Brief Definitive Report

Identification of Amino Acid Residues Important for Ligand Binding to Fas

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Summary

The interaction of Fas (CD95), a member of the tumor necrosis factor receptor (TNFR) family, and its ligand (FasL) triggers programmed cell death (apoptosis) and is involved in the regulation of immune responses. Although the Fas–FasL interaction is conserved across species barriers, little is currently known about the molecular details of this interaction. Our aim was to identify residues in Fas that are important for ligand binding. With the aid of a Fas molecular model, candidate amino acid residues were selected in the Fas extracellular domain 2 (D2) and D3 and subjected to serine-scanning mutagenesis to produce mutant Fas molecules in the form of Ig fusion proteins. The effects of these mutations on FasL binding was examined by measuring the ability of these proteins to inhibit FasL-mediated apoptosis of Jurkat cells and bind FasL in ELISA and BIAcore assays. Mutation of two amino acids, R86 and R87 (D2), to serine totally abolished the ability of Fas to interact with its ligand, whereas mutants K84S, L90S, E93S (D2), or H126S (D3) showed reduced binding compared with wild-type Fas. Two mutants (K78S and H95S) bound FasL comparably to wild type. Therefore, the binding of FasL involves residues in two domains that correspond to positions critical for ligand binding in other family members (TNFR and CD40) but are conserved between murine and human Fas.

Programmed cell death (apoptosis) mediated by the Fas–FasL system is a mechanism used to control immune responses. The Fas (CD95) antigen, a 45-kD protein of the TNF receptor (TNFR) family, is widely expressed and binds a TNF-like ligand (FasL) (1). Perturbations of the Fas–FasL interaction have drastic functional consequences in lpr and gld mice, leading to lymphadenopathy and severe immune disregulation (2, 3). A human lymphoproliferative disorder, the Canale-Smith syndrome, appears to be due to mutation of the signal transduction domain of Fas (4). Although FasL is expressed as a cell surface molecule, it is also released after cleavage by metalloproteinases (5), enabling FasL to act as a soluble mediator of cell death. Fas-mediated cell death is thought to be involved in the pathology of a number of disease states, including fulminant hepatitis and chronic liver disease (6, 7), multiple sclerosis (8), and it may also have a role in neutrophil-mediated tissue destruction (9). In addition, some tumors are able to escape immune surveillance by releasing FasL, which kills activated T cells infiltrating the tumor (10, 11).

Molecular details of the Fas–FasL interaction have yet to be determined. Fas is a type I membrane protein, consisting of three TNFR-like extracellular domains (D1, D2, and D3), a hydrophobic transmembrane region, and a cytoplasmic tail containing a death domain. The death domain binds Fas death domain–binding protein (FADD, MORT1), which links Fas to a cascade of IL-1β-like proteolytic enzymes known as caspases (12). Recently, the three dimensional structure of the Fas death domain was solved using NMR spectroscopy, and was shown to consist of six antiparallel, amphipathic α helices arranged in a novel fold (13). Fas binds to FasL across the murine and human species barrier, suggesting the conservation of amino acid residues important for binding. We wished to investigate the structural basis for the Fas–FasL interaction. Using the TNFR three-dimensional structure as a template, we were able to generate a model of the Fas extracellular domains (14). On this model, we were able to map residues conserved between human and murine Fas, and positions implicated in the interaction of TNFR with TNF (15), and/or positions implicated by mutagenesis analysis in the interaction of another family member, CD40, with the CD40L (16, 17). A surface was identified on the extracellular D2 of Fas and a part of D3, which consists of residues conserved in murine and human Fas, but not conserved between Fas and TNFR or CD40. Residues in this region were considered potential candidates for FasL binding and were subjected to serine-scanning mutagenesis. We found that binding of FasL is centered on D2 of Fas and involves a region that corresponds to the ligand binding sites in TNFR and CD40.
Materials and Methods

Monoclonal Antibodies and Fusion Proteins. Soluble FasL was produced in a manner similar to that described for a closely related TNF family member, gp39, the ligand for CD40 (18), by fusing the extracellular domain of FasL to the extracellular domain of murine CD8. cDNA encoding for the extracellular domain of human FasL (amino acids 105–281) was amplified by PCR from monocyte cDNA using the primers CGC CGC GGA TCC CTT CCA CCT ACA AAA GGA GGT CTT G (forward primer containing a BamHI site) and GCC TCC TCT AGA CCC AAA GTG CTT AAG TAA GCT ATA TAA GCC (reverse primer containing a XbaI restriction enzyme site). Amplified cDNA was digested with BamHI and XbaI, gel purified, and ligated into the pCDM7 vector containing cDNA encoding for murine CD8. CD8–FasL was produced in COS cells following transfection by the DEAE-Dextran chloroquine method. Supernatants containing CD8–FasL fusion proteins were harvested and passed through a 0.22-μm filter. CD8–FasL was affinity purified on an anti-CD8 (53-6) column as previously described for CD8–CD40L (19).

A soluble protein (FasL–FasRγ1), consisting of the extracellular domain of Fas fused to the hinge, CH2, and CH3 regions of human IgG1 containing a thrombin cleavage site, was constructed. In brief, cDNA was amplified by PCR using oligonucleotide primers 5′-CGC CCC AAG CTT CGG AGG ATT GCT CAA (containing a HindIII site) and 3′-GGG TGC TCT AGA CCC AAA GTG CTT AAG TAA GCT ATA TAA GCC (reverse primer containing a XbaI restriction enzyme site). Amplified cDNA was digested with BamHI and XbaI, gel purified, and ligated into the pCDM7 vector containing cDNA encoding for murine CD8. CD8–FasL was produced in COS cells following transfection by the DEAE-Dextran chloroquine method. Supernatants containing CD8–FasL fusion proteins were harvested and passed through a 0.22-μm filter. CD8–FasL was affinity purified on an anti-CD8 (53-6) column as previously described for CD8–CD40L (19).

Three anti-Fas mAbs were used to confirm the structural integrity of the mutant Fas fusion proteins. SM 3/1 (IgG2a), SM 3/17 (IgG2a), and DX-2 (IgG1) were purchased from Biosource International (Camarillo, CA) for ELISA. These were unable to immobilize FasL or FasL–FasRγ1 on a BIAcore instrument (Pharmacia Biosensor, Uppsala, Sweden) at 25°C using PBS, pH 7.4, containing 0.005% surfactant P20 (Pharmacia Biosensor) as the running buffer. CD8–FasL was immobilized on research-grade CM5 sensor chips (Pharmacia Biosensor) using standard CNBr coupling, excess activated carboxyl groups were inactivated with ethanolamine. Immobilization of ~6,000 RU of FasL was achieved.

Apparent association and dissociation rates for wild-type FasLγ1 were determined using concentrations ranging from 10 to 200 nM. After the injection, flow of running buffer alone was established to allow observation of the dissociation of bound protein. Dissociation to baseline was obtained in the allotted time, so it was not necessary to inject any reagent to regenerate the ligand surface. Apparent association and dissociation rates were determined by curve-fitting using BIAevaluation 2.1 (Pharmacia Biosensor).

Results and Discussion

Generation of FasLγ1 M utants. Fas belongs to the TNFR superfamily and its extracellular region includes three domains (D1–D3) with distinct sequence homology to TNFR

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On this basis, a detailed three-dimensional model was generated using the TNFR x-ray structure as a template. Eight residues that were spatially adjacent in the model (K78, K84, R86, R87, L90, E93, H95 in D2, and H126 in D3) were selected as candidates for mutagenesis. These eight residues are conserved in murine and human Fas, but they are not conserved between family members.

Six of these positions (all except R86 and H95) correspond to residues that are implicated in the TNFR and/or CD40–ligand interactions. The molecular model and mutated residues are shown in Fig. 1. Color-coding of this model is based upon the results of the mutagenesis studies described below and discussed at the end of this report. Mutant proteins were generated, expressed as Ig fusion proteins (FasthrRγ1), and purified for use in FasL binding and functional assays.

Binding of Mutant Proteins to mAb. The binding of each mutant protein to a panel of anti-Fas mAbs, which recognize three epitopes based on the following: (a) their ability to block CD8–FasL-mediated apoptosis (DX-2); (b) their ability to induce apoptosis (SW1/1); or (c) their inability to block or induce apoptosis (SW1/17) in our system (data not shown), was examined. Each mAb was unable to immunoblot FasthrRγ1 when the protein was reduced before loading on the gel, indicating that the epitopes the mAb recognize are conformationally sensitive. Therefore, these mAbs are suitable to monitor the overall structural integrity of expressed mutant proteins. Each mAb bound to each mutant and to wild-type FasthrRγ1 equivalently as determined by ELISA (Fig. 2), indicating that the overall structural integrity of the proteins was not significantly compromised as a consequence of the mutations.

FasL Binding. The binding characteristics of FasthrRγ1 mutants were compared with wild-type FasthrRγ1 in vitro.

Supernatants of CD8–FasL were able to kill Jurkat cells in a dose-dependent manner (data not shown), and the killing could be inhibited by wild-type FasthrRγ1. FasthrRγ1 wild-type and mutants were titered at a constant concentration of CD8–FasL, and the viability of the Jurkat cells was determined by measuring the change in color of Alamar Blue.

Figure 2. A panel of anti-Fas mAbs bind FasthrRγ1 and FasthrRγ1 mutants. Fusion proteins were immobilized, and binding was detected by anti-Fas mAbs at various concentrations. Data is shown for mAb at 1 μg/ml, but similar relative binding was observed at lower concentrations of mAb. Open bar, SW1/17; shaded bar, SW1/1; closed bar, DX-2. Negative control mAb OD values for each fusion protein were ~0.1.

Figure 3. Mutation of FasthrRγ1 at positions R86 and R87 abrogates its ability to inhibit FasL-mediated killing of Jurkat cells. Jurkat cells were incubated with a 1:8 dilution of CD8–FasL-containing supernatant in the presence of wild-type and mutant FasthrRγ1 in triplicate wells for each concentration of fusion protein. Increased cell death is represented by low OD values.
Blue added to the microtiter wells. In this assay, FasthrR-g1 inhibited apoptosis in a concentration-dependent manner, whereas mutant proteins showed a range of inhibitory activities (Fig. 3). Mutation of residues K78 and H95 to serine had little effect on the ability of the fusion proteins to inhibit killing, whereas mutation of residues, K84, L90, E93, and H126 to serine markedly reduced the ability of FasthrR-g1 to inhibit the apoptotic activity of CD8-FasL. Mutation to serine at positions R86 and R87 completely abolished the inhibitory effect of the fusion proteins, suggesting they are critical for the Fas–FasL interaction. A control fusion protein, mCD6D1thrR-g1, had no protective effect in these assays.

To measure directly the binding of FasthrR-g1 mutants to FasL, an ELISA assay was developed. FasthrR-g1 and mutants were captured on ELISA plates using donkey anti-human IgG. CD8-FasL was added to plates, and binding of FasL was detected using the NOK-2 mAb and HRP-conjugated anti-mouse IgG.

Surface Plasmon Resonance Analysis of FasthrR-g1 and Mutant Binding to FasL. Surface plasmon resonance analysis of the Fas–FasL interaction was undertaken by BIAcore analysis to obtain an approximation of the binding constant for this interaction. Purified CD8-FasL was immobilized on a sensor chip and various concentrations of FasthrR-g1 in fluid phase was passed over the chip. Association and dissociation rates were calculated, and a K_d of value of ~7 × 10^{-8} M was obtained from the ratio of dissociation/association. This is a relatively weak interaction compared with K_d values obtained for the TNFR(p55)lg-TNF interaction (6.5 × 10^{-11} M for TNF-α and 6.4 × 10^{-10} M for TNF-β) obtained by Scatchard analysis (22). The K_d of the FasthrR-g1-CD8-FasL interaction is also considerably weaker than that reported for CD40-CD40L (K_d, ~5 × 10^{-10} M)
(23]) but similar to another TNFR–TNF family member, 4-1BB/4-1BBL (K_d of low affinity sites using recombinant ligand ~7 × 10^{-8} M and K_d of high affinity sites ~3 × 10^{-10} M (24)), each K_d value obtained by binding Ig fusion proteins to ligand expressed on cells.

Equilibrium binding curves (Fig. 5) showed results equivalent to the ELISA. K78S saturation binding was higher than wild type due to a slower off rate from FasL. H95S binding was similar to wild type; however, the curves were steeper on each side of the equilibrium plateau, suggesting faster on and off rates. R 86S and R 87S showed no binding to the chip, whereas mutants K84S, L90S, E93S, and H 126S showed reduced binding to the chip-bound FasL. Again, the hierarchy of the intermediate mutants was consistent between different assays, with K84S, L90S, E93S having roughly equivalent RU values, whereas binding H 126S was markedly lower than the other three mutants.

Outline of the Fas Ligand-binding Site. Mutants with deleterious effects on ligand binding were mapped into the three-dimensional Fas model (see Fig. 1). Two adjacent arginine residues (R 86 and R 87; colored magenta in Fig. 1) are critical for binding. Four other residues support binding (K84, L90, E93, H 126; colored in gold in Fig. 1) but are not critical as mutation of these residues does not obliterate binding to ligand. In the model, these six residues form a surface that is likely to constitute a center of Fas–FasL interactions. Therefore, binding of ligand is centered on extracellular D2, but residues in D3 may support ligand binding. Mutation of residues K 78 and H 95 have no deleterious effects on ligand binding and have been colored in green in the model (see Fig. 1). Positions equivalent to H 95 have not been implicated in the ligand binding of TNFR or CD40. Residues critical for ligand binding in Fas (R 86 and R 87) are adjacent to a disulfide bond, which is conserved in TNFR and CD40 (14); however, positions equivalent to only one of these residues (R 87) are implicated in ligand binding for the other family members (15). Although the surface of ligand binding for TNFR, CD40, and Fas are located in a similar location, the amino acid composition of the binding surfaces differ. Different residues at equivalent positions alter the ligand binding surface and therefore determine the ligand binding specificity. At present, it is unclear whether all of the identified residues are directly involved in FasL recognition, or whether some mutations introduce local conformational changes sufficient to compromise binding to ligand but not to mAb. Additionally, other residues may be expected to contribute to the interaction.

In summary, individual residues in Fas have been identified as critical for ligand binding and an important role of charged residues in mediating Fas–FasL interactions has been demonstrated. Residues important for binding are conserved in murine and human Fas, which provides a rationale for the observed cross-species Fas–ligand interactions. However, these residues are not conserved in TNFR or CD40, thus explaining the specificity of the Fas receptor–ligand interaction. On the basis of our study, Fas D2 includes two residues that are critical for ligand binding and three residues that support ligand binding. Four of these residues (except R 86) correspond to residues that contribute to CD40 (17) and TNFR ligand binding (15). This suggests that although specific residue contributions differ, equivalent regions are utilized by these receptors for mediating different ligand binding specificities.

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