A murine monoclonal antibody (mAb A23-16) was produced that recognizes a glycoprotein antigen preferentially expressed on the surface of human small cell lung carcinoma cells. This antibody is of IgG1 isotype, has an association constant of \( 5 \times 10^5 \text{ M}^{-1} \), and reacts preferentially with human small cell lung carcinoma cell lines and fresh frozen sections in enzyme-linked immunosorbent assays and immunoperoxidase assays, respectively. The antigen recognized by A23-16 is a sulfated glycoprotein with phosphorylated threonine residues. The mature 90-kDa molecule has intrachain disulfide bonds and appears to be derived from a 78-kDa precursor, which is neither sulfated nor phosphorylated, but contains N-linked oligosaccharides. Conversion of the 78-kDa precursor to the mature form is accompanied by processing of these oligosaccharides from the high mannose to the complex type, although the increase in molecular mass from 78 to 90 kDa cannot be accounted for by this modification alone. MAb A23-16 reacts with its target antigen independent of the N-linked oligosaccharides, but requires intact intrachain disulfide bond(s) for reactivity. These studies on the molecular characterization of a monoclonal antibody-defined glycoprotein, preferentially expressed by small cell lung cancer, provide a basis for further structural and functional studies that may eventually lead to a delineation of its biological relevance for neoplastic transformation.

Lung cancer is the most common human malignancy today and the leading cause of cancer-related death with more than 130,000 new cases diagnosed yearly in the United States (1, 2). The disease is highly malignant as the majority of patients survive less than 1 year. Lung cancer is generally divided into two major categories: small cell lung carcinoma (SCLC) and non-small cell carcinoma of the lung. SCLC comprises about 20–25% of lung cancer patients and is characterized by rapid metastatic spread and poor prognosis, although it shows some responsiveness to chemotherapy (3, 4).

The use of immunological approaches to manipulate the immune response of small cell lung cancer patients offers an alternative means for therapy possibly by using monoclonal antibodies directed to SCLC-associated antigens for targeting of chemotherapeutic drugs or radionuclides. The development of hybridoma technology resulted in a number of reports describing monoclonal antibodies reacting with SCLC (5–8). However, the majority of these publications dealing with these potential glycoprotein target antigens stressed mainly the relative specificity of the monoclonal antibodies detecting them rather than an in depth biochemical characterization of their target antigens. This is in contrast to other areas of cancer research where both glycolipid and glycoprotein antigens targeted by monoclonal antibodies have been more extensively characterized by biochemical and immunological means as summarized in recent reviews (9–11).

In this report, we focus mainly on the biochemical characterization of a target antigen for a monoclonal antibody reactive with small cell carcinoma of lung that may be of potential use for therapy of this neoplasm.

**MATERIALS AND METHODS**

**Cells**—Tumor cell lines and lymphoblastoid cell lines were propagated in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 mM glutamine, and 25 \( \mu \text{g/mL} \) gentamycin. Hybridomas were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM glutamine, and 25 \( \mu \text{g/mL} \) gentamycin, or ascites in pristane-primed BALB/c mice.

The following human cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD; HPB-ALL (lymphoblastoid); CALU-1 and SK-MES-1 (squamous lung carcinoma); PAN-C1 (pancreatic carcinoma); and WI-38 (diploid lung fibroblast). All other cell lines used were obtained as follows: T293 (SCLC) and T291 (lung adenocarcinoma) from Dr. H. Masui and Dr. G. Sato, University of California, San Diego; NCI-H69C, NCI-H69, NCI-H128, NCI-H82, and NCI-146 (SCLC) from Dr. J. Minna, National Cancer Institute, Bethesda, MD; and SHP-77 (SCLC) from Dr. A. Korsa, University of Pittsburgh; UCLA-P3 (lung adenocarcinoma); L14 (lymphoblastoid) as well as M14 and M21 (melanoma) from Dr. D. L. Morton, UCLA; MELUR (melanoma) from Dr. Ursula Koldovsky, Dusseldorf, W. Germany; COLO 357 (pancreatic carcinoma) from Dr. R. S. Metzgar, Duke University; SK-N-RA and SK-N-AS (neuroblastoma) from Dr. L. Nelson, Memorial Sloan Kettering Institute, New York.

**Tissues**—Portions of fresh normal and malignant tissue were obtained from the Surgical Pathology Department of the Ida M. Green Hospital of Scripps Clinic, La Jolla, CA. Specimens were embedded in tissue Tek Medium (Scientific Products) and frozen in blocks in isopentane at liquid nitrogen temperature. They were then stored at \(-70\, ^\circ\text{C}\).

**Production of Monoclonal Antibodies**—Monoclonal antibody A23-16 was produced against the human SCLC cell line T293 by standard hybridoma technology (12, 13). Details relevant to this report are as follows: BALB/c mice were injected intraperitoneally with \( 10^7 \) T293...
cells at weekly intervals. Three days after the final injection, the splenocytes were fused with the M5 variant of the nonsecretor murine myeloma cell line SP2/0 and cultured in 96-well plates with 2 × 10⁶ murine thymocytes per ml. Hybridoma A23-16 was selected by growth in Dulbecco's modified Eagle's medium containing 10% horse serum, hypoxyantheme, aminopterin, and thymidine and subcloned by limiting dilution.

**Ascites Production**—BALB/c mice were primed by injecting 0.5 ml of pristane. Two weeks after priming, 10 × 10⁶ hybridoma cells were injected intraperitoneally into the mice and ascites fluid collected repeatedly every 2–3 days.

**Purification**—Purification was achieved by precipitating the antibody from the ascites by 45% saturation with (NH₄)₂SO₄, dialyzing against 10 mM Tris-HCl, pH 8.0, and application to a DEAE-cellulose column equilibrated with this buffer (13). The antibody was eluted from the column with a linear gradient of NaCl (0.04 M), dialyzed against PBS, and stored at −70 °C.

**Isotyping**—The isotype of the monoclonal antibody was determined to be IgG1 by ELISA, using aliquots of diluted, affinity-purified rabbit anti IgG1 for different murine light chains (Southern Biotechnology Associates, Birmingham, AL) that were dived into 96-well microtiter plates (Dynatech, Alexandria, VA).

**Antibody Determination**—The concentration of the gp antibody was measured by ELISA. Briefly, 5 × 10⁵ target cells/well were plated in polyvinyl microtiter plates (Dynatech). Prior to ELISA, the plates were rehydrated by washing them twice with 10 mM PBS, pH 7.4, containing 0.1% Tween 20, 0.2% Thimerosal to remove any non-specific binding sites. Supernatants were diluted 1:2 in washing buffer containing 0.1% bovine serum albumin as diluent. Diluted test supernatant (50 µl) was added to each well and plates were incubated for 1 h at 4 °C. Following three washes, 50 µl of peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) was added to each well and the reaction mixture was again incubated for 1 h at 4 °C. After two final washes, 50 µl of substrate solution (400 µl of 0.1 M o-phenylenediamine plus 0.12% H₂O₂) was added to each well. The reaction was stopped at 15 min by addition of 25 µl of 4 N H₂SO₄ to each well. Optical absorbance at 492 nm was measured with an ELISA plate reader.

**Indirect Immunoprecipitation and SDS-PAGE**—Purified antibodies were preincubated for 15 min with PBS containing 10% goat serum and 0.1% bovine serum albumin, and incubated at room temperature. After two washes in HBSS and one wash in PBS, the sections were overlayed with horseradish peroxidase conjugated to goat anti-mouse immunoglobulin (Bio-Rad) diluted 1:500 with immunoperoxidase dilution buffer (PBS containing 10% goat serum, 0.1% bovine serum albumin), and incubated for 1 h at room temperature. Finally, the tissue sections were incubated for 15 min at room temperature with immunoperoxidase substrate buffer (10 mM Tris, pH 7.6, containing 0.6 µg/ml 3,3'-diaminobenzidine and 0.005% H₂O₂) after washing twice in HBSS and once in PBS. The sections were counterstained briefly in 1% methylene blue, dehydrated through graded ethanol, washed in Histoclear (National Diagnostics Somerville, NJ), mounted with Pro-Texx (Lerner Laboratories, New Haven, CT), and examined by microscopy.

**Glycoprotein Antigen on Human Small Cell Lung Cancer**

**RESULTS**

**Production, Selection, and Characterization of MAb A23-16**—Twenty-seven hybridomas that reacted in ELISA with the immunizing T293 cells, but not with the lymphoblastoid cell line L14, were selected from approximately 1000 primary hybridoma clones. From these hybridomas, 19 specifically stained fresh frozen sections of SCLC in immunoperoxidase assays. MAb A23-16 was selected for further study and subjected to extensive analyses with ELISA cell binding assays against cell lines derived from SCLC, other solid tumor
cell lines, as well as lymphoblastoid and fibroblastoid cell lines.

MAb A23-16 (IgG 1) binds to the surface of T293 SCLC cells in a saturable fashion with an association constant \( K_a \) of \( 5 \times 10^8 \) M\(^{-1}\). Moreover, Scatchard analysis revealed that T293 SCLC cells have \( 4.7 \times 10^5 \) binding sites for this antibody per cell.

**ELISA Cell Binding Analysis**—Table I summarizes the reaction of mAb A23-16 with various human cell lines. MAb A23-16 reacted with all SCLC cell lines tested but not with those derived from other solid tumors, with the exception of the lung adenocarcinoma cell line UCLA-P3 and the neuroblastoma cell line SK-N-RA. It is possible that the non-SCLC cell lines that reacted positively with mAb A23-16 may share a common antigen; however, immunoprecipitation analysis will be needed for confirmation. The antibody did not react with a series of lymphoblastoid cell lines of either T- or B-cell origin, human red blood cells, or WI-38 human lung fibroblasts.

**Immunohistochemical Staining of Frozen Tissue Sections**—Table II summarizes the reactivity of the A23-16 antibody with fresh frozen sections of tumors and normal fetal and adult tissues. MAb A23-16 reacted only with sections of SCLC and failed to stain sections of other tumors and most normal tissues. Of the normal tissues tested, sections of liver, kidney, colon, and spleen. Among normal adult tissues that showed no detectable immune staining were lung, pancreas, spleen, colon, and brain cortex.

**Molecular Characterization of the A23-16 Antigen**—Indirect immunoprecipitation and Western blotting were employed for...
SDS-PAGE analysis was performed on unlabeled detergent extracts of T293 SCLC cells following Western blotting of proteins from SDS gels to nitrocellulose membranes. Under nonreducing conditions, mAb A23-16 reacts with the 76-kDa form of the antigen, but does not react on Western blots following chemical reduction of the samples with 2-mercaptoethanol (Fig. 1B). In addition to the 76-kDa component detected on Western blots under nonreducing conditions, mAb A23-16 also detects a molecule of 200 kDa in extracts of T293 cells, the nature of which is currently unknown.

Bioynthesis of the A23-16 Antigen—A pulse-chase labeling experiment was performed with T293 cells to assess the metabolic processing of the A23-16 antigen and determine which biosynthetic forms, if any, are recognized by mAb A23-16. Following a 10-min pulse of T293 cells with [35S]methionine and subsequent chase in unlabeled culture medium, aliquots were removed and detergent extracts prepared for immunoprecipitation and SDS-PAGE analysis. Under reducing conditions, the first molecule precipitated by A23-16 after a 10-min labeling period is one of 76 kDa, which appears to gradually give rise to the mature 90-kDa form previously found on the cell surface (Fig. 2). The kinetics with which the 76-kDa molecule disappears and is replaced by the 90-kDa form are suggestive of a precursor-product relationship. A parallel analysis of the same cell extracts on nonreducing SDS-PAGE gels indicates an initial reactive molecule of 66 kDa, that gives rise to one of 76 kDa, previously noted by surface iodination analyzed on nonreducing SDS gels.

In order to determine whether the 76- and 90-kDa molecules observed under reducing conditions contain N-linked oligosaccharides, T293 cells were metabolically labeled with [35S]methionine for 1 h in methionine-free medium, such that both components can be immunoprecipitated. Immunoabsorbants were then digested with Endo-H prior to SDS-PAGE, under conditions outlined under "Materials and Methods." According to the resulting reaction pattern shown in Fig. 3, the 76-kDa component exhibits an increased electrophoretic migration as a result of Endo-H digestion, indicating the presence of high-mannose type, N-linked oligosaccharides. In contrast, the 90-kDa molecule is not affected by Endo-H digestion. Endo-F digestion of the 90-kDa molecule, following iodination and immunoprecipitation from T293 cell extracts, reveals approximately the same drop in apparent molecular mass as that seen when the 76-kDa putative precursor is digested with Endo-H (Figs. 1 and 3). These data further support the precursor-product relationship between these two molecules.

In order to determine whether N-linked oligosaccharides are required on the antigen molecule for the formation of the A23-16 determinant, T293 cells were pre-treated for 3 h in medium containing various concentrations of tunicamycin, then labeled for 1 h in the appropriate concentration of this drug prior to extraction with RIPA lysis buffer. Tunicamycin inhibits the addition of N-linked carbohydrates to glycoproteins by preventing the formation of the lipid-linked sugar intermediate prior to its addition to asparagine residues. Lysates of T293 cells treated only with the solvent, dimethyl sulfoxide, contain primarily the 76-kDa precursor molecule and a trace of the mature 90-kDa product when immunoprecipitated with the A23-16 antibody (Fig. 4). An increase in the concentration of tunicamycin to 1 μg/ml results in immunoprecipitation of both the 76-kDa molecule and the nonglycosylated form of 70 kDa. At tunicamycin concentrations of 3 and 9 μg/ml, only the nonglycosylated 70-kDa form is immunoprecipitated, suggesting that the N-linked oligosaccharides are not required for reactivity with mAb A23-16.

Phosphorylation and Sulfation—To assess whether the molecule recognized by mAb A23-16 is phosphorylated, T293 SCLC cells were labeled with [32P]orthophosphate for 3 h at 37 °C, and then its detergent lysate was subjected to immunoprecipitation with mAb A23-16. Subsequently the 90-kDa molecule was found to be phosphorylated, as indicated by the autoradiogram seen in Fig. 5A. Phosphoamino acid analysis of the 32P-labeled 90-kDa antigen (Fig. 5B) demonstrates that this phosphorylation occurs at threonine residue(s).

Further analyses of post-translational modification were performed to determine whether the 90-kDa component is sulfated. To this end, T293 SCLC cells were cultured in the
The data presented here indicate that a murine monoclonal antibody A23-16 of the IgG 1 isotype reacts specifically with a glycoprotein antigen that is preferentially expressed on cell lines and tissues derived from small cell lung carcinoma. ELISA assays of a variety of human tumor cell lines, as well as fibroblastoid and lymphoblastoid cell lines, indicate that the antibody reacts with a glycoprotein antigen that is preferentially expressed on cell lines and tissues derived from small cell lung carcinoma.

of indirect immunoprecipitation analyses, pulse-chase biosynthetic experiments, and enzymatic digestion studies. Initial immunoprecipitation/SDS-PAGE experiments revealed that mAb A23-16 precipitates a single molecule of 90 kDa under nonreducing conditions (Fig. 1A). This demonstrates that the native molecule contains one or more intrachain disulfide bonds, and the Western blot analysis suggests that these bonds must be intact for A23-16 reactivity, since the antibody reacted with its target antigen in Western blots only under nonreducing conditions (Fig. 1B).

An evaluation of the biosynthesis of this antigen molecule by pulse-chase studies with \([^{35}S]\)methionine revealed a component of 76 kDa reacting with mAb A23-16 immediately following a 10-min pulse label and the subsequent appearance of 90 kDa within 30 min. The 90-kDa molecules labeled during the 10-min pulse remained associated with the cells for as long as 21 h (Fig. 2). These results suggest that the 90-kDa component is the mature form of biosynthetic product of this antigen that is derived from a 76-kDa precursor.

Conversion of the 76- to the 90-kDa form apparently involves the processing of N-linked oligosaccharides from the high-mannose to the complex type. Following immunoprecipitation, treatment of the respective components with Endo-H suggests that the 76-kDa precursor molecule contains N-linked oligosaccharides of the "high-mannose" type, whereas the 90-kDa product was unaffected by Endo-H digestion (29). Digestion of the 90-kDa molecule with Endo-F resulted in an increase in electrophoretic migration, while Endo-H digestion had no such effect, therefore, this component contains N-linked oligosaccharide residues of the complex type. The increase in migration of the 90 kDa following Endo-F digestion was approximately the same as that observed when the 76-kDa molecule was digested with Endo-H and when the 76 kDa were analyzed in the presence of tunicamycin. These
data, along with the pulse-chase data, support the hypothesis that a precursor-product relationship exists between the 76- and 90-kDa molecules, since they appear to contain approximately the same amount of N-linked carbohydrate; however, the increase in apparent molecular mass from the 76-kDa putative precursor to the 90-kDa form cannot totally be accounted for by the addition and processing of N-linked oligosaccharides. Other possible explanations for this difference in molecular mass include O-linked glycosylation, phosphorylation, and sulfation, especially since the mature, 90-kDa molecule, in contrast to the 76-kDa molecule, appears to be phosphorylated at threonine residues, as well as sulfated (Fig. 5). Although a structure-function relationship among these post-translational modifications is not immediately apparent, they are nevertheless of interest and may become more defined once more structural information is obtained on their protein backbone.

A number of tumor-associated antigens have been reported in the literature that range in molecular mass between 90 and 100 kDa, including p97, a melanoma-associated glycoprotein and the transferrin receptor that is present in a variety of tumor tissues (11). At present one cannot discern any apparent relationship between these molecules and the 90-kDa glycoprotein expressed on SCLC cells and specified by mAb A23-16.

MAb A23-16 failed to mediate either antibody-dependent cellular cytotoxicity or complement-dependent lysis of tumor cells in vitro (data not shown) and consequently appears less likely to be useful for any immunological manipulations of the host immune response to small cell lung carcinoma. However, the 90-kDa target antigen of mAb A23-16 is preferentially expressed on the surface of SCLC cells with a relatively high density of 4.7 x 10^5 per cell. For this reason, and because the 90-kDa target antigen is apparently not shed, mAb A23-16 may well be useful to target chemotherapeutic drugs or radionuclides to this antigen epitope and aid in suppressing the growth of SCLC, and thereby contributing to the development of new treatment modalities for small cell carcinoma of the lung.

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