Marek's Disease Herpesviruses

IV. Molecular Characterization of Marek's Disease Herpesvirus A Antigen†

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Received 3 May 1982/Accepted 29 November 1982

Marek's disease herpesvirus A antigen (MDHV-A) was identified as a 61,000- to 65,000-dalton glycoprotein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after immunoprecipitation from the culture medium of both [³⁵S]methionine- and [¹⁴C]glucosamine-labeled infected cells by specific rabbit serum directed against MDHV-A. Rigorous identification was accomplished by selective blocking of this specific immunoprecipitation of the glycoprotein with purified MDHV-A that was isolated at its characteristic isoelectric point. These results identify and characterize MDHV-A in terms of the previously determined physical and chemical properties of the antigen. A molecule of similar size was immunoprecipitated from the culture medium of cells infected with herpesvirus of turkeys, extending previous observations about the identity of a potentially important common antigen shared by MDHV and the nonpathogenic vaccine virus, herpesvirus of turkeys.

Two antigens, A and B, are detected most prominently and consistently in immunodiffusion analysis of extracts of cells infected with Marek's disease herpesvirus (MDHV) and of concentrated culture medium from infected cells with sera from naturally infected chickens (3, 4). The A antigen (MDHV-A) is found primarily in the culture medium and also in the extracts of infected cells; the B antigen (MDHV-B) is found primarily in the extracts of infected cells. These two antigens continue to be of considerable interest for a number of virological and immunological reasons, as summarized previously (14, 15, 27).

Among the more important reasons for this interest are that both antigens are glycoproteins (14, 25, 27) and are common to MDHV and the vaccine virus, herpesvirus of turkeys (HVT) (20, 23, 24, 27, 30). The latter point becomes especially intriguing in view of the observations that the two viruses share only about 1 to 4% genetic homology (12), but that HVT can still effectively protect chickens against Marek's disease (MD). Clearly, an important objective of further studies in the MD system will be to obtain a better understanding of the structure and expression of the genomes of both viruses. One obvious starting point is a molecular analysis of the common

antigens MDHV-A and MDHV-B since they must result from expression of both genomes.

Other reasons for interest in these two glycoprotein antigens are their possible relationships to membrane phenomena and to protection, which in turn may be interrelated. The former include virion envelope antigens and membrane antigen, which is detected on the surface of MDHV-infected cells by immunofluorescence and is also a common antigen between MDHV and HVT (2, 19). Although the likely role of cell-mediated immunity against lymphoid tumor cell surface antigens (18, 27) cannot be ignored, there are reports of successful immunization against MD with purified plasma membranes (7, 8), noninfectious nonionic detergent-solubilized and -insoluble material (13), and solubilized glycoproteins from membrane-rich fractions (31) from MDHV- or HVT-infected fibroblast cells or both. Other studies suggest the following two-step mechanism of resistance in vaccinated birds (21, 22): humoral and cell-mediated antiviral responses first reduce the replication and spread of MDHV, and a cell-mediated response is then directed against tumor cells. Consistent with this two-step model are the more recent observations that immunization with viral antigens inhibits both replication of challenge virus and tumor formation, whereas immunization with tumor antigens inhibits only tumor formation (17). However, in none of the above studies was

† Journal article no. 10306 from the Michigan Agricultural Experiment Station.

the protective antigen(s) defined or characterized (7, 8, 13, 17, 21, 22, 31).

In this laboratory a long-term study was undertaken to produce sufficient quantities of both MDHV-A and -B for their immunological, physical, and chemical characterization, using immunodiffusion as a means of identification (14, 15, 27). The fact that the two antigens are both glycoproteins and share many other properties, and the observation that their sizes, although different, are sufficiently similar for the methods used up to that time, served to explain the confusion that previously existed in the literature regarding "common" antigens (27). On the other hand, a key difference is their separation by more than 2 pH units by isoelectric focusing (15, 27), which made it possible to purify each antigen free of the other and then to use the highly purified antigen to prepare rabbit antisera reactive with one but not with the other antigen (15, 27). Current studies are designed to extend the earlier work by using the two purified antigens and their respective antisera, rabbit anti-A (R α A) and rabbit anti-B, for further immunological as well as molecular characterization of MDHV-A and -B by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this paper, we report the rigorous immunological identification and molecular characterization of MDHV-A as a 61,000- to 65,000-dalton glycopeptide by specific immunoprecipitation of that molecule with R α A and also by blocking that immunoprecipitation with highly purified MDHV-A.

(This paper was presented in part at the Cold Spring Harbor Workshop on Herpesviruses, 26–31 August 1980, Cold Spring Harbor, N.Y.; the International Workshop on Herpesviruses, 27–31 July 1981, Bologna, Italy; and the Fifth International Congress of Virology, 2–7 August 1981, Strasbourg, France.)

Primary duck embryo fibroblast cells were prepared and propagated according to the procedures established by Long et al. (15), with minor modification to facilitate optimal antigen synthesis in small-scale cultures. Cells were seeded in 100-mm-diameter plastic tissue culture dishes (10^7 cells per plate) with 10 ml of standard growth medium per plate. MDHV strain GA-infected cells in passage 25 (15) and HVT strain FC-126-infected cells in passage 16 (27) were used to generate stock supplies of MDHV- and HVT-infected duck embryo fibroblast cells in passages 26 and 17, respectively. Virus-infected cells frozen in liquid nitrogen were used to infect 85% confluent duck embryo fibroblast monolayers at a 1:8 dilution so that cytopathic effect was barely noticeable at 24 h postinfection and reached a maximum at approximately 48 to 72 h postinfection. Infected-cell monolayers were

maintained in 10 ml of growth medium without calf serum.

Infected cells were labeled starting at 48 h postinfection, when more than 50% of the cells demonstrated cytopathic effect. Uninfected cells of the same age in culture were handled in the same manner as infected cells. Labeling of protein with [35 S]methionine in 100-mm dishes was accomplished by washing cell monolayers three times with warm Hanks balanced salt solution (GIBCO Laboratories) and incubating for 24 h in 5 ml of Dulbecco minimal essential medium (GIBCO) containing 1/20 of the normal concentration of unlabeled methionine and [35 S]methionine (New England Nuclear Corp.) at 50 μ Ci/ml. Glycoproteins were labeled by incubating cell monolayers for 24 h in 5 ml of growth medium containing 1% calf serum and [14 C]glucosamine (New England Nuclear) at 1 μ Ci/ml.

Culture medium from infected and uninfected cells was collected, clarified at 3,000 rpm (International PR6) for 10 min, and then ultracentrifuged at 30,000 rpm for 1 h in a type 30 rotor (Beckman Instruments, Inc.). The clarified medium was stored at -20°C (15) until used for immunoprecipitation. For the determination of radioactive isotope incorporation in culture medium, 100 μ l of [14 C]glucosamine-labeled material or 10 μ l of [35 S]methionine-labeled material was assayed for trichloroacetic acid-precipitable radioactivity by standard methods (5). Protein concentrations were determined by the method established by Lowry et al. (16) with crystalline bovine serum albumin as the standard.

For immunoprecipitation analysis, clarified infected culture medium was used as a source of MDHV-A. Immunoprecipitations were performed according to the methods of Witte and Wirth (29), with antigen-antibody complexes collected by the Cowan I strain of *Staphylococcus aureus* (10). R α A, negative for MDHV-B by immunodiffusion, was prepared by inoculating rabbits with highly purified MDHV-A (15). As a control, normal rabbit serum (NRS) was also used in immunoprecipitation analyses. Optimal immunoprecipitation was obtained with culture medium containing specific activities of 10^3 to 10^4 [35 S]methionine cpm/ μ g of protein and 10^3 to 10^5 [14 C]glucosamine cpm/ μ g of protein. Generally, 0.1 ml of culture medium labeled to these specific activities contained sufficient total counts per minute for rapid visualization of MDHV-A with small amounts of antisera. (The counts per minute used in each experiment are given in the figure legends.) Before incubation with immune serum, the antigen-containing material was clarified with NRS and *S. aureus* to remove labeled molecules that nonspecifically adsorb to either immunoglobulin G or the *S. aureus* immunoabsorbant.

To block the immunoprecipitation of MDHV-A, 40 μ l of immune serum was incubated with 1 U (14, 15) of MDHV-A for 24 h at 0°C. Immunoprecipitated material was removed by centrifugation at 30,000 rpm for 1 h in a type 50.1 rotor at 4°C. The MDHV-A preparation was partially purified by isoelectric focusing as described previously (15).

Discontinuous stack SDS-PAGE was done according to the methods of Laemmli (11) in a vertical slab gel apparatus (Hoeffer Scientific Instruments; model SE-520). Washed *S. aureus* pellets containing immunoprecipitates were prepared for electrophoresis as described by Witte and Wirth (29). Electrophoresis was carried out at a constant voltage of 150 V (ISCO Electrophoresis Power Supply model 1493) until the marker dye ran off the gel, usually after 1.5 to 2 h. Detection of labeled proteins and glycoproteins was by fluorography as described by Bonner and Laskey (1). Standard molecular weight markers included 14 C-labeled phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (22,000) (New England Nuclear). Molecular weight estimations were calculated by interpolation between standard proteins, according to the method of Weber and Osborn (28).

The preliminary observation (data not shown) that a 61,000- to 65,000-dalton molecule was the major [35 S]methionine- and [14 C]glucosamine-labeled species shed into the culture medium of infected, but not uninfected, cells suggested that the molecule might be MDHV-A. However, to rigorously confirm the identity of this molecule as MDHV-A, specific immunological analysis was required; an analysis was made possible by R α A, a rabbit antiserum highly specific for MDHV-A (15). A [35 S]methionine-labeled polypeptide with a molecular weight range of 61,000 to 65,000 was immunoprecipitated from culture medium with R α A (Fig. 1A). This polypeptide was not detected by SDS-PAGE analysis of immunoprecipitates of infected cell culture medium with NRS, even with an intentional overexposure of the gel (Fig. 1B). The virus-specific nature of this polypeptide was confirmed by its complete absence in culture medium from uninfected cells, even upon intentional overexposure (unpublished data).

To unequivocally confirm the identity of the 61,000- to 65,000-dalton polypeptide as MDHV-A, blocking experiments were performed by preincubating R α A with MDHV-A that was purified free of MDHV-B through isoelectric focusing (15). Blocking immune serum with the MDHV-A preparation selectively and completely eliminated the 61,000- to 65,000-dalton polypeptide (Fig. 1C). The narrow band remaining at about 66,000 daltons after blocking is a slightly

larger, unrelated polypeptide that is occasionally resolved from the broader MDHV-A band and is present in reduced amounts in culture medium from uninfected cells (Fig. 1A).

MDHV-A has previously been characterized as a glycoprotein by [14 C]glucosamine labeling and immunodiffusion analysis (14, 15). Therefore, if the 61,000- to 65,000-dalton polypeptide is the glycosylated form of MDHV-A, rather than an unglycosylated precursor polypeptide, it should also be labeled with the same sugar. The preliminary observation that a 61,000- to 65,000-dalton molecule contained glucosamine (data not shown) strongly suggested that the molecule is MDHV-A. However, to unequivocally confirm the identity of the 61,000- to 65,000-dalton polypeptide as glycosylated MDHV-A, culture medium from [14 C]glucosamine-labeled cells was examined by immunoprecipitation analysis. A virus-specific molecule of 61,000 to 65,000 molecular weight was immunoprecipitated with R α A (Fig. 2) but was not visible in control precipitates obtained with NRS. In addition, the immunoprecipitation of this glucosamine-labeled molecule with R α A could be blocked by purified MDHV-A (Fig. 2).

Immunodiffusion studies have demonstrated that HVT and MDHV infections result in the appearance of antigenically related A antigens (20, 23, 24, 27, 30). When culture medium from [35 S]methionine-labeled cells infected with HVT was examined by immunoprecipitation analysis with R α A, a virus-induced polypeptide was observed with a molecular weight similar to that of the previously identified MDHV-A antigen (Fig. 3). This polypeptide was not visible in immunoprecipitations performed with NRS.

The glycoprotein A and B antigens have previously been defined only by immunodiffusion (4, 14, 15, 25), and all previous attempts at biochemical and biophysical characterization (14, 15, 24, 25, 27) were based on this criterion. In this study, the antigen was identified by an entirely different and more sensitive approach combining radioimmunoprecipitation and SDS-PAGE. The results of specific immunoprecipitations with two MDHV-A-reactive sera and blocking studies with highly purified MDHV-A show that MDHV-A is a glycoprotein with an apparent molecular weight of 61,000 to 65,000 on SDS-PAGE. Results similar to those obtained with R α A (Fig. 1 and 2) were also obtained with MDHV-A-reactive immune chicken serum collected from birds naturally infected with MDHV (C. Glaubiger, unpublished data). The identification of MDHV-A with R α A is very important because this serum was prepared with highly purified antigen obtained from isoelectric focusing. Thus, its successful use provides a more rigorous means of identification than immune

FIG. 1. Immunoprecipitation analysis of culture medium from [^{35}S]methionine-labeled MDHV-infected (INF) and uninfected (CON) cells with R α A. (A) Culture medium (100 μl) from INF and CON cells containing 4.8×10^5 and 6.6×10^5 cpm, respectively, was reacted with R α A. Immunoprecipitates were analyzed by SDS-PAGE (8.5%; 48-h fluorographic exposure). (B) Culture medium (100 μl) from INF and CON cells containing 3.0×10^5 and 4.7×10^5 cpm, respectively, was reacted with R α A NRS, and immunoprecipitates were analyzed by SDS-PAGE (7.5%; 7-day fluorographic exposure). (C) Culture medium (100 μl) from INF cells was reacted with R α A blocked with MDHV-A, and the immunoprecipitates were analyzed by SDS-PAGE (7.5%; 48-h fluorographic exposure).

chicken serum, which has other reactivities including anti-MDHV-B activity (14, 27). Furthermore, the immunoprecipitation of this polypeptide with both sera could be selectively blocked by incubating the two antisera with purified MDHV-A before immunoprecipitation. This blocking was observed with both [^{35}S]methionine- and [^{14}C]glucosamine-labeled material and with all immune sera used (Fig. 1 and 2; C. Glaubiger, unpublished data) and contributes further to the rigorous identification of MDHV-A. The specificity of this blocking is best shown with the [^{35}S]methionine-labeled polypeptide, since only the MDHV-A band is eliminated whereas nonspecifically bound normal cell polypeptides remain (Fig. 1C). In summary, the use of R α A and purified MDHV-A for immunoprecipitation and blocking, respectively, not only assures more rigorous identification, but also relates the current work to previous physical and chemical analyses of the antigen (14, 15).

Van Zaane and Gielkens (34) reported the detection of a virus-specific glycoprotein with an apparent molecular weight of 60,000 to 65,000 on SDS-PAGE that was secreted into the culture medium of MDHV-infected duck embryo fibroblast cells, but they recognized that their data

did not allow them to reach a firm conclusion about its identity or function. Ikuta et al. (6) also reported the immunoprecipitation of a heterogeneous, 64,000-dalton virus-specific glycoprotein among six polypeptides selectively released into the culture medium. In both cases the glycoprotein detected was immunoprecipitated with complex sera obtained from MDHV-immunized birds by a double antibody procedure, but no further attempt was made to rigorously confirm its identity as MDHV-A. Whereas both studies (6, 34) provide preliminary data suggesting that the similar-sized glycoprotein might be MDHV-A, largely because of its appearance in the culture medium of infected cells, neither identification is conclusive. In contrast, the more rigorous identification of MDHV-A as a glycoprotein of 61,000 to 65,000 molecular weight presented here was made possible due to the availability of the two key reagents discussed above, as a result of earlier studies in this laboratory (14, 15), and should prove useful by confirming the identity of MDHV-A in the one-dimensional analysis performed by both Ikuta et al. (6) and Van Zaane and Gielkens (34). Additionally, these data should facilitate the identification of MDHV-A in the two-dimensional analyses of

FIG. 2. Immunoprecipitation analysis of culture medium from [^{14}C]glucosamine-labeled MDHV-infected (INF) and uninfected (CON) cells with R α A. (A) Culture medium (600 μl) from INF and CON cells containing 2.4×10^5 and 1.0×10^5 cpm, respectively, was reacted with R α A and NRS. Immunoprecipitates were analyzed by SDS-PAGE (8.5%; 16-day fluorographic exposure). (B) Same as (A) but with R α A blocked with MDHV-A and an 8-day fluorographic exposure.

virus-specific polypeptides performed by the latter workers, as they use molecular biological approaches in an attempt to further characterize several groups of herpesviruses found in the MD system (32, 33).

The work reported here is an extension of the initial purification and characterization of MDHV-A by Long et al. (14, 15). Analysis of the purified antigen by immunodiffusion and by SDS-PAGE and staining showed that it was not purified to homogeneity (15). However, absorption of the rabbit serum with sonicated extracts of uninfected cells, cell culture medium, and calf serum removed antibody to the contaminants, resulting in the antiserum monospecific for MDHV-A in immunodiffusion analysis. This antiserum is the R α A used in this study. When purified MDHV-A was analyzed by SDS-PAGE, four polypeptides with approximate molecular weights of 21,000, 52,000, 57,000, and 82,000 were observed, but the one(s) due to MDHV-A could not be identified (15). A reexamination of those data now suggests that the two polypeptides with molecular weights of 52,000 and

57,000 are actually two poorly resolved peaks of a broad band (15) that may represent the 61,000- to 65,000-dalton MDHV-A observed in this study. This is significant in light of the observation that under certain gel conditions, MDHV-A sometimes appears as a doublet (C. Glaubiger, unpublished data). If the broad 52,000- to 57,000-dalton band previously seen (15) is the same as the 61,000- to 65,000-dalton band identified as MDHV-A (Fig. 1 and 2), then the difference between the two molecular weight estimates could be accounted for by the different SDS-PAGE buffer systems used.

Long et al. (14) also found that purified MDHV-A had an apparent molecular weight of 44,800 by gel filtration in Sephadex G-200 and an apparent molecular weight of 53,160 by calibration from sedimentation coefficients. The differences between the molecular weight values observed with gel filtration and those found in this study by SDS-PAGE are probably a consequence of the technical differences involved in these two different analytical procedures for determining molecular weight. Furthermore,

FIG. 3. Immunoprecipitation analysis of culture medium from [35 S]methionine-labeled HVT- and MDHV-infected cells with R α A. Culture medium (100 μ l) from MDHV-infected cells containing 4.0×10^5 cpm and culture medium (200 μ l) from HVT-infected cells containing 8.0×10^5 cpm were reacted either with R α A that had been absorbed with unlabeled, normal cell culture medium or with NRS. Immunoprecipitates were analyzed by SDS-PAGE (7.5%; 4-day fluorographic exposure).

these differences are very likely compounded by the fact that MDHV-A is a glycoprotein. It is difficult to achieve an accurate assessment of the molecular weight of glycosylated proteins by SDS-PAGE because glycosylated polypeptides may bind SDS anomalously, resulting in migration rates that are not strictly inversely proportional to the logarithms of the molecular weights (26). Thus, as indicated above for the previous work, the size estimates resulting from this molecular characterization study must also be considered apparent molecular weights. Nevertheless, the size values obtained are quite reproducible, and once a molecule has been rigorously identified as described above, separation according to apparent molecular weight provides a rapid, sensitive, and dependable means of identifying MDHV-A in future studies. Furthermore, the broadness of the MDHV-A band, and the possibility that it may exist as a poorly resolved doublet, can be explained by the existence of MDHV-A polypeptides in different stages of glycosylation.

It has been previously demonstrated by immunodiffusion that HVT- and MDHV-infected cells share an immunologically similar A antigen (20, 23, 24, 27). SDS-PAGE immunopre-

cipitation analysis of culture medium from HVT-infected cells with sera specific for MDHV-A antigen also demonstrated a 61,000- to 65,000-dalton polypeptide similar to that of MDHV (Fig. 3). This result also agrees with the preliminary immunoprecipitation studies of MDHV- and HVT-infected cells by Van Zaane and Gielkins (34). Available evidence (Fig. 3 and data not shown) indicates that MDHV-A and HVT-A antigen are very similar in size, but the problems of analyzing glycoproteins by SDS-PAGE precludes determining whether they are identical in size. This will require size determination and tryptic peptide analysis of their polypeptide portions. The data presented in this report indicate that such studies are feasible.

The fact that HVT- and MDHV-infected cells share an immunologically similar antigen of very similar size is significant in light of hybridization studies which indicate that HVT and MDHV share less than 5% (9, 12) and possibly as little as 1% (12) DNA homology. This would allow MDHV and HVT genomes of about 10^8 daltons to share coding potential for up to only three or four proteins of approximately 5×10^4 daltons (12). Presumably MDHV-A, or at least a portion of the polypeptide responsible for its common antigenic determinant, would be encoded by this region of homology. The same is likely true for the other common antigen, MDHV-B. Since vaccination with HVT confers a highly effective immunity against MD, these common proteins, or at least the part(s) bearing the common antigenic determinant, may be of major importance when considering antiviral immunity to MD. A complete analysis of these common molecules, and the determination of their importance in the relationship between the two viruses, will require extensive molecular biological analysis of the polypeptides in question, the genomes of the two viruses (especially the common or shared regions), and expression of these genomes. The molecular identification and characterization of MDHV-A described in this report, and the similar studies in progress for MDHV-B, provide the information and methods needed to continue with molecular biological studies of the common antigens and the expression of the MDHV and HVT genomes.

We thank Jon Allan, Judith Benyi, and Michelle Williams for assistance with cell culture.

This research was supported by Public Health Service grant CA 23408 from the National Cancer Institute. L.F.V. was the recipient of Public Health Service Research Career Development Award CA-70808 from the National Cancer Institute.

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