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AMINO-TERMINAL VARIATION IN MELANOMA ANTIGENS

John J. Marchalonis and Christian Schwabe
Department of Biochemistry,
Medical University of South Carolina, Charleston, SC 29425

Douglas M. Gersten
Department of Pathology, National Biomedical Research Foundation,
Georgetown University, Washington, DC 20007

Vincent J. Hearing
Department of Dermatology, National Cancer Institute,
Bethesda, MD 20205

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Melanoma tumors express both common antigenic determinants and individually specific markers. A melanoma-specific glycoprotein antigen (B700) with a molecular weight of approximately 65,000 daltons was detected on murine B16 melanoma cells but appears on other murine and human melanoma tumors. In order to determine the relationship between the B700 antigen and other melanoma antigens which have been described and to elucidate molecular changes that have taken place in the transformation from melanocyte to melanoma, we have purified the B700 glycoprotein to homogeneity. We have carried out amino acid composition analysis and partial sequence determinations and report that the B700 melanoma antigen shows similarities to serum albumin, but is not identical to this normal component. Moreover, amino-terminal variation occurs in the first 15 residues of the B700 antigen produced by separate B16 tumors.

Melanoma tumors express both common antigenic determinants and individually specific markers (1). We have reported the detection and isolation of a cross-reacting, melanoma-specific glycoprotein antigen (termed B700) that has a molecular weight of approximately 65,000 daltons (2-6) and which was originally detected on murine B16 melanoma cells but is also present on other murine melanoma tumors. Two other recently described melanoma-associated antigens are related to products normally expressed by other cell types, notably p97 which is related to transferrin (7-9), and Ia antigens (10-13). In order to aid in elucidating the molecular changes that have taken place in the transformation from melanocyte to melanoma, we have purified the B700 protein to homogeneity. We have now carried out amino acid composition analyses and partial sequence determinations in order to compare this marker with the two other melanoma antigens as well as with normal membrane or serum components. Our data support the conclusion that the B700 melanoma antigen shows similarities to serum albumin, but is not identical to this normal component, and, moreover, that amino-terminal variation occurs in the B700 antigen as it is produced by separate B16 tumors.

Materials and Methods

Isolation of B700 Melanoma Antigen: The B700 antigen was isolated from solid B16 tumors carried in C57Bl/6N mice by density gradient centrifugation, gel filtration chromatography, and by preparative gel electrophoresis, carried out under previously described

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conditions (2,5). It has been possible to isolate B700 proteins in quantities ranging from 100 µg to 1 mg from a starting material of 50 g Bl6 (wt). Identification of the material as B700 was confirmed by binding of either goat or rabbit antisera produced against the B700 marker (2,5). Purity of the antigens was established by polyacrylamide gel electrophoresis and isoelectric focusing (5).

Polyacrylamide gel electrophoresis and immunoblot transfer: Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (23) was carried out under reducing conditions. Immunoblot analysis was performed with separation by polyacrylamide gel electrophoresis in sodium dodecylsulfate containing buffers under reducing conditions (7.5% gel) and the separated proteins were transferred to nitrocellulose (21,22). The transferred proteins were incubated with either 5 µl of normal rabbit serum or 5 µl of rabbit antiserum to B700 and visualized by the immunoperoxidase reaction.

Amino acid composition and sequence analysis: The amino acid content of the purified B700 antigen was obtained using a Beckman 119CL amino acid analyzer equipped with a model 126 data integrator (2) with the protein being hydrolyzed under standard conditions using constant boiling HCl. The amino terminal sequence of individual B700 antigens and murine serum albumin were determined using a Beckman 890C automatic sequencer employing a 0.1 M quadrol program with polybrene (16, 17); PTH residues were identified by high performance liquid chromatography using a Dupont liquid chromatograph.

Results

Figure 1 presents a comparison by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate between purified B700 antigen and murine serum albumin. The

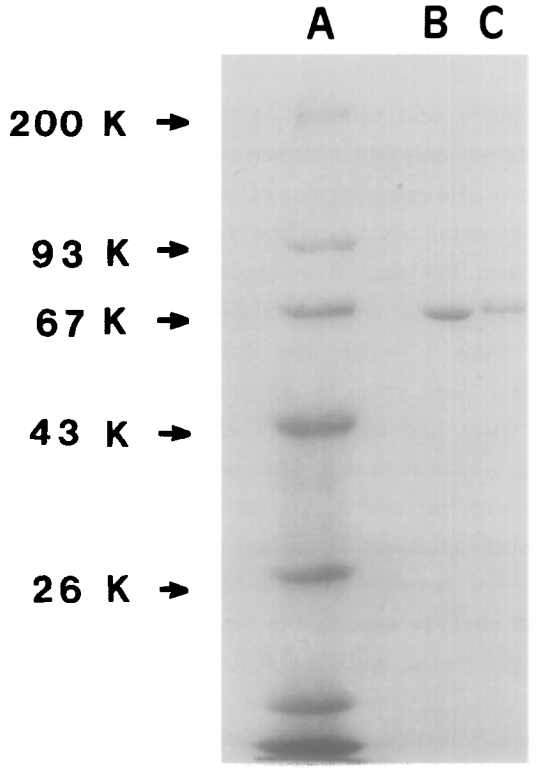

Figure 1. Resolution by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (23) under reducing conditions of molecular weight standards (Lane A), purified melanoma B700 antigen (Lane B) and purified murine serum albumin (Lane C). The load of B700 and albumin was 2 µg. The molecular weights of the standards in kilodaltons are indicated to the left of Lane A. 7.5% polyacrylamide slab gel.

TABLE 1. Amino Acid Composition of B700 and Comparison to Other Selected Proteins

RES	(MOLE %)	PROTEIN	SDQ
ASX	9.8	B700 Antigen	—
THR	6.3		
SER	5.5	Bovine Serum Albumin	22
GLX	13.7	Murine Serum Albumin	22
Pro	5.4	Rat Serum Albumin	37
GLY	5.1	Human Serum Albumin	37
ALA	8.9	Complement C-4	47
CYS	1.5		
VAL	5.5	αfeto-protein (murine)	51
MET	1.5	HLA-DR(α)	56
ILE	3.5	Human TLV p24	63
LEU	9.3	Actin	70
TYR	3.1	HLA-DR(β)	76
PHE	4.1	Human μ Chain (IgM)	82
HIS	2.5	Fetuin	99
LYS	7.7		
ARG	3.3	Random Composition	494
TRP	2.7		

> 200 PROTEINS WERE ANALYZED; CLOSEST MATCHES REPORTED.

SDQ was calculated as described by Marchalonis and Weltman (14).

B700 antigen is free of significant contamination by this technique, and it is also apparent that the mobility of the B700 antigen on the gel is slightly greater than that of the murine serum albumin.

Comparison of the amino acid composition of B700 with other proteins was by the statistical method of Marchalonis and Weltman (14,15). The residue content of B700 was compared with the amino acid compositions of more than 200 other proteins in the data bank. The SDQ values reported are a quantitative measure of the comparison among the proteins; values less than 100 suggest relatedness (less than 2% likelihood that the observed relationship arose by chance), but values less than 50 invariably indicate closely related proteins. In Table 1 (which lists only the closest matches), the strongest indications of relatedness were given by comparison with the albumins. Although the tumors used in this study had never been exposed to bovine serum albumin, the compositional similarity is closest to this molecule and to mouse serum albumin. The SDQ values of 37 obtained with rat and human serum albumin also indicate a relatedness. While a relationship to albumins was suggested by this comparison, it is clear that the compositions are different because the observed comparison values are considerably higher than those which would be expected in the case of identity (less than 5 SDQ units) or close homology, e.g. 14 SDQ units for the comparison between human and bovine albumins.

Table 2 shows the N-terminal peptide structure of our initial B700 preparation which has thus far been sequenced for the first 15 residues. A comparison of this sequence with those of other proteins by computer analysis using the Protein Sequence Database computer bank of Dayhoff et al (18), again indicated significant homologies to the albumins. This table also shows that there is no homology between the B700 antigen and

TABLE 2. Amino Terminal Sequence of B700 and Its Homology to Other Selected Proteins

PROTEIN	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	Identities	
B700 Antigen[a]	G T F I L E I A H S F K D R G	15/15	
Bovine Serum Albumin	D T H K S E I A H R F K D L G	9/15	
Human Serum Albumin	D A H K S E V A H R F K D L G	7/15	
Murine Serum Albumin[a]	E A H K S E I A H R Y N D L G	6/15	
H-2K[b]	- P H S L R Y F V T A V S R P	2/15	
αfetoprotein	A L H E N E F G I A S T L D S	1/15	
HLA-DR(β)	R D S P E D F V Y Q F K G M C	2/15	
Glycoprotein 70	A A P G S S P H E V Y N I T W	0/15	
p97 Melanoma Antigen	- - G M E V R W C A T S D X E	1/12	10/10
Transferrin	V P D K T V R W C A T S D X E	1/12	7/10
Lactotransferrin	G R R R R S V Q W C A V S Q P E	0/12	6/10

[a] N-terminal sequence determined using the Beckman 890C automatic sequencer and identification of PTH residues was made by high performance liquid chromatography (16,17). p97 melanoma antigen, transferrin and lactotransferrin data are from references 7-9. Other sequence data were taken from the Protein Sequence Database computer bank of Dayhoff et al. (18). Recovery of N-terminal residue was 70-75% of initial sample mass loaded. Repetitive yields averaged 92%.

the p97 melanoma antigen (9), nor is there homology to common surface components such as major histocompatibility complex antigens, α-fetoprotein, IA antigen or viroglycoprotein. Discounting the albumins, no other proteins in the database showed significant homologous primary structure to B700. The degree of partial homology with the conserved regions of albumin is striking, as are the differences at several points in those regions. An analagous situation has recently been reported for p97, which shows significant homology with transferrins (9), and the data illustrating that are given at the bottom of Table 2.

We carried out N-terminal sequence analysis on three separate preparations of B700 which had been obtained at three different times from B16 solid tumor preparations in order to assess the possibility of individual variation within this defined tumor marker. Comparison of the N-terminal sequences of the three distinct B700 preparations with those of mouse serum albumin is given in Table 3. All three sequences are clearly homologous to one another and to serum albumin, yet each sequence differs from the others to some degree. For example, sequences 1 and 2 are identical at 67% of their positions, sequences 2 and 3 are identical at 50% of their positions, but sequences 1 and 3 are identical only at 20% of residues; yet they all show greater than 40% homology to mouse serum albumin. The sequence Glu-Ile-Ala (EIA) at positions 6 through 8 is conserved in all four molecules. Others such as positions 3, 4, 5, 9, 10, 13 and 14 are shared by three of the four molecules. In addition to the individual substituents, B700 (# 3) shows a length variation; in order to put its sequence into register with that of the other molecules it is necessary to either shift the N-terminal GLU (E) to position 2 or to introduce a gap at position 3 with the N-terminal residues being E and Alanine (A). All three of the B700 sequences are clearly homologous, but not identical, to that of albumin. The degree of variability observed is reminiscent of that observed in the two other

TABLE 3. N-Terminal Variation in B700 Melanoma Antigen

	Residue Position														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
B700(1)	G	T	H	I	L	E	I	A	H	S	F	K	D	R	G
B700(2)	D	T	H	K	S	E	I	A	H	R	F	K	D	L	G
B700(3)*	E	A	-	K	S	E	I	A	A	R	Y	N	P	L	-
MSA	E	A	H	K	S	E	I	A	H	R	Y	N	D	L	G

	Number of Identities		
	1	2	3
1			
2	10/15		
3	3/14	7/14	
MSA	7/15	11/15	11/14

* A gap was introduced in B700(3) to allow N-terminal alignment with mouse serum albumin. N-terminal sequence of all proteins given here was obtained using a Beckman 890C automatic sequencer employing a 0.1 M quadrol program with polybrene and identifying PTH residues by high performance liquid chromatography (16,17).

systems which have been documented to show antigen variation and N-terminal sequence variability; the immunoglobulins (19) and the variant surface glycoproteins of trypanosomes (20).

Despite the similarities between B700 and albumins in primary sequence and in amino acid composition, we have not found evidence for serological relationships between albumin and the tumor marker. This is illustrated by immunoblot transfer (21,22) as shown in Figure 2 which employs a potent polyclonal rabbit antiserum to the isolated B700 antigen. This antiserum was reacted against different protein fractions which had been separated by gel electrophoresis and stained with a conjugated immunoperoxidase to illustrate specific binding of antibodies. The normal rabbit serum control showed no specific binding. The rabbit antiserum to the B700 antigen, by contrast, showed specific staining of the B700 antigen in the total B16 cell homogenate, of B700 in the partially purified fraction, and of the purified B700 antigen. The specific antiserum reacted with neither of the tyrosinase isozymes tested which are melanocyte specific proteins, nor with purified bovine serum albumin.

Discussion

Melanoma cells synthesize and express tumor-associated glycoproteins that are similar to proteins normally synthesized by other types of cells, but not usually expressed by melanocytes. Taking our data in the context of other studies, this has now been shown to occur for the B700, p97 (7-9) and IA antigens (10-13). Depending upon their degree of homology with normal components, these glycoproteins might appear to be cross-reactive with the normal cellular products, or they might seem to be immunologically unrelated as in the case of B700. If it were not for the amino acid composition and sequence data obtained for B700, we would not have suspected any homology with albumin, since we have not found cross-reactivity between B700 and albumins using both conventional and monoclonal antisera. We emphasize that the antisera used in the identification of the B16

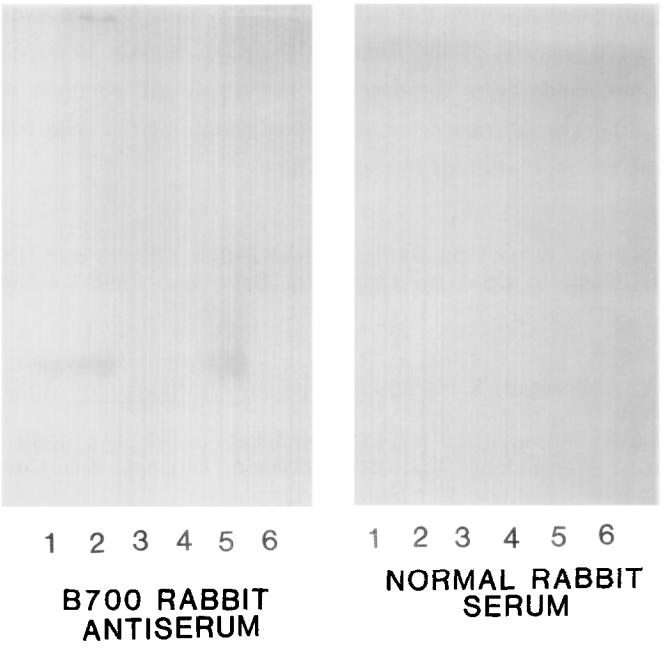

Figure 2. Immunoblot analysis of specificity of rabbit antiserum to murine B700 melanoma antigen. Proteins were separated by polyacrylamide gel electrophoresis in sodium docylsulfate containing buffers (23) under reducing conditions (7.5% gel) and transferred to nitrocellulose (21,22). The transferred proteins were then incubated with either 5 µl of normal rabbit serum or 5 µl rabbit antiserum to B700 and visualized by an immunoperoxidase reaction. Lane 1, B-16 cell homogenate (30 µg). Lane 2, Sephacryl S300 purified B700 (20 µg). Lane 3, purified T4 tyrosinase (2 µg). Lane 4, purified T1 tyrosinase (2 µg). Lane 5, purified B700 (2 µg). Lane 6, bovine serum albumin (2 µg).

antigen recognized a "common determinant" which is shared by antigens on six different melanoma lines and has also been shown to occur on human melanomas. These antisera do not react with non-melanoma tumors (2-6). Furthermore, by virtue of glycosylation and of metabolic labeling studies, we have determined the unique nature of B700 (2,5).

The mechanism of formation of these tumor-associated antigens by malignant melanocytes is as yet undefined. They probably represent the activation of genes normally dormant in melanocytes, but the gene products clearly are distinct from those of the normal homologous genes. Our data show that the primary structure of B700 proteins is both abnormally specified with respect to albumin sequence and perhaps more importantly, variable from tumor to tumor. The primary sequence and serological data thus far obtained regarding the B700 antigen suggest the existence of both common and variable sequences within the melanoma-specific antigen. The variability so far has been demonstrated at the N-terminal region, and the extent of variation in the rest of the molecule remains to be determined. We feel that at this time our data support the hypothesis that a high degree of gene duplication has taken place in the B16 genome. Further, it appears that during the course of serial transplantation of the tumor cell line (over a 12 month period), we have selected for several subclones of the heterogeneous tumor cell population, each with different activated B700 genes. Which environmental

pressures have significance towards this selection in the host, and the overall importance of these factors in the survival of the tumor in the host, remain to be elucidated. Our results suggest a molecular basis for observed individual and common determinants of tumor cells, it should prove interesting to study this system at the gene level to elucidate the mechanisms and the regulation of this variability.

Acknowledgments

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