Characterization of Phosphoproteins and Protein Kinase Activity of Virions, Noninfectious Enveloped Particles, and Dense Bodies of Human Cytomegalovirus

CLINTON ROBY AND WADE GIBSON*

Virology Laboratories, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 10 February 1986/Accepted 14 May 1986

Phosphorylation of the proteins of human cytomegalovirus (CMV) virions, noninfectious enveloped particles (NIEPs), and dense bodies was investigated. Analyses of particles phosphorylated in vivo showed the following. (i) Virions contain three predominant phosphoproteins (i.e., basic phosphoprotein and upper and lower matrix proteins) and at least nine minor phosphorylated species. (ii) NIEPs contain all of these and one additional major species, the assembly protein. (iii) Dense bodies contain only one (i.e., lower matrix) of the predominant and four of the minor virion phosphoproteins. Two-dimensional (charge-size) separations in denaturing polyacrylamide gels showed that the relative net charges of the predominant phosphorylated species ranged from the basic phosphoprotein to the more neutral upper matrix protein. In vitro assays showed that purified virions of human CMV have an associated protein kinase activity. The activity was detected only after disrupting the envelope; it had a pH optimum of approximately 9 to 9.5 and required a divalent cation, preferring magnesium to manganese. In vitro, this activity catalyzed phosphorylation of the virion proteins observed to be phosphorylated in vivo. Peptide comparisons indicated that the sites phosphorylated in vitro are a subset of those phosphorylated in vivo, underscoring the probable biological relevance of the kinase activity. Casein, phosphitin, and to a minor extent lysine-rich histones served as exogenous phosphate acceptors. Arginine-rich and lysine-rich histones and protamine sulfate, as well as the polyamines spermine and spermidine, stimulated incorporation of phosphate into the endogenous viral proteins. Virions of all human and simian CMV strains tested showed this activity. Analyses of other virus particles, including three intracellular capsid forms (i.e., A, B, and C capsids), NIEPs, and dense bodies, indicated that the active enzyme was not present in the capsid. Rate-velocity sedimentation of disrupted virions separated the protein kinase activity into two fractions: one that phosphorylated exogenous casein and another that phosphorylated primarily the endogenous virion proteins.

Virions of primate strains of cytomegalovirus (CMV) contain three predominant phosphorylated proteins which, like the phosphoproteins of herpes simplex virus (HSV) (15, 30, 40), appear to be located in the tegument region of the particle (10, 11, 13). Two of these, the basic phosphoprotein (human CMV [HCMV], 149 kilodaltons [kDa]; Colburn, 119 kDa) and the upper matrix protein (HCMV, 74 kDa; Colburn, 69 kDa), are underrepresented in the infected cell (10, 53) compared with their relative abundance in virions. It was also found that the intracellular form of the upper matrix protein of strain Colburn CMV undergoes a late posttranslational phosphorylation that alters its electrophoretic mobility (53). Although neither the explanation of these findings nor the significance of tegument protein phosphorylation is known, both may reflect late maturational events of virus assembly and be related to the activity of a virion-associated protein kinase.

The presence of virion-associated protein kinases is typical of enveloped viruses (1, 17, 18, 36, 41, 45, 46, 49, 52) and has been reported for many members of the herpes group (8, 39, 43, 48, 51), including CMV (31). Although the function of kinases contained within virions is unclear, there is evidence from several systems of a physical association between kinase enzymes and the proteins which they appear to phosphorylate. For example, the HSV virion kinase partitions predominantly with the detergent-insoluble capsid teg-

ument structure, which contains its putative substrates (30). Similarly, it has been shown with vesicular stomatitis virus that a capsid-tegument-like core structure (i.e., the vesicular stomatitis virus ribonucleoprotein complex) contains a protein kinase activity (L protein; 44) as well as its substrate, the NS protein (21, 44). An association between kinase enzyme and protein substrate has also been shown with murine leukemia virus (57), in which it has been suggested that the phosphorylation of Pr65^{gag} is related to cleavage of this polyprotein and maturation of the virion (35, 56).

For purposes of further characterizing the architectural and intermolecular associations of the virion protein kinase(s), CMV provides several attractive and comparatively unusual features. First, CMV infection gives rise to three distinct species of enveloped particles: (i) complete particles containing DNA, capsid, tegument, and envelope (e.g., virions); (ii) particles that are structurally and compositionally similar to virions but contain no DNA (i.e., noninfectious enveloped particles [NIEPs]); and (iii) large enveloped spherical aggregates composed primarily of lower matrix protein and containing neither DNA nor a capsid (i.e., dense bodies) (13, 22). The absence of specific architectural elements from these enveloped particles, as well as from intracellular capsid forms (e.g., nucleocapsid from dense bodies, DNA from NIEPs, and envelope and tegument from intranuclear capsids), offers an alternative to virion fractionation experiments for studying protein kinase associations. Second, the CMV virion appears to be more

* Corresponding author.

completely disrupted by treatment with nonionic detergents (e.g., 0.5% Nonidet P-40 [NP-40]) than is that of HSV. This property might provide an advantage in attempts to fractionate the enzyme from the particle and determine whether virions of CMV, like those of pseudorabies virus (48), contain multiple protein kinase activities.

In this report we partially characterized the gross protein kinase activity associated with virions of CMV, used comparative analyses of different particle types to obtain information about its structural localization, and demonstrated that the virion-associated protein kinase can be separated into at least two physically and enzymatically distinguishable activities or forms.

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MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were prepared, maintained, and infected with virus as described elsewhere (10). HCMV strain AD169 (42) was used in most of the experiments reported here. Other viruses used were HCMV strains Towne, Davis, CMT-6, and 751 (11); strain Colburn (simian CMV-like isolate; 10; L. J. Charamella, R. B. Reynolds, L. T. Ch'ien, and C. A. Alford, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V373, p. 256); and HSV type 1 (HSV-1), F prototype (6).

Recovery of extracellular virus particles. Except where noted, virions, dense bodies, and NIEPs were recovered from infected cell culture media as described elsewhere (22). Medium was clarified of cells, layered onto glycerol-tartrate gradients prepared in 50 mM Tris hydrochloride–100 mM NaCl (TN buffer; pH 7.4) (2, 50), and subjected to centrifugation (25,000 rpm, 40 min, Sorvall AH-627 rotor [Ivan Sorvall, Inc., Norwalk, Conn.] or 40,000 rpm, 20 min, Beckman SW41 rotor [Beckman Instruments, Inc., Fullerton, Calif.]) at 4°C in a Sorvall OTD-50 ultracentrifuge set on slow acceleration and braking modes. Particles were located by their light-scattering properties, removed with a 23-gauge needle, diluted with TN buffer, and separately rebanded as described above. Particles were finally subjected to equilibrium centrifugation (15 to 20 h) and collected as described above, diluted with TN buffer, concentrated by either pelleting (35,000 rpm, 4°C, 3 h, SW50.1 rotor) or vacuum dialysis (11) where indicated, and stored at –80°C until used.

Recovery of intracellular virus particles. The procedure used was essentially the same as that described elsewhere (10). C capsids were recovered from the cytoplasmic fractions of NP-40-disrupted infected cells. A and B capsids were recovered from the NP-40 nuclear fraction (treated sequentially with deoxycholate, DNase, urea, and BRIJ 58). Virus particles were separated from the resulting cytoplasmic and nuclear extracts by rate-velocity sedimentation in sucrose gradients.

Protein kinase assay. Except where otherwise noted in the text, the protein kinase reaction mixture (total of 20 µl) contained the following: 0.1% NP-40, 50 mM Tris (pH 9.0), 20 mM MgCl₂, 10 mM dithiothreitol, 1 to 5 µCi of [γ -³²P]ATP, and sample (all final concentrations). Typically, 5 µl of the sample to be assayed, along with 5 µl of dH₂O or other addition (i.e., exogenous acceptor proteins, polyamines, etc.), was added to 10 µl of a twofold-concentrated reaction mixture. Exogenous proteins were present at final concentrations of 1 mg/ml. Reactions were incubated at 37°C

for 60 min and terminated by adding 25 µl of twofold-concentrated solubilizing buffer (see below) and 5 µl of 100 µM EDTA (pH 7.0).

Gel electrophoresis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by Laemmli (27). Samples were solubilized by adding an equal volume of twofold-concentrated solubilizing buffer (4% SDS, 20% beta-mercaptoethanol, 10% glycerol, 100 mM Tris [pH 7.0], 0.01% bromophenol blue); they were then heated in a boiling water bath for 3 min and stored at –80°C. Except where otherwise noted, resolving gels were 10% acrylamide and stacking gels were 4% acrylamide (both 28.0:0.735, acrylamide to methylene-bis acrylamide), and Pierce SDS (no. 28365) was used in gel and electrode buffers to enable resolution of the matrix proteins of strain Colburn (53). Gels were stained with Coomassie brilliant blue (7), dried onto Whatman 3MM filter paper (Whatman, Inc., Clifton, N.J.), and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) either at –70°C with an intensifying screen (Coronex Lighting Plus, Du Pont Co., Wilmington, Del.) (29) or at room temperature without a screen. Detection of [³⁵S]methionine-labeled proteins was enhanced by fluorography (3, 28). Fluorograms and autoradiograms were scanned at 540 nm with an EC910 transmission densitometer (E-C Apparatus Corp., St. Petersburg, Fla.), and relative band intensities were calculated ($0.5 \times \text{peak height} \times \text{peak width}$).

Peptide analysis. Gels containing ³²P-labeled proteins for peptide analysis were soaked briefly with a mixed bed resin (AG 501-x8; Bio-Rad Laboratories, Richmond, Calif.), dried onto Whatman 3MM filter paper, and exposed to XAR film at –70°C with an intensifying screen. Protein bands of interest were cut out of the gel, treated with TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin, and subjected to two-dimensional separation on thin-layer cellulose plates. Thin-layer electrophoresis was done at pH 1.9 (acetic acid-formic acid-water, 78:25:897); chromatography was in butanol-pyridine-acetic acid-water (15:10:3:12). Further details are presented elsewhere (53). Fluorograms were prepared by exposing the dried plates to XAR film at –70°C with an intensifying screen.

Materials. Phosvitin (P-1253), protamine sulfate (P-4020), casein (C-7891), lysine-rich histones (H-5505), arginine-rich histones (H-4380), putrescine (P-7505), spermidine (S-2501), spermine (S-2876), and ATP (A-2383) were purchased from Sigma Chemical Company, St. Louis, Mo. Bovine serum albumin (no. 902506) was from Schwarz/Mann, Orangeburg, N.Y. [γ -³²P]ATP (>2,000 Ci/mmol; PB.10168) was from Amersham Corp., Arlington Heights, Ill. RNase A and DNase I were from Worthington Diagnostics, Freehold, N.J.; proteinase K (161-519) was from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

RESULTS

In vivo phosphorylated proteins of extracellular HCMV particles. In addition to virions, two noninfectious particles can be recovered from the medium of HCMV-infected HFF cells (22). These have been designated as dense bodies (24, 32) and NIEPs (22). For purposes of corroborating and extending earlier descriptions of virion and NIEP phosphoprotein patterns (11, 22) and comparing them directly with that of dense bodies, the biosynthetically phosphorylated proteins of all three extracellular HCMV particles were identified in the following experiment. HFF cells infected

with strain AD169 HCMV were labeled with $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$ of complete medium) for 5 days, beginning 2 days after infection. Extracellular particles were recovered from the medium by glycerol-tartrate gradient centrifugation, concentrated by vacuum dialysis, solubilized, and subjected to SDS-PAGE, all as described in Materials and Methods. A fluorogram prepared from the resulting gel (Fig. 1) demonstrates the following. First, HCMV virions contain three major phosphoproteins, referred to as the basic phosphoprotein (BPP, 149 kDa) and the upper matrix (UM, 74 kDa) and lower matrix (LM, 69 kDa) proteins. In addition, they contain at least nine minor phosphorylated bands ranging from about 122 down to 24 kDa (i.e., 122, 110, 94, 88, 80, 75, 53, 43, and 24 kDa). Second, NIEPs contain these same phosphorylated proteins and one extra major species (i.e., 36-kDa assembly protein; 22, 23). The relative intensities of the major virion and NIEP phosphoproteins varied somewhat from preparation to preparation but were generally $\text{BPP} > \text{LM} > \text{UM} > 24 \text{ kDa}$. Finally, the dense body phosphoprotein pattern was consistent with earlier descriptions based on staining and biosynthetic radiolabeling with amino acids (22). The LM protein was the predominant band, and the other two major phosphorylated species found in virions and NIEPs (i.e., UM, BPP) were absent or present at less than 1% of their relative amounts in the other particles. Four of the minor phosphorylated bands present in virions and NIEPs (i.e., 94, 88, 80, and 75 kDa) were also found in dense bodies. Much of the radioactivity seen at the bottom of the virion and NIEP lanes was incorporated into the phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in the approximate relative ratio of 16:1:1:7 (P. McSorley and W. Gibson, unpublished findings).

When *in vivo*-phosphorylated NIEP preparations were subjected to two-dimensional (charge-size) separation in denaturing polyacrylamide gels, the pattern observed for the three predominant phosphoproteins (Fig. 2) was essentially the same as that reported for ^{14}C -labeled virion preparations (11). (i) The BPP was the most basic in net charge. (ii) The UM protein was the most acidic, showing little charge heterogeneity. (iii) The LM protein, slightly more basic in charge than the UM protein, resolved into three major charge isomers. Figure 2 also shows that the net charges of the assembly protein and the 24-kDa protein were in the same range as those of the matrix proteins. The assembly protein band consisted of three discrete charge isomers, whereas the 24-kDa band contained two more heterogeneous charge isomers. And finally, it was noted that the most basic isomers of the LM protein, the assembly protein, and the 24-kDa protein were present at the same position in the first-dimension gel, suggesting that they were associated during electrofocusing.

Purified HCMV virions exhibit protein phosphorylating activity. The following experiment was done to establish whether preparations of purified HCMV virions exhibit a protein kinase activity and, if so, to determine which of the virion proteins serve as phosphate acceptors *in vitro*. Three cultures of HFF cells were infected with HCMV strain AD169. Two days after infection, the growth medium of one culture was replaced with fresh medium; that of another, with medium containing ^{35}S methionine (10 $\mu\text{Ci}/\text{ml}$ of complete medium); and that of a third, with fresh medium containing $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$ of complete medium). Six days later, extracellular virions were collected by using glycerol-tartrate gradients. The two biosynthetically radiolabeled preparations were concentrated by vacuum dialysis, solubilized,

FIG. 1. Phosphoproteins of CMV virions, NIEPs, and dense bodies labeled *in vivo*. Extracellular virions, NIEPs, and dense bodies (DB) were recovered from the infected cell growth medium by rate-velocity sedimentation in glycerol-tartrate gradients. The concentrated, solubilized particles were subjected to SDS-PAGE in a 10% polyacrylamide gel. Shown here is a fluorographic image of the resulting gel, including the stacking gel (Stacker). The arrow at the top of the figure indicates the position of virion DNA that has moved into the resolving gel (seen more clearly in Fig. 3); the arrow at the bottom indicates position of phospholipids described in text. AP, 36-kDa assembly protein. For an explanation of the abbreviations used in this and subsequent figures, see reference 11.

ized, and stored at -80°C until analyzed. The nonlabeled virions were concentrated by pelleting (35,000 rpm, 3 h, 4°C ; Sorvall AH650 rotor), suspended in 50 mM NaCl-50 mM Tris (pH 8.0), reacted *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in Materials and Methods, and then solubilized. All three

FIG. 2. Two-dimensional separation of NIEP proteins phosphorylated in vivo. NIEP phosphoproteins were solubilized and subjected to two-dimensional separation, first by charge (Electrofocusing) and then by size (SDS-PAGE), as described previously (20). Major protein charge isomers are included within brackets; basic proteins are on the right, and acidic proteins are on the left. AP, 36-kDa assembly protein.

preparations were subjected to SDS-PAGE. A 10% "high bis" gel was used to resolve the major capsid protein and the BPP (22). An autoradiogram prepared from the resulting gel (Fig. 3) showed that (i) HCMV virions purified by this procedure contain protein kinase activity (i.e., protein bands were labeled in vitro); (ii) proteins phosphorylated in vitro comigrated during SDS-PAGE with proteins phosphorylated in vivo; (iii) the relative intensities of these bands were similar for the in vivo- and in vitro-labeled preparations; however, the 74-kDa UM protein was generally phosphorylated better in vitro than in vivo; and (iv) all three of the major phosphate acceptors comigrated during electrophoresis with abundant virion constituents (compare ^{35}S and ^{32}P channels, Fig. 3). Treatment of the products phosphorylated in vitro with DNase, RNase, or proteinase K (all at 100 $\mu\text{g}/\text{ml}$ of reaction mixture; 37°C, 30 min) showed that they were unaffected by the nucleases but, as expected for proteins, were eliminated by the protease. Based on these properties and others discussed below (e.g., peptide comparisons [see Fig. 7]), the proteins phosphorylated in vitro were given the same names as those phosphorylated in vivo

FIG. 3. Comparison of HCMV virion proteins labeled in vivo and in vitro. Virions radiolabeled with [γ - ^{32}P]ATP (In Vitro) or $^{32}\text{P}_i$ (In Vivo) or [^{35}S]methionine were subjected to SDS-PAGE with a 10% "high-bis" gel (22) to resolve the major capsid protein and BPP. Shown here is a fluorogram prepared from the resulting gel. HMWP, high-molecular-weight protein (212 kDa); MCP, major capsid protein (153 kDa); mCP, minor capsid protein (34 kDa); gp, glycoprotein.

with which they comigrate during SDS-PAGE (e.g., BPP, 80-kDa protein, UM and LM proteins, assembly protein, and 24-kDa protein).

All three extracellular HCMV particles contain protein kinase activity. Since NIEPs and dense bodies differ from virions in both structure and composition (22), it was of interest to determine whether they also have an associated kinase activity and, if so, which of their proteins are phosphorylated in vitro. This was done by recovering extra-

cellular virions, dense bodies, and NIEPs from the medium of HCMV-infected cells and testing each for protein kinase activity, as described in Materials and Methods. The results of this experiment showed that each of the three purified particle types has an associated kinase activity (Fig. 4). The pattern of protein phosphorylation in vitro was similar to that seen with particles phosphorylated in vivo (compare with Fig. 1, 3, and 6). The reduced intensity of the NIEP LM protein, phosphorylated in vitro or in vivo, is consistent with the 30 to 60% lower amount of this protein in NIEPs, relative to virions (22). One clear difference, however, is that the NIEP assembly protein, which was strongly labeled in vivo (see Fig. 1 and 6), labeled only weakly in vitro (about twofold lower than the faint 24-kDa band; seen better in Fig. 5). Thus, kinase activity is present in particles that contain no DNA but otherwise closely resemble virions (i.e., NIEPs) and also in enveloped particles that contain neither DNA nor a capsid (i.e., dense bodies).

All CMV strains tested exhibit similar protein kinase activity. Additional strains of CMV were tested for protein kinase activity to determine whether there are differences in the level expressed or in the pattern of proteins phosphorylated in vitro. Virions and NIEPs of HCMV strains AD169, 751, Towne, Davis, and CMT were prepared and assayed for kinase activity as described above. Virions of strain Colburn

FIG. 4. Proteins of HCMV virions, NIEPs, and dense bodies phosphorylated in vitro. Virions (Vir.), NIEPs, and dense bodies (DB) were assayed in vitro for protein kinase activity (see Materials and Methods), and the radiolabeled proteins were separated by SDS-PAGE. Shown here is a fluorogram prepared from the resulting gel. The 24,000-molecular-weight (24K) phosphoprotein could be seen in the original fluorogram but did not photograph well.

FIG. 5. Protein kinase assay of virions from different strains of HCMV, a simian strain of CMV, and HSV. Extracellular virions and NIEPs were recovered from cells infected with HCMV strains CMT-6, Towne, Davis, 751, and AD169. Virions from the simian CMV strain Colburn (Colb.) and from HSV-1 were similarly prepared. The preparations were assayed in vitro for protein kinase activity (see Materials and Methods) and then subjected to SDS-PAGE. Fluorograms of the resulting gels are presented here. Panels A (HCMV NIEPs and HSV virions; a composite of two exposures of same gel) and B (HCMV and Colburn virions) show the results of two separate experiments. VP#, HSV virion protein number (19, 47); X and Y, HSV virion proteins discussed in text. Dot adjacent to AD169 lane indicates position of AD169 assembly protein (AP) (i.e., 36 kDa versus 35 kDa for other strains).

CMV (simian strain) and HSV-1 were also assayed for comparison.

The results of this experiment showed that virions and NIEPs of all CMV strains tested contained protein kinase and provided no evidence for HCMV strain-specific differences in the amount or specificity of the particle-associated activity. These comparisons were based on the pattern and intensity of the bands phosphorylated in vitro (Fig. 5; data not shown) relative to the amount of protein present which was estimated by silver staining. Furthermore, the only notable difference between the virion and NIEP patterns was the presence of the assembly protein band (i.e., 35 or 36

FIG. 6. Stability of phosphate added to HCMV virion, NIEP, and dense body proteins *in vivo* during *in vitro* protein kinase reaction. Virions, NIEPs, and dense bodies phosphorylated *in vivo* were reacted *in vitro* with nonlabeled ATP (protein kinase assay) and analyzed after SDS-PAGE, all as described in the text. Shown here is a collage prepared from different fluorographic exposures of the resulting gel. Assay conditions (lanes) were as follows: ^{32}P , labeled *in vivo* with no further incubation (a); ^{32}P -labeled *in vivo* with 75 μM unlabeled ATP present during *in vitro* reaction (b); ^{32}P , labeled *in vivo* and no ATP present during *in vitro* reaction (c); and not labeled *in vivo* but $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ present during *in vitro* reaction (d). AP, 36-kDa assembly protein.

kDa) in NIEPs (compare Fig. 5A and B). The NIEP patterns shown here, along with two virion patterns for comparison, demonstrate the following. (i) The slower electrophoretic mobility of the AD169 assembly protein is observed in preparations phosphorylated *in vitro* (Fig. 5A). (ii) The level of assembly protein phosphorylation, relative to that of the other NIEP phosphoproteins, is dramatically lower *in vitro* (about 15-fold) than *in vivo* (see Fig. 1 and 6). (iii) The HSV-1 protein designated Y is larger than the 36-kDa assembly protein in the adjacent lane.

We previously established that the counterpart proteins of HCMV and strain Colburn virions differ in their respective electrophoretic mobilities (11). These differences are seen here (Fig. 5B) in the comparatively smaller BPP (119 kDa)

and UM (69 kDa) and LM (66 kDa) proteins of Colburn virions. In contrast to the relative labeling of the two HCMV matrix proteins, the UM protein of Colburn virions was consistently a better phosphate acceptor *in vitro* than was the LM protein. The HSV-1 pattern closely resembles earlier patterns of HSV-1 virion proteins phosphorylated *in vivo* (15) or *in vitro* (30). Based on their estimated molecular weights, relative intensities, and banding patterns, seven of the nine predominant HSV bands were designated as virion proteins 1, 2, 4, 12, 13, 14, 16, and 18. As discussed below, there is some uncertainty about the identity of the other two major bands, here indicated as X and Y, which appear to correspond to the VP6A and VP22 bands in biosynthetically phosphorylated virions (15, 25).

Phosphate added *in vivo* is not removed during *in vitro* protein kinase reaction. The following experiment was done to determine whether *in vitro* phosphorylation occurs at existing unoccupied sites or requires the removal of phosphate added *in vivo*. Nonlabeled and ^{32}P -labeled (100 $\mu\text{Ci/ml}$ of complete medium, 2 to 5 days postinfection) virions, NIEPs, and dense bodies were prepared by glycerol-tartrate gradient centrifugation. Particles phosphorylated *in vivo* were subjected to *in vitro* kinase reactions in the presence of 75 μM cold ATP or in the absence of added ATP (Fig. 6, lanes b and c). A sample of each nonreacted preparation was kept for comparison (Fig. 6, lanes a). Particles not labeled *in vivo* were reacted *in vitro* in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 6, lane d). A fluorogram prepared from a gel containing the resulting proteins separated by SDS-PAGE is shown in Fig. 6. Densitometric measurements of these patterns showed that essentially none of the ^{32}P label acquired *in vivo* was lost from the proteins during the *in vitro* reaction. Under the same reaction conditions, phosphate was transferred from

FIG. 7. Peptide comparison of HCMV proteins phosphorylated *in vivo* and *in vitro*. The BPP and UM and LM proteins from HCMV virions phosphorylated *in vivo* or *in vitro* were subjected to tryptic peptide comparison, as described in the text. Shown here are fluorograms prepared from the resulting thin-layer plates. Selected spots were given letter designations for purposes of discussion (see text). Both electrophoretic (first dimension, left [+] to right [-]) and chromatographic (second dimension, bottom to top) separations were slightly longer for the *in vitro*-labeled preparations (processed together as a set).

[γ - ^{32}P]ATP to the proteins of nonlabeled virus particles (Fig. 6, lanes d), demonstrating that kinase-mediated phosphorylation had occurred.

Fidelity of phosphorylation in vitro. As a means of further comparing the proteins phosphorylated in vitro with their size counterparts in biosynthetically labeled particles and determining whether the same or different sites are labeled in vivo and in vitro, the phosphopeptides of both sets of proteins were analyzed. Tryptic hydrolysates of the BPP and the UM (74 kDa) and LM (69 kDa) proteins, radiolabeled either in vivo with $^{32}\text{P}_i$ or in vitro with [γ - ^{32}P]ATP, were prepared and analyzed by two-dimensional separations on thin-layer cellulose plates as described in Materials and Methods. A set of fluorograms prepared from the resulting plates (Fig. 7) demonstrated the following. First, the phosphopeptide patterns are distinct for each of the three predominant HCMV phosphoproteins, whether labeled in vivo or in vitro. Second, most of the peptides phosphorylated in vitro were also labeled in vivo: the only apparent exception was an UM peptide (Fig. 7, asterisk). Third, the relative intensities of some of the peptides phosphorylated in vitro differed from those labeled in vivo (e.g., Fig. 7, e and n). Fourth, while most, if not all, of the LM protein peptides phosphorylated in vivo were also labeled in vitro, fewer than half of the BPP peptides phosphorylated in vivo were labeled in vitro. Finally, the peptides of the UM protein labeled in vitro corresponded with only the minor spots labeled in vivo (e.g., Fig. 7, f, g, h, and i). The predominant phosphopeptide labeled in vivo (Fig. 7, j) was absent from the in vitro

FIG. 8. Influence of pH, temperature, and divalent cations on HCMV virion-associated protein kinase activity. Virions were subjected to in vitro protein kinase assays (see Materials and Methods) in which the pH (A), temperature (B), and divalent cation concentration (C and D) were varied as described in the text. Samples were subjected to SDS-PAGE, and the extent of protein phosphorylation was calculated from fluorographic exposures of the resulting gels, as described in Materials and Methods. Additional details are as follows. (A) Protein kinase assays were done with the following buffers (50 mM) at the indicated pHs: MES (pH 5, 5.5, 6, 6.5, 7), MOPS (pH 6, 6.5, 7, 7.5, 8), Tris (pH 7, 7.5, 8, 8.5, 9), glycylglycine (pH 7, 7.5, 8, 8.5, 9), and glycine (pH 8, 8.5, 9, 9.5, 10). Just prior to SDS-PAGE, 5 μl of 1 M Tris (pH 7.0) was added to each sample to reduce pH differences. In order to construct a continuous pH curve with data from different experiments and different buffers, the data were normalized for all buffers containing an assay point at pH 8 (MOPS, Tris, glycylglycine, and glycine) by setting all phosphate incorporation levels at pH 8 to the same value. Phosphate incorporation over the range of each buffer was then normalized to its adjusted value at pH 8.0. MES data points were normalized to those of MOPS over the pH range 6 to 7. Multiple values for each pH (obtained from different experiments, and overlapping buffer ranges within an experiment) were averaged, and the standard deviation was calculated (vertical lines through data points). (B) Abscissa indicates incubation temperature of protein kinase assay. Changes in protein kinase activity at different assay incubation temperatures are indicated ($\circ$ - $\circ$). 37°C was set as 100% since that is the physiological temperature for the reaction. Symbols below the curve (arrow) indicate relative protein kinase activity in virion preparations heated for 5 min at 41 (Δ), 45 (*), 50 ($\square$), and 55°C ($\diamond$) before assay for 1 h at 37°C (i.e., thermal lability of enzyme). (C) Abscissa scale was broken at 5 mM so that all data points could be plotted. The manganese (Mn^{++}) curve represents the average of phosphate incorporation in the presence of either MnCl_2 or MnSO_4 . (D) Fluorogram shown here is representative of experiments summarized in panel C. Mg, MgCl_2 ; Mn-C, MnCl_2 ; Mn-S, MnSO_4 ; and control (no divalent cation added).

FIG. 9. Effect of exogenous proteins on HCMV virion-associated protein kinase activity. Virions were reacted in protein kinase assay mixtures containing the following additions (all 1 mg/ml final concentration except lanes f and g, 0.8 mg/ml) heated 5 min at 90°C: phosvitin (lane b), casein (Cas.) (lane c), protamine sulfate (Pr.S.) (lane d), lysine-rich histones (L-R Hist.) (lane e), casein plus an equal volume of water (Cas. + H₂O) (lane f) as a control for lane g, and casein plus an equal volume of protamine sulfate (Cas. + Pr.S.) (lane g). Shown here are the resulting phosphorylated proteins separated on a 14% acrylamide gel cross-linked with diallyltartardiamide in the ratio 1.09:28 (diallyltartardiamide:acrylamide). The four predominant bands (Cas.-1 through Cas.-4) in the casein preparation were detected by Coomassie brilliant blue staining or, as shown here, by phosphorylation. Dots adjacent to lane e indicate the three weakly labeled lysine-rich histone bands.

pattern, but a new (related?) spot was detected in the same area (see Fig. 7, asterisk).

Factors affecting *in vitro* kinase reaction. A series of experiments was done to investigate the parameters that

affect the virion protein kinase reaction *in vitro*, with endogenous viral proteins serving as phosphate acceptors. In the first experiment, the effect of pH on the reaction was determined. It was necessary to use a series of buffers to test activity over a broad range of pH, and the following were compatible with the kinase reaction: 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 5 to 7); 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH 6 to 8); Tris (pH 7 to 9); glycylglycine (pH 7 to 9); and glycine (pH 8 to 10). Phosphate incorporation averaged 105% in glycylglycine (pH 7 to 9), 86% in glycine (pH 8 to 9), and 180% in MOPS (pH 7 to 8), relative to incorporation at equivalent pH values in Tris buffer. Incorporation in MES averaged 108% of that of MOPS over the pH range 6 to 7. Both phosphate and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffers were less efficient at supporting the protein kinase reaction and were not used. *In vitro* protein kinase reactions were carried out over the pH range of 5 to 10, and samples of each reaction mixture were subjected to SDS-PAGE. Calculations based on densitometric measurements of autoradiograms prepared from the resulting gels were used to construct the curve shown in Fig. 8A. The results of this experiment indicate that the pH optimum for the kinase activity is approximately 9 to 9.5, with 81% optimal activity at pH 8 and 72% optimal activity at pH 10. Relative labeling of the three major protein acceptors did not vary appreciably with pH, but that of the 24-kDa protein did. As pH was changed from 9 to 10, phosphorylation of the 24-kDa protein remained about the same, while that of the major acceptors decreased. Thus, at pH 9.5 and 10, the relative extent of 24-kDa protein labeling increased.

The second experiment was done to determine the temperature optimum of the *in vitro* kinase reaction and the thermal stability of the protein kinase activity. Glycylglycine was used as the buffer since its pH is more stable to changes in temperature than that of Tris buffer. The temperature optimum for the reaction was tested by incubating kinase reactions for 60 min at 24, 37, 41, 45, 50, or 55°C (Fig. 8B, connected circles). Compared with the level of phosphorylation at 24°C, incorporation of phosphate into virion acceptor proteins was threefold greater when the assays were done at 37°C, 3.3-fold greater when done at 41°C (maximal), and 1.5-fold greater at 45°C. Assays done at 50°C or above showed less than 10% of the radiolabeling observed at 37°C. The sensitivity of the kinase activity itself to thermal inactivation was tested by heating samples of purified virions for 5 min at 37, 41, 45, 50, or 55°C and then measuring the residual kinase activity by incubating each heated virus sample for 60 min at 37°C in the kinase reaction mixture. The results of this procedure are shown in Fig. 8B (note symbols below arrow) and indicate that half of the enzymatic activity was still present after heating virions at 50°C for 5 min (square symbol), and 19% of the kinase activity survived heating at 55°C (diamond symbol), relative to the activity of virions heated 5 min at 37°C.

In the third experiment, the effect of divalent cations on the kinase activity was tested. The results presented in Fig. 8C and D show the following. First, the presence of a divalent cation was required for incorporating phosphate into virion acceptor proteins. Second, the optimal concentration of magnesium was found to be 20 mM with 50 and 20% of optimum activity at 1 and 100 mM, respectively. Third, of the concentrations of manganese tested, 1 mM gave the highest level of kinase activity. At this concentration the manganese and magnesium were equally effective in the kinase assay. However, at the optimal concentration for

magnesium (20 mM), assays containing manganese showed only 25% as much kinase activity. The decreased effectiveness of manganese at 20 mM, compared with that of magnesium, was mainly due to a selective decrease in labeling of the LM protein. No significant level of protein kinase activity was detected in the presence of the other divalent cations tested (i.e., Ca, Co, Zn, Ni [data not shown]). Sodium chloride was used to test the response of this kinase activity to monovalent cations and was found to inhibit the reaction at concentrations above 100 mM. Furthermore, NaCl inhibited *in vitro* phosphorylation of the UM protein to a greater extent than that of the other principal acceptors (data not shown).

The influence of several other factors on the protein kinase reaction was tested with the following results. Disruption of the virion envelope was required for activity; this could be achieved mechanically (e.g., pelleting particles by centrifugation) or more effectively by treating the particles with a suitable detergent (see Fig. 11, Vir +, Vir - NP-40). We used NP-40 for this purpose and found that kinase activity was greater in the presence of 0.1% NP-40 than in the presence of either 1 or 0.01% NP-40. Dithiothreitol, although not absolutely required, stimulated the protein kinase activity fourfold when present at a concentration of 10 mM (optimal; 1 mM, 86% optimal; 100 mM, 60% optimal). Kinase activity was not affected by the cyclic nucleotides cAMP and cGMP at concentrations of 0.25 to 5.0 μ M, nor was it affected by the PP_i analogs phosphonoformate and phosphonoacetate (50 to 400 μ g/ml).

Exogenous acceptor proteins. The ability of exogenous (i.e., nonvirion) proteins to serve as phosphate acceptors for the virion-associated enzyme was tested by adding specific proteins (final concentration, 1 mg/ml) to separate kinase reaction mixtures both in the presence and in the absence of added virus (Fig. 9 and data not shown). Of the proteins tested, the two acidic species, phosvitin (Fig. 9, lane b) and casein (Fig. 9, lane c), were the best exogenous substrates, but their addition to the reaction mixture resulted in a decreased phosphorylation of the endogenous viral proteins. This inhibitory effect was stronger for casein and, unlike that of phosvitin, could be reduced by heating the casein preparation before use (heated preparation shown in Fig. 9). In contrast, the basic proteins protamine, lysine-rich histones, and arginine-rich histones, although not utilized as substrates themselves (except minor labeling of lysine-rich histones), stimulated the phosphorylation of the endogenous virion acceptor proteins. The magnitude of stimulation produced by these basic proteins was estimated to be three- to sixfold. The presence of protamine sulfate stimulated the virion-kinase-mediated phosphorylation of both exogenous casein and the virion acceptor proteins (Fig. 9, lanes f and g). None of the exogenous acceptor protein preparations used here showed protein kinase activity themselves, as deter-

FIG. 10. Effect of polyamines on HCMV virion-associated protein kinase activity. Virions were reacted in protein kinase assay mixtures with different concentrations of the added polyamines putrescine (PUT), spermidine (SPD), spermine (SPM), spermidine plus spermine (SPD/SPM), or without added polyamines (control). (A) Protein phosphorylation was measured after SDS-PAGE; the results of three similar experiments were averaged. (B) Fluorogram prepared from a gel representative of those used to obtain the data summarized in panel A. Polyamine concentrations (millimolar) are shown above gel lanes (SPD/SPM contained 0.5 mM spermidine and 0.5 mM spermine).

FIG. 11. Protein kinase activity of virions and intracellular capsid forms of strain Colburn CMV. Extracellular virions (Vir.), cytoplasmic C capsids (C), and nuclear A and B capsids (A and B) were recovered from cells infected with strain Colburn CMV. All particles were taken directly from sucrose rate-velocity gradients and reacted in protein kinase assay mixtures in the presence (+) or absence (–) of NP-40 and with or without added casein. The remaining volume of nonreacted particles was concentrated, subjected to SDS-PAGE, and silver stained (55). Details of the experiment are presented in the text. Shown here are autoradiographic images prepared from the samples assayed for protein kinase activity and a direct duplicate (12) of the silver-stained gel showing the protein composition of each preparation. Dots adjacent to the B capsid lane in the silver-stained gel panel indicate the position of the 37-kDa assembly protein of strain Colburn B capsids. MCP, Major capsid protein; mCP, minor capsid protein; Cas.-2, casein band 2.

mined by the absence of phosphorylation in reactions lacking virions (data not shown).

Stimulation by polyamines. Since the CMV protein kinase was stimulated by basic molecules (see above) and since the polyamines spermidine and spermine have been shown to be present in the CMV virion (16), an experiment was done to test their influence on the CMV particle-associated kinase activity. The efficiency of the protein kinase at phosphorylating virion proteins in the absence of added polyamines was compared with its activity in the presence of added putrescine, spermidine, or spermine. Results from densitometric measurements of three separate polyamine assays, of which the experiment shown in Fig. 10B is an example, showed that spermidine and spermine had a significant stimulatory effect on the phosphorylation of virion proteins. The strongest effect was produced by 5 mM spermidine, which increased the phosphorylation of viral acceptor proteins by 3.5-fold. At concentrations of 1 mM, however, the stimulatory effect of spermine (2.4-fold) was

greater than that of spermidine (1.3-fold). Putrescine had little effect at the concentrations tested, showing a maximal stimulation of only 1.3-fold at 5 mM. The stimulation of phosphorylation by spermidine was not selective (relative increase was nearly the same for each of the viral protein acceptors), whereas spermine enhanced the phosphorylation of the BPP and LM protein to a greater extent than it did the UM protein (Fig. 10B). Neither ornithine, the amino acid precursor of polyamines, nor the smaller basic ammonium ion (NH_4Cl) had a noticeable stimulatory effect (i.e., less than that of putrescine).

Protein kinase activity associated primarily with enveloped particles. The finding that dense bodies have protein kinase activity (Fig. 4) provided evidence that since these enveloped particles do not contain a nucleocapsid (22), the activity may not reside in the capsid structure. This possibility was tested directly by recovering intracellular capsid forms (10, 23) and assaying them for protein kinase activity. Strain Colburn CMV (simian strain) was used in this experiment, since intracellular capsids are recovered in comparatively lower yield from HCMV-infected HFF cells (23). The experiment was done as follows. C capsids were recovered from the NP-40 cytoplasmic fraction of infected cells, A and B capsids were recovered from a lysate of the NP-40 nuclear fraction, and virions were recovered from clarified infected-cell maintenance medium—all by sedimentation in 15 to 50% sucrose gradients as described in Materials and Methods and elsewhere (10, 23). Equal aliquots of the gradient bands containing these particles were assayed for protein kinase activity, and the remaining material was concentrated by vacuum dialysis. All preparations were solubilized and subjected to electrophoresis in a 10% polyacrylamide gel. An autoradiogram of the samples reacted in vitro with [$\gamma\text{-}^{32}\text{P}$]ATP and a silver-stained portion of the same gel containing the concentrated original samples are shown in Fig. 11. The results of this experiment can be summarized as follows. First, neither of the intranuclear capsid forms (i.e., A and B capsids) exhibited significant levels of protein kinase activity as measured by phosphorylation of endogenous and exogenous (i.e., casein) proteins (compare Fig. 11, capsids, left-hand and middle sets). Second, cytoplasmic C capsids showed low kinase activity (i.e., about 1% that of disrupted virions), which was not appreciably increased by treating the particles with NP-40 before the reaction (compare Fig. 11, C – NP-40 with C + NP-40, left-hand set). Similarly, exogenous casein was only weakly phosphorylated by the C capsid kinase activity (i.e., about 5% of the virion level), and the lower casein band was labeled more strongly than the upper (Fig. 11, middle panel). Third, the virion preparation showed essentially no kinase activity in the absence of detergent but showed strong phosphorylation of both endogenous and exogenous acceptor proteins when NP-40 was added (compare Fig. 11, Vir. channels, left and middle panels). The same protein species were labeled in C capsids and virions (i.e., 119-kDa BPP, 69-kDa matrix protein, and [not visible here] 23-kDa protein), and these have been shown elsewhere to be the Colburn counterparts of the HCMV BPP, UM or 74-kDa protein, and 24-kDa protein (10, 11, 13; unpublished data on 23- and 24-kDa proteins). It is noted that all three capsid preparations contained the three predominant acceptor proteins for in vitro phosphorylation; namely, the BPP and the UM and LM proteins. In particular, C capsids had approximately as much of these three proteins as did virions (Fig. 11, right panel).

Fractionation of virion-associated protein kinase activity by rate-velocity sedimentation. An attempt was made to frac-

FIG. 12. Distribution of protein kinase activity after rate-velocity sedimentation of disrupted virions. Virions of strain Colburn CMV were recovered from the medium of infected cells by sedimentation through 15 to 50% (wt/vol) sucrose (in 150 mM NaCl–40 mM sodium phosphate buffer [pH 7.4]) gradients (9-ml linear gradient with 3-ml overlay of medium) in a Beckman SW41 rotor spun at 40,000 rpm and 4°C for 20 min (slow acceleration and stop). The light-scattering virion bands were collected; half was diluted 1:1 with the NaCl-phosphate buffer described above and kept on ice as a control, and half was diluted 1:1 with the same buffer but containing 1% NP-40. After incubation at 0°C for 10 min, the two virus preparations were layered above 15 to 50% sucrose gradients (11-ml linear gradient with 0.75-ml sample overlay), prepared, and spun as described above. The resulting gradients were collected by displacement from the tube (density gradient fractionator no. 185; ISCO, Lincoln, Nebr.) and samples of each fraction were assayed for protein kinase activity as described in Materials and Methods. Casein (1 mg/ml final concentration) was added to each reaction mixture as an exogenous substrate (Fig. 9). After the reaction, samples were solubilized and subjected to SDS-PAGE. Fluorograms of the resulting gels, presented here, show the distribution of protein kinase activity in the gradients containing nondisrupted (Virions) and disrupted (Virions + NP-40) virions.

tionate the kinase activity away from other proteins by treating virions with NP-40 (0.5%) and then subjecting the disrupted virions to rate-velocity sedimentation in a 15 to 50% sucrose gradient (Fig. 12). The results show that essentially all of the protein kinase in the gradient containing nondisrupted, rebanded virions, sedimented with the particles and also that the strongest activity was in fractions 7 and 8. Both virion proteins and casein (exogenous acceptor added to all reactions) were labeled. In contrast, only a small proportion of the kinase activity in the gradient containing NP-40-disrupted virions sedimented as far into the gradient as fraction 7. Instead, the enzymatic activity shifted toward the top of the gradient and resolved into two regions. The upper region (e.g., fractions 1 and 2) contained an activity that phosphorylated casein and, to a lesser extent, the BPP and UM and LM proteins. The second region of the gradient (e.g., fractions 4 and 5) contained an activity that phosphorylated the BPP and UM and LM proteins but labeled the casein bands only weakly, showing preference for the lower of the two, as was noted above for C capsids (Fig. 11).

DISCUSSION

In this report we present the results of studies to characterize the *in vivo* and *in vitro* phosphorylated proteins of HCMV virions, NIEPs, and dense bodies and to examine the

virion-associated protein kinase activity. As discussed below, the data support the following conclusions. (i) Each of these virus particles has a distinctive phosphoprotein composition. (ii) All three enveloped particles contain protein kinase activity; the gross activity requires a divalent cation and envelope disruption for activity, has a basic pH optimum, is stimulated by basic proteins and polyamines, and phosphorylates exogenous, nonviral substrates. (iii) The protein kinase is not present in an active form in the capsid and can be fractionated from virions into one activity that phosphorylates casein and another that does not phosphorylate casein but shows good activity toward viral acceptors.

Proteins phosphorylated *in vivo* and *in vitro*. The first series of experiments was done to identify and compare the CMV virion, NIEP, and dense body proteins phosphorylated *in vivo* and *in vitro*. The results showed that all three particle types have distinctive phosphoprotein patterns that reflect their overall compositional differences (22). The three virion proteins phosphorylated most strongly *in vivo* were the BPP and the UM and LM proteins. NIEPs contained these same proteins but were distinguished by the presence of the abundant phosphorylated assembly protein (22). Dense bodies lack the BPP, the UM protein, and the assembly protein, but contain the LM protein and at least two minor phosphoproteins also found in virions and NIEPs, i.e., the 80- and 24-kDa species. Based on their comigration during SDS-PAGE (e.g., Fig. 3), their similar distribution after

separation by two-dimensional SDS-PAGE (charge-size; data not shown), and their common phosphopeptides (Fig. 7), it was concluded that the virion proteins phosphorylated *in vitro* by the particle-associated protein kinase were the same species phosphorylated *in vivo*.

Among the predominant bands (i.e., BPP, UM and LM proteins, assembly protein), the BPP was the most highly phosphorylated (i.e., ^{32}P -radioactivity/protein) both *in vitro* and *in vivo*. In contrast, the NIEP assembly protein, which like the BPP was highly phosphorylated *in vivo* (e.g., $\geq 60\%$ total protein radioactivity; Fig. 1), was phosphorylated poorly *in vitro* (e.g., $\leq 4\%$ total protein radioactivity; Fig. 5, strain AD169). Several explanations of this comparatively unusual behavior have been considered, including the following possibilities. (i) Assembly protein phosphorylation sites are saturated *in vivo* and therefore are unavailable for phosphorylation *in vitro*. (ii) The phosphorylation sites are conformationally or architecturally sequestered from the enzyme. (iii) Assembly protein phosphorylation is catalyzed by an enzyme different from the one(s) described here. Each of these possibilities is compatible with probable functional differences between the assembly protein and the other major virion phosphoproteins. Specifically, the assembly protein is tightly associated with one of the compositionally simplest capsid forms (i.e., B capsids) and appears to have an early role in capsid formation and possibly in DNA packaging (10, 23). This protein species, which has counterparts in HSV (14, 38), pseudorabies (26), equine herpesvirus (37), and varicella-zoster virus (9), is not present in the mature virion (10, 22, 23). The major virion phosphoproteins, like those of HSV (15, 25, 30, 40), appear to be constituents of the tegument region of the particle (10, 11, 13) and involved with later stages of virion assembly, such as nucleocapsid envelopment.

An analysis of the *in vitro* phosphorylation of HSV-1 virion proteins (Fig. 5) was done to establish a point of reference for comparing these results with those of similar experiments with HSV (30, 43). Only two differences were noted from the patterns shown by LeMaster and Roizman (30). First, our pattern showed an additional major band, indicated as X in Fig. 5, with an estimated molecular weight of 135,000. This band migrated faster during SDS-PAGE than did VP6 (i.e., 146 kDa), which is one of the virion proteins phosphorylated most strongly *in vivo* (15, 25). It may, however, correspond to the less intensely phosphorylated band just smaller than VP6 (Fig. 6 in reference 15). Detection of this protein is attributed to procedural differences between the two studies (e.g., virus isolation technique and *in vitro* phosphorylation conditions). The second difference involves the band designated as Y in Fig. 5. Based on its intense radiolabeling *in vitro* and its comparatively broad banding pattern, Y appears to correspond to the protein designated as VP23 by LeMaster and Roizman (30). Our estimates, however, indicate that the size of this protein (i.e., about 40 kDa) is closer to that of VP22 than VP23 (i.e., 37 and 33 kDa, respectively [47]). Since VP22 was the virion protein phosphorylated most strongly *in vivo*, whereas VP23, an integral capsid protein (14), was nonphosphorylated in the same preparation, as well as in capsids (Fig. 5 and 6 in reference 15), we suggest that this band corresponds to VP22. If so, then CMV and HSV share the characteristics that none of their integral capsid proteins are phosphorylated *in vivo* or *in vitro* and that the protein phosphorylated most strongly *in vivo* is also the one most strongly phosphorylated *in vitro*.

Characterization of virion-associated protein kinase activ-

ity. The results of experiments to characterize the gross (i.e., nonfractionated) protein kinase activity associated with CMV virions showed the following. (i) The specificity and amount of activity was similar for all strains tested, including five human and one simian strain. (ii) The activity has a requirement for divalent cations, preferring Mg^{++} to Mn^{++} . (iii) The pH optimum of the activity is approximately 9 to 9.5. (iv) Phosphorylation was best at a reaction temperature of about 41°C. (v) The activity was stimulated by the polyamines spermidine and spermine, both of which are present in the particle (16), and also by the basic proteins protamine and the histones. (vi) The activity was not affected by the presence of cyclic nucleotides. (vii) In addition to the endogenous virion proteins, the exogenous acidic proteins casein and phosvitin also served as phosphate acceptors. (viii) No phosphatase activity was detected in virions, NIEPs, or dense bodies.

These properties are generally similar to those of virion-associated protein kinase activities described for other herpes group viruses (8, 30, 31, 39, 43, 48, 51). It should be noted, however, that the activity reported here did differ in at least two respects from that initially described for CMV (31). First, in the earlier report the pH optimum for the reaction was found to be broader and more neutral (i.e., 6.5 to 8.5; we observed only 28% optimal activity at pH 6.5). Second, whereas the former study concluded that casein was a poor exogenous acceptor for the virion kinase activity, the results presented here show that casein, although a poor acceptor for the capsid-tegment-bound enzyme (Fig. 12, fractions 4 and 5) as found with HSV (30), was a good substrate for the nonfractionated virion-associated protein kinase (Fig. 9) and also for the detergent-released activity (Fig. 12, fractions 1 and 2) described in this report.

A protein kinase activity immunoprecipitated from infected cells by HCMV-specific monoclonal antibodies has also been described (33, 34), but its characteristics are very different. Unlike the virion-associated activity described here, the immunoprecipitated protein kinase had a pH optimum near 7, used Mn^{++} only 10% as effectively as Mg^{++} , did not show an enhanced activity in the presence of polyamines, and was not detected in disrupted virions. The protein precipitated by this monoclonal antibody is 68,000 in molecular weight, abundant, and specific to HCMV-infected cells (33, 34). A second study, again using HCMV-specific monoclonal antibodies to recover the activity, identified an abundant, nuclear and virion, 67-kDa phosphoprotein that showed protein kinase activity (4, 5). Since the HCMV LM protein (11, 54) exhibits many of these same properties (e.g., abundant, nuclear and virion, 69-kDa phosphoprotein), it is important to establish whether the protein kinase activity characterized (4, 5) is intrinsic to the immunologically identified protein rather than copurified with it. In this connection, our recent efforts to fractionate the HCMV virion-associated protein kinase activity reported here have shown that it does not copurify with the LM protein or with any other abundant virion protein, under moderately denaturing conditions (i.e., 0.5% NP-40, 500 mM NaCl, 10 mM dithiothreitol) (Abstr. 10th Int. Herpesvirus Workshop, 1985; manuscript in preparation).

Localization of protein kinase activity within particles. Two series of experiments were done to investigate the architectural localization and intermolecular associations of the virion protein kinase. The results of the first showed that the presence of protein kinase activity correlated only with the presence of an envelope. All three particles having envelopes (i.e., virions, NIEPs, dense bodies) showed compara-

ble levels of activity (Fig. 4). The most informative of these were dense bodies, which do not contain a capsid (or its constituent proteins) and lack two of the three predominant virion phosphoproteins (i.e., BPP and UM protein). Of the intracellular capsid forms tested, neither nuclear A nor B capsids contained detectable protein kinase activity, and the small amount found in cytoplasmic C capsids was less than 5% of the amount present in virions. Thus, it is concluded that the activity is not resident in the nucleocapsid. Second, it seems likely that none of the three predominant virion phosphoproteins is itself a protein kinase since dense bodies lack both the BPP and the UM protein (Fig. 1; 22), yet contain protein kinase activity and C capsids, which have all three of these phosphoproteins, exhibited little protein kinase activity (Fig. 11).

The results of the second experiment showed that after treatment with NP-40, the virion-associated protein kinase activity of strain Colburn could be separated into a comparatively soluble form (Fig. 12, fractions 1 and 2) and a more particulate form (Fig. 12, fractions 4 and 5). These activities were distinguished by their ability to phosphorylate casein: the more soluble form phosphorylated casein well, whereas the faster-sedimenting form showed little activity toward casein but showed good phosphorylation of the cosedimenting BPP and UM and LM proteins. Virions of HCMV were more completely disrupted by the conditions used for this experiment but also yielded both a soluble activity that phosphorylated casein and a faster sedimenting activity that did not (data not shown). These findings could be accounted for by the presence of a single protein kinase enzyme that exhibits different properties in its bound and free forms, as has been suggested for HSV (30). However, they are also compatible with the possibility that CMV virions contain more than one protein kinase, as has been reported for pseudorabies virus (48). Although the data presented here do not distinguish between these alternatives, recent experiments showed that when HCMV virions are subjected to more stringent disruption conditions (i.e., 0.5% NP-40, 0.5 M NaCl, 10 mM dithiothreitol), two distinct soluble protein kinase activities are found. Like the material at the top of the gradient shown in Fig. 12, both of these activities were able to phosphorylate exogenous substrates (e.g., casein and phosphovitin) but were distinguished by their size, casein band specificity, stimulation by protamine sulfate, inhibition by heparin, and chromatographic behavior on phosphocellulose (manuscript in preparation).

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