Characterization of the putative protein kinases specified by varicella-zoster virus genes 47 and 66

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The proteins predicted to be encoded by varicella-zoster virus (VZV) genes 47 and 66 display sequence similarity to the serine/threonine family of protein kinases. Homologues of gene 47 exist in α-, β- and γ-herpesviruses but homologues of gene 66 are specific to the α-herpesviruses. Monospecific rabbit antisera were raised against two separate fusion proteins constructed from a portion of each protein fused to the carboxy terminus of β-galactosidase. These antisera were used to characterize the 47 and 66 proteins in VZV-infected cells and in cells infected with vaccinia virus recombinants expressing each protein. The 47 protein is a 54K phosphoprotein which is distributed between the cytoplasmic and nuclear compartments of VZV-infected cells and is associated with the capsid/tegument fraction of purified VZV particles. Gene 66 encodes a 48K phosphoprotein when expressed by VZV or a vaccinia virus recombinant, and, in the latter case, the 66 protein was located exclusively in the cytoplasm. The 47 protein immunoprecipitated from VZV-infected cells could be phosphorylated in vitro, but the same protein produced by in vitro transcription and translation could not. This and other evidence indicates that additional proteins induced or encoded by VZV may be involved in the phosphorylation of the 47 protein.

Introduction

Protein kinases are involved in a wide variety of functions in eukaryotic cells, these include metabolism, cell cycle control, hormone response and control of transcription and translation. The most commonly studied eukaryotic protein kinases are those which modify serine or threonine residues (reviewed by Edelman et al., 1987) and those which modify tyrosine residues (reviewed by Hunter & Cooper, 1985). The consensus sequence for the 240 amino acid catalytic domain of these proteins contains several highly conserved motifs (Hanks et al., 1988), and members of the serine/threonine and tyrosine protein kinase families may be differentiated by subtle differences in these regions. The presence of such motifs, predicted from DNA sequence data, has led to the identification of a large number of putative protein kinases. Two distinct families of virus-encoded serine/threonine protein kinase genes have been proposed in the herpesviruses, homologous to either gene US3 or gene UL13 of herpes simplex virus type 1 (HSV-1).

HSV-1 gene US3 and its counterparts in herpes simplex virus type 2 and varicella-zoster virus (VZV), genes US3 and 66 respectively, were identified as potentially encoding protein kinases by McGeoch & Davison (1986). Smith & Smith (1989) then identified HSV-1 gene UL13, VZV gene 47 and Epstein-Barr virus (EBV) gene BGLF4 as members of a second family of putative herpesvirus protein kinase genes, and Chee et al. (1989) additionally noted a homologous gene in human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6). Neither β-herpesviruses (e.g. HCMV and HHV-6) nor γ-herpesviruses (e.g. EBV) appear to have homologues of US3. Recent sequence data for the α-herpesviruses pseudorabies virus (PRV) (van Zijl et al., 1990; Zhang et al., 1990; de Wind et al., 1992), Marek's disease virus (Ross et al., 1991), equine herpesvirus 1 (EHV-1) (Colle et al., 1992; Telford et al., 1992), equine herpesvirus 4 (Nagesha et al., 1993) and the γ-herpesvirus, herpesvirus saimiri (Albrecht et al., 1992), confirm the presence of a UL13 counterpart in the α-, β- and γ- herpesviruses and a US3 counterpart only in the α-herpesviruses.

In addition to sequence homology, there is some experimental evidence to support the notion that HSV-1 genes US3 and UL13 encode protein kinases. A monospecific rabbit antiserum directed against a predicted peptide from the US3 protein reacted with a 68K protein in extracts of cells infected with HSV-1 and with a protein of identical Mr, which could be phosphorylated in vitro in highly purified fractions of the HSV-1 cytoplasmic protein kinase (Frame et al., 1987). Deletion of the US3 gene resulted in loss of the 68K protein and the cytoplasmic protein kinase activity (Purves et al.,...
In vitro phosphorylation of nuclear extracts from cells infected with HSV-1 or of disrupted HSV-1 virions yielded a 57K phosphoprotein which was identified as the UL13 protein by a monospecific rabbit antiserum directed against a UL13 fusion protein (Cunningham et al., 1992). In experiments carried out using a mutant containing a disrupted UL13 gene, the 57K protein was not detected by in vitro phosphorylation of nuclear or virion extracts, and a marked reduction in the phosphorylation of other HSV-1 proteins was noted (Coulter et al., 1993).

In this paper we describe the characterization of the protein products of genes 47 and 66, the putative protein kinases of VZV, by heterologous expression and antibody techniques.

Methods

Cells and viruses. Monolayers of CV-1 cells were grown in 5% CO₂ at 37 °C in Dulbecco’s modification of Eagle’s medium supplemented with 4 mm-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5% fetal calf serum (FCS). The VZV strain described by Dumas et al. (1981) was passaged in CV-1 cells by trypan blue-stained infected monolayers showing 20 to 50% c.p.e, and adding the cells at a ratio of 1:5 to 1:1 to uninfected subconfluent monolayers. Infected monolayers were incubated in 5% CO₂ at 37 °C until 20 to 50% c.p.e, was observed (usually 1 to 2 days), and were then used for extraction of proteins or for immunofluorescence. Vaccinia virus strain WR and recombinants derived from it were grown in CV-1 cells and proteins were extracted 16 to 20 h after infection.

Construction of plasmids. The plasmid pDK8 was constructed for the expression of amino acid residues 95 to 322 of the VZV 66 protein fused to the carboxy terminus of β-galactosidase. 5'-phosphorylated BamHI linkers (CGGATCCG) were ligated to a 923 bp SphI fragment obtained from a plasmid containing the VZV SsrI g fragment. After digestion with BamHI and SsrI, the resultant 686 bp fragment was purified and inserted into pUR289 (Rüther & Müller-Hill, 1983) digested with BamHI and SsrI.

The plasmid pDK15 was constructed for the expression of the entire VZV 47 protein (residues 1 to 510) fused to the carboxy terminus of β-galactosidase. A 2285 bp PstI fragment obtained from a plasmid containing VZV SsrI d fragment was ligated into PstI-digested pUR292 (Rüther & Müller-Hill, 1983). The VZV sequence encodes residues 16 to 510 of the 47 protein. To allow expression of the entire VZV protein as a β-galactosidase fusion, the region encoding residues 1 to 15 was reconstituted by inserting a synthetic oligonucleotide duplex between BamHI and PstI sites, so that the ATG codon of gene 47 is positioned in the context of BamHI (GGATCC), ApIII (CTTAGG) and Ncol (CCATGG) sites: GGATCCTAAAGGTACCACCATGG.

For the expression of the entire 66 protein (residues 1 to 393) from vaccinia virus, a synthetic oligonucleotide duplex comprising the first 18 codons of gene 66 was inserted into pMJ601 (Davison & Moss, 1990) digested with SalI and NarI, such that the ATG codon is immediately preceded by a SalI site. The NarI-KpnI fragment from VZV SsrI g fragment which encodes the carboxy-terminal 375 amino acids of the 66 protein and contains downstream sequences was then inserted between the NarI and KpnI sites to form pMJ702.

For expression of the entire 47 protein from vaccinia virus, a 1768 bp fragment produced by digesting pDK15 with ApIII and BspMI (located downstream from gene 47) was cloned into pMJ601 digested with the same two restriction enzymes. This plasmid, pMJ703, was then digested with BamHI and the resultant 1768 bp fragment containing gene 47 was inserted in the required orientation into BamHI-digested pTZ18R (Pharmacia). The resultant plasmid, pTZ47, was employed for in vitro or transient expression of the 47 protein under control of the T7 RNA polymerase promoter.

Construction of vaccinia virus recombinants expressing the 47 and 66 proteins. Plasmids pMJ703 and pMJ702 were used to generate vaccinia virus recombinants capable of expressing the 47 and 66 proteins at late times as described by Davison & Moss (1989). The vaccinia virus recombinants were denoted VV47 and VV66.

Preparation of rabbit antiserum directed against the 47 and 66 proteins. β-Galactosidase fusion proteins expressed in Escherichia coli containing pDK15 and pDK8 were found to be insoluble following induction by 1 mM-IPTG for 2 h at 37 °C. Consequently, the 66 fusion protein was prepared by SDS-PAGE and electroelution essentially as described by Mole & Lane (1987). A sample of purified fusion protein was re-examined by SDS-PAGE to assess recovery and the remainder was stored at −70 °C for a few days until used as immunogen.

The 47 fusion protein was partially purified by isolating insoluble inclusion bodies enriched for the 47 fusion protein by a modification of the method described by Harlow & Lane (1988). Briefly, pelleted bacteria from a 20 ml culture were resuspended in 2 ml buffer A (50 mM-Tris–HCl pH 8.0, 1 mM-EDTA, 100 mM-NaCl) containing 1 mg/ml lysozyme, incubated for 30 min at room temperature then centrifuged at 3000 g for 10 min. The pellet was resuspended in 2 ml ice-cold buffer A containing 0.1% sodium deoxycholate and incubated at 4 °C for 10 min. Then 16 μl of 1 M-MgCl₂ and 30 μl of 1 mg/ml DNase I were added, and incubation was continued at 4 °C for 30 min. After centrifugation at 600 g for 10 min at 4 °C, the pellet was resuspended in 1 ml 50 mM-Tris–HCl pH 8.0, 2 mM-urea, re-centrifuged, washed once with 1 ml buffer A and finally resuspended in 1 ml buffer A. After analysis by SDS-PAGE and assessment of protein concentration, the inclusion body preparations were stored at −20 °C and used for immunization within 1 to 2 days.

Antisera to the 66 fusion protein were prepared by Serotech. Two sandy half-top rabbits were injected subcutaneously with 150 μg of electroeluted fusion protein in 0.5 ml PBS emulsified with an equal volume of Freund’s complete adjuvant. The rabbits were boosted on days 22 and 36 with 150 μg of fusion protein in 0.5 ml PBS emulsified with an equal volume of Freund’s incomplete adjuvant. Final antisera were collected on day 70.

Antisera to the 47 fusion protein were prepared by injecting two New Zealand white rabbits with 150 μg of fusion protein in an inclusion body preparation in 0.3 ml PBS emulsified with an equal volume of Freund’s complete adjuvant. The rabbits were boosted on days 18, 41 and 82 with 150 μg of fusion protein from fresh inclusion body preparations in 0.3 ml PBS emulsified with an equal volume of Freund’s incomplete adjuvant. Final antisera were collected on day 125.

Radiolabelling of infected cells. For labelling VZV proteins with ³²P, 37 mm wells in Nunc six-well multidish plates containing VZV-infected cells showing 20 to 50% c.p.e, were rinsed twice with phosphate-free Eagle’s medium lacking FCS and then incubated overnight at 37 °C with the same medium supplemented with 100 μCi/ml [³²P]orthophosphate (PBS 11; Amersham).

For labelling recombinant vaccinia virus proteins, 37 mm wells of CV-1 cells were infected with virus at an m.o.i. 10 and incubated at 37 °C for 1 h. The infected monolayers were washed twice with phosphate-free Eagle’s medium lacking FCS and incubated with the same medium supplemented with 100 μCi/ml [³²P]orthophosphate at 37 °C for 16 to 20 h.
For labelling proteins expressed under the control of the T7 RNA polymerase promoter, cells were infected with a vaccinia virus recombinant expressing the T7 RNA polymerase (vTF7-3) and then transfected with plasmid pTZ47 as described by Elroy-Stein & Moss (1992). After incubation at 37 °C for 16 h, infected cell monolayers were washed twice with Eagle's medium lacking FCS and containing 20% of the normal amount of methionine. The monolayers were then incubated with the same medium supplemented with 100 μCi/ml [35S]methionine (SJ 204; Amersham) for 2 to 4 h. For labelling with 32P, infected cell monolayers were washed twice with phosphate-free Eagle's medium lacking FCS and incubated with the same medium supplemented with 100 μCi/ml [32P]orthophosphate at 37 °C for 2 to 4 h.

**Immunoblotting.** Infected cell monolayers from two 37 mm wells were washed twice with ice-cold PBS and then scraped into 1 ml PBS. Cells were pelleted by brief centrifugation in a benchtop microcentrifuge at 4 °C, resuspended in 0.5 ml of a 1:1 mixture of PBS and boiling mixture (80 mM-Tris-HCl pH 6.8, 2% SDS, 0.1 mM-DTT, 10% glycerol, 0.5 mM-β-mercaptoethanol), heated at 100 °C for 5 to 10 min and stored at −20 °C.

Proteins were subjected to SDS-PAGE, electroblotted onto nitrocellulose and probed with anti-47 or anti-66 sera (each at a dilution of 1:1000) using an enhanced chemiluminescence kit supplied by Amersham.

**Immunoprecipitation.** Cell extracts from two 37 mm wells of radiolabelled cells were prepared in 0.5 ml lysis buffer (0.1 M-Tris-HCl pH 8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate and 0.2% PMSF), and immunoprecipitation was performed as described by Murphy et al. (1989).

**Fractionation of cells.** Mock- or virus-infected cells were fractionated into cytoplasm, 0.1 M-, 0.4 M- and 2.0 M-NaCl nuclear wash fractions and the final 20 μM-NaCl resistant nuclear pellet as described by Stevenson et al. (1992). Nuclear and cytoplasmic extracts were prepared for in vitro phosphorylation reactions as described by Coulier et al. (1993).

**Isolation of VZV particles and fractionation into capsid/tegument and envelope.** Cells from sixteen 175 cm2 flasks of VZV-infected cells showing advanced c.p.e. were detached by shaking and collected by centrifugation at 600 g for 10 min at 4 °C. Virus particles were recovered from the cell pellet by a modification of the method of Dumas et al. (1980). Briefly, the cells were resuspended gently in 2 ml of trypsin–EDTA solution (0.05% trypsin, 0.02% EDTA in PBS) and incubated at room temperature for 20 min. Trypsinized cells were pelleted by centrifugation at 600 g for 10 min at 4 °C and the supernatants were loaded onto 11 ml gradients of 5 to 15% Ficoll in Eagle’s medium lacking phenol red. The gradients were centrifuged at 18 000 g for 2 h at 4 °C in a Sorvall OTD-Combi ultracentrifuge using a TST41 rotor. The virus band was recovered, diluted in Eagle’s medium lacking phenol red, and virus particles were pelleted at 100 000 g for 1 h at 4 °C and resuspended at 4 °C overnight in 100 μl PBS.

Resuspended virus particles were rapidly mixed with an equal volume of de-envelopment buffer (50 mM-Tris–HCl pH 8.0, 0.5 M-NaCl, 20 mM-EDTA, 2% NP40) and incubated on ice for 15 min. The sample was then centrifuged for 5 min in a benchtop microcentrifuge at 4 °C to produce a detergent-soluble membrane fraction (the supernatant) and a capsid/tegument fraction (the pellet).

**Indirect immunofluorescence.** Cell monolayers were fixed and permeabilized as described by Randall & Dinwoodie (1986). Incubations with antisera and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma) were carried out at dilutions of 1:20 and 1:80, respectively. Slides were viewed using a Nikon Microphot SA fluorescence microscope and photographed using a Nikon FX-35DX camera with Kodak Tri-X-Pan 400 film.

**Preparation of acetone powders and absorption of antisera.** Acetone powders of E. coli expressing β-galactosidase and the 47 or 66 fusion proteins were prepared as described by Harlow & Lane (1988). Briefly, 350 ml cultures of IPTG-induced E. coli were harvested and then washed with 0.9% NaCl. Each pellet was weighed, resuspended in 0.9% NaCl (at 1 ml/g wet weight), and 4 volumes of acetone at −20 °C were added. After incubation on ice for 30 min with occasional vigorous mixing, the acetone precipitate was collected by centrifugation at 600 g for 10 min at 4 °C, resuspended in fresh acetone at −20 °C, incubated on ice for 10 min and again collected by centrifugation. The pellet was spread onto a Whatman no. 1 filter circle and dried overnight at room temperature. Powders were stored at room temperature.

Absorption of antisera against the acetone powders was performed by adding acetone powder at 1% to a 1 ml volume of antisera and incubating at 4 °C on a rotary mixer for 30 min. After centrifugation for 5 min in a benchtop microcentrifuge at 4 °C, the supernatant was retained and the whole process was repeated a further two to five times. Absorbed sera were stored at −20 °C.

**Gel electrophoresis of proteins.** SDS-PAGE was conducted using 9% polyacrylamide gels cross-linked with 1:37.5 N,N’-methylene-bis-acrylamide unless otherwise indicated. Gels from immunoprecipitation experiments were incubated in fixative (10% acetic acid, 50% methanol), treated with En'Hance (New England Nuclear), dried and autoradiographed using Kodak X-6-6 film with a Dupont Cronex Lightning Plus intensifying screen at −70 °C.

**In vitro expression of the 47 protein.** The 47 protein was expressed in vitro using a Tnt-coupled reticulocyte lysate system (Promega). Plasmid pTZ47 was used as template for T7 RNA polymerase and proteins were labelled with [35S]methionine according to the manufacturer’s protocol.

In vitro phosphorylation of proteins. In vitro phosphorylation reactions were carried out using crude nuclear or cytoplasmic extracts or immunoprecipitated proteins. Volumes of 10 μl of extracts or 20 μl of packed Protein A–Sepharose beads used in immunoprecipitations were mixed with an equal volume of assay mixture containing 50 mM-Tris–HCl pH 7.5, 1 mM-EDTA, 1 μCi [γ-32P]ATP and either 0.1 M- or 1.5 M-NaCl in the presence of 10 mM-MgCl2 or 10 mM-MnCl2. Reactions were incubated for 30 min at 37 °C and terminated by addition of an equal volume of SDS–PAGE boiling mixture. Samples were heated at 100 °C for 5 min and analysed by SDS–PAGE.

**Results**

The products of VZV genes 47 and 66 in VZV-infected cells

Fig. 1(a) shows an immunoblot of mock- and VZV-infected cells probed with rabbit antiserum directed against a β-galactosidase fusion with the entire 47 protein (anti-47 serum). A band with an apparent Mr of 54K was evident in extracts of VZV-infected cells (lane 2) but not in extracts of mock-infected cells (lane 1). This protein was not recognized by pre-immune serum from the same rabbit (data not shown). Anti-47 serum also immunoprecipitated a 54K phosphoprotein from extracts of VZV-infected cells labelled with [32P]orthophosphate (Fig. 1b, lane 1). The
Fig. 1. (a) Autoradiograph showing immunoblotting with anti-47 serum. Extracts of mock- (lane 1) and VZV-infected cells (lane 2) were subjected to SDS-PAGE, blotted and probed with anti-47 serum. Mr markers are shown to the left and the 47 protein is indicated (○). (b) Autoradiograph showing immunoprecipitation of a2P-labelled extracts of VZV- (lane 1) and mock-infected cells (lane 2) with anti-47 serum. Mr markers are shown to the left and the 47 protein is indicated (○).

lower Mr bands which are evident in the VZV-infected cell extracts in Fig. 1 may represent breakdown products of the 47 protein. The 47 protein was not detected in extracts of a3S-labelled VZV-infected cells, possibly as a result of a lower specific activity of incorporated a3S relative to a32P.

Fig. 2 shows the result of an immunoprecipitation experiment using serum from a rabbit immunized with a β-galactosidase fusion protein containing residues 95 to 322 of the 66 protein (anti-66 serum). Two major phosphoprotein species with apparent Mr's of 48K and 54K were precipitated by immune serum from a32P-labelled extracts of VZV-infected cells (lane 4) but not from extracts of mock-infected cells (lane 3). These proteins were immunoprecipitated in variable relative proportions in different experiments, but neither was precipitated by the preimmune serum (lanes 1 and 2). In contrast to the anti-47 serum, the anti-66 serum did not react convincingly with immunoblots of extracts from VZV-infected cells (data not shown), but did react with extracts of cells infected with VV66 (as described below). This may indicate that a relatively low level of target antigen is present in VZV-infected cells.

Expression of the 47 and 66 proteins by recombinant vaccinia viruses

Expression of the 47 and 66 proteins by recombinant vaccinia viruses, VV47 and VV66, was assessed by immunoblotting and immunoprecipitation experiments. Transient expression of the 47 protein was also achieved by transfection of pTZ47 into cells infected with vTF7-3, a vaccinia virus recombinant which expresses T4 RNA polymerase. Fig. 3(a) shows that the anti-47 serum reacted in an immunoblot experiment with a 54K protein which was present in extracts of cells infected with VV47 (lane 1) but not in extracts of cells infected with a vaccinia virus recombinant expressing a portion of VZV gene 61 (lane 2). This protein was not detected by immunoprecipitation of a32S- or a32P-labelled extracts of VV47-infected cells (data not shown). The 54K protein was immunoprecipitated from extracts of a32S-labelled cells transfected with pTZ47 after infection by vTF7-3 (Fig. 3b, lane 1), but not from parallel extracts of cells...
Fig. 3. (a) Autoradiograph showing recognition of the 47 protein expressed by VV47. Extracts of cells infected by VV47 (lane 1) or a vaccinia virus recombinant expressing a fragment of VZV 61 protein (lane 2) were subjected to SDS-PAGE (on a 12.5% gel), blotted and probed with anti-47 serum. M_r markers are shown to the left and the 47 protein is indicated (●). (b) Autoradiograph showing recognition of the 47 protein expressed transiently from pTZ47 in cells infected by vTF7-3. Cells were labelled with 3^5_S (lane 1) or 32p (lane 2), extracts immunoprecipitated with anti-47 serum and proteins were analysed by SDS-PAGE. M_r markers are shown to the left and the 47 protein is indicated (●).

labelled with 32p (Fig. 3b, lane 2). These results imply that the 47 protein is present at low levels in VV47-infected cells (as indeed may be the case in VZV-infected cells) and that phosphorylation of the 47 protein is reduced when it is expressed in isolation from other proteins induced or encoded by VZV.

Fig. 4(a) shows that probing an immunoblot of mock- and VV66-infected cell extracts with anti-66 serum led to recognition of a VV66-specific 48K protein. Bands of higher M_r in the VV66 lane are probably derived from β-galactosidase (the major band of 116K) expressed by the recombinant vaccinia virus. Immunoprecipitation of 32P-labelled extracts with anti-66 serum (Fig. 4b) also showed recognition of a 48K protein in extracts of VV66-infected cells (lane 3), which comigrated with the 48K protein precipitated from extracts of VZV-infected cells (Fig. 2, lane 4; Fig. 4b, lane 2). In contrast, the 54K protein precipitated from VZV-infected cell extracts was not detected in extracts of VV66-infected cells.

Confirmation of the identity of the 66 protein

As a result of the differences between the proteins immunoprecipitated by anti-66 serum from extracts of VZV- and VV66-infected cells, experiments were conducted using anti-66 serum which had been absorbed six times against acetone powders of E. coli expressing the 47 fusion protein, the 66 fusion protein or β-galactosidase. These sera were then used in immunoprecipitation experiments with 32P-labelled extracts of mock-, VZV- or VV66-infected cells. It is clear from Fig. 5 that only
antiserum absorbed against the 66 fusion protein exhibited a marked reduction in immunoprecipitation of the 48K protein from extracts of VZV- or VV66-infected cells. In contrast, only the non-absorbed antiserum was able to immunoprecipitate the additional 54K protein from extracts of VZV-infected cells. These results indicate that the 48K protein is the true product of gene 66 and that the 54K protein is precipitated in a non-specific manner.

Subcellular localization of the 47 and 66 proteins

Fig. 6(a) indicates that the 47 protein was found in both the cytoplasmic and nuclear fractions of VZV-infected cells, with part of the latter being resistant to elution by 2 M-NaCl. As indicated above, the anti-66 serum did not react convincingly in immunoblotting experiments with VZV-infected cell extracts, but did react with extracts of cells infected with VV66 (e.g. see Fig. 4a). When VV66-infected cells were analysed, the 66 protein was located exclusively in the cytoplasm (Fig. 6b).

Indirect immunofluorescence experiments confirmed that at least a proportion of the 47 protein is present in the nuclei of VZV-infected cells. To ensure that fluorescence was specific, anti-47 serum was absorbed three times against acetone powders. Absorbed sera were tested by immunoblotting extracts of VZV-infected cells, and only anti-47 serum absorbed against the 47 fusion protein failed to recognize the 54K protein (result not shown). In immunofluorescence experiments, anti-47
serum (Fig. 7c) and anti-47 serum absorbed against β-galactosidase (Fig. 7d) or the 66 fusion protein (Fig. 7e) showed association of the 47 protein with large nuclear inclusions in VZV-infected cells. Similar immunofluorescence was not detected in mock-infected cells probed with anti-47 serum (Fig. 7b) or in VZV-infected cells probed with pre-immune serum (Fig. 7a) or with anti-47 serum absorbed against the 47 fusion protein (Fig. 7f). It was not possible to confirm from these results that the majority of the 47 protein was cytoplasmic (as was inferred from subcellular fractionation of VZV-infected cells), since mock-infected cells exhibited non-specific cytoplasmic fluorescence when probed with anti-47 serum (Fig. 7b). Nevertheless, the lack of nuclear fluorescence observed when VZV-infected cells were probed with anti-47 serum absorbed against the 47 fusion protein (Fig. 7f) confirmed that the nuclear fluorescence was specific to the 47 protein.

Mock-infected cells also exhibited non-specific cytoplasmic fluorescence when probed with anti-66 serum (result not shown). Consequently, this serum proved to be of little use in immunofluorescence experiments and the cytoplasmic localization of the 66 protein indicated by immunoblotting experiments could not be confirmed by this method.

Association of the 47 protein with virus particles

Virus particles removed from infected cells by trypsin treatment were separated into capsid/tegument and envelope fractions, and samples were analysed by immunoblotting (Fig. 8). The 47 protein was found to be associated with VZV-infected cells and virus particles (lanes 2 and 3), and reaction with virus particles from which the envelopes had been removed indicated a likely association with the capsid/tegument fraction (lane 4). Removal of the viral envelope was confirmed by immunoblotting parallel fractions with monoclonal antibodies directed against the envelope glycoproteins gpI, -II and -III (data not shown).
The 66 protein was not detected in virus particles by immunoblotting (results not shown), but it is not possible to draw a firm conclusion from this because of the poor reactivity of the anti-66 serum with extracts from VZV-infected cells.

In vitro phosphorylation of the 47 and 66 proteins

As the predicted products of genes 47 and 66 show homologies to protein kinases, the ability of each of these proteins to be phosphorylated in isolation was investigated. Proteins were immunoprecipitated from infected cell extracts and incubated in vitro with [γ-32P]ATP (Fig. 9a). The 47 protein immunoprecipitated from VZV-infected cells was phosphorylated in the presence of 10 mM-Mg2+ (lane 3) or 10 mM-Mn2+ (lane 7), though more efficiently with the latter. The autoradiograph was over-exposed in order to reveal faint bands in lanes other than lane 7. Similar experiments have confirmed a greater degree of incorporation of label from [γ-32P]ATP into the 47 protein in the presence of Mn2+ relative to Mg2+ (data not shown). In contrast, the 47 protein produced by in vitro transcription/translation was not phosphorylated in a parallel experiment (results not shown). Similarly, the 66 protein was not convincingly phosphorylated in immunoprecipitates obtained from VZV-infected cell extracts in the presence of either Mg2+ or Mn2+ (lanes 1 and 5) or from VV66-infected cell extracts (result not shown). The 66 protein was one of several phosphorylated proteins in crude cytoplasmic extracts of VV66-infected cells (Fig. 9b, lane 1), indicating either that phosphorylation of the 66 protein occurs via a cellular protein or that the antibodies inhibit phosphorylation of the 66 protein. The 48K phosphoprotein was not seen in a parallel experiment using extracts of cells infected with an unrelated vaccinia virus recombinant (result not shown).

Discussion

Monospecific antisera were used in an immunological analysis of the two putative protein kinases of VZV, the products of genes 47 and 66. The 47 and 66 proteins have apparent Mr of 54K and 48K respectively, similar to their predicted Mr of 54K and 44K (Davison & Scott, 1986). As anticipated from their predicted function as
protein kinases, these proteins are phosphorylated in VZV-infected cells. However, the 47 protein appears to be hypophosphorylated when expressed transiently or by a vaccinia virus recombinant. Also, the 47 protein immunoprecipitated from unlabelled extracts of VZV-infected cells could be phosphorylated in vitro but the 47 protein produced by in vitro transcription/translation could not. These results imply that other proteins induced or encoded by VZV may be involved in phosphorylation of the 47 protein. Experiments by Coulter et al. (1993) on the HSV-1 UL13 protein (the homologue of the VZV 47 protein) support this interpretation. These workers found that a transformed cell line which expressed large amounts of UL13 mRNA did not yield extracts in which the UL13 protein could be phosphorylated. On infection with a UL13 insertion mutant, however, phosphorylation of the UL13 protein was detected in nuclear extracts of infected cells and in cell-released virions. It should be noted, however, that the corresponding protein of PRV can be phosphorylated in vitro after transient expression and immunoprecipitation (de Wind et al., 1992).

Our results confirm observations made by Ng & Grose (1992) that the 47 protein is a 54K phosphoprotein in VZV-infected cells and that it is distributed largely in the cytoplasm (as assessed by indirect immunofluorescence). We have confirmed, by fractionation of VZV-infected cells, that the major fraction of the 47 protein is cytoplasmic, but have also demonstrated by two independent methods that a proportion is associated with the nuclei of infected cells. This difference may be due to the alternative methods of fixation and permeabilization used in immunofluorescence. Our result is consistent with the presence of the 47 protein in the capsid/tail fragment of VZV particles purified from the surfaces of infected cells.

The targets of the HSV-1 UL13 and US3 protein kinases have been reported to be the US1 (Purves & Roizman, 1992) and UL34 proteins (Purves et al., 1991, 1992), respectively. The site of phosphorylation of the UL34 protein was initially proposed on the basis of studies of in vitro phosphorylation of synthetic peptides by the US3 protein kinase (Purves et al., 1986), and has been investigated by mutagenesis of the UL34 protein (Purves et al., 1991). Comparable sites are not present in the homologous VZV (Davison & Scott, 1986) or EHV-1 proteins (Telford et al., 1992). It is possible that the US3 protein kinases of VZV and EHV-1 have different targets from that of the HSV-1 US3 protein kinase. More probably, the family of US3 protein kinases may have a common unidentified target but also have additional targets specific to each virus (e.g. the UL34 protein of HSV-1) which may or may not be of biological significance. Similar comments apply to the UL13 protein kinase family, which has members in all herpesvirus subfamilies but whose reported target in HSV-1 has counterparts only in α-herpesviruses.

We are grateful to John Subak-Sharpe for his continued interest in this work, to Bernard Moss for supplying the vaccinia virus recombinant vTF7-3, to John McAulachlan for synthesis of oligonucleotides and to Ron Ellis for supplying monoclonal antibodies directed against VZV glycoproteins I, II and III. This work was generously supported by the Robertson Trust and the Hugh Fraser Foundation.

References


(Received 10 May 1993; Accepted 16 September 1993)