Ribosomal protein S2/Sa kinase purified from HeLa cells infected with vaccinia virus corresponds to the B1R protein kinase and phosphorylates in vitro the viral ssDNA-binding protein

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A ribosomal protein S2 kinase was purified 6000-fold from cytoplasmic extracts of HeLa cells infected with vaccinia virus, using 80S ribosomes or 40S ribosomal subunits as a substrate. Although the preparation was not homogeneous, a 34K component was identified, the chromatographic behaviour of which correlated with enzyme activity. During its purification the ribosomal protein S2 kinase was resolved from a less abundant ribosomal protein S13 kinase, demonstrating the two to be different entities. A second protein kinase activity against a 43K ribosomal protein comigrated with the ribosomal protein S2 kinase activity during all five chromatographic procedures employed, and we conclude that the two activities are properties of a single species. Two-dimensional gel electrophoresis demonstrated that this second substrate was the acidic ribosomal protein Sa, of isoelectric point approximately 5.2, previously shown to be phosphorylated during infection with vaccinia virus. Another substrate for the ribosomal protein S2/Sa kinase in vitro was the 36K viral ssDNA-binding protein, of isoelectric point approximately 5.0, which is also known to be phosphorylated in vivo. The 34K protein correlating with the catalytic activity in the most purified preparations of the ribosomal protein S2/Sa kinase was recognized by an antibody specific for a protein expressed in Escherichia coli from vaccinia virus gene B1R. This and other evidence suggest strongly that the ribosomal protein S2/Sa kinase is the product of this gene.

Introduction

It is now established that the employment of protein phosphorylation as a regulatory mechanism extends to the viruses of eukaryotic cells, particularly to large DNA viruses such as herpesviruses and poxviruses (Leader & Katan, 1988; Leader, 1993). When cells are infected with vaccinia virus, S2 and S13 ribosomal proteins which are not phosphorylated in uninfected cells become phosphorylated (Kaerlein & Horak, 1976, 1978). It was shown that an acidic ribosomal protein, Sa, is also specifically phosphorylated in vaccinia virus-infected cells (Buendia et al., 1987). The phosphorylation of ribosomal protein S2 occurs early in infection and involves one seryl residue and two or three threonyl residues; that of ribosomal protein S13 lags behind by about an hour, and involves a single seryl residue (Kaerlein & Horak, 1978; Buendia et al., 1987). The unphosphorylated form of ribosomal protein S2 has completely disappeared 1 h after infecting Ehrlich ascites tumour cells with vaccinia virus, strain Copenhagen (Buendia et al., 1987). The roles of these specific and efficient phosphorylations are unknown, but the phosphorylation of ribosomal protein S2, although not that of ribosomal protein S13, correlates temporally with the shut-off of host protein synthesis and it was proposed that it may release the translational block induced by the basic 11K protein, a major component of vaccinia virus (Buendia et al., 1987).

Viral proteins are also phosphorylated during the early stage of the infection. Notable among these is an abundant 34K to 35K phosphoprotein found in the sites of viral DNA replication, and is the single protein from the viral factories found to be phosphorylated (Nowakowski et al., 1978a, b). It is an acidic protein with an isoelectric point of 5.2 to 5.5, with multiple phosphorylation sites on threonine residues (Nowakowski et al., 1978a). This phosphoprotein binds ssDNA cellulose and is eluted with 0.25 M-NaCl. Recently, it has been reported that it is encoded by the gene I3 of vaccinia virus (Davis & Mathews, 1993; S.C. Rochester & P. Traktman, personal communication).
We have previously identified protein kinase activities in extracts of HeLa cells infected with vaccinia virus. These are able to catalyse the multiple phosphorylation in vitro of ribosomal protein S2 on seryl and threonyl residues, and the phosphorylation of a single seryl residue on ribosomal protein S13 (Beaud et al., 1989). The activities, which were partially resolved by chromatography on DEAE–cellulose, were candidates for the enzyme(s) that catalyses the ribosomal protein phosphorylations that had been observed in vivo. Since that initial work, a vaccinia virus gene, B1R, has been shown to encode a protein kinase (Howard & Smith, 1989; Traktman et al., 1989; Banham & Smith, 1992; Lin et al., 1992; Rempel & Traktman, 1992) so that it was also important to determine the relationship between this enzyme and those we had isolated from infected cells. Here we report further purification of the latter enzymes which has allowed us to address these questions. We show that the ribosomal protein S2 and S13 kinases are separate entities, and that the ribosomal protein S2 kinase can also catalyse the phosphorylation of ribosomal protein Sa and the viral 36K ssDNA-binding protein. We present strong evidence that the ribosomal protein S2 kinase synthesized in infected HeLa cells is equivalent to the protein kinase encoded by vaccinia virus gene B1R.

**Methods**

**Cells and virus.** HeLa S-3 cells were maintained in suspension culture in minimum Eagle’s medium containing 5% horse serum. Vaccinia virus, strain WR, was titrated on monolayers of HeLa cells. HeLa S-3 cells and the WR strain of vaccinia virus were obtained from B. Moss.

**Assay of protein kinase activity.** The standard assay mixture for ribosomal protein kinase activity contained, in a total volume of 40 μl, 20 mM-Tris-HCl pH 7.5, 10 mM-MgCl₂, 1 mM-DTT, 50 μM-ATP containing 1 μCi [γ-32P]ATP and 1 A₄₅₀ unit of washed 80S ribosomes from uninfected cells or, where indicated, 0.3 A₄₅₀ unit of rat liver 40S ribosomal subunits. Incubations were at 30 °C for 30 min. For assay of casein, histone or protamine kinase activities, 40 μg of the particular protein was substituted for ribosomes. One unit of protein kinase activity is that amount of enzyme which catalyses the incorporation of 1 pmol phosphate into protein under standard assay conditions.

**Gel electrophoretic analysis of phosphorylated ribosomal proteins.** In our initial studies we established the identity of ribosomal proteins using standard two-dimensional PAGE (O’Farrell, 1975). As two-dimensional PAGE is not suitable for analysis of multiple column fractions, a method was developed using the first dimension of that system (Madjar et al., 1979a) which, being performed at pH 8.3 (in 8 M-urea), eliminates proteins that are not chemically basic. The details of this method (referred to as urea–PAGE) are as follows.

The 40 μl reaction mixture was cooled on ice and to it was added 137 μl of a mixture of 0.1 volume of 1 M-magnesium acetate and 1 volume of acetic acid. Carrier ribosomes (1 A₄₅₀ unit, 5 μl) were then added and the ribosomal RNA was allowed to precipitate for at least 2 h. This was then subjected to centrifugation at 12000 r.p.m. for 15 min, the supernatants containing the extracted protein were collected, and 800 μl of 5% TCA was added. The protein was precipitated for at least 2 h, subjected to centrifugation, and the sedimented protein washed with 350 μl of acidified acetone (1 volume of acetic acid, 4 volumes of H₂O, 25 volumes of acetone), dried in a desiccator, and dissolved in 40 μl sample buffer (20 mM-Tris–boric acid pH 8.3, 2 mM-EDTA, 40 mM-DTT, 8 M-urea). Polyacrylamide slab gels (7%) were prepared in 0.2 M-Tris–boric acid pH 8.6, 8 M-urea and 10 mM-EDTA (lower electrode buffer: 36 mM-Tris–boric acid pH 8.6, 3 mM-EDTA). The samples were layered under the upper electrode buffer (60 mM-Tris–boric acid pH 8.3, 6 mM-EDTA) and electrophoresis was carried out at 7 V/cm for 16 h. The gel was stained with Coomassie blue, dried on Whatman 3MM paper and exposed to X-ray film. The bands corresponding to ribosomal proteins S2 and S13 were excised and their radioactivity was measured by Cerenkov counting or using a Molecular Dynamics PhosphorImager. This laborious method allowed us to measure S2 and S13 ribosomal protein kinase activities unambiguously during the first chromatographic steps. We then used SDS–PAGE to measure incorporation of the 32P label into ribosomal proteins S2 (36K), S13 (17K) and Sa (43K). Incorporation of radioactivity into ribosomal proteins S2 and S13 was also found to be approximately linear during the 30 min incubation period (data not shown). In certain instances two-dimensional gel electrophoresis was used to characterize individual acidic phosphoproteins. The systems used employed SDS–PAGE in the second dimension and, in the first dimension, either electrophoresis in 4% acrylamide at pH 5.5 (Madjar et al., 1979a) or isoelectric focusing in a gel containing ampholines in the pH range 3.5 to 9.5 (O’Farrell, 1975).

**Preparation of post-ribosomal supernatant.** HeLa cells (3 × 10⁵ cells from a 6 l culture) were infected with vaccinia virus at 7 p.f.u./cell (1 h adsorption period at not less than 10⁵ cells/ml). After 4 h of infection (or overnight incubation in the presence of 50 μg/ml cytosine arabinoside) the cells were centrifuged and washed in PBS. All subsequent operations were carried out at 0 °C or in the cold room. One volume of hypotonic buffer (10 mM-Tris–HCl pH 7.5, 10 mM-KCl, 1.5 mM-magnesium acetate, 1 mM-EGTA) containing 40 μg/ml PMSF, 20 μg/ml each of aprotinin, pepstatin A and leupeptin, was added to the packed cells which were allowed to swell at 0 °C for 5 min. The cells were broken with 20 strokes of a Dounce homogenizer and concentrated isotonic buffer was added to give a final concentration of 25 mM-Tris–HCl pH 7.5, 125 mM-KCl, 5 mM-magnesium acetate and 1 mM-DTT. The lysate was subjected to centrifugation for 30 min at 10000 r.p.m., and after discarding the surface lipid, the post-mitochondrial supernatant was removed and subjected to centrifugation for 90 to 105 min at 50000 r.p.m. in a Beckman 50Ti rotor. This sedimented the ribosomes, and the resulting post-ribosomal supernatant was then dialysed against DE buffer (20 mM-Tris–HCl pH 7.5, 1 mM-EDTA, 1 mM-EGTA, 10 mM-2-mercaptoethanol, 10% glycerol) and stored in liquid nitrogen.

**Preparation of ribosomes for use as substrate.** Ribosomes were prepared from uninfected HeLa cells as described above for infected cells and the ribosomes suspended in a buffer of high ionic strength (30 mM-Tris–HCl pH 7.5, 0.5 M-KCl, 5 mM-MgCl₂, 2 mM-DTT) and maintained for 30 min at 0 °C. They were then layered over a cushion (7 ml) of 1 M-sucrose in the high ionic strength buffer and subjected to centrifugation for 17 h at 35000 r.p.m. in a Beckman 50Ti rotor. The washed ribosomes were rinsed and then suspended in TM buffer (20 mM-Tris–HCl pH 7.5, 5 mM-magnesium acetate, 1 mM-DTT) and stored at −70 °C. These were used as the substrate for the ribosomal protein kinases except where it is indicated that rat liver 40S ribosomal subunits (Parkar et al., 1985) were employed.

**Chromatographic purification of ribosomal protein kinases.** All buffers used for chromatography or dialysis were supplemented with 10 mM-2-mercaptoethanol and 40 μg/ml PMSF.
(i) **Step 1: DEAE-cellulose chromatography.** Dialysed post-ribosomal supernatant from 121 of infected cells was thawed and applied to Whatman DE-52 DEAE-cellulose pre-equilibrated with DE buffer and packed in a 1 x 8 cm column. The column was washed with 110 ml of DE buffer and protein eluted with a 360 ml linear gradient of 0 to 0.35 M-KCl in the same buffer at a flow rate of 36 ml/h. The peak of ribosomal protein S2 kinase activity was eluted at 0.5 M-KCl, and fractions 29 to 37, between 25 and 80 mM (Fig. 1), were pooled and dialysed against DE buffer.

(ii) **Step 2: phosphocellulose chromatography.** The pooled DEAE-cellulose fractions were applied to phosphocellulose (Bio-Rad Cellex P) pre-equilibrated with DE buffer and packed in a 1 x 5.8 cm column. The column was washed with 65 ml of 0.1 M-KCl in DE buffer and then eluted with a 80 ml linear gradient of 0.1 to 0.7 M-KCl in the same buffer at a flow rate of 20 ml/h. The peak of ribosomal protein S2 kinase activity was usually eluted at 0.3 M-KCl and fractions 37 to 41, between 0.3 and 0.41 M (Fig. 1), were pooled and dialysed against DC buffer (150 mM-Tris-HCl pH 8.7, 50 mM-NaCl, 1 mM-EDTA).

(iii) **Step 3: DNA-cellulose chromatography.** This was performed according to the procedure of Nowakowski et al. (1978). A 1.6 x 5 cm column was packed with DNA-cellulose (Sigma; 6.2 mg of ssDNA per ml of column), and BSA (Pentex; 10 mg in DC buffer) was first passed through the column. The pooled phosphocellulose fractions were then applied to the DNA-cellulose column, which was washed with DC buffer. The proteins retained were then eluted at a flow rate of 12 ml/h with either a 100 ml linear gradient of 0.05 to 1.0 M-NaCl in DC buffer (Fig. 1) or, more usually, in a single step of 0.5 M-NaCl. The fractions (1.2 ml) containing the bound ribosomal protein kinase activity were pooled and dialysed against CA buffer (20 mM-Tris-HCl pH 7.5, 50 mM-KCl, 1 mM-EGTA, 1 mM-EDTA, 1 mM-EGTA, 0.1% NP40, 10% glycerol).

(iv) **Step 4: casein-agarose chromatography.** The pooled dialysed DNA-cellulose fractions were then applied to a column of casein-agarose (Sigma; 8 mg a-casein per ml gel), packed in a 1.6 x 8 cm column and equilibrated with CA buffer. The column was then washed with 15 ml of CA buffer and eluted with a 50 ml linear gradient of 0.05 to 0.8 M-KCl in the same buffer at a flow rate of 15 ml/h, and 1 ml fractions were collected. The fractions containing most of the S2 kinase activity but not the S13 kinase were pooled (0.4 M-KCl, Fig. 4a) and dialysed against AA buffer (40 mM-Tris-Cl pH 7.5, 50 mM-KCl, 1 mM-EGTA, 1 mM-EGTA, 0.1% NP40, 10% glycerol).

(v) **Step 5: ATP-agarose chromatography.** The casein-agarose fractions were applied to a 1 x 2.6 cm column of ATP-agarose (Sigma; spacer 11 atoms and 3 gmol ATP per ml gel) which was equilibrated with AA buffer containing 10 mM-magnesium acetate. The column was then washed with 15 ml of AA buffer containing 1 mM-magnesium acetate and eluted with a 25 ml linear gradient of 0 to 50 mM-EDTA in AA buffer at a flow rate of 7.5 ml/h, and 0.75 ml fractions were collected. A step elution of 10 mM-ATP with 10 mM-magnesium acetate in AA buffer was then carried out to release the ATP-binding proteins not eluted with EDTA.

**Immunoblotting.** After SDS-PAGE, the proteins from the gel were transferred onto a membrane (Schleicher & Schüll BA85 0.45 μm or Pharmacia Immobilon P) using an ANSCO 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 filter was then blocked for 1 h at room temperature in 20 ml TBS (50 mM-Tris-Cl pH 7.5, 0.2 M-NaCl) containing 5% non-fat dried milk and 0.05% Tween 20. It was then incubated overnight in a cold room with the anti-BIR serum (Rempel & Traktman, 1992; Banham & Smith, 1992), diluted 100-fold in TBS containing 5% non-fat dried milk. The filter was then washed three times with TBS containing 1% non-fat dried milk and incubated with a goat anti-rabbit IgG (H, L) coupled to horseradish peroxidase (Caltag) for 1 h at room temperature. After several washes in TBS buffer, the BIR protein present on the filter was revealed with 4-chloro-1-naphthol.

**DNA-binding protein assay.** Aliquots of the chromatographic fractions (3 μl) were spotted on an Immobilon P (Pharmacia) membrane equilibrated with SSC buffer (15 mM-sodium citrate, 0.15 M-NaCl, 1 mM-EDTA). 32P-labelled DNA, a kind gift from A. Domi, was prepared by incubating T4 polynucleotide kinase, [γ-32P]ATP and dephosphorylated bacteriophage λ DNA digested with BstEII. The unbound ATP was removed by dialysis on a Millipore filter (VMVP 0.05 μm) and the labelled DNA was then denatured by boiling for 10 min, followed by rapid cooling in ice water. The filter with bound proteins was then incubated for 30 min at room temperature with 2 ml of SSC containing 104 to 106 c.p.m. of the 32P-labelled ssDNA and 0.5 mM-ATP, to prevent incorporation of any residual ATP. After washing five times with SSC, the filter was dried and the radioactivity present in each spot was measured using the PhosphoImager.

**Results**

**Chromatographic purification of the ribosomal protein S2 and S13 kinase activities**

The chromatographic separations used in the purification of the ribosomal protein S2 and S13 kinases from HeLa cells infected with vaccinia virus are shown in Fig. 1. The DEAE–cellulose step (Fig. 1a) separates the ribosomal kinases from casein kinase II (the second peak of casein kinase), but not from casein kinase I (the first peak) or protein kinase C (Beaud et al., 1989). The latter was removed by the succeeding phosphocellulose step, which partly separated the ribosomal protein kinases from the casein kinase I and a large portion of the protein (Fig. 1b). The third step, DNA-cellulose, was originally introduced in an attempt to separate the protein kinases from a comigrating protein, thought to be the vaccinia virus ssDNA-binding protein (see below). Although it failed to achieve this separation and only produced a small increase in the overall purification, it was convenient to include it as it removed the remaining casein kinase I (Fig. 1c). There is residual casein kinase activity associated with the ribosomal S2 kinase (Fig. 1c), but this activity is distinct from casein kinase I (which phosphorylates both α- and β-casein to similar extents) as it had a marked preference for α-casein over β-casein (not shown, but cf. Fig. 2a). The ribosomal protein S2 kinase not retained on this column probably resulted from slight overloading.

Both the ribosomal protein S2 and S13 kinase activities copurified up to the DNA–cellulose chromatographic step, for which reason only the more abundant ribosomal protein S2 kinase activity has been presented in Fig. 1(a, c). However, significant resolution of the ribosomal protein S2 and S13 kinase activities was obtained by casein–agarose chromatography (Fig. 2a), and this also separated the ribosomal protein S13 kinase and part of the ribosomal protein S2 kinase from the casein kinase.
Fig. 1. Purification of ribosomal protein kinases by column chromatography. Post-ribosomal supernatant from infected cells was purified by chromatography as described in Methods. The columns shown are (a) DEAE-cellulose, (b) phosphocellulose and (c) DNA-cellulose. $2$ and S13 kinase activities were analysed using urea-PAGE; casein kinase activity was measured with a mixture of $\alpha$- and $\beta$-caseins and analysed on filters or by SDS-PAGE.

Fig. 2. (a) Casein-agarose chromatography of ribosomal protein kinases. Ribosomal kinases were purified from $1.3 \times 10^9$ HeLa cells infected overnight with vaccinia virus in the presence of cytosine arabinoside, as described in Methods, but step 3 (DNA-cellulose) was omitted and the fractions from the phosphocellulose chromatography containing the $S2$ kinase activity were then subjected to casein-agarose chromatography. The $0.2 \text{ M-KCl}$ and $0.4 \text{ M-KCl}$ fractions correspond to pooled fractions 32 to 38 and 39 to 53, respectively. (b) ATP-agarose chromatography of ribosomal protein kinases. The $0.4 \text{ M-KCl}$ fraction from the casein-agarose column was applied to a DNA-cellulose column and the proteins eluted at $0.5 \text{ M-NaCl}$ were deposited on an ATP-agarose column that was processed as described in Methods. Protein kinase assays were carried out with 40S subunits as ribosome substrate and $S2$, S13, $\alpha$- and $\beta$-casein kinase activities were analysed using SDS-PAGE. The $10 \text{ mm-magnesium acetate}$, $20 \text{ mm-EDTA}$ and $40 \text{ mm-EDTA}$ fractions correspond to pooled fractions 8 to 24, 42 to 58 and 59 to 66, respectively.

Kinase activity eluted from the casein-agarose column in a manner identical to the $S2$ kinase activity (Fig. 2a).

Fractions from the casein-agarose column were subjected to ATP-agarose chromatography. Protein kinases would be expected to bind to the column only in the presence of magnesium ions, and therefore should be specifically released by EDTA. The elution profiles from Fig. 2(b) showed that the majority of ribosomal protein $S2$ kinase activity was retained on the ATP-agarose column, and that it was eluted by an EDTA gradient. Once again, ribosomal $S4/43K$ protein kinase activity co-eluted with the ribosomal protein $S2$ kinase activity. SDS-PAGE analysis of the fractions from the ATP-agarose column (Fig. 3) revealed that a considerable
Vaccinia virus protein kinase

Fig. 3. SDS-PAGE analysis of proteins separated by ATP-agarose chromatography. The numbered fractions from Fig. 2(b) (90 µl) were applied to the gel, subjected to electrophoresis, and then stained with silver nitrate. A photograph is shown. Fraction 86 is from the final 10 mM-ATP step elution. The right lane contained 100 ng of the indicated markers (from Bio-Rad). The amount of ribosomal protein S2 kinase activity corresponding to each fraction is indicated below the fraction number. The stained bands corresponding to 60K and 65K are artefacts due to the high sensitivity of the staining procedure used, because they are present when no sample has been applied.

purification of the S2 kinase had been achieved as only a few proteins were released by EDTA. Although the presence of three artefactual bands (between 53K and 64K) in all lanes somewhat obscured the analysis, it was clear that one protein of Mr 34000 appeared to correlate with the peak of ribosomal protein S2 kinase activity (Fig. 3, fractions 49 to 57). As expected, most of the 36K protein was not retained on ATP-agarose (Fig. 3, fractions 10 to 20), although that which did bind was also released by EDTA (Fig. 3, fractions 45 to 53). Finally, it should be noted that an entirely different set of proteins was released by a further step elution with ATP (Fig. 3, fraction 86), and none of the ribosomal protein kinases activities released by EDTA could be detected in the corresponding pooled and concentrated fractions (data not shown).

At the ATP-agarose stage, the yield of S2 kinase activity was approximately 10% of that in the DEAE-cellulose fraction, with a specific activity of 23 nmol per mg protein and corresponding to about 6000-fold purification. It is thus clear that only low amounts of S2 kinase are synthesized in HeLa cells infected with vaccinia virus.

Characterization of the ribosomal protein S2 kinase as the product of the vaccinia virus B1R gene

As mentioned above, the peak of ribosomal protein S2 kinase activity eluted from ATP-agarose coincided with a 34K protein (Fig. 3). To investigate this correlation further, we used SDS-PAGE to analyse the protein composition of different fractions from the ATP-agarose column. In each case we used a volume of the different fractions that contained the same amount of S2 kinase activity, so that a protein band corresponding to the S2 kinase activity would exhibit a constant staining with silver nitrate whereas that of unrelated proteins would be expected to vary. Fig. 4(a) shows that several bands (migrating as 64K, 56K, 53K and 34K proteins) were present with similar intensity in each of the three fractions containing equal amounts of ribosomal protein S2 kinase activity (lanes 1 to 3). However, the three upper bands are artefacts usually seen after silver nitrate staining because they are present when sample buffer without protein is applied to the gel and thus they are also present in the samples that do not contain S2 kinase activity (lanes 4 and 5, Fig. 4a). Therefore, these artefactual bands may obscure the presence of protein bands with identical migration from the S2 kinase. However, it is clear that the only detectable protein band with similar intensity in lanes 1 to 3 from Fig. 4(a) was a 34K protein, strongly suggesting that it is the ribosomal protein S2 kinase. Furthermore, if we assume that about 25 ng of the 34K protein (the same amounts of markers were applied to lane 5) corresponds to the 5 units of S2 kinase present in each fraction shown in Fig. 4(a), lanes 1 to 3, we can estimate that the specific activity of the ribosomal protein S2 kinase is approximately 200 pmol/min per mg protein. This is in the range expected for pure p70 or p90 ribosomal protein S6 kinases (Erikson &
Characteristics of ribosomal protein S2 kinase

(i) Sedimentation coefficient of ribosomal kinases
When analysis of the size of the ribosomal protein kinases was attempted using a Superose-6 FPLC column, complete loss of activity was observed. Therefore casein–agarose fractions corresponding to the 0.2 M-KCl and 0.4 M-KCl fractions from Fig. 2(a) were not investigated by gelatin gradient centrifugation (data not shown). The ribosomal protein S2 and S13 kinase activities in the pooled 0.2 M-KCl region had similar sedimentation coefficients of approximately 5 S and 4 S, respectively. However the ribosomal protein S2 kinase from the pooled 0.4 M-KCl region of the casein–agarose column sedimented as two discrete peaks, one sedimenting roughly as before at approximately 5 S, and one at approximately 2.4 S. The 2.4 S form of the ribosomal protein S2 kinase corresponds to a monomeric form of the B1R protein (34 K) and the 5 S form to a homomeric or heteromeric protein. The protein composition of silver nitrate-stained SDS gels from fractions of the glycerol gradients revealed that the abundant 36 K protein also sedimented as a 5 S and a lighter 2 S to 3 S form (data not shown), suggesting that it may aggregate. The cosedimentation of S2 ribosomal kinase and 36 K protein is in agreement with a probable association between these proteins, as already suggested by co-elution after successive chromatographic analyses.

(ii) Ionic conditions of S2 kinase activity
The optimum conditions for assaying the ribosomal protein S2 kinase were investigated. Fig. 5(a) shows that increasing concentrations of KCl gradually inhibited the ribosomal protein S2 kinase, although the enzyme still retains about half of its activity at an ionic strength at which ribosomes are active in protein synthesis. The KCl dependence for the S13 kinase appeared qualitatively similar, although the lower activity of this did not permit quantification. The magnesium optimum was between 5 and 15 mM (Fig. 5b). The activity of the ribosomal protein S2 kinase declined as the pH was increased above pH 8.0, but about 50% activity still remained at pH 9.5 (Fig. 5c).

(iii) $K_m$ values for ATP and 40 S ribosomal subunits
Some enzymatic kinetic characteristics of the purified ATP–agarose fraction were investigated. Measurements of the activities of the ribosomal protein S2 and S13...
kinases as a function of ATP concentration (results not shown) revealed identical $K_m$ values for ATP (10 $\mu$M) with either ribosomal protein S2 or Sa as co-substrate, consistent with our conclusion that these activities are properties of the same enzyme. As regards ribosomal substrates, it is implicit in the fact that 40S ribosomal subunits (Fig. 2), as well as 80S ribosomes, were used to assay the ribosomal protein kinases that both these serve as substrates. No additional proteins were phosphorylated when 40S ribosomal subunits were used as substrate for ribosomal kinase from the DNA–cellulose step, although there was an approximately twofold increase in the phosphorylation of ribosomal protein S13 relative to ribosomal protein S2, suggesting that ribosomal protein S13 may be slightly masked in the 80S ribosome (not shown). The S2 and Sa kinase activities were also measured as a function of the concentration of 40S subunits as substrate (results not shown). The S2 and Sa kinase activities levelled off at a concentration of between 100 and 600 nM. Although accurate values could not be obtained from these data, they allow the $K_m$ to be placed in the range of 0.1 to 0.3 $\mu$M, significantly lower than that of the ribosomal S6 kinases (about 5 $\mu$M; Erikson & Maller, 1986; Jenö et al., 1989). A possible reason for the higher affinity of vaccinia virus S2 kinase for 40S ribosomes might be its specific localization in the cytoplasmic viral factories (Banham & Smith, 1992), where ribosomes are expected to be present in relatively low concentrations.

**Other substrates of purified ribosomal protein S2 kinase**

(i) **Protein Sa from 40S ribosomal subunits**

Throughout the purification, a protein kinase activity that strongly phosphorylated a second ribosomal protein of 43K comigrated with the ribosomal kinases. This activity was eluted on casein–agarose chromatography and ATP–agarose chromatography in a similar manner to the ribosomal protein S2 kinase, and differently from the ribosomal protein S13 kinase (Fig. 2a). Furthermore, the S2 and 43K kinase activities were thermally inactivated at 40 °C with the same kinetics (data not shown). We conclude, therefore, that the ribosomal protein S2 kinase and the 43K protein kinase activities are properties of the same enzyme. We had, in fact, detected the phosphorylation of this 43K protein during earlier studies with less purified enzyme preparations and 80S ribosomes (see Fig. 3 of Beaud et al., 1989), but as it was an acidic protein it was not visualized in the system of two-dimensional gel electrophoresis used to analyse the basic ribosomal proteins S2 and S13. We now address the question of its identity.

The 43K phosphoprotein coincided with a stained band on SDS–PAGE of highly purified 40S subunits, and there is only a single ribosomal protein, Sa, of this $M_r$ (Collatz et al., 1977; Madjar et al., 1979b). Sa is an acidic ribosomal protein and is phosphorylated during infection with vaccinia virus in vivo (Buendia et al., 1987). The acidic nature of the 43K phosphoprotein was consistent with its being Sa, and the fact that it was phosphorylated to at least the same extent as ribosomal protein S2 suggested it was present in a stoichiometrically comparable amount, as would be expected if it were a ribosomal protein. Equal phosphorylation of Sa and S2...
Fig. 6. Two-dimensional analysis of phosphorylated ribosomal protein Sa. 40S ribosomal subunits were phosphorylated using S2 kinase from fractions 47 to 55 of the casein-agarose column of Fig. 2(a), and the proteins extracted were subjected to two-dimensional gel electrophoresis by the method of O'Farrell (1975). A portion of the stained gel (a) and an autoradiograph (b) are illustrated. The Mr values are from Mr 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. Only the relevant portions of the gels are shown.

had not been apparent in vivo when analysed by a two-dimensional system with an acidic first dimension (Buendia et al., 1987), but this can be explained by a failure of much of the protein to enter the second dimension, as found in experiments (not shown) in which the 43K phosphoprotein was analysed in this system. Better analysis of the protein was obtained using the two-dimensional system of O'Farrell (1975), with an isoelectric focusing gradient in the first dimension. Fig. 6 shows a radioactively labelled major protein of 43K with an isoelectric point of about 5.2. Only a single phosphorylated species is apparent in the figure, although over exposure revealed two additional minor species (not shown). Phospho-amino acid analysis indicated that Sa was phosphorylated on both threonine and serine residues, with phosphothreonine being much the more predominant (results not shown).

The two-dimensional system used here to analyse ribosomal protein Sa has the advantage that it clearly identifies the protein on the basis of size, isoelectric point and relationship to a group of proteins of similar low Mr but different charges. This has allowed unequivocal identification of Sa as the 43K ribosomal protein phosphorylated by the product of vaccinia virus gene B1R, expressed in E. coli (Banham et al., 1993). The latter work showed by two-dimensional electrophoresis of basic proteins that ribosomal protein S2 was the other ribosomal protein phosphorylated by the viral gene product, providing further support for our conclusion that it is equivalent to the enzyme that we have described here.

(ii) Vaccinia virus 36K ssDNA-binding protein

It appeared that there was a third substrate for the ribosomal protein S2 kinase. This is a dominant 36K viral protein that was visible in the stained gel of fractions from casein–agarose chromatography, which was the subject of endogenous phosphorylation [corresponding to peak fractions 34 to 35 of Fig. 2(b), data not shown]. It was not clear which of the protein kinases is responsible for phosphorylating the 36K protein. As none of the latter was purified free of the ribosomal protein kinases an experiment was performed in which
Fig. 8. Two-dimensional analysis of phosphorylated 36K DNA-binding protein. Proteins were phosphorylated as described in Methods and subjected to two-dimensional gel electrophoresis by the method of O’Farrell (1975). The DNA-binding protein in fractions 33 to 37 of the casein-agarose column of Fig. 2(a) was phosphorylated in vitro by endogenous enzyme and then processed as described in Fig. 7. The relevant portions of the stained gel (a) and autoradiograph (b) are shown.

the protein kinases in a fraction (fraction 33, Fig. 2a) containing the protein were denatured by heating at 85 °C for 5 min and then combined with ribosomal protein S2 kinase from a fraction (fraction 51, Fig. 2a) lacking the 36K protein, and free of both the ribosomal protein S13 and α-casein kinases. It can be seen from Fig. 7 that the 36K protein was phosphorylated, indicating that this protein is a substrate for the ribosomal protein S2 kinase. Furthermore, the ribosomal protein S2 kinase and the 36K protein kinase were thermally inactivated with the same half-life, arguing strongly that they are the same enzyme (data not shown). As expected, the 36K protein kinase activity comigrated with ribosomal protein S2/Sa kinase activity on the ATP-agarose column (Fig. 2b).

The stained gels from casein-agarose fractions (not shown) indicated that the 36K protein correlated with a ssDNA-binding activity, measured by a dot blot assay (Fig. 2a). These observations suggested that the 36K protein was identical to the 34K to 35K ssDNA-binding phosphoprotein previously described by Nowakowski et al. (1978a) and designated FP11. Other properties were also shared by both proteins: they are early proteins, they are eluted at a similar salt concentration (0.25 M-NaCl) from DNA-cellulose columns because the 36K protein co-eluted with the S2 kinase (see Fig. 1c), and they are relatively abundant proteins. Because Nowakowski et al. (1978a) showed that FP11 is an acidic protein, we also performed two-dimensional gel electrophoresis (O’Farrell, 1975) of the phosphorylated 36K protein. Fig. 8(a) shows the major stained 36K protein of isoelectric point approximately 5.0 and there is also a minor 36K species, not shown, of pI between 5.8 and 6.0. The isoelectric point of 5.0 observed here is slightly lower than the 5.2 to 5.5 reported previously for FP11 but, given the different experimental conditions employed, we regard this as being sufficiently similar to corroborate the identification. It can be seen that the stained 36K protein species resolved by isoelectric focusing was radioactively labelled, a single spot being observed on autoradiography (Fig. 8a). Altogether, these results strongly suggest that the vaccinia virus ssDNA-binding protein is a third substrate for the S2/Sa kinase.

Discussion

We have achieved about 6000-fold purification of the ribosomal protein S2 kinase activity from cytoplasmic extracts of HeLa cells infected with vaccinia virus. Although not complete, the considerable degree of purification of this enzyme has allowed us to obtain strong evidence that it corresponds to the product of vaccinia virus gene B1R: ribosomal protein S2 kinase activity correlates with the presence of a 34K protein (Fig. 3 and 4a) and concentrated fractions of ribosomal protein S2 kinase contain a 34K protein recognized by antibodies specific for the 34K B1R protein (Fig. 4b). The conclusion that the ribosomal protein S2 kinase purified from infected cells is the product of gene B1R is supported by separate parallel experiments showing that the B1R kinase expressed in E. coli can phosphorylate the ribosomal proteins S2 and Sa (but not ribosomal protein S13) on the 40S ribosomal subunits in vitro (Banham et al., 1993). Such a ribosomal substrate specificity has not, to our knowledge, been described for any other cellular or viral protein kinase. Furthermore, the two enzymes share a third physiological substrate, the viral 36K DNA-binding protein, as the protein kinase expressed from vaccinia virus gene B1R also catalyses the phosphorylation of this protein (A. Banham & D. P. Leader, unpublished). Taken together, the immunochemical evidence and substrate characterization argue overwhelmingly that the two enzymes are equivalent.

Although the product of vaccinia virus gene B1R had previously been shown to have protein kinase activity against artificial substrates such as casein (Banham & Smith, 1992; Lin et al., 1992; Rempel & Traktman, 1992), no potential physiological substrate had been
identified. Our work with the authentic enzyme from infected HeLa cells has shown that casein is, in fact, an indifferent substrate for the protein kinase compared to ribosomes and the viral DNA-binding protein. It is of interest to compare the ribosomal protein S2/Sa kinase with an activity isolated from vaccinia virions by Kleiman & Moss (1975), which phosphorylated certain virion proteins in vitro. That enzyme exhibited a weak casein kinase activity that was greatly stimulated by protamine. However, the weak casein kinase activity of the ribosomal protein S2/Sa kinase was not stimulated by protamine (results not shown), and is, thus, unlikely to be equivalent to the enzyme described by Kleiman & Moss (1975).

The clear resolution by casein-agarose chromatography of the ribosomal protein S2 kinase from the ribosomal protein S13 kinase shows that the two kinases are distinct molecular entities and is consistent with the fact that the phosphorylation of S13 occurs later than that of S2 in vivo (Kaarlein & Horak, 1978; Buendia et al., 1987). The suggestion (Kaarlein & Horak, 1978) that this time lag might reflect a requirement for the phosphorylation of ribosomal protein S2 before ribosomal protein S13 can be phosphorylated is unsupported by the similar rates of phosphorylation that were observed in vitro (not shown). Furthermore, the observation that the S13 kinase activity in vitro was lower than S2 kinase activity by about 10-fold suggests that the phosphorylation of S13 in vivo may be a slow process, which could account for the apparent time lag between S2 and S13 phosphorylations. The origin of the ribosomal protein S13 kinase remains an enigma. One possibility is that it is a viral gene which is either unrelated to the major eukaryotic cellular protein kinase family or so far diverged that it has not been recognized from the complex sequence of the genome of vaccinia virus. That some ribosomal protein S2 kinase activity remains associated with the ribosomal protein S13 kinase activity during casein–agarose chromatography suggests an alternative possibility: ribosomal protein S13 kinase activity may be conferred on ribosomal protein S2 kinase through an association with another protein.

We have shown that the purified ribosomal protein S2 kinase can also phosphorylate another potential physiological substrate, an abundant 36K protein thought to be the ssDNA-binding phosphoprotein (Nowakowski et al., 1978a, b). This protein copurifies with the kinase during much of the separation and although an artefactual association cannot be excluded, the ribosomal protein S2 kinase may bind to the 36K protein because the latter is a protein substrate for the enzyme. It is known that the B1R protein is localized in cytoplasmic virus factories where viral replication occurs (Banham & Smith, 1992), and it is tempting to speculate that the binding of the ribosomal protein S2 kinase to the 36K protein may have the physiological function of directing the enzyme to this location. The viral ssDNA-binding phosphoprotein does appear to have the ability to associate with different proteins, as it was recently shown that it interacts with the vaccinia virus ribonucleotide reductase (Davis & Mathews, 1993). The finding that the ssDNA-binding phosphoprotein is most probably a substrate for the ribosomal protein S2/Sa kinase is interesting for another reason. This is that viral DNA replication ceases when vaccinia virus with a temperature-sensitive mutation in B1R is shifted to the non-permissive temperature (Condit et al., 1983; Rempel & Traktman, 1992), suggesting a role for the product of this gene in DNA replication. Thus, it is possible that, in addition to any role in protein synthesis, the ribosomal protein S2/Sa kinase is involved in regulating viral DNA replication by modulating the phosphorylation of the ssDNA-binding protein.

We are indebted to P. Traktman and G. Smith for the gift of antisera, and we thank M. Kohiyama for advice and G. Thomas for an initial gift of 40S ribosomes. We thank A. Banham and G. Smith for communication of their results before publication. We are grateful to R. Beaud for growing the HeLa cells and vaccinia virus, and also for preparing the cell extracts and carrying out several experiments. We thank Miss L. Irvine and Mrs I. Gall for performing the two-dimensional protein analysis, and R. Schwartzman for photographic work. This study was supported by grants from the D.R.E.D., Direction de la Recherche et des Etudes Doctorales du Ministère de l'Education Nationale, grant 'Virologie Fondamentale' (to G.B.) and from the Wellcome Trust and CIBA-Geigy (to D.P.L.).

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(Received 8 June 1993; Accepted 6 September 1993)