Human Cytomegalovirus Virion-Associated Protein with Kinase Activity

WILLIAM J. BRITT* AND DENISE AUGER

Department of Pediatrics, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35294

Protein kinase activity was detected in immunoprecipitates of human cytomegalovirus virions and infected cells by using a monoclonal antibody directed against an abundant 68,000-dalton virion structural protein. Purification of this protein by electrophoresis confirmed that the kinase activity was associated with this protein. The kinase activity was dependent on divalent cations (Mg^{2+}, Mn^{2+}) and cyclic nucleotide independent and exhibited optimal activity at pH 7 to 8. The kinase phosphorylated threonine and serine but not tyrosine.

Protein kinase activity has been demonstrated in a number of different viruses (1, 2, 13, 17). In some cases this kinase activity was demonstrated in purified virions, while in others specific structural proteins have been shown to have enzymatic activity. The function(s) of most virion-associated protein kinases remains unknown; however, many are believed to be directly involved in viral replication. Possible functions of virus-associated protein kinases include regulation of viral nucleic acid replication and transcription (13, 17, 19), modification of virion structural proteins leading to either uncoating or encapsidation of viral nucleic acid (6, 19, 20), and as yet undefined changes in host cell proteins involved in viral replication. In addition, several studies have detailed the apparent role of virus-encoded protein kinases in neoplastic transformation (4, 15). Several members of the herpesvirus family have been shown to contain a virion-associated protein kinase (10, 11, 16). Epstein-Barr virus and human cytomegalovirus (CMV) have also been shown to induce protein kinase activity in infected cells (9, 12). In this report we used murine monoclonal antibodies reactive with a 68-kilodalton (kDa) CMV virion structural protein (p68) (18) to identify and characterize the kinase activity of this virion protein in vitro. Our findings are in contrast to those in a previous report which detailed the presence of a CMV-induced infected-cell protein kinase (12).

Initially, we screened a large number of murine monoclonal antibodies reactive with a variety of different virion structural proteins in an attempt to identify CMV-encoded proteins with kinase activity. Preliminary studies indicated that detectable kinase activity was consistently associated with precipitation of an abundant 68-kDa virion protein. Two antibodies, 28-19 and 28-103 (3a), that were reactive with p68 were used to further characterize this kinase activity in both infectious virions and infected cells. Soluble proteins were precipitated from extracellular virions and the cytoplasmic and nuclear enriched fractions of infected cells with either normal mouse serum (NMS), monoclonal antibody 7-17, which is reactive with a major CMV envelope glycoprotein (3), or antibody 28-103. The antigen-antibody complexes were collected, washed extensively, and suspended in kinase buffer containing [γ-^{32}P]ATP (ICN, Irvine, Calif.). After a 20-min incubation, the pellets were washed and the antigen-antibody complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In contrast to NMS and antibody 7-17, antibody 28-103

precipitated kinase activity from virions and both the cytoplasmic and nuclear fractions of infected cells (Fig. 1). This kinase activity phosphorylated at least two identifiable proteins, p68 and the heavy chain of mouse immunoglobulin (MIg) (Fig. 1). A third, as yet uncharacterized, protein of estimated size 70 to 80 kDa was also phosphorylated (Fig. 1). Although the p68 was phosphorylated only minimally in the virion, ^{32}P incorporation into MIg was readily detectable (Fig. 1), suggesting either that limiting amounts of the phosphate acceptor p68 were available in immunoprecipitates of solubilized virions or that the virion phosphate acceptor site was phosphorylated at saturating levels.

In previous studies, considerable controversy surrounded the initial characterization of several virus-associated kinases because of the possibility of copurification of cellular kinases with the putative virus-encoded kinases. Because of this possibility, we attempted further purification of p68. Initially, immunoaffinity columns were prepared by coupling antibody 28-103 to Sepharose 4b (Sigma Chemical Co., St. Louis, Mo.). Although use of this column allowed efficient collection of significant amounts of p68, the need to use mild denaturing conditions resulted in contamination of the p68 with other unidentified proteins (data not shown). In addition, differences in migration between reduced and nonreduced immunoprecipitates containing p68 in SDS-PAGE suggested that this protein was linked to other infected-cell proteins by disulfide bonding (data not shown). For these reasons, we used a combination of immunoprecipitation and preparative SDS-PAGE to further purify p68 for use in the kinase reaction. Following immunoprecipitation and separation by SDS-PAGE, p68 was visualized and eluted from the gel. After a cycle of denaturation and renaturation, the purified p68 was used in the kinase reaction. When purified p68 was incubated with [γ-^{32}P]ATP and MIg, which was added to ensure the presence of an adequate amount of phosphate acceptor, ^{32}P was detected in p68 and the heavy and light chains of MIg (Fig. 2). With the exception of the lane containing 0.5 µg of p68, the level of ^{32}P incorporation into both p68 and MIg appeared to increase linearly with increasing concentrations of p68 (Fig. 2). Although several possible explanations exist for the nonlinearity of ^{32}P incorporation obtained with 0.5 µg of p68, the most likely would seem to be an error in accurately sampling the solution which contained a low concentration of purified p68. Interestingly, 25 ng of p68 phosphorylated the heavy and light chains of MIg very efficiently, but very little ^{32}P was incorporated into the p68 (Fig. 2). This result was similar to our
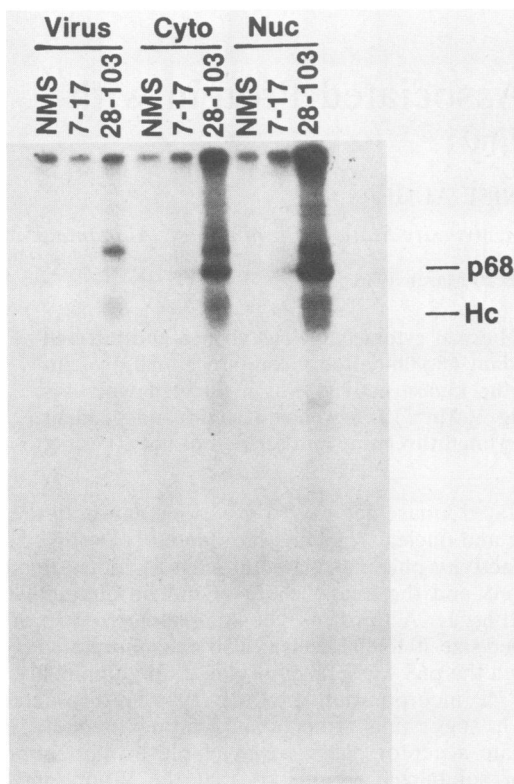
---

* Corresponding author.

FIG. 1. Extracellular virions were purified from the supernatant of 10^6 CMV AD169-infected human fibroblast cells by sedimentation through density gradients (3). Cytoplasmic (Cyto) and nuclear (Nuc) enriched fractions of the infected cells were prepared as described by Gibson (7). The virions and the nuclear fraction were suspended in 0.05 M Tris–0.15 M NaCl, pH 7.3 (TBS) containing 0.1% SDS and incubated for 20 min at 4°C. Deoxycholate, SDS, and Nonidet P-40 were added to the virion, nuclear, and cytoplasmic lysates to final concentrations of 0.5, 0.1, and 1.0%, respectively. The lysates were then precleared with NMS and Formalin-fixed *Staphylococcus aureus* (Bethesda Research Laboratories, Gaithersburg, Md.) as described (3). The lysates were incubated with NMS, antibody 7-17, or antibody 28-103 at 4°C for 6 h, and the precipitates were collected and washed as previously described (3). Following the final wash, the precipitates were suspended in 100 μl of kinase buffer (0.05 M Tris, 0.15 M NaCl, 0.01 M Mg^{2+}, pH 7.5), and 10 μCi of [γ-^{32}P]ATP was added. The kinase reaction was incubated at room temperature for 20 min, washed two times, and analyzed by SDS-PAGE. Molecular weights were determined by migration of the standards bovine serum albumin and MIg heavy (Hc) and light chains (Lc), and phosphorylated proteins were detected by autoradiography (Britt and Auger, in press).

findings when virions were a source of p68 (Fig. 1), suggesting that in this experiment the p68 was also present in limiting amounts as a phosphate acceptor.

Using antibody 28-103 and immunoprecipitation to collect p68 from infected cells, we characterized the in vitro enzymatic properties of this protein kinase. The activity of this enzyme was dependent on the presence of the divalent cations Mg^{2+} and Mn^{2+} (Table 1). The enzyme was active over a broad pH range, with maximal incorporation of ^{32}P occurring between pH 7.0 and 8.0 (Table 1). Enzymatic activity was not dependent on the presence of either cyclic AMP or cyclic GMP (Table 1). In addition, nonradiolabeled ATP completely inhibited the incorporation of ^{32}P. The enzyme could transfer phosphate to a number of different substrates, including casein, protamine, and mouse immunoglobulin M (Table 1); however, histones were phosphorylated minimally, if at all (Table 1). Because 50 μg of histones caused a reduction in the ^{32}P incorporated into the pellet, it appeared that this preparation of histones may have inhibited the activity of the enzyme. The mechanism and significance of this apparent inhibition of enzymatic activity by histones is unknown. Finally, phosphoamino acid analysis revealed that the amino acids phosphorylated in the in vitro kinase reaction migrated with both phosphoserine and phosphothreonine but not phosphotyrosine (Fig. 3).

In this report we have identified and characterized a virion-associated protein kinase of CMV. Previously, Mar and Huang also reported that CMV virions contain a protein kinase with several properties similar to the p68 kinase (11); however, these authors failed to identify the virion protein exhibiting this enzymatic activity. More recently, Davis et al. mapped the genetic sequences encoding a 67-kDa phosphoprotein with apparent kinase activity between 0.37 and 0.39 map units on the genome of CMV (5). However, these authors did not demonstrate kinase activity of the purified protein. Michelson et al. also described a 68-kDa CMV-
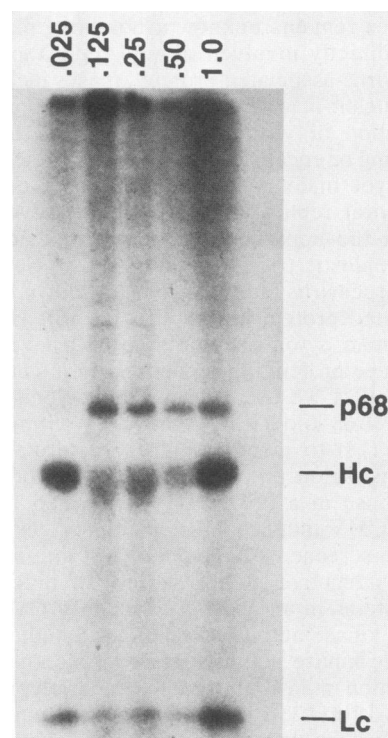

FIG. 2. Infected-cell lysates were prepared from 5 × 10^7 CMV-infected human fibroblast cells as described in the legend to Fig. 1. After preclearing, p68 was precipitated with antibody 28-103 and electrophoretically separated by SDS-PAGE. The p68 was visualized by staining with Coomassie blue, cut from the gel, and eluted by the method of Hager and Burgess (8). The p68 was precipitated with cold acetone, denatured with 8 M guanidine, and allowed to renature in TBS. Between 0.025 and 1.0 μg of p68 was added to 100 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP. Fifty micrograms of MIg was added as a phosphate acceptor. After a 20-min incubation at room temperature, 10% trichloroacetic acid (TCA) was added to precipitate the phosphorylated proteins. Following a wash with 10% TCA and acetone, the insoluble material was solubilized and analyzed by SDS-PAGE. The amount (in micrograms) of p68 used in the kinase reaction is shown at the top of the figure. Molecular weights were determined as described in the legend to Fig. 1.

TABLE 1. In vitro characteristics of the CMV p68 protein kinase

Divalent cation dependence[a]			pH dependence[b]		Nucleotide dependence[c]		cpm (% of control)	Substrates for p68 Kinase[d]	cpm	
Cation	Concn (mM)	cpm (% of control)	pH	cpm	Nucleotide	Concn (μM)		Substrate (mM)	Supernatant	Pellet
None		1,053 (10)	4.0	1,890	None		6,019 (100)	None	1,243	32,311
EDTA	10	696 (6)	5.0	3,047	cAMP	5	4,695 (78)	EDTA (10)	763	1,464
Mg^{2+}	10	10,701 (100)	6.0	6,295		50	3,890 (65)	IgM	2,610	40,570
	50	7,311 (75)	7.0	7,394		500	6,978 (115)	Casein	2,221	25,132
	100	7,311 (75)	8.0	8,428	cGMP	5	5,078 (84)	Protamine	1,920	32,821
Mn^{2+}	10	6,837 (68)	9.0	1,460		50	7,411 (123)	Histone	982	16,725
	50	7,075 (70)				500	7,493 (124)			
	100	8,883 (88)			ATP	1	271 (2.5)			
CA^{2+}	10	886 (8)								
	50	839 (8)								
	100	491 (5)								

[a] Immunoprecipitates of antibody 28-103 and p68 were obtained as described in the legend to Fig. 1. After extensive washing, the pellets were suspended in 100 μl of TBS, pH 7.5, containing either no divalent cations, EDTA, or one of the divalent cations listed. After addition of 1 μCi of [γ-^{32}P]ATP, the reaction mixtures were incubated at room temperature for 20 min. The pellets were then washed three times, and the antigen-antibody complexes were eluted as described previously (3). The eluted proteins were precipitated with 10% trichloroacetic acid (TCA) at 4°C as described in the legend to Fig. 2 and counted. Control activity was defined as the incorporation of ^{32}P after addition of 10 mM Mg^{2+}.

[b] Immunoprecipitates were obtained, and the pellets were suspended in 0.05 M HEPES (N-Z-hydroxyethylpiperazine-N'-Z-ethanesulfonicacid)–0.15 M NaCl at the pH listed. Approximately 1 μCi of [γ-^{32}P]ATP was added, and the reaction mixtures were incubated for 20 min at room temperature. The incorporation of ^{32}P into TCA-precipitable material was determined as described above.

[c] Standard immunoprecipitation reaction mixture was obtained. Various amounts of cAMP, cGMP, or nonradiolabeled ATP were then added. The reaction was allowed to proceed for 20 min at room temperature, and the ^{32}P incorporation into TCA-precipitable material in the eluted antigen-antibody complexes was determined.

[d] Immunoprecipitates were prepared as described above. Mouse IgM, protamine, casein, or histones (50 μg) were used. A control tube containing 10 mM EDTA was included to determine the background level of incorporation of ^{32}P in the absence of kinase activity. After addition of 10 μCi of [γ-^{32}P]ATP, the reactions were incubated for 20 min at room temperature. The staphylococcal protein A-containing immune complexes were pelleted, and the supernatants were removed. The ^{32}P incorporation into TCA-precipitable material was determined for the supernatants and the antigen-antibody complexes bound to the protein A pellets as described in the legend to Fig. 2.
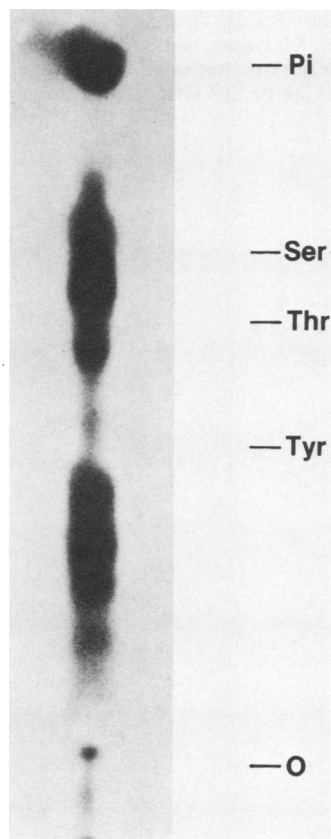
induced nuclear protein with kinase activity (12). Because these authors concluded that the p68 CMV-induced protein kinase was a nonvirion protein, the possibility remains this kinase activity was associated with a cellular protein induced by CMV. In contrast to this report, our findings clearly indicated that the p68 kinase recognized by antibody 28-103 was a structural protein of the CMV virion (3a) and therefore was virus encoded. In addition, we also detected kinase activity in the cytoplasm of infected cells (Fig. 1). Furthermore, biochemical evidence indicated that the p68 virion-associated kinase activity could function efficiently in the presence of either Mg^{2+} or Mn^{2+} and had a pH optimum of 7.0 to 8.0 (Table 1). These results would suggest that the protein kinase activity associated with p68 of the virion was distinct from the CMV-induced kinase activity described previously (12) and was more closely related to the kinase activity associated with purified virions (11). Because virion protein kinases in other viruses have been shown to play a role in such critical replicative functions as uncoating of

FIG. 3. Kinase reaction carried out as described in the legend to Fig. 1 with p68 immunoprecipitated from infected cells. Antigen-antibody complexes were eluted and precipitated with 10% TCA as described in the legend to Fig. 2. The precipitated protein was resuspended in 2 M HCl, heated at 100°C for 60 min, and then lyophilized. Approximately 1,000 cpm was spotted on cellulose thin-layer chromatography plates (Sigma) along with 2 μg of nonradiolabeled phosphoserine, phosphothreonine, and phosphotyrosine standards (Sigma). Following electrophoresis at 600 V for 2 h in 0.5% pyridine–5% acetic acid–94.5% water, pH 3.5, the plates were stained with ninhydrin. The migration from the origin (O) of the most cathodal position of the standards of phosphoserine (Ser), phosphothreonine (Thr), and phosphotyrosine (Tyr) are shown in the right margin.

intact nucleocapsids, nucleic acid replication, and encapsidation (6, 14, 18–21), the presence of protein kinase activity in the CMV virion suggested the possibility of an analogous role(s) for this abundant CMV structural protein. Future studies will be necessary to clarify the function of this protein.

LITERATURE CITED

1. **Albin, C., and W. S. Robinson.** 1980. Protein kinase activity in hepatitis B virus. J. Virol. **34:**297–302.
2. **Blair, G. E., and W. C. Russell.** 1978. Identification of a protein kinase activity associated with human adenoviruses. Virology **86:**157–166.
3. **Britt, W. J.** 1984. Neutralizing antibodies detect a disulfide-linked glycoprotein complex within the envelope of human cytomegalovirus. Virology **135:**369–378.
3a. **Britt, W. J., and D. Auger.** 1985. Identification of a 65,000 dalton virion envelope protein of human cytomegalovirus. Virus Res. **4:**31–36.
4. **Collett, N. S., and R. L. Erickson.** 1978. Protein kinase activity associated with the avian sarcoma virus SRC gene product. Proc. Natl. Acad. Sci. USA **75:**2021–2024.
5. **Davis, M. G., and E. S. Huang.** 1985. Nucleotide sequence of a human cytomegalovirus DNA fragment encoding a 67-kilodalton phosphorylated virion protein. J. Virol. **56:**7–11.
6. **Garrcea, R. L., K. Ballmer-Hofer, and T. L. Benjamin.** 1985. Virion assembly defect of polyomavirus hr-t mutants: underphosphorylation of major capsid protein VP_1 before viral DNA encapsidation. J. Virol. **54:**311–316.
7. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. Virology **111:**516–537.
8. **Hager, D. A., and R. F. Burgess.** 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate and renaturation of enzymatic activity: results with single subunit of Escherichia coli RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. **109:**76–86.
9. **Kamla, T., K. Takaki, Y. Hinuma, and Y. Watanabe.** 1981. Protein kinase activity associated with Epstein-Barr virus-determined nuclear antigen. Virology **113:**512–520.
10. **Lemaster, S., and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. J. Virol. **35:**798–811.
11. **Mar, E. C., P. C. Patel, and E. S. Huang.** 1981. Human cytomegalovirus-associated DNA polymerase and protein kinase activities. J. Gen. Virol. **57:**149–156.
12. **Michelson, S., M. Tardy-Pain, and O. Barzu.** 1984. Properties of a human cytomegalovirus induced protein kinase. Virology **134:**259–268.
13. **Paoletti, E., and B. Moss.** 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. **10:**417–424.
14. **Sanchez, A., B. P. De, and A. K. Banerjee.** 1985. In-vitro phosphorylation of NS protein by the L protein of vesicular stomatitis virus. J. Gen. Virol. **66:**1025–1036.
15. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20:**807–816.
16. **Stevely, W. S., M. Katan, V. Stirling, G. Smith, and D. P. Leader.** 1985. Protein kinase activities associated with the virions of pseudorabies and herpes simplex virus. J. Gen. Virol. **66:**661–673.
17. **Strand, M., and J. T. August.** 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature (London) New Biol. **233:**137–140.
18. **Tsiapalis, C. M.** 1977. Chemical modification of DNA poly-merased phosphoprotein from avian myeloblastosis virus. Nature (London) **266:**27–31.
19. **Wilson, M., and R. A. Consigli.** 1985. Functions of a protein kinase activity associated with purified capsids of the granulosis virus infecting Plodia interpunctella. Virology **143:**526–535.
20. **Witt, D. J., and D. F. Summers.** 1980. Relationship between virion associated kinase effected phosphorylation and transcription activity of vesicular stomatitis virus. Virology **107:**34–49.
21. **Witt, D. J., C. W. Naeue, and D. F. Summers.** 1981. Phosphorylation of vesicular stomatitis virus proteins as a possible contributing factor in virion uncoating. J. Gen. Virol. **56:**383–391.