# BIOPHYSICS AND BIOCHEMISTRY

# Epitope Mapping of Human Fas Using Peptide Phage Display

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Sandwich EIA for measurement of soluble Fas was developed on the basis of SA-7 and SA-8 monoclonal antibodies to full-length human Fas. The threshold sensitivity of the test system is 0.3 ng/ml. Several isoforms of soluble Fas were identified. The structure of SA-7 and SA-8 antibody epitopes was determined using the peptide phage library. It was shown that SA-7 antibody epitope is determined by amino acid residues 129-134 (CKPNFF), while SA-8 antibody epitope is determined by amino acid residues 94-99 (KAHFSS) of full-length Fas. Hence, sandwich EIA on the basis of SA-7 and SA-8 monoclonal antibodies for detection of soluble Fas in human serum is to detect the following Fas isoforms: FasExo6Del, FasExo4Del, FasExo4,6Del, FasExo4,7Del, and FasExo8Del.

**Key Words:** *Fas; monoclonal antibodies; Sandwich EIA; peptide phage display; epitope mapping*

Fas/APO-1/CD95 is a transmembrane glycoprotein belonging to the TNF/NGF receptor family [12].

Fas is expressed in the thymus, kidneys, liver, heart, is exposed by activated lymphocytes, natural killers, virus-infected and tumor cells. Fas triggers cell apoptosis after interactions with the ligand (FasL) or agonistic monoclonal antibodies (MAb) to Fas. Despite extensive expression of Fas in malignant tumors of different genesis, many tumor cells are resistant to Fas-dependent apoptosis. The mechanisms of this resistance can be mutations in Fas genes and genes of proteins involved in the transfer of apoptotic signal from activated Fas and hyperexpression of proteins inhibiting Fas-dependent apoptosis [12]. One of these proteins is soluble Fas (sFas), a product of alternative splicing of full-length fas transcript. sFas inhibits FasL-induced cytotoxicity and renders advantages for survival and multiplication to sFas-secreting tumor cells [8].

Isoforms of sFas capable of *in vitro* inhibiting Fas-dependent apoptosis are described [7,14].

We previously obtained MAb to full-length human Fas: SA-7 ($IgG_1(k)$; $K_a=(5.8\pm0.7)\times10^8$ $M^{-1}$) and SA-8 ($IgG_1(k)$; $K_a=(4.0\pm0.6)\times10^7$ $M^{-1}$). Based on these MAbs, we developed a sandwich EIA for measurement of sFas in human serum; the minimum sensitivity of the test system is 0.3 ng/ml sFas [13].

This test system was used for measuring sFas concentrations in patients with malignant and benign tumors of different location. High levels of sFas

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were observed in malignant tumors of the bones (osteogenic sarcoma, chondrosarcoma, Ewing's tumor), in ovarian cancer, breast cancer, gastric and colorectal cancer, prostatic, adrenal, and thyroid cancers [1-6]. High levels of serum sFas in cancer patients were associated with unfavorable prognosis.

In order to clear out which Fas splicing isoforms are detected by our test system, we evaluated the structure of SA-7 and SA-8 MAb epitopes.

## MATERIALS AND METHODS

The *random* peptide library in the phage display format [9] was used. The PH.D.-7™ phage library (New England Biolab) with presentation of about $2\times10^9$ possible variants of 7-component peptides was chosen for location of minimum epitopes of antibodies. The *random* peptides in this library are expressed in the N-terminal part of g3 minor protein of M13 phage envelope and are separated from g3 protein by a flexible spacer (-G-G-G-S) for improving the interactions with the target ligands.

Phage particles reacting with SA-7 and SA-8 MAb were selected from the PH.D.-7™ library (Bio-Labs, Cat. NE8100S) as follows: SA-7 or SA-8 MAb were adsorbed overnight on EIA plates (0.5 μg/well) in 0.05 M carbonate buffer (pH 9.6) at 4°C. Free binding sites on the plastic were blocked with 1% BSA (320 μl/well). The phages were incubated with adsorbed antibodies in PSB containing 0.1% Twin-20 (PSB-T) and 0.1% BSA. After incubation free phage particles were washed 10-20 times with PSB-T, bound phage particles were eluted with 0.1 N HCl and then neutralized with 1 M NaOH and used for infection of exponential culture of *E. coli* XL-1 strain carrying fertility factor ($A_{600HM}$=0.6-0.8). Cells infected with the phages were cultured for 16-20 h and phages were isolated from culture fluid by precipitation with 20% polyethylenglycol (mol. weight 8000 kDa) solution with 2.5 M NaCl. Phages isolated after the 1st round of selection were used for selection cycle 2, *etc.* In order to select phage particles with higher affinity, the number of phage particles and the time of their incubation with MAb were reduced with each cycle: $10^{11}$ phages were taken for round 1, $10^{10}$ for round 2, $10^9$ for rounds 3 and 4, the duration of incubation being reduced from 60 to 30 min. During selection round 4, the bound phages were affinely displaced with the same antibodies as on the underlayer in order to select phage particles with higher affinity.

*E. coli* strain XL-1 cells were infected with phages of selection rounds 3 and 4 during the exponential growth phase and inoculated in Petri dishes with 1% agar with tetracycline. The dishes with 100-300 colonies were then used for the analysis of clones by indirect EIA. Nitrocellulose replicas were removed from the dishes and incubated in 1% BSA, then in solution of the corresponding antibodies (10 μg/ml), and washed 10 times in PSB-T. The replicas were then incubated with peroxidase-conjugated antibodies to mouse immunoglobulin (Amersham) and after washing the replicas were treated with 1.3 mM diaminobenzidine solution with 0.02% $H_2O_2$. Colonies giving positive signals were selected for further analysis.

Affinity constants of peptides exposed by the phages were determined.

The peptides were synthesized by the solid-phase method in a manual solid-phase reactor [3] using Fmoc strategy. The synthesis was carried out using a polymer underlayer (Wang's polymer). The polypeptide chain was elongated using 2-(1H-benzotriasole-1-yl)-1,1,3,3-tetramethyluronium tetraborate in the presence of 1-hydroxybenzotriasole and N-methylmorpholine in 4:3.6:2:4 ratio of equivalents, respectively, for polymer-bound amino acid. The Fmoc group, was removed with 20% piperidine solution in dimethylformamide (20 min). After attachment of the last amino acid and removal of the Fmoc group the peptidylpolymer was washed, removed from the reactor, and dried. The peptide was separated from polymer carryer and amino acid residues (AR) were deblocked with trifluoroacetic acid:water:ethanedithiol:triisopropylsilane mixture (94:2.5:2.5:1, v/v).

Peptides were isolated by HPLC in an ULTRDHERE OCTYL column (21.2 mm×25 cm).

DNA of individual phages was sequenced by Sanger's method using -28 gIII (5'-$^{HO}$GTA TGG GAT TTT GCT AAA CAA C-3') primer.

Interactions between SA-8 and SA-7 MAbs with peptide 116-135 of Fas extracellular part were analyzed by indirect EIA as follows. The synthetic peptide 116-135 of extracellular part of Fas was adsorbed overnight on an EIA plate (Costar No. 9017, 0.5 μg/well) in 0.05 M carbonate buffer (pH 9.6) at 4°C. Free binding sites were blocked with 1% BSA for 30 min. SA-7 or SA-8 MAb were then added in serial double dilutions from 40 to 0.3 μg/ml in PSB-T (100 μl/well). The plates with antibodies were incubated for 1 h at ambient temperature and washed several times in PSB-T solution. Antibodies to mouse immunoglobulins conjugated with horseradish peroxidase were then added and the mixture was incubated for 40 min. Orthophenylene diamine in citrate-phosphate buffer (4 mM, pH 5.0) with 0.003% $H_2O_2$ served as the substrate. After staining development, the reaction was stopped with 10% sulfuric acid. Optical density was measured at 490 nm.

Analysis of the specificity of SA-7 and SA-8 MAb interactions with synthetic peptides 94-99 and 129-134 was carried out by competitive EIA with full-length Fas antigen (Coultronics) as follows. Fas in a concentration of 1 μg/ml in 0.05 M carbonate buffer (pH 9.6) was adsorbed on an EIA plate (50 μl/well) overnight at 4°C. Free binding sites were blocked with 1% BSA for 30 min. Then SA-7 or SA-8 MAb were added in a concentration of 1 μg/ml and synthetic peptides in different volumes (from Fas equimolar ratio to 50-fold molar excess). Incubation was carried out for 2 h at ambient temperature, the plates were washed in PSB-T, and polyclonal antibodies to mouse immunoglobulins conjugated with horseradish peroxidase (Amersham) were added. The mixture was incubated for 40 min at ambient temperature, washed, and 4 mM orthophenylenediamine solution in citrate-phosphate buffer (pH 5.0) with 0.003% $H_2O_2$ was added. After staining development, the reaction was stopped with 10% $H_2SO_4$. Optical density was detected at $\lambda$=490 nm.

## RESULTS

We previously showed that SA-7 and SA-8 MAb react with denatured Fas in immunoblotting (the data not presented). This fact suggests that epitopes of each of these antibodies are not conformational, but linear.

Two peptides of the Fas extracellular part were identified (116-135 and 126-145) interacting with agonistic MAb to Fas CH-11 [10]. Hence, the epitope of CH-11 antibodies was located determining AR of full-length Fas 126-135. We synthesized peptide 116-135 and found that SA-7 (but not SA-8) MAb react with this peptide in EIA in a dose-dependent manner (Fig. 1).

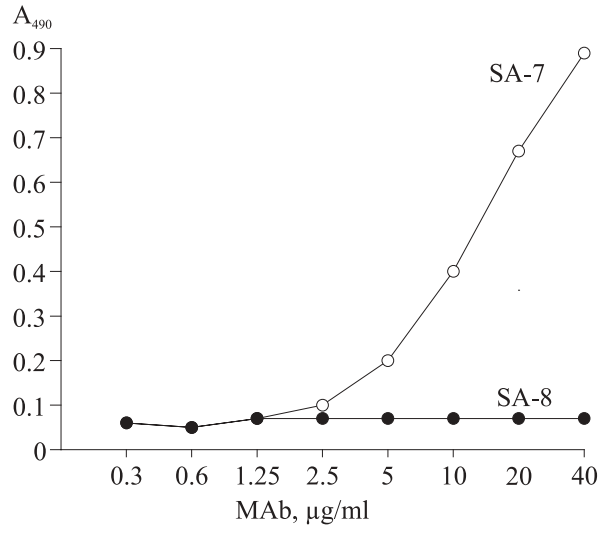


**Fig. 1.** Reactions of SA-7 and SA-8 MAb with peptide 116-135 of extracellular part of Fas.

The structure of SA-7 and SA-8 MAb epitopes was determined by 4 rounds of affine selection of phage particles from PH.D.-7™ library. After cloning the phages reacting with SA-7 and SA-8 MAb were selected, which were used for immunochemical analysis and evaluation of the structure of amino acid sequence of peptides, presented by these phages (Table 1).

The antibody consensus mimotopes were calculated and the incidence of each AR in the peptides presented by the phages was analyzed.

Histidine AR was detected in all peptides exposed by phages reacting with SA-8 MAb. The incidence of phenylalanine AR was 76.5% (82.4% with consideration for the homologous replacement by tyrosine AR). Isoleucine and lysine AR were equally incident (64.7%). Serine AR was detected in 29.4% cases (in 53% peptide sequences with consideration for equivalent replacement by threonine AR). Hence, the following AR determined the SA-8 MAb consensus mimotope: XKIHFXS. Comparison of the consensus mimotope with amino acid sequence of full-length Fas showed that SA-8 MAb epitope is determined by AR 94-99 of the Fas extracellular part (Table 2). Four of seven consensus mimotope AR corresponded to the structure of full-length Fas, while isoleucine AR between lysine and histidine AR can be considered as a homologous replacement of alanine AR in the structure of presumable SA-8 MAb epitope.

The following AR were most incident in peptides exposed by phages reacting with SA-7 MAb: phenylalanine AR (95% incidence, 100% with consideration for equivalent replacement with tyrosine AR), lysine AR (95% incidence), histidine AR (86%), isoleucine AR (76%), proline AR (53%), and phenylalanine 2nd AR (29% incidence, 43% with consideration for equivalent replacement with tyrosine AR). Hence, SA-7 MAb consensus mimotope is determined by XKIHFFP AR (Table 2).

Coincidence of SA-7 MAb consensus mimotope with the structure of full-length Fas suggests that the epitope of these antibodies is determined by AR of Fas extracellular part 130-134. Of 6 consensus mimotope sequence AR, 3 residues coincided with the structure of the presumable epitope. Histidine AR was determined in the consensus mimotope instead of $N^{132}$, both AR being neutral by charge. The hypothesis about the structure of SA-7 MAb epitope is confirmed by the data on their specific reaction with synthetic peptide 116-135 of Fas extracellular part (Fig. 1).

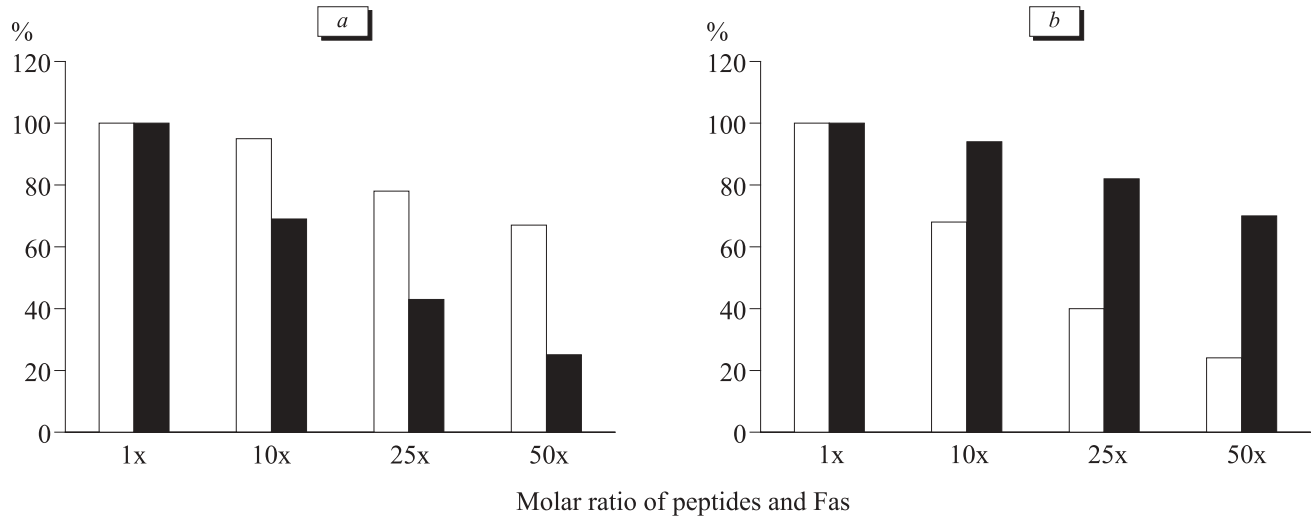


**Fig. 2.** Inhibition of interactions between SA-7 (*a*) and SA-8 MAb (*b*) and Fas with peptides No. 1 (light bars) and No. 2 (dark bars).

The structures of SA-7 and SA-8 MAb consensus mimotopes are similar, and therefore we determined the affinity constants of all selected phages for MAb used for selection. No direct correlations between the number of AR in the mimotope sequence coinciding with the structure of presumable epitope and the affinity constant value were detected for SA-8-specific phages (Table 1). On the other hand, it was shown that affinity constants of phage particles carrying X**K**XX**FF**X peptides (3

**TABLE 1.** Structure of Peptides Exposed by Phages of PH.D.-7™ Library, Reacting with SA-7 and SA-8 MAb to Full-Length Human Fas

| Clone | Peptide structure in phage | $K_a$, $M^{-1}$ | Clone | Peptide structure in phage | $K_a$, $M^{-1}$ |
|---|---|---|---|---|---|
| SA-8/III-1 | A**K**I**HF**Y**S** | $3.9*10^6$ | SA-7/IV-1 | G**K**I**HFF**R | $7*10^7$ |
| SA-8/III-2 | **DK**I**HF**K**S** | $5.6*10^6$ | SA-7/IV-2 | G Y**KP**T**F**S | $1.1*10^7$ |
| SA-8/III-3 | V N**K**P**HF**H | $7.0*10^6$ | SA-7/IV-3 | G Y**KP**T**F**S | $1.1*10^7$ |
| SA-8/V-1 | AY**I****HF**LP | $4*10^7$ | SA-7/IV-4 | S**K**I**H****F**HP | $8.0*10^6$ |
| SA-8/IV-2 | SE**I****H**LMG | $5.0*10^6$ | SA-7/IV-5 | G**K**I**HFF**R | $7*10^7$ |
| SA-8/IV-3 | A**K**I**HF**H**S** | $4.5*10^6$ | SA-7/IV-6 | G**K**I**HFF**R | $7*10^7$ |
| SA-8/IV-4 | H**K**P**HF**P**S** | $2.6*10^6$ | SA-7/IV-7 | N**K**I**H****F**Y**P** | $6.6*10^6$ |
| SA-8/IV-5 | A LQI**H**TM | $3.9*10^6$ | SA-7/IV-8 | G**K**I**HFF**R | $7*10^7$ |
| SA-8/IV-6 | AQI**HF**LP | $4.0*10^6$ | SA-7/IV-9 | S**K**I**H****F**LS | $9.4*10^6$ |
| SA-8/IV-7 | **D**NP**H**YTT | $3.6*10^6$ | SA-7/IV-10 | S**K**I**H****F**HP | $8.0*10^6$ |
| SA-8/IV-8 | G**K**P**HF**TT | $3.4*10^6$ | SA-7/IV-11 | G**K**I**H****F**HP | $9.8*10^6$ |
| SA-8/IV-9 | SF**H**IYFL | $7.5*10^6$ | SA-7/IV-12 | S**K**I**H****F**PE | $1.8*10^7$ |
| SA-8/IV-10 | S**K**I**HF**HT | $5.0*10^6$ | SA-7/IV-13 | C**K**I**HFF**R | $1.7*10^8$ |
| SA-8/IV-11 | R**K**P**HF**TG | $6.8*10^6$ | SA-7/IV-14 | D**K**I**H****F**Y**P** | $1.0*10^7$ |
| SA-8/IV-12 | L**K**L**HF**NT | $4.2*10^6$ | SA-7/IV-15 | RYYPY**F**P | $1.1*10^7$ |
| SA-8/IV-13 | A**K**I**HF**YS | $5.8*10^6$ | SA-7/IV-16 | A**K**I**H****F**PP | $1.5*10^7$ |
| SA-8/IV-14 | R**K**I**HF**HP | $3.6*10^6$ | SA-7/IV-17 | E**KP**H**F**LV | $9.8*10^6$ |
| | | | SA-7/IV-18 | A**K**I**H****F**LP | $7.0*10^6$ |
| | | | SA-7/IV-19 | H**K**I**H****F**LP | $1.5*10^7$ |
| | | | SA-7/IV-20 | R**K**LH**F**LP | $9.3*10^6$ |
| | | | SA-7/IV-21 | S**K**I**H****F**Y**H** | $5.3*10^6$ |

**Note.** Here and in Table 2: AR coinciding with Fas structure in presumable antibody epitope are shown with bold letters; AR homologous to those in the structure of presumable epitope are underlined.

**TABLE 2.** Structure of SA-7 and SA-8 MAb Consensus Mimotopes

| Structure of full-length Fas | Structure of MAb consensus mimotope | Incidence of AR, % |
|---|---|---|
| SA-7 | | |
| $C^{129}$ | X | — |
| $K^{130}$ | **K** | 95 |
| $P^{131}$ | I | 76 |
| $N^{132}$ | H | 86 |
| $F^{133}$ | **F** | 95(100%—F+Y) |
| $F^{134}$ | **F** | 29(43%—F+Y) |
| $C^{135}$ | P | 53 |
| SA-8 | | |
| $D^{93}$ | X | — |
| $K^{94}$ | **K** | 65 |
| $A^{95}$ | I | 65 |
| $H^{96}$ | **H** | 100 |
| $F^{97}$ | **F** | 76(82%—F+Y) |
| $S^{98}$ | X | — |
| $S^{99}$ | **S** | 29(53%—S+T) |

coinciding residues of 7) were among the highest ($7\times10^7$ $M^{-1}$) for SA-7-specific phages. The highest affinity constant ($1.7\times10^8$ $M^{-1}$) was determined for phages exposing the **CK**XX**FF**X mimotope sequence (4 coinciding AR of 7) (Table 1). It seems that cysteine AR is very important for interactions between SA-7 MAb and Fas, and hence, we included the cysteine AR in SA-7 MAb presumable epitope. Hence, we hypothesized that SA-7 epitope was determined by AR 129-134 of Fas extracellular part.

Hexamer synthetic peptides were created: peptide No. 1 (94-99) corresponding to presumable SA-8 MAb epitope (KAHFSS) and peptide No. 2 (129-134) corresponding to presumable SA-7 antibody epitope (CKPNFF).

The structure of antibody epitopes was confirmed in experiments on competitive inhibition of interactions between SA-7 and SA-8 MAbs with full-length Fas by synthetic peptides No. 1 and No. 2 (Fig. 2).

Peptide No. 1 corresponding to presumable SA-8 MAb epitope preferably inhibited the reaction of these antibodies with full-length Fas and did not modify the reaction between SA-7 MAb and Fas (Fig. 2). On the other hand, peptide No. 2 corresponding to presumable SA-7 MAb epitope preferably inhibited the reaction between SA-7 MAb with full-length Fas. We therefore concluded that peptide No. 1 was specific for SA-8 MAb and peptide No. 2 for SA-7 MAb.

Hence, the structure of SA-7 and SA-8 MAb to full-length human Fas is determined. SA-7 MAb epitope is determined by Fas AR 129-134 (CKPNFF), while SA-8 MAb epitope is determined by Fas AR 94-99 (KAHFSS).

The sFas isoforms are a result of alternative splicing of full-length Fas mRNA consisting of 9 exons. Several variants of Fas spliced mRNA with deletions of different exons are identified: FasExo6-Del, FasExo3,4Del, FasExo3,4,6Del, FasExo4Del, FasExo4,6Del, FasExo4,7Del, FasExo8Del, and FasExo3Del. The main sFas isoform is FasExo6-Del, in which only the transmembrane domain is deleted, while the rest sFas isoforms are presented in minor amount [7]. All sFas isoforms contain exons 1 and 2. SA-8 MAb epitope is determined by AR 94-99 in Fas exon 2, that is, SA-8 MAb react with all sFas isoforms. Exon 3 is deleted with nucleotides 391-529 excised from full-length Fas mRNA (AR 131-200 of full-length Fas primary amino acid sequence are removed). SA-7 MAb epitope is determined by full-length Fas AR 129-134 at the interface of exons 2 and 3, and therefore SA-7 MAb cannot react with sFas isoforms with deleted exon 3. Hence, sandwich EIA based on SA-7 and SA-8 MAb should detect the following sFas isoforms: FasExo6Del, FasExo4Del, FasExo4,6Del, FasExo4,7Del, and FasExo8Del.

## REFERENCES


1. S. G. Abbasova, N. E. Kushlinskii, A. N. Murashev, *et al.*, *Byull. Eksp. Biol. Med.* **127**, No. 3, 328-331 (1999).
2. N. E. Kushlinskii, T. A. Britvin, S. G. Abbasova, *et al.*, *Ibid.*, **131**, No. 4, 430-433 (2001).
3. N. E. Kushlinskii, T. A. Britvin, G. A. Polenova, *et al.*, *Ibid.*, **132**, No. 10, 443-445 (2001).
4. N. E. Kushlinskii, T. A. Britvin, G. A. Polenova, *et al.*, *Ibid.*, **134**, No. 8, 197-201 (2002).
5. N. E. Kushlinskii, Yu. N. Solovyov, I. V. Babkina, *et al.*, *Ibid.*, **129**, No. 5, 583-586 (2000).
6. N. E. Kushlinskii, Yu. N. Solovyov, I. V. Babkina, *et al.*, *Vestn. Rossiisk. Akad. Med. Nauk*, No. 3, 3-8 (2001).
7. I. Cascino, G. Papoff, A. Eramo, and G. Ruberti, *Front. Biosci.*, **1**, d12-d18 (1996).
8. J. Cheng, T. Zhou, C. Liu, *et al.*, *Science*, **263**, No. 5154, 1759-1762 (1994).
9. R. Cortese, F. Fellcl, G. Galfre, *et al.*, *Trends Biotechnol.*, **12**, No. 7, 262-267 (1994).
10. B. Fadeel, J. Thorpe, and F. Chiodi, *Int. Immunol.*, **7**, No. 12, 1967-1975 (1995).
11. G. Fisher, F. J. Rosenberg, S. E. Straus, *et al.*, *Cell*, **81**, No. 6, 935-946 (1995).
12. S. Nagata, *Ibid.*, **88**, No. 3, 355-365 (1997).
13. I. L. Rodionov, M. B. Baru, and V. T. Ivanov, *Pept. Res.*, **5**, No. 2, 119-125 (1992).
14. H. Schumann, H. Morawietz, K. Hakim, *et al.*, *Biochem. Biophys. Res. Commun.*, **239**, No. 3, 794-798 (1997).