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# Virion basic phosphoprotein from human cytomegalovirus contains O-linked *N*-acetylglucosamine

(herpesvirus structural proteins/viral protein processing/galactosyltransferase)

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**ABSTRACT** **A 149-kDa virion protein of human strains of cytomegalovirus is the principal acceptor for galactose added *in vitro* by bovine milk galactosyltransferase. Peptide comparisons with other biochemical characteristics of the galactosylated protein identified it as the virus-encoded basic phosphoprotein. This protein is an abundant constituent of the virion and is located in the tegument region, between the capsid and the envelope, rather than in the envelope layer with the recognized virion glycoproteins. The galactosylated carbohydrate was resistant to a commercial preparation of endoglycosidase F but was sensitive to removal by alkali-induced β-elimination, indicating an O-linkage to the protein. Chromatographic and electrophoretic determinations identified the β-eliminated material as the alditol of Galβ1-4GlcNAc, establishing that the human cytomegalovirus virion basic phosphoprotein contains single O-linked residues of *N*-acetylglucosamine.**

Human cytomegalovirus (human herpesvirus 5, HCMV) is a large, enveloped, double-stranded DNA-containing agent that is one of six herpesgroup viruses known to infect man. It is distinguished from other members of this group by the 50% larger size of its genome (i.e., ≈240 kilobase pairs), its comparatively high degree of species specificity and slow replication cycle, and its ability to cross the placental barrier. Clinically, HCMV is a causative agent of congenital birth defects and can produce fatal infections in immunosuppressed individuals, including patients with acquired immunodeficiency syndrome (1–3).

Like those of other herpesviruses, the virion of HCMV contains an icosahedral nucleocapsid surrounded by a tegument layer, composed primarily of phosphorylated proteins, and a membrane envelope. The envelope is the last layer added to the particle as it leaves the nucleus during maturation, and the first to be removed as the particle enters another host cell. The presumed involvement of the virion envelope glycoproteins in both of these processes, in addition to their role in eliciting an immune response, has made them the focus of considerable interest.

During the course of studies to determine the structure and linkage of oligosaccharides attached to HCMV virion glycoproteins, we used the enzyme galactosyltransferase (GalTase) and UDP-[$^3$H]Gal to label terminal *N*-acetylglucosamine (GlcNAc) residues (i.e., in the absence of α-lactalbumin; refs. 4 and 5). Unexpectedly, results of initial experiments showed that the principal galactosylated band corresponded in size to a major virion phosphoprotein, designated earlier as the basic phosphoprotein (BPP, 149 kDa, ref. 6), rather than to one of the identified glycoproteins (6–16). Unlike the virion glycoproteins, the BPP migrates as a comparatively tight band during electrophoresis (6, 17), is not recognized by any of the lectins, glycosidases, or sialyltransferases tested (ref. 15, ‡), is phosphorylated both *in vivo* (6, 12, 17–19) and *in vitro* (6, 11, 17, 18), and is thought to be located internally rather than in the envelope (6, 11).

Single O-linked residues of GlcNAc have been discovered as a covalent modification of a number of cytoplasmic and nuclear proteins (20–29) and shown to be good substrates *in vitro* for GalTase (20, 23, 24, 26–29). Given the modest influence that a few such residues might have on the electrophoretic mobility and size heterogeneity of a large protein, it seemed plausible that the observed GalTase labeling of the BPP was due to the presence of these O-linked monosaccharides. This report presents the results of experiments that verify this possibility, and a discussion of the results as they bear on efforts to understand the functional significance of this modification.

## METHODS

**Cells, Viruses, and Virus Isolation.** Human foreskin fibroblast (HFF) cell cultures were prepared, maintained, and infected as described (30). Pertinent information about the HCMV strains 751, AD169, and Towne, the simian cytomegalovirus (SCMV)-like strain Colburn, and the herpes simplex virus type 1 (HSV-1) strain F is presented elsewhere (6, 31–33). Virions were recovered from the maintenance medium of infected cell cultures by a single sedimentation in negative viscosity–positive density (glycerol–tartrate) gradients (34–36) and concentrated by pelleting (15). Pellets were resuspended in $H_2O$ and stored at −80°C.

**Polyacrylamide Gel Electrophoresis.** The ratio of acrylamide to crosslinker was 25.7:1 (wt/vol) for gels with a high concentration of *N*′,*N*′-methylenebisacrylamide (high bis gels) (36) and diallyltartardiamide (DATD) gels (30) and was 38:1 (wt/vol) for all other gels. Details are presented elsewhere for the specific conditions of $NaDodSO_4$/PAGE (15, 36, 37), fluorography (38–40), and densitometry (30).

**Autogalactosylation of GalTase.** Autogalactosylated GalTase (catalog no. G-5507, Sigma) was prepared as described (20), and then precipitated twice in 85% (vol/vol) saturated ammonium sulfate (at 4°C), and resuspended and stored in a solution of 25 mM Hepes, 5 mM $MnCl_2$, and 50% (vol/vol) glycerol (pH 7.3) at −20°C (23). The resulting enzyme concentration was determined (41) to be ≈16 units/ml.

**GalTase Assay.** Virions isolated as described above were labeled essentially as described (23) in 20 μl of reaction mixture containing 10 mM Gal, 15 mM NaCl, 0.5% Nonidet

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Abbreviations: HCMV, human cytomegalovirus; BPP, basic phosphoprotein; HSV-1, herpes simplex virus type 1; DATD, diallyltartardiamide; GalTase, galactosyltransferase; SCMV, simian cytomegalovirus; O-GlcNAc, O-linked *N*-acetylglucosamine; endo F, endogylcosidase F.

‡Benko, D. M. & Gibson, W., 10th Herpesvirus Workshop, August 11–16, 1985, Ann Arbor, MI, abstr. 150; and 12th Herpesvirus Workshop, July 30–August 4, 1987, Philadelphia, PA, abstr. 149.

P-40, 5 mM $MnCl_2$, 10 mM Hepes (pH 7.3), and 0.2% aprotinin (catalog no. A6012, Sigma). The labeling reaction (37°C for 30 min) was initiated by adding 8 milliunits of GalTase, followed by 1 μCi UDP-[$^3$H]Gal (catalog no. TRK.513, 17.3 Ci/mmol; 1 Ci = 37 GBq; Amersham) that had been dried under a stream of $N_2$ and resuspended in water containing enough 5′-AMP to give a final concentration of 2.5 mM in the reaction mixture. Increasing the amount of enzyme by up to 8-fold or the nucleotide sugar by up to 50-fold did not substantially increase incorporation of [$^3$H]Gal into the BPP.

## RESULTS

**GalTase Selectively Labels One HCMV Virion Protein.** Virions of HCMV (strains 751, Towne, and AD169) and, for purposes of comparison, of SCMV (strain Colburn) and HSV-1 were isolated and labeled with UDP-[$^3$H]Gal by using GalTase, then solubilized and separated by PAGE. Fluorograms made from the gel (Fig. 1) showed that HCMV, SCMV, and HSV-1 virions each contained distinctly different sets of [$^3$H]galactosylated proteins.

Of the six principal HCMV glycoproteins (gp) (i.e., gp163, gp145, gp130, gp70, gp62, and gp57) identified in lectin binding assays (15), only gp62 and gp130 were detectably labeled (results of two-dimensional separations; data not shown). Neither the primary [$^3$H]Gal acceptor, a 149-kDa band containing ≥90% of the incorporated radioactivity, nor other weakly labeled bands at 225, 75, 65, 60, 46, 35, and 18 kDa (Fig. 2, dots), corresponded to identified glycoproteins. Labeled bands in the enzyme preparation were detected only with prolonged fluorographic exposure and did not correspond to bands in any of the virion preparations (Fig. 2*A*). No bands were labeled if GalTase was omitted from the reaction (i.e., virion does not contain a GalTase); and [$^3$H]galactosylation of the 149-kDa band was lower by a factor of ≈40 (normalized to intensity of stained band) if Nonidet P-40 was omitted from the reaction mixture, consistent with an internal localization in the virion (data not shown).

The pattern of SCMV virion proteins visualized by this method resembled those obtained earlier with the lectin wheat germ agglutinin as a probe (15). Two-dimensional (nonreducing/reducing) PAGE separations indicated that the two diffuse [$^3$H]galactosylated bands correspond to gp163 and gp119. These two glycoproteins have been shown to contain both N- and O-linked oligosaccharides (ref. 15; ‡; and unpublished data).

HSV-1 virions contained two comparatively weak acceptors that correspond in position to virion glycoproteins gC (115–130 kDa) and gD (59–65 kDa) (32).

**HCMV [$^3$H]Gal Acceptor Identified as the BPP.** The electrophoretic mobility of the HCMV BPP (149 kDa), relative to

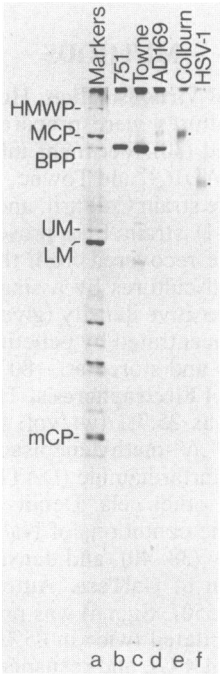


FIG. 1. Virion acceptors for GalTase. Extracellular virions of HCMV (strains 751, Towne, and AD169 in lanes b–d), SCMV (strain Colburn in lane e), and HSV-1 (lane f) were isolated, [$^3$H]galactosylated, solubilized, and subjected to PAGE in a 10% high bis gel (36). A fluorogram of the gel is shown. AD169 virion proteins labeled biosynthetically with [$^{35}$S]methionine (25 μCi/ml; no. NEG-009A, New England Nuclear) were included as molecular size markers (lane a). The relative mass (by staining) of major capsid protein present in the [$^3$H]galactosylated preparations (lanes b–f) was ≈1:1.4:1.6:2.8:3.3 (HSV/AD169/751/Towne/Colburn). HMWP, 212-kDa high-molecular-weight protein; MCP, 153-kDa major capsid protein; UM and LM, 74-kDa and 69-kDa upper and lower matrix proteins, respectively; mCP, 34-kDa minor capsid protein (6). Dots to right of lanes e and f indicate positions of galactosylated bands described in text.

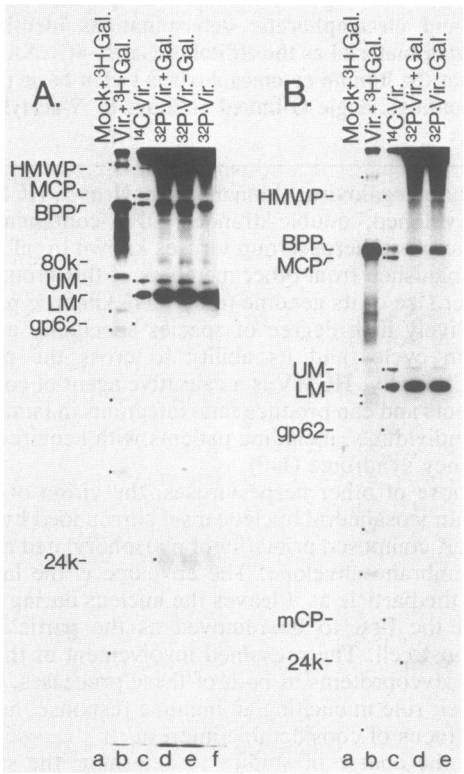


FIG. 2. BPP and galactosylated bands comigrate and exhibit gel-dependent changes in mobility. Biosynthetically $^{32}$P-labeled (100 μCi/ml; no. PBS.11A, Amersham) and nonlabeled HCMV (strain 751) virions were isolated, and the following GalTase assay mixtures were prepared and incubated, and products were separated in a 10% high bis gel (*A*) and in a 12% DATD gel (*B*). Lanes: a, GalTase + UDP-[$^3$H]Gal (i.e., no viral acceptor); b, nonlabeled virions + GalTase + UDP-[$^3$H]Gal; d, $^{32}$P-labeled virions + GalTase + UDP-galactose; e, $^{32}$P-labeled virions + GalTase; c and f, biosynthetically $^{32}$P- and [$^{14}$C]amino acid (25 μCi/ml; no. NEC445, New England Nuclear)-labeled virions, respectively. Fluorograms from the gels are shown. Abbreviations are as in Fig. 1 with the following additions: 80K, 80-kDa phosphoprotein (6, 17); gp62, 62-kDa glycoprotein (6, 15); 24K, 24-kDa phosphorylated protein (11, 17). Dots indicate the respective positions of galactosylated bands from 225 kDa to 18 kDa described in the text.

the major capsid protein, depends on the PAGE system used (6, 19, 36). To determine whether the $^{3}$H-galactosylated 149-kDa protein also exhibited this characteristic, both biosynthetically $^{32}$P-labeled and nonlabeled HCMV (strain 751) virions were isolated and incubated *in vitro* with GalTase: $^{32}$P-labeled virions in the presence of nonradioactive UDP-Gal, and nonlabeled virions in the presence of UDP-[$^{3}$H]Gal. The reaction products were compared following separation by PAGE in both high bis and DATD gels. In the high bis gel, the [$^{3}$H]galactosylated band and BPP comigrated in a position slightly ahead of the 153-kDa major capsid protein (Fig. 2*A*). The 149-kDa [$^{3}$H]galactosylated band also comigrated with the BPP in the DATD gel, but in this system both proteins moved more slowly than the major capsid protein (Fig. 2*B*). This parallel and unusual behavior of the two bands strongly suggested that they were the same protein.

This was verified by comparing the proteins by partial proteolysis. Biosynthetically $^{32}$P-labeled, *in vitro* [$^{3}$H]galactosylated, or radioiodinated virions were prepared and subjected to PAGE, and their 149-kDa proteins were analyzed following partial proteolysis (Fig. 3). The [$^{3}$H]galactosylated 149-kDa band (Fig. 3*A*) gave rise to two main peptides of 55 kDa and 40 kDa. These corresponded to the two predominant peptides in the phosphorylated (Fig. 3 *B* and *C*) and iodinated preparations (Fig. 3*D*), thereby establishing the identity of the 149-kDa galactosylated band as the BPP.

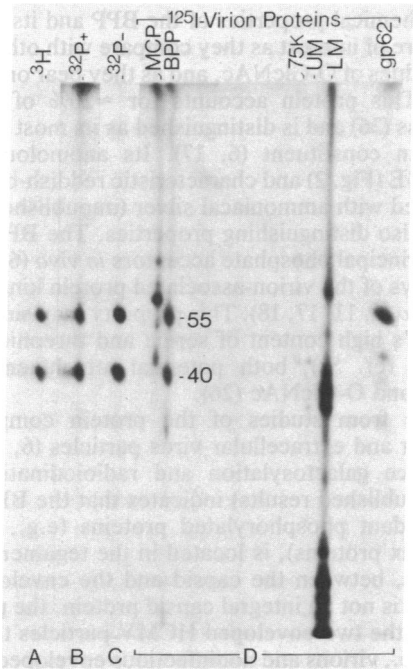


FIG. 3. Peptide comparisons. Radiolabeled HCMV (strain 751) proteins were compared following partial cleavage at tryptophan residues by *N*-chlorosuccinimide (catalog no. 0762, Sigma) (42). Proteins were resolved in an 8% high bis gel. The lanes of interest were cut from the first gel, treated with 15 mM *N*-chlorosuccinimide in a solution of 1 g of urea, 1 ml of $H_2O$, and 1 ml of acetic acid at 23°C for 1 hr, applied to the top of a second-dimension gel, and subjected to PAGE. After electrophoresis, the second-dimension gel was stained with Coomassie brilliant blue (43) and fluorographed. Peptides from the 149-kDa protein are shown. Lanes: A, [$^{3}$H]galactosylated *in vitro*; B, $^{32}$P-labeled *in vivo* and galactosylated; C, $^{32}$P-labeled *in vivo*; D, $^{125}$I-labeled *in vitro* after adding Nonidet P-40 to 1.0%. A larger segment of the gel lane is shown in lane D to enable comparison of the BPP peptide pattern with those of several other virion proteins. All samples were separated in the same gel during respective protein (first dimension) and peptide (second dimension) separations.

**BPP Terminal GlcNAc Is O-linked.** The following series of experiments was done to determine the nature of the glycopeptide linkage and the structure of the carbohydrate attached to the BPP. To determine whether the acceptor GlcNAc residues of the BPP are on N-linked oligosaccharides, portions of assay mixtures prepared for the experiment summarized in Fig. 2 were incubated with or without a commercial endoglycosidase F (endo F) preparation, under conditions favoring the enzyme preparation's peptide: *N*-glycosidase F activity (44, 45), solubilized, and analyzed by PAGE (Fig. 4). Comparison of the endo F-treated and nontreated preparations (Fig. 4 *Right*) shows that the intensity and position of the [$^{3}$H]galactosylated BPP band was not affected by the treatment, indicating that its GlcNAc residues are not N-linked. Likewise, the glycosidase had no effect on the intensity or electrophoretic mobility of the $^{32}$P-labeled BPP. Loss of $^{32}$P radioactivity from the lower matrix protein, and occasionally from others including the BPP, appears to be due to a phosphatase activity in the endo F preparation (unpublished observations). The silver-stained gel (Fig. 4 *Left*) shows that the reaction mixtures contained approximately equal amounts of virions (e.g., intensities of major capsid protein were about the same), that the endo F

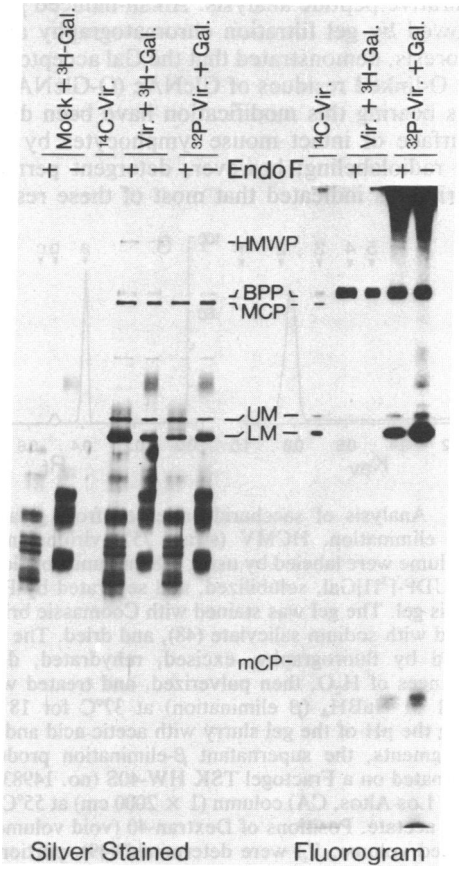


FIG. 4. Linkage of carbohydrate to BPP is endo F resistant. Five-microliter portions of denatured [with 0.4% $NaDodSO_4$ (Bio-Rad) at 100°C for 1 min] GalTase assay mixtures listed in the legend to Fig. 2 were brought to final volumes of 20 μl containing 50 mM EDTA, 50 mM 2-mercaptoethanol, 2 mM *o*-phenanthroline, 0.5% Nonidet P-40, and 40 mM sodium phosphate (pH 8.5), and either 0.7 unit commercial endo F preparation (no. NEE-150, New England Nuclear) (lanes marked +) or $H_2O$ (lanes marked −); incubated at 37°C for 7 hr; and then solubilized, and subjected to PAGE in a 12% DATD gel. A direct duplicate image (46) made from the silver-stained gel (47) and a composite of fluorograms made from the gel after completely removing the silver (47) are shown. Abbreviations and sample order are as in Figs. 1 and 2, respectively.

was active (e.g., changed position of bands in the GalTase preparation), and that there was no evidence of proteolysis.

To determine whether the BPP acceptors are O-linked, the [$^3$H]galactosylated protein was subjected to $\beta$ elimination. At least 67% of the total radioactivity was released from the gel slice by this treatment, consistent with the majority of the terminal GlcNAc residues of BPP being attached through an O-linkage. Gel filtration chromatography of the $\beta$-eliminated material showed that 85% of the radioactivity was contained in a single peak that eluted at the expected position for the alditol of Gal$\beta$1-4GlcNAc (i.e., $K_{av}$ = 0.82) (Fig. 5*A*). These results indicated that the protein's acceptor GlcNAc residues are linked directly to the protein. High-voltage paper electrophoresis of the $\beta$-eliminated, size-fractionated material, along with three structurally defined disaccharide alditols, showed that the released disaccharide comigrated with the authentic alditol of Gal$\beta$1-4GlcNAc, as expected from the specificity of GalTase (Fig. 5*B*).

## DISCUSSION

A major virion protein of HCMV is selectively radiolabeled *in vitro* by bovine milk GalTase. The protein's identity as the 149-kDa BPP was established by several techniques, including comparative peptide analysis. Alkali-induced $\beta$ elimination, followed by gel filtration chromatography and paper electrophoresis, demonstrated that the Gal acceptors of BPP are single O-linked residues of GlcNAc (O-GlcNAc).

Proteins bearing this modification have been discovered on the surface of intact mouse lymphocytes by GalTase-catalyzed radiolabeling; however, detergent permeabilization experiments indicated that most of these residues are internally located (20). Subsequent fractionation studies established that the majority of proteins bearing O-GlcNAc in rat liver cells are present in the cytosol and nuclear envelope (23). Examples of proteins having this modification are (*i*) a group of eight nuclear-pore-complex proteins (24–26, 28, 29), whose saccharides appear to be exposed on the cytoplasmic and nucleoplasmic sides of the nuclear envelope (22, 24, 25, 28), rather than in the lumenal space, typical of the oligosaccharides of other proteins; (*ii*) a major cytoskeletal protein (i.e., band 4.1) and a 65-kDa cytosolic protein of human erythrocytes (27); and (*iii*) proteins of adult *Schistosoma mansoni* worms (51).

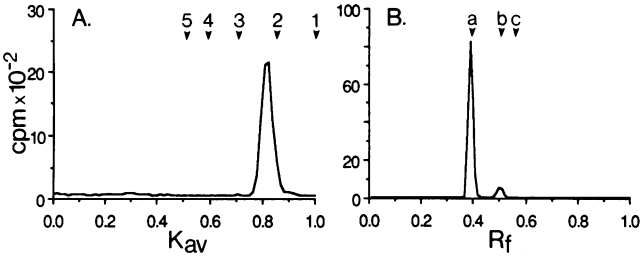


FIG. 5. Analysis of saccharide released from galactosylated BPP by $\beta$ elimination. HCMV (strain 751) virions in a 160-$\mu$l reaction volume were labeled by using 128 milliunits of GalTase and 40 $\mu$Ci of UDP-[$^3$H]Gal, solubilized, and separated by PAGE in a 10% high bis gel. The gel was stained with Coomassie brilliant blue (43), treated with sodium salicylate (48), and dried. The BPP band was located by fluorography, excised, rehydrated, desalted in several changes of $H_2O$, then pulverized, and treated with 0.1 M NaOH in 1 M $NaBH_4$ ($\beta$ elimination) at 37°C for 18 hr. After neutralizing the pH of the gel slurry with acetic acid and removing the gel fragments, the supernatant $\beta$-elimination products were size-fractionated on a Fractogel TSK HW-40S (no. 149836, Alltech Associates, Los Altos, CA) column (1 × 2000 cm) at 55°C in 50 mM ammonium acetate. Positions of Dextran-40 (void volume, $V_o$) and Gal (included volume, $V_i$) were determined (49), portions of fractions between $V_o$ and $V_i$ were analyzed by scintillation spectrometry, and $K_{av}$ values were calculated. The resulting elution profile is shown in *A*. Arrows indicate the respective elution positions of polymers composed of one to five GlcNAc residues. Fractions containing the disaccharide peak shown in *A* were pooled, lyophilized, resuspended in $H_2O$, and subjected to high-voltage paper electrophoresis in 1% sodium tetraborate on Whatman 3MM paper at 3000 V for 3.5 hr (50). Following electrophoresis, the paper was cut into 0.5-cm strips and subjected to scintillation spectrometry, and $R_f$ values were calculated. (*B*) Migration profile for the released BPP disaccharide alditol, and the relative positions of three structurally defined disaccharide alditols, [$^{14}$C]Gal$\beta$1-4GlcNAc, [$^3$H]-Gal$\beta$1-3GalNAc, and [$^3$H]Gal$\beta$1-4GalNAc (arrowheads labeled a, b, and c, respectively) that served as reference standards (23).

Although there has been speculation as to the function of O-GlcNAc (e.g., targeting signal, regulatory alteration, or protease resistance), its significance remains unknown. The BPP has attractive features as a model system for approaching such questions. Its gene has been identified and sequenced (52) and, as an element of the viral genome, can be more conveniently manipulated than essential cellular genes. In addition, the BPP appears to contain only one or a small number of GlcNAc residues, since (*i*) its electrophoretic mobility was not altered by galactosylation (Fig. 2, compare lanes d and e), in contrast to those of other proteins known to contain multiple residues of GlcNAc (26, 29), and (*ii*) two-dimensional separation (17) of tryptic peptides from [$^3$H]galactosylated BPP revealed that ≥90% of the radioactivity was present in a single spot (data not shown). This should simplify efforts to identify the attachment site, alter it at the genetic level, and assess the functional consequences.

The biochemical properties of the BPP and its location in the virion are of interest as they compare with other proteins having residues of O-GlcNAc, and as they bear on the role of the BPP. This protein accounts for ≈20% of the virion protein mass (36) and is distinguished as its most electropositive protein constituent (6, 17). Its anomolous mobility during PAGE (Fig. 2) and characteristic reddish-brown color when stained with ammoniacal silver (unpublished observations) are also distinguishing properties. The BPP is one of the three principal phosphate acceptors *in vivo* (6, 12, 17–19) and in assays of the virion-associated protein kinase activity done *in vitro* (6, 11, 17, 18). This property may simply reflect the protein's high content of serine and threonine residues (i.e., 19%; ref. 52), both potential attachment sites for phosphate and O-GlcNAc (26).

Evidence from studies of the protein composition of intracellular and extracellular virus particles (6, 30, 53) and from surface galactosylation and radioiodination experiments (unpublished results) indicates that the BPP, like the other abundant phosphorylated proteins (e.g., upper and lower matrix proteins), is located in the tegument region of the particle, between the capsid and the envelope (6, 11). Although it is not an integral capsid protein, the presence of the BPP in the two-enveloped HCMV particles that contain a capsid (i.e., virions and noninfectious enveloped particles), and its absence from an enveloped particle lacking a capsid (i.e., dense bodies; refs. 17 and 36) indicates that it is more directly associated with the capsid structure than with the envelope. Further, its presence in normal amounts in noninfectious enveloped particles, which are structurally similar to virions but contain no DNA (36), suggests that its role does not involve an interaction with DNA. Given this apparent internal location, it was somewhat unexpected that the BPP was the most highly reactive virion protein in nitrocellulose immunoblot assays when probed with high titer sera from individuals infected with HCMV (e.g., acquired immunodeficiency syndrome patients; refs. 11 and 19). It will be important to determine whether this property is related to the presence of O-GlcNAc, as appears to be the case with the nuclear-pore-complex proteins (25, 26, 28). In this connection it is interesting that the BPP was recognized

by two nuclear-pore-complex-specific monoclonal antibodies (i.e., RL1 and RL2), whose binding requires O-GlcNAc residues (refs. 25 and 26; S. Adams, L. Gerace, D.B., and W.G., unpublished results).

Although the BPP of all HCMV strains examined contained this modification, neither SCMV nor HSV-1 virions contained a protein that was comparably well-labeled by GalTase. This was somewhat surprising since these viruses are grossly similar in structure, were produced in the same cell type, are generally considered to mature through similar assembly pathways, and, at least in the case of SCMVs, have a BPP homologue (e.g., ref. 6; L. Robson, J. Y. Lee, and W.G., unpublished results of DNA cross-hybridization). It is unknown whether the SCMV homologue and hypothetical HSV-1 counterpart are not labeled by GalTase because they have no O-GlcNAc, because they have additional saccharides attached to the O-GlcNAc, or because their O-GlcNAc residues are otherwise sterically inaccessible to GalTase.

Since capsids lacking DNA are generally not enveloped in SCMV- or HSV-infected cells (36, 54), it is possible that the tegument proteins of these viruses selectively promote maturation and envelopment of only DNA-containing capsids. If so, a modification such as reported here could interfere with this process and lead to indiscriminate envelopment of both filled and empty capsids, as happens with strains of HCMV (36). Whatever the explanation is of this apparently differential modification of the HCMV BPP, it is of interest both in connection with its influence on virus replication and assembly and as it lends itself to more general studies of the broader significance of this protein modification.

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