The Molecular Interaction of Fas and FAP-1

A TRIPEPTIDE BLOCKER OF HUMAN Fas INTERACTION WITH FAP-1 PROMOTES Fas-INDUCED APOPTOSIS*

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Fas (APO-1/CD95), which is a member of the tumor necrosis factor receptor superfamily, is a cell surface receptor that induces apoptosis. A protein tyrosine phosphatase, Fas-associated phosphatase-1 (FAP-1), that was previously identified as a Fas binding protein interacts with the C-terminal 15 amino acids of the regulatory domain of the Fas receptor. To identify the minimal region of the Fas C-terminal necessary for binding to FAP-1, we employed an in vitro inhibition assay of Fas/FAP-1 binding using a series of synthetic peptides as well as a screen of random peptide libraries by the yeast two-hybrid system. The results showed that the C-terminal three amino acids (SLV) of human Fas were necessary and sufficient for its interaction with the third PDZ (GLGF) domain of FAP-1. Furthermore, the direct cytoplasmic microinjection of this tripeptide (Ac-SLV) resulted in the induction of Fas-mediated apoptosis in a colon cancer cell line that expresses both Fas and FAP-1. Since t(S/T)V(L/I) motifs in the C termini of several other receptors have been shown to interact with PDZ domain in signal transducing molecules, this may represent a general motif for protein-protein interactions with important biological functions.

Fas (APO-1/CD95) and its ligand have been identified as important signal mediators of apoptosis (1). The structural organization of Fas (APO-1/CD95) indicates that it is a member of the tumor necrosis factor receptor superfamily, which also includes the p75 nerve growth factor receptor (2), the T-cell activation marker CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3), the Hodgkin-lymphoma-associated antigen CD30 (4), the human B cell antigen CD40 (5), and T cell antigen CD27 (3). The third PDZ repeat of FAP-1 was first identified as a domain showing the specific interaction with the C terminus of Fas receptor (10). In the present study, we first demonstrated that the C-terminal three amino acids (SLV) of human Fas were necessary and sufficient for its interaction with the third PDZ domain of FAP-1. More important, we were able to induce Fas-mediated apoptosis in a colon cancer cell line by the direct cytoplasmic microinjection of this tripeptide (Ac-SLV).

MATERIALS AND METHODS

Constructions of Libraries and Screenings—To create numerous mutations in a restricted DNA sequence, PCR mutagenesis with degenerate oligonucleotides was employed according to a protocol described elsewhere (27). Based on the homology between human and rat, two palindromic sequences were designed for construction of a semi-random library. The two primers used were 5'-CGAATTCCNNNNNNAATGAANNNCAAAGTCTGNNNTGAGGATC-3' and 5'-CGAATTCTAGAANNNNNNAACTGCNNNNNNGTCTGAGGATCTCA-3'. Briefly, the two primers (200 pmol each), purified by high pressure liquid chromatography, were annealed at 70 °C for 5 min and cooled at 23 °C for 60 min. A Klenow fragment (5 units) was used for filling in with a dNTP mix (final concentration, 1 mM per each dNTP) at 23 °C for 60 min. The reaction was stopped with 1 μl of 0.5 M EDTA, and the DNA was purified with ethanol precipitation. The resulting double-stranded DNA was digested with EcoRI and BamHI and re-purified by electrophoresis on non-denaturing polyacrylamide gels. The double strand oligonucleotides were then ligated into the EcoRI-BamHI sites of the pBTM116 vector.
plasmid (28). The ligation mixtures were electroporated into the E. coli XL1-Blue MRF' (Stratagene) for the plasmid library. The large scale transformation was carried out as previously reported. The plasmid library was transformed into L40 strain cells (MATa, trp1, leu2, his3, ade2, LYS2:(lexAop)4-HIS3, URA3::(lexAop)8-lacZ) carrying the plasmid pVP16–31 containing a FAP-1 cDNA (10). Clones that formed on histidine-deficient medium (His+) were transferred to plates containing 40 μg/ml X-gal to test for a blue reaction product (β-gal+) in plate and filter assays. The His+ β-gal+ clones were cured of the LexA/Fas plasmid by growing cells in tryptophan-containing medium and then mated against a panel of α-type yeast, strain NA87–11A (MATa, leu2, his3, trp1, pho3, pho5), containing the plasmid pBMT116 that produced LexA DNA binding domain fusion protein containing Fas(191–335), portions of the CD40 cytosolic domain, Bcl-2 protein, lamin, and mutant Ha-Ras.

**FIG. 1.** Mapping of the minimal region of the C-terminal of Fas required for the binding to FAP-1. **A**, strategy for screening of a random peptide library by the yeast two-hybrid system. **B**, the amino acid sequences of positive clones that showed interaction with FAP-1 in yeast; alignment of the C-terminal 15 amino acids of Fas between human, rat, and mouse (a) and the results of screening a semi-random peptide library (b). **Top row** indicates the amino acids that were fixed based on the homology between human and rat. **Dashed lines** show unchanged amino acids. The results of screening a random peptide library are show in c. Numbers at right show each independent clone.
proteins (29). Mated cells were selected for growth in medium lacking tryptophan (pBMT116 plasmid) and leucine (pVP16 plasmid) and tested for ability to trans-activate a lacZ reporter gene by a β-gal colorimetric filter assay. The clones selected by His+ and β-gal+ assay were tested for further analysis. The palindromic oligonucleotide, 5′-GGAATTC-(NNN)4–15-TGAGGATCCTCA-3′, was used for the construction of the random peptide library.

In Vitro Binding Assay—HFAP-1 cDNA (10) subcloned into the Bluescript vector pSK-II (Stratagene) was in vitro-translated from an internal methionine codon in the presence of [35S]-methylmethionine using a coupled in vitro transcription/translation system (Promega, TNT coupled). The resulting 35S-labeled protein was incubated with GST-Fas fusion proteins that had been immobilized on GST-Sepharose 4B affinity beads (Pharmacia Biotech Inc.) in a buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM dithiothreitol, 2 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 1 mM benzamidine, and 7 μg/ml pepstatin for 16 h at 4 °C. After washing vigorously 4 times in the same buffer, associated proteins were recovered with the glutathione-Sepharose beads by centrifugation, eluted into boiling Laemmli buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Inhibition Assay of Fas/FAP-1 Binding—In vitro translated [35S]HFAP-1 was purified with a NAP-5 column (Pharmacia) and incubated with 1 μg GST fusion proteins for 16 h at 4 °C. After washing 4 times in the binding buffer, radioactivity incorporation was determined in a β-counter. The percentage of binding inhibition was calculated as follows: percent inhibition = [radioactivity incorporation using GST-Fas (191–335) with peptides] – [radioactivity incorporation using GST-Fas (191–320) with peptides]/[radioactivity incorporation using GST-Fas (191–335) without peptides] 

Reverse Transcriptase-PCR analysis and Flow Cytometry—Total RNA was isolated from DLD-1 cells and 3 μg was reverse transcribed using a FAP-1 specific primer (5′-AGGTCTGGAGAGAGCGA- GAACTAC). PCR amplification was then performed for 25 cycles using the same reverse primer and a forward primer (5′-GAACTACGTTG- CAGAATGGG-3′) (10). The resulting PCR products (607 base pairs) were subjected to agarose gel-electrophoresis and analyzed by ethidium bromide staining. Fas expression on DLD-1 cells was determined by staining with either FITC-conjugated anti-human Fas mouse IgG antibody (UB2, MBL International, MA) or FITC-conjugated anti-mouse IgG antibody (Pharmingen, CA) as a control and analyzed on a FACScan flow cytometer (Becton Dickinson and Company).

Immunoprecipitation of Native Fas with GST-FAP-1—GST-fusion proteins with or without FAP-1 were incubated with cell extracts from Jurkat T-cells expressing Fas. The bound Fas was detected by Western analysis using anti-Fas monoclonal antibody (F22120, Transduction Laboratories) or FITC-conjugated anti-mouse IgG antibody (Pharmingen, CA) as a control and analyzed on a FACScan flow cytometer (Becton Dickinson and Company).

Microinjection—DLD-1 human colon cancer cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. For microinjection, cells were plated on CELLocate (Eppendorf) at 1 × 10^5 cells/ml in a 35-mm plastic culture dish and grown for 1 day. Just before microinjection, Fas monoclonal antibodies CH11 (MBL International, MA) were added at the concentration of 500 ng/ml. All microinjection experiments were performed with a 0.4-second injection time and 40 hPa injection pressure using an automatic microinjection system (Eppendorf transjector 524R, micro-manipulator 5171 and Femtotips) (30). Synthetic tripeptides were suspended in 0.1% (v/v) FITC-dextran (Sigma/KPBS) at the concentration of 100 mM. The samples were microinjected into the cytoplasmic region of DLD-1 cells. 16 to 20 h postinjection, the cells were washed with PBS and stained with 10 μg/ml Hoechst 33342 in PBS. After incubation at 37 °C for 30 min, the cells were photographed, and the cells showing condensed chromatin were
counted as apoptotic. For each experiment, 65–150 cells were microinjected. Apoptosis of microinjected cells was determined by assessing morphological changes of chromatin using phase contrast and fluorescence microscopy (31, 32).

Peptide Synthesis—Peptides were automatically synthesized on an Advanced ChemTech ACT357 by analogy to published procedures (33). Wang resin (0.2–0.3 mmol scale) was used for each run and N\textsuperscript{\text{\textprime}}-Fmoc (N-(9-fluorenylethoxycarbonyl) protection) was employed for all amino acids. Deprotection was achieved by treatment with 20% piperidine/DMF, and coupling was completed using DIC/HOBt and subsequent HBTU/DIEA. After the last amino acid was coupled, the growing peptide on the resin was acetylated with Ac2O/DMF. The peptide was cleaved from the resin with concomitant removal of all protecting groups by treating with TFA. The acetylated peptide was purified by high pressure liquid chromatography and characterized by FAB-MS groups by treating with TFA. The acetylated peptide was purified by high pressure liquid chromatography and characterized by FAB-MS.

RESULTS

Screenings of Random Peptide Libraries by Yeast Two-hybrid System—To identify the minimal peptide stretch in the C-terminal region of the Fas receptor necessary for FAP-1 binding, we employed an in vitro inhibition assay using a series of synthetic peptides as well as a yeast two-hybrid system comprising of random peptide libraries (Figs. 1A and 2, A and B). First, semi-random libraries (based on the homology between human and rat Fas) (Fig. 1B, a and b) of 15 amino acids fused to a LexA DNA binding domain were constructed and cotransformed into yeast strain L40 with a plasmid pVP16–31 (10) that produces a VP16 transactivation domain fused to the third PDZ domain of FAP-1. After the selection of 200 His\textsuperscript{+} colonies from an initial screen of 5.0 \times 10\textsuperscript{5} transformants, 100 colonies that were \(\beta\)-galactosidase positive were picked for further analysis. Sequence analysis of the library plasmids encoding the C-terminal 15 amino acids revealed that all of the C termini were either valine, leucine, or isoleucine residues. Second, a random library of 4–15 amino acids fused to a LexA DNA binding domain was constructed and screened according to this strategy (Fig. 1B, c). All of the third amino acid residues from the C terminus were serine. As before, the results of C-terminal amino acids were all either V, L, or I. No other consistent, non-random amino acid sequences were found by these library screenings, suggesting that S-X-V/L/I represents the motifs important for the association of Fas with the third PDZ domain of FAP-1.

To further confirm whether the last three amino acids are necessary and sufficient for Fas/FAP-1 binding, plasmids encoding LexA-SLV, -PLV, -PLY, -SLY, and -SLA fusion proteins were constructed and cotransformed into yeast with pVP16-FAP-1 (PDZ3). The results showed that only LexA-SLV specifically reacted with FAP-1, whereas LexA-PLV, -PLY, -SLY, and -SLA did not (Fig. 3A). In vitro binding studies using various GST-tripeptide fusions and in vitro-translated FAP-1 were consistent with these results (Fig. 3B).

In Vitro Inhibition Assay of Fas/FAP-1 Binding Using Synthetic Peptides—In addition to yeast two-hybrid approaches, we also employed an in vitro inhibition assay of Fas/FAP-1 binding. First, we tested whether a synthetic peptide representing the C-terminal 15 amino acids of Fas could inhibit the binding of a GST-Fas protein to in vitro-translated FAP-1. As shown in Fig. 2A, the binding of FAP-1 to GST-Fas was dramatically reduced by the 15-amino acid peptide in a concentration-dependent manner. In contrast, a control peptide (human PAMP) (34) had no effect on Fas/FAP-1 binding under the same conditions. Second, we examined the effect of shorter Fas C-terminal peptides on Fas/FAP-1 binding. As shown in Fig. 2B, a peptide comprised of only the last three amino acids of Fas (SLV) was able to bind to FAP-1 with the same efficiency as 4–15 synthetic peptides. Amino acid substitution analysis revealed that the 3rd amino acid residue from the C terminus was either serine or threonine, and the C-terminal amino acid valine, leucine, or isoleucine. In contrast, there was no amino acid preference for the amino acid 2 residues from the C terminus with respect to inhibitory effects on Fas/FAP-1 binding. These results were consistent with those of the yeast two-hybrid system (Fig. 1B, b and c).

Immunoprecipitation of native Fas with GST-FAP-1—To further substantiate our proposal that the PDZ domain interacts with the tripeptide motif (S/T)-(V/L/I), GST-fusion protein containing the 3rd PDZ domain of FAP-1 was tested for interactions with Fas expressed in Jurkat T-cells in the presence or absence of peptide inhibitors. The results revealed that the tripeptide Ac-SLV, but not Ac-SLY, abolished in a dose-dependent manner, the binding of FAP-1 to Fas in cell lysates prepared from Jurkat T-cells (Fig. 3C). This corroborates the data obtained above with recombinant Fas, which suggested that the C-terminal three amino acids SLV define the minimum binding site for FAP-1 and that the amino acids serine and valine are critical for this physical association.
To examine the physiological significance of the association between the C-terminal three amino acids of Fas and the third PDZ domain of FAP-1, a microinjection experiment was employed with synthetic tripeptides in a colon cancer cell line, DLD-1. DLD-1 cells express both Fas and FAP-1 and are resistant to Fas-induced apoptosis. As shown in Fig. 4A, treatment of DLD-1 cells with actinomycin D significantly reduced the expression levels of FAP-1 mRNA and sensitized DLD-1 cells to Fas-induced apoptosis. In contrast, the expression levels of Fas in DLD-1 cells were not affected by actinomycin D treatment. Similar results were obtained with cycloheximide treatment (data not shown). These results are
consistent with the idea that FAP-1 negatively regulates Fas-induced apoptosis in DLD-1 cells. To test this possibility more directly, the tripeptide Ac-SLV was microinjected at 0.5–2.5 mM per cell (a final concentration) into the cytoplasm of DLD-1 cells. Microinjection of Ac-SLV into DLD-1 cells dramatically enhanced the ability of Fas-monoclonal antibody (CH11, 500 ng/ml) to induce apoptosis (Fig. 4B, a and e, and Fig. 4C). In contrast, microinjection of an equivalent amount of Ac-SLY peptide or the PBS/K injection buffer did not permit Fas-induced apoptosis of these cells (Fig. 4B, b and f, and Fig. 4C).

**DISCUSSION**

We demonstrated that the C-terminal three amino acids (SLV) of human Fas alone were necessary and sufficient for binding to the third PDZ domain of FAP-1 by using a series of synthetic peptides as well as a screening of random peptide libraries. We also revealed that the consensus motif for binding to PDZ domain is t(S/T)-X-(V/L/I), rather than the previously proposed t(S/T)-X-V sequence. It is possible, therefore, that FAP-1 binds to the C termini of other receptors besides Fas. Moreover, it will be of interest to explore whether other PDZ domain-containing proteins such as inducible nitric-oxide synthetase can also bind to the C terminus of Fas.

PDZ repeats have been found previously in guanylate kinases, as well as in neuronal nitric-oxide synthetase (35), and the rat post-synaptic density protein (PSD-95), which is a homolog of the Drosophila tumor suppressor protein, lethal-(1)-disc-large-1 (dlg-1) (36, 37). These domains may be capable of homo- and heterodimerization but are better known for their ability to interact with the C termini of receptors such as the NMDA, Shaker-type K+ channel, and the APC proteins (Table I) (38–40). Although FAP-1 has six PDZ domains in both the N- and the C-terminal regions, only the third PDZ domain of FAP-1 can interact with the C-terminal three amino acids (SLV) of human Fas (data not shown). The recent reports on crystal structures of PDZ domains in dlg and PSD-95 strongly suggested that the C-terminal three amino acids sequence t(S/T)-X-V recognized a Gly-Leu-Gly-Phe loop and an arginine side chain of PDZ domains (41). However, the third PDZ domain of FAP-1 has the amino acid sequence SLGI instead of GLGF. When taken together with our data and previous reports, we would like to propose that the consensus motif of PDZ domain necessary for the binding to the C-terminal three amino acids sequence t(S/T)-X-(V/L/I) is (K/R/Q)-X_{n+2}-2X_{n+4}(G/S/A/E)-L-G-(F/I/L), where X and n represent any amino acid and at least 2, but not more than 4, respectively.

The analysis of the scanned tripeptides revealed that the third amino acid residue from the C terminus and the C-terminal amino acids having the strongest binding effect were either serine or threonine; and either Valine, leucine, or isoleucine, respectively. We therefore conclude that while the C-terminal three amino acids of human Fas are essential for binding to the third PDZ domain of FAP-1 protein, only the first and last residues in this tripeptide sequence are critical. These findings are similar to the situation recently reported for the x-ray crystal structure of the PDZ domain of PSD95 bound to its peptide ligand (42), where it was shown that the side-chain of a penultimate residue does not make contacts with the binding pocket in the PDZ domain, unlike the side-chains of the C-terminal and the third residue from the C terminus. However, a glycine residue in the second position from the C terminus is relatively disfavored among other amino acids residues, implying that the second amino acid residue may also require some kind of side chain in this particular interaction. Furthermore, we could not obtain any threonine residue from a screening of random peptide libraries by a yeast two-hybrid system, suggesting that a preference for serine over threonine in the third position from the C terminus of human Fas.

Another important finding in this paper is that the C-terminal consensus sequence identified in human Fas for interaction with the third PDZ domain of human FAP-1 is not conserved in mouse and rat Fas receptors. As shown in Fig. 1B (a), the C-terminal three amino acids of Fas in human (SLV), mouse (CLE), and rat (SLE) are not conserved, and only human Fas has a motif of t(S/T)-X-(V/L/I) sequence for the binding of PDZ domain. Thus, it is possible that other regulators of Fas-mediated signal transduction exist in mouse and rat. Our preliminary studies suggest that the third PDZ domain of human FAP-1 can interact with only human Fas, not with mouse and rat Fas. However, the third PDZ domain of mouse FAP-1 can interact with human Fas (data not shown). Previously, it has been shown that the C-terminal 15 amino acids of human Fas receptor represent a negative regulatory domain when human Fas is expressed in the mouse fibroblast L929 cell line (15). Furthermore, mouse L929 cells express FAP-1 endogenously (data not shown). These findings, therefore, indirectly support the hypothesis that the physical association of FAP-1 with the C terminus of human Fas is essential for protecting at least some types of cells from Fas-induced apoptosis. However, it is also possible that some other murine protein besides FAP-1 interact with mouse Fas via the C-terminal 15 amino acids negative regulatory domain. Experiments are in progress to explore these alternative possibilities. Of note, is the observation that not all PDZ domains bind to the t(S/T)-X-(V/L/I) consensus motif (43), raising the possibility that each PDZ domain may recognize other peptide sequences in the C termini of receptors such as mouse and rat Fas, which do not end in t(S/T)-X-(V/L/I). Thus, it will be interesting in future experiments to determine whether any of their PDZ domains are capable of binding to the tripeptide sequences found in the tail of mouse and rat Fas.

In our previous report (10), we correlated the expression level of FAP-1 with relative resistance to Fas-induced apoptosis in a variety of human tumor cell lines that express Fas. In this study, we observed that treatment of DLD-1 cells with actinomycin D reduced the expression level of FAP-1 mRNA and enhanced Fas-induced apoptosis dramatically. These results are consistent with the recent report that actinomycin D treatment of AIDS-associated Kaposi’s sarcoma cells down-regulates FAP-1 mRNA and also sensitized these cells to Fas-induced apoptosis (44).

Using a tripeptide blocker (Ac-SLV) of interaction of human Fas with FAP-1, we provide further evidence here that the interaction of FAP-1 with Fas can be an important contribution to Fas-resistance at least in some human cells. However, it is important to note that other mechanisms of Fas-resistance can also occur, such as overexpression of bcl-2 and secretion of soluble Fas from tumor cells. Further investigations, including the identification of substrates for FAP-1, elucidation of species differences in the Fas/FAP-1 interaction, and structure-function analysis of the FAP-1 protein will provide insights to the potential therapeutic implications of the Fas/FAP-1 interaction in cancer, autoimmune diseases, AIDS, and allograft rejection.
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