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Analysis of Phosphorylation Sites of Herpes Simplex Virus Type 1 ICP4

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The herpes simplex virus ICP4 protein is required for induction of early and late viral gene transcription as well as for repression of expression of its own gene and several other viral genes. Several electrophoretic forms of ICP4 have been observed, and phosphorylation is thought to contribute to this heterogeneity and possibly to the multiple functions of ICP4. To define the complexity of the site(s) of phosphorylation of ICP4 and to initiate mapping of this site(s), we have performed two-dimensional phosphopeptide mapping of wild-type and mutant forms of ICP4 labeled in infected cells or in vitro. Wild-type ICP4 labeled in infected cells shows a complex pattern of phosphopeptides, and smaller mutant forms of ICP4 show progressively fewer phosphopeptides, arguing that multiple sites on ICP4 are phosphorylated. The serine-rich region of ICP4, residues 175 to 198, was shown to be a site for phosphorylation. Furthermore, the serine-rich region itself or the phosphorylation of this region increases phosphorylation of all phosphopeptides. A mutant ICP4 molecule lacking the serine-rich region showed low levels of phosphorylation by protein kinase A or protein kinase C in vitro. These results suggest that there may be a sequential phosphorylation of ICP4, with phosphorylation of the serine-rich region stimulating phosphorylation of the rest of the molecule. In addition, purified ICP4 showed an associated kinase activity or an autophosphorylation activity with properties different from those of protein kinase A or protein kinase C.

Herpes simplex virus (HSV) infected-cell polypeptide 4 (ICP4) is an immediate-early viral protein and the major transcriptional regulatory protein encoded by the virus. ICP4 is required for early and late viral transcription (7–9, 11, 12, 21, 32, 38, 40, 55, 56) and for repression of its own expression and that of other immediate-early viral genes (6, 7, 33, 44, 49). Although the mechanisms of these effects are not completely established, the abilities of ICP4 to bind to specific DNA sequences (10, 20, 24, 28, 31) and to cellular transcription factors (13, 14, 52) likely contribute to these functions.

ICP4 has been shown to undergo phosphorylation in infected cells (57, 58) and to undergo ADP-ribosylation (39) and adenylation and guanylation (2) in isolated nuclei or nuclear extracts. The only biochemical evidence available regarding ICP4 phosphorylation is that a temperature-sensitive mutant form of ICP4 labeled at the nonpermissive temperature contained phosphoserine and phosphothreonine residues (10). As many as three species of ICP4 are resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37), and as many as seven species are resolved by two-dimensional isoelectrofocusing (1). The precise nature of the posttranslational modifications of ICP4 and the origin of this heterogeneity have not been described, but these modifications may regulate the activities of ICP4 (29, 34, 41, 53).

Genetic analyses of the functional organization of the ICP4 protein have shown the following: residues 143 to 210 and residues 800 to 1298 are required for transactivation (35, 49); residues 263 to 487 are required for DNA binding (49, 60); residues 723 to 732 are required for nuclear localization (7, 30, 51); residues 309 to 489, including the DNA-binding domain, are required for dimerization (50, 59); and residues 171 to 251 are important for phosphorylation (7). Within this last portion of ICP4, residues 175 to 198 constitute a serine-rich region that is conserved among the ICP4 homologs and contains 19 serine residues and 1 threonine residue in a 24-residue sequence. This region also contains consensus sites for phosphorylation by cellular protein kinase A (PKA), protein kinase C (PKC), and casein kinase II. The importance of the serine-rich region in phosphorylation, as a site of action for PKA, and for growth of the virus in vivo, especially in sensory neurons, is demonstrated in the accompanying paper (62).

Despite the extensive genetic analysis of the ICP4 gene, there is no information available about the specific sites of phosphorylation on the molecule. We have used two-dimensional phosphopeptide mapping to attempt to localize the sites of phosphorylation on the ICP4 molecule. These studies demonstrated that multiple sites on ICP4 are phosphorylated, that the serine-rich region is a site of phosphorylation, and that the serine-rich region or its phosphorylation stimulates phosphorylation of other sites on the molecule.

MATERIALS AND METHODS

Virus and cells. The KOS1.1 strain of HSV type 1 (HSV-1) was used as the wild-type (wt) virus (7). The procedures for the propagation and plaque assay of KOS1.1 on Vero cells were as described previously (22). The HSV-1 KOS ICP4 mutant viruses *n*12, *d*2, *n*214, and *n*208 (7) and *d*8-10 (49) were propagated on E5 cells, a Vero cell-derived cell line that expresses complementing levels of the wt ICP4 upon HSV infection (6, 7). The genotypes of these viruses are described in Table 1. The HSV-1 KOS1.1 *d*27 mutant virus was propagated on V27 cells (42).

Labeling of viral proteins in infected cells and immunoprecipitation. Approximately 2×10^6 Vero cells were incubated in phosphate-free Dulbecco's modified Eagle's medium (Flow Laboratories) containing 2% inactivated fetal bovine serum for 3 h prior to infection. Cells were then infected with wt or mutant

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TABLE 1. Genotypes of virus strains used in this study

Virus	ICP4 gene	Reference
wt	wt gene with 1,298 codons	
*d*2	Deletion of codons 185–309	7
*d*8-10	Deletion of codons 142–210	49
*n*208	Nonsense codon at codon 777	7
*n*214	Nonsense codon at codon 592	7
*n*12	Nonsense codon at codon 251	7

viruses at a multiplicity of infection of 5 to 10 PFU per cell and labeled with 100 μCi of $^{32}P_i$ (New England Nuclear) in 2 ml of phosphate-free Dulbecco's modified Eagle's medium–2% fetal bovine serum from 2.5 to 5.5 or 6.0 h postinfection. At the end of the labeling, the cells were washed four times in phosphate-buffered saline containing the protease inhibitor TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) (0.1 mM) and the phosphatase inhibitors sodium orthovanadate (0.1 mM) and sodium PP_i (5 mM). Washed cell pellets were either lysed in SDS-containing gel sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.3% [wt/vol] SDS, 10% glycerol, 5.0% [vol/vol] 2-mercaptoethanol, 0.00125% [wt/vol] bromophenol blue) and directly subjected to SDS-PAGE or resuspended in 0.5 ml of lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40) containing 1 mM TLCK, 0.1 mM sodium orthovanadate, and 5 mM sodium PP_i for immunoprecipitation. For immunoprecipitation, the cell lysates were subjected to centrifugation in a microcentrifuge for 15 min, and portions of the solubilized lysate were incubated for 2 to 3 h at 4°C with 3 to 4.5 μl of ICP4-specific monoclonal antibody 58S (immunoglobulin G2a subclass) (51) for wt, *d*2, and *d*8-10 virus-infected cell extracts or with polyclonal antibody N15 (54) for *n*12, *n*214, and *n*208 virus-infected cell extracts. Different antibodies were used for the different ICP4 proteins because 58S is directed against an epitope in the carboxy-terminal portion of ICP4 (51), while N15 recognizes the amino-terminal half of the molecule (54). At the same time, 80 μl of Pansorbin cells (Calbiochem) was incubated with 5 to 10 μg of rabbit anti-mouse immunoglobulin G2a for 1 h at 4°C. At the end of the incubation, the excess antibody was washed away with lysis buffer. Immune complexes formed between ICP4 and ICP4-specific antibody were collected on rabbit anti-mouse immunoglobulin G2a-conjugated Pansorbin cells for monoclonal antibody or on Pansorbin cells for polyclonal antibody by incubation for 1 h at 4°C. The immunoprecipitates were washed three times with phosphate-buffered saline wash buffer (20 μM Tris-HCl [pH 8.0], 50 mM NaCl, 0.2% Nonidet P-40, 1 mM TLCK, 0.1 mM sodium orthovanadate, 5 mM sodium PP_i) and analyzed by SDS-PAGE with 9% polyacrylamide gels.

In vitro phosphorylation of purified ICP4 proteins with PKA or without added kinase. Approximately 20 ng of ICP4 protein purified as described previously (18) was added to a solution containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM dithiothreitol, and 20 μM [γ-^{32}P]ATP (0.15 mCi). The phosphorylation reaction was initiated by adding 60 U of PKA type I catalytic subunit purified from bovine heart (Sigma Chemical Co.; 1,000 U/0.016 mg of protein). Reaction mixtures were incubated at 30°C for 30 min and stopped by addition of SDS sample buffer. Equal portions of the reaction mixtures were analyzed by SDS-PAGE and transferred onto a nitrocellulose sheet for exposure to Kodak X-AR film and for staining with an ICP4-specific antibody. In some cases, the reactions were terminated by addition of 0.5 ml lysis buffer, and immunoprecipitation was conducted exactly as described above.

In vitro phosphorylation of purified ICP4 proteins with PKC. Approximately 20 ng of purified ICP4 protein was added to a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mg of phosphatidylserine per ml, 6 μg of diolein per ml, and 20 mM [γ-^{32}P]ATP (0.15 mCi). Reactions were initiated by addition of 50 to 100 U of PKC purified from rat brain (Promega).

Two-dimensional phosphopeptide analysis. (i) Preparation of tryptic peptides. Regions of unfixed polyacrylamide gels containing ^{32}P-labeled ICP4 proteins were excised by using the autoradiogram as a template. The gel slices were then crushed, boiled for 5 min in 1 ml of 50 mM ammonium bicarbonate (pH 7.3) containing 0.1% SDS and 5% 2-mercaptoethanol, and then shaken overnight at room temperature. The eluted proteins were then precipitated on ice for 2 h by addition of 200 μl of 100% trichloroacetic acid in the presence of 20 μg of RNase A as carrier. The pellet was washed with 100% ethanol (−20°C), resuspended in 50 μl of performic acid (8 parts 99% formic acid, 1 part 30% hydrogen peroxide, 1 part deionized H_2O), and incubated for 1 h on ice. The oxidized protein was then lyophilized and washed twice with water before digestion with 20 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Worthington Biochemical Corp.) in 50 μl of 50 mM ammonium bicarbonate, pH 8.0. The digested protein was again lyophilized and washed with distilled water four times. Approximately equal amounts of protein as determined by Western blots (immunoblots) (corresponding to approximately 1,000 Cerenkov cpm of the wt protein) were resolved in two dimensions on 100-μm cellulose thin-layer chromatography (TLC) plates (20 by 20 cm) (EM Science).

(ii) Separation of tryptic peptides. Peptides were dissolved in 10 μl of a pH 1.9 solution (88% formic acid–acetic acid–water [25:78:897, by volume]) and spotted on TLC plates along with 0.5 μl of tracking dye (a mixture of 5 mg of ε-dinitrophenyl-lysine per ml and 1 mg of xylene cyanol blue FF per ml). Electrophoresis was performed towards the cathode in the pH 1.9 solution for 28 min at 1,000 V and was followed by ascending chromatography in 1-butanol–acetic acid–pyridine–water (75:15:50:60, by volume). The positions of labeled peptides were determined by autoradiography.

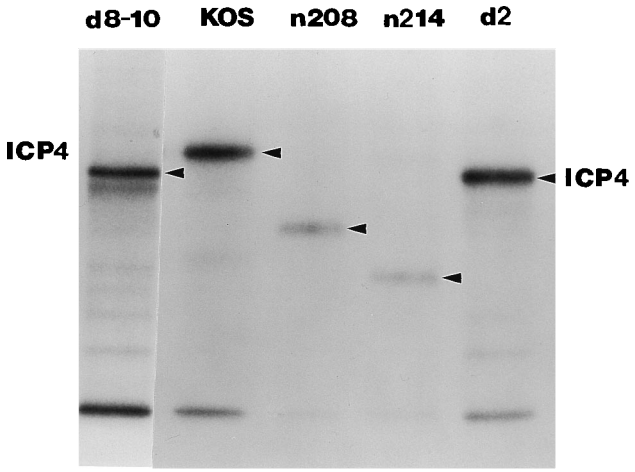

FIG. 1. Immunoprecipitation of ^{32}P-labeled ICP4. Vero cells were infected with the indicated viruses and labeled with [^{32}P]phosphoric acid. The cell lysates were immunoprecipitated with either monoclonal antibody 58S (for *d*8-10, KOS1.1, and *d*2) or polyclonal antibody N15 (for *n*208 and *n*214). The immunoprecipitates were subjected to SDS–9% PAGE. Arrowheads indicate ICP4 polypeptides.

Determination of phosphoamino acids. ICP4 proteins or tryptic peptides recovered from TLC plates were hydrolyzed in 50 to 100 μl of 6 N HCl at 110°C for 70 min. The hydrolysate was then lyophilized and resuspended in 7 μl of pH 1.9 solution containing 1 mg of cold phosphoamino acid markers (phosphoserine, phosphothreonine, and phosphotyrosine) per ml. Routinely, four samples of 30 to 100 Cerenkov cpm each were spotted on a TLC plate, and electrophoresis was performed for 20 min at 1,500 V in the pH 1.9 solution for the first dimension and for 16 min at 1,300 V in a pH 3.5 solution (acetic acid-pyridine-water [10:1:189, by volume]) for the second dimension. The positions of nonradioactive marker phosphoamino acids were detected by staining with 0.25% ninhydrin.

Peptide sequencing. The phosphopeptides of interest were recovered from TLC plates, eluted from the cellulose with pH 1.9 buffer, and then washed once with deionized water as described elsewhere (3). The peptides were then repeatedly washed and lyophilized to remove any contaminants. N-terminal amino acid sequencing of phosphopeptides was performed at the Core Facility of the Dana-Farber Cancer Institute (Boston, Mass.) according to procedures reported previously (43) with an Applied Biosystems Gas-Phase Model 470A sequenator.

RESULTS

Phosphopeptide analysis of ICP4 from infected cells. A previous study had shown that the ICP4 protein is phosphorylated on serine and threonine residues (10), but no information was available on the sites of phosphorylation on ICP4. Therefore, we used two-dimensional phosphopeptide mapping to examine the complexity of ICP4 phosphorylation and to attempt to determine the sites on ICP4 that are phosphorylated. We infected Vero cells with the wt virus or ICP4 mutant virus *n*12, *n*214, *n*208, *d*2, or *d*8-10 (Table 1). The infected cells were then labeled with $^{32}P_i$ from 2.5 to 6.0 h postinfection. Cell extracts were prepared under conditions that solubilize more than 90% of ICP4 (49). ICP4 was immunoprecipitated from each extract and resolved by SDS-PAGE and autoradiography (Fig. 1 and results not shown). The bands corresponding to ICP4 polypeptides (indicated by arrowheads) were excised from the gel, eluted, digested exhaustively with trypsin, and analyzed by two-dimensional phosphopeptide mapping. A representative autoradiographic map of wt ICP4 is shown in Fig. 2A. Fourteen

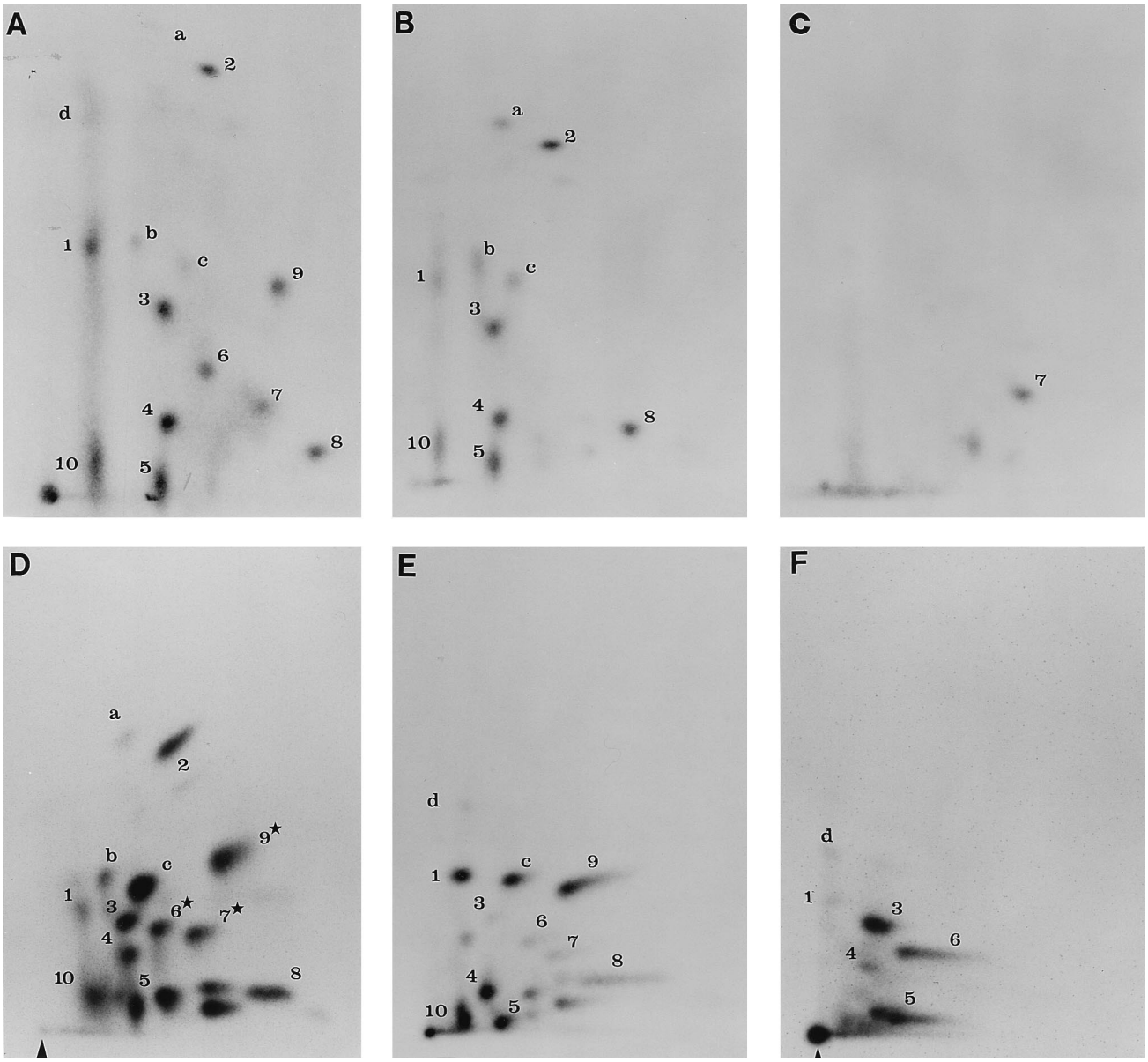

FIG. 2. Two-dimensional phosphotryptic peptide maps of ICP4 proteins. Vero cells were infected with KOS1.1 (A), *d*8-10 (B), *n*12 (C), *d*2 (D), *n*208 (E), or *n*214 (F) at a multiplicity of infection of 10. At 2.5 h postinfection, the cells were labeled for 3 h with $^{32}P_i$, and total cell extracts were prepared. ICP4 proteins were immunoprecipitated and run on SDS-polyacrylamide gels as shown in Fig. 1 (and results not shown) (61). The ICP4 proteins were then eluted from gel slices, digested with TPCK-trypsin, concentrated by lyophilization, and analyzed on TLC plates. Electrophoresis was carried out for 28 min at 1,000 V in a pH 1.9 solution, with the origin (arrowheads) at the lower left and the cathode to the right. The plates were dried and then chromatographed in an ascending buffer from bottom to top. The plates were autoradiographed with intensifying screens to shorten exposure times.

spots were consistently observed in independent experiments, and they were designated numerically (1 to 10) or alphabetically (a to d). The 14 phosphopeptides were placed into three groups according to their relative intensities. Spots 1 to 5 were consistently darker than spots 6 to 10, whereas spots a to d were always lighter than spots 6 to 10. The material at the origin was likely from undissolved peptides, because in some experiments the sample was completely dissolved and separated, and no additional spots were observed. The difference in the intensities of these spots indicated that the stoichiometries and/or the turnover rates of the phosphates at different sites were not equivalent. In addition, some spots appeared to be related to each other. For example, the intensities of spots 6 and 7 varied in a reciprocal manner. Spots 9 and 6 lie on a diagonal sloping towards the anode, whereas spots 6 and 7 lie on an opposite diagonal. These observations suggested that spots 9 and 6 may be phosphoisomers, with spot 9 being a less phosphorylated form. Spot 7 possibly represented a partial trypsin digestion product of spot 6. Partial digestion products can be caused by a proline residue immediately C terminal to an arginine residue, by tandemly arranged arginine or lysine residues, or by the presence of phosphorylated serines or threonines adjacent to the cleavage site (3, 57). Therefore, the phosphopeptide pattern of ICP4 is complex.

To identify the phosphorylated amino acid residues in each phosphopeptide, ^{32}P-labeled ICP4 was purified and subjected

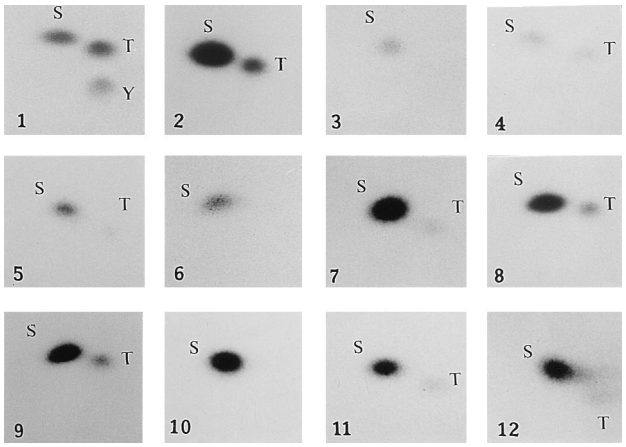

FIG. 3. Phosphoamino acid analysis of ICP4 protein and individual phosphotryptic fragments. The wt ICP4 protein was labeled in vivo with $^{32}P_i$ and isolated by immunoprecipitation and SDS-PAGE. Either the purified ICP4 protein was directly subjected to HCl hydrolysis, or it was first subjected to phosphopeptide mapping and then individual spots were recovered and subsequently subjected to HCl hydrolysis. The hydrolysates were analyzed by electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. (1) Unlabeled standards stained with 0.25% ninhydrin. Labeled species were visualized by autoradiography with intensifying screens. (2) Phosphoamino acid residues in the wt ICP4. (3 through 12) Tryptic phosphopeptides (spots) 1 through 10, respectively. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

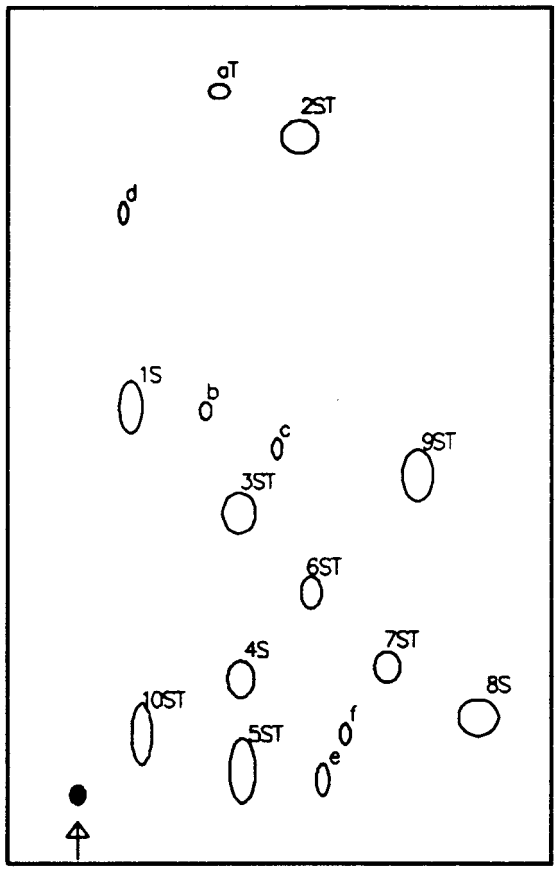

FIG. 4. Composite phosphotryptic peptide map of the wt ICP4 protein. The most intense spots were labeled 1 to 10, and the fainter six spots were labeled a to f. S, contains phosphoserine; T, contains phosphothreonine. Arrow, origin.

to HCl hydrolysis or first subjected to phosphopeptide mapping, and then each individual spot was recovered and subsequently subjected to acid hydrolysis. The phosphoamino acids were resolved by two-dimensional electrophoresis. Phosphorylation of wt ICP4 occurred on both serine and threonine residues but not on tyrosine residues (Fig. 3, panel 2). Phosphoserine was the major phosphoamino acid, with the ratio of phosphoserine to phosphothreonine being about 10:1. These results were consistent with the observations of Faber and Wilcox (10), who used a temperature-sensitive mutant form of ICP4 labeled at the nonpermissive temperature. The composition of phosphoamino acids in each of the individual spots is shown in Fig. 3, panels 3 to 12, and summarized in a composite map of the ICP4 phosphopeptides (Fig. 4). Panels 8, 9, and 11 of Fig. 3 represent the samples recovered from spots 6, 7, and 9, respectively. They were all phosphorylated on serine and threonine residues, although with relatively different ratios. The other phosphopeptides containing both phosphoserines and phosphothreonines are spots 2, 3, 5, and 10 (Fig. 3, panels 4, 5, 7, and 12, respectively). Spots 1, 4, and 8 (panels 3, 6, and 10, respectively) contained only phosphoserines, while spot a contained only phosphothreonines (data not shown). The phosphoamino acid residues in spots b to d were not examined because insufficient radioactivity was recovered. The complex phosphopeptide pattern on serine and threonine residues could be due to heterogeneous phosphorylation of one or a few sites or to phosphorylation of numerous sites.

Phosphorylation of mutant ICP4 molecules. We analyzed the phosphorylation of several mutant ICP4 molecules in an attempt to provide initial mapping of sequences required for phosphorylation. The results from two-dimensional phosphopeptide analysis of mutant ICP4 molecules are shown in Fig. 2B to F and summarized in Table 2. Several different patterns of phosphorylation emerged, in that the mutant proteins were missing different numbers of the identified phosphopeptides. The mutant *n*208 ICP4, containing residues 1 to 776, was missing only phosphopeptides 2, a, and b. This result indicated that the majority of the phosphopeptides were contained within the amino-terminal 776 residues of ICP4. The *d*8-10 ICP4, missing residues 142 to 210, lacked phosphopeptides 6, 7, and 9, while the *n*214 ICP4 molecule contained only phosphopeptides 1, 3, 4, 6, and d. Missing spots could be due to deletion of phosphorylation sites or to conformational changes in the protein that make the sites unavailable or less available for phosphorylation. Certain mutant ICP4 molecules, the *d*2 protein in particular, generated unique phosphotryptic peptides (Fig. 2D, spots 6*, 7*, and 9*), with the simultaneous disappearance of the original spots 6, 7, and 9. The new spots may be due to phosphorylation of the serine-rich region from residue 175 to 185 juxtaposed to the new sequence brought in by the deletion of the residues. Thus, these may be new phosphopeptides containing the serine-rich region. It should be pointed out that the nonsense mutations in *n*208 and *n*214 and the deletion in *d*2 have been shown to affect the ICP4 activities of transactivation, nuclear localization, and DNA binding, respectively (7). These results show that the same mutant ICP4 molecules exhibit altered phosphorylation. The relationships between the abnormal phosphorylation, altered protein structure, and altered activities of these mutant ICP4 molecules remain to be elucidated.

The phosphorylation pattern of *n*12 ICP4, an ICP4 protein with only the amino-terminal 250 residues, showed phosphorylation on only two spots, one of which comigrated with spot 7 (Fig. 2C). This result supports the idea that the phosphopeptide in spot 7 is contained within the amino-terminal 250 res-

TABLE 2. Summary of phosphopeptide mapping

Virus[a]	Presence of phosphopeptide[b]:													
	1	2	3	4	5	6	7	8	9	10	a	b	c	d
wt	+	+	+	+	+	+	+	+	+	+	±	+	+	+
*d*8-10 (Δ142–210)	+	+	+	+	+	−	−	+	−	+	+	++	++	+
*n*208 (1–776)	+	−	±	+	+	±	±	+	+	+	−	−	++	±
*n*214 (1–591)	±	−	++	±	+	+	−	−	−	−	−	−	−	+
*d*2 (Δ185–309)	±	+	+	+	+	*	*	+	*	+	+	+	++	−
*n*12 (1–250)	−	−	−	−	−	−	+	−	−	−	−	−	−	−

[a] Codons present or deleted (Δ) in the mutant viruses are indicated in parentheses.
[b] +, spot present; −, spot absent; ++, spot with increased intensity; *, new spot; ±, weak spot.

idues of ICP4. It should be noted that *n*12 ICP4 exhibits multiple electrophoretic forms on SDS gels (7), so the phosphorylation pattern of *n*12 ICP4 may be dependent on which of these forms was isolated.

In summary, the multiple patterns of phosphorylation exhibited by the mutant ICP4 molecules showed a decreasing number of phosphopeptides as more of the ICP4 protein was deleted. This argues strongly that multiple sites on ICP4 are phosphorylated and that the complex phosphopeptide pattern is not due simply to heterogeneous phosphorylation of one site or region.

Phosphorylation of the serine-rich region of ICP4. As described above, phosphotryptic peptide analysis of *d*8-10 ICP4 showed that spots 6, 7, and 9 were missing (Fig. 2B). However, when equal molar amounts of *d*8-10 and wt ICP4 were analyzed in parallel, we observed that all of the remaining phosphopeptide spots were underrepresented in *d*8-10 ICP4 compared with wt ICP4. For example, at least threefold-longer exposure times were required for *d*8-10 peptides to give spots with intensities equal to those of the spots for wt ICP4 (Fig. 2A and B). From SDS-PAGE analyses, it has been shown that the deletion of the serine-rich region causes about a 90% reduction in ICP4 phosphorylation (62). Given that only a limited number of phosphopeptides were missing in *d*8-10, it appeared that the large reduction in phosphorylation was due also to decreased phosphorylation of all of the phosphopeptides in the ICP4 molecule. This raised the possibility that ICP4 is differentially phosphorylated at multiple sites and that the fully phosphorylated state of ICP4 depends upon a conformational change introduced by the serine-rich region and/or by phosphorylation of the serine-rich region. Thus, the phosphorylation of the multiple sites in ICP4 could be due to a sequential or stepwise mode with phosphorylation of the serine-rich region being the primary event. Alternatively, some of the missing spots may be caused by a conformational change resulting from the deletion of the serine-rich region, so that these phosphorylation sites become inaccessible to protein kinases.

To confirm that the serine-rich region was indeed phosphorylated, we eluted spots 7 and 9 from TLC plates for N-terminal peptide sequencing. The sequencing data showed that spot 7 had the sequence N-Arg-Arg-Arg-His-Gly-Arg-Trp-Arg, which corresponds to residues 166 to 173 of the serine-rich region of ICP4 (Fig. 5). However, spot 9, although isolated in the same manner as spot 7, could not be sequenced because of either an N-terminal block or insufficient recovery, possibly because spot 9 is more hydrophobic and the efficiency of elution from cellulose was rather low. Given that spots 6, 7, and 9 were all phosphorylated on the serines and threonines with relatively different ratios (Fig. 3, panels 8, 9, and 11, respectively) and from an examination of the sequence of the serine-rich region, we know that there are 19 serines and only 1 threonine between amino acids 166 and 215. We therefore concluded that the single threonine at residue 180 must be phosphorylated on at least some molecules and that multiple serines were also phosphorylated in the serine-rich region. Deletion of the entire serine-rich region has been shown to reduce the activity of ICP4 in transactivation of early genes (36, 49) as well as late genes (62). Our data show that this deletion not only removes several phosphorylated peptides but also decreases phosphorylation of other parts of ICP4.

In vitro phosphorylation of ICP4. We wished to also define the sites of phosphorylation in in vitro reactions because we had shown that PKA phosphorylation of purified ICP4 was dependent on the presence of the serine-rich region (62). Purified wt or *d*8-10 ICP4 used in these reactions showed a single band on stained gels (Fig. 6). PKA or PKC labeled wt ICP4 in

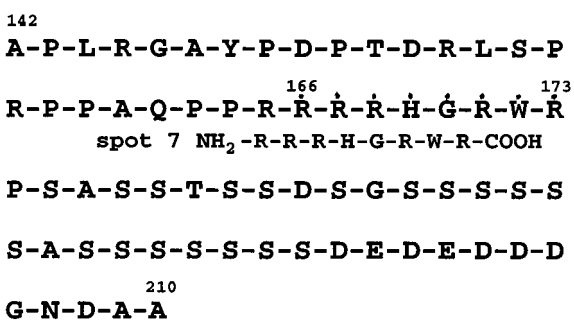

FIG. 5. Amino acid sequence of the serine-rich region (residues 142 to 210) of HSV-1 ICP4. The amino acid residues marked with dots correspond to the N-terminal sequence of spot 7 (shown).

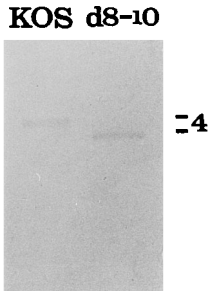

FIG. 6. Purified wt and *d*8-10 ICP4 proteins. ICP4 proteins were purified from KOS- or *d*8-10-infected Vero cells as described previously (18). Approximately 0.3 μg of each sample was subjected to SDS–9% PAGE and stained with Coomassie brilliant blue R250 to examine the purity. 4, ICP4.

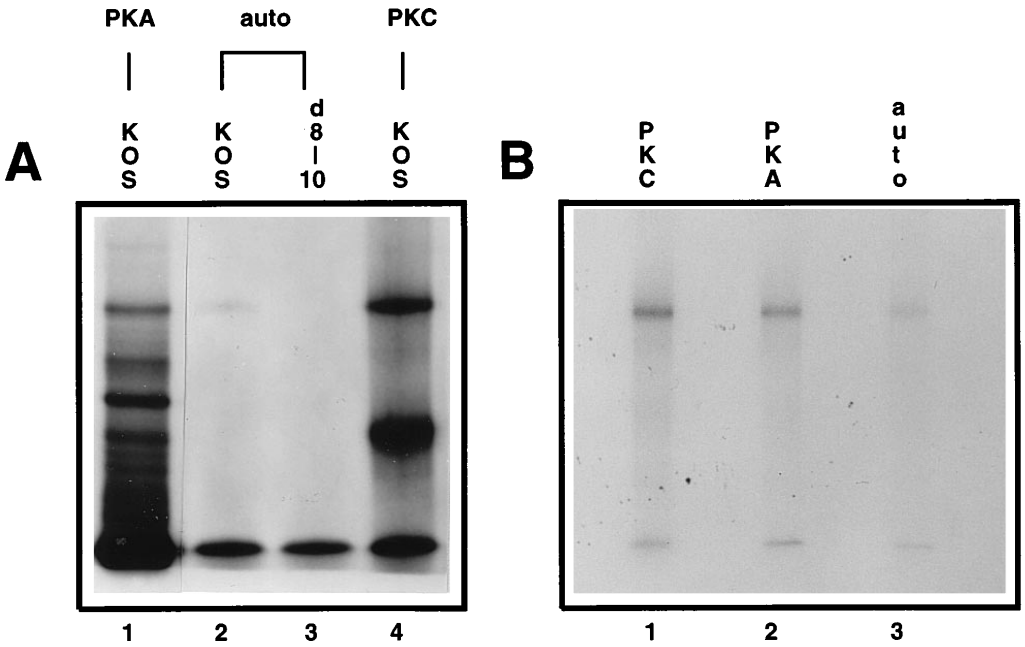

FIG. 7. Phosphorylation of ICP4 proteins with PKA, with PKC, or without added kinase (auto). (A) Purified wt (KOS) or *d*8-10 ICP4 was incubated with or without added kinase as indicated. (B) Phosphorylated wt ICP4 proteins labeled by incubation with PKA or PKC or without added kinase were immunoprecipitated with the ICP4-specific 58S monoclonal antibody to show the differences in phosphorylation.

these in vitro reactions (Fig. 7A, lanes 1 and 4, respectively), and surprisingly, there was some labeling of ICP4 in the reaction mixes without added kinase (Fig. 7A, lane 2). To determine whether phosphorylation of ICP4 by PKA, by PKC, or without exogenous kinase actually occurred at the in vivo sites, we conducted two-dimensional phosphopeptide mapping on labeled ICP4 molecules immunoprecipitated from the reactions (Fig. 7B). Figure 8 shows the maps of phosphotryptic peptides of ICP4 labeled by incubation in the absence of added kinase (Fig. 8A), by incubation with PKA (Fig. 8B), and by incubation with PKC (Fig. 8C). The positions of individual phosphopeptides in each case were then determined by mixing equal numbers of counts of the in vitro tryptic peptides with the in vivo-labeled peptides and observing whether the spots comigrated (Fig. 8D to F). With ICP4 labeled in vitro with PKA, eight phosphopeptides were apparent (Fig. 8B), and two of them comigrated with in vivo spots 6 and 7 (Fig. 8E). Phosphopeptide 6 was the peptide most efficiently labeled by PKA (Fig. 8B). However, for ICP4 phosphorylated in vitro by PKC, seven phosphopeptides were observed (Fig. 8C), and three of them comigrated with in vivo spots 6, 7, and 10 (Fig. 8F). Spot 7 and an adjacent novel spot were the peptides most efficiently phosphorylated by PKC. The in vitro phosphopeptide corresponding to in vivo spot 10 exhibited some variability in terms of intensity from experiment to experiment. This variability may be caused by the instability of phosphate groups on this peptide. Furthermore, some new phosphopeptides, such as 9*, were observed in the in vitro-phosphorylated proteins. In contrast, in vitro phosphorylation of ICP4 without added kinase showed only one predominant phosphopeptide (Fig. 8A), and this phosphopeptide colocalized with the in vivo spot 7 (Fig. 8D). Thus, the phosphorylation of ICP4 with PKA or PKC or without added kinase appeared to phosphorylate ICP4 at some of the in vivo sites. More importantly, these results indicated that phosphopeptide 7 may contain sequences needed for each of the three phosphorylation events. As described above, in vivo phosphopeptide 7 represents a fragment between residues 166 and 215 from the serine-rich region. Therefore, sequences from the serine-rich region are likely to be a target for in vitro phosphorylation.

Phosphoamino acid analysis of in vitro-labeled ICP4 peptides. To further characterize the sites of phosphorylation from the in vitro reaction, we determined the identities of the phosphoamino acids from the in vivo-labeled wt ICP4 and for the phosphopeptide 7 generated by in vivo or in vitro phosphorylation. Phosphorylation of the wt ICP4 protein occurred at both serine and threonine residues but not at tyrosine residues (Fig. 9). Phosphoserine was the major phosphoamino acid, with the ratio of phosphoserine to phosphothreonine being about 10:1. The in vitro-"autophosphorylated" peptide (Fig. 9, AUTO) contained only phosphoserine residues, while the peptide 7 in vitro phosphorylated by PKA or PKC contained both phosphoserines and phosphothreonines. Notably, the ratios of phosphoserines to phosphothreonines were different for PKA-, PKC-, or in vivo-phosphorylated peptide 7 (Fig. 9, SPOT 7). In the serine-rich region, there are 19 serines and only 1 threonine at amino acid residue 180. Therefore, this single threonine must be phosphorylated, in addition to multiple serines in the serine-rich region of ICP4 from infected cells. These results also indicated that in the serine-rich region, more than one serine residue and the threonine residue were modified by PKA, whereas at least one serine and one threonine were modified by PKC in the in vitro phosphorylation. Alternatively, the different ratios between phosphoserines and phosphothreonines might reflect different turnover rates of phosphate groups on these two amino acid residues. The data obtained from the phosphopeptide mapping and phosphoamino acid analysis suggested that the phosphorylation of ICP4 by PKA or PKC occurs at some sites that are similar to and some that are different from those phosphorylated by the kinase activity associated with ICP4.

Protein kinase activity associated with ICP4. While per-

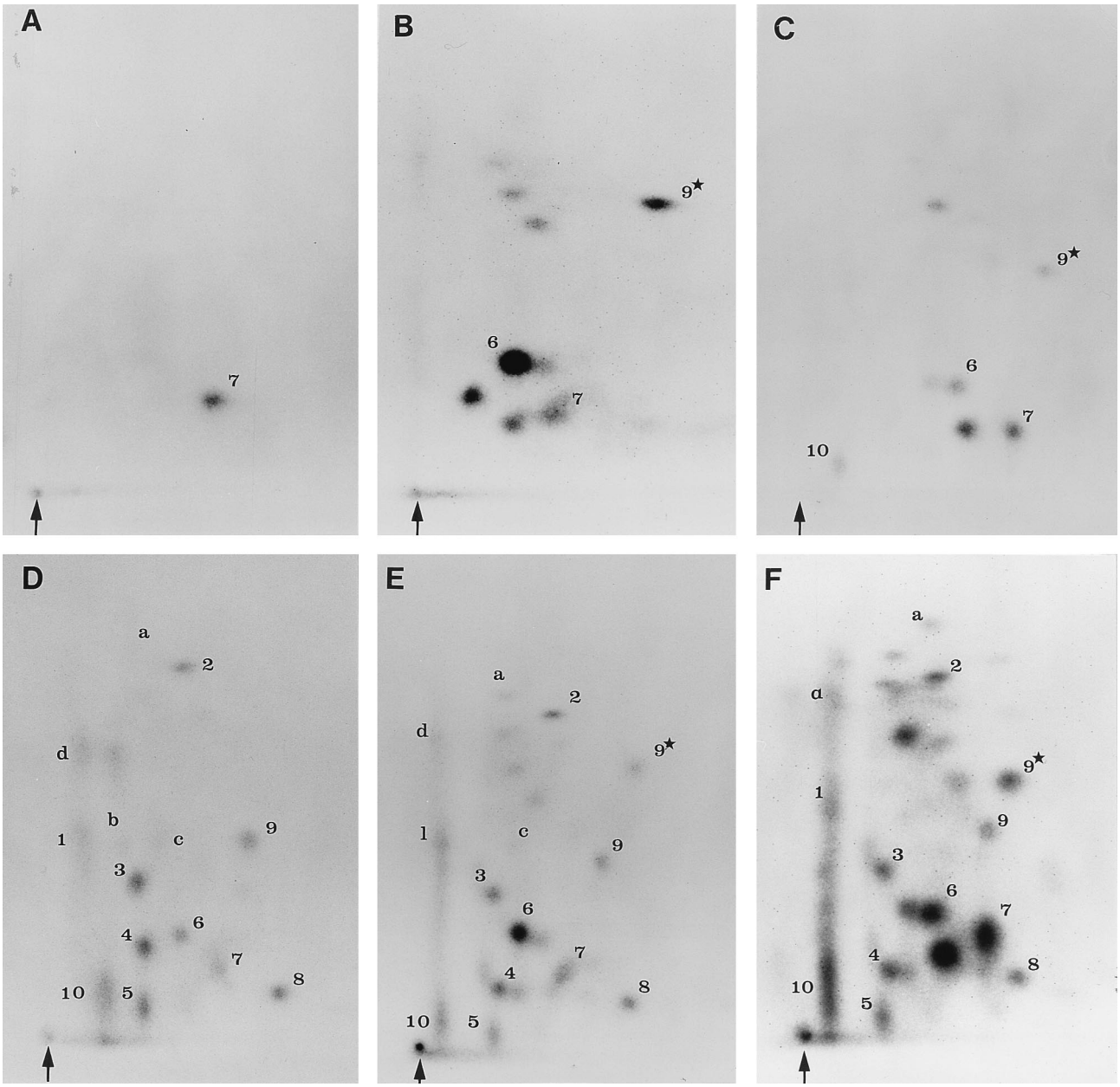

FIG. 8. Phosphotryptic maps of ICP4 phosphorylated in vitro and in vivo. Tryptic phosphopeptides, generated and resolved as described in Materials and Methods, were from ICP4 immunoprecipitated from in vitro reactions performed as follows: (A) ICP4 incubated alone; (B) ICP4 incubated with PKA; and (C) ICP4 incubated with protein kinase C. (D to F) tryptic phosphopeptides generated from in vivo-labeled ICP4 were mixed with those in panels A, B, and C, respectively, to identify the peptides phosphorylated in the absence of added kinase (D), by PKA (E), or by PKC (F). Cerenkov counts per minute loaded on thin-layer plates and exposure times (at −80°C with intensifying screens) were as follows: (A) 300 cpm, 5 days; (B) 1,300 cpm, 48 h; (C) 1,100 cpm, 48 h; (D) 300 cpm of each, 5 days; (E) 900 cpm of each, 3 days; and (F) 1,100 cpm of each, 4 days. The arrows indicate the sample origins. In panels A, B, and C, only the spots corresponding to the in vivo ones were labeled, with spot 9* as an exception.

forming the in vitro phosphorylation experiments, we observed that incubation of the highly purified wt ICP4 protein with [γ-^{32}P]ATP in the absence of added kinase resulted in the incorporation of low levels of label into ICP4 (Fig. 7A, lane 2; Fig. 7B, lane 3). When an equivalent amount of *d*8-10 ICP4 protein was incubated, no labeled band was observed (62) (Fig. 7A, lane 3). The amount of phosphorylation in the absence of added enzyme was approximately 1/10 of the level of phosphorylation in the presence of PKA (Fig. 7A, lanes 1 and 2). This phosphorylation in the absence of added enzyme may have resulted from an enzyme tightly bound to ICP4 or from an activity instrinsic to ICP4 itself. Different reaction conditions were also examined to determine the optimal conditions. We found that this reaction was dependent upon 10 mM Mg^{2+} but inhibited by the presence of 1 mM Mn^{2+}, conditions which appear to be different from those observed for many cellular or viral proteins undergoing autophosphorylation (6, 21, 24). We also found that purified ICP4 protein incubated at a very low concentration (about 0.1 ng/25-μl reaction mixture) was still phosphorylated (data not shown), consistent with a monomo-

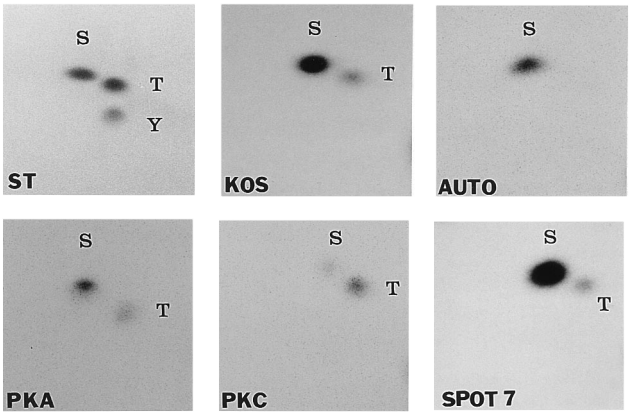

FIG. 9. Phosphoamino acid analysis of in vitro-labeled ICP4. Both in vivo- and in vitro-^{32}P-labeled ICP4 proteins were purified and either directly subjected to HCl hydrolysis or first subjected to phosphopeptide mapping. Spot 7 was then recovered and subsequently subjected to HCl hydrolysis. The hydrolysates were analyzed by electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. ST, internal phosphoamino acid standard stained with 0.25% ninhydrin to indicate the corresponding positions for phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y). KOS, hydrolysates of the in vivo-labeled wt ICP4 protein. About 200 Cerenkov cpm was used for phosphoamino acid analysis. The remainder of the panels show phosphoamino acid analyses of phosphopeptide 7 obtained from a reaction with ICP4 and no added kinase (AUTO), a reaction with ICP4 and PKA (PKA), a reaction with ICP4 and PKC (PKC), and ICP4 labeled in infected cells (SPOT 7). About 100 Cerenkov cpm of each was loaded onto TLC plates. Following electrophoresis, the plates were exposed to Kodak X-AR film for at least 4 days at −80°C with intensifying screens.

lecular reaction. We conclude that there is an unusual kinase activity associated with ICP4.

ICP4 from ICP27 mutant-infected cells shows altered phosphorylation. Rice and Knipe (41) first showed that the electrophoretic mobility of ICP4 was decreased in cells infected with ICP27 temperature-sensitive mutants at the nonpermissive temperature, and Su and Knipe (53) showed that expression of ICP27 with ICP4 in transfected cells increased the mobility of ICP4. McMahan and Schaffer (28) later observed a similar effect in cells infected with ICP27 null mutants. To investigate whether ICP27 indeed leads to an alteration of phosphorylation of ICP4, we infected Vero cells with wt or ICP27 null mutant (*d*27-1) virus, labeled the cultures with $^{32}P_i$, and purified the ICP4. Two-dimensional phosphopeptide mapping was conducted for the ICP4 proteins isolated from wt- or *d*27-1-infected Vero cells to more closely examine the effect of ICP27 on the decrease of ICP4 electrophoretic mobility. Figure 10 shows a comparison of the phosphopeptide maps of KOS1.1 ICP4 (Fig. 10A) and *d*27-1 ICP4 (Fig. 10B). Several changes were apparent in ICP4 from *d*27-1-infected cells compared with ICP4 from wt virus-infected cells. A new spot (labeled with a star) appeared, and the intensities of spots a, 2, 3, 4, 8, and 9 were significantly increased. However, the intensities of spots 1, 5, 10, and d were decreased. A larger amount of material remained near the origin with *d*27-1 ICP4 (Fig. 10B), but the differences in the phosphopeptide patterns were reproduced in other experiments (61). Therefore, ICP27 leads to a change in the phosphopeptide pattern of ICP4, indicating that a viral factor in addition to host factors can affect ICP4 phosphorylation.

DISCUSSION

Although there is evidence that the state of phosphorylation may affect the properties of the HSV ICP4 regulatory protein (29, 34, 41, 46, 53), there has been little detailed information about the sites of phosphorylation on ICP4. We have examined the tryptic phosphopeptide patterns of wt and mutant ICP4 proteins as a first step towards an understanding of ICP4 phosphorylation sites and how phosphorylation at these sites affects the functions of ICP4. A complex pattern of phosphopeptides was observed with ICP4 labeled in infected cells, and some of these phosphopeptides appeared to be structurally related. Therefore, despite the complexity of the phosphopeptide pat-

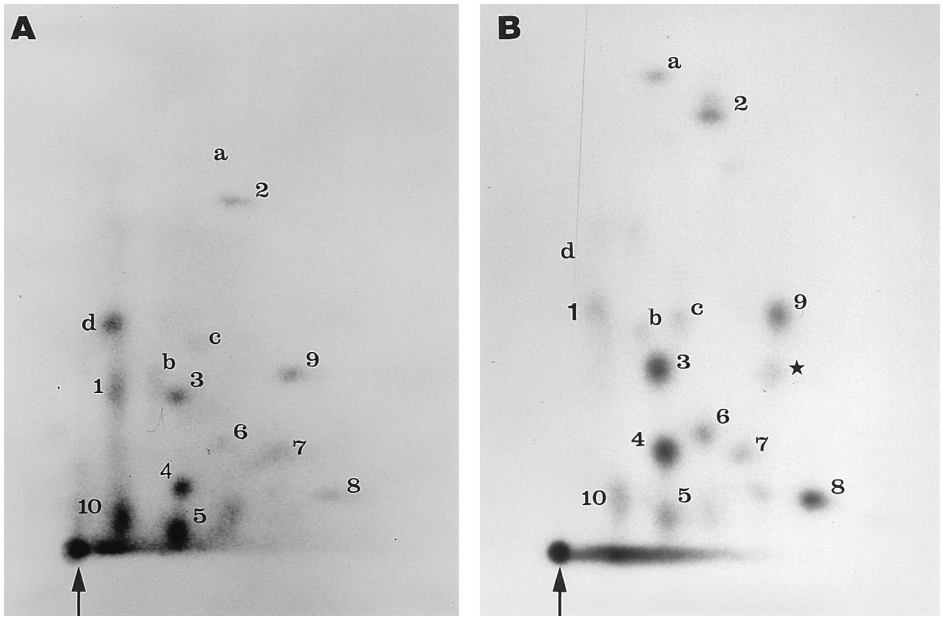

FIG. 10. Two-dimensional phosphotryptic peptide maps of ICP4 proteins from wt- or ICP27 null mutant-infected cells. Vero cells were infected with KOS1.1 (A) or *d*27-1 (B) at a multiplicity of infection of 10. The labeling and isolation of ICP4 tryptic peptides and the two-dimensional mapping were performed exactly as described in the legend to Fig. 3. Plates were autoradiographed with intensifying screens to shorten exposure times.

tern, we could not determine from this analysis if multiple sites on ICP4 were phosphorylated.

Phosphopeptide analysis of mutant ICP4 molecules containing various portions of the molecule showed at least five different peptide profiles, with the general pattern being that smaller ICP4 proteins showed fewer phosphopeptides. This was consistent with the idea that ICP4 has several sites of phosphorylation. These data did not precisely map the sites of phosphorylation on ICP4, but the results from two-dimensional phosphopeptide mapping of the wt and *n*208 ICP4 proteins indicate that most of the phosphorylation sites reside in the N-terminal half of the molecule. Furthermore, within this part of the molecule there are several functionally important domains, such as the DNA-binding domain, regions important for transactivation and the formation of complexes with TFIID and TBP, and a nuclear localization signal. These domains contain consensus motifs for phosphorylation by cellular PKA, PKC, or casein kinase II. In particular, the serine-rich transactivation domain contains consensus motifs for both PKA and casein kinase II. It will be important to determine if these sites are indeed phosphorylated by the corresponding protein kinases and how the individual phosphorylation events affect the properties and activities of ICP4.

Phosphorylation of the serine-rich region. The serine-rich region of ICP4, residues 142 to 210, was identified as one target for phosphorylation on ICP4 in both infected cells and in vitro reactions. The *d*8-10 ICP4, lacking these residues, did not contain the major phosphopeptides, spots 6, 7, and 9, when labeled in infected cells. N-terminal sequencing of spot 7 showed that it arose from the serine-rich region, directly proving that this region is phosphorylated. Phosphoamino acid analysis showed that multiple serine residues and the one threonine residue in this region were phosphorylated. The specific serine residues that were phosphorylated in this region were not determined.

Despite the observation that only a few spots were missing in the *d*8-10 ICP4 phosphopeptide pattern, other studies have shown that total ^{32}P incorporation into *d*8-10 ICP4 was reduced by 90% relative to that for wt ICP4 (62). In addition, nearly all of the phosphopeptide spots were reduced in intensity in *d*8-10 ICP4 compared with wild-type ICP4. Thus, while part of the loss of phosphorylation in *d*8-10 ICP4 can be attributed to the loss of phosphorylation in the serine-rich region, the rest appears to be due to a decrease in phosphorylation of other sites. The serine-rich region of ICP4 seems to stimulate phosphorylation of the rest of the molecule, possibly through its own phosphorylation or by changing the conformation of the rest of the protein. Thus, there may be a sequential phosphorylation of multiple sites on ICP4, which could explain the multiple electrophoretic forms of ICP4 seen on SDS gels.

In vitro phosphorylation of purified ICP4 in the absence of added protein kinases. It was unexpected to find that highly purified ICP4 proteins could undergo phosphorylation without exogenous kinases. This activity could be either an intrinsic property of ICP4 or a tightly associated cellular or viral protein kinase(s). In either case, the same activity may play a role in phosphorylation of ICP4 in vivo, because the same phosphopeptide was labeled in vivo. Further insight into this property could be gained by studies on ICP4 proteins purified from an in vitro translation system or from an *Escherichia coli*- or a baculovirus-based expression system. Both cellular proteins, in particular, cell surface receptors (17, 48, 63), and viral proteins, for instance, the Src protein of Rous sarcoma virus (5), undergo autophosphorylation. Structural analysis revealed that these proteins usually contain a specific ATP-binding motif (Gly-X-Gly-X-X-Gly) in their catalytic domains (15, 25). Examination of the predicted ICP4 amino acid sequence indicates that ICP4 also contains such a motif (Gly-Tyr-Gly-Ala-Ala-Gly) in residues 515 to 520. If ICP4 does undergo autophosphorylation, this region and its flanking sequences may be part of a catalytic domain. Alternatively, if this phosphorylation is due to an associated protein kinase, dissociation of the complex under appropriate conditions should allow purification and identification of the enzyme.

Effects of HSV ICP27 on ICP4 phosphorylation and function. Studies with infected cells (28, 41) and transfected cells (53) have shown that ICP27 leads to an electrophoretic shift in ICP4 and stimulation of late gene expression. The electrophoretic shift was hypothesized to be due to changes in phosphorylation of ICP4. The phosphopeptide analysis presented here indicates that ICP4 phosphorylation is indeed altered in cells infected with an ICP27 mutant virus. We hypothesize that ICP27, either directly or indirectly, alters protein kinase levels or phosphatase levels so that phosphorylation of ICP4 is altered. The altered ICP4 may then be a more efficient transactivator of late gene expression (26, 41, 42, 45), likely at the level of transcription (19). The presence of ICP27 may also affect the phosphorylation of ICP4 such that the ability of ICP4 to bind to DNA and repress transcription is affected. Recently, it has been shown that the DNA-binding and repression activity of a mutant ICP4 protein that cannot activate transcription is altered by the presence of ICP27 (46). ICP27 has also been observed to be required for viral inhibition of RNA splicing (16) and for inhibition or activation of expression of genes containing specific 3′ termini or processing signals (4, 27, 47). If ICP27 does lead to a general change in protein phosphorylation in infected cells, this could be responsible for the pleiotropic effects of ICP27.

Phosphorylation of ICP4 in the infected cell. On the basis of the results reported in this paper and the accompanying paper (62), we postulate that the following events occur in the infected cell. During productive infection of a permissive cell, ICP4 is first phosphorylated in the serine-rich region by PKA, PKC, or another kinase. This modification allows or promotes a change in conformation in ICP4 so that it can be phosphorylated at other sites in the molecule, fully activating it to interact with cellular transcription factors or DNA and activate transcription. Alternatively, phosphorylation of the serine-rich region may activate a kinase activity associated with or intrinsic to ICP4 which phosphorylates other sites on the ICP4 molecule in *cis* or *trans*. Although the serine-rich region of ICP4 stimulates phosphorylation of ICP4 and viral growth, it is not absolutely essential for phosphorylation of ICP4 or viral growth in permissive cells. Therefore, in these cells there are mechanisms for phosphorylation of ICP4 which are independent of the serine-rich region.

In contrast, there is a greater restriction of growth of the serine-rich region mutant *d*8-10 in trigeminal ganglion cells (10^3- to 10^4-fold reduction compared with wt) than in the corneal epithelium (10^1- to 10^2-fold reduction compared with wt (62). Thus, there is increased dependence on the serine-rich region of ICP4 for viral replication in the neuronal cells. This may be due to decreased levels in neurons of kinases that phosphorylate ICP4 in the absence of the serine-rich region. The stimulation of phosphorylation of ICP4 by the serine-rich region may have evolved as a means to accomplish efficient phosphorylation of ICP4 in the neuronal cell, where levels of critical kinases are low, or it may have evolved as part of a mechanism for sensing the levels of protein kinase activity in a neuronal cell as part of the decision to undergo a productive infection or establish a latent infection in the neuron. Recent results (23) have shown a low level of ICP4 gene transcripts in

latently infected ganglia. ICP4 may be expressed at low levels during latent infection but be poorly phosphorylated. Activation or damage of the neuron could activate kinases so that the ICP4 is phosphorylated, initially in the serine-rich region. This would lead to a stimulation of the ICP4 transactivation ability, possibly leading to reactivation. Given the possibility that ICP4 may be present in latently infected ganglia, it may also affect the latent state through its repression activity. Because the ability of ICP4 to bind DNA and repress transcription may also be a function of phosphorylation or the presence of other immediate-early proteins, these activities may also be subject to the same regulatory mechanisms as activation. In these ways, phosphorylation of ICP4 would serve as part of a signal transduction pathway serving to regulate the latent genome in the sensory neuron.

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