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The phosphorylation of ribosomal protein S6 by protein kinases from cells infected with pseudorabies virus

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We examined the ability of protein kinase activities from BHK (baby-hamster kidney) cells infected with pseudorabies virus to catalyse the phosphorylation of ribosomal protein S6 *in vitro*. When the cytosol from infected cells was fractionated on DEAE-cellulose, 40S ribosomal protein kinase activity was found associated with the two isoforms of the cyclic AMP-dependent protein kinase, protein kinase C and a protein kinase (ViPK, virus-induced protein kinase) only detected in infected cells. The phosphorylation of ribosomal protein by ViPK was of particular interest because the appearance of the protein kinase and the increase in the phosphorylation of protein S6 in infected cells shared a similar time course. At moderate concentrations of KCl the major ribosomal substrate for ViPK was ribosomal protein S7, a protein not found to be phosphorylated *in vivo*. However, at 600 mM-KCl, or in the presence of 5–10 mM-spermine at 60–150 mM-KCl, the phosphorylation of ribosomal protein S7 was suppressed and ribosomal protein S6 became the major substrate. The maximum stoichiometry of phosphorylation obtained under the latter conditions was 1–2 mol of phosphate/mol of S6, and only mono- and di-phosphorylated forms of S6 were detected on two-dimensional gel electrophoresis. As the infection of BHK cells by pseudorabies virus results in the appearance of phosphorylated species of S6 containing up to 5 mol of phosphate/mol of S6 protein, it appears unlikely that ViPK alone can be responsible for the multiple phosphorylation seen *in vivo*. Nevertheless, tryptic phosphopeptide analysis did indicate that *in vitro* ViPK catalysed the phosphorylation of at least one of the sites on ribosomal protein S6 phosphorylated *in vivo*, so that a contributory role for the enzyme in the phosphorylation *in vivo* cannot be excluded.

INTRODUCTION

Ribosomal protein S6 of higher eukaryotes can acquire up to five phosphoryl groups per molecule, stimulation of the extent of phosphorylation above basal occurring in a variety of circumstances [see Leader (1980) and Parker *et al.* (1985) for reviews]. Although the function of this phosphorylation is still unclear, there is a suggestive correlation between the rate of protein biosynthesis and the extent of phosphorylation of S6 in many tissue-culture cells under conditions of rapid growth. However, there are also other circumstances in which there is increased phosphorylation of S6 where this correlation does not hold, e.g. when inhibitors of protein biosynthesis are added to cells. Another possible exception involves the phosphorylation of ribosomal protein S6 during lytic infection of cells by vaccinia virus (Kaerlin & Horak, 1978), or by human and swine herpes viruses (Kennedy *et al.*, 1981). These latter systems are of particular interest because the availability of herpes virus mutants could possibly facilitate a genetic approach to the function of the phosphorylation.

We have been studying protein kinases (ATP: protein phosphotransferases, EC 2.7.1.37) extracted from cells infected with the swine herpes virus pseudorabies virus, and have isolated an apparently novel protein kinase (ViPK) which could not be detected in uninfected cells (Katan *et al.*, 1985). This protein kinase catalysed the phosphorylation of serine residues of the basic proteins, protamine and histones (but not those of the acidic proteins casein and phosvitin), used ATP (but not GTP) as a phosphoryl donor, and its activity was not stimulated by a variety of molecules that serve as effectors for other protein kinases. It is not yet known whether the ViPK is encoded by the host or viral genome, although the present evidence most favours the latter possibility (Purves *et al.*, 1986).

The appearance of ViPK in cells infected with pseudorabies virus coincided with the increased phosphorylation of ribosomal protein S6. It was thus clearly of interest to examine whether this or other protein kinase activities from infected cells could catalyse the multiple phosphorylation of ribosomal protein S6 *in vitro*. Certain protein kinases [such as the cyclic AMP-dependent protein kinase (Wettenhall & Morgan, 1984; Wettenhall *et al.*, 1982)] can, *in vitro* and *in vivo*, only phosphorylate a limited subset of the five available sites on ribosomal protein S6. We therefore paid particular attention to the stoichiometry and sites of the phosphorylation in the study, the results of which are described here.

MATERIALS AND METHODS

Materials

General laboratory chemicals were of analytical grade, where appropriate, and obtained from standard commercial suppliers unless otherwise indicated. Mixed histones (type IIA), lysine-rich histones (type IIIS), protamine

Abbreviations used: BHK cells, baby-hamster kidney fibroblasts; ViPK, virus-induced protein kinase.

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sulphate, spermine and phosphatidylserine were from Sigma; cyclic AMP was from Boehringer; DEAE-cellulose (DE-52) was from Whatman; Blue A was from Amicon; [γ-^{32}P]ATP was either synthesized as described by Maxam & Gilbert (1980) or purchased from Amersham International, from whom [^{32}P]P_i was also obtained. Trypsin (EC 3.4.21.4, tosyl-phenylalanyl-chloromethane-treated) was from Worthington.

Cells and virus

BHK-21/C13 cells were maintained in monolayer cultures in modified Eagle medium containing 10% (v/v) calf serum, and infected at or just before confluence with pseudorabies virus at a multiplicity of approx. 20 plaque-forming units/cell (Kennedy *et al.*, 1981).

Preparation of ribosomes and ribosomal subunits

Ribosomes and ribosomal subunits were isolated from rat liver or from BHK cells as previously described (Parker *et al.*, 1985). ^{32}P-labelling of BHK cells before isolation of ribosomes was also as previously described (Kennedy *et al.*, 1981), except that the Eagle medium contained undialysed serum, and Earle's salts completely lacking phosphate was used.

Preparation and anion-exchange chromatography of cellular post-ribosomal supernatant.

The post-ribosomal supernatant of BHK cells (100–150 mg of protein) was prepared and subjected to chromatography on DEAE-cellulose on a 6 cm × 1.6 cm column, eluted with a linear gradient of 0–0.4 M-KCl exactly as previously (Katan *et al.*, 1985; see Fig. 1).

Preparation of partially purified ViPK

In certain experiments (indicated in the text) the DEAE-cellulose column fractions containing ViPK were further purified on Blue A to yield a preparation free of other known protein kinases (Katan *et al.*, 1985).

Assay of protein kinase activity

In order to relate ribosomal protein S6 kinases to known protein kinases, DEAE-cellulose column fractions were assayed with histones or protamine as a substrate (as described by Katan *et al.*, 1985), as well as with 40S ribosomal subunits as substrate (as described by Parker *et al.*, 1985). Mixed histones were used for the assay of cyclic AMP-dependent protein kinase; protamine was used for the ViPK, and lysine-rich histone for protein kinase C. When ribosomes were used as substrate for protein kinases, 40S subunits from rat liver (which already contain a small proportion of protein S6 in a monophosphorylated state) were used where subsequent analysis was by one-dimensional gel electrophoresis; but 40S ribosomal subunits from BHK cells grown for 6 days (in which protein S6 is almost completely dephosphorylated; Parker *et al.*, 1985) were used where subsesquent analysis was by two-dimensional gel electrophoresis.

A unit of protein kinase activity is defined as that amount of enzyme that catalyses the incorporation of 1 nmol of phosphate/min into the appropriate optimal substrate under standard assay conditions.

Polyacrylamide-gel electrophoresis

One-dimensional gel electrophoresis of phosphorylated ribosomal protein in the presence of SDS, or two-dimensional gel electrophoresis as described by Lastick & McConkey (1976), was performed as previously (Parker *et al.*, 1985). Ribosomal proteins separated by two-dimensional gel electrophoresis were designated according to the standard nomenclature of McConkey *et al.* (1979).

Calculation of stoichiometry of phosphorylation

The stoichiometry of the phosphorylation of ribosomal protein S6 was calculated from the radioactivity in the latter protein, after excision from one-dimensional gels, and the specific radioactivity of the [γ-^{32}P]ATP, assuming that 1 A_{260} unit of 40S ribosomal subunits is equivalent to 66.3 pmol (1 A_{260} unit is the quantity of material contained in 1 ml of a solution which has an A_{260} of 1 when measured in a cell with a 1 cm light-path).

Analysis of tryptic phosphopeptides

Ribosomal 40S subunits were phosphorylated *in vitro* or *in vivo*, and their ribosomal proteins (200 μg) were fractionated by one-dimensional gel electrophoresis in SDS. The region containing ribosomal protein S6 was extracted, subjected to tryptic digestion, and fractionated by reversed-phase h.p.l.c. on a Vydak TPC18 column, as previously described (Parker *et al.*, 1985).

RESULTS

Analysis of DEAE-cellulose column fractions

As the majority of ribosomes of BHK cells are not bound to membranes, it seemed appropriate to examine their phosphorylation *in vitro* by protein kinases present in the cellular cytosol fraction. This was prepared from cells infected for 6 h with pseudorabies virus, and fractionated by chromatography on DEAE-cellulose. Column fractions were assayed with the appropriate histone or protamine substrates for different protein kinases (results not shown, but see Katan *et al.*, 1985), and then their ability to catalyse the phosphorylation of rat liver 40S ribosomal subunits *in vitro* was analysed by one-dimensional gel electrophoresis (Fig. 1). The reaction conditions were varied to accommodate the requirements of the different protein kinases known to be present.

Fig. 1(*a*) shows the results of assay under ionic conditions shown to be suitable for the protein-synthetic function of 40S ribosomal subunits (Voorma *et al.*, 1979), with the additional inclusion of cyclic AMP. The column fractions in the region where the two isoforms of the cyclic AMP-dependent protein kinase are eluted catalysed the phosphorylation of a band corresponding to a protein of 31 kDa apparent molecular mass. We, like others before us (Del Grande & Traugh, 1982), have shown that this corresponds to ribosomal protein S6, and that the maximal extent of phosphorylation achieved with the cyclic AMP-dependent protein kinase *in vitro* is approx. 2 mol of phosphate/mol of ribosomes (Parker *et al.*, 1985). Another band phosphorylated by these column fractions corresponded to an apparent molecular mass of 14.5 kDa. Two-dimensional gel electrophoresis showed this to be ribosomal protein L35, a protein of the 60S ribosomal subunit contaminating our 40S subunit preparation (results not shown). This protein is not phosphorylated in either virus-infected or uninfected BHK cells *in vivo* (Kennedy *et al.*, 1981).

Phosphorylation of the 31 kDa protein was also obtained by column fractions containing protein kinase

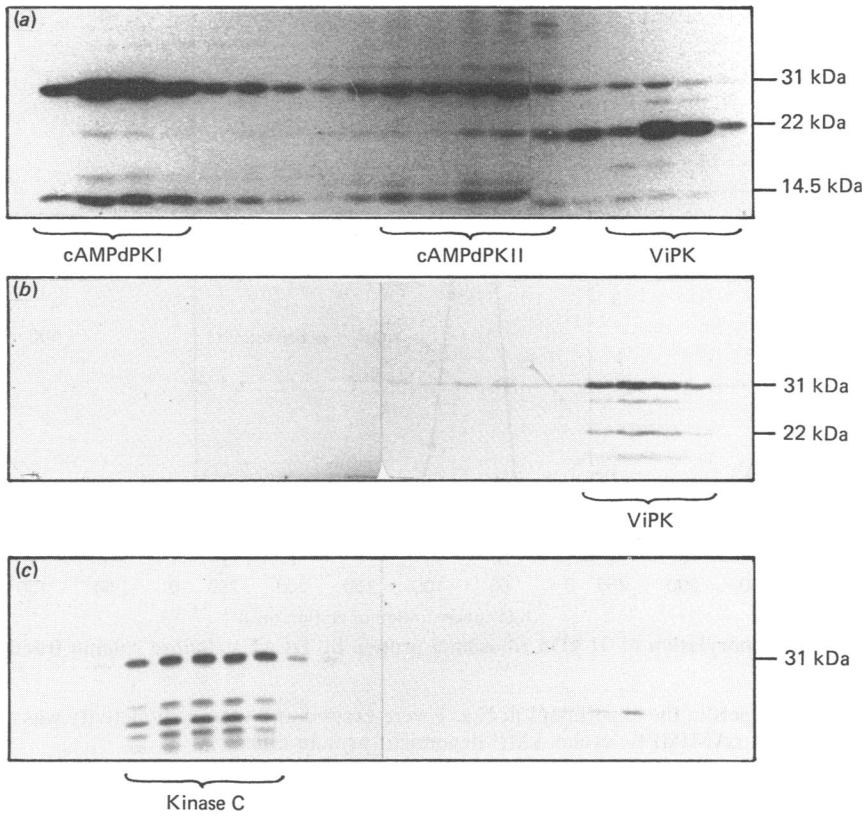

Fig. 1. DEAE-cellulose chromatography of ribosomal protein kinases in BHK cells infected with pseudorabies virus

BHK cells were infected with pseudorabies virus and harvested 6 h later, and post-ribosomal supernatant was prepared and subjected to column chromatography on DEAE-cellulose, as described in the Materials and methods section. Column fractions (30 μl) were assayed for the phosphorylation of rat liver 40S ribosomal subunits (1 A_{260} unit) at 10 mM-$MgCl_2$ in a reaction volume of 120 μl, incubation being at 30 °C for 30 min (*a* and *b*) or 15 min (*c*). The ionic conditions and other additions were: (*a*) 100 mM-KCl, in the presence of 10 μM-cyclic AMP; (*b*) 600 mM-KCl; (*c*) 0.15 mM-$CaCl_2$ and 500 μg of phosphatidylserine/μl. The reaction mixtures (80 μl samples) were then subjected to one-dimensional gel electrophoresis in SDS, followed by drying and autoradiography (see the Materials and methods section). The positions of elution of the relevant known protein kinases (assayed with histones or protamine) are indicated, as are the apparent molecular masses of the major phosphorylated proteins. Abbreviation: cAMPdPK, cyclic AMP-dependent protein kinase.

C activity when these were assayed in the presence of Ca^{2+} and phosphatidylserine (Fig. 1*c*). We have presented a detailed characterization of the phosphorylation *in vitro* of ribosomal protein S6 by pure protein kinase C from bovine brain (Parker *et al.*, 1985).

When assayed at 100 mM-KCl, column fractions in the region at which ViPK was eluted catalysed predominantly the phosphorylation of a 22 kDa protein, there being weak phosphorylation of the 31 kDa band. As it had previously been found that the activity of ViPK against protamine and histone is stimulated by increasing ionic strength (Katan *et al.*, 1985), we also examined the phosphorylation of 40S subunits by ViPK at 600 mM-KCl (Fig. 1*b*). Under these conditions the 31 kDa protein became the main substrate, and the phosphorylation of the 22 kDa protein decreased markedly. The photographic exposures of the autoradiographs in the different frames of Fig. 1 were not identical, and so Fig. 1 does not allow a quantitative comparison of the different ribosomal-protein-S6 kinase activities. Quantitative data from this experiment are presented in Fig. 2, and these show that the phosphorylation of the 31 kDa protein by ViPK at 600 mM-KCl was comparable with that presumed to be due to the cyclic AMP-dependent protein kinases and protein kinase C.

We further investigated the phosphorylation of the 31 kDa protein with a more purified preparation (see the Materials and methods section) of ViPK (Fig. 3). When this preparation was assayed with 40S ribosomes at low ionic strength, the minor phosphorylated proteins observed in addition to that of 22 kDa in Fig. 1(*a*) were much less apparent [see also Fig. 8B of Katan *et al.* (1985) for a comparison under conditions identical with those in Fig. 1*a*]. They presumably represent endogenous substrate for ViPK or other protein kinases (e.g. casein kinase II) present in the DEAE-cellulose fraction. The phosphorylation of the 31 kDa protein, seen at 600 mM- and 700 mM-KCl, was not yet evident at 350 mM-KCl; and in other experiments (results not shown) we have found that at 440 mM-KCl this phosphorylation is only about one-quarter as great as at 660 mM-KCl. Similar results to those at 700 mM-KCl were obtained at 700 mM-NaCl (Fig. 3). For various reasons the conditions of low ionic strength, used for the experiments presented here, vary from 60 to 150 mM-KCl and from 5 to 10 mM-$MgCl_2$. However, as should be apparent from the

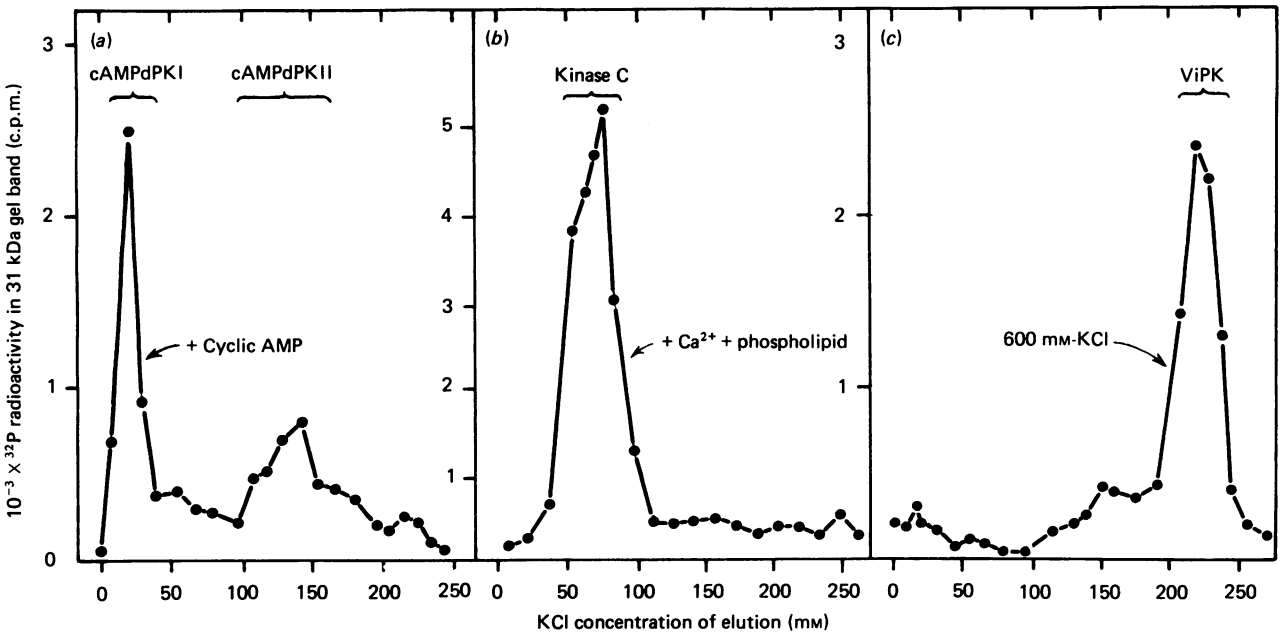

Fig. 2. Quantification of phosphorylation of 31 kDa ribosomal protein by DEAE-cellulose column fractions from BHK cells infected with pseudorabies virus

The 31 kDa bands from the gels in the experiment in Fig. 1 were excised and their radioactivity was determined by scintillation spectrometry. Abbreviation: cAMPdPK, cyclic AMP-dependent protein kinase.

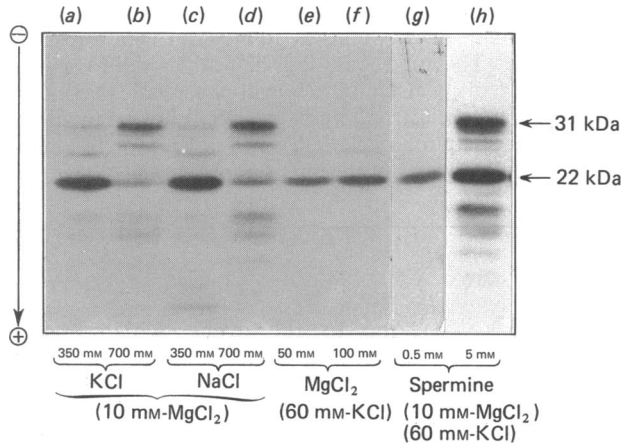

Fig. 3. Effect of ionic conditions on the phosphorylation of 40S ribosomal subunits by ViPK

A partially purified preparation of ViPK (0.1 unit) was used to phosphorylate rat liver 40S ribosomal subunits (1 A_{260} unit) in a reaction volume of 120 μl at 30 °C for 30 min (see the Materials and methods section) and the ionic composition of the reaction mixture was varied: (*a*) 350 mM-KCl, 10 mM-$MgCl_2$; (*b*) 700 mM-KCl, 10 mM-$MgCl_2$; (*c*) 350 mM-NaCl, 10 mM-$MgCl_2$; (*d*) 700 mM-NaCl, 10 mM-$MgCl_2$; (*e*) 60 mM-KCl, 50 mM-$MgCl_2$; (*f*) 60 mM-KCl, 100 mM-$MgCl_2$; (*g*) 60 mM-KCl, 10 mM-$MgCl_2$, 0.5 mM-spermine; (*h*) 60 mM-KCl, 10 mM-$MgCl_2$, 5 mM-spermine. The reaction mixtures were subjected to one-dimensional polyacrylamide gel electrophoresis in SDS. The Figure shows an autoradiograph of the dried gel, the apparent molecular masses of the major phosphoproteins being indicated.

results in Fig. 3 and elsewhere (Katan *et al.*, 1985), these variations did not influence the quantitative value or qualitative nature of the results. High ionic strength was not the only condition under which ViPK could catalyse the phosphorylation of the 31 kDa protein: spermine also provided a suitable environment for this. The effect could be detected at 5 mM-spermine, but at this concentration did not produce suppression of the phosphorylation of the 22 kDa protein. More extensive phosphorylation of the 31 kDa protein was seen at slightly higher concentrations (see Figs. 4 and 5). The effect of spermine could not be reproduced by increasing the concentration of Mg^{2+} in the assay mixture.

Identification of ribosomal proteins phosphorylated by ViPK

Two-dimensional gel electrophoresis was performed to determine the identity of the 31 kDa and 22 kDa ribosomal proteins phosphorylated by ViPK. The 22 kDa protein was identified as ribosomal protein S7 (Fig. 4), a protein that, like L35, is not phosphorylated in infected cells *in vivo*. We assume that these and similar phosphorylations (Parker *et al.*, 1985) are artifacts of the conditions of the reaction *in vitro*. Nevertheless it is worth pointing out that, because ribosomal protein S7 has not been reported to be a substrate for other protein kinases, its phosphorylation *in vitro* is a useful distinguishing feature of ViPK.

The results of Fig. 4 also demonstrate that S6 is the ribosomal protein phosphorylated by ViPK in the presence of spermine. As the relative molecular mass of purified ribosomal protein S6 is 31000 (Collatz *et al.*, 1976), S6 most likely corresponds to the 31 kDa band of Figs. 1–3. (We cannot, of course, exclude the

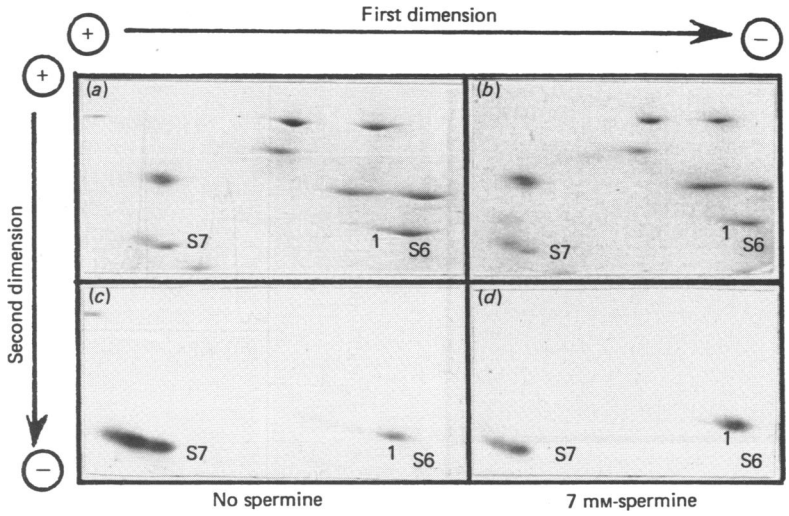

Fig. 4. Two-dimensional gel analysis of ribosomal proteins phosphorylated by ViPK

A partially purified preparation of ViPK (1 unit) was used to phosphorylate a mixture of 40S and 60S BHK ribosomal subunits (15 A_{260} units) in a reaction volume of 800 μl at 30 °C for 3.5 h. The reaction contained 150 mM-KCl and 5 mM-$MgCl_2$, and in one condition (*b*, *d*) spermine (7 mM) was present. The protein was isolated from the ribosomes and subjected to two-dimensional gel electrophoresis as described in the Materials and methods section. (*a*, *b*) Stained gels; (*c*, *d*) autoradiographs. The numeral '1' indicates the position of the monophosphorylated derivative of ribosomal protein S6.

possibility that in addition the 31 kDa band contains a non-ribosomal contaminant of the 40S subunits, the phosphorylation of which is also stimulated by spermine.)

Stoichiometry of phosphorylation of ribosomal protein S6

We adopted two different approaches to the question of the stoichiometry of the phosphorylation of ribosomal protein S6. The first involved analysis by two-dimensional gel electrophoresis, in which the five different phosphorylated species of S6 migrate to discrete positions on the anodic side of the unphosphorylated species (Parker *et al.*, 1985). In the experiment of Fig. 4 only the monophosphorylated species of S6 was seen on the autoradiograph (Fig. 4*d*), and an examination of the stained gel pattern (Fig. 4*b*) showed most of the protein to be unphosphorylated. We suspected that this might be an underestimate of the extent of phosphorylation, because of the relatively large amount of 40S ribosomal subunits required for two-dimensional gel electrophoresis, and the limited amount of purified ViPK available. We therefore repeated the phosphorylation (this time at 10 mM-spermine) using a large amount of the (concentrated) DEAE-cellulose fractions containing ViPK. Although the resulting two-dimensional gel pattern is somewhat streaky (a result, we believe, of the quantity of protein in the enzyme preparation that had to be added to the ribosomes), it is clear that in this case both mono- and di-phosphorylated forms of ribosomal protein S6 were produced (Fig. 5). In no experiment have we obtained evidence that the ViPK can give rise to more extensively phosphorylated species.

The other approach to the question of the stoichiometry involved calculation of the mol of phosphate/mol of S6 after separating ribosomal proteins by one-dimensional gel electrophoresis (see the Materials and methods section). This approach (which has yielded values of up to 4.5 mol of phosphate/mol of S6 when protein kinase

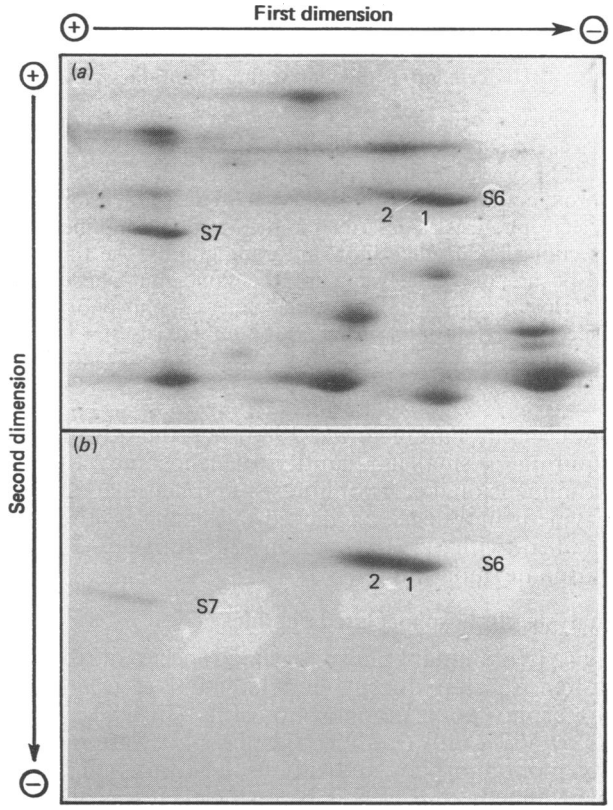

Fig. 5. Phosphorylation of ribosomal protein S6 by ViPK

The experiment described in Fig. 4 (panels *b* and *d*) was repeated, but with a concentrated preparation (800 units) of ViPK from DEAE-cellulose chromatography, and with 150 mM-KCl, 5 mM-$MgCl_2$ and 10 mM-spermine in the incubation. (*a*) Stained gel; (*b*) autoradiograph. The numerals '1' and '2' indicate the mono- and di-phosphorylated forms of ribosomal protein S6.

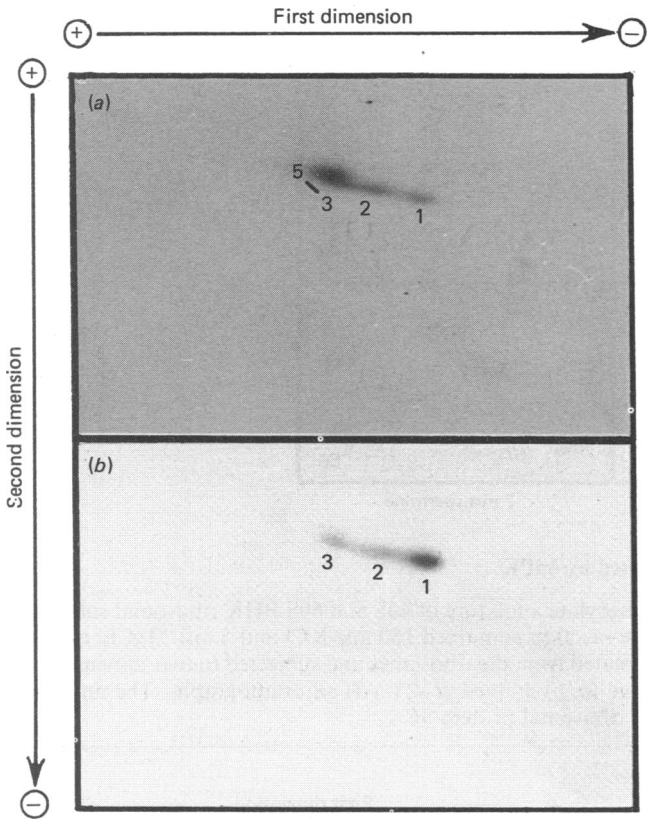

Fig. 6. Phosphorylated derivatives of ribosomal protein S6 in BHK cells after infection with pseudorabies virus

Uninfected cells (*b*) or BHK cells infected with pseudorabies virus for 8 h (*a*) were incubated with [^{32}P]P_i (5 mCi) for the 3 h before harvesting. The ribosomes were isolated, and protein was extracted and subjected to two-dimensional gel electrophoresis as described in the Materials and methods section. The frames show autoradiographs, the times of exposure of which were not the same, but chosen for clarity of representation of the different phosphorylated species of ribosomal protein S6 (numbered 1–5).

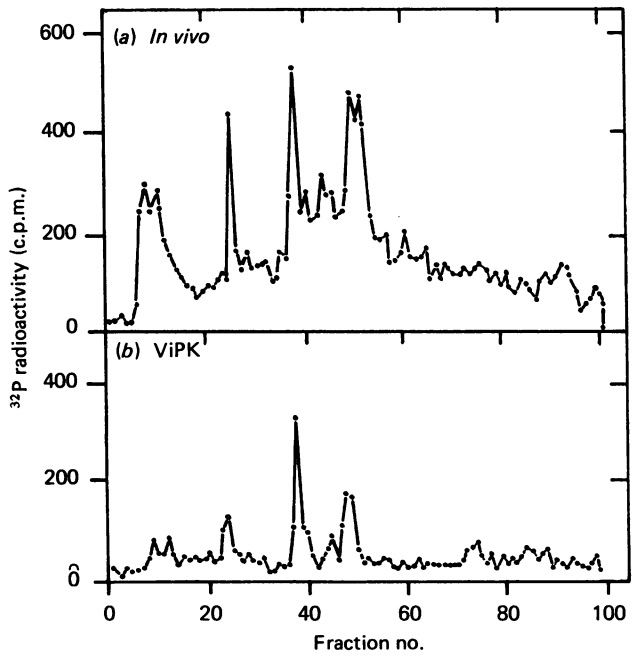

Fig. 7. Analysis of tryptic peptides from ribosomal protein S6

^{32}P-labelled ribosomal protein S6 was obtained from BHK cells infected with pseudorabies virus in the experiment described in Fig. 6(*a*) (*a*), or from 40S ribosomal protein (0.2 A_{260} unit) incubated with partially purified ViPK (1 unit) *in vitro* for 2 h in a reaction containing 150 mM-KCl, 5 mM-$MgCl_2$ and 10 mM-spermine (*b*), and subjected to h.p.l.c. as described in the Materials and methods section.

C was used; Parker *et al.*, 1985) has the advantage of requiring a smaller quantity of ribosomes than for two-dimensional electrophoresis, and hence an excess of enzyme is more easily achieved. Nevertheless the values obtained with ViPK were in the range 1–2 mol of phosphate/mol of S6.

Analysis of phosphorylated peptides

The predominant phosphorylated species of ribosomal protein S6 seen during viral infection *in vivo* are the triphospho- to pentaphospho-forms (Fig. 6), whereas *in vitro* ViPK only catalysed the phosphorylation of S6 to give monophospho- and diphospho-forms (Fig. 5; see also Kennedy *et al.*, 1981). Nevertheless it was of interest to know whether the sites that were phosphorylated *in vitro* corresponded to any of these found *in vivo*. Tryptic phosphopeptides from ribosomal protein S6 were therefore analysed by h.p.l.c. (Fig. 7). The major phosphopeptide from ribosomal protein S6 phosphorylated by ViPK *in vitro* (that eluted at fraction 37) corresponded to one from ribosomal protein S6 phosphorylated *in vivo* in cells infected with pseudorabies virus.

DISCUSSION

In our efforts to identify the protein kinase responsible for the multiple phosphorylation of ribosomal protein S6 in cells infected with pseudorabies virus it was natural for attention to focus on the newly described ViPK, the appearance of which occurred at a similar time to the phosphorylation (Katan *et al.*, 1985). Admittedly, the ViPK phosphorylated ribosomal protein S6 only poorly at low or moderate ionic strength (Figs. 1 and 3), where the major ribosomal substrate was protein S7. However, at high ionic strength the ViPK became specific for protein S6 as a ribosomal substrate, and the phosphorylation of ribosomal protein S6 was generally stimulated. Furthermore a similar specificity could be obtained under the more physiological salt conditions of 150 mM-KCl and 5 mM-$MgCl_2$ when 5–10 mM-spermine was included in the assay (Figs. 3–5), and these concentrations of spermine may also be within the physiological range (Tabor & Tabor, 1976). Thus the candidacy of ViPK as the S6 kinase could not be lightly disregarded.

It is interesting that the so-called 'protease-activated kinase II' (Lubben & Traugh, 1983), an enzyme that may in fact be equivalent to protein kinase C (Parker *et al.*, 1985), also becomes specific for ribosomal protein S6 as the ionic strength is increased, the phosphorylation of protein S10 (which is not phosphorylated *in vivo*) being suppressed. This might suggest that increasing the ionic strength alters the conformation of the ribosome. Nevertheless, because spermine (albeit at lower concen-

trations) and high ionic strength also stimulate the phosphorylation of histones by ViPK (Katan *et al.*, 1985), an effect on the enzyme cannot be excluded.

The question of whether ViPK acts as a ribosomal protein S6 kinase *in vivo* rests not only on the above considerations of the conditions used *in vitro* to obtain phosphorylation, but also on the extent to which this phosphorylation encompasses the five sites on ribosomal protein S6 that become phosphorylated during viral infection *in vivo*. The fact that under no condition could we observe the phosphorylation of more than two sites per ribosome (Fig. 5), and that 2 mol of phosphate/mol of ribosome was the maximum stoichiometry achieved, make it extremely unlikely the ViPK is the protein kinase responsible for the multiple phosphorylation of S6 *in vivo*. The finding that the major tryptic phosphopeptide phosphorylated *in vitro* corresponds to one phosphorylated *in vivo* (Fig. 7) allows the possibility that ViPK could contribute (along with other protein kinases) to the phosphorylation of ribosomal protein S6 *in vivo*. However, the fact that this major tryptic phosphopeptide phosphorylated *in vitro* by ViPK is also phosphorylated by the cyclic AMP-dependent protein kinase (Parker *et al.*, 1985; Katan, 1985) argues against a unique contributory role for ViPK.

If the ViPK does not appear a strong candidate for the S6 kinase of viral infection, what of the other kinases in BHK cell extracts that can use ribosomal protein S6 as a substrate? The cyclic AMP-dependent protein kinase can phosphorylate two sites on the protein (Wettenhall *et al.*, 1982), only one of which may be a substrate *in vivo* (Wettenhall & Morgan, 1984). Hence this kinase can also, at best, only be assigned a contributory role, even though increases in the concentration of cyclic AMP in cells infected with herpes virus hominis (Bittlingmaier *et al.*, 1977) or pseudorabies virus (Kennedy, 1982) have, in fact, been observed.

The other protein kinase that we detected in BHK cells with the ability to phosphorylate ribosomal protein S6 is protein kinase C (Nishizuka, 1983). We have previously shown (Parker *et al.*, 1985) that, at least *in vitro*, protein kinase C from bovine brain can phosphorylate five sites on ribosomal protein S6; and we have found that this phosphorylation includes most of the tryptic phosphopeptides of Fig. 7(*a*) (Katan, 1985). The key question is therefore whether protein kinase C is activated during viral infection *in vivo*. We cannot give a definite answer here, for we have not determined whether there is increased phospholipid turnover in infected cells, as would be required to generate the diacylglycerol thought to activate protein kinase C *in vivo*. However, we can say that viral infection does not cause a translocation of protein kinase C from the cytoplasm to the cell membrane (Katan *et al.*, 1985; Katan, 1985), as is frequently found when the enzyme is activated (Anderson *et al.*, 1985).

Our results therefore leave open the possibility that the protein kinase responsible for the phosphorylation of ribosomal protein S6 in virus-infected cells remains undetected. This could have been due to the conditions chosen for assay, which, for example, would not have allowed detection of calmodulin-dependent protein kinases, some of which have been reported to phosphorylate ribosomal protein S6 *in vitro* (Gorelick *et al.*, 1983). Alternatively, the S6 kinase may have been inactivated during the isolation of the cellular extract. The recent detection of an S6 kinase in extracts of cells, previously stimulated by serum, was achieved by the judicious use of phosphatase inhibitors (Novak-Hofer & Thomas, 1984, 1985). This suggests an avenue for future approaches to the problem.

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