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Purification and characterization of a 90 kDa protein released from human tumors and tumor cell lines

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A novel tumor-associated protein, termed 90K, and recognized by mAb SP-2 was purified from serum of breast cancer patients, ovarian cancer ascitic fluid and conditioned medium of human breast cancer cells. In these three sources, native 90K is present as a high molecular weight complex that was dissociated by SDS-PAGE into a major band of approximately 90,000 Da. On the basis of electrophoretic mobility, buoyant density value, amino acid composition, and immunoreactivity, the 90K from the different sources appeared to be identical. NH_2-terminal amino acid sequence revealed no homology to known protein.

Tumor-associated antigen; Monoclonal antibody; Breast cancer; Protein purification; NH_2-terminal amino acid sequence

1. INTRODUCTION

Various proteins which are shed or secreted by tumor cells had been previously identified in the serum of patients with different forms of cancers. These proteins can be used as diagnostic–prognostic indicators and for therapy surveillance. To obtain mAbs against circulating breast cancer-associated antigens, mice were immunized with a mixture of proteins released into conditioned medium of CG-5 human breast cancer cells [1]. One of the mAbs generated, SP-2, recognized an antigen of molecular weight 90,000, termed 90K. This protein was found in more than 80% of breast cancers but not in non-cancerous normal mammary gland surrounding the cancer tissue [1]. Quantitation of 90K protein in the serum has been made possible by development of an ELISA assay with SP-2 mAbs [2]. Approximately 50% of patients with breast cancer, 40% of patients with gastrointestinal malignancies, and 30% of patients with gynecological malignancies, had elevated serum levels of the 90K protein [2]. More importantly, the assay has demonstrated that the fraction of patients showing elevated serum levels is greater for individuals with metastatic disease and that the level of 90K in serum correlates with cancer progression [2–4]. Since 90K protein is distinct from other circulating antigens such as CA 15-3, CEA, and CA 125 [2,4], it may represent an additional useful diagnostic tool for the surveillance of breast cancers and other malignant diseases.

The 90K protein has not yet been characterized, so it is not known if it has previously been described under a different name or it is a new undiscovered entity. In this report, we describe the purification and characterization of SP-2-reactive 90K protein from conditioned medium of CG-5 cells, breast cancer patient serum, and ovarian cancer patient ascitic fluid.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IRMA, immunoradiometric assay; mAb, monoclonal antibody; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

2. MATERIALS AND METHODS

CG-5, an estrogen-supersensitive variant of the MCF-7 human breast cancer cell line, was previously described [5]. The other human breast cancer cell lines are from The American Tissue Culture Collection. The murine mAb SP-2 produced by hybridomas grown in pristane-primed Balb/c mice [1] was purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange chromatography [2]. Purified SP-2 was labeled with sodium-^{125}I using lactoperoxidase [6]. The proteases and other enzymes were purchased from Sigma. Electrophoresis reagents were from Bio-Rad Laboratories. All other reagents were of the highest commercially available grade.

2.1. *Solid-phase radioimmunoassay*

A 'two-step' sandwich IRMA was developed to quantitate 90K. The assay uses polystyrene beads coated with mAb SP-2 as solid phase and ^{125}I-SP-2 as the labeled antibody. Results are expressed in arbitrary units. The detailed procedure is described elsewhere [7].

2.2. *PAGE and immune blotting analyses*

SDS-PAGE was performed according to [8]. Samples were heated at 50°C for 30 min in the presence of sample buffer (63 mM Tris-HCl, pH 6.8, 4% SDS, 5% mercaptoethanol and 20% glycerol) and electrophoresed for 4 h at 40 mA. A 9% separating gel and a 4% stacking gel were used. The separating gel was silver stained using a Bio-Rad kit. Native PAGE was performed on 4–20% gradient gel for 16 h at 20 mA. Sample buffer was 63 mM Tris-HCl plus 0.25% NP-40. The gel was either silver stained or electroblotted onto nitrocellulose mem-

branes at 50 V for 2 h as described by Towbin et al. [9], except that the transfer buffer did not contain methanol. The membranes were blocked with bovine skim milk, followed by incubation with mAb SP-2 (10 μg/ml) for 2 h at room temperature. The membranes were washed thoroughly with PBS and stained with avidin–biotin staining kit (Sigma) according to the manufacturer's instructions.

2.3. *Radiolabeling of cells and immunoprecipitation*

For metabolic labeling, 2×10^6 cells were incubated at 37°C for 6 h in Dulbecco modified Eagle's medium containing 250 μCi/ml [^{35}S]methionine. Culture fluids containing the radioactive proteins and pre-clarified as described [1], were incubated with one SP-2-coated polystyrene bead at 4°C for 16 h. The beads were washed with distilled water, treated with 100 μl of SDS-sample buffer during 30 min at 50°C. Extracts were analysed by SDS-PAGE. As controls, aliquots of culture fluid were incubated with polystyrene beads that have been coated with a mAb against alpha-fetoprotein. [^{35}S]methionine-labeled protein bands were visualized by fluorography. In certain experiments, the cells were incubated in the presence of 5 μg/ml tunicamycin (Sigma) for 2 h prior to the addition of [^{35}S]methionine.

2.4. *Purification of 90K protein*

(a) From *conditioned medium*. CG-5 cells were grown in Dulbecco's modified Eagle's medium supplemented with 3% FCS using Cell Factory plastic chambers (Nunc, Roskilde, Denmark). Conditioned medium from confluent cell layers was collected. Then fresh medium was added and collected after 24 h incubation periods for additional 3–4 days. The concentration of 90K protein produced under these conditions ranged from 100 to 400 U/ml. Pooled culture supernatants (10 l) were centrifuged at $4{,}000 \times g$ followed by a 10-fold concentration using a Minitan apparatus (Millipore). Solid ammonium sulfate was slowly added (final concentration 43%) and allowed to stand overnight at 4°C, protein precipitates were collected by centrifugation at $10{,}000 \times g$. The precipitates were stored at −20°C. Under these conditions the immunoprecipitation of 90K activity by SP-2 antibody is stable for at least 2 months.

(b) From *human serum*. Serum from a patient with advanced breast cancer containing high concentrations of 90K protein as revealed by IRMA analysis was cleared by centrifugation at $10{,}000 \times g$ for 20 min, then diluted 1:1 with PBS and precipitated with ammonium sulfate as described above.

(c) From *ascitic fluid*. Ascitic fluid was obtained by paracentesis from a patient with advanced ovarian carcinoma. The fluid was cleared by centrifugation at $10{,}000 \times g$ for 20 min and precipitated with ammonium sulfate as described.

The ammonium sulfate precipitates were dialyzed extensively against PBS and poured into a Sepharose CL-6B column (4.2 × 85 cm) equilibrated and eluted with PBS plus 0.5 M NaCl, pH 8.1, at a flow rate of 18 ml/h. Fractions (5 ml) were collected and assayed for 90K protein by IRMA analysis. Fractions containing 90K protein were pooled, dialyzed against 0.005 M Na-phosphate buffer, pH 7.4, and applied to a DE 52-cellulose column (2 × 8 cm) equilibrated in the same buffer. The column was washed extensively with buffer and the absorbed proteins were eluted using a stepwise sodium chloride gradient (0.062 to 1.0 M). Fractions containing the 90K protein activity were pooled and mixed with SP-2 antibody conjugated to Sepharose CL-4B (4 mg antibody/ml resin) at a volume ratio of 8:1 (sample/resin). mAb SP-2 was coupled to Sepharose by the method of Schneider et al. [10]. The mixture was mixed overnight at 4°C and the 90K protein was eluted with 3 M $MgCl_2$. The amount of protein was estimated with the Bio-Rad protein assay based on the method of Bradford [11].

2.5. *Density gradient centrifugation*

Centrifugation of the 90K protein isolated from either CG-5-conditioned medium, breast cancer serum, or ovarian cancer ascitic fluid after desorption from the immuno affinity matrix, was performed in 5 ml of CsCl isopicnic density gradient. The protein was dissolved in a CsCl solution in PBS with a starting density of 1.4 g/ml, and the gradients were formed by centrifugation in a Beckman SW 50.1 rotor at $145{,}000 \times g$ for 72 h at 4°C. Fractions (0.25 ml) were collected, diluted 1:10 with PBS and assayed for antigenic activity by IRMA. The density of each fraction was determined by weighing a known volume.

2.6. *Biochemical characterization of the 90K protein*

The 90K protein was applied on microtiter plates. Microplates (Dynatecs) were coated with 50 μl of purified protein (100 ng/ml of 0.05 M carbonate buffer, pH 9.6) and incubated overnight.

(a) *Chemical treatment*. Methanol treatment was carried out at 4°C for 30 min. Denaturation was performed with either 6 M urea and 6 M guanidine-HCl or 1% SDS at 45°C for 1 h. Periodate oxidation was accomplished for 1 h at room temperature with 10, 20, 30, 40, 50 mM $NaIO_4$ in acetate buffer (50 mM, pH 4.5) in the dark according to Stahl et al. [12]. Reduction was performed with dithiothreitol (10 mM in 50 mM Tris, pH 8.1) or 5% 2-mercaptoethanol at 37°C for 1 h. Alkylation was performed with 20 mM iodacetic acid at 30°C for 30 min.

(b) *Proteolytic enzymes*. Microplates were exposed at 37°C for 90 min to either trypsin (2 mg/ml), chymotrypsin (2 mg/ml), pronase (1 mg/ml) in 50 mM Tris, 2 mM $CaCl_2$, pH 8.1, or to papain (0.2 mg/ml) in 50 mM cysteine-HCl, pH 6.0.

(c) *Exoglycosidases*. Microplates were exposed to either neuraminidase, fucosidase, α-glucosidase and β-glucosidase in 50 mM acetate buffer, pH 5.0, or to chondroitinase-ABC in 250 mM Tris, 176 mM CH_3COONa, 250 mM NaCl, pH 8.0. Incubations were carried out at 37°C for 90 min. Appropriate concentrations of exoglycosidases were chosen to ensure complete digestion of the oligosaccharide residues. This was verified in separate experiments in which the substrates were shown to be completely hydrolyzed as detected by thin-layer chromatography.

After treatment, microplates were washed and treated with 1% gelatin in PBS and 50 μl of ^{125}I-labeled SP-2 (approximately 50,000 cpm) were added to each well and incubated at 37°C for 1 h. After 3 washes with PBS, the bound radioactivity was determined in a gamma-counter.

2.7. *Amino acid analysis and amino-terminal sequencing*

Purified 90K protein was electrophoresed in a 9% SDS polyacrylamide gel under reducing conditions using a Mini-gel apparatus. Proteins were electroblotted to polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Beaford, MA), stained with Coomassie blue, and the bands corresponding to an M_r of 90 kDa excised. For amino acid analysis, 3–4 bands for a total of approximately 50 μg of 90K were hydrolyzed under vacuum in 6 N HCl at 110°C for 22 h. After hydrolysis, the amino acids were analyzed on a Beckman analyzer using a pH gradient system [13]. For amino-terminal sequencing, approximately 20–30 μg of protein were subjected to microsequence analysis utilizing a pulsed liquid sequencer (Applied Biosystems Inc., Mod. 475).

3. RESULTS

The purification procedure used for the isolation the 90K protein from either conditioned medium of CG-5 cells or from breast cancer patient serum, and ovarian cancer patient ascitic fluid is summarized in Table I. At each step of purification, the total amount of proteins was determined, and the amount of 90K protein was quantitated by an IRMA. Nearly all 90K protein immunoactivity was recovered in a 43% ammonium sulfate precipitate, resulting in about 4-fold enrichment. This step removed the large majority of albumin present in the initial preparation. The ammonium sulfate-precipitated protein was next subjected to size exclusion

chromatography using a Sepharose CL-6B column (Fig. 1). The 90K protein from CG-5 cells, breast cancer serum, and ovarian cancer ascitic fluid was identified in a major peak eluting immediately behind the void volume of the column, indicating that it is a high molecular weight complex. In a few experiments, a lower molecular weight species probably representing a degradation product was also observed. Low molecular weight proteins found at the end of the elution were unreactive with the SP-2 antibody. Treatment of the samples with either 6M urea or 6M guanidine-HCl before chromatography gave identical elution profiles (not shown). The high molecular weight peak (corresponding to fractions 21–28 of Fig. 1) was further purified by DEAE-cellulose chromatography. The 90K protein obtained from each of the three different sources eluted from the column at a NaCl concentration of 0.25 M (data not shown). The final purification was accomplished by immunoaffinity on Sepharose CL-4B coupled with SP-2. Bound mAb was extracted with 3 M $MgCl_2$. Other eluting solutions which were used including glycine-HCl (pH 2.4), 1 M NaOH (pH 11.2), and 3 M KSCN were less effective for protein elution.

Based on specific activity (U/μg protein), the purification of 90K protein from CG-5 tissue culture fluid, breast cancer serum, and ovarian cancer ascitic fluid was approximately 84,300-, 52,300- and 83,400-fold, respectively. These enrichments were calculated by measuring the 90K immunoreactivity in the 3 M $MgCl_2$ extracted from the affinity matrix with IRMA and determining the amount of protein by comparing the silver-staining intensity of the 90K band on SDS-PAGE gels with BSA standards of known concentration. Starting from 50 ml of serum (about 3.5 g protein) from a breast cancer patient, approximately 13 μg of protein were recovered in the final purified preparation. Based on the purification and the calculated recovery, we estimate that the concentration of 90K protein in the serum of a breast cancer patient is approximately 1.5 μg/ml.

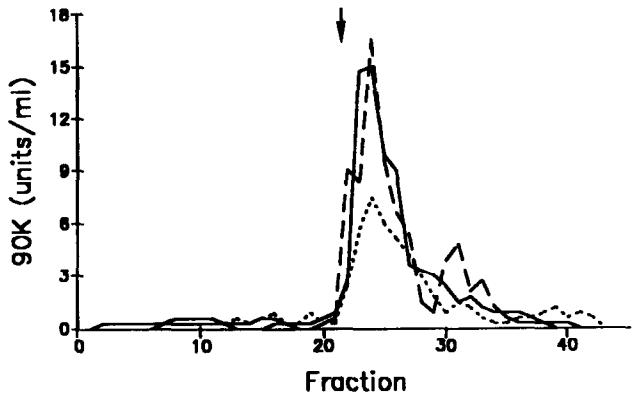

Fig. 1. Sepharose CL-6B column chromatography of 90K isolated from CG-5 tissue culture fluid (—), breast cancer patient serum (...), and ovarian cancer patient ascitic fluid (--). Fractions were assayed for 90K activity by IRMA. The arrow indicates the elution volume of Dextran blue 2000.

3.1. *PAGE and immunoblotting analyses of 90K protein isolated from different sources*

In agreement with previous data [1], 90K protein released into the conditioned medium of [^{35}S]methionine-labeled CG-5 cells and other breast cancer cell lines migrates as a single band with an apparent molecular weight 90 kDa as revealed by SDS-PAGE analysis (Fig. 2A). The mobility of [^{35}S]methionine-labeled protein was identical under reducing or non-reducing conditions, suggesting that the protein does not contain intra-

Table I

Purification of 90K

Sample	Protein (mg)	Activity (U × 10^{-6})	Yield (%)	Purification	Specific activity (U/μg)
CG-5 tissue culture					
Fluid (10 l)	14,100	2.51	(100)	(1)	0.18
$(NH_4)_2SO_4$ precipitate	3,700	2.61	(104)	4.0	0.7
Sepharose CL-6B	230	1.92	76.4	46.8	8.3
DEAE-cellulose	61	1.71	68.1	157	28
Immunoaffinity	0.029	0.44	17.5	84,300	15,174
Breast cancer serum					
(50 ml)	3,100	1.28	(100)	(1)	0.41
$(NH_4)_2SO_4$ precipitate	950	1.38	106	3.5	1.4
Sepharose CL-6B	58	0.91	71	38	15.6
DEAE-cellulose	15	0.71	55.4	115	47.3
Immunoaffinity	0.013	0.28	21.8	52,277	15,538
Ovarian cancer					
Ascitic fluid (1 liter)	13,500	0.62	(100)	(1)	45.9
$(NH_4)_2SO_4$ precipitate	4,250	0.71	114	3.6	167
Sepharose CL-6B	282	0.58	93.5	44.7	2,056
DEAE-cellulose	282	0.58	93.5	44.7	2,056
Immunoaffinity	0.11	0.21	34	83,380	19,090

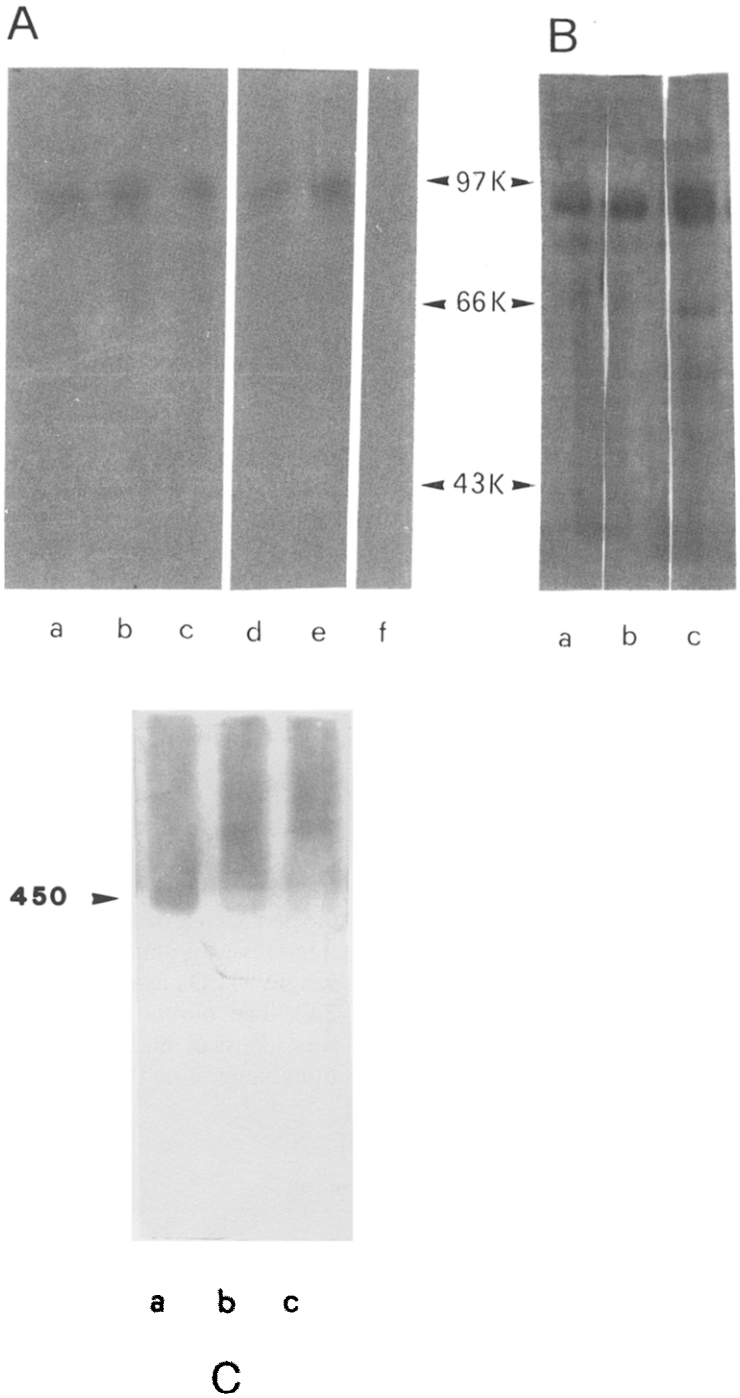

Fig. 2. PAGE and Western blot analysis of 90K. (A) Immunoprecipitates of radioactive 90K from human breast cancer cells. Aliquots (200,000 trichloroacetic acid precipitable cpm) of [^{35}S]methionine-labeled culture fluid were immunoprecipitated with mAb SP-2 (lanes a–e) or mAb against α-fetoprotein (lane f), and analyzed by SDS-PAGE in the presence (lanes a–c,e) or absence (lane d) of 2-mercaptoethanol, followed by fluorography. Lanes a and d, CG-5 cells; lane b, MCF-7 cells; lane c, T47D cells; lane e, tissue culture fluid from CG-5 cells after the cells were exposed to tunicamycin before [^{35}S]methionine labeling. (B) SDS-PAGE analysis of 90K purified from CG-5 culture fluid (lane a, 1,220 U), breast cancer patient serum (lane b, 1,550 U), and ovarian cancer patient ascitic fluid (lane c, 1,320 U). The gels were silver stained. (C) Western blot of purified 90K from CG-5 culture fluid (lane a), breast cancer serum (lane b), and ovarian cancer ascitic fluid (lane c). Purified 90K was analyzed on 4–20% gradient gel containing 0.1% NP-40 and proteins electroblotted onto a nitrocellulose membrane. The arrowhead indicates migration of ferritin (M_r = 450 kDa). Positions of size markers are indicated with arrowheads in kDa.

chain disulfide bonds. Moreover, tunicamycin treatment of CG-5 cells prior to labeling with [^{35}S]methionine did not influence the electrophoretic mobility of the 90K protein isolated from the conditioned medium

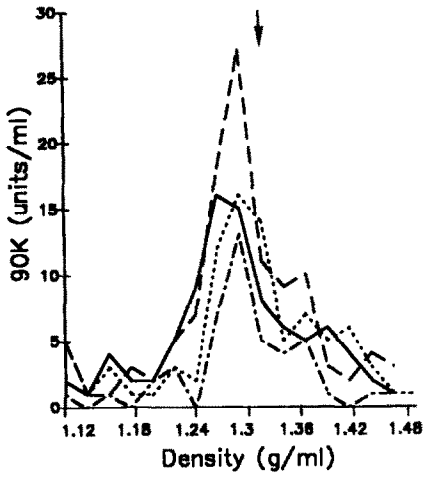

Fig. 3. Density gradient centrifugation of 90K. Purified 90K from CG-5 culture fluid (—), breast cancer serum (...), ovarian cancer ascitic fluid (---), and unfractionated breast cancer serum (-·-) were subjected to equilibrium ultracentrifugation in cesium chloride. Fractions were assayed for 90K activity by IRMA, and density was determined by weighting a known volume. Arrow, buoyant density of β-galactosidase.

(Fig. 2A, lane c). It appears therefore that the 90K protein does not contain detectable amounts of N-linked carbohydrates. Fig. 2B depicts a comparison of the electrophoretic mobility on SDS-PAGE of 90K protein purified from CG-5 conditioned medium, breast cancer patient serum, and ovarian cancer patient ascitic fluid. This experiment shows that a protein with apparent molecular weight of 90,000 was detected in silver-stained gels. Co-electrophoresis of the purified protein from breast cancer patient serum detected by silver staining and [^{35}S]methionine-labeled immunoprecipitates from CG-5-conditioned medium analyzed by autoradiography gave a superimposable band with apparent molecular weight of 90,000 (data not shown).

Immunoblot analysis of the purified 90K protein transferred from a native 4–20% polyacrylamide gel containing 0.25% NP-40, demonstrated the presence of similarly immunoreactive species in CG-5-conditioned medium, breast cancer serum, and ovarian cancer ascitic fluid (Fig. 2C). The SP-2 antibody bound in these three sources to a high molecular weight (M_r > 450 kDa) diffuse band. The SP2 antibody poorly recognized the 90K polypeptide transferred from SDS-gels (data not shown). These results are consistent with the Sepharose CL-6B elution profiles shown in Fig. 1 and indicate that the native 90K protein isolated from different sources exists as a high molecular weight aggregate.

3.2. *Analysis of purified 90K by density gradient centrifugation*

Samples of protein eluted from SP-2 affinity matrix were subjected to density gradient centrifugation. This procedure did not reveal different average buoyant density for the antigen obtained from the three different sources which ranged between 1.28 g/ml and 1.31 g/ml (Fig. 3). Moreover, the 90K protein in unfractionated breast cancer serum produced essentially an identical density profile, indicating that 90K isolated by this procedure did not represent a subset of the original antigen.

3.3. *Amino acid analysis and amino-terminal sequence of 90K*

Table II shows that 90K protein purified from CG-5 cells conditioned medium, breast cancer patient serum, and ovarian cancer patient ascitic fluid have similar amino acid composition. The antigen was relatively rich in glutamic acid/glutamine, in addition to serine and leucine. The NH_2-terminal sequence of 90K from the three different sources was determined for the first 22 amino acids by automated Edman degradation (Table III). The repetitive yield during the sequencing was ≥91%. A single amino acid sequence was found in all preparations. The sequence was compared with all protein sequences in the Genebank/EMBL databases with the aid of the tFasta program. No significant homology with any known protein in the database was observed.

The biochemical nature of the SP-2 determinant on 90K proteins was investigated using several chemical and enzymatic treatments (Table IV). Exposure to methanol strongly reduced 90K immunoreactivity as did 6 M guanidine-HCl, 6 M urea or 1% SDS, lyophilization and heat. Neither reduction with dithiothreitol and 2-mercaptoethanol, nor alkylation with iodoacetamide or treatment with non-ionic detergents NP-40, Tween 20, and Triton X-100 affected the immunoreactivity of 90K protein. Exposure to sodium-*m*-periodate

Table II

Amino acid composition of 90K

Amino acid	Molar percentage		
	CG-5 cells	Breast cancer serum	Ovarian cancer ascitic fluid
Glx	11.8	10.7	11.1
Asx	7.6	6.9	8.3
Ser	12.4	11.9	11.9
Thr	4.3	4.8	4.3
Gly	8.8	9.1	8.9
Pro	5.1	4.9	4.7
Val	4.9	4.2	5.1
Leu	12.1	13.3	13.2
Ile	1.1	0.9	1.3
Ala	8.1	7.9	6.9
Phe	2.8	2.4	2.5
Met	1.1	1.3	0.9
His	3.1	3.3	2.9
Lys	2.5	2.7	2.8
Arg	4.1	3.9	3.2
Tyr	3.5	3.7	3.7
Trp	N.D.	N.D.	N.D.
Cys	N.D.	N.D.	N.D.

N.D. = not determined.

Table III

Amino acid sequence of the NH_2-terminal end of 90K from three sources

CG-5 culture medium
V N D G D M (C) L A D G G A T N Q G R V E I F
Breast cancer serum
V (N) D G D M P L A D G G (A) T N Q G R V E I F
Ovarian cancer ascitic fluid
V N D G D M (C) L A D G G A T N Q G (L) V E I F

Sequence determined by Edman degradation as described in section 2. Residues in parentheses are uncertain.

had a small effect at high concentrations (50 mM). All the proteases tested reduced 90K immunoreactivity, the most effective were pronase and papain. Treatment with exoglycosidases did not affect 90K immunoreactivity. In fact, the ability of the immobilized antigen to bind ^{125}I-labeled SP-2 following treatment with neuraminidase and β-galactosidase, was increased suggesting that removal of terminal carbohydrate moieties may increase accessibility towards the SP-2 epitope on the 90K protein.

4. DISCUSSION

mAb SP-2 specially binds to a 90 kDa protein which can be immunoprecipitated with this antibody from conditioned medium of breast cancer cells and from sera and ascitic fluid of tumor bearing patients. Native 90K protein purified from conditioned medium, human breast cancer serum, and ascitic fluid from ovarian cancer exists as a high molecular weight complex composed of several 90 kDa molecules. This suggests that in its native state this protein tends to form an oligomeric structure. The 90K protein isolated from either conditioned medium of CG-5 cells or breast cancer serum and ovarian cancer ascitic fluid all have similar behaviour when analysed by size-exclusion chromatography, ion-exchange chromatography, PAGE and immunoblotting analyses as well as buoyant density ultracentrifugation. Moreover, the 90K protein isolated from these three sources have similar amino acid composition and NH_2-terminal amino acid sequence indicating that they are encoded by the same gene. It appears therefore that the 90K protein isolated from either established long-term cancer cell lines, from cancer patients's serum or ascites represent similar if not identical molecules.

Various chemical and physical treatments of 90K protein were undertaken in order to shed light on the properties of this molecule. Denaturating agents or treatments greatly reduced antibody binding, further indicating that the SP-2 binds to a conformational determinant of the 90K protein. Furthermore, dissociation of the oligomeric structure of the antigen into monomers under denaturating conditions resulted in a nearly complete loss of SP-2 binding activity.

Other tumor-associated antigens shown to be expressed by breast cancers with apparent molecular weight of 90 kDa are likely to represent distinct molecules for the following reasons. The antigen recognized by mAb B6.2 [14,15] is a cell surface glycoprotein and contrary to 90K, is restricted to breast cancer cells. The antigen recognized by mAb 465.12S [16] is a cell surface glycoprotein. This glycoprotein is shed by the cells, and similarly to 90K can be isolated from the conditioned medium of cultured cells and from the serum of cancer patients. However, unlike the 90K this protein is an N-glycosylated glycoprotein [16]. The melanoma-associated antigen termed p97, gp87, or gp95 [17–19] is a membrane protein which is structurally related to transferrin [20]. Another melanoma antigen, FD, is also a cell surface glycoprotein whose expression is restricted to a very limited number of cells [21]. Finally, the antigen defined by mAb 3G2-C6 [22] is a cell surface component expressed in a significant number of bladder cancers but rarely present in breast cancers [23].

In summary, this report describes the purification of

Table IV

Effects of chemical and physical treatments on 90K activity

Treatment	Relative binding activity
Control	1
Methanol	0.04
Guanidine-HCl 6M	0.18
Urea 6M	0.19
SDS	0.14
Dithiothreitol	0.89
2-Mercaptoethanol	1.1
Iodacetic acid	0.93
NP-40	1.13
Tween-20	1.05
Triton X-100	0 88
Lyophylization	0
Heat (100°C, 5 min)	0
Periodate concentration (mM)	
0	1
10	1.05
20	0.91
30	0.95
40	0.90
50	0.71
Proteases	
Pronase	0.05
Papain	0.07
Trypsin	0.38
Chymotrypsin	0.42
Exoglycosidases	
Neuraminidase	1.28
Fucosidase	0.98
Chondroitinase ABC	0.98
α-Galactosidase	0.98
β-Galactosidase	1.62

a 90K protein by a four-step procedure involving ammonium sulfate precipitation, size-exclusion chromatography, ion-exchange chromatography, and immunoaffinity chromatography with SP-2 mAb. The degree of purity of the 90K protein is established by SDS-PAGE analysis and silver staining and by a unique amino terminal sequence. The availability of purified 90K protein with information on partial amino acid sequence will facilitate cDNA cloning and detailed analysis of expression in normal and various pathological tissues.

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REFERENCES

[1] Iacobelli, S., Arno', E., D'Orazio, A. and Coletti, G. (1986) Cancer Res. 46, 3005–3010.
[2] Iacobelli, S., Arno', E., Sismondi, P., Natoli, C., Gentiloni, N., Scambia, G., Giai, M., Cortese, P., Benedetti-Panici, P. and Mancuso, S. (1988) Breast Cancer Res. Treat. 11, 19–30.
[3] Scambia, G., Benedetti-Panici, P., Baiocchi, G., Perrone, L., Iacobelli, S. and Mancuso, S. (1988) Anticancer Res. 8, 761–764.
[4] Benedetti-Panici, P., Scambia, G., Baiocchi, G., Iacobelli, S. and Mancuso, S. (1989) Gynecol. Oncol. 35, 286–289.
[5] Natoli, C., Sica, G., Natoli, V., Serra, A. and Iacobelli, S. (1983) Breast Cancer Res. Treat. 3, 23–32.
[6] Thorell, J.T. and Johansson, B.G. (1971) Biochim. Biophys. Acta 251, 363–368.
[7] Natoli, C., Dianzani, F., Mazzotta, F., Balocchini, E., Pierotti, P., Antonelli, G. and Iacobelli, S., J. AIDS (in press).
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982) J. Biol. Chem. 257, 10766–10769.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Stahl, P., Six, H., Rodman, J.S., Schlesinger, P., Tulsiani, D.P.R. and Touster, O. (1976) Proc. Natl. Acad. Sci. USA 73, 4045–4049.
[13] Hirs, C.H.W. (1983) in: Methods in Enzymology, vol. 91, pp. 3–8, Academic Press Inc. New York.
[14] Kufe, D.W., Sargent, N.L., Shapiro, H., Hand, P., Austin, F., Colcher, D. and Schlom, J. (1983) Cancer Res. 43, 851–857.
[15] Schlom, J., Greiner, J., Horan-Hand, P., Colcher, D., Inghirami, G., Weeks, M., Petska, S., Fisher, P.B., Noguchi, P. and Kufe, D. (1984) Cancer 54, 2777–2794.
[16] Natali, P.G., Wilson, B.S., Imai, K., Bigotti, A. and Ferrone, S. (1982) Cancer Res. 42, 582–589.
[17] Brown, J.P., Nishiyama, A.K., Hellstrom, I. and Hellstrom, K.E. (1981) J. Immunol. 127, 539–546.
[18] Dippold, W.G., Lloyd, K.O., Li, L.T.C., Ikeda, H., Oettgen, H.F. and Old, L.J. (1980) Proc. Natl. Acad. Sci. USA 77, 6114–6118.
[19] Liao, S.-K., Kwong, P.C. and Khosravi, M.J. (1985) J. Cell. Biochem. 27, 303–316.
[20] Brown, J.P., Herwick, R.M., Hellstrom, I., Hellstrom, K.E., Doolittle, R.F. and Dreyer, W.J. (1982) Nature 296, 171–173.
[21] Mattes, M.J., Reaò, F.X., Furukawa, K., Old, L.J. and Lloyd, K.O. (1987) Cancer Res. 47, 6614–6619.
[22] Zhang, D. and Lin, C.W. (1989) Cancer Res. 49, 6621–6628.
[23] Young, D.A., Prout Jr., G.R. and Lin, C.-W. (1985) Cancer Res. 45, 4439–4446.