Site-Specific Glycosylation of the Human Cytomegalovirus Tegument Basic Phosphoprotein (UL32) at Serine 921 and Serine 952

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The virion basic phosphoprotein (BPP), UL32, of the human cytomegalovirus (HCMV) is a 149-kDa tegument protein that represents about 15% of the virion protein mass and is modified by O-linked *N*-acetylglucosamine (O-GlcNAc). O-GlcNAc has been postulated to mediate subunit-subunit interaction in many different types of intracellular protein complexes, while BPP may play a role in viral assembly and/or envelopment. This report describes the identification of the major O-GlcNAc attachment sites on the HCMV (AD169) BPP. Because the amount of BPP isolated from infectious virions was insufficient to determine the site(s) of glycosylation, the full-length protein has been characterized following overexpression in recombinant baculovirus-infected insect cells. The recombinant protein (rBPP) was electrophoretically (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunologically (by Western immunoassaying) indistinguishable from the BPP isolated from HCMV virions. In addition, the rBPP was modified by O-GlcNAc, and a comparison of the tryptic glycopeptides from the rBPP and native virion BPP indicated that their O-GlcNAc sites are the same. Furthermore, the major sites of O-GlcNAc attachment to the rBPP were mapped on high-performance liquid chromatography-purified glycopeptides by gas phase microsequencing, manual Edman degradation, and electrospray-mass spectrometry. The results demonstrate that the major sites of O-GlcNAc attachment to the BPP are Ser-921 and Ser-952. Identification of these sites will now enable mutagenesis studies to determine the influence of O-GlcNAc on the intracellular location, protein-protein interaction, and biological function of BPP. Finally, the fidelity of the addition of O-GlcNAc to rBPP in insect cells compared with native virion BPP is documented to demonstrate the possible general applicability of the baculovirus expression system to study O-GlcNAc on other low-abundance proteins.

Human cytomegalovirus (HCMV [also called human herpesvirus 5]) is a ubiquitous human pathogen that causes severe congenital abnormalities in newborn infants and a variety of life-threatening conditions in transplant recipients, patients with cancer or AIDS, and other immunocompromised individuals (1). Structurally, HCMV, like the other members of the herpesvirus family, consists of a double-stranded DNA genome enclosed within an icosahedral nucleocapsid. The nucleocapsid is surrounded by a phosphoprotein matrix known as the tegument and a membrane envelope (21, 61). The viral envelope contains several glycoproteins that often elicit antibody production presumably because of their surface location on the particle. Some of these envelope glycoproteins are involved in viral attachment and/or entry of the nucleocapsid into host cells (21, 50, 61, 67); therefore, they have been studied as targets for immunotherapy or as potential HCMV vaccine candidates (1, 6, 22, 57, 58, 72).

In addition to the envelope glycoproteins, the major tegument phosphoprotein, which is known as the basic phosphoprotein (BPP [also called pp150 or UL32]), is highly immunogenic (33, 35, 42, 43) even though it is not exposed on the surface of the enveloped virion (20, 33, 60). Recent studies have demonstrated the use of synthetic BPP peptides and

fusion proteins expressed in bacteria as substrates for immunoassays of patient serum as a means of rapid diagnosis of HCMV (44, 54, 63).

Although the function of BPP is unknown, its abundance and location in the tegument of the virion suggest that it may play a role in viral assembly and/or envelopment or during the initial stages of infection. Prior work on BPP has characterized it to the following extent. (i) The open reading frame (ORF) of BPP from HCMV strain AD169 has been identified elsewhere (35). It codes for a polypeptide of 1,048 amino acids corresponding to a molecular mass of about 113 kDa, but migrates anomalously in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (60) with an apparent molecular mass of about 149 kDa. (ii) BPP is present in all HCMV strains that have been tested, including both laboratory strains and clinical isolates (22a, 33), but has no apparent counterpart in either herpes simplex virus type 1 or 2, as judged by protein comparisons in SDS-PAGE gels (20) or by gene sequence analysis (35). (iii) BPP has been shown to be multiply phosphorylated *in vivo* and can act as an *in vitro* substrate for a virion-associated protein kinase activity (60). (iv) BPP has been shown to be modified by O-linked *N*-acetylglucosamine (O-GlcNAc) residues (3, 5) but not by other forms of N- or O-linked glycans (4).

O-GlcNAc is a unique posttranslational glycosylation of nuclear and cytosolic proteins first described structurally by Torres and Hart (70). This modification has since been demonstrated for a wide range of cytosolic and nuclear proteins, including RNA polymerase II (38, 39), RNA polymerase II

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transcription factors (34), nuclear pore proteins (17, 27, 30, 52, 65, 66), neurofilaments (16), oncoproteins (14, 15, 55), and viral fiber proteins (49). Although these proteins serve different functions in the cell, they are all phosphorylated, and those whose function is known can form reversible multimeric complexes based on their state of phosphorylation. These observations suggest that O-GlcNAc may also be involved in modulating the assembly and/or disassembly of such complexes. While the function of O-GlcNAc remains obscure, several lines of evidence also suggest that it is a dynamic regulatory modification much more akin to phosphorylation than to classical O- and N-linked glycosylation (23, 26, 28, 29). For example, the levels of O-GlcNAc on nuclear and cytosolic proteins fluctuate transiently after mitogen stimulation of B lymphocytes (36), and the turnover rate of O-GlcNAc on cytokeratins has been shown to be much faster than the rate of protein turnover (12, 13). In addition, O-GlcNAc levels of various nuclear and cytosolic proteins appear to change dramatically in a cell cycle-dependent manner (37).

As a first step toward mutagenesis studies to determine the function of O-GlcNAc, it is necessary to identify the sites of glycosylation. However, the low level of abundance of many O-GlcNAc-modified proteins often precludes isolation of sufficient mass for analysis of glycosylation sites. In this report, we describe the identification of two major O-GlcNAc attachment sites on the HCMV BPP expressed to a high level as a recombinant product (rBPP) in baculovirus-infected insect cells. The two major glycosylation sites are Ser-921 and Ser-952. In addition, the fidelity of the addition of O-GlcNAc to rBPP in baculovirus-infected insect cells, compared with the native HCMV virion BPP, is documented to demonstrate the possible general applicability of the baculovirus expression system to study O-GlcNAc modifications of other low-abundance proteins.

MATERIALS AND METHODS

Cells, viruses, and culture conditions. Human foreskin fibroblasts (HFF) were prepared, cultured, and infected as previously described (19). Details of HCMV strain AD169 are described elsewhere (20). *Spodoptera frugiperda* (Sf9) insect cells were obtained from Invitrogen and maintained in Grace's supplemented insect medium (Gibco no. 11605-011) containing an additional 1.2 mg of glutamine per liter and 10% fetal bovine serum (Gibco no. 200-6140AJ) either in monolayers or in spinner cultures at 27°C as described elsewhere (53). Monolayer cultures of Sf9 cells were infected with wild-type (Invitrogen no. B825-03) or recombinant *Autographa californica* multiply enveloped nuclear polyhedrosis virus (baculovirus) at a multiplicity of infection (MOI) of 0.1 for the production of high virus titer stocks or at a multiplicity of infection of 2 to 5 for overexpression of recombinant proteins. Virus titers were determined by plaque assays on six-well culture plates in Grace's complete medium containing 1.5% SeaPlaque agarose (FMC Bioproducts) (53).

Subcloning of HCMV UL32. The entire ORF of HCMV UL32, which codes for the virion BPP, was obtained within the 12.3-kb *HindIII*-J restriction fragment from the HCMV strain AD169 genome as part of a plasmid carrying ampicillin resistance from Bart Barrell and Mark S. Chee (Cambridge, United Kingdom). The location of UL32 in the HCMV genome is described elsewhere (11, 35). In order to minimize the amount of excess DNA that accompanied the BPP ORF in expression vectors, the entire ORF was subcloned from the 12.3-kb *HindIII*-J restriction fragment into the *Bam*HI polylinker of pGem4Z (Promega) as follows (see Fig. 1 for

schematic). (i) A unique *Mun*I site 119 bp downstream from the stop codon was converted to a *Bam*HI site by the addition of *Bam*HI linkers (NEB no. 1015) after cutting with *Mun*I (New England Biolabs). Restriction mapping confirmed the conversion of the *Mun*I site to a *Bam*HI site. The majority of the BPP ORF could then be excised from this plasmid via a unique *Sst*I (NEB) site 168 bp from the start codon and the newly generated *Bam*HI site downstream from the stop codon. This 3,105-bp *Sst*I-*Bam*HI fragment was purified and extracted from a 0.8% agarose gel. (ii) The first 168-bp fragment of the ORF was generated by a PCR strategy that also added a *Bam*HI site and an *Nde*I site near the start codon. Two DNA primers were synthesized for the PCR. One spanned the *Sst*I site 168 bp from the start codon, and the other contained the engineered *Bam*HI and *Nde*I sites and the first 17 bases downstream from the start codon. An Ampligene PCR kit (Perkin-Elmer Cetus) was used to amplify the first 168 bp of the BPP ORF for 30 cycles with the *HindIII*-J fragment containing plasmid as the template. A 1.4% agarose gel demonstrated a PCR product with the appropriate size which was subsequently digested with *Bam*HI and *Sst*I and purified in an agarose gel. (iii) The *Bam*HI-*Sst*I fragment (168 bp) from step ii and the *Sst*I-*Bam*HI fragment (3,105 bp) from step i described above were then ligated into the *Bam*HI polylinker of pGem4Z in a three-way ligation and transformation. Plasmids from some of the transformants were demonstrated to contain the entire BPP ORF by restriction mapping, and the fidelity of the PCR was confirmed by sequence analysis. This pGem4Z plasmid containing the BPP ORF in the *Bam*HI polylinker was designated pKG3.

Production of recombinant baculovirus expressing the BPP. The *Bam*HI cassette containing the BPP ORF was excised from pKG3 and subcloned into the *Bam*HI polylinker of pBlueBacIII (Invitrogen) and designated pKG8. Sf9 cells were cotransfected with linearized baculovirus DNA and pKG8 in a cationic liposome mixture as described by the suppliers (Invitrogen no. B825-03). At 3 days posttransfection, the infectious medium was harvested and used in plaque assays to detect recombinant baculovirus. Recombinant plaques, as judged by the expression of β -galactosidase and the loss of intracellular occlusions, were purified by several rounds of plaque assays and were stored at 4°C. This recombinant baculovirus expressing BPP instead of polyhedrin was designated KGrUL32.

Isolation of infected cell fractions. Sf9 cells were infected with either KGrUL32 or wild-type baculovirus at a multiplicity of infection of 2 to 5 in Grace's complete medium in the absence of serum. After 90 min, the inoculum was removed and replaced with complete medium containing 10% fetal bovine serum. At various times postinfection, the infected cells were harvested by centrifugation, washed with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline solution (Gibco) and solubilized as either whole-cell lysates or as Nonidet P-40 (NP-40) cytosolic and nuclear fractions. For whole-cell lysates, the infected cells were washed with HEPES-buffered saline solution, solubilized in 2× Laemmli solubilization buffer (41), and stored at -80°C until further use. For NP-40 cytosolic and nuclear fractions, the infected cells were washed with HEPES-buffered saline solution, solubilized with 0.5% NP-40-150 mM NaCl in 10 mM HEPES, pH 7.4, and separated into cytosolic (supernatant) and nuclear (pellet) fractions by centrifugation in a Brinkmann model 5412 microcentrifuge. A protease inhibitor cocktail containing aprotinin, leupeptin, antipain, benzamidin, chemostatin, papstatin, and phenylmethylsulfonyl fluoride (31) was included in all fractions to prevent excess proteolysis.

SDS-PAGE, Western blots (immunoblots), and fluorography. Proteins were separated by SDS-PAGE as described by Laemmli (41) and were stained with Coomassie brilliant blue (18). Western blotting was performed on proteins transferred to polyvinylidene difluoride as described previously (71), but with the following modifications. Rabbit polyclonal antiserum against BPP (see below) was used as a primary antibody at a 1:1,000 dilution with donkey anti-rabbit immunoglobulin G coupled to horseradish peroxidase at a 1:5,000 dilution as the secondary antibody. Detection of the horseradish peroxidase activity was by enhanced chemiluminescence and fluorography as described by the supplier (Amersham). Tritium-labeled glycoproteins were visualized by impregnation of gels with 1 M sodium salicylate and fluorography (10).

Rabbit antiserum. Polyclonal antibodies to the carboxyl terminus of the BPP were generated in New Zealand White rabbits as follows. (i) A synthetic peptide representing the last 12 carboxyl-terminal amino acids of the BPP from HCMV strain AD169 was synthesized with the addition of a single cysteine residue at the amino-terminal end. (ii) This peptide was coupled via the amino-terminal cysteine to either bovine or chicken egg albumin through the heterobifunctional cross-linker N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) as described by the supplier (Pierce). (iii) These conjugates were used as immunogens to produce polyclonal antisera to BPP in New Zealand White rabbits. The resulting antiserum after three challenges with immunogen (about 200 µg each) was shown to be specific for the BPP (see Fig. 2).

GTase labeling of the BPP. BPP from HCMV virions or from Sf9 cells infected with recombinant baculovirus (KGrUL32) was labeled with UDP-[4,5-³H]galactose (³H]Gal) (38.5 Ci/mmol; NEN) and autogalactosylated bovine milk galactosyl-transferase (GTase) to probe for O-GlcNAc as described previously (73).

Carbohydrate characterization. The acceptor for the enzymatic transfer of [³H]galactose to BPP was demonstrated to be exclusively O-GlcNAc by its resistance to hydrolysis with peptide N-glycosidase F (PNGase F) and its sensitivity to alkaline β-elimination and reduction both as described previously (5, 25). The β-elimination products were identified by high-pH anion-exchange chromatography with pulsed-ampereometric detection (HPAE-PAD) by isocratic elution in 200 mM NaOH at 1.0 ml/min for 25 min on a Dionex Bio-LC equipped with a CarboPac-MA1 column.

Enzymatic and chemical digestion of GTase-labeled BPP. Labeled BPP was digested with trypsin (Boehringer Mannheim sequencing grade or Worthington tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] trypsin) in 100 mM ammonium bicarbonate, pH 7.9, for 16 h at 37°C. The glycopeptides were high-performance liquid chromatography (HPLC) purified and further digested with proline-specific endopeptidase (Seikagaku) in 100 mM ammonium bicarbonate (pH 7.9) for 16 h at 37°C. GTase-radiolabeled BPP glycopeptides were also compared by SDS-PAGE after partial chemical hydrolysis at tryptophan residues by treatment with N-chlorosuccinamide (NCS) (45) as described previously (3, 5).

RP-HPLC. Tritium-labeled glycopeptides were isolated by several rounds of reverse-phase HPLC (RP-HPLC) on a Rainin model (48-00XL) HPLC system equipped with either a Vydac 5-µm C18 column (4.6 by 250 mm) or a Vydac narrow-bore C18 column (2.1 by 150 mm). Glycopeptides were bound to the column in either 0.07% trifluoroacetic acid or 0.1% phosphoric acid containing 100 mM sodium perchlorate (47) and eluted with an increasing gradient of acetonitrile as indicated in the figure legends. Elution of peptides was monitored by A₂₁₄, and the glycopeptides were detected by liquid

scintillation spectrometry of a small aliquot of each fraction collected.

Gas phase sequencing and manual Edman degradation. The primary sequences of HPLC-purified glycopeptides were determined by gas phase automated Edman degradation on a Porton Instruments (Tarzana, Calif.) model PI 2090E micro-sequencing system equipped with an on-line PTH amino acid analyzer. The sites of glycosylation were determined from an aliquot of each of the sequenced glycopeptides by manual Edman degradation (68) with modifications as described by Kelly et al. (39). Briefly, each labeled glycopeptide was covalently coupled via the COOH terminus to an aryl amine membrane disk with aqueous carbodiimide (Millipore). Single amino acids were then removed from the NH terminus by standard Edman degradation chemistry. The released amino acid derivative from each cycle was collected, dried, neutralized, and assayed by scintillation spectrometry to determine the cycle at which the [³H]Gal-labeled amino acid was released.

ESMS of glycopeptides. The electrospray-mass spectrometry (ESMS) spectra of HPLC-purified glycopeptides were recorded, following acetonitrile elution from a capillary C18 RP-HPLC column equilibrated in 0.1% formic acid at a flow rate of 4 µl/min on a Sciex API-III triple quadrupole mass spectrometer equipped with an atmospheric-pressure ion source. Positive-ion mass spectra were acquired at an orifice potential of 70 V by scanning the first quadrupole (Q1) over a mass/charge (*m/z*) range of 325 to 2,200 at a rate of 6 s per scan. Data were acquired and processed with Sciex API-MacSpec software version 3.22.

RESULTS

Expression of full-length rBPP in *S. frugiperda* cells. The subcloning of the BPP ORF into pBlueBacII and the generation of the KGrUL32 with the BPP ORF replacing the polyhedrin gene are summarized in Fig. 1. To determine whether the recombinant virus could express full-length BPP, Sf9 cells were infected with KGrUL32 or wild-type baculovirus. Lysates of these infected cells were then assayed for the production of BPP by SDS-PAGE (Fig. 2A). Coomassie blue staining demonstrated the presence of a recombinant protein with a molecular mass of 149 kDa (lane 2) that comigrated with HCMV (AD169) virion BPP (lane 1) and was not present in lysates of wild-type baculovirus-infected cells (lane 3). The absence of the polyhedrin gene product (molecular mass, 29 kDa) in the KGrUL32 lysate (lane 2) compared with the wild-type lysate (lane 3) also established that the recombinant virus was produced by the expected homologous recombination. This 149-kDa Coomassie-stained band was confirmed as BPP by a Western immunoassay using a rabbit polyclonal antibody directed against a carboxyl-terminal synthetic peptide of BPP (Fig. 2B). The polyclonal peptide antiserum reacted specifically with HCMV (AD169) virion BPP (lane 1) and with the rBPP from infected Sf9 cell lysates (lane 2), but not with any proteins in the wild-type baculovirus-infected cells (lane 3). The faster-migrating bands that react with antiserum in the recombinant lysate (lane 2) are believed to be proteolytic fragments of the full-length protein rather than truncated forms (because of incomplete transcription or translation), since they did not appear in all lysate preparations and since the rabbit antiserum is directed against the last 12 amino acids of the carboxyl terminus of BPP.

Maximizing production and extraction of rBPP from infected Sf9 cells. Infected cells were fractionated into crude nuclear and cytosolic fractions by extraction with 10 mM

FIG. 1. Schematic of the subcloning of BPP ORF (UL32) and the generation of a recombinant baculovirus (KGrUL32). The entire ORF of the HCMV strain AD169 BPP was subcloned from the *Hind*III-J genome fragment into pGem4Z and denoted pKG3. The insert was amplified in pKG3, excised with *Bam*HI, inserted into the *Bam*HI polylinker of pBlueBacIII, and used to generate a recombinant baculovirus (designated KGrUL32) as described in detail in Materials and Methods.

HEPES, pH 7.4, containing 0.5% NP-40 and 150 mM NaCl and then by centrifugation to produce a nuclear pellet and a cytosolic supernatant. Greater than 90% of the rBPP was present in the cytosolic supernatant fraction of these extracts, as judged by Western immunoassays (data not shown); therefore, subsequent preparations of rBPP were prepared from the NP-40 cytosolic supernatants which showed a marked enrichment of the rBPP to total protein (compare Fig. 2, lane 1, with Fig. 3A, lane 1). A time course of infection with the KGrUL32 also revealed that the maximum production of rBPP occurs by 3 days postinfection (data not shown); therefore, all subsequent preparations of rBPP were harvested at 3 days postinfection.

The rBPP is modified by O-GlcNAc. Bovine milk GTase can specifically transfer [3 H]galactose from the nucleotide sugar UDP-[3 H]Gal to any accessible terminal GlcNAc residues, thus providing a means to covalently tag terminal GlcNAc residues on glycoproteins (62, 73). One can then distinguish between N-linked and O-linked glycans by a combination of enzymatic digestion with PNGase F to release N-linked glycans and chemical hydrolysis by alkaline β -elimination to release O-linked glycans. Therefore, we used GTase labeling to probe for O-GlcNAc on rBPP. The NP-40 cytosolic supernatants from KGrUL32-infected Sf9 cells at 3 days postinfection were

FIG. 2. KGrUL32 expresses the full-length BPP. HCMV virions and baculovirus-infected Sf9 cells were solubilized in Laemmli buffer and resolved by SDS-10% PAGE as described in Materials and Methods. Proteins were visualized by Coomassie staining (A) or by Western immunoassaying (B) of duplicate lanes of the same gel. Lanes: 1, HCMV (strain AD169) virions; 2, lysate of KGrUL32-infected Sf9 cells; 3, lysate of wild-type baculovirus-infected Sf9 cells. The migration positions of prestained protein molecular weight markers (sizes in kilodaltons) are indicated between the panels.

GTase labeled, and the resulting radiolabeled glycoproteins were resolved by SDS-PAGE and visualized by Coomassie staining and by fluorography (Fig. 3A). The rBPP (lanes 1 and 3) and the ovalbumin (lanes 2 and 4) were readily labeled with the [3 H]Gal, thus demonstrating the existence of terminal GlcNAc residues as substrates for GTase. In addition, two other prominent bands from the NP-40 cytosolic fraction at about 90 and 41 kDa were also substrates for the [3 H]Gal transfer (lane 3). Since the 41-kDa band was also labeled in extracts of the wild-type baculovirus-infected Sf9 cells but not in the mock-infected cells (data not shown), it was concluded that it is a baculovirus-encoded protein. The most likely candidate is a 41-kDa baculovirus tegument protein (gp41) that has recently been reported to be modified by O-GlcNAc (74). The 90-kDa band was only occasionally present in preparations of KGrUL32-infected cells, which is consistent with data in Fig. 2B and 4, suggesting that this band is a proteolytic product of the full-length rBPP. The rBPP was also completely resistant to digestion with PNGase F, unlike the N-linked glycan of ovalbumin (Fig. 3B), thereby demonstrating that the glycans of rBPP are most likely O linked. This was confirmed by alkaline β -elimination and reduction of SDS-PAGE gel-purified HCMV virion BPP and rBPP. In both cases, all of the released radiolabeled glycans migrated with the disaccharide alditol of Gal β 1,4GlcNAc by gel filtration chromatography on Bio-Gel P4 (data not shown) and by HPAE-PAD (Fig. 3C) under conditions that clearly resolve related reduced disaccharides. Thus, rBPP expressed in insect cells, like HCMV virion BPP, is modified by O-GlcNAc.

Comparison of GTase-labeled glycopeptides from rBPP and HCMV BPP. In order to effectively use the overexpressed rBPP as a tool to map the major sites of glycosylation on HCMV BPP, it was important to demonstrate that the O-GlcNAc-modified glycopeptides from rBPP are the same as those in BPP isolated from HCMV virions. Because of the limited mass of native BPP that could be obtained from isolated virions, equally radiolabeled quantities of GTase-labeled glycopeptides of rBPP and HCMV virion BPP were

FIG. 3. rBPP is modified by O-GlcNAc. Ovalbumin and NP-40 cytosolic supernatants from KGrUL32-infected Sf9 cells were GTase labeled as described in Materials and Methods. (A) The radiolabeled proteins resolved by SDS-10% PAGE and visualized by Coomassie staining (lanes 1 and 2). Proteins tagged with [3 H]Gal were detected by fluorography of a duplicate gel impregnated with 1 M sodium salicylate (lanes 3 and 4). The migration positions of protein molecular weight markers (sizes in kilodaltons) are shown between the panels. (B) GTase-labeled ovalbumin (lanes 1 and 2) and NP-40 cytosolic supernatant (lanes 3 and 4) pretreated with PNGase F and resolved by SDS-7.5% PAGE. The radiolabeled proteins were visualized by fluorography as described above. (C) The alkaline β -elimination products of GTase-labeled HCMV BPP and rBPP pooled after Bio-Gel P4 gel filtration chromatography (not shown) and then subjected to HPAE-PAD to determine the structure of the released glycan. The elution positions of the β -elimination products were demonstrated by liquid scintillation spectrometry of each 0.5-min fraction. The elution positions of common disaccharide alditol are indicated by arrowheads. a, Gal β 1,3GalNAcitol; b, Gal β 1,3GlcNAcitol; c, Gal β 1,4GalNAcitol; d, Gal β 1,4GlcNAcitol. Closed triangles, HCMV BPP; open triangles, rBPP.

directly compared following either chemical or enzymatic digestion. First, chemical cleavage by partial hydrolysis at tryptophan residues with NCS and then separation of the resulting fragments by SDS-PAGE revealed that the fragments from both BPP samples gave identical radiolabeled products of

FIG. 4. Glycopeptide comparisons of HCMV BPP and rBPP after chemical digestion. The GTase-labeled BPP samples were separated by SDS-7.5% PAGE. The lanes of interest were excised from the first gel, treated with NCS, applied to the top of a second-dimension 12% gel, and subjected to PAGE all as described in Materials and Methods. After electrophoresis, the second-dimension gel was impregnated with 1 M sodium salicylate and fluorographed. The fluorograph of the 7.5% gel stripes across the top of the 12% gels represents a duplicate lane from the same gel in addition to those that were treated with NCS and used to overlay the second-dimension gels. The arrow on the rBPP gel highlights the presence of identical NCS products produced from a lower-molecular-weight species from the first-dimension gel (see text for details).

about 40 and 55 kDa (Fig. 4). This result suggested that the sites of glycosylation were on the same domains of rBPP and HCMV native virion BPP. Treatment with NCS also revealed labeled fragments with molecular masses of 40 and 55 kDa at lower molecular retentions from the first-dimension gel (Fig. 4, arrow). These results are consistent with the presence of proteolytic fragments of rBPP in the NP-40 cytosolic fraction prior to GTase labeling as seen in Fig. 2B and 3A.

A more detailed comparison of the GTase-labeled glycopeptides was achieved by extensive trypsin treatment of equivalent counts of rBPP and HCMV BPP, followed by C18 RP-HPLC of the resulting glycopeptides. Separation of the glycopeptides at either pH 2 (Fig. 5) or pH 7 (not shown) resolved three tritium-labeled peaks. Although the stoichiometry of labeling with GTase in the latest eluting peak was different for the rBPP (Fig. 5B) versus the HCMV BPP (Fig. 5A), in both cases the retention times of all [3 H]Gal-labeled peaks were identical, thus suggesting that rBPP expressed in insect cells contains the same major O-GlcNAc-modified glycopeptides as BPP isolated from HCMV virions.

Purification of GTase-labeled glycopeptides from rBPP. About 200 μ g of GTase-labeled rBPP from infected Sf9 cell NP-40 cytosolic fractions was digested with 20 μ g of trypsin, and the resulting peptides were separated by C18 RP-HPLC. A_{214} (Fig. 6A) showed an abundance of peptides produced by the tryptic digestion, while liquid scintillation spectrometry of an aliquot from each 1-min fraction collected demonstrated three peaks of tritium, with peak b as the major radiolabeled species (Fig. 6B). Subsequently, the radiolabeled peaks (a, b, and c) were pooled separately and further resolved by RP-HPLC using a more shallow acetonitrile gradient to further

FIG. 5. Glycopeptide comparisons of HCMV BPP and rBPP after extensive trypsin digestion. GTase-labeled BPP samples were excised from an SDS-7.5% PAGE gel, equilibrated in 100 mM ammonium bicarbonate, macerated through a fine-mesh screen, and digested with a fivefold excess of TPCK-trypsin. The resulting labeled glycopeptides were separated by RP-HPLC on a C18 column (4.6 by 250 mm) equilibrated in 0.07% TFA with a linear gradient of 0 to 60% acetonitrile at 0.75 ml/min for 60 min. Each 1-min fraction was collected, dried, and quantified by liquid scintillation spectrometry. HCMV BPP (A) and rBPP (B) tryptic glycopeptides are shown.

purify the glycopeptides away from nonlabeled peptides. Peaks a and b were resolved into multiple peptides as judged by A_{214} (Fig. 7a and b), with only one peak in each corresponding to the [^3H]Gal label (Fig. 7a' and b'). Further separation of peak c, however, resulted in multiple peptides corresponding to the [^3H]Gal label (Fig. 7c and c'). The low abundance and continued coelution of the multiple peaks from pool c with nonglycosylated peptides precluded additional characterization. The asymmetry of the A_{214} profile from peak a (Fig. 7a) suggested that the peptide was not pure after the second-dimension HPLC gradient; therefore, it was finally resolved by a third gradient in a different solvent system. This gradient resulted in a single, symmetrical A_{214} peak (Fig. 8a) that corresponded to the [^3H]Gal-labeled peptide (Fig. 8a').

Identification of O-GlcNAc attachment sites on rBPP. The primary sequences of the purified glycopeptides from peaks a and b were determined by gas phase automated Edman sequencing (Fig. 9A, glycopeptides 1 and 2). The exact sites of O-GlcNAc attachment could then be established by manual Edman degradation (see Materials and Methods) of a portion of each HPLC-purified, [^3H]Gal-labeled glycopeptide. This method allows for the collection of the N-terminal amino acid derivative after each cycle of the Edman degradation. The positions of the [^3H]Gal-labeled glycoamino acids are then determined by liquid scintillation spectrometry of the sample from each cycle. However, the manual Edman degradation of glycopeptides 1 and 2 revealed no labeled glycoamino acids after 10 cycles. This suggested that the sites of glycosylation were located toward the carboxyl termini of glycopeptides 1 and 2. Therefore, a separate preparation of these tryptic glycopeptides was subjected to digestion with proline-specific endopeptidase (prolidase) prior to the second-dimension

HPLC purification to generate smaller glycopeptides. Gas phase sequencing of these doubly digested glycopeptides gave the expected prolidase product (Fig. 9A, glycopeptides 3 and 4) from glycopeptides 1 and 2, respectively. In addition, the manual Edman degradation demonstrated that these peptides both contained O-GlcNAc modifications at cycle number 2 (Fig. 9B), corresponding to glycosylation of Ser-921 and Ser-952, respectively. Consistent with the presence of O-GlcNAc at Ser-921 and Ser-952, the amount of serine recovered during automated sequencing was considerably less at the sites of glycosylation than that for the surrounding amino acids (Fig. 9B). Furthermore, the absence of any detectable serine at the glycosylation site of glycopeptide 4 during gas phase sequencing suggests that the stoichiometry of the O-GlcNAc modifications at Ser-952 is 1:1.

HPLC-ESMS of glycopeptides 1 and 2. To confirm that the tryptic glycopeptides isolated from peaks a and b contained a single O-GlcNAc modification extended with a galactose, a portion of these peptides was analyzed by HPLC-ESMS as described in Materials and Methods. Glycopeptide 1 with a single O-GlcNAc-Gal attached to Ser-921 has a calculated mass of 2,055 Da. Consistent with this mass, the spectrum generated by HPLC-ESMS demonstrated that this peptide contained major peaks with m/z ratios of 1,029 and 686, corresponding to the doubly protonated and triply protonated ions of the molecular ion (2,055 Da) (Fig. 10A). Furthermore, the remaining major peaks at m/z 845 and 366 are likely to be the doubly protonated forms of the nonglycosylated peptide (1,690 Da) and its corresponding free GlcNAc-Gal (366 Da), which was partially released during ionization of the glycopeptide. Release of both O- and N-linked glycans from glycopro-

FIG. 6. Preparative isolation of rBPP tryptic peptides by RP-HPLC. GTase-labeled rBPP was digested with TPCK-trypsin as described in Materials and Methods and applied to a Vydac C18 column (4.6 by 250 mm) in 0.1% phosphoric acid-100 mM NaClO_4 (47). The peptides were eluted at 0.75 ml/min with a linear gradient of 0 to 60% acetonitrile for 90 min. (A) UV A_{214} ($\text{Abs}_{214\text{nm}}$) (the output expressed in millivolts); (B) liquid scintillation spectrometry of an aliquot of each 1-min fraction. Arrows indicate the elution positions of the [^3H]labeled peaks a, b, and c.

FIG. 7. Second-dimension RP-HPLC purification of GTase-labeled tryptic glycopeptides of rBPP. The radiolabeled peaks (a, b, and c) from Fig. 6 were pooled, concentrated, and further resolved by RP-HPLC as described in the legend to Fig. 6, but with a linear gradient of 12 to 48% acetonitrile for 80 min. (a, b, and c) A_{214} (Abs_{214nm}); (a', b', and c') liquid scintillation spectrometry of an aliquot of each 0.5-min fraction from profiles a, b, and c, respectively. Arrows indicate the elution positions of 3H -labeled glycopeptides.

teins and peptides by collision-induced fragmentation due to ionization conditions has been reported elsewhere (9). Similarly, glycopeptide 2 with a single GlcNAc-Gal attached to Ser-952 has a calculated mass of 2,575 Da. The HPLC-ESMS spectrum of this peptide contained both the doubly protonated (m/z 1,289) and the triply protonated (m/z 859) ions of the molecular ion (2,575 Da) (Fig. 10B). As described above, the ionization energy converted a portion of this glycopeptide into the nonglycosylated peptide (2,211 Da), which is seen in its doubly protonated form (m/z 1,107) and as the free GlcNAc-Gal (m/z 366). Tryptic digestion of the BPP predicts no other peptides or glycopeptides that could account for these mass profiles. Therefore, this HPLC-ESMS analysis of glycopeptides 1 and 2 confirms that each of these peptides is modified by a single O-GlcNAc-Gal unit. These results also preclude the possibility that these peptides are phosphorylated to a great extent, since no mass ions were detected that could account for a phosphorylated peptide. Furthermore, when these results are taken together with the sequence analysis and the manual Edman degradation from Fig. 9, it is clear that Ser-921 and Ser-952 are the major sites of O-GlcNAc attachment to BPP.

DISCUSSION

The primary findings of this report are that (i) the full-length BPP from HCMV strain AD169 can be overexpressed as a recombinant baculovirus in Sf9 insect cells, (ii) baculovirus-infected insect cells contain the necessary enzymes and precursors to attach O-GlcNAc to the overexpressed rBPP, (iii) the glycopeptides produced by chemical and enzymatic digestion of rBPP are the same as those found in native BPP isolated from HCMV virions, and (iv) the major sites of O-GlcNAc attachment to rBPP are Ser-921 and Ser-952.

The baculovirus expression system has been widely used to overexpress and study numerous proteins; however, it is limited in studies of mammalian cell-derived glycoproteins be-

cause of the variations in glycoprotein processing known to occur in insect cells (2, 7, 8, 32, 48). Unlike classical N-linked and O-linked glycosylation which occurs in the lumen of the endoplasmic reticulum and in the Golgi apparatus and requires the concerted action of multiple glycosidases and glycosyltransferases, O-GlcNAc attachment occurs in the cytosol and requires only a single, peptide-specific O-GlcNAc transferase (23, 24, 26, 28, 29). As a result, if insect cells contain sufficient O-GlcNAc transferase activity, then they should support this posttranslational modification and enable O-GlcNAc modifications on proteins overexpressed from recombinant baculoviruses during infection of Sf9 cells to be studied. A recent study which demonstrated that gp41, a baculovirus tegument protein, is modified by O-GlcNAc provided evidence for a functional O-GlcNAc-transferase activity in Sf9 insect cells (74). In addition, insect cells have been reported to produce only a limited amount of N-linked glycoproteins that terminate with GlcNAc (7, 8, 32). This is consistent with a recent report demonstrating that Sf9 cells express a much lower level of GlcNAc transferase I and GlcNAc transferase II activities than HepG2 cells (2). This characteristic makes the baculovirus system particularly attractive to study O-GlcNAc, since lower levels of terminal GlcNAc on N-linked glycoproteins would prevent the background labeling with GTase-UDP-[3H]Gal, thus eliminating a competing reaction when probing for O-GlcNAc.

Expression of O-GlcNAc-modified proteins in the baculovirus system has been reported previously (51, 59); however, in these reports, no attempt was made to demonstrate that the sites of glycosylation are the same as those in the glycoproteins from their natural source. Recently, Ku and Omary (40) presented evidence that cytokeratins 8 and 18 expressed as recombinant baculovirus in Sf9 cells are glycosylated with O-GlcNAc and that the major sites of glycosylation are the same as those found in human HT29 cells, as judged by tryptic

peptide mapping. In addition, they noted that there were some quantitative differences in the intensity of labeled glycopeptides in insect versus human cells. Consistent with these results, we demonstrate here that rBPP is glycosylated in insect cells and that the glycopeptides produced by chemical or enzymatic digestion are the same as those from native BPP isolated from HCMV virions. However, as is indicated in Fig. 5, attachment of O-GlcNAc to the glycopeptides in the later eluting peak appears to be more favorable in insect cells, resulting in more incorporation of the [^3H]Gal compared with the HCMV BPP. A likely explanation for these results is that the O-GlcNAc transferase(s) in insect cells may have slightly different peptide specificities for O-GlcNAc attachment or that the overexpressed protein is folded in a different manner in insect cells, such that additional O-GlcNAc sites are exposed to the GlcNAc transferase enzyme. The acceptor peptide specificities of O-GlcNAc transferase in insect cells and other cell types are being investigated to address these questions.

The major sites of O-GlcNAc attachment to rBPP have been mapped to Ser-921 and Ser-952 near the carboxyl terminus of this 1,048-amino-acid protein. The amino acid contexts of these O-GlcNAc attachment sites are similar to those described for other O-GlcNAc-modified proteins; however, an exact amino acid consensus sequence for O-GlcNAc attachment to all proteins has not yet been identified (23, 28, 29). This suggests that there may be many O-GlcNAc transferase activities for different sites of attachment, which is similar to the presence of many kinases with different phosphorylation site specificities. When the known sites of O-GlcNAc attachment are examined, it is clear that a majority of sites contain a proline within three amino acids of the glycoamino acid, and in many cases a valine is also nearby (23, 28, 29). Consistent with this trend, both of the major sites of O-GlcNAc attachment to

FIG. 8. Third-dimension RP-HPLC purification of peak a. The radiolabeled peak a from Fig. 7 was applied to a Vydac C18 column (2.1 by 150 mm) equilibrated in 0.07% TFA. The peptides were eluted at 0.15 ml/min with a linear gradient of 0 to 60% acetonitrile for 90 min. (A) UV A_{214} (Abs_{214nm}), with the output expressed in millivolts; (B) liquid scintillation spectrometry of an aliquot of each 1-min fraction.

FIG. 9. Gas phase microsequencing and identification of the O-GlcNAc attachment sites on glycopeptides from rBPP. The primary sequences of RP-HPLC-purified glycopeptides were determined by gas phase microsequencing as described in Materials and Methods. (A) The primary sequences of glycopeptides from peaks a and b (glycopeptides 1 and 2) and peaks a and b after digestion with proline-specific endopeptidase (prolidase) and RP-HPLC purification of the resulting glycopeptides (glycopeptides 3 and 4); (B) aliquots of glycopeptides 3 and 4 subjected to manual Edman degradation as described in Materials and Methods to determine the sites of O-GlcNAc attachment. Picomoles recovered from each cycle of the gas phase sequencing of glycopeptides 3 and 4 in panel A are also given. D, the amount of tritium that remained bound to the aryl amine disks after 9 cycles of manual Edman degradation.

BPP contain a proximal proline (Pro-919 and Pro-950, respectively), and Ser-921 also has an adjacent valine, Val-920. Within the same region of the BPP there are several other potential sites of glycosylation which may account for the other very low-abundance tryptic glycopeptide peaks that were partially resolved but could not be identified from peak c in Fig. 6 and 7.

The function of the O-GlcNAc modification of BPP is presently unknown; however, the location of this protein in the tegument region of the virion suggests that BPP may be important during the initial stages of infection or during envelopment of the infectious virion. Immunolocalization of BPP in infected human foreskin fibroblast cells has shown that BPP is located primarily in the cytosol and appears to be excluded from the nucleus (46, 63). These findings provide some insight into the debate over the site of final envelopment of HCMV virions during egress from the cell. By analogy with the envelopment of herpes simplex virus types 1 and 2 (61), the envelopment of HCMV was believed to occur at the inner nuclear membrane. However, considerable evidence is now

FIG. 10. HPLC-ESMS extracted ion profiles of glycopeptides 1 and 2. The mass spectra of glycopeptides 1 (A) and 2 (B) from Fig. 9A were recorded by HPLC-ESMS as described in Materials and Methods. The predicted structure and corresponding calculated mass for each peptide are shown above the extracted ion profile. % int., percent relative intensity; m/z , mass-to-charge ratio of extracted ions.

available to support an alternative mechanism of HCMV envelopment in which envelopment and/or de-envelopment of the nucleocapsid across the inner and/or outer nuclear envelope is followed by final envelopment at some cytosolic vesicle (56, 64, 69). Consistent with a mechanism of cytosolic envelopment of the nucleocapsid, the cytosolic location of the BPP puts it in a topologically correct compartment and suggests that it may be involved in the envelopment process. Furthermore, a role for the O-GlcNAc-modified BPP in this mechanism is possible, since many other O-GlcNAc-modified glycoproteins are involved in the formation of protein-protein complexes (23, 28, 29). Therefore, the BPP may interact with other cytosolic proteins, the capsid proteins, or perhaps integral membrane proteins as a signal for envelopment. Identification of the major sites of glycosylation on BPP will now enable mutagenesis studies to be done to determine the influence of O-GlcNAc modifications on the intracellular location, protein-protein interaction, and other biological and biochemical properties of this interesting HCMV protein.

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