Characterization of a Protein Kinase and Two Phosphate Acceptor Proteins from Vaccinia Virions

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SUMMARY

The phosphorylation of two purified vaccinia virus proteins (Acceptors I and II) by a protein kinase isolated from vaccinia virus cores has been studied. Phosphorylation of viral acceptor proteins by the purified enzyme was dependent on the presence of ATP, Mg\(^{2+}\), protamine or other basic proteins, and was maximal at alkaline pH values. Cyclic mononucleotides did not stimulate the vaccinia protein kinase under a variety of conditions. Protamine, however, was shown to function as an enzyme activator. In its presence, the purified vaccinia protein kinase phosphorylated mainly serine residues in Acceptor I, and predominantly threonine residues in Acceptor II. Phosphorylation of protamine accounted for less than 1% of the total \(^{32}\)P incorporation. Tryptic peptide maps prepared from \(^{32}\)P-labeled Acceptors I and II demonstrated that they contained different labeled peptide sequences and were, therefore, distinct protein species. From additional studies on both purified and virus-associated protein kinase it was concluded that various proteins affected the protein kinase reaction in one of three ways. One class of proteins served as phosphate acceptors, but only when another activator protein was present. A second class consisted of proteins that were strong activators but poor phosphate acceptors. The third class contained proteins that were fair phosphate acceptors, but which also activated the phosphorylation of other acceptor proteins.

In the accompanying report, we described the purification of a protein kinase and two phosphate acceptor proteins from infectious vaccinia virus particles (1). A remarkable feature of this system is that the enzyme requires a basic protein as an activator in order for phosphorylation of viral acceptor proteins to occur. We now present further studies on the characterization of the purified protein kinase and phosphate acceptor proteins from vaccinia virus.

EXPERIMENTAL PROCEDURES

Materials and Methods

Purification of Protein Kinase and Viral Phosphate Acceptor Proteins—The protein kinase was purified from vaccinia virus cores through the DEAE-cellulose III column chromatography step, and the two viral phosphate acceptor proteins, designated Acceptor I and Acceptor II, were purified through the CM-cellulose column chromatography step as described in the accompanying report (1).

**Protein Kinase Assay**—Assays for protein kinase were performed as previously described. Standard reaction mixtures contained 50 mM Tris-HCl, pH 10.25, 0.01% Nonidet P-40, 5 mM dithiothreitol, 5 mM MgCl\(_2\), 0.5 mM \[^{32}\text{P}\]ATP (30 to 100 cpn/pmol), 2 to 5 \(\mu\)g of purified Acceptor I or Acceptor II, and 1.5 to 4 \(\mu\)g of protamine and 25 \(\mu\)l of purified enzyme in a volume of 0.2 ml. Background values determined by incubating the total reaction mixture without enzyme for the appropriate time, then adding enzyme and immediately stopping the reaction, were always subtracted. Separate background values were determined for different substrate and activator proteins. Because of the low concentrations of purified viral proteins and the presence of detergent and reducing agents, protein concentrations were frequently determined by using [\(^{3}\text{H}\)]leucine-labeled virus as described in the preceding report (1). A typical preparation of purified protein kinase was found to have a protein concentration of about 5 \(\mu\)g/ml in this manner, whereas Acceptor I and Acceptor II solutions contained about 100 \(\mu\)g/ml.

**Polyacrylamide Gel Electrophoresis**—The procedures for acid urea and dodecyl sulfate polyacrylamide gel electrophoresis have also been demonstrated (1).

**Demonstration of Phosphoserine and Phosphothreonine Residues**—Purified acceptor proteins were labeled with \[^{33}\text{P}\]ATP (100 to 200 cpn/pmol) in 0.4 ml standard protein kinase reactions. The products were collected by precipitation with 25% trichloroacetic acid containing 0.05 M NaPP\(_3\), and washed three times by dissolving them in 1 N NaOH and 0.05 M NaPP\(_3\), and reprecipitating each time with trichloroacetic acid. After one wash with ethanol, the pellets were dried and hydrolyzed at 100°C under nitrogen in 2 N HCl in sealed glass tubes for 6 hours or 16 hours. The hydrolysates were diluted with water, lyophilized over NaOH pellets, and dissolved in 20 to 30 \(\mu\)l of water containing 10 \(\mu\)g each of phosphothreonine and phosphoserine standards. Ten-microliter aliquots were spotted on thin layer cellulose sheets (20 \(\times\) 20 cm). Electrophoresis was carried out on a cold plate apparatus in 0.1 M phosphoric acid, pH 2.5, for 2 hours at 600 volts as previously described (2). Amino acid spots were localized by spraying with ninhydrin and \[^{33}\text{P}\]labeled compounds were detected by autoradiography for 1 to 7 days. Phosphoserine, phosphothreonine, and \[^{33}\text{P}\], were also run individually on the same plates as the composites. For preparative purposes, electrophoresis was performed at 800 volts for 2 hours in a volatile buffer containing acetic acid-formic acid-water (15:5:100). Labeled materials were identified by autoradiography, scraped from the plate, eluted with several washes of 0.1 N acetic acid, and lyophilized.

**Tryptic Phosphopeptide Maps**—Purified acceptor proteins were labeled with \[^{32}\text{P}\]ATP (200 cpn/pmol) in an enlarged (0.5 ml) standard reaction mixture. The reaction was stopped by addition of 0.2 mg of bovine serum albumin, 1 \(\mu\)mol of unlabeled ATP, and 1 mg of bovine serum albumin, 1 \(\mu\)mol of unlabeled ATP, and 1 mg of bovine serum albumin, 1 \(\mu\)mol of unlabeled ATP, and
at saturating concentrations of protamine and ATP. Activity described in the following experiments.

When the phosphate acceptor protein concentration was measured was proportional to the concentration of Acceptor I or Acceptor II, and little or no activity was obtained in their absence (Fig. 1A). Greater activity was observed in the presence of Acceptor II, however insufficient quantities of purified phosphate acceptor proteins were available to carry the experiment to saturation. In subsequent experiments 2.8 µg of phosphate acceptor proteins per 0.2 ml of assay were used where appropriate. In some experiments only Acceptor II, the major component, was tested.

Protamine Concentration—Negligible protein kinase activity occurred with Acceptor I or II in the absence of protamine (Fig. 1B). Activity was proportional to the protamine concentration over only a narrow range and saturation was achieved at 1 µg per 0.2-ml reaction.

ATP Concentration—The effect on the reaction rate of varying the concentration of ATP is shown in Fig. 2. Saturation was approached at an ATP concentration of 0.5 mM and 1 mM ATP was not inhibitory. A straight line was obtained by a double reciprocal plot of the data (inset, Fig. 2). Under these conditions, an apparent Kₐ of 8.8 × 10⁻⁴ M was calculated.

Mg²⁺ Concentration—The addition of Mg²⁺ was an absolute requirement for enzyme activity. Maximum stimulation was observed when the Mg²⁺ concentration was 3 to 5 mM and only a small inhibition occurred when the Mg²⁺ concentration was 10 mM (Fig. 3A). Calcium ion was totally ineffective in replacing Mg²⁺, whereas Mn²⁺, at concentrations between 0.1 and 10 mM, allowed less than 10% of the activity obtained with Mg²⁺.

pH—A broad pH optimum between 9.5 and 10.5 with either Acceptor I or Acceptor II was obtained (Fig. 3B). Lack of Effect of Cyclic Mononucleotides—The addition of 5 × 10⁻⁴ M cyclic AMP, CMP, GMP, or UMP to the protein kinase reaction in the presence of Acceptor II, either with or without added protamine, did not stimulate phosphorylation. Because many cyclic AMP-dependent protein kinases are active at pH values between 6 and 7 (4–6), these experiments were repeated at pH 6.0. Again cyclic mononucleotides were without demonstrable stimulatory effect. The four cyclic mononucleotides also had no effect on the phosphorylation of lysine-rich histone either at pH 10.2 or pH 6.0 (data not shown).

Time Course and Enzyme Concentration—Under the established optimal conditions, protein kinase activity was linear with time and the concentration of ATP is shown in Fig. 2. Saturation was approached at an ATP concentration of 0.5 mM and 1 mM ATP was not inhibitory. A straight line was obtained by a double reciprocal plot of the data (inset, Fig. 2). Under these conditions, an apparent Kₐ of 8.8 × 10⁻⁴ M was calculated.

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The abbreviation used is: cyclic AMP, etc., cyclic adenosine 3':5'-monophosphate.
FIG. 3. Effect of Mg\textsuperscript{2+} concentration and pH on protein kinase activity. Protein kinase activity was measured in standard reactions containing (A) 2.8 μg of Acceptor II, 4 μg of protamine, and the indicated Mg\textsuperscript{2+} concentrations or (B) in reactions containing 0.05 M Tris buffer at the noted pH values, 4 μg of protamine, and 2.8 μg of either Acceptor I (---O) or Acceptor II (O- -O).

FIG. 4. Time course and effect of varying amounts of enzyme. Protein kinase activity was measured in standard reaction mixtures containing 2.8 μg of Acceptor II and 1.5 μg of protamine for the times indicated (A). Varying amounts of enzyme were assayed for 15 min in the presence of Acceptor II and protamine in standard protein kinase reactions (B). The enzyme solution used contained approximately 5 μg/ml of protein.

for 15 min and continued for more than 60 min (Fig. 4A). Activity was nearly proportional to the amount of enzyme added over a 5-fold range (Fig. 4B).

Demonstration of Phosphate Incorporation into Purified Acceptor I and Acceptor II—Our initial observation that the soluble protein kinase required protamine as an activator in order to phosphorylate viral phosphate acceptor proteins was made using a partially purified preparation of unseparated acceptor proteins. In order to extend this observation, the reaction products obtained by incubating \[^32\text{P}\]ATP, protein kinase, and protamine with purified \[^3\text{H}\]leucine-labeled Acceptor I or Acceptor II were analyzed by dodecyl sulfate and acid urea polyacrylamide gel electrophoresis. The pattern of radioactivity obtained from acid urea gel analysis is shown despite the aggregation of Acceptor I in this system because the acceptor proteins were very well separated from protamine (Fig. 5). Labeled phosphate was incorporated into peaks of tritiated viral protein, both when Acceptor I (Fig. 5, upper panel) or Acceptor II (Fig. 5, lower panel) were used. Less than 1% of the total \[^32\text{P}\] counts were associated with the stained protamine band. The patterns obtained when similar reactions were analyzed by autoradiography following dodecyl sulfate gel electrophoresis supported these findings. The \[^32\text{P}\]-labeled bands of Acceptor I and Acceptor II corresponded to the stained bands shown in the previous paper. Acceptor I, which aggregated in the acid urea gel system, appeared as one main radioactive band by dodecyl sulfate gel electrophoresis (data not shown).

We considered that protamine, despite its apparent low level of phosphorylation, might serve as an active intermediate in the transfer of ATP to virus acceptor proteins. As a preliminary test of this hypothesis, \[^31\text{P}\]-labeled protamine containing more than \(2 \times 10^6\) cpm/mg was prepared with beef heart protein kinase. The isolated protamine was then incubated in a reaction mixture containing vaccinia protein kinase, Acceptor II, Mg\textsuperscript{2+}, and with and without unlabeled ATP. Reaction products were analyzed by acid urea gel electrophoresis but no transfer of \[^31\text{P}\] to Acceptor II was detected.

Demonstration of Phosphoserine and Phosphothreonine Residues in Acceptor I and Acceptor II—Purified Acceptor I and Acceptor II were each labeled with \[^31\text{P}\] in an enlarged standard kinase reaction. The products were precipitated and washed, hydrolyzed in 2 M HCl under nitrogen for 6 hours or 16 hours at 100°, and then analyzed by thin layer electrophoresis. Radioautographs from such an experiment are shown in Fig. 6. Radioactive material which was subjected to co-electrophoresis with the phosphoserine standard was present in the 6- and 16-hour hydrolysate of Acceptor I (Fig. 6, I-A, I-B), whereas only smaller amounts of phosphothreonine were observed. In contrast, phosphothreonine was the dominant labeled amino acid residue generated by 6-hour or 16-hour hydrolysis of Acceptor II (Fig. 6, II-A, II-B). As expected, considerable amounts of P\(_1\) were formed by the hydrolysis of phosphorylated amino acids. A significant portion of the radioactivity was recovered in material having slower mobility than phosphoserine or phosphothreonine. Upon isolation and rehydrolysis, these materials gave rise to additional phosphoserine in the case of Acceptor I, and phosphothreonine in the case of Acceptor II, indicating that they were incompletely hydrolyzed phosphopeptides (Fig. 6, I-C, II-C). The faint spots which migrate ahead of phosphoserine and ahead of Pi seen in autoradiographs from Acceptor II may be phosphothreonine oligomers which are relatively resistant to acid hydrolysis. Oligomers of phosphoserine which are resistant to acid hydrolysis have been recovered from \[^31\text{P}\]-labeled phosvitin and casein (7).
These experiments demonstrated that the vaccinia protein kinase catalyzed the phosphorylation of serine and threonine residues in viral phosphate acceptor proteins. In addition, they suggested that Acceptor I and Acceptor II were distinct proteins, because predominantly serine was phosphorylated in the first, whereas threonine was phosphorylated almost exclusively in the second.

**Tryptic Peptide Mapping of Acceptor I and Acceptor II**—In order to confirm that Acceptor I and Acceptor II were different viral proteins, two-dimensional tryptic peptide mapping of \(^{32}\)P-labeled Acceptor I and Acceptor II was performed as described under “Materials and Methods.” Each acceptor protein was labeled in a kinase reaction in which protamine served as the enzyme activator. We have shown that under these conditions, protamine is not phosphorylated to a significant degree (Fig. 5) and, consequently, does not contribute to the pattern of radioactive phosphopeptides. The tryptic peptide map of Acceptor I demonstrated one major phosphopeptide with an isoelectric point close to pH 6.4 (Fig. 7). This result suggests that primarily a single site on Acceptor I was phosphorylated. In contrast, the peptide map of Acceptor II showed a cluster of negatively charged phosphopeptides. Because Acceptor II gives rise to what appear to be oligomers of phosphothreonine on acid hydrolysis, the multiple tryptic phosphopeptides could be the result of different degrees of phosphorylation of one peptide sequence. These findings further demonstrated that Acceptor I and Acceptor II are discrete proteins, rather than multiple forms of the same polypeptide.

**Interaction of Other Activator and Substrate Proteins with Vaccinia Protein Kinase**—The ability of a variety of proteins, either by themselves or in combination, to act as phosphate acceptors or activators of the protein kinase reaction was tested. The re-
The reaction products obtained by incubating \( ^{32} \text{P} \)-ATP, protein kinase, and lysine-rich histone, either alone or with Acceptor II, were analyzed by acid urea gel electrophoresis. A similar set of incubations in which protamine was used in place of lysine-rich histone served as the control. The radioactivity profile obtained from sliced gels is shown in Fig. 9. The upper panel corresponds to stained bands of Acceptor II, whereas the small peak between them corresponded to histone. Analysis of a reaction in which Acceptor II was incubated with protamine and enzyme demonstrated the same two major peaks of phosphorylated Acceptor II but no peak between them (Fig. 9B). Only slight \( ^{32} \text{P} \) incorporation occurred in a reaction containing protamine, without Acceptor II, as shown immediately below. These experiments indicated that histones, as well as protamine, have the capacity to activate the vaccinia protein kinase and stimulate phosphorylation of viral acceptor proteins.

**Analysis of Products of Kinase Reaction in Presence of Lysine-rich Histone and Acceptor II**—We have shown that protamine activates the protein kinase and allows phosphorylation of viral acceptor proteins. It was now of interest to determine whether the stimulation of the kinase reaction effected by histone fractions in the presence of Acceptor II was also due to phosphorylation of viral acceptor proteins rather than enhanced phosphorylation of the histone fraction. The effect of lysine-rich histone was studied because it was the purest of the readily available histones, and it also could be separated from viral proteins by electrophoresis. The reaction products obtained by incubating \( ^{32} \text{P} \)-ATP, protein kinase, and lysine-rich histone, either alone or with Acceptor II, were analyzed by acid urea gel electrophoresis. A similar set of incubations in which protamine was used in place of lysine-rich histone served as the control. The radioactivity profile obtained from sliced gels is shown in Fig. 9. The upper panel corresponds to stained bands of Acceptor II, whereas the small peak between them corresponded to histone. Analysis of a reaction in which Acceptor II was incubated with protamine and enzyme demonstrated the same two major peaks of phosphorylated Acceptor II but no peak between them (Fig. 9B). Only slight \( ^{32} \text{P} \) incorporation occurred in a reaction containing protamine, without Acceptor II, as shown immediately below. These experiments indicated that histones, as well as protamine, have the capacity to activate the vaccinia protein kinase and stimulate phosphorylation of viral acceptor proteins.

**Analysis of Products of Kinase Reaction in Presence of Protamine and Casein**—As shown in Table I, protein kinase activity could be detected when the enzyme was incubated under standard conditions in the presence of both protamine and casein, but no activity was demonstrated in reactions to which only casein was added. This observation suggested that protamine might activate the enzyme and allow phosphorylation of casein. To test this model, the products obtained from a standard enzyme reaction containing both protamine and casein were analyzed by acid urea gel electrophoresis, as shown in Fig. 10. At least 90% of the total \( ^{32} \text{P} \) incorporation occurred into casein. Although casein...
in a 0.4-ml reaction mixture with \( [\gamma^{32P}] \text{ATP} \) (150 cpm/pmol), lysine-rich histone (20 pg), and the purified protein kinase to catalyze the phosphorylation of activity. The finding that protamine or histones were required for electrophoretic analysis of the products from control reactions were analyzed by acid urea polyacrylamide gel electrophoresis as described under “Materials and Methods.” The position of lysine-rich histone is indicated by an arrow. A similar analysis of a reaction in which lysine-rich histone alone was incubated with protein kinase and \( [\gamma^{32P}] \text{ATP} \) is shown directly below. B, acid urea gel electrophoretic analysis of the products from control reactions containing protamine and Acceptor II (above) or protamine alone (below).

**Fig. 9.** Polyacrylamide gels demonstrating the phosphorylation of purified Acceptor II by the vaccinia protein kinase when activated by lysine-rich histone. A, protein kinase was incubated in a 0.4-ml reaction mixture with \( [\gamma^{32P}] \text{ATP} \) (150 cpm/pmol), lysine-rich histone (20 pg), and Acceptor II (7.5 pg). The products were analyzed by acid urea polyacrylamide gel electrophoresis as described under “Materials and Methods.” The position of lysine-rich histone is indicated by an arrow. A similar analysis of a reaction in which lysine-rich histone alone was incubated with protein kinase and \( [\gamma^{32P}] \text{ATP} \) is shown directly below. B, acid urea gel electrophoretic analysis of the products from control reactions containing protamine and Acceptor II (above) or protamine alone (below).

**Fig. 10.** Polyacrylamide gel demonstrating the phosphorylation of casein by vaccinia protein kinase when activated by protamine. Casein and protamine were incubated in an enlarged standard protein kinase reaction mixture under the conditions described in Table I and the products were analyzed by acid urea gel electrophoresis (see “Materials and Methods”). The mobility of each protein was determined from stained gels run simultaneously, and is indicated above the radioactive profile. The scale on the ordinate is expanded because of the lower activities obtained using casein as phosphate acceptor and consequently incorporation into protamine appears greater than in previous analyses.

was a less active substrate than Acceptor II, the qualitative effect of protamine on the enzyme appeared similar with either substrate.

Effect of Basic Proteins on Virus-associated Protein Kinase Activity—The finding that protamine or histones were required for the purified protein kinase to catalyze the phosphorylation of purified viral acceptor proteins made it of interest to determine whether the enzyme could be activated in a similar manner while associated with virus. In the experiments described in the remaining sections of the paper, whole vaccinia virions were added to reaction mixtures as both the source of enzyme and phosphate acceptor proteins. It had been previously demonstrated that protamine and histones stimulated the kinase reaction catalyzed by virus \textit{in vivo} by 3- to 6-fold (2). The products of a protein kinase reaction obtained by incubating \([\text{H}]\text{leucine-labeled virus and } [\gamma^{32P}] \text{ATP alone, with protamine, or with lysine-rich histone were analyzed by dodecyl sulfate and acid urea polyacrylamide gel electrophoresis. Panel A of Fig. 11 demonstrates the pattern of phosphate incorporation into viral protein in the absence of added basic activator protein. This was best seen in the dodecyl sulfate gel, in which \( ^{32P} \) was localized predominantly in low molecular weight viral polyglycine. When protamine was included in the incubation, a marked stimulation of phosphate incorporation into similar low molecular weight material was observed in the dodecyl sulfate gel. However, the position of protamine, which is marked by an arrow in panel C, coincided with that of the viral polypeptides of interest. Analysis of an identical reaction by acid urea gel electrophoresis clearly showed that phosphate label was incorporated into primarily one main band of \([\text{H}]\text{leucine-labeled viral protein}, whereas only about 5% of the total \( ^{32P} \) uptake was associated with protamine. Thus, protamine acts primarily as an activator or co-factor even when virus-associated protein kinase activity is measured. Furthermore, the major viral protein phosphorylated in the presence of protamine has the same electrophoretic mobility on both acid urea and dodecyl sulfate gels as purified Acceptor II. This was confirmed by double label experiments (not shown). Experiments were also performed using lysine-rich histone in place of protamine. In the dodecyl sulfate gel (Fig. 11B), \( ^{32P} \) was incorporated into proteins having the same electrophoretic mobility as lysine-rich histone as well as into more rapidly migrating low molecular weight viral polyglycine as noted previously (2). Analysis of a duplicate reaction by acid urea gel electrophoresis also showed that lysine-rich histone stimulated the reaction both by serving as a phosphate acceptor itself and by stimulating \( ^{32P} \) incorporation into Acceptor II. Lysine-rich histone was clearly more effective as a phosphate acceptor than was protamine, although both of these basic proteins markedly stimulated phosphorylation of the same viral polypeptide.

**Tryptic Peptide Mapping of Products of Virus-associated protein Kinase**—The tryptic peptide map of the products obtained from a reaction catalyzed by virus-associated protein kinase was examined in order to compare it to the peptide maps which had been obtained using phosphorylated purified Acceptor I and Acceptor II. Vaccinia virions were incubated with \([\gamma^{32P}] \text{ATP} \) either alone or in the presence of protamine in a standard kinase reaction, and tryptic peptide mapping of the products followed by radioautography was performed. Fig. 12A demonstrates the radioactive phosphopeptides derived from the reaction with whole virions. Two labeled peptides (Fig. 12A, arrow) were in the region of the map where the major phosphopeptide of Acceptor I was found (see Fig. 7). Analysis of the labeled products from an incubation of \([\gamma^{32P}] \text{ATP}, \text{virions, and protamine demonstrated an additional peptide (Fig. 12B, arrow) at a position close to that of the major peptides obtained by similar analysis of labeled Acceptor II (see Fig. 7). Major tryptic phosphopeptides that do not correspond to those obtained with Acceptor I and II were also found indicating that additional peptides were phosphorylated by the virus-associated enzyme.
FIG. 11. Polyacrylamide gels demonstrating the effect of protamine and lysine-rich histone on the products phosphorylated by vaccinia virions. [H]leucine-labeled vaccinia virions (300 μg) were incubated with [γ-32P]-ATP without exogenous basic protein (Panel A), with 50 μg of lysine-rich histone (Panel B), or with 50 μg of protamine (Panel C). After 15-min incubation at 37°, the reaction products were precipitated with trichloroacetic acid and prepared for electrophoretic analysis on both dodecyl sulfate and acid urea polyacrylamide gels as described under "Materials and Methods." Unreacted whole virus, lysine-rich histone, and protamine were run on parallel gels. The positions of the stained bands of lysine-rich histone and protamine are indicated by arrows. The gels were sliced and their radioactivity profiles were determined. Note the difference in scale of the ordinates.

DISCUSSION

In the accompanying report we described the purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. We have now characterized more fully the purified enzyme, the two purified phosphate acceptor proteins, and the requirement for basic proteins. Gel electrophoretic analysis of the products obtained by incubating the protein kinase with Acceptor I or Acceptor II in standard reaction mixtures containing ATP, Mg2+, and protamine confirmed that phosphate is incorporated almost exclusively into the purified acceptor proteins, rather than into protamine. This suggested that protamine serves as an enzyme activator. Thin layer electrophoresis of acid hydrolysates of labeled Acceptor I and Acceptor II demonstrated that the former contains mainly [32P]serine, whereas the latter contains mainly [32P]threonine. This result indicated that the two acceptors are distinct proteins, because each contains a different phosphorylated amino acid. This conclusion was supported by the finding that the tryptic peptide maps of [32P]-labeled Acceptor I and Acceptor II are also different. The major phosphoprotein, isolated from vaccinia virus grown in cells incubated with [32P], contains exclusively phosphoserine and a single major tryptic phosphopeptide distinct from that of either phosphorylated Acceptor I or Acceptor II (3).

The ability of a number of proteins to activate the vaccinia
protein kinase or to serve as substrate proteins themselves was explored using both purified enzyme and virion-associated enzyme. These experiments suggested that different proteins interact with the vaccinia protein kinase in one of several ways: purely as an activator, purely as a substrate, or as both activator and substrate. In the first case, the protein stimulates the phosphorylation of a different substrate protein, without being extensively phosphorylated itself. Protamine best fit into this category because it activated the phosphorylation of Acceptor I, Acceptor II, and casein. Both viral acceptor proteins, as well as casein, fit into the second category because they served as substrate proteins only when another activator proteins were present. Finally, some proteins such as lysine-rich histone are moderately active substrates by themselves and can also activate the protein kinase to phosphorylate Acceptor II.

The mechanism by which protamine or other basic proteins activate the vaccinia protein kinase remains to be established. Several models may be considered: a, the basic protein activator might form an active phosphorylated reaction intermediate necessary for the transfer of phosphate from ATP to the substrate; b, the activating protein might bind to the substrate protein so as to expose certain sites to phosphorylation; or finally, c, binding between the enzyme and basic protein might occur with consequent activation. As a preliminary test of the first model we attempted without success to transfer azP from protamine that had been previously labeled with beef heart protein kinase, to Acceptor II. The negative result, however, does not rule out this mechanism because protamine was labeled with a heterologous enzyme. The second model would require that the binding of protamine be relatively nonspecific so as to activate several different acceptor proteins. We consider the third model most attractive because protamine has been found to activate cyclic-AMP-dependent protein kinases purified from rabbit erythrocytes by dissociating the regulatory subunit from the catalytic subunit (8, 9). Miyamoto et al. (10, 11) likewise demonstrated that incubation of histones with a cyclic-AMP-dependent protein kinase from bovine brain, or with a cyclic-GMP-dependent protein kinase from lobster tail effected the dissociation of these enzymes into subunits. It is important to determine whether the vaccinia protein kinase, which has a molecular weight of 62,000 is also composed of regulatory and catalytic subunits and whether another viral protein can serve as an activator.

The biological roles of the protein kinase and acceptor proteins are unknown. It is possible that the enzyme we have isolated is responsible for phosphorylation of the polypeptides that are labeled when vaccinia virus is grown in cells incubated with 32P (3, 12). Studies have not yet been carried out to determine whether Acceptor I and Acceptor II are phosphorylated in vivo during the initiation of virus infection. Enzymes such as RNA polymerase (13, 14), poly(A) polymerase (16, 17), and RNA methylase (17), which are contained within the core, are needed for initiation of infection. A role for the core-associated protein kinase in the regulation of the activities of one or more of these viral enzymes might be considered. Another unexplored possibility is that the protein kinase phosphorylates host proteins. Following infection, vaccinia virus rapidly shuts off host cell protein synthesis, and it is conceivable that this inhibition is achieved by modification of host cell proteins by phosphorylation.

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