The ULI3 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase

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Herpes simplex virus type 1 (HSV-1) induces a protein kinase (PK) activity in infected cell nuclei. In vitro, the enzyme is able to phosphorylate exogenous casein (albeit inefficiently) but not protamine, can use ATP or GTP as a phosphate donor, is stimulated by high salt concentrations and is insensitive to inhibition by heparin. On the basis of these properties, the PK appears to be distinct from previously described cellular enzymes and from the cytoplasmic PK encoded by the viral US3 gene. A major substrate of the enzyme in vitro is a virus-induced protein with an Mr of 57000 (Vmw57). The gene encoding Vmw57 was mapped using recombinants between HSV-1 and HSV-2 to a region of the virus genome containing genes UL9 to UL15. Use of a monospecific rabbit antiserum showed that Vmw57 is a virion structural protein encoded by gene UL13. These results, in conjunction with previous reports that the UL13 protein contains PK sequence motifs, support the notions that the nuclear PK and Vmw57 are identical, and that the observed reactivity is due to autophosphorylation.

Introduction

Protein kinase (PK) activity is associated with several herpesviruses (Rubenstein et al., 1972; Randall et al., 1972; Tan, 1975; Lemaster & Roizman, 1980; Blue & Stobbs, 1981; Flügel & Darai, 1982; Michelson et al., 1984; Katan et al., 1985, 1986; Stevely et al., 1985; Montalvo & Grose, 1986; Purves et al., 1986). The most extensively characterized examples are cytoplasmic PKs induced after infection of cells by the alphaherpesviruses herpes simplex virus type 1 (HSV-1) and pseudorabies virus (reviewed by Leader & Katan, 1988). The HSV-1 PK can utilize ATP but not GTP to phosphorylate serine or threonine residues in basic substrates such as protamine, but not acidic substrates such as casein. It is active in high salt concentrations and appears to be insensitive to effectors. The denatured subunit detected in autophosphorylation experiments has an Mr of 68000.

DNA sequence data have provided additional strong evidence that alphaherpesviruses encode distinct PKs. Several motifs characteristic of PKs have been identified in the predicted translation products of HSV-1 gene US3 and its counterpart, gene 66, in varicella-zoster virus (VZV) (McGeoch & Davison, 1986). Two reports have demonstrated unequivocally that the HSV-1 cytoplasmic PK is the product of US3. Frame et al. (1987) have shown that a polyclonal rabbit antiserum directed against a peptide containing the eight amino acid residues at the carboxy terminus of the US3 protein reacts specifically with an HSV-1-induced protein with an Mr of 68000. It also recognizes a protein of identical Mr, in an extensively purified preparation of the HSV-1 PK, which could be phosphorylated by incubation with [γ-32P]ATP. Purves et al. (1987) have shown that an HSV-1 mutant in which part of the US3 coding region has been deleted does not induce the enzyme, in contrast to wild-type virus and a derivative of the mutant in which US3 is reinserted. The role of the US3 cytoplasmic PK in virus infection is unknown, but it is not required for lytic growth of virus in cell culture.

Smith & Smith (1989) and Chee et al. (1989) have noted the presence in the HSV-1 genome of a second gene encoding a protein with PK sequence motifs. This gene, UL13, has counterparts not only in other alphaherpesviruses such as VZV (Davison & Scott, 1986; McGeoch et al., 1988), but also in betaherpesviruses, such as human cytomegalovirus (Chee et al., 1990) and human herpesvirus 6 (Lawrence et al., 1990), and in the gammaherpesvirus Epstein–Barr virus (Baer et al., 1984). This is in contrast to the US3 PK, which has counterparts only in the alphaherpesviruses. The protein specified by UL13 has a predicted Mr of 57193 (McGeoch et al., 1986, 1988) and, like the US3 PK, has sequence motifs indicating that it has the ability to
phosphorylate serine and threonine, rather than tyrosine, residues.

In this paper, we report the properties of a novel HSV-1 PK, and show that a major substrate of the enzyme is a virion structural protein encoded by UL13. We discuss the possibility that the latter protein is identical to the PK.

Methods

Preparation of nuclear and virion extracts. Baby hamster kidney (BHK) C13 cells were either mock-infected or infected (usually for 8 h) with HSV-1 strain 17 (Brown et al., 1973), HSV-2 strain HGS2 (Timbury, 1971) or HSV-1/HSV-2 intertypic recombinants (Marsden et al., 1978; Chartrand et al., 1981) at a multiplicity of 20 in Eagle’s medium containing 2% (v/v) foetal calf serum (FCS).

Proteins were extracted from nuclei using salt washes and ammonium sulphate precipitation as described by Piette et al. (1985). Precipitated proteins were resuspended in 0.4 ml PK assay buffer (50 mM-Tris-HCl pH 8.0, 50 mM-magnesium acetate, 0.5 M-NaCl, 0.1% (v/v) NP40, 1 mM-DTT) and stored at −70 °C.

Virion extracts were prepared as described by Whittaker & Meredith (1990). HSV-1 virions were prepared by sucrose density centrifugation, and their purity was ascertained by SDS-PAGE. Virions were treated with ether, extracted with 1% (w/v) 3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulphphonate for 5 min at 0 °C, centrifuged at 12500 g for 10 min through a 35% (w/v) sucrose cushion and the supernatant was retained.

PK assay. Nuclear extract (5 to 25 μl containing approximately 0.1 to 0.5 μg protein) was incubated for 20 min at 37 °C with 50 μl PK assay buffer containing 5 μCi [γ-32P]ATP or [γ-32P]GTP (Amersham; PB10218 or PB10244) in a total volume of 100 μl. NaCl was included at a concentration of 1 M in earlier experiments, and in later experiments at 1.5 M. Casein, protamine or heparin were added in experiments at 1.5 M.

In vitro transcription reactions were carried out for 2 h at 37 °C in 100 μl volumes containing 2 μg linearized plasmid, 40 mM-Tris-HCl pH 7.5, 6 mM-MgCl₂, 2 mM-stermiphenidimine, 10 mM-NaCl, 2.5 mM of each NTP (Promega), 100 units (U) RNasin (Boehringer Mannheim), 2 μCi [γH]UTP (Amersham; TRK412) and 400 U T7 RNA polymerase (Bethesda Research Laboratories). The yield of RNA was estimated by measuring radioisotope incorporation. Synthesized RNA was extracted using 1:1 (v/v) phenol:chloroform, ethanol-precipitated, dissolved in water at 2 mg/ml and stored at −20 °C.

Preparation of polyclonal antisera against the UL13 protein. Amino acid residues 149 to 207 of the UL13 protein were expressed as a fusion protein in the carboxy terminus of Escherichia coli β-galactosidase. This region contains PK motifs I and II (Smith & Smith, 1989). A 3-kbp XhoI fragment of KpnI containing the carboxy-terminal portion (codons 149 to 518) of UL13 was inserted in the appropriate orientation into the Sall site of the fusion protein expression vector pUR288 (Rüther & Müller-Hill, 1983). It was found that the fusion protein specified by this plasmid was degraded rapidly. An alternative construction was made by digesting the plasmid with XbaI and SstII, thus removing codons 208 to 518, treating with T4 DNA polymerase in the presence of the four dNTPs and ligating. A clone containing the appropriate plasmid was selected. The fusion protein generated after induction with 1 mM IPTG was insoluble, and therefore could not be purified by affinity chromatography. Instead, extracts of induced bacteria were prepared by lysozyme treatment and sonication, and insoluble material containing the fusion protein was pelleted by centrifugation. Proteins were separated by SDS-PAGE, and the fusion protein was visualized by treatment of the gel with 1 M-KCl, excised and electrophoresed in 5 to 15% SDS-polyacrylamide gels. The extracted fusion protein was lyophilized, washed with acetone, resuspended in PBS and stored at −70 °C.

Two sandalplo rabbits were immunized with purified fusion protein by Serotec Ltd., Oxford, U.K. as follows. Initially, preimmune sera were obtained and each rabbit was inoculated subcutaneously with 40 μg protein in Freund's incomplete adjuvant. On day 28, sera were prepared and each rabbit was boosted with 120 μg protein in Freund's complete adjuvant. Further sera were obtained on days 35 and 49, and the rabbits were boosted with 40 μg protein each on days 43 and 63. Final sera were prepared on day 70.
Immune precipitation reactions. Immune precipitation reactions were carried out essentially as described by Zweig et al. (1980). In some experiments, antisera were preincubated with exogenous proteins for 2 h at 4 °C. Precipitated proteins were subjected to SDS-PAGE.

Gel electrophoresis of proteins. SDS-PAGE was conducted using 10% or 12% (w/v) polyacrylamide gels cross-linked with 1:37.5 (w/w) N,N'-tetramethylene-bisacrylamide or 1:57 (w/w) N,N'-diallyltartardiamide. Gels from immune precipitation experiments were fixed in 10% (v/v) acetic acid, 50% (v/v) methanol, treated with En3Hance (New England Nuclear), dried and autoradiographed using Kodak XS-6 film with a Dupont Cronex Lightning Plus intensifying screen at −70 °C. Mr standards were included in all gels.

Results

Induction of a nuclear PK by HSV-1

Endogenous protein phosphorylation was studied in nuclear extracts of mock- or HSV-1-infected cells. Nuclear PK activity was measured at various times after infection by monitoring incorporation of 32P from [γ-32P]ATP into TCA-precipitable material (Fig. 1a). The level of activity in mock-infected cells was indistinguishable from the background, but enzyme activity in HSV-1-infected cells increased until about 5 h after infection and then remained approximately constant. SDS–PAGE of the samples showed that nuclear extracts of HSV-1-infected cells were able to phosphorylate a major endogenous protein species with an apparent Mr of 57000 (Vmw57; Fig. 1b, lanes 5 to 8). (The estimated Mr depended on the cross-linker used in polyacrylamide gels; when N,N'-diallyltartardiamide was used instead of N,N'-methylene-bisacrylamide it was 51000.) In contrast, very little PK activity was detected in nuclear extracts prepared from mock-infected cells (Fig. 1b, lanes 1 to 4). When cells were infected with HSV-1 and labelled with 32p in vivo, a minor phosphoprotein with the same Mr, as Vmw57 was detected in nuclear extracts (Fig. 1c).

Biochemical properties of the nuclear PK

The PK which phosphorylates Vmw57 was characterized with respect to salt dependence, substrate specificity, phosphate donor and inhibition by heparin. Phosphorylation was detected in the presence of NaCl at concentrations ranging from 0 to 2.5 mM, but was optimal at 1.5 mM (Fig. 2a, lanes 8 to 14). A concentration of 1.5 mM was used in subsequent assays. A cellular PK able to phosphorylate exogenous casein (Mr, 23600) in the presence of 1.5 mM-NaCl was also detected (Fig. 2b, lane 3). This activity was rather variable in different experiments, and was optimal at 0.2 mM-NaCl (data not shown). The substantial enhancement of casein kinase activity in infected cells indicates that the virus-induced PK is able to phosphorylate casein (Fig. 2b, lane 4). Phosphorylation of Vmw57 was also stimulated moderately by casein and markedly by protamine (Fig. 2b, lanes 4 and 6). Protamine, which has an Mr of less than 10000, was shown not to be phosphorylated using polyacrylamide gels (data not shown) of higher concentration. The HSV-1-induced PK was able to use ATP or GTP as a phosphate donor (Fig. 2b, lanes 2 and 8), and was insensitive to inhibition by heparin at concentrations up to 25 µg/ml (Fig. 2c).
Fractionation of the nuclear PK on DNA–cellulose

The nuclear location of the PK suggested a possible interaction with DNA, so nuclear extracts of HSV-1-infected cells were fractionated further by chromatography on DNA–cellulose. Fractions were eluted with a 0·1 to 1·0 M-NaCl gradient and assayed for PK activity in the presence of 1·5 M-NaCl. The fraction that eluted at approximately 0·5 M-NaCl gave maximal incorporation of $^{32}$P into TCA-precipitable material (Fig. 3a). The activity in this fraction was assayed at NaCl concentrations ranging from 0·3 to 2·0 M in the presence of exogenous casein, and phosphorylated proteins were examined by SDS–PAGE (Fig. 3b). The eluted PK promoted phosphorylation of Vmw57 and casein to a similar degree at each NaCl concentration. The optimal NaCl concentration was similar to that observed previously in unfractionated nuclear extracts. Coomassie blue staining indicated that the elution profile of total Vmw57 was identical to that of Vmw57 which could be phosphorylated, and that several proteins were present in the peak fraction (data not shown). Despite the last point, the results imply that Vmw57 and the virus-induced PK co-elute from DNA–cellulose. No activity capable of phosphorylating Vmw57 or casein was eluted when nuclear extracts from mock-infected cells were subjected to DNA–cellulose chromatography (data not shown).

Viral origin of Vmw57

Nuclear extracts were prepared from HSV-2-infected cells and assayed for activity in the presence of 1·5 M-

Fig. 2. Biochemical properties of the nuclear PK. (a) Effect of NaCl. Nuclear extracts of mock- (lanes 1 to 7) or HSV-1-infected (lanes 8 to 14) cells were labelled in vitro in the presence of 0·1, 0·2, 0·5, 1·0, 1·5, 2·0 or 2·5 M-NaCl, respectively. Samples were subjected to SDS–PAGE and the gel was autoradiographed. (b) Effect of exogenous substrates. Nuclear extracts of mock- (lanes 1, 3, 5 and 7) or HSV-1-infected (lanes 2, 4, 6 and 8) cells were labelled in vitro in the presence of $[\gamma^{32}P]ATP$ (lanes 1 and 2), $[\gamma^{32}P]ATP$ plus 1 µg casein (lanes 3 and 4), $[\gamma^{32}P]ATP$ plus 1 µg protamine (lanes 5 and 6) or $[\gamma^{32}P]GTP$ (lanes 7 and 8). All reactions included 1·5 M-NaCl. Samples were subjected to SDS–PAGE and the gel was autoradiographed. The position of casein is indicated by a vertical line. (c) Effect of heparin. PK activity in the presence of 1·5 M-NaCl was monitored by TCA precipitation.

Fig. 3. Fractionation of the nuclear PK on DNA–cellulose. (a) Fractions were eluted from DNA–cellulose using a 0·1 to 1·0 M-NaCl gradient, and PK activity in the presence of 1·5 M-NaCl was monitored by TCA precipitation. (b) Autoradiograph showing proteins phosphorylated in vitro by incubation of the peak fraction from (a) in the presence of 5 µg casein and 0·3, 1·0, 1·5 or 2·0 M-NaCl (lanes 1 to 4, respectively).
The observation that the major phosphorylated species had a slightly greater Mr than HSV-1 Vmw57 (Fig. 4b, compare lanes 1 and 3) indicates that the protein is virus-encoded. A series of HSV-1/HSV-2 intertypic recombinants was analysed in order to identify the region of the HSV-1 DNA molecule encoding Vmw57. The genome structures of the recombinants are shown in Fig. 4(a). Nuclear extracts of cells infected with each recombinant were prepared, and the Mr’s of major phosphorylated species were compared with those of cells infected with HSV-1 or HSV-2 (Fig. 4b). This analysis revealed that Vmw57 maps to a region between the HSV-1 BamHI c-a (defined in R12-4) and BglII o-p sites (defined in RE6), which contains genes UL9 to UL15 (McGeoch et al., 1988).

**Characterization of a rabbit antiserum against the HSV-1 UL13 protein**

Of the primary translation products of genes UL9 to UL15, that of UL13 is nearest in Mr to Vmw57. To ascertain whether UL13 encodes Vmw57, antisera were raised in two rabbits which had been inoculated with a fusion protein of β-galactosidase linked to amino acid residues 149 to 207 of the predicted UL13 protein. As a substrate for testing the antisera, UL13 was transcribed and translated in vitro. When pTZUL13(Kpn) was cut downstream from UL13 using NcoI, a UL13-specific protein with an apparent Mr of 57000 was produced (Fig. 5a, lane 1). When pTZUL13(Xho) was cut downstream from UL13 using EcoRI, two UL13-specific proteins were detected. One corresponded in size to that produced by pTZUL13(Kpn) and comigrated with Vmw57 (Fig. 5a, lane 2; compare Fig. 5c, lanes 1 and 2). The other had a greater Mr, and was presumably the result of translation initiation at the upstream in-frame ATG codon present in the multiple cloning site. When pTZUL13(Xho) was cut within UL13 using XhoI, neither UL13-specific protein was produced; instead, truncated proteins with Mr’s of approximately 18000 were noted (Fig. 5a, lane 3).

Antiserum obtained from one rabbit 49 days after the initial inoculation precipitated the UL13-specific proteins, whereas preimmune serum did not (Fig. 5b, lanes 1 to 3, 5 and 7; Fig. 5c, lanes 3 and 4); preincubation with purified UL13 fusion protein inhibited antibody reactivity (Fig. 5c, lane 5). These results indicate that one rabbit produced antibodies which reacted specifically with the UL13 protein. Antibodies against the UL13 protein were not detected in sera from the other rabbit. It should be noted that the heavy chains of IgG migrated just above the UL13 proteins and distorted this region in the gels, causing the two UL13-specific proteins to migrate as a single band at a greater rate than in samples not subjected to immune precipitation (compare Fig. 5b, lanes 2 and 6).

**Identification of the UL13 protein as Vmw57**

Nuclear extracts of HSV-1-infected cells were phosphorylated in vitro and immune-precipitated using

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**Fig. 4. Viral origin of Vmw57.** (a) Genome structures of intertypic recombinants between HSV-1 and HSV-2 that were used to locate the region of the genome encoding Vmw57. The HSV genome structure is shown at the top, with horizontal lines and open rectangles denoting unique and inverted repeat regions, respectively. Recombinant structures are shown as solid lines superimposed on dashed lines indicating HSV-1 (upper) and HSV-2 (lower) sequences. Crossover limits are shown as solid vertical lines. The scale shows the fractional genome length. (b) Autoradiograph showing electrophoretic mobilities of HSV-1 Vmw57 (lanes 1, 4, 7, 10 and 13), its counterpart in HSV-2 genome length. (b) Autoradiograph showing electrophoretic mobilities of HSV-1 Vmw57 (lanes 1, 4, 7, 10 and 13), its counterpart in HSV-2 genome length. (b) Autoradiograph showing electrophoretic mobilities of HSV-1 Vmw57 (lanes 1, 4, 7, 10 and 13), its counterpart in HSV-2 genome length.
reactive antisera. Results of the more important of a range of experiments are described below. It should be noted that the pattern of labelling varied between different nuclear extracts. In some, Vmw57 was the major phosphorylated protein detected, but in others a major phosphoprotein with an $M_r$ of 38000 was also detected (e.g. Fig. 5c, lane 1). In addition, minor phosphoproteins were visualized after over-exposure of autoradiographs (Fig. 6a, lane 1). Similar attempts to detect Vmw57 in [$^{35}$S]methionine-labelled cells were not successful (data not shown).

When sera obtained 28, 35, 49 and 70 days after the initial immunization were assayed for their ability to react with $^{32}$P-labelled Vmw57, antibodies were detected in the earliest sample (data not shown). Preincubation of antiserum obtained at 70 days with 2 µg or more of purified UL13 fusion protein inhibited immune precipitation of $^{32}$P-labelled Vmw57 (Fig. 6a, lane 5; b, lanes 2 to 6). Preincubation with PBS (Fig. 6a, lane 4), pure β-galactosidase (Fig. 6a, lane 6) or a fusion protein of β-galactosidase linked to a portion of UL15 (Fig. 6a, lane 7) or UL17 protein (Fig. 6b, lane 7) failed to inhibit binding to Vmw57. The results of these experiments show that an antiserum raised against the UL13 protein reacted specifically with Vmw57, and demonstrate unequivocally that UL13 encodes Vmw57.

Identification of Vmw57 as a virion protein

SDS–PAGE of an extract of purified virions which had been phosphorylated in vitro revealed a major phosphoprotein which comigrated with Vmw57 (Fig. 7, lanes 1 and 10). Like Vmw57, this protein was immune-precipitated by the UL13 antiserum (Fig. 7, lanes 3 and 7), and binding was inhibited by preincubation of the antiserum with the UL13 fusion protein but not with the UL17 fusion protein (Fig. 7, lanes 4, 5, 8 and 9). These results show that Vmw57 is a structural component of the virion, and strongly suggest that a PK responsible for its phosphorylation is also present in virus particles.

Discussion

HSV-1 induces a PK activity in the nuclei of infected cells. The activity is similar to the cytoplasmic PK
encoded by HSV-1 gene US3 characterized previously in that it has the unusual property of being stimulated in vitro by high salt concentrations. However, the two enzymes differ in subcellular location, and in the ability of the nuclear PK to utilize GTP as a phosphate donor and to phosphorylate exogenous casein but not protamine. The nuclear PK has properties somewhat similar to those described for a PK isolated from liver nuclei (Baydoun et al., 1986; Delpech et al., 1986), except that it is significantly less sensitive to inhibition by heparin (Baydoun et al., 1986). Thus, the HSV-1-induced nuclear PK cannot be identified with either the US3 cytoplasmic PK or cell nuclear PKs characterized previously. It may correspond to the virion kinase described by Lemaster & Roizman (1980), but comparative studies to confirm this have not been carried out.

Vmw57 is a major substrate of the nuclear PK in vitro. This protein was shown by analysis of intertypic recombinants to be encoded by one of HSV-1 genes UL9 to UL15, and additional experiments using a specific antiserum showed it to be encoded by the UL13 gene. Moreover, Vmw57 was shown to be present in virions. It is rather more difficult to determine whether the nuclear PK is viral or cellular in origin, since several PK activities that are probably cellular in origin have been detected in HSV-1 virions (Stevely et al., 1985). Our analyses were carried out using partially purified
enzyme, and we have by no means ruled out the possibility that the activity pertains to a cellular enzyme induced by HSV-1 infection rather than to a virus gene product. There are, however, three lines of circumstantial evidence supporting the hypothesis that Vmw57 is the PK and that the observed labelling of Vmw57 in vitro represents autophosphorylation. First, Vmw57 and the PK fractionated together during DNA–cellulose chromatography. Second, virions appear to contain Vmw57 and the PK because virion extracts were able to phosphorylate endogenous Vmw57. Third, the UL13 protein has been predicted to function as a serine–threonine protein kinase on the basis of its primary amino acid sequence (Smith & Smith, 1989; Chee et al., 1989).

Proof that Vmw57 is the PK will involve further extensive studies. One approach is to undertake purification to homogeneity of the activity from HSV-1-infected cells or a heterologous expression system. In preliminary experiments we have been unsuccessful in retaining the PK activity from HSV-1-infected cells in purification steps beyond DNA–cellulose chromatography. Also, we have been unable to isolate a recombinant vaccinia virus expressing the UL13 protein by transcription from a powerful late promoter. We interpret this as indicating that high levels of Vmw57 are lethal to growth of vaccinia virus. An alternative approach to the problem which has met with initial success involves isolating an HSV-1 mutant with a lesion in UL13 (L. Coulter, H. Moss & D. McGeoch, unpublished data). Such a mutant is currently being used to characterize the PK.

References


(Received 5 September 1991; Accepted 6 November 1991)