JOURNAL OF VIROLOGY, May 1992, p. 2717–2723
0022-538X/92/052717-07$02.00/0

Vol. 66, No. 5

The Vaccinia Virus B1R Gene Product Is a Serine/Threonine Protein Kinase†

SIQI LIN, WEN CHEN, AND STEVEN S. BROYLES*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-6799

Received 16 December 1991/Accepted 31 January 1992

The nucleotide sequence of the vaccinia virus open reading frame B1 predicts a polypeptide with significant sequence similarity to the catalytic domain of known protein kinases. To determine whether the B1R polypeptide is a protein kinase, we have expressed it in bacteria as a fusion with glutathione *S*-transferase. Affinity-purified preparations of the fusion protein were found to undergo autophosphorylation and also phosphorylated the exogenous substrates casein and histone H1. Mutation of lysine 41 to glutamine within the conserved kinase catalytic domain II abrogated protein kinase activity on all three protein substrates, supporting the notion that the protein kinase activity is inherent to the B1R polypeptide. Casein and histone H1 were phosphorylated on serine and threonine residues. The B1R fusion protein was phosphorylated on a threonine residue(s) by an apparently intramolecular mechanism. The autophosphorylation reaction resulted in phosphorylation of the glutathione *S*-transferase portion of the fusion and not the protein kinase domain. The protein kinase activity of B1R was specific for ATP as the phosphate donor; GTP was not utilized to a detectable extent. Immunoblotting experiments with anti-B1R antiserum showed that the protein kinase is located in the virion particle. Chromatography of virion extracts resulted in separation of the B1R protein kinase from the bulk of the total protein kinase activity, indicating that multiple protein kinases are present in the virion particle and that B1R is distinct from the previously described vaccinia virus-associated protein kinase.

Protein phosphorylation is a common mechanism for the regulation of physiological function. Metabolic pathways, cell division, ion transport, and hormone response are among the processes controlled by protein phosphorylation (reviewed in references 4 and 11). In each case, the function of key proteins participating in these processes is modulated by the phosphorylation state. Phosphate moieties on proteins can dramatically affect the catalytic activity of an enzyme by allosteric means or by altering the ability of a protein to interact with ligands or other proteins. The phosphorylation of proteins is directly mediated by protein kinases and protein phosphatases that are often targets for regulation themselves by protein phosphorylation as part of a cascade in signal transduction pathways.

Viruses often utilize many of the same regulatory mechanisms as cells, and this is particularly evident for the poxviruses, such as vaccinia virus. This virus multiplies exclusively within the cytoplasm of the host cell, and accordingly has evolved to high complexity to cope with its environment. This is evidenced by the fact that vaccinia virus encodes some 200 different proteins, many of which are normally associated with cellular functions (reviewed in references 5 and 17). Most, if not all, of the components of the replication and transcription machinery are virus encoded. Many vaccinia virus proteins are dispensable for virus multiplication but nevertheless appear to function in the interference with normal host cell activity and host immunity.

Mounting evidence suggests that protein phosphorylation may be an important regulatory mechanism for vaccinia virus. Several proteins that constitute the virion particle are phosphorylated on serine and threonine residues (30). In addition, a protein kinase is known to be packaged within the virion particle (13, 31). This enzyme was shown to phosphorylate a variety of proteins, of both viral and nonviral origin, on serine and threonine residues (14). It is not known whether this protein kinase is encoded by vaccinia virus. Recent evidence indicates that vaccinia virus may encode enzymes that mediate protein phosphorylation. The H1L gene was shown to encode a protein phosphatase that can remove phosphate from tyrosine and serine residues (7). The biological role of this enzyme remains uncertain. In addition, DNA sequence analysis suggests that vaccinia virus may encode a protein kinase. The 34-kDa protein predicted from the sequence of the viral gene B1R bears a striking similarity to the catalytic domains of known protein kinases (6, 10, 29). Most of the amino acids known to be conserved in the protein kinase family are located in the appropriate sites of the sequence predicted for the B1 protein.

In order to determine whether the B1 protein is a protein kinase, we have expressed it in a bacterial expression system. The purified fusion protein was shown to phosphorylate itself as well as exogenous proteins with ATP as a phosphate donor.

MATERIALS AND METHODS

Plasmid constructions. The vaccinia virus B1 gene was cloned by polymerase chain reaction from purified vaccinia virus virion (Western Reserve strain) DNA by using the oligonucleotides GCGCCATATGAACTTTCAAGGACTTG TG and GGCCGGATCCTTAATAATATACACCCTGC as primers. The 0.9-kb DNA product was purified by agarose gel electrophoresis, cleaved with *Nde*I and *Bam*HI endonucleases, and repurified by gel electrophoresis. This fragment was inserted into the bacteriophage T7 expression vector pET-3a (20) to assemble the plasmid pSB100. A glutathione

* Corresponding author.

† Paper 13179 from the Purdue University Agricultural Experiment Station.

S-transferase (GST)-B1 fusion vector was constructed by rendering the *Nde*I-*Bam*HI fragment blunt at both ends with DNA polymerase I Klenow fragment (21) and inserting the fragment into the *Sma*I site of the GST vector pGEX-KG (8) to create pSB101. For site-directed mutagenesis, the B1 gene was excised from pSB100 with *Sal*I and *Bam*HI and inserted into the corresponding sites of bacteriophage M13mp18 DNA. Mutagenesis was performed with a kit from Amersham according to the manufacturer's recommendations. The mutated gene was transferred back into pGEX-KG as described above.

Protein production and purification. Native B1 polypeptide was synthesized in *Escherichia coli* BL21 harboring pSB100 by using bacteriophage lambda CE6 as the source of the bacteriophage T7 RNA polymerase (27). The GST-B1 fusion protein was synthesized in strain HB101 harboring pSB101 by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) as described previously (7) except that cells were grown to an A_{600} of 0.5 at 30°C. IPTG was added to induce fusion protein synthesis, and after 30 min, the cells were shifted to 15°C for 16 h. Soluble protein was extracted from induced cells, and the fusion protein was purified by affinity chromatography on a glutathione-agarose column (7). Protein was quantitated by the method of Bradford (1).

Immunological methods. The 34-kDa B1 protein produced from pSB100 was used to raise antiserum. An insoluble fraction from cells induced for protein synthesis was prepared, and B1 protein was purified by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and injected into rabbits as described previously (2). Immunoblotting of proteins was performed by the method of Towbin et al. (28) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG). Immunoprecipitation experiments were performed as described previously (18) with goat anti-rabbit IgG-agarose beads (Bio-Rad Laboratories).

Protein kinase assays. Standard protein kinase assays were performed in a solution containing 50 mM Tris (pH 9.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM [γ-^{32}P]ATP, and 0.05 to 0.25 μg of GST-B1 protein. Reactions were run for 10 min at 37°C, at which time they were terminated by the addition of SDS gel sample buffer. Proteins were run on 12% polyacrylamide–SDS gels (16), dried, and exposed to X-ray film for autoradiography. Where indicated, 2 μg of dephosphorylated casein (Sigma Chemical Company) or chicken erythrocyte histone H1 or 5 μg of poly(tyrosine-glutamate) (Sigma) was included in the reaction mix. Histone H1 was a generous gift from J. Lauderdale and A. Stein, Purdue University, and was purified as described previously (25). For quantitation of phosphorylation of a specific polypeptide substrate, ^{32}P-labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membranes (Immobilon; Millipore Corp.), and stained with amido black to locate the protein band of interest. The bands were excised, and radioactivity was quantitated by scintillation counting.

Phosphoamino acid analysis. Proteins were phosphorylated with [γ-^{32}P]ATP as described above, subjected to SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred onto a polyvinylidene difluoride membrane. Membranes were stained with amido black to localize the polypeptide of interest. Protein bands were excised and hydrolyzed in 5.7 N HCl at 110°C for 1 h, and phosphoamino acids were resolved by electrophoresis on thin-layer cellulose plates at pH 3.5 as described previously (12). The positions of phosphoserine, phosphothreonine, and phosphotyrosine standards were identified by ninhydrin staining.

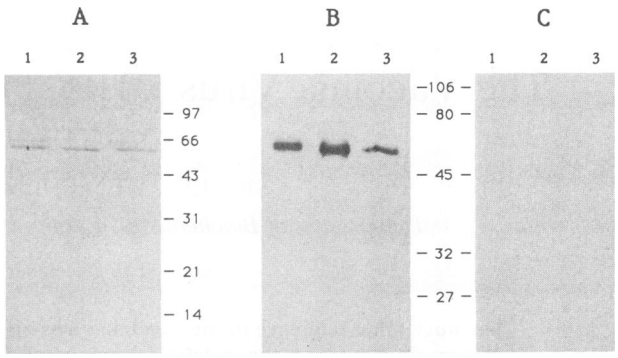

FIG. 1. Recombinant B1 protein expressed in bacteria. The GST-B1 fusion protein (lane 1), the GST-B1(K41Q) mutant (lane 2), and GST-B1(K45Q) mutant (lane 3) were purified by affinity chromatography and analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (A) or immunoblotting with B1 antiserum (B) or preimmune serum (C). The electrophoretic mobilities of molecular size standards are indicated (in kilodaltons).

Fractionation of vaccinia virion extracts. Vaccinia virus was propagated on HeLa cells and purified as detailed previously (3). The virus envelope was solubilized in 50 mM Tris (pH 8.0)–0.5% Nonidet P-40–50 mM dithiothreitol for 1 h at 37°C (23), and the core-associated enzymes were solubilized in 50 mM Tris (pH 8.4)–0.25 M NaCl–0.2% sodium deoxycholate–10 mM dithiothreitol for 1 h at 4°C (3). Core extracts were chromatographed on DEAE-cellulose as described previously (13) with the NaCl concentrations described in the text. The NaCl concentration in the column fraction was determined by using a conductivity meter.

RESULTS

Expression of recombinant B1 protein. The vaccinia virus B1 gene was cloned by polymerase chain reaction and inserted into bacterial expression vectors to direct protein synthesis. Initially, the B1 gene was placed behind a bacteriophage T7 promoter. This construction directed high-level expression of a 34-kDa polypeptide, as predicted from the nucleotide sequence of the gene (data not shown). This protein was found to be produced exclusively as an insoluble product, and attempts to obtain soluble B1 protein were unsuccessful. The recombinant 34-kDa protein was used to raise antiserum but could not be characterized biochemically. The B1 gene was then inserted into the vector pGEX-KG and expressed as a protein fusion with GST. A fraction of GST-B1 fusion protein was determined to be soluble when bacterial cells were grown and induced for protein production at 37°C. It was noted that a significantly greater fraction of the GST-B1 protein was found to be soluble if the protein was synthesized at 15°C. Similar effects of temperature on the solubility of recombinant proteins made in bacteria have been observed by others (22).

The GST-B1 fusion protein was purified by affinity chromatography on glutathione-agarose. The protein was determined to have a mass of 56 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1), close to that predicted by the fusion of the 27-kDa GST and the 34-kDa B1 protein. Typical preparations were estimated to be approximately 70% pure. B1 was shown to be part of the 56-kDa protein by immunoblotting. Antibody directed against the 34-kDa B1 protein

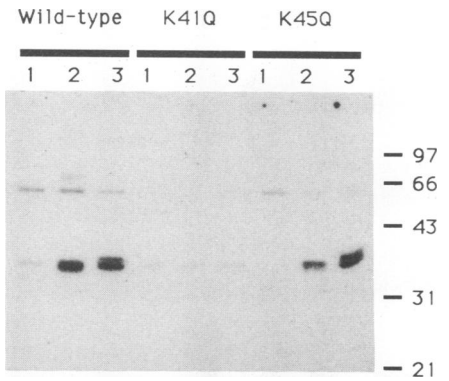

FIG. 2. Protein kinase activity of GST-B1 fusion protein. Purified wild-type GST-B1, GST-B1(K41Q), or GST-B1(K45Q) were incubated alone (lanes 1) or with casein (lanes 2) or histone H1 (lanes 3) in the presence of [γ-^{32}P]ATP, and phosphorylated products were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographed. The positions of molecular size standards are shown on the right (in kilodaltons).

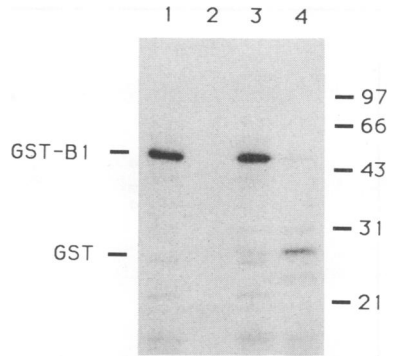

FIG. 3. Localization of the site of autophosphorylation in the GST-B1 fusion protein. Protein kinase reactions were performed in the presence of 0.15 μg of GST-B1 (lane 1), 2.0 μg of GST (lane 2), or 0.15 μg of GST-B1 plus 2.0 μg of GST (lane 3). Lane 4 is autophosphorylated GST-B1 cleaved with thrombin. The positions of GST-B1 and GST are indicated.

reacted with the 56-kDa polypeptide, but preimmune serum did not (Fig. 1).

Protein kinase activity. To determine whether the B1 protein possessed protein kinase activity, the GST-B1 protein was incubated both alone and with exogenous candidate protein substrates in the presence of [γ-^{32}P]ATP and $MgCl_2$. Phosphorylated polypeptides were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Reaction of GST-B1 alone resulted in phosphorylation of a 56-kDa polypeptide, which comigrated with the GST-B1 fusion protein, as well as a minor polypeptide of 32 kDa (Fig. 2), demonstrating that the GST-B1 preparation contains protein kinase activity. The 56-kDa phosphoprotein was confirmed to be the fusion protein by immunoprecipitation with anti-B1 antiserum (data not shown). The GST-B1 preparation was also capable of phosphorylating exogenous proteins. Coincubation of the GST-B1 protein with casein under kinase assay conditions resulted in more intense phosphorylation of a 32-kDa polypeptide which precisely comigrated with casein, as determined by Coomassie blue staining. A 66-kDa polypeptide that was a contaminant of the casein was also phosphorylated to a lesser degree. Addition of chicken erythrocyte histone H1 to the protein kinase assay mix resulted in the phosphorylation of a protein doublet of 34 and 33 kDa, corresponding to the a and b forms of histone H1, respectively.

To distinguish whether the protein phosphorylation was due to the GST-B1 fusion protein or a contaminating bacterial protein kinase, we sought a null mutant of the B1 kinase. The conserved lysine residue in domain II of several protein kinases has been strongly implicated as functioning in catalysis (9). Alignment of the predicted amino acid sequence of the vaccinia virus B1 gene product with that of other protein kinases has indicated that either lysine 41 (10) or lysine 45 (29) of the vaccinia virus sequence could correspond to the conserved lysine in domain II of other protein kinases. We used site-directed mutagenesis to replace either lysine 41 or 45 of the B1 kinase with a glutamine residue to assess the importance of each residue for protein kinase activity. Each mutant was expressed as a GST fusion protein and purified by affinity chromatography (Fig. 1). The GST-B1(K41Q) mutant protein was found to be incapable of phosphorylating the 56-kDa polypeptide, nor was it able to phosphorylate casein or histone H1 (Fig. 2), directly demonstrating that the B1 gene product is a protein kinase. This preparation did phosphorylate the minor 32-kDa protein observed for the wild-type enzyme. This same pattern of protein phosphorylation was produced by GST with no B1 fusion (not shown). The GST-B1(K45Q) mutant was capable of phosphorylation of the 56-kDa polypeptide and phosphorylation of casein and histone H1 in a manner indistinguishable from the wild-type enzyme. These results demonstrate that lysine 45 is dispensable but lysine 41 of the B1 protein is vital to the function of a protein kinase that must be inherent to the B1 protein. Therefore, phosphorylation of the 56-kDa polypeptide was suggested to be the result of autophosphorylation of the GST-B1 fusion protein. The minor phosphorylated 32-kDa protein observed in the absence of exogenous substrates must be due to a contaminating bacterial protein kinase.

Having established that the GST-B1 fusion protein undergoes autophosphorylation, the question remained whether the phosphates were attached to the GST or the B1 domain of the protein. Two approaches were used to localize the phosphates on the fusion protein. First, a mixing experiment was performed to test the ability of the protein kinase to phosphorylate GST having no fusion. The GST-B1 fusion protein was mixed with GST and reacted with [γ-^{32}P]ATP, and phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The mixture of GST-B1 and GST resulted in phosphorylation of the 56-kDa GST-B1 fusion protein but only weakly phosphorylated the 27-kDa GST polypeptide (Fig. 3), even though GST was over 10-fold in excess of GST-B1. This result suggested that GST is not a good substrate for the B1 protein kinase. The GST-B1 fusion protein was further analyzed by separation of the GST and B1 domains after autophosphorylation. The fusion protein expression vector is designed to produce a thrombin cleavage site at the fusion junction (7). Thrombin cleavage of the ^{32}P-labeled GST-B1 fusion protein resulted in reduction of the 56-kDa phosphoprotein and the appearance of a new ^{32}P-labeled 27-kDa polypeptide (Fig. 3). Little or no phosphorylated 34-kDa polypeptide corresponding to the B1 domain was observed. The 27-kDa product is consistent with the size of GST, indicating that autophosphorylation of the GST-B1 fusion protein occurred predominantly on the GST domain.

The phosphorylation of GST when fused to the B1 protein

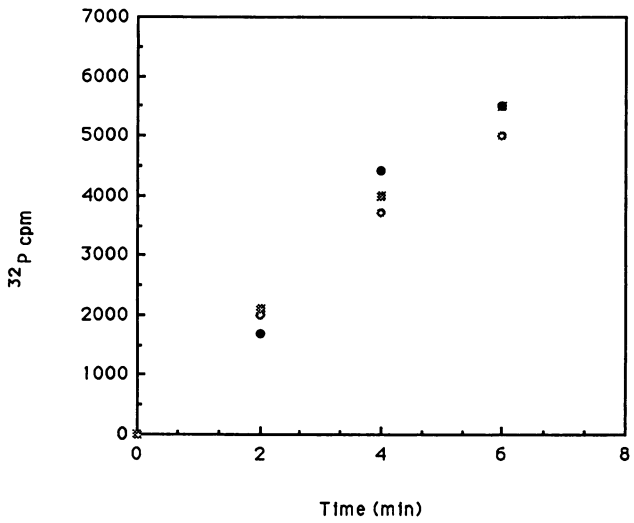

FIG. 4. Effect of dilution on the rate of autophosphorylation of the GST-B1 protein. The fusion protein (0.75 μg of protein) was reacted with [γ-^{32}P]ATP at enzyme concentrations of 1.3 μM (○), 2.6 μM (●), or 3.9 μM (□) protein kinase. Phosphorylation of GST-B1 was quantitated as described in Materials and Methods.

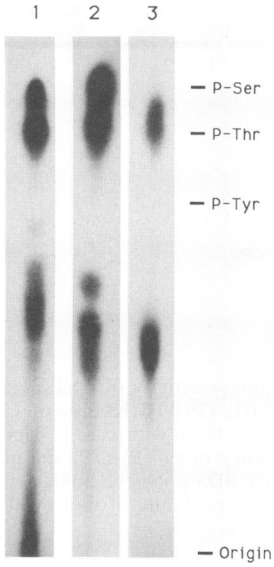

FIG. 5. Phosphoamino acid analysis of proteins phosphorylated by GST-B1. The GST-B1 protein was used to phosphorylate casein (lane 2), histone H1 (lane 1), or itself (lane 3) with [γ-^{32}P]ATP. Phosphorylated polypeptides were isolated and acid hydrolyzed, and the phosphoamino acids were separated by electrophoresis on thin-layer cellulose plates. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) are indicated on the right.

kinase but not when separate suggests that the autophosphorylation of the GST-B1 fusion protein proceded by an intramolecular reaction. This notion is supported by the lack of effect of dilution on the autophosphorylation reaction. Under conditions in which enzyme was not saturating, variation of the enzyme concentration over a threefold range did not alter the rate of autophosphorylation of the GST-B1 protein (Fig. 4). This result is most easily explained as a diffusion-independent reaction, expected for an intramolecular event.

Amino acid specificity of the B1 protein kinase. The types of amino acid phosphorylated by the B1 protein kinase were determined for the exogenous substrates casein and histone H1 as well as for the autophosphorylation of GST-B1. Polypeptides were phosphorylated with a GST-B1 preparation containing a very low level of contaminating bacterial protein kinase and [γ-^{32}P]ATP. Phosphoprotein products were separated by electrophoresis and blotted onto polyvinylidene difluoride membranes. Individual polypeptides were acid hydrolyzed, and phosphorylated amino acids were analyzed by thin-layer electrophoresis (12). For both casein and histone H1, phosphoserine and phosphothreonine were detected among the ^{32}P-labeled products (Fig. 5). For the GST-B1 polypeptide, phosphothreonine was the major phosphoamino acid observed. No phosphotyrosine was detected in any of these proteins.

As a final attempt to detect tyrosine phosphorylation, the random copolymer poly(tyrosine-glutamate) was tested as a substrate for the B1 protein kinase. This polypeptide has been reported to be a substrate for kinases that may otherwise appear to be specific for serine and threonine residues (26). We were unable to detect any phosphorylation of this polypeptide by the B1 protein kinase (data not shown), indicating that it cannot phosphorylate the tyrosine residues in the copolymer.

Nucleotide specificity of the B1 protein kinase. As shown above, the vaccinia virus B1 protein kinase can utilize ATP as a phosphate donor. Determinations of casein kinase activity as a function of nucleotide concentration and analysis by a Lineweaver-Burk plot indicate that the K_m for ATP is 30 μM (not shown). GTP was not utilized as a phosphate donor by the B1 protein kinase. Using [γ-^{32}P]GTP, we were unable to detect autophosphorylation of GST-B1 or the phosphorylation of casein or histone H1. In addition, the ATP analog ^{35}S-adenosine 5′(3-thiotriphosphate) was tested as a substrate and was found not to label protein substrates in the B1 kinase reaction.

Factors influencing B1 protein kinase activity. In order to determine the relationship between the B1 protein kinase and a previously described protein kinase activity that is packaged within the vaccinia virus particle (13, 14), we have examined the effect of various solution conditions on the ability of the B1 protein kinase to phosphorylate casein. The B1 protein kinase activity was optimal in buffer of pH 8.0 to 9.0. In solutions containing 10 μM ATP, there was an absolute requirement for $MgCl_2$, and maximal activity was achieved above 2 mM $MgCl_2$. Manganese could replace magnesium in the protein kinase reaction, but calcium and zinc were completely ineffective. These properties are similar to those described previously for the vaccinia virus-encapsidated protein kinase (13, 14). That protein kinase was markedly stimulated by protamine to an extent that was dependent upon the type of protein substrate. Phosphorylation of casein reportedly was stimulated some 90-fold by protamine (14). We have not observed pronounced stimulation of the B1 protein kinase. Protamine was found to increase the phosphorylation of casein by different GST-B1 preparations by 0.5- to 2-fold when present at concentrations ranging from 10 to 100 μg/ml (data not shown).

Characterization of the B1 protein kinase in virions. To determine what relationship, if any, the B1R protein kinase has to the previously described protein kinase in virions, virions were fractionated by differential detergent extraction and column chromatography, and the various fractions were

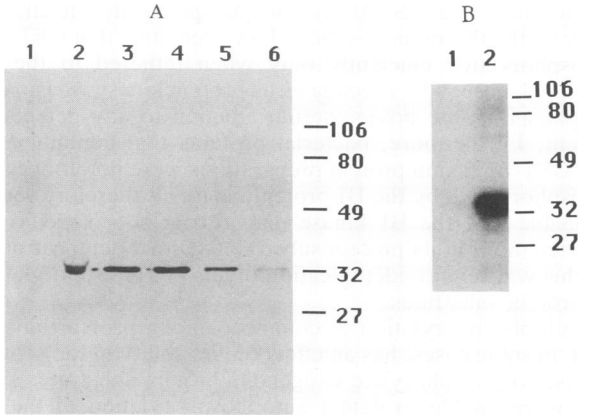

FIG. 6. Localization of the B1 protein in the vaccinia virion. (A) Immunoblot of vaccinia virus proteins probed with anti-B1 antiserum. Purified vaccinia virions (lane 2) were extracted with Nonidet P-40 to solubilize the membrane fraction (lane 3) and subsequently extracted with 0.2% (lane 4) and 0.5% (lane 5) sodium deoxycholate to solubilize the core particle. Lane 6 is material not solubilized by sodium deoxycholate treatment. Lane 1 is total vaccinia virus protein probed with preimmune serum. (B) Casein kinase activity of Nonidet P-40-solubilized envelope fraction (lane 1) and 0.2% sodium deoxycholate-solubilized core fraction (lane 2). The mobilities of molecular size standards are given on the right (in kilodaltons).

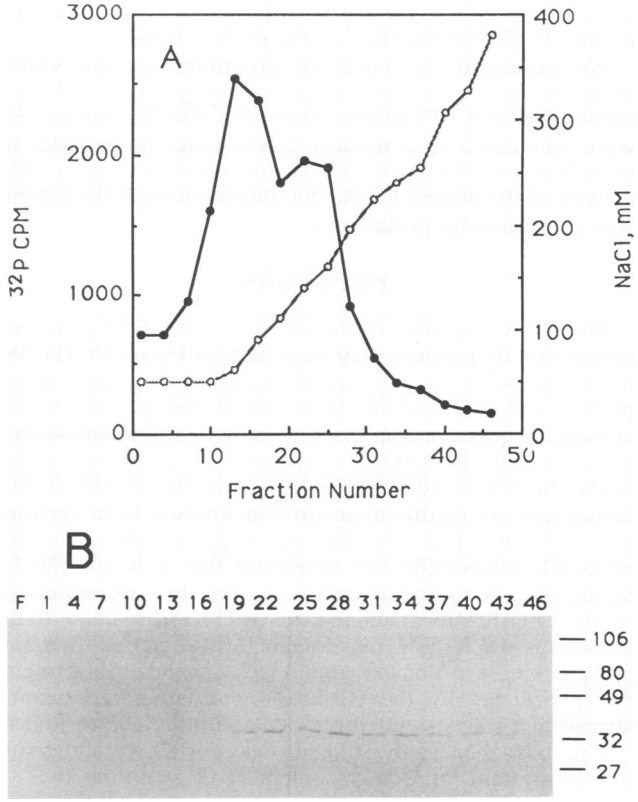

FIG. 7. DEAE-cellulose chromatography of vaccinia virion-associated protein kinase activity. The sodium deoxycholate-soluble fraction of virus cores was applied to a DEAE-cellulose column in buffer containing 50 mM NaCl. After the column was washed with the same buffer, the bound proteins were eluted with a linear gradient of 50 to 400 mM NaCl. Fifteen microliters of each column fraction was tested for casein kinase activity (panel A, ●) NaCl concentration (○), and the presence of the B1 polypeptide by immunoblotting (panel B). Lane F is the flowthrough fraction of the column. The casein kinase assay with 15 μl of the 280-ml flowthrough fraction gave 1,378 cpm. The positions of protein size standards are shown to the right in panel B (in kilodaltons).

monitored for casein kinase activity and the B1 polypeptide by immunoblotting with anti-B1 antiserum. Virion particles were extracted with Nonidet P-40 to solubilize the membranes and then extracted with sodium deoxycholate to solubilize the core-associated enzymes. Immunoblotting of these fractions demonstrated the presence of comparable amounts of the B1 polypeptide in both the envelope and core fractions (Fig. 6). Casein kinase activity was also detected in both fractions. Approximately 40 times more total protein kinase activity was found in cores than in the envelope fraction. The finding of the B1 protein kinase in both the envelope and core fractions does not seem to be the result of incomplete solubilization of the membrane or partial solubilization of the core by Nonidet P-40. Also, addition of deoxycholate to the Nonidet P-40-soluble fraction did not stimulate its activity. Several virion polypeptides were found to be quantitatively solubilized by the Nonidet P-40 treatment (not shown). In addition, immunoblotting of these fractions with antiserum directed against the small subunit of the vaccinia virus early transcription factor showed it to be solubilized with deoxycholate but not with Nonidet P-40, as described previously (2).

The virion core extract was further fractionated by chromatography on DEAE-cellulose. The previously described core-associated protein kinase was reported to flow through DEAE-cellulose in buffers containing 50 mM NaCl (13). When we passed core extracts over DEAE-cellulose in the presence of 50 mM NaCl, protein kinase activity was detected in the fraction that did not bind to the column, in agreement with the earlier study. Development of the column with a 50 to 400 mM NaCl gradient resulted in the elution of protein kinase activity that was biphasic in shape (Fig. 7). The column profile consisted of two apparently overlapping peaks of activity. The major peak eluted first, and a minor peak was released at about 125 mM NaCl. In order to locate the B1 polypeptide, the DEAE column fractions were analyzed by immunoblotting. No B1 polypeptide was detected in the flowthrough fraction of the column (Fig. 7B). After concentration of this fraction 10-fold, we still failed to detect B1. Immunoblotting of column fractions released by the salt gradient revealed the B1 polypeptide beginning at fraction 19, coinciding with the minor peak of activity. Importantly, fractions 10 to 16, which contained the major peak of protein kinase activity in the profile, did not have detectable B1 polypeptide. This result indicates that there are multiple protein kinases in the vaccinia virion particle. Furthermore, the B1 protein kinase represents a minor fraction of the total activity extracted from virions.

From the yield of activity in the minor peak of the DEAE-cellulose column profile, we estimate that the B1 protein kinase constituted about 4% of the total activity in the extract. Because these protein fractions are not pure enzymes, it is not known whether the differences in activity are due to numbers of enzyme molecules or inherent specific activities. We also do not know the relationship between the protein kinase that did not bind to the DEAE column and the major peak of activity released with the salt gradient. We suspect they may be the same, since the bound enzyme was released immediately upon increase of the ionic strength of

the column buffer. Resolution of these uncertainties will require further purification of the protein kinases.

We examined the effect of protamine on the virion-associated protein kinases fractionated by DEAE-cellulose chromatography. Fractions containing the B1 polypeptide were stimulated to a modest extent (one- to twofold) by protamine, similar to that of the recombinant enzyme. The activity of the protein kinase that flowed through the column was unaffected by protamine.

DISCUSSION

The DNA sequence of the vaccinia virus B1R gene predicted that its product may be a protein kinase (9–11). We have expressed the product of the gene as a GST fusion protein and demonstrate here that it has protein kinase activity. Sequence comparison of known protein kinases has revealed that all of these enzymes have conserved amino acids; however, sequence motifs can be identified that distinguish serine/threonine protein kinases from tyrosine protein kinases (24). The deduced sequence of the vaccinia virus B1 polypeptide has suggested that it is specific for serine and threonine residues (9, 10). For example, the sequence DIKASN at amino acids 147 to 152 is similar to the consensus DLKPEN for domain VI of serine/threonine protein kinases. Phosphoamino acid analysis of proteins phosphorylated by the B1 fusion protein indeed demonstrated that it is specific for serine and threonine residues.

The most thoroughly studied region of the catalytic subunit of protein kinases is domain II (24). Within this sequence is an invariant lysine that is generally 14 to 23 amino acids downstream of the last conserved glycine of domain I. Mutagenesis and nucleotide affinity labeling studies have shown that this lysine is absolutely essential for the protein kinase activity and likely participates in the phosphotransfer reaction. The crystal structure of the catalytic subunit of cyclic AMP-dependent protein kinase placed this lysine at the catalytic site of the enzyme (15). The vaccinia virus B1 protein kinase sequence suggested that either lysine 41 or lysine 45 could be a candidate for the essential lysine in domain II of the enzyme. Site-directed mutagenesis of the two demonstrated that lysine 41 is essential for protein kinase activity, whereas lysine 45 is not. Therefore, lysine 41 of the vaccinia virus B1 protein kinase likely plays a role in the phosphotransfer reaction. Lysine 41 is just 13 amino acids downstream of the last conserved glycine in domain I of the B1 protein kinase. This is the shortest spacing between domains I and II that has been reported.

The catalytic domains of protein kinases span about 300 amino acids but generally have carboxyl- or amino-terminal extensions that may regulate protein kinase activity (24, 28). The sequence of the vaccinia virus B1 gene predicts that the B1 protein kinase catalytic subunit is exactly 300 amino acids in length (9, 10), making it one of the smallest protein kinase catalytic subunits known. It seems doubtful that the B1 polypeptide will be found to possess additional regulatory sequences. This does not preclude the existence of another viral polypeptide which could interact with the B1 protein to regulate its protein kinase activity.

The patterns of protein phosphorylation produced by the B1 kinase are indicative of some degree of selectivity toward protein substrates. The B1 protein kinase phosphorylated both the acidic protein casein and the basic histone H1 on both serine and threonine residues. Phosphate attachment on two types of residues on a given protein suggests that multiple phosphorylation events occurred on each protein. In addition, the GST-B1 fusion phosphorylated itself, but only on the threonine residues. It is interesting that GST was phosphorylated efficiently only when tethered to the B1 protein kinase. The vaccinia virus protein kinase was unable to phosphorylate bovine serum albumin to any detectable extent. Furthermore, bacterial proteins that contaminated the GST-B1 fusion protein preparations were not noticeably phosphorylated by the B1 protein kinase. It therefore seems probable that the B1 kinase may recognize a specific sequence motif in its protein substrates. Direct demonstration of this will require identification of sites of phosphorylation in protein substrates.

Autophosphorylation is common among protein kinases and, in some cases, has an effect on the ability of the protein kinase to phosphorylate exogenous protein substrates (24). We were unable to detect autophosphorylation of the B1 protein kinase domain of the fusion protein. Whether autophosphorylation of this enzyme occurs in vivo and whether this serves to regulate the activity of the B1 protein kinase remains to be determined. It is interesting that vaccinia virions contain an abundant phosphoprotein with a mass similar to that of the B1 polypeptide (30).

Analysis of vaccinia virions with antibody directed against the B1 polypeptide has demonstrated that the B1 protein kinase is a component of the virion particle. Chromatography of virion extracts resulted in separation of protein kinase activity into a fraction containing the B1 polypeptide and another with no detectable B1. The simplest interpretation of this result is that multiple protein kinases are present in the virion. This raises a question as to the origin of the other kinase(s). Inspection of the deduced protein sequences encoded within the entire vaccinia virus genome (6) reveals that the B1 protein is the only protein encoded by vaccinia virus that satisfactorily conforms to the conserved sequence elements of the catalytic domains of protein kinases (9). The other protein kinase activity we have detected is therefore likely to be a host cell protein kinase. Confirmation of this notion will require purification and direct analysis of the protein kinase.

The function and physiologically significant protein substrates for the vaccinia virus B1 protein kinase are, at this time, a matter of conjecture. While the B1 protein kinase appears to be packaged within the virion particle, we do not yet know whether this packaging is specific or whether the localization to the virion is essential for virus function. The fact that conditionally lethal mutations map to the B1R open reading frame indicates that the protein kinase is an essential gene product (29). B1 mutants do not synthesize DNA at normal levels under permissive conditions (19). This would suggest that the protein kinase must phosphorylate a component of the DNA replication machinery or, alternatively, a protein functioning in the synthesis of the proteins required for DNA replication.

ACKNOWLEDGMENTS

We are grateful to James Lauderdale and Arnold Stein for histone H1 and to Marrietta Harrison, Robert Geahlen, and Harry Charbonneau for critical commentary on the manuscript.

This work was supported by grant AI 28432-01 from the National Institutes of Health, Junior Faculty Award JFRA-329 from the American Cancer Society, and Institutional Research Grant IN-17-30 from the American Cancer Society.

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