Brief Definitive Report

A UNIQUE ANTIGENIC EPITOPE OF HUMAN MELANOMA IS CARRIED ON THE COMMON MELANOMA GLYCOPROTEIN gp95/p97

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The identification of tumor-specific antigens has been an elusive goal in human tumor immunology. Antibodies present in the sera of some cancer patients that react with autologous cultured tumor cells have provided reagents for the initial detection of restricted tumor cell surface antigens (reviewed in reference 1). We termed this approach "autologous typing." The antigens detected by this method that are restricted to the autologous tumor were designated "class 1" or "unique" tumor antigens. Of the six class 1 melanoma antigens that have been detected using this approach however, only one (FD) has been amenable to biochemical analysis (2). In previous studies we demonstrated that the FD specificity in the autologous cell line SK-MEL-131 is carried on a glycoprotein of ~90 kD (gp90). We have also produced mouse polyclonal (3) and monoclonal (4) antibodies to this glycoprotein. These mouse mAbs detected gp90 in a large variety of cultured human cells and in some normal tissues, showing that the FD specificity (recognized by the patient's serum) is a unique determinant carried on a common molecule (detected by mouse antibodies). We now show that this common molecule, gp90, is a previously recognized glycoprotein that was originally identified as a melanoma antigen using mouse mAbs and designated gp95 (5) or p97 (6, 7).

Materials and Methods

Cell Lines and Antibodies. Melanoma cell lines SK-MEL-28 (FD epitope negative) and SK-MEL-131, clone 1.36 (FD epitope positive), have been described (2), as have the conditions for their culture (2). Mouse mAbs I12 and KF23 have also been described previously (4, 5). Human serum from patient FD detects a unique epitope on SK-MEL-131 cells (2).

Radioimmunoprecipitation and PAGE. Methods for the immunoprecipitation of ^{125}I-labeled antigens, as well as sequential immunoprecipitation procedures, are described in references 2 and 3. SDS-PAGE and IEF were described previously (3, 8).

Purification of gp90 and Determination of its NH_2-terminal Amino Acid Sequence. SK-MEL-28 melanoma cells were cultured in Eagle's MEM containing insulin-transferrin-selenium (Collaborative Research, Lexington, MA) for 3 d. The spent medium (5 liters) was concentrated 10-fold and proteins were precipitated with 50% saturated ammonium sulfate. The precipitate was dissolved in PBS, dialyzed against the same buffer, and applied to a con A-Sepharose column (10 ml; Pharmacia Fine Chemicals, Piscataway, NJ). Unbound proteins were removed by washing with PBS and bound glycoproteins were eluted with 1.0 M methyl α-D-mannoside in PBS (compare reference 3). After dialyzing the eluate against 0.1 M NaCl, 0.01 M Tris-

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HCl, pH 7.0, the sample was passed through a column of normal mouse Ig (prepared from Tresyl-Sepharose; Pharmacia Fine Chemicals). The unbound sample was then applied to a column of mAb KF23-Sepharose (3.5 mg/ml), also prepared from Tresyl-Sepharose. The column was washed with 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.0, and 0.5 M NaCl, 0.01 M Tris, pH 8.5, and antigen was then eluted with 0.1 M NaCl, 50 mM diethylamine-HCl (pH 11.5). For further purification, the column eluate was dialyzed against water, lyophilized, and separated by SDS-PAGE on a 7% gel. After electrophoresis, the antigen (~10 μg) was transferred to polyvinylidene difluoride membrane (Millipore Continental Water Systems, Bedford, MA) in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid containing 10% methanol) as described by Matsudaira (9). The membrane was rinsed in water and air dried and the 90–95-kD region was located using prestained standards in adjacent lanes and cut out. The sample was used for amino acid sequencing using a gas phase sequenator (Applied Biosystems, Inc., Foster City, CA) at the Harvard Microchemistry facility (Boston, MA).

Tryptic Peptide Mapping. Proteins, isolated by antibody affinity chromatography and SDS-PAGE as described above, were radioiodinated with ^{125}I by the chloramine T procedure (10) and further purified by SDS-PAGE. Gel slices containing the antigen were washed successively with 10% acetic acid and 10% methanol. After drying by lyophilization they were placed in a solution of 50 μg/ml of DPCC-trypsin (Sigma Chemical Co., St. Louis, MO) in 0.13 M ammonium bicarbonate. After incubating overnight at 37°C, the trypsin solution was removed, the slices were washed with water, and the combined extracts were lyophilized. The tryptic digests were analyzed by two-dimensional (2D) mapping as described by Elder et al. (11) and the peptides were detected by autoradiography with XAR-5 film (Kodak).

Results

Two lines of evidence demonstrated the relationship between the FD epitope–bearing gp90 and gp95/p97. Sequential radioimmunoprecipitation experiments showed that preclearing of a ^{125}I-labeled antigen preparation from the allogeneic melanoma cell line SK-MEL-28 with mAb KF23 (detecting gp90) removed antigen reacting with mAb I12 (detecting gp95) (Fig. 1). Also, mAb I12 removed all the antigen from SK-MEL-131 reacting with human serum FD (detecting the unique epitope). Furthermore, we determined the partial NH_2-terminal amino acid sequence of purified gp90 glycoprotein from SK-MEL-28 cells. The antigen was isolated from the spent culture medium of SK-MEL-28 cells by con A–agarose and mAb KF23 antibody affinity chromatography, followed by preparative SDS-PAGE. After the affinity chromatography step, the sample contained three components in the 90-kD region (data not shown), which were reduced to one component in 2D IEF-SDS-PAGE after neuraminidase treatment (Fig. 2 *A*). Immunoprecipitation with mAb KF23 confirmed that this component was gp90. Determination of the partial NH_2-terminal amino acid sequence by a gas phase sequenator showed identity to the previously reported sequence for p97 derived either from NH_2-terminal amino acid sequencing (12) or deduced from the nucleotide sequence of the cloned gene (13) (Fig. 2 *B*). From these data we conclude that gp90 is identical to the previously described gp95 and p97 antigens; in addition, the FD epitope is carried on the gp95/p97 glycoprotein. This molecule has amino acid sequence homology to transferrin and also binds iron and has been termed melanotransferrin (12).

In an attempt to determine the biochemical basis for the expression of the FD determinant in gp95/p97 from SK-MEL-131 cells, but not SK-MEL-28 cells, tryptic peptide maps of the purified antigens from the two cell lines were compared (Fig. 3). No reproducible differences were found between the tryptic peptides of the antigen from FD^+ and FD^- cells.

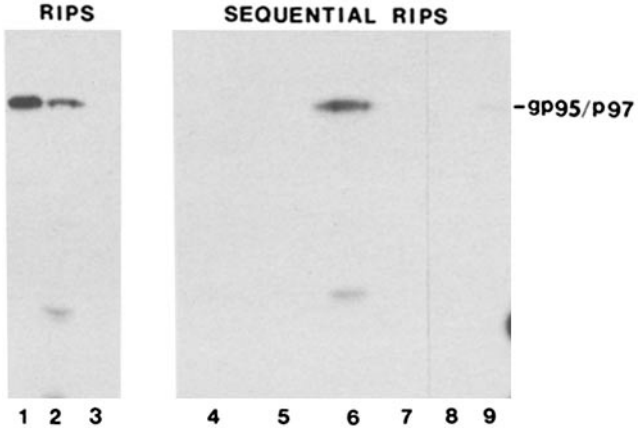

FIGURE 1. Direct and sequential radioimmunoprecipitation (RIPS) experiments comparing antigens reactive with mAbs KF23 (anti-gp90), I12 (anti-gp95), and FD serum. (*Left panel*) Direct immunoprecipitation of ^{125}I-labeled antigen from SK-MEL-28 with mAb I12, (lane *1*), mAb KF23, (lane *2*), and control mAb (lane *3*). (*Right panel*) Sequential immunoprecipitation experiments. ^{125}I-labeled antigen from SK-MEL-28 (lanes *4–7*) was precleared four times with the first mAb and protein A–agarose (Repligen, Boston, MA). The remaining sample was reacted with the second antibody and protein A–agarose and the immune precipitates were analyzed by SDS-PAGE. The antigen preparation was precleared with mAb KF23 (lanes *4* and *5*) or control antibody (lanes *6* and *7*) and was subsequently immunoprecipitated with mAb I12 (lanes *4* and *6*) or control antibody (lanes *5* and *7*). The results indicate that preclearing with mAb KF23 removes antigen reacting with mAb I12. Preclearing SK-MEL-131 (clone 1.36; reference 2) antigen with mAb I12 (lane *8*) and control mAb (lane *9*) and subsequent immunoprecipitation with FD serum (lanes *8* and *9*) showed that the FD determinant is carried on gp95. The unidentified components in some of the lanes are nonspecific components.

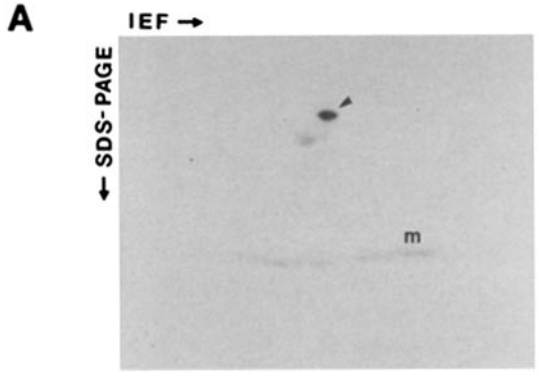

FIGURE 2. Purification and partial amino acid sequence of gp90. (*A*) The two-dimensional IEF-SDS-PAGE analysis of the sample purified as described in Materials and Methods. The sample was treated with neuraminidase (0.3 U/ml for 3 h at 37°C) before separation. Gp90 is indicated with an arrow; the other components are albumin, which was later removed by preparative SDS-PAGE, and IEF markers (m). (*B*) the N terminal amino acid data on the purified protein and the sequence is compared with the published sequence for p97 (12, 13).

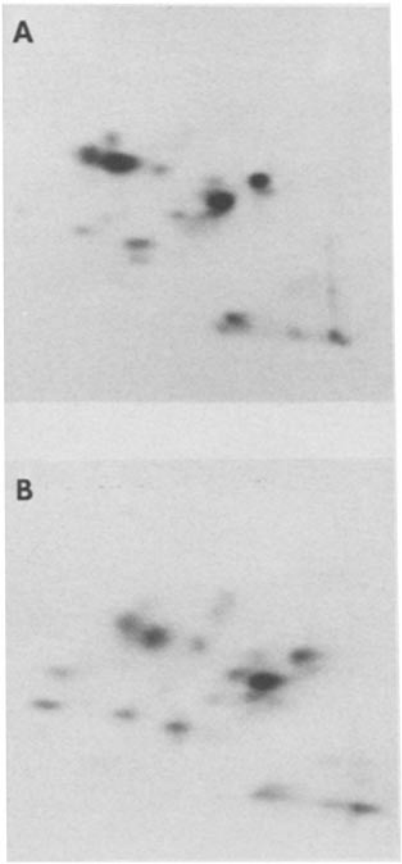

FIGURE 3. Comparison of tyrosine-containing tryptic peptides of gp95/p97 from SK-MEL-28 (*A*) and SK-MEL-131 (*B*) cells. The patterns are essentially identical; the minor differences observed in the two patterns were not reproducible.

Discussion

Although gp95/p97 is preferentially expressed on melanoma cell lines, it is found on a range of other cultured cell types (5–7). In vivo gp95/p97 is expressed strongly in the majority of melanoma tumors but only weakly on epithelial cancers (14, 15). The expression of gp95/p97 on normal tissues is generally limited to sweat gland ducts and the linings of blood vessels in some tissues (4). Using double-determinant assays, Brown et al. (16) demonstrated a range of 0.1–610 ng/mg tissue in melanoma tissues and a range of 0.1–10 ng/mg in normal adult tissues.

The fact that the FD specificity is heat labile and trypsin sensitive suggests that FD is a protein epitope. Both the original gp95/p97 from SK-MEL-131 and SK-MEL-28 cells and neuraminidase-, endo-*N*-acetylglucosaminidase-, or *N*-glycanase-treated products had identical molecular sizes as determined by SDS-PAGE (data not shown), indicating that there are no major molecular differences, which would have resulted in differences in mobility, in either the polypeptide or carbohydrate moieties of gp95/p97 from FD^+ and FD^- cell sources. No significant differences were detected in 2D maps of ^{125}I-labeled tryptic peptides of gp95/p97 from SK-MEL-131 and -28 cells. These results suggest that discrete differences, such as single amino acid substitutions, distinguish the two species. The observation made previ-

ously (4) that FD serum only partially removes molecules reacting with KF26 (another mAb detecting gp95/p97) could be explained by the low affinity of FD antibodies, but it also raises the interesting possibility that a mixed population of gp95/p97 molecules, corresponding to common and unique forms, may be produced by SK-MEL-131.

This demonstration that the autoimmunogenic melanoma epitope FD resides on a common melanoma antigen (gp95/p97) raises a number of interesting questions for tumor immunology. As only a single melanoma cell line in the series tested expressed the FD determinant, the expression of this novel epitope in gp95/p97 is uncommon. However, it focuses attention on the possibility of other changes in gp95/p97 in melanoma, and the available molecular probes for this glycoprotein (13) will permit analyses of these modifications and their frequency. In contrast to the modification resulting in the FD epitope, other possible transformation-related alterations in gp95/p97 may not elicit humoral immunity. The possibility that such changes in gp95/p97 could be targets for cellular immune responses against melanoma needs to be examined.

Summary

Analysis of antibodies present in the serum of melanoma patient FD has shown that they detect a unique tumor epitope present only on the autologous melanoma cell line SK-MEL-131. Previous results had shown that the unique FD epitope is carried on a common glycoprotein of ~90 kD, widely expressed on melanoma and a few other cell types. We now show by sequential radioimmunoprecipitation and partial amino acid sequencing that this common molecule is a previously recognized melanoma antigen, originally identified by mouse mAbs, designated gp95 or p97 (and also known as melanotransferrin). Thus, FD is the first of the class I (unique) melanoma antigens that has been characterized and related to a known cell surface molecule.

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