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Identification of a Human Homologue of the Murine Tumor Rejection Antigen GP96[1]

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ABSTRACT

A family of cell surface glycoproteins with a molecular weight of 96,000 (gp96) has recently been implicated in the individually distinct immunogenicity of chemically induced sarcomas of inbred mice. Rabbit antiserum to murine gp96 detects an antigenically related M_r 96,000 cell surface glycoprotein on two cultured human melanoma cell lines, SK-MEL-13 and SK-MEL-177. Molecular probes for 5′ and 3′ ends of the murine gp96 gene detect a 3.5-kilobase transcript in RNA preparations from melanoma cells, similar to the murine gp96 transcript. While 5′ probes do not hybridize to Southern blots of genomic human DNA, the 3′ probes identify several distinct bands under stringent hybridization and washing conditions. This suggests that the 3′ end of the gp96 gene is more conserved than its 5′ end. No gross alterations in gp96 gene organization were detected in melanoma cells. B-lymphoblastoid cell lines derived from four different individuals also showed no restriction fragment polymorphism in the gp96 gene.

INTRODUCTION

Identification of tumor cell surface antigens recognized by autologous or syngeneic host remains the central issue of tumor immunology. In the study of human cancer, evidence for humoral (1) and cellular (2) immune recognition with specificity for human cancer cells continues to accumulate and a number of the antigens detected serologically have been characterized as gangliosides (3–5) and glycoproteins (5–8). In the case of experimental tumors, the clearest evidence for tumor-specific immunity comes from the demonstration of tumor rejection antigens that elicit transplantation immunity in syngeneic animals (9, 10). Recent studies have implicated M_r 96,000 glycoproteins as the tumor rejection antigens of antigenically distinct chemically induced sarcomas of inbred mice (11). The present communication describes the identification of a human cell surface antigen which shares homology with the murine tumor rejection antigen gp96, in terms of its serologically detected determinants and its molecular features.

MATERIALS AND METHODS

Cell Lines. Human melanoma cell lines SK-MEL-13 and -177 have been described (5).

Erythrocyte Rosetting. Detection of binding of antibodies to melanoma cell lines was carried out as described (5).

Preparation of Protein Extracts. Cells were scraped from the surface and washed three times with phosphate buffered saline. A cell pellet of 0.2 ml was suspended in 1 ml phosphate buffered saline containing 0.1 mM phenylmethyl sulfonylfluoride and the suspension was sonicated three times for 10 s each time while on ice. The cell lysate was centrifuged at 1000 × *g* for 10 min to remove whole cells and nuclei. The supernatant was collected and was solubilized by addition of 0.1 volumes of 2% sodium deoxycholate. The suspension was kept on ice for 20 min with occasional shaking and was centrifuged at 15,000 × *g* for 15 min. The supernatant was collected and frozen at −70°C in several aliquots.

Generation of Rabbit Serum to Murine gp96. Murine gp96 antigen was obtained from BALB/c Meth A sarcoma as described (11). Rabbit antisera were generated by repeated weekly injections, 20 μg each, of purified gp96. The antigen preparation was emulsified with Freund's complete adjuvant for the first two injections and with Freund's incomplete adjuvant for the next four injections. Subsequent antigen injections were in phosphate buffered saline. Two rabbits were immunized with this procedure and sera from both rabbits tested positive with the murine as well as human gp96.

Western Blotting. Protein extracts were applied to sodium dodecyl sulfate-polyacrylamide gels and were blotted to nitrocellulose and probed with a rabbit serum as described (12).

Southern and Northern Blotting. Genomic DNA and RNA were extracted, blotted to nitrocellulose, and probed as described (13).

RESULTS AND DISCUSSION

Detection of a Murine gp96-related Antigen on Human Melanoma. Generation of a rabbit antiserum to purified murine gp96 has been described in "Materials and Methods." In erythrocyte rosetting assays for detection of cell surface antigens, this serum reacted (titer, 1:128) with two human melanoma cell lines SK-MEL-13 and -177; no reactivity was seen with nonimmune rabbit serum. A surface-iodinated extract of SK-MEL-13 was immunoprecipitated with this serum and the precipitate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A single band of M_r 96,000 was observed (Fig. 1). Protein extracts from the two cell lines were also analyzed by Western blotting. Partially purified murine gp96 prepared from the BALB/c sarcoma Meth A was also applied to the gels for comparison. The Western blots were probed with rabbit anti-gp96 antiserum and the nonimmune rabbit serum. The results show (Fig. 1) that rabbit anti-gp96 antiserum detects a molecule with 96,000 molecular weight in both melanoma cell lines. The murine and human melanoma gp96 molecules appear indistinguishable in size.

Northern Blot Analysis. Total RNA preparations from the two melanoma cell lines were probed with the murine gp96 probe pMA2 which defines the 5′ end of the gp96 gene (14) and pV3, which defines the 3′ end of the gene.[3] In both melanomas, a 3.5-kilobase transcript, identical to the murine gp96 transcript, was detected with pMA2 and pV3 (Fig. 2).

Southern Blot Analysis. High molecular weight DNA from the cell line SK-MEL-13 was digested with restriction endonucleases *Bam*HI, *Eco*RI, or *Pst*I. Southern blots of these samples were probed with the 5′ probe pMA2: under high and low stringency conditions, no reproducibly hybridizing bands were observed. However, when the same blots were probed with pV3, a cDNA fragment which defines the relatively 3′ end of the murine gp96 gene, several bands were found to hybridize even under conditions of high stringency (Fig. 3). This observation suggests that the 5′ region of the gp96 gene is less

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[3] P. K. Srivastava, C. F. Cahill, and L. J. Old, manuscript in preparation.

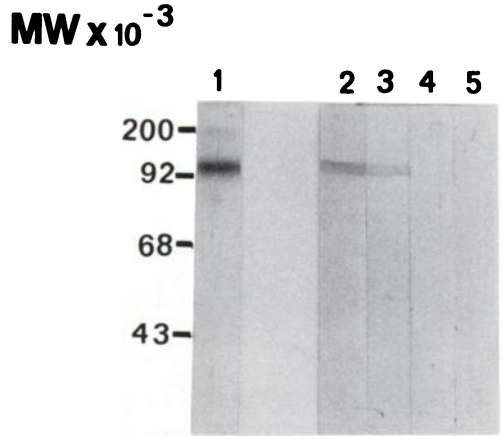

Fig. 1. Immunochemical identification of human gp96. *Lane 1*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immunoprecipitate of ^{125}I-surface-labeled SK-MEL-13 cells with rabbit anti-murine gp96 antiserum. *Lanes 2* and *3*, Western blots of extracts of BALB/c tumor Meth A (*Lane 2*) and SK-MEL-13 cells (*Lanes 3*) probed with rabbit anti-murine gp96. *Lanes 4* and *5*, Western blots of the same extracts probed with nonimmune rabbit serum.

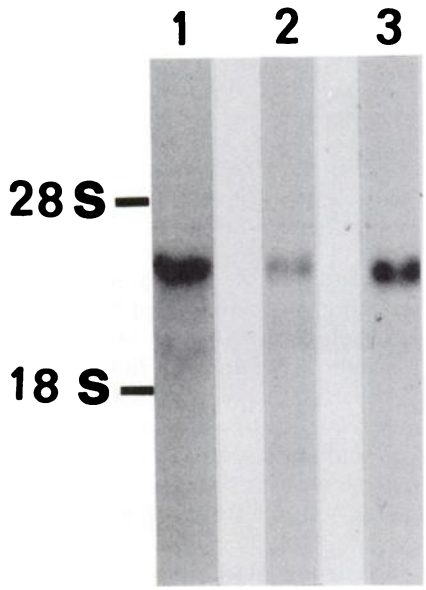

Fig. 2. Northern blot hybridization of ^{32}P-labeled pMA2 with poly(A) + RNA from BALB/c sarcoma Meth A (*Lane 1*) and total RNA from SK-MEL-13 (*Lane 2*) and SK-MEL-177 (*Lane 3*).

conserved than the relatively 3′ region. Also, the discordance between the Northern and Southern blot analyses with the 5′ probe pMA2 is perhaps due to the differential stability of RNA-DNA and DNA-DNA hybrids. Detection of several distinct bands with pV3 suggests that there may be several gp96 copies in the human genome; this suggestion is strengthened by the large size of the hybridizing DNA fragments and the fact that same number of bands are observed in DNA digested with different restriction enzymes. However, the possibility that some bands are the result of cross-hybridization with genes related to but not identical with gp96 needs to be ruled out. The question of the number of gp96 encoding genes in human genome requires further probing of genomic Southern blots with more defined probes for the human gp96 gene.

In order to investigate the possibility of allelism in the human gp96, genomic DNA from B-lymphoblastoid cell lines NANN, Boleth, KT12, and Poco, derived from four different individuals was digested with *Bam*HI, *Eco*RI, or *Pst*I and probed with the pV3 probe. No differences were observed nor was there any difference between the pattern of bands in lymphoblastoid cell

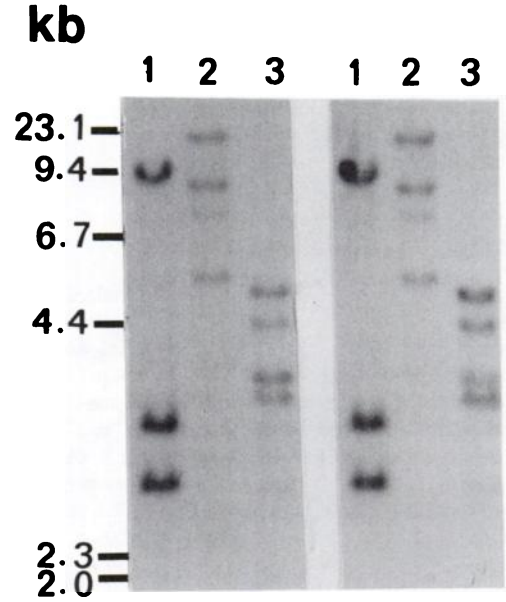

Fig. 3. Southern blot hybridization of ^{32}P-labeled pMA2 with genomic DNA from SK-MEL-13 (*left*) and B-lymphoblastoid cell line NANN (*right*) derived from peripheral blood lymphocytes of a healthy individual. Southern blots of genomic DNA from SK-MEL-177 and Boleth, KT12, and Poco cell lines showed an identical pattern. DNA (15 μg/lane) was digested with *Bam*HI (*Lane 1*), *Eco*RI (*Lane 2*) and *Pst*I (*Lane 3*).

lines and melanoma cells SK-MEL-13 and -177 (Fig. 3). Thus, there is no evidence for tumor-related rearrangement nor for individual-related structural variation in the gp96 gene.

Gp96 is also present in several other animal species[4] and may perform an essential function conserved across species. In this regard, the observed homology between murine gp96, the yeast heatshock protein hsp90, hamster glucose-regulated protein grp94, and the endoplasmic reticular protein ERp99 (15, 16) may be of significance.

Tumor transplantation studies in mice demonstrate that gp96 preparations elicit immunity specific to the tumor from which they are derived. This indicates that gp96 molecules have the potential for variability and hence, immunogenicity in the host of origin. Analysis of gp96 polymorphism in normal and malignant cells and its potential use as an immunogen in human cancer immunotherapy need to be explored.

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