The large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is a multifunctional protein. It consists of a ribonucleotide reductase and a serine/threonine protein kinase (PK) domain, which has three proline-rich motifs consistent with SH3-binding sites at positions 140, 149, and 396. We used site-directed mutagenesis to identify amino acids required for kinase activity and interaction with signaling proteins. Mutation of Lys176 or Lys259 reduced PK activity (5–8-fold) and binding of the 14C-labeled ATP analog p-fluorosulfonylbenzoyl 5′-adenosine (FSBA) but did not abrogate them. Enzymatic activity and FSBA binding were abrogated by mutation of both Lys residues, suggesting that one can bind ATP. Mutation of Glu209 (PK catalytic motif III) virtually abrogated kinase activity in the presence of Mg2+, or Mn2+ ions, suggesting that Glu209 functions in ion-dependent PK activity. ICP10 bound the adaptor protein Grb2 in vitro. Mutation of the ICP10 proline-rich motifs at positions 396 and 149 reduced Grb2 binding 20- and 2-fold, respectively. Binding was abrogated by mutation of both motifs. Grb2 binding to wild type ICP10 was competed by a peptide for the Grb2 C-terminal SH3 motif, indicating that it involves the Grb2 C-terminal SH3.

Ribonucleotide reductase (RR) is an essential enzyme for the conversion of ribonucleotides to the corresponding deoxyribo nucleotides in eukaryotic and prokaryotic cells, and its activity may represent the rate-limiting step in DNA synthesis and concomitant cell growth (1). Several herpesviruses including HSV-1, HSV-2, Epstein-Barr virus, varicella zoster virus, pseudorabies virus, and equine herpesvirus types 1 and 3, induce a novel distinct RR activity (2–7) that may be required for virus growth in nondividing cells (8–11). The HSV RR differs from the cellular enzyme in that it is insensitive to dTTP and dATP inhibition and does not have an absolute Mg2+ requirement (12). However, like the mammalian and bacterial enzymes, the HSV RR activity is formed by the association of two distinct subunits, the coding regions of which do not overlap. The large subunit (RR1) is a 140-kDa protein, designated ICP6 for HSV-1 and ICP10 for HSV-2. The small subunit (RR2) is a 38-kDa protein encoded by a 1.2-kb mRNA overlapping the 3′ end of the 5.0-kb mRNA that encodes RR1 (13, 14).

The HSV RR1 genes differ from their counterparts in eukaryotic and prokaryotic cells and in other viruses in that they possess a unique one-third 5′-terminal domain (15, 16). The ICP10 unique domain (ICP10 protein kinase (PK) oncogene) causes neoplastic transformation of immortalized cells (17–21). Its protein product (amino acids 1–411) has conserved PK catalytic motifs characteristic of serine/threonine (Ser/Thr)-specific kinases, which are preceded by a transmembrane (TM) helical segment (21–24). Immunogold electron microscopy indicates that ICP10 is localized on the cell surface and is internalized by the endocytic pathway. A TM deletion mutant does not localize to the cell surface, indicating that the TM is a membrane-spanning domain (21, 25, 26). Three proline rich regions consistent with core Src homology region 3 (SH3)-binding motifs (27, 28) are present in the ICP10 PK oncoprotein at positions 140–167 and 396–410. They may be involved in the binding of signaling proteins, which ultimately results in the ICP10-mediated activation of the ras signaling pathway (21, 25).

A wealth of evidence indicates that the PK activity is an intrinsic property of ICP10. Thus, (i) expression of the ICP10 PK oncogene in eukaryotic or bacterial expression systems permits the synthesis of an enzymatically active protein (22, 29); (ii) ICP10 binds the 14C-labeled ATP analogue FSBA, and binding is specifically competed by another ATP analogue, AMP-PNP (24); (iii) ICP10 kinase activity is retained after electrophoresis on denaturing gels and renaturation on a nitrocellulose membrane (24); (iv) ICP10 mutants deleted in the conserved PK catalytic motifs (amino acids 106–411) or the TM segment are PK-negative although the TM mutant retains all known target sites for cellular kinases (21, 24, 25); (v) unlike casein kinase, a putative contaminant,2 ICP10 PK favors Mn2+ ions, does not require monovalent cations, and is not inhibited by zinc sulfate (30, 31); and (vi) treatment with epidermal growth factor activates the kinase activity of a chimERIC protein consisting of the ligand-binding domain of the epidermal growth factor receptor and the PK domain of ICP10 (32). Recent studies of the HSV-1 RR1 (ICP6) also concluded that the PK activity is intrinsic (33, 34). However, the role of specific protein sites in kinase activity and the binding of signaling proteins involved in ras activation (21) are still unclear. The
studies described in this report were designed to address this question.

MATERIALS AND METHODS

Cells—African Green monkey (Vero) cells and human embryonic kidney (293) cells were obtained from American Type culture collection and, respectively, grown in minimal essential medium or Eagle's minimum essential medium with 10% fetal calf serum.

Mutation of ICP10 Eukaryotic Expression Vectors—The construction of the expression vectors pJW17 and pJW32 was described (22). pJW32 was created by inserting a 14-base pair XbaI triple terminating linker into the unique SmaI site immediately after the first codon for residue 446 of pJH17 (22). Mutants pJH19 and pHL15, which are, respectively, deleted in all the conserved PK catalytic motifs (amino acids 106–411) or the TM segment of the expression vectors pJW17 and pJW32 was described (22). pJW17 and pJW32 respectively, were established in transfected 293 cells by G418 selection as described (21, 24). They were grown in Dulbecco's minimal essential medium with 10% fetal bovine serum.

Antibodies—Monoclonal antibodies (mAbs) specific for ICP10 (30 and H3) and polyclonal antibody anti-L-A-1 to a peptide located at amino acid residues 13–26 were previously described (22, 29, 36, 37).

Metabolic Labeling of Cell Extracts and Immunoprecipitation—Labeling and preparation of whole cell extracts was as described previously (21, 24, 25). Briefly, cells were labeled with [35S]methionine (100 μCi/ml; specific activity, 1120 Ci/mmol; DuPont NEN) in methionine-free Dulbecco's minimal essential medium with 10% fetal bovine serum (18 h at 37 °C) and resuspended in ice-cold radiomunno precipitation buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 100 Kallikrein units/ml aprotinin (Sigma)) and cleared of cell debris by centrifugation at 20,000  g for 30 min. Cells were incubated with 15 μl of antibody h, 4 °C and 100 μl of protein A-Sepharose CL-4B beads (50% (v/v), Sigma) (30 min at 4 °C). Beads were extensively washed in ice-cold radiomunno precipitation buffer, and bound proteins were eluted by boiling (for 5 min) in 50 μl of denaturing solution (150 mM NaCl, 20 mM Tris-HCl, pH 7.0, 5% SDS, 14% 2-mercaptoethanol, 17% sucrose, 0.04% bromthymol blue). Proteins were resolved by SDS-PAGE on 8.5% polyacrylamide gels and visualized by autoradiography. Quantitation was by densitometric scanning using the NIH Image program version 1.44, and results are expressed as densitometric integration units.

Immunocomplex PK Assay/Immunoblotting—In vitro PK assays, unlabelled cell extracts in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 20 Kallikrein units/ml aprotinin (Sigma) and 50 μg of protein) in a buffer containing 50 mM HEPES, 0.15 mM NaCl, 10% glycerol, 1% Triton, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium or-

| MUTATION | SEQUENCE | PLASMID |
|----------|----------|---------|
| Lys176   | gagaagagctcccgggggtgggactggggcggcgggagc-3' (pJN29), 5'-GAGAACAGC-CTTCCGAACCCGAGCC-3' (pJN5), 5'-ATGGAAAAGGGGGCAGCA-CGGGCGCGCGGACATCTGGGTG-3' (pJN21), and 5'-TATGCTTGGGGCGGACCGGAGATGATGG-3' (pJN22). Mutation created novel Apal (pJN29), SfuI (pJN5), EagI (pJN21), and SadI (pJN22) sites used for initial identification of the mutant phage progeny. Mutation was confirmed by sequence analysis (24, 29, 35). To generate mutant pJN23, both the pJN21 and pJN22 mutagenic primers were annealed with the template DNA. The mutated pJN29, pJN5, pJN21, pJN22, and pJN23 constructs were restricted with HindIII/RsrI, and the resulting 1.35-kb fragments were cloned into pJW17 to replace the corresponding wild type sequences. In pJN29, Lys176 (adjacent to Lys178) was mutated to Gly. In pJN5, Glu209 (PK catalytic motif II) was mutated to Lys. In pJN21, the wild type sequence 5'-GAGAACAGCCTTCCGAACCCGAGCCGAC-3' (pJH9) was mutated to 5'-GAGAACAGCCTTCCGAACCCGAGCC-3' (pJHL4). In pJN22, the wild type sequence 3'-GAGAACAGCCTTCCGAACCCGAGCC3' (pJHL4) was mutated to 3'-GAGAACAGCCTTCCGAACCCGAGCC3' (pJHL4). Mutation was identified by restriction digestion with SfiI and confirmed by sequence analysis. The mutated construct was restricted with HindIII/StuI, and the resulting 1.5-kb fragment was cloned into pJW17 replacing the wild type sequences. pJZ15 expresses an ICP10 mutant in which Lys176 was replaced with Leu and Lys259 was replaced with Gly (Table I). Establishment of Constitutively Expressing Cell Lines—Lines J HLal, J HL15, and J H9 that, respectively, express ICP10, a TM-deleted ICP10 mutant (pJ HL15) or an ICP10 mutant deleted in the conserved PK catalytic motifs (pJH9) were previously described (21, 24, 25). Cell lines J N10, J N29, J Z15, J N21, J N22, and J N23 that constitutively express the ICP10 mutants pJH4, pJN29, pJZ15, pJN21, pJN22, and pJN23 respectively, were established in transfected 293 cells by G418 selection as described (21, 24). They were grown in Dulbecco's minimal essential medium with 10% fetal bovine serum.
thovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 Kallikrein units/ml aprotinin were incubated (2 h, 4 °C) with 10 μg of glutathione-agarose beads coated with glutathione S-transferase (GST) or the GST-Grb2 fusion protein. Beads were washed 3 times in binding buffer (20 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40), resuspended in 100 μl denaturing solution, and heated at 90 °C for 5 min. Supernatants were electrophoresed on SDS-PAGE (7% polyacrylamide), electrotransferred to nitrocellulose membranes, and immunoblotted with anti-LA-1 antibody as described above. Molecular weight markers are listed.

RESULTS

Autophosphorylation Activity of Site-directed Mutants in Lys Residues—Computer-assisted analysis of the predicted amino acid sequence of ICP10 PK identified eight conserved catalytic motifs (22). One of these (motif II) includes the so-called invariant Lys believed to be essential for PK activity because it is the ATP binding site (39, 40). Studies of cells transfected with an ICP10 mutant in this Lys residue (Lys176) concluded that the residue is involved but is not essential for PK activity (24). Since mutation of the invariant Lys of a yeast cAMP kinase did not abrogate its PK activity, and a similar reduction in kinase activity was also achieved by mutation of two adjacent Lys residues (41), the present studies were designed to determine whether a similar situation also holds for ICP10 PK. In addition to Lys176 (pHL4), we studied a mutant in Lys259 (pN29) (Table I), selected because it is the only Lys residue adjacent to Lys259 within residues 1–283 previously shown to be essential for kinase activity (29). A mutant in both of these Lys residues (pZ15) was also studied. To avoid potentially confounding results due to different transfection efficiencies, we used cells transformed with expression vectors for wild type ICP10 or its Lys mutants. mAb H3 immunoprecipitation of [35S]methionine-labeled expressing JHLa1 (lanes 1 and 6), N10 (lanes 2 and 7), N29 (lanes 3 and 8), Z15 (lanes 4 and 9), or 293 (lanes 5 and 10) cells were precipitated with mAb H3 (lanes 1–5) or preimmune serum (lanes 6–10).

Duplicate samples of the transformed cell extracts were subjected to mAb H3 immunocomplex PK assays. The levels of phosphorylated protein were significantly lower in lines expressing the Lys176 (Fig. 2A, lane 2) or Lys259 (Fig. 2A, lane 3) mutants than in the ICP10-expressing cell line (Fig. 2A, lane 1). Densitometric integration units were 4578, 4187, and 3727 for the Lys176 and Lys259 mutants, respectively. The Km for ATP (determined in immunocomplex PK assays) was 1.2 μM for wild type ICP10. It was 5.5-fold higher for the Lys176 mutant (Km = 6.6 μM) and 7.8-fold higher for the Lys259 mutant (Km = 9.4 μM), PK activity was abrogated by mutation of both of these Lys residues (Fig. 2A, lane 4), indicating that either Lys residue can function in kinase activity. The reduction/loss of PK activity by the Lys mutants is not due to different protein levels in the assay itself, since immunoblotting of the precipitates from the immunocomplex PK assays with anti-LA-1 antibody revealed similar levels of protein in all the precipitates (Fig. 2B, lanes 1–4). Densitometric integration units in the immunoblots were 4150, 4578, 4187, and 3727 for ICP10, Lys176, Lys259, and Lys176/Lys259 mutants, respectively. Reduction/loss of PK activity by the Lys mutants is also not a rare event resulting from the use of a rare transformed clone or a unique cell type used to establish constitutively expressing cells, since similar results were obtained in independently established transfected cell lines, as shown in Fig. 3 for Lys259, and in transfected Vero cells as shown in Fig. 4 for Lys176 and Lys259. It is also not due to conformational changes such as those responsible for altering the intracellular localization of the TM-deleted mutant and causing its null phenotype (21, 24), since fluorescence-activated cell sorting analysis indicated that all three Lys mutants localize to the cell surface, and their secondary structure is similar to that of ICP10 as determined according to the method of Garnier et al. (61) (data not shown).

Transphosphorylation Activity of Site-directed Mutants in Lys Residues—To examine the role of Lys176 and Lys259 in ICP10 kinase activity, we determined the transphosphorylation potential of the mutants for calmodulin (CaM), which had been previously shown to be a substrate for ICP10 kinase (23, 24). Consistent with previous reports, CaM was phosphorylated by ICP10 (Fig. 2A, lane 1). Its phosphorylation was sig-
ATP and SH3 Binding Sites in ICP10

significantly decreased by mutation of Lys176 (Fig. 2A, lane 2) or Lys259 (Fig. 2A, lane 3), and it was not phosphorylated by the double Lys mutant (Fig. 2A, lane 4). Densitometric integration units for the phosphorylated CaM were 3100, 620, 480, and 0 for ICP10, Lys176, Lys259, and Lys176/Lys259, respectively. PK activity (auto- and transphosphorylation) was also not evidenced by the TM-deleted mutant (Fig. 2A, lane 6) or the mutant deleted in all eight PK catalytic domains (Fig. 2A, lane 5), although protein expression was similar to that of ICP10, as determined by immunoblotting of the precipitates from the PK assays with anti-LA-1 antibody (Fig. 2B). These findings indicate that CaM transphosphorylation by the Lys mutants is decreased proportionally to the decrease in their autophosphorylating potential, thereby confirming that these Lys residues are essential for ICP10 kinase activity.

The Lys176/Lys259 Mutant Does Not Bind [14C]FSBA—To

examine whether loss of PK activity by mutation of residues Lys176 and Lys259 is due to the failure to bind ATP, we utilized the ATP analog, FSBA, which inactivates many kinases by covalently binding to the ATP binding lys (42, 43). Immunoprecipitates of JHLa1 (ICP10), 4-JN10 (Lys176), JN29 (Lys259) and 7 JZ15 (Lys176/Lys259) cells were incubated with [14C]FSBA for 30 min in the presence or absence of the competitor AMP-PNP, and the proteins were resolved by SDS-PAGE. ICP10 bound FSBA (Fig. 5, lane 1) and binding was competed by AMP-PNP (Fig. 5, lane 2). [14C]FSBA binding was significantly (4–6-fold) lower for the Lys176 (Fig. 5, lane 3) and Lys259 (Fig. 5, lane 5) mutants, and the residual binding was competed by AMP-PNP (Fig. 5, lanes 4 and 6). The double Lys mutant did not bind FSBA (Fig. 5, lane 7). We interpret these findings to indicate that ATP binds both Lys residues at positions 176 and 259.

Glu209 Is Required for Auto- and Transphosphorylation Activity—The carboxyl group of the Glu residue in PK catalytic motif III is believed to form a salt bridge with the ATP binding lys, which helps stabilize the interaction between the latter and the α- and β-phosphates of ATP (40). Mutation of this Glu residue has a severe effect on PK activity (41). To examine whether the conserved Glu residue in ICP10 PK catalytic motif III (Glu209) is required for kinase activity, we studied a mutant (pN5) in which Glu209 was replaced by Lys. In a first series of experiments, immunocomplex PK assays were done with mAb H3 in the presence of 4 μg of exogenously added CaM used as a phosphorylation substrate. Auto- and transphosphorylating activity was severely (20-fold) decreased (Fig. 6A, lane 2) relative to that of ICP10 (Fig. 6A, lane 1), suggesting that Glu209 plays a major role in ICP10 kinase activity.

Because previous studies had shown that ICP10 kinase activity has a preference for Mn2+ (23), we asked whether Glu209 has a different role in MnATP as compared with MgATP-dependent PK activity. PK assays were done (in the absence of CaM) in a buffer containing 2 mM MnCl2, 5 mM MgCl2, or both cations. These concentrations were selected because they support optimal levels of PK activity (30). The ICP10 PK activity was significantly (5–6-fold) higher in the presence of Mn2+ (Fig. 6B, lane 3) than Mg2+ (Fig. 6B, lane 2) ions. Confirming the conclusion that ICP10 favors Mn2+. Activity in the presence of both cations (Fig. 6B, lane 1) was similar to that seen
with only Mn\(^{2+}\). The kinase activity of the Glu\(^{209}\) mutant was significantly lower than that of the wild type ICP10 under all three experimental conditions. In presence of Mg\(^{2+}\), the PK activity of the Glu\(^{209}\) mutant (Fig. 6B, lane 5) was 18-fold lower than that of ICP10 (densitometric integration units 2150 and 120 for ICP10 and Glu\(^{209}\), respectively). In the presence of Mn\(^{2+}\) (Fig. 6B, lane 6), the activity was 15-fold lower than that of ICP10 (densitometric integration units 5250 and 350 for ICP10 and Glu\(^{209}\), respectively), and in the presence of both cations (Fig. 6B, lane 4) it was 20-fold lower than that of ICP10 (densitometric integration units 4200 and 210 for ICP10 and Glu\(^{209}\), respectively). These findings indicate that Glu\(^{209}\) is required for both MgATP- and MnATP-dependent PK activity of ICP10.

ICP10 Proline-rich Domains Are Required for Grb2 Binding in Vitro—ICP10 PK has two proline-rich domains. The first domain is located at position 140–167 and consists of two consensus SH3-binding motifs. One (at position 140–147) is specific for Src in that it has the PXXXPX motif and an Arg residue at the N terminus. The second (AVPPPPPFFWGH) is at position 149–159, and it is similar to the motif recently shown to bind Abl, Src, Crk, and Fyn SH3 domains (28). The second proline-rich domain in ICP10 is located at position 396–410. It is a class II binding site similar to those shown to bind adaptor proteins (44–48) in which the motif PXXP can be in either of two positions with basic residues at the C terminus (His\(^{308}\) and Arg\(^{210}\)). Since (i) similar proline-rich motifs bind adaptor proteins such as Grb2 (44–46, 48) and (ii) ICP10 was previously shown to bind the Grb2-hSOS complex in immunoprecipitation/immunoblotting experiments (21), the question arises whether Grb2 binding to ICP10 involves interaction between its SH3 domains and these ICP10 proline-rich regions. To address this question we used ICP10 mutants in the proline-rich motifs that are similar to those shown to bind adaptor proteins at positions 149–159 (pJN21), 396–410 (pJN22), or both (pJN23). To control for potential artefacts due to transfection efficiency, all experiments were done with cell lines that constitutively express these mutants. Expression levels were similar in all three cell lines and in cells expressing wild type ICP10 (Fig. 1) as determined by mAb H3 immunoprecipitation of \(^{35}\)S-methionine-labeled cell extracts.

Extracts from cells that constitutively express ICP10 or its mutants were incubated with glutathione-agarose beads coated with GST-Grb2 or GST proteins (equilibrated for protein concentration), and the bound proteins were identified by immunoblotting with anti-LA-1 antibody. GST-Grb2 bound ICP10 (Fig. 7, lane 12). Binding was significantly decreased by mutation of the proline-rich motif at position 396 (Fig. 7, lane 7) and minimally reduced by mutation of the proline-rich motif at position 149 (Fig. 7, lane 5). It was abrogated by mutation of both proline-rich motifs (Fig. 7, lane 9). Quantitative analysis was done by densitometric scanning, and the results are expressed as percentage of binding = densitometric units with Grb2/densitometric units of extract. According to this analysis, Grb2 binding to ICP10 was 21%. It was reduced 2-fold (12% binding) by mutation of the first proline-rich motif (at position 149) and 20-fold (1.2% binding) by mutation of the second proline-rich motif (at position 396). GST did not bind ICP10 (Fig. 7, lane 11) or its proline-rich site mutants (Fig. 7, lanes 4, 6, and 8).

The Grb2 C-terminal SH3 Binds ICP10—Previous studies had shown that the Grb2-hSOS complex couples activated growth factor receptor Tyr kinases to ras signaling (49–52) involving interaction of the Grb2 SH2 motif with the activated growth factor receptor and its SH3 motif(s) with hSOS (51, 52). Immunoprecipitation/immunoblotting experiments indicated that the Grb2-hSOS complex also couples ICP10 to ras activation (21). However, ICP10 is a Ser/Thr kinase in which Grb2 binding appears to involve interaction of SH3 motifs with ICP10 proline-rich sites. To determine the region(s) of Grb2 responsible for binding ICP10, extracts of ICP10-expressing cells were incubated with GST-Grb2 in the presence of increasing concentrations of peptides that represent the Grb2 SH3 or SH2 motifs. Binding was reduced (60% inhibition) by the Grb2 C-terminal SH3 peptide 156–199 at a concentration of 30 μM, and it was virtually abrogated by 120 μM of this peptide (Fig. 8). Peptides 1–68 and 54–164, which respectively represent the Grb2 N-terminal SH3 and SH2 motifs, did not inhibit binding (Fig. 8), even at concentrations as high as 500 μM (data not shown). These data indicate that Grb2 binding to ICP10 involves the interaction of the Grb2 C-terminal SH3 motif with the ICP10 proline-rich sites, particularly at position 396.

**DISCUSSION**

The HSV-2 RR1 gene differs from its counterparts in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique 5’-terminal domain that has transforming activity (ICP10 PK oncogene) and encodes a growth factor receptor Ser/Thr PK that appears to interact with signaling proteins to activate ras (21). Although a wealth of evidence indicates that its kinase activity is intrinsic (24, 30, 32), concern arose about the ability of ICP10 to function with only 8 (22) of the 12 catalytic motifs conserved by previously studied PKs (39). The studies described in this report were designed to identify specific amino acids that are required for kinase activity and those involved in ICP10 interaction with signaling proteins. The following comments seem pertinent with respect to our findings.

The basic shared functions common to all PKs are ion-dependent ATP binding and catalysis. In all PKs studied to date,
three conserved sequence motifs are associated with MgATP binding. They include: (i) the Gly-rich loop (catalytic motif I), which appears to be involved in stabilizing nontransferable ATP β-phosphates, and (ii) the two conserved charged residues that respectively constitute catalytic motifs II (Lys) and III (Glu). These amino acids presumably form an ion pair that, in the ternary complex, provides a docking site for MgATP (40). In tyrosine kinases, which are typically expressed at low levels (40), replacement of the Lys residue in catalytic motif II may be sufficient to show a null phenotype (53). However, in the yeast cAMP kinase catalytic subunit (41) and in isocitrate dehydrogenase PK (54), both of which are expressed at high levels, replacement of this Lys residue reduced PK activity but was insufficient to cause a null phenotype. The kinase activity of the yeast cAMP kinase was equally reduced by replacement of two adjacent nonconserved Lys residues, and it was severely compromised by replacement of the Glu residue in catalytic motif III (41).

Our findings for ICP10 are similar. The first three conserved catalytic motifs associated with ion-dependent ATP binding are present in ICP10 PK. They include the Gly-rich loop (at positions 106–110), Lys176 (motif II), and Glu209 (motif III) (22). Mutation of one Gly residue within the Gly-rich loop did not significantly reduce the ICP10 kinase activity (24), as also reported for several other PKs that lack one or all three Gly residues (55). It seems that the Gly residues are not the most important requirement for PK activity, but rather several critical backbone amides are needed in order to hydrogen bond to nontransferable β-phosphates of ATP, thereby locking them into place (56). On the other hand, replacement of the Lys residue in PK catalytic motif I (Lys176) reduced the ICP10 PK activity (K_m = 1.2 and 6.6 μM for ICP10 and Lys176 mutant, respectively) to a similar extent as that previously reported for the yeast cAMP kinase (41). Also, as described for the yeast cAMP kinase (41), a similar reduction in ICP10 PK activity (K_m = 9.4 μM) was achieved by replacement of Lys259, which is the only Lys residue close to Lys176. We conclude that both Lys residues bind ATP, since (i) the Lys176 and Lys259 mutants evidenced a similar decrease in FSBA binding relative to wild type ICP10, (ii) FSBA binding was specifically competed with another ATP analogue (AMP-PNP), and (iii) a null phenotype (no PK activity or FSBA binding) was achieved when both Lys residues were mutated. As also described for the yeast cAMP kinase (41), replacement of Glu209 caused a severe decrease in both MnATP-dependent and MgATP-dependent PK activity, suggesting that the ion pair that is presumably formed between the two charged residues (Lys and Glu) provides a docking site for either MnATP or MgATP. Significantly, background activity was not observed in immunoprecipitates from mock-transfected cells or from cells stably transfected with ICP10 and precipitated with preimmune serum. If present, the contaminating activity in this study is less than 0.1% of the specific activity of the wild type ICP10, since the TM-deleted mutant was sometimes detectable within this range. Also, the double Lys and TM-deleted mutants retain all known target sites for cellular PKs, but they totally lack kinase activity. Thus, by identifying three amino acid sites that are essential for kinase activity, the present studies confirm previous conclusions that the ICP10 PK activity is intrinsic.

Sequence similarities exist throughout the core region of all eukaryotic PKs, but many of the enzymes also contain inserts, sometimes very large ones, in the core region itself (39). If the structural framework implied for all PKs by present understanding of the function of conserved catalytic motifs is indeed correct, these inserts must be accommodated without perturbing the general folding of the core. In this context it may be significant that in known eukaryotic PKs, catalytic motifs I and II are separated by a short (14–23 amino acids) β-sheet structure (40), while in ICP10 PK these motifs are separated by a bulky insert that consists of 63 amino acids and encompasses the proline-rich site at position 140–167. This insert is interspersed with α-helices (23) that may cause suboptimal folding of the core, thereby interfering with efficient ATP binding. According to this interpretation, the high levels of ICP10 PK activity observed in J HLA1 cells must reflect the existence of an alternate ATP binding site, which fulfills the structural constraints for optimal core folding and ATP binding efficiency. Lys259, which is separated by 14 amino acids that form a short β-strand (23) from a potential nucleotide binding consensus motif (Gly271–X-Gly274–X-X-Gly276) provides such an alternate site. However, our studies fall short from demonstrating ATP occupancy of both Lys259 and Lys259 on wild type ICP10 because we cannot exclude the possibility that each Lys residue substitutes for the lost one in the single Lys mutants.

Another basic shared function common to all PKs, including ICP10, is catalysis, which involves the Asp and Asn residues in PK catalytic motif VI. Asp is believed to accept the proton from the attacking substrate hydroxyl group during phosphotransfer, and Asn chelates the secondary Mg2+ ion and may serve to stabilize the loop (39, 40). In ICP10 PK, the Asp residue is replaced by Glu (Glu124), which has a similar charge and is likely to fulfill the same function. Alternatively, catalysis is accomplished by an alternate motif (265DSPGN269), which contains Asp and Asn residues in a functionally appropriate configuration. Indeed, the bacterially expressed truncated ICP10 protein (pp2934), which lacks catalytic motif VI, retains PK activity (29, 30), which is lost by replacement of Asp265.

Proline-rich motifs are consensus binding sites for SH3 domains involved in protein-protein interaction for epidermal growth factor receptor signaling (51), cellular localization of

FIG. 8. Mapping of the Grb2 region that binds ICP10. Extracts of J HLA1 cells were reacted with GST-Grb2 fusion protein bound to glutathione-agarose beads after preincubation (1 h, 4°C) with 0, 30, or 120 μM of synthetic peptides representing the N-terminal (amino acids 1–68) or C-terminal (amino acids 156–199) SH3 domains or 120 μM of a peptide representing the SH2 domain (amino acids 54–164) of Grb2. Results for 500 m of peptides were similar to those obtained with 120 μM. Bound proteins were analyzed by immunoblotting with anti-LA-1 antibody, and data are expressed as percentage of binding of the untreated (0) control.

M. Kulka, C. C. Smith, and L. Aurelian, manuscript in preparation.
cytoplasmic proteins (57), activation of phosphatidylinositol kinase by IgM cross-linking (58), and up-regulation of the GTPase activity of dynamin (59). SH3-binding proline-rich motifs share a common PXXP motif and have residues that contribute to specificity. Class I motifs have an amino to carboxyl-terminal binding orientation and include sites specific for Src. Class II motifs are more promiscuous in their binding. They have a carboxyl- to amino-terminal binding orientation, PXXP can be in either of two positions, and they have basic residues at the C terminus (28, 44–48).

The first ICP10 proline-rich domain is located in the insert between catalytic motifs I and II. It consists of a class I, Src-specific SH3 binding motif (140TPETPQP147) followed by a promiscuous class II motif (149AVPPPPPPPPFWGH159) similar to that recently shown to bind Abl, Src, Fyn, and Crk (28). The second ICP10 proline-rich domain located between catalytic motifs I and II. It consists of a class I, Src-related SH3 or SH2 motifs. The affinity of Grb2 binding to the specific SH3 binding motif (140RTPEPQGP147) followed by a proline-rich site at position 149 caused only minimal (2-fold) reduction in Grb2 binding. We therefore tested the possibility that proline-rich motif at position 149 plays a more important role in Grb2 binding in vivo or, for example, when the site at position 96 is missing. Indeed, Grb2 binding is abrogated only by mutation of both class II sites, and Grb2 binds to a truncated ICP10 protein that lacks the site at position 96.4

We conclude that the Grb2 C-terminal SH3 binds ICP10 because binding was competed by a peptide that represents this SH3 motif but not by peptides that represent the N-terminal SH3 or SH2 motifs. The affinity of Grb2 binding to the ICP10 proline-rich sites is still unclear, but binding is competed by peptide concentrations within the range of those that compete the high affinity hSOS binding (52, 63). Since Grb2 is a plausible candidate for recruiting ICP10 to the cytoplasmic proteins (57), activation of phosphatidylinositol kinase by IgM cross-linking (58), and up-regulation of the GTPase activity of dynamin (59). SH3-binding proline-rich motifs share a common PXXP motif and have residues that contribute to specificity. Class I motifs have an amino to carboxyl-terminal binding orientation and include sites specific for Src. Class II motifs are more promiscuous in their binding. They have a carboxyl- to amino-terminal binding orientation, PXXP can be in either of two positions, and they have basic residues at the C terminus (28, 44–48).

We conclude that the Grb2 C-terminal SH3 binds ICP10 because binding was competed by a peptide that represents this SH3 motif but not by peptides that represent the N-terminal SH3 or SH2 motifs. The affinity of Grb2 binding to the ICP10 proline-rich sites is still unclear, but binding is competed by peptide concentrations within the range of those that compete the high affinity hSOS binding (52, 63). Since Grb2 binds hSOS at its N-terminal SH3 (52, 60), these findings suggest that Grb2 couples ICP10 to ras activation by binding both ICP10 and hSOS at SH3 sites, consistent with the structural flexibility of this molecule (28). Taken in toto our data indicate that ICP10 PK is a primitive protein in which minimal genetic information can adapt to a relatively wide functional diversity and has the necessary flexibility to use additional and alternate catalytic sites as required.

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