

THE MELANOMA ANTIGEN gp75 IS THE HUMAN
HOMOLOGUE OF THE MOUSE *b* (*BROWN*)
LOCUS GENE PRODUCT

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The molecular identification of immunogenic determinants on human cancer cells provides a basis for understanding the immune response to cancer. Two classes of antigens on human melanoma cells have received particular attention as targets for immune recognition: (a) unique antigens expressed only by autologous melanoma cells (1), and (b) differentiation antigens expressed normally by cells of the melanocyte lineage (2, 3). Differentiation antigens of melanocytes have been defined by their relationship to other well-defined phenotypic traits expressed during melanocyte differentiation (2, 3), the most distinctive being the synthesis of the pigment melanin within melanosomes.

Clinical observations have suggested, albeit indirectly, that an immune response directed against differentiation antigens expressed by normal pigment cells might influence the course of metastatic melanoma (4, 5). In this regard, melanosomal antigens potentially could be recognized by the immune system. This has been demonstrated directly by immunoprecipitation of a gp75 antigen from autologous melanoma cells by serum IgG antibodies of a patient with metastatic melanoma (6). The gp75 antigen is a melanosomal polypeptide that is the most abundant glycoprotein synthesized by pigmented melanocytes and melanomas (7; Vijayasradhi, S., and Houghton, A. N., unpublished observations). Epidermal melanocytes, benign pigmented lesions, and primary and metastatic melanomas express gp75, but other cell types do not (8). Thus, gp75 is a prominent tissue-specific glycoprotein in melanocytic cells and is potentially autoimmunogenic. In this study, we set out to identify the gp75 antigen, and report that it is the human counterpart of a product of a gene at the mouse *b* locus, a region that determines the type of melanin synthesized.

Materials and Methods

Tissue Culture. The melanoma cell line SK-MEL-19 cell line was grown in MEM plus 1% nonessential amino acids and penicillin and streptomycin, except that SerumPlus 10% (vol/vol) (Hazelton Research Products Inc., Lenexa, KS) was substituted for FCS.

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Isolation and Purification of gp75. Postnuclear membrane fraction from melanoma cell line SK-MEL-19 was isolated. High salt (3 M KCl) insoluble, detergent (0.5% deoxycholate) soluble protein fraction was collected and applied to a Mono Q (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) anion-exchange column. Bound proteins were eluted with a linear gradient of 0–1.0 M NaCl. Fractions were assayed for gp75 by a competitive inhibition assay that measured inhibition of binding of mouse mAb TA99 (300 ng/ml) (9) to SK-MEL-19 cells fixed in methanol/acetone (1:1 vol/vol) by enzyme immunoassay (3). Fractions containing gp75 were pooled, applied to a Con A-Sepharose column, and bound proteins were eluted with 0.25 M α -D-methylmannopyranoside. Fractions containing gp75 were pooled and applied to a mAb TA99 Affi-Gel 10 affinity column. Bound gp75 was eluted from the column with 0.1 M glycine-HCl, pH 3.1.

Immunoprecipitation, Gel Electrophoresis, and Peptide Mapping. Iodination of Con A-Sepharose-bound protein fraction of SK-MEL-19 by chloramine T method and immunoprecipitation with mAb TA99 and AU serum was done as described (6). Proteins were analyzed by one- and two-dimensional SDS-PAGE (10, 11). For endoglycosidase H (Endo H) digestion, the immunoprecipitates were suspended in 0.4% SDS, heated at 100°C for 5 min, and digested with 1 mU of Endo H (Genzyme Corp., Boston, MA) in 100 mM citrate buffer, pH 5.5, for 16 h at 37°C. Peptide mapping of immunoprecipitated gp75 was performed by limited proteolysis with *Staphylococcus aureus* V8 protease (V8 protease) (Boehringer Mannheim Biochemicals, Indianapolis, IN) in SDS-PAGE according to Cleveland et al. (12).

Peptide Sequencing. Peptide sequencing was performed at the Harvard University Microchemistry Facility. Purified gp75 (12 μ g) electroblotted onto nitrocellulose was digested with V8 protease (1:20 wt/wt) according to Aebersold et al. (13), and resulting peptides were separated on a Brownlee RP-300 C8 column. Peaks from V8 protease digests were pooled. Reduced and alkylated peptide fractions were digested with trypsin (1:25 wt/wt) overnight at 37°C. Fractions were sequenced on an ABI 477A protein sequencer (Applied Biosystems Inc., Foster City, CA).

Cloning and Sequencing of gp75 cDNA. A cDNA library was constructed from a melanoma cell line SK-MEL-19. Oligonucleotide probe was used to isolate a gp75 cDNA clone according to methods previously described (14). The sequence of the oligonucleotide probe was: 5' CTCGAAGGTGAAGCCCAGGTTGTCGGGGCGGTCACGAACATCTC 3'. A 2.0-kb cDNA clone, designated GP75-1, was sequenced (Stratagene Cloning Systems, La Jolla, CA). The nucleotide sequence of GP75-1 has been deposited with EMBL data bank under the accession number X51455.

Results and Discussion

Both mouse mAb TA99 and serum from melanoma patient AU immunoprecipitate a 75-kD antigen, and mAb TA99 preclears the gp75 antigen precipitated by AU serum (9). Since mAb TA99 was used to purify gp75 from the melanoma cell line SK-MEL-19, it was important to confirm that mAb TA99 detected only the gp75 antigen recognized by AU serum. In previous studies, we had shown that mAb TA99 does not react with human tyrosinase, a 75–80-kD glycoprotein also expressed in pigmented melanocytes (14). However, from these experiments, we could not rule out the possibility that mAb TA99 crossreacts with other polypeptides expressed by SK-MEL-19.

Proteins immunoprecipitated by mAb TA99 and AU serum antibody were analyzed by one- and two-dimensional SDS-PAGE and by peptide maps using limited proteolysis with *S. aureus* V8 protease. Proteins precipitated with both mAb TA99 and AU serum antibodies had identical molecular masses (75 kD), isoelectric points (5.5–5.9) (data not shown), and peptide composition (Fig. 1), confirming that TA99 and AU serum recognized the same gp75 molecule.

The gp75 antigen was purified as described in the Materials and Methods. Affinity-

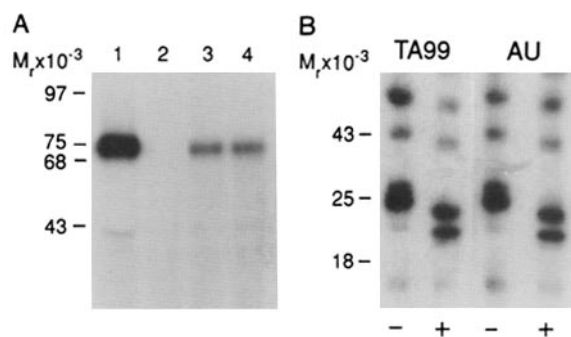


FIGURE 1. Immunoprecipitation and peptide composition analysis of proteins recognized by mAb TA99 and AU serum. (A) One-dimensional SDS-PAGE of immunoprecipitates from ^{125}I -labeled lysates of SK-MEL-19 by mAb TA99 (lane 1), normal human serum (lane 2), and two serum specimens from patient AU drawn 22 mo apart (lanes 3 and 4). (B) Bands corresponding to gp75 or to gp75 treated with Endo H (partially deglycosylated) were excised from SDS-polyacrylamide gel and the peptide composition was analyzed by limited digestion with *S. aureus* V8 protease on SDS-PAGE. (-) gp75; (+) gp75 partially deglycosylated with Endo H. Autoradiographic exposure was 2 d for TA99 and 5 d for AU peptides.

purified gp75 was used for peptide sequencing. The amino acid sequences of three internal peptides were obtained by proteolytic cleavage of purified gp75 with V8 protease and trypsin. The sequences of the three peptides are shown in Fig. 2. There was 90% identity with amino acid sequences deduced from the mouse cDNA clone, pMT4, isolated by Shibahara et al. (at amino acid positions 247–260, 333–349, and 428–441 of pMT4) (15). Oligonucleotide probes were derived from peptide sequences of p75 and used to screen a cDNA library constructed from the human melanoma cell line SK-MEL-19. A cDNA clone (2.0 kb) was isolated from a cDNA library of SK-MEL-19. Partial nucleotide sequence of the cDNA clone (GP75-1) showed 88.6% identity with pMT4 (between nucleotides 649 and 1395 within the open reading frame). The derived amino acid sequence of GP75-1 showed 93.6% identity with the derived amino acid sequence of pMT4 between amino acid residues 193 and 441. There was 55.3% identity between the cDNA sequences of gp75 and human tyrosinase (14) (between nucleotides 615 and 1359 of tyrosinase; data not shown).

The close homology between human gp75 and the deduced product of the mouse pMT4 gene sheds some light on the possible structure and function of gp75. The pMT4 clone was isolated from mouse melanocytic cells (15). Originally pMT4 cDNA was thought to code for mouse tyrosinase, which maps to the mouse *c* (*albino*) locus. However, further studies showed that pMT4 is distinct from tyrosinase (14, 16). Likewise, the sequence of gp75 is distinct from the sequence of human tyrosinase, although there is limited identity (43.1%) between gp75 and the derived amino acid sequence of human tyrosinase (between amino acid residues 187 and 434) (14). The

1. Asn-Thr-Val-Glu-Gly-Tyr-Ser-**Asp**-Pro-Thr-Gly-Lys-Tyr-**Asp**-Pro-Ala-Val
2. Met-Phe-Val-Thr-Ala-Pro-**Asp**-Asn-Leu-Gly-Tyr-Thr-Tyr-Glu
3. Asn-Phe-**Asp**-Ser-Thr-Leu-Ileu-Ser-Pro-Asn-Ser-Val-Phe-Ser

FIGURE 2. Amino acid sequence of three peptides derived from gp75. (**Bold face**) Amino acid residues that differ from residues predicted from the mouse cDNA pMT4.

function of gp75 and the pMT4 product is suggested by the finding that pMT4 maps to the *b* (*brown*) locus in the mouse (17), a region that regulates coat color (18). The homology between gp75 and the deduced amino acid sequence of pMT4 permits the formal identification of the human homologue of the mouse *brown* locus gene product. Based on its melanosomal localization and structural similarity to tyrosinase, the gp75 molecule may regulate melanin synthesis, and determine the type of melanin synthesized.

It has been perceived that melanosomal determinants and other intracellular antigens are not potential targets for immunotherapy of melanoma. However, recently it has become evident that intracellular proteins can be processed and presented as peptides to CTL by APCs (19). This finding opens up the theoretical possibility that T cell responses against melanoma could be directed against molecules expressed within the tumor cell. Alternatively, melanosomal components, which are normally transported outside the melanocyte during maturation, could accumulate in the extracellular space around tumor cells, or local tissue necrosis could lead to release and deposition of intracellular products. In support of the accessibility of gp75, radiolabeled TA99 mAb specifically localizes to human melanoma xenografts in *nu/nu* mice (20), indicating the availability of the antigen to antibody within tumor sites.

IgG antibodies in the melanoma patient AU recognized determinants on gp75 that were shared by melanoma cells and normal melanocytes (6). With the isolation of cDNA clones that code for gp75, it should be possible to study strategies for active immunization against gp75. There are at least two requirements for effective induction of CTL responses to gp75: (a) immune tolerance to gp75 must be broken, and (b) gp75 peptides must be processed and effectively presented by major histocompatibility antigens on melanoma cells. Alternatively, it is possible that differentiation antigens of melanocytes could carry unique determinants. Genes encoding products expressed by normal melanocytes could be mutated or rearranged during malignant transformation, generating novel epitopes for recognition by CTL and antibodies. In this regard, the best characterized unique antigen on human melanoma cells is a determinant on melanotransferrin, a 95–97-kD glycoprotein expressed on cultured melanocytes and melanoma cells (1, 21). In support of this possibility, genetic alterations have been detected at high frequency and widely throughout the genome of human melanoma cells (22, 23).

Summary

The gp75 antigen is an abundant intracellular glycoprotein expressed in melanosomes of human pigmented melanocytes and melanomas. IgG antibodies in sera of a patient with metastatic melanoma have been shown to immunoprecipitate gp75, suggesting that immunological tolerance against gp75 can be broken. The mouse mAb TA99, which specifically recognizes gp75, was used to isolate and purify the antigen. Amino acid sequences of three internal peptides were determined from the purified gp75 polypeptide. cDNA clones were isolated by screening with oligonucleotides based on these peptide sequences. The gp75 peptides and cDNA had ~90% identity with, respectively, the derived amino acid and nucleotide sequences of a mouse gene that maps to the *b* (*brown*) locus. The *brown* locus determines coat color in the mouse, suggesting that gp75 regulates or influences the type of melanin synthesized.

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