Interactions of Cellular Polypeptides with the Cytoplasmic Domain of the Mouse Fas Antigen*

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The mouse Fas/APO-1 antigen represents a 45-kilodalton transmembrane receptor that initiates apoptosis by a poorly defined signaling mechanism. The cytoplasmic domain of Fas does not display any known enzymatic activities but is capable of interacting with a number of proteins that were identified recently using the yeast interactive cloning method. To investigate direct biochemical interactions from cellular lysates prepared from Fas-responsive cells, a series of recombinant glutathione S-transferase-mouse Fas fusion proteins representing different regions of the mouse Fas cytoplasmic domain was used. Polypeptides of 25, 50, and 70 kilodaltons were found to associate with the Fas intracellular domain, and this binding was stable in the presence of 1 M NaCl. These interactions were also detected using a mouse Fas fusion protein containing an Ile to Asn mutation, which is responsible for a lymphoproliferative disorder in certain strains of mice (Iprcg). Furthermore, the binding of cellular proteins to Fas could be blocked upon incubation with a polyclonal antibody directed against the cytoplasmic domain of Fas. The strong association of cellular proteins with the cytoplasmic region implies that constitutive interactions may exist to regulate apoptotic signaling through the Fas antigen.

The activation of the Fas/APO-1 antigen by the Fas ligand or specific agonist antibodies results in a rapid induction of apoptosis (1–3). The resulting condensation and fragmentation of nuclei, loss of plasma membrane, and cell shrinkage are common morphological features of the apoptotic process. Analysis of a mouse lymphoproliferation mutation, Iprcg, provides genetic evidence that the Fas molecule is critically involved in T-cell regulation (4). The Iprcg mutation has been mapped to a point mutation in the cytoplasmic domain of the mouse Fas antigen, which abrogates its ability to initiate apoptosis (4, 5).

The Fas antigen is a member of a large family of cell surface molecules, which includes the p55 and p75 TNF-α receptors, CD27, CD30, CD40, the lymphotoxin-β receptor, and the p75 neurotrophin receptor (6, 7). While the homology among these receptors has been dictated primarily by similar extracellular cysteine-rich repeats, functional analysis of the p55 TNF receptor and the Fas antigen in transducing apoptosis has defined a cytoplasmic region of 89 amino acids, which has been designated the “cell death” domain (5, 8). A similar sequence has also been discovered in the cytoskeletal protein ankyrin and in a number of proteins that bind to Fas and the p55 TNF receptor death domains. These proteins, MORT1/ADD (9, 10), RIP (11), and TRADD (12), were identified using the yeast interactive cloning technique. Each protein can independently initiate cell death when transiently overexpressed in heterologous cells. The ubiquitous expression patterns of these proteins, the finding of a similar motif in ankyrin, and in vitro analysis (9–11) suggest that the death domain serves as a protein association motif.

The biochemical mechanisms responsible for Fas action are incompletely understood. An acidic sphingomyelinase activity is induced upon cross-linking of Fas with monoclonal antibodies in U937 cells (13) that gives rise to higher intracellular ceramide levels. A similar activity has also been detected upon TNF-α binding to the p55 TNF receptor (14); however, recent evidence suggests an important role for a neutral sphingomyelinase in Fas and p55 TNF receptor signal transduction. In addition, cellular tyrosine phosphorylation is increased within minutes after engagement of the Fas antigen in Jurkat and U937 cells (15). The potential involvement of tyrosine phosphorylation in Fas signal transduction is supported by the identification of FAP-1/BAS, a protein tyrosine phosphatase, which negates Fas-mediated apoptosis (16). The effects of FAP-1 upon anti-Fas antibody-induced cell death have been mapped to an interaction of FAP-1 with the C terminus of the Fas antigen, a region known to attenuate Fas-mediated apoptosis (5).

Since the cytoplasmic domain is clearly necessary for Fas function as a receptor (4, 5) and numerous binding interactions have been defined for Fas (17), we have investigated the binding properties of the intracellular domain of Fas using recombinant fusion proteins. Our results indicate that strong protein-protein associations exist that map to distinct regions of the Fas molecule. The protein binding interactions are specific, because an anti-Fas antibody can effectively block the binding. Surprisingly, the predominant binding interactions are still observed in a cytoplasmic Fas mutation (Iprcg), suggesting that the Fas receptor can participate in constitutive protein-protein interactions that regulate its apoptotic function.

**MATERIALS AND METHODS**

GST Fusion Expression Constructs—The mouse Fas cDNA (courtesy of S. Nagata) was subcloned into the mammalian expression vector pCMV5 using BglII and Clal sites in the polylinker. The resulting pCMV5-mFas plasmid was purified by alkaline lysis and equilibrium centrifugation through a CsCl gradient. All GST fusion bacterial expression constructs were prepared using the pgSTag vector (18). Deletion mutations of the Fas wild-type cytoplasmic sequence similar to those described by Itoh and Nagata (5) were generated by PCR. The Fas deletion (FD) mutants were obtained by using a common 5′ primer (5′-CGGGATCCAAATGCTTATGCTTATGCT-3′), unique 3′ primers

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1. The abbreviations used are: TNF, tumor necrosis factor; GST, glutathione S-transferase; IPTG, isopropylthiogalactoside; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; FD, Fas deletion; PCR, polymerase chain reaction; TBST, Tris-buffered saline/Tween 20.
Cell Culture—All cell lines were cultured at 37 °C in 5% CO2. HeLa cells were grown in Dulbecco’s modified Eagle’s medium + 10% fetal calf serum; L929 cells were grown in minimum essential medium with Earle’s salts + 10% horse serum; and Jurkat and Hut78 cells were grown in RPMI 1640 + 10% fetal calf serum (heat-inactivated). All cultures were maintained in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin, 1 μg/ml lincomycin, and 1 μg/ml gentamicin.

Metabolic Labeling and Preparation of Cell Lysates—Metabolic labeling of mouse Fas was performed using 1μCi [35S]methionine and -cysteine (DuPont NEN). HeLa and L929 cells were grown until the cultures reached midlogarithmic growth phase (0.4–0.5 × 106/plate) and plated onto 10-cm tissue culture dishes. Jurkat and Hut78 cells were also grown on 10-cm tissue culture dishes at 10 × 106/plate. The cells were then washed twice with 10 ml of phosphate-buffered saline and then washed with 2 ml of methionine- and cysteine-free medium. The cells were then incubated at 37 °C in 5 ml of methionine- and cysteine-free medium for 20 min in order to reduce intracellular stores of methionine and cysteine. To each plate of cells, [35S]methionine and -cysteine were added to 0.1 μCi/ml, and the cells were incubated for 3 h at 37 °C.

After labeling, the [35S]-containing medium was removed, and the cells were washed four times with 10 ml of phosphate-buffered saline. HeLa and L929 cells were harvested by scraping, and Cells were harvested from mouse Fas expressing cultures by centrifugation at 900 × g and lysed for 10 min on ice in Lysis Buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 25 μg/ml PMSF) and vortexed briefly. The lysed cells were then centrifuged at 900 × g for 3 min to remove insoluble debris. Supernatants were removed and stored at −80 °C until use. In experiments where anti-GST-mFas or anti-GST IgG were preincubated with the GST-fusion proteins prior to the addition of lysates, 2-mercaptoethanol was not present in the Lysis Buffer used to lyse or wash the protein complexes.

Binding Assay—Cell lysates from 35S-labeled cells were thawed and precleared with 50 μl of Sepharose-GSH (Sepharose-GSH/Lysis Buffer, 1:1 v/v) and 25 μg GST for 2 h at 4 °C. The Sepharose matrix was removed by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge. The supernatants were subsequently incubated with 50 μl of Sepharose-GSH (Sepharose-GSH/Lysis Buffer, 1:1 v/v) and 10 μg GST protein or the GST-fusion protein for 2 h at 4 °C. The matrix was recovered by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge, and the supernatants were then removed. The matrix was washed four times with 1 ml of ice-cold Lysis Buffer, followed by 2 washes with 1 ml of 50 mM Tris, pH 8.0. The GST- or GST-fusion protein was then eluted from the Sepharose-GSH matrix by incubating twice with 50 μl of 20 mM GSH, 50 mM Tris, pH 8.0 (10-min incubation at room temperature) and then centrifugation at 14,000 rpm and pooling the supernatants. Purified GST or GST-fusion proteins were stored at −80 °C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Protein samples were resolved on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked in Tris-Buffered Saline (150 mM NaCl, 50 mM Tris, pH 8.0, 2.5 mM CaCl2, 1 mM dithiothreitol) for 2 h at 37 °C. This was then incubated for 12% SDS-PAGE along with 1 μg of GST and GST-mFas (221–306) (no thrombin cleavage). The separated proteins were then blotted onto a polyvinylidene difluoride membrane. The membrane was blocked using 10% dry milk/TBST for 1 h at 4 °C, followed by anti-GST-Fas serum at 1:100 dilution. Membranes were then incubated with primary antibody in blocking buffer, followed by incubation with a secondary antibody conjugated to HRP. Membranes were then developed using a chemiluminescent substrate (ECL, Amersham).

Cell-surface shear force analysis and metabolic labeling and preparation of cell lysates were performed as described above except for the presence of different salt concentrations in the Lysis Buffer used to wash the protein complexes on the matrix. After incubation of precleared lysates with GST or GST-mFas (221–306), the Sepharose matrix was subjected to washes with Lysis Buffer containing 200, 300, 400, 500, or 1000 μg/ml NaCl. The remaining proteins were eluted with 25 μg of 2-mercaptoethanol, and 1 μg of [35S]methionine and -cysteine was added, and each preparation was heated to 100 °C for 5 min. Equal volumes of the preparations were analyzed by SDS-PAGE followed by fluorography (ENHANCE, DuPont NEN). The dried gels were exposed to radiographic film for 2–10 days at −80 °C.

Salt Gradient—The binding assay to GST or GST-mFas (194–306) and analysis of associated cellular proteins were performed as described above except for the presence of different salt concentrations in the Lysis Buffer used to wash the protein complexes on the matrix. After incubation of precleared lysates with GST or GST-mFas (194–306), the Sepharose matrix was subjected to washes with Lysis Buffer containing 200, 300, 400, 500, or 1000 μg/ml NaCl. The remaining proteins were eluted with 25 μg of 2-mercaptoethanol, and 1 μg of [35S]methionine and -cysteine was added, and each preparation was heated to 100 °C for 5 min. Equal volumes of the preparations were analyzed by SDS-PAGE followed by fluorography (ENHANCE, DuPont NEN).

The dried gels were exposed to radiographic film for 2–10 days at −80 °C.
a 1:1000 dilution in 1% dry milk/TBST for 1 h at 4°C and goat anti-rabbit IgG horseradish peroxidase (Boehringer Mannheim) at a 1:5000 dilution in 1% dry milk/TBST for 1 h at 4°C. The blot was developed using ECL (Amersham Corp.).

Antibody Blocking Experiment—The binding assay to GST or GST-mFas-(194–306) and analysis of associated cellular proteins was performed as described above, except that labeled lysates were prepared without 2-mercaptoethanol. Anti-GST or anti-GST-mFas at 10, 33, or 100 µg was preincubated with 50 µl of Sepharose-GSH (Sepharose-GSH/Lysis Buffer, 1:1, v/v) and 10 µg of GST-mFas-(194–306) for 30 min at 4°C. Binding assays and analysis of associated cellular proteins were performed as described above.

RESULTS

The cytoplasmic domain of the Fas antigen contains an 89-amino acid motif (residues 204–292) required for antibody-induced apoptosis in murine L929 cells (5). To characterize potential binding interactions of the intracellular sequence of mFas, a series of glutathione S-transferase proteins were prepared that contained different sequences from the cytoplasmic domain of the mouse Fas antigen. Fig. 1 shows the FD mutants, which are based upon the work of Itoh and Nagata (5) who assessed the cytotoxic activities of the human Fas deletion mutants after transfection in murine L929 cells. The GST-mFas FD mutants share a common N terminus fused in frame to GST but differ by sequential deletions at the C terminus of the mFas intracellular domain. The longest construct, GST-mFas-(194–306), contained part of the juxtamembrane domain (amino acids 194–203), the entire death domain (amino acids 204–292), and the C-terminal region (amino acids 293–306). The last 14 amino acids define an inhibitory domain by analogy to studies performed with the human Fas antigen (5).

Two other constructs, GST-mFas-(221–306) and GST-mFas lpr<sup>ca</sup>, were also generated. The fusion protein, GST-mFas-(221–306), differs from GST-mFas-(194–306) by lacking 27 amino acids at the N terminus of the mFas coding region that includes 17 amino acids of the death domain and the 10 residues of the juxtamembrane domain found in GST-mFas-(194–306).
replaced by an asparagine residue (Fig. 1). Certain strains of mice carrying this mutation in the Fas antigen develop lymphadenopathy and an autoimmune syndrome. This point mutation has been shown to interfere with the ability of Fas to transmit an apoptotic signal (4).

**Interactions of the Fas Cytoplasmic Domain**—A variety of Fas-responsive cell lines have been defined, in which expression of Fas antigen results in the initiation of apoptosis upon cross-linking the receptor with agonist antibody (1, 2, 4, 5, 20). To evaluate binding interactions of Fas, two cell lines that are negative for Fas antigen expression, L929 and HeLa cells, and two lines that express Fas, Jurkat and Hut78, were analyzed for binding using affinity-purified bacterial GST-mFas fusion proteins. All four cell lines are responsive to agonist antibody (sensitivity for L929 and HeLa cells has been demonstrated after transfection of Fas cDNA). Lysates from [35S]methionine and -cysteine-labeled HeLa, L929, and Jurkat cells were prepared and screened for GST or GST-mFas binding proteins as in Fig. 2. GST-mFas-(194–306) was incubated for 30 min at 4°C with different amounts of anti-GST-mFas IgG or anti-GST IgG prior to incubation with HeLa lysates. The eluted material was subjected to 12% SDS-PAGE and fluorography. The indicated band represents the mFas fragment cleaved from the GST carrier after thrombin treatment.

**Specificity of Protein Interactions**—In order to assess the strength of the protein interactions detected with the GST-fusion proteins, the GST-mFas-polypeptide complexes were subjected to washes with increasing salt concentrations. The p25, p50, and p70 polypeptides were found to be associated with GST-mFas-(194–306) over a range of salt concentrations from 200 mM to 1 M (Fig. 3). These results demonstrate that strong interactions exist between these cellular polypeptides and mFas cytoplasmic amino acids 194–306. The specificity of these interactions was tested using an anti-GST-mFas antibody. A polyclonal antibody was generated to the recombinant GST-mFas-(221–306) protein. The antibody recognized the Fas intracellular domain by Western blot analysis (Fig. 4A). The antibody was affinity-purified and then used to assess the nature of the binding interactions between the GST-mFas-(194–306) recombinant protein and p25, p50, and p70. The GST-mFas-(194–306) protein was preincubated with increasing amounts of the anti-GST-mFas or anti-GST antibody and used to screen HeLa cell metabolically labeled lysates as described under “Materials and Methods.” Incubation with the anti-GST-mFas IgG blocked binding of p25 and p70 to GST-mFas (Fig. 4B). A longer exposure indicated that the p50 protein was similarly disrupted. Control experiments indicated...
that the anti-GST antibody had no effect up to 100 μg of IgG. These findings indicate that the three polypeptides associate specifically with the mFas intracellular domain containing amino acid residues 194–306.

Mapping Interactions with the Fas Cytoplasmic Domain—To characterize the association of cellular proteins with the Fas cytoplasmic domain, two other GST-mFas fusion proteins were used to assay metabolically labeled HeLa cell lysates. The first, GST-mFas(lprcg), represents a protein with identical primary structure to GST-mFas-(194–306) except for a single amino acid change at residue 225, where an isoleucine was replaced by an asparagine (see Fig. 1). The mouse Fas antigen harboring such a mutation has been shown to lack biological activity. The second fusion protein, GST-mFas-(221–306), differs from GST-mFas-(194–306) in that the N-terminal 27 amino acids of the mFas coding region are missing, creating a deletion of 17 amino acids at the N terminus of the death domain (see Fig. 1).

Binding experiments with HeLa and L929 cell extracts indicated that while both p25 and p70 interacted strongly with both GST-mFas lprcg and GST-mFas-(194–306), only p25 was found to be strongly associated with GST-mFas-(221–306) (Fig. 5). These findings imply that p25 and p70 interact with mFas via distinct domains. Binding of p70 to Fas appears to require a region between residues 194 and 221, while p25 binds to more C-terminal sequences. Furthermore, the fact that both p25 and p70 bind to GST-mFas lprcg is intriguing as the lprcg mutant is believed to represent an inactive form of Fas.

To define further the binding of p50 to the cytoplasmic domain of wild-type Fas, a series of C-terminal mutations (FD) were assayed, which corresponded to deletions made in the human Fas antigen (5). Metabolically labeled HeLa cell lysates were assayed for p50 binding using these GST-fusion proteins (Fig. 1). The p50 protein was found to strongly associate with all the FD mutants except GST-mFas FD2, which contains residues 194–221 of mouse Fas (Fig. 6A). Since p50 binds tightly to the FD4 mutant (mFas-(194–267)), the binding domain for p50 spans residues 222 to 268 of mFas.

In addition, all the FD mutant proteins used in this analysis were found to bind equally well to labeled p70 from HeLa cells (Fig. 6B). These results are consistent with the mapping of p70 binding to a region of Fas between amino acids 194 and 221 (Fig. 5). Attempts to visualize binding of p25 to the FD mutants did not provide conclusive mapping information; however, the binding of p25 to GST-mFas-(221–306) (Fig. 5) indicated that p25 interacts with the C-terminal 85 amino acids of Fas. Taken together, the GST-fusion protein data demonstrate that each cellular protein displays a distinctive binding interaction with the cytoplasmic domain of mouse Fas. Binding of p70 has been localized to residues 194–221; p50 binds to residues 222–268; and p25 binds to a region between residues 221 and 306. The mouse Fas intracellular domain is therefore capable of multiple protein-protein interactions.

DISCUSSION

The Fas antigen belongs to a group of cell surface receptors, the TNF receptors, CD40, CD30, and the p75 neurotrophin
receptor, for which intracellular receptor signaling pathways are not well defined. A major question regarding the mechanism of Fas-mediated cell killing is how activation of the receptor leads to the biochemical reactions responsible for apoptosis. A number of activities, including sphingomyelin turnover (13), tyrosine phosphorylation and dephosphorylation (15, 16), and activation of ras (21) have been implicated. However, none of these enzymatic activities have been physically linked to the Fas receptor.

To begin to understand Fas receptor function, we have utilized recombinant GST-fusion proteins to assay for proteins that associate with cytoplasmic sequences of Fas. In contrast to the yeast two-hybrid approach, this approach provides direct biochemical evidence for interacting proteins from cell lysates. Three proteins of apparent molecular masses 25, 50, and 70 kilodaltons, from HeLa, J urkat, L929, and Hut78 cells, display strong interactions with the intracellular domain of mouse Fas. These proteins are not restricted to Fas-expressing cells, since HeLa and L929 cells do not express the Fas antigen (5, 20); these two cell lines are rendered Fas-sensitive upon introduction of the Fas cDNA by transfection. The binding interactions are stable even in the presence of 1 m NaCl and could be specifically blocked by polyconal antibodies against the Fas intracellular domain. Mapping data suggest that each protein binds to a different Fas cytoplasmic sequence (Fig. 7). Since these proteins were found to bind to the Fas intracellular domain in the absence of ligand, it is possible that these interactions are constitutive in nature. However, given that the intracellular domain of Fas has been demonstrated to self-associate (9, 22), it is also possible that the resultant recombinant fusion protein complexes contain the Fas intracellular domain in dimeric or oligomeric form. This suggests that the recombinant Fas proteins used here may mimic the behavior of the receptor upon agonist binding.

Supporting the idea that these interactions are constitutive in nature is the behavior of the labeled HeLa cell proteins toward the mFas lprcg mutation mutant fusion protein. A comparable level of binding was observed to the lprcg and wild-type fusion proteins, suggesting that the lprcg mutation does not interfere with these associations. These results are of interest because mouse experiments clearly demonstrate that the Ile to Asn lprcg mutation (Ile to Asn) is somewhat less effective than the Lpr mutation (Val to Asn). Also, p70 was found to interact with the Fas intracellular domain. Mapping data suggest that each protein binds to a different Fas cytoplasmic sequence (Fig. 7). Since these proteins were found to bind to the Fas intracellular domain in the absence of ligand, it is possible that these interactions are constitutive in nature. However, given that the intracellular domain of Fas has been demonstrated to self-associate (9, 22), it is also possible that the resultant recombinant fusion protein complexes contain the Fas intracellular domain in dimeric or oligomeric form. This suggests that the recombinant Fas proteins used here may mimic the behavior of the receptor upon agonist binding.

The 25- and 70-kilodalton proteins are similar in size to two cellular proteins, MORT1/FADD (9, 10) and RIP (11), respectively, both of which were identified using a yeast interaction screening technique with the human Fas cytoplasmic domain. The MORT1/FADD and RIP proteins were shown to induce apoptosis when overexpressed in heterologous cells. In contrast to FADD and RIP proteins, the p25 and p70 proteins were found to interact with the lprcg mutant form of Fas. Also, p70 maps to a different binding domain than the RIP protein (11). Several reasons may account for the differential results observed with these metabolically labeled proteins. First, the p25 and p70 proteins may be different from MORT1/FADD and RIP proteins. Second, there may be species-specific differences, because the mouse Fas lprcg mutation (Ile to Asn) is somewhat different from the human Fas lprcg mutation (Val to Asn). Third, the p25 and p70 proteins may bind to the Fas lprcg protein through different conformational states, which may be exhibited either by Fas or the interacting proteins. In this regard, a potential disadvantage with the use of recombinant fusion proteins is that the exact folding of the cytoplasmic domains may not be reproduced.

For many receptor systems, ligand binding leads to dimerization or aggregation of receptors, which facilitates post-translational modifications such as tyrosine phosphorylation. The action of agonist anti-Fas antibodies indicates that aggregation of Fas may serve as a key step in initiating its apoptotic function. This model of Fas action has been borne out by experiments demonstrating that the intracellular domains of both Fas and the p55 TNF receptor self-associate (22, 23). The experiments here demonstrate that strong and multiple protein-protein interactions exist in the absence of antibody or Fas ligand. Similar sized proteins to p25 and p50 using Fas cross-linking followed by immunoprecipitation were recently reported (24). Our results suggest that ligand-independent protein interactions are possible for Fas. If the cell death proteins that have been identified as potential partners for the Fas antigen and the p55 TNF receptor (17) function as protein association domains, the binding of the Fas ligand or agonist antibodies to the Fas receptor may lead to one of several possibilities: (a) additional protein interactions; (b) dissociation and relocalization of Fas-associated proteins to their cellular site of action; or (c) appropriate conformational changes that unmask a cell death activity in the constitutively associated protein complex. Direct assays of receptor proximal events will be required in order to discriminate among these possibilities.

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