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Cutting Edge: Two Distinct Motifs within the Fas Ligand Tail Regulate Fas Ligand-Mediated Costimulation

Mingyi Sun, * Shinhee Lee, * Saoussen Karray,2† Matthieu Levi-Strauss,3† Kristina T. Ames, *, and Pamela J. Fink4*

The cytoplasmic domain of Fas ligand is sufficient to costimulate CD8+ T cells by driving Fas ligand recruitment into lipid rafts and association with select Src homology 3-containing proteins, activating PI3K and MAPK pathways, mediating nuclear translocation of the transcription factors NFAT and AP-1, and enhancing IFN-γ production and Ag-specific CD8+ T cell proliferation. We now show that Fas ligand molecules lacking amino acids 45–54 in the proline-rich region of the cytoplasmic domain fail to costimulate but serve as effective death inducers. Death induction and costimulation by Fas ligand are therefore clearly separable functions. Further, upon Fas ligand-mediated costimulation, casein kinase I phosphorylates Fas ligand, in which two conserved casein kinase I binding sites regulate NFAT activation and costimulation. These results help resolve how one molecule can serve as a double-edged immunomodulator by directing discrete biological consequences. The Journal of Immunology, 2007, 179: 5639–5643.

Optimal T cell activation generally requires costimulation in addition to a signal delivered through the TCR. Three main classes of costimulatory molecules have been identified to date: Ig superfamily members (1), TNF receptor (TNFR)5 family members (2), and TNF family members that are newly emerging mediators of costimulation through reverse signaling (3). Although the molecular mechanisms of costimulation delivered by Ig and TNFR family members have been studied extensively (4, 5), the underlying mechanism of costimulation transduced by TNF family members has only recently come under scrutiny (6).

Fas ligand (FasL, CD178) is a type II transmembrane protein belonging to the TNF family. Although well characterized for its capacity to deliver a death signal through Fas (CD95), previous studies demonstrate that this TNF family member can also transmit a positive reverse signal to costimulate CD8+ T cells (6–10). FasL-mediated costimulation is required for optimal thymocyte maturation (10) and for Ag-driven proliferation of mature T cells in vivo and in vitro (7–9), with a stronger influence on CD8+ than on CD4+ T cells (8). The cytoplasmic tail of FasL is sufficient to costimulate by the recruitment of FasL into lipid rafts and association with select Src homology 3 (SH3)-containing proteins such as Fyn, PI3K, and Grb2 but not Lck, by enhancement of the phosphorylation of Akt, Erk1/2, JNK, and FasL itself at serine residues, and by activation of the transcription factors NFAT and AP-1 (6, 11, 12).

The Fasl tail contains two distinct motifs: a striking proline-rich region consisting of several SH3 binding domains and two conserved casein kinase I (CKI) binding sites. Using mutagenesis, we now identify residues within both motifs that are crucial for FasL costimulation but are not required for protein expression or Fas-induced death. These results offer the first evidence that costimulation and death induction are separable functions and demonstrate that two distinct motifs within the FasL cytoplasmic domain regulate costimulation through multiple signaling pathways.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6), B6.MRL-FasL+/− (lpr), and BALB/c mice were purchased from The Jackson Laboratory or bred on site. B6.FasL+/−/− mice were generated by cross/backcross breeding of B6.lpr to B6.FasL+/−/− mice (13). All mice were used at 6–9 wk of age and in accordance with the Institutional Animal Care Use Committee guidelines of the University of Washington (Seattle, WA).

Reagents

Hamster anti-CD3 (clone 145-2C11), mouse anti-Ly49a (clone A1), and 7-aminoactinomycin D (7-AAD) were purchased from BD Pharmingen. Rabbit anti-Erk1/2, anti-phospho-Erk1/2, and anti-phospho-Akt were obtained from Cell Signaling Technology. Mouse anti-GFP (clone JL-8) was purchased from Clontech Laboratories. Santa Cruz Biotechnology supplied rabbit anti-Fasl (clone Q-20). Affinity-purified goat anti-mouse IgG and anti-hamster IgG were obtained from Rockland. Molecular Probes supplied streptavidin-AF488 and goat anti-rabbit AF546.

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5 Abbreviations used in this paper: TNFR, TNF receptor; 7-AAD, 7-aminoactinomycin D; B6, C57BL/6; CID, chemical inducers of dimerization; CKI, casein kinase I; EC, extracellular; Fasl, Fas ligand; FKBP3, three FK506 binding domains; HA, hemagglutinin; lpr, MRL-FasL+/−; M, myristoylation-target domain; SH3, Src homology 3; TM, transmembrane domain; WT, wild type.

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Cell lines

The generation of H-2<sup>R</sup>-reactive FasL<sub>mut</sub>-/lpr CTLs and the maintenance of H-2L<sup>D</sup>-reactive L3, Jurkat, and Phoenix E packaging cells were described previously (6). To generate stable infectants, LZRS-GFP-IRES-based retroviruses (provided by Dr. Philip Greenberg, University of Washington) expressing target genes were isolated from Phoenix E stable transfectants and concentrated to infect L3 or FasL<sub>mut</sub>-/lpr CTLs 4 days after restimulation.

Recombinant DNA constructs

The murine ζ and FasL chemical inducers of dimerization (CID) constructs were generated as described (6). In brief, the myristoylation-target domain (M) is linked to the murine ζ and FasL cytoplasmic domains, three FK506 binding protein domains (FKBP3), and the hemagglutinin (HA) epitope. For the FasL/Ly49a chimeric construct, the extracellular (EC) and transmembrane (TM) domains of murine Ly49a (also a type II protein) were fused to the cytoplasmic domain of murine FasL. This chimeric molecule lacks the FasL trimerization domain and cannot associate with endogenous FasL molecules. Deletional mutations in the proline-rich region and alanine substitutions in the CID binding sites of the FasL intracellular domain were performed by PCR on the FasL CID, full-length FasL, or FasL/Ly49a chimeric constructs by using the QuickChange multisite-directed mutagenesis kit (Stratagene) as directed. All DNA constructs were verified by sequencing.

CID, immunoblotting, and proliferation assays

CID, immunoblotting and [<sup>3</sup>H]Tdr proliferation assays were performed as described (6).

Flow cytometry and microscopy

Surface expression of FasL/Ly49a fusion proteins was determined by flow cytometric analysis of L3 infected cells stained with biotinylated anti-Ly49a and streptavidin-PE. For monitoring the kinetics of FasL/Ly49a expression, lot-dependent differences in anti-Ly49a brightness were accommodated by normalizing the geometric mean fluorescence intensity of the day 0 time point. To assay killing by FasL<sub>mut</sub>-/lpr CD8<sup>+</sup> T cell lines stably infected with either WT or mutated FasL molecules, B6.WT and B6. <i>lpr</i> thymocytes were used as targets and incubated with effector CD8<sup>+</sup> T cells for 4 h in the presence of 4 mM MgCl<sub>2</sub> and 4 mM EGTA. Apoptosis of CD4<sup>-</sup>CD8<sup>-</sup> thymocytes was assessed by 7-AAD staining.

To detect the intracellular expression of CID constructs, Jurkat cells were transfected with CID constructs 2 days before fixation with 4% paraformaldehyde and 0.1% saponin and staining with biotinylated anti-HA and rabbit anti-FasL, followed by streptavidin-AF488 and goat anti-rabbit AF546, respectively. Stained cells were mounted on slides with Fluoromount-G medium (Southern Biotechnology Associates) and examined under a laser capture microdissection microscope (AS-LCM; Leica Microsystems). Images magnified by ×500 were captured with a Hamamatsu Orca ER digital camera using Wasabi software (Hamamatsu) and further processed using Photoshop (Adobe).

In vitro CKI assay

L3 cells were lysed and FasL was immunoprecipitated as described (6). The beads with protein complexes were incubated with and without 200 μM CKI inhibitor CKI-7 (US Biological) in 20 μl of Assay Dilution Buffer I (Upstate) containing 500 ng of recombinant CKI (Upstate) and 10 μCi of [<sup>32</sup>P]ATP (PerkinElmer) per sample at 30°C for 10 min before boiling for 5 min. Supernatants were blotted onto P81 paper and washed three times with 0.75% phosphoric acid and once with acetone before transfer to a vial containing 5 ml of a scintillation fluid.

Results and Discussion

Deleting aa 45–54 in the proline-rich region blocks delivery of a costimulatory signal

Previous results showed that the cytoplasmic domain of FasL is sufficient to mediate costimulation by driving NFAT nuclear translocation and AP-1 activation (6). A CID assay was used to further determine whether the proline-rich region is critical for FasL-mediated signal transduction (6). In brief, the ζ and FasL cytoplasmic tails, lacking both TM and ligand binding EC domains, were targeted to the inner leaflet of the plasma membrane of transfected Jurkat cells through an M motif (Fig. 1A). These membrane-bound domains were then crosslinked by chemical inducers that oligomerize molecules containing FK506 binding domains. To assess the delivery of signals dependent on CID, Jurkat cells were cotransfected with the relevant ζ and FasL CID constructs and firefly and Renilla luciferase reporters were subjected to CID assay. The combinations were empty vector alone (V), p plus empty vector (p), FasL plus empty vector (F), and p plus FasL (p+F). Values are presented as the ratio of emission from the firefly reporter relative to the Renilla transfection control. The ratio of reporter to transfection control for cells incubated with medium alone was 4.0–2.9 × 10<sup>−3</sup>. Each signal was normalized to the medium control and represents the mean ± SD of two experiments. Asterisks denote significant differences relative to the ζ-only control. Using the two-tailed Student’s t test on data from the optimal dimerization concentration of WT transfectants, <i>p</i> ≤ 0.006 for NFAT, <i>p</i> ≤ 0.002 for AP-1, and <i>p</i> ≤ 0.022 for IFN-γ.

Deleting aa 45–54 or aa 45–50 in the FasL tail does not affect FasL surface expression but inhibits FasL-mediated costimulation of CD8<sup>+</sup> T cell proliferation

The Δ45–54 mutation was incorporated into a construct (Fig. 2A) encoding a fusion protein of the murine FasL cytoplasmic...
Deletion of aa 45–54 does not affect the timing or extent of FasL surface expression, revealing that this domain is not required for regulating FasL transport in CD8\(^+\) T cells.

However, Δ45–54 failed to costimulate the TCR-induced proliferation of L3 infectants (Fig. 2C), whereas crosslinking the WT fusion protein boosted the anti-CD3-induced proliferation of the infectants in an anti-Ly49a dose-dependent manner. These data clearly indicate that aa 45–54 are critical for costimulation and that the defective function of the mutant may be associated with the inhibition of NFAT and AP-1 activation detected by the CID assay (Fig. 1).

A more refined deletional mutation (Δ45–50) was incorporated into the FasL/Ly49a chimeric construct (Fig. 2D). Surface expression of Δ45–50 followed the same kinetics as both WT and Δ45–54 fusion proteins (Fig. 2E). However, crosslinking Δ45–50 FasL molecules failed to costimulate CD8\(^+\) T cell proliferation (Fig. 2), indicating that aa 45–50 of the FasL cytoplasmic tail comprise one or more SH3 binding domains required to transmit a costimulatory signal delivered through FasL.

Δ45–54 deletes normal killing of Fas\(^+\) target cells

The cytoplasmic domain of FasL has been reported to regulate the localization of FasL in lipid rafts, where it induces death through binding to Fas. However, it is controversial whether the proline-rich domain is required for optimal cytolysis (15–17). To determine whether Fasl lacking aa 45–54 in the tail can...
bind to Fas and induce the apoptosis of Fas$^+$ cells, FasLnull/lpr

CTL lines were stably infected with constructs encoding murine FasL molecules with either WT or mutated cytoplasmic domains. In these CTL lines, the only FasL molecules were those provided by infection, and the absence of Fas avoided both suicide and fratricide. CD4$^+$ CD8$^+$ thymocytes were used as target cells because they express high levels of Fas and are sensitive to Fas-mediated apoptosis (18). In addition to exhibiting efficient surface expression but impaired costimulation for Ag-specific CD8$^+$ T cell proliferation (not shown), cells expressing $\Delta 45–54$ were as cytolytic as those expressing WT FasL over a range of E: T ratios (Fig. 4A), delivering death signals upon interaction with Fas on WT but not on lpr target cells (Fig. 4B). $\Delta 45–54$ therefore does not influence death induction but selectively blocks FasL-mediated costimulation. This deletion mutation should be useful for generating a knockin mouse to study FasL costimulation in vivo in the absence of lymphoproliferative disease (19).

FasL is phosphorylated by CKI and FasL molecules with mutant CKI binding sites mediate defective NFAT activation

Serine residues in the FasL tail can be rapidly phosphorylated when L3 cells are stimulated through the TCR in the presence of plate-bound FaslgG (6), suggesting that CKI binding motifs provide a docking site that influences the costimulatory signal transduced by the FasL cytoplasmic domain. To investigate the role of CKI in FasL phosphorylation, an in intro CKI assay was performed using FasL immunoprecipitated from L3 cells as a substrate. Recombinant CKI can phosphorylate FasL in vitro, and this phosphorylation can be blocked by the CKI inhibitor CKI-7 (Fig. 5A). FasL immunoprecipitated from cells after stimulation with anti-CD3 and FaslgG showed a marked reduction in sensitivity to CKI phosphorylation in vitro (Fig. 5A). This suggests that CKI-sensitive sites are phosphorylated in vivo upon TCR and FasL co-crosslinking and are therefore unavailable to exogenous CKI in vitro. To further identify the influenace of CKI binding sites on FasL costimulation, four point mutations were introduced by alanine substitution of S17, S18, S19, and S20 residues in the CKI binding sites of the FasL cytoplasmic domain with alanine substitutions of serine/threonine residues (S/T→A) in the CKI binding sites, FKBP3, and a C-terminal HA epitope. C. The localization of CID proteins was detected as described in Fig. 1B. D. The CID assay was performed and analyzed as described in Fig. 1C. Each signal represents the mean ± SD of two independent experiments. Asterisks denote significant differences relative to the $\xi$-only control. Using the two-tailed Student’s t test on the data from the optimal dimerizer concentration, $p \geq 0.05$ (WT) for NFAT, $p \leq 0.025$ (WT) and $p \geq 0.054$ (S/T→A) for AP-1, and $p \leq 0.05$ (WT) for IFN-γ. Incorporating these four alanine substitutions into the FasL/Ly49a construct resulted in apparent protein degradation (not shown), and the influence of the CKI sites in L3 costimulation could not be further investigated.

![Figure 4](http://www.jimmunol.org) Cells expressing FasL molecules lacking as 45–54 in the tail induce Fas-mediated death. A. FasLnull/lpr CD8$^+$ T cell lines stably infected with the indicated constructs on day 3 after allantoicogenic restimulation were incubated with WT thymocytes at the indicated E: T ratios for 4 h in the presence of EGTA to chelate Ca$^{2+}$ and prevent perforin-mediated cytolysis. Apoptosis of CD4$^+$ CD8$^+$ thymocytes was assessed by 7-AAD staining. B. The indicated effectors were cultured with either WT or lpr thymocytes at an E: T ratio of 8:1. The percentage (%) of the specific lysis of targets by effectors was normalized by subtracting the percentage of 7-AAD$^+$ lpr thymocytes from the percentage of 7-AAD$^+$ WT thymocytes. Data represent the mean ± SD of three experiments. Using the two-tailed Student’s t test on the data from the WT and $\Delta 45–54$ effectors, $p = 0.52$.

![Figure 5](http://www.jimmunol.org) Recombinant CKI phosphorylates FasL, and mutating serine/threonine residues in the CKI binding sites of the FasL cytoplasmic domain inhibits NFAT activation. A. Equal numbers of L3 cells were incubated for 1 h with PBS or 0.72 µg/ml plate-bound anti-CD3 plus 5 µg/ml plate-bound FaslgG. FasL immunoprecipitated from the cell lysates was used as a CKI substrate in the presence (■) or absence (□) of CKI-7 as indicated. Equal amounts of FasL immunoprecipitated under each condition were subjected to SDS-PAGE to normalize CKI activity. Data represent the mean ± SD of three experiments. B. The murine FasL CID construct encodes an N-terminal M motif, the FasL cytoplasmic domain with alanine substitutions of serine/threonine residues (S/T→A) in the CKI binding sites, FKBP3, and a C-terminal HA epitope. C. The localization of CID proteins was detected as described in Fig. 1B. D. The CID assay was performed and analyzed as described in Fig. 1C. Each signal represents the mean ± SD of two independent experiments. Asterisks denote significant differences relative to the $\xi$-only control. Using the two-tailed Student’s t test on the data from the optimal dimerizer concentration, $p \geq 0.05$ (WT) for NFAT, $p \leq 0.025$ (WT) and $p \geq 0.054$ (S/T→A) for AP-1, and $p \leq 0.05$ (WT) for IFN-γ. Incorporating these four alanine substitutions into the FasL/Ly49a construct resulted in apparent protein degradation (not shown), and the influence of the CKI sites in L3 costimulation could not be further investigated.
NFAT activation and the readout of this NFAT activation upon FasL costimulation are still not well defined. It is possible that the phosphorylation status of these serine/threonine residues is important for regulating the function of GSK3, a molecule involved in the PI3K pathway and known to promote nuclear export of NFAT (20). In contrast, costimulation through CD28 results in the phosphorylation of tyrosine residues and the further activation of downstream transcription factors (e.g., NF-κB) essential for cell survival but not for cell proliferation or IL-2 production (4, 21). These results further demonstrate that the costimulatory pathways transduced by FasL and CD28 are distinct.

The existence of costimulation transduced through reverse signaling is well established by now (3, 22, 23). However, a main challenge to studying this signaling pathway is that although the biological effects are clear, the underlying molecular mechanisms have proven difficult to trace. The identification of specific residues within the FasL cytoplasmic domain regulating costimulation confirms that FasL is a positive regulator of CD8+ T cells and can be used as a model for studying the costimulatory signaling induced by other TNF family molecules. In addition, the specific residues we identified are not essential for Fas-induced death, indicating that costimulation and death induction are separable functions of FasL involving distinct residues within the FasL tail.

Disclosures
The authors have no financial conflict of interest.

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