A Novel Protein Domain Required for Apoptosis

MUTATIONAL ANALYSIS OF HUMAN Fas ANTIGEN*

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The Fas antigen is a cell surface protein that can mediate apoptosis and that belongs to the tumor necrosis factor receptor family. Murine fibroblast L929 cells or T-cell lymphoma WR19L cells expressing the human Fas antigen were killed within 4-6 h by anti-human Fas antibody in a concentration-dependent manner. Human Fas antigen cDNAs with various mutations in the cytoplasmic region were constructed and expressed in L929 cells. A deletion of 15 amino acids from the C terminus of the Fas antigen enhanced the Fas antibody-induced killing activity, whereas a further deletion abolished its activity. This suggests the presence of an inhibitory as well as a signal-transducing domain in the cytoplasmic region of the Fas antigen. A 68-amino acid portion of the signal-transducing domain significantly conserved in the Fas antigen as well as in the type I tumor necrosis factor receptor was considered to be the novel protein domain required for apoptotic signal transduction.

During embryonic and postembryonic development, many cells die by programmed cell death, which plays a major role in determining morphologic and functional maturity in a variety of systems, including neural network formation and clonal deletion of autoreactive T-cells (1-3). Programmed cell death generally occurs by a process called apoptosis. Apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, and extensive degradation of chromosomal DNA into oligomers of about 180 base pairs (1-3).

The Fas antigen is expressed on the surface of various cell lines, including activated T- and B-cells. The mouse monoclonal anti-human Fas antibody is cytotoxic to some cells expressing the Fas antigen (4). We isolated a cDNA encoding the human Fas antigen by expression cloning (5). The human Fas antigen consists of 325 amino acids with a single transmembrane domain. The cytoplasmic region (145 amino acids) does not contain domains for kinases, phosphatases, or any other apparent enzymes. However, the extracellular domain was similar to that of the tumor necrosis factor (TNF) receptor family, which includes the low affinity nerve growth factor receptor family. Murine fibroblast L929 cells expressing the human Fas antigen cDNA were killed within 4-6 h by anti-human Fas antibody (5). After expression of the human Fas antigen cDNA in mouse fibroblast L929 and T-cell lymphoma WR19L cells, the anti-human Fas antibody kills these cells by apoptosis, which indicates that the Fas antigen mediates an apoptotic signal into the cells (5).

Recently, we have identified a mouse Fas antigen gene (16) as the structural gene for mouse lymphoproliferation (lpr) mutation (17). The lpr mutation is a recessive and autosomal mutation, and mice homozygous at this locus produce multiple autoantibodies, accumulate nonmalignant CD4+CD8+ T-lymphocytes, and develop an autoimmune disease resembling systemic lupus erythematosus (18). These findings indicate that the Fas antigen plays an important role in the programmed cell death of T-lymphocytes in the thymus. Here, we identified a domain in the cytoplasmic region of the Fas antigen, which is required to transduce the death signal. A significantly conserved sequence of this domain was found in the cytoplasmic region of the TNF type I receptor, which also transduces the apoptotic signal into cells (19).

MATERIALS AND METHODS

Plasmid Construction—The deletion mutants, FD2, FD4, and FD5, were constructed by digesting the 2.6-kilobase XhoI DNA fragment of the plasmid pEF5 (5) carrying the full-length human Fas antigen cDNA with TaqI (at 942), BglII (at 1083), or SpeI (at 1150). After treatment with the Klenow fragment of Escherichia coli DNA polymerase, an XbaI linker (CTAGTCTAGACTAG, New England Biolabs) was added to the DNA ends, which were then digested with XhoI. The XbaI fragments containing the Fas antigen sequence were then transferred to the pEF-BOS expression vector (20), and the plasmids carrying the Fas antigen sequence in the correct orientation were designated as pEF-FD2, pEF-FD4, and pEF-FD5, respectively. The other two deletion mutants, FD7 and FD8, were constructed by PCR (21). In brief, oligonucleotides HFD2 (GGAAATTCTAGACTAGGGACAAAGATTGGCTTTTTTGAGAT, nucleotides 1084-1108 of the human Fas antigen cDNA; the extra sequence not present in human Fas antigen cDNA) and HFD3 (GGAAATTCTAGACTAGGGTCAAAAGATTGGCTTTTTTGAGAT, nucleotides 1084-1108 of the human Fas antigen cDNA; the extra sequence not present in human Fas antigen cDNA) were used as reverse primers for construction of FD7 and FD8 deletion mutants, respectively. The oligonucleotide HF1 (GAAGATCCAGATCTAACTT) carrying the nucleotide sequence of each dNTP, 2 ng of the 1.8-kilobase XhoI-EcoRI fragment of pEF5 (5), and 2.5 units of Taq polymerase in a total volume of 50 μl. The conditions for PCR were 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C for 30 cycles. The products were digested with BglII and EcoRI, and then the BglII-EcoRI DNA fragment carrying an in-frame termination codon, as well as XbaI and EcoRI sites, were synthesized and used as reverse primers for construction of FD7 and FD8 deletion mutants, respectively. The oligonucleotide HF1 (GAAGATCCAGATCTAACTT) carrying the nucleotide sequence from 696 to 715 of human Fas antigen cDNA was the forward primer for both constructions. PCR proceeded in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 20 μM of each dNTP, 200 μM of each primer, 2 ng of the 1.8-kilobase XhoI-EcoRI fragment of pEF5 (5), and 2.5 units of Taq polymerase in a total volume of 50 μl. The conditions for PCR were 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C for 30 cycles. The products were digested with BglII and EcoRI, and then the BglII-EcoRI DNA fragment carrying the truncated Fas antigen cDNA replaced the C-terminal BglII-XbaI fragment of pEF5 (5).

The mutant carrying a point mutation in the cytoplasmic region of the Fas antigen was also constructed by PCR. Primers HF9 (TGTAATCTAGACTAGTGT) and nucleotides from 597 to 614 of the human Fas antigen cDNA, HF10 (GCCATTTCCTCTAGGACGC, 1265-1282), HFP1 (TTCTAAAAGTGTGACATACATGGCCCAA, 1256-1272) were used as reverse primers for construction of the C-terminal BglII-XbaI fragment of pEF5 (5).

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1 The abbreviations used are: TNF, tumor necrosis factor; PCR, polymerase chain reaction.

10932
A Protein Domain Required for Apoptosis

Rapid Cell Death Induced by Anti-Fas Antibody or TNF—We previously showed that mouse L929 cell transformants expressing the human Fas antigen are killed within 16 h by anti-human Fas antibody in a dose-dependent manner in the presence of 0.5 μg/ml actinomycin D (5). In Fig. 1A, semi-confluent L929 cell transformants expressing the human Fas antigen were incubated with anti-Fas antibody in the presence of actinomycin D. The number of viable cells stained with crystal violet started to decrease after a 3-h incubation with anti-Fas antibody, and no viable cells remained after 6 h. The cells were not killed by the Fas antibody in the absence of actinomycin D (see below), which suggests the presence of a factor in L929 cells that inhibits the Fas antigen-mediated apoptotic process.

The Fas antigen can also transduce the apoptotic signal in mouse T-cell lymphoma WR19L cells. In this cell line, actinomycin D was not necessary to induce Fas antigen-mediated apoptosis. As shown in Fig. 1B, all transformant cells expressing the human Fas antigen were killed within 4 h by incubation with the anti-Fas antibody.

TNF has cytocytic activity on L929 cells in the presence of actinomycin D (22) and on WR19L cells in the absence of metabolic inhibitors. The kinetics of the cell death process induced by TNF in these cell lines was similar or slightly slower than that observed with the anti-Fas antibody (Fig. 1).

Expression of the Mutant Fas Antigen in L929 Cells—The cytoplasmic region of the Fas antigen consists of 145 amino acids, but it does not contain an enzymatic domain for tyrosine kinase or serine/threonine kinases. To clarify the region responsible for apoptotic signal transduction, a set of deletion mutants of the Fas antigen cDNA was prepared by progressive deletion from the C-terminal end as described under “Materials and Methods.” Five mutant cDNAs (FD5, FD8, FD7, FD1a, and FD2) coded for the cytoplasmic region of the Fas antigen, up to amino acid positions 304, 296, 289, 281, and 234, respectively (Fig. 2). These mutated cDNAs were placed under the promoter of the human elongation factor 1α (EF-1α) and introduced into mouse L929 cells together with the neomycin resistance gene.

Fluorescence-activated cell sorting analysis of mutant Fas antigen expression in stable transformants using anti-Fas monoclonal antibody indicated that more than 80% G-418-resistant transformant clones expressed the human Fas antigen on the cell surface. The expression levels of the Fas antigen in the positive clones were similar among different mutant proteins. Fig. 3 shows representative results. Western blotting analysis of the cell lysates with anti-human Fas antibody also indicated that all of the transformed clones expressed similar amounts of the mutated Fas antigen (data not shown).

Signal-transducing Domain in the Fas Antigen—The ability of the mutated Fas antigen to transduce the apoptotic signal was then examined with the transformants established above.
To eliminate clonal variations, four to six independent transformant clones were chosen for each mutant. Fig. 4 shows the anti-Fas antibody-dependent cell death of the transformants expressing the wild-type Fas antigen and the mutants. Clones expressing the wild-type Fas antigen and FD5 clones expressing the mutant lacking the C-terminal 15 amino acids were killed by the anti-Fas antibody (Fig. 4, A and B). FD5 clones were more sensitive to the anti-Fas antibody than the clones of wild type. On the other hand, clones expressing mutants carrying a further deletion (FD8, FD7, FD4, and FD2) were unaffected by the anti-Fas antibody. Fig. 4C shows typical results using FD8 clones. These findings indicate that the 130-amino acid portion from 175 to 304 in the cytoplasmic region of the human Fas antigen is essential for the Fas antibody-triggered apoptotic signal transduction in L929 cells. Similar results were obtained with WR19L cell transformants expressing the mutated Fas antigen (data not shown).

Mice with the allelic lpr<sup>+</sup> defect carry a point mutation in the cytoplasmic region of the mouse Fas antigen, which converts Ile-225 to Asn-225. To confirm that this mutation abolishes the apoptotic signal transduction of the Fas antigen, the corresponding valine residue of the human Fas antigen at amino acid 238 was mutated to asparagine, and the mutant human Fas antigen was expressed in mouse L929 cells. As shown in Fig. 4D, the transformants expressing the Fas antigen with this point mutation were unaffected by anti-Fas antibody.

The C-terminal Domain of the Fas Antigen Is an Inhibitory Domain—As described above, the deletion of the C-terminal 15 amino acids from human Fas antigen up-regulated the ability of the Fas antigen to transduce the apoptotic signal. As shown in Fig. 4, A and B, the half-maximal response of anti-Fas antibody with transformant clones expressing the deletion mutant occurred at a concentration of 5–30 ng/ml, whereas 80–300 ng/ml anti-Fas antibody was required for the half-maximal response with transformant clones expressing the wild-type Fas antigen. Furthermore, unlike the wild type, the mutated Fas antigen transduced the apoptotic signal in L929 cells in the absence of actinomycin D. As shown in Fig. 5, the L929 cell transformants expressing the wild-type Fas antigen were unaffected by the anti-Fas antibody at concentrations of up to 3 μg/ml in the absence of actinomycin D, whereas 60–80% of the cells expressing the FD5 mutants were killed by 3 μg/ml anti-Fas antibody under these conditions. These findings indicate that the C-terminal 15 amino acids of the Fas antigen function as an inhibitory domain, the deletion of which up-regulates the ability of the Fas antigen to transduce the apoptotic signal.
A Protein Domain Required for Apoptosis

DISCUSSION

We showed that the Fas antigen/antibody system induces rapid cell death. All WR19L cell transformants expressing the Fas antigen were killed within 4 h, which is much faster than the death caused by the depletion of growth factors from dependent cell lines (usually more than 10 h) (23-25). This suggests that the Fas antigen actively transduces the death signal, while the cell death caused by factor depletion is an indirect process. Previously, we found that the Fas antigen gene is the structural gene for lpr and suggested an important role of the Fas antigen in the development of T-cells in the thymus (17). During the thymic development of T-cells, more than 90% of the T-cells die by apoptosis at the CD4+ CD8+ stage, which is the major fraction of thymocytes (26). Nevertheless, only a small percentage of the cells were positive for apoptotic bodies, indicating that only a small percentage of the thymocytes is at the death stage. This might be due to the rapid dying process. That is, the CD4+ CD8+ T-cell precursors stay in the thymus for 3.5 days before they become single positive cells (27). If the dying process requires only 4 h, as does Fas antibody-triggered cell death, a rough estimation suggests that only 4.7% (4/84) cells are at the dying stage.

Previously, we observed that a domain in the cytoplasmic region of the Fas antigen is homologous to the part of the cytoplasmic region of the TNF type I receptor (5). Between two TNF receptors, type I (55 kDa) and type II (75 kDa), the former but not the latter is responsible for the cytotoxic activity of TNF (19). Deletion analysis of the human Fas antigen from the C terminus indicated that the region homologous to the TNF type I receptor is essential for the Fas antigen to transduce the death signal into cells. As shown in Fig. 6, alignment of the amino acid sequences of the cytoplasmic region of the Fas antigen and TNF type I receptor indicates that a domain of 68 amino acids (231-298 of the human Fas antigen) is significantly conserved between them (24% identity). Deletions of this domain at the C terminus and a point mutation of the Val-238 in the middle of this domain completely abolished the ability of the Fas antigen to mediate apoptosis. Recent mutational analysis of TNF type I receptor has also suggested that the corresponding domain of the TNF type I receptor is necessary and sufficient to mediate the TNF-triggered cytotoxicity into cells.² This domain of the

² D. V. Goeddel, personal communication.
Fas antigen and TNF type I receptor might have enzymatic activity for apoptosis or interact with similar cytoplasmic proteins to transduce the cell death signal. The cytotoxic activities mediated by the Fas antigen and TNF type I receptor in L929 but not WR19L cells were seen only in the presence of actinomycin D. This indicates that some factors transformants (O, U, A) or the FDS mutant (B) were incubated with various concentrations (0-3 μg/ml) of anti-Fas antibody in the absence of actinomycin D at 37 °C for 16 h and stained with crystal violet. The percentage of viable cells was calculated as described in Fig. 1. Three independent clones for the wild-type transformants (C, D, E) or four independent clones for the mutant transformants (O, B, H, D) were analyzed.

platelet-derived growth factor and epidermal growth factor receptors, contain a tyrosine residue near the C terminus of the molecule (28). The tyrosine phosphorylation of this residue inhibits the tyrosine kinase activity of the receptor, whereas G-protein-coupled receptors carrying seven transmembrane regions such as adrenergic receptors contain a phosphorylation site for CAMP-dependent protein kinase in the last cytoplasmic domain (29). Phosphorylation of this residue desensitizes the receptor function. The C-terminal amino acid residues (29), whereas G-protein-coupled receptors carrying seven transmembrane regions such as adrenergic receptors contain a phosphorylation site for CAMP-dependent protein kinase in the last cytoplasmic domain (29). Phosphorylation of this residue desensitizes the receptor function. The C-terminal region of the Fas antigen may regulate the Fas antigen-mediated apoptotic signal transduction by a similar mechanism. In this respect, it is noteworthy that human and mouse Fas antigens have a sequence (XSXXEX in human Fas, XSXXDXX in mouse Fas antigen) similar to the consensus phosphorylation site motif for casein kinase II (30) near the C terminus of the molecule. More detailed analyses of the Fas antigen-mediated apoptotic signal transduction may clarify the mechanism for apoptosis.

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A Protein Domain Required for Apoptosis

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