Lyons, C. M. Jones, B. L. M. Hogan, *Trends Genet.* **7**, 408 (1991).
2. R. W. Padgett, R. D. St. Johnston, W. M. Gelbart, *Nature* **325**, 81 (1987).
3. F. A. Spencer, F. M. Hoffmann, W. M. Gelbart, *Cell* **28**, 451 (1982); D. Segal and W. M. Gelbart, *Genetics* **109**, 119 (1985); V. F. Irish and W. M. Gelbart, *Genes Dev.* **1**, 868 (1987).
4. D. A. Hursh, R. W. Padgett, W. M. Gelbart, *Development* **117**, 1211 (1993).
5. R. W. Padgett, J. M. Wozney, W. M. Gelbart, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2905 (1993).
6. T. K. Sampath, K. E. Rashka, J. S. Doctor, R. F. Tucker, F. M. Hoffmann, *ibid.*, p. 6004.
7. S. Cheifetz *et al.*, *Cell* **48**, 409 (1987).
8. X.-F. Wang *et al.*, *ibid.* **67**, 797 (1991); F. Lopez-Casillas *et al.*, *ibid.*, p. 785.
9. L. S. Mathews and W. W. Vale, *ibid.* **65**, 973 (1991); H. Y. Lin, X.-F. Wang, E. Ng-Eaton, R. A. Weinberg, H. F. Lodish, *ibid.* **68**, 775 (1992); M. Estevez *et al.*, *Nature* **365**, 644 (1993).
10. L. L. Georgi, P. S. Albert, D. L. Riddle, *Cell* **61**, 635 (1990); R. Ebner, R.-H. Chen, S. Lawler, T. Zioncheck, R. Derynck, *Science* **262**, 900 (1993); R. Ebner *et al.*, *ibid.* **260**, 1344 (1993); K. Tsuchida, L. S. Mathews, W. W. Vale, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11242 (1993); L. Attisano *et al.*, *Cell* **75**, 671 (1993); P. Franzén *et al.*, *ibid.*, p. 69; C. H. Bassing *et al.*, *Science* **263**, 87 (1994).
11. F. T. Boyd and J. Massagué, *J. Biol. Chem.* **264**, 2272 (1989).
12. R. Weiser, L. Attisano, J. L. Wrana, J. Massagué, *Mol. Cell. Biol.* **13**, 7239 (1993).
13. H. Y. Lin and H. F. Lodish, *Trends Cell Biol.* **3**, 14 (1993).
14. To amplify DNA from the kinase domain of TGF-β–like receptors, we made primers corresponding to two conserved regions of known kinases (Fig. 2). The sequences of the primers are as follows: primer 1, CA(C/T) CG(A/C/G/T) GA(C/T) AT(A/C/T) AA(A/G) TC(A/C/G/T) AA(A/G) AA; and primer 2, TC(A/C/G/T) AG(A/C/G/T) A(C/T)(C/T) TC(A/C/G/T) GG(A/C/G/T) GCC AT(A/G) TA. Genomic DNA and DNA from a 4- to 8-hour embryonic complementary DNA (cDNA) library (*24*) were amplified by PCR and cloned into pBluescript (Stratagene), which had been treated with Eco RV, TAQ polymerase, and dTTP. Conditions for PCR were as follows: three cycles at 94°C for 1 min, 40°C for 1 min, 72°C for 3 min, followed by 27 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Random colonies were sequenced (*2, 23*), analyzed by computer, and placed into groups on the basis of sequence identity. After all the clones were compared and grouped, cDNA clones were isolated from the positive inserts and sequenced to verify that they belonged in the TGF-β–like receptor family.
15. G. von Heijne, *Nucleic Acids Res.* **14**, 4683 (1983).
16. T. Xie, A. L. Finelli, R. W. Padgett, unpublished data.
17. A description of *Drosophila* genetic markers can be found in D. L. Lindsley and G. G. Zimm [*The Genome of* Drosophila melanogaster (Academic Press, New York, 1992)] as well as in FlyBase. FlyBase is a database of genetic and molecular data for *Drosophila* (W. M. Gelbart, Harvard University) and can be accessed at Indiana University with the Internet Gopher. A description of dpp^{hr93} can be found in K. A. Wharton, R. P. Ray, and W. M. Gelbart [*Development* **117**, 807 (1993)]. The transposon P20 (*4*) contains embryonic and larval regulatory elements and coding sequences of *dpp*. A shorter transposon, P6.5, contains only the embryonic and coding sequences of *dpp* (R. W. Padgett, unpublished data).
18. T. Schüpbach and E. Wieschaus, *Genetics* **121**, 101 (1989).
19. S. Govind and R. Steward, *Trends Genet.* **7**, 119 (1991).
20. On the basis of an examination of the genomic organization of the *sax* gene, we amplified the *sax* coding sequences in two overlapping fragments by PCR. The products were cloned (*14*) and sequenced with a series of oligonucleotides corresponding to the *sax* coding region. Because PCR amplification can introduce mutations into DNA, we amplified each fragment twice and sequenced multiple clones derived from the two independent PCR reactions. We compared these sequences with those from the two parental strains, *cn bw* and *cn bw sp*. The entire coding sequence of all of the alleles was examined.
21. D. R. Knighton *et al.*, *Science* **253**, 407 (1991); D. R. Knighton *et al.*, *ibid.*, p. 414.
22. E. L. Ferguson and K. V. Anderson, *Development* **114**, 583 (1992).
23. A. L. Finelli, C. A. Bossie, T. Xie, R. W. Padgett, *ibid.*, in press.
24. N. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988).
25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K. Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. S. Poole, L. M. Kauvar, C. Drees, T. Kornberg, *Cell* **40**, 37 (1985).
27. Genetics Computer Group, Program Manual for the GCG Package, version 7 (April 1991); 575 Science Drive, Madison, WI 53711, USA.
28. S. R. Childs *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9475 (1993); T. Xie and R. W. Padgett, unpublished data.
29. L. Attisano, J. L. Wrana, S. Cheifetz, J. Massagué, *Cell* **68**, 97 (1992).
30. R. D. St. Johnston *et al.*, *Genes Dev.* **4**, 1114 (1990).
31. E. Wieschaus and C. Nüsslein-Volhard, in Drosophila: *A Practical Approach*, D. M. Roberts, Ed. (IRL, Oxford, 1986), pp. 199–227.
32. We thank the members of our laboratory for advice during the course of these studies, T. Schüpbach and E. Wieschaus for providing *sax* stocks, the Developmental Group at Rutgers and Padgett laboratory for improving earlier versions of this manuscript, G. Montelione for examining the placement of *sax* mutations on the three-dimensional structure of cAMP-dependent protein kinase, and Y. Jin for isolation of *sax* cDNAs. We also thank V. Twombly and W. M. Gelbart for exchanging unpublished information. Supported by research grants from the Council for Tobacco Research (to R.W.P.), from the National Institutes of Health (to R.W.P.), and by a Rutgers Research Council grant (to R.W.P.). T. X. is a Busch Predoctoral Fellow and A.L.F. is a Benedict-Michael Predoctoral Fellow.

26 January 1994; accepted 28 February 1994

Protection from Fas-Mediated Apoptosis by a Soluble Form of the Fas Molecule

Jianhua Cheng, Tong Zhou, Changdan Liu, John P. Shapiro, Matthew J. Brauer, Michael C. Kiefer, Philip J. Barr, John D. Mountz*

Fas is an apoptosis-signaling receptor molecule on the surface of a number of cell types. Molecular cloning and nucleotide sequence analysis revealed a human Fas messenger RNA variant capable of encoding a soluble Fas molecule lacking the transmembrane domain because of the deletion of an exon encoding this region. The expression of soluble Fas was confirmed by flow cytometry and immunocytochemical analysis. Supernatants from cells transfected with the variant messenger RNA blocked apoptosis induced by the antibody to Fas. Levels of soluble Fas were elevated in patients with systemic lupus erythematosus, and mice injected with soluble Fas displayed autoimmune features.

The Fas/Apo-1 molecule (designated as CD95) is a cell-surface receptor belonging to the nerve growth factor receptor–tumor necrosis factor–α receptor family of apoptosis-signaling molecules (*1*). Defects in the mouse *Fas* gene lead to autoimmune features in mice (*2*), and the human Fas molecule has been implicated in the abnormally high levels of lymphocyte apoptosis seen in human immunodeficiency virus (HIV)–infected humans (*3*). To investigate the possible presence of abnormal Fas transcripts in human subjects with autoimmune disease, we isolated cellular RNA from the peripheral blood mononuclear cells (PBMC) of two systemic lupus erythematosus (SLE), two angioimmunoblastic lymphadenopathy (AILD) patients, and two normal controls. The Fas mRNA transcripts were then analyzed after amplification with the use of oligonucleotide primers derived from the 5′ untranslated region (UTR) starting at nucleotide (nt) 170 and 3′ UTR and ending at nt 1336 (Fig. 1A). The amplified product was expected to be 1167 base pairs (bp) in size and to encompass the entire translation region, as well as portions of the 5′ and 3′ UTRs of the Fas mRNA. However, in addition to the anticipated full-length Fas complementary DNA (cDNA) fragment, a distinct smaller 1104-bp DNA fragment was also observed in patients with SLE and AILD and in normal controls.

The structure of the smaller polymerase chain reaction (PCR) product was determined by cloning (Fig. 1B), DNA se-

J. Cheng, T. Zhou, C. Liu, J. D. Mountz, University of Alabama at Birmingham and the Birmingham Veterans Administration Medical Center, 473 LHR Building, Birmingham, AL 35294, USA.
J. P. Shapiro, M. J. Brauer, M. C. Kiefer, P. J. Barr, LXR Biotechnology Inc., 1401 Marina Way South, Richmond, CA 94804, USA.

*To whom correspondence should be addressed.

quence analysis, and comparison of the deduced amino acid sequence with that of the previously reported human Fas molecule (*1*). A schematic representation of the alternately spliced Fas mRNA is shown in Fig. 2. The DNA sequence analysis confirmed that the longer insert represented the anticipated 1167-bp Fas cDNA fragment and contained the intact open reading frame. The smaller 1104-bp clone, designated as FasΔTM, had a 63-bp deletion starting at nucleotide sequence (700) GA TCC AGA and ending at nucleotide sequence GTT TGG G (762) (Fig. 2B). This deletion does not affect the open reading frame but results in a Fas protein product lacking the 21 amino acid residues corresponding to the last 5 amino acid residues of the Fas extracellular domain and 16 of the 17 amino acid residues of the Fas hydrophobic transmembrane (TM) domain. Thus, this product is likely to be expressed as a soluble, secreted form of Fas.

To determine whether FasΔTM arose by translation of an alternately spliced transcript, genomic DNA surrounding the TM-encoding region was amplified by PCR and cloned. DNA sequence analysis revealed (i) a 152-bp intron and (ii) a 1183-bp intron flanking the TM-encoding exon (Fig. 2A). Splicing of the intron A donor site to the intron B splice acceptor would accurately yield the FasΔTM transcript. The presence of FasΔTM transcript was also confirmed by RNA protection analysis.

To confirm the existence of the soluble Fas translation product, the Fas cDNA and FasΔTM coding region were inserted into a mammalian expression vector (pcDNA-1) and transfected into COS cells. Flow cytometry and immunohistochemical analysis were performed by staining of the transfected COS cells with antibody to human Fas. Reverse transcription PCR analysis indicated that the transfected cells expressed the corresponding Fas transcript. Immunofluorescence (Fig. 3A) and flow cytometry analysis (Fig. 3B) revealed that the antibody stained the surface of cells transfected with the full-length Fas cDNA but not the surface of cells transfected with the FasΔTM expression plasmid. This analysis also revealed that there were no apparent differences in the degree of cytoplasmic staining between cells transfected with the full-length Fas cDNA and cells transfected with the FasΔTM variant. Taken together, these data confirm that

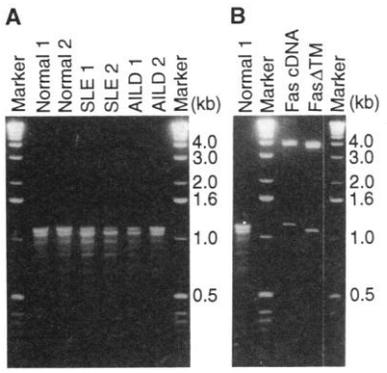

Fig. 1. Reverse transcriptase–PCR analysis of the human Fas mRNA species. (**A**) First-strand cDNA prepared from human PBMC from patients referred to the National Institutes of Health as described in (*13*) and meeting the criteria for SLE (*14*) or AILD (*15*), from normal control subjects and from patients subjected to PCR with primers corresponding to sequences in the 5′ and 3′ UTRs of Fas mRNA (*1*, *16*). The PCR products were separated in a 1.2% agarose gel. (**B**) The recombinants derived by the cloning of DNA fragments amplified from the normal subject 1, shown for comparison in the first lane of the gel. The two cloned inserts and the number of base pairs in each insert are Fas cDNA, 1167-bp insert; and FasΔTM, 1104-bp insert. Standard DNA size markers are shown.

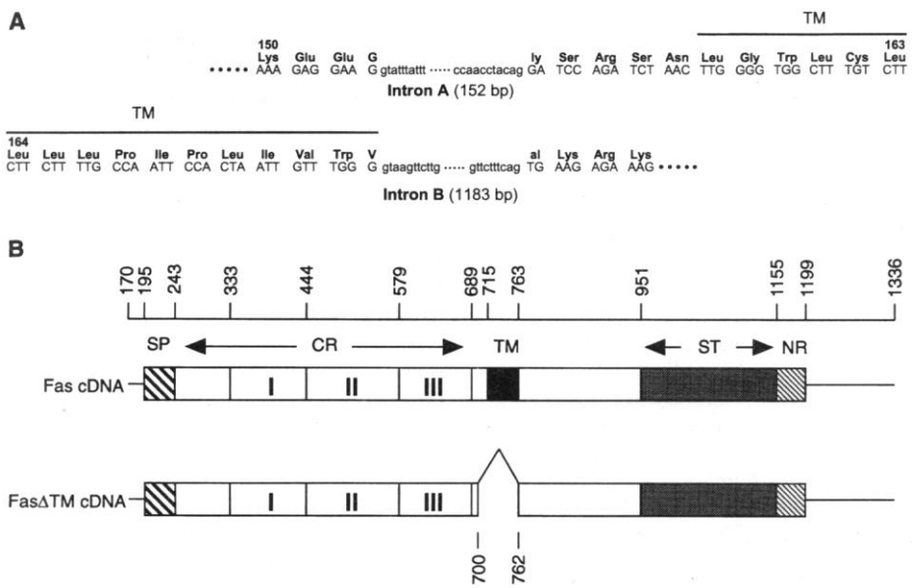

Fig. 2. Human FasΔTM structure. (**A**) Genomic DNA sequence surrounding the TM-encoding region (*17*). The intron DNA sequence is shown in lowercase letters. Amino acid numbering is according to (*1*). The TM region is overlined. (**B**) Schematic representation of alternatively spliced Fas mRNA variants. The translated and UTR regions are indicated by boxes and thick solid lines, respectively. Regions lacking in the FasΔTM variant are indicated by broken lines and nucleotide positions. Abbreviations: SP, signal peptide; CR, cysteine-rich subdomains; TM, transmembrane domain; ST, signal transduction domain; and NR, negative regulation domain, respectively (*1*). Nucleotide regions encoding each domain are shown above.

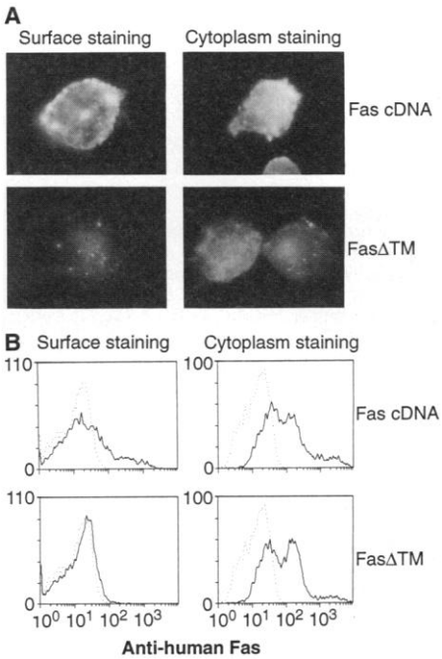

Fig. 3. Expression analysis of Fas molecule encoded by the Fas cDNAs. (**A**) Indirect immunofluorescence analysis of expression of Fas molecule in the transfected COS cells (*18*). The transfected COS cells were incubated with mouse monoclonal antibody to human Fas (IgM) and then stained with FITC-conjugated goat anti-mouse IgM. The stained cells were cytospun onto slides, mounted with mounting fluid, and photographed with a Zeiss fluorescence microscope and camera (magnification, ×1000). (**B**) Flow cytometry analysis of expression of Fas molecule in the transfected COS cells. COS cells were transfected with either the Fas cDNA or FasΔTM and cultured for 72 hours. Single cell suspensions were stained with IgM mouse monoclonal antibody to human Fas followed by FITC-conjugated goat anti-mouse IgM. Cytoplasmic staining was carried out by pretreatment of the cells with 70% ethyl alcohol to allow permeabilization and entry of the antibody. Purified mouse IgM was used as a negative control. The representative histograms show (solid trace) the specific binding and (dotted trace) non-specific binding).

the FasΔTM transcript produced the expected Fas protein product, as did the Fas cDNA. In addition, they suggest that FasΔTM protein was not retained in the membrane of the cell but was present in the cytoplasm as a soluble form.

Further confirmation of the presence of a secreted FasΔTM protein was sought by the use of an inhibition assay for apoptosis induced by the antibody to Fas. Phytohemagglutinin (PHA-P)–stimulated PBMC from normal subjects and the CEM-6 human T cell line were used for this experiment, because it has been shown that stimulated cells undergo apoptosis after culture with the antibody to Fas (*4*) and that CEM-6 is highly sensitive to anti-Fas–induced apoptosis. The PHA-P–stimulated PBMC and CEM-6 cells were cultured with antibody to Fas in the presence of supernatant derived from COS cells transiently expressing the FasΔTM molecule from the FasΔTM expression recombinant or expressing the Fas molecule from the full-length Fas cDNA expression recombinant. There was a dose-dependent increase in cell survival rate when the former, but not the latter, supernatant was used (Fig. 4) (*5*). This higher survival rate observed in cells treated with the FasΔTM supernatant can be explained by the secretion of the soluble FasΔTM molecule into the supernatant, resulting in neutralization of the antibody to Fas and, consequently, in the inhibition of the antibody-mediated apoptosis.

A soluble form of Fas was present at increased concentration in approximately 60% of patients with SLE (Fig. 5). In normal controls and patients with rheumatoid arthritis, serum levels of soluble Fas were less than 100 ng/ml. Although soluble Fas is only increased approximately twofold in 60% of SLE patients, much higher physiological concentrations may be present at limited sites where there is high expression of Fas and Fas ligand and in certain lymphoid organs (*1*, 6).

To demonstrate that soluble Fas is biologically active, we produced a mouse-soluble Fas to determine the role of this molecule in vivo. The soluble Fas was injected (2.5 μg per gram of body weight intraperitoneally) every day for 3 days. This treatment gave rise to an average serum concentration comparable to that observed in the SLE patients described above. There was an increase in $CD4^-CD8^-$ as well as $CD4^+$ and $CD8^+$ (single positive) thymocytes with a decrease in $CD4^+CD8^+$ (double positive) thymocytes (Fig. 6A). There was a threefold increase in the number of spleen cells, primarily due to an increase in B cells (Fig. 6B). These data demonstrate that levels of soluble Fas observed in SLE patients can have a dramatic effect on lymphocyte development in vivo. These alterations in phenotypic development resulted in increased autoreactivity, as evidenced by a fourfold increase in the response of syngenetic, mixed cell culture proliferation by thymocytes and spleen cells from soluble Fas–treated mice, compared to those from control-treated mice (Fig. 6C). These results demonstrate that soluble Fas in vivo can lead to altered lymphocyte development and increased proliferation in response to self antigens.

Soluble forms of receptors are produced either through the proteolytic cleavage of membrane-bound receptors, as is the case for the interleukin-2 (IL-2) receptor (*7*) and the human tumor necrosis factor receptor (*8*), or as translation products of alternatively spliced mRNA, as is the case for the mouse IL-4 receptor (*9*) and the mouse and human IL-7 receptors (*10*). The secreted receptors represent truncated

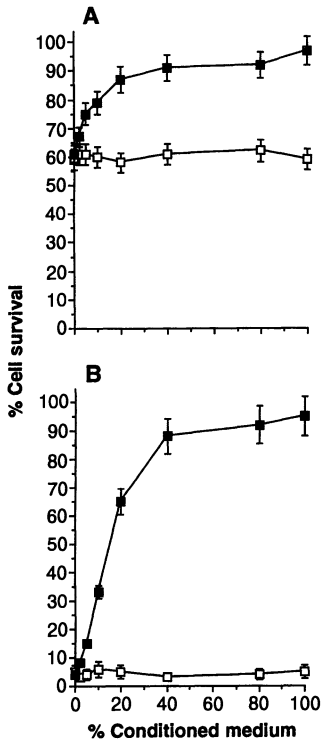

Fig. 4. Inhibition of apoptosis in vitro. (**A**) Normal human PBMC stimulated with PHA-P, then incubated in RPMI 1640 with antibody to human Fas in the presence of supernatant harvested from COS cells transfected with either full-length Fas cDNA or FasΔTM (*19*). (**B**) CEM-6 cells incubated with antibody to human Fas in the presence of supernatant as described above. The percent (mean ± SEM of three independent experiments) of surviving cells not undergoing apoptosis is indicated. Fas cDNA, open squares; FasΔTM, filled squares.

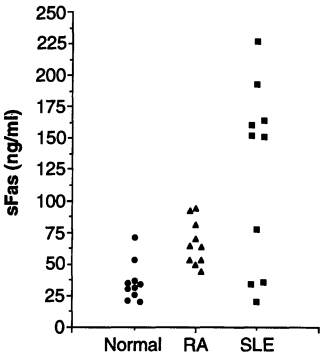

Fig. 5. Increased serum levels of soluble Fas in SLE. Sera from 10 normal individuals, 10 RA patients, or 10 SLE patients assayed for the presence of soluble Fas (sFas) by enzyme-linked immunosorbent assay (*20*). The concentrations were determined by the inclusion of dilutions of purified recombinant soluble Fas to create a standard curve.

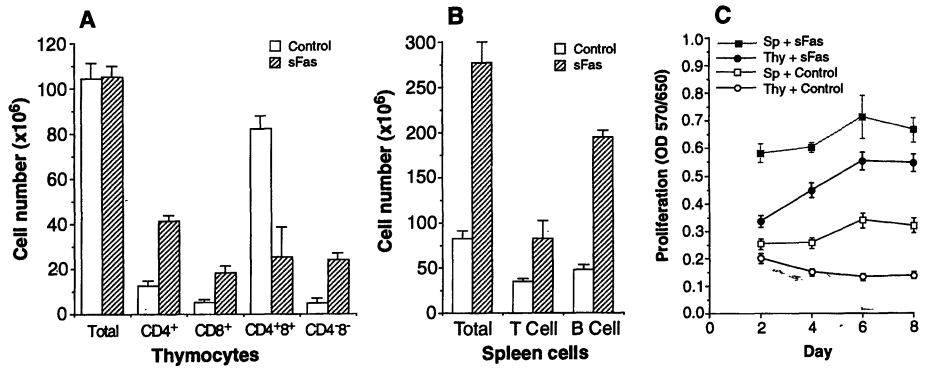

Fig. 6. Effect of sFas in vivo. (**A**) Effect of sFas on thymocyte development. Mice (8-week-old CD1 females) were treated with either a mouse sFas extracellular-domain human IgG1 fusion protein (*21*) or the control human IgG1 (2.5 μg per gram of body weight intraperitoneally) daily for 3 days. Single cell suspensions of thymocytes were counted and stained with FITC-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD8 and analyzed (10,000 events per thymus) by two-color flow cytometry analysis. (**B**) Single cell suspensions of spleen cells counted and stained with FITC-conjugated anti-CD3 and phycoerythrin-conjugated anti-B220 and analyzed (10,000 events per spleen) by two-color flow cytometry analysis. (**C**) The syngeneic mixed lymphocyte response of thymocyte or spleen cells (5×10^5) from each mouse, determined in separate cultures and assayed for proliferation at the indicated times. The proliferation was measured by MTT colorometric assay (*22*). All data represent the number of cells (mean ± SEM) of three mice per group.

forms of the membrane-bound receptors that can bind ligand in a way similar to that of their membrane-bound counterparts. As a result, these receptors play an important role in the regulation of normal receptor activity and autoimmune disease (*11*). Elevated serum concentrations of a soluble Fas were found in over one-half of patients with SLE, and soluble Fas is capable of inhibiting Fas-mediated apoptosis in vitro and altering lymphocyte development and proliferation in response to self antigen in vivo. These findings suggest a critical role for this molecule in autoimmune diseases. Therapies such as plasmapheresis that may remove soluble Fas from sera of SLE patients may restore normal apoptosis and reduce autoimmune disease (*12*). Also, in light of the fact that Fas-mediated apoptosis has been implicated in lymphocyte apoptosis in HIV infection (*3*), it is of great importance to investigate a role for FasΔTM in immunodeficiencies and whether blockade of Fas-mediated apoptosis in vivo, with the use of recombinant FasΔTM molecules, is of clinical use in such disease conditions.

REFERENCES AND NOTES

1. N. Itoh *et al.*, *Cell* **66**, 233 (1991); R. Watanabe-Fukunaga *et al.*, *J. Immunol.* **148**, 1274 (1992); A. Oehm *et al.*, *J. Biol. Chem.* **267**, 10709 (1992); N. Itoh and S. Nagata, *ibid.* **268**, 10932 (1993).
2. R. Watanabe-Fukunaga, C. I. Brannan, N. G. Copeland, N. A. Jenins, S. Nagata, *Nature* **356**, 314 (1992); M. L. Watson *et al.*, *J. Exp. Med.* **176**, 1645 (1992); M. Adachi, R. Watanabe-Fukunaga, S. Nagata, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1756 (1993); J. Wu, T. Zhou, J. He, J. D. Mountz, *J. Exp. Med.* **178**, 461 (1993).
3. J. F. Krowka, B. Cuevas, M. S. Ascher, H. W. Sheppard, unpublished data.
4. L. B. Owen-Schaub, S. Yonehara, W. L. Crump III, E. A. Grimm, *Cell. Immunol.* **140**, 197 (1992).
5. Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, *J. Cell Biol.* **119**, 493 (1992).
6. S. Takashi, T. Takahashi, P. Golstein, S. Nagata, *Cell* **75**, 1169 (1993).
7. O. Josimovic-Alasevic, T. Herrmann, T. Diamantstein, *Eur. J. Immunol.* **18**, 855 (1988).
8. T. J. Schall *et al.*, *Cell* **61**, 361 (1990).
9. B. Mosley *et al.*, *ibid.* **59**, 335 (1989).
10. R. G. Goodwin *et al.*, *ibid.* **60**, 941 (1990).
11. R. Fernandez-Botran, *FASEB J.* **5**, 2567 (1991).
12. D. J. Wallace, *N. Engl. J. Med.* **327**, 1029 (1992).
13. D. M. Klinman *et al.*, *J. Exp. Med.* **163**, 1292 (1986).
14. E. M. Tan *et al.*, *Arthritis Rheum.* **25**, 1277 (1982).
15. G. Frizzera, Y. Kaneko, M. Sakurai, *J. Leukemia* **3**, 1 (1989); A. Ohsaka *et al.*, *J. Cancer* **69**, 1259 (1992).
16. RNA (10 μg) isolated with the use of the guanidine isothiocyanate–acid phenol method was used for first-strand cDNA syntheses. Two oligonucleotides, 5′-CACTTCGGAGGATTGCTCAACA-3′ (nt 170 to 191) and 5′-TATGTTGGCTCTTCAGC GCTA-3′ (complementary to nt 1316 to 1336), were used to amplify human Fas mRNA by PCR. The PCR products were analyzed and recovered from agarose gels and subcloned into a pCR vector (Invitrogen, San Diego, CA).
17. For genomic PCR, primers were designed to flank each of the putative introns. Human genomic DNA (0.5 μg) was used as a template in each PCR reaction. All nucleotide sequences were determined by the dideoxy chain termination method with the use of modified bacteriophage T7 DNA polymerase (United States Biochemical).
18. The full-length Fas and the FasΔTM cDNAs were inserted into the Eco RI cloning site of pcDNA I (Invitrogen). The orientations of the insert were determined with Hind III digestion. COS-7 cells were transfected with the use of DEAE-dextran. After 72 hours, single cell suspensions were stained with the mouse monoclonal antibody to human Fas [immunoglobulin M (IgM)] (Upstate Biotechnology, Lake Placid, NY). After washing, the cells were stained with fluorescein isothiocyanate (FITC)–conjugated goat IgM to mouse (Southern Biotechnology, Birmingham, AL) for 30 min. As a control, the same cells were similarly treated, except that the primary antibody was replaced with an unrelated mouse IgM.
19. Cells were stimulated with PHA-P (10 μg/ml) (Pharmacia, Piscataway, NJ) for 48 hours. Approximately 5×10^5 stimulated cells were then cultured in a flat-bottom 96-well plate in RPMI 1640–10% fetal calf serum and IgM (200 ng/ml) in the presence of serial dilutions of supernatants derived from the COS-7 cells that were transfected with either Fas cDNA or FasΔTM expression plasmids and cultured for 3 days. After 12 hours of incubation, 2×10^5 cells were cytospun onto poly-L-lysine–pretreated slides, fixed in 10% (v/v) formalin at room temperature for 12 hours, and stained for in situ apoptosis. A minimum of 200 lymphocytes were counted in 10 randomly selected microscopic fields by an observer unaware of the cell culture condition. The apoptosis analysis of CEM-6 cells was carried out in a similar manner.
20. Sera were first diluted to a ratio of 1:5 in borate-buffered saline (BBS), and Ig complexes were absorbed with anti-human Ig beads at 4°C for 12 hours. Plates were precoated with IgM (2 μg/ml). After blocking, the absorbed sera were added and incubated for 4 hours at room temperature. After extensive washing, the bound antibody was detected with an antibody to mouse IgG1 that is isotype-specific and reports alkaline phosphatase, and the plates were incubated with *p*-nitrophenylphosphate substrate.
21. A 530-bp extracellular Fas sequence (nt 26 to 556) (*1*) was PCR-amplified from a mouse *fas* cDNA clone and ligated into a pSP65 vector that contained the hinge, CH2 and CH3 sequence of human IgG1 (hIgG1) [R. G. Goodwin *et al.*, *Cell* **73**, 447 (1993)]. The fusion gene was sequenced to confirm that the *fas* extracellular domain was in frame with the hIgG1 fragment. The *fas-hIgG1* fusion gene was transfected into COS cells and transiently expressed as a secreted fusion protein. This protein was purified with an anti-hIgG1 agarose affinity column and could bind to the Fas ligand (*5*).
22. MTT (Sigma) was dissolved in BBS to make a solution (5 mg/ml). The MTT-BBS solution (10 μl) was added to each well (containing 100 μl of cells) and incubated for 4 hours at 37°C in a 5% CO_2 humidified incubator. 100 μl of 0.04 M HCl in isopropanol was added to each well and was vigorously mixed by pipeting. The plates were read in a microtiter plate reader (Emax, Molecular Division, Menlo Park, CA), with the use of a 570-nm filter. Background noise was subtracted with a dual-wavelength setting of 650 nm.

23. We thank W. J. Koopman for helpful discussions, V. Prochazka for genomic DNA sequencing, T. F. Tedder for providing the hIgG1-pSP65 plasmid, J. F. Krowka for communicating results before publication, and B. K. Bunn for expert secretarial assistance in typing the manuscript. Supported in part by a Veterans Administration (VA) Merit Review Award; a VA Career Development Award; and grants P60 AR20614, P50 AI23694, P01 AR03555, and R01 AI30744 from the National Institutes of Health.

19 November 1993; accepted 4 February 1994

High-Resolution Solution Structure of the β Chemokine hMIP-1β by Multidimensional NMR

Patricia J. Lodi, Daniel S. Garrett, John Kuszewski, Monica L.-S. Tsang, James A. Weatherbee, Warren J. Leonard, Angela M. Gronenborn,* G. Marius Clore*

The three-dimensional structure of a member of the β subfamily of chemokines, human macrophage inflammatory protein–1β (hMIP-1β), has been determined with the use of solution multidimensional heteronuclear magnetic resonance spectroscopy. Human MIP-1β is a symmetric homodimer with a relative molecular mass of ~16 kilodaltons. The structure of the hMIP-1β monomer is similar to that of the related α chemokine interleukin-8 (IL-8). However, the quaternary structures of the two proteins are entirely distinct, and the dimer interface is formed by a completely different set of residues. Whereas the IL-8 dimer is globular, the hMIP-1β dimer is elongated and cylindrical. This provides a rational explanation for the absence of cross-binding and reactivity between the α and β chemokine subfamilies. Calculation of the solvation free energies of dimerization suggests that the formation and stabilization of the two different types of dimers arise from the burial of hydrophobic residues.

Human macrophage inflammatory protein–1β, also known as Act-2, is a member of the β chemokine (chemotactic cytokine) subfamily of proteins (*1*, *2*). These proteins, as well as the related α chemokines, comprise a polypeptide chain of ~8 to 10 kD and contain four cysteine residues at near-identical positions. The distinction between the α and β chemokine subfamilies was initially based on whether the first two cysteine residues are separated by one residue (α) or are adjacent (β). This division also extends to chromosomal location (chromosomes 4 and 17 for the α and β chemokines, respectively) and function. Thus, whereas the α chemokines (for example, IL-8) are potent chemoattractants