A Structural Investigation of the Epstein–Barr (EB) Virus Membrane Antigen Glycoprotein, gp340

By A. J. MORGAN, A. R. SMITH, R. N. BARKER AND M. A. EPSTEIN*

Department of Pathology, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

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SUMMARY

Epstein–Barr (EB) virus membrane antigen (MA) glycoprotein (gp340) purified by a molecular weight-based technique has been subjected to biochemical analysis. Following treatment with glycosidases or tunicamycin during synthesis, the carbohydrate moiety was found to be made up of both O-linked and N-linked types and to constitute about 50% of the molecular mass. Digestion studies with neuraminidase and oligosaccharidase have indicated that the molecule is heavily sialated with most of the sialic acid located on the O-linked sugars. The high carbohydrate content of gp340 appears to confer resistance to proteolysis; thus, V8 protease was only effective at concentrations above 1 mg/ml when three large fragments of mol. wt. 330K, 190K and 160K were generated. Removal of sialic acid before V8 protease digestion did not alter this pattern nor affect the antigenicity of the digestion fragments. Antigenicity of the intact molecule was likewise unaffected by removal of sialic acid nor were the O-linked and N-linked carbohydrate moieties essential for this property. The binding of virus-neutralizing human sera and monoclonal antibody by gp340 from which either O-linked or N-linked sugars had been removed seems to indicate that the sites on the molecule that generate the neutralizing antibodies are present in the protein component. The significance of these results is discussed in relation to the development of a subunit vaccine against EB virus.

INTRODUCTION

The need for a subunit vaccine to protect against, or modify, Epstein–Barr (EB) virus infection has been recognized for some years (Epstein, 1976, 1979) and the relevance as an immunogen in this connection of the EB virus-determined membrane antigen (MA), which induces virus-neutralizing antibodies, has been discussed elsewhere (Epstein, 1976; Strnad et al., 1979; Thorley-Lawson & Edson, 1979; North et al., 1980; Epstein & Morgan, 1983).

The MA complex contains two major high molecular weight components of 340K and 270K (gp340/270) which share antigenic determinants (Hoffman et al., 1980; Thorley-Lawson & Geilinger, 1980; Franklin et al., 1981) and are responsible for eliciting the virus-neutralizing antibodies (Hoffman et al., 1980; Thorley-Lawson & Geilinger, 1980; North et al., 1982a; Morgan et al., 1983a). Although highly purified, antigenic, gp340 has been prepared (North et al., 1982a, b; Thorley-Lawson & Poodry, 1982; Morgan et al., 1983b) information has been lacking as to which elements of this large molecule contain the immunologically active sites. Understanding of the structure of gp340 and the role, if any, of the sugar moiety is obviously a prerequisite for the preparation in the long term of a subunit vaccine based on recombinant DNA technology or chemical synthesis of peptides.

The present paper describes experiments in which gp340 has been analysed after treatment with glycosidases and V8 protease.
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METHODS

Cell cultures. The EB virus-carrying, marmoset B95-8 lymphoblastoid cell line (Miller et al., 1972) was grown in the presence of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) by methods already described (North et al., 1982b).

Antiserum to MA. A human antiserum (HS 77) with high titre naturally occurring antibodies to MA and a control human serum (HS 15) lacking such antibodies were obtained from healthy donors. A monoclonal antibody to MA (72A1) which both binds gp340 and neutralizes EB virus was kindly provided by Dr. G. J. Hoffman (Johns Hopkins School of Medicine, Baltimore, Md., U.S.A.) (Hoffman et al., 1980).

Radiolabelling of gp340. Glycoprotein gp340 was purified by the molecular weight-based method of Morgan et al. (1983b) and iodinated with $^{125}$I (North et al., 1982b). In addition, B95-8 cells were labelled internally with L-[4,5-$^{3}$H]leucine as reported elsewhere (North et al., 1982a) and with $^{3}$H]galactose. For incorporation of the $^{3}$H]galactose, 4 x $10^{6}$ cells were suspended in 10 ml RPMI 1640 medium with 250 $\mu$Ci of the label and incubated for 72 h at 37 °C. In each case the cells were collected by centrifugation, lysed in buffer, centrifuged to deposit the nuclei, and the supernatant was then dialysed against phosphate-buffered saline. B95-8 cells were also surface-labelled with $^{125}$I using a lactoperoxidase technique (North et al., 1980). All the radiochemicals were obtained from Amersham International.

Treatment with tunicamycin. In some cases, B95-8 cells were exposed to 10 $\mu$g/ml tunicamycin during culture for 3 h and this treatment was then continued throughout the period of incorporation of [3$^{3}$H]leucine and [3$^{3}$H]galactose. A 3 h exposure to this concentration of tunicamycin was also applied on some occasions before surface radioiodination.

Treatment with V8 protease. $^{125}$I-labelled gp340 was bound to HS 77 antibody and immobilized on 20 $\mu$l of packed Sepharose-Protein A (North et al., 1980) and was then digested with V8 protease (Miles Laboratories) by the method of Cleveland et al. (1977). Samples were incubated with 20 $\mu$l V8 protease in buffer (0.5% SDS, 0.124 m-Tris–HCl pH 6.7, 0.5 mg/ml bovine serum albumin) at concentrations of 0-1, 1, 2 or 4 mg/ml. The incubation was at 37 °C for 30 min and the reaction was stopped by the addition of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer.

Treatment with glycosidases

$^{125}$I-labelled gp340 was immobilized as for V8 protease digestion before treatment with the following.

Neuraminidase. Samples were incubated with 10$\mu$l neuraminidase (Bethesda Research Laboratories) in buffer (0.15% Triton X-100, 0.1 m-sodium acetate, pH 5) at a concentration of 8 units/ml.

$\alpha$-D-N-Acetyl galactosaminyl oligosaccharidase (NAGOS). Samples were incubated with 20 $\mu$l NAGOS (lot 1211, specified as protease-free; Bethesda Research Laboratories) in buffer (0.1% Triton X-100, 0.05 m-potassium phosphate, pH 6.5) at a concentration of 0.24 units/ml.

Endo-$\beta$-N-acetyl glucosaminidase H (endo H). Samples were incubated with 10 $\mu$l endo H (Miles Laboratories) in buffer (0.1% Triton X-100, 0.1 m-sodium acetate, pH 5.2) at a concentration of 12 units/ml.

Endo-$\beta$-N-acetyl glucosaminidase D (endo D). Samples were incubated with 5 $\mu$l endo D (Miles Laboratories) in buffer (0.1% Triton X-100, 0.025 m-Tris–HCl, pH 6.8) at a concentration of 4 units/ml.

N-Acetyl galactosaminidase (NAGAL). Samples were incubated with 10 $\mu$l NAGAL (Bethesda Research Laboratories) in buffer (0.1% Triton X-100, 0.05 m-potassium phosphate, pH 6.8) at a concentration of 10 units/ml.

$\beta$-Galactosidase. Samples were incubated with 5 $\mu$l $\beta$-galactosidase (Sigma) in buffer (0.1% Triton X-100, 0.05 m-potassium phosphate, pH 6.8) at a concentration of 1667 units/ml.

The incubations were at 37 °C for 30 min and all the reactions were stopped by centrifugation and removal of the supernate, followed either by the addition of SDS–PAGE sample buffer, or by washing the Sepharose–Protein A with a buffer appropriate for further digestions.

SDS–PAGE, fluorography and autoradiography. These procedures were carried out as in earlier work (North et al., 1980; Morgan et al., 1983b). Molecular weights were estimated by comparison with standard markers.

RESULTS

Treatment of $^{125}$I-labelled gp340 with V8 protease

When $^{125}$I-gp340 was digested with various concentrations of the enzyme and the products were analysed by SDS–PAGE, several polypeptide fragments were produced (Fig. 1). These polypeptides had a wide range of apparent molecular weights and the number of fragments and intensity of their individual bands depended on the concentration of enzyme used (Fig. 1). As the concentration of V8 protease was increased from 0.1 mg/ml to 4 mg/ml the amount of $^{125}$I-gp340 was progressively reduced giving a corresponding increase in the amount of smaller fragments. These had apparent mol. wt. of 330K, 190K and 130K and were designated fragments I, II and III respectively (Fig. 1). Boiling of the samples before digestion, or
Structure of Epstein-Barr virus gp340

Fig. 1. Autoradiograph of SDS-PAGE showing fragments of $^{125}$I-gp340 resulting from treatment with various concentrations of V8 protease. It can be seen that very high concentrations of the enzyme were required to achieve more than minimal digestion. The concentrations used were: lane 1, 0.1 mg/ml; lane 2, 1 mg/ml; lane 3, 2 mg/ml; lane 4, 4 mg/ml; lane 5, enzyme-free control.

Increasing the digestion time to 18 h, did not alter this pattern. $^{125}$I-gp340 and fragments I, II and III could be eluted from the gel, renatured and immunoprecipitated with an anti-MA serum (HS 77) but not with monoclonal antibodies. The lower molecular weight fragments (V to VII, Fig. 1) were not immunoprecipitable following elution and renaturation (Table 1); there was too little of fragment IV for testing.

Treatment of $^{125}$I-gp340 and fragments I, II and III with neuraminidase

$^{125}$I-gp340 and fragments I, II and III were recovered from gels and immobilized. Paired samples were incubated with or without neuraminidase and then analysed by SDS-PAGE. Digestion with neuraminidase modified the mobility of the molecules such that $^{125}$I-gp340 and fragment I showed a surprising increase in apparent molecular weight of 50K and 80K respectively (Fig. 2). A similar increase in molecular weight was observed with fragment II but not with fragment III; however, both these fragments had become more heterogeneous merely as a result of re-running in SDS-PAGE (Fig. 2, lanes 5 to 8) giving, in each case, several separate bands. Removal of the sialic acid by treatment with neuraminidase before V8 protease digestion gave very similar results. In either case, de-sialated fragments I, II and III retained their antigenicity (Table 1).

Treatment of $^{125}$I-gp340 with endoglycosidases

After NAGOS digestion of $^{125}$I-gp340, the apparent mol. wt. of the molecule in SDS-PAGE was reduced by about 80K (Fig. 3). This reduction occurred irrespective of whether or not the molecule had been pretreated with neuraminidase, indicating that most of the sialic acid is attached to O-linked oligosaccharides.

Treatment of $^{125}$I-gp340 with either endo D or endo H did not result in any change in molecular weight after SDS-PAGE (data not shown).

However, if the $^{125}$I-gp340 was treated sequentially with neuraminidase, β-galactosidase and NAGAL the molecule was then rendered susceptible to endo D digestion as indicated by a subsequent small decrease in molecular weight (Fig. 4).
Fig. 2. Autoradiograph of SDS-PAGE showing the effect of neuraminidase on the mobility of $^{125}$I-gp340 and its V8 protease fragments I to III. Fragment III was the only material whose components showed no change in mobility after treatment with the enzyme, indicating that little sialic acid was present. Lane 1, treated $^{125}$I-gp340; lane 2, untreated $^{125}$I-gp340; lane 3, treated fragment I; lane 4, untreated fragment I; lane 5, treated fragment II; lane 6, untreated fragment II; lane 7, treated fragment III; lane 8, untreated fragment III.

Table 1. Immunoprecipitation of eluted, renatured, V8 protease fragments*

<table>
<thead>
<tr>
<th>125I-labelled material tested</th>
<th>Treatment</th>
<th>Immunoprecipitation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp340</td>
<td>None</td>
<td>62</td>
</tr>
<tr>
<td>Fragment I</td>
<td>None</td>
<td>52</td>
</tr>
<tr>
<td>II</td>
<td>None</td>
<td>61</td>
</tr>
<tr>
<td>III</td>
<td>None</td>
<td>71</td>
</tr>
<tr>
<td>IV</td>
<td>None</td>
<td>ND†</td>
</tr>
<tr>
<td>V</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td>VI</td>
<td>None</td>
<td>18</td>
</tr>
<tr>
<td>I</td>
<td>Neuraminidase</td>
<td>66</td>
</tr>
<tr>
<td>II</td>
<td>Neuraminidase</td>
<td>60</td>
</tr>
<tr>
<td>III</td>
<td>Neuraminidase</td>
<td>63</td>
</tr>
</tbody>
</table>

* Fragments I, II and III show similar antigenicity to that of the undigested gp340 irrespective of whether neuraminidase treatment was applied.
† ND, Not determined.

Binding of virus-neutralizing monoclonal antibody to sugar-depleted gp340

B95-8 cells were grown in the presence of tunicamycin prior to and during $[^3]$H]leucine labelling to prevent N-linked glycosylation. When lysates of these cells were immunoprecipitated with human antiserum to MA (HS 77) or with a virus-neutralizing monoclonal antibody (72A1), two high molecular weight bands were observed (Fig. 5, lanes 2 and 4) despite a somewhat raised background. Comparison of these bands with control precipitations from lysates of cells which had not been treated with tunicamycin (Fig. 5, lanes 1 and 3) indicates that the smaller polypeptide represents gp340 lacking N-linked oligosaccharide. $[^3]$H]Galactose was incorporated into gp340 in the presence of tunicamycin, indicating synthesis of sugars which were not N-linked (data not shown).
B95-8 cells radiolabelled by the lactoperoxidase method were also used to prepare lysates, and when the latter were treated with NAGOS, binding of gp340 to the virus-neutralizing antibody (72A1) was not prevented (Fig. 6) although some increase in heterogeneity was observed.

Taken together, these results show that N-linked oligosaccharides are not required in the reaction of gp340 with specific, virus-neutralizing, antibody (72A1), and the same appears to be the case for O-linked oligosaccharides.

DISCUSSION

In view of the successful elaboration of a subunit vaccine to EB virus based on the virus-determined MA gp340 (Morgan et al., 1983a) and current evaluation of its biological effectiveness, the determination of the structure of this molecule is of considerable relevance. Thus, it is important to know which components of the molecule, protein or carbohydrate, are essential in generating virus-neutralizing antibodies and whether fragments can be made which retain this property.

As regards the production of polypeptide fragments, the present experiments have succeeded in obtaining limited digestion of $^{125}$I-gp340 by taking advantage of the restricted specificity of V8 protease (Cleveland et al., 1977). However, it is of interest that very high concentrations of the enzyme were required, probably reflecting a considerable degree of protection of the
molecule by the carbohydrate portion. The observation that fragment I had only a slightly lower apparent molecular weight than the original molecule suggests that a small peptide was cleaved from some substrate molecules while others were broken down more completely to give fragments II and III, representing, perhaps, variations in glycosylation. In any event, all three fragments were equally antigenic and sialic acid was clearly not involved in this function since fragments I, II and III retained their antigenicity after treatment with neuraminidase (Table 1).

The production of polypeptide fragments from gp340 provides material likely to prove useful for the location of important antigenic sites.

The surprising increase in apparent molecular weight of gp340 and fragments I and II after neuraminidase digestion calls for comment. Carbohydrates affect the migration of glycoproteins in SDS–PAGE in a complex manner. Chains of uncharged sugar residues decrease the migration of such molecules and can therefore lead to an over-estimation of molecular weight (Segrest et al., 1971), but sialic acid has the opposite effect. It has been found in studies on cell surface glycoproteins that neuraminidase digestion did not affect the mobility of molecules containing fewer than six sialic acid residues per hundred amino acids, whereas similar treatment of a glycoprotein with five times this amount of sialic acid resulted in a change of apparent mol. wt. from 95K to 105K (Brown et al., 1981). By analogy, the marked apparent molecular weight increase of $^{125}$I-gp340 and fragments I and II after neuraminidase treatment would seem to indicate that these molecules contain large amounts of sialic acid.

Previous investigations in which cells expressing gp340 were grown in the presence of tunicamycin indicated that part of the carbohydrate was of the N-linked type (Edson & Thorley-
Lawson, 1981) whilst the sensitivity of the molecule to NAGOS digestion (Fig. 3) now appears to demonstrate substantial amounts of O-linked sugars as well. As regards the specificity of the NAGOS digestion, any possible contaminating protease activity would have been mopped up by the considerable amount of unlabelled protein (antibody and Protein A) used in the immobilization. Furthermore, the gp340 molecule is resistant to both V8 protease (Fig. 1) and proteases in cell lysates (North et al., 1980) and would not therefore respond to traces of contaminating protease if these were present in the NAGOS. Also arguing against any significant proteolysis is the discrete molecular weight shift with only a small increase in band width (Fig. 3), and the absence of appreciable amounts of material in the gel front (Fig. 3, lanes 3 and 4). The fact that the apparent molecular weight of $^{125}$I-gp340 remains the same after NAGOS treatment whether or not neuraminidase was first applied indicates that the NAGOS itself did not contain any neuraminidase, whilst the shift was too large for other minor glycosidase contaminants to be responsible. Furthermore, [3H]galactose was incorporated into gp340 in the presence of tunicamycin, suggesting that O-linked sugars were indeed synthesized. The present conclusion that O-linked carbohydrate forms part of the gp340 molecule is strongly supported by the findings of Edson & Thorley-Lawson (1983) that the difference in apparent molecular weights between nascent and mature gp340 cannot be accounted for by N-linked glycosylation alone. Finally, it should be noted that other herpesviruses express antigens containing both N-linked and O-linked sugars (Johnson & Spear, 1983).

It has been inferred from pulse-labelling experiments that the N-linked carbohydrate moiety of mature gp340 is of the 'complex' type (Edson & Thorley-Lawson, 1983) and the present paper establishes and extends this supposition by showing the sensitivity of gp340 to endo D digestion following sequential treatment with a set of exoglycosidases (Fig. 4). Although the reaction was unlikely to have reached completion, it is clear that the molecule was rendered susceptible to endo D.

The changes observed in the apparent molecular weight of gp340 after removal of N- and O-linked sugars (Fig. 3 and 5) show that carbohydrate accounts for at least 50% of the molecule. In this connection, it was of considerable significance that the EB virus-neutralizing monoclonal antibody (72A1) bound gp340 depleted of N-linked oligosaccharides and seemingly also O-linked oligosaccharides since this provides strong evidence for the presence of an epitope important for virus neutralization in the polypeptide chain itself. This interpretation again rests on the assumption that the NAGOS was free of contaminating enzymes. Thus, the same batch of NAGOS (specified protease-free) was used in all experiments, and although there is an increase in band width after digestion (Fig. 6, lane 2) this may represent proteolysis by cellular enzymes in the lysate subjected to NAGOS digestion which were unable to act on the control material (Fig. 6, lane 1) because of protection afforded by the presence of a full complement of carbohydrate. Alternatively, the increased band width could reflect incomplete removal of O-linked sugars, although the presence of low-molecular weight material would argue against this. That the carbohydrate is not essential for virus neutralization is also suggested by the findings that only polyspecific sera bind gp340 eluted from SDS gels, whereas the monoclonal antibody does not, indicating the likely presence of a conformational epitope.

The present investigations have thus determined the overall structure of gp340 and demonstrated the relevance of the polypeptide alone for use as a vaccine. These observations greatly simplify further long-term detailed studies on gp340 structure since they need now only concentrate on this part of the molecule. Future sequencing of the polypeptide will open up the possibility of producing synthetic and/or genetically engineered material.

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