

Human Cytomegalovirus Carries Serine/Threonine Protein Phosphatases PP1 and a Host-Cell Derived PP2A

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Received 25 August 1995/Accepted 16 November 1995

Human cytomegalovirus (CMV), a herpesvirus, is an important cause of morbidity and mortality in immunocompromised patients. When studying hyper-immediate-early events after contact between CMV virions and the cell membrane, we observed a hypophosphorylation of cellular proteins within 10 min. This can be explained in part by our finding that purified CMV contains serine/threonine protein phosphatase activities. Biochemical analyses indicate that this protein phosphatase activity has all characteristics of type 1 and 2A protein phosphatases (PP1 and PP2A). Specifically, PP1 accounts for approximately 30% and PP2A accounts for the remaining 70% of the phosphorylase phosphatase activity found. CMV produced in astrocytoma cells stably expressing an amino-terminally tagged PP2A catalytic subunit contained tagged enzyme, thus demonstrating the cellular origin of CMV-associated PP2A. PP2A is specifically found inside the virus, associated with the nucleocapsid fraction. Western blot (immunoblot) analysis of purified virus revealed the presence of the catalytic subunits of PP2A and PP1. Furthermore, the catalytic subunit of PP2A appears to be complexed to the regulatory subunits PR65 and PR55, which is also the most abundant configuration of this enzyme found in the host cells. Incubation of virus with okadaic acid before contact of CMV with cells prevented hypophosphorylation of cellular proteins, thus demonstrating the role of CMV-associated phosphatases in this phenomenon. CMV can thus transport an active enzyme from one cell to another.

Human cytomegalovirus (CMV) is a member of the family of *Herpesviridae*. It is one of the principal causes of congenital malformation and a major cause of disease and death in transplant recipients and patients with AIDS. During the acute phase of infection, CMV induces a profound, prolonged immunosuppression. This immunosuppression cannot be explained solely by CMV infection of immunocompetent cells. During viremia, leukocytes circulating in the peripheral blood carry the viral genome but display a very restricted set of viral proteins. Various studies (reviewed in reference 48) indicate that the major viral protein (ppUL83) present in these leukocytes may result not from viral genome expression but rather from passive uptake of virus particles, although there is still some debate on this issue (11).

CMV is composed of some 30 to 35 structural proteins (reviewed in reference 44). Many of these enter the cell within the minute following contact between virus with the cell membrane (49). Some glycoproteins and the most abundant matrix proteins (ppUL32 and ppUL83) can be detected immunologically at the cell surface or internally before any expression of CMV genes occurs. This finding suggests that viral structural proteins may be able to modify cell functions in the absence of viral replication. Indeed, *in vitro* studies by Boldogh et al. (4) have demonstrated proto-oncogene induction and biochemical modifications of host cell metabolism (reviewed in reference 2) by incoming viral structural proteins. In addition, Liu and Stinski (24) showed that incoming ppUL82 plays a role in the

activation of the CREB-responsive element in the CMV major immediate-early enhancer.

We have been studying hyper-immediate-early modifications of cell metabolism by CMV, defined as events occurring within the first 5 to 10 min following contact between the virus and the host cell membrane, in the absence of any virus replication and before the onset of viral immediate-early protein synthesis. We observed a hypophosphorylation of host cell proteins. An analysis of purified virus for the presence of protein phosphatase (PPase) activity revealed the presence of serine/threonine (S/T) phosphatase activities.

Reversible phosphorylation is a crucial mechanism in intracellular signal transduction antagonistically controlled by a large set of protein kinases and PPases (reviewed in references 30 and 36). Four classes of protein S/T phosphatases have been biochemically identified: PPase 1 (PP1), PP2A, PP2B (calcineurin), and PP2C (reviewed in reference 42). PP1 and PP2A are the predominant PPase activities found in cells. *In vivo*, both PP1 and PP2A exist as oligomeric holoenzymes with different intracellular localizations and/or substrate specificities. Specifically, catalytic subunits of PP1 and PP2A (PP1_C and PP2A_C) associate with regulatory subunits (also termed targeting subunits) (20, 27). PP1_C and PP2A_C have molecular masses of 37.5 and 35.6 kDa, respectively. Molecular cloning revealed the existence of more than one isoform for each (6, 39, 46). Regulatory subunits are not shared between the different classes of PPases (5). In particular, the set of regulatory subunits found for PP2A enzymes is very extensive: PP2A_C is always found associated with a constant regulatory subunit of 65 kDa (PR65). Additionally, other regulatory subunits (with molecular masses ranging from 54 to 130 kDa) variably associate with this core dimer (reviewed in reference 42; see also reference 27).

In vitro, PP1 and PP2A have broad, often overlapping sub-

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strate specificities. Assignment of measured activities to either one of the enzymes is facilitated by the selective use of specific inhibitors during the assay (10). PP1 can be distinguished from PP2A on the basis of its inhibition by two thermostable proteins, termed inhibitor 1 and inhibitor 2 (7, 42). Furthermore, PP2A and PP1 were recently identified as the intracellular targets of the tumor promoter okadaic acid (OA), a polyketal fatty acid. OA binds and inhibits PP2A and PP1 in extracts with 50% inhibitory concentrations of 0.2 and 20 nM, respectively, and can therefore be used to distinguish PP2A from PP1 as well (9).

We found two PPase activities associated with CMV virions, one of which was due to incorporation of a cellular PPase into the virion. Inhibition of virion-associated phosphatases prevented hyper-immediate-early hypophosphorylation of cellular proteins.

MATERIALS AND METHODS

Cells. Human diploid fibroblasts and human astrocytoma cells (U373MG) were grown and maintained in Dulbecco's modified medium (DME) supplemented with 2 mM glutamine and 10 and 5% fetal calf serum (FCS), respectively. All cells were free of mycoplasma contamination.

Virus. Infection experiments were performed with the Ad169 strain of CMV, originally obtained from the ATCC (strain VR538). Virus stocks were mycoplasma free. Two strains of virus, Towne and Ad169, were purified. For purification, extracellular virus was collected 7 to 10 days after infection of fibroblasts. After clarification for 1 h at 3,500 rpm and 4°C, polyethylene glycol 6000 (PEG; Sigma) was added to a final concentration of 5%, and precipitation was carried out overnight at 4°C with stirring. Virus was collected by centrifugation at 3,500 rpm for 1 h at 4°C. The pellet was resuspended in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) with or without 0.5 M urea. Virus was then purified on 40 to 70% sorbitol gradients (44a) by centrifugation for 1 h at 22,500 rpm and 4°C. The band of virus at 55% sorbitol was collected and diluted in TBS. In some cases, virus was ultrapurified on a discontinuous cesium chloride gradient (densities of 1.2 g/cm³ over 1.4 g/cm³) for 1 h at 4°C. Virus at the interface was collected and dialyzed against 25 mM Tris (pH 7.5)–1 mM EDTA. In one instance, two bands were seen. Both were submitted to electron microscopic examination, and several areas of the grid were counted to roughly estimate the ratio of virus to dense bodies. The upper band consisted mainly of dense bodies (only 15% virions), while the lower band contained 79% virions. Nucleocapsids with adherent tegument proteins were prepared and examined in an electron microscope after negative staining as described by Landini and Ripalti (23).

Alternately, extracellular virus was clarified of cell debris at 10,000 rpm for 1 h, precipitated overnight with PEG as described above, and pelleted at 3,500 rpm for 1 h. The resulting pellet was centrifuged for 1 h through a 20% sucrose cushion at 4°C and 30,000 rpm in a Beckman SW41 rotor, resuspended at 1/100 of the original volume of culture supernatant in 50 mM Tris (pH 7.4)–1 mM EDTA–150 mM NaCl containing 20% glycerol, and held at –20°C.

Measurement of PPase activity. S/T phosphatase activity was measured by determining the release of ³²P from a phosphorylase *a* substrate labeled on serine 14 by phosphorylase kinase in the presence of [γ -³²P]ATP (Gibco, Paris, France). Briefly, assays were performed in 50 mM Tris (pH 7.5)–50 mM NaCl–0.67 mg of bovine serum albumin per ml–0.1 mM EDTA–0.1% β -mercaptoethanol–3 mM caffeine–10 μ M ³²P-labeled phosphorylase *a* in a final assay volume of 30 μ l and incubated for 10 to 30 min at 30°C. The assay was stopped by the addition of cold 50% trichloroacetic acid. Preparations were incubated for 10 min on ice and then centrifuged for 5 min at 10,000 rpm at 4°C. A portion of the supernatant was counted in Amersham BCS scintillation liquid. Inhibitor 1 specific for PP1 was purified as described by Aitken et al. (1) and phosphorylated as described by Waelkens et al. (53), and deinhibitor (DI) was purified as described by Goris et al. (14).

Stable transfection of astrocytoma cells with tagged PP2A_C. U373MG astrocytoma cells were plated in 25-cm² culture flasks and transfected with 5 μ g of DNA per 10⁶ cells, using the calcium phosphate technique. One series of cells received an expression vector (pN^{pp1}-PP2A-tag) encoding PP2A_C tagged at the NH₂ terminus with a nonapeptide (YPYDVPDYA) of influenza virus under the control of the Moloney leukemia virus promoter and carrying the neomycin resistance gene (52) (generous gift of B. E. Wadzinski and G. L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). Another series of cells was transfected with the same vector DNA (pCW1-neo) devoid of cDNA encoding the tagged PP2A_C. Five hours after transfection, cells were washed twice with phosphate-buffered saline (PBS) and refed growth medium containing serum. Forty-eight hours later, cells were trypsinized, replated at 50,000 cells per well in 48-well culture plates, and fed growth medium containing serum and 400 μ g of the neomycin analog G418 per ml. Resistant cells appeared within 2 to 3 weeks. PP2A_C-transfected cell lines and neomycin-resistant control cells were analyzed by Western blotting (immu-

noblotting) for the presence of the tagged PP2A_C, using the monoclonal antibody (MAb) 12CA5, specific for the nonapeptide tag (Berkeley Antibody, Co. Inc., Richmond, Calif.), or a polyclonal anti-PP2A_C antibody (generous gift from M. Mumby, University of Texas Southwestern Medical Center, Dallas). Two PP2A_C-carrying cell lines (P2.3 and P2.6) and one neomycin-resistant cell line (N1.2) were used in the studies described below. No further cloning of cells was performed.

Labeling of cells with ³²P_i. Astrocytoma cells were seeded (10⁵ per well of 24-well plates). They were washed with PBS, incubated in phosphate-free DME for 1 h, and then labeled with 100 μ Ci of ³²P_i (Amersham) per ml. Cells were labeled for 5 min at 37°C, then immediately placed on ice and washed once rapidly with ice-cold Tris buffer (25 mM Tris-HCl [pH 7], 5 mM EDTA, 5 mM sodium fluoride), and lysed in electrophoresis sample buffer containing a mixture of phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium vanadate, 200 mM OA, 10 mM β -glycerol phosphate, 10 mM *p*-nitrophenyl phosphate [pNPP]).

Western blot analysis of cells stably expressing tagged PP2A_C, virus produced on these cells, and PPase subunits in purified virus. Cells or virus were dissociated in electrophoresis buffer (62.5 mM Tris [pH 6.8], 5% β -mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 15% glycerol) by heating at 95°C for 5 min. Proteins were separated in SDS–10% polyacrylamide (Electran) minigels (Hofer), using prestained molecular weight markers (Sigma). Gels were immediately transferred to reinforced nitrocellulose filters (Sartorius, France), using a semidry transfer apparatus (Biometra, Paris, France) at 100 mA for 2 h. Following transfer, filters were stained with Ponceau S red (2% in 3% trichloroacetic acid) to control the quality of transfer. Filters were blocked in 5% skimmed milk in TBS (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) containing 0.1% Tween 20 (TBS-Tween) for 30 min at room temperature and then incubated in 5% milk–TBS-Tween containing anti-tag MAb 12CA5 (1/500) overnight at 4°C with shaking. Following 3 washes in 5% milk–TBS-Tween, filters were incubated for 1 h at room temperature with peroxidase-labeled anti-mouse immunoglobulin (1/1,000; Amersham) for the detection of MAb 12CA5 or with peroxidase-labeled anti-rabbit immunoglobulin (1/400; Amersham) for detection of the anti-PP2A_C polyclonal antibody. After further washing, peroxidase-labeled substrates were detected by the enhanced chemiluminescence luminescence method (Amersham) as instructed by the manufacturer. In some cases, blots were stripped of antibody by incubation in 100 mM β -mercaptoethanol–2% SDS–62.5 mM Tris-HCl (pH 6.7) at 50°C for 1 h followed by blocking with TBS–5% milk–0.5% Nonidet P-40 (NP-40)–0.1% Tween 20 for 1 h.

The antibodies used to study PPase subunits in immunoblots of purified virus were polyclonal antisera raised in rabbits against either peptides or recombinant proteins by techniques previously described (19, 50). AbPP1^{317/330} is directed against the α isoform of PP1_C (PP1_C α) (35). Anti-PP2A_C antibodies included AbC^{recomb} (50) and AbC^{302/309} (51). Anti-PR65 (anti-A subunit) antibodies Ab65^{recomb} and Ab65^{177/196} are both described in reference 50. PR55 (B subunit) was sought with the isoform-specific antibodies Ab55 α ¹²⁰ and Ab55 α ¹²⁰ (19). In addition, we generated antibodies against recombinant PR55 α . This protein was expressed in *Escherichia coli* JM109/DE3 by using the T7 promoter driving expression plasmid pRK172 (29) as previously described (19). The recombinant protein was retrieved from insoluble inclusion bodies, solubilized in 8 M urea in 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–10 mM dithiothreitol, and dialyzed against TBS. This material was then injected into rabbits. The immunoglobulin G fraction of the resulting antisera was isolated by using protein A-Sepharose CL-4B (Pharmacia). Subsequently, Ab55 α ^{recomb} was affinity purified on immobilized recombinant PR55 α . The antiserum was found to be specific for PR55 α . Anti-PR72 antisera have been described by Hendrix et al. (18).

As positive controls, purified PPase subunits or holoenzymes were used (28, 50). Recombinant PP1_C was kindly provided by E. Y. C. Lee (University of Miami, Miami, Fla.). For competition experiments, peptide antibodies were diluted and preincubated with the antigenic peptide at a final concentration of 50 μ M for 30 min. After immunodetection with the antisera described above, proteins were revealed with ¹²⁵I-coupled donkey anti-rabbit antibodies (Amersham) at a dilution of 1:2,000. Blots were analyzed by using a PhosphorImager and the ImageQuant software (Molecular Dynamics). Image files of gel scans were processed, mounted, and printed as figures.

RESULTS

CMV-induced hypophosphorylation of cellular proteins. Astrocytoma cells labeled with ³²P_i in the presence of virus showed a global hypophosphorylation of proteins during the first 5 min of viral contact compared with uninfected cells labeled in parallel (Fig. 1; compare lanes 1 and 3). The extent of this hypophosphorylation was 3.6-fold higher than that of uninfected cells as determined by densitometric scanning. This effect could be prevented by pretreating pelleted CMV with 300 nM OA (an S/T phosphatase inhibitor) for 1 h at room temperature (threefold difference between lanes 1 and 2). OA by itself had only a small effect (1.3-fold) on cellular phosphorylation during the short incubation time (compare lanes 3 and 4). This is due to the slow accumulation of OA in living cells

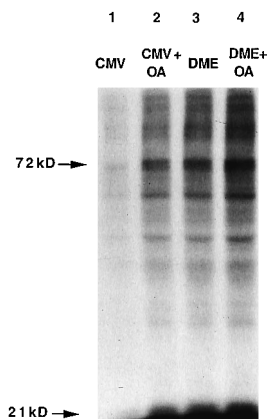


FIG. 1. Protein phosphorylation in cells after contact with CMV. Human astrocytoma cells were incubated in phosphate-free DME in the absence of serum for 1 h. During this time, pelleted CMV was incubated with (lane 2) or without (lane 1) 300 nM OA at room temperature. Serum (5% FCS) and 100 μ Ci of 32 P_i per ml were added. Medium on cells was replaced with CMV (lane 1), OA-treated CMV (lane 2), DME-5% FCS (lane 3), or DME-5% FCS plus 300 nM OA (lane 4). After 5 min of contact, all cells were washed with ice-cold Tris buffer containing NaF (5 mM), lysed in electrophoresis buffer, and separated in SDS-10% polyacrylamide gels. Densitometric scanning of the autoradiograph showed a 3-fold increase in phosphorylation between lanes 1 and 2 and only a 1.3-fold increase between lanes 3 and 4.

(10a). This finding indicated that our CMV preparations contain S/T phosphatase activity which induced a global cellular hypophosphorylation as a hyper-immediate-early effect of CMV infection on the cell.

Characterization of CMV-associated phosphatase activity.

We therefore measured S/T phosphatase activity in preparations of purified Ad169 and Towne strains of CMV. As can be seen in Fig. 2, all sorbitol gradient-purified preparations of virus (AD1, AD2, AD3, T1, and T2) contained an S/T phosphatase activity. The level of activity varied from one preparation to another (100 to 900 pmol/min/mg). Preparation T3, which was ultrapurified on a cesium gradient after sorbitol purification, had as much specific PPase activity (800 pmol/min/mg) as any of the other preparations. We also measured PPase activities in two dense-body-enriched preparations which contained <15% virus particles and found practically no spontaneous activity (results not shown).

That the release of phosphate was an enzymatic reaction catalyzed by S/T phosphatases specifically found in CMV preparations was corroborated by the following set of experiments. The enzymatic nature of the phosphatase activity associated with purified virus was reflected in its linearity as a function of protein concentration (Fig. 3A) and of time of incubation (Fig. 3B). Although the OA sensitivity of the activity (Fig. 4) suggested that the release of low-molecular-weight phosphate was catalyzed by PPases, we further ensured that our observations were not due to nonspecific proteolysis of the labeled substrate; we measured PPase activity by measuring P_i liberated as a phosphomolybdate complex specifically extracted by isobutanol (41). We found that more than 95% of the liberated phosphate was P_i. To control for contamination by nonspecifically associated cellular enzyme, we measured PPase activity in crude, PEG-precipitated proteins from the supernatants of infected cells as well as from uninfected cell cultures (see Materials and Methods). PEG precipitates from uninfected

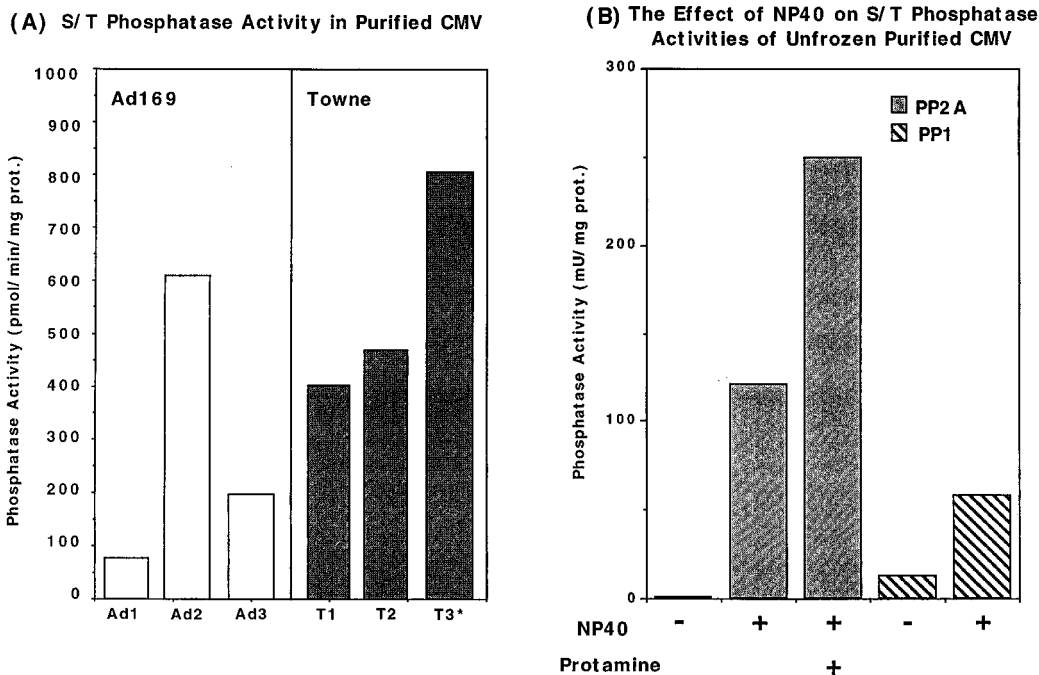


FIG. 2. S/T phosphatase activity in purified CMV. (A) Two strains of CMV, Ad169 (Ad1 to Ad3) and Towne (T1 to T3), were purified on sorbitol gradients. Preparation T3 was ultrapurified on a cesium chloride gradient. PPase activity was measured as described in Materials and Methods, using 32 P-labeled phosphorylase *a* as the substrate. Results are expressed in picomoles of phosphate liberated per minute per milligram of protein (prot.). (B) CMV (Ad169 strain) was purified by PEG precipitation and passage through a sorbitol density gradient as described in Materials and Methods except that virus was processed with no freezing from beginning to end. PP2A and PP1 activities were then measured in the absence or the presence of NP-40 (0.3 or 0.03%), as indicated below the graph. Results correspond to the averages of two experiments. PP2A activity, undetectable in the absence of NP-40, increased to 121.2 mU/mg of protein following NP-40 treatment and to 250 mU/mg in the presence of protamine (10 μ g/ml). PP1 activity was increased sixfold by NP-40 treatment of freshly purified virus.

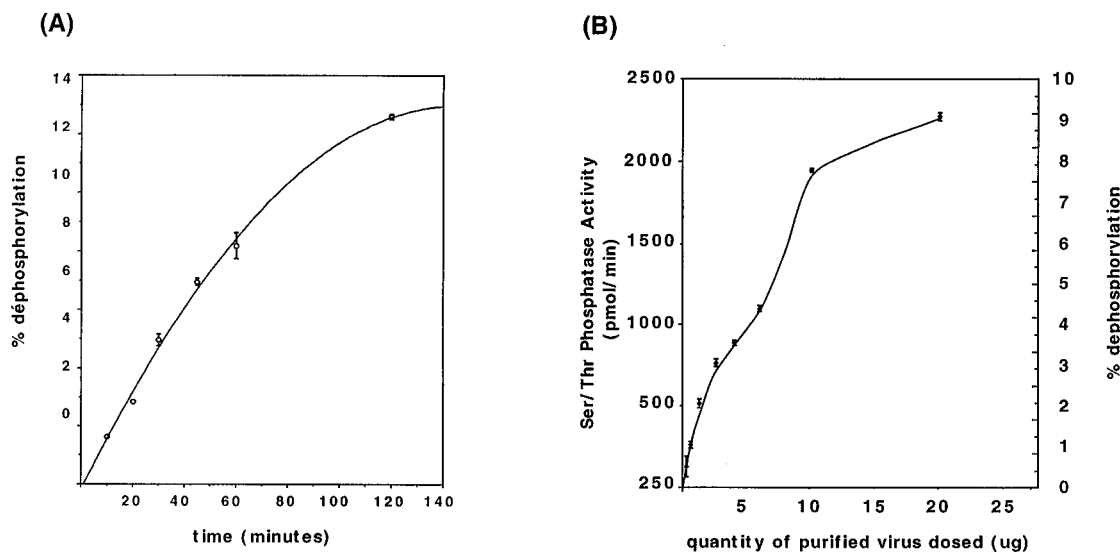


FIG. 3. S/T phosphatase activity as a function of time and concentration of protein. (A) Sorbitol-purified Ad169 (4 μg) was incubated with phosphorylated phosphorylase *a* substrate for increasing times (10 min to 2 h) at 30°C. PPase activity is expressed as percentage of phosphate released from the substrate in the presence of virus compared with release in the absence of virus at each time point. Release increased linearly with time of incubation. (B) Various concentrations of purified Ad169 were incubated with the ^{32}P -labeled phosphorylase *a* for 30 min at 30°C. PPase activity (left axis), expressed in picomoles of phosphate released per minute per milligram, increased with increasing protein concentration. Results are expressed as percent dephosphorylation of substrate on the right axis.

(Fig. 4A) and infected (Fig. 4B) cell supernatants were similar in content when examined in an electron microscope except for the presence of virions and dense bodies in the latter preparation. No PPase activity was detected for an equivalent amount of PEG-precipitated proteins from uninfected cell supernatants, while there was considerable activity in PEG precipitates of infected cell supernatants (Fig. 4C). In addition, the infected cell supernatant activity was progressively inhibited by increasing concentrations of OA, a specific inhibitor of S/T phosphatases PP2A and PP1 (3) which has no effect on proteases.

To further characterize the PPase activity present in the virion, the phosphorylase phosphatase activity was measured in the presence of several effectors of the S/T phosphatases PP2A and PP1 (Table 1). Protamine stimulates the S/T phosphatase PP2A and inhibits PP1 (21). DI is a specific stimulator of PP1 activity in the presence of pNPP (13). pNPP stimulates PP1 activity while inhibiting PP2A activity (12). Both protamine and DI-pNPP stimulated the basal activity. Thus, both PP1- and PP2A-type phosphatases were present. Protamine was only slightly stimulatory (and in some virus preparations even slightly inhibitory), which is further evidence for the presence of a mixture of the two PPases. Inhibitor 1, a specific inhibitor of PP1, inhibited virion-associated PPase activity by 30%. The

concentration of inhibitor 1 used in experiment 5 of Table 1 was 10-fold higher than the concentration that inhibits free PP1_C by 95%. Thus, 30% of the total measured phosphorylase phosphatase activity can be attributed to a type 1 PPase, while the remaining activity appears to be catalyzed by a type 2A phosphatase. However, the ratio of PP2A to PP1 seemed to differ from one preparation to another, since stimulation and inhibition by protamine and DI-pNPP were variable. The presence of both PPases is reinforced by the dose-response curve to OA of phosphorylase phosphatase activity in the presence and absence of DI-pNPP. In the presence of DI-pNPP, the 50% inhibitory concentration is about 10^{-8} M (Fig. 5), whereas in its absence, 10^{-12} M OA inhibits about 70%, whereas the remaining 30% is inhibited only by a concentration of greater $>10^{-9}$ M.

These results indicate that the phosphorylase phosphatase activity measured in the presence of DI-pNPP is a PP1-type activity which is less sensitive to OA. DI could stimulate virion-associated PPase on its own (Table 1, experiment 6), which indicates that the PP1 activity measured is somewhat suppressed.

Location of PPase activity within CMV virions. We suspected that the variability of the CMV-associated PPase activity described above might be due to the different freezing-thawing cycles to which the different preparations were subjected during the preparation procedures. Since CMV is an enveloped virus, it was likely that the continuous freezing and thawing broke open the virions and released PPases from the inside, thus giving rise to a higher spontaneous specific activity. Therefore, we purified virus on sorbitol gradients without freezing the preparations from start to finish and then measured PPase activity. These preparations were then treated with NP-40 to open the viral envelope. In the absence of NP-40, PP2A activity (defined as activity sensitive to 3 nM OA) was undetectable (Fig. 2B), while PP1 activity (activity that was resistant to 3 nM OA) was 12.9 mU/mg of protein. In the presence of 0.03 to 0.3% NP-40, an average of 121 mU of PP2A per mg was present; this level increased to 250 mU/mg

TABLE 1. Effectors of the phosphorylase phosphatase activity of purified CMV

Effector	pmol/min/mg (or mU/mg) of viral protein					
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
None	78	588	543	828	1,038	1,023
DI-pNPP	287	512	559	1,429		1,518
DI						2,272
pNPP						1,136
Protamine		792	688	1,492		
Inhibitor 1					748	

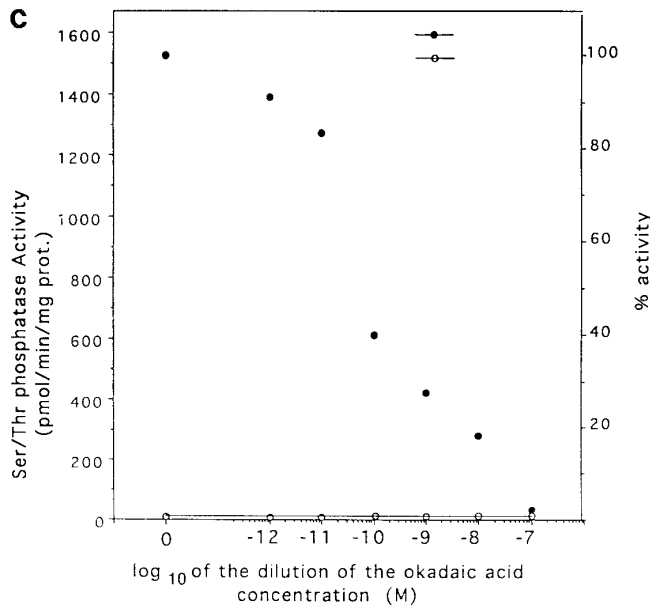
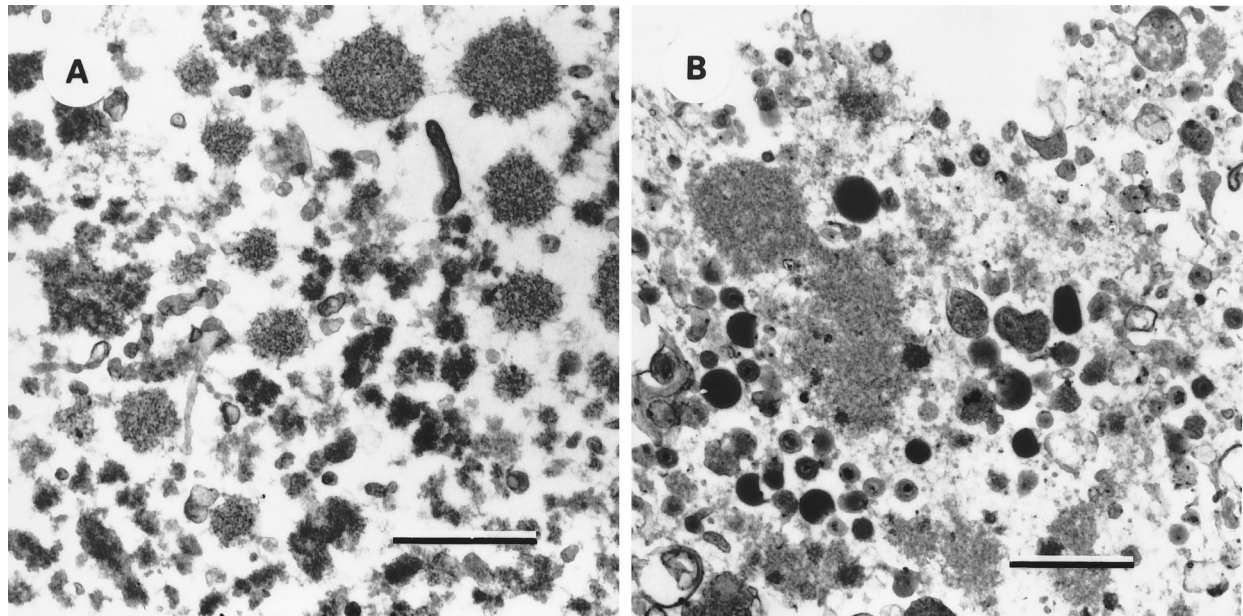


FIG. 4. S/T phosphatase activity in PEG precipitates of uninfected and infected cell supernatants. Eight hundred milliliters of 5-day-old human fibroblast culture supernatant and 1,200 ml of infected cell supernatant were clarified and precipitated overnight with PEG as described in Materials and Methods. Precipitated material from uninfected (A) and infected (B) cell supernatants was fixed and embedded for electron microscopy. (C) PPase activity was measured on 4 μ g of protein (prot.). No activity was found in PEG precipitates of uninfected cells (open circles). PPase activity was present in supernatants of infected cells (closed circles) and was progressively reduced by increasing concentrations of OA.

cifically with PP2A in pelleted nucleocapsids and adherent tegument proteins, as well as in whole virus and uninfected cell extracts. Reactivity with the same amount (10 μ g per lane) of purified whole virus pelleted in parallel was slightly less intense than with the nucleocapsid preparation. Thus, we could also identify PP2A immunochemically in CMV nucleocapsid preparations.

The PP2A_C detected in CMV virions is of cellular origin. We performed experiments to further define the characteristics of the PP2A found in CMV virions. It was still unclear whether CMV encodes its own PP2A. A computer search of the CMV genome did not reveal any sequences homologous to known S/T or dual-specificity PPases (8). As described above (Fig. 6B), we were able to detect a protein with our antibody to human PP2A_C (AbC^{299/309}). Therefore, it seemed unlikely that CMV encodes its own PP2A; rather, this virus-associated PPase was likely of cellular origin. To show this unambiguously, we developed a cell system stably expressing a tagged PP2A_C (see Materials and Methods). An astrocytoma cell line (U373MG) permissive for CMV replication was stably transfected with the PP2A_C tag expression vector or with the control vector (pCW1-neo). We chose two PP2A-expressing cell lines (P2.3 and P2.6) and a neomycin-resistant cell (N1.2) for the experiments described below. The cell lines were infected at a multiplicity of infection of 0.01 with CMV strain Ad169. Supernatants produced on infected P2.3, P2.6, and N1.2 cells were designated virus-tagP23, virus-tagP26, and virus-tagneg, respectively. Supernatants produced on uninfected cells were called control-tagP23, control-tagP26, and control-tagneg. Culture supernatants collected 6 days after infection were PEG precipitated (see Materials and Methods). The PEG precipi-

in the presence of 30 mg of protamine chloride per ml. As with other CMV preparations, PP1 represented about 30% of the total phosphatase activity detected. NP-40 at the highest concentration used (0.3%) had a 10% inhibitory effect on cellular PP2A activity, which increased to 30% when polycations were included. Thus, NP-40 appears to expose PP2A located within the virion rather than stimulating an activity on the surface of the particles.

This conclusion was substantiated by the immunoblot detection of PP2A in nucleocapsid preparations which were devoid of envelopes as demonstrated by electron microscopy (Fig. 6A). For this purpose, purified virus treated with 0.5% NP-40 for 30 min at 4°C was ultracentrifuged for 1 h through a 15% sorbitol cushion as described by Landini and Ripalti (23). The pellet was immunoblotted with a rabbit polyclonal antiserum specific for PP2A_C, AbC^{299/309} (50), as described in Materials and Methods. As shown in Fig. 6B, this antibody reacted spe-

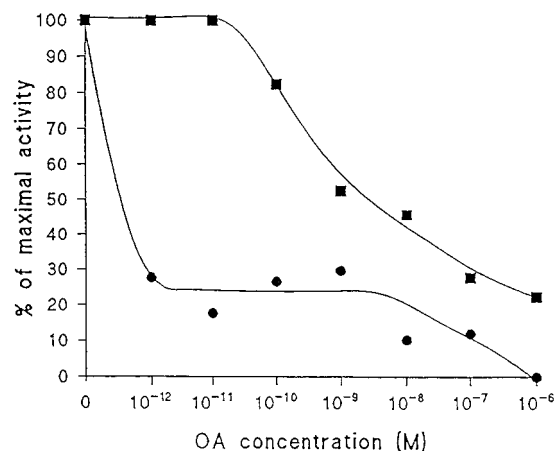


FIG. 5. Inhibition of the basal and DI-pNPP-stimulated phosphorylase phosphatase activity of purified CMV by OA. Highly purified CMV was preincubated with (squares) and without (circles) DI-pNPP as described in Materials and Methods, and the phosphorylase phosphatase activity was measured for 40 min at 30°C in the presence of the indicated concentrations of OA. The control value in the absence of OA (543 mU/mg) was taken as 100% for the basal activity, whereas the control level (100%) of DI-pNPP-stimulated activity was 559 mU/mg.

tates were then tested for the presence of the PP2A_C tag by Western blotting with a MAb against the tag. The results (Fig. 7) show the detection of a 38-kDa protein in PEG precipitates of virus produced on cells stably expressing the PP2A_C tag but not on N1.2 cells. No band was detected in PEG-precipitated material from uninfected cell supernatants. The 38-kDa band corresponds to the PP2A tag, as can be seen in the extracts from the P2.3 and P2.6 cell lines. When the membrane shown in Fig. 7 was stripped and subsequently incubated with an anti-PP2A_C serum (generous gift from M. Mumby), we detected the 36-kDa PP2A_C in PEG-precipitated virus produced on all cells, whether or not they constitutively expressed the PP2A tag (results not shown).

CMV replication in constitutively PP2A_C tag-expressing cell lines did not differ from that in control cells. Virus produced on PP2A_C tag-expressing stabilized cell lines, as well as on the neomycin-resistant control cells, was titered by plaque reduction. The titers were 8.7×10^3 PFU/ml for line P2.3, 10^4 PFU/ml for line P2.6, and 10^4 PFU/ml for line N1.2.

As mentioned in the introduction, PP2A exists as trimeric, oligomeric holoenzymes *in vivo*. Therefore, we examined whether CMV is taking up holoenzymes or just parts of PP2A from its host cell. For this analysis, we compared the presence of regulatory subunits in virus preparations with that in host cell extracts. We investigated the presence of PP2A and PP1 subunits in the virion by immunoblot analysis of define quantities of purified CMV or dense-body-enriched preparations, as well as in uninfected and infected fibroblasts, using antisera specific to the catalytic and the various noncatalytic subunits of PP2A and to PP1_C.

The presence of PP1 was investigated by using AbPP1^{317/330}, which reacts only with the α isoform (39). In fibroblast extracts and purified CMV, this antibody detected a band at 38 kDa which migrated slightly faster than purified PP1_C α (Fig. 8A, lane 1). PP1_C α was also detected in a dense-body-enriched preparation (lane 2), which raises the question of whether the presence of PP1_C in CMV virions is a characteristic of infectious virus. In addition, this antibody specifically detected a band of 33 kDa which was as prominent in purified CMV as the 38-kDa protein (lane 3). If this is a degraded form of PP1_C, it

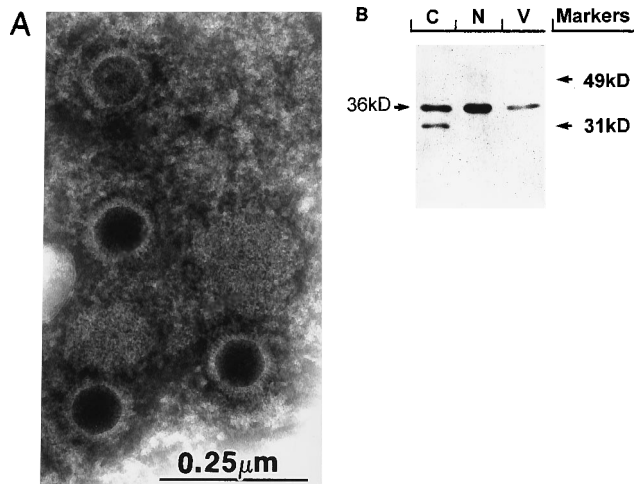


FIG. 6. Electron microscopy and Western blot analysis of nucleocapsids. (A) Nucleocapsids were derived from purified virus by treatment with NP-40 followed by centrifugation through a 15% sorbitol cushion as described by Landini and Ripalti (23). Shown is an electron micrograph of negatively stained nucleocapsids. No enveloped particles were observed in these preparations. (B) Ten-microgram samples of uninfected cell lysate (C), nucleocapsid (N), and whole virus (V) were separated in an SDS-12.5% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a polyclonal antiserum specific for PP2A_C as described in Materials and Methods. The 36-kDa PP2A_C is indicated on the left, and the positions of molecular mass markers are shown on the right.

must be an N-terminal degradation product, since AbPP1^{317/330} was generated against the carboxyl terminus of PP1_C (35). The signals were specific, since they were competed for by the corresponding peptide.

The presence of PP2A subunits was examined by using antisera generated against either the recombinant PP2A_C (AbC^{recomb}) or a C-terminal peptide (AbC^{302/309}) (39). Both antibodies detected PP2A_C in purified virus and fibroblast extracts (Fig. 8B, lanes 1 and 3). The doublet seen in the cell extract is often seen in immunoblots of PP2A_C and might be caused by posttranslational modifications. It comigrated with purified PP2A_C (lane C) at an apparent molecular mass of 36 kDa. Detection by AbC^{302/309} was specifically competed for by the peptide.

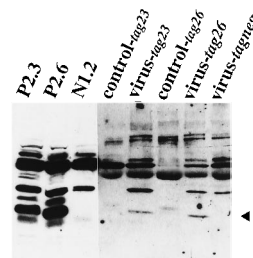


FIG. 7. Western blot analysis of astrocytoma cell lines stably expressing PP2A_C and of virus produced on these cells. Western blots of astrocytoma cell lines stably expressing tagged PP2A_C (P2.3 and P2.6) were probed with a MAb directed against the tag (12CA5). The control cell line (N1.2) consisted of cells stably transfected with the empty vector and selected in G418. The arrow indicates the 38-kDa tagged phosphatase. CMV produced in cell lines P2.3 and P2.6 was PEG precipitated and analyzed in Western blots probed with the anti-tag MAb. A band (arrowhead) comigrating with the 38-kDa band in P2.3 and P2.6 cell extracts is seen in virus preparations labeled virus-tag26 and virus-tag23. No corresponding band is seen in N1.2 cell extracts or in virus-tagneg produced on this cell line. No bands are seen in PEG precipitates of supernatants of uninfected P2.3 and P2.6 cells (control-tag23 and control-tag26).

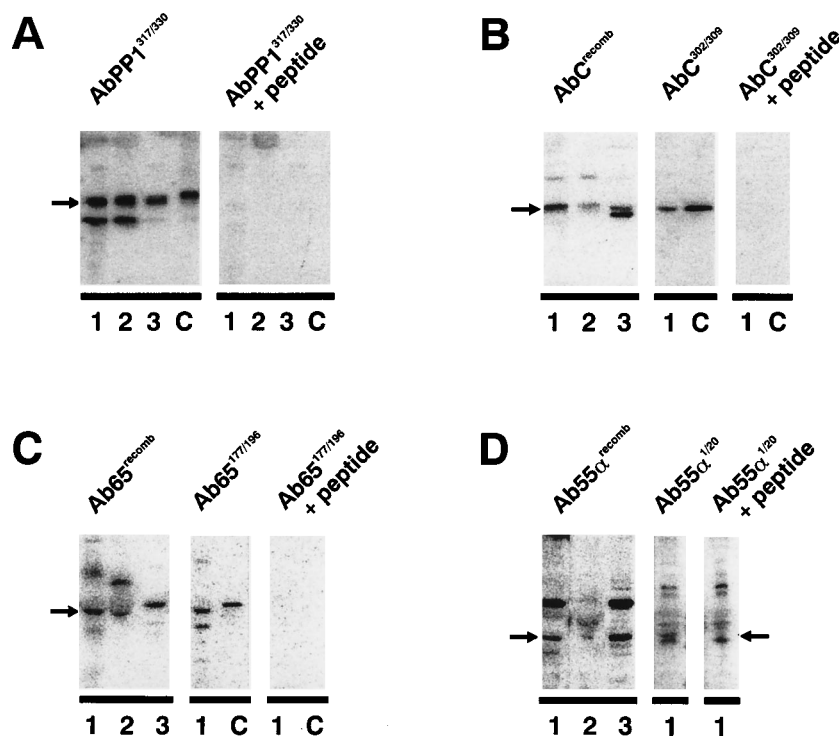


FIG. 8. Identification of PPase subunits in extracts of purified CMV, dense bodies, and the CMV host cell FSF. Proteins were immunoblotted as described in Materials and Methods. Lane 1, 8 μ g of purified CMV; lane 2, 8 μ g of a dense-body preparation; lane 3, 8 μ g of FSF cells infected for 72 h with CMV. The primary antibodies used to detect the PPase subunits are indicated above the blots and described in detail in Materials and Methods. Competition experiments (indicated by +peptide) were always performed with 50 μ M of the antigenic peptide. (A) Presence of PP1_C. The arrow indicates an apparent molecular mass of 39 kDa. The positive control (lane C) was 5 ng of homogeneous, recombinant PP1_C α . (B) Presence of PP2A_C. The arrow indicates an apparent molecular mass of 36 kDa. The positive control (lane C) was 5 ng of PP2A_C purified from MCF-7 cells. (C) Presence of PR65. The arrow indicates an apparent molecular mass of 57 kDa. The positive control (lane C) was 9 ng of homogeneous, recombinant PR65 α . (D) Presence of PR55 α . The arrow indicates an apparent molecular mass of 52 kDa.

The presence of the PR65 subunit was analyzed with antibodies to recombinant PR65 (Ab65^{recomb}) and to a specific peptide (Ab65^{177/196}) (50). PR65 protein was identified in purified virus and fibroblast extracts (Fig. 8C, lanes 1 and 3). Both antibodies recognized protein which comigrated with purified, recombinant PR65 α (lane C) at an apparent molecular mass of 63 kDa. In purified virus, the protein migrated faster (approximately 57 kDa). Again, a weak band was seen in dense-body preparations with an intensity equivalent to 60% of that in purified virus. We attribute the lower apparent molecular weights of these bands to the presence in CMV preparations of protein ppUL83 (M_r , 65,000), which represents 15% of the protein mass of virions and 95% of that of dense bodies. Again, the signal obtained with antipeptide antiserum Ab65^{177/196} was sensitive to competition by peptide. In addition, both antibodies detected a band with an apparent molecular mass of approximately 55 kDa in viral preparations. Its detection was also sensitive to antigen competition. Since this band is not present in host cell extracts, we suspect that this is a proteolysed form of the PR65, but we cannot exclude entirely that CMV encodes a protein with some homology to PR65.

PR55 α was also detected in CMV preparations. Although virus preparations exhibited considerable nonspecific background, the use of independently raised antisera enabled us to unambiguously demonstrate the presence of this protein in immunoblots. Antisera raised against recombinant protein (Ab55 α ^{recomb}) and against a specific peptide (Ab55 α ^{1/20}) (Fig. 8D) recognized specifically a protein with an apparent molecular mass of 52 kDa (lanes 1 and 3). Neither PR55 β nor any regulatory subunit of the PR72 class (18) was detected in either

fibroblasts or virus preparations (results not shown). Therefore, it appears that the CMV-associated PP2A is the form which is also the most abundantly found in cells (7).

The levels of the PPase subunits (per mass of total protein) in the virus were similar to those found in fibroblasts used to generate virus stocks. The catalytic subunits of PP1 and PP2A (approximately 5 ng/8 μ g), as well as PR65 (approximately 9 ng/8 μ g), represent about 0.06% of the viral proteins (Fig. 8C, lane 3). PR55 α was also present within this range (data not shown). Interestingly, there was no change in the levels of these subunits in cell extracts before and after infection. Thus, it appears that the majority of PP1 and PP2A in both virus and the host cell are represented by the same subunits and in similar proportions. These results lend support to the cellular origin of PP2A. It was interesting to find that PP2A subunits were specifically associated with purified virus and not with dense bodies, whereas PP1_C was present to the same extent in both.

DISCUSSION

The initial steps of virus infection presumably require modification of some host cell processes to create conditions conducive for eventual viral replication. The strategy of some viruses, like herpes simplex virus (34) and vaccinia virus (37), results in dramatic alterations of host cell metabolism, while others interfere with intracellular signal transduction, for example, papovavirus middle T interaction with cellular phosphatase activity (reviewed in reference 33). We show here that CMV induces hypophosphorylation of host cell proteins within minutes of contacting the cell membrane, in the absence of any

CMV genome expression. This appears to be largely elicited by S/T phosphatases associated with the virus, since CMV incubated with OA before infection of cells no longer caused protein hypophosphorylation.

Results of enzymatic assays using specific effectors and inhibitors indicate that the phosphatase activities present in the virion resemble those of PP2A and PP1. PP2A is responsible for about 70% of the virus-associated phosphatase activity, with PP1 being responsible for the rest. Indeed, immunoblot analyses revealed the presence of both phosphatases in purified virus preparations. The phosphatases appear to be specifically associated with the virus, since the detection of PP2A activity and the increase in PP1 activity upon NP-40 treatment of purified virus, as well as the immunodetection of PP2A in nucleocapsid preparations, show that the phosphatases are located within the virion.

Roby and Gibson (38) looked for phosphatase activity while studying virion protein kinases but did not detect any such activity. We assayed PPase in purified virus by using the kinase buffer described by them and did not detect PPase activity (data not shown). However, inclusion of ATP in the reaction at the concentration that they used (100 μ M) reduced PPase activity by 73%, which might explain why they did not detect any such activity. The 50% inhibitory concentration of ATP for PP2A is 120 μ M (51). In addition, they purified virus on gradients containing tartrate, a classic inhibitor of acid phosphatases. We were unable to detect phosphatase activity in virus purified on such gradients (results not shown).

The presence of cellular PP2A in CMV virions is unusual but not unprecedented since other cellular enzymes and proteins have been shown to be incorporated into the virus particle. These include a DNase activity (23) and a DNA polymerase I activity (25) and host cell annexin II (54). The CMV-associated PP2A activity is at least partially due to the presence of a cellular enzyme, since virus incorporated tagged, cellular PP2A_C. This was not due to an overabundance of PP2A, since neither of the PP2A tag-expressing cell lines overexpressed PP2A_C, as corroborated by immunoblotting with a polyclonal anti-PP2A_C antibody (data not shown). In transfected cells, the tagged version of PP2A_C apparently replaces endogenous PP2A_C; thus, the total level of PP2A_C remains constant. This is in agreement with previous reports that the PP2A_C cannot be overexpressed (26).

To our knowledge, this work is the first to describe incorporation of a cellular PPase into a virus particle. Vaccinia virus (16, 17) and a baculovirus (17) encode dual-specificity PPases. Other animal viruses encode proteins that interact with cellular PPases and modify their enzymatic activities. Human papovavirus 16 (43) and simian virus 40 encode proteins that modulate the activity of PP2A (reviewed in reference 33).

In light of our present knowledge, one can only suggest possible roles for virion-associated PPases in these events. CMV contacts a receptor on fibroblasts which has recently been described as a 92.5-kDa phosphoglycoprotein (22). Dephosphorylation of this protein following contact with the virus could play a role in the fusion of the viral envelope with the cell membrane. One viral structural phosphoprotein (pp65) is immediately transported to the nucleus (15), where it presumably plays an important role in the initiation of viral genome expression. pp65 has two nuclear localization signals (40) and is in a phosphorylated form in the virion (38). Resident PPases in the virus might dephosphorylate pp65, thereby unmasking its nuclear localization signal. Our preliminary results (not shown) indicate that, indeed, CMV-associated PPase activity can dephosphorylate pp65 previously autophosphorylated in vitro as an immune complex with a specific MAb (32).

In any event, as shown here, the CMV virion can transport enzymatically active phosphatases into its host cell, where they are partially responsible for a hypophosphorylation of cellular proteins observed immediately after contact between the cell and the virion. Thus, enveloped virus particles, like CMV, can transport a cellular enzyme from one cell and "inoculate" it into another cell in an active form. Such passively inoculated material introduced into nonpermissive cells, in the absence of any viral gene expression, could affect host cell metabolism, albeit transiently. As concerns CMV, this notion is important in light of the fact that during active infection in vivo, CMV causes a profound immunosuppression which cannot be explained solely by viral replication in immunocompetent cells (31). CMV particles do penetrate these cells, as evidenced by the presence of CMV DNA (45), but viral gene expression does not occur (47). Our studies thus raise the possibility that the mere entrance of structural components of virions into the cell can elicit changes in the metabolism of immunocompetent cells.

ACKNOWLEDGMENTS

We are grateful to M. C. Mumby for providing the antiserum used in preliminary experiments. We thank B. Wadsinski and G. Johnson for providing the tagged PP2A_C plasmid and F. Arenzana-Seisdedos for helpful discussions.

This work was supported by grant 930609 from the Institut National de la Santé et de la Recherche Médicale (S.M.), by the Agence Nationale de Recherche sur le SIDA, and by EC (ROCIO) contract BMH1-CT93-1007.

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