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Tyrosine Phosphorylation of the Herpes Simplex Virus Type 1 Regulatory Protein ICP22 and a Cellular Protein Which Shares Antigenic Determinants with ICP22

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At least eight herpes simplex virus type 1 (HSV-1) and five HSV-2 proteins were tyrosine phosphorylated in infected cells. The first viral tyrosine phosphoprotein identified was the HSV-1 regulatory protein ICP22. Also, two novel phosphotyrosine proteins were bound by anti-ICP22 antibodies. H_{R22} is a cellular protein, while the F_{R10} protein is observed only in HSV-1-infected cells.

Tyrosine phosphorylation of HSV-1(F) and HSV-2(G) proteins in human and mouse cells. Members of the herpesvirus family can cause severe disease in neonates and in immunologically deficient patients. Herpes simplex virus (HSV), the prototype of the family, is a neurotropic herpesvirus. HSV causes a variety of infections in humans, remains latent in neuronal tissue in its host for life, and can be reactivated to cause lesions at or near the initial site of infection. Generally, lytic HSV infection in healthy individuals results in benign lesions while infections in neonates obtained from the mother and infections in immunocompromised patients may become life-threatening. These infections usually result from lytic replication (reviewed in reference 17) following reactivation of virus from the latent state. While the mechanism of viral reactivation from the latent site remains unclear, the process may involve the transfer of a neuronal signal to the viral replication machinery. Since protein tyrosine kinases have been implicated in orchestrating several cellular signaling processes (5), the goal of this study was to determine whether viral proteins were tyrosine phosphorylated following the infection of mammalian cells with either HSV type 1 (HSV-1) or HSV-2.

HSV-1(F) and HSV-2(G), the prototypes of HSV-1 and HSV-2, respectively, used in this study, were obtained from Bernard Roizman (University of Chicago), and stocks of these strains were prepared with Vero cells (4). HeLa, Vero, and NIH 3T3 tissue culture cells, originally obtained from the American Type Culture Collection, were grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. Subconfluent cell cultures containing approximately 10^6 cells were exposed to 5 PFU per cell for 1 h and then incubated at 37°C in medium containing 2% newborn calf serum. Uninfected and infected cells were lysed immediately by exposure to RIPA (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 1% Triton X-100, 1% trasylol, 1 mM phenylmethylsufonyl fluoride, 1 mM Na_3VO_4) buffer and sonicated with a Branson sonifier, prior to clearing of the extracts by microcentrifugation. Electrophoretic separations of denatured infected cell proteins were done in polyacrylamide gels cross-linked with *N*,*N*′-methylenebisacrylamide (BIS; Sigma, St. Louis, Mo.) containing 0.1% sodium dodecyl sulfate at 1 mA/cm for 15 h. The separated polypeptides were electrically transferred to nitrocellulose sheets in a gel buffer containing 0.025% sodium dodecyl sulfate at 120 V for 5 h at 4°C. Polypeptides were visualized on nitrocellulose after being stained with RC20, a mouse monoclonal antibody cross-linked with alkaline phosphatase, which is specific for phosphotyrosine (Transduction Laboratories, Lexington, Ky.), in an immunoalkaline phosphatase-coupled reaction (Bio-Rad, Duarte, Calif.). Immunoblots were scanned at a resolution of 300 dots per inch with AGFA Arcus II linked to a Macintosh model 9500 PowerPC workstation. Raw digital images, saved as TIF (tagged image files) files with no alterations to the image itself by using Adobe Photoshop (version 4.0), were organized into figures with Microsoft PowerPoint (version 4.0). Grey-scale prints of figures were obtained with a Tecktronix Phaser II DSX printer (maximum resolution, 300 dots per inch). The results from this experiment (Fig. 1) showed that as many as eight HSV-1 (lane 6) and five HSV-2 (lane 4) proteins were recognized by the phosphotyrosine-specific antibody at levels which were discernible above that of the cell protein-specific background (lanes 1 and 5). In comparing HSV-1 and HSV-2 proteins in HeLa cells, at least four polypeptides (A to D) with similar mobilities were recognized by the antibody (lanes 3 and 4). In contrast, in NIH 3T3 cells, at least eight HSV-1 bands (A to H) were observed (lane 6). While band C, which migrated near the 68,000-molecular-weight marker, showed the darkest staining in all lanes with virus-infected cells, it was especially intense with HSV-1-infected NIH 3T3 cells (lane 6). Immunoblotting with an antibody which recognizes both the HSV-1- and HSV-2-infected cell protein 4 (ICP4) showed that equal amounts of viral proteins were present in the lanes (data not shown), indicating that NIH 3T3 and HeLa cells were equally permissive for HSV-1 and HSV-2 infection. Finally, in uninfected HeLa cells, the most reactive antiphosphotyrosine band, H_{RC20}, was a large protein which migrated between the 97,000- and 200,000-molecular-weight markers (lane 2). From these results we conclude that HSV-1 and HSV-2 infection results in a novel pattern of tyrosine-phosphorylated proteins, inasmuch as it is different from that which existed prior to infection. Although the presence of numerous virus-specific bands with similar mobilities in the two cell types suggests that viral proteins are tyrosine phosphorylated, these results cannot exclude the possibility that the infected-cell-specific bands correspond to cellular proteins whose expression or modification was induced by viral infection. Since the amounts of the viral proteins added to each lane were essentially the same, the differences in the intensities of

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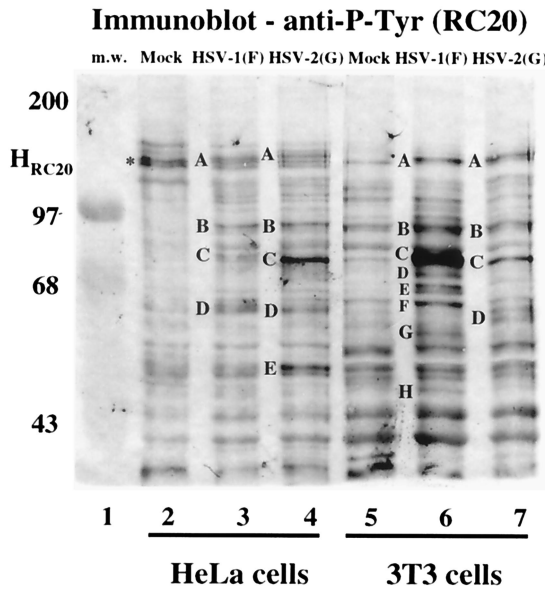

FIG. 1. Digital scanning image of antiphosphotyrosine (P-Tyr) antibody immune reactivities. Uninfected (Mock) and HSV-1(F)- and HSV-2(G)-infected HeLa and NIH 3T3 cell proteins were separated in a 10% BIS-acrylamide denaturing gel, transferred to nitrocellulose, and probed with antibody RC20. m.w., prestained molecular weight markers whose sizes (in thousands) are listed on the left; A to H, immune reactive bands present in the infected cell lanes beginning with the slowest migrating band at the top and ending with the fastest observed in each lane; *, the major tyrosine-phosphorylated protein in HeLa cells (H_{RC20}).

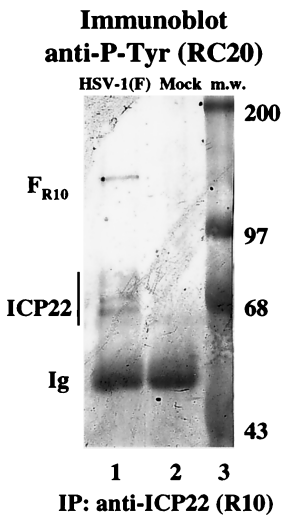

FIG. 2. Digital scanning image of an antiphosphotyrosine (P-Tyr) immunoblot. Uninfected (Mock) and HSV-1(F)-infected HeLa cell proteins were immunoprecipitated (IP) with antibody R10 prior to electrophoretic separation on a 10% BIS-acrylamide denaturing gel, transfer to nitrocellulose, and probing for reactivity with antibody RC20. The locations of immunoprecipitated proteins (ICP22 and F_{R10}) are indicated on the left. m.w., prestained molecular weight markers whose sizes (in thousands) are on the right; Ig, location of the immunoglobulin band.

immunostaining likely reflect various amounts of modification, suggesting that the levels of tyrosine phosphorylation in infected cells is determined by the particular cell type.

Tyrosine phosphorylation of the HSV-1(F) ICP22 protein. While the results presented in Fig. 1 show alteration of the RC20 profile after infection, possibly reflecting changes to the amount, or phosphorylation state, of cellular and viral proteins, they do not confirm whether viral proteins were modified by tyrosine phosphorylation. The brightest band observed for infected cells in Fig. 1 migrated near the 68,000-molecular-weight marker, and this was the location at which we would predict the viral regulatory protein ICP22 to migrate (7, 8). Anti-ICP22 immunostaining showed that band C and ICP22 migrate with similar mobilities (data not shown). To definitively determine whether ICP22 was tyrosine phosphorylated, HeLa cells were infected with HSV-1(F) and extracted as described for Fig. 1. ICP22 was then immunoprecipitated with rabbit polyclonal antibody R10 (obtained from Bernard Roizman), specific for ICP22 amino acids 21 to 36 (1). Approximately 500 μg of cleared whole-cell protein was mixed with 1 μg of antibody and incubated with rocking for 2 h. Goat anti-rabbit secondary antibody (Sigma) was added (to 1 μg/ml), and the mixture was incubated for an additional 1 h. Protein A-Sepharose beads (20 μl; Sigma) were added and incubated for 1 h with rocking, prior to being pelleted and washed three times with 1 ml of RIPA buffer. Precipitated proteins were separated in a denaturing gel, transferred to nitrocellulose, and probed for reactivity with antibody RC20 specific for phosphotyrosine.

The results (Fig. 2) from this experiment were as follows. (i) Lane 1 shows that in the region of the blot near the 68,000-molecular-weight marker, a protein species consisting of two dark bands and several lighter, slower-migrating bands reacted with the RC20 antibody. ICP22 was previously shown to exist in multiple forms (1, 14), and Western blotting analysis of a parallel blot with the R10 antibody confirmed that these bands were ICP22 (data not shown). (ii) In addition to ICP22, a slower-migrating band, termed F_{R10}, was also precipitated with the R10 antibody and reacted with the RC20 antibody (lane 1). Graphic analysis was used to calculate the approximate size of this band, and based on an average of three similar experiments, the observed molecular weight of F_{R10} was 133,000. Thus, the F_{R10} protein was similar in size to the H_{RC20} protein (Fig. 1). Neither F_{R10} nor the ICP22 protein was observed in the uninfected cells (lane 2). From these results, we conclude that the ICP22 protein of HSV-1(F) is tyrosine phosphorylated in infected cells and that multiple forms of the protein contain this modification. In addition, a second tyrosine-phosphorylated protein, F_{R10}, precipitates along with ICP22. From these data it cannot be distinguished whether F_{R10} (i) binds ICP22 and therefore coprecipitates with it or (ii) cross-reacts with the R10 antibody and precipitates independently of ICP22. That F_{R10} was observed only in the infected cell extract suggests that either it is a viral protein or it is a cellular protein whose expression or modification was induced by the viral infection. The ICP22 protein was of particular interest since viruses which contain deletions of, and insertions in, the gene encoding ICP22 (12) have reduced plating efficiencies in rodent cell lines and in primary human cell strains but not in continuously passaged primate cell lines (10, 11, 16, 18). In addition, these viruses have a reduced capacity to replicate in the central nervous systems of mice (10, 18). It has also been reported that in most cells, ICP22-deletion mutants (12) affect the expression of the gene encoding ICP0, as well as a subset of late viral genes, suggesting a role for ICP22 in the regulation of HSV-1 gene expression (13, 14, 18). Recently, ICP22 was implicated in the posttranslational processing of cellular RNA polymerase II (16), which results as a consequence of HSV-1 infection (15), and accordingly, ICP22 was shown to localize with RNA polymerase II inside infected cells (6). The α22 gene contains two

open reading frames, which are independently transcribed, encoding the full-length ICP22 and a truncated version ($U_S1.5$) whose amino acid sequence is identical to the carboxy-terminal domain of ICP22 (3). Finally, ICP22 was shown to be a highly posttranslationally modified protein, and these modifications include nucleotidylylation (2, 9) and [^{32}P]orthophosphorylation (14, 20). Our results now show that specific tyrosine phosphorylation also occurs on the protein inside infected cells.

Generation of a rabbit polyclonal antibody against full-length ICP22. The antibody described above, R10, was raised against a synthetic peptide consisting of only 17 ICP22 amino acids (1). In this series of experiments, an antibody raised against the entire ICP22 protein fused to glutathione *S*-transferase (GST) was generated. pJB130 is a recombinant plasmid which was designed to encode the full-length α22 gene fused to the carboxy-terminal end of the GST gene. The protocol for the growth, induction, and purification of herpesviral fusion proteins in *Escherichia coli* was a modification of procedures described previously (19). Following induction of *E. coli* BL21/pJB130 cells, the fusion protein was purified, separated on a denaturing gel, and stained with Coomassie blue. The results (data not shown) with four independent protein preparations showed that, in each case, almost all of the GST-ICP22 fusion protein migrated near the 97,000-molecular-weight marker, indicating that the fusion between full-length ICP22 and GST was stable. This result was in contrast to that from earlier studies showing high levels of degradation when smaller portions of ICP22 were used for GST chimeras (1a, 9). In another experiment, the GST-ICP22 fusion protein reacted with the R10 antibody on an immunoblot, confirming that the ICP22 portion was in frame with GST (data not shown). Purified GST-ICP22 protein was then used to generate rabbit polyclonal antibodies. Polyclonal sera were obtained from rabbits by Cocalico Biologicals, Inc. (Reamstown, Pa.). Antibody titers were determined in a horizontal, slotted hybridization chamber (Life Technologies) by using electrophoretically separated HSV-1(F)-infected cell extracts. The extracts were transferred to nitrocellulose, and high-titer samples were termed RGST22.

Antibody RGST22 binds a tyrosine-phosphorylated cellular protein. The experiment for which the results are shown in Fig. 2 was repeated with the RGST22 antibody in place of the R10 antibody for the immunoprecipitation reactions. Products from parallel immunoprecipitation reactions were loaded onto a denaturing gel and transferred to nitrocellulose, and one half of the blot was reacted with antibody RC20 while the other was reacted with RGST22. The results (Fig. 3) were as follows. (i) Figure 3B shows that RGST22 precipitated multiple forms of ICP22 from HSV-1(F)-infected cells, as expected (lane 2). In addition, RGST22 precipitated a higher-molecular-weight protein (H_{R22}) from both infected and uninfected cells. The H_{R22} protein had a molecular weight of ~133,000 (determined as described for Fig. 2), which was similar to those of H_{RC20} (Fig. 1) and F_{R10} (Fig. 2). (ii) Comparison of the results in Fig. 3A and B showed that both ICP22 and H_{R22} reacted with the RC20 antibody, following precipitation with the RGST22 antibody (both lanes 2). Some other cellular and/or viral bands coprecipitated with the RGST22 antibody. However, in the case of ICP22, the fastest migrating form of the protein observed in panel B, lane 2, was not reactive with RC20 (panel A, lane 2), suggesting that it represents an unmodified form of the protein. All of the slower-migrating forms of ICP22 reacted with the RC20 antibody (panel A, lane 2). From these results we conclude that (i) multiple forms of ICP22 are tyrosine phosphorylated in HSV-1(F)-infected HeLa cells and (ii) the form of ICP22 which migrates fastest in a denaturing gel represents a non-tyrosine-phosphorylated species. This observation is intriguing in light of the fact that the incorporation of [^{32}P]orthophosphate into ICP22 was shown to involve the viral U_L13 gene product (14). It is not known whether U_L13 plays a role in the tyrosine phosphorylation of ICP22. (iii) The antibody RGST22, which was generated against a full-length version of ICP22, binds a cellular protein, H_{R22}, which appears to be tyrosine phosphorylated.

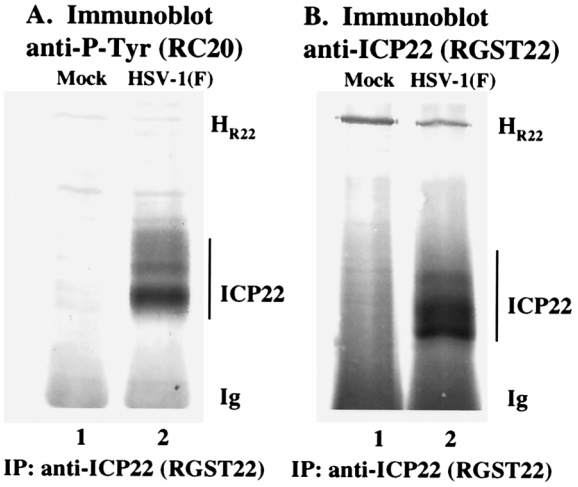

FIG. 3. Digital scanning images of antiphosphotyrosine (A) and anti-ICP22 (B) immunoblots. Uninfected (Mock) and HSV-1(F)-infected HeLa cell proteins were immunoprecipitated (IP) with antibody RGST22 prior to electrophoretic separation on a 10% BIS-acrylamide denaturing gel, transfer to nitrocellulose, and probing for reactivity with either antibody RGST22 or RC20. The locations of immunoprecipitated proteins (ICP22 and H_{R22}) are indicated on the right. P-Tyr, phosphotyrosine; Ig, location of the immunoglobulin band.

Comparison of R10 and RGST22 immune reactivities with immobilized cellular, HSV-1, and HSV-2 proteins. The results in Fig. 2 and Fig. 3 differ in that the anti-ICP22 antibody R10 did not immunoprecipitate a tyrosine-phosphorylated protein from uninfected cells. To directly compare the reactivities of antibodies RGST22 and R10 with denatured protein, duplicate samples of mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cell extracts in PBSA* buffer (phosphate-buffered saline containing 1% deoxycholate, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM 1-chloro-3-tosylamido-7-amino-2-heptone, 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone) were separated in a denaturing gel cross-linked with *N,N′*-diallyltartardiamide (DATD; Sigma) and transferred to nitrocellulose, and each half of the blot was probed with either R10 or RGST22. Since the results in Fig. 3 show higher RGST22 immune reactivities with ICP22 late in infection compared to that with H_{R22}, a viral multiplicity of infection of 0.5 PFU/ml was used, and the infected cells were harvested at 12 h. postinfection in an attempt to obtain equal levels of staining. The results (Fig. 4) from this study were as follows. (i) As expected, both RGST and R10 reacted with ICP22 from HSV-1-infected cells (Fig. 4A, lane 3, and B, lane 2). While R10 did not bind ICP22 from HSV-2 (Fig. 4B, lane 3), multiple HSV-2 bands, migrating both above and below the HSV-1 ICP22 protein, were recognized by RGST22 (Fig. 4A, lane 4). As this data is the first immunological characterization of HSV-2 ICP22, it is unclear whether all of these bands represent a population of ICP22; it is conceivable that the slower-migrating forms are intact protein while the faster form is a truncated or proteolytically cleaved ICP22. Comparison of the results in Fig. 3 and 4 show that multiple ICP22 species were observed when BIS was used as the polyacrylamide cross-linker, while a

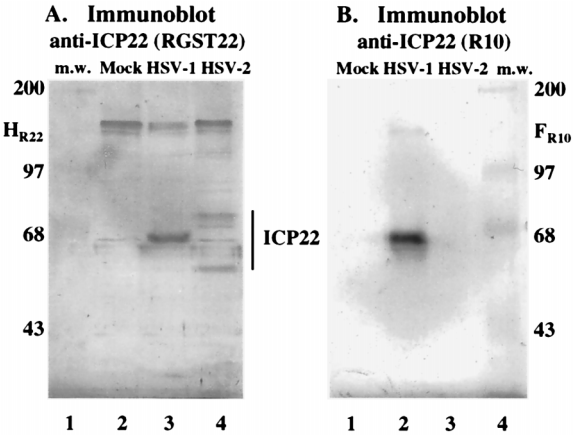

FIG. 4. Digital scanning images of immunoblots with anti-ICP22 antibodies RGST22 (A) and R10 (B). Uninfected (Mock) and HSV-1(F)- and HSV-2(G)-infected HeLa cell proteins were separated in a denaturing 12% DATD-acrylamide gel, transferred to nitrocellulose, and probed with either RGST22 or R10 antibody. The locations of ICP22, H_{R22}, and F_{R10} are indicated. m.w., prestained molecular weight markers whose sizes (in thousands) are listed.

single band was observed with the DATD gel. Various extents of resolution of ICP22 had been observed previously (2, 14) and most likely relate to the different properties of the two cross-linkers. (ii) RGST22 reacted with a series of bands, H_{R22} (~133 kDa), in uninfected and HSV-1- and HSV-2-infected cells (Fig. 4A, lanes 2 to 4), and the viral infections had no obvious significant effects on the electrophoretic properties of H_{R22}. From these results we conclude that the RGST22 antibody cross-reacts with a cellular protein. Thus, the antibody raised against the full-length ICP22 fusion protein specifically bound H_{R22} on immunoblots, as well as in immunoprecipitation reactions (Fig. 3). H_{R22} seemed to be tyrosine phosphorylated based on its reactivity with the antiphosphotyrosine antibody (Fig. 3), and it was similar in size to the major tyrosine-phosphorylated protein (Fig. 1) in HeLa cells, H_{RC20}. No immune reaction with H_{R22} was observed with the use of preimmune serum (data not shown). Although we have not observed H_{R22} reactivity using other antibodies prepared with GST-HSV protein immunogens or an anti-GST-specific antibody (data not shown), we cannot exclude the unlikely possibility that modified H_{R22} cross-reacts with an anti-GST antibody. (iii) While the similar mobilities of F_{R10} and H_{R22} might suggest that F_{R10} is of cellular origin, also, the current data is unable to confirm this prediction, and two additional observations seem inconsistent with this hypothesis. First, consistent with the results shown in Fig. 2, R10 reacted with F_{R10} only in HSV-1(F)-infected cells (Fig. 4B, lane 2) while H_{R22} was present in both uninfected and infected cells. Second, comparison of the results in Fig. 4A and B indicated that the F_{R10} protein migrated with a mobility similar to that of the fastest H_{R22} band. While these differences could be attributable to the various specificities of the R10 and RGST22 antibodies, we cannot exclude the possibility that F_{R10} represents a protein, either expressed or induced by the virus, which is unique from H_{R22}. It is also possible that the anti-ICP22 antibody recognizes a phosphotyrosine epitope present in ICP22, as well as in F_{R10} and H_{R22}. Experiments designed to clone both of these proteins should resolve these issues. The discovery that the cellular H_{R22} protein was also recognized by an ICP22-specific antibody, combined with the fact that both proteins appear to be tyrosine phosphorylated, raises the possibility that H_{R22} may be the cellular homolog of ICP22 or, at least, might share some functional properties with ICP22. While recent computational alignment analyses (1a) have identified several proteins containing domains homologous to those of ICP22, the function of the H_{R22} protein in cells is still unknown. Tyrosine phosphorylation of cellular proteins was shown to play several significant roles in intracellular signaling (reviewed in reference 5). As ICP22 is an important viral regulatory protein, it is conceivable that it uses tyrosine phosphorylation as a means to tap into the cellular signal transduction pathways, and this possibility might help to explain some of the early events leading up to the reactivation of HSV from its latent state.

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