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Identification of induced protein kinase activities specific for the ribosomal proteins uniquely phosphorylated during infection of HeLa cells with vaccinia virus

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We have examined the ribosomal protein kinase activities in partially purified cytoplasmic extracts from HeLa cells infected with vaccinia virus. We found an activity or activities, absent from mock-infected cells, that was capable of phosphorylating the proteins S2 and S13 in vitro. The ribosomes phosphorylated in vitro exhibited the same multiple phosphorylation of S2 found in vivo, at least 3 phosphoryl residues being seen, and the same mono-phosphorylation of S13. Also as in vivo, ribosomal protein S2 contained phosphothreonine as well as phosphoserine, whereas S13 contained only phosphoserine. This strongly suggests that these new protein kinase activities are responsible for the ribosomal protein phosphorylations that occur during infection with vaccinia virus.

Protein kinase; Ribosomal protein S2; Ribosomal protein S13; Vaccinia virus

1. INTRODUCTION

The importance of protein phosphorylation in the infectious strategy of many lytic eukaryotic viruses is now becoming appreciated [1]. In the case of vaccinia virus, changes in the phosphorylation of specific 40 S ribosomal proteins during infection of HeLa cells were originally detected some years ago [2,3]. In addition to an increase in the phosphorylation of ribosomal protein S6, normally the only phosphorylated protein of the 40 S ribosomal subunit, there was phosphorylation de novo of two proteins which had never been reported to be phosphorylated in uninfected cells. One of these was clearly identified as ribosomal protein S2. The other – initially designated as ribosomal protein S16 [3] – is, in fact, ribosomal protein S13, as was established using several systems of two-dimensional gel analysis in our more recent study of the phosphorylation of Ehrlich ascites tumour cells infected with vaccinia virus [4]. We found 3–4 sites of phosphorylation on ribosomal protein S2, and confirmed the phosphorylation of one phosphate group per molecule of S13. With the cells and conditions that we used there was no increase in the phosphorylation of ribosomal protein S6, suggesting that a protein kinase(s) distinct from a ribosomal protein S6 kinase might be responsible for the phosphorylation of ribosomal proteins S2 and S13.

The function of these new ribosomal protein phosphorylations is not known, but their temporal association with the switch to predominant translation of viral mRNA raises the possibility that they could have a role in modulating this switch. A prerequisite for studying the effects of these phosphorylations on the function of the ribosomes is the identification and purification of the protein kinase(s) that catalyzes them. Although a protein kinase activity has previously been isolated from vaccinia virus cores [5,6], it is not known whether it can phosphorylate ribosomes. In our own previous work we did observe some phosphorylation of S2 when ribosomes were incubated with vaccinia virus cores; but this was limited to a single phosphoryl group, and our attempts to solubilize a ribosomal protein kinase activity of the appropriate specificity from the virions were unsuccessful [4]. We therefore decided to focus our attention on cytoplasmic extracts isolated from infected cells. We report here the identification of a new protein kinase activity, or activities, capable of reproducing the phosphorylations of ribosomal proteins S2 and S13 that occur during infection by vaccinia virus in vivo.

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2. EXPERIMENTAL

HeLa cells were maintained in suspension culture and infected (or mock-infected) with vaccinia virus (WR strain) at 7 plaque-forming units per cell. Cells and virus were kindly provided by B. Moss. Cells were harvested 4 h later and ribosomes and post-ribosomal supernatants were isolated as previously described [7]. In brief, the cells were washed with phosphate-buffered saline, resuspended in 1 vol. of hypertonic buffer (10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM magnesium acetate, 1 mM EGTA and 40 μg/ml phenylmethylsulphonyl fluoride), and then ruptured with a Dounce homogenizer. The crude extract was centrifuged for 30 min at 10000 rpm in a Sorvall SS34 rotor, and the supernatant re-centrifuged for 90 min at 50000 rpm in a Beckman Ti50 rotor. Ribosomes for analysis of endogenous protein kinase activity were resuspended at a concentration of 100 A_{260} units per ml, and those for use as substrate for exogenous protein kinase (in this case only those from uninfected cells) were washed in 1 M KCl, recentrifuged, resuspended and stored at −70°C. The post-ribosomal supernatant was either stored at −70°C, or, before DEAE-cellulose chromatography (as described in the legend to fig.2), dialyzed overnight against 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol and 40 μg/ml phenylmethylsulphonyl fluoride.

The protein kinase assays were essentially as previously described [7]. The standard assay mixture contained 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μM ATP (1 μCi of [γ-^{32}P]ATP) and substrate and enzyme (and any variations from the foregoing) as indicated in the figure legends. Incubation was for 30 min at 30°C.

The radioactively-labelled reaction products were analyzed either by one-dimensional gel electrophoresis under denaturing conditions, or by two-dimensional gel electrophoresis in 'system III', which allows resolution of the phosphorylated forms of ribosomal proteins S2 and S13 [4,8,9].

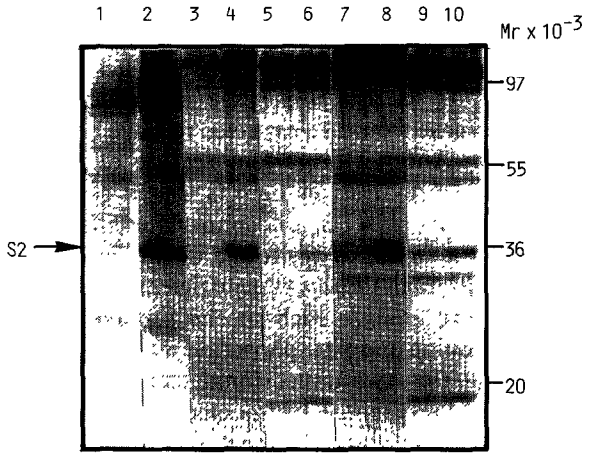

Fig.1. Endogenous and exogenous ribosomal protein kinase activities induced in cells infected with vaccinia virus. Ribosomes and post-ribosomal supernatants were isolated from HeLa cells and assayed for protein kinase activity in an 80 μl reaction mixture as described in section 2, but at 110 mM KCl, and subjected to SDS gel electrophoresis. The figure shows a composite autoradiograph from mixtures containing either 4 A_{260} units of crude ribosomes from cells infected with vaccinia virus (lane 2) or mock-infected cells (lane 1); or mixtures containing 3.2 A_{260} units of salt-washed ribosomes from mock-infected cells (lanes 4, 6, 8 and 10), or mixtures containing no ribosomes (lanes 3, 5, 7 and 9) to which were added 20 μl undialyzed (lanes 3–6) or dialyzed (lanes 7–10) post-ribosomal supernatant from cells infected with vaccinia virus (lanes 3, 4, 7 and 8) or from mock-infected cells (lanes 5, 6, 9 and 10).

3. RESULTS

First we examined the endogenous ribosomal protein kinase activity of crude ribosomes from mock-infected cells and cells infected with vaccinia virus. It can be seen from fig.1 (lanes 1 and 2) that there was a clear difference between these: the ribosomes from infected cells had much greater endogenous protein kinase activity against a protein of about 34000 relative molecular mass. This protein kinase activity was also present in the post-ribosomal supernatant, as shown by the exogenous phosphorylation of salt-washed ribosomes from uninfected cells (fig.1, lanes 3–6). The size of the phosphorylated protein was consistent with it being either or both of ribosomal proteins S2 or S6. However, it should be noted that after dialysis of the supernatants an endogenous phosphoprotein of a similar electrophoretic mobility became apparent (fig.1, lanes 7–10). Preliminary two-dimensional gel electrophoresis did, in fact, demonstrate phosphoryla-

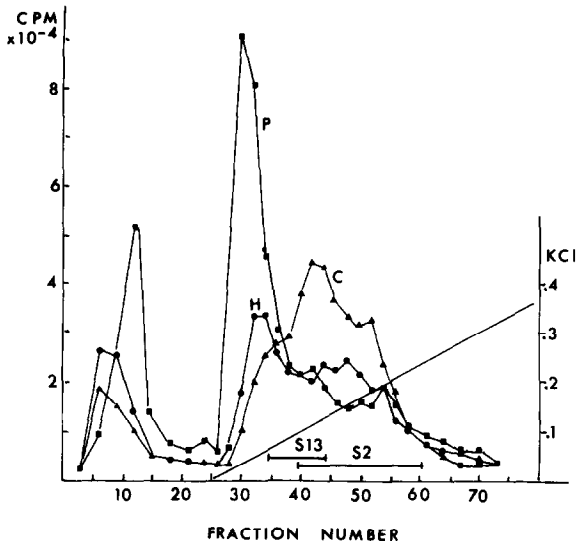

Fig.2. DEAE-cellulose chromatography of post-ribosomal supernatant from HeLa cells infected with vaccinia virus. A dialyzed extract from 6 × 10^8 infected cells (about 30 mg protein) was applied to an equilibrated 2.5 × 1.0 cm DEAE-cellulose column, washed with 25 ml of dialysis buffer, and then eluted with a 50 ml gradient of 0–350 mM KCl at a flow rate of 12 ml/h, corresponding to the fractions 26–75. Protein kinase assays were performed on 40 μl column fractions as described in section 2 in 120 μl reaction mixtures containing 0.8 mg/ml of either protamine (P, ■—■), mixed histones (H, ●—●) or casein (C, ▲—▲), and radioactivity precipitated by trichloroacetic acid was measured. An identical chromatographic separation of an extract of mock-infected cells (not illustrated) showed a similar pattern for these substrates. The horizontal bars indicate the position of ribosomal protein S2 and S13 kinase activity, assayed as described in the legend to fig.3. The protein kinase not bound to the column probably results from overloading in order to maintain a high concentration of protein.

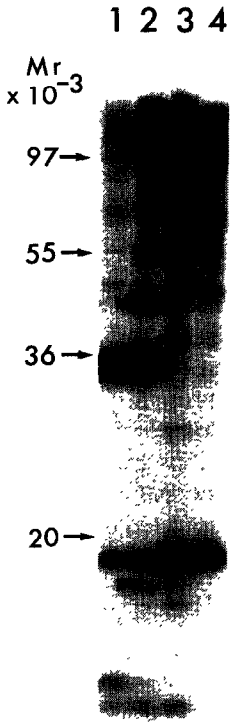

Fig.3. Polyacrylamide gel electrophoresis of ribosomal proteins phosphorylated in vitro by protein kinase activity partially purified by DEAE-cellulose chromatography. Assays were performed in 25 μl reaction mixtures as described in section 2, but at 36 mM Tris-HCl (pH 7.5) and 100 mM KCl, and polyacrylamide gel electrophoresis performed in the presence of SDS. The lanes 2 and 3 of the resulting autoradiograph correspond to reaction mixtures containing 0.3 A_{260} units of salt-washed ribosomes as substrate (lanes 1 and 4 were from reactions with no exogenous substrate); lanes 1 and 2 correspond to reactions containing 10 μl of DEAE-cellulose fraction 38 from cells infected with vaccinia virus (fig.2), and lanes 3 and 4 to reactions containing 10 μl of the corresponding fraction from an identical chromatographic separation of an extract of mock-infected cells. The relative molecular masses of protein standards are indicated, and arrowheads in lane 2 point to the 48 kDa and 17 kDa proteins mentioned in the text.

tion of protein S2 (and S13) when ribosomes were incubated with dialyzed post-ribosomal supernatant from infected cells (not shown, but cf. fig.4). However it was clearly desirable to obtain a more purified preparation of the activities in the post-ribosomal supernatant.

To this end we subjected the post-ribosomal supernatant to DEAE-cellulose chromatography, using the same KCl gradient that we had previously employed to determine the positions of migration of some of the cellular protein kinases (fig.2). In doing this we encountered a technical problem in assaying the S2 kinase by one-dimensional gel electrophoresis. This was because the extract of infected cells contained a similarly sized 34 kDa phosphoprotein substrate which became apparent during the purification (fig.3, lane 1). Nevertheless it was possible to judge that the S2 kinase was present in fractions 39–60 (ca. 0.08–0.25 M KCl – see fig.2), starting to elute just after the main peak of protamine kinase activity, which corresponds to protein kinase C [7]. Also apparent to a greater extent in DEAE-cellulose fractions from infected than uninfected cells was a phosphorylated protein of 17 kDa, the size of ribosomal protein S13 (fig.3, lane 2). This assignation, and that of S2, were confirmed by two-dimensional gel electrophoresis – see below. In contrast to the S2 kinase activity, the S13 kinase activity eluted as a single peak at about 0.08 M KCl (fractions 34–44 – see fig.2). We were aided in locating the S13 kinase by another substrate on the salt-washed ribosomes: a protein of 48 kDa (fig.3, lane 2), that was not detected in the two-dimensional separation of basic proteins in fig.4, presumably because it has an isoelectric point of less than 8.6.

For unequivocal identification of the ribosomal proteins phosphorylated, and to determine the number of phosphorylated forms, two-dimension gel electrophoresis was performed (fig.4). It can be seen (frames A and C) that the ribosomal proteins phosphorylated by column fraction 39 from fig.2 were S2 and S13, whereas the corresponding fraction from an experiment with mock-infected cells showed no kinase activity against these proteins. The phosphorylated forms of S2 and S13 could also be seen on inspection of gels after staining with Coomassie blue (results not shown). In all cases there was minor phosphorylation of ribosomal protein S6, but this was no greater in the fraction from infected cells than in that from the mock-infected cells. (It should be mentioned that β-glycerol phosphate was not included during extraction, so that any pre-existing or induced cellular S6 kinase would be expected to have been inactivated [10].) Another important feature of the two-dimensional gel analysis in fig.4 is that it reveals the presence of at least 3 phosphorylated forms of ribosomal protein S2, as are found during infection in vivo [3,4]. Although there was insufficient radioactivity incorporated to analyze each species separately, both phosphothreonine and phosphoserine were found in S2 eluted from the two-dimensional gel (results not shown), also consistent with the characteristics of the phosphorylation in vivo. Similarly only one phosphate group was added to protein S13 during phosphorylation in vitro (fig.4), and was present on seryl residues, as previously found in vivo [4].

The clearest evidence that different protein kinases might be responsible for phosphorylating ribosomal proteins S2 and S13, as mentioned above, is also provided by fig.4. Thus, S2 kinase activity, but almost no S13 kinase activity, was present in column fractions 46 (fig.4, frame D), 53 and 60 (results not shown). The observation that the S2 kinase activity did not elute as a single discrete peak indicates the presence of multiple forms on the column, although we do not suggest that these necessarily exist in the infected cell.

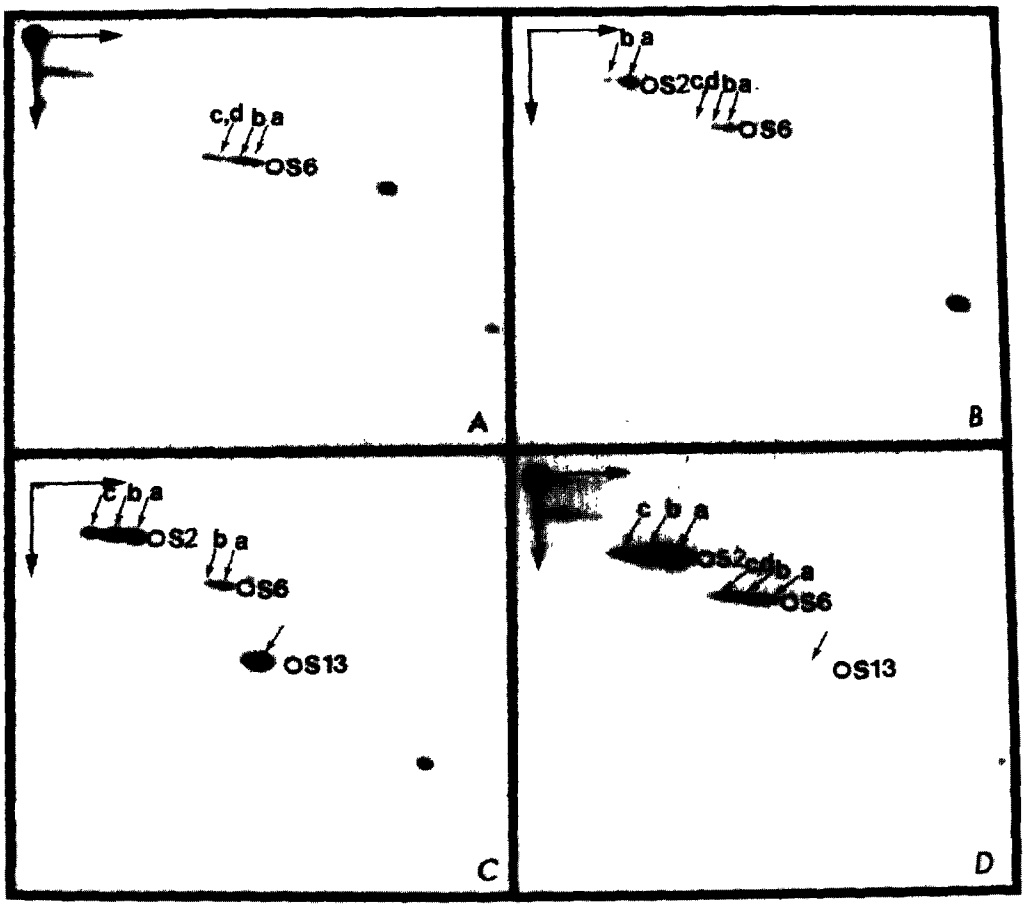

Fig.4. Two-dimensional gel electrophoresis of ribosomal proteins phosphorylated in vitro by protein kinase activity partially purified by DEAE-cellulose chromatography. The protein kinase assay was similar to that described in the legend to fig.3, but contained 7.5 A_{260} units of ribosomes. The ribosomes phosphorylated in vitro were sedimented through a cushion containing 1 M KCl, and the proteins analyzed by two-dimensional gel electrophoresis using 'system III' [4,8,9]. The figure shows autoradiographs from reactions with different enzyme fractions (10 μl): (A) DEAE-cellulose fraction 39 from mock-infected cells; (B, C and D) DEAE-cellulose fractions 32, 39 and 46, respectively, from cells infected with vaccinia virus. The position of the unphosphorylated forms of ribosomal proteins S2, S6 and S13 are indicated by circles, and the different phosphorylated forms of S2 and S6 are designated a, b, c and d, each corresponding to the addition of a single phosphoryl residue, as the pH in the first dimension (pH 8.6) is close to the p*I* of the basic ribosomal proteins. Autoradiographs corresponding to fractions 32 and 46 from mock-infected cells are not illustrated as these were similar to that shown in frame A.

4. DISCUSSION

This work demonstrates that the soluble fraction of cells infected with vaccinia virus contains a protein kinase or kinases, absent from uninfected cells, that can reproduce in vitro the phosphorylations of ribosomal proteins S2 and S13 occurring during infection in vivo. It is therefore most likely that we have identified the kinase(s) responsible for catalyzing these phosphorylations during infection. It should be stressed that no known cellular protein kinase has specificity for either or both of these ribosomal proteins in vitro (see e.g. [11–14]).

The results presented here also raise some other interesting questions about the protein kinase activity. One is the indication, already discussed, that there is more than one kinase activity induced; the other is that the kinase activity or activities also have other physiological substrates, such as the 34 kDa phosphoprotein (presumed to be viral) detected in fig.3. These questions can only be answered through further purification, as can that of the genetic origin of the kinase(s) – virus or host. Until recently protein kinases were not known to be part of the stable genetic repertoire of eukaryotic viruses [1], but it is now clear that members of another family of large DNA viruses, the α-herpes viruses, encode at least one [15], if not two [16], such enzymes. It is therefore possible that the ribosomal protein kinase activities induced during infection by vaccinia virus are encoded by the viral genome. If this proves to be the case, the combination of viral genetics with enzyme purification will be powerful tools in elucidating the function of the phosphorylation of these ribosomal proteins.

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