Protein Kinase Activity from Vaccinia Virions: Solubilization and Separation into Heat-Labile and Heat-Stable Components

JAY KLEIMAN AND BERNARD MOSS

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

Received for publication 18 May 1973

A protein kinase was solubilized from whole vaccinia virions by using a solution containing deoxycholate, dithiothreitol, and sodium or potassium chloride. The released enzyme was completely dependent on Mg^{2+} and was greatly stimulated by added basic proteins such as protamine or histones. Dithiothreitol was also stimulatory, whereas GTP, CTP, UTP, and P_i at concentrations equimolar with ATP had little or no effect. Attempts to purify the protein kinase were initially unsuccessful, leading us to consider that either the enzyme was extremely labile or that two readily separable components were required for activity. The observation that the material extracted with NP-40 detergent during the preparation of viral cores stimulated the protein kinase activity of the intact cores supported the second possibility. As the protein kinase, now solubilized from viral cores, was passed through successive DEAE-cellulose columns, it became increasingly dependent for activity on addition of the NP-40 extract. A 30- to 40-fold stimulation of protein kinase activity, which afforded recovery of essentially all starting activity, could be effected by addition of the NP-40 extract to the partially purified enzyme. The NP-40 extract was shown to contain a heat stable, trypsin-sensitive protein, whose action could not be duplicated by cyclic nucleotides.

Protein kinase activities are associated with many enveloped viruses including RNA tumor viruses (6, 14), vesicular stomatitis virus (14), herpesvirus (10, 13), vaccinia virus (11), and frog virus 3 (5). The true nature of these enzymes, as host or virus coded components, and their biological roles remain unknown. One function of the vaccinia enzyme might be to phosphorylate virion polypeptides since recent *in vivo* studies (H. Rosemond, J. Kleiman, and B. Moss, *Fed. Proc.* 32:462, 1973; H. Rosemond, and B. Moss, *J. Virol.*, in press) demonstrated that at least one virion protein contains phosphoserine residues. Peptide mapping of tryptic digests further suggested that a single site on the protein was phosphorylated. Additional work with this system depends on the isolation and characterization of the viral enzyme, and determination of the function of the virion phosphoprotein. In this communication we describe the solubilization of the protein kinase activity from vaccinia virions and the unex-

pected finding that it consists of heat-stable and heat-labile components.

MATERIALS AND METHODS

Virus purification. Vaccinia virus (strain WR) was purified from HeLa cells by sedimentation through a sucrose cushion and two cycles of sucrose gradient centrifugation as previously described (7, 9).

Protein kinase assay. The basic reaction contained 50 mM Tris-hydrochloride, pH 10.25, 3 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.01% Nonidet P-40 (NP-40), and 0.50 mM γ - ^{32}P -adenosine triphosphate in a total volume of 0.1 or 0.2 ml. The reaction was initiated by addition of viral protein, and allowed to proceed for 15 min. The reaction was terminated by the addition of 0.1 ml of 0.1 M disodium EDTA containing 250 μ g of bovine serum albumin followed by 2.5 ml of trichloroacetic acid containing 0.05 M sodium pyrophosphate ($NaPP_i$). After standing at room temperature for 15 min, the precipitate was collected by centrifugation and redissolved in 0.1 ml of 1 N NaOH and 0.05 M $NaPP_i$. The material was reprecipitated with trichloroacetic acid, and the product was collected and washed on HA membrane filters

(0.45 μ m pore size; Millipore Corp.). The filters were counted in 10 ml of toluene-based scintillation fluid.

Preparation of viral cores and NP-40 soluble fraction. Cores were prepared by incubation of purified virus, at a final concentration of 60 to 120 optical density at 260 nm (OD_{260}), at 37 C for 30 min in 50 mM Tris-hydrochloride, pH 8.4, 0.5% NP-40, and 50 mM DTT as previously described (2, 11). At the end of this incubation, the reaction was cooled on ice and sonically treated with a Branson microtip probe at one-half maximal setting for two 30-s periods. The material was then layered on a 36% sucrose cushion in 10 mM Tris-hydrochloride, pH 8.4, and 1 mM DTT, and centrifuged in a Beckman SW-50L rotor at 25,000 rpm for 30 min. The pellet of viral cores was suspended by brief sonic treatment in 10 mM Tris-hydrochloride, pH 8.4. The material remaining above the sucrose cushion was collected, and will be referred to as the NP-40 soluble fraction. This material was stored at -20 C until use.

Disruption of vaccinia virus and virus cores with sodium deoxycholate. Vaccinia virions, or viral cores, were treated at 4 C for 30 min with 0.3 M Tris-hydrochloride, pH 8.4, 0.1% sodium deoxycholate, 50 mM DTT, and KCl or NaCl at concentrations of 0.25 or 0.5 M. The material was then centrifuged at 4 C at $130,000 \times g$ in a Beckman SW50-L rotor for 1 h, and the supernatant fraction was decanted.

DEAE-cellulose chromatography. DEAE-cellulose was equilibrated with buffer containing 0.15 M Tris-hydrochloride, pH 8.4, 0.2% NP-40 or 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 2 mM DTT, and 0.25 M NaCl or KCl (high salt buffer) or 0.05 M NaCl or KCl (low salt buffer). Columns (0.9 by 3-4 cm) were operated at 4 C at flow rates of about 10 ml/h.

Viral protein determination. Radioactive vaccinia virus, grown in the presence of 3 H-leucine, was counted in a toluene-Triton X-100 based scintillation fluid. A conversion factor of 64 μ g of protein per OD_{260} of whole virions was used to calculate the specific activity of viral proteins. The concentration of isolated viral proteins was then determined by using this value.

Source of materials. Total histone was purchased from Worthington Biochemicals and calf-thymus lysine-rich histone, protamine, arginine-rich histone, phosvitin, α -casein, and bovine serum albumin were purchased from Sigma Chemical Co. γ - 32 P-ATP was obtained from New England Nuclear Corp. DEAE-cellulose (DE-52) was purchased from the Reeve Angel Co., and was not recycled before use. DNase, RNase, and trypsin were obtained from Worthington Biochemicals.

RESULTS

Solubilization of protein kinase. The protein kinase was solubilized by treating vaccinia virions with a solution containing sodium deoxycholate, salt, DTT, and Tris buffer, as described in Materials and Methods. Liberation of the enzyme resulted in a 5- to 10-fold increase in

measurable protein kinase activity. Approximately 50 to 60% of this heightened activity was recoverable in the supernatant fraction obtained by centrifugation at $130,000 \times g$. Extractions performed with virus in which the DNA was labeled with 3 H-thymidine showed that this procedure liberated essentially all viral DNA. The nucleic acids were removed by diluting the extract to reduce the salt concentration to 0.25 M and then passing it through a DEAE-cellulose column in high salt buffer. This step separated greater than 97% of the viral nucleic acid from the protein kinase, which was not absorbed to the DEAE-cellulose under these conditions. Between 65 and 75% of the activity applied was recovered in the flow through fractions when glycerol and EDTA were present in the buffers.

The unsolubilized protein kinase in whole virions was able to incorporate γ - 32 P from ATP into endogenous acceptor proteins and was stimulated several fold by addition of histones or protamine (11). In contrast, the soluble protein kinase was almost completely dependent on added basic proteins for activity. Protamine sulfate was the most active of the proteins sampled at the tested concentrations, but several others including total histone and lysine-rich histone were also active. The reaction was saturated with protamine at concentrations of less than 10 μ g/ml (Fig. 1). Under conditions in which ATP was also saturating, protein phosphorylation was proportional to the amount of enzyme added and was linear with respect to time for at least 15 min. Protein kinase activity was completely dependent upon addition of Mg^{2+} and was stimulated by dithiothreitol. Activity was not inhibited by GTP, CTP, UTP, or P_i at concentrations equimolar with ATP. Cyclic AMP at a concentration of 1 μ M had neither stimulatory nor inhibitory effect.

Separation of the protein kinase into heat-labile and heat-stable components. Attempts to purify the protein kinase were initially thwarted by repeated losses of enzyme activity during standard chromatographic fractionation procedures. We considered that either the enzyme was extremely labile, or that two readily separable components were needed for activity. The solution to this problem came from experiments in which viral cores prepared with NP-40 detergent, rather than whole virions, were used as the source of enzyme. As previously described (11), most of the protein kinase activity of whole virions was recovered in viral cores, and the NP-40 soluble fraction possessed negligible enzyme activity. However, when the cores

FIG. 1. Protamine saturation curve. Solubilized protein kinase containing approximately 10 μ g of viral protein was incubated in standard 0.2-ml assays modified to contain between 0 and 4 μ g of protamine sulfate. Appropriate zero time values were determined for each point. With 10 to 40 μ g of protamine per assay, a decrease of approximately 30 to 40% from the maximum values shown above was observed. (Data not shown).

were treated with deoxycholate the protein kinase activity was not stimulated 5- to 10-fold as noted with intact virions. These observations suggested that two components, one of which was partially released by nonionic detergent treatment, were needed for the increased activity of the soluble enzyme. Initial experiments in which viral cores were recombined with the NP-40 soluble fraction demonstrated this to be the case. Recombination of the NP-40 soluble fraction with cores resulted in an augmentation of protein kinase activity, and the extent of stimulation was proportional to the amount added over at least a fivefold range (Fig. 2). The basal activity without added NP-40 soluble fraction was presumably due to the incompleteness of extraction of the factor. The factor that was released during preparation of cores was entirely stable to heat treatment at 90 C for 10 min, and consequently will be referred to as heat-stable factor (HSF). The core-associated activity was completely destroyed during identical treatment, and will be referred to as heat-labile factor (HLF).

Partial purification of HLF. Since initial experiments demonstrated that a soluble protein kinase could be obtained by treating whole virions with deoxycholate and salt, attempts were now made to solubilize the heat-labile component from viral cores. The enzyme was assayed in the presence of protamine and heat-stable factor prepared from NP-40 extracts of whole virus. The results of this solubilization are shown in Table 1. All of the activity was obtained in the high-speed supernatant fraction. Addition of HSF stimulated soluble protein kinase approximately 12-fold. Essentially all of the soluble HLF activity passed through two successive DEAE-cellulose columns in 0.25 M NaCl and 0.05 M NaCl buffer under the

FIG. 2. Effect on protein kinase activity of recombination of core and NP-40 fractions prior to solubilization of HLF. Viral cores and NP-40 soluble fractions were prepared as in Materials and Methods, except that the centrifugation step used to separate cores from the soluble fraction was done at 17,500 rpm in a Sorvall refrigerated centrifuge without use of a sucrose cushion. Cores were resuspended in 10 mM Tris-hydrochloride, pH 8.4, at a concentration of approximately 20 mg/ml. The NP-40 soluble fraction was at an approximate concentration of 3.2 mg/ml. Cores were recombined at 4 C with varying amounts of NP-40 soluble fraction, and then incubated with the solution containing sodium deoxycholate used for solubilization of the protein kinase for 30 min at 4 C. Samples containing approximately 2 μ g of cores recombined with between 1.0 and 8.0 μ g of NP-40 soluble fraction were assayed.

TABLE 1. Dependence of protein kinase reaction on added HSF^a

Fraction	Protein kinase activity (pmol ³² P/15 min)		Stimulation
	-HSF	+HSF	
Whole virus	92		
Cores	64	140	2.2
Total DOC	52	336	6.4
DOC supernatant fluid	32	381	11.8
High salt DEAE (0.25 M NaCl)	27	358	13.4
Low salt DEAE (0.05 M NaCl)	11	335	30

^a Cores from approximately 60 mg of purified whole virions were prepared as described in Materials and Methods. The HLF was solubilized from cores with deoxycholate (DOC) and salt, and passed through DEAE-cellulose columns in high and low salt buffers as described. A similar fraction of the total material recovered at each stage of the procedure was assayed in the presence or absence of approximately 40 μ g of heat-treated HSF.

conditions described in Materials and Methods. These steps apparently removed residual HSF, and the resulting enzyme was then stimulated as much as 30- to 40-fold (Table 1, Fig. 3) by HSF. These steps also removed essentially all of the viral DNA. On storage at -20 C, the extent to which HLF was active in the absence of added HSF increased slightly, although the total activity in the presence of both HSF and HLF remained constant for at least 6 weeks.

Preliminary characterization of HSF. Treatment of vaccinia virions with NP-40 detergent and with a reducing agent was originally used by Easterbrook to separate the lipoprotein envelope from the DNA-containing core (2). Initial characterization of the HSF was, therefore, directed toward determining whether it was protein or lipid.

The NP-40 soluble fraction prepared from vaccinia virus was incubated in a 90 C water bath for 10 min and then rapidly cooled on ice. A large flocculent precipitate, representing 60% of the initial protein, was removed by centrifugation at 2,000 rpm for 15 min, and the supernatant fluid was used for further studies. Greater than 90% of the initial HSF activity remained in the supernatant fluid, and activity was stable at 4 C for at least 4 weeks.

Digestion of HSF with trypsin for 30 min resulted in complete loss of stimulatory activity (Table 2). Control experiments ensured that the trypsin was completely inactivated after treat-

FIG. 3. Dependence of HLF on added HSF for protein kinase activity. HSF was prepared as described in the text from NP-40 soluble fraction at an initial concentration of 1.5 mg/ml by treatment at 90 C. HLF was prepared as described in the text through the low-salt (0.05 M NaCl) buffer DEAE-cellulose column step and an amount that contained approximately 10 μ g of viral protein was used per assay. Indicated samples of HSF were added directly to the assay mix prior to incubation at 37 C and HLF was added to initiate the reaction. A zero time blank was determined by adding both HLF and HSF and immediately stopping the reaction. HLF and HSF were also incubated individually. HSF alone, dashed lines; HLF plus 0 to 80 μ liters of HSF, solid line.

TABLE 2. Inactivation of HSF with trypsin

Treatment of HSF	Protein kinase activity (% control)
Trypsin ^a	5
Trypsin + untreated HSF ^b	133
Heat-inactivated trypsin ^c	91

^a HSF (approximately 100 μ g) was incubated in a buffer containing 50 mM Tris-hydrochloride, pH 8.4, 50 mM DTT, and 0.5% NP-40 with 10 μ g of trypsin for 30 min at 37 C in a volume of 0.2 ml. Trypsin was then inactivated by heating the entire mixture at 90 C for 10 min. A sample of the reaction was assayed for protein kinase activity with about 10 μ g of HLF in a standard reaction mixture for 15 min. Control values (300 pmol/15 min) were obtained by incubating HSF without trypsin.

^b HSF was incubated with 10 μ g of trypsin at 37 C as in *a* and then trypsin was inactivated at 90 C. After cooling, an equal amount of untreated HSF was added to the mix, and a sample of this final product assayed as in *a*.

^c HSF was incubated at 37 C in a volume of 0.1 ml at 37 C for 30 min, then at 90 C for 1 min. While the HSF was at 90 C, trypsin at a concentration of 50 μ g/ml was added, and the incubation was allowed to proceed for 10 min. A sample of this material was then assayed as in *a*.

ment of HSF and that protein kinase activity was restored by addition of fresh HSF. In contrast, RNase A (Worthington) digestion for 1 h produced no loss of HSF activity, whereas pancreatic DNase (Worthington) produced a 35% inhibition. The significance of the latter observation is not clear but contamination of the DNase with a small amount of protease is possible. Lipid is not an essential component of HSF since 50 to 90% of the activity was recovered after extraction with CHCl_3 :MeOH (2:1). HSF activity was not dialyzable, and was excluded from Sephadex G-25. Approximately 50% of the activity was recovered after precipitation with 5% trichloroacetic acid by dissolving the precipitate in water and dialyzing exhaustively against water at 4 C. The action of HSF could not be duplicated by incubation of HLF preparations with cyclic-AMP, cyclic-GMP, cyclic-CMP, or cyclic-UMP in final concentrations of 1, 10, or 100 μM . From these experiments it was concluded that a protein was responsible for HSF activity.

Effect of added proteins on protein kinase activity of HSF and HLF. The protein kinase activity resulting from recombination of HLF and HSF exhibited a requirement for specific exogenous proteins qualitatively similar to that of the unfractionated soluble protein kinase from whole virions (Table 3). Essentially, no reaction occurred in the absence of added basic proteins. The reaction was most dependent on the presence of HSF when protamine was used. In the absence of HSF, HLF possessed greater protein kinase activity in reactions utilizing total histone or lysine-rich histone rather than protamine, but was still markedly stimulated by addition of HSF. In contrast, when arginine-rich histone was added to the reaction, HLF exhibited greater activity alone than it did in the presence of HSF. With these more purified preparations, phosvitin, α -casein, and bovine serum albumin were inactive (Table 3).

DISCUSSION

The vaccinia virus protein kinase has been solubilized by using salt, DTT, and the ionic detergent, deoxycholate. The properties of soluble enzyme are similar to those of protein kinase in partially disrupted virions, but soluble enzyme is more highly dependent for activity on added protamine or histones. Further investigation and isolation of vaccinal protein kinase suggested some novel features. At least two components were necessary for expression of enzymatic activity. One of these was entirely associated with viral cores, and was heat labile. The second could be partially released during

the stripping of envelopes from viral cores with NP-40 detergent by the method of Easterbrook (2). However, since 30 to 40% of the total virus protein is released by this procedure, the HSF may be a loosely bound internal component. This factor (HSF) was heat stable, trichloroacetic acid precipitable, excluded from Sephadex G-25, and totally inactivated by tryptic digestion. These data suggested that HSF was a protein and that the activity was extremely stable.

Since the NP-40 extraction procedure only partially removed HSF, it was necessary to further purify HLF to make it more highly dependent on recombination with the former for expression of enzymatic function. This was accomplished by passage through a DEAE-cellulose column. The combination of HSF and HLF was still insufficient for protein kinase activity unless protamine or histones were

TABLE 3. *Effect of added proteins on protein kinase activity of heat-stable and heat-labile factors*

Nonviral protein	Components of the reaction ^a		Pmol of ³² P incorporated
	HLF	HSF	
None	+	—	0
None	—	+	0
None	+	+	1.2
Protamine	+	—	17.2
Protamine	—	+	0.2
Protamine	+	+	196
Total histone	+	—	36.8
Total histone	—	+	0.2
Total histone	+	+	126
Arginine-rich histone	+	—	41.7
Arginine-rich histone	—	+	0.47
Arginine-rich histone	+	+	24.7
Lysine-rich histone	+	—	24.9
Lysine-rich histone	—	+	0.25
Lysine-rich histone	+	+	135.9
Phosvitin	+	—	0
Phosvitin	—	+	0
Phosvitin	+	+	0
α -Casein	+	—	0
α -Casein	—	+	0
α -Casein	+	+	0
Bovine serum albumin	+	—	0
Bovine serum albumin	—	+	0
Bovine serum albumin	+	+	0

^a Approximately 5 μg of HLF, partially purified through the low-salt DEAE chromatography step, and 20 μliters of HSF prepared as described in the text were incubated individually with each added nonviral protein (4 μg) or in combination. For each nonviral protein species, zero time values were obtained by adding both HSF and HLF and immediately stopping the reaction.

added. The dependence on HSF was most marked when protamine was used.

Protein kinases that are stimulated by cyclic nucleotide monophosphates have been purified from a variety of tissues and characterized (8). It has been documented by a number of investigators that such enzymes consist of two subunits which are associated in an inactive holoenzyme. This holoenzyme is not dissociated by routine chromatographic procedures. Activation of such protein kinases by cyclic-AMP or cyclic-GMP is effected by the binding of nucleotide to a regulatory subunit, resulting in dissociation of the regulatory subunit from the catalytically active subunit (3, 4, 12). The resulting solitary catalytic subunit is enzymatically active. In contrast, the solubilized protein kinase activity from vaccinia virions is not stimulated by cyclic nucleotides, and there is no evidence for an inhibitory subunit.

An additional type of regulation of protein kinase activity has recently been described by Donnelly et al. (1). A protein kinase modulator that is heat stable and trichloroacetic acid precipitable was isolated from lobster tail and other tissues. It had either stimulatory or inhibitory properties, depending on the type of substrate and the tissue source of kinase. It is possible that the HSF which we have isolated from vaccinia virions is analogous to this modulator protein. However, the modulator protein isolated by Donnelly et al. required cyclic nucleotides for expression of its activity, and only had the capacity to stimulate protein kinase activities several fold.

It will be necessary to further purify and characterize both the heat-stable and heat-labile components to understand their relationship to each other. Determination of whether the HSF becomes physically associated with either the HLF or the added protamine, and particularly whether HSF itself is phosphorylated are important questions that remain to be answered. These studies take on special biological significance since protein kinases are usually regulatory enzymes.

ACKNOWLEDGMENTS

We wish to thank Norman Cooper for growing and purifying vaccinia virus, Edith Rosenblum for technical help, and

Enzo Paoletti and Hortencia Rosemond for their helpful discussions.

ADDENDUM IN PROOF

It has now been shown that HSF acts as substrate (phosphate acceptor) in the vaccinia protein kinase reaction. Protamine is required to activate this reaction by some as yet unknown mechanism, but in contrast to several previously described protein kinase systems (E. G. Krebs, 1972, *Curr. Top. Cell. Reg.*, 5:99-132) is not appreciably phosphorylated during the reaction.

LITERATURE CITED

1. Donnelly, T., J. F. Kuo, P. Reges, Y. Liu, and P. Greengard. 1973. Protein kinase modulator from lobster tail muscle. *J. Biol. Chem.* **248**:190-198.
2. Easterbrook, K. B. 1966. Controlled degradation of vaccinia virus in-vitro: an electron microscopic study. *J. Ultrastruct. Res.* **14**:484-496.
3. Ehlichman, J., A. Hirsch, and O. Rosen. 1971. Interconversion of cyclic nucleotide independent forms of a protein kinase from beef heart. *Proc. Nat. Acad. Sci. U.S.A.* **68**:731-735.
4. Gill, G., and L. Garren. 1971. Role of the receptor in the mechanism of action of adenosine 3'5' cyclic monophosphate. *Proc. Nat. Acad. Sci. U.S.A.* **68**:786-790.
5. Gravell, M., and T. L. Cromeans. 1972. Virion-associated protein kinase and its involvement in nongenetic reactivation of frog polyhedral cytoplasmic deoxyribovirus. *Virology* **48**:847-851.
6. Hatanaka, M., E. Twiddy, and R. V. Gilden. 1972. Protein kinase associated with RNA tumor viruses and other budding RNA viruses. *Virology* **47**:536-538.
7. Joklik, W. K. 1962. The preparation and characteristics of highly purified radioactively labeled poxvirus. *Biochim. Biophys. Acta* **61**:290-301.
8. Jost, D. P., and H. V. Rickenberg. 1971. Cyclic AMP. *Ann. Rev. Biochem.* **40**:741-774.
9. Moss, B., and N. P. Salzman. 1968. Sequential protein synthesis following vaccinia virus infection. *J. Virol.* **2**:1016-1027.
10. Randall, C. C., H. W. Rogers, D. N. Downer, and G. A. Gentry. 1972. Protein kinase activity in equine herpesvirus. *J. Virol.* **9**:216-222.
11. Paoletti, E., and B. Moss. 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. *J. Virol.* **10**:417-424.
12. Rieman, E., G. Brostrom, J. Corbin, C. King, and E. Krebs. 1971. Separation of regulatory and catalytic subunits of the cyclic 3'5' adenosine monophosphate-dependent protein kinase(s) of rabbit skeletal muscle. *Biochem. Biophys. Res. Commun.* **42**:187-194.
13. Rubenstein, A. S., M. Gravell, and R. Darlington. 1972. Protein kinase in enveloped herpes simplex virions. *Virology* **50**:287-290.
14. Strand, M., and J. T. August. 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. *Nature N. Biol.* **233**:137-140.