Molecular Characterization of the Melanocyte Lineage-specific Antigen gp100*

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The glycoproteins recognized by monoclonal antibody (mAb) NKI-beteb are among the best diagnostic markers for human melanoma because their expression is restricted to melanocytic cells. Recently, we isolated a cDNA clone, termed gp100-cl, which confers immunoreactivity not only to mAb NKI-beteb, but also to two other mAbs used to diagnose malignant melanoma, HMB-50 and HMB-45. In this report, we demonstrate that gp100-cl cDNA encodes glycoproteins of 100 kDa (gp100) and 10 kDa (gp10) which are recognized by these mAbs in human melanoma cells. The translation product deduced from the open reading frame present in gp100-cl cDNA is highly homologous to another melanocyte-specific protein, Pmel17. Nucleotide sequence analysis of genomic DNA indicates that the transcripts corresponding to gp100 and Pmel17 cDNAs originate from a single gene via alternative splicing. In all normal and malignant melanocytic cells analyzed, gp100 and Pmel17 RNAs are simultaneously expressed.

Melanoma is a neoplasm that originates from melanocytes, pigment-producing cells in the skin. Melanoma is a relatively immunogenic tumor, as demonstrated by the presence of both cytotoxic T lymphocytes (CTL)† (Knuth et al., 1992) and antibodies (Mattes et al., 1983) reacting with melanoma cells in patients. Characterization of the antigens recognized revealed that they include both tumor-specific antigens (van der Bruggen et al., 1991) and the melanocyte differentiation antigens tyrosinase (Brichard et al., 1993) and gp75 (Vijayasaradhi et al., 1990). Both these differentiation antigens are localized in a distinct cellular organelle, the melanosome, and are involved in the synthesis of the pigment melanin (Hearing and Tsukamoto, 1991). To understand the potential role of immunological events in the pathogenesis and clinical course of melanoma, it is important to identify more of these antigens. This will not only result in the identification of potential targets for immune responses against melanoma, but may also lead to the identification of antigens involved in melanocyte differentiation and possibly transformation.

Many monoclonal antibodies have been raised against melanoma cells. Clinical and pathological analyses using these mAbs have led to the description of different steps in the transformation and progression of melanoma (reviewed by Ruiter et al., 1990)). Some of these mAbs define important markers in the initial diagnosis of melanoma, while others define melanoma progression antigens. Most of the antigens are expressed by both melanoma cells and melanocytes and are probably expressed during melanocyte differentiation. The melanocyte lineage-specific antigens recognized by mAb NKI-beteb are among the best diagnostic markers for human melanoma (Vennegoor et al., 1988). NKI-beteb reacts with melanoma cells throughout tumor development and does not cross-react with other tumor cell types or normal cells, except for cells of the melanocytic lineage. The antigens recognized by NKI-beteb are glycoproteins of approximately 10 kDa (gp10) and 100 kDa (gp100). Ultrastructural analysis of NKI-beteb binding sites indicates that the antigens are localized in premelanosomal vesicles (Vennegoor et al., 1988). Recently, we isolated a cDNA clone, gp100-c1, which confers immunoreactivity to NKI-beteb after expression in gp100-negative BLM melanoma cells (Adema et al., 1993). Interestingly, two other mAbs used to diagnose malignant melanoma, HMB-50 (Vogel and Esclamado, 1988) and HMB-45 (Gown et al., 1986), also react with BLM cells transfected with gp100-c1 cDNA (Adema et al., 1993), indicating that all three mAbs recognize proteins encoded by a single cDNA. Herein, we report the molecular characterization of gp100-c1 cDNA and the melanocyte lineage-specific protein it encodes and discuss oncological and cell-biological aspects of the gp100 antigen.

MATERIALS AND METHODS

Cells and Monoclonal Antibodies—The melanoma cell line Mel-2a, M14, MEWO, and BLM (Adema et al., 1990) and the uveal melanoma cell line Mel 202 (Ksander et al., 1991) have been described. Isolation of human melanocytes from breast or foreskin was performed by the method of Elsinger and Marko (1982) with modifications by (Smit et al., 1993). NKI-beteb and HMB-50 have been described previously (Vennegoor et al., 1988; Vogel and Esclamado, 1988). HMB-45 was purchased from Enzo Biochem.

DNA Constructs, Transfections, and Immunofluorescence—gp100-c1 cDNA was cloned in both orientations (pSVLgp100+ and pSVLgp100−) in the Smal site of the eukaryotic expression vector pSVL (Pharmacia Biotech Inc.). pSVL contains the SV40 late promoter and polyadenylation site, as well as the SV40 origin of replication, allowing a very high copy number during transient expression in COS-7 cells. For the construction of the 3′ truncated gp100 transcription unit pSVLgp100+ (Δ3S), we deleted the sequence between the BgII site in the 3′ part of gp100-c1 cDNA and the SstI site in the vector. The resulting construct encodes a truncated gp100 protein in which the carboxyl-terminal 133 amino acids of gp100 are replaced by 4 amino acids (Arg-Ile-Gln-Thr) encoded by vector sequences. Transient expression of the constructs in COS-7 cells was performed by using 40 μg/ml Lipofectin reagent from Life Technologies, Inc. (Felgner et al., 1987) and 7.5 μg of DNA. Transfected COS-7 cells were prepared for immunofluo-
rescence as described previously (Adema et al., 1993) and examined using a confocal laser scanning microscope at 488 nm (Bio-Rad MRC 600).

**Metabolic Labeling, Immunoprecipitations, and V8 Protease Mapping**—Immunoprecipitation experiments were performed on metabolically labeled ([35S]methionine/cysteine; Amersham Corp.) cells as described by Vennegoor et al. (1988) using either NKI-beteb or HMB-50 covalently linked to protein A-Sepharose CL-4B beads (Pharmacia). In some experiments, tunicamycin (75 μg/ml, Calbiochem) was added during the prelabeling period and remained present during the metabolic labeling reaction (12.5 min). Immunoprecipitates were analyzed under reducing conditions by SDS-PAGE using 5–17.5% gradient gels. The relative molecular weight of the proteins was determined using coelectrophoresed, prestained markers (Life Technologies, Inc.). Gels were treated with 1 N sodium salicylate (pH 5.4) prior to autoradiography (Kodak XAR).

V8 protease mapping was performed using the procedure described by Cleveland et al. (1977). Briefly, gel slices containing the 100-kDa proteins were placed in the wells of a second SDS gel (10%) and overlaid with *Staphylococcus aureus* V8 protease (2.5 μg/sample, Miles Laboratories). After electrophoresis, gels were treated as described above.

**Molecular Cloning of Part of the gp100/Pmel17 Gene**—Part of the gp100/Pmel17 gene was amplified by PCR on human genomic DNA isolated from peripheral blood lymphocytes using the following primers: 5'–TCTTGACCAGGCATGATA-3') or a Pmel 17-specific oligonucleotide (5'-CTCTTCTCACAGGT-3') was included as a control. The PCR products were subsequently cloned into the EcoRI site (underlined) (5'-TATCTAGAATTCTGC ACC AGATACTGAAG-3') and cloned into the EcoRI site of pUC 18.

**RNA Isolation and Analysis**—Total RNA was isolated using the guanidine thiocyanate/cesium chloride procedure (Chirgwin et al., 1979). cDNA was prepared using the GeneAmp RNA PCR kit (Perkin-Elmer), and PCR analysis was performed for 35 cycles in the presence of 3 mM MgCl2 using primers 1497/1516 and 1889/1897 (see above) as described (Adema and Baas, 1991). As probes, we used either a gp100-specific exon/exon junction oligonucleotide (5'-CTCTTCTCACAGGCATGATA-3') or a Pmel17-specific oligonucleotide (5'-CTCTTCTCACAGGCATGATA-3'). The PCR products were subsequently amplified using a nested set of primers containing an additional EcoRI site (underlined) (5'-TATCTAGAATTCTGC ACC AGATACTGAAG-3' and 5'-TATCTAGAATTCTGC ACC AGATACTGAAG-3') and cloned into the EcoRI site of pUC 18.

**RESULTS**

**Expression of gp100-c1 cDNA in Non-pigmented COS-7 Cells**

Results in immunoreactivity with mAbs NKI-beteb, HMB-50, and HMB-45—Expression of gp100-c1 cDNA in gp100-negative BLM melanoma cells results in immunoreactivity with the melanocyte lineage-specific mAbs NKI-beteb, HMB-50, and HMB-45 (Adema et al., 1993). To determine whether expression of gp100-c1 cDNA in non-melanocytic cells also results in immunoreactivity with these mAbs, we performed transient expression experiments in COS-7 cells (monkey kidney fibroblasts) with constructs containing gp100-c1 cDNA in the coding or non-coding orientation. As shown in Fig. 1, only COS-7 cells transfected with the construct containing the cDNA in the coding orientation (COS-7/pSVLgp100+) reacted with all three mAbs. These data demonstrate that immunoreactivity with NKI-beteb, HMB-50, and HMB-45 after expression of gp100-c1 cDNA is not restricted to melanocytic cells. In addition, these data show that the COS expression system can be used for further biochemical characterization of the proteins encoded by gp100-c1 cDNA.

**Analysis of the Proteins Encoded by gp100-c1 cDNA**—To characterize the proteins encoded by gp100-c1 cDNA, COS-7/ pSVLgp100+ cells were metabolically labeled and subjected to immunoprecipitation with NKI-beteb or HMB-50. As shown in Fig. 2, NKI-beteb (panel A) and HMB-50 (panel B) specifically detected proteins of approximately 100 kDa (95–110 kDa) in extracts of COS-7/pSVLgp100+ cells. The molecular mass of these proteins is similar (see also below) to those detected in extracts of metabolically labeled MEWO cells (Fig. 2), which express the antigens endogenously (Vennegoor et al., 1988). Consistent with previous reports (Vennegoor et al., 1988; Vogel and Esclamado, 1988), both mAbs also recognize a protein of 10 kDa in extracts of MEWO cells (Fig. 2, lanes 6 and 7). A protein of the same size reacted with NKI-beteb in COS-7/pSVLgp100+ cells (Fig. 2A, lane 4) and could be discerned with HMB-50 after prolonged exposure (not shown). No specific proteins were immunoprecipitated by either of the mAbs from extracts prepared.
from COS-7 cells transfected with the construct containing the cDNA in the non-coding orientation (Fig. 2). Comparison of the culture medium of metabolically labeled COS-7/pSVLgp100+ cells and MEWO cells revealed that both mAbs also recognized proteins of about 100 kDa (see also below) in the culture medium of these cells (Fig. 3). No proteins of 10 kDa were immunoprecipitated by the mAbs from the culture medium of COS-7/pSVLgp100+ cells, as has been shown for melanoma cells.

To exclude the possibility that the proteins detected by the mAbs are derived from endogenous genes induced after transfection with gp100-c1 cDNA, we performed immunoprecipitation experiments with COS-7 cells expressing a 3' truncated gp100 transcription unit (see “Materials and Methods”). As shown in Fig. 4, proteins of approximately 85 kDa were detected by both mAbs in COS-7 cells expressing this construct, consistent with a deletion of 129 amino acids. This finding provides direct evidence that the 100-kDa protein recognized by NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells is encoded by gp100-c1 cDNA.

The 100-kDa Protein Encoded by gp100-c1 cDNA Is Identical to gp100—The proteins of about 100 kDa identified by NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells versus MEWO cells had a slightly different mobility when analysed by SDS-PAGE (Fig. 2). This difference could be due to altered glycosylation, an event frequently observed in the COS expression system. Analysis of the proteins immunoprecipitated from MEWO cells and COS-7/pSVLgp100+ cells cultured in the presence of the glycosylation inhibitor tunicamycin demonstrated that in both cell types the size of the proteins of about 100 kDa was reduced to two protein bands of 90 and 85 kDa, confirming that the difference in mobility is due to altered glycosylation (not shown).

To provide further evidence that the proteins recognized by NKI-beteb in COS-7/pSVLgp100+ cells and MEWO cells are identical, we performed a V8 protease mapping experiment. As shown in Fig. 5, the same protein fragments were obtained after V8 protease digestion of the major 100-kDa protein isolated from COS-7/pSVLgp100+ cells or MEWO cells. We conclude from these data that gp100-c1 cDNA encodes the melanocyte lineage-specific glycoprotein gp100 recognized by NKI-beteb and HMB-50 in melanoma cells.
**gp100 Is a Type I Transmembrane Protein Highly Homologous to Pmel17**—The nucleotide sequence of gp100-c1 cDNA was determined. It contains 2115 base pairs and terminates with a poly(A) tract of 15 nucleotides, preceded by the consensus polyadenylation sequence AATAAA (Proudfoot and Brownlee, 1976). An open reading frame extending from position 22 through 2007 is present in gp100-c1 cDNA. This open reading frame starts with an ATG codon within the appropriate sequence context for translation initiation (Kozak, 1987) and predicts a protein of 661 amino acids (Fig. 6). The amino-terminal 20 amino acids fit all criteria for signal sequences, including a stretch of 7 amino acids absent in gp100 at position 455—546) are present. Furthermore, a histidine-rich domain (position 567 (see also Table II). A single nucleotide difference at position 762 (C→G/Arg→Pro) and a stretch of 7 amino acids absent in gp100 at position 567 (see also Table II). A single nucleotide difference at position 762 (C→T) does not result in an amino acid substitution. gp100 is also 80% homologous to a putative protein deduced from a partial bovine cDNA clone (RPE-1) (Kim and Wistow, 1992) and 42% homologous to a chicken melanosomal matrix protein, MMP115 (Mochii et al., 1991).

**gp100 and Pmel17 Are Encoded by a Single Gene**—The most striking difference between gp100 and Pmel17 cDNAs is the in-frame deletion of 21 bp in gp100 cDNA. Possibly, both cDNAs correspond to transcripts generated by alternative splicing of a single primary transcript. To test this hypothesis, we used PCR to analyze the genomic DNA corresponding to the part of the gp100 gene surrounding the putative alternative splice site. Comparison of the nucleotide sequence of this genomic DNA with the sequence of gp100-c1 cDNA revealed the presence of an intron (102 bp) just at the position of the 21-bp insertion in Pmel17 cDNA (Table I). The exon/intron boundaries nicely fit the consensus 5' donor and 3' acceptor splice site sequences (Padgett et al., 1986). In the genomic DNA, the sequence comprising the additional 21 bp in Pmel17 cDNA is located directly upstream of this 3' cleavage site and is preceded by an alternative 3' acceptor splice site (Table I). Whereas the gp100-specific 3' acceptor splice site fits the consensus sequence, the Pmel17-specific 3' acceptor splice site appears to be suboptimal, in that it lacks a pyrimidine-rich region (Table I). Suboptimal.
The deduced amino acid sequence contains a putative signal peptide (underlined). The first amino acid of mature gp100 is designated +1. Putative N-linked glycosylation sites are indicated by an asterisk, whereas the transmembrane region is underlined.

Table I
Genomic organization of part of the human gp100/Pmel17 gene

A and A' represent the introns that are removed in transcripts corresponding to gp100-cl cDNA and Pmel17 cDNA, respectively. Exon sequences are indicated in uppercase and intron sequences as lowercase letters. The best fit to the branch point sequence (Ruskin et al., 1984) is underlined.

<table>
<thead>
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<th>Intron</th>
<th>Size</th>
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<tr>
<td>A</td>
<td>102</td>
</tr>
<tr>
<td>A'</td>
<td>81</td>
</tr>
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</table>

Expression of gp100 and Pmel17 RNAs in Cells of the Melanocytic Lineage—The finding that gp100 and Pmel17 RNAs arise by alternative splicing of a single primary transcript raises the question of whether this occurs in a regulated manner. Previously, we showed that an RNA species of 2.5 kilobases is the major RNA product detected by gp100-cl cDNA on Northern blots of human melanoma cells. The poly(A) addition site is indicated in italics. The deduced amino acid sequence contains a putative signal peptide (underlined). The first amino acid of mature gp100 is designated +1. Putative N-linked glycosylation sites are indicated by an asterisk, whereas the transmembrane region is underlined.
DISCUSSION

Herein, we demonstrate that gp100-c1 cDNA encodes the melanocyte lineage-specific antigens recognized by mAbs NKI-beteb, HMB-50, and HMB-45, which are valuable diagnostic markers for melanoma. Expression of gp100-c1 cDNA in COS-7 cells demonstrates that NKI-beteb and HMB-50 recognize the same proteins of 100 and 10 kDa. Comparison of the 100-kDa protein in these cells and MEWO melanoma cells by V8 protease digestion demonstrates that they have the same protein backbone. The relationship between the 100- and 10-kDa proteins is not clear at present. The 10-kDa protein may be derived from the 100-kDa protein either by specific proteolytic processing or by degradation. Two findings lend support to the latter explanation. (a) The amount of 10-kDa proteins relative to the amount of 100-kDa proteins varies considerably between experiments, and (b) the 10-kDa protein can only be detected after a labeling period of 2 h while the 100-kDa protein is already present in the culture medium after 1 h (Esclamado et al., 1986; results not shown).

In addition to NKI-beteb and HMB-50, HMB-45 also reacts with gp100-transfected COS-7 cells. HMB-45 has been reported to immunoprecipitate proteins of 10 kDa from extracts and of 100 kDa from culture medium of melanoma cells, both of which comigrate with those detected by HMB-50 and NKI-beteb (Esclamado et al., 1986; Vennegoor et al., 1988). In addition, an additive enzyme immunoassay revealed an additivity index of 91% for the mixture of HMB-45 and NKI-beteb (Vennegoor et al., 1988). For our study, HMB-45 could not be obtained in sufficient amounts to directly analyze the proteins it detects in COS-7/pSVLgp100+ cells. However, the combined data of the authors mentioned above and those described herein indicate that the antigens recognized by HMB-45 are also encoded by gp100-c1 cDNA.

Proteins Homologous to gp100—A data base search revealed that gp100 is almost identical with the melanocyte-specific protein Pmel17. The cDNA encoding Pmel17 was isolated from a λ gt11 melanocyte cDNA library (Kwon et al., 1987; 1991). The most striking difference between gp100-c1 and Pmel17 cDNA consists of an in-frame deletion of 21 bp in gp100-c1 cDNA. Nucleotide sequence analysis of part of the gene encoding gp100 demonstrates that both cDNAs correspond to transcripts originating from a single gene via alternative splicing. A single 5' donor splice site is used in combination with two different, partially overlapping, 3' acceptor splice sites. No regulated expression of gp100 and Pmel17 mRNAs in cells of the melanocytic lineage has been observed; cells either expressed neither of the mRNAs or both. These data are consistent with previous results obtained by Kwon et al. (1987, 1991), indicating that the gene encoding Pmel17, and hence the gp100 gene, is a single-copy gene that maps to human chromosome 12 (region 12 pter-q21). Three other nucleotide differences have been detected between gp100 and Pmel17, two of which give rise to an amino acid substitution. They may represent allelic variations or polymorphisms, but we cannot exclude the possibility that they result from mutations.

In addition to Pmel17, gp100 was found to be 80% homologous to the putative protein (RPE1) product encoded by a partial bovine cDNA isolated from retinal pigment epithelium (RPE) (Kim and Wistow, 1992). Bovine RPE has been shown to react with HMB-50 (Kim and Wistow, 1992) and human RPE with HMB-45 (Kapur et al., 1992). Since the RPE1 protein is 80% homologous to gp100 and lacks the 7 amino acids present in Pmel17 (Table II), it may well represent the bovine homologue of gp100.

Another data base entry showing significant homology (42%) to gp100 is the melanosomal matrix glycoprotein MMP115 isolated from chicken RPE (Mochii et al., 1991). MMP115 localizes, as gp100, in melanosomal vesicles. No function has been reported for MMP115. The amino-terminal sequence (670 amino acids) of MMP115 is 42% homologous to the corresponding part in gp100. The carboxy-terminal part of MMP115 (84 amino acids) is only 13% homologous to gp100 and does not contain a transmembrane domain. Strikingly, the homology between gp100 and MMP115 decreases just at the site of the 7-amino acid insertion in Pmel17 (Table II). Perhaps MMP115 represents a soluble form of the chicken homologue of gp100 still to be discovered in man.

A data base search with the gp100 amino acid sequence or parts of this sequence did not reveal the presence of any known functional domain. The spacing of the cysteines in the cysteine-rich region that determines its tertiary fold is highly conserved between gp100/Pmel17, RPE1, and MMP115 (Table II) but is distinct from the ones found in other protein families, e.g. the integrin (Kishimoto et al., 1989) or nerve growth factor receptor families (Mallett et al., 1991). Therefore, the cysteine-rich domain present in the Pmel17/gp100 family may represent a novel structural or functional (interaction with other proteins) domain.
**Molecular Characterization of Melanocyte Markers**

### Table II

<table>
<thead>
<tr>
<th>Gp100</th>
<th>Pmel17</th>
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<tbody>
<tr>
<td>RPE1</td>
<td>MMP115</td>
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Alignment of the carboxyl-terminal part of members of the gp100/Pmel17 family

Identical amino acids (—) and gaps (•) are conserved. Conserved cysteine residues (#) are indicated as well.

### Melanocyte-specific Antigen gp100: Cell-biological and Oncological Significance

The synthesis of melanin in melanocytes is a multistep process regulated at different levels (reviewed by Hearing and Tsukamoto (1991)). Tyrosinase (EC 1.14.10.1) is the key enzyme in this pathway and catalyzes the initial steps in the cascade of reactions leading to the production of melanin from the amino acid tyrosine. Two other members of the tyrosinase family are TRP-1 or gp76 (Shibahara et al., 1986; Vijayasuradhi et al., 1990) and TRP-2 (Jackson et al., 1992), the latter of which contains 3,4-dihydroxyphenylalanine (DOPA)chrome tautomerase (EC 5.3.2.3) activity (Tsukamoto et al., 1992). The combined data on gp100 and Pmel17 imply that they are also related to this process; 1) both proteins are only expressed in pigmented cells (Kwon et al., 1987; Vennecoor et al., 1988), 2) gp100 is present in melanosomal vesicles (Vennecoor et al., 1988), and 3) an increase in the amount of transcripts derived from the gp100/Pmel17 gene correlates with increasing levels of melanization (Kwon et al., 1987). Preliminary data indicate that Pmel17 also reacts with the anti-gp100 mAbs and is localized in melanosomes. Cumulatively, these data support a role of both gp100 and Pmel17 in melanization, either as enzymes regulating the quality or quantity of melanin synthesis or as structural components of the melanosome. The possibility that these proteins play a role in malignant transformation is not likely, but it cannot be excluded.

Melanoma is a relatively immunogenic tumor, demonstrated by the presence of both CTL (reviewed by Knuth et al., 1992) and antibodies (Mattes et al., 1983) against melanoma cells in patients. The recent finding that some CTL clones react not only with melanoma cells but also with normal melanocytes raises the possibility that these proteins play a role in malignant transformation is not likely, but it cannot be excluded.

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