Characteristics of the Induction of a New Protein Kinase in Cells Infected with Herpesviruses

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SUMMARY

The appearance of a recently described protein kinase activity (virus-induced protein kinase, ViPK) has been studied during infection of hamster fibroblasts with pseudorabies virus or with herpes simplex virus type 1 (HSV-1). An enzyme activity with comparable catalytic properties was induced in both cases, and had broadly similar kinetics of appearance to that of the viral DNA polymerase. The amount of active ViPK detected depended on the multiplicity of infection, and no ViPK was induced after the viruses had been subjected to irradiation with u.v. light. When cells were infected with the tsK mutant of HSV-1, ViPK was induced at the permissive but not at the restrictive temperature. The ViPK preparations obtained from cells infected with each virus differed in chromatographic properties on anion-exchange and gel-permeation resins. These results indicate that expression of the viral genome is required for induction of ViPK. They suggest that the enzyme may be encoded by the viral genome, but do not provide proof of this.

INTRODUCTION

During the infection of cells by herpesviruses there is a change in the profile of phosphorylated proteins, encompassing both proteins absent from uninfected cells, generally virus-coded proteins (Stevely, 1975; Pereira et al., 1977; Marsden et al., 1978; Wilcox et al., 1980), and pre-existing host cell proteins (Fenwick & Walker, 1979; Kennedy et al., 1981). There is as yet no direct evidence that any of these phosphorylations is of functional importance in the life-cycle of herpesviruses, but the diversity of the regulatory systems involving protein phosphorylation would suggest that this is highly likely.

The protein phosphorylations occurring during viral infection could, in principle, be catalysed by pre-existing active protein kinases of the host cell, or by protein kinases present or active only in infected cells. We have been directing our attention towards the latter category of enzyme, and have recently reported the partial purification and biochemical characteristics of a new protein kinase (virus-induced protein kinase, ViPK) isolated from the cytosol of hamster fibroblasts infected with pseudorabies virus (PRV) (Katan et al., 1985; Katan, 1985). This enzyme catalyses the transfer of phosphate from ATP (but not GTP) to the seryl residues of basic (but not acidic) proteins, and its activity is not dependent upon molecules that can serve as effectors for the well characterized cellular protein kinases. The feature of the ViPK that perhaps most strikingly distinguishes it from these latter enzymes is a KCl optimum of approx. 0.5 M.

The induction of the enzyme during viral infection, and the difference in its properties from known cellular protein kinases, raised the possibility that the ViPK might be encoded by the viral genome. As a first step towards answering this question (which may ultimately require genetic methods for its resolution), we have examined the relationship of the induction of the ViPK to the virus life cycle, comparing cells infected with PRV with those infected with herpes simplex virus type 1 (HSV-1).

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METHODS

Materials. General laboratory chemicals were of analytical grade, where appropriate, and obtained from standard commercial suppliers unless otherwise indicated. Protamine sulphate and cycloheximide were from Sigma; DEAE-Sephacel, Sephacryl S-200 and the Mono Q column were from Pharmacia; $^{33}$P, was from Amersham and was used to synthesize $[\gamma-^{32}P]ATP$ according to Maxam & Gilbert (1980).

Viruses. Pseudorabies virus was originally derived from a stock preparation (Kaplan & Vatter, 1959) and has been plaque-purified several times. Virus stocks were prepared from infected BHK monolayer cultures as previously described (Chantler & Stevely, 1973). The tsK mutant of HSV-1 Glasgow strain 17 (Marsden et al., 1976), and the parent strain, were kindly provided by Dr J. Macnab, MRC Virology Unit, Institute of Virology, University of Glasgow.

Irradiation of viruses. Inactivation by u.v. light was as follows. Stock virus was diluted tenfold with phosphate-buffered saline containing 1% (w/v) glucose. Aliquots (2.5 ml) received 5 kJ/m² of radiation in 50 mm Petri dishes under a UVSL-58 Mineralight (Ultra-Violet Products, San Gabriel, Ca., U.S.A.). Dosage was calculated from the distance between source and sample and the time of irradiation.

Cells and infection. BHK-21/C13 cells were maintained in monolayer cultures in modified Eagle’s medium containing 10% calf serum, and infected at or just before confluence with PRV at a multiplicity of approximately 20 p.f.u./cell, or HSV-1 at a multiplicity of approximately 10 p.f.u./cell, unless otherwise indicated. The times of infection were 8 h for PRV and 18 h for HSV-1, except where stated otherwise.

Preparation and anion-exchange chromatography of cellular post-ribosomal supernatant. The post-ribosomal supernatant of BHK cells was prepared and subjected to chromatography on DEAE-Sephacel in a 2.5 × 1 cm column, eluting with a linear gradient of 0 to 0.4 M-KCl exactly as previously described (Katan et al., 1985).

Assay of protein kinase activity. The standard assay mixture for protein kinase activity contained, in a total volume of 0.12 ml, 20 mM-Tris–HCl pH 7.4, 50 mM-KCl, 10 mM-MgCl₂, 10 mM-2-mercaptoethanol, 0.1 mM-ATP containing 0.5 to 1 μCi ($[\gamma-^{32}P]ATP$, and protamine sulphate (0.8 mg/ml). The protein kinase (40 μl) was then added and incubation was at 30 °C for 30 min. At the end of the incubation 100 μl samples were spotted onto Whatman 3MM paper discs which were washed for 15 min periods twice in 20% TCA, four times in 10% TCA, and then rinsed in absolute ethanol, dried, and their radioactivity was measured by scintillation spectrometry.

In some cases the products of the phosphorylation reaction were analysed by one-dimensional gel electrophoresis in the presence of SDS, performed according to Parker et al. (1985).

Assay of DNA polymerase. This was performed according to Weissbach et al. (1973).

Determination of minimum concentration of cycloheximide for inhibition of protein biosynthesis. This was performed according to Leader & Barry (1971). The percentage inhibitions at 0.2 μg, 0.6 μg, 2 μg, 6 μg and 20 μg cycloheximide per ml were 74%, 88%, 94%, 95% and 92%, respectively.

RESULTS

ViPK is induced by infection with PRV or HSV-1

It is necessary at the outset to explain how we identify and quantify ViPK. In order to distinguish it from other protein kinases (e.g. protein kinase C) that can also phosphorylate protamine (the preferred artificial substrate of ViPK), we routinely subject the post-ribosomal supernatant of infected cells to anion-exchange chromatography and assay the protein kinase activity of column fractions. As previously reported (Katan et al., 1985) the ViPK from BHK cells infected with PRV elutes at a higher ionic strength (approx. 220 mM on DEAE-cellulose) than the other protamine kinases, i.e. protein kinase C (50 to 80 mM) and the so-called protein kinase M, its presumed proteolytic derivative (approx. 150 mM). The relative proportions of protein kinase C and 'protein kinase M' vary in different experiments (compare Fig. 1 a and c) because the stability of protein kinase C depends on the concentration of protein applied to the column. However, we stress that viral infection does not influence the yield or relative proportion of these latter enzymes, and that we have previously excluded the possibility that ViPK might be a proteolytic cleavage product of protein kinase C (Katan et al., 1985).

Fig. 1 (a, b) illustrates the induction of ViPK 8 h after infection of BHK cells with PRV, the chromatography on DEAE-Sephacel rather than DEAE-cellulose resulting in elution of the enzyme at a slightly higher ionic strength than that cited above. A new protamine kinase activity was also found in cells infected for 18 h with HSV-1, although this eluted at a somewhat lower ionic strength than the enzyme from cells infected with PRV, and hence it was incompletely resolved from the peak of 'protein kinase M'. In view of this difference (further considered below) it was necessary to determine the extent to which these enzymes had similar catalytic
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properties. In addition to their preference for protamine as an artificial substrate, two features argue their similarity. Firstly, they were both active at concentrations of KCl up to 1 M (Fig. 2), and secondly they were both able to phosphorylate ribosomal protein S7 on 40S ribosomal subunits in vitro (Fig. 3), a distinguishing characteristic documented in more detail elsewhere in the case of PRV (Katan, 1985). In particular we stress that protein kinase C does not have these characteristics (Parker et al., 1985). The preparation of ViPK from cells infected with HSV-1 also phosphorylated ribosomal protein S6 (Fig. 3), but we have not excluded the possibility that this may be a consequence of cross-contamination with 'protein kinase M' rather than an intrinsic difference from the enzyme isolated from cells infected with PRV. It should also be added that the phosphorylation of 40S ribosomal subunits by the enzymes from cells infected with either HSV-1 or PRV was affected similarly by high ionic strength or 10 mM-spermine (see Katan, 1985).

As well as evidence in support of the similarity of the two enzymes, these characteristics have been used routinely to confirm that chromatographic fractions, designated in this paper as containing ViPK, do indeed contain this particular protein kinase.

Correlation of induction of ViPK with viral infection

We examined the time at which ViPK appeared in cells infected with PRV (Fig. 4). There was a steady increase in activity during the virus growth cycle that roughly paralleled that of the β or early protein, viral DNA polymerase, and preceded the release of virus from cells. (The relationship of ViPK and HSV-1 protein synthesis is addressed in Fig. 8, below.) The amount of ViPK detected depended on the multiplicity of infection with either PRV or HSV-1 (Fig. 5), although there was a decreased yield with PRV at the highest multiplicities. Similar effects at high multiplicities have been observed for other enzymes induced by HSV (Perera, 1970).
Fig. 2. Effect of KCl on the activity of ViPK from BHK cells infected with herpesviruses. Partially-purified ViPK preparations obtained by chromatography on DEAE Sephacel (Fig. 1) were used to phosphorylate protamine at the concentrations of KCl indicated. Infection was with PRV (●) or HSV-1 (■).

Fig. 3. Phosphorylation of ribosomal proteins by ViPK from BHK cells infected with herpesviruses. Partially purified ViPK preparations obtained by chromatography on DEAE-Sephacel (Fig. 1) were used to phosphorylate rat liver 40S ribosomal subunits in vitro, and these were then subjected to SDS-PAGE (see Methods). (a) ViPK from cells infected with PRV; (b) ViPK from cells infected with HSV-1. An autoradiograph of the dried gel is illustrated.

Subcellular location of ViPK

Our assays of ViPK have been on the cytoplasmic enzyme. This was because we have found no ViPK in the particulate fraction of cells infected with PRV. We have previously described some of the protein kinase activities present in virions of PRV (Stevely et al., 1985), and these include a peak of protamine kinase activity eluting from DEAE-cellulose at approx. 200 mM-KCl (see Fig. 4 in Stevely et al., 1985). This is most likely ViPK, but we would stress that, if this is so, only a minor portion of ViPK is associated with the virion, and that ViPK is not enriched in the virion relative to other protein kinases (among which, cellular casein kinase II and protein kinase C have been tentatively identified).

Requirement for viral gene expression in the induction of ViPK

The induction of ViPK was prevented if cells infected with PRV were treated with doses of cycloheximide just sufficient to produce maximum inhibition of protein biosynthesis (Fig. 6), or if the virus was inactivated by irradiation with u.v. light (Fig. 7). The results of these
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Fig. 4. Temporal relationship of appearance of ViPK to other events during the infection of BHK cells with PRV. BHK cells were infected with PRV as described in Methods, and the post-ribosomal supernatants isolated at the times indicated. The major portions of these were subjected to chromatography on DEAE-Sephacel as in Fig. 1, and column fractions assayed for protein kinase activity. The total ViPK activity (●) was estimated and expressed as a percentage of the maximum value, as was the DNA polymerase activity (○) assayed on a small portion of the original post-ribosomal supernatant. (The initial DNA polymerase activity is that of the host enzyme.) The number of p.f.u. of virus in the growth medium was also determined (■).

Fig. 5. Effect of multiplicity of infection with PRV (●) or HSV-1 (○) on the yield of ViPK. BHK cells were infected for 8 h with PRV or 18 h with HSV-1 at the multiplicities indicated; ViPK was isolated and quantified as described in the legend to Fig. 4.

Fig. 6. Effect of cycloheximide on the induction of ViPK in BHK cells infected with PRV. Cells were infected with PRV in the absence (a) or presence (b) of cycloheximide (2 µg/ml) and harvested 8 h later. Their post-ribosomal supernatants were isolated, subjected to chromatography on DEAE-Sephacel and fractions, the KCl concentrations of which are indicated (---) were assayed for protein kinase activity (●) as described in Methods. Addition of cycloheximide (2 µg/ml) to the assay had no effect on the ViPK activity.

Fig. 7. Effect of irradiation with u.v. light on the ability of PRV to cause the induction of ViPK in BHK cells. Cells were infected for 8 h with 20 p.f.u./cell PRV (a), or with an equivalent amount of virus that had been subjected to 5 kJ/m² u.v. radiation (b). The post-ribosomal supernatants were isolated, subjected to chromatography on DEAE-Sephacel and fractions, the KCl concentrations of which are indicated (---), were assayed for protein kinase activity (●).
Fig. 8. Induction of ViPK in BHK cells infected with the tsK mutant of HSV-1. Cells were infected with tsK (10 p.f.u./cell) at either 31 °C (a) or 38.5 °C (b), or with the parent wild-type virus at 38.5 °C (c), and harvested 18 h later. The post-ribosomal supernatants were isolated, subjected to chromatography on DEAE-Sephacel using a somewhat shallower gradient than elsewhere, and fractions, the KCl concentrations of which are indicated (---), were assayed for protein kinase activity (●). The DNA polymerase activity of a portion of the post-ribosomal supernatants was also determined, confirming that there was no escape into the synthesis of early viral proteins at 38.5 °C (the restrictive temperature).

experiments are consistent with there being a need for viral gene expression for the induction of ViPK, although neither technique used in the experiments is without its limitations. In the case of HSV-1 a less equivocal approach was possible because of the availability of temperature-sensitive mutants. We used tsK, in which a mutation in the 175000 mol. wt. immediate-early protein, ICP4, prevents the synthesis of mRNA for the early and late proteins (Watson & Clements, 1980). It can be seen from Fig. 8 that ViPK was induced at the permissive temperature (31 °C) but not at the restrictive temperature (38.5 °C), although the enzyme was induced by infection with the parent virus at the latter temperature. It should be noted that the amount of kinase detected in this experiment was less than in other experiments because circumstances dictated the use of only $5 \times 10^8$ cells (rather than the $5 \times 10^9$ cells of the experiment of Fig. 1).

**Different physical characteristics of ViPKs**

The different chromatographic properties on DEAE-Sephacel of ViPKs from cells infected with each of the viruses (Fig. 1) could indicate that the two enzymes are distinct, or it might
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Fig. 9. Resolution of ViPK activities by chromatography on Mono Q. Equal activities of ViPK, partially purified from BHK cells infected with PRV or HSV-1 by chromatography of post-ribosomal supernatants on DEAE-Sephacel (Fig. 1) were combined and subjected to chromatography on Mono Q. Fractions, the NaCl concentrations of which are indicated (---), were assayed for protein kinase activity (○). The absorbance at 280 nm (---) is also shown. The inset shows the result of phosphorylation of 40S ribosomal subunits by the ViPK in peaks 1 and 2 performed as described in the legend to Fig. 3.

merely be an artefact caused by non-specific association of an identical molecule with different viral proteins. To investigate this point further, we mixed preparations of ViPK isolated by DEAE-Sephacel chromatography of cells infected with PRV or HSV-1, and subjected these to rechromatography on the high-performance Mono Q anion-exchange column. This resolved the mixture into two peaks of activity (Fig. 9), and these eluted at positions similar to the individual preparations analysed separately, ViPK from cells infected with HSV-1 again eluting at lower ionic strength (results not shown). Furthermore, the first peak of activity showed the same ability to phosphorylate both ribosomal proteins S6 and S7 at moderate ionic strength exhibited by cruder preparations from cells infected with HSV-1 (Fig. 3), whereas under the same conditions the second peak of activity only catalysed the phosphorylation of ribosomal protein S7 (see inset).

Because the enzymes from the two sources showed apparently genuine differences in anion-exchange chromatography, we subjected them (separately) to gel-permeation chromatography on Sephacryl S-200 (Fig. 10). The apparent relative molecular mass (approx. 200000) for ViPK from cells infected with HSV-1 was much greater than that (approx. 90000) from cells infected with PRV. [This latter value is somewhat greater than that of 68000 obtained previously by three different methods (Katan et al., 1985), perhaps reflecting different behaviour of the enzyme on this resin.] These values, especially that for the enzyme from cells infected with HSV-1, may, of course, reflect different oligomeric structures in the two cases, and the subunit size could be quite similar. However, the fact that the chromatography was conducted at 0.5 M-KCl would argue against the differences being due to non-specific aggregation.
Fig. 10. Gel-permeation chromatography of ViPKs on Sephacryl S-200. Partially purified preparations (see Fig. 1) of ViPK from BHK cells infected with PRV (○) or HSV-1 (■) were separately subjected to chromatography on Sephacryl S-200 in a buffer containing 500 mM-KCl, 1 mM-EDTA, 10 mM-2-mercaptoethanol, 20 mM-Tris-HCl pH 7.6. The positions of elution of proteins of known relative molecular mass (Mr) are indicated, and the inset shows a semilogarithmic plot of $M_r \times 10^{-3}$ against $K_v [(V_e - V_0)/(V_t - V_0)]$, where $V_e$ is the elution volume, $V_0$ the void volume and $V_t$ the column volume.

One trivial explanation of the different physical properties of the ViPK preparations is partial proteolytic cleavage of one of the enzymes during extraction from the cells and subsequent chromatography. However, when isolation was performed in the presence of protease inhibitors (2 mM-EGTA, 40 μg/ml phenylmethylsulphonyl fluoride, 100 μg/ml leupeptin) no difference in the position of elution from DEAE-Sepharose was observed (results not shown). Specific intracellular partial proteolysis cannot, however, be excluded (see Discussion).

DISCUSSION

We have previously shown on the basis of its catalytic properties that ViPK is distinct from known cellular protein kinases (Katan et al., 1985). The present study demonstrates that the appearance of ViPK in cells infected with PRV or HSV-1 both requires and correlates with the expression of the viral genome. Thus, there can be little doubt that ViPK must play a role in the interaction of these alpha herpesviruses with their host cells. This is a new finding of considerable potential importance.

The appearance in infected cells of new proteins dependent on viral gene expression does not necessarily indicate that the proteins are viral. Examples of cellular proteins in this category include stress proteins (Notarianni & Preston, 1982) and the proteins induced by interferon (Friedman et al., 1984), although these are unlikely to be relevant in this particular case. Comparison of the SDS–polyacrylamide gel mobilities of similar proteins from different strains of HSV has frequently been used to provide an indication that these are of viral origin. As we have not yet obtained homogeneous preparations of ViPK that would allow us to identify them on polyacrylamide gels, we were obliged to adopt a modification of this strategy using alpha herpesviruses that infect different species and comparing the elution of enzyme activity on different chromatographic media (Fig. 9 and 10). This approach has the limitation that interaction of the kinase with other proteins could affect its chromatographic behaviour. Nevertheless, the results of the co-chromatography on Mono Q (Fig. 9) provide a strong indication that the ViPK activities from cells infected with each of the two viruses represent
different species. The simplest interpretation of this result is that the ViPK is encoded by the viral genome.

Further experiments are nevertheless required before a viral origin for the enzyme can be considered absolutely proven. For example, it could be argued that the two different ViPK species might arise from proteolytic activation of an inactive cellular protein kinase catalysed by viral proteases (Dierich et al., 1979) of different cleavage specificity. Although such a possibility may appear unlikely, it is important that we obtain rigorous immunochemical and genetic evidence before concluding that ViPK is a virus-encoded protein kinase. Apart from the protein kinases of oncogenic retroviruses, no viral protein kinase has yet been reported that meets these criteria.

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