Int. J. Cancer: **57**, 90–97 (1994)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

MOLECULAR CLONING AND EXPRESSION OF A cDNA ENCODING A PROTEIN DETECTED BY THE K1 ANTIBODY FROM AN OVARIAN CARCINOMA (OVCAR-3) CELL LINE

Kai CHANG and Ira PASTAN[1]

Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Building 37, Room 4E16, Bethesda, MD 20892, USA.

MAb K1 recognizes a cell-surface glycoprotein (MW ~40 kDa) present in ovarian carcinomas, malignant mesotheliomas, squamous-cell carcinomas and normal mesothelial cells. In this study, expression screening was used to isolate cDNA clones encoding an antigen recognized by MAb K1 from a cDNA library made from a human ovarian carcinoma cell line (OVCAR-3). Subsequently, other clones were isolated by DNA hybridization using a cDNA probe derived from one of the initial clones. The sequence of all the clones was similar. The longest cDNA contains 2,444 base pairs, and encodes a polypeptide of 263 amino acids with a calculated molecular weight of 30,511 daltons. The nucleotide sequence and deduced amino-acid sequence of the protein show no homology to other sequences in current data bases. *In vitro* translation of RNA transcripts from the cDNA inserts yielded polypeptides of 29 and 30 kDa. Similar-sized proteins were obtained upon expression of the cDNA in *Escherichia coli*, and these proteins were reactive with MAb K1. The protein(s) expressed in *E. coli* were purified and used to make rabbit or mouse antisera. These antisera reacted strongly with a soluble cytosolic protein in OVCAR-3 cells, but not with the membrane-bound antigen. Soluble cytosolic proteins of a similar size, recognized with MAb K1, were found in OVCAR-3 and N87 (gastric cancer) cells but not in 10 other cancer cell lines. These data indicate that the cloned cDNA encodes a cytosolic protein that reacted with MAb K1. This soluble protein is expressed only in cells containing the CAK1 surface glycoprotein, suggesting that the 2 proteins could be structurally related.

MAb K1 is a murine $IgG1_\kappa$ monoclonal antibody (MAb) produced by a hybridoma obtained from mice immunized with the ovarian carcinoma cell line, OVCAR-3 (Chang *et al.*, 1992*d*). This antibody recognizes a differentiation antigen present on normal mesothelium, but it also reacts strongly and homogeneously with most human epithelial ovarian cancers (Chang *et al.*, 1992*a, d*), many squamous-cell carcinomas of various origins (Chang *et al.*, 1992*c*), and all tested malignant mesotheliomas of epithelial or mixed type (Chang *et al.*, 1992*b*). Further characterization of MAb K1 has demonstrated that MAb K1 reacts with an antigen different from those recognized by other MAbs that react with ovarian cancers. These include OC125 (Bast *et al.*, 1981), B72.3 (Colcher *et al.*, 1981), Mov18/Mov19 (Miotti *et al.*, 1987)/MW207 (Mattes *et al.*, 1987), OVB-1 (Kurrasch *et al.*, 1989) and OVB-3 (Willingham *et al.*, 1987).

The antigen recognized by MAb K1, CAK1, is a cell-surface glycoprotein with a molecular weight of ~40 kDa (Chang *et al.*, 1992*a*). CAK1 is associated with cell membrane via a glycosyl-phosphatidylinositol (G-PI) tail. Both PI-specific phospholipase C and n-butanol remove CAK1 from the surface of OVCAR-3, HeLa and H-meso cells, as well as from human squamous-cell-carcinoma tissue samples (Chang *et al.*, 1992*a, b, c*). Unlike many PI-anchored cell-surface proteins and tumor-associated antigens, CAK1 is not shed into the serum of cancer patients or the medium from K1-positive cultured cells (Chang *et al.*, 1992*a*). The epitope is protease-sensitive, and exoglycosidase-insensitive (Chang *et al.*, 1992*a*), suggesting that MAb K1 reacts with the polypeptide backbone of the antigen. Furthermore, MAb K1 does not react with a panel of 34 neoglycoproteins whose carbohydrate residues are commonly present on tumor-associated antigens (data not shown).

The CAK1 antigen is present in relatively low amounts on the membrane of cultured tumor cells; we estimate that cultured cell lines contain between 15,000 and 50,000 molecules/cell (data not shown). However, expression of CAK1 is increased when tumor cells are grown in animals (Chang *et al.*, 1992*b*). In addition, the presence of the CAK1 antigen was detected in the cytoplasm of normal mesothelia (data not shown) as well as in some human squamous-cell carcinomas (Gown, A., personal communication) by sensitive immunohistochemical methods, suggesting that other forms of CAK1 may exist in the cytosol. The present study has 2 principal aims. One is to characterize the cell-surface-bound CAK1 glycoprotein. The second is to investigate the relationship of the cell-surface CAK1 with the cytoplasmic K1-reactive protein.

MATERIAL AND METHODS

Material

MAb K1 was isolated as previously described (Chang *et al.*, 1992*a, d*). MOPC21 (mouse myeloma $IgG1_\kappa$ protein) was used as a negative control antibody for MAb K1 (Sigma, St. Louis, MO). Peroxidase-conjugated goat anti-mouse IgG (H+L) was used in primary screening and Western blots (Jackson ImmunoResearch Laboratories, West Grove, PA). *E. coli* strain XL1-blue, poly d(T) cellulose columns, *in vitro* transcription kits with T3 and T7 RNA polymerases, and helper phage were purchased from Stratagene (La Jolla, CA). *E. coli* strain DH5α, agarose, protein standards, DNA markers, RNA ladders, isopropyl-β-thiogalactoside and the 5'-RACE kit were obtained from GIBCO (Gaithersburg, MD). LB broth, superbroth, SOC and NZCYM media were supplied by Digene (Silver Spring, MD), and the plasmid DNA purification kit by Qiagen (Chatsworth, CA).

Exoglycosidases (neuraminidase, β-galactosidase) and endoglycosidases (endoglycosidase F and peptide-N-glycosidase F) were purchased from Sigma and Oxford GlycoSystem (Rosedale, NY). Restriction endonucleases, T4 ligase, calf intestinal alkaline phosphatase and *in vitro* translation kit were obtained from New England BioLab (Beverly, MA) and Promega (Madison, WI). Sequenase DNA sequencing kit and PCR/RT-PCR kits were purchased from United States Biochemical (Cleveland, OH) and Perkin Elmer Cetus (Norwalk, CT) respectively.

Cell culture

The human ovarian cancer cell line, OVCAR-3, and other cell lines A431, KB3-1, HUT-102, Daudi, FLEM, HepG2, Huh7, HTB103, N87, MCF-7, and MB-MDA231 were ob-

[1]To whom correspondence and reprint requests should be addressed. Fax: (301) 402-1344.

Received: August 19, 1993 and in revised form December 6, 1993.

tained from the ATCC (Rockville, MD). Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with L-glutamine (2 mM), penicillin (50 μg/ml), streptomycin (50 units/ml), and 5–10% FBS (GIBCO). Cells were used when they reached 80–90% confluence after 3 washes with ice-cold PBS (GIBCO). These cells were then used immediately as the source of total RNA or protein preparations.

Solubilization of the CAK1 antigen from postnuclear supernatant or membrane preparation of OVCAR-3 cells

Approximately 1×10^8 OVCAR-3 cells grown to 90% confluence were rinsed 3 times with cold PBS- and scraped into 50-ml conical tubes. The cells were then pelleted and washed once in PBS without Ca^{++} and Mg^{++} (PBS^-) and once in hypotonic buffer (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$ and 1 mM EDTA). After resuspension in 5 ml hypotonic buffer for 10 min at 4°C, the cells were homogenized by 20 to 40 strokes of a tight Dounce homogenizer. The post-nuclear supernatant (PNS) was isolated by centrifugation at 200 *g* at 4°C for 10 min. The membrane-rich preparation was made from the PNS by ultracentrifugation at 100,000 *g*. The soluble and cytosolic fraction was used directly for Western blotting and the PNS or the membrane-enriched preparation was further used for solubilization experiments.

To solubilize the CAK1 protein, 10% octylglucoside in 0.25 M sodium phosphate, pH 7.5, 0.02% sodium azide, 0.05 M EDTA, 0.1% SDS, 1% β-mercaptoethanol, and 1 mM PMSF were added to the PNS or membrane preparation at a final detergent concentration of 1 to 1.5% and incubated with gentle rotation for 60 to 120 min at 4°C. The octylglucoside-solubilized proteins were collected after ultracentrifugation at 100,000 *g* for 30 min at 4°C. Both pellet and supernatant were adjusted to equal volumes before applying to SDS-PAGE.

Enzymatic digestion of CAK1 from OVCAR-3 cells and Western analysis

One ml of the octylglucoside-solubilized OVCAR-3 extract was dialyzed at 4°C or 24°C for 5 to 8 hr against (1) neuraminidase buffer (0.05 M sodium acetate, pH 5.5, 0.02% sodium azide and 2 mM PMSF); or (2) neuraminidase/β-galactosidase buffer (0.05 M sodium citrate, pH 3.5, 0.02% sodium azide and 1 mM PMSF); or (3) O-glycanase buffer (0.25 M sodium phosphate pH 6.0, 0.02% sodium azide, 0.05% SDS, 1 mM PMSF and 1% β-mercaptoethanol); or (4) endoglycosidase F buffer (0.1 M sodium phosphate pH 6.0, 0.05 M EDTA, 0.1% SDS, 1% β-mercaptoethanol, 0.02% sodium azide and 1 mM PMSF); or (5) N-glycanase (peptide-N-glycosidase F) buffer (0.25 M sodium phosphate pH 7.5, 0.02% sodium azide, 0.05 M EDTA, 0.1% SDS, 1% β-mercaptoethanol and 1 mM PMSF). After a 500-μl aliquot of each of these dialyzed samples had been heated at 100°C for 3 min, the samples were cooled on ice and 10% Triton X-100 or 10% octylglucoside was added to a final concentration of 1%, and PMSF to 1 mM, followed by a 37°C incubation with 1 unit of neuraminidase (*Clostridium perfringens*, Sigma) for 1 to 2 hr; or 1 unit of neuraminidase plus 4 units of β-galactosidase (jack bean, Sigma) for 4 hr; or 6 mU of O-glycanase (glycopeptide α-N-acetylgalactosaminidase, Oxford Glycosystem) overnight; or 5 units of endoglycosidase F overnight; or 8 units of peptide-N-glycosidase F overnight. The reactions were terminated by adding 300 μl of 3 × SDS-PAGE sample buffer, and the samples were resolved on a 10 or 12.5% SDS-PAGE reducing gel, stained with either Coomassie blue or silver stain (Sigma, Bio-Rad), and transferred to nitrocellulose for Western blotting.

Western blotting was performed as described (Chang *et al.*, 1992*a*) with minor modifications. After SDS-PAGE and transfer, nitrocellulose papers were first soaked in 3% milk blotto (3% Carnation non-fat milk powder, 2% glycine, 1 mM PMSF in PBS) at room temperature for 30 to 60 min, followed by incubation with 5 μg/ml MAb K1 or MOPC-21 at 4°C for 12 to 18 hr. The blots were washed in PBS containing 0.05% Tween 20 (PBS/T) 5 times for 60 min, then incubated with 10 μg/ml of peroxidase-conjugated goat anti-mouse IgG (H+L) in blotto at 4°C for 8 to 16 hr, or at 23°C for 1 to 2 hr. After 5 washes in PBS/T for 60 min, the nitrocellulose blots were developed using 0.4 mg/ml of diaminobenzidine (Sigma) and 0.01% hydrogen peroxide in PBS for 10 min. The reaction was terminated by rinsing the filters in distilled water. Western analysis for expression screening of the Uni-ZAP-XR OVCAR-3 cDNA library was performed in a similar manner.

Poly $(A)^+$ RNA isolation, and cDNA library construction and screening

Total cellular RNA from OVCAR-3 cells was extracted as described (Sambrook *et al.*, 1989*a*). Poly $(A)^+$ RNA was isolated with oligo(dT)-cellulose affinity chromatography according to the Stratagene protocol. The cDNA synthesis and library constructions were performed by Stratagene. Poly $(A)^+$ RNA (5 μg) were used as templates for synthesis of the cDNA using the ZAP cDNA™ synthesis kit. The cDNAs containing 3′ XhoI and 5′ EcoRI cohesive termini were size-fractionated, ligated into phosphorylated Lambda ZAP II arms, and packaged with Gigapack II packaging extracts, and 3.5×10^6 primary phages were obtained.

Both 2.5×10^6 plaques of an unamplified and 1×10^6 pfu of an amplified library were screened at approximately 50,000 pfu/150-mm plate by the method of Young and Davis (1983) using protein-A-purified MAb K1 (5 μg/ml) and peroxidase-conjugated goat anti-mouse IgG (H+L) (10 μg/ml). Positive plaques were isolated and the phages were purified to homogeneity by at least 3 rounds of screening. *In vivo* excision of the positive phage clones with R408 helper phage was carried out as described (Arcot and Deininger, 1992), and the circularized phagemid DNAs were extracted using the Qiagen plasmid DNA isolation kit and protocol. Restriction mapping using XhoI, EcoRI, SalI, BamHI and NcoI revealed that 4 clones were identical. The fifth clone contained an internal EcoRI site and reacted weakly with the control MOPC-21 antibody. One clone, g17(3), which was chosen for DNA sequence analysis, contained a cDNA insert of 2,100 bp with a 668-bp open reading frame sequence at its 5′ terminus. A cDNA probe (specific activity = 8.5×10^5 cpm/μl) spanning the 668-bp ORF sequence was made by random priming and the same OVCAR-3 library was re-screened to isolate a full-length cDNA clone using the filter hybridization method (Benton and Davis, 1977) with minor modifications.

Sequencing analysis, restriction mapping, and Northern and Southern analyses

Using T3, T7 and 12 17-bp synthetic primers, the entire cDNAs were sequenced according to the method of Sanger (1977). Restriction sites were determined with several restriction endonucleases (XhoI, SalI, NcoI and EcoRI) and the nucleotide sequence and the deduced amino-acid sequence were analyzed for homology to sequences in GenBank/EMBL.

To determine the size of the mRNA, total RNA (20 μg) and/or poly$(A)^+$ mRNA (2 μg) were electrophoresed on a 1% agarose gel containing MOPS buffer (0.04 M 3-morpholinopropanesulfonic acid, 0.01 M sodium acetate, 0.01 M EDTA) and 16.6% formaldehyde. All gels were stained with ethidium bromide to assess the integrity of the RNA and the quantity of each loading. The RNA was then transferred to nitrocellulose paper in 10× SSC for 18 to 36 hr and cross-linked using a UV cross-linker (Stratalinker 1800, Stratagene). The blots were pre-hybridized, then hybridized with the appropriate cDNA probe. Northern blots were also probed with radiolabeled human β-actin cDNA as an internal control. In Southern-blot analyses, human placental genomic DNA was digested with

EcoRI, HindIII, BamHI, PstI and BglII, electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose paper (Human Geno-Blot, Stratagene). After prehybridization with salmon sperm DNA, the blots were hybridized with the probe as described above.

In vitro *transcription and translation*

To study the polypeptide encoded by the cDNA clones, the purified g5 and g26 pBluescript plasmid DNAs were linearized by digestion with XhoI or EcoRI. The *in vitro* synthetic and capped RNAs were transcribed with T3 or T7 RNA polymerase in the presence of $m^7G(5')ppp(5')G$ analog of GTP to obtain either sense or anti-sense RNA transcripts following the method supplied by the manufacturers (Stratagene and Promega). The yield of the synthetic RNAs ranged from 40 to 50 μg per reaction and the integrity and size of the transcripts were determined by gel electrophoresis on a 1% agarose gel and ethidium bromide staining. Synthetic RNAs (2–4 μg/ml) were translated *in vitro* in a 50-μl reaction solution containing 10 μl (0.8 mCi/ml) of L-[^{35}S]-methionine, 10 μl of cocktail, 4 μl of 1M potassium acetate, 1 μl of 32.5 mM magnesium acetate, 1 μl RNasin and 20 μl of reticulocyte lysate in the presence and absence of 0.5 μl of dog pancreatic microsomal membrane following the protocol provided by the manufacturer (New England Nuclear and Promega). Translation products were resolved on a 12.5% SDS-PAGE reducing gel, the proteins were fixed and the unincorporated radioactivity was removed by soaking the gel 3 times in 200 ml of fixation buffer (40% methanol and 10% acetic acid in deionized water) for 30 min. The gels were then soaked in 200 ml of Amersham's Amplify enhancement solution for 30 min. After drying, the translated products were visualized by autoradiography.

Expression of the cloned cDNA in E. coli

Oligonucleotide primers (1. 5'ACGTTGCAACGTCATATG GGACCAC TTCACAAAAGC3' and 2. 5'ACGTTGCAAC-GTGAATTCTTAGGTCAGCTT CAAGCC3') were synthesized by automated phosphoramidite chemistry on a model 380A DNA synthesizer (Applied Biosystems). NdeI and EcoRI restriction sites were introduced 5' to the first initiation codon and 3' to the termination codon of the open reading frame (ORF) of g26 (2,444 bp) using a recombinant PCR method. PCR was performed for 35 cycles (denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec and elongation at 72°C for 60 sec), the final elongation step being extended by 3 min, using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). The reaction products were filtered through a Centricon 100 and extracted with phenol/chloroform. The amplified DNA fragment was analyzed in a 1% agarose gel stained with ethidium bromide.

pVEX11 plasmid DNA and the PCR-engineered ORF of g26 DNA were digested with NdeI and EcoRI. This was followed by 2 units of calf intestine alkaline phosphatase (CIAP) treatment at 37°C for 30 min (pVEX11 only) and phenol/chloroform extraction. The DNA fragments were then isolated and purified to homogeneity using LMP agarose (SeaPlaque-GTG from FMC, Rockland, ME) as described (Sambrook *et al.*, 1989*a*). The NdeI/EcoRI containing ORF of g26 (referred to as g26NE) fragment was directionally cloned to the pVEX11 vector with T4 ligase in an insert:vector molar ratio of 3:1 using a rapid ligation method (Sambrook *et al.*, 1989*b*). The ligated product (pAPK1) was transformed into competent DH5α *E. coli* cells and the insert-containing clones were isolated and verified by restriction mapping of DNA minipreps. The cDNA was then used to transform BL21 (λDE3) *E. coli* in an effort to express the cloned gene following the procedure described (Studier and Moffatt, 1986).

A single colony of the pAPK1, or pVEX11-transformed BL21 cells was inoculated into a 5-ml LB culture containing 100 μg/ml of ampicillin, and grown at 37°C until it reached an OD_{600} of 0.8 to 1.0; this was followed by addition of 1 mM IPTG and continuous growth at 37°C for 1 to 2 hr. An aliquot of each culture was dissolved in SDS-PAGE sample buffer and the rest of the culture was pelleted. The bacterial pellet was resuspended in 250 μl of TE buffer, frozen and thawed 3 times on dry ice and sonicated for 30 sec ×2 at 50 w. The lysate was microcentrifuged and the pellet was suspended in 500 μl 1× sample buffer, while the supernatant was mixed with 250 μl of 2× SDS-PAGE sample buffer. The whole cell extract, and insoluble and soluble fractions were analyzed by 10% SDS-PAGE and Western blotting as described above.

Rabbit and mouse sera against recombinant APK1 protein

Cytosolic recombinant antigenic protein (APK1) encoded by pAPK1 was precipitated with 20% ammonium sulphate and resolved in a 10% SDS-PAGE preparative gel and stained with Coomassie brilliant blue. The APK1 bands were electroeluted. APK1 (0.3 mg) in 0.5 ml was mixed with 0.5 ml complete Freund's adjuvant (CFA) and injected into rabbits s.c. at multiple sites on day 1, then a second boost of antigen in incomplete Freund's Adjuvant (IFA) was given on day 21 and a third boost of 0.3 mg/0.5 ml APK1 on day 42. The sera were collected on day 49.

Renatured APK1 (20 μg in CFA) was injected into BALB/c mice both i.p. and s.c. Three weeks later each mouse was boosted with 10 μg of APK1 in IFA, then 5 boosts of 10 μg/mouse of APK1 in PBS were given once a week. Blood was collected within 3 days after the final boost, and the serum was saved.

RESULTS

Characterization of CAK1 from OVCAR-3 cells

We have previously used intact OVCAR-3 cells to show that CAK1 is a glycoprotein probably anchored to the cell membrane by phosphotidylinositol (Chang *et al.*, 1992*a, b, c*). To further characterize the protein, we needed to release it in a soluble form. We have previously tried several detergents without success (Chang *et al.*, 1992*a*). In this study, we evaluated octylglucoside. Figure 1 shows that over 80% of CAK1 was released into the supernatant after treatment with 1% or 1.5% octylglucoside (Fig. 1, lanes 1 and 3 respectively),

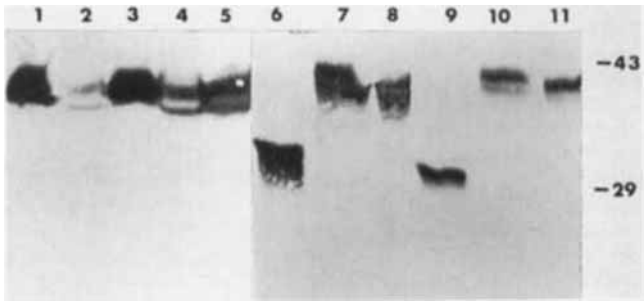

FIGURE 1 – Octylglucoside solubilization of the CAK1 antigen and evidence of N-linked glycosylation. Octylglucoside-solubilized OVCAR-3 membrane proteins, 50–100 μg per lane, were separated in 10% acrylamide gels and immunoblotted as described in the text. Lane 1, soluble proteins in 1% octylglucoside; lane 2, insoluble pellet from 1% octylglucoside treatment; lane 3, soluble protein in 1.5% octylglucoside; lane 4, insoluble pellet from 1.5% octylglucoside-treated material; lane 5, control without octylglucoside treatment. Digestion of the ocylglycoside-solubilized CAK1 with endoglycosidases was performed as described in the text. Lane 6, Endo-F-digested CAK1; lane 7, CAK1 treated in an EndoF buffer without Endo F; lane 8, control CAK1 before treatment; lane 9, PNGase-F-digested CAK1; lane 10, CAK1 treated in a PNGase F buffer without PNGase F; and lane 11: control CAK1 before treatment. Right: kDa values.

while less than 20% of CAK1 remained associated with membranes (Fig. 1, lanes 2 and 4). The CAK1 band was often a doublet. However, treatment with endoglycosidase F generates a single, smaller band (lanes 6 and 9) indicating that the doublet may be due to different glycosylated forms. In addition, an abundant membrane protein with an MW ~39 kDa is evident upon Coomassie blue staining which may contribute to the generation of the 2 bands. We then subjected the octylglucoside solubilized CAK1 to a series of exo- and endoglycosidases. Extensive digestion with neuraminidase or neuraminidase and β-galactosidase or O-glycanase had no effect on the size of CAK1, whereas digestion with endoglycosidase F (Fig. 1, lanes 6–8) or peptide-N-glycosidase F (Fig. 1, lanes 9–11) decreased the apparent molecular weight of the glycoprotein. This indicates that CAK1 is an N-linked glycoprotein. The apparent MW of CAK1 decreased from 40 kDa to 30 kDa after deglycosylation with either of the endoglycosidases (Fig. 1, lane 7 or 8 *vs.* 6, and lane 10 or 11 *vs.* 9, respectively). Peptide-N-glycosidase F deglycosylated CAK1 more completely than endoglycosidase F (Fig. 1, lane 9 *vs* 6). After removal of carbohydrates, MAb K1 reacted with the deglycosylated form of CAK1 with the same intensity as with glycosylated CAK1 (Fig. 1, lanes 7, 8, 10, and 11). This result confirms our previous data that MAb K1 recognized the core peptide rather than the carbohydrate moiety present on the protein (Chang *et al.*, 1992*a*). Based on the observation that the antibody reacted with a denatured form of CAK1 on an immunoblot, we screened a λ cDNA expression library for a cDNA encoding the 30-kDa protein reactive with MAb K1.

Isolation and characterization of the pAPK1 cDNA clones

The human ovarian carcinoma cell line, OVCAR-3, was chosen as a source of poly $(A)^+$ mRNA for construction of a lambda ZAP cDNA library because OVCAR-3 cells were the original immunogens for generation of MAb K1 and they contain CAK1 on their surface as demonstrated by immunofluorescence (Chang *et al.*, 1992*a, d*). We screened about 2×10^6 recombinant phages with MAb K1 and obtained 5 positive clones from an amplified library. These K1-positive clones were purified and screened simultaneously with MAb K1 and a control antibody, MOPC-21, of the same isotype, to verify their specificity. Four of the 5 clones were specifically reactive with MAb K1. One clone showed weak reactivity with the control MOPC-21 antibody and was not further investigated. All 4 K1-specific cDNA clones had inserts about 2,100 bp long (Fig. 2) and had similar restriction patterns when analyzed by the restriction enzymes XhoI, EcoRI, SalI, BglI, BamHI and NcoI, indicating they may have come from a single primary phage clone.

The complete nucleotide sequence was determined on both DNA strands. The g17(3) clone is 2,100 bp long, and contains a 668-bp open reading frame followed by a 3′ non-coding segment of 1,432 bp. Neither a 5′ non-coding sequence nor a consensus initiation sequence (Kozak, 1987) could be found in the cDNA fragment. Therefore, we assumed that this clone is missing sequences at the 5′ end of the cDNA. To determine the size of the messenger RNA, the RNAs extracted from tumor-cell lines and normal tissues were resolved by electrophoresis and a Northern-blot was performed. This experiment showed that the RNA was about 3,800 bp in size (Fig. 3). To obtain the sequence of the 5′ end, RNA from OVCAR-3 cells was retrotranscribed into cDNA, which was then extended in the 5′ direction using the 5′ RACE method (Frohman *et al.*, 1988). The result showed that a segment of about 1,000 to 1,400 bp was missing at the 5′ end of the g17(3) insert (data not shown). To obtain a full-length cDNA, the OVCAR-3 cDNA library was rescreened with an EcoRI/BamHI cDNA fragment as a probe [g17(3)EB]. This probe came from the 5′ end of the g17(3) cDNA insert. Using a random-primed cDNA probe, we obtained 30 candidate clones from the primary screening; 11 clones were subjected to a second round of subcloning and all had inserts that ranged from 400 bp to 2,500 bp in size. Two of the longest clones (g5 and g26) were sequenced as shown in Figure 2. The nucleotide sequences of g5 (2335 bp) and g26 (2444 bp) were identical except that clone g26 contained an

```
   1  TGGATAGAGCGAGGAGAGGTCAACCGTCGTAGCGCCAATAACTTCTACTCCATGATCCAG
  61  TCGGCCAACAGCCATGTCCGCCTGCCTGGTGAACGAGAAAGCTGCCCATGAGAAAGATAT
 121  GGAAGAAGCAAAGGAGAAGTTCAAGCAGGCCCTTTCTGGAATTCTCATTCAATTTGAGCA

 181  GATAGTGGCTGTGTACCATTCCGCCTCCAAGCAGAAGGCATGGGACCACTTCACAAAAGC
                                                  M  G  P  L  H  K  S

 241  CCAGCGAAGAACATCAGCGTGTGGTGCAAACAAGCTGAGGAAATTCGCAACATTCATAAT
      P  A  K  N  I  S  V  W  C  K  Q  A  E  E  I  R  N  I  H  N

 301  GATGAATTAATGGGAATCAGGCGAGAAGAAGAAATGGAAATGTCTGATGATGAAATAGAA
      D  E  L  M  G  I  R  R  E  E  E  M  E  M  S  D  D  E  I  E

 361  GAAATGACAGAAACAAAAGAAACTGAGGAATCAGCCTTAGTATCACAGGCAGAAGCTCTG
      E  M  T  E  T  K  E  T  E  E  S  A  L  V  S  Q  A  E  A  L

 421  AAGGAAGAAAATGACAGCCTCCGTTGGCAGCTCGATGCCTACCGGAATGAAGTAGAACTG
      K  E  E  N  D  S  L  R  W  Q  L  D  A  Y  R  N  E  V  E  L

 481  CTCAAGCAAGAACAAGGCAAAGTCCACAGAGAAGATGACCCTAACAAAGAACAGCAGCTG
      L  K  Q  E  Q  G  K  V  H  R  E  D  D  P  N  K  E  Q  Q  L

 541  AAACTCCTGCAACAAGCCCTGCAAGGAATGCAACAGCATCTACTCAAAGTCCAAGAGGAA
      K  L  L  Q  Q  A  L  Q  G  M  Q  Q  H  L  L  K  V  Q  E  E

 601  TACAAAAAGAAAGAAGCTGAACTTGAAAAACTCAAAGATGACAAGTTACAGGTGGAAAAA
      Y  K  K  K  E  A  E  L  E  K  L  K  D  D  K  L  Q  V  E  K

 661  ATGTTGGAAAATCTTAAAGAAAAGGAAAGCTGTGCTTCTAGGCTGTGTGCCTCAAACCAG
      M  L  E  N  L  K  E  K  E  S  C  A  S  R  L  C  A  S  N  Q

 721  GATAGCGAATACCCTCTTGAGAAGACCATGAACAGCAGTCCTATCAAATCTGAACGTGAA
      D  S  E  Y  P  L  E  K  T  M  N  S  S  P  I  K  S  E  R  E

 781  GCACTGCTAGTGGGGATTATCTCCACATTCCTTCATGTTCACCCATTTGGAGCAAGCATT
      A  L  L  V  G  I  I  S  T  F  L  H  V  H  P  F  G  A  S  I

 841  GAATACATCTGTTCCTACTTGCACCGTCTTGATAATAAGATCTGCACCAGCGATGTGGAG
      E  Y  I  C  S  Y  L  H  R  L  D  N  K  I  C  T  S  D  V  E

 901  TGTCTCATGGGTAGACTCCAGCATACCTTCAAGCAGGAAATGACTGGAGTTGGAGCCAGC
      C  L  M  G  R  L  Q  H  T  F  K  Q  E  M  T  G  V  G  A  S

 961  CTGGAAAAGAGATGGAAATTCTGTGGCTTCGAGGGCTTGAAGCTGACCTAAATCTCTTTG
      L  E  K  R  W  K  F  C  G  F  E  G  L  K  L  T  *

1021  CCTAACAACTTGGGACTCCTGAAGATAAATATGTGTTGGACAAGCATAGAAAGTGATTTA
1081  TATTTTTAATGGTTTTCAAGTGGAAGTTCCTTTGAATTTGTCAGTTCATTCCTGGAAAAT
1141  CTTTTGAGTTAAAATAAGGATCCTAGGACAGCACCTCGAACTACAGGCCCTAAAGAGAAA
1201  TTGCCTCAAACCACAAGTGCTGTAACTTCCTCCCCTTTCTGTCAATTGGTTGTCTTTAAA
1261  TATTGCAAAAGTCCTGATGCTAAACAGTATTTGGAGTGTTTTCAGTGTCTGTACTACTGT
1321  TGTAGACCTTGGTATTTTTTTAAACACTGTTAACTGAAATGTTTTGATGATTTGTATGTG
1381  ATTTGTGTTTCTAAACTTCTCTTTACATTAATGTTGTTACTGGTGAAAGGCATGAGAGCA
1441  GCACTAAGTCCTCTGTGTAACTGCCATTGTCTTTCCAATCCCCAGTAGACCAGTAAATAA
1501  ATAACACATCAGTGTCTTCTAGAAGGTGCCTGACCAGGTTCACCTTTTAAACGACAAAGC
1561  ATGGTTTGTGGCTTTTTGCAAAATTACTATGAACCAAAAGTTGACAAATGTTCCAAAGTT
1621  ATTTTCTCTAACATATCACATTAAAGATCTGTTTCAGAATTGTAAAAAGTACATCTAGAT
1681  GTGTTTACAGAAAGCAAGTATCCAGTATGACTGGCATGTGTTCATGCTATTCAGAATCAC
1741  TTGTAAATAGTCTGCTTTTAAAGGAGGGCATGTTCAGTTTTCTGTGAATTAAAATATGCT
1801  CATGTGTGGGCACACACGCACAAACACACACACGCACGCACACAGTGGCAGAAGGATTTA
1861  TATTAATATTCTTTCCCCTCTGGCCTTCTTACAGTCTGTTGGTCCCTTTGCTTCTGTTGT
1921  CAGTGTGTTGAATTGCAAACCGAGTACTGCTGTAAATACTATGTTTACTTCATGCTGAAT
1981  GTTTGCAAAGACTTGATATAAGTATTAATAGTAATGAATCAATGAATAAATAATGAGCTA
2041  GGGTTTGTGAGGCTTTCTACAAATAGGTCAGCTCCACCTGGAGTGCGAATTGCCAGAGAC
2101  ACCTTGGTAGTGCCCATCGGCAAATCGCAATGGCAGCATGTGAGTGGACCCATTCAGAAA
2161  CTTTCTGCTTGGTGGAAAGTAAACAGAGAGGATGGAGGTTTGGGGCGAATGTCCTGAGGC
2221  AGAGATGGTCTTTATTGTGTGTGGTGGTGGTTGTGGTATTTATAATAATGCAAGCATACC
2281  CTCCCTTGAGTCTCAATTGAAGATAAAAGAATGTACTGAGCAAGCAAAGCCAATGGAGAG
2341  TATTTCACAAAAATACTTTGTAAATGAGATGCCAGTAGTGTTCAAAGTTGTATTTTTAAA
2401  AGATAAATATTCCTTTTTATACCTCAAAAAAAAAAAAAAAAAA  2444
```

FIGURE 2 – Nucleotide sequence of the cDNA and deduced amino-acid sequence of the open reading frame. The complete nucleotide sequence (upper line) and deduced amino-acid sequence designated as single letters (lower line) for the g26 cDNA clone are shown. Numbers (left) represent nucleotide bases. The translation of CAK1 starts at position 219 with a typical eukaryotic consensus sequence and ATG codon, and terminates at position 1009 (TAA). Three potential glycosylation sites are underlined. Termination signals (AATAAA or GATAAA) were located at positions 1496, 2024 and 2402 and then followed by a polyadenylation tail. EcoRI and Xhol I insertion sites (not shown) are immediately adjacent to the 5′ and 3′ termini.

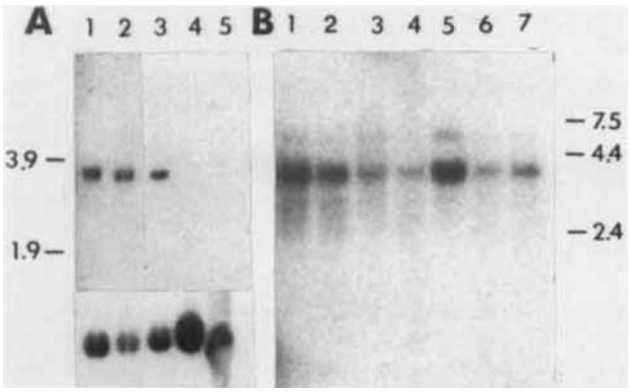

FIGURE 3 – *(a)* RNA blot analysis of normal and tumor cell lines. Poly(A)$^+$ mRNAs were isolated as described. The sources of poly(A)$^+$ mRNA are as follows: Lane 1, OVCAR-3; lane 2, A431; lane 3, N87; lane 4, FEMX; lane 5, WI38 cells. β-actin probe was used as an internal control and showed on bottom figure. Left: kb values. *(b)* Normal human tissue RNA blotting analysis. Two micrograms of poly(A)$^+$ RNA from human heart, placenta, lung, liver, muscle, kidney and pancreas, as indicated in lanes 1 to 7, were electrophoresed and transferred to charge-modified nylon membrane. A major 3.8-kb band was evident for all of the tissue RNAs tested and a minor band of 5.5 kb was also present. Right: kb values.

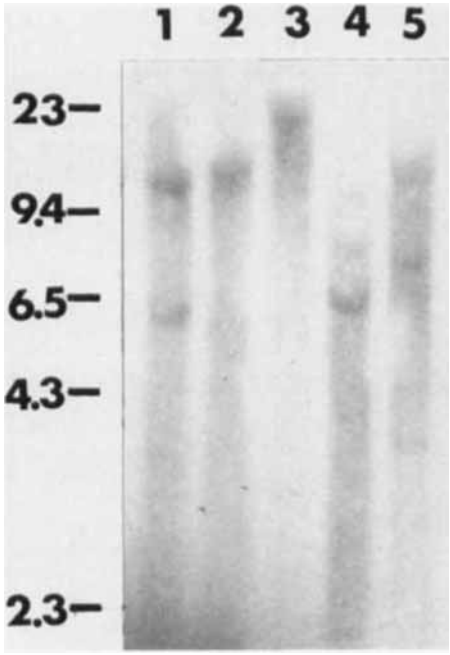

FIGURE 4 – DNA analysis of human genomic DNA. Eight micrograms of placental genomic DNA were digested with EcoRI, HindIII, BamHI, PstI and BglII as shown in lanes 1 to 5. Internal restriction sites are seen for all endonucleases except for HindIII in the sequence. Left: kb values.

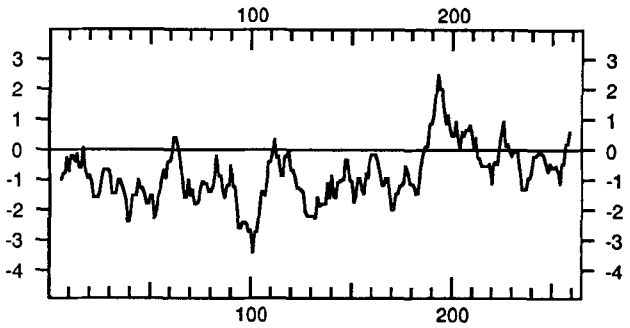

FIGURE 5 – Hydropathy plot of CAK1. A Kyte and Doolittle (1982) analysis of the deduced amino-acid sequence using a window size of 11.

extra 109 bp at the 5′ end. In clone g5, the coding region was preceded by 97 bp, while in clone g26, it was preceded by 219 bp. Both contained a 789-bp open reading sequence. The open reading frame is initiated by an ATG codon at position 220 with a purine base (G) at positions 217 and 221 consistent with a eukaryotic consensus initiation sequence (GXXATGG) (Kozak, 1987). The in-frame ATG is preceded by 2 in-frame stop codons at positions 109 and 175. The 3′ non-coding segment contains 2 polyadenylation signals (AATAAA) at positions 1496 and 2024 and a GATAAA (2402) 17 bp upstream of poly (A) tail.

Northern- and Southern-blot analysis

As shown in Figure 3*a*, a single 3,800 mRNA species from cells of OVCAR-3, A431 and N87 (lanes 1, 2 and 3) was found to hybridize with the cDNA probe, but it was not detectable in FEMX melanoma cells or WI38 human fibroblast cells (lanes 4 and 5). Based on ethidium bromide staining of the gel and on similar hybridization with the control β-actin probe (lanes 1–5, bottom), the RNA from each of these sources appeared to be intact and equally loaded and transferred. Using the same probe, we also tested poly(A)$^+$ mRNA from various normal human tissue samples (Fig. 3*b*). mRNA of the same size was present in heart, placenta and skeletal muscle (lanes 1, 2 and 5, respectively). It was also present, but in lower amounts, in lung, liver, kidney and pancreas (lanes 3, 4, 6 and 7, respectively). Small amounts of mRNA, about 5.5 kb in size, hybridized with the cDNA probe in all the normal tissues tested. The origin of this signal is not known. A Southern blot of the digested genomic DNA from human placental tissue is shown in Figure 4. The probe hybridizes strongly with a prominent band of about 16 kb in genomic DNA cleaved with HindIII (lane 2). Other less prominent bands are present in other digestions. The simple pattern of the Southern blot suggests that the CAK1 antigen is not a member of a large gene family.

Amino-acid sequence analysis

The deduced amino-acid sequence of the cloned cDNA is shown in Figure 2. The protein is 263 amino acids in length with a calculated MW of 30,511 kDa. It is an acidic protein with a pI = 4.9 and contains 3 potential N-linked glycosylation sites, Asn-X-Ser or Asn-X-Thr as underlined in Figure 2 and multiple potential phosphorylation sites (Ser, Thr, and Tyr). An amino-acid hydropathy plot of the deduced amino acid identifies one very hydrophobic region from amino-acid residues 185 to 200 (Fig. 5). Homology analysis was performed for both nucleotide and amino-acid sequences using FASTA and TFASTA of the GCG program against EMBL/Genebank. No significant homology was found with any known sequence at either the nucleotide or amino-acid level. At the carboxyl terminus of proteins that are attached to the plasma membrane by PI, there are usually non-polar amino acids forming a hydrophobic region (Low and Saltiel, 1988). However, we could not identify such a region in the deduced peptide sequence of the cDNA we had isolated. Furthermore, a typical signal peptide sequence could not be recognized at the N-terminus of the coding sequence, even though CAK1 is known to be a cell-surface-associated glycoprotein (Chang *et al.*, 1992*a, b, c, d*).

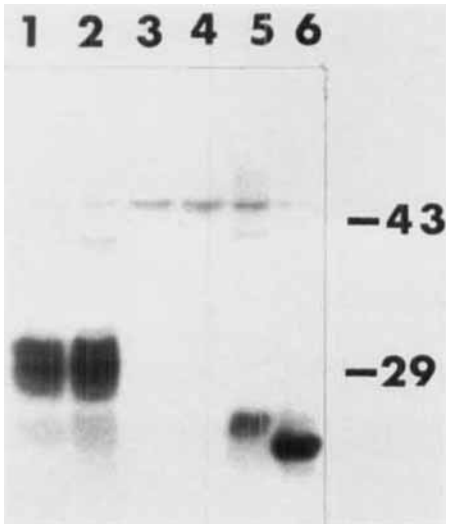

FIGURE 6 – *In vitro* translation of the protein encoded for by the cloned cDNA. Following *in vitro* transcription of synthetic RNA from the pBluescript plasmid containing the g26 cDNA insert, 2 μg of the synthetic RNA were translated *in vitro*. *In vitro* translation products were resolved on a 12.5% reducing SDS-PAGE and autoradiographed (see text). Lane 1 manifests the total ^{35}S-labeled protein following *in vitro* translation of g26 cDNA transcript; lane 2 contains the total ^{35}S-labeled product translated in the presence of microsomal membranes. Lanes 3 and 4 represent the products of the control *in vitro* translation to which an anti-sense transcript or no RNA transcript was added, respectively; lanes 5 and 6 were control (human placenta lactogen mRNA) *in vitro* translation products translated in the absence (lane 5) or presence (lane 6) of microsomal membranes. Right: kDa values.

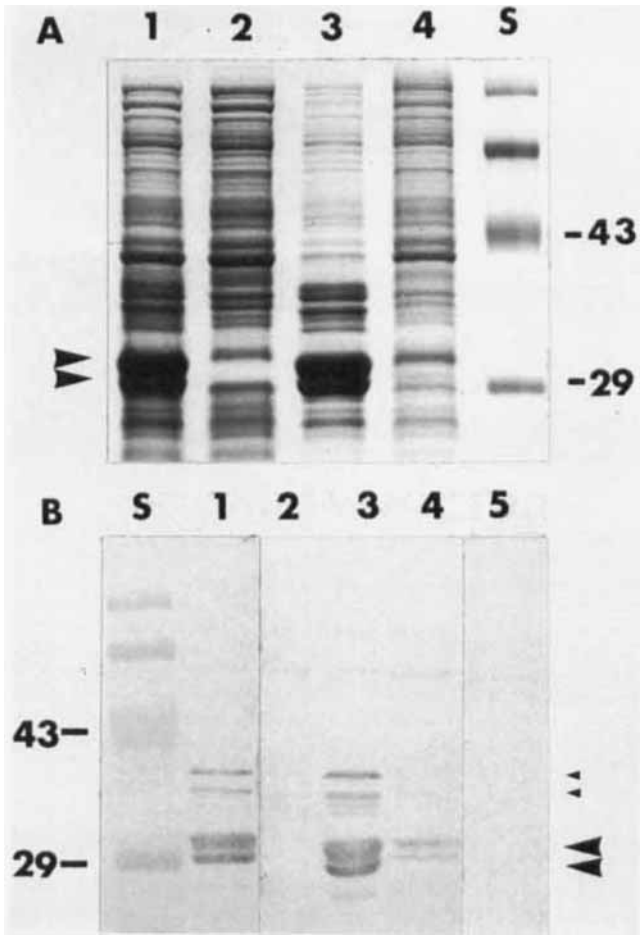

FIGURE 7 – *(a)* Analysis of the *E. coli* expressed recombinant APK1. Whole-cell lysates, soluble and insoluble fractions of *E. coli* transformed with T7 driven expression vector (pVEX11) with or without gAPK1 insertion are shown on reducing SDS-PAGE. Lane 1, whole-cell lysate of the gAPK1-transformed *E. coli*, in which bands of apparent MW 29 and 30 kDa are seen (arrowheads); lane 2, whole-cell lysate of the pVEX11 transformed cells; lane 3, soluble fraction; lane 4, insoluble fraction of gAPK1-transformed cells. Lane S, molecular weight standard. Right: kDa values. *(b)* Immunoblotting analysis of recombinant APK1. Immunoblotting was performed on nitrocellulose transferred with revolving bands from reducing SDS-PAGE in similar setting as in *(a)*, using MAb K1 (lanes 1 to 4) and a negative control MOPC-21 (lane 5, for whole-cell lysate of gAPK1 transformant). Two major bands with apparent MW of 29 and 30 kDa were moderately reactive with MAb K1 (large arrowheads). Two minor bands, (small arrowheads) were not specific for gAPK1 expression, since they were also present in lower amounts in pVEX11-transformed cells (lane 2) and in similar amounts in TGFα-PE40-transformed cells (not shown). Lane S, MW standards. Left: kDa values.

In vitro *translation analysis*

Even though the cDNA clone we had isolated did not have a typical signal sequence as expected for a membrane-associated protein, we wanted to determine if the sequence at the amino end might still function as a signal sequence and enable the protein to reach the cell surface. Therefore, we used a reticulocyte lysate system and pancreatic microsomes to determine if the protein encoded by clone g26 could be introduced into microsomes. First, mRNAs were prepared, using T3 or T7 RNA polymerase; both sense and anti-sense transcripts were detected as a single band (data not shown) with apparent lengths of 2,400 bp for the g26 transcripts. As shown in Figure 6, *in vitro* translation of the synthetic sense RNA of g26 yields a polypeptide with an apparent MW of approximately 29–30 kDa in the absence (lane 1) or presence (lane 2) of microsomal membranes, whereas *in vitro* translation products of the anti-sense RNA transcript (lane 3) or a negative control without addition of exogenous RNA (lane 4) did not produce detectable quantities of the 29- to 30-kDa radiolabeled polypeptide. There was no apparent change in MW of the *in vitro*-translated g26 in the presence of microsomal membranes, indicating that neither translocation nor glycosylation of the cDNA encoded product had occurred (Fig. 6, lane 2). In addition, we performed protease protection and microsome sedimentation experiments (data not shown) which verified that the cDNA-encoded product was not translocated into microsomes.

Expression of recombinant APK1 antigen in E. coli

To prepare a sufficient amount of APK1 for further characterization, we expressed the antigen in *E. coli* using a T7-based expression system (Studier and Moffatt, 1986). The g26NE segment was ligated into a T7-based vector (pVEX11) as described above and the resultant pAPK1 was expressed in *E. coli* BL21 (λDE3). As shown in Figure 7*a*, the whole-cell lysate (lane 1) and the cytosolic fraction (lane 3) of the transformed BL21 cells showed 2 bands with apparent MWs of 29 and 30 kDa (arrowheads). Similar bands were not evident either in BL21 cells transformed with pVEX11 (lane 2) or in insoluble inclusion bodies and membranes (lane 4). These results indicate that these proteins are a result of transformation of BL21 with pAPK1 and are soluble in the cytosol. The formation of doublet bands instead of a single prominent band for pAPK1 expression is probably due to a Shine-Dalgarno (SD) sequence present at positions 107 to 112 (AAGAAG) 2 bp upstream from the third ATG codon in the open reading

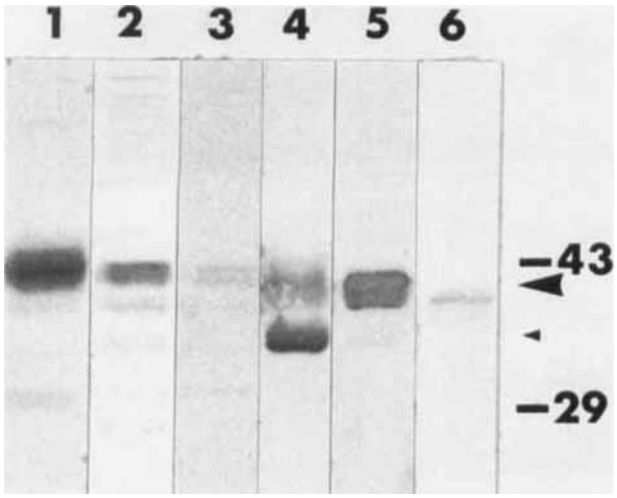

FIGURE 8 – Antisera raised from mouse immunized with recombinant APK1 found in OVCAR-3 cells. Fifty micrograms of octylglucoside-solubilized membrane (lanes 1–3) and cytosolic preparations (lanes 4–5) of OVCAR-3 cells were electrophoresed and transferred to nitrocellulose, and a Western blot was performed as described in text. Lanes 1 and 4, incubated with MAb K1; lanes 2 and 5, incubated with the mouse antiserum to recombinant APK1; lanes 3 and 6, incubated with normal mouse serum. Large arrowhead: cytosolic protein reactive with both MAb K1 and mouse antiserum; small arrowhead: another cytosolic species reactive only with MAb K1. Right: kDa values.

frame. The BL21 T7 RNA polymerase may recognize this internal SD sequence. This was confirmed by immunoblotting which showed that both bands reacted with MAb K1 with a similar intensity (Fig. 7*b*, lanes 1 and 3, large arrowheads). The reactivity of MAb K1 with the 29-kDa and 30-kDa recombinant proteins was specific, since an isotype-matched control antibody MOPC-21 did not react with these bands (lane 5). Other minor bands (small arrowheads) of a higher MW also reacted with MAb K1, but these bands were also present in BL21 lysates transformed with either pVEX11 (Fig. 7*a*, lane 2) or TGFα-PE40 (data not shown), suggesting they may be products related to transformation of BL21 cells and unrelated to pAPK1-specific expression. Therefore, we conclude that MAb K1 reacts with a 29-kDa and 30-kDa protein encoded by g26NE.

Reactivity of rabbit and mouse antisera with tumor-cell lines as compared with MAb K1

To investigate the nature of the cloned antigen, both rabbit and mouse antisera against the recombinant antigen encoded by pAPK1 were raised as described above. These antisera were then analyzed for their immunoreactivity with membrane and soluble proteins present in several different human tumor cell lines. As shown in Figure 8 and Table I, the mouse antiserum strongly reacted with a cytosolic protein(s) found in both OVCAR-3 (Fig. 8, lane 5, large arrowhead) and N87 cells (Table I). The mouse antiserum was poorly reactive with the membrane-associated octylglucoside-solubilized 40-kDa CAK1 (lane 2). As expected, MAb K1 reacted with the membrane (CAK1) antigen (lane 1). In addition, MAb K1 reacted with 2 bands of 34 kDa and 39 kDa, respectively, in the cytosolic fraction of OVCAR-3 cells (lane 4). One of these bands (MW ~39 kDa) was in the same location as the band reacting with the mouse antisera. The 39-kDa protein that reacted with the mouse antiserum was not detected in any of the 10 other tumor-cell lines. These cells also failed to react with MAb K1 (Table I). Neither the control MOPC 21 antibody (data not shown) nor the normal mouse serum reacted with membrane (lane 3) or cytosolic (lane 6) proteins from OVCAR-3 cells. It is evident that clone g26 encodes a soluble protein found in OVCAR-3 and N87 tumor cells but not in many other human cancer-cell lines including A431, KB3-1, HUT-102, Daudi, HTB103, HepG2, Huh7, MCF-7, MB-MDA231 and JMN (Table I). Both OVCAR-3 and N87 cell lines also contain substantial amounts of CAK1, while the other lines do not.

TABLE I – IMMUNOBLOTTING ANALYSIS OF TUMOR-CELL LINES WITH MAb K1 AND MOUSE ANTISERUM TO APK1

Cell line	Immunoblotting analysis (CAK1/APK1)		
	PNS	Membrane	Cytosol
OVCAR-3	+++/+++	+++/+	+++/+++
N87	+++/+++	+++/+	+++/+++
JMN	−/−	−/−	−/−
KB3-1	−/−	−/−	−/−
A431	−/−	−/−	−/−
HTB103	−/−	−/−	−/−
Daudi	−/−	−/−	−/−
HUT102	−/−	−/−	−/−
HepG2	−/−	−/−	−/−
Huh7	−/−	−/−	−/−
MCF7	−/−	−/−	−/−
MB-MDA231	−/−	−/−	−/−

PNS = postnuclear supernatant. Immunoblotting analyses were performed as described in Chang *et al.* (*1992d*) and in the text (this report). +++, Very strong reactivity; +, moderate reactivity and −, no reactivity. The values for reactivity with MAb K1 (CAK1) are on the left and reactivity with mouse anti APK1 are on the right in each column.

DISCUSSION

Characteristics of the cloned cDNA and its encoded protein

The cDNA clones encoding a protein recognized by MAb K1 were isolated from an expression library made from OVCAR-3 cells. These clones differ in size, but all encode an identical peptide of 263 amino-acid residues. A computer-assisted search at both the nucleotide and amino-acid levels showed no homology of the cloned molecule with known sequences. The protein encoded by the cloned cDNA, now designated APK1, has the following characteristics: (1) the 2,444-bp cDNA contains a 789-bp open reading frame which encodes a polypeptide of 263 amino acids; (2) both *in vitro* translation and *in vivo E. coli* expression of the K1-reactive clone g26 yield polypeptides of 29 and 30 kDa, which agrees with the calculated MW value of the peptide of ORF; (3) recombinant antigen(s) expressed in *E. coli* has an MW of 29 and 30 kDa and is specifically reactive with MAb K1; and (4) mouse antisera to the recombinant protein reacted strongly with the same cytosolic proteins in OVCAR-3 with which MAb K1 reacts, and reacted weakly with a 39- to 40-kDa membrane protein that migrates in the same location as the MAb-K1-reactive CAK1 band. These characteristics show that the isolated cDNA sequence encodes an antigenic protein reactive with MAb K1. However, CAK1 is a membrane-bound protein whereas APK1 is a soluble protein.

Relationship of the CAK1 to APK1

The complexity of expression of oncoproteins, including tumor antigens and oncogene products, has been explored in many human malignancies. Tumor antigen can be an oncoprotein resulting from an over-expressed oncogene, as in the case of HER-2/*neu* in breast carcinomas (Slamon *et al.*, 1987; Chang *et al.*, 1991). A normal functional gene can be mutated during carcinogenesis leading to "creation" of tumor-specific epitope(s) such as mutant p53 (Finlay *et al.*, 1989; Lane and

Benchimol, 1990; Gannon *et al.*, 1990; Bartek *et al.*, 1990; Chang *et al.*, 1991). Tumor antigen can share epitope(s) with a normal protein as was shown in CEA and its related antigens in colon cancer and normal colon (Kuroki *et al.*, 1981; Henslee *et al.*, 1992). It is not surprising that 3 protein species were found to be immunologically reactive with MAb K1, even though the complicated mechanism which lies behind them remains to be discovered.

We have found that the cDNA we isolated encodes a 39- to 40-kDa cytosolic protein which is recognized by MAb K1. The protein detected by MAb K1 on immunoblots was only detectable in 2 of 12 tumor-cell lines examined (Table I). Moreover, both of these (OVCAR-3 and N87) strongly reacted with MAb K1 when analyzed by immunofluorescence. In addition, the amount of APK1 expression correlated with the level of CAK1 in these tumor-cell lines. Another K1-reactive 34-kDa species (now designated as CAK2) is also present in the cytosol of both OVCAR-3 and N87 cells, but it does not react with either rabbit or mouse sera raised against recombinant APK1. CAK2 and APK1 are usually present together in cells that are strongly K1-positive, but absent in most K1-negative or weakly positive cells, indicating that they may be coexpressed with the CAK1 antigen in these tumor cells.

In summary, we have isolated a cDNA that encodes a 39- to 40-kDa protein (CAK1) found in OVCAR-3 and N87 tumor-cell lines. APK1 is a cytosolic protein whereas CAK1 is bound to the plasma membrane. Nevertheless, APK1 is immunologically related to the CAK1 antigen. Now that a K1-reactive antigen has been cloned, epitope mapping can be carried out to pin-point the K1-reactive peptide sequence and should be very useful in cloning the other antigens such as CAK1 or CAK2 that are recognized by MAb K1.

REFERENCES

ARCOT, S.S. and DEININGER, P.L., Large-scale subcloning of bacteriophage λ ZAP clones. *BioTechniques,* **12,** 370–371 (1992).

BARTEK, J., IGGO, R., GANNON, J. and LANE, D.P., Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene,* **5,** 893–899 (1990).

BAST, R.C., FREENEY, M., LAZARRUS, H., NADLER, L.M., COLVIN, N.B. and KNAP, R.C., Reactivity of a monoclonal antibody with human ovarian carcinoma. *J. clin. Invest.,* **68,** 1331–1337 (1981).

BENTON, W.D. and DAVIS, R.W., Screening lamda gt recombinant clones by hybridization to single plaques *in situ. Science,* **196,** 180–183 (1977).

CHANG, K., DING, I., KERN, F.G. and WILLINGHAM, M.C., Immunohistochemical analysis of p53 and HER-2/neu proteins in human tumors. *J. Histochem. Cytochem.,* **39,** 1281–1291 (1991).

CHANG, K., PAI, L.H., BATRA, J.K., PASTAN, I. and WILLINGHAM, M.C., Characterization of the antigen (CAK1) recognized by monoclonal antibody K1 present on ovarian cancers and normal mesothelium. *Cancer Res.,* **52,** 181–186 (1992*a*).

CHANG, K., PAI, L.H., PASS, H., POGREBNIAK, H.W., TSAO, M-S., PASTAN, I. and WILLINGHAM, M.C., Monoclonal antibody K1 reacts with epithelial mesothelioma but not with lung carcinoma. *Amer. J. Surg. Pathol.,* **16,** 259–268 (1992*b*).

CHANG, K., PASTAN, I. and WILLINGHAM, M.C., Frequent expression of the tumor antigen CAK1 in squamous-cell carcinomas. *Int. J. Cancer,* **51,** 548–554 (1992*c*).

CHANG, K., PASTAN, I. and WILLINGHAM, M.C., Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int. J. Cancer,* **50,** 373–381 (1992*d*).

COLCHER, D., HORAN HAND, P., NUTI, M. and SCHLOM, J., A spectrum of monoclonal antibodies reactive with human mammary tumor cells. *Proc. nat. Acad. Sci. (Wash.),* **78,** 3199–3208 (1981).

FINLAY, C.A., HINDS, P.W. and LEVINE, A.J., The p53 proto-oncogene can act as a suppressor of transformation. *Cell,* **57,** 1083–1093 (1989).

FROHMAN, M.A., DUSH, M.K. and MARTIN, G.R., Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. nat. Acad. Sci. (Wash.),* **85,** 8998–9002 (1988).

GANNON, J.V., GREAVES, R., IGGO, R. and LANE, D.P., Activating mutation in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.,* **9,** 1595–1602 (1990).

HENSLEE, J.G., VACHULA, E.J., PAXTON, R.J., MATIAS, M.S., SHIVELY, J.E., RITTENHOUS, H.G. and TOMITA, J.T., Identification and characterization of new antigenic fragments related to carcinoembryonic antigen in adult feces. *Cancer Res.,* **52,** 4175–4182 (1992).

KOZAK, M., An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.,* **15,** 8125–8148 (1987).

KUROKI, M., KOGA, Y. and MATSUOKA, Y., Purification and characterization of carcinoembryonic antigen-related antigens in normal adult feces. *Cancer Res.,* **41,** 713–720 (1981).

KURRASCH, R.H., RUTHERFORD, A.V., RICK, M.E., GALLO, M.G., LOVELACE, E.T., PASTAN, I. and WILLINGHAM, M.C., Characterization of a monoclonal antibody, OVB1, which binds to a unique determinant in human ovarian carcinoma and myeloid cells. *J. Histochem. Cytochem.,* **37,** 57–67 (1989).

KYTE, J. and DOOLITTLE, R.F., A simple method for displaying the hydropathic character of a protein. *J. mol. Biol.,* **157,** 105–132 (1982).

LANE, D.P. and BENCHIMOL, S., p53: oncogene or anti-oncogene? *Genes Devel.,* **4,** 1–8 (1990).

LOW, M.G. and SALTIEL, A.R., Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science,* **239,** 268–275 (1988).

MATTES, M.J., LOOK, K., FURUKAWA, K., PIERCE, V.K., OLD, L.J., LEWIS, JR., J.L. and LLOYD, K.O., Mouse monoclonal antibodies to human epithelial differentiation antigens expressed on the surface of ovarian carcinoma ascites cells. *Cancer Res.,* **47,** 6741–6750 (1987).

MIOTTI, S., CANEVARI, S., MÉNARD, S., MEZZANZANICA, D., PORRO, G., PUPA, S.M., REGAZZONI, M., TAGLIABUE, E. and COLNAGHI, M.I., Characterization of human ovarian-carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. *Int. J. Cancer,* **39,** 297–303 (1987).

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., *Molecular cloning, a laboratory manual,* 2nd ed., pp. 7.19–7.22, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989*a*).

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., *Molecular cloning, a laboratory manual,* 2nd ed., pp. F2–F9, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989*b*).

SANGER, F., NICKLEN, S. and COULSON, A.R., DNA sequencing with chain-terminating inhibitors. *Proc. nat. Acad. Sci. (Wash.),* **74,** 5463–5467 (1977).

SLAMON, D.J., CLARK, G.M., WONG, G.G., LEVIN, W.J., ULLRICH, A. and MCGUIRE, W.L., Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science,* **231,** 177–181 (1987).

STUDIER, F.W. and MOFFATT, B.A., Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. mol. Biol.,* **189,** 113–130 (1986).

WILLINGHAM, M.C., FITZGERALD, D.J. and PASTAN, I., *Pseudomonas* exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model. *Proc. nat. Acad. Sci. (Wash.),* **84,** 2474–2478 (1987).

YOUNG, R.A. and DAVIS, R.W., Efficient isolation of genes by using antibody probes. *Proc. nat. Acad. Sci. (Wash.),* **80,** 1194–1198 (1983).