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Bacterial death induced by expression of the intracellular portion of human Fas

Yili Yang[1], June S. Hong[1], Astrid Eder[1] and Jonathan D. Ashwell*[,1]

[1] Laboratory of Immune Cell Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, MD 20892, USA

* corresponding author: J.D. Ashwell, Building 10, Room 1B-40, National Institutes of Health, Bethesda, MD 20892, USA. tel: 301-496-4931; e-mail: jda@box-j.nih.gov

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Abstract

In attempting to produce the intracellular portion of human Fas ($IC_{175-319}$) as a GST-fusion protein we found that expression of GST-$IC_{175-319}$, but not GST alone or GST-$IC_{231-298}$ (containing the Fas death domain), rapidly caused the death of host *E. coli* cells. Expression of GST-$IC_{175-319}$ with a single amino acid substitution (V238N) corresponding to the mouse *lpr*^{cg} mutation, or E245A, which abolishes the ability of Fas to self-associate, did not kill bacteria. Deletional analysis identified a 20-amino acids region ($Asp_{210}-Lys_{230}$) as essential for the killing activity, and introduction of a single amino acid substitution (T225P) in this 20 amino acid region markedly decreased the ability of Fas- $IC_{175-319}$ to cause bacterial death. These data indicate that Fas can deliver a death signal in prokaryotic organisms by a means that shares some features with eukaryotic cells, and raise the possibility that certain mechanisms leading to programmed cell death may be conserved from bacteria to mammalian cells.

Keywords: Fas; CD95; bacteria; programmed cell death

Abbreviations: Fas-IC, intracellular portion of Fas; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactoside

Introduction

Fas (CD95) is a type I transmembrane protein that belongs to the TNF/NGF receptor superfamily.[1,2] Engagement of Fas with anti-Fas antibody or Fas ligand results in apoptotic cell death.[3–5] Fas-mediated apoptosis is essential for lymphocyte homeostasis, contributes to killing by cytotoxic T cells and NK cells, and is required for the maintenance of immune privilege in certain tissues.[6–8] Comparison of the amino acid sequence of Fas and the type 1 TNF receptor (p55) revealed a conserved region of approximately 68 amino acids, corresponding to human Fas 231-298, that is required for induction of apoptosis by both receptors, and hence was termed the 'death domain'.[9,10] Subsequent studies have shown that the death domain of Fas binds with another death domain-containing protein (MORT1 or FADD) whose N-terminus in turn interacts with caspase-8 (also known as FLICE or MACH).[11,12] The formation of this complex activates caspase-8 and initiates the intracellular cascade that results in apoptosis.[13–15] It appears that the aggregation of caspase-8 is sufficient for this purpose, since heterologous oligomerization of caspase-8 is sufficient to activate its activity and trigger apoptosis.[16,17]

Simply expressing Fas does not necessarily confer susceptibility to Fas-mediated death. For example, human peripheral blood T lymphocytes express high levels of Fas within 24 h of activation, yet are resistant to killing by anti-Fas antibodies, although FADD and caspase-8 seem to be constitutively expressed.[11,12,18] Fas-mediated signaling also requires tyrosine phosphatase SHP-1 and can be regulated by agonists for PKA and PKC.[19,20] It is not clear how these molecules interact with the Fas/FADD/caspase-8 axis. Furthermore, Fas interacts with a number of other proteins, such as RIP, FAF-1, FAP-1, and Daxx,[21–24] raising the possibility that molecules other than FADD may be involved in proximal transduction of Fas-mediated signals. Indeed, it has been shown that Daxx enhances Fas-mediated apoptosis and appears to transduce Fas-mediated death signals by activating JNK and caspases.[24]

As part of a study to identify molecules that interact with Fas, we attempted to produce a recombinant protein consisting of the intracellular portion of Fas ($IC_{175-319}$) fused to GST. Unexpectedly, expression of the intracellular portion of Fas caused death of host bacteria. Since it has been shown that, like mammalian cells and single cell eukaryotic organisms, bacteria can undergo programmed cell death under certain circumstances,[25] this prompted us to characterize the molecular requirements for this inducible form of bacterial cell death.

Results

Induction of Fas-$IC_{175-319}$ in *E. coli* causes death of the bacteria

The *E. coli* strain DH5α was transformed with the plasmids pGEX-2T (encoding GST alone), pGEX-$IC_{175-319}$ (containing the entire intracellular portion of Fas), or pGEX-$IC_{231-298}$ (containing the classic Fas death domain) (Figure 1). A single colony of each plasmid-containing bacterium was inoculated into Super Broth medium and treated with IPTG to induce protein expression by the transforming plasmid. After incubation with glutathione Sepharose 4B, the precipitated proteins were separated by SDS–PAGE and stained with Coomassie Blue. While the expression of GST and GST-$IC_{231-298}$ were greatly induced by IPTG, reproducibly very low levels of GST-$IC_{175-319}$ were detected (data not shown).

Because overexpression of Fas has been shown to induce eukaryotic cell death in the absence of ligand,[16,17] we considered the possibility that the GST-Fas-IC fusion protein was causing the death of these prokaryotic cells. To determine if expression of GST-$IC_{175-319}$ was killing the bacteria, viable cells in the culture were counted using a spread-plate method. As shown in Figure 2A, IPTG had no effect on the number of colonies from bacteria containing pGEX-2T or pGEX-$IC_{231-298}$ (pGEX-2T: 2.3×10^9 c.f.u./ml in medium alone, 2.0×10^9 c.f.u./ml after treatment with IPTG; pGEX-$IC_{231-298}$: 2.7×10^9 c.f.u./ml in medium alone, 2.9×10^9 c.f.u./ml after treatment with IPTG). In contrast, the number of pGEX-$IC_{175-319}$-containing bacteria was reduced approximately 2000-fold by treatment with IPTG (from 2.3×10^9 c.f.u./ml to 1.0×10^6 c.f.u./ml). The decrease of viable bacteria was dependent on the concentration of IPTG, with an ED_{50} of approximately 10 μM (Figure 2B).

To directly determine whether the bacterial cells expressing cells were being killed by IPTG induction of GST-$IC_{175-319}$ synthesis, use was made of the fluorescent vital dye rhodamine 123.[26,27] This water-soluble cationic dye is highly concentrated by mitochondria in an energy-dependent manner[28] and has been used to directly assess the viability of bacteria.[27] As shown in Figure 3, EDTA-permeabilized bacteria took up rhodamine 123 (compare Figure 3A, B and C with D, E and F respectively). The staining profiles of pGEX-2T- and pGEX-$IC_{231-298}$-containing bacterial showed little change in the presence of IPTG (compare Figure 3D with G; F with I). In contrast, the mean fluorescence of pGEX-$IC_{175-319}$-containing bacteria decreased in the presence of IPTG (compare Figure 3E and H), yielding a profile that was similar to unstained bacteria (compare Figure 3B and H). Thus, induction of Fas-$IC_{175-319}$

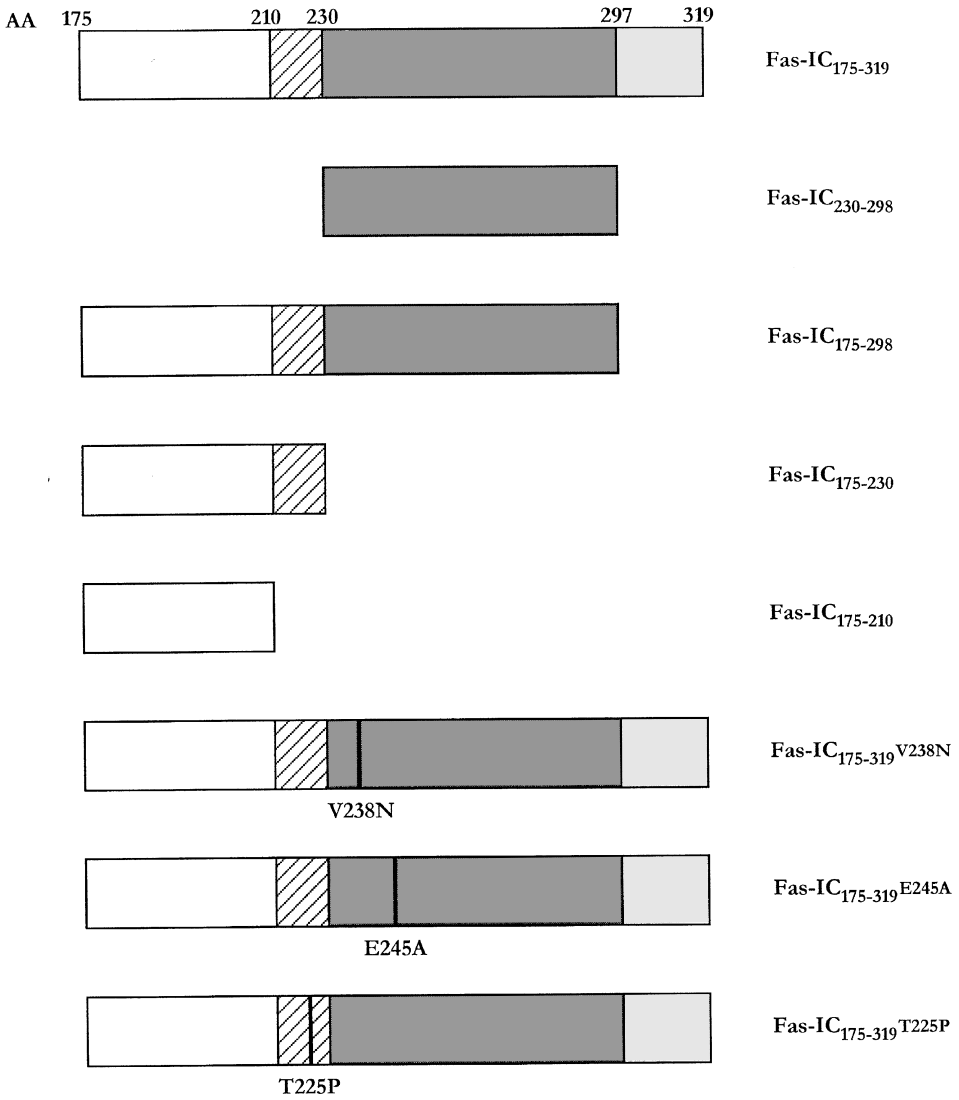

Figure 1 Diagram of wild-type and mutated human Fas-IC

expression specifically resulted in death of the host bacteria.

Fas-IC with point mutation V238N or E245A does not kill host bacteria

The intracellular region of Fas from *lpr*^{cg} mice harbors a single amino acid substitution, valine to asparagine, at residue 225 (V225N).[6] Ligation of this Fas variant cannot transduce apoptotic signals, resulting in the accumulation of aberrant T cells in *lpr*^{cg} mice. Human Fas with the corresponding mutation, V238N, is also unable to deliver apoptotic signals when expressed in mouse or human cells.[9] To determine whether the ability of Fas-IC to kill bacteria is related to induction of apoptosis by Fas in mammalian cells, bacteria were transformed with pGEX-2T, pGEX-IC$_{175-319}$, or pGEX-IC$_{175-319}$V238N. The growth curves of bacteria transformed with pGEX-2T were similar in the presence or absence of IPTG, while bacteria transformed with pGEX-IC$_{175-319}$ were killed by induction with IPTG (Figure 4A and B). In contrast to pGEX-IC$_{175-319}$-transformed bacteria, bacteria transformed with pGEX-IC$_{175-319}$V238N grew vigorously after IPTG treatment (Figure 4C).

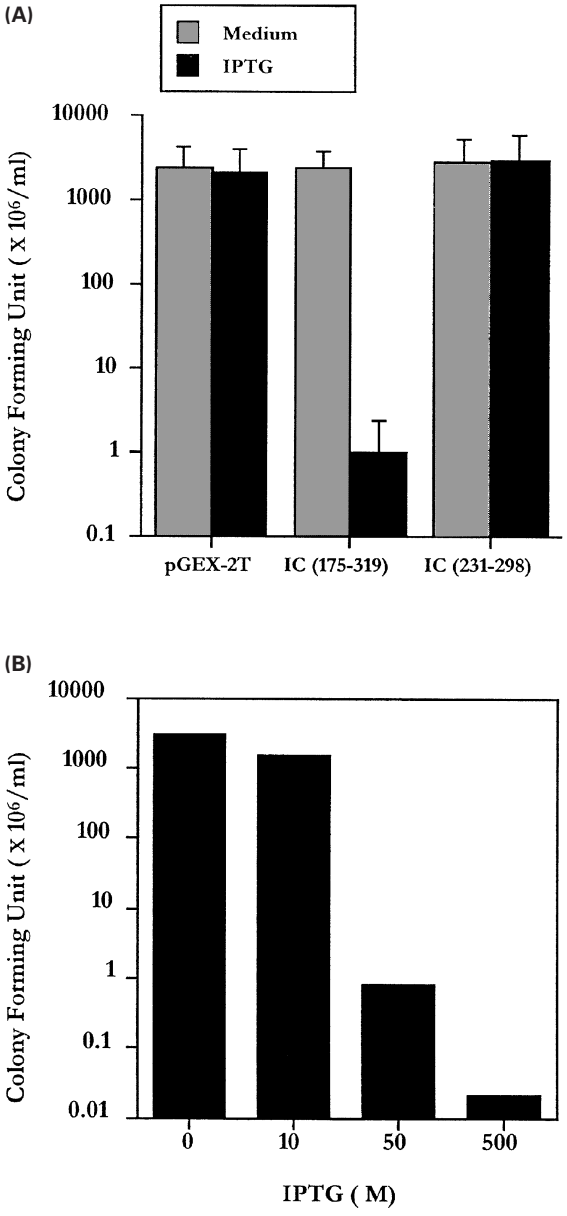

Figure 2 IPTG induces death of bacteria transformed with pGEX-IC$_{175-319}$. (**A**) Numbers of viable bacteria transformed with pGEX-2T, pGEX-IC$_{175-319}$, or pGEX-IC1$_{231-298}$ were determined after incubation for 30 min in the absence or presence of 100 μM IPTG. The data represent the mean $\pm$ S.E.M. of five independent experiments. (**B**) Culture of bacteria transformed with pGEX-IC$_{175-319}$ was divided into several tubes and treated with 0, 10, 50 and 500 μM IPTG for 30 min. Numbers of viable cells were calculated from the number of colonies on each plate

Induction of apoptosis by ligation of Fas with anti-Fas antibody or Fas ligand requires aggregation of Fas and its association with FADD.[15] *In vitro* analysis has demonstrated that human Fas-IC containing the V238N mutation can not self-associate and bind FADD.[29,30] To determine if self-association, association with a FADD-like molecule, or both is required for Fas-IC to kill bacteria, use was made of Fas-IC containing the substitution E245A, which does not readily self-associate but still can interact with FADD.[29] As shown in Figure 4D, the growth of DH5α transformed with pGEX-IC$_{175-319}$E245A was not significantly affected by treatment with IPTG. Together with the data obtained with the V238N mutation, these results indicate that the ability to self-associate is required for Fas-IC to kill bacteria, whereas association with a putative FADD-like molecule alone is not sufficient to lead to bacterial death.

A 20-amino acid region of the intracellular portion of Fas is required for Fas-induced bacterial death

To determine what region(s) of intracellular Fas contribute to bacterial death, cDNAs encoding truncated forms of Fas-IC were cloned into pGEX-2T. Expression of Fas-IC$_{175-298}$ resulted in death of host bacteria, although about tenfold less efficiently than full length Fas-IC$_{175-319}$ (note a decrease of approximately 100-fold in cell number compared to the approximately 1000-fold decrease caused by Fas-IC$_{175-319}$) (Figure 5). Interestingly, Fas-IC$_{175-230}$ killed bacteria as efficiently as IC$_{175-298}$, indicating amino acids 230–298 (the classic death domain) are not essential for Fas-IC-mediated toxicity. However, further deletion of 20 amino acids from the COOH-terminus of IC$_{175-230}$ resulted in high levels of expression of Fas-IC$_{175-210}$ in bacteria treated with IPTG (data not shown) but complete loss of the ability to kill bacteria (Figure 5). Therefore, amino acids 210–230 are required for Fas-IC-induced death of bacteria. These residues are, however, not sufficient, because expression of GST-IC$_{210-230}$ or even GST-IC$_{210-319}$ did not result in loss of bacterial viability (data not shown).

A single amino acid substitution, T225P, in this required 20-amino acid region of Fas has been shown to prevent Fas from signaling for apoptosis in human cells.[31] Therefore, the intracellular portion of this mutated Fas was cloned into pGEX-2T and used to transform DH5α. The number of viable bacteria containing pGEX-IC$_{175-319}$T225P

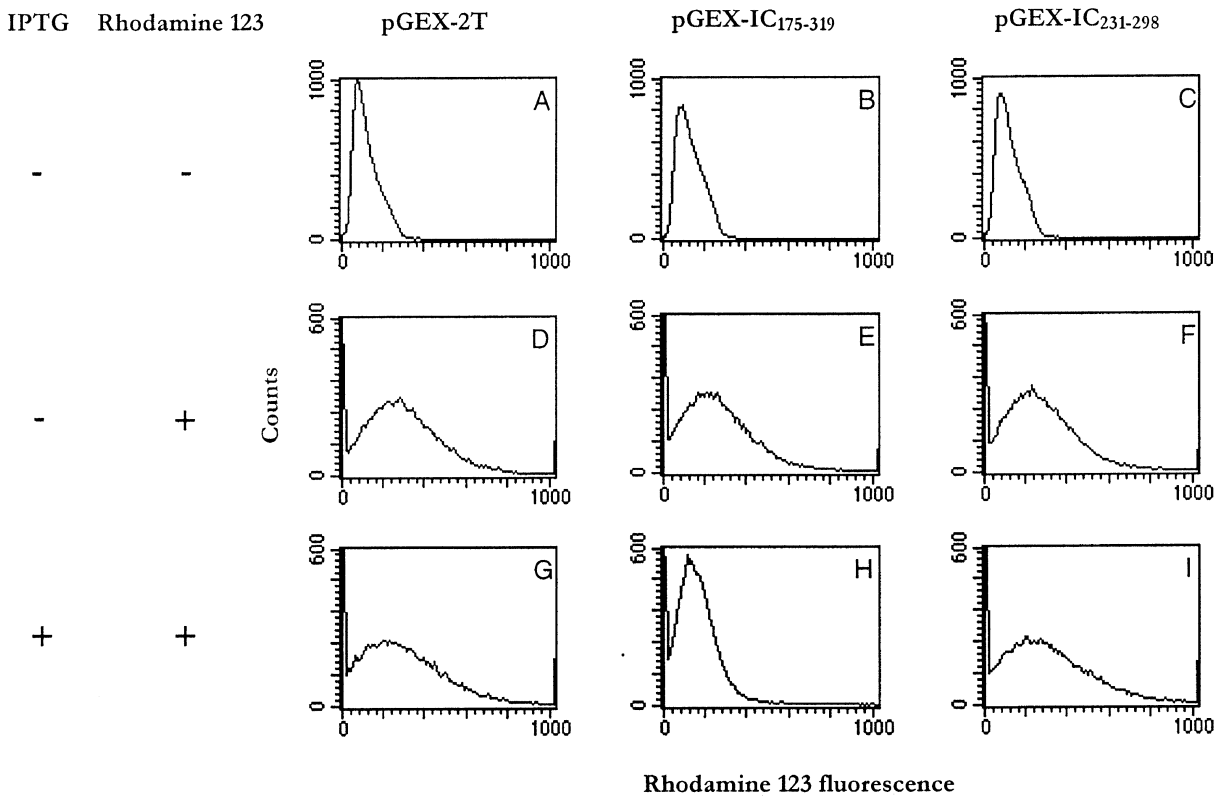

Figure 3 Histograms of Rhodamine 123 staining of bacteria transformed with pGEX-2T, pGEX-IC$_{175-319}$, and pGEX-IC$_{231-298}$

was only slightly affected when treated with IPTG (Figure 6A). This is not due to lack of expression of fusion protein because GST-IC$_{175-319}$T225P was induced well by IPTG (Figure 6B). Consistent with this, bacteria containing pGEX-IC$_{175-231}$ with the T225P mutation were also not killed by incubation with IPTG (data not shown). Therefore, the ability of Fas-IC to kill bacteria is closely related to its ability to mediate apoptosis in eukaryotic cells.

Discussion

Apoptosis, or programmed cell death, is an important mechanism for multicellular organisms to remove unwanted or dangerous cells.[32] Over the last few years it has been realized that eukaryotic unicellular organisms can also undergo programmed cell death that is similar if not identical to apoptosis.[33] Even more striking, primitive forms of programmed cell death have been found in prokaryotes. For example, some bacteria carry plasmids that encode stable 'toxin' and labile 'antidote' pairs, such as a restriction enzyme and its cognate DNA methylase.[34,35] When the plasmid is lost, labile antidotes decrease more rapidly than toxins and the plasmid-free bacteria die. Examples of lethal/protective gene pair systems that ensure the retention of the plasmid on which they reside ('selfish genes') in which the antidote is a protein (rather than antisense RNA) have been termed 'proteic killer gene systems'.[36] Endogenous suicide pathways can also be triggered by some bacteriophages, affording a protective mechanism to limit the spread of infection.[37] Bacteria can also commit suicide in response to environmental stress. One such example is bacteria carrying a plasmid encoding colicin that synthesize this toxin in response to DNA damage and die,[25] reminiscent of mammalian cells that respond to DNA-damaging agents by producing p53 and undergoing apoptosis.[38,39] Bacterial death in response to environmental stresses (nutrient deprivation, pH changes, heat shock, etc.) has some similarities to apoptosis (for example, cell shrinkage and protein and RNA degradation), leading to the suggestion that this form of death be termed 'proapoptosis'.[40] Programmed cell death of unicellular organisms is considered 'altruistic' in that it benefits neighboring cells and maintains the genetic stability of the population. These observations have raised the question of whether these cells have similar cell death machinery and utilize similar 'suicide' mechanisms as cells from multicellular organisms.

Caspases, cysteine proteases that cleave substrates at aspartate residues, are mediators of Fas-mediated apoptosis in eukaryotic cells.[13,14] We used a panel of fluorogenic substrates that are preferentially cleaved by caspases -2, -3, -6, -8 and -9, to ask if any caspase-like activity was induced in dying bacteria. None of these substrates was cleaved when added to lysates of pGEX-IC$_{175-319}$-transformed bacteria made before or after induction with IPTG (data not shown). Consistent with this, we found that the caspase-specific inhibitors z-VAD-FMK, YVAD-FMK, and Ac-DEVD-CHO, and a more general cysteine protease

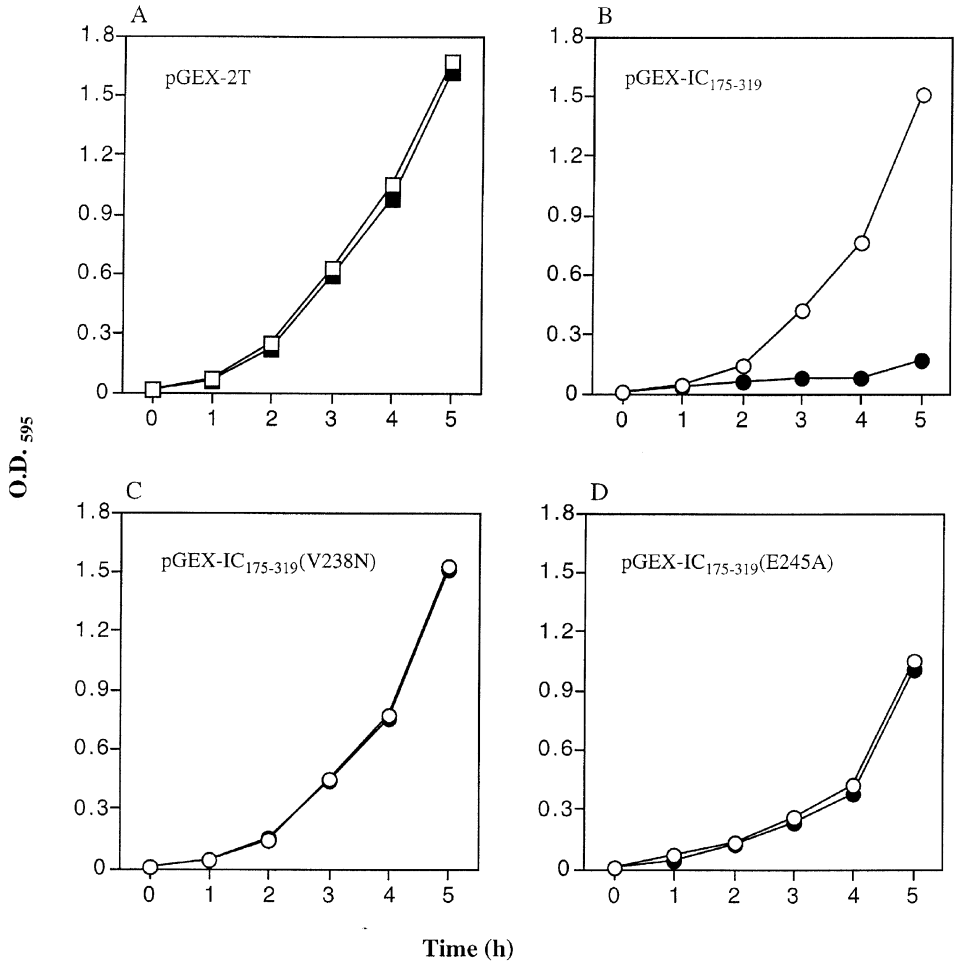

Figure 4 Fas-IC with the single amino acid substitution V238N or E245A does not kill bacteria. Single colonies of bacteria transformed with pGEX-2T, pGEX-$IC_{175-319}$, pGEX-$IC_{175-319}$V238N, or pGEX-$IC_{175-319}$E245A were inoculated into 2 ml of SuperBroth. After 16 h of incubation, 100 μl of each culture were transferred into 10 ml of fresh medium with or without 100 mM IPTG. Absorption at 595 nM was determined at the indicated times

inhibitor Boc-D-FMK, did not prevent Fas-IC-induced bacterial death (data not shown). Furthermore, co-expression of cowpox virus protein CrmA and baculovirus protein p35, both of which have been shown inhibit caspase activity and block Fas-mediated apoptosis,[13,41] did not rescue bacteria from Fas-IC-induced death (data not shown). These results strongly support the notion that caspase-like enzymes are not involved in Fas-induced bacterial cell death. It is noteworthy in this regard that no molecules homologous to pro-apoptotic or anti-apoptotic molecules such as ced-3, ced-4, ced-9, caspases, or Fas have been identified in the genomes of *S. cerevesiae* and *E. coli*. Furthermore, caspase-independent Fas-mediated death of mammalian cells has also been observed.[42–44] Interestingly, in yeast expression of the mammalian pro-apoptotic molecules Bax and Bak resulted in death while expression of mutants of Bax and Bak that cannot kill mammalian cells did not cause yeast cell death.[45–47] Furthermore, coexpression of Bcl-2 blocked the cytotoxicity

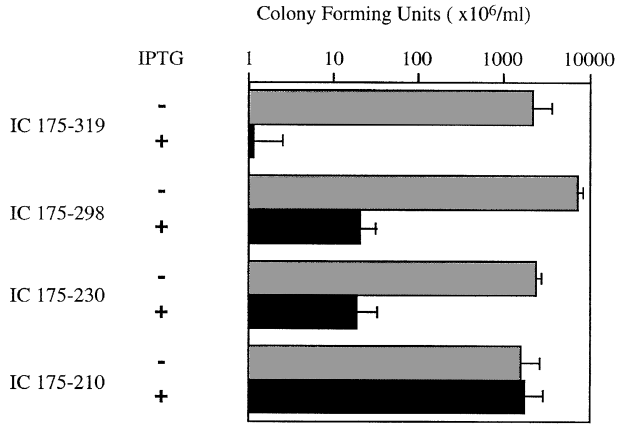

Figure 5 Toxicity of COOH-terminal truncated Fas-ICs in bacteria. Wild-type and COOH-terminal-truncated Fas-IC cDNAs were cloned into pGEX-2T and transformed into DH5α. The numbers of viable cells in the cultures were determined as described in Material and Methods

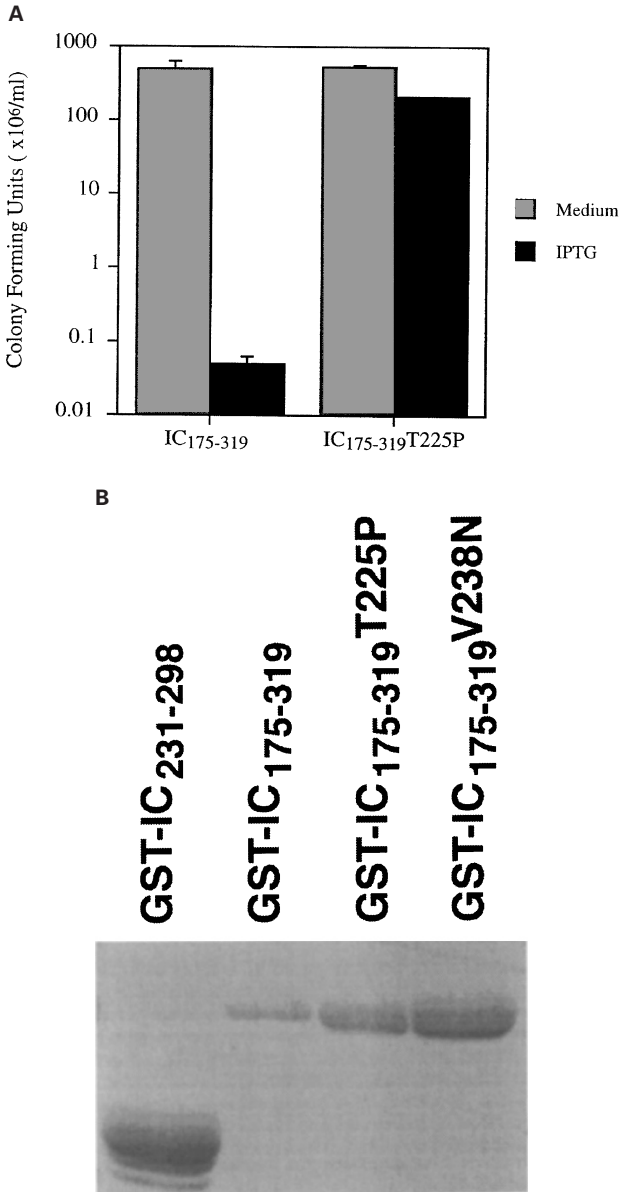

Figure 6 T225P mutation abolishes the ability of Fas-IC to kill host bacteria. (**A**) Numbers of viable bacteria transformed with pGEX-$IC_{175-319}$ or pGEX-$IC_{175-319}$T225P after treatment with 100 μM IPTG. (**B**) Bacteria transformed with pGEX-$IC_{231-298}$, pGEX-$IC_{175-319}$, pGEX-$IC_{175-319}$T225P, and pGEX-$IC_{175-319}$V238N were grown in medium until O.D.$_{595}$ reached 0.4. After induction with 100 μM IPTG for 30 min, pGEX-$IC_{231-298}$, GST-$IC_{175-319}$, GST-$IC_{175-319}$T225P, and GST-$IC_{175-319}$V235N were purified by binding to glutathione Sepharose 4B and examined by SDS–PAGE and Coomassie Blue staining

of Bax and Bak in yeast, indicating that the ability of Bax and Bak to kill yeast cells is related to their ability to induce apoptosis in mammalian cells. Similar results were obtained when pro-apoptotic ced-4 and anti-apoptotic ced-9 were expressed in *S. pombe*.[48] Intriguingly, the effects of Bax and ced-4 on yeast cells are also somewhat different from their action in mammalian and *C. elegans* cells. For example, ced-4 exerts its pro-apoptotic action in *C. elegans* through activation of ced-3, whereas the cytotoxicity of ced-4 in yeast cannot be blocked by coexpression of p35, a potent inhibitor of ced-3.[48] This is similar to our observation Fas-IC-induced bacterial death shares some but not all features with that induced in mammalian cells. In addition, we have found that induction of Bax also results in death of host bacteria, which can not be inhibited by coexpression of p35 (our unpublished data). Given the complexity of eukaryotic cells, expression of pro-cell death molecules in bacteria could be a very useful way to identify and characterize critical cellular components.

An important issue to consider with regard to the observation that expression of intracellular Fas kills bacteria is whether death is induced by a Fas-specific mechanism. Bacteria are commonly used for the production of heterologous proteins, and when proteins are greatly overexpressed bacteria may cease growth and die.[49] This is thought to be due to a starvation response that leads to, among other things, destruction of ribosomes.[50] This does not appear to be the mechanism responsible for Fas-$IC_{175-319}$-induced bacteria death for several reasons. First, the expression level of Fas-$IC_{175-319}$ is low compared to that of Fas-$IC_{230-298}$, Fas-$IC_{175-210}$, Fas-$IC_{175-319}$V238N, none of which affected the growth of *E. coli*. Second, the effect of expressing Fas-$IC_{175-391}$ on host bacteria was evident by direct staining or plating after treatment with IPTG for only 30 min, whereas growth inhibition caused by enforced expression of exogenous protein takes hours. Third, the ability of Fas-$IC_{175-319}$ to kill bacteria was associated with a 20-amino acid region and correlates well with its ability to induce apoptosis in mammalian cells, indicating that bacterial death is due to the specific cytotoxicity of Fas-IC. In fact, it has been known for more than a decade that expression of certain eukaryotic proteins in *E. coli* can result in death despite low protein yield.[51–53] Much like the early days in the study of apoptosis, however, the mechanisms underlying this type of death have received little attention and are largely unknown. Understanding the means by which Fas-IC kills bacteria may provide insights into the more general mechanisms of bacterial death.

An interesting issue is whether killing of bacteria by Fas-IC requires interaction with a FADD-like molecule. Fas-IC containing the V238N mutation is unable to bind FADD in eukaryotic cells,[30] and in fact was unable to kill bacteria. However, although it is able to bind FADD in mammalian cells, Fas-$IC_{175-319}$ with a single amino acid substitution E245A did not kill host bacteria. Moreover, expression of Fas-$IC_{175-230}$, which cannot bind to FADD,[18] does cause the death of bacteria. Together, these results suggest that association with FADD-like molecule is not required for Fas-IC to induce death of bacteria. It is interesting to note that although V238 and E245 are not located in the minimal required region ($IC_{175-230}$) required for killing activity, Fas-IC with single amino acid substitutions at these sites are inactive with regard to bacterial death. This is likely because these mutations disrupt the structure of Fas-IC, as suggested NMR data that they cannot efficiently self-associate and the V238N mutant had many $^{15}N/^{1}H$ amide chemical shifts compared with wild-type Fas-IC.[29] The

NMR studies indicated that Fas-IC has six α-helices, and that amino acids 210–230, which are required for the majority of the bactericidal activity, includes the NH_2-proximal helices α1 and α2.[29] It is conceivable that this region binds evolutionarily-conserved components of the Fas-mediated apoptosis signaling pathway. It is hoped that exploration of the molecular requirements for Fas-induced killing of bacteria may shed light on conserved mechanisms for the induction of death in eukaryotic cells.

Materials and Methods

Bacteria strains, cells, medium, and reagents

DH5α *E. coli* were purchased from Gibco- BRL (Gaithersburg, MD, USA) and cultured in Super Broth medium. Isopropylthiogalactoside (IPTG) and Rhodamine 123 were purchased from Sigma (St. Louis, MO, USA). Jurkat cells were cultured in RPMI 1640 (Biofluids Inc., Rockville, MD, USA) supplemented with 10% FCS, 100 U/ml penicillin, 150 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol. The anti-human Fas antibody CH-11 was purchased from Kamiya (Seattle, WA, USA).

Plasmids constructs

The prokaryotic expression vector pGEX-2T was obtained from Pharmacia (Uppsala, Sweden). Human Fas cDNA was kindly provided by Drs. John Mountz and Jianhua Cheng (University of Alabama at Birmingham Station, Birmingham, AL, USA). cDNAs of intracellular portion of Fas ($IC_{175-319}$) and death domain ($IC_{231-298}$) were generated by PCR using primers (1) 5′-CGGGATCCA AGAGAAAGGAAGTACAGAA-3′ and (2) 5′-GGAATTCCTAGAC-CAAGCTTTGGATTT-3′, and (3) 5′-CGGGATCCGGCTTTGTTCG AAAGAATGG-3′ and (4) 5′-GGAATTCCTAGATG ATAGTCT-GAATTTTCT-3′ respectively. They were cloned into *Bam*HI and *Eco*RI double-digested pGEX-2T and the sequences of the constructs were confirmed by DNA sequencing. Similarly, cDNAs of $IC_{175-298}$, $IC_{175-230}$, and $IC_{175-210}$ were made using primers (1) and primer (4), (1) and (5) 5′-GGAATTCTATTTAACTTGACTTAGTGTCAT-3′, and (1) and (6) 5′-GGAATTCCT AAGATAAATTTATTGCCA-3′ respectively. Human Fas cDNA with the T225P substitution was kindly provided by Dr. Charles Zacharchuk (National Cancer Institute, NIH, Bethesda, MD, USA). Fas-IC with the V238N and E245A single amino acid substitutions were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and confirmed by DNA sequencing.

Protein expression and detection

Intracellular portions of Fas were expressed as GST-Fas-IC fusion proteins following induction with 100 μM of IPTG. They were purified with glutathione Sepharose 4B according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). After separation on 12% SDS–PAGE, proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Viable bacteria enumeration

A single bacterial colony transformed with the indicated plasmid was inoculated in medium and incubated for approximately 4 h. After further incubation in the presence or absence of IPTG for 30 min, a small amount of the culture was diluted and spread over the surface of an LB plate containing 100 mg/ml of ampicillin. After overnight incubation, the number of viable cells was determined from the number of colonies on the plates.

Assessment of bacterial viability by flow cytometry

Viability of bacteria was assessed by staining with the vital fluorescent dye rhodamine 123 as described.[27] Briefly, a single colony of DH5α cells transformed with the indicated plasmid was grown in Super Broth medium for approximately 4 h. After further incubation in the absence or presence of IPTG for 30 min, bacteria were harvested and washed three times with 50 mM Tris buffer (pH 7.5) containing 5 mM EDTA. The pellets were resuspended in medium containing 100 ng/ml of rhodamine 123 and incubated at room temperature for 10 min. Flow cytometric analysis was carried out with a FACScan (Becton Dickinson, San Jose, CA, USA).

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