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Vaccinia Virus Gene H5R Encodes a Protein That Is Phosphorylated by the Multisubstrate Vaccinia Virus B1R Protein Kinase

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Vaccinia virus gene B1R encodes a protein kinase, the previously identified substrates of which include the proteins S2 and Sa of 40S ribosomal subunits. This work characterizes another substrate of the B1R kinase: a 36-kDa protein induced at the early stage of infection. Partially purified 36-kDa protein, eluted from a single-stranded DNA-cellulose column by 0.5 M NaCl, was separated by two-dimensional gel electrophoresis. Phosphorylation in vitro yielded multiple forms of the 36-kDa protein with approximate isoelectric points (pI) of 5.5, 5.7, 5.9, and 6.3, in addition to the apparently unphosphorylated form with a pI of approximately 6.8. The tryptic peptides derived from 36-kDa proteins with pI values of 5.7, 5.9, and 6.3 yielded almost identical high-pressure liquid chromatography profiles, strongly suggesting that the 36-kDa protein was modified by the phosphorylation of at least four sites, which were characterized as threonine residues. The amino acid sequence of several tryptic peptides derived from the 36-kDa protein showed that the 36-kDa protein was encoded by gene H5R of vaccinia virus. Consistent with this, the B1R kinase—either expressed in *Escherichia coli* or highly purified from HeLa cells—phosphorylated a recombinant trpE-H5R fusion protein in vitro. Fingerprints of the trpE-H5R and 36-kDa proteins phosphorylated by recombinant B1R kinase revealed common sites of phosphorylation, although some tryptic peptides were specific to either protein. Comparison was made of fingerprints of tryptic phosphopeptides derived from 36-kDa single-stranded DNA-binding protein labelled in vivo or in vitro. A common subset of peptides was observed, suggesting that some sites on H5R protein are phosphorylated by the B1R kinase in infected cells. These results suggest that some of the multiple threonine sites in the H5R protein are phosphorylated in vivo by the B1R protein kinase.

Vaccinia virus replicates its DNA and expresses its genome in the cytoplasm of the infected cell (27). That protein phosphorylation may play a role in cell-virus interactions or in the replication of vaccinia virus is suggested by the fact that the protein encoded by gene B1R of vaccinia virus contains the conserved sequences present in cellular serine/threonine protein kinases (17, 42). B1R protein was expressed in *Escherichia coli* and shown to phosphorylate artificial substrates, including caseins (2, 24, 35). Far better substrates for the recombinant B1R protein in vitro are proteins S2 and Sa from 40S ribosomal subunits (1, 23). It is pertinent to emphasize that ribosomal proteins are potential physiological substrates of the B1R kinase, as they become phosphorylated during infection of cells with vaccinia virus (6, 19, 20). Phosphorylation occurs during the early stage of the infection and correlates temporally with the shutoff of host protein synthesis, although a functional role for the phosphorylation of ribosomal subunits remains to be established. An S2-Sa protein kinase activity has been purified from HeLa cells infected with vaccinia virus and was shown to correspond to the B1R protein (3, 4).

The B1R kinase is also likely to be involved in vaccinia virus DNA replication: two temperature-sensitive (*ts*) mutants, *ts*2 and *ts*25, defective for DNA replication at the nonpermissive temperature (7, 8), are mutated in the B1R gene (42). Although the B1R kinase appears to be vital for virus replication (35), the phenotype of the *ts* mutants is complex because the extent of the deficiency in viral DNA replication depends on the host (34) and also because the *ts* proteins, especially that from the *ts*2 mutant, are labile even at the nonpermissive temperature (35).

In previous work, we observed a further potential physiological substrate for the S2-Sa kinase purified from HeLa cells infected with vaccinia virus (4). This is a 36-kDa protein, induced in infected cells, which almost certainly corresponds to a major phosphoprotein previously isolated from the virosomes, designated protein FP11 by Nowakowski et al. (28, 29). Common properties of the two proteins are high abundance in proteins synthesized in the absence of viral DNA replication, similar migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34 to 36 kDa), elution from single-stranded DNA (ssDNA)-cellulose at a relatively low salt concentration (0.2 to 0.3 M NaCl), acidic isoelectric point (5.0 to 5.5), and phosphorylation exclusively on threonine residues (22a). Recently, Davis and Mathews (10) described a DNA-binding protein of vaccinia virus which they showed to be the product of gene I3L. They had some evidence which suggested that the I3L protein might correspond to the major phosphoprotein from virosomes, despite the fact that it eluted from ssDNA-cellulose at a considerably higher ionic strength. It was therefore important to characterize the molecular nature of the 36-kDa protein substrate of the S2-Sa protein kinase and to determine whether it is a substrate of the kinase in vivo. We present evidence here that the 36-kDa protein is, in fact, the product of vaccinia virus gene H5R and that it is indeed phosphorylated in vivo by the B1R kinase.

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MATERIALS AND METHODS

Materials. Culture media were from Eurobio (Paris, France), and newborn calf serum was from Gibco (Grand Island, N.Y.). Urea and glycerol were from Appligène (Illkirch, France). Ampholine (pH 5 to 8) was from LKB (Copenhagen, Denmark), ampholytes 3 to 9 were from Pharmacia (Uppsala, Sweden), and all other products were from Sigma (St. Louis, Mo.). Tran-^{35}S label is a mixture of [^{35}S]methionine and [^{35}S]cysteine from ICN Biomedicals (Irvine, Calif.).

Cells, viruses, and plasmids. HeLa cells were maintained in monolayer culture in minimum Eagle medium containing 10% newborn calf serum. Vaccinia virus (wild-type WR strain, obtained from B. Moss) was titrated on monolayers of HeLa cells. Plasmid pATH11-Ag35 (25a) was constructed by inserting an *Spe*I-*Nsi*I DNA fragment bearing H5R (truncated by 31 nucleotides on the 5′ side) into the pGEM3zf(+) plasmid (Promega), digested with *Xba*I and *Pst*I. This plasmid was then digested with *Bam*HI and *Hin*dIII, and the 826-bp fragment so generated was cloned into the same sites of pATH11 (21) to generate plasmid pATH11-Ag35. The trpE-H5R fusion protein was expressed in *E. coli* C600 transformed with pATH11-Ag35, as previously described (21). The cells (4-ml culture) were then washed twice with phosphate-buffered saline (PBS), suspended in 0.4 ml of extraction buffer (50 mM Tris-Cl [pH 7.5], 10 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. The extract was then centrifuged for 15 min in a microcentrifuge at 4°C, and the supernatant was dialyzed overnight against 20 mM Tris-Cl (pH 7.5)–10 mM 2-mercaptoethanol–1 mM EDTA–10% glycerol. The B1R kinase was expressed after transformation of competent cells of *E. coli* BL21(DE3)pLysE (41) with plasmid pAB6 (2) and induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial extracts were prepared as described above and stored at −70°C.

Analysis of tryptic peptides. For one-dimensional electrophoresis, approximately 6 μg (for fingerprints) or 50 μg (for microsequence determinations) of 36-kDa protein present in the 0.2 M KCl casein-agarose fraction (4) were subjected to standard SDS gel electrophoresis (22), and the proteins from the gel were transferred onto a membrane (0.45-μm pore size; BA85, Schleicher & Schuell) with an electrophoretic transfer system with horizontal graphite electrodes. The transfer was carried out in a buffer containing 39 mM glycine, 48 mM Tris-base, 0.0375% SDS, and 20% methanol for 90 min at 10 to 20 V. The nitrocellulose filter was then lightly stained with Amidoschwarz 10B (Serva), and the protein spots of interest were cut from the filter and digested with modified trypsin (Boehringer) or with trypsin (sequencing grade, Boehringer) as described previously (25). The tryptic phosphopeptides were then analyzed by reverse-phase high-pressure liquid chromatography (HPLC) as described below or applied to thin-layer cellulose plates (Eastman Kodak) and separated by electrophoresis at pH 6.5 (10% pyridine, 0.4% acetic acid) in the first dimension and by chromatography (*n*-butanol–pyridine–acetic acid–water, 75:50:15:60) as described by Boyle et al. (5).

For two-dimensional electrophoresis (30), approximately 20 μg of 36-kDa protein present in the 0.5 M NaCl DNA-cellulose fraction was precipitated with 5 volumes of acetone at −20°C for 2 h. After centrifugation at 12,500 × *g* for 15 min, the precipitate was dried and dissolved in 30 μl of isoelectric focusing sample buffer (9.5 M urea, 1% Nonidet P-40, 50 mM dithiothreitol). Isoelectric focusing was carried out on horizontal slab gels (7 cm long and 0.9 mm thick) containing 4.45% acrylamide, 0.12% bisacrylamide, 8 M urea, and 2% (wt/vol) Ampholines 5 to 8 or 3 to 9 (Pharmacia). The gel was preelectrophoresed on a cooled apparatus for 15 min at 700 V (constant voltage), and samples (10 μl each) were applied on a small piece of Whatman 3MM paper, placed on the acidic side of the gel. It should be noted that, for some unknown reason, we observed a much slower migration of the 36-kDa protein (but not of the other proteins) through the pH gradient when the sample was applied on the basic side. Electrophoresis was carried out at 700 V for 120 min and then 1,000 V for 30 min. The pH gradient was measured on pieces of the isoelectric focusing gel that were put into 1 ml of boiled water. The isoelectric focusing gel was then dried on Whatman 1 paper (under vacuum without heating), and the relevant part of the gel was excised. It was equilibrated with SDS sample buffer for 10 min and applied to a 12.5% SDS gel for electrophoresis. The gel was then lightly stained with Amidoschwarz and washed with water, and pieces of gel containing the protein to be analyzed (approximately 3 to 10 μg) were excised. Tryptic digestion in situ, HPLC purification of peptides, and protein microsequencing were then carried out by J. d'Alayer at the Laboratory of Protein Microsequencing (Department of Biotechnology, Institut Pasteur, Paris, France). Briefly, each piece of gel was processed as described previously (37) and then digested in situ in 0.1 M Tris-Cl (pH 8.8)–0.01% Tween 20, with 1 μg of porcine pancreatic trypsin (Sigma), at 30°C overnight. Tryptic peptides were isolated by reverse-phase HPLC (C_{18} Vydac column, 2 by 25 mm), with a 0 to 45% gradient of acetonitrile prepared in 0.1% trifluoroacetic acid. The amino acid sequences of the peptides indicated were then established with an Applied Biosystems sequencer.

^{32}P labelling in vivo and in vitro of the 36-kDa ssDNA-binding protein. HeLa cells (in 25-cm^2 flasks) were infected with vaccinia virus at a multiplicity of infection of 10 PFU per cell. After 1 h of adsorption, the cell monolayer was washed three times with PBS and then incubated with 1.5 ml of radioactive medium (minimum Eagle medium without phosphate salts and supplemented with 34 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-Na [pH 7.5], 34 μg of cytosine arabinoside per ml, 8% dialyzed calf serum, and 0.75 mCi of [^{32}P]orthophosphoric acid without carrier) for 5 h. Mock-infected cells were also labelled as a control. The cells were then detached from the flask with trypsin, extensively washed with PBS, and lysed in 1 ml of DC-50 buffer (0.15 M Tris-Cl [pH 8.7], 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 40 μg of phenylmethylsulfonyl fluoride per ml, 20 μg of leupeptine per ml, 20 μg of pepstatin per ml, 20 μg of aprotinin per ml, 1 mM ATP). The lysate was centrifuged for 10 min at 650 × *g*, and the cytoplasmic extract was applied to an ssDNA-cellulose column (1 ml) equilibrated with DC-50 buffer and washed with 10 ml of DC-50 buffer. The DNA-binding proteins were eluted with the same buffer containing 0.5 M NaCl, concentrated on Centricon 10 filters (Amicon), and heated for 5 min at 85°C to inactivate endogenous protein kinase activity. The labelled proteins were then separated by SDS gel electrophoresis and transferred to a nitrocellulose filter, and the prominent radioactive 36-kDa band was then excised and digested with modified trypsin (Boehringer) as described above. The 36-kDa protein labelled in vitro was prepared in the following way. Approximately 2 μg of 36-kDa protein from the fraction eluted at 0.2 M KCl from casein-agarose was incubated in 32 mM Tris-Cl (pH 7.5)–12 mM $MgCl_2$–8 mM KH_2PO_4–1 mM dithiothreitol–40 μM [γ-^{32}P]ATP (specific activity, 4,500 Ci/mol)–2.2 U of S2/Sa kinase—purified on ATP-agarose (4)—for 1 h at 33°C.

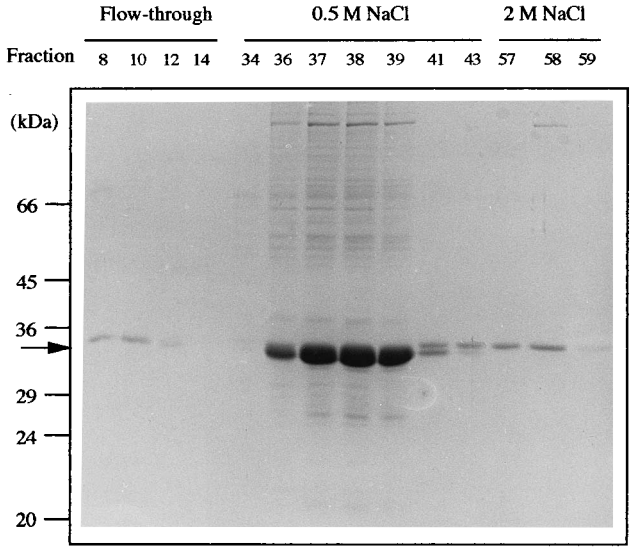

FIG. 1. SDS-PAGE of proteins eluted from ssDNA-cellulose. Approximately 1 mg of protein from the 0.2 M KCl fraction of casein-agarose chromatography (Fig. 2 in reference 4) was applied to a column (1.6 by 11 cm) of ssDNA-cellulose (Sigma). Step elutions were carried out by successively raising the NaCl concentration from 50 mM (sample and wash) to 0.5 M and then 2 M. Aliquots of column fractions indicated were analyzed by SDS-PAGE, and a photograph of the gel stained with Coomassie blue is shown. The arrow indicates the migration of the 36-kDa protein.

RESULTS

The ssDNA-binding protein is the product of gene H5R of vaccinia virus. We previously found that the 36-kDa protein copurified with the S2-Sa kinase activity during DEAE-cellulose and phosphocellulose chromatography of extracts of infected cells. However, casein-agarose chromatography separated the major part of 36-kDa protein, eluted at 0.2 M KCl, from most of the S2-Sa kinase activity, which was eluted at a higher salt concentration (4). The 0.2 M KCl casein-agarose fraction contained ssDNA-binding activity, and we therefore purified the 0.2 M KCl casein-agarose fraction by ssDNA-cellulose chromatography. Consistent with previous work (4), SDS-PAGE analysis of proteins from column fractions confirmed that most of the 36-kDa protein was eluted from ssDNA-cellulose by a 0.5 M NaCl step-elution (Fig. 1) as the major band in a complex mixture of proteins. After incubation with [γ-^{32}P]ATP and purified S2-Sa kinase, the radioactive 36-kDa protein comigrated with the major protein labelled

with Coomassie blue on SDS gel electrophoresis (results not shown). It should be noted that, in recent experiments, the 36-kDa protein often migrated between 34 and 35 kDa, as shown in Fig. 1, but we continue to designate it a 36-kDa protein to distinguish the substrate of the S2-Sa kinase from the previously described proteins Ag35 (35 kDa) and FP11 (34 to 35 kDa) mentioned below. (As will be seen, the molecular weight predicted for the protein from the sequence of its gene is approximately 23,000.) It is interesting that there was also a protein with slightly lower electrophoretic migration than the 36-kDa protein, most of which was eluted at 2 M salt concentration. This was not phosphorylated in vitro by purified S2 kinase (results not shown) and, because of its high affinity for ssDNA, may correspond to the vaccinia virus I3L protein. However, it was not further characterized.

We then used two-dimensional gel electrophoresis to isolate the 36-kDa protein which was previously characterized as an acidic protein with an isoelectric point (pI) of approximately 5 (4), similar to the value of 5.2 to 5.5 reported for the 34- to 35-kDa viral phosphoprotein isolated from the virosomes (28). However, using a pH gradient of 3.5 to 8.5 in the first dimension and SDS gel electrophoresis in the second dimension (30), we observed that the 0.2 M KCl casein-agarose fraction contained several 36-kDa proteins that stained with Coomassie blue, and these had isoelectric points varying from approximately pH 5 to pH 7 (results not shown).

Although we could not exclude the presence of different protein species in these multiple spots, it was more likely that the 36-kDa protein was phosphorylated on several sites and that some of them had been dephosphorylated during the prolonged storage (several months at 0°C) of the preparation of 0.2 M KCl casein-agarose fraction that was used. Moreover, the 36-kDa protein was prepared after infecting HeLa cells in the presence of cytosine arabinoside, and it is possible that the absence of DNA replication resulted in an incomplete phosphorylation of the 36-kDa protein. Indeed, in agreement with the single spot found before (and probably with an isoelectric point of approximately 5.5 instead of 5.0, as shown below), we also found that a single form of the 36-kDa protein was present in cells infected with vaccinia virus (results not shown). We took advantage of this partial dephosphorylation to confirm that the 36-kDa protein contains multiple sites of phosphorylation. To this end, we phosphorylated in vitro the 0.5 M NaCl DNA-cellulose fraction with highly purified S2-Sa kinase and analyzed the ^{32}P-labelled protein by two-dimensional gel electrophoresis, with a pH 5 to 7 gradient to improve the separation between the different forms of the 36-kDa phosphoprotein. The two-dimensional gel shown in Fig. 2 reveals that the ^{32}P label was also present in multiple 36-kDa proteins with approximate isoelectric points of 5.5, 5.7, 5.9, and, to a lesser extent, 6.3, strongly suggesting at least four sites of phosphorylation. These labelled proteins were also detectable by staining with Coomassie blue, as was one with a pI of approximately 6.8 that did not contain detectable associated radioactivity and presumably corresponded to the unphosphorylated form of the 36-kDa protein.

Attempts at Edman degradation of the intact 36-kDa protein were unsuccessful, indicating that the N terminus of the protein is blocked. The three 36-kDa proteins with approximate isoelectric points of 5.7, 5.9, and 6.3 (Fig. 2B) were therefore digested in situ with trypsin as described in Materials and Methods, and the peptides were analyzed by reverse-phase HPLC to determine whether they were in fact modified forms of the same protein. Almost identical A_{214} profiles were obtained for the three proteins (Fig. 3B to D), confirming their interrelationship and strongly suggesting the presence of multiple sites of phosphorylation in the 36-kDa ssDNA-binding protein. The 36-kDa protein was also isolated after SDS gel electrophoresis of the 0.2 M KCl casein-agarose fraction. It was then transferred onto a nitrocellulose filter and digested with modified trypsin as described in Materials and Methods. Examination of the A_{214} profile of the corresponding tryptic peptides separated by reverse-phase HPLC (Fig. 3A) showed that many tryptic peptides eluted from the HPLC column at the same positions as those from the profiles shown in Fig. 3B to D. The different relative amounts of these peptides in Fig. 3A can be ascribed mainly to differences in the trypsin used and in the digestion procedures. This confirmed that the 36-kDa protein substrate was the major component of the less-purified casein-agarose 0.2 M NaCl fraction.

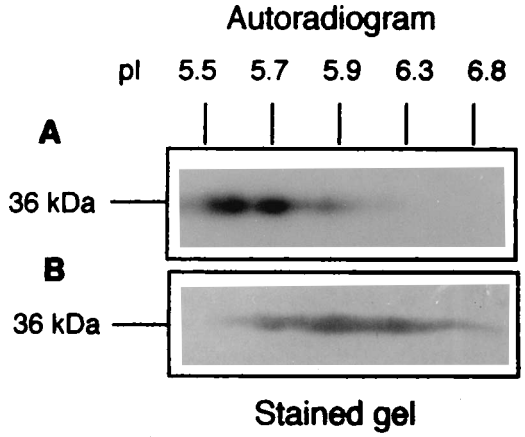

FIG. 2. Two-dimensional analysis of phosphorylated 36-kDa protein. Protein (4.5 μg) eluted at 0.5 M NaCl from ssDNA-cellulose was phosphorylated in vitro in the presence of [γ-^{32}P]ATP as described in Materials and Methods and dialyzed on Millipore filters (VSWP, 0.025-μm pore size). Then, 36 μg of the same protein fraction, not phosphorylated in vitro, was added, and the mixture was analyzed by two-dimensional gel electrophoresis. The gel was stained with Coomassie blue and then dried. The relevant portions of the autoradiogram (A) and stained gel (B) are shown.

Amino acid sequence determination of peptides from peaks present in all the profiles of Fig. 3 revealed four sequences that are represented in the genome of vaccinia virus: ADTSSFTK, ISAVSTV, IITR, and SDLDS. These sequences (preceded by a lysine or an arginine residue) are present in open reading frame (ORF) H5R from the Copenhagen strain of vaccinia virus (14, 18), from the WR strain (designated H6R ORF in reference 36), or from the IHD strain (designated Ag35 protein in reference 15), as is shown in Fig. 4. The three sequences of 203 amino acids predicted from these ORFs are identical except that the glycine residue at position 91 in H5R of the vaccinia virus Copenhagen strain is changed to a serine residue in the other two strains. A homologous gene of 220 amino acids (with 88% identity) is also present in variola virus, strain India-1967 (I5R ORF, reference 39). There was one peak in Fig. 3 (found in the protein separated in one dimension [Fig. 3A], although mainly in the less acidic form of the 36-kDa protein [Fig. 3D] separated in two dimensions) that yielded a sequence, GYADT, not represented as a possible tryptic peptide in H5R or elsewhere in the genome of vaccinia virus. This must have arisen from a cellular contaminant protein.

It was interesting to compare the pI values corresponding to the different phosphorylated forms (6.3, 5.9, 5.7, and 5.5) of the 36-kDa protein with those calculated for phosphorylated H5R protein. Unphosphorylated H5R protein, assuming a neutral N-terminal residue (because we know it is blocked), has a

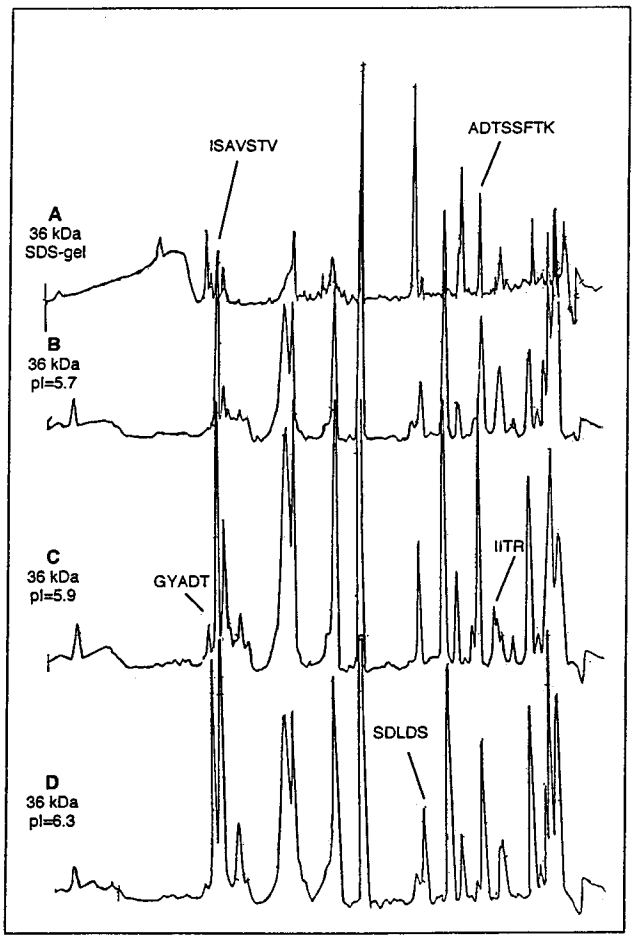

FIG. 3. HPLC profiles of peptides produced after tryptic digestion of the 36-kDa protein isolated by one- or two-dimensional gel electrophoresis. The tryptic peptides were prepared and purified as described in Materials and Methods, and the A_{214} profiles are shown. The 36-kDa protein was isolated by SDS gel electrophoresis (A) or separated by two-dimensional gel electrophoresis into components with approximate pI values of 5.7 (B), 5.9 (C), or 6.3. (D).

calculated pI of 6.8 (11, 12). Furthermore, and assuming pK values of 2 and 6.7 for the two acidic protons of the phosphate esters of amino acids (5), we calculated isoelectric points of 6.2, 5.8, 5.5, 5.3, 5.15, and 5.0 for the H5R protein with a neutral N terminus and phosphorylated at one to six sites, respectively. The first two values coincided with the experimental determinations, and the differences observed for the more acidic forms may result from unpredictable modification of pK values due to clustering of electric charges, for example (9). It should be noted that the addition of more than four phosphates would only slightly decrease the calculated pI value and consequently more than four threonine residues may be phosphorylated in the most acid form of the H5R protein.

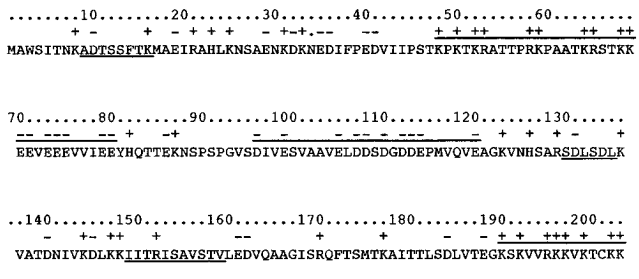

FIG. 4. Amino acid sequence of the vaccinia virus H5R protein. The sequences of the tryptic peptides described in the text are underlined. Regions containing exclusively either acidic (− sign, positions 70 to 80 and 96 to 120) or basic (+ sign, 48 to 69 and 190 to 203) residues are indicated by a line above the sequence.

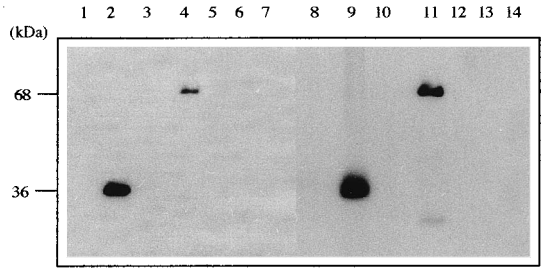

FIG. 5. Phosphorylation of vaccinia virus H5R protein by B1R protein kinase in vitro. Lanes 1 to 7 show autoradiographs of one-dimensional SDS gels from an experiment in which recombinant B1R kinase (5 μl of extract from bacteria transformed with pAB6) was used to phosphorylate either recombinant DNA-binding protein (7 μl of bacterial extract transformed with pATH11-Ag35) or heat-inactivated DNA-binding protein isolated from HeLa cells infected with vaccinia virus (2 μl of material purified to the casein-agarose step), and lanes 8 to 14 show the results of an analogous experiment using S2-Sa kinase isolated from HeLa cells infected with vaccinia virus (2 μl of material purified to the casein-agarose step). Protein substrates were DNA-binding protein from infected cells (lanes 2, 3, 9, and 10), pATH11-Ag35 extract (lanes 4, 5, 11, and 12), and control pATH11 extract (lanes 6, 7, 13, and 14). Enzyme was omitted from tubes 3, 5, 7, 10, 12, and 14. Other details are given in Materials and Methods.

The B1R kinase phosphorylates in vitro the H5R protein (fused to trpE) expressed in *E. coli*. To confirm that the H5R protein is a substrate of the B1R kinase, we expressed in *E. coli* the H5R protein with the first 10 amino acids deleted and fused to the trpE protein, by using cells transformed with plasmid pATH-Ag35 (25a). A 68-kDa soluble fusion protein was expressed in large amounts in cells induced by tryptophan starvation, as judged by an intense band stained with Coomassie blue. It is interesting to note that the fusion protein did not show such an anomalous electrophoretic migration: the trpE-H5R protein would be expected to migrate as a protein of 60 kDa (approximately 37 kDa for trpE protein [21] plus 23 kDa for H5R protein). The trpE-H5R fusion protein was a good substrate for the highly purified S2-Sa kinase isolated from HeLa cells and for the B1R kinase expressed in *E. coli* (Fig. 5). The trpE protein alone did not serve as a substrate for either preparation of the kinase.

The trpE-H5R fusion protein or the 36-kDa protein isolated from the 0.2 M KCl casein-agarose fraction was phosphorylated in vitro with recombinant B1R kinase and transferred to nitrocellulose, and the tryptic peptides of the ^{32}P-labelled proteins were analyzed by two-dimensional separation on thin-layer cellulose plates. The autoradiogram of the fingerprints corresponding to the trpE-H5R recombinant protein revealed the presence of 13 major spots (Fig. 6A) and of 15 spots in that of the 36-kDa protein (Fig. 6B), suggesting multiple phosphorylation sites in vitro. Among them, seven peptides (labelled A to G) did comigrate, as can be seen from careful analysis of the fingerprint corresponding to the simultaneous application of both samples, which contained 21 corresponding labelled tryptic peptides (Fig. 6A+B). There are several possible explanations for the existence of the nonidentical phosphopeptides. Phosphorylation sites present only in the recombinant H5R protein may result from any of the following causes. (i) As shown above (Fig. 2), the 36-kDa protein was only partially

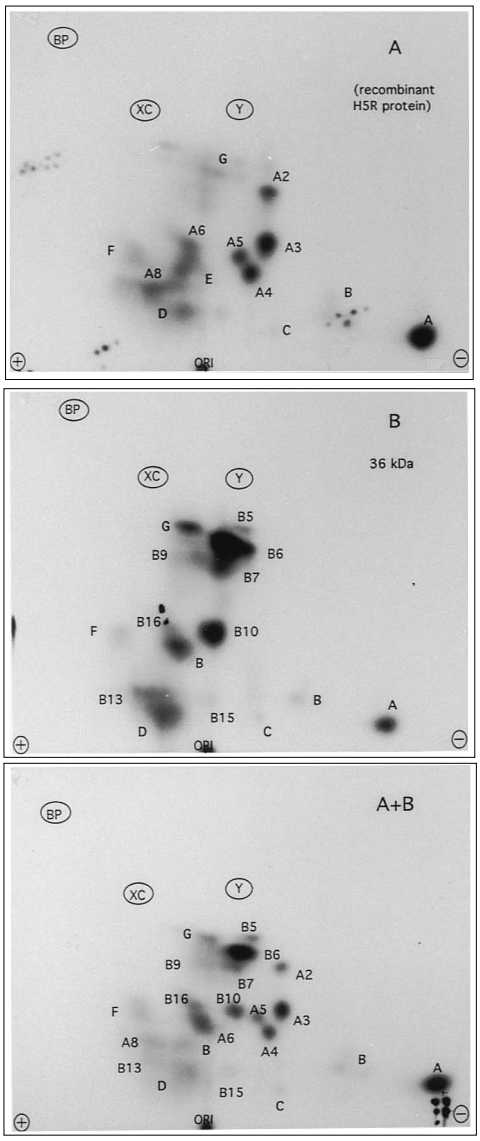

FIG. 6. Tryptic phosphopeptide maps of recombinant vaccinia virus H5R protein (A) and of 36-kDa protein (B), phosphorylated by recombinant B1R protein kinase. Recombinant trpE-H5R fusion protein and 36-kDa protein were phosphorylated with recombinant B1R kinase as described in the legend to Fig. 5, and the corresponding proteins labelled with ^{32}P were isolated by SDS gel electrophoresis and transferred to nitrocellulose membrane. Relevant radioactive bands were then treated with trypsin and processed as described in Materials and Methods. For panel A+B, a mixture of peptides derived from recombinant trpE-H5R fusion protein and 36-kDa protein in vivo and in vitro was analyzed. In the autoradiograms shown, the letters A to G alone designate peptides with identical migrations, whereas those assigned both a letter and a number designate peptides that are unique to either panel A or panel B (the spot marked B and migrating between spots B10 and B16 in panels B and A+B should read E). BP, XC, and Y indicate the migration of bromophenol blue, xylene cyanol (electrical charge = −1), and ε-DNP-lysine (no charge), respectively.

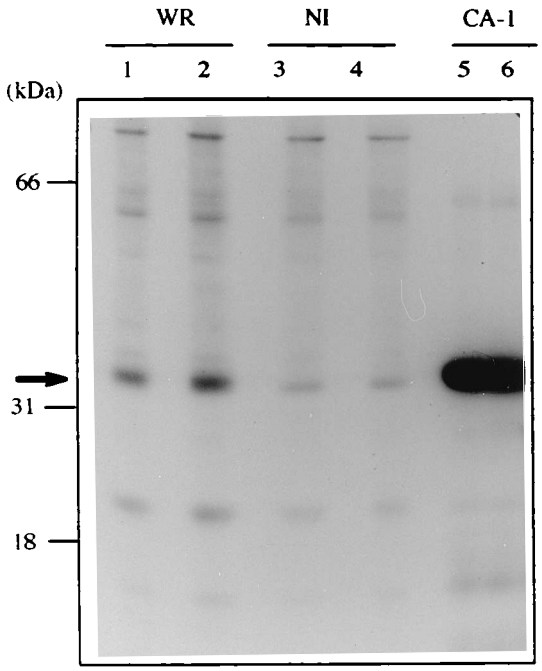

FIG. 7. SDS gel electrophoresis of 36-kDa protein labelled with ^{32}P in vivo or in vitro. The ssDNA-binding proteins labelled in vivo and in vitro with casein agarose fraction CA-1 (4) were prepared as described in Materials and Methods. The autoradiogram of the blot of the gel is shown. The applied samples (analyzed in duplicate) were DNA-binding proteins from cells infected with vaccinia virus (lanes 1 and 2, WR), from control uninfected cells (lanes 3 and 4, NI), or from casein-agarose fraction CA-1 phosphorylated in vitro (lanes 5 and 6, CA-1). The arrow indicates the migration of the 36-kDa protein.

dephosphorylated, and therefore, some sites may not be phosphorylatable in vitro because they are already in a phosphorylated state. (ii) The 36-kDa protein may be associated with cellular proteins masking some phosphorylation sites, whereas such interactions are less likely to occur with the recombinant trpE-H5R protein expressed in *E. coli*. Conversely, the first 10 amino-terminal amino acids from the H5R protein were deleted in the trpE-H5R protein, and phosphorylation sites present in this region of the 36-kDa protein would be absent in the recombinant H5R protein, with corresponding labelled tryptic peptides present only in the digestion product of the 36-kDa protein. (iii) A different conformation of the H5R protein when fused to the trpE protein might mask or create phosphorylation sites in a manner that cannot be predicted.

The finding that about half of the tryptic phosphopeptides were identical in the 36-kDa protein and the recombinant H5R protein labelled in vitro by recombinant B1R kinase strongly suggests that the B1R kinase phosphorylates both proteins at several identical sites. Taken together with the amino acid sequence data presented above, these results prove that the 36-kDa protein substrate of the B1R kinase is encoded by the vaccinia virus H5R gene.

The sites phosphorylated in the 36-kDa protein in vivo and in vitro do overlap. We next investigated whether the phosphorylation occurring in vivo was at sites similar to those phosphorylated by the B1R kinase in vitro by analyzing the corresponding tryptic peptides. The 36-kDa protein labelled with ^{32}P in vivo was purified by ssDNA-cellulose chromatography of a cytoplasmic extract of infected cells incubated with radioactive phosphate, and SDS-PAGE was performed. Figure 7 reveals a much more intense 36-kDa band in labelled DNA-binding proteins from infected cells (lanes 1 and 2) compared with the corresponding fraction of proteins labelled in uninfected cells (lanes 3 and 4). Tryptic peptide maps from the 36-kDa protein labelled in vivo (Fig. 7, lanes 1 and 2) or in vitro (Fig. 7, lanes 5 and 6) were prepared as described in Materials and Methods and are shown in Fig. 8. In both cases, about a dozen tryptic phosphopeptides were separated, suggesting multiple phosphorylation sites. A common subset of

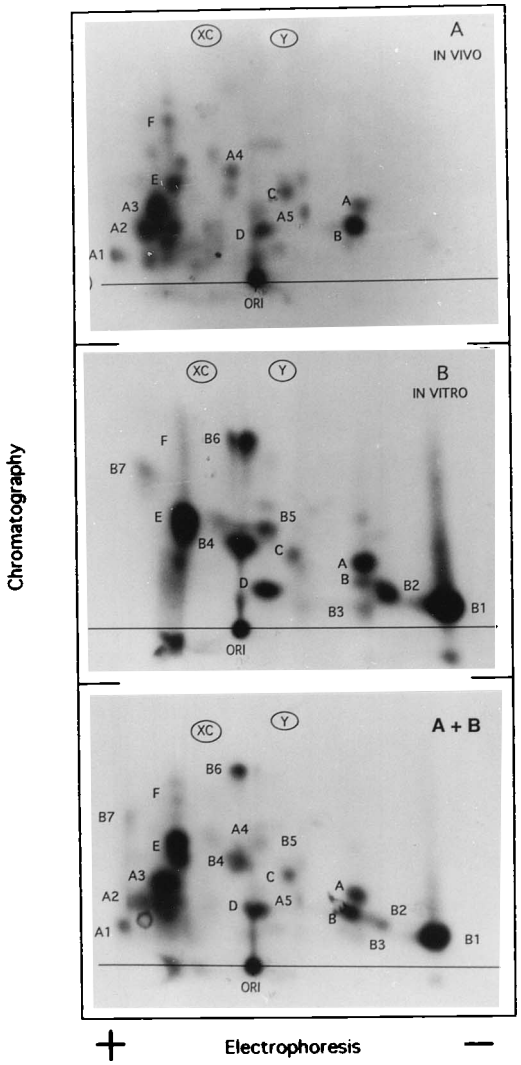

FIG. 8. Tryptic peptide maps of 36-kDa protein labelled with ^{32}P in vivo (A) or in vitro (B). Labelled 36-kDa protein was isolated after SDS gel electrophoresis and digested with trypsin as described in Materials and Methods. For panel A+B, a mixture of peptides derived from the 36-kDa protein labelled with ^{32}P in vivo and in vitro was analyzed. Autoradiograms are shown in which the letters A to F alone designate peptides with identical migration in panels A and B, whereas those assigned both a letter and a number designate peptides that are unique to either panel A or panel B. XC and Y indicate the migration of xylene cyanol and ε-DNP-lysine, respectively.

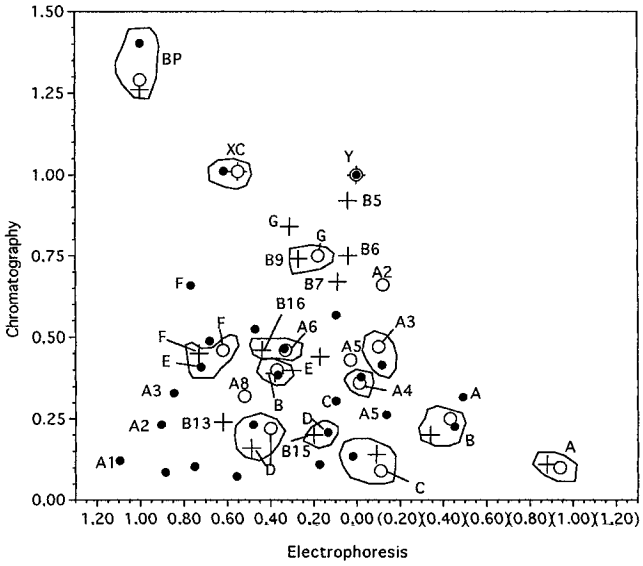

FIG. 9. Schematic representation of the migration of tryptic phosphopeptides derived from trpE-H5R protein (open circles, Fig. 6A) or 36-kDa protein (plus sign, Fig. 6B) phosphorylated by recombinant B1R kinase and from the 36-kDa ssDNA-binding protein labelled in vivo (filled circles, Fig. 8A). The coordinates of major spots from autoradiograms presented in Fig. 6 and 8A were measured relative to the position of ε-DNP-lysine of coordinates (0 and 1), and to that of the abscissa of the bromophenol blue marker as unit value. The phosphopeptides having similar migrations are included in a polygon.

phosphopeptides was observed, designated A to F in Fig. 8, establishing that in vivo the B1R kinase could be responsible for the phosphorylation of several sites on the 36-kDa protein. It was of interest to compare the fingerprint of the 36-kDa protein labelled in vivo (Fig. 8A) with that of the trypE-H5R fusion protein or the 36-kDa protein phosphorylated by recombinant B1R kinase (Fig. 6). To this end, the coordinates of the different spots, relative to the migrations of the bromophenol blue marker (abscissa value equal to +1) and the (uncharged) ε-2,4-dinitrophenol (DNP)–lysine (coordinate values of 1 in the ordinate and 0 in the abscissa because it bears no electrical charge and its migration results only from electroendosmosis), were measured on the autoradiograms and are presented in Fig. 9. It is clear that most of the tryptic peptides from recombinant H5 protein or from 36-kDa protein phosphorylated in vitro by the recombinant B1R kinase can be related to a peptide from the 36-kDa protein labelled in vivo of similar mobility, strongly suggesting phosphorylation at identical sites. However, several peptides phosphorylated in vivo (notably those corresponding to the acidic phosphopeptides A1 to A3 in Fig. 8A) were not significantly phosphorylated in vitro by highly purified S2-B1R kinase (Fig. 8B) or by recombinant B1R kinase (Fig. 6), suggesting that a second protein kinase must also be involved in phosphorylating the 36-kDa protein in vivo.

DISCUSSION

In this paper, we demonstrate that the 36-kDa protein substrate of the B1R protein kinase, which also phosphorylates proteins S2 and Sa from 40S ribosomal subunits (1, 4), is encoded by gene H5R of vaccinia virus. This identification was made by determination of amino acid sequences of tryptic peptides derived from the 36-kDa protein substrate separated by two-dimensional gel electrophoresis. Furthermore, H5R expressed in *E. coli* as a trpE fusion protein was also an efficient substrate for the B1R kinase either expressed in *E. coli* or purified from extracts of HeLa cells infected with vaccinia virus. This new substrate of the B1R kinase is an early protein because it was synthesized in cells infected with vaccinia virus in the presence of cytosine arabinoside, and this is consistent with the presence of an early RNA start site at about 76 nucleotides upstream from the H5R ORF (designated H6R by Rosel et al. [36]). Although the molecular weight predicted for the H5R protein is 22,270, it was previously shown that the product of the translation of H5R mRNA in vitro migrates in SDS gels as a 40-kDa protein (36) and that the H5R protein synthesized in *E. coli* migrates as a protein of 35 kDa (15). Our identification of the 36-kDa protein as the product of gene H5R is, therefore, consistent with the anomalous migration that others have found. Gordon et al. (16) suggested that the

anomalous migration of H5R protein may result from the presence of a relatively proline-rich region in the amino acid sequence (five residues present between positions 45 and 116), but there are several regions with either acidic or basic residues (Fig. 4) which may also contribute to the anomalous migration.

As described in the Introduction, our previous evidence argues strongly that the 36-kDa protein is identical to FP11 (28, 29), which is a major phosphoprotein isolated from the virosomes, which are rapidly sedimenting cytoplasmic protein-DNA complexes containing newly replicated DNA. Furthermore, an antibody prepared against the H5R protein strongly labelled virus factories seen by immunoelectron microscopy of thin sections of cells infected with vaccinia virus (9a). We isolated the 36-kDa protein as a soluble protein present in cytoplasmic extracts from HeLa cells infected in the presence of cytosine arabinoside, presumably because the virosomes could not be formed. FP11 protein appears to be similar to polypeptide B (32, 33, 38) or VDP12 (40). It is clearly useful to be able to assign genes to vaccinia virus proteins that had been studied before the DNA sequence of the virus became available, and from our present results, we now argue that the FP11 protein (polypeptide B) is the product of gene H5R. In this respect, it should be noted that polypeptide B has been shown to be an early protein but continues to be synthesized at the late stage (32, 33, 38), which is in agreement with the presence of a late RNA start site about 35 nucleotides downstream from the early promoter of the H5R gene (36). As already mentioned, however, it has also been suggested that the FP11 phosphoprotein is equivalent to a 34-kDa DNA-binding phosphoprotein which is the product of vaccinia virus gene I3L (10). That assignment was based heavily upon the assumption that there is a single vaccinia virus DNA-binding phosphoprotein migrating at about 34 kDa. Although the I3L protein appears to be phosphorylated in cells infected with vaccinia virus, the recombinant protein is not a substrate for the B1R kinase in vitro and I3L is phosphorylated in vivo on serine residues, in contrast with FP11, which is phosphorylated on threonine residues (35a). It is therefore clear that distinct phosphoproteins with DNA-binding properties are encoded by genes I3L and H5R. However, the I3L protein binds much more strongly to ssDNA-cellulose than FP11 and the H5R–36-kDa protein (it was eluted at 0.6 to 2 M NaCl), and this and their other common physical characteristics suggest that it is the H5R protein which is equivalent to FP11.

It was previously shown that the H5R protein corresponds to a prominent antigen, designated Ag35 protein, of vaccinia virus envelope (15, 16, 26). It was not determined whether the Ag35 protein from the virus membrane is phosphorylated, but an acidic 35-kDa phosphoprotein was present among ^{32}P-labelled vaccinia virus proteins separated by two-dimensional electrophoresis, although it is difficult to judge whether it was a bona fide component of the virion (31). It is interesting that these authors found a similar 35-kDa protein to be immunoprecipitated by an anti-vaccinia virus serum prepared against live virus, suggesting that it is highly immunogenic, as is the case for the Ag35 envelope protein (43). Recently, the H5R protein was expressed in insect cells by a baculovirus recombinant, and dissemination of Ag35 protein throughout the cell was observed. It is of note that higher concentrations of Ag35 were found on the cell surface, the nuclear perimeter, and the intranuclear virogenic stroma, revealing a dual targeting of the H5R protein towards membranes and DNA-protein foci of virus replication (26).

As we found the 36-kDa–H5R protein as a soluble component of cytoplasmic extracts prepared by mechanical disruption of infected cells, it is likely (at least at the early stage of the infection) that the association of the H5R protein with the nascent internal viral membrane is relatively weak and that most of it was solubilized, with only a minor fraction remaining associated with membranes. However, it is also possible that it did not derive from the viral membrane, as Mohandas et al. (26) showed that the H5R protein expressed by a baculovirus vector is also targeted towards intranuclear DNA-protein foci, in addition to the viral lipoprotein membranes. It is thus tempting to speculate that the H5R phosphoprotein is also involved in virus assembly, perhaps by allowing virus DNA incorporation into virus particles.

Although the 36-kDa–H5R protein does bind to ssDNA, it is not known at present whether it possesses an intrinsic ssDNA-binding activity or whether it binds to ssDNA by association with another protein which possesses an intrinsic ssDNA-binding activity. It is possible that the H5R protein might associate with vaccinia virus I3L protein, which binds strongly to ssDNA, and perhaps other proteins that remain to be characterized. In favor of this hypothesis, we observe that the 36-kDa–H5R protein was released from the ssDNA-cellulose column at a relatively low salt concentration and together with several proteins (Fig. 1) (4), suggesting that protein subunit dissociation occurs at 0.2 to 0.5 M NaCl, a salt concentration that would cause the H5R protein to elute from the DNA-cellulose column but leave most of the I3L protein still bound to ssDNA. Furthermore, most of the 36-kDa phosphoprotein migrates in glycerol gradients together with several proteins (results not shown). The amino acid sequences of H5R proteins contain clusters of either basic or acid amino acid residues (Fig. 4) that may promote protein-protein interactions and are also present in the 32-kDa subunit of RPA, the cellular ssDNA-binding protein (13).

The other question addressed in this paper is whether the B1R kinase mediates the phosphorylation of the H5R protein during infection, given that both proteins are expressed at the early stage of infection. We showed that several tryptic phosphopeptides derived from the 36-kDa protein phosphorylated in vitro by highly purified S2-Sa (B1R) kinase and in vivo with radioactive phosphate were identical. As anticipated, many of them had migration similar to that of those corresponding to H5R-trpE fusion protein or to 36-kDa protein phosphorylated by the recombinant B1R kinase. Several acidic tryptic peptides were uniquely present in the 36-kDa protein labelled in vivo, suggesting that other sites on the H5R protein must be phosphorylated by a different protein kinase, presumably of cellular origin.

In conclusion, we have demonstrated that the H5R protein is a new substrate for the B1R kinase and provided evidence that the protein is also phosphorylated in vivo. It remains to be established whether this phosphorylation plays a role in vaccinia virus replication, given that *ts* mutants of the B1R kinase fail to replicate viral DNA at the nonpermissive temperature (34, 35). The determination of the residues modified by the B1R kinase and their subsequent site-directed mutagenesis hold the prospect of answering this question.

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