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Phosphorylation of ribosomal proteins by the vaccinia virus B1R protein kinase

Alison H. Banham[a,*], David P. Leader[b], and Geoffrey L. Smith[a]

[a]*Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK* and [b]*Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, UK*

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Two proteins of the 40S ribosomal subunit were shown to be phosphorylated in vitro by a vaccinia virus-encoded serine/threonine protein kinase. These were identified by two-dimensional gel electrophoresis as ribosomal proteins Sa and S2 and were shown by phosphoamino acid analysis to both be phosphorylated on serine and threonine residues. The three phosphorylated forms of S2 produced by the B1R protein kinase in vitro matched the phosphorylated forms of S2 previously observed in cells infected with vaccinia virus. These data strongly suggest that this enzyme is responsible for the phosphorylation of S2 and Sa which occurs early during vaccinia virus infection

Vaccinia virus; Protein kinase; Phosphorylation; Ribosomal protein S2; Ribosomal protein Sa

1. INTRODUCTION

The protein encoded by vaccinia virus open reading frame (ORF) B1R is a serine/threonine protein kinase that is expressed early during infection and is packaged into virions [1–3]. A role for the B1R protein kinase in virus DNA replication has been proposed since two temperature-sensitive mutants, *ts*2 and *ts*25, which have a DNA negative phenotype at the non-permissive temperature, map to the B1R gene [4,5]. Infection of L cells with these *ts* mutants at the non-permissive temperature arrests prior to DNA replication and when cells infected at the permissive temperature are shifted to the non-permissive temperature in the midst of DNA replication, DNA synthesis ceases [3,5]. Thus the B1 protein is required directly or indirectly throughout DNA replication. Studies with other protein kinases have shown that these enzymes frequently have more than one substrate and biological role in vivo, so that although the B1R protein kinase plays an essential role in DNA replication, other functions are possible.

The activity of the B1R protein kinase has been demonstrated in vitro with artificial substrates such as casein and histones but not with proteins that are phosphorylated during virus infection. These potential physiological substrates include both viral and cellular proteins [6,7]. Previous studies have demonstrated the phosphorylation of 40S ribosomal proteins Sa, S2 and S13 in cells early after infection with vaccinia virus and for S2 and S13 the phosphorylation occurs on serine and threonine residues [8–10]. Virus infections in the presence of cycloheximide and cordycepin and the in vitro phosphorylation of ribosomes with protein kinase(s) derived from viral cores, have suggested a viral origin for the protein kinase responsible for the phosphorylation of S2 and S13 [9,10]. Furthermore, protein kinase activities capable of phosphorylating S2 and S13 in vitro have been partially purified from the cytoplasm of infected cells [11]. The function of these ribosomal protein phosphorylations is unknown, but their temporal association with the switch to translation of viral mRNA suggests that they could have a role in this switch. The rat S2 ribosomal protein is encoded by a highly conserved repetitive gene family LLRep3 and is related to *S. cerevisiae* S4 and prokaryotic S5 ribosomal proteins [12]. These latter ribosomal proteins are involved in the binding of aminoacyl-tRNA to ribosomes and in conditioning the fidelity of translation and S2 may serve a similar function in mammalian ribosomes [12]. Thus modification of translation seems a likely potential function of the vaccinia virus-induced ribosomal protein phosphorylation.

In this study we have investigated whether the B1R protein kinase is able to phosphorylate ribosomal proteins Sa, S2 or S13 in vitro and consequently might be the enzyme responsible for the ribosomal protein phosphorylations observed early during vaccinia virus infection.

Correspondence address: G.L. Smith, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK. Fax: (44) (865) 275 501.

**Present address:* Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK.

2. MATERIALS AND METHODS

2.1. *Preparation of 40S ribosomal subunits*

Ribosomes were isolated from rat liver, treated with 0.1 mM puro-

mycin for 15 min at 37°C and dissociated into subunits by centrifugation through a gradient of 10–30% sucrose in 10 mM Tris-HCl (pH 7.6), 0.5 M KCl, 5 mM $MgCl_2$ and 20 mM 2-mercaptoethanol at 27 000 rpm for 4 h at 28°C in a Beckman SW28 rotor [13]. Samples were aliquoted and stored at −70°C.

2.2. *Preparation of crude* E.coli *extracts containing the B1R protein kinase*

E coli harboring plasmid pAB6, which contains the B1R ORF under control of the bacteriophage T7 promoter [1], were grown in the presence of 0.2% maltose to an OD_{550} of 0.5. The cells were then infected with phage λ CE6 [14] at 10 plaque forming units (pfu) per cell to induce B1R expression and were left unshaken for 20 min at room temperature for the phage to adsorb before being shaken at 37°C for 2 h. Cells were harvested by centrifugation, washed in ice-cold PBS, resuspended in ice-cold extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM DTT, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 23 mg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml chymostatin) and lysed by 2 × 2 s bursts from an ultrasonic disintegrator (MSE 100W) Cell debris was removed by centrifugation at 13 000 rpm in a microfuge for 5 min at 4°C and the protein concentration of extracts was determined [15].

2.3. *In vitro ribosomal protein phosphorylation*

Ribosomal 40S subunits (0.05 A_{260} units/reaction) were phosphorylated with 5 μg of the bacterial extract containing B1R in a 10 μl assay volume comprising, 50 mM Tris pH 7, 5 mM $MgCl_2$, 5 mM DTT, 1 μCi [γ-^{32}P]ATP and 5 μM ATP The reaction was stopped after 15 min at 37°C by the addition of 10 μl of 2 × protein sample buffer (125 mM Tris-HCl pH 6.8, 4% sodium dodecylsulfate (SDS), 40% glycerol, 1 M 2-mercaptoethanol, 0.002% Bromophenol blue). Samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) [16] and autoradiography of the dried gel.

2.4. *Phosphoamino acid analysis*

Ribosomal proteins of the 40S subunit phosphorylated by bacterially expressed B1R were resolved by SDS-PAGE on a 12% gel and electrophoretically transferred to an Immobilon-P blotting membrane The ^{32}P-labeled bands were excised and briefly rinsed with distilled water before hydrolysis in 5 7 M HCl for 1 h at 110°C. The supernatants were lyophilized, resuspended in pH 1.9 buffer (50: 156: 1794, 88% formic acid, glacial acetic acid: H_2O) and phosphoamino acids were resolved by one-dimensional electrophoresis on a thin layer cellulose plate at pH 3.5 [17, 18] and detected by autoradiography Unlabeled phosphoamino acid standards were located by ninhydrin staining

2 5. *Two-dimensional analysis of phosphorylated ribosomal proteins*

Basic ribosomal proteins were resolved by PAGE first on a 15% gel at pH 8.7 (separating mainly on the basis of charge) and second on a 15% gel at pH 4 5 (separating mainly on the basis of size) [19] Acidic ribosomal proteins were resolved by isoelectric-focusing in a polyacrylamide gel containing ampholines in the pH range 3 5 9.0 followed by SDS-PAGE in the second dimension [20].

3. RESULTS

To determine whether the vaccinia virus B1R protein kinase expressed in *E. coli* was able to phosphorylate ribosomal proteins of the 40S subunit, bacterial extracts were incubated with 40S ribosomal subunits in the presence of [γ-^{32}P]ATP. Fig. 1 shows that two ribosomal proteins are specifically phosphorylated by *E. coli* extracts containing the B1R protein kinase (lane 2), but not by control extracts lacking this enzyme (lane 1). The sizes of these two proteins correspond well with the sizes

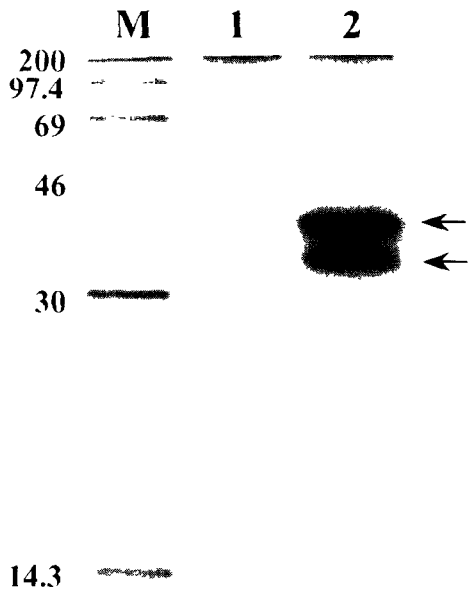

Fig. 1 Ribosomal protein phosphorylation by the vaccinia virus B1R protein kinase 40S ribosomal subunits were incubated with extracts from *E. coli* cells expressing B1R (lane 2) or control cells (lane 1). Phosphorylated proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography. The positions of migration of ^{14}C-labeled protein standards are shown (lane M) in kDa.

of ribosomal proteins Sa and S2 which are 43 kDa and 33 kDa, respectively. There was no ^{32}P-labeled protein of 17 kDa corresponding to ribosomal protein S13 which is phosphorylated during vaccinia virus infection.

The identities of the phosphorylated ribosomal proteins were examined by two-dimensional gel electrophoresis (Figs. 2 and 3). In each case panel A shows the proteins stained by Coomassie brilliant blue and panel B is an autoradiograph of the gel in panel A. Fig. 2 shows the analysis of basic ribosomal proteins. Although the majority of ribosomal protein S2 (which is predominantly unlabelled carrier) migrates as a single form (panel A) there are three radioactive spots which correspond to phosphorylated derivatives of ribosomal protein S2 of increasing acidity (panel B). These forms of S2 are very similar to the three major phosphorylated species of S2 found within vaccinia-infected cells [10]. There was no detectable phosphorylation of ribosomal protein S13 (Fig. 2) consistent with the results of one-dimensional electrophoresis (Fig. 1). Ribosomal protein Sa is acidic [21,22] and consequently was not observed using this gel electrophoresis system. However, it was observed under conditions previously used to detect Sa in virus-infected cells [10] although due to the low pH of electrophoresis in the first dimension the protein barely entered the gel (data not shown). Therefore, the number of phosphorylated derivatives of Sa were assessed by isoelectric focusing [20]. An M_r 43 K protein

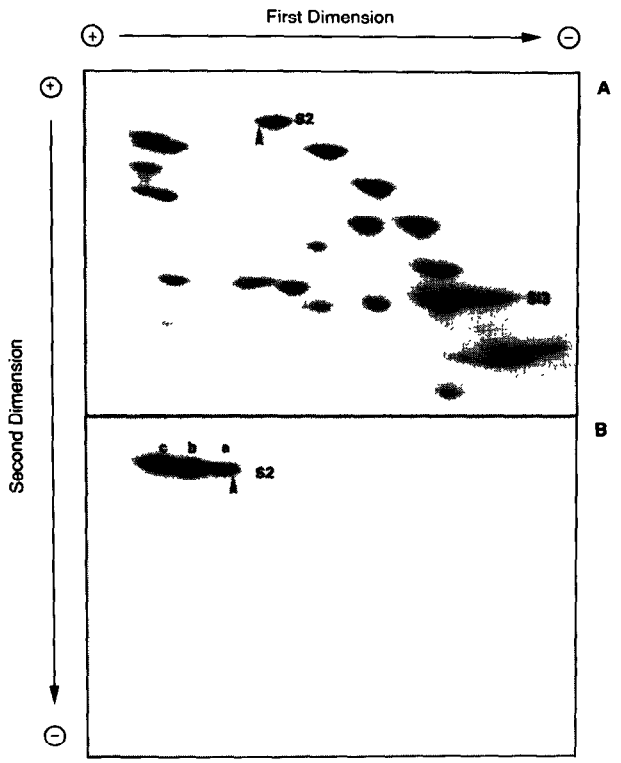

Fig. 2. Characterization of basic ribosomal proteins phosphorylated by the vaccinia virus B1R protein kinase. 40S ribosomal subunits were incubated with extracts of *E coli* expressing B1R (section 2), carrier 40S ribosomal subunits were added (10 A_{260} units) and the subunits sedimented through a cushion of 0.5 M sucrose at 50 000 rpm for 4 h at 4°C in a Beckman Ti70 rotor. Protein was extracted [13] and subjected to two-dimensional gel electrophoresis [19] Panel A. Gel stained with Coomassie brilliant blue Panel B Autoradiograph. The arrows indicate corresponding positions in the two frames.

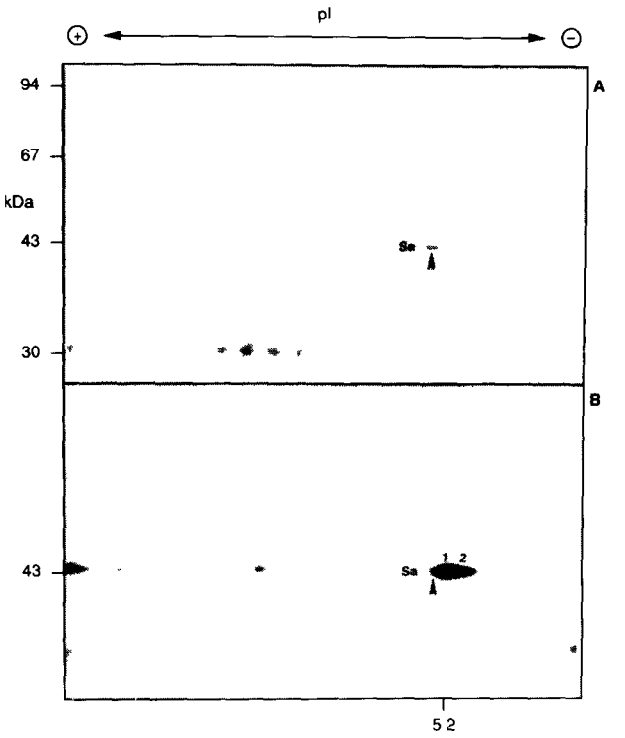

Fig. 3. Characterization of acidic ribosomal proteins phosphorylated by the vaccinia virus B1R protein kinase. 40S ribosomal subunits were treated as described in Fig. 2, but subjected to two-dimensional analysis (section 2) [20]. Panel A. Photograph of gel stained with Coomassie brilliant blue. Panel B Autoradiograph. The arrows indicate corresponding positions in the two frames. The M_r values are from molecular weight standards present in the second dimension and the isoelectric points were determined by measuring the pH of slices of a parallel first-dimension gel after these had been equilibrated with boiled distilled water.

with an isoelectric point of approximately 5.0 was visualized by staining with Coomassie blue (Fig. 3A) and one major and one minor phosphorylated derivative migrated with slightly higher isoelectric points (Fig. 3B).

Next the amino acid residues which were phosphorylated on ribosomal proteins Sa and S2 by the B1R protein kinase were determined. These two ribosomal proteins were resolved by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane, located by autoradiography, excised and acid hydrolysed. Fig. 4 shows the resultant phosphorylated amino acids which were resolved by high voltage electrophoresis and identified using unlabeled standards located by ninhydrin staining. The vaccinia virus protein kinase clearly phosphorylated both ribosomal proteins Sa and S2 on serine and threonine residues. Ribosomal protein Sa (lane 1) was phosphorylated equally on serine and threonine residues whilst ribosomal protein S2 (lane 3) was phosphorylated predominantly on threonine residues.

4. DISCUSSION

Three ribosomal proteins of the 40S subunit, Sa, S2 and S13, are specifically phosphorylated early during infection with vaccinia virus [8–10]. Although protein kinase activities capable of phosphorylating ribosomal proteins S2 and S13 in vitro have been partially purified from vaccinia virus infected cells [11], the origin and identity of these enzymes is currently unknown. Here the vaccinia virus B1R protein kinase is shown to phosphorylate ribosomal proteins Sa and S2, but not S13, on serine and threonine residues in vitro.

Phosphorylation of ribosome protein S2 occurs early during vaccinia virus infection in the presence of cytosine arabinoside, an inhibitor of virus DNA replication [9]. This protein is initially phosphorylated on a serine residue within 10 min of infection and this event is independent of continuing transcription and protein synthesis, suggesting that a virion-associated protein kinase is responsible [9,10]. By 1 h post-infection the S2 protein is further phosphorylated on three threonine

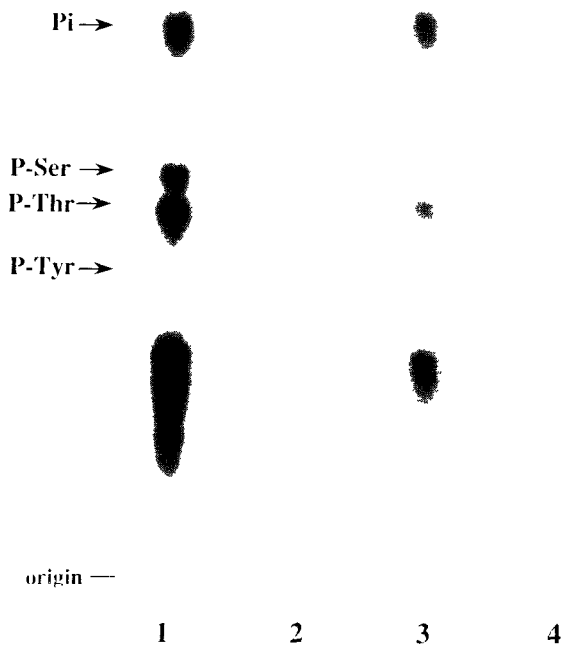

Fig. 4. Phosphoamino acid analysis of phosphorylated ribosomal proteins Sa and S2. Extracts from *E. coli* cells expressing B1R (lanes 1 and 3) or from control cells (lanes 2 and 4) were used to phosphorylate 40S ribosomal proteins in vitro. Phosphoamino acids were analysed as described in section 2 for both ribosomal proteins Sa (lanes 1 and 2) and S2 (lanes 3 and 4). The positions of phosphoamino acid standards are indicated by arrows, phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr).

residues and this event is dependent upon early virus protein synthesis since it is inhibited by both cordycepin and cyclohexamide [9,10]. Data presented here suggest that the product of the B1R gene can perform these phosphorylations. Furthermore, the presence of the B1R protein kinase within virus particles and its expression early during infection [1–3] are consistent with such activity. Surprisingly, although B1R can phosphorylate S2 on both serine and threonine residues in vitro, protein kinase activity from vaccinia virus cores only phosphorylates S2 on serine residues. This might be due to the kinase activity from virus cores being mostly attributable to proteins other than B1R which may be serine-specific [2]. Other possibilities are that B1R might only phosphorylate threonine residues after it has been modified or the substrate might be inaccessible to the virion-associated enzyme.

Little is known of the phosphorylation of the acidic ribosomal protein Sa which occurs early during infection [10]. In the in vitro assay described here Sa is more highly phosphorylated than S2 by B1R and the phosphorylation of Sa occurs with equal intensity on both serine and threonine residues. The S2 kinase purified from infected cell extracts also phosphorylates Sa in vitro [26].

The B1R protein kinase did not phosphorylate ribosomal protein S13 under the conditions of this investigation. In vivo S13 phosphorylation occurs on a serine residue 1 to 2.5 hours post-infection during viral DNA synthesis [10] suggesting that the S13 protein kinase is not packaged in the virion and is, or requires, a virus-encoded early or intermediate protein. It is unlikely that there is another vaccinia-encoded protein kinase responsible for the phosphorylation of S13 in vivo because B1R is the only ORF in the vaccinia virus genome (strain Copenhagen) that is likely to encode a protein with protein kinase activity [23]. Although there is another vaccinia ORF designated B12R, that is related to B1R and to some other protein kinases, the gene product was considered unlikely to have protein kinase activity due to the poor conservation of several important motifs [24]. Moreover, the B12R protein expressed in *E. coli* does not have protein kinase activity under conditions in which the vaccinia B1R protein kinase and a serine protein kinase from African swine fever virus are active [1,25] (A.H. Banham and G.L. Smith unpublished data). Another possibility is that the multiple threonine phosphorylations of S2 are a prerequisite to S13 phosphorylation perhaps by the S2 kinase [10]. Certainly this is not the case in vitro as the B1R kinase phosphorylates S2 on multiple threonine residues without any subsequent phosphorylation of S13. It is very probable that different protein kinases are responsible for phosphorylating ribosomal proteins S2 and S13 because the S2 protein kinase activity purified from infected cells (which also phosphorylates three sites on the ribosomal protein S2) has been separated from S13 kinase activity, although it still retains the ability to phosphorylate ribosomal protein Sa [26].

This in vitro study shows that the vaccinia virus-encoded B1R protein kinase is able to reproduce the phosphorylated forms of ribosomal proteins Sa and S2 observed in vaccinia virus-infected cells. This is the first identification of potential in vivo substrates for this protein kinase. It is noteworthy that although certain ribosomal proteins (e.g. ribosomal protein S6) are substrates for several protein kinases in vitro, ribosomal protein S2 has not been reported to be phosphorylated by any cellular kinases including the cyclic AMP-dependent protein kinase, protein kinase C or casein kinases I and II [13,27].

A possible function for the phosphorylation of ribosomal proteins early during vaccinia infection is to enhance selective translation of virus mRNAs. The mechanism by which vaccinia virus shuts off host protein synthesis is unclear, but it does not involve alteration of the eIF-4 cap-binding complex [28,29] and cap-dependent translation is not inhibited by mechanisms similar to those used by adenovirus and picornviruses (reviewed by [30]). Poxvirus mRNAs synthesized late during infection have an unusual 5′ poly(A) sequence of up to 35 nucleotides [31–34] and similarly, some early virus mRNAs also have a 5′ oligo(A) sequence downstream

of the cap structure [35,36]. Perhaps ribosomal phosphorylation is a novel mechanism for the selective translation of such virus mRNAs.

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