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Isolation of the Melanoma-Associated Antigen p23 Using Antibody Phage Display

Jian Li,* Sarita Pereira,* Patricia Van Belle,‡ Ping Tsui,‡ David Elder,‡ David Speicher,* Keith Deen,‡ Alban Linnenbach,* Rajasekharan Somasundaram,* Rolf Swoboda,* and Dorothee Herlyn2*

The general responsiveness of human melanoma to immunotherapy has been well established, but active immunotherapy of melanoma has been hampered by insufficient information on the immunogenicity of melanoma-associated Ags in patients. In this study, we isolated a recombinant phage-Fab clone (A10-5) from a phage-Fab library derived from the B cells of a melanoma patient in remission after immunotherapy. Purified A10-5 Fab bound at high levels to cultured melanoma cell lines and to tissue sections of metastatic and vertical growth phase primary melanoma, but not to radial growth phase primary melanoma, nevi, or normal skin. A10-5 Fab bound to both the surface and the cytoplasm of cultured melanoma cells, but only to the cytoplasm of cultured fibroblasts. Western blot analysis revealed A10-5 Fab reactivity with a 33- and a 23-kDa glycoprotein under nonreducing conditions, and with a 23-kDa protein only under reducing conditions. A cDNA with an open reading frame predicted to encode a 23-kDa protein was cloned by screening a melanoma cell cDNA library with A10-5 Fab. This protein (p23) is the human homologue of the murine tumor transplantation Ag P198 that interacts with the cytoplasmic domain of ErbB-3 expressed by melanoma cells. Thus, the Ab phage display method has identified a novel, stage-specific melanoma-associated Ag that may have therapeutic and diagnostic value. The Journal of Immunology, 2001, 166: 432–438.

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tudies have demonstrated that lymphocytic infiltration of melanoma is accompanied by increased survival of the patient (1), suggesting the involvement of host immune responses in the growth control of these tumors. A beneficial role for Abs has been demonstrated in melanoma patients with vitiligo (2), as well as in patients immunized with GM2 ganglioside (3), anti-idiotypic Ab mimicking the high m.w. melanoma-associated proteoglycan (4), or allogeneic tumor cells (5). Defined tumor Ags have advantages over whole tumor cells because they provide highly standardized reagents, induce immune responses more reproducibly, and can be selected for expression of not only B cell epitopes, but also helper and cytolytic T cell epitopes (6). Thus far, clinical trials with isolated tumor Ags in melanoma patients have primarily included gangliosides (reviewed in Ref. 3). These carbohydrates have elicited humoral, but not cellular, immune responses in melanoma patients (3). Because both arms of the immune response have been implicated in the control of tumor growth (reviewed in Ref. 6), defined protein Ag vaccines are needed that induce both humoral and cellular immune responses.

We have used the Ab (Fab) phage display approach to identify melanoma-associated protein Ags as candidate vaccines for melanoma patients. A Fab library was established from the B cells of a melanoma patient in complete remission after vaccination with allogeneic tumor cells (7). From this library, a Fab (A-18) was isolated that bound not only to melanoma tissues but also to cells derived from various normal tissues (7). Because of its expression by normal tissues, the A-18 Ag was not identified. During the course of those studies, another combinatorial Fab (A10-5) was isolated, which is described in detail in this study.

Materials and Methods

Cell lines

Table I lists the cell lines used in this study. The EBV-transformed B cell line RS, established from the lymphocytes of a healthy donor as described (8), was grown in RPMI 1640 medium supplemented with 10% FBS. Melanocyte cell lines FM1024, -2404, and -2408; fibroblast cell lines FF1065 and FF2207; and melanoma cell lines WM1158, -1552C, -35, and -793 were obtained from Drs. Meenhard Herlyn and Elliot Levine (The Wistar Institute, Philadelphia, PA). These cell lines were propagated in MCDB 153 medium supplemented with 2% FBS. Melanoma cell lines Mel-2, -3, -4, and -B used for vaccination of the patient have been described (9) and were maintained in RPMI 1640 medium supplemented with 10% FBS. Melanoma cell line DM196 was provided by Dr. Timothy Darrow (Duke University, Durham, NC) and was maintained in MCDB 153/10% FBS medium. Melanoma cell line A375, colorectal carcinoma cell line SW1116, breast carcinoma cell lines MCF-7 and MDA231, and glioma cell line U87 MG were purchased from American Type Culture Collection (Manassas, VA). These cell lines were maintained in MCDB 153/2% FBS medium, with the exception of breast and glioma cell lines, which were maintained in RPMI 1640/10% FBS medium. African green monkey kidney COS-7 cells were maintained in DMEM/10% FBS (LifeTech, Rockville, MD).

Construction of human combinatorial Fab library

The phage-Fab library derived from the PBMC of melanoma patient GL has been described (7). This patient demonstrated serum Abs that bound specifically to melanoma cell lines, but not to colorectal carcinoma cell lines (7). Briefly, total RNA was isolated from 109 PBMC of patient GL. The presence of human Fab on the surface of the phage was determined by immunoblotting using mouse anti-human Fab-specific IgG and donkey anti-mouse IgG conjugated to HRP.

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2 Address correspondence and reprint requests to Dr. Dorothee Herlyn, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. E-mail address: dherlyn@wistar.upenn.edu
Phage biopanning
Pools of phage (10^9 PFU) were absorbed to confluent monolayers of adherent normal melanocytes in a T75 flask for 2.5 h at room temperature. Unbound phage in the supernatants were collected, titered in Escherichia coli XL-1 blue cells, and subjected to five rounds of selection for binding to HLA nonmatched (with respect to vaccine cell lines) melanoma lines A375 and DM196. Bound phage were eluted at pH 2.2 and selected for binding to the four vaccine lines Mel-2, Mel-3, Mel-4, and Mel-B. Finally, bound phage were eluted at pH 2.2 and selected for binding to tunicamycin-treated (50 μg/ml in DMSO, for 2 h at 37°C) melanoma cell lines DM196 and Mel-B. Phage were eluted and amplified by injection of E. coli XL-1 blue cells. Individual phage were isolated for further testing.

Phage cell binding screening
Cultured tumor or normal cells in suspension (5 × 10^6 cells/microtiter plate well) were incubated for 30 min at 4°C with 5% BSA or human AB serum (EBV-B cells only) in HBSS and with 10^6 PFU of specific phage or control VCS M13 phage. Cells were washed in HBSS supplemented with 0.1% BSA or 0.1% human AB serum (EBV-B cells only), and binding was determined by flow cytometry using biotinylated sheep anti-M13 Ab (1:100 dilution) and streptavidin-PE (1:75 dilution). Binding of specific phage to cells was considered significant at levels at least 4 times those of the control phage and with SD of the mean less than 10%.

DNA preparation and sequencing of clone A10-5
Phagemid DNA was prepared essentially as described (11). Briefly, E. coli XL-1 blue cells (Stratagene, La Jolla, CA), infected with A10-5 phagemid, were grown overnight at 37°C in super broth (SB; 3 g tryptone, 20 g yeast extract, 10 g MOPS (Sigma, Denver, CO) per liter, pH 7.0), containing 1% glucose and 50 μg/ml carbenicillin. Phagemid DNA was purified using a Qiagen Maxi-prep kit (Qiagen, Valencia, CA) and both genes were sequenced using the following sequencing primers: sense, 5′-CCCACTGAAAAGTCTGAGTCT-3′; antisense, 5′-TGGTCAAGGGCA CCGAGTTCACGACAGCCG-3′ (VE); sense, 5′-CAGAATTTTCA TATGTCGCC-3′; and antisense, 5′-GACCTTCTCCCCGAGA-3′ (VE). A10-5 Fab production and purification
Following phage screening, phage clone A10-5 was amplified by infection of E. coli XL-1 blue cells. Phage-infected cells were cultured overnight at 37°C in SB containing 50 μg/ml carbenicillin and 1% glucose. Phagemid DNA was purified using a Qiagen kit. To excise gene III and juxtapose the hexahistidine tag at the C terminus of the heavy chain, 10 μg phagemid DNA was digested with SpeI and Nhel. After gel purification, fragments containing the heavy and light chains were self-ligated overnight at 16°C using a ligation kit (Stratagene). The ligated DNA was purified by phenol/chloroform extraction and used to transform E. coli JM109. Single colonies were selected, and expression of heavy and light chains was confirmed by restriction enzyme digestion and sequencing.

For Fab purification, the clone containing heavy and light chains was incubated overnight at 37°C in 100 ml SB with 50 μg/ml carbenicillin and 1% glucose. The cell pellet was suspended in 100 ml of 100 mM NaCl and 20 mM Tris (pH 8.0), and lysed by sonication. Cell debris was removed by centrifugation at 10,000 × g for 5 min. NaCl was added to 0.8 M followed by 0.5 ml of 50% nitrotriacetic acid slurry (Qiagen) and the sample was incubated on a rotator for 2 h at room temperature. The Fab-bound resin was pelleted by centrifugation at 10,000 × g for 15 min at 4°C. The pellet was washed once with 10 ml phosphate buffer (100 mM sodium phosphate, 50% (pH 8.0)), and Fab was eluted by incubation in 0.5 ml of 300 mM imidazole (Sigma) for 30 min at room temperature. After centrifugation, the supernatant containing Fab was dialyzed extensively against PBS overnight at 4°C. The Fab was analyzed by immunoblotting using HRP-labeled goat anti-human IgG Fab (Cappel, Chester, PA). Approximately 50% purity of the Fab was achieved by the one-step nitrotriacetic acid purification procedure.

Biotinylation of phages and Fab
Phage (10^10 PFU/ml) or Fab (1 mg/ml) were suspended in biotinylation buffer (150 mM sodium borate, 1 M NaCl (pH 9.5)) containing 5 μl Biotinyl-7-normal human serum (20 mg/ml in DMSO; Boehringer Mannheim, Indianapolis, IN) and incubated for 15 min at room temperature. The reaction was stopped with 15 μl of stop solution (3 N HCl; final concentration of 50 mM). Labeled phage or Fab were dialyzed against PBS overnight at 4°C.

Screening for Fab cell binding
For detection of Fab binding to the surfaces of cultured tumor and normal cells, 5 × 10^6 cells were incubated with specific A10-5 Fab or control 43 Fab (both at 2.5 μg/ml) in 1% BSA in PBS for 30 min at 4°C. Control 43 Fab binds to respiratory syncytial virus (12). Cells were washed in PBS/1% BSA and Fab binding to cells was determined by flow cytometry using PE-labeled streptavidin (1:75 dilution; Immunology Research, Grove, PA). To determine inhibition of binding of biotinylated A10-5 Fab to Mel-B melanoma cells, the cells (10^6/well) were incubated with serum (reciprocal serum dilutions 2, 6, 18, 54) derived from patient GL after vaccination, or with human AB serum pool derived from healthy donors for 2 h on ice. Biotinylated A10-5 Fab (40 μg/ml) was then added to the wells for 2 h on ice. To detect binding of A10-5 Fab to Mel-B cells, PE-labeled streptavidin (1:75 dilution) was added to the wells and binding of biotinylated A10-5 Fab to the cells was determined relative to buffer controls. For detection of Fab binding to intracellular structures, cells were grown on 12-mm coverslips, washed with PBS, and fixed with 1% paraformaldehyde in PBS for 5 min on ice. Coverslips were washed in PBS and cells were permeablized with 0.25% (v/v) Triton X-100 in PBS for 20 min on ice. Cells were incubated with biotinylated A10-5 Fab or control 43 Fab (both at 50 μg/ml) for 1 h at room temperature and rinsed with PBS. Cells were then incubated with streptavidin-fluorescein isothiocyanate (diluted 1:100 in PBS; Lifetech) for 30 min at room temperature in the dark and rinsed with PBS. Coverslips were mounted onto glass slides with 5 μl Fluoro-mount G (Fisher Scientific, Pittsburgh, PA) and analyzed with a Leica (Deerfield, IL) TCS 4D confocal microscope.

To determine Fab binding to tissue, formalin-fixed, paraffin-embedded sections were cut (5-μm thickness), mounted on ProbeOn Plus slides (Fisher Scientific), and air-dried in a fume hood (7). The assay was performed by manual capillary action using the MicroProbe (13, 14). Briefly, tissue sections were rapidly dewaxed, cleared with absolute ethanol, and rehydrated with 1× Tris-based buffer, pH 7.4 (10× automation buffer; Biomeida, Foster, PA). Ag retrieval was conducted using an EDTA buffer heat-induced-epitope retrieval (HIER), 10 mM; NeoMarkers, Union City, CA) diluted to a final concentration of 1.0 mM. The manufacturer’s protocol was followed completely.

The primary biotinylated A-10 Fab or control biotinylated 43 Fab (both at 10 μg/ml) were applied to the sections, and slides were incubated overnight at 4°C. The biotinylated complex was detected with an alkaline phosphatase-linked streptavidin complex (Boehringer Mannheim) at 1 U/ml in 10% automation buffer following a 10-min incubation at 45°C. The red signal was developed by incubating with an indol chromogen system (300 mM 5-bromo, 4-chloro, 3-indolylphosphate/iodoacte blue tetrazolium (BCIP/INT; Amresco, Solon, Ohio), for 10 min at 50°C. Sections were lightly counterstained with a 1:3 (v/v) solution of aqueous hematoxylin (Biomeida) in water for 10 s at room temperature, rinsed in water, mounted with Crystal Mount (Biomeida), and heated at 80°C to harden the medium.

Immunofinityng of Ag
Protein extracts of Mel-B melanoma cells and FF2207 fibroblasts (10^6 cells each) were prepared in cold Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), containing 0.5% Nonidet P-40, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM PMSF, 25 mg/ml aprotinin, and 25 mg/ml leupeptin). Extracted proteins were absorbed to VCS M13 wild-type phage (10^7 PFU) and simultaneously incubated with biotinylated A10-5 phage (10^9 PFU) in 1% BSA/PBS on ice for 2 h. Streptavidin-agarose beads (50 μl; Sigma) were added to the absorbed phages and mixtures were incubated on a rotator at 30–50 rpm for 1 h at room temperature, followed by centrifugation at 10,000 rpm for 5 min at 4°C. Cell pellets were washed three times with PBS and protein was eluted with 50 μl of 0.1 N HCl. The eluate was separated from the heads by centrifugation (10,000 rpm) for 5 min at room temperature and 4 μl of 2 M Tris base (pH 10.5) was added. The purified protein was stored at −70°C.

Western blot analysis of purified protein
Purified protein samples (see above) were electrophoresed on a 10–16% SDS-PAGE (Bio-Rad, Richmond, CA) under nonreducing and reducing (β-mercaptoethanol, Sigma; boiling for 5 min) conditions and transferred to polyvinylidene difluoride membranes (Amersham, Piscataway, NJ). After blocking with 5% BSA and 4% milk in PBS at 4°C overnight, membranes were washed in PB buffer (0.05% Tween 20/PBS), incubated with A10-5 Fab or control 43 Fab (both at 0.2 μg/ml) for 2 h at room temperature, washed again, and incubated with HRP-labeled goat anti-human F(ab)’2 (diluted 1:2000; Cappel) for 1 h at room temperature. Finally,
membranes were washed three times and developed using the ECL detection kit (Amersham).

Expression cloning

For expression cloning of the Ag recognized by A10-5 Fab, a cDNA library was constructed from WM1158 melanoma cells in the CDM8 vector (15) and transfected into COS-7 monkey kidney cells using the Lipofectamine Plus kit (Lifetech). After 24 h, transfected COS-7 cells were detached with EDTA, washed, and immunoselected on microtiter plates coated sequentially with rabbit anti-human Fab and A10-5 Fab. Plasmid DNA was recovered from the bound COS-7 cells, amplified in E. coli MC1061/F5 cells (Lifetech), and reintroduced into COS-7 cells. Three rounds of enrichment were performed to isolate positive clones. The isolated cDNA was reintroduced into COS-7 cells and reactivity of the transfected cells with biotinylated A10-5 Fab was confirmed by flow cytometry using PE-labeled streptavidin. One cDNA clone (clone 2) was characterized in detail.

Nucleic acid sequencing and gene database analysis of the Ag

Nucleic acid sequencing was performed with a model 373A automated DNA sequencer (Applied Biosystems, Foster, CA) using the Applied Biosystems BioDye terminator cycle sequencing ready reaction kit. The sequencing primers for the Ag clones were 5'-ATGCTGACAACCTCCGCC-3' and antisense, 5'-AGCCAGCA CAATGATCTCGAGGA-3') derived from the nucleotide sequence of clone 2. The RT-PCR product was sequenced.

Results

Selection of phage A10-5

Pools of phage from the combinatorial Fab library were absorbed to normal melanocytes followed by panning against and elution from melanoma cells. To eliminate phage binding to N-linked carbohydrates Ags, the phage were bound to and eluted from tumic melanoma cells (7). Greater than 109-fold enrichment for melanoma-specific phage was achieved. Four phage were selected for detailed binding specificity studies because they showed high-level binding to cultured melanoma cells (MEL-2, -3, -4, and -B) used to vaccinate the patient, and to HLA nonmatched (with respect to the vaccine cell lines) melanoma cell lines A375, DM196, WM793, WM35, and WM1552C (results not shown). Phage pool A10 did not bind to fibroblasts and melanocytes and, therefore, this phage population was cloned. Clone A10-5 was selected for detailed study because it showed high-level binding to melanoma cells. The deduced amino acid sequence of the heavy chain of the Fab is 94.2% identical with a published human anti-melanoma Fab bound to FF2207 fibroblasts (Fig. 1). A10-5 Fab bound to intracytoplasmic structures and nuclei of Mel-B melanoma cells as well as FF2207 fibroblasts (Fig. 2). Control 43 Fab did not bind to these cells (results not shown). The binding of A10-5 Fab to Mel-B melanoma cells was inhibited by GL patient’s serum (diluted 1:3) by 82%, whereas the AB serum pool inhibited this reaction by 11%. In this assay, circulating Ag most likely does not interfere because serum-free supernatant derived from Mel-B cells did not significantly inhibit binding of A10-5 Fab to the cells (not shown). Thus, the Ag recognized by A10-5 Fab is not shed by melanoma cells in vitro and therefore, it most likely is also not shed into the patient’s circulation in vivo.

Binding reactivity of A10-5 Fab to tissues

Expressed A10-5 Fab, but not 43 Fab, bound to all of 10 metastatic melanoma lesions (Fig. 3A) and to three of five vertical growth phase (VGP) primary melanoma lesions (Fig. 3A). There was strong cytoplasmic staining of the cells. However, A10-5 Fab did not bind to any of five radial growth phase (RGP) primary melanoma or five compound nevic tissues tested (Fig. 3A). Up to 60% of the cells within metastatic lesions specifically bound A10-5 Fab, whereas only up to 20% of the cells within VGP lesions specifically bound Fab (Fig. 3B). Fab, Fig. 4 shows the staining of metastatic melanoma (A) and VGP primary melanoma (C) lesions, and the absence of staining of RGP primary melanoma lesion (D) with A10-5 Fab. None of the lesions demonstrated staining with control 43 Fab, as shown for metastatic melanoma in Fig. 4B. In some tissues, fibroblasts and infiltrating macrophages were stained, whereas endothelial cells, melanocytes, and keratinocytes were not stained.

Binding reactivity of A10-5 Fab to extracts of melanoma cells

Western blot analysis under nonreducing conditions using A10-5 Fab and immunoafinity-purified Mel-B melanoma cell extract revealed an intense 23-kDa band and a weaker 33-kDa band (Fig. 5). However, under reducing conditions, only the 23-kDa band was

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### Table 1. Binding of human A10-5 phage to tumor and normal cell lines assessed by flow cytometry

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Phage A10-5 % Cells Positive (mean of three independent assays ± SD)</th>
<th>Phage VCS M13 % Cells Positive (mean of three independent assays ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mel-B&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>94.4 ± 2.4</td>
<td>93.1 ± 1.2</td>
</tr>
<tr>
<td>Mel-2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>74.4 ± 3.2</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>Mel-3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>71.3 ± 2.6</td>
<td>2.6 ± 1.7</td>
</tr>
<tr>
<td>DM196&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.5 ± 2.4</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>A375&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.8 ± 2.1</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>WM1158&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93.1 ± 1.4</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>WM1552&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.4 ± 2.3</td>
<td>2.0 ± 1.6</td>
</tr>
<tr>
<td>WM35&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>39.3 ± 4.2</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>WM793&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>30.2 ± 3.0</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>MCF-7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.0 ± 2.7</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>MDA231&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.6 ± 3.2</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>SW1116&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.1 ± 2.5</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>U87MG&lt;sup&gt;g&lt;/sup&gt;</td>
<td>10.5 ± 2.7</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>Normal cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FM1024&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 1.4</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>FM2404&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 1.3</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>FM2408&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.1 ± 1.0</td>
<td>2.4 ± 1.4</td>
</tr>
<tr>
<td>FF1065&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.2 ± 2.3</td>
<td>2.8 ± 1.6</td>
</tr>
<tr>
<td>FF2207&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.4 ± 1.8</td>
<td>2.2 ± 1.2</td>
</tr>
<tr>
<td>EBV-RS&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.8 ± 2.2</td>
<td>3.2 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Melanoma vaccine cell line.  
<sup>b</sup> Metastatic melanoma cell line.  
<sup>c</sup> HLA nonmatched (with respect to vaccine cell lines) melanoma cell line.  
<sup>d</sup> Primary melanoma cell line (RGP/VGP: WM 1532 and WM 35; VGP: WM 793).  
<sup>e</sup> Breast carcinoma cell line.  
<sup>f</sup> Colon carcinoma cell line.  
<sup>g</sup> Glioma cell line.  
<sup>a</sup> Melanocyte cell line.  
<sup>b</sup> Fibroblast cell line.  
<sup>c</sup> EBV-transformed B cell line.
visualized. In extracts of FF2207 fibroblasts, A10-5 Fab reacted only with the 23-kDa protein under nonreducing conditions. The control 43 Fab did not react with any of those proteins. A10-5 Fab did not react with extracts of human melanocytes (results not shown).

**Characteristics of the cloned Ag**

Eight candidate cDNA clones were investigated after three rounds of enrichment by panning for A10-5 Fab reactivity. One of these, clone 2, reacted specifically with A10-5 Fab after reintroduction into transfected COS-7 cells as determined by flow cytometry (Table II). Thus, >88% of the clone 2-transfected COS-7 cells specifically reacted with A10-5 Fab, whereas <5% of the cells reacted with 43 Fab. Furthermore, <5% of control vector-transfected COS-7 cells reacted with A10-5 Fab. The nucleotide sequence of clone 2 is 100% identical with the sequence of the human ribosomal L13a (18) protein, previously termed p23 (19). p23 interacts with ErbB-3 (20) and is the human homologue of the murine p198 transplantation protein (21). Both proteins show 88% identity at the nucleotide level and 94% identity at the amino acid level.

The sequence of the RT-PCR product of WM1158 melanoma RNA using clone 2 primers was identical with the sequences of clone 2, confirming the origin of clone 2 from the WM1158 cDNA library (not shown).

**Discussion**

In this study, we have identified a novel melanoma-associated Ag expressed by metastatic and VGP primary melanoma, but not by RGP primary melanoma, nevi, or normal skin, using a previously described (7) phage-Fab library derived from a melanoma patient in complete remission after vaccination with an allogeneic tumor cell vaccine.

The deduced amino acid sequence of the heavy chain of the Fab is 94.2% identical with a published human anti-HSV heavy chain (16), and the light chain is 100% identical with a human antistaphylococcus protein A light chain (17). Thus, the combination of a known heavy and light chain sequence results in a Ab with a new Ag specificity. Binding of the Fab to tumor cells was significantly inhibited by the patient’s serum, but not by control serum, suggesting that original light and heavy chain pairing occurred during the assembly of the combinatorial Fab, although we...
cannot exclude the possibility that the Fab and the serum Abs bind to spatially close, but distinct determinants.

The Ag recognized by A10-5 Fab is expressed on the surface of cultured melanoma cells and, to a lesser extent, on colorectal cancer and glioma cells, but is not surface-expressed by normal cultured melanocytes, fibroblasts, or EBV-transformed B cells. However, permeabilized cultured fibroblasts did reveal the Ag in the cytoplasm and nucleus. The Ag is also expressed in the cytoplasm and nucleus of cultured melanoma cells. In tissue sections, the Ag is expressed in metastatic and VGP primary melanomas, but not in RGP primary melanoma, nevi, or normal skin.

The Ag appeared as 33- and 23-kDa protein bands in Western blots of nonreduced melanoma cell extracts, but as a single 23-kDa protein in reduced extracts. In fibroblast extracts, only the 23-kDa protein was detected under nonreducing conditions. Thus, in melanoma cells, the Ag consists of a 23-kDa protein that might be disulfide-linked to a 10-kDa protein or might be presented to the cell surface by electrostatic interaction with acidic proteins, because the Ag is a highly basic protein. Alternatively, it is possible that the 33- and 23-kDa moieties are not related, but both express the determinant recognized by A10-5 Fab. In contrast, the Ag in fibroblasts is expressed only in the cytoplasm and only the 23-kDa protein is found. Thus, processing and/or association of the protein with other proteins in melanoma cells differs from that in fibroblasts. Differences in protein processing in tumor cells vs normal cells also have been described in the cathepsin system (22, 23). Ovarian carcinoma patients raised Abs to both the immature (procathepsin D) and the mature (cathepsin) form of the enzyme (24, 25).

Table II. Binding of A10-5 Fab to COS-7 cells transfected with clone 2 cDNA

<table>
<thead>
<tr>
<th>Target Cell</th>
<th>Expt. No.</th>
<th>% Cells Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS-7 + clone 2 cDNA</td>
<td>1</td>
<td>88.4</td>
</tr>
<tr>
<td>COS-7 + control cDNA</td>
<td>2</td>
<td>89.9</td>
</tr>
<tr>
<td>COS-7 + control cDNA</td>
<td>1</td>
<td>3.9</td>
</tr>
<tr>
<td>COS-7 + control cDNA</td>
<td>2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* NT, Not tested.
The detection of p23 expression on the tumor cell surface was unexpected because p23 lacks a transmembrane domain. However, it is possible that association of p23 with a 10-kDa protein targets p23 to the membrane, although this has not been directly investigated. Moreover, transfection of COS cells with p23 cDNA resulted in membrane expression of the protein, as evidenced by A10-5 Fab binding to the surface of transfected COS cells. Again, p23 may be associated with another anchoring-facilitating protein in COS cells.

In light of the preferential expression of the combinatorial Fab-defined Ag by metastatic melanoma, this Ag may be of prognostic and/or therapeutic value. Expression of the Ag by cultured fibroblasts and fibroblasts in tissue sections does not preclude its use as a therapeutic target because the Ag was not expressed on the surface of these cells. Molecular cloning of the Ag by screening a melanoma cDNA library with A10-5 Fab showed that the cloned Ag is identical with the human ribosomal protein L13a, located on chromosome 1a (18) and previously termed p23 (Ref. 19; GenBank no. X56932). p23 is expressed by breast and lung carcinomas and sarcomas (20). Here, we demonstrate that p23 is expressed in human melanoma cells, both in isolated form and in association with a 10-kDa protein. p23 binds with its N-terminal end to the 26-aa juxtamembrane cytoplasmic domain of ErbB-3 (20). Melanoma cells express ErbB-3 (26), and p23 may bind to ErbB-3 in these cells. The interaction of p23 with ErbB-3 has been proposed to play a role in negative growth regulation of ErbB-3-expressing cells, based on observations that human breast cancer cells transfected with p23 show suppressed colony formation of ErbB-3-overexpressing cells, decreased growth rate, and induction of differentiation and apoptosis (20). Our findings implicating preferential expression of p23 in metastatic vs primary melanomas contrast with those findings; however, it is possible that the association of p23 with the 10-kDa protein in melanoma cells inhibits its growth-suppressive function. The identity of the 10-kDa species is presently unknown.

Human p23 demonstrates >88% amino acid sequence identity with the murine p198 transplantation Ag (21), and the bovine (19) and rat (27) p23 L13a. A mutated epitope of murine p198 is the target of cytolytic T cells in the rejection of murine P815 mastocyte in vivo (21). Here, we demonstrate the immunogenicity of the human p23 homologue in a vaccinated melanoma patient, as we have successfully isolated a Fab from the B cell library of this patient. Both the human p23 of melanoma cells described here and the murine p198 homologue are expressed on the human and murine tumor cell surface, respectively. Although p23 mRNA has been detected in human breast and lung carcinomas and in sarcomas (20), that study did not investigate cell surface expression of p23.

In addition to the ribosomal protein p23, other tumor-associated ribosomal proteins (e.g., the cell-surface-expressed 67-kDa protein with a 37-kDa core protein (28, 29) and the ribosomal protein L7 (30)) are associated with tumor cells. Thus, ribosomal proteins can be expressed on the tumor cell surface. In addition, p23 is also found in the nucleus of permeabilized melanoma cells and fibroblasts, consistent with the previously demonstrated nuclear location of ribosomal proteins (31). p23 mRNA was detected in normal human tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (20). However, we did not find p23 protein expression in normal tissues of the skin, colon, stomach, and endometrium using the A10-5 phage in immunohistochemical analysis of these tissues (not shown), although we did not determine RNA levels in those tissues. This finding is surprising because ribosomal proteins are expected to be expressed in most, if not all, normal tissues. It is possible that protein expression in normal tissues is below the level of detection by A10-5 Fab/phage binding in immunohistochemistry. The one-step immunohistochemical technique used may have been insufficiently sensitive to detect low protein levels in situ. In contrast, the protein is expressed at relatively high levels in tumor cells, allowing its detection by A10-5 Fab.

Other investigators (32, 33) have isolated Ab fragments with melanoma cell reactivity from the B cell libraries of melanoma patients using the Ab phage display approach. However, the tissue specificity of the Fab was not determined and the Ags recognized by the Fab were not identified in those studies. To our knowledge, our study presents the first successful cloning of an Ag using phase-display Ab fragments. The p23 Ag is an immunotherapeutic target on melanoma cells for B cells (because it is recognized by A10-5 Fab) and, possibly, for T cells (analogous to the murine p198 homologue, a CTL target Ag; Ref. 21), making p23 a vaccine candidate Ag for melanoma patients. Furthermore, p23, like integrin β1 (34), is a marker that may allow the distinction between RGP and VGP primary melanoma. Additional studies with higher numbers of tissues using sensitive Fab staining techniques are required to critically evaluate the potential of p23 as a diagnostic marker and therapeutic target.

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References


