A Physical Interaction between the Cell Death Protein Fas and the Tyrosine Kinase p59\textsuperscript{fyn}*

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The Fas antigen (Apo1/CD95) is a transmembrane protein belonging to the nerve growth factor receptor family. It is expressed on a variety of cells, including activated lymphocytes. Ligation of Fas leads to apoptotic death of the cells upon interaction with each other (see Ref. 21 for a review) and is also suggestive of a binding site for TNF. Both Fas and TNFR appear to be coupled to a sphingomyelinsase, ceramide-producing pathway, which could explain at least in part, the apoptotic outcome of ligating these receptors (12–15). Whether this coupling is direct or not is unknown.

Fas contains no previously known signaling motifs or catalytic activities. In an effort to identify molecules that might interact with Fas and link it to signal transduction pathways, a number of groups have employed the yeast 2-hybrid system, with the Fas cytoplasmic region as “bait.” This approach has identified three different proteins that can interact with Fas: FADD (MORT1) (16, 17), RIP (18), and FAP-1 (PTP-BAS) (19), although evidence that these proteins interact in a physiologically relevant system is lacking. FADD and RIP both contain death domain-like motifs, and interaction with Fas may occur via dimerization of this region. The functions of FADD and RIP are not known, although RIP may be a protein kinase. FAP-1 is a tyrosine phosphatase and, interestingly, interacts with the Fas carboxyl terminus, which has been shown to be involved in negatively regulating Fas killing (9). The level of endogenous FAP-1 seems to correlate with the Fas sensitivity of a cell, and transfection of cells with FAP-1 encoding DNA also leads to a reduction in Fas sensitivity. This suggests that one or more tyrosine kinases may play a role in Fas killing. Indeed, it has previously been demonstrated that Fas activity is inhibited by tyrosine kinase inhibitors, and ligation of Fas leads to the tyrosine phosphorylation of a number of cellular proteins (20). Therefore, there is now strong evidence from two different sources that tyrosine kinase activity is involved in Fas signal transduction.

Although it has not been shown that Fas itself is tyrosine-phosphorylated, we examined the context of the tyrosine residues in the cytoplasmic domains of both human and mouse Fas for any similarities to those in other signaling molecules that are known to interact with tyrosine kinases. Interestingly, one of the tyrosines is found in the death domain within a conserved YXXL motif. This sequence is reminiscent of half of an immunoreceptor tyrosine-based activation motif (ITAM) found in the T cell receptor CD3 complex chains and elsewhere (see Ref. 21 for a review) and is also suggestive of a binding site for proteins with SH2 domains (22). ZAP-70 and p59\textsuperscript{fyn} are two tyrosine kinases known to interact with similar sequences, although p59\textsuperscript{fyn} is much more flexible in its binding sequence requirements than ZAP-70 (23). Also, since disruption of the fyn gene has been correlated with lack of programmed cell death in certain regions of the mouse brain (24) (which, along with T lymphocytes, expresses the highest level of p59\textsuperscript{fyn}), we decided to investigate the possibility that p59\textsuperscript{fyn} might interact with Fas and contribute to Fas signal transduction. Here we report that there is indeed a specific physical interaction between Fas and p59\textsuperscript{fyn}.

Deletion of activated peripheral T cells is important in the down-regulation of immune responses and controlling T cell numbers. The mechanisms by which this is accomplished are unclear, but the expression and action of a transmembrane death protein known as the Fas antigen has recently been implicated (1–5). It is now believed that activated T cells eventually co-express Fas and Fas ligand, leading to the apoptotic death of the cells upon interaction with each other (see Refs. 6–8 for recent reviews). The nature of the Fas death signal is currently unknown and has become the focus of intense research. This article must therefore be hereby marked "publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. §§Medical Scientist of the Alberta Heritage Foundation for Medical Research. ¶¶Postdoctoral Fellow of the Alberta Heritage Foundation for Medical Research. **Postdoctoral Fellow of the Alberta Heritage Foundation for Medical Research.

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tween p59\(^{fn}\) and Fas in Fas-sensitive cell lines. The biological relevance of this interaction was confirmed by the observation of an increased survival and decreased rate of apoptosis in alloantigen-stimulated splenocytes from fny knockout mice. Furthermore, fny-deficient activated T cells are resistant to killing when their surface Fas is ligated by either anti-Fas antibody or Fas ligand presented by Fas-dependent cytotoxic T cells. This provides the first report of a protein with known catalytic activity capable of linking Fas to a signal transduction pathway that has been shown to occur in a physiologically relevant system.

### MATERIALS AND METHODS

**Cell Culture and Stimulation—**YAC-1 targets and PMM-1 cytolytic hybridomas were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with antibiotics, sodium pyruvate, 2-mercaptoethanol, HEPES, and 5% fetal calf serum as described previously (25). PMM-1 cells were activated with PMA and ionomycin as described (25). For mixed lymphocyte cultures, H-2\(^{b}\) splenocytes were obtained from fny\(^{-}\)\(^{-}\) mice (kindly provided by Drs. P. R. Plummer and M. Appleye, University of Washington, see Ref. 26) and C57Bl/6 (fnym\(^{-}\)\(^{-}\)) mice (Jackson Laboratories) and stimulated with an equal number (10^7/ml) of H-2\(^{b}\) \(^{-}\)irradiated splenocytes from Balb/c mice. Cultures were grown in RPMI medium supplemented with 10% fetal calf serum and 60 units/ml recombinant interleukin 2.

Immunoprecipitations and Western Blotting—5 \(\times\) 10^5 YAC-1 cells alone or with an equivalent number of activated PMM-1 cells were incubated for 10 min at 37°C in serum-free medium (RPMI A1-M V, 1:1, Life Technologies, Inc.) and lysed in 1 ml of lysis buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonfyl fluoride, and multi-protease inhibitor mixture, Ref. 27) for 30 min on ice. Postnuclear supernatants were preclared with 20 \(\mu\)l of protein A/G-agarose (Pierce) and subjected to immunoprecipitation using the indicated antibodies. Hamster anti-mouse Fas (J2, Pharmingen) and rat anti-mouse CD45 (130) were used at 5 \(\mu\)g/ml and immunoprecipitated with 20 \(\mu\)l of protein A/G-agarose. 5 \(\mu\)g of agarose-conjugated anti-fyn antibody (FYN15, Santa Cruz Biotechnology) per ml was used to immunoprecipitate p59\(^{fn}\).

Immunoprecipitates were washed extensively in lysis buffer without EDTA and either dissociated in SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis (6%), and electroblotted onto nitrocellulose, or subjected to in vitro kinase assays. Western blotting was performed using either rabbit-anti-fyn antibody (FYN3, Santa Cruz Biotechnology) or rabbit anti-Fas peptide Fab antibody (kindly provided by Dr. K. Elkon, Ref. 28) and horseradish peroxidase-conjugated donkey anti-rabbit (Amersham) and visualized using the ECL method (Amersham).

Analysis of DNA Fragmentation—DNA fragmentation gels were done as described previously (29). Genomic DNA was isolated from 10^6 Ficoll-purified splenocytes from MLCs on the days indicated and electrophoresed in 1% agarose gels in Tris-borate-EDTA buffer. DNA was visualized by ethidium bromide staining and UV fluorescence.

**Determination of Sensitivity of Cells to Fas-mediated Killing—**C57BL/6 and Fyn\(^{-}\)\(^{-}\) splenocytes were examined for cell viability and sensitivity to PMM-1 induced cell death after stimulation with irradiated Balb/c splenocytes (1:1 ratio). On day 5 poststimulation, the splenocytes were exposed to either cross-linked anti-Fas antibody or PMM-1 cells pretreated with PMA and ionomycin. The cell viability assays were carried out in 96-well microtiter plates in the following manner. The antibody was diluted to a final concentration of 40 \(\mu\)g/ml in PBS, then 50 \(\mu\)l of this suspension was added per well. Plates were allowed to sit for 2 h at room temperature or at 4°C overnight before being washed twice with 100 \(\mu\)l of PBS. Triplicate wells with and without anti-Fas antibody containing 2 \(\times\) 10^5 cells each were incubated for 24 h at 37°C. Cell viability was determined by staining with eosin or trypan blue.

**Standard cytotoxicity assays were carried out with PMM-1 effector cells stimulated with PMA (10 ng/ml) and ionomycin (3 \(\mu\)g/ml). Effector cells were stimulated for 3 h at 37°C and then washed in RPMI media. Target cells, C57BL/6 or Fyn\(^{-}\)\(^{-}\) splenocytes were labeled with chromium sulfate for 60 min at 37°C before being washed in RPMI and PBS. Assays were set up in 200-\(\mu\)l volumes containing a maximum of 5 \(\times\) 10^4 effector cells and 1 \(\times\) 10^5 target cells. To examine the impact of effector to target cell ratios, the concentration of the PMM-1 cells was serially diluted from 5 \(\times\) 10^4 cells per well to 0.625 \(\times\) 10^4 cells per well.

**RESULTS AND DISCUSSION**

To determine if p59\(^{fn}\) associates with the Fas antigen, co-immunoprecipitation experiments were performed. We chose to study YAC-1 cells which express relatively high levels of both Fas and p59\(^{fn}\) and are very sensitive to killing by PMM-1, a killer cell that has been shown to kill via a Fas-dependent pathway (25). Either Fas or p59\(^{fn}\) were immunoprecipitated from extracts of YAC-1 target cells. Western blot analysis revealed that p59\(^{fn}\) was co-immunoprecipitated with anti-Fas antibody, but not with an isotype-matched control or antibodies to another cell surface molecule CD45 (Fig. 1). Interestingly, ligation of Fas on YAC-1 cells does not appear to be required for the association of p59\(^{fn}\), as we obtained the same results from YAC-1 cells preincubated in the presence or absence of PMM-1 Fas-dependent killer cells (data not shown).

In order to establish the reciprocity of this interaction, extracts were first immunoprecipitated with either anti-Fas or anti-fyn antibodies. The resulting precipitates were then probed with both antibodies. As can be seen in Fig. 2, fny is present in the Fas precipitates, and Fas can be detected in the anti-fyn precipitated sample. Together, these experiments establish an association between Fas and fny.

Elimination of activated T cells is believed to occur via Fas/ Fas ligand interactions (1-8). Therefore, in order to determine if p59\(^{fn}\) plays a role in Fas function, we studied mixed lymphocyte cultures (MLCs) of splenocytes obtained from normal and fny\(^{-}\)\(^{-}\) mice (26). Throughout the early stages of MLC, the T cells from normal and fny\(^{-}\)\(^{-}\) mice exhibited nearly identical growth characteristics (Fig. 3A). [3H]thymidine uptake experiments also indicated comparable levels of proliferation within the early stages of culture (data not shown). However, by day 17 after stimulation, the cultures of fny-deficient T cells had substantially more viable cells than control cultures, indicating an impairment in the normal (presumably Fas-based) mechanisms of activated T cell deletion (Fig. 3A). Additionally, analysis of several MLCs indicated an increase in fny\(^{-}\)\(^{-}\) lymphocyte lifespan manifested as a greatly elevated cloning efficiency

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**Fig. 2. Reciprocity of Fas-fyn interaction.** Western blot showing co-immunoprecipitation of Fas and fyn from PMM-1 stimulated YAC-1 cell lysates. The right lane of each panel is present solely as a positive control for the blotting antibodies and to show the mobilities of fyn and Fas, but is not meant to be quantitative. In the left panel, the positive control was a small portion of an anti-fyn immunoprecipitate from a RIPA buffer lysate of YAC-1 cells previously determined to contain specifically immunoprecipitated p59<sup>yn</sup> using two separate anti-fyn antibodies. In the right panel, the positive control is 20 μl of a non-immunoprecipitated YAC-1 postnuclear cell lysate.

**Fig. 3.** fyn-deficient lymphocytes stimulated with alloantigen exhibit increased lifespans and cloning efficiency. A, elevation of viable cell numbers at late time points of fyn<sup>−/−</sup> mixed lymphocyte cultures. Viable cell numbers were recorded over an extended time course after stimulation with alloantigen by eosin exclusion staining. The cultures were equilibrated and restimulated on day 17 (arrow). B, increased cloning efficiency of fyn<sup>−/−</sup> T cells. Cells were removed from the primary MLCs on day 7, set up at the indicated densities in 96-well plates, and stimulated with 5 × 10<sup>5</sup> irradiated Balb/c splenocytes per well. Plates were scored for growth 7 days after plating. The results are typical of 4 separate cloning experiments from independent MLCs.

(Fig. 3B). A representative cloning experiment is shown. In 3 other cloning experiments, the actual number of clones obtained varied, but an enhancement in cloning efficiency of the fyn<sup>−/−</sup> T cells of approximately 5:1 was consistently observed. This apparent increase in fyn<sup>−/−</sup> T cell lifespan was dosely paralleled by a reduction in the level of apoptosis as assessed by characteristic DNA fragmentation (Fig. 4). No discernable differences in the relative amounts of Fas surface expression on fyn<sup>−/−</sup> and control splenocytes was observed upon flow cytometric analysis (data not shown). These data are all consistent with a role for p59<sup>yn</sup> in Fas signal transduction.

Finally, we sought to directly determine if fyn played a role in Fas-mediated cell death. To do this, we utilized alloantigen-stimulated splenocytes from fyn<sup>−/−</sup> and fyn<sup>+/+</sup> mice and determined their susceptibility to anti-Fas antibody. Wells of 96-well plates were coated with either anti-Fas or bovine serum albumin as a control, and day 5 alloantigen-activated splenocytes were incubated in them overnight. The next day, cell viability was assessed by vital dye exclusion. As seen in Fig. 5A, the fyn<sup>−/−</sup> splenocytes were resistant to the effects of anti-Fas antibody while the control cells were susceptible. We also determined the relative sensitivities of fyn<sup>−/−</sup> and fyn<sup>+/+</sup> splenocytes to killing by PMM-1. Normal and fyn-deficient splenocytes were used as targets in a 51Cr-release lytic assay. The fyn<sup>−/−</sup> cells were found to be far less sensitive to PMM-1 than their normal counterparts (Fig. 5B). These results provide strong and direct evidence that fyn activity is involved in the Fas death pathway in these cells.

We are currently working on determining how the fyn tyrosine kinase interacts with Fas. As mentioned previously, the conserved YXXL motif in the death domain of Fas is intriguing in that it is very similar to ITAM sequences involved in signal transduction via the T cell receptor-associated CD3 chains. We have so far not been able to demonstrate tyrosine phosphorylation of Fas. Furthermore, to our knowledge, no death-domain-containing protein has ever been shown to be phosphorylated on tyrosine, even though many of these proteins, including TNFRI and RIP, possess conserved YXXL motifs in their death domains. Clearly, the importance of these sequences in binding fyn and/or relaying an apoptotic signal needs further study.

It should be noted that lack of phosphorylation of the tyrosine residue within the YXXL motif of Fas would not preclude the possibility that p59<sup>yn</sup> interacts with Fas at this sequence. These sequences can bind tyrosine kinases in at least two different ways: via SH2 domain interactions with the phosphorylated tyrosine residues or via a mechanism independent of both SH2 domains and tyrosine phosphorylation. An intact tyrosine activation motif consists of 2 YXXL motif of Fas would not preclude the possibility that p59<sup>yn</sup> interacts with Fas at this sequence. These sequences can bind tyrosine kinases in at least two different ways: via SH2 domain interactions with the phosphorylated tyrosine residues or via a mechanism independent of both SH2 domains and tyrosine phosphorylation. An intact tyrosine activation motif consists of 2 YXXL sequences separated by approximately 10 amino acids. Binding of ZAP-70 to the CD3e ITAM has recently been shown to be dependent on very specific sequence criteria (23). These include the requirement for an intact ITAM, accurately spaced tyrosine residues, and prior phosphorylation of the tyrosine residues. Binding of ZAP-70 to the ITAM is likely achieved via its 2 SH2 domains (23, 30, 31). Binding of fyn to an ITAM is much more flexible, 

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be able to partially compensate for p59<sup>fyn</sup> in its absence, as may be the case in CD3 signal transduction (26). To our knowledge, fyn-deficient mice have not been shown to exhibit an lpr-like phenotype, with large numbers of T lymphocytes accumulating with age. This has not yet been specifically studied, however, and our data would predict that in older fyn<sup>-/-</sup> mice, there may be some degree of lymphoproliferative disease apparent. Our observations are also important in that they predict that some types of human lymphoproliferative and autoimmune diseases could result in mutations that affect Fas-Fas interactions or p59<sup>fyn</sup> activity. Clearly, the identification of a physiologically relevant kinase associating with the apoptosis-inducing Fas antigen opens up numerous new avenues of investigation for elucidating the entire nature of the death signal.

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