Identification of a target protein of US3 protein kinase of herpes simplex virus type 2

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Herpes simplex virus type 2 (HSV-2) gene US3 has been shown to encode a serine-threonine protein kinase. In this study, we have tried to identify target proteins of the US3 protein kinase using a US3 lacZ insertion mutant of HSV-2. When permeabilized cells were labelled with [γ-32P]ATP under the optimum conditions for the US3 enzyme, the most striking difference between wild-type HSV-2 strain 186- and mutant-infected cells was observed in the phosphorylation of proteins ranging in Mr values from 14K to 22K. Studies of in vitro phosphorylation with purified virions and with cells infected with a US9-defective HSV-1 mutant suggested that a tegment phosphoprotein encoded by the US9 gene may be a target of HSV-2 US3 protein kinase.

It has been shown that two genes (US3 and UL13) of herpes simplex virus (HSV) encode protein kinases (McGeoch et al., 1985; McGeoch & Davison, 1986; Smith & Smith, 1989; Cunningham et al., 1992). The N-terminal domain of the large subunit (UL39 gene product) of viral ribonucleotide reductase has also been shown to have protein kinase activity (Chung et al., 1989; Paradis et al., 1991). Although all of these activities are non-essential for viral replication in cell cultures, the phosphorylation of viral and cellular proteins by these kinases is thought to be important in various aspects of virus-cell interaction in vivo. The HSV-1 US3 protein kinase has been identified as an enzyme present in the cytoplasm of infected cells (Purves et al., 1986; Frame et al., 1987) and virions (Zhang et al., 1990). The purified enzyme has an apparent Mr of 68K and preferentially phosphorylates model substrates containing arginyl residues on the N-terminal side of a target seryl or threonyl residue (Frame et al., 1987; Leader et al., 1991). In addition, a recent study by Purves et al. (1991) has shown that the US3 protein kinase is involved in the phosphorylation of a virion 30K protein encoded by UL34. However, the biological role of the US3 protein kinase is not well understood.

We have previously reported the construction of a US3 lacZ insertion mutant of HSV-2 and its phenotype in vitro and in vivo (Nishiyama et al., 1992). Although the US3-disrupted mutant (L1BR1) grew as efficiently as the parent virus in Vero cells and mouse embryo fibroblasts, the mutant was more than 10000-fold less virulent than the parent virus following intraperitoneal and footpad infection in mice, and failed to grow in peritoneal macrophages (Nishiyama et al., 1992; Kurachi et al., 1993). The biochemical properties of the HSV-2 US3 protein kinase were similar to those of the HSV-1 counterpart and the pseudorabies virus-encoded 38K protein kinase (Daikoku et al., 1993; Katan et al., 1985; Purves et al., 1987), but were different in several other respects (Daikoku et al., 1993). In this paper, we report that the US3 protein kinase of HSV-2 may be involved in phosphorylation of the US9 gene product, a tegment phosphoprotein with Mr values ranging from 14K to 22K.

To identify target proteins of the HSV-2 US3 protein kinase, confluent monolayers of Vero cells were infected with either wild-type HSV-2 strain 186 or the recombiant mutant L1BR1 at a multiplicity of 5 p.f.u./cell. At 12 and 24 h post-infection, the cells were harvested and permeabilized by freezing and thawing once. The permeabilized cells were subjected to in vitro phosphorylation with [γ-32P]ATP. The reaction was carried out under optimum conditions for the HSV-2 US3 protein kinase except that KCl concentrations were varied. The 32P-labelled phosphoprotein profiles were then analysed by SDS-PAGE and autoradiography. The most striking differences between 186- and L1BR1-infected cells was observed in the phosphorylation of proteins ranging in Mr values from 14K to 22K (Fig. 1a and b). The phosphorylation of these proteins in the permeabilized 186-infected cells was enhanced by the addition of 50 or 200 mM-KCl, but inhibited by the addition of 500 mM. A protein with an apparent Mr of 76K was also more strongly phosphorylated at high concentrations of KCl in 186-infected cells than in...
Fig. 1. In vitro phosphorylation in permeabilized infected cells. Confluent monolayers of Vero cells were mock-infected (lanes 1 to 4) or infected with wild-type HSV-2 (lanes 5 to 8 and 13 to 16) or the US3 lacZ insertion mutant L1BR1 (lanes 9 to 12 and 17 to 20) at a multiplicity of 5 p.f.u./cell. After incubation at 37 °C for 12 h (lanes 1 to 12) or 24 h (lanes 13 to 20), cells were harvested, permeabilized by freezing and thawing, and incubated at 30 °C for 30 min in reaction mixtures containing 20 mM-Tris-HCl pH 9.2, 20 mM-MgCl₂, 10 mM-2-mercaptoethanol, 20 gM-ATP and 1 ~tCi [7-z2p]ATP. The KC1 concentrations were varied: lanes 1, 5, 9, 13 and 17, 0 raM; lanes 2, 6, 10, 14 and 18, 50 raM; lanes 3, 7, 11, 15 and 19, 200 raM; lanes 4, 8, 12, 16 and 20, 500 raM. The reactions were terminated by adding SDS, and 32P-labelled proteins in each sample were separated in denaturing gels containing 10% (a) and 15% (b) acrylamide. The gels were dried and exposed to BAS III imaging plates (Fuji) for about 24 h. These were analysed with a Fuji Bioimaging Analyzer BAS 2000 system. The white and black squares and brackets indicate the positions of protein bands whose intensity was markedly reduced in L1BR1-infected cells.

L1BR1-infected cells (Fig. 1a, marked by white square). These results indicate that the HSV-2 US3 protein kinase was directly or indirectly involved in the phosphorylation of these proteins.

Since our previous study revealed that the HSV-2 US3 protein kinase is very sensitive to quercetin, a bioflavonoid, we examined the effect of this inhibitor on the phosphorylation of the 14K to 22K proteins. As shown in Fig. 2, the in vitro phosphorylation of these proteins was strongly inhibited by the addition of 50 μM-quercetin. The addition of 1 mM of synthetic oligopeptide (amino acid sequence RRRRAAVA) moderately suppressed the phosphorylation. The sensitivities were very similar to those of the purified US3 protein kinase (Daikoku et al., 1993). These observations further support the involvement of the enzyme in the phosphorylation of the proteins.

The profiles of the 14K to 22K phosphoprotein species in an autoradiograph appeared very similar to a well characterized tegment phosphoprotein of HSV-1 (Frame et al., 1986). The protein is encoded by the US9 gene and its primary translation product has an apparent Mr of 13K. The phosphorylation of the original product results in at least 12 electrophoretically distinct polypeptides ranging from 12K to 20K. Furthermore, these species are subdivided into two groups, the lower and higher Mr forms, possibly because of dimerization (Frame et al., 1986). Experiments were therefore performed to determine whether the phosphorylated 14K to 22K proteins corresponded with the US9-encoded tegment phosphoprotein. Since neither the nucleotide sequence of the US9 region of HSV-2 nor a US9-defective mutant of HSV-2 was available, we used an HSV-1 mutant for in vitro phosphorylation experiments. The HSV-1 mutant N38 has a deletion of four U₅ genes including US9, but has no defect in the US3 region (Nishiyama et al., 1993). As shown in Fig. 3 (lane 2), phosphorylated protein bands of Mr values ranging from 14K to 22K were observed in the profiles from cells infected with the wild-type parental virus SP23. In cells infected with the US9-defective mutant, however, no distinct bands were observed at the corresponding positions. On the other hand, slightly phosphorylated bands representing Mr values ranging from 14K to 22K could be recognized in the in vitro phosphorylated protein profile from cells infected with L1BR1 (Fig. 3, lane 5), although the intensity of the
labelling was much reduced compared with that observed in the profiles from cells infected with the wild-type or the rescued virus L1B-11 (Fig. 3, lanes 4 and 6).

Protein phosphorylation was also examined in detergent-treated virions. As shown in Fig. 4 the in vitro phosphorylation of purified HSV-2 virions gave similar profiles of proteins, ranging from 14K to 22K, to those of the infected cells. There was a significant reduction in L1BR1 virions in the degree of phosphorylation of these species. In addition, when an in vitro phosphorylation experiment was performed using de-enveloped capsid-tegment structures of the wild-type virions, similar profiles were obtained (data not shown). These observations are consistent with the phosphorylated proteins ranging from 14K to 22K being the tegment phosphoproteins encoded by US9. We further examined the effects of KCl and quercetin on protein phosphorylation in the wild-type virions (Fig. 4, lanes 4 to 9). The labelling of the 14K to 22K bands increased in intensity with increasing KCl concentration up to 200 mM, but decreased at 500 mM-KCl. Quercetin strongly inhibited the phosphorylation of these proteins at a concentration of 50 μM. The sensitivities to the salt and the inhibitor coincided with those obtained with the permeabilized cells infected with the wild-type HSV-2.

Recently, we have purified the US3-encoded protein kinase of HSV-2 and characterized its biochemical properties (Daikoku et al., 1993). In the present study, we have tried to identify the target proteins of the US3 protein kinase of HSV-2 using information obtained from the above study. We found that when permeabilized cells were labelled with [γ-32P]ATP under the optimum conditions for the HSV-2 US3 protein kinase, there was a striking difference between wild-type- and L1BR1-infected cells in the degree of phosphorylation of proteins with M_r values from 14K to 22K. A similar difference was also observed when purified virions were used. The migration pattern of the 14K to 22K phosphoproteins in SDS-PAGE was very similar to that of a tegment protein encoded by the US9 gene (Frame et al., 1986). No corresponding bands could be observed in the in vitro phosphorylated protein profiles with US9-defective mutant N38-infected cells. Therefore it seems very likely that the 14K to 22K proteins are the US9-encoded tegment protein. However, further experiments will be necessary to confirm this identification.

Our data do not necessarily exclude the involvement of other protein kinases in the phosphorylation of the US9
tectment protein. The protein of HSV-1 is known to contain conserved potential phosphorylation sites for tyrosine kinases and casein kinases I and II (Petrovsks & Post, 1987; Leader & Katan, 1988). In fact, we could detect some phosphorylation of the 14K to 22K proteins even in L1BR1-infected cells and in the L1BR1 virions. The US9 tegument protein may be phosphorylated by several kinds of protein kinases of both viral and cellular origin.

The US3 gene, unlike UL13, has counterparts only in alphaherpesviruses such as varicella-zoster virus (VZV) and pseudorabies virus (McGeoch & Davison, 1986). In the VZV genome, the counterpart gene (gene 65) of US9 exists adjacent to the counterpart gene (gene 66) of US3. This observation appears interesting when considering the functional relationship of the two gene products. The US3 protein kinase of HSV-1 has been shown to be involved in the phosphorylation of the essential UL34 gene product, but phosphorylation is not essential for the replication of HSV-1 in Vero or baby hamster kidney cells (Purves et al., 1991; Purves & Roizman, 1992). Our recent studies have shown that L1BR1, unlike wild-type HSV-2, cannot grow in peritoneal macrophages of mice (Nishiyama et al., 1992; Kurachi et al., 1993) or in macrophage-like cell lines such as RAW264 and U-937 (data not shown). The phosphorylation of the target proteins by US3 protein kinase might be indispensable for virus replication in selected cells.

We thank T. Tsuruguchi and F. Ivata for technical assistance. This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

References


(Received 20 December 1993; Accepted 3 March 1994)