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Protein Kinase Activity Associated with the Large Subunit of Herpes Simplex Virus Type 2 Ribonucleotide Reductase (ICP10)

THEODORE D. CHUNG,[1] JAMES P. WYMER,[1] CYNTHIA C. SMITH,[1]
MICHAEL KULKA,[2] AND LAURE AURELIAN[1,2]*

Virology/Immunology Laboratories, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Division of Biophysics, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205[2]

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The large subunit of the herpes simplex virus type 2 (HSV-2) ribonucleotide reductase (RR1) is demonstrated to possess serine/threonine-specific kinase activity. Computer-assisted sequence analysis identified regions within the amino terminus of ICP10 that are homologous to the catalytic domain of known protein kinases (PKs). An in vitro kinase assay confirmed the ability of ICP10, immunoprecipitated from either HSV-2-infected cells or from cells transfected with an ICP10 expression vector, to autophosphorylate and transphosphorylate exogenous substrates in the presence of ATP and Mg^{2+}. The HSV-1 RR1 was shown to be negative for PK activity under these conditions. PK activity was localized to a 57-kDa amino-terminal region within ICP10 that lacked RR activity.

Ribonucleotide reductase (RR) plays a key role in DNA synthesis of eucaryotic and procaryotic cells. Mammalian and *Escherichia coli* RRs consist of two subunits, and their activities are under stringent allosteric control (52). Mammalian cells infected with herpes simplex virus (HSV) contain a different RR activity that is insensitive to dTTP or dATP inhibition and does not have an absolute Mg^{2+} requirement (27). Like the mammalian and bacterial enzymes, the HSV RR consists of two heterologous subunits. The large subunit (RR1), molecular size 140 kilodaltons (kDa), designated ICP6 for HSV-1 and ICP10 for HSV-2, is encoded within the U_L region of the viral genome (map units 0.554 to 0.584). The small subunit (RR2), molecular mass of 38 kDa, is encoded by a 1.2-kilobase (kb) mRNA that overlaps the 3′ end of the 5.0-kb transcript of RR1. However, the coding regions for the two subunits do not overlap (2, 4, 12, 34).

The HSV RR1 protein, localized primarily to the cytosolic fraction, is phosphorylated, undergoes phosphate cycling, and exists as several distinct species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (55). Comparison of the DNA sequences of the HSV RR1 to counterparts in eucaryotic and procaryotic cells or in other viruses, including Epstein-Barr virus, varicella-zoster virus, and pseudorabies virus (28), has identified an additional amino-terminal domain in the HSV RR1 that produces a 50% increase in molecular weight (37, 51). Proteolytic degradation studies have associated RR activity with the carboxyl two-thirds of the HSV RR1 molecule, leading to the suggestion that the amino-terminal domain unique to the HSV protein is functionally distinct (19).

The following observations are consistent with this interpretation. Fragments covering only the promoter and/or proximal one-third of the HSV-2 RR1 (ICP10) coding region have transforming potential (15, 21–23). The transforming potential of HSV-1 maps at a site distant from the ICP6 gene (9), and the HSV-unique amino-terminal domain of RR1 shows only 38% intertypic (HSV-1 versus HSV-2) homology, as compared to 93% for the remaining carboxyl two-thirds of the molecule (37). Additionally, the expression of RR1 and enzymatic activity behave temporally as delayed-early genes (reviewed in reference 16). However, *cis*-response elements of the ICP10 promoter include those associated with regulation of immediate-early gene expression, as well as consensus AP-1, octamer transcription factor 1, and potential SP-1 binding elements (56) which may be related to the postulated distinct function of the amino terminus.

The studies described in this report sought to obtain a better definition of this putative function. In vitro kinase assays of ICP10-containing immunoprecipitates from HSV-2-infected cells and from cells transfected with an ICP10 expression vector or an ICP10-negative frameshift mutant indicate the presence of a protein kinase (PK) activity associated with ICP10. This activity was localized to the one-third amino-terminal region of ICP10 that shares homology with conserved features of PK catalytic domains (PK motifs).

MATERIALS AND METHODS

Computer analysis of sequences. Computer analysis was performed with an IBM personal System 2 model 60 with the PC/Gene program (Intelligenetics, Inc., Mountain View, Calif.). The Cyborg data base manager (International Biotechnologies, Inc., New Haven, Conn.) was used for homology searches with the GenBank data base (version 52). Identification of PK motifs was based on the analysis of Hanks et al. (14).

Cells and viruses. Human epidermoid carcinoma no. 2 (HEp-2) cells were grown in medium 199 with 10% newborn calf serum, 150 U of penicillin G per ml and 150 μg of streptomycin sulfate per ml. Vero (African green monkey kidney) and 293 (adenovirus type 5-transformed human embryonal kidney) cells were grown, respectively, in minimum essential medium or Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. Stocks of HSV-2 (strain G) and HSV-1 (strain F) were prepared and titers were determined as described previously (50).

Plasmids. The construction of the ICP10 constitutive

* Corresponding author.

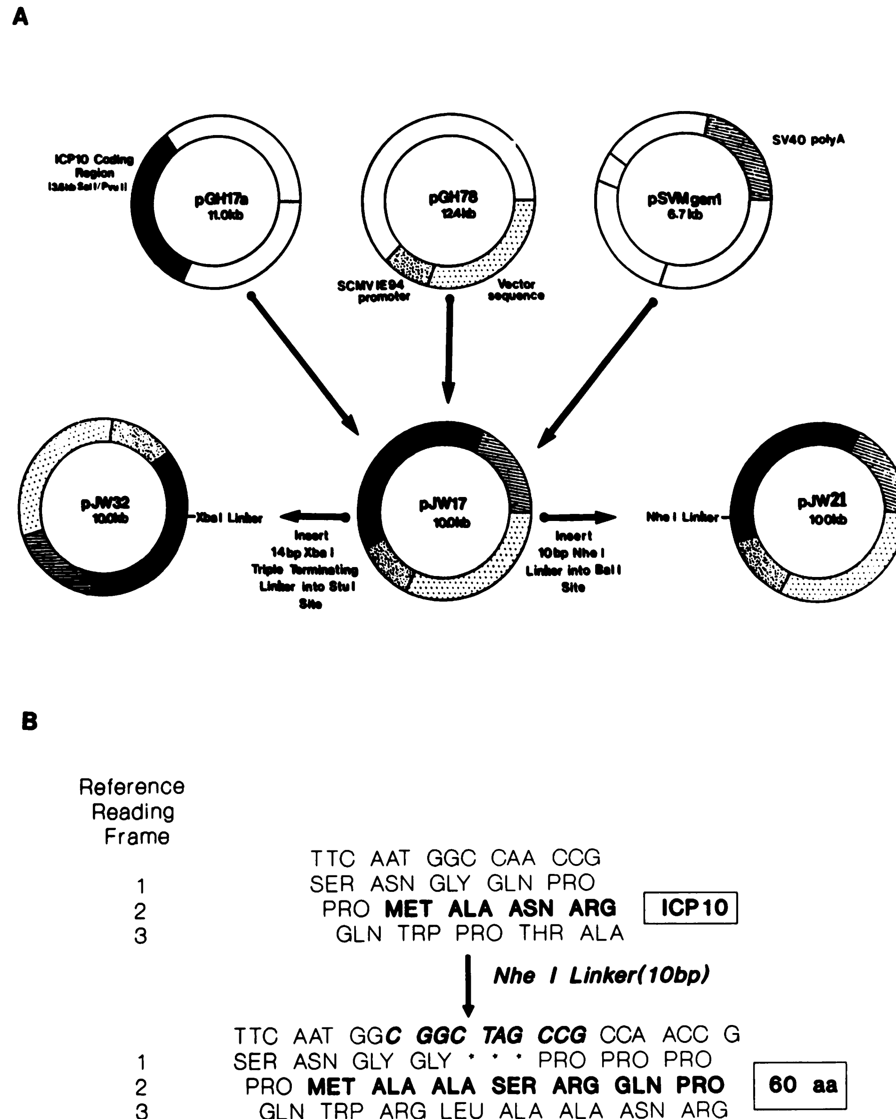

FIG. 1. Construction of ICP10 constitutive expression vector pJW17, the amino-terminal mutant pJW32, and frameshift mutant pJW21. (A) The 3.6-kb *Sal*I-*Pvu*I ICP10 coding region from pGH17a and the simian virus 40 poly(A) from pSVM-gen1 were colinearly inserted into pGH78 behind the SCMV IE94 promoter where it replaced the IE175 gene. pJW32 was constructed by inserting a 14-bp *Xba*I triple terminating linker into the *Stu*I site of pJW17. pJW21 was generated by inserting a 10-bp *Nhe*I oligonucleotide linker into the *Bal*I site of pJW17. (B) Schematic representation of the frameshift mutation in pJW21. The initiation codon of ICP10 directs translation (bold lettering) in reference reading frame 2. Insertion of the 10-bp *Nhe*I linker[d(CGGCTAGCCG)] at the *Bal*I site (after the first base pair of the triplet codon for residue 2) results in a frameshift mutant that theoretically directs the synthesis of an unrelated 60-amino-acid-long polypeptide.

expression vector pJW17, the amino-terminal mutant pJW32, and the frameshift mutant pJW21 is shown in Fig. 1. Parent plasmids were pGH78, containing the constitutive simian cytomegalovirus IE94 promoter regulating the expression of HSV-1 IE175 gene (44); pGH17a, containing the ICP10 coding region (15); and pSVM-gen1, containing the simian virus 40 poly(A) domain (43). A 3.75-kb *Pvu*I fragment of pGH17a was subcloned into the *Xba*I site of pUC18 after the addition of an *Xba*I linker. The 1.5-kb *Bgl*II-*Eco*RI fragment of pSVM-gen1, containing the simian virus 40 middle T-antigen splice site and the poly(A) signal, was subsequently inserted distal to the ICP10 coding region in order to provide for efficient termination of transcription. *Sal*I partial and *Eco*RI complete digestion of the resulting plasmid generated a 5.1-kb fragment encompassing the ICP10 mRNA leader and coding sequences with the simian virus 40 poly(A) site. Replacement of the IE175 gene in pGH78 with this fragment generated the constitutive expression vector. The amino-terminal mutant pJW32 was created by inserting a 14-base-pair (bp) *Xba*I triple terminating linker [d(CTAGTCTAGACTAG); New England BioLabs, Inc., Beverly, Mass.] into the unique *Stu*I site of pJW17. *Stu*I cuts the ICP10 gene immediately after the codon for amino acid residue 446. The frameshift mutant pJW21 was created by inserting a 10-bp *Nhe*I oligonucleotide linker [d(CG-GCTAGCCG); New England BioLabs, Inc.] into the *Bal*I site of pJW17, which interrupts the codon for the second amino acid. This frameshift mutant should express a 60-residue peptide unrelated to ICP10.

Monoclonal antibodies (MAbs). The preparation and partial

characterization of MAb 30 has been described elsewhere (L. Aurelian, P. Terzano, C. C. Smith, T. Chung, A. Shamsuddin, S. Costa, and C. Orlandi, Cancer Cells, in press). It specifically precipitates ICP10 from HSV-2-infected cells but does not react in Western blot (immunoblot) assays (L. Aurelian et al., in press). MAb 27, specific for the HSV major DNA-binding protein ICP8, was prepared from a tertiary clone of hybridomas derived from the same fusion used to obtain MAb 30. MAb 48S, which recognizes HSV-type common determinants on the HSV-1 (ICP6) and HSV-2 (ICP10) RR1 (47), was obtained from M. Zweig (Laboratory of Molecular Virology, National Cancer Institute, Frederick, Md.).

Peptide synthesis and antiserum preparation. The synthesis of peptide LA-1, corresponding to residues 13 to 26 of the HSV-2 ICP10 protein, and the preparation of monospecific antibody (anti-LA-1) were described elsewhere (L. Aurelian et al., in press). Two additional peptides with amino acids corresponding, respectively, to residues 165 to 179 (LA-3) and 355 to 369 (LA-2) of ICP10 were synthesized by Merrifield solid-phase methods (49). Cysteine was added to the carboxyl termini, and the peptides were incorporated into keyhole limpet hemocyanin, emulsified in complete Freund adjuvant, and used to prepare specific antisera by subcutaneous injection into New Zealand White rabbits as described elsewhere (L. Aurelian et al., in press). All antibodies recognize ICP10 but do not inhibit RR activity (Chung and Aurelian, unpublished data).

Metabolic labeling, immunoprecipitation, and subcellular fractionation. HEp-2 cells mock infected with phosphate-buffered saline, pH 7.4, or infected with 20 PFU of HSV-2 (G) per cell were labeled with [^{35}S]methionine (50 μCi/ml, specific activity 1,120 Ci/mM; Dupont, NEN Research Products, Boston, Mass.) in minimum essential medium containing 0.1× the normal concentration of methionine and 1% dialyzed fetal calf serum at various times postinfection (p.i.). Transfected cells were similarly labeled with [^{35}S]methionine 30 to 40 h posttransfection. Cells were harvested by scraping; they were washed in cold phosphate-buffered saline and were used in immunoprecipitation. In some experiments, they were fractionated as described by Boss et al. (6) into soluble (SOL), cytoskeletal (CSK), and nuclear (NUC) components prior to immunoprecipitation. The purity of the fractions was previously established (6). The method of preparation and purity of plasma membranes were as previously described (53). Briefly, cells were hypotonically lysed in a solution consisting of 0.02 M boric acid, pH 10.2, with 0.2 mM EDTA, and they were purified by centrifugation (24,000 × g; 1 h) on a 35% (wt/wt) sucrose cushion. Purity was confirmed by glucose-6-phosphatase assays, indicating that the enzymatic activity in membrane preparations was less than 5% of that in whole cell extracts (data not shown). Whole cell pellets, isolated nuclei, and membrane fractions (5 × 10^6 cell equivalents/ml) were suspended in cold radioimmunoprecipitation buffer (0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 0.15 M NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 15 min, and disrupted by sonication (5 10-s pulses at maximum setting; Ultrasonics model 220F Sonifier). Extracts were clarified by centrifugation (30 min) at 16,000 × g and incubated (1 h, 4°C) with 25 μl of antibody and 100 μl of protein A-Sepharose CL4B (50% [vol/vol]; Sigma Chemical Co., St. Louis, Mo.). Beads were washed with cold radioimmunoprecipitation buffer, and bound proteins were eluted by boiling (3 min) in 100 μl of denaturing solution (150 mM Tris hydrochloride [pH 7.0], 5.7% SDS, 14% 2-mercaptoethanol, 17% sucrose, 0.04% bromothymol blue). Proteins were resolved by SDS-PAGE on 7% polyacrylamide gels and visualized by autoradiography on Kodak X-Omat AR film as previously described (20).

DNA transfection. Cells were plated 24 h prior to transfection into 6-well cluster dishes (6 by 35 mm; Costar, Cambridge, Mass.) at 1 × 10^5 cells/well and were transfected with supercoiled plasmid DNA (2.5 μg/well) by the calcium phosphate precipitation method by using a glycerol boost (56). They were harvested 40 to 44 h posttransfection, washed twice with phosphate-buffered saline, and stored as a dry pellet at −80°C until used.

Western blot assay. Cells were extracted in radioimmunoprecipitation buffer, and extracts standardized for protein concentration (31) were subjected to SDS-PAGE on 8.5% polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose and analyzed by immunoblotting with anti-LA-1 serum as previously described (Aurelian et al., in press).

Immune complex kinase assay. Unlabeled cell extracts were standardized for protein concentration and immunoprecipitated with antibody and protein A-Sepharose CL4B. The beads were washed with TS buffer (20 mM Tris hydrochloride [pH 7.4], 0.15 M NaCl), suspended in 50 μl of kinase reaction buffer consisting of 10 μCi of [γ-^{32}P]ATP (0.1 μM; Dupont, NEN Research Products), 5 mM $MgCl_2$, and 20 mM Tris hydrochloride (pH 7.4), with or without 5 μg of histone type III-S (from calf thymus; Sigma) and incubated at 30°C for 10 min (38). In competition experiments, the immune complexes were incubated for 30 min at 4°C with a second (competing) antibody before addition of the kinase reaction buffer. The kinase reaction was terminated by boiling in 100 μl of 0.5× denaturing solution, and the proteins were resolved by SDS-PAGE.

Phosphoamino acid analysis. Extracts of HSV-2-infected cells were immunoprecipitated with MAb 30 (specific for ICP10) and assayed for in vitro kinase activity. Gels were exposed to Kodak X-Omat AR film to locate the ^{32}P-labeled ICP10 band. Gel sections corresponding to ICP10 were excised and homogenized in 2.0 ml of 50 mM ammonium bicarbonate with 0.1% SDS–1 mM EDTA–10 μg of bovine serum albumin per ml (16 h, 37°C). The eluted proteins were cleared of gel debris by filtration through a 0.22-μm cellulose acetate filter (Spin-X; Costar) and trichloroacetic acid (20% final concentration) precipitated. Following centrifugation (15 min; 16,000 × g; 4°C), the precipitates were washed with cold absolute ethanol and cold ethanol-diethyl ether (50% [vol/vol]), suspended in 100 μl of boiling 6 N HCl, and incubated 1 h at 110°C. The hydrolysates were lyophilized and resolubilized in 10 μl of a marker mixture containing 1 mg each of phosphoserine, phosphothreonine, and 04-phosphotyrosine (Sigma) per ml. Phosphoamino acids were separated by two-dimensional paper electrophoresis on Whatman no. 3 chromatography paper at pH 2.3 for 2.5 h at 400 V in 1 M acetic acid (first dimension) and at pH 5.0 for 2.5 h at 400 V in 0.1 M pyridine-acetic acid (second dimension). The position of the markers was ascertained by staining with ninhydrin spray reagent (Sigma), and the ^{32}P-labeled amino acids were visualized by autoradiography (46).

RESULTS

Conserved PK motifs in the ICP10 amino-terminal domain. Computer-assisted analysis of the predicted amino acid sequence of ICP10, aligned as described by Hanks et al. (14), revealed the presence of PK motifs (subdomains I through

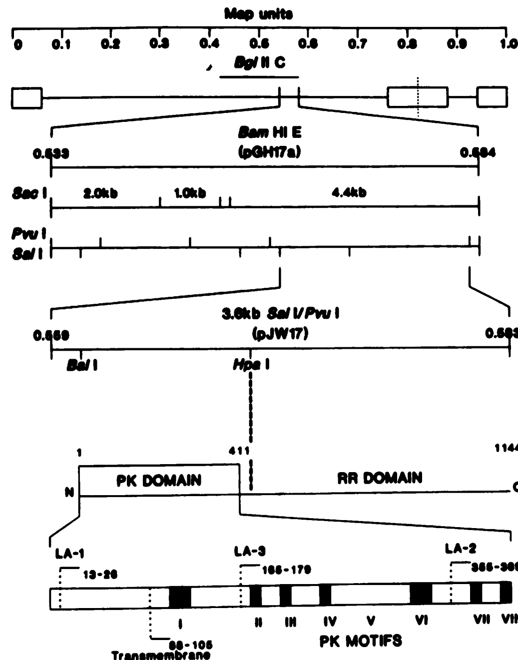

FIG. 2. Physical map of the location of the ICP10 coding region cloned in pJW17, and schematic representation of ICP10 as a multifunctional protein. Two domains are identified, an amino-terminal PK domain (residues 1 to 411) and a carboxy-terminal RR domain (residues 411 to 1144). The unique *Hpa*I site within the ICP10 coding region represents the 3′ end of the minimal transforming region of HSV-2 (21) and cuts the gene after the codon for residue 417. The *Stu*I site cuts the gene after the codon for residue 446. The expanded PK domain highlights regions of homology with catalytic motifs conserved in PKs (PK motifs [■]; 14). The location of a potential transmembrane helical segment (42) and of the synthetic peptides LA-1, LA-3, and LA-2 are identified by respective residue numbers.

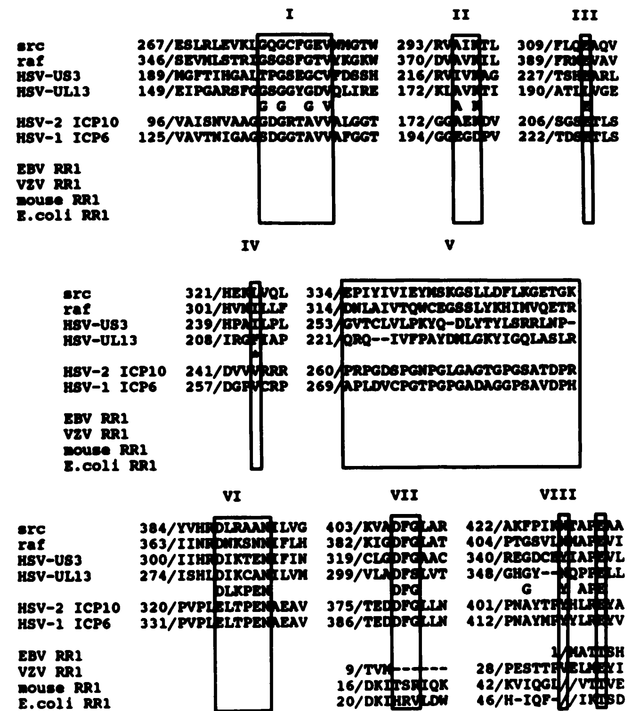

FIG. 3. Amino acid sequence alignment of ICP10 (from HSV-2 strain 333 [51, 56]) and ICP6 (from HSV-1 strain 17 [37]) with PK motifs (14). src and raf are prototype protein-tyrosine and protein-serine/threonine kinases, respectively. The gene product of HSV US3 is a previously characterized HSV PK (41). HSV UL13 was recently identified by computer-assisted analysis (48). Sequences of the RR1 of EBV, VZV, mouse, and *E. coli* are aligned with the HSV sequences as previously described (37, 51). Boxed regions highlight residues that are highly conserved in ICP10.

VIII) clustered within the HSV-unique amino terminus at residues 1 to 411 (Fig. 2). The motifs (Fig. 3) include, at residues 105 to 110 (subdomain I), a near-consensus Gly-X-Gly-X-X-Gly motif found in many nucleotide-binding proteins (54) that is followed at residue 112 by Val, consistent with other PKs (14). Residues 174 to 176 (subdomain II) include the invariant Lys that appears to be involved in phosphotransfer reactions (24). In v-*src* (25) and other PKs (14), this Lys residue reacts with the ATP analog *p*-fluorosulfonyl 5′-benzoyl adenosine, thereby inhibiting enzyme activity. All substitutions at this site, including Arg, have resulted in loss of PK activity (14). Neither of these subdomains is conserved in the HSV-1 RR1 protein (Fig. 3). Other motifs include subdomains III and IV, the functions of which are unknown, and the invariant or nearly invariant residues in subdomains VI and VII that have also been implicated in ATP binding (7). Subdomain VI contains residues that are specifically conserved in either the protein-serine/threonine or the protein-tyrosine kinases. Our motif is similar to the consensus Asp-Leu-Lys-Pro-Glu-Asn, considered a strong indicator of serine/threonine specificity (14). Glu-324 is a conservative substitution for the near-consensus Asp that is common among evolutionarily related proteins (10). Thr-326 (substituting Lys) is situated within the poorly conserved domain of this motif (14). The triplet Asp-Phe-Gly in subdomain VII is of particular interest, as it represents the most highly conserved short stretch in the PK catalytic domains (14). It is flanked on the right side by nearly neutral residues (Fig. 3). Subdomain VIII contains the invariant residue Glu that is part of the Ala-Pro-Glu triplet, considered a key PK catalytic domain indicator (18).

Additional features revealed by this computer-assisted analysis are a potential transmembrane helical segment at residues 88 to 105, based on the algorithm of Rao and Argos (42), four potential N-glycosylation sites (residues 69, 609, 716, and 944), and features of a signal peptide, including a positively charged residue (Arg-4), followed by a short (nine residues) hydrophobic core ending with the Ala-X-Ala motif (Ala-11–Gly-12–Ala-13), the most common signal peptidase recognition site found in membrane-associated proteins (39). Finally, sequences associated with rapid intracellular protein degradation were identified at residues 141 to 161 and 177 to 212 according to the PEST SCORE algorithm (45). It is noteworthy that these PEST SCORES (7.57 and 23.15, respectively) are similar to those observed for oncogenes E1A and c-*myc* and are consistent with a short half-life (45). Similar sequences were also identified in the HSV-1 protein at residues 130 to 154 and 212 to 243 (PEST SCORES 9.31 and 6.65, respectively), but not in the HSV RR2 nor in the mammalian or the EBV or VZV RR1 proteins.

Detection of a serine/threonine-specific PK activity in ICP10 containing immunoprecipitates. To determine whether immunoprecipitated ICP10 contains phosphorylating activity, extracts of HEp-2 cells mock infected with phosphate-buffered saline or infected with HSV-2 or HSV-1 (4 or 8 h p.i.) were precipitated with MAbs 30 or 48S specific for ICP10 and

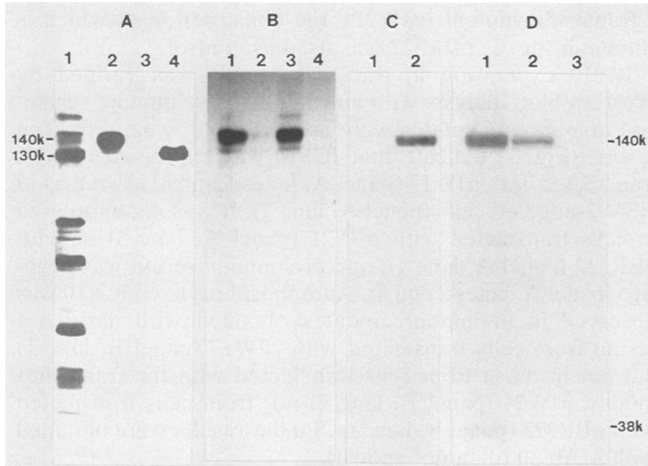

FIG. 4. ICP10-associated PK activity. (A) MAb 30 immunoprecipitation of ICP10. Extracts of HSV-2 (lanes 1, 2, and 4)- or mock (lane 3)-infected cells labeled with [^{35}S]methionine (4 to 8 h p.i.) were immunoprecipitated with MAb 30 (lanes 2 and 3) or MAb 27 (lane 4). Total extract proteins are shown in lane 1. (B) MAb 48S immunoprecipitation of ICP10 and ICP6. Extracts of cells labeled with [^{35}S]methionine at 4 to 8 h p.i. with HSV-2 (lanes 1 and 2), HSV-1 (lane 3), or mock infected (lane 4) were precipitated with MAb 48S (lanes 1, 3, and 4) or normal ascites (lane 2). (C) PK activity is associated with ICP10. In vitro kinase activity of immune complexes precipitated by MAb 30 (lane 2) or MAb 27 (lane 1) from 4-h HSV-2-infected cells ([γ-^{32}P]ATP and Mg^{2+}). (D) PK activity is not associated with ICP6. In vitro kinase activity of immune complexes precipitated from HSV-2-infected cells (8 h p.i.) with MAb 30 (lane 1) or by MAb 48S from cells infected with HSV-2 (lane 2) or from cells infected with HSV-1 for 8 h and precipitated with MAb 48S (lane 3). Gels were run separately and are not directly comparable.

subjected to standard PK assays ([γ-^{32}P]ATP and Mg^{2+}) in the absence of exogenously added protein substrates. Extracts similarly immunoprecipitated with antibody (MAb 27) to an unrelated protein (ICP8) were used as controls.

As summarized in Fig. 4 (panels A and B) for extracts of mock-infected or HSV-infected cells labeled from 4 to 8 h p.i. with [^{35}S]methionine, the immunoprecipitation of ICP10 by MAbs 30 and 48S is specific. Thus, only one 140-kDa protein, consistent with RR1, was precipitated from HSV-2-infected cells by MAbs 30 (panel A, lane 2) and 48S (panel B, lane 1) and from HSV-1-infected cells by MAb 48S (panel B, lane 3). Normal ascites was negative (panel B, lane 2) and the protein was not precipitated from similarly labeled mock-infected cells (MAb 30, panel A, lane 3; Mab 48S, panel B, lane 4). MAb 27 specifically precipitates ICP8 from HSV-2-infected (panel A, lane 4) but not mock-infected (data not shown) cells.

In duplicate samples subjected to standard PK assays, phosphorylation of a single protein band consistent with ICP10 (140 kDa) was observed only in extracts of cells infected with HSV-2 and immunoprecipitated with MAb 30 (Fig. 4, panel C, lane 2; panel D, lane 1) or MAb 48S (Fig. 4, panel D, lane 2) but not MAb 27 (Fig. 4, panel C, lane 1). Significantly, phosphorylation was not observed in ICP6-containing precipitates of HSV-1-infected cells with MAb 48S (Fig. 4, panel D, lane 3), even though they were positive for ICP6 (Fig. 4, panel B, lane 3).

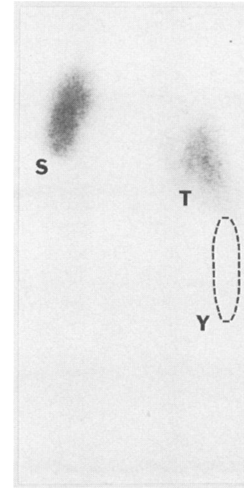

FIG. 5. Phosphoamino acid analysis of in vitro-phosphorylated ICP10. An acid hydrolysate of ICP10 phosphorylated in an in vitro kinase assay was subjected to two-dimensional paper electrophoresis, and the labeled phosphoamino acids were visualized by autoradiography. The positions of unlabeled markers phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were determined by ninhydrin staining.

Phosphoamino acid analysis of in vitro-phosphorylated ICP10 confirmed the prediction that the ICP10-associated PK activity is serine/threonine specific (Fig. 5).

Subcellular localization of ICP10-associated PK activity. To define the intracellular localization of the ICP10-associated PK activity, HSV-2-infected HEp-2 cells, unlabeled or labeled with [^{35}S]methionine (8 to 12 h p.i.), were fractionated into SOL, CSK, and NUC compartments and immunoprecipitated with MAb 30. Labeled precipitates were analyzed by SDS-PAGE. Unlabeled precipitates were used for in vitro kinase assays.

[^{35}S]methionine-labeled ICP10 was detected in the SOL (Fig. 6, panel A, lane 1) and CSK (Fig. 6, panel A, lane 2), but not in the NUC (Fig. 6, panel A, lane 3) fraction. Consistent with these findings, autophosphorylated ICP10 was also detected in the SOL (Fig. 6, panel B, lane 3) and CSK (Fig. 6, panel B, lane 2) but not the NUC (Fig. 6, panel B, lane 1) fraction. As described previously for ICP6 (55), NUC fractions were positive for ICP10 (and PK activity) at 4 to 8 h p.i. (data not shown). Phosphorylating activity was not observed in precipitates of SOL fractions with MAb 27 (Fig. 6, panel B, lane 4). The 38-kDa RR2, coprecipitated by MAb 30 from cells infected with HSV-2 for $\geq$ 8 h (Fig. 6, panel A, lanes 1 and 2), was not (Fig. 6, panel B), or only minimally (Fig. 4, panel C, lane 1), phosphorylated.

A 140-kDa protein consistent with ICP10 was also precipitated by MAb 30 from purified plasma membrane fractions of [^{35}S]methionine-labeled HSV-2-infected cells (Fig. 6, panel C, lane 2). Three other proteins (60, 45, and 38 kDa) were also precipitated. The 38-kDa protein presumably represents the coprecipitated RR2. The identity of the 60- and 45-kDa proteins is unknown. A major (140-kDa) and a minor (38-kDa) phosphorylated band were also observed in the in vitro kinase assays of the membrane precipitates. The 60- and 45-kDa proteins were apparently not phosphorylated (Fig. 6, panel D, lane 2). MAb 27 precipitates of membrane fractions were negative for PK activity (Fig. 6, panel D, lane 1).

ICP10-associated PK activity is independent of other viral proteins. The presence of PK activity in immunoprecipitates

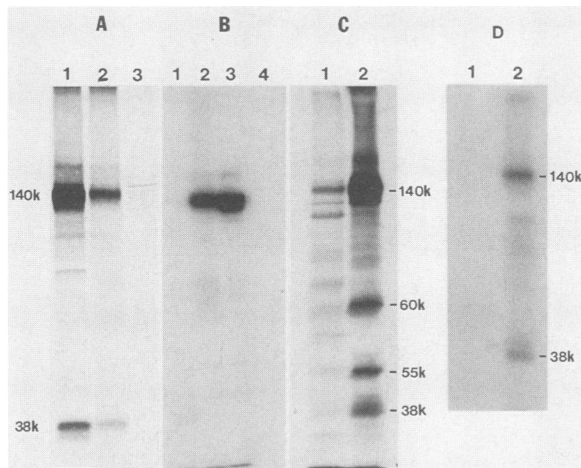

FIG. 6. Subcellular localization of ICP10-associated PK. (A) Compartmentalization of ICP10 in HSV-2-infected cells. ICP10 was immunoprecipitated by MAb 30 from the SOL (lane 1), CSK (lane 2), and NUC (lane 3) fractions of HSV-2-infected cells labeled with [^{35}S]methionine at 8 to 12 h p.i. (B) Compartmentalization of ICP10-associated PK activity. PK activity of immune complexes precipitated by MAb 30 from SOL (lane 3), CSK (lane 2), and NUC (lane 1) or by MAb 27 from SOL (lane 4) fractions of HSV-2-infected cells (8 h p.i.). (C) ICP10 localization in purified plasma membranes. Total proteins from plasma membranes of HSV-2-infected cells labeled with [^{35}S]methionine at 4 to 8 h p.i. (lane 1) or immunoprecipitated with MAb 30 (lane 2). (D) ICP10-associated PK activity is present in purified plasma membranes. Immune complexes precipitated by MAb 27 (lane 1) or MAb 30 (lane 2) from purified plasma membranes of HSV-2-infected cells (8 h p.i.) were assayed for PK activity. Gels were run separately and are not directly comparable.

from HSV-2-infected cells may reflect contamination of the precipitates by trace amounts of a viral protein, other than ICP10, that has kinase activity but does not autophosphorylate. To address this question, 293 cells were transfected with the ICP10 constitutive expression vector pJW17 or with a frameshift mutant (pJW21), the construction of which is shown in Fig. 1. pBR322 was used as control.

ICP10 expression in transfected cells was verified by Western blot analysis with anti-LA-1 or preimmune serum, and immunoprecipitates were assayed for PK activity. The results (Fig. 7) indicate that ICP10 was expressed in cells transfected with pJW17 (panel A, lanes 5 and 6) as well as in HSV-2-infected cells (panel A, lane 7). It was not expressed in cells transfected with pJW21 (panel A, lane 3) or with pBR322 (panel A, lane 1), and preimmune serum was negative (panel A, lanes 2 and 4). Autophosphorylated ICP10 was observed in immunoprecipitates obtained with anti-LA-1 serum from cells transfected with pJW17 (panel B, lane 1) but not in those from cells transfected with the frameshift mutant pJW21 (panel B, lane 2) nor from cells transfected with pBR322 (panel B, lane 3). Similar results were obtained with MAb 30 (data not shown).

ICP10-associated PK activity is independent of the carboxyl two-thirds of ICP10. To determine whether the one-third amino-terminal domain that presumably contains PK activity could be functionally dissociated from the RR-containing carboxyl two-thirds of ICP10 (19), we constructed an amino-terminal expression vector, pJW32, by inserting a triple terminating linker after the codon for amino acid 446 (Fig. 1). Cells were transfected with pJW32, pJW17, or pBR322. At 30 h posttransfection, they were each split into two samples. One of the samples was labeled with [^{35}S]methionine and precipitated with MAb 30 in order to determine gene expression. The unlabeled duplicate sample was immunoprecipitated (with MAb 30) and assayed for PK activity. ICP10 was precipitated from cells transfected with pJW17 (Fig. 7, panel C, lane 2), while a 57-kDa protein was precipitated from cells transfected with pJW32 (Fig. 7, panel C, lane 3). Extracts of pBR322-transfected cells were negative (Fig. 7, panel C, lane 1). Consistent with the interpretation that PK activity resides within the amino-terminal region of ICP10, the 57-kDa protein in precipitates of pJW32-transfected cells was autophosphorylated (Fig. 7, panel D, lane 3). ^{32}P incorporation in this protein was 5-fold higher than that observed in ICP10

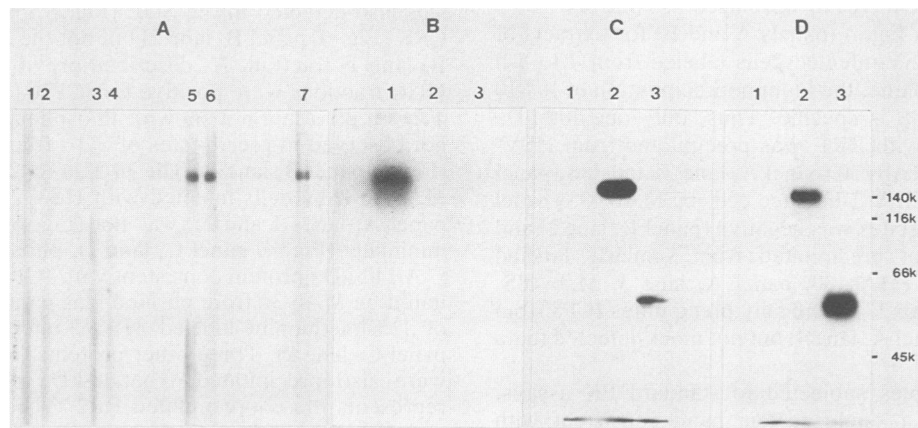

FIG. 7. ICP10-associated PK activity expressed in cells transfected with the ICP10 constitutive expression vector. (A) ICP10 expression in 293 cells transfected with the constitutive expression vector pJW17 (lanes 4, 5, and 6), the frameshift mutant pJW21 (lanes 2 and 3), or control vector pBR322 (lane 1) or infected with HSV-2 for 8 h (lane 7) as detected by Western blot analysis with anti-LA-1 (lanes 1, 3, 5, 6, and 7) or preimmune (lanes 2 and 4) serum. (B) PK activity associated with constitutively expressed ICP10. Immunoprecipitates of extracts from cells transfected with pJW17 (lane 1), pJW21 (lane 2), or pBR322 (lane 3) were analyzed for PK activity. Similar results were obtained with MAb 30. (C) Expression of a 57-kDa protein in pJW32-transfected cells (lane 3), as determined by immunoprecipitation of [^{35}S]methionine-labeled 293 cells with MAb 30. Immunoprecipitates of cells transfected with pJW17 were positive for ICP10 (lane 2); cells transfected with pBR322 were negative (lane 1). (D) PK activity associated with the amino-terminal 57-kDa protein. Immunoprecipitates of extracts from cells transfected with pBR322 (lane 1), pJW17 (lane 2), or pJW32 (lane 3) were analyzed for PK activity.

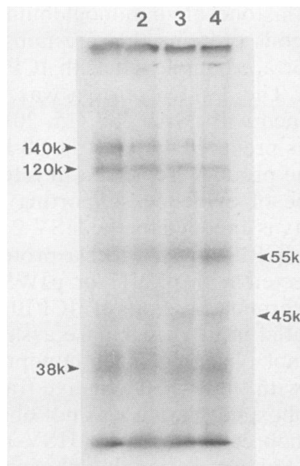

FIG. 8. PK activity of ICP10 proteolytic degradation products. HSV-2-infected Vero cells (8 h p.i.) were extracted in the absence of protease inhibitors and incubated at 25°C in the absence of inhibitors for 0 (lane 1), 0.5 (lane 2), 2 (lane 3), or 5 (lane 4) h. Extracts were immunoprecipitated with anti-LA-1 serum and the resulting immune complexes were assayed for PK activity.

(pJW17-transfected cells; Fig. 7, panel D, lane 2). The exact significance of this finding is unclear at present.

PK activity is associated with a 55-kDa species generated by endogenous proteolytic degradation. Previous studies have shown that ICP6 is degraded by a Vero cell-specific protease and have correlated RR activity with a 93-kDa degradation product that presumably represents the RR1 carboxy-terminal domain (19). To determine whether the amino-terminal region of the protein could be functionally dissociated from the RR activity by endogenous proteolysis, Vero cells infected with HSV-2 for 8 h were extracted in the absence of protease inhibitors and incubated at 25°C for 0.5, 2, or 5 h without addition of inhibitors. Extracts were precipitated with anti-LA-1 serum (which recognizes the ICP10 amino-terminal residues 13 to 26) and assayed for in vitro kinase activity.

The levels of autophosphorylated ICP10 decreased as a function of time of in vitro incubation (Fig. 8; Table 1). This decrease was accompanied by a concomitant increase in the levels of two lower-molecular-mass species (lanes 2 through 4). Thus, at 0 h, ICP10 represented 25.9% of the total labeled

TABLE 1. PK activity of ICP10 and related proteolytic degradation products recognized by anti-LA-1[a]

Band (kDa)	Activity during incubation period[b]							
	0 h		0.5 h		2.0 h		5.0 h	
	Peak area[c]	% Total area[d]	Peak area	% Total area	Peak area	% Total area	Peak area	% Total area
140	3.23	25.9	2.31	17.8	1.10	9.60	—[e]	—
120	2.67	21.4	3.27	25.2	1.58	13.8	1.94	17.0
55	1.80	14.4	2.19	16.9	3.03	26.3	4.04	35.4
45	—	—	—	—	0.72	6.29	0.83	7.31
38	4.79	38.4	5.19	40.1	5.06	44.0	4.59	40.3

[a] Densitometric scanning of autoradiograph in Fig. 8.
[b] Incubation of extract at 25°C in absence of protease inhibitors before immunoprecipitation with anti-LA-1 (see legend to Fig. 8).
[c] Unit area under peak detected with baseline subtraction.
[d] Percent area of total peak area.
[e] —, Not detectable by densitometric scanning.

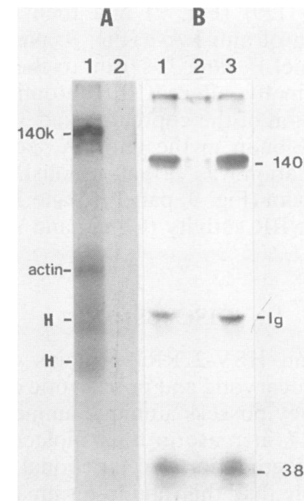

FIG. 9. (A) Phosphorylation of exogenous substrate by ICP10-associated PK. PK activity of immune complexes precipitated by MAb 30 from HSV-2 (lane 1)- or mock (lane 2)-infected cells was assayed in the presence of 5 μg of histone type III-S (H). Actin is an integral component of the cell extracts which is sometimes coprecipitated. (B) Inhibition of ICP10-associated PK activity by antipeptide antibody. Immune complexes precipitated by MAb 30 from HSV-2-infected cells were incubated with secondary antibody anti-LA-1 (lane 1), anti-LA-2 (lane 2), or anti-LA-3 (lane 3) for 30 min at 4°C before addition of the PK reaction buffer. Transphosphorylation of immunoglobulin (Ig) from polyclonal sera has been previously observed (36).

proteins as detected by densitometric scanning (Table 1). At 5 h, it was no longer detectable. On the other hand, a 55-kDa species recognized by anti-LA-1 serum increased from 14.4% at 0 h to 35.4% at 5 h, and a 45-kDa species was first observed at 2 h and was slightly increased by 5 h. The levels of the 38-kDa protein that presumably represents the coprecipitated RR2 remained constant throughout the incubation period (Fig. 8; Table 1). We interpret these data to indicate that ICP10 undergoes proteolytic cleavage, resulting in the generation of the 55- to 45-kDa amino-terminal species that retain the kinase activity. Consistent with this interpretation and the previous reports for ICP6 (19), RR activity decreased by 50% at 30 min of in vitro incubation and was completely lost by 5 h (data not shown). The source of the 120-kDa protein is not clear. It may be analogous to the 110-kDa degradation product of ICP6 (19), or, alternatively, it may represent a cellular protein nonspecifically recognized by anti-LA-1 serum.

Inhibition of ICP10-associated PK activity with antipeptide antibody. Two series of experiments were done to further characterize the ICP10-associated PK activity. In the first series, extracts of HSV-2-infected cells (4 h p.i.) were immunoprecipitated with MAb 30, and the precipitates were assayed for PK activity in the presence of the exogenously added substrate histone that is specific for serine/threonine kinases (36). Autophosphorylated ICP10 as well as phosphorylated histone were observed in precipitates from HSV-2-infected (Fig. 9, panel A, lane 1) but not mock-infected (Fig. 9, panel A, lane 2) cells. In addition, actin, a frequent contaminant of immunoprecipitates, was also phosphorylated.

In the second series of experiments, the precipitates were first incubated with antisera to synthetic peptides LA-1 (residues 13 to 26), LA-2 (residues 355 to 369), or LA-3

(residues 165 to 179) (Fig. 2) and then assayed for PK activity. Addition of anti-LA-1 (Fig. 9, panel B, lane 1) or LA-3 (Fig. 9, panel B, lane 3) serum to the precipitates did not, or only minimally, affect ICP10 autophosphorylation or the phosphorylation of the coprecipitated 38-kDa protein or of the immunoglobulin in the anti-LA sera. On the other hand, phosphorylation was virtually abolished by addition of the anti-LA-2 serum (Fig. 9, panel B, lane 2). None of these antibodies inhibit RR activity (Chung and Aurelian, unpublished data).

DISCUSSION

The HSV-1 and HSV-2 RR1 proteins differ from their counterparts in eucaryotic and procaryotic cells and in other viruses in that they possess a unique amino-terminal region that causes a 50% increase in their molecular weights (37, 51). Proteolytic degradation and functional studies have led to the conclusion that this amino-terminal domain is not required for RR activity (19; Chung and Aurelian, unpublished data) and may, in fact, represent a functionally distinct domain. Indeed, DNA sequences that encode the amino terminus of the HSV-2 (but not HSV-1) RR1 have neoplastic potential (9, 15, 20–23), and the amino termini of the ICP6 and ICP10 proteins show relatively little homology (37). Additionally, while the RR activity behaves temporally as a delayed-early gene (reviewed in reference 16), ICP10 contains *cis*-response elements associated with the regulation of immediate-early gene expression and consensus AP-1 elements (56) potentially associated with regulation of this putatively distinct function.

The salient feature of our data is the observation that a PK activity is the product of the HSV-2 RR1 gene (presumably at the 5′ end) or is very closely associated with it. Indeed, biosynthetic labeling has permitted the detection (by immunoprecipitation with MAbs 30 or 48S) of only one protein in HSV-2- or HSV-1-infected cells. This protein, consistent with HSV RR1 (140 kDa), is not present in mock-infected cells and is not recognized by another antibody (MAb 27, specific for the major HSV DNA-binding protein [ICP8]). By using MAbs 30 and 48S, we show here that a PK activity is associated only with HSV-2-infected cells, that the expression of this activity is dependent on the expression of the HSV-2 RR1 coding region, and that the PK activity is localized in the one-third amino-terminal region of ICP10.

Viral and cellular proteins are phosphorylated during productive infection with HSV, and there have been several reports of phosphorylating activities that might correspond to a viral enzyme (30, 41; reviewed in reference 29). To date, only one HSV-1-coded PK has been characterized experimentally. This PK, encoded by the US3 gene, appears to be independent of known regulatory molecules and can utilize ATP (but not GTP) to phosphorylate serine or threonine residues of basic substrates. It is not essential for HSV-1 growth in culture (41) nor for the establishment of latency in mice (35). PK catalytic motifs were also identified in the US3 gene of HSV-2 by DNA-sequencing studies (33), and more recently, PK motifs were similarly identified in the HSV-1 UL13 gene (48). However, to the extent of our knowledge, PK enzymatic activity has not been experimentally described in HSV-2-infected cells.

The following lines of evidence support our conclusion that the ICP10-associated PK activity is encoded by the HSV-2 RR1 gene. First is the phosphorylating ability of the specifically immunoprecipitated ICP10, the substrate being ICP10 itself (autophosphorylation reaction), exogenously added substrate (histone or immunoglobulin from anti-LA sera) or heterologous proteins that are nonspecifically precipitated (actin) or are complexed with ICP10, such as the 38-kDa RR2 (19). The 38-kDa protein was not detected in precipitates obtained with MAb 48S (15, 20) nor in precipitates from extracts prepared 4 h p.i. (the 38-kDa protein is a delayed-early gene product [reviewed in reference 16]).

The second line of evidence supporting our conclusion that the PK activity is encoded by the HSV-2 RR1 gene is the finding that both ICP10 and a 57-kDa protein precipitated from cells transfected with pJW17 or pJW32 (encodes the one-third amino-terminal region of ICP10), respectively, were phosphorylated in in vitro kinase assays, while phosphorylation was not observed in immunoprecipitates from cells transfected with the ICP10-negative frameshift mutant pJW21. Finally, phosphorylation was not observed in ICP6-containing immunoprecipitates from HSV-1-infected cells, consistent with the interpretation that phosphorylation is not due to an as yet unidentified cellular protein. In this context, it seems particularly significant to point out that PK motifs in subdomains I and II are not conserved in ICP6 (Fig. 3). This is not a feature unique to the HSV-1 strain used in our studies (strain F) but, rather, appears to be characteristic of all other studied strains (37). Possibly ICP6 has kinase activity, but it is unable to autophosphorylate under the experimental conditions used in these studies. Alternatively, the absence of PK activity in ICP6 may simply represent evolutionary divergence.

We believe that the ICP10-associated PK activity is localized to the amino terminus of ICP10 because (i) all the PK motifs are clustered within the first 411 amino acid residues of the protein, (ii) DNA sequence analysis has shown that the region represented by residues 1 to 300 is entirely unique to HSV (37, 51), and (iii) PK activity was associated with a 57-kDa protein encoded within residues 1 to 446 of the ICP10 protein (pJW32). In this context, it may be important to point out that consistent with the presence of PEST sequences, the two enzymatic activities (PK and RR) were functionally dissociated by endogenous proteolytic degradation. PK activity was associated with 45- to 55-kDa degradation products that were free of RR activity.

The conclusion that the PK activity is localized in the amino-terminal domain of ICP10 is further supported by the observation that antibody to the synthetic peptide LA-2 (residues 355 to 369) specifically out competed the ICP10-associated PK activity. The exact mechanism of this competition is not immediately evident. The observation that anti-LA-2 serum inhibited phosphorylation of both ICP10 and heterologous substrates suggests that the antibody does not simply interfere with a phosphorylation accepting site in the LA-2 region. This conclusion is also supported by the failure of the anti-LA-1 serum to cause a similar inhibition, even though LA-1 contains two serine residues. The inhibition is likely to be specific, since anti-LA-3 serum, which also binds in the catalytic domain, did not inhibit PK activity. Final conclusions as to the role, if any, of the LA-2 epitope (and other regions within the PK domain [residues 1 to 446] of the ICP10 protein) must await the results of ongoing site-directed mutagenesis studies. However, it should be pointed out that the LA-2 epitope is immediately N terminal to PK subdomain VII that contains the highly conserved Asp-Phe-Gly triplet. This triplet has been implicated in ATP binding and is thought to represent the central core of the catalytic domain (14). Secondary-structure analysis of the ICP10 amino acid sequence by the method of Garnier et al. (13) identified the LA-2 region as a major

beta-turn conformation. If this turn is important for proper orientation of the ATP-binding motif with other motifs critical for catalysis, anti-LA-2 may inhibit kinase activity by disrupting the enzymatically active conformation of the protein.

It is tempting to consider the potential functional significance of the PK activity within the context of previous studies indicating that (i) protein phosphorylation may be involved in the regulation of cellular and viral processes (reviewed in reference 17) and (ii) DNA sequences that code for the amino-terminal region of ICP10 have neoplastic potential for established hamster cells (15, 20–23). Indeed, studies of a HSV-2 mutant that is temperature sensitive for ICP10 expression and PK activity suggest that either RR or PK activities, or both, are required for virus growth in cultured cells at the nonpermissive temperature. Cells transformed by this mutant are defective for anchorage-independent growth at the nonpermissive temperature, albeit only at early passages (Smith et al., submitted for publication). DNA sequences that code for the HSV-1 RR1 (ICP6) are negative for both PK activity (Fig. 4, panel D, lane 3) and transforming potential (9). This is not due to strain differences since the same F strain was used in both studies. Significantly anchorage-independent growth is induced by pJW17 and pJW32 but not by the frameshift mutant pJW21 nor by a construct expressing only the carboxyl two-thirds of ICP15 (J. P. Wymer and L. Aurelian, manuscript in preparation).

Nearly half of all known retroviral oncogene products identified to date have been shown to possess PK activity (5). The transforming abilities of v-*src* and v-*abl* depend on the intrinsic PK activities of their protein products (40, 46). The case of v-*abl* is particularly interesting since the gene encodes a 130-kDa protein, but only the amino-terminal 45-kDa PK domain is required for transformation by Abelson murine leukemia virus (40). Also attractive within the context of this analogy is the observation that the ICP10-associated PK activity is associated with the CSK fraction and the plasma membrane. Both of these fractions were highly purified, suggesting that the ICP10-associated PK activity was not due to contamination by soluble proteins. Furthermore, sequence analysis revealed the presence in the ICP10 amino-terminal region of a transmembrane helical segment and features of a signal peptide (39), both consistent with protein membrane localization. HSV-2-infected cells stained with MAb 30 in membrane immunofluorescence (Chung and Aurelian, unpublished data) and ICP10 had been previously associated with plasma membranes of HSV-2-infected (3) or -transformed (11) cells. It may be more than coincidental that the transforming potential of v-*src* has been shown to require association of its protein product with membranes (26), and localization of pp60^{src} to the CSK fraction has been suggested as an important determinant for its transforming ability (8). Several other viral transforming proteins, the PKs encoded by v-*abl*, v-*fps*, and v-*fgr*, have also been shown to fractionate with the CSK and the plasma membrane (1, 6, 32).

The structural similarity of the ICP10 PK domain to cellular PKs makes it reasonable to assume that it evolved from a cellular gene (29). The uniqueness of the amino-terminal domain of ICP10 and its associated PK activity suggests that the gene encoding this domain was expressed by an ancestral HSV because it conferred some selective advantage. This advantage may be related to its ability to catalyze the phosphorylation of some cellular substrate. Final conclusions pertaining to the potential role of the ICP10-associated PK activity in productive infection or transformation or both must await the results of the ongoing site-directed mutagenesis studies.

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