Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7653–7657, August 1994
Biochemistry

Vaccinia protein kinase 2: A second essential serine/threonine protein kinase encoded by vaccinia virus

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Communicated by Bernard Moss, April 7, 1994

ABSTRACT **The major protein kinase activity from vaccinia virus core particles was purified to near homogeneity. The protein kinase is a 50-kDa polypeptide that is shown here to phosphorylate primarily seryl residues in α-casein, a casein kinase I-specific peptide substrate, and itself through autophosphorylation. The sequence of four peptides derived from the protein kinase demonstrated that it is encoded by the vaccinia virus *F10L* gene. Expression of the *F10L* gene product in bacteria as a fusion with glutathione *S*-transferase confirmed that the vaccinia *F10L* gene encodes the protein kinase. We have termed this enzyme vaccinia protein kinase 2 (VPK2) to distinguish it from the protein kinase encoded by the vaccinia *B1R* gene. Targeted disruption of the VPK2 gene with a positive selectable marker demonstrated that all viruses with a disrupted gene also possessed a wild-type gene, suggesting that VPK2 is essential for viability. The discovery of a second essential protein kinase encoded by vaccinia virus, in addition to a protein phosphatase, underscores the importance of protein phosphorylation in poxvirus biogenesis.**

Protein phosphorylation is a common mechanism for regulation of cellular processes (reviewed in refs. 1 and 2). The phosphorylation state of an enzyme can influence profoundly its catalytic activity, and phosphorylation can impact the ability of a protein to interact with other proteins or ligands. Proteins influenced by phosphorylation are often ones functioning at critical junctures of biochemical pathways or proteins that are integral parts of subcellular structures. The protein kinases and protein phosphatases that control the phosphorylation states of proteins can function as molecular switches to activate or deactivate cellular events in response to various stimuli and may themselves be regulated by phosphorylation.

There is mounting evidence that protein phosphorylation is essential for the replication of vaccinia virus. Vaccinia is a member of the poxvirus family and characteristically has a large DNA genome encoding ≈200 different proteins (3). This virus replicates exclusively in the host cell cytoplasm, and consequently has evolved to a high level of complexity. Two vaccinia gene products previously have been described as potential modulators of protein phosphorylation. The *H1L* gene encodes a serine/threonine/tyrosine protein phosphatase (4) that appears to be essential for virus viability (S.S.B., unpublished observations). The *B1R* gene product is a serine/threonine protein kinase (5–7) that is also essential for viability (6, 8). Several vaccinia gene products are known phosphoproteins (9–11), and the virion core structure contains abundant protein kinase activity (12). The partial purification and characterization of a virion-associated protein kinase have been reported (13, 14). We recently have described evidence that the major virion core protein kinase activity is distinct from the protein kinase encoded by the *B1R* gene (5). In this report, we describe the purification and characterization of the major vaccinia core protein kinase, the second protein kinase known to be encoded by vaccinia virus.

MATERIALS AND METHODS

Cells and Viruses. Vaccinia virus strain WR was grown on HeLa S3 cells and purified by sucrose gradient centrifugation as described (15). BSC40 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Purification of the Major Vaccinia Core Protein Kinase. Five thousand A_{260} units of purified vaccinia virus were extracted with 50 mM Tris·HCl, pH 8.0/50 mM dithiothreitol (DTT)/0.5% Nonidet P-40 (NP-40) to isolate core particles (16). Vaccinia cores contain at least two distinguishable protein kinase activities (5). Total protein kinase activity was determined by using α-casein, which is an efficient substrate for both the major virion protein kinase as well as the protein kinase encoded by the vaccinia *B1R* gene (5). The major protein kinase activity was determined specifically by its ability to phosphorylate the peptide D4 (see below). Cores were solubilized in 0.2% sodium deoxycholate/0.25 M NaCl/20 mM Tris·HCl, pH 8.4/1 mM DTT, and nucleic acid was removed by passage over a 20-ml DEAE-cellulose column equilibrated in the same buffer lacking sodium deoxycholate (17). Unadsorbed proteins were diluted to 50 mM NaCl with buffer B [20 mM Tris·HCl, pH 8.0/0.1 mM EDTA/1 mM DTT/0.01% NP-40/10% (vol/vol) glycerol] and applied to a second 20-ml DEAE-cellulose column linked in tandem to a 20-ml single-stranded DNA-cellulose column (DEAE-cellulose II/DNA-cellulose), both equilibrated with 50 mM NaCl in buffer B. The flow-through fraction was concentrated by an Amicon concentrator equipped with a YM-30 membrane, diluted with buffer B to a NaCl concentration of <10 mM, and loaded onto a third DEAE-cellulose column (15 ml) equilibrated with buffer B. The column was developed with a 60-ml linear gradient of 0–0.25 M NaCl in buffer B. The protein kinase eluted at ≈70 mM NaCl. Protein kinase peak fractions were pooled and applied to a 10-ml phosphocellulose column equilibrated with 0.1 M NaCl in buffer B. The column was developed with a 40-ml gradient of 0.1–0.6 M NaCl in buffer B. The major protein kinase eluted from this column at 0.3 M NaCl. Peak protein kinase fractions were concentrated by centrifugation in a Centricon-30 concentrator, and one-half of the preparation was subjected to centrifugation on a 15–35% glycerol gradient containing 0.2 M NaCl, 50 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 0.01% NP-40 at 40,000 rpm for 53 hr in a Beckman SW41 rotor. Protein was quantitated according to Bradford (18) with bovine serum albumin as the standard.

Protein Kinase Assays. Routine protein kinase assays were performed in a 20-μl solution containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 10 μg of dephosphorylated α-casein (Sigma), 100 μM [γ-^{32}P]ATP, and 5 μl of column

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Abbreviations: DTT, dithiothreitol; GST, glutathione *S*-transferase; VPK2, vaccinia protein kinase 2.

fraction. Reactions were conducted at 37°C for 10 min and were terminated by addition of SDS/PAGE sample buffer. Proteins were separated by SDS/PAGE and electroblotted onto poly(vinylidene difluoride) membranes. Casein bands were excised, and radiolabel was quantitated by scintillation counting (5).

Peptide phosphorylation assays were conducted at 30°C for 10 min with 1.5 mM peptide D4 (ref. 19; amino acid sequence, DDDDVASLPGLRRR), a generous gift from Peter Roach (Indiana University School of Medicine). Incorporation of label into peptide was determined by P81 paper binding (20). Kinase activity was quantitated as pmol of phosphate transferred from ATP to peptide per min. The protein kinase inhibitor CK1-7 (Seikagaku America, Rockville, MD) was included in reactions as indicated.

Phosphoamino Acid Analysis. Phosphoproteins were purified by SDS/PAGE and electroblotting as described above and hydrolyzed in 5.7 M HCl at 110°C for 1 hr. Phosphoamino acids were resolved by thin-layer electrophoresis at pH 3.5 (21).

Amino Acid Sequence Analysis. The protein kinase purified to the phosphocellulose step was concentrated 3-fold by vacuum centrifugation and precipitation with 10% trichloroacetic acid. The proteins were subjected to SDS/PAGE, and the 50-kDa polypeptide band corresponding to the protein kinase was excised and digested *in situ* with endoproteinase Lys-C (Wako BioProducts, Richmond, VA) as described (22) with the following modifications. Proteinase digestions were performed at 37°C for 4 hr in a solution containing 100 mM Tris·HCl (pH 9.0) and 0.5 μg of Lys-C. Peptides eluted from the gel were fractionated by reverse-phase HPLC on an Applied Biosystems separation system (model 130A) equipped with a Vydac RP-18 microbore column (250 × 2.1 mm). A linear gradient from 0% to 60% acetonitrile in 0.1% trifluoroacetic acid was run for 75 min at a flow rate of 150 μl/min. Elution was monitored at 214 nm, and peaks were collected manually. Amino acid sequence analysis was performed on an Applied Biosystems gas-phase sequencer (model 470A).

Recombinant Protein Synthesis and Purification. The vaccinia virus *F10L* gene was cloned by PCR from purified vaccinia virus (strain WR) DNA using Vent DNA polymerase (New England Biolabs) and the oligonucleotides GCCGGATCCATGGGTGTTGCCAATGATTCG and GCGTCTAGATTAGTTTCCGCCATTTATCCA as primers. The 1.3-kbp DNA product was cleaved with restriction endonucleases *Bam*HI and *Xba* I and inserted into the corresponding sites of the glutathione *S*-transferase (GST) fusion vector pGEX-KG (23). Fusion protein synthesis was induced by isopropyl β-D-thiogalactoside in *Escherichia coli* strain TG1 as described (23). Soluble fusion protein was purified from cell extracts by affinity chromatography on glutathione-agarose (23).

Targeted Disruption of the Vaccinia *F10L* Gene. The F10L PCR product described above was inserted into the *Sma* I site of the vector pUC118 (24). Nucleotides 426–516 of the *F10L* open reading frame were deleted by cleavage with *Nco* I and replaced with a vaccinia neomycin-resistance cassette from plasmid pVVneo (25). Recombinant virus was produced by transfection of the interrupted *F10L* gene construct into HeLa cells that had been infected with vaccinia virus essentially as described (26). Virus resulting from this procedure subsequently was plated on BSC40 cells and incubated for 3 days in medium containing G418 (2 mg/ml) (Life Technologies, Grand Island, NY). Antibiotic-resistant plaques were isolated and subjected to two more rounds of plaque purification under the same conditions. The DNA from isolated plaques was analyzed by PCR using the *F10L* flanking primers described above to determine the structure of the *F10L* gene.

RESULTS

Purification of the Major Protein Kinase from Vaccinia Virions. Vaccinia virus particles contain an abundant protein kinase activity (12). The activity is localized to the virus core structure and can be solubilized with deoxycholate (13). Using casein as a phosphoacceptor, we have demonstrated previously that the protein kinase activity from vaccinia cores is due to at least two different enzymes (5). The enzyme responsible for the majority of the activity is chromatographically distinct from the protein kinase encoded by the vaccinia *B1R* gene. We have purified the major protein kinase to determine its identity.

The major protein kinase and the B1R protein kinase both efficiently utilize casein as a protein substrate. We have determined that the major protein kinase efficiently phosphorylates the casein kinase I substrate peptide D4, whereas B1R protein kinase does not (data not shown). The major protein kinase has a K_m for the D4 peptide of ≈1.8 mM, similar to that observed for casein kinase I (19). Thus, the D4 peptide substrate provides a means to distinguish the major core protein kinase from the B1R protein kinase.

The major vaccinia protein kinase was purified from a deoxycholate extract of core particles by sequential chromatography on DEAE-cellulose, DNA-cellulose, and phosphocellulose. The first DEAE-cellulose chromatography resulted in increased activity, possibly due to removal of an enzyme inhibitor (Table 1). The B1R protein kinase was quantitatively separated from the major protein kinase after the second DEAE-cellulose step. After sequential passage over DNA-cellulose and chromatography on phosphocellulose, final purification of the major protein kinase was achieved by sedimentation on a glycerol gradient after concentration with a Centricon filter. The casein kinase activity was found in gradient fractions 15–20 and peaked in fraction 17 (Fig. 1). The enzyme was purified >500-fold from the crude core extract with an overall yield of ≈18% (Table 1). Analysis of the glycerol gradient fractions by SDS/PAGE revealed that the fractions with maximal casein kinase activity contained a polypeptide of 50 kDa with a sedimentation profile that paralleled that of the protein kinase (Fig. 1). Many protein kinases undergo autophosphorylation. When the glycerol gradient fractions were tested for autophosphorylation capability, a 50-kDa phosphoprotein was detected (Fig. 1). The extent of phosphorylation of this protein was inde-

Table 1. Protein kinase activity was measured as phosphate transferred to the peptide D4 at a peptide concentration of 1 mM

Step	Protein, mg	Activity, pmol·min^{-1}	Specific activity, pmol·min^{-1}·mg^{-1}	Purification, -fold	Yield, %
DEAE I	68.7	70,090	1,020		
DEAE II/DNA-cellulose	10.56	61,320	5,807	5.7	87.5
DEAE III	1.24	34,296	27,658	27.1	48.9
Phosphocellulose	0.293	21,950	74,915	74.4	31.3
Centricon-30	0.096	6,453	67,219	65.9	9.2
Glycerol gradient	0.023	4,582	199,217	195.3	6.5

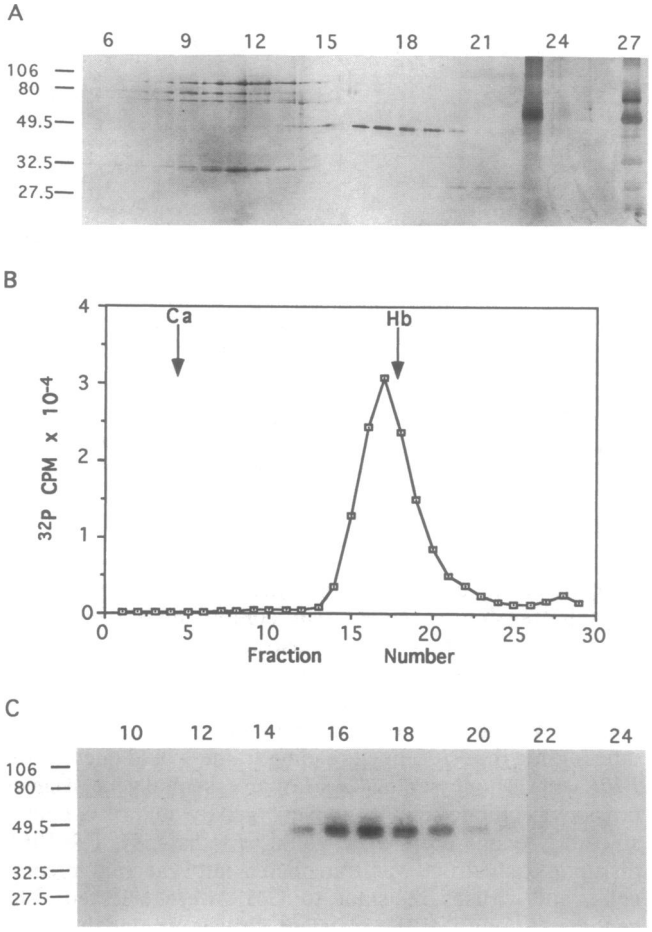

FIG. 1. Glycerol gradient sedimentation of the major vaccinia virion protein kinase. Protein kinase purified to the phosphocellulose step was pooled, concentrated, and sedimented on a glycerol gradient as described. Gradient fractions were collected from tube bottoms, and samples of each fraction were analyzed for polypeptide content by SDS/PAGE and silver staining (*A*), phosphorylation of α-casein (*B*), and autophosphorylation by SDS/PAGE and autoradiography (*C*). Electrophoretic mobilities of protein standards are indicated (kDa) on the left in *A* and *C*. Sedimentation positions of 11S catalase and 4.6S hemoglobin in a parallel gradient are indicated in *B*.

pendent of protein concentration (data not shown), indicative of an intramolecular phosphorylation. From the results described above, we conclude that the protein kinase activity was due to the 50-kDa protein. The protein kinase sedimented at a rate of ≈4.7 S relative to marker proteins, indicating a monomeric protein.

Properties of the Protein Kinase. Because the major core protein kinase appeared to have substrate specificity similar to that of casein kinase I, its enzymatic properties were characterized. The protein kinase from vaccinia virions was specific for ATP as the phosphodonor. GTP was not utilized to a detectable extent. The enzyme was found to be inhibited by the casein kinase inhibitor CK1-7. Half-maximal casein phosphorylation was observed at ≈200 μM CK1-7 with casein or the D4 peptide as substrates (data not shown). This was ≈10 times the concentration of inhibitor needed to reduce casein kinase I activity to a similar extent (27).

Amino acids phosphorylated by the protein kinase were determined by phosphoamino acid analysis. Autophosphorylated protein kinase contained only phosphoserine and no detectable phosphothreonine or phosphotyrosine (Fig. 2). Casein was phosphorylated by the vaccinia protein kinase

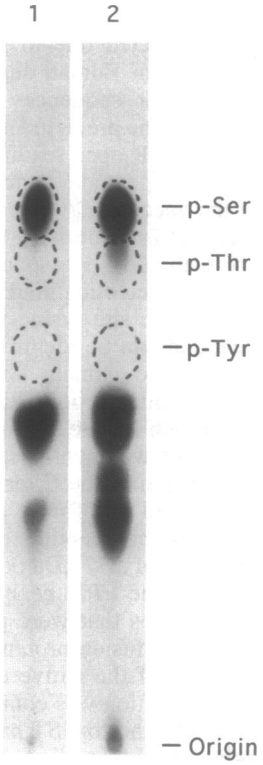

FIG. 2. Phosphoamino acid analysis of proteins phosphorylated by the major vaccinia core protein kinase. Purified protein kinase was incubated with [γ-^{32}P]ATP alone in an autophosphorylation reaction mixture (lane 1) or with [γ-^{32}P]ATP and α-casein (lane 2). Phosphoamino acids released by acid hydrolysis were resolved by thin-layer electrophoresis and were visualized by autoradiography. Positions of migration of phosphoamino acid standards phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) are circled.

primarily on a seryl residue(s), but a small amount of phosphothreonine was also detected.

Identification of the Protein Kinase Gene. Amino acid sequence information was used to identify the gene encoding the protein kinase. Because of protein losses incurred in the Centricon concentration step, enzyme purified to the phosphocellulose step was used for sequencing. The protein was subjected to SDS/PAGE (Fig. 3), and the 50-kDa polypeptide band was excised and digested in the gel slice with endoproteinase Lys-C. Peptide fragments were isolated by reverse-phase HPLC, and the amino acid sequence of four peptides was determined by automated Edman degradation. The peptide sequences were IEENDYINSSFFQQK, SALNDFDF-

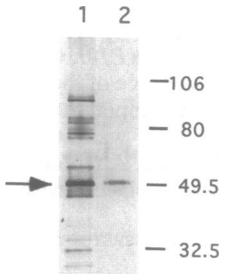

FIG. 3. SDS/PAGE analysis of protein kinase used for amino acid sequencing. Phosphocellulose-purified protein kinase (lane 1) was run on a 10% gel, and the 50-kDa polypeptide designated by the arrow was excised from the gel and cleaved with endoproteinase Lys-C for generation of peptides for sequencing. Lane 2, protein kinase from fraction 17 of the glycerol gradient shown in Fig. 2. Molecular masses of marker proteins are given on the right in kDa.

SQVAGII, VEHN?YYDFHFF (? indicates a predicted tryptophan that was not detected due to interference by the diphenyl urea by-product of Edman degradation), and VSLIHPISFLEK. These four sequences were found to be perfect matches to that of the predicted M_r 52,158 product of the vaccinia virus strain WR open reading frame *F10L* (Fig. 4) (J. Sisler and B. Moss, personal communication).

The sequence of the vaccinia *F10L* gene product contains several recognizable motifs comprising parts of the protein kinase catalytic domain (28). Reasonable candidates for domain I that forms the nucleotide binding fold and domain II that contains the active site lysine participating in the phosphotransfer reaction can be identified. There is also a particularly good match to domain VI that contains the signature for serine/threonine protein kinases.

Expression of the Protein Kinase in Bacteria. The vaccinia virus *F10L* gene product was synthesized in recombinant form in bacteria as a fusion to the C terminus of GST. The fusion protein was purified by affinity chromatography on glutathione-agarose. The polypeptide composition of the purified protein consisted of a 77-kDa polypeptide expected for the fusion of GST to the *F10L* gene product as well as several smaller polypeptides that were presumed to be the result of proteolysis of the fusion protein (data not shown). The substrate specificity of the native enzyme purified by glycerol gradient centrifugation was compared to that of the GST–F10L fusion protein and to GST having no fusion that was similarly expressed and purified. The native protein kinase was capable of phosphorylating α-casein and weakly phosphorylated phosvitin and myelin basic protein (Fig. 5). Phosphorylation of core histones, histone H1, and histone H3 was not detected. The recombinant GST–F10L fusion protein similarly phosphorylated α-casein as well as phosvitin to a much lesser extent (Fig. 5*B*). The fusion protein phosphorylated myelin basic protein at a significantly higher rate than did the native protein kinase. We attributed this higher rate of phosphorylation of myelin basic protein to be the result of artifactual affinity of GST for basic proteins. We previously observed that the GST–B1R protein kinase fusion efficiently phosphorylated histone H1, whereas the B1R protein kinase liberated of the GST by proteolysis did not (ref. 5; unpublished observations). The GST–F10L fusion protein did not phosphorylate any of the histones. A weakly phosphorylated 77-kDa polypeptide was observed in all reaction mixtures containing the fusion protein and likely was the result of autophosphorylation of the fusion protein. Recombinant GST devoid of the F10L protein fusion had no detectable protein kinase activity with any of the candidate protein substrates described above, indicating that the observed protein kinase activity is a property of the F10L polypeptide. The similarity in substrate specificity of the native protein kinase and the recombinant fusion protein confirmed that the vaccinia virus *F10L* gene encodes the protein kinase.

Targeted Disruption of the *F10L* Gene. Little information is available as to the importance of the vaccinia *F10L* gene, and no mutations have been mapped therein. Targeted disruption of the protein kinase gene was attempted as a test of the essential nature of the gene. An internal segment of the *F10L* gene was deleted and replaced with a cassette having a vaccinia promoter directing the synthesis of aminoglycoside phosphotransferase (Neo^R) that confers resistance to the antibiotic G418. This plasmid construct permitted insertion of the Neo^R gene into the *F10L* gene of the virus by homologous recombination. Under positive selection by the antibiotic G418, a double cross-over on both sides of the Neo^R gene interrupting the *F10L* open reading frame would occur if the *F10L* gene is nonessential (26). For an essential gene, a single cross-over on one side of the Neo^R gene would be expected since the second cross-over would be lethal (29). The F10L disruption construct was transfected into vaccinia-infected cells, and viruses resistant to G418 were selected. The genome structure of the resistant viruses was analyzed by PCR with primers that flank the *F10L* gene. A PCR DNA product of 1.3 kb was expected for an intact *F10L* gene, and a 3.0-kb product would be produced from the *F10L* gene interrupted by the Neo^R cassette. For all G418-resistant viruses isolated, both the wild-type and interrupted versions of the *F10L* gene were present (data not shown), indicating that a single homologous cross-over in one of the flanking regions in the *F10L* gene had occurred. These results suggest that vaccinia protein kinase 2 is essential for vaccinia viability.

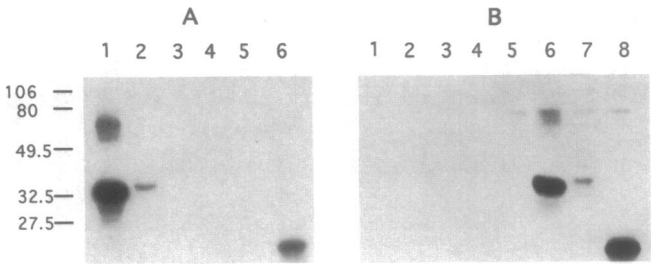

FIG. 5. Protein kinase activity of native VPK2 and recombinant GST–F10L fusion protein. (*A*) Glycerol gradient-purified VPK2 was incubated under protein kinase assay conditions with 2 μg of α-casein (lane 1), 10 μg of phosvitin (lane 2), 10 μg of histone H1 (lane 3), 10 μg of core histones (lane 4), 10 μg of histone H3 (lane 5), and 10 μg of myelin basic protein (lane 6). Numbers on left are kDa. (*B*) Affinity-purified GST (lanes 1–4) and GST–F10L fusion protein (lanes 5–8) were incubated alone (lanes 1 and 5) or with α-casein (lanes 2 and 6), phosvitin (lanes 3 and 7), or myelin basic protein (lanes 4 and 8). Phosphorylation products were resolved by SDS/PAGE and visualized by autoradiography.

DISCUSSION

Protein kinase activity was detected in vaccinia virions >20 years ago (12). Previous work suggested that the majority of the protein kinase activity was due to a single protein (13, 14) that subsequently was suggested to be separate from the protein kinase encoded by the vaccinia *B1R* gene (5). In this report, purification of the major vaccinia core protein kinase is described, and the enzyme is shown to be encoded by the vaccinia *F10L* gene. Thus, the *F10L* gene product is the second known protein kinase encoded by vaccinia virus. We therefore suggest that the vaccinia *F10L* gene product be designated vaccinia protein kinase 2 (VPK2) and the vaccinia

1 MGVANDSSPEYQWMSPHRLSDTVILGDCLYFNNIMSQLDLHQNWAPSVRLLNYFKNFNRETLLKIEENDYINSSFFQQKDKRFYPINDDF

91 YHISTGGYGIVFKIDNYVVKFVFEATKLYSPMETTAEFTVPKFLYNNLKGDEKKLIVCAWAMGLNYKLTFLHTLYKRVLHMLLLLIQTMD

181 GQELSLRYSSKVFLKAFNERKDSIDFVKLLSHFYPAVINSNINVINYFNRMFHFFEHEKRTNYEYERGNIIIFPLALYSADKVDTELAIK

271 LGFKSLVQYIKFIFLQMALLYIKIYELPCCDNFLHADLKPDNILLFDSNEPIIIHLKDKKFVFNERIKSALNDFDFSQVAGIINKKIKNN

361 FKVEHNWYYDFHFFVHTLLKTYPEIEKDIEFSTALEEFIMCTKTDCDKYRLKVSILHPISFLEKFIMRDIFSDWINGGN

FIG. 4. Predicted amino acid sequence of the vaccinia *F10L* gene product. Peptides identified by sequencing of protein kinase are underlined. Protein kinase motifs are bracketed with motif numbers as given in ref. 28.

B1R gene product be referred to as vaccinia protein kinase 1 until the functional roles of these enzymes are clarified.

The amino acid sequence of VPK2 suggests that it is an atypical protein kinase. Comparison of a large number of protein kinase sequences has identified several sequences that are highly conserved within the catalytic domain (28). While acceptable matches to domains II and VI can be identified in the sequence of VPK2 (Fig. 4), the other domains are either unusual in their sequence or unrecognizable. For example, domain I that forms the ATP binding pocket of protein kinases has the almost invariant sequence GXXGXG. In VPK2, the first glycine of domain I is replaced with a serine. Deviations from the consensus domain I sequence also have been observed in other protein kinases (30–32). In addition, domain VII of protein kinases usually has the very highly conserved sequence DFG that is not identifiable in VPK2.

Little information is currently available on the vaccinia *F10L* gene product. The sequence of the *F10L* gene suggests that it is transcribed as a late class vaccinia gene. The consensus sequence TAAATG that is the hallmark of vaccinia late promoters (33) is found at the initiation codon of the *F10L* open reading frame as is a T-rich sequence upstream that is also characteristic of late gene promoters (33). The *F10L* open reading frame contains four early gene transcription termination signals that are of the sequence TTTTTNT (34), making it unlikely that this gene is transcribed early in the infectious cycle. This is in contrast to the vaccinia *B1R* protein kinase gene, which is transcribed as an early class gene (8, 35).

The function and biological substrates of VPK2 are unknown. Phosphorylation of the casein kinase I-specific peptide substrate D4 by VPK2 suggests that it may have substrate specificity that is similar to casein kinase I. It is noted, however, that the sequence elements in the D4 peptide that encourage its use as a substrate for phosphorylation by VPK2 may be very different from those for casein kinase I. The localization of abundant quantities of VPK2 to the virus core structure suggests the possibility that protein phosphorylation may have a role in early gene transcription. Vaccinia early mRNA synthesis takes place in an intact but permeable core structure inside the infected cell (reviewed in refs. 36 and 37). Most of the enzymes that have been localized to the vaccinia core particle are believed to have roles in mRNA synthesis. It is, therefore, conceivable that VPK2 could regulate components of the transcription machinery through protein phosphorylation. Further experimentation will be required to test this notion.

We are grateful to Mary Bower for the amino acid sequence analyses, to Paul Graves and Peter Roach for the gift of the D4 peptide, to Dennis Hruby for plasmid pVVneo, and to Bernard Moss for sharing the unpublished sequence of the *F10L* gene. This work was supported by grants from the National Institute of Allergy and Infectious Diseases, the American Cancer Society, and the Purdue Research Foundation. This is publication no. 14098 from the Purdue University Agricultural Experiment Station.

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