Molecular cloning of mesothelin, a differentiation antigen present on mesothelial, mesotheliomas, and ovarian cancers

(glycosylphosphatidylinositol anchor/plasma membrane/antibody/cell adhesion)

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ABSTRACT  Monoclonal antibody MAB K1 recognizes a 40-kDa glycoprotein present on the surface of mesothelial cells, mesotheliomas, and ovarian cancers. We have used MAB K1 to isolate a 2138-bp cDNA that encodes this antigen. The cDNA has an 1884-bp open reading frame encoding a 69-kDa protein. When the cDNA was transfected into COS and NIH 3T3 cells, the antigen was found on the cell surface and could be released by treatment with phosphatidylinositol-specific phospholipase C. The 69-kDa precursor is processed to the 40-kDa form. The protein has been named mesothelin because it is made by mesothelial cells. Mesothelin may play a role in cellular adhesion.

Monoclonal antibodies are currently being used to diagnose and treat cancer (1, 2). To be useful for therapy, the antibody should recognize an antigen that is present in large amounts on the cancer cells and in negligible amounts on normal cells. Alternatively, the antigen can be present in substantial amounts on normal cells, if the normal cells are not components of an essential organ. This approach has been useful in developing new treatments for leukemias and lymphomas. Several differentiation antigens have been identified on lymphomas and leukemias which are good targets for immunotherapy because they are not present on the stem cells which give rise to differentiated lymphocytes (2). Thus, normal lymphocytes that are killed by immunotherapy can be regenerated. Some examples of lymphocyte antigens of this type are CD19, CD22, CD25, and CD30 (2, 3). Clearly, it would be very useful to have antibodies that recognize differentiation antigens on solid tumors, but only a small number of these are available. One reason contributing to the paucity of such antibodies is that efforts to identify differentiation antigens on various types of epithelial cells have been relatively modest compared with the intense efforts made to identify differentiation antigens on cells of the hematopoietic system. Ovarian cancer represents one of the diseases which could be treated by immunotherapy, because the ovaries are always removed during surgery for this disease and reactivity with normal ovarian tissue is not a problem. Several antibodies that recognize differentiation antigens on ovarian cancer cells have been generated. One of these is OC125, which recognizes the CA125 antigen (4). CA125 is a high molecular weight glycoprotein that is shed by ovarian cancer cells and has been useful in the diagnosis of ovarian cancer. However, antibodies to CA125 are not useful for immunotherapy because the CA125 antigen is shed into the bloodstream (4). Another is MOV18, which recognizes the folate-binding protein. This protein is abundant in ovarian cancers as well as in some other tumors. Unfortunately, this protein is also abundantly expressed in kidney (5). We have isolated an antibody termed MAB K1 that reacts with many ovarian cancers and many mesotheliomas. Like OC125, the antibody also reacts with normal mesothelial cells, but it does not react with other cell types except for weak reactivity with some cells in the trachea (6, 7). The antigen recognized by MAB K1 appears to be a differentiation antigen present on mesothelium and is expressed on cancers derived from mesothelium, such as epithelioid type mesotheliomas, as well as on most ovarian cancers. Thus immunotherapy directed at the CAK1 antigen will run the risk of damaging normal mesothelial cells and perhaps cells of the trachea (6–9).

In the ovarian cancer cell line OVCAR-3 as well as HeLa cells, the antigen has been shown to be an ~40-kDa glycoprotein that is attached to the cell surface by phosphatidylinositol. The protein is released when cells are treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (7). We have previously attempted to clone a cDNA encoding the CAK1 antigen but instead have cloned cDNAs encoding two intracellular proteins which also react with MAB K1 (10). Neither of these is the cell surface antigen recognized by MAB K1. Here we describe the cloning of a cDNA that encodes the CAK1 antigen that is expressed on the cell surface.* We have expressed the cDNA in 3T3 cells and characterized the protein made by these cells.

MATERIALS AND METHODS

Cells and Antibodies. Human ovarian tumor cell line OVCAR-3 and cell lines A431, KB, MCF-7, COS-1, WI-38, and NIH 3T3 were obtained from the American Type Culture Collection. Cells were cultured either in RPMI 1640 or Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with L-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), and 5–10% fetal bovine serum (FBS) (GIBCO). NIH 3T3 transfectants were grown in DMEM with the neomycin analogue G418 (GIBCO) at 0.8 mg/ml. Cells were used when they reached 80–90% confluency, after three washes with ice-cold phosphate-buffered saline (PBS) (GIBCO). MAB K1 and antibody MOPC-21 have been described (6) and were used at 5–10 μg/ml.

Isolation of the cDNA Clones. The HeLa S3 cDNA library (Clonetech) was screened as described (10) at ~50,000 plaques forming units per 150-mm filter with protein A-purified MAB K1 (5 μg/ml) and peroxidase-conjugated goat anti-mouse IgG (heavy- and light-chain specific, 10 μg/ml; Jackson ImmunoResearch). Two positive plaques (A6-1 and A6-2) were isolated and the phages were purified to homogeneity by three or more rounds of screening. After verification of their specificity with MAB K1 by showing they did not react with a control MOPC-21 antibody, single-plaque isolates of A6-1 and A6-2 were used for preparation of 5–10 plaque plates, followed by extraction and purification of phage DNA with a λ phage

Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U40434).

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DNA kit (Qiagen, Chatsworth, CA). Phage DNA was then digested with EcoRI and the insert was subcloned into the EcoRI site of a pcDNA1/Amp (Invitrogen) vector by a rapid ligation protocol (10). Plasmid DNAs were isolated by use of Qiagen’s plasmid DNA isolation kit (10). Restriction mapping using XhoI, EcoRI, SalI, BamHI, and NcoI, as well as DNA sequencing, revealed that the two plasmid clones (p6-1 and p6-2) had identical 1500-bp inserts.

To isolate a longer clone, the insert of p6-1 was purified to make a cDNA probe (specific activity, 8.5 x 10^6 cpm/ml) by random priming. The HeLa S3 cDNA library was rescreened by a filter hybridization method (10). Fourteen clones were isolated and purified, and their insert sizes were assessed by digestion with EcoRI. Four large inserts were subcloned into a pcDNA1/Amp plasmid vector (p9, p13-1, p16, and p18-1). p9 contained the largest insert with a long open reading frame.

**DNA Sequence Analysis.** By the use of T3 and T7 promoter primers and twenty 17-bp synthetic primers, the entire cDNA insert of p9 was sequenced by the method described by Sanger et al. (11) and an automatic cycle sequencing method (Taq DyeDeoxy Terminator Cycle sequencing kit; Applied Biosystems).

**Northern Blot Analysis.** Total RNAs (20 µg) from OVCAR-3, KB, MCF-7, A431, and WI38 cells were electrophoresed in a 1% agarose gel in Mops buffer with 16.6% formaldehyde and then transferred to a nylon paper. Northern hybridization was done with a method described before (10). The blot was washed and reprobed with a 32P-labeled human β-actin cDNA as an internal control to assess the integrity and quantity of the RNA samples loaded.

**In Vitro Transcription and Translation.** The TNT coupled reticulocyte lysate system, canine pancreatic microsomal membrane, 2 µg of plasmid DNAs of pcDICA1-9, pAPK1 (10), and [3H]leucine were used in an in vitro transcription and translation and translocation/processing experiment according to the protocol of the manufacturer (Promega). Translation products were resolved by SDS/10% PAGE under reducing conditions. The proteins were fixed and the unincorporated label was removed by soaking the gel three times for 30 min in 200 ml of buffer containing 40% methanol and 10% acetic acid in deionized water. The gels were then soaked for 30 min in 200 ml of ENTENSIFY Part A and Part B (NEN). After drying, the translated products were visualized by autoradiography.

**Expression of the Cloned cDNAs in Mammalian Cells.** Transient transfections of COS cells were performed with pcDICA1-9 (p9) and LipofectAMINE (GIBCO) according to the manufacturer’s protocol. COS-1 cells were plated a day before the experiment at 2.5 x 10^5 cells per 60-mm dish. Twenty-four microliters of LipofectAMINE and 76 µl of OptiMEM I medium were mixed with 10 µg of pcDNA1/Amp vector or pcDICA1-9 in 100 µl of OptiMEM I medium at room temperature for 30 min. After the COS-1 cells were washed twice with OptiMEM I 2.4 ml of OptiMEMI was added and the transfection mixtures were overlaid onto COS-1 cells and incubated at 37°C. After 5 hr, 2.6 ml of DMEM with 20% FBS was then added into each dish. Forty-eight hours after transfection, the dishes were subjected to immunofluorescence labeling (6, 7) or other treatments. The insert from pcDICA1-9 (in pcDNA1/Amp) was also subcloned into a pcDNA3 (Invitrogen) vector for stable transfection. Plasmid minipreps were made with Qiagen’s Miniprep plasmid DNA kit and orientation of the insert in individual clone was determined by restriction mapping. The resulting plasmid, pcDCAK1-9, was then used to transfect NIH 3T3, MCF-7, A431, and OVCAR-3 cells by DNA–calciu phosphate precipitation (12). After overnight exposure to the precipitate, the cells were washed with PBS three times and fed with fresh DMEM/10% FBS for 2–3 days. Geneticin (G418 sulfate; 0.8 mg/ml) was added and the cultures were maintained until colonies 2–3 mm in diameter were formed. Colonies were then transferred into wells of a 96-well plate and then into a 35-mm dish when they were 80% confluent. Transfected cells were screened by immunofluorescence (6, 7) and positive cells were further subcloned by limited dilution as described (6). One of the NIH 3T3 transfectant clones, NIH 3T3 K20, was chosen for further study. To localize the expression of CAK1, both cell surface and intracellular immunofluorescence labeling was also performed according to methods described before (7).

**Treatment of Transfected Cells with PI-PLC.** CAK1 cDNA-transfected NIH 3T3 cells (NIH 3T3 K20 cells) were grown in 175-mm² flasks, and when they reached 90% confluency, the cells were washed in PBS three times. The cells were incubated with either 5 ml of PI-PLC (1.25 units/ml, from Bacillus cereus; Boehringer Mannheim) or 0.05% trypsin/0.052 mM EDTA for 30 min at 37°C and 30 min at room temperature with shaking. The supernatants were collected and after centrifugation at 1000 x g and concentrated about 10-fold with a Centricon 30 unit (Amicon). The concentrated supernatants were used in SDS/PAGE and immunoblot analysis. The enzyme-treated cells can be recultured and the recovery of CAK1 expression can be seen after overnight culture. Treatment with PI-PLC was done in a similar manner using 35-mm diameter dishes followed by immunofluorescence labeling of the treated cells (7).

**Immunoblot Analysis of Transfected NIH 3T3 Cells.** Membrane and cytosolic fractions from transfected NIH 3T3 K20 cells (10) were subjected to SDS/12.5% PAGE and the resolved proteins were transferred to nitrocellulose. Immunoblotting was performed as described (8, 10).

**RESULTS**

Expression cloning was used to isolate the CAK1 cDNA. We previously observed that MAb K1 reacts with OVCAR-3 and HeLa cells. Because we were unable to isolate the cDNA from an OVCAR-3 library (10), we screened a HeLa cDNA library expressed in Agt11 as described in Materials and Methods. A total of 1 x 10^6 phages were screened and 2 phage clones (A6-1 and A6-2) were identified. DNA sequencing showed that both phages contained the same 1.5-kb insert. The insert hybridized to mRNA from OVCAR-3 and KB cells (a HeLa subclone which also reacts with MAAb K1) but not to RNA from many other cell lines, indicating that the cDNA is specific for cells reacting with MAAb K1 (Fig. 1). The mRNA detected was 2.2 kb long, indicating that the insert isolated was not full-length. The insert contained an open reading frame, a stop codon, and a poly(A) tail but the 5' end appeared to be missing. Therefore, the phage library was rescreened with one of the inserts and 14

**FIG. 1.** CAK1 RNA levels in cell lines. Samples (20 µg) of total RNA from OVCAR-3 cells (lane 1), MCF-7 cells (lane 2), KB cells (a HeLa subclone; lane 3), A431 cells (lane 4), and WI38 cells (lane 5) were resolved by electrophoresis, transferred to nylon paper, and probed with a 32P-labeled CAK1 cDNA. Hybridization with an actin cDNA probe showed that the lanes were equally loaded.
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of the CAKI-1 cDNA. The nucleotide sequence (upper line) and the deduced amino acid sequence (lower line) of the CAKI-1 cDNA subjected to amino acid sequencing. The translation of CAKI-1 cDNA starts at nucleotide 100–101 (ATG) and terminates at 186–188 (TGA). The putative signal peptide is underlined and a typical hydrophobic sequence for glycosylphosphatidylinositol anchor is doubly underlined. A possible furin cleavage sequence, RPRFRR, is underlined and the cleavage site is shown by an arrow. There are four potential N-linked glycosylation sites (in boldface letters). A variant polyadenylation signal (AGTAAA) is present 22 bp upstream from the poly(A) tail. The original p6-1 cDNA sequence spans nucleotides 721–2138.

new phages with cDNA inserts of various sizes were isolated. The largest insert (no. 9) was 2138 bp long and when sequenced contained an open reading frame of 1884 bp (Fig. 2). It contains a typical Kozak sequence (13) (AXXATGG) followed by an open reading frame that encodes an 69-kDa protein.

The sequence was not present in various data bases examined (EMBL/GenBank release 89.0, June 1995). Because the CAKI-1 antigen was originally found to be present in 40 kDa in size, several experiments were carried out to determine whether clone 9 encoded CAKI.

In Vitro Translation. Insert 9 was cloned into a pcDNAI/Amp vector to make pcDICAK1-9 and used in the TNT reticulocyte system. A 69-kDa protein was produced (Fig. 3, lane 1). In the presence of pancreatic microsomes a slightly larger protein was observed (lane 2), indicating that the protein had been inserted into microsomes and glycosylated. As a control, a cDNA encoding a 30-kDa cytosolic protein that also reacts with MAb 1B1 (10) was subjected to the same analysis. The size of that protein was unaffected by the presence of microsomes (lanes 3 and 4).

Expression in Cultured Cells. pcDICAK1-9 was transfected into COS cells for transient expression. Fig. 4 shows the specific MAb K1 labeling pattern of COS cells transfected with insert 9. In nonpermeabilized cells, a typical cell surface fluorescent pattern was detected (Fig. 4A). In permeabilized cells, strong staining of the Golgi region was evident (Fig. 4B). No cytosolic staining was detected. Also, no immunoreactivity was detected in cells transfected with vector without insert (Fig. 4C) or with control inserts (data not shown). Thus, insert 9 encodes a cell surface protein that is also present in the Golgi compartment.

Size and Processing of CAKI Antigen. To determine the size of the antigen produced by cells transfected with insert 9, NIH 3T3 cells were transfected with pcD3CAKI-1 to make stable cell lines. Stably transfected clones were produced as described in Materials and Methods and the presence of antigen on the surface was confirmed by immunofluorescence. Then mem-
brane and cytosolic fractions were prepared from NIH 3T3 K20 cells and from OVCAR-3 cells, subjected to SDS/PAGE, and analyzed by immunoblotting with MAb K1 (Fig. 5). As previously reported, the major reactivity in OVCAR-3 cells was with a doublet of about 40 and 43 kDa that was present in membranes but not in the cytosol. In the transfectants, two bands of equal intensity were detected in the membrane fraction; one of about 40 kDa and a second of about 71 kDa. No signal was detected in the cytosol. These data suggest that CAKI is made as a high molecular weight precursor that is processed by proteolysis to an ~40-kDa form.

Nature of Cell Surface Attachment. To determine whether CAKI was attached to the transfectants via a phosphatidylinositol linkage as it is in OVCAR-3 cells (7), the NIH 3T3 transfectant cell line K20 was treated with PI-PLC for 60 min. Fig. 6A shows the strong cell surface labeling pattern in untreated cells. Fig. 6C shows that fluorescence was absent after treatment with PI-PLC. Fig. 6 B and D show phase-contrast images before and after treatment, respectively. The treated cells were still attached to the dish but were slightly altered in shape. The medium from PI-PLC-treated cells was concentrated, subjected to SDS/PAGE, and analyzed with MAb K1. A band of about 70 kDa was detected (data not shown), but no lower molecular weight bands were detected.

DISCUSSION

This paper describes the molecular cloning of the CAKI antigen, which is found on mesothelium, mesotheliomas, ovarian cancers, and some squamous cell carcinomas. We have named this antigen mesothelin to reflect its presence on mesothelial cells. One unexpected feature of mesothelin is that its cDNA encodes a 69-kDa protein, whereas the antigen present on OVCAR-3 cells, used to isolate MAb K1, has a molecular mass of ~40 kDa. The DNA sequence and the deduced amino acid sequence of CAKI are shown in Fig. 2. The cDNA is 2138 bp long and contains an open reading frame of 1884 bp. The protein it encodes contains 628 amino acids with a calculated molecular mass of 69,001 Da. A homology analysis was performed with nucleotide or amino acid sequences and none was detected with EMBL/GenBank accession by the Genetics Computer Group program. The protein contains four potential N-linked glycosylation sites, NXS or NXT, that are shown in boldface letters. A typical signal sequence is not present at the amino terminus. However, a short hydrophobic segment is located 15 amino acids from the first methionine (Fig. 2). This sequence might function as a signal sequence for membrane insertion, because the protein is found on the cell surface (Figs. 4 and 5) and is inserted into microsomes during cell-free translation (Fig. 3). Also present is a putative proteolytic processing site, RPRFR, beginning at amino acid 293 (Fig. 2). This site is recognized by furin, a protease important in the processing of several membrane proteins as well as in the activation of Pseudomonas and diptheria toxins (14). The 40-kDa form appears to be derived from a 69-kDa precursor by several processing steps. These are summarized in Fig. 7. Initially, mesothelin is made as a 69-kDa polypeptide with a hydrophobic tail which is probably removed and replaced by phosphatidylinositol (7). After glycosylation at one or more of its four putative N-linked glycosylation sites, it is cleaved by a protease to yield the 40-kDa fragment (or doublet) found on the surface of OVCAR-3 cells and a smaller (~31-kDa) fragment. The latter could be released into the medium and/or further degraded. The amino-terminal fragment has recently been detected in the medium of OVCAR-3 cells (unpublished data). In transfected NIH 3T3 and MCF-7
Differentiated ovarian poorly published mesothelin have altered adhesive expressing mesothelin should develop. Very succumb patients through the peritoneal cavity. Also traffic of Mesothelial cells (unpublished data). From we ovarian carcinoma cells that and invade will make Mesothelin adhesion, since Mesothelin is membranous and mesothelin have a role in the adhesion and/or adhesion (15, 16). Glycosylphosphatidylinositol-linked proteins may interact with tyrosine kinases (17, 18). Currently we have very little information on the function of mesothelin. One possibility is that it has a role in adhesion, since CAK1 transfsects are more slowly removed from culture dishes than nontransfected cells (unpublished data). Mesothelial cells are extremely flat and regulate the traffic of molecules and cells in and out of the peritoneal cavity. Mesothelin may have a role in these processes. Mesothelin could also be responsible for the adhesion and implantation of ovarian carcinoma cells that frequently occur throughout the peritoneal cavity. The isolation of a cDNA clone that encodes mesothelin will make investigation of these phenomena possible.

Mesothelin is very abundant in normal mesothelial cells from which malignant mesotheliomas and ovarian cystadenocarcinomas are derived. These two types of tumors share a unique biological characteristic that distinguishes them from other solid tumors. In the early stages, both types of tumors spread aggressively throughout the peritoneal (or thoracic) cavity and invade locally but do not metastasize distally through the lymphatic system or the bloodstream. In fact, many patients succumb to their cancer before distant metastases develop. Very little is known about the molecules involved in the dissemination of mesotheliomas and ovarian cancers. Mesothelin may have a role in this process, since cells overexpressing mesothelin have altered adhesive properties (unpublished data) and mesothelin expression is diminished in poorly differentiated ovarian cancers (8, 9). Investigation of mesothelin should not only advance our knowledge of mesothelial cell function but also promote our understanding of the role of this glycoprotein in carcinogenesis, invasion, and metastasis of ovarian cancers and malignant mesotheliomas.

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