, as determined by Northern (RNA) blotting, were sensitive to variable extents to induced cell death caused by antibody to Fas (Fig. 3A). In contrast, all three tumor lines tested which expressed FAP-1 (RS11846, 380, and COS-Fas) were completely resistant to antibody to Fas. This resistance could not be explained by differences in the relative levels of Fas antigen expressed on the surface of the cells, as determined by immunofluorescence flow cytometric analysis.

Next, a cDNA encoding the full-length FAP-1 protein was expressed in a Fas-sensitive clone from the T cell leukemia line Jurkat. Analysis of several independent transfected clones revealed a correlation between the levels of FAP-1 expression and relative resistance to Fas-mediated cytotoxicity. Some representative clones were treated with antibody to Fas for 4 hours and DNA fragmentation indicative of apoptosis was detected by TUNEL assay (*19*); resistance to Fas-mediated apoptosis was demonstrated in clones with higher levels of FAP-1 expression (Fig. 3B). Transfected Jurkat clones with high levels of FAP-1 expression withstood higher concentrations of antibody than control cells did (Fig. 3C) and remained viable for longer periods of time when cultured with antibody to Fas (Fig. 3D). In contrast to Jurkat cells which expressed full-length FAP-1, transfectants expressing a truncated version of FAP-1 lacking the catalytic domain were not protected from Fas-induced DNA degradation (Fig. 3E). Relative levels of Fas expression were equivalent for all subclones shown, on the basis of immunofluorescence flow cytometric assays.

Although other factors besides FAP-1 may contribute to the inhibition of Fas signal transduction events involved in the induction of apoptosis (*7*, *20*), the data presented here support the idea that FAP-1 is a negative regulator of Fas-induced pathways that lead to cell death. This finding therefore implies the involvement of a protein tyrosine kinase (PTK) in some aspect of Fas-mediated cytotoxicity. It has been reported that Fas-induced apoptosis is accompanied by rapid tyrosine phosphorylation of proteins in T cells and can be blocked by pharmacological inhibitors of PTKs (*21*). Presumably, therefore, an antagonistic relation between this unknown PTK and FAP-1 influences the relative sensitivity of cells to apoptosis induced by antibody to Fas.

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10. Two-hybrid screens were performed essentially as described (*9*) in L40 strain cells [*MATa, trp1, leu2, his3, ade2, LYS2::(lexAop)*^{4}-HIS3, URA3::(lexAop)^8-lacZ] with plasmid pBTM116 containing a human Fas cDNA (amino acids 191 through 335) subcloned in frame with the LexA open reading frame (ORF) and a mouse embryo cDNA library cloned into pVP16. Clones that formed on His-deficient media (His$^+$) were transferred to plates containing X-gal (40 μg/ml), resulting in 84 clones that produced a blue reaction product (β-Gal$^+$) in plate and filter assays (*22*).
11. We cured the 84 His$^+$, β-Gal$^+$ clones of the LexA-Fas plasmid by growing cells in Trp-containing medium and then mating them against a panel of α type yeast, strain NA87-11A (*MATα, leu2, his3, trp1, pho3, pho5*) containing plasmid pBTM116 that produced LexA DNA binding domain fusion protein containing Fas (amino acids 191 through 335), portions of the CD40 cytosolic domain, Bcl-2 protein, lamin, and mutant Ha-Ras proteins (*22*). Mated cells were selected for growth in medium that lacked Trp (pBTM116 plasmid) and Leu (pVP16 plasmid) and tested for the ability to trans-activate a *lacZ* reporter gene by a β-Gal colorimetric filter assay (*22*).
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13. The original description of PTP-BAS (*12*) indicated only three GLGF repeat domains, but our analysis with the DNA Strider program (version 1.2) (CEA, France) suggests the presence of six GLGF repeat domains in this PTP.
14. The cDNA sequences encoding various fragments of human Fas were generated by PCR with the following forward (F) and reverse (R) primers containing Eco RI (underlined) and Bcl 1 (italic) sites (bold indicates the stop codon, TCA); F1, 5′-GGAATTCAGATTATCGTCCAAAAGTG-3′; F2, 5′-GGAATTCAAGAGAAAGGAAGTACA-3′; F3, 5′-GGAATTCAAAGGCTTTGTTCGAAAG-3′; R1, 5′-GTGA**TCA**GTTAGATCTGGATCCTTC-3′; R2, 5′-GTGA**TCA**CGCTTCTTTCTTTCCATG-3′; R3, 5′-GTGA**TCA**CTAGACCAAGCTTTGGAT-3′; HFAS-*lpr* (Asn^{254}, 5′-TTCGAAAGAATGGTAACAATGAAGCCAAA-3′; HFAS-15F, 5′-AATTCGACTCAGAAAATTCAAACTTCAGAAATGAAATCCAAAGCTTGGTCTAG-3′; and HFAS-15R, 5′-TCGACTAGACCAAGCTTTGGATTTCATTTCTGAAGTTTGAATTTTCTGAGTCG-3′. We used the following combinations of primers to produce the indicated human Fas cDNA fragments: (a) F1 and R1, amino acids 17 through 173; (b) F2 and R3, 191 through 335; (c) F2 and R3 and Fas-*lpr* (Asn^{254}); (d) F3 and R3, 246 through 335; (e) F2 and R2, 191 through 290; (f) F3 and R2, 246 through 290; (g) Fas/APO-1 (191 through 320), which was generated by restriction endonuclease digestion with Spe I, followed by treatment with T4 DNA polymerase to generate a stop codon; and (h) Fas-15F and Fas-15R, 321 through 335. These Fas deletion mutant cDNAs were subcloned into the Eco RI and Bam HI sites of pEG202 [A. S. Zervous, J. Gyuris, R. Brent, *Cell* **72**, 223 (1993)] in frame with the LexA ORF, the results were confirmed by DNA sequencing, and then the cDNAs were excised with Eco RI and Sal I and subcloned into pBTM116 in frame with LexA.
15. The Fas cDNAs described in (*14*) were subcloned into the Eco RI and Xho I sites of either pGEX-2T-1 or pGEX-4T-1 in frame with the ORF of GST and expressed in DH5αF′ or HB101 cells (BRL/Gibco) by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 to 16 hours at 30°C. GST fusion proteins were purified from bacterial lysates with glutathione–Sepharose 4B (Pharmacia, Piscataway, NJ).
16. The HFAP10 cDNA was subcloned into the Eco RV site of Bluescript pSK-II and in vitro translated from an internal Meth (amino acid 1323) in the presence of [^{35}S]-L-Meth with a coupled transcription-translation system (TNT lysate, Promega) and T7 RNA polymerase, resulting in the production of a human FAP-1/β-Gal fusion protein that was incubated with GST-Fas fusion proteins immobilized on glutathione-Sepharose in 50 mM tris (pH 8.0), 150 mM NaCl, 5 mM dithiothreitol (DTT), 2 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsufonyl fluoride, and leupeptin (1 μg/ml) for 16 hours at 4°C. The beads were then vigorously washed five times in the same solution, pelleted by centrifugation, and boiled in Laemmli sample buffer before analysis by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography.
17. A human fetal brain cDNA library in λgt11 was screened with the ^{32}P-labeled insert from pVP16-31 used as a hybridization probe, resulting in four independent human Fas partial cDNA clones; HFAP10 is the longest.
18. The cDNA sequences encoding the human CD40 were generated by PCR with the following F and R primers containing Eco RI (underlined) and Bcl I (italic) sites (bold indicates the stop codon, TCA): CD40 (amino acids 216 through 277), 5′-GGAATTCAAAAAGGTGGCCAAG-3′ (F2) and 5′-TGATCA**TCA**CTGTCTCTCCTGCAC-3′ (R2); CD40 (225 through 269), 5′-GGAATTCAAGGCCCCCCACCCCAAG-3′ (F1) and 5′-TGA**TCA**ACTCTCTTTGCCATCCTC-3′ (R1). The PCR products were digested with Eco RI and Bcl I, then directly cloned into the Eco RI and Bam HI site of pBTM116. The Eco RI and Sal I fragments from pBTM116-CD40 were also subcloned into the Eco RI and Sal I sites of pGEX4T-1 (Pharmacia) for the expression of GST-CD40 proteins.
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24. L40 strain cells containing pVP16-31 or pVP16-43 were cured of plasmid pBTM116-Fas (amino acids 191 through 335) by growth on Trp and then mated with NA87-11A cells transformed with various pBTM116 plasmids producing LexA DNA binding domain fusion proteins containing portions of the Fas, CD40, Bcl-2, lamin, or mutant Ras proteins as indicated (*11*). Interactions of LexA and VP16 fusion proteins were detected by growth on His-deficient media and by a β-galactosidase colorimetric filter assay (*22*) based on the ability to trans-activate HIS3 and *lacZ* reporter genes containing LexA operators. L40 cells producing a VP16-Raf fusion protein served as a positive control when mated with NA87-11A cells containing pBTM116-Ras(V12) (*9*).
25. The Fas cDNAs described in (*14*) in pEG202 was cotransformed with pVP16-31 into EGY48 cells (*MATα trp1 ura3 his3 LEU2::pLexAop6-LEU2*) containing pSH18-34 (Gal1 promoter–*lacZ* with eight LexA operators), and β-galactosidase activity

was assessed by colorimetric plate and filter assays (*22*).

26. Total RNA (20 μg) isolated from various cell lines was subjected to Northern blot assay with ^{32}P-labeled HFAP10 probe, and the results were scored as detectable or undetectable. All cell lines expressed Fas antigen as determined by both flow cytometric immunofluorescence assay with antibody to Fas DX2 (*23*) and by Northern blotting with a ^{32}P-labeled Fas cDNA probe.
27. A cDNA encoding the full-length FAP-1 protein was constructed with a series of four overlapping PCR reactions and DNA derived from a λgt11 fetal brain cDNA library. The 5′- and 3′-flanking primers contained Not I sites that were used for subcloning downstream of the cytomegalovirus (CMV) promoter in pRc/CMV, an expression plasmid that contains a G418 resistance gene (Invitrogen). Jurkat cells were electroporated with 25 μg of pRc/CMV or pRc/CMV–FAP-1, and stable transfectants were obtained by selection in G418 (0.8 mg/ml). Independent clones were obtained by limiting dilution. In addition, a cDNA encoding a COOH-terminal–truncated FAP-1 protein was created by introduction of a stop codon after position 2225. The cDNAs encoding the full-length and truncated FAP-1 proteins were subcloned into pREP-9 (Invitrogen) and expressed in Jurkat cells.
28. Total RNA was isolated from individual transfectant clones and 3 μg was reverse transcribed with a FAP-1–specific primer (5′-AGGTCTGCAGAGAAGCAAGAATAC-3′). PCR amplification was then performed for 25 cycles with the same R and a F primer (5′-GAATACGAGTGTCAGACATGG-3′). The resulting PCR products (607 bp) were subjected to agarose gel electrophoresis and analyzed by Southern (DNA) blotting with a ^{32}P–end-labeled internal FAP-1 oligonucleotide probe (5′-CTAACTCCATTGACAGCTAGGA-3′).

29. We thank P. Krammer for Fas cDNA; C. Ware for GST-TNFRI and TNFRII (University of California at Riverside); S. Hollenberg and E. Golemis for the two-hybrid systems; N. Iwama, H. Kobayashi, and L. Fong for technical assistance; and the Council for Tobacco Research (CTR-3113) for its support. T.S. is fellow of the U.S. Army Medical Research and Development Command, Breast Cancer Research Program. J.C.R. is a Scholar of the Leukemia Society of America.

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