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Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumor Necrosis Factor/Nerve Growth Factor Receptor Superfamily

SEQUENCE IDENTITY WITH THE Fas ANTIGEN*

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The APO-1 antigen as defined by the mouse monoclonal antibody anti-APO-1 was previously found to be expressed on the cell surface of activated human T and B lymphocytes and a variety of malignant human lymphoid cell lines. Cross-linking of the APO-1 antigen by anti-APO-1 induced programmed cell death, apoptosis, of APO-1 positive cells. To characterize the APO-1 cell surface molecule and to better understand its role in induction of apoptosis, the APO-1 protein was purified to homogeneity from membranes of SKW6.4 B lymphoblastoid cells by solubilization with sodium deoxycholate, affinity chromatography with anti-APO-1 antibody, and reversed phase high performance liquid chromatography. Each purification step was followed by an APO-1-specific solid phase enzyme-linked immunosorbent assay using the monoclonal antibody anti-APO-1. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the APO-1 antigen was found to be a membrane glycoprotein of 48-kDa. Endoproteinase-cleaved peptides of the APO-1 protein were subjected to amino acid sequencing, and corresponding oligonucleotides were used to identify a full-length APO-1 cDNA clone from an SKW6.4 cDNA library. The deduced amino acid sequence of APO-1 showed sequence identity with the Fas antigen, a cysteine-rich transmembrane protein of 335 amino acids with significant similarity to the members of the tumor necrosis factor/nerve growth factor receptor superfamily. The APO-1 antigen was expressed upon transfection of APO-1 cDNA into BL60-P7 Burkitt's lymphoma cells and conferred sensitivity towards anti-APO-1-induced apoptosis to the transfectants.

Apoptosis is the most common form of eucaryotic cell death. It is found, *e.g.* in tissue replacement, in organ development and metamorphosis, in tissue atrophy, and in tumor regression. Apoptosis is induced by diverse agents such as glucocorticoids, cytostatic drugs, cytolytic cytokines such as tumor necrosis factor (TNF)[1] and lymphotoxin, and in target cells of various killer cells including cytotoxic T lymphocytes. Its most prominent morphological features are condensation of the chromatin and membrane blebbing (zeiosis). In many cells undergoing apoptosis, an endonuclease is induced that cuts the genomic DNA into polynucleosomal fragments revealed on agarose gels as a "DNA ladder" (1, 2).

We have recently raised the mouse monoclonal antibody anti-APO-1, which defined a novel cell surface antigen, APO-1, on activated human T and B lymphocytes and on malignant human lymphoid cell lines. Anti-APO-1 binding to the APO-1 antigen abrogated growth by induction of apoptosis of sensitive cells *in vitro*. Likewise, *in vivo*, a single injection of anti-APO-1 into nu/nu mice with human lymphoid tumor xenotransplants eliminated these tumors by induction of apoptosis (3–5).

To further understand anti-APO-1-induced apoptosis, the APO-1 antigen was characterized. We report here the purification of the APO-1 antigen and the isolation of a cDNA clone for this protein. The deduced amino acid sequence for APO-1 revealed a significant sequence similarity to the members of the tumor necrosis factor/nerve growth factor receptor superfamily. Comparison of the APO-1 antigen with the recently published sequence of the Fas antigen (6) showed that both molecules are identical. The Fas antigen was originally defined by an other cytolytic antibody named anti-Fas (7).

MATERIALS AND METHODS

APO-1-specific ELISA—For detection and quantification of APO-1, each purification step was followed by an ELISA. Serial dilutions (in H_2O) of APO-1-containing samples were coated into flat bottom 96-well immunoassay plates (ICN Biomedicals, Meckenheim, Ger-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X63717.

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[1] The abbreviations used are: TNF, tumor necrosis factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); NGF, nerve growth factor.

many) (100 μl final volume/well) with 0.1 volume of 1 M $NaHCO_3$, pH 9.6. After 2 h incubation, the liquid was removed, and the plates were washed four times with 200 μl of 0.05% Tween 40 in PBS, pH 7.3. The washing buffer was removed, and the bound APO-1 antigens were incubated with anti-APO-1 IgG2b monoclonal antibody (2 μg/ml in culture medium) for 1 h (100 μl/well). The IgG2b anti-APO-1 class switch variant was derived from the original IgG3 anti-APO-1 secreting hybridoma by sequential sublining. The IgG2b and the IgG3 anti-APO-1 showed identical binding specificity toward APO-1.[2] Plates were washed again before an horseradish peroxidase-conjugated goat anti-mouse IgG2b antiserum (0.5 mg/ml, Dianova, Hamburg, Germany) 1:1000 diluted in PBS, 5% fetal calf serum, 0.05% Tween 40, pH 7.3, was added at 100 μl/well for 1 h. The plates were washed again before 75 μl of substrate buffer were added to each well (100 mM Na_2HPO_4, 50 mM citric acid, 0.1% orthophenylenediamine, 0.003% H_2O_2). After 10 min, the reaction was stopped by adding 25 μl of 3 N H_2SO_4/well. The intensity of a given coloration was measured by extinction at 492 nm (Titertek Multiscan plus, Flow Laboratories, Meckenheim, Germany). The ELISA was carried out in duplicates. One unit of APO-1 antigenic activity was arbitrarily defined as the amount of antigen needed to give half-maximal absorbance in the ELISA.

Purification of the APO-1 Antigen—Membranes from 5 × 10^{10} SKW6.4 cells prepared by a Tween 40 method (9) were solubilized with 2% sodium deoxycholate, 0.01 mM Tris-HCl, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride, pH 8.1, incubated for 1 h at 4 °C, and centrifuged at 100,000 × *g* for 1 h. The supernatant was first applied to a mouse IgG3 (FII23, IgG3 nonbinding control antibody) Sepharose 4B column (3 mg of monoclonal antibody, 4 ml of beads) to adsorb nonspecific binding material. The flow-through was then applied to an IgG3 anti-APO-1 column (9 mg of monoclonal antibody, 12 ml of beads). The anti-APO-1 affinity column was washed first with 0.1% sodium deoxycholate, 0.01 mM Tris-HCl, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride, pH 8.1 (buffer 1), followed by 0.15 M NaCl in the above buffer and finally with buffer 1 again until absorbance at 280 nm returned to baseline. Bound material was eluted with 0.05 M diethylamine/HCl, 0.1% sodium deoxycholate, 0.1% NaN_3, 0.1% phenylmethylsulfonyl fluoride, pH 11.5, and neutralized immediately. Fractions with high APO-1 antigenic activity were pooled, brought to 10% acetonitrile, and chromatographed at 1 ml min^{-1} on a reversed phase HPLC column (PLRP-S column, 300 A, 8 μm, 250 × 4.6 mm, Polymer Laboratories, Shropshire, UK) using a linear gradient from 5–70% acetonitrile in water containing 0.1% trifluoroacetic acid. Peak fractions were pooled again and subjected to a second round of HPLC under the above conditions.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels was performed as described (10). Proteins in the gel were visualized by silver staining (11).

Protein Determination—For protein determination, gels were silver-stained, and the amount of APO-1 antigen was estimated by comparing the intensity of the stain with a standard protein (bovine serum albumin) band stained with similar intensity. Protein concentration in extracts was determined by the method of Bradford (12).

Deglycosylation—200 ng of lyophilized protein samples were dissolved in 20 μl of 100 mM Tris/HCl, containing 0.5% SDS, 1% CHAPS, 5% β-mercaptoethanol, 10 mM EDTA, pH 7.0, denatured at 100 °C for 2 min, and incubated with 0.3 units of endoproteinase F/*N*-glycosidase F (Boehringer, Mannheim, Germany) at 37 °C for 4 h. Reactions were analyzed by 12% SDS-PAGE.

Immunoblot Analysis—100-ng aliquots of pure APO-1 antigen were lyophilized and resuspended in SDS-PAGE sample buffer containing 3% SDS. The samples were heated to 95 °C for 5 min and analyzed by 12% SDS-PAGE under nonreducing conditions. After electrophoresis, proteins in the gel were immunoblotted (13) onto an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) with anti-APO-1 (IgG2b).[2] The immobilized monoclonal antibody was detected with horseradish peroxidase-labeled goat-anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL.

Biological Assay for APO-1 Antigenic Activity—2 × 10^4 SKW6.4 cells were incubated in duplicates in flat bottom 96-well microtiter plates (Technomara, Fernwald, Germany) for 24 h with various amounts of purified APO-1 antigen in the presence of either 20 ng/ml isotype-matched nonbinding control monoclonal antibody FII23 (3) or 20 ng/ml anti-APO-1. Prevention of cell death (%) was measured by [^{3}H]thymidine deoxyribose incorporation after pulsing the cells with 0.5 μCi of[^{3}H]thymidine deoxyribose (Amersham, Braunschweig, Germany) for the last 6 h of the culture according to the formula $cpm_{APO\text{-}1\text{-}anti\text{-}APO\text{-}1}/cpm_{APO\text{-}1\text{-}FII23} \times 100$.

Amino Acid Sequencing—10 μg (210 pmol) of purified APO-1 were digested with Asp-N endoproteinase (Boehringer, Mannheim, Germany) according to the manufacturer's instructions to generate peptides that were separated by C8 reversed phase using a 2.1 × 100-mm Brownlee Aquapore RV-300 column and a nonlinear 0–85% acetonitrile gradient. The purified peptides were submitted to automated Edman degradation using an ABI 470A gas phase Sequenator (Applied Biosystems, Weiterstadt, Germany). The program cycles supplied by ABI were modified (one additional coupling step) to increase the repetitive yield. The phenylthiohydantoin derivatives of amino acids were identified by HPLC in an ABI 120A PTH Analyzer with an ABI 900 Data Analysis Module. The HPLC injection vent was equipped with a 100-μl sample loop, allowing the analysis of 83% of the phenylthiohydantoin samples, in order to improve the sensitivity of the system. Sequence analyses were carried out on 30–50-pmol samples, and repetitive yields of 83–93% were obtained.

cDNA Cloning—The primers 5′-CCGCTGCAGA(T,C)AC(A,G,C,T)GC(A,G,T,C)GA(A,G)CA(A,G)AA(A,G)GT (upstream primer, sense) and 5′GGGGAATTCTA(A,G,T,C)GC(T,C)TC(T,C)TT(T,C)TT(A,G,T,C)CC(A,G)TG (downstream primer, anti-sense) were constructed according to the peptide sequences DTAEQKV (upstream, residues 268–275) and HGKKEAY (downstream, residues 285–291). Reaction mixes of 50 μl (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dGTP, dATP, dGTP, dTTP) contained 50 ng of cDNA prepared from poly(A^+)-enriched SKW6.4 RNA (Amersham Corp., Braunschweig, Germany, cDNA Synthesis System Plus-Kit), 100 pmol of each primer mixture and 1 unit of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus Instruments). 35 cycles (94 °C, 1 min; 48 °C, 2 min; 72 °C, 2 min) were performed (14). The amplified DNA (68 base pairs) was inserted into the vector pBluescript-KS (Stratagene, La Jolla, CA), taking advantage of the restriction endonuclease sites present in the 5′-ends of the primers. Transformed bacteria carrying the APO-1-specific insert were identified by colony hybridization probing with oligonucleotide mixtures derived from internal sequences AA(T,C)TGGCA(T,C)CA(A,G)(TT(A,G);CT(A,G,T,C))CA(T,C)GG corresponding to residues 279–286 (NWHQLHG). A SKW6.4 cDNA library was synthesized from 5 μg of Poly(A^+) RNA of SKW6.4 cells using a cDNA synthesis kit (Pharmacia LKB Biotechnology Inc.). Fragments longer than 0.5 kb were selected by Sepharose CL-4B chromatography and inserted into the *Eco*RI cut vector λ gt10. The cDNA library comprised 10^6 independent clones and had an average insert size of 1 kb. The library was amplified once.

DNA Sequence Analysis—The 2.55-kb fragment was inserted into the pBluescript-KS vector, and unidirectional deletions were generated using exonuclease III/S1 nuclease (Boehringer, Mannheim, Germany). Sequences were determined by the dideoxy sequencing method (15) using universal sequencing primers or APO-1-specific primers and the Sequenase DNA Sequencing Kit (U. S. Biochemical Corp.). Computer analyses were made with the HUSAR software package of the German Cancer Research Center, Heidelberg, Germany.

Northern Hybridization—Total RNA (10 μg) in sample buffer containing 2.2 M formaldehyde were electrophoresed on a 1% agarose gel, blotted, and hybridized with the 2.55-kb cDNA fragment of APO-1. The fragment was labeled using the Random Primed DNA Labeling Kit (Boehringer, Mannheim, Germany).

Generation of Stable Transfectants—The Burkitt's lymphoma cell line BL 60-P7 (16) was used for electrotransfection, which was performed as described (17). Briefly, 2 × 10^7 cells and 20 μg DNA in a total volume of 200 μl of PBS were placed in an electroporation chamber with an electrode distance of 4 mm. A high voltage of 200 V (capacitance 960 microfarads) was applied with an electropulsing device (Bio-Rad). After 5 min on ice, 20 ml of growth medium were added, and cells were incubated for 24 h. Then, 2.5 × 10^5 cells/ml were transferred to 96-, 48-, and 24-well microtiter plates for selection of hygromycin B-resistant clones (250 μg/ml, Sigma). APO-1 expression was measured 22–31 days after transfection. 10^6 cells were treated with anti-APO-1 (IgG3) or the isotype-matched nonbinding control monoclonal antibody FII23. The second antibody was goat anti-mouse Ig fluorescein isothiocyanate-conjugated F(ab′)$_2$ (70 μg/ml) fragment (Dianova, Hamburg, FRG). Fluorescence was measured on a FACScan (Becton Dickinson).

DNA Fragmentation Analysis—Cells (10^6/ml) were incubated either with anti-APO-1 (1 μg/ml) or with FII23 monoclonal antibody

[2] J. Dhein, P. T. Daniel, B. C. Trauth, A. Oehm, P. Möller, and P. H. Krammer, submitted for publication.

(1 μg/ml) in culture medium at 37 °C. At various times, aliquots of 10^6 cells were removed, and DNA was prepared. Cells were washed with cold PBS and disrupted with NTE buffer, pH 8 (100 mM NaCl, 10 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) containing 1% SDS and proteinase K (0.2 mg/ml). After incubation for 24 h at 37 °C, samples were extracted twice with phenol plus chloroform (1:1, v/v) and precipitated by ethanol. The DNA was dissolved in 50 ml of NTE buffer and digested with ribonuclease (1 mg/ml) at 37 °C for 30 min. Samples were analyzed on a 1.8% agarose gel in the presence of 0.5 μg/ml ethidium bromide (3).

RESULTS

Purification of the APO-1 Antigen—APO-1 was purified following the purification procedure outlined in Fig. 1. Membranes of SKW6.4 B lymphoblastoid cells were solubilized with sodium deoxycholate using a deoxycholate:protein ratio (w/w) of 7.5:1. The sodium deoxycholate extract with no detectable APO-1 antigenic activity was loaded onto an anti-APO-1 affinity column and 3.3×10^5 units of APO-1 antigenic activity were eluted with diethylamine/HCl, pH 11.5 (Fig. 2*A*). Purification to homogeneity was achieved by two consecutive chromatographic separations by reversed phase HPLC (Fig. 2*B*). There was some loss of antigenic activity at this step, since only 1.5×10^4 units of APO-1 antigenic activity was eluted from the second HPLC column (Table I). Fig. 2*C* shows the analysis of the purified APO-1 antigen by SDS-PAGE and silver staining after affinity chromatography (*lane 1*) and the first (*lane 2*) and the second (*lane 3*) round of reversed phase HPLC. A single band of apparent M_r 48,000 was revealed for the purified APO-1 antigen under nonreducing conditions. After reduction, the APO-1 protein had a M_r of 51,000 indicating that the APO-1 molecule consists of a single polypeptide chain with intramolecular disulfide bonds (Fig. 2*D*, *lane 1*). The purified APO-1 molecule was identified as a glycoprotein with 8-kDa *N*-glycosidic-linked polysaccharides, representing 16% of the total protein. Fig. 2*D*, *lane 2* shows the 43-kDa APO-1 protein after treatment with endoproteinase F/*N*-glycosidase F under reducing conditions. Deglycosylation of the APO-1 protein under nonreducing conditions showed no decrease in molecular weight (data not shown). A 48-kDa band specific for APO-1 was also stained in a Western blot analysis under nonreducing conditions (Fig. 3*A*, *lane 2*). Under reducing conditions, the Western blot did not develop (data not shown), indicating that the epitope recognized by anti-APO-1 is dependent on intramolecular disulfide bonds. Chromatofocusing on Mono-P revealed that APO-1 has a pI of 5.4–5.7 (data not shown). The purified APO-1 protein was capable of preventing anti-APO-1 induced growth inhibition and apoptosis of SKW6.4 cells (Fig. 3*B*).

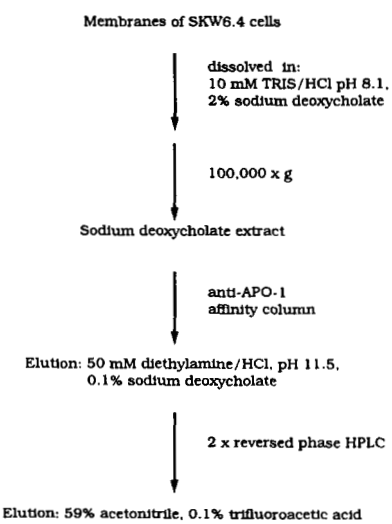

FIG. 1. **Flow sheet for the purification of the APO-1 antigen.**

Isolation and Characterization of a cDNA Clone for the APO-1 Antigen—An N-terminal amino acid sequence of the purified APO-1 protein was not obtained, indicating that the N terminus was blocked. Therefore, 10 μg of APO-1 protein were cleaved by Asp-N endoproteinase. Resulting peptides were separated by reversed phase HPLC and were subjected to amino acid analysis (Table II). The sequence information of peptide 1 was used to synthesize short degenerate oligonucleotides. These oligonucleotides were used as primers in polymerase chain reaction with SKW6.4 cDNA, and an unambiguous cDNA probe matching one of the sequenced APO-1 peptides was obtained. With this probe, four hybridizing plaques out of 1×10^6 plaques were identified in an SKW6.4 cDNA library. The clone with the longest insert was analyzed further. A single open reading frame of 1005 nucleotides was found, starting with an ATG (nucleotide 221) that is preceded by an in-frame TGA termination codon (nucleotides 62–64). The sequence flanking the assigned ATG (CAACCATGC) contains 7 of 9 residues identical with the translation initiation consensus sequence (18). The open reading frame predicts a protein containing 335 amino acids with typical features of a transmembrane protein (Fig. 4). Amino acids 1–16 are hydrophobic and probably constitute a leader peptide with a putative cleavage point between A^{16} and R^{17} (19). The mature protein has a predicted molecular mass of 36 kDa. A second hydrophobic region was found at position 172–190, flanked on both sides with positively charged residues (R^{171} and KRK^{193}) indicative of a transmembrane region. The N-terminal 155 amino acids of the mature protein contain 18 cysteine residues and two potential *N*-linked glycosylation sites. The C-terminal 145 amino acids represent the putative intracellular part of APO-1.

The deduced APO-1 protein sequence revealed significant sequence similarity to the cysteine-rich region of the low affinity nerve growth factor (NGF) receptor (20), the B cell activation antigen CD40 (21), both types of TNF receptor (22–24), the rat T cell activation antigen OX40 (25), the deduced protein sequence of the murine cDNA 4-1BB (26) expressed upon T cell activation, and the Shope fibromavirus T2 protein (27). Furthermore, the APO-1 protein sequence was found to be identical with the recently published sequence of the Fas antigen (6). A consensus alignment of the cysteine-rich domains is shown in Fig. 5*A*. Sequence similarity was also found when the intracellular domains of APO-1, CD40, NGF receptor, and the TNF receptor sequences were compared (Fig. 5*B*). A threonine residue implicated in CD40 signaling (T^{254}) (28) is also conserved in TNF receptor 1 (T^{300}) and in APO-1 (T^{241}).

Functional Expression of the APO-1 Antigen—The cDNA of APO-1 was used to generate stable transfectants in Burkitt's lymphoma BL60-P7 cells (primarily APO-1 negative and APO-1 mRNA negative (see Fig. 7, *lane 2*). A 1070-base pair *Sst*II-*Xba*I fragment comprising 70 base pairs of the 5′-untranslated and the APO-1 coding region was inserted into the eucaryotic expression vector pKEX-2-XR (29) yielding pKEX-2-XR-APO-1. 34 hygromycin B-resistant transfectants were isolated. About 40% of these clones showed immunofluorescence staining specific for APO-1. One APO-1-positive (K50) and one APO-1-negative clone (K04) were chosen for further analysis. K04 showed no APO-1 surface expression, and its growth was not inhibited by the anti-APO-1 monoclonal antibody (Fig. 6, *A* and *B*). K50 showed APO-1 cell surface expression, and its growth was inhibited by anti-APO-1 (Fig. 6, *C* and *D*). Growth inhibition of K50 was due

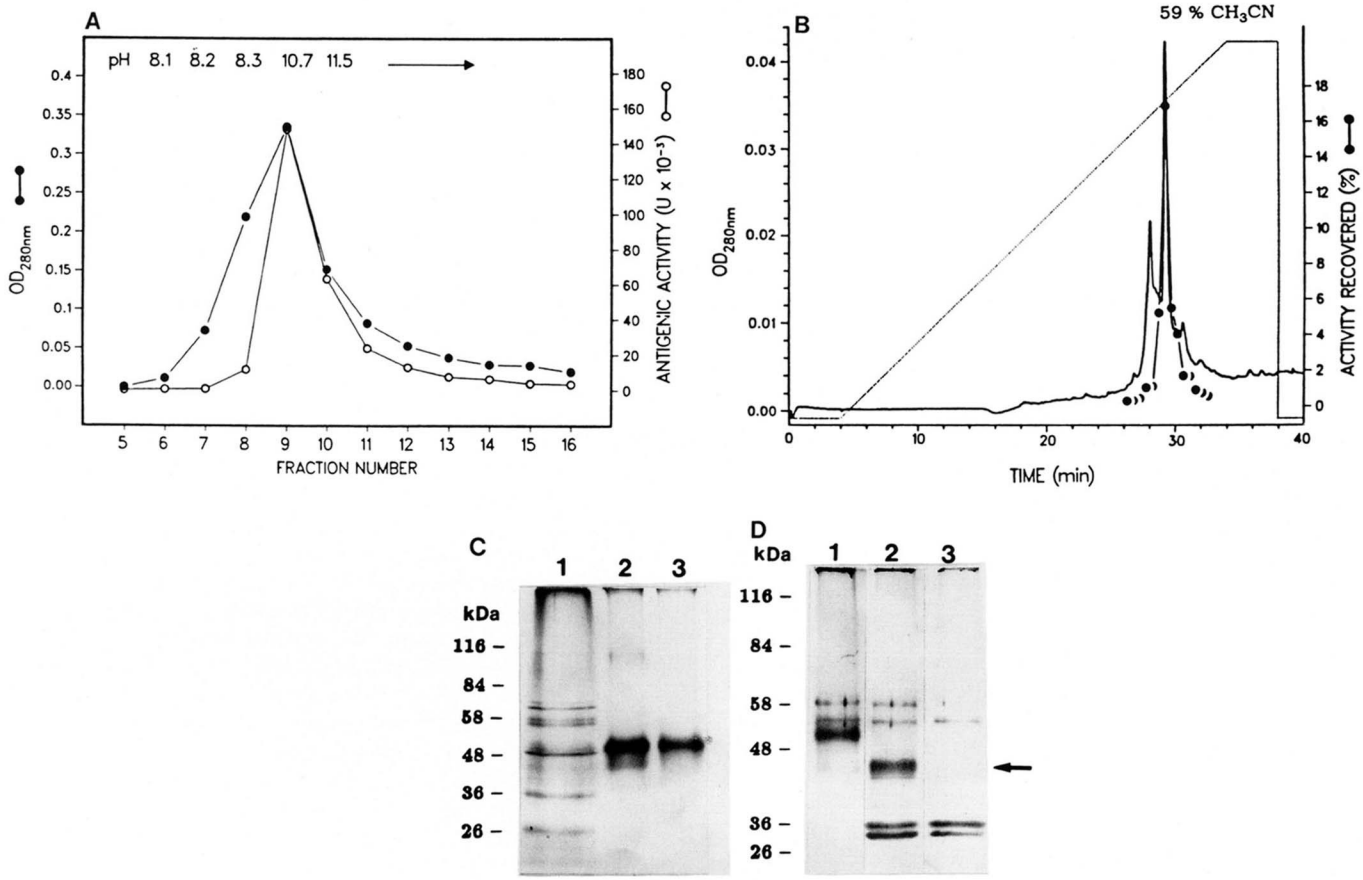

FIG. 2. **Purification of APO-1.** *A*, elution of APO-1 from an anti-APO-1 affinity column. A sodium deoxycholate extract from membranes of 5×10^{10} SKW6.4 cells was loaded onto an anti-APO-1 IgG3 Sepharose 4B affinity column and eluted with 0.05 M diethylamine/HCl, 0.1% sodium deoxycholate, 0.1% NaN_3, pH 11.5. Two-ml fractions were collected and assayed for pH, absorbance at 280 nm (●), and antigenic activity (○). *B*, reversed phase HPLC of APO-1. Pooled APO-1-containing fractions adjusted to 10% acetonitrile from the anti-APO-1 affinity column were chromatographed on a PLRP-S column using a linear gradient from 5–70% acetonitrile in 0.1% trifluoroacetic acid. The chromatographic separation of first round reversed phase fractions with high antigenic activity is shown. 0.5-ml fractions were collected and assayed for antigenic activity (●). *C*, SDS-PAGE showing the purified APO-1. Pooled fractions (100 ng) with high antigenic activity of the anti-APO-1 affinity column (*lane 1*), of the first round HPLC (*lane 2*), and of the second round HPLC (*lane 3*) were analyzed by 12% SDS-PAGE under nonreducing conditions. Proteins were visualized by silver-staining. *D*, deglycosylation of APO-1. 200 ng samples of purified APO-1 antigen were incubated for 4 h at 37 °C in the presence of 5% β-mercaptoethanol (*lane 1*) and 0.3 units of endoglycosidase F/*N*-glycosidase F (*lane 2*). Samples were analyzed by 12% SDS-PAGE. *Lane 3*, enzymes in reaction buffer alone.

TABLE I

Purification of APO-1

APO-1 antigenic activity was determined by solid phase ELISA with the anti-APO-1 monoclonal antibody

Fraction	Total protein	Antigenic activity	Specific activity	Yield
	mg	*units* × 10^{-3}	*units* × 10^{-3} mg^{-1}	%
Crude membrane extract	1167.40[a]			
Deoxycholate extract	699.20[a]			
Eluted from affinity column	0.410[b]	334.00	814.63	(100)
Eluted from first reversed phase HPLC	0.060[b]	55.45	924.17	16.60
Eluted from second reversed phase HPLC	0.0225[b]	15.00	666.67	4.49

[a] Assayed by the Bradford method (12).

[b] Determined from the staining intensity of a standard bovine serum albumin band in a silver-stained acrylamide gel.

to induction of apoptosis, and a DNA ladder was revealed after incubation with anti-APO-1. The isotype-matched monoclonal antibody FII23, however, did not induce fragmentation of the genomic DNA (see Fig. 7). These data indicate that the recombinant protein is expressed and functionally active in the transfected cells.

Fig. 8 shows in a Northern hybridization that APO-1 cDNA hybridized predominantly to an endogenous 2.7-kb and weakly to an endogenous 2.0-kb mRNA from SKW6.4 cells. The APO-1-expressing K50 transfectant showed a major band of 2.1 and a minor band of 2.3 kb, as expected for the spliced and unspliced APO-1 RNA transcribed from the transfected cDNA expression construct. APO-1 cDNA did not hybridize to the mRNA of APO-1-negative BL60-P7 cells or to mRNA of the APO-1 expression negative transfectant clone K04. This shows that APO-1 expression of K50 was due to transcription of the transfected cDNA and not due to up-regulation of expression of an endogenous gene.

DISCUSSION

Purification of the APO-1 antigen was achieved from membranes of SKW6.4 cells by a combination of membrane solu-

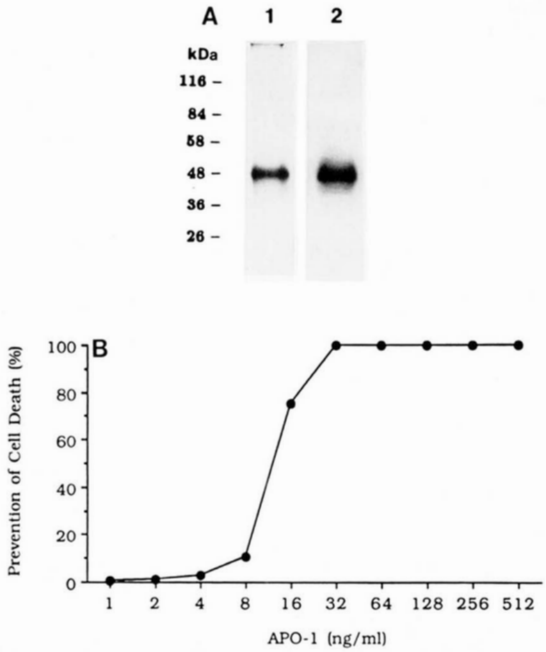

FIG. 3. **Functional analysis of the purified APO-1 antigen.** *A*, Western blot analysis. 100-ng aliquots of pure APO-1 antigen eluted at 59% acetonitrile from the second reversed phase HPLC column were lyophilized and resuspended in SDS-PAGE sample buffer containing 3% SDS. The samples were heated to 95 °C for 5 min and analyzed by 12% SDS-PAGE under nonreducing conditions. After electrophoresis, protein samples on the gel were either silver-stained (*lane 1*) or immunoblotted (*lane 2*) onto an Immobilon polyvinylidene difluoride membrane with anti-APO-1 (IgG2b). The immobilized monoclonal antibody was detected with horseradish peroxidase-labeled goat-anti-mouse IgG2b. *B*, prevention of anti-APO-1-induced cell death by purified APO-1. 2×10^4 SKW6.4 cells were incubated in duplicates in flat bottom 96-well microtiter plates for 24 h with various amounts of purified APO-1 antigen in the presence of either 20 ng/ml isotype-matched nonbinding control monoclonal antibody FII23 or 20 ng/ml anti-APO-1. Prevention of cell death (%) was measured by [^{3}H]thymidine deoxyribose incorporation after pulsing the cells with 0.5 μCi of [^{3}H]thymidine deoxyribose for the last 6 h of the culture according to the formula $cpm_{APO\text{-}1\text{-}anti\text{-}APO\text{-}1}/cpm_{APO\text{-}1\text{-}FII23} \times 100$.

TABLE II

Peptide sequences of the APO-1 protein

X, no clear amino acid was determined for this sequencing cycle.

Peptide	Peptide length	Amino acid sequence
	-mer	
Peptide 1	23	DTAEQKVQLLRNWHQLHGKKEAY
Peptide 2	15	DSENSNFRNEIQSLV
Peptide 3	15	DVDLSKYIXXIAXVM

bilization with sodium deoxycholate, affinity chromatography on immobilized anti-APO-1, and reversed phase HPLC. The purified APO-1 protein consisted of a single polypeptide chain of M_r 48,000 in the nonreduced and M_r 51,000 in the reduced state. The purified APO-1 antigen was found to be a glycoprotein with 8-kDa *N*-glycosidic-linked polysaccharides, representing 16% of the total protein. The purified polypeptide is the antigen recognized by the anti-APO-1 antibody. This is shown by Western blotting and in a biological assay where purified APO-1 was functionally active and capable to prevent anti-APO-1 induced cell death of SKW6.4 cells. Anti-APO-1 did not bind to the reduced APO-1 antigen, indicating that the APO-1 epitope was dependent on intramolecular disulfide bonds. The enrichment required to yield pure APO-1 antigen

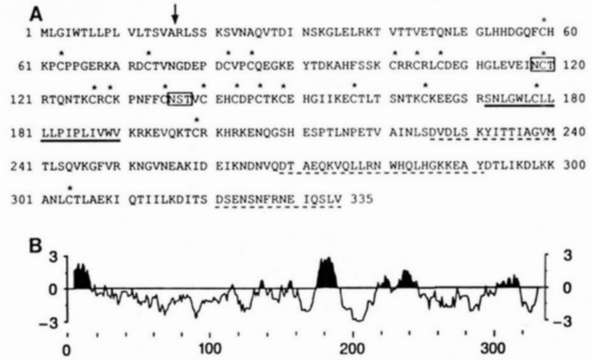

FIG. 4. **APO-1 amino acid sequence and hydropathy plot.** *A*, the deduced APO-1 amino acid sequence (*single letter code*). Cysteine residues are marked by an *asterisk*; the putative leader peptide cleavage site is indicated by an *arrow*. The putative transmembrane domain is *underlined*, and potential *N*-linked glycosylation sites are *boxed*. *Dashed lines* represent peptide sequences identified by Edman degradation. The entire nucleotide sequence is available upon request and has been deposited in the EMBL Data Bank, accession number X63717. *B*, hydropathy profile of the predicted APO-1 amino acid sequence. Values were determined using the sequence analysis software package of the University of Wisconsin genetic computer group according to Ref. 8. *Horizontal axis* is amino acid number.

from membranes of SKW6.4 cells could not be determined, since the starting material was not active in the APO-1 specific ELISA. We assume that lack of antigenic activity may be due to a masking of the epitope by contaminants in the APO-1 preparation. Taking into account that 22.5 μg of APO-1 antigen were purified from 5×10^{10} SKW6.4 cells at an epitope density of 4×10^4 APO-1 molecules/SKW6.4 cell (3), a theoretical yield for the APO-1 purification of 14% can be calculated.

The purified APO-1 protein was digested to generate peptides that were sequenced by Edman degradation. The sequence information of one of the peptides obtained was used to synthesize oligonucleotides. These oligonucleotides were used in a polymerase chain reaction to synthesize an unambiguous cDNA probe for APO-1. With this probe, a cDNA was cloned with a single open reading frame of 1005 nucleotides, predicting a 335-amino acid protein with a putative 16 amino acid leader peptide at the N-terminal end and a single transmembrane domain. The predicted molecular weight of the mature protein is 36,000 and is in good agreement with the 43,000 deglycosylated APO-1 protein.

The deduced APO-1 protein sequence revealed significant sequence similarity to the cysteine-rich region of the NGF receptor, the B-cell activation antigen CD40, both types of TNF receptors, the rat T cell activation antigen OX40, the deduced protein sequence of the murine cDNA 4–1BB expressed upon T cell activation, and the Shope fibromavirus T2 protein (20–27). While this paper was written, Itoh *et al.* (6) described the sequence of the Fas antigen obtained after expression cloning. Sequence comparison of APO-1 and Fas showed complete identity. The Fas antigen was previously defined by the cytolytic monoclonal antibody anti-Fas as a 200-kDa cell surface protein expressed on various human cells, including myeloid cells, T lymphoblastoid cells, and diploid fibroblasts. The anti-Fas antibody (IgM) was established by using the human diploid fibroblast FS-7 cell line as an immunogen in mice (7). The cDNA for Fas was stably expressed in murine T cell lymphoma WR19L and fibroblast L929 cells. The transformed cells were shown to be killed by anti-FAS antibody by induction of apoptosis (6).

A common feature of all members of the NGF/TNF receptor superfamily are repeated cysteine-rich extracellular do-

FIG. 5. **Sequence comparison of APO-1 to other members of the TNF receptor superfamily.** The Multialign and Clustal programs of the University of Wisconsin genetic computer group were applied. *A*, consensus alignment of the cysteine-rich sequences of APO-1, CD40, NGF receptor, TNF receptor type I, and TNF receptor type II. Residues are *numbered* starting with the initiating methionine. Identical residues are *boxed*. Conserved cysteine residues are shown by *shading*. *B*, alignment of intracellular protein regions. The abbreviations and references are as in *A*.

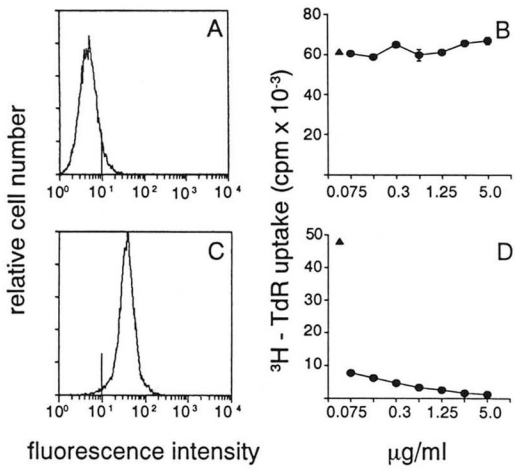

FIG. 6. **Functional expression of the APO-1 antigen in BL60-P7 cells.** Detection of APO-1 cell surface expression in BL60-P7 transfectants. Transfected BL60-P7 cells were stained by indirect immunofluorescence with anti-APO-1 (IgG3, κ) monoclonal antibody. *A*, K04 transfectant; *C*, K50 transfectant. A control antibody FII23 (IgG3, κ) did not bind to the transfectants (data not shown). Anti-APO-1-mediated growth inhibition of pKEX-2-XR-APO-1-transfected BL60-P7 cells. K04 cells (*B*) and K50 cells (*D*) were treated with anti-APO-1 monoclonal antibody.

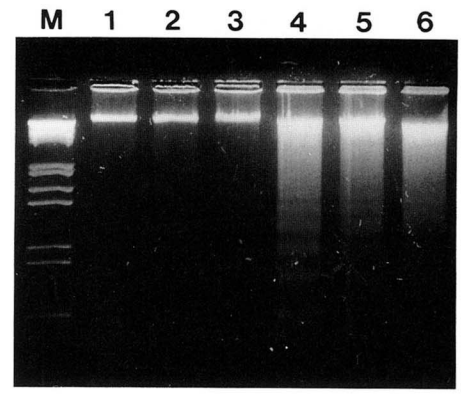

FIG. 7. **Anti-APO-1 induced DNA fragmentation.** BL60-P7 cells (10^6/ml) (*lanes 1* and *2*) and K50 cells (10^6/ml) (*lanes 3–6*) were incubated with 1 µg/ml monoclonal antibody for 2 h (*lane 4*), 4 h (*lane 5*), and 8 h (*lanes 1–3* and *6*) before cells were lysed and DNA was prepared. DNA was analyzed on a 1.8% agarose gel. *M*, marker; *lanes 1* and *3*, isotype-matched control monoclonal antibody FII23; *lanes 2* and *4–6*, anti-APO-1 monoclonal antibody.

mains. Whereas the two types of TNF receptor, the NGF receptor, and the CD40 antigen comprise four cysteine-rich extracellular domains, the APO-1 antigen and the OX40 antigen have only three such related domains. A lower degree of a new sequence similarity was also found when the intracellular domains of APO-1, CD40, NGF receptor, and the TNF receptor were compared. Interestingly, a threonine res-

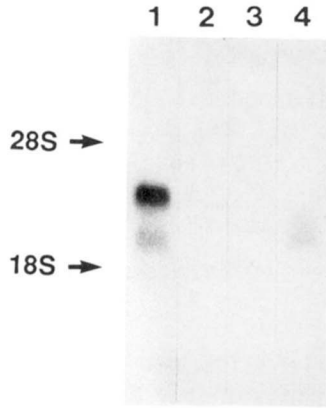

FIG. 8. **Northern blot analysis of APO-1 mRNA.** Total RNA (10 μg) from SKW6.4 cells (*lane 1*), from BL60-P7 cells (*lane 2*), from K04 cells (*lane 3*), and from K50 (*lane 4*) cells were fractionated on a 1% agarose gel, blotted, and hybridized with the 2.55-kb cDNA fragment of APO-1.

idue implicated in CD40 signaling (T*254*) (28) is also conserved in TNF receptor 1 (T*300*) and in APO-1 (T*241*). Such similarities might be indicative of a common mechanism of signal transduction by these molecules.

The APO-1 cDNA was introduced into the human Burkitt's lymphoma line BL60-P7, and APO-1-expressing transfectants were found to be susceptible toward anti-APO-1 mediated apoptosis. Thus, these cells seem to provide all cellular components necessary for signaling through APO-1.

Future studies will have to address the intracellular signal pathway and the physiological ligand of APO-1. Such a ligand might be a cytotoxic molecule, directly inducing apoptosis, or a survival factor, whose removal leads to apoptosis.

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