Journal of Virology, Aug. 1995, p. 4979–4985 Vol. 69, No. 8
0022-538X/95/$04.00+0
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Characterization of the Novel Protein Kinase Activity Present in the R1 Subunit of Herpes Simplex Virus Ribonucleotide Reductase

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Received 1 March 1995/Accepted 17 May 1995

We have compared the protein kinase activities of the R1 subunits from herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) ribonucleotide reductase following expression in *Escherichia coli*. Autophosphorylation activity was observed when kinase assays were performed with immunoprecipitated R1 or proteins purified to homogeneity, and the activity was stimulated by the basic protein protamine. Transphosphorylation of histones or calmodulin by purified or immunoprecipitated HSV-1 and HSV-2 R1 was not observed, and our results suggest that the activities of these two proteins are similar. We further characterized the protein kinase activity of HSV-1 R1 by producing insertion and deletion mutants constructed with a plasmid expressing R1 amino acids 1 to 449. C-terminal deletion analysis identified the catalytic core of the enzyme as comprising residues 1 to 292, and this polypeptide will be useful for structural determinations by X-ray crystallography. Insertion of a 4-amino-acid sequence at sites within the protein kinase domain identified regions essential for activity; insertions at residues 22 and 112 completely inactivated activity, and an insertion at residue 136 reduced activity sixfold. Similar insertions at residues 257, 262, 292, and 343 had no effect on activity. The ATP analog 5 -fluorosulfonylbenzoyladenosine, which covalently modifies conventional eukaryotic kinases at an essential lysine residue within the active site, did label HSV R1, but this labelling occurred outside the N-terminal domain. These data indicate that the HSV R1 kinase is novel and distinct from other eukaryotic protein kinases.

Ribonucleotide reductase (EC 1.7.1.4.1) catalyzes the conversion of ribonucleotides to the corresponding deoxyribonucleotides, a reaction essential for the de novo synthesis of DNA (42). The enzyme encoded by most herpesviruses consists of homodimeric R1 and R2 subunits in an $\alpha_2\beta_2$ configuration (for a review, see reference 12). The herpes simplex virus (HSV) enzyme is essential for viral pathogenesis in mice and rats (3, 25, 41) and is a target for antiviral chemotherapy (7, 16, 29, 32). Deletion of the R1 subunit from pseudorabies virus and varicella-zoster virus results in viruses which are avirulent in pigs (15) or impaired for growth in vitro (22).

Evidence suggests that HSV R1, in addition to its role in ribonucleotide reduction, may have another function in viral pathogenesis. R1 mRNA is produced under immediate-early conditions in the presence of cycloheximide, whereas R2 mRNA is not expressed until early times postinfection (6). Strikingly, the R1 promoter is only weakly transactivated by Vmw65 in transient-transfection assays, and optimal expression is obtained with Vmw110; the major transactivator of early and late virus genes Vmw175 does not stimulate the R1 promoter (14, 45, 47, 48). The R1 promoter has binding sites for the AP-1 activator protein and transactivation of HSV type 2 (HSV-2) R1 by AP-1 has been demonstrated (47, 48). We (8) and others (14, 45) have proposed a role for R1 during immediate-early times postinfection which requires a protein kinase activity present in the unique N-terminal domain of the protein.

Sequence comparisons of R1 subunits of prokaryotic and eukaryotic origin have shown that HSV-1 and HSV-2 possess a unique N-terminal extension of approximately 300 amino acids (38, 44). The N-terminal extension of HSV-1 R1 (termed 1R1) is dispensable for ribonucleotide reduction and is linked to the remainder by a protease-sensitive loop (9, 11, 23, 28). Protein kinase activity of the HSV-2 R1 (termed 2R1) N terminus was first described by Chung et al. (5); sequence comparisons with eukaryotic protein kinases identified putative catalytic motifs, and Chung et al. (5) reported activity in an immunocomplex kinase assay. Despite lack of homology between 1R1 and 2R1 in the N terminus, subsequent studies demonstrated protein kinase activity of 1R1 (1, 8, 40), which suggested that the HSV R1 protein kinase was distinct from other conserved eukaryotic kinases. A lysine residue, essential for activity and invariant in eukaryotic kinase when mutated, did not affect 2R1 activity (30), and this lysine is absent from 1R1. Luo and Aurelian (30) reported the covalent modification of 2R1 by the ATP analog 5′-fluorosulfonylbenzoyladenosine (FSBA), which affinity labels eukaryotic protein kinases at the active-site lysine, which to them suggested the presence of an alternative catalytic lysine within the N terminus.

Transphosphorylation of histones and calmodulin by 2R1 and properties characteristic of growth factor receptor kinases, such as membrane association, myristylation, and activation by basic proteins, have been reported (4, 5, 30), and a putative transmembrane segment located between residues 86 and 106 is essential for activity (30). These observations have been used to suggest (1, 43) that the protein kinase activity of 2R1 is associated with the ability of a HSV-2 DNA region, termed *mtrIII* (21, 24, 26), to transform cells. Association of 2R1 with components of the transformation-related signalling pathway has been reported (43) and malignant transformation by *mtrIII* was enhanced by kinase expression (1): the equivalent region of HSV-1 DNA does not transform (2). Initial observations with 1R1 suggested that the N-terminal protein kinase could transphosphorylate (40), but subsequent observations made with protein purified following expression in *Escherichia coli* indicated that transphosphorylation was the result of a copurifying contaminant kinase (8); surprisingly, this prokaryotic kinase was able to transphosphorylate several proteins present in HSV-infected cell extracts.

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In this report we address several issues related to HSV R1 protein kinase activity. Both HSV-1 and HSV-2 R1, purified after expression in *E. coli* exhibited autophosphorylation which was stimulated by protamine, and there was no transphosphorylation of histones or calmodulin. Insertion and deletion mutants were generated within the N terminus of 1R1, and certain mutants inactivated autophosphorylation and thus identified regions essential for activity. These R1 regions shared no amino acid homology with catalytic motifs of eukaryotic kinases, indicating that HSV R1 contains a novel class of protein kinase.

MATERIALS AND METHODS

Expression of HSV-1 and HSV-2 R1 in *E. coli*. Expression of 1R1 in *E. coli* by using the T7 system has been described previously (19). An identical strategy was adopted for the construction of a vector expressing 2R1 with the exception that the 2R1 open reading frame was removed from plasmid pRR1-SD by digestion with *Xba*I and *Bam*HI and ligated into pET8c. Conditions for protein expression, lysozyme extraction, and preparation of the 33% ammonium sulfate fraction were as described previously (19). The ammonium sulfate fraction was applied directly to a 5-ml Cibacron Blue column, and bound R1 eluted in a 0 to 2.5 M NaCl gradient developed over 5 column volumes. Fractions containing R1, identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were dialyzed overnight against 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–2 mM dithiothreitol (DTT), pH 7.5, and further purified by heparin chromatography (10).

Construction of N-terminal insertion and deletion mutants. Vector pC449R1, which expresses 1R1 amino acids 1 to 449 (8) was used for the construction of insertion and deletion mutants. Mutations were made throughout this region by insertion of a 12-bp oligonucleotide (pCCCGAATTCGGGp), containing an *Eco*RI site, into blunt restriction sites; prior to this insertion, an *Eco*RI site in the vector backbone was destroyed by filling in the ends. Partial enzyme digests were made in the *Eco*RI-negative plasmid with restriction endonucleases *Nae*I, *Rsa*I, and *Sma*I, each of which has several sites within the 1.4-kb R1 fragment. Digest products were resolved with 1% Tris-acetate agarose gels, and linear bands were isolated, treated with Gene Clean, and treated with calf intestinal phosphatase to reduce vector background. The oligonucleotide was annealed, phosphorylated, and ligated into the linearized plasmids; deletion mutants were spontaneously produced by this method. Clones containing inserts were identified by screening with *Eco*RI, the site of insertion was crudely mapped by double digestion with *Xba*I and *Eco*RI or *Hin*dIII and *Eco*RI, and the precise location of the insert or extent of the deletion was determined by double-stranded dideoxy DNA sequencing. Induction of protein expression, lysozyme extraction, and ammonium sulfate precipitation were performed as described previously (19), except that ammonium sulfate was added to 45% saturation. Ammonium sulfate fractions from 0.5 liter of culture were resuspended in 2 ml of 25 mM HEPES–2 mM DTT, pH 7.6.

Protein kinase assays and immunoprecipitation. Protein kinase assays using purified 1R1 or 2R1 were performed in the presence or absence of 0.1 mg of protamine per ml with 1 mM $MnCl_2$, 250 mM NaCl, and 1 μl of [^{32}P]ATP (specific activity, 1,000 to 3,000 Ci/mmol; Amersham) (8). Histones (Sigma) or calmodulin (Calbiochem) as substrates for transphosphorylation were added to the assay mixture to give a final concentration of 0.1 mg/ml. In the case of 2R1, 1R1, and 1R1 mutants, immunoprecipitation of R1 polypeptides from 200 μl of ammonium sulfate fractions was achieved with 10 μl of antiserum 106, raised against dN245R1 (10). In some instances, 1R1 and 2R1 were precipitated with monoclonal antibody (MAb) 7602 (37) which recognizes a conformational epitope within the reductase domain of HSV R1. Prior to the addition of antibodies, the ammonium sulfate fractions were diluted 1:1 with 25 mM HEPES with 2 mM DTT and 500 mM NaCl, pH 7.6. Immunoprecipitation was performed overnight at 4°C or for 2 h at 25°C, after which 100 μl of a 50% slurry of protein A-Sepharose (Sigma) in the above buffer with 0.1% Nonidet P-40 was added and incubated for a further 2 h at 25°C. In addition to protein A-Sepharose, 5 μl of sheep anti-mouse immunoglobulin G was added to immunoprecipitations performed with MAb 7602. Protein A-Sepharose pellets were obtained by brief centrifugation at 13,000 × *g* and washed three times with 20 volumes of 25 mM HEPES–2 mM DTT buffer with 1 M NaCl and 0.5% Nonidet P-40. Prior to kinase assay, the matrix was washed once with 1 ml of 25 mM HEPES, pH 7.6, with 1 mM $MnCl_2$ and 250 mM NaCl, suspended in 30 μl of this buffer with 1 μl of [^{32}P]ATP, with or without 0.1 mg/ml protamine and incubated for 30 min at 25°C with constant agitation. The matrix was then washed once with kinase assay buffer, and ^{32}P incorporation was observed by SDS-PAGE and autoradiography.

Quantification of immunoprecipitated R1 polypeptides and levels of autophosphorylation. The amount of 1R1 or 2R1 immunoprecipitated by 10 μl of antiserum 106 was determined by measuring the R1 concentration before and after incubation with the antiserum plus protein A-Sepharose by the R2 affinity enzyme-linked immunosorbent assay (ELISA) described by Conner et al. (11). Purified 1R1 or 2R1 was used to calibrate the ELISA. The amounts of immunoprecipitated 1R1 or 1R1 mutated polypeptides were quantified by limiting dilutions on Western blots (immunoblots) using antiserum F1, which recognizes 1R1 amino acids 3 to 181 (11, 28); the amount of immunoprecipitated 1R1 was known from ELISA allowing determination of the amount of immunoprecipitated 1R1 mutated polypeptides to be calculated. Immunocomplex kinase assays were performed, and the 1R1 bands were identified by Western blotting with F1, and ^{32}P incorporation was assayed by excision of the relevant band and liquid scintillation counting.

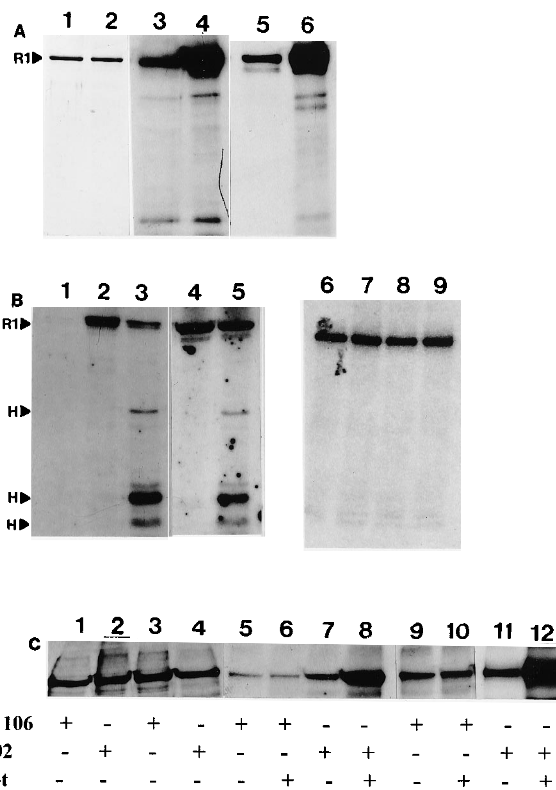
Labelling of proteins with [^{14}C]FSBA. The ATP analog [^{14}C]FSBA (specific activity, 40 to 60 mCi/mmol; NEN-Dupont) was used at 100 μM to label 5 to 10 μg of purified proteins under optimum 1R1 kinase assay conditions (without protamine). The proteins used were 1R1, 2R1, dN245R1, an N-terminally truncated 1R1 polypeptide (10), and *E. coli* R1, which was a kind gift from JoAnne Stubbe. Labelling was performed at 25°C for 30 min, and [^{14}C]FSBA incorporation was determined by SDS-PAGE and autoradiography. Peptide mapping with trypsin was performed as described by Conner et al. (8) with [^{14}C]FSBA-labelled R1. Limited proteolysis of 1R1 and dN245R1 before or after covalent modification was performed as described by Conner et al. (9) with 0.4 μg of trypsin per ml.

RESULTS

Protein kinase activities of HSV 1R1 and HSV 2R1. Purification of 1R1 and 2R1 was achieved by a combination of Cibacron blue and heparin chromatography, and the proteins are shown in Fig. 1A (lanes 1 and 2). Incubation of 300 ng of 1R1 and 2R1 under optimum 1R1 kinase assay conditions resulted in autophosphorylation of both proteins (Fig. 1A, lanes 3 to 6), and this activity was stimulated by 0.1 mg of protamine per ml (lanes 4 and 6). Neither purified 1R1 nor 2R1 was capable of transphosphorylating histones (Fig. 1B, lanes 2 and 4), although the same histone preparation was transphosphorylated by a contaminant *E. coli* protein kinase (Fig. 1B, lanes 3 and 5) present in R1 purified by a single heparin column (8); the additional Cibacron blue step removes this activity. No ^{32}P incorporation was observed when histones alone were incubated under optimum 1R1 conditions with 0.1 mg of protamine per ml (Fig. 1B, lane 1). No transphosphorylation of calmodulin was observed when 1R1 or 2R1 was incubated with this substrate (data not shown).

Autophosphorylation by 1R1 was demonstrated by immunocomplex kinase assays performed on protein immunoprecipitated from the 33% ammonium sulfate fraction by antiserum 106 (Fig. 1B, lanes 6 to 9). The level of ^{32}P incorporation by 1R1 was not affected by extensive washing of the immunoprecipitated protein with 0.5 (lane 6), 1 (lane 7), 2 (lane 8), or 3 (lane 9) M NaCl prior to kinase assay; these high-salt washes would be sufficient to remove any contaminant kinases. Interestingly, when immunocomplex kinase assays were performed on 1R1 and 2R1 immunoprecipitated by antiserum 106, no stimulation by protamine was observed (Fig. 1C, compare lanes 5 and 6 and lanes 9 and 10). Stimulation by protamine was observed when immunocomplex kinase assays were performed on 1R1 and 2R1 immunoprecipitated by MAb 7602 (Fig. 1C, compare lanes 7 and 8 and lanes 11 and 12). We conclude that antiserum 106, which is raised against 1R1 amino acids 245 to 1137 acts to prevent protamine stimulation of R1 kinase activity. No transphosphorylation of histones or calmodulin was observed with immunoprecipitated 1R1 or 2R1 (data not shown).

Kinase activities of various mutant 1R1 polypeptides. Using a plasmid constructed to express amino acids 1 to 449 of R1 and an oligonucleotide designed to insert four amino acids at blunt-end restriction sites, a number of N-terminal proteins with deletions and insertions within the protein kinase domain were obtained. The sites of insertion and the extents of deletions, as determined by sequencing, are shown in Fig. 2, and expression of these polypeptides in *E. coli* was demonstrated by Western blotting (Fig. 3A). The nomenclature of these proteins identifies the location of the insertion or extent of the deletion, for example, polypeptide in22R1 has a 4-amino-acid



C	1	2	3	4	5	6	7	8	9	10	11	12
As 106	+	-	+	-	+	+	-	-	+	+	-	-
7602	-	+	-	+	-	-	+	+	-	-	+	+
prot	-	-	-	-	-	+	-	+	-	+	-	+

FIG. 1. (A) Coomassie blue-stained SDS-polyacrylamide gel showing purified 1R1 (lane 1) and 2R1 (lane 2). Autophosphorylating activities (lanes 3 to 6) of purified 1R1 (lanes 3 and 4) and 2R1 (lanes 5 and 6) incubated without (lanes 3 and 5) or with 0.1 mg protamine per ml (lanes 4 and 6) are shown. (B) Activity of 1R1 (lane 2) and 2R1 (lane 4), purified by Cibacron blue and heparin chromatography following incubation with 0.1 mg of histones per ml. Lanes 3 and 5 show transphosphorylation of histones by preparations of 1R1 and 2R1 purified by heparin chromatography only. Histones alone are shown in lane 1. R1, 1R1; H, histones. Lanes 6 to 9 show immunocomplex kinase assays of 1R1 immunoprecipitated with antiserum 106 and washed with 0.5 (lane 6), 1 (lane 7), 2 (lane 8), or 3 (lane 9) M NaCl prior to assay. (C) Autophosphorylation of immunoprecipitated 1R1 or 2R1. Immunocomplex kinase assays were performed on 1R1 (lanes 1, 2, 5 to 8) or 2R1 (lanes 3, 4, 9 to 12) immunoprecipitated with antiserum 106 (As 106) or MAb 7602 (7602) in the presence (+) or absence (−) of 0.1 mg of protamine (prot) per ml as indicated.

insert at residue 22, and in polypeptide dC345R1, all residues downstream of amino acid 345 had been deleted.

Ammonium sulfate fractions were prepared for all of the mutant proteins, 1R1, 2R1, and a 1R1 N-terminally truncated polypeptide dN871R1 which comprises residues 871 to 1137 (10). Immunoprecipitations were performed with antiserum 106, and the precipitated proteins are shown in Western blots (Fig. 3A) probed with antiserum F1 (lanes 1 to 14), which recognizes R1 amino acids 3 to 181, or with antiserum 106 (lanes 15 and 16). Antiserum 106 successfully immunoprecipitated all proteins, and a number of degradation products are visible with dC449R1 and polypeptides with insertions (Fig. 3A, lanes 5 to 12). Distortion of some of the bands in Fig. 3A is caused by comigration of the polypeptides with immunoglobulin G heavy chains which are present in large amounts following immunoprecipitation. Antiserum 106 is raised against 1R1 amino acids 245 to 1137, and despite the fact that few of these residues are present in dC257R1, this polypeptide was successfully immunoprecipitated and recognized by this antiserum in Western blots (lane 15). However, antiserum 106 reacted poorly with dN871R1, and this polypeptide is only

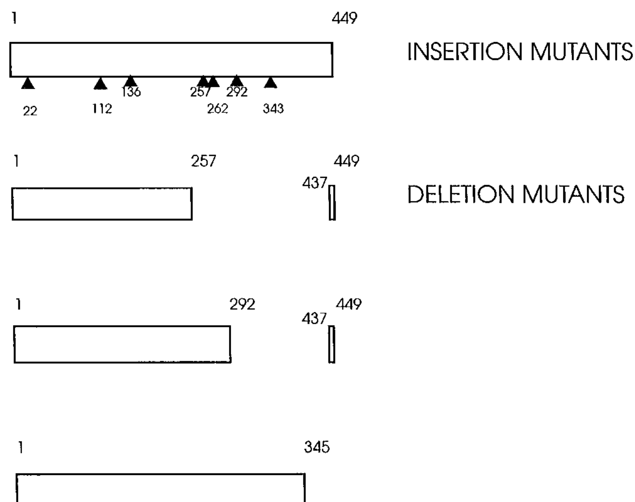


FIG. 2. Line diagram, drawn to scale, showing locations of insertions and deletions in the N-terminal domain of 1R1. The numbers are residue numbers.

faintly visible in lane 16. A number of *E. coli* proteins, which also reacted with antiserum F1, were nonspecifically precipitated by antiserum 106 (Fig. 3A, lanes 3 and 4). Autophosphorylation of immunoprecipitated 1R1 and 2R1 was observed following immunocomplex kinase assays (Fig. 3B, lanes 1 and 2). A number of *E. coli* proteins also incorporated ^{32}P under these conditions, but this activity was not related to R1, as these proteins were also detected in extracts from *E. coli* which did not express any R1-related polypeptides (Fig. 3B, lane 3) or which expressed the N-terminal deletion dN871R1 (Fig. 3B, lane 4).

Autophosphorylating activity was detected with immunoprecipitated dC292R1 (Fig. 3B, lane 5) and with dC345R1 (Fig. 3B, lane 8) but not with dC257R1 (Fig. 3B, lane 6) or dN871R1 (Fig. 3B, lane 7). These data determine that residues 1 to 292 of HSV-1 R1 form the catalytic core of the 1R1 protein kinase.

Specific autophosphorylation was detected with immunoprecipitated dC449R1 (Fig. 3C, lane 1) but was not observed in immunoprecipitates from control *E. coli* extracts (Fig. 3C, lane 2). Activity was also observed with dC449R1 polypeptides possessing inserts at amino acids 257 (lane 4), 262 (lane 6), 292 (lane 8), and 343 (lane 9). No kinase activity was detected with immunoprecipitated polypeptides in22R1 (lane 3), in112R1 (lane 5), and in136R1 (lane 7), indicating that 4-amino-acid inserts in these regions of 1R1 abolish autophosphorylation. The diffuse band faintly visible in lanes 5 and 7 originates from *E. coli*, as it can also be observed in control lanes (Fig. 3C, lane 2; Fig. 3B, lanes 3 and 4). In addition to full-length dC449R1, antiserum 106 also immunoprecipitated a number of degradation products present in the ammonium sulfate fractions (Fig. 3A) and phosphoprotein bands corresponding to these products were not observed on Fig. 3C but were seen on some autoradiographs (data not shown). We were unable to conclude if any truncated products possessed kinase activity, as degradation may have occurred following autophosphorylation.

Quantification of protein kinase activity by immunoprecipitated 1R1 proteins. The data presented in Fig. 3B and C suggest that there is variation in levels of autophosphorylation which could reflect the effects of mutations or be the result of variation in the amounts of polypeptides immunoprecipitated by antiserum 106. Using an R2 affinity ELISA and limiting dilutions in Western blots, we were able to estimate the

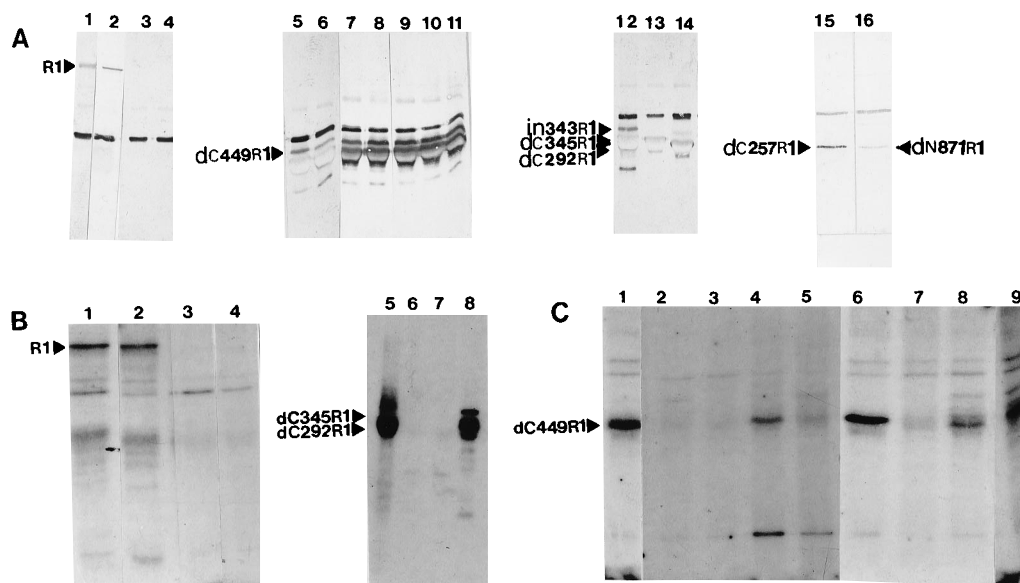


FIG. 3. (A) Western blots, probed with antiserum F1 (lanes 1 to 14) or 106 (lanes 15 and 16) showing proteins immunoprecipitated by antiserum 106 from *E. coli* extracts containing polypeptides 1R1 (lane 1), 2R1 (lane 2), dC449R1 (lane 5), in22R1 (lane 6), in112R1 (lane 7), in136R1 (lane 8), in257R1 (lane 9), in262R1 (lane 10), in292R1 (lane 11), in343R1 (lane 12), dC345R1 (lane 13), dC292R1 (lane 14), dC257R1 (lane 15), and dN871R1 (lane 16). Proteins immunoprecipitated from control *E. coli* extracts are shown in lanes 3 and 4. (B) Autophosphorylation by immunoprecipitated 1R1 polypeptides. Immunocomplex kinase assays were performed on 1R1 (lane 1), 2R1 (lane 2), dN871R1 (lanes 4 and 7), dC292R1 (lane 5), dC257R1 (lane 6), and dC345R1 (lane 8). An *E. coli* control is shown in lane 3. (C) Kinase activity of immunoprecipitated 1R1 insertion mutants: dC449R1 (lane 1), in22R1 (lane 3), in257R1 (lane 4), in112R1 (lane 5), in262R1 (lane 6), in136R1 (lane 7), in292R1 (lane 8), and in343R1 (lane 9). An *E. coli* control is shown in lane 2. In all panels, proteins related to R1 are indicated by arrowheads.

amounts of R1 proteins immunoprecipitated by antiserum 106 and thereby standardize the amount of R1 protein in our kinase assay. Quantification of 1R1 and 2R1 before and after immunoprecipitation by ELISA indicated that 10 μl of antiserum 106 reacted with 1.1 and 0.55 μg of 1R1 and 2R1, respectively; antiserum 106 was raised against an N-terminal deletion of 1R1 and presumably does not recognize 2R1 as efficiently. The ratios of immunoprecipitated, mutated proteins to the known amount of immunoprecipitated 1R1 were determined by limiting dilutions and Western blotting with antiserum F1. Examples of these dilutions with polypeptides 1R1, dC345R1, dC257R1, and in292R1 are presented in Fig. 4. These ratios were used to estimate the amounts of polypeptides with insertions or deletions used in immunocomplex kinase assays. Kinase assays were performed in parallel, R1 bands were identified by Western blotting, and ^{32}P incorporation was determined by liquid scintillation counting of excised bands. The results are summarized in Table 1 and confirm that in22R1, in112R1, and dC257R1 are inactive, the activity of in136R1 is reduced approximately sixfold, and in257R1, in262R1, in292R1, in343R1, dC345R1 and dC292R1 retain full

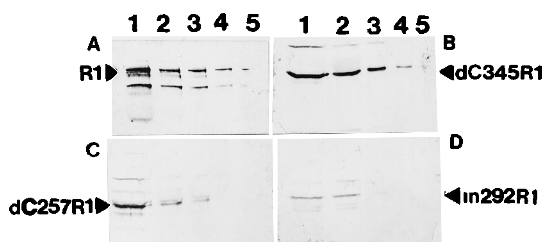


FIG. 4. Western blots, probed with antiserum F1, showing limiting dilution analysis of polypeptides 1R1 (A), dC345R1 (B), dC257R1 (C) and in292R1 (D). Dilutions are 1:1, 1:2, 1:4, 1:8, and 1:16 (lanes 1 to 5, respectively).

R1 autophosphorylation. A second experiment, performed under identical conditions, produced results totally compatible with those in Table 1. This method of quantification assumes that the reactivity with antiserum F1 is similar for all mutated 1R1 polypeptides and is minimally affected by insertions at amino acids 22, 112, and 136.

Affinity labelling of R1 by [^{14}C]FSBA. Both 1R1 and 2R1 were covalently modified by [^{14}C]FSBA as shown by autoradiography in Fig. 5A (lanes 1 and 2) suggesting the presence of an ATP binding site. However, dN245R1, in which most of the N-terminal domain has been deleted, and the R1 subunit from

TABLE 1. Quantification of 1R1 protein levels and protein kinase activity

Protein	Ratio of protein to 1R1	Amt (μg) precipitated by antiserum 106	cpm [^{32}P][a]	Kinase activity (pmol of ^{32}P/mg of protein)
1R1	1	1.10	1,457	3.30
2R1	0.5	0.55	710	3.23
dC449R1	0.5	0.55	620	2.82
in22R1	1	1.10	126	0.00
in112R1	1	1.10	113	0.00
in136R1	0.5	0.55	181	0.45
in257R1	0.25	0.28	390	3.90
in262R1	1	1.10	1,316	2.99
in292R1	0.25	0.28	325	2.90
in343R1	1	1.10	1,478	3.36
dC345R1	1	1.10	1,796	4.08
dC292R1	1	1.10	1,497	3.40
dC257R1	0.5	0.55	63	0.00
dN871R1	ND[b]	ND	80[c]	0.00

[a] 400 cpm is approximately equivalent to 1 fmol of ^{32}P.
[b] ND, not determined.
[c] This value was used to correct other values for background counts per minute.

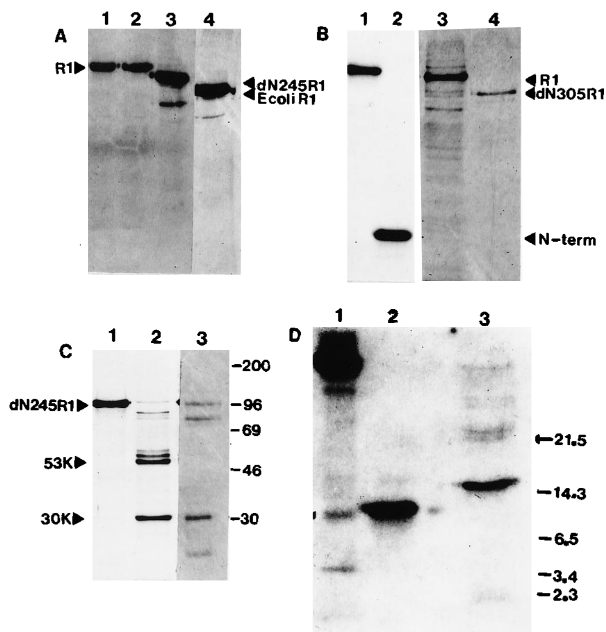


FIG. 5. (A) Autoradiograph showing labelling of polypeptides 1R1 (lane 1), 2R1 (lane 2), dN245R1 (lane 3), and *E. coli* R1 (lane 4) with [^{14}C]FSBA. (B) Autophosphorylating activity of the N-terminal domain of 1R1 produced by proteolytic cleavage of intact 1R1 with 0.4 mg of trypsin per ml. Kinase assays were performed on intact 1R1 (lane 1) and trypsin-treated 1R1 (lane 2). Lanes 3 and 4 show [^{14}C]FSBA labelling of intact 1R1 (lane 3) and dN305R1 produced by treatment of 1R1 with 0.4 mg of trypsin per ml (lane 4). (C) Coomassie blue-stained gel showing intact dN245R1 (lane 1) and fragments generated by treatment of dN245R1 with 0.4 mg of trypsin per ml (lane 2). Lane 3 shows labelling of trypsin-treated dN245R1 with [^{14}C]FSBA. (D) Lane 1, [^{14}C]FSBA-labelled 2R1; lanes 2 and 3, ^{14}C-labelled peptides from tryptic digestion of covalently modified 2R1 (lane 2) and *E. coli* R1 (lane 3). The positions (in kilodaltons) of molecular mass markers are indicated.

E. coli RR, which has no unique N-terminal domain, were also labelled by this reagent (Fig. 5A, lanes 3 and 4). Incubation of 1R1 with 0.4 μg of trypsin per ml results in cleavage at residue 305 (11) to release the unique N-terminal domain which is active in autophosphorylation assays (Fig. 5B, lane 2) and dN305R1 containing the reductase domain, only the latter of which is labelled with [^{14}C]FSBA (Fig. 5B, lane 4). Treatment of dN245R1 with 0.4 μg of trypsin per ml results in cleavage at residues 305 and 575 to produce subdomain fragments of 30 and 53 kDa (Fig. 5C, lane 2), which contain residues 305 to 574 and 575 to 1137, respectively (9). Incubation of trypsin-treated dN245R1 with [^{14}C]FSBA results in labelling of the 30-kDa fragment (Fig. 5C, lane 3); larger fragments corresponding to dN305R1 and dN245R1 were also modified in this reaction. Peptide mapping of covalently modified 2R1 (Fig. 5D, lane 2) and *E. coli* R1 (lane 3) indicated that [^{14}C]FSBA labelling was at a single site in these proteins. We conclude from these results that covalent attachment of FSBA to R1 occurs outside the N-terminal kinase domain and within the ribonucleotide reductase catalytic domain at a site located between 1R1 residues 305 and 575.

DISCUSSION

We have further characterized the novel protein kinase activity present in the unique N terminus of HSV R1. Following expression in *E. coli* and purification, both 1R1 and 2R1 had autophosphorylation activity which was stimulated by the basic protein protamine. Previously we have shown stimulation of 1R1 activity by protamine (8), and others have demonstrated stimulation of 2R1 activity by polylysine (4); stimulation of activity by basic proteins is a feature of several eukaryotic kinases (36). Immunocomplex kinase assays have been used to identify a region of 1R1 and 2R1 involved in activation by basic proteins. MAb 7602, which binds within the reductase domain, immunoprecipitated both 1R1 and 2R1, and their kinase activities were stimulated by protamine. By contrast, autophosphorylation of 1R1 and 2R1 following immunoprecipitation with antiserum 106 (raised against 1R1 amino acids 245 to 1137) was not stimulated by protamine. A proteolytic truncation of 1R1 comprising amino acids 1 to 305 of the unique N-terminal domain retains protein kinase activity (8); thus, the protamine interaction site presumably lies within residues 245 to 305 and is conserved between 1R1 and 2R1. Binding of 106 antibody molecules within this region prevents protamine stimulation by blocking the interaction site. A second polyclonal antiserum raised in rabbits against full-length 1R1 immunoprecipitated 1R1 and 2R1, but the proteins were inactive in kinase assays, presumably because binding of some of the antibodies present in this antiserum inhibits activity (data not shown).

Neither 1R1 nor 2R1, purified from *E. coli* extracts by either standard chromatographic procedures or immunoprecipitation, was capable of transphosphorylating histones or calmodulin. When purified by heparin chromatography alone, 1R1 and 2R1 preparations contain an *E. coli* protein kinase which can transphosphorylate histones: an additional purification step, Cibacron blue chromatography, and immunoprecipitation remove this contaminant. We have considered the possibility that our preparations of 1R1 and 2R1 could be contaminated with a second *E. coli* kinase which is responsible for the observed autophosphorylation. Indeed we estimate our preparations of 1R1 and 2R1 to be only 90% pure, and other kinases could be present. However, results presented here and in previous publications argue against this possibility. We have demonstrated here that 4-amino-acid insertions at residues 22, 112, and 136 abrogate kinase activity, whereas similar insertions at residues 257, 262, 292, and 343 have no effect. In addition, our C-terminal deletion analysis identifies residues 257 to 292 as being essential for activity. Luo and Aurelian (30) have demonstrated kinase activity of 2R1 following SDS-PAGE and renaturation in situ and have shown that deletion of 2R1 residues 85 to 106 results in loss of kinase activity. Genetic evidence suggests therefore that the kinase activity is intrinsic to 1R1 and 2R1, but it is possible that the R1 mutants are inactive because they fail to interact with a protein kinase. This interaction would have to be specific to R1, since it is inhibited by mutations within defined regions of the N terminus and stable to 2 to 3 M NaCl. Kinase activity of 1R1 and 2R1 isolated from mammalian and bacterial cells has been reported elsewhere (1, 4, 5, 8, 30, 31, 40, 43), and the same peptide is phosphorylated in reactions performed with 1R1 isolated from infected cell and *E. coli* extracts (8), indicating that the interacting protein kinase would have to be conserved between prokaryotes and eukaryotes. If such a kinase existed, then its properties, interactions with HSV R1, and role in viral pathogenesis would be of considerable interest.

Although we cannot exclude totally the possibility that our observations are the result of a contaminant activity specifically interacting with HSV R1, the balance of evidence to date argues for the presence of a novel protein kinase activity in the unique N-terminal domain. Other nonconventional eukaryotic protein kinases have been reported (33, 39), but HSV R1 would contain the first novel herpesvirus protein kinase. In particular, we note that the activity of the novel protein kinase

FIG. 6. Amino acid sequence alignments of 1R1 and 2R1 showing locations of inserts in 1R1 (arrowheads) which inactivate kinase activity.

encoded by the BCR gene (33) is similar to HSV R1 kinase in that it is inhibited by FSBA but not affinity labelled by this reagent. HSV-1 also encodes two other protein kinases which show amino acid homology with conventional protein kinases (34, 35). Our inability to detect transphosphorylation activity with 1R1 or 2R1 expressed in *E. coli* contradicts results from others who have demonstrated transphosphorylation of calmodulin and histones by 2R1 (4, 5, 43). However, these results were generated with proteins immunoprecipitated from infected or transfected cell extracts, and additional cellular factors could be required to stimulate transphosphorylation. Our results demonstrate that protein kinase activities of 1R1 and 2R1 are similar and observed experimental differences may be due to interactions specific to mammalian cells.

The original identification of 2R1 as a protein kinase (5) was based on apparent amino acid homologies with eukaryotic kinases which have highly conserved catalytic motifs. Despite the absence of these motifs from the unique N terminus of 1R1, this domain of the protein exhibited protein kinase activity (8, 40). Studies with 2R1 have shown that a motif essential for eukaryotic protein kinase function was dispensable for 2R1 activity (30). In this study, mutations produced by insertion of a 4-amino-acid sequence at residues 22, 112, and 136 totally or partially inactivated 1R1 autophosphorylation activity. The amino acid sequences around these regions are shown in Fig. 6, in alignment with the 2R1 sequence. The insert at position 112 disrupts a potential GxGxxG motif associated with nucleotide binding in other proteins including protein kinases (46). A similar motif is present in 2R1 at residues 105 to 110, but replacement of Gly-106 by Pro did not affect 2R1 activity (30). Residues around amino acids 22, 112, and 136 show no conservation with other protein kinase motifs (20), and insertions at these sites which abrogate activity may directly affect catalytic residues, have local effects on active-site conformation, or disrupt the global folding of the N-terminal domain. Effects of the insertions on the overall conformation of the N terminus seem unlikely, as the amounts of protein immunoprecipitated by antiserum 106 were similar to that obtained with the wild-type polypeptide.

Luo and Aurelian (30) demonstrated affinity labelling of 2R1 with [^{14}C]FSBA, and we have shown that this reagent inhibits 1R1 kinase activity (8). Here we show that this ATP analog does not covalently modify residues within the active site of the R1 protein kinase domain, again indicating that the catalytic mechanism is novel and distinct. [^{14}C]FSBA modified HSV R1 within the reductase domain of the protein at a discrete site located between residues 305 and 575. *E. coli* R1 was also labelled at a single site by this reagent. *E. coli* RR activity is regulated by nucleotide effectors, including ATP, which bind to two separate sites in R1 (17), and direct photoaffinity labelling by dTTP to cysteine 292 has been demonstrated (18). HSV RR is not allosterically regulated (27), and effector binding sites are absent from R1 (17). Modification of R1 subunits by [^{14}C]FSBA at an ATP site is therefore unlikely, and it is probable that the reagent attaches to an exposed residue, such as serine, tyrosine, lysine, or histidine, susceptible to electrophilic attack. In support of this hypothesis, we were able to inhibit modification of R1 by [^{14}C]FSBA, using fluorosulfonylbenzoyl chloride (data not shown).

C-terminal deletion analysis has defined the catalytic core of 1R1 protein kinase. Removal of residues 345 to 449 and 292 to 437 did not affect activity, whereas deletion of residues 257 to 437 abolished autophosphorylation. Residues located between amino acids 257 and 292 may be essential for activity, or the autophosphorylation site may be located within this region, and we are locating the site of autophosphorylation by phosphopeptide mapping. The ability of R1 amino acids 1 to 292 to autophosphorylate is in agreement with previously published data, which demonstrated that a proteolytic cleavage product of 1R1 comprising residues 1 to 305 and a truncated domain of 2R1 consisting of residues 1 to 270 or 13 to 283 were fully active (8, 31). The levels of expression of the truncated polypeptides dC292R1 and dC345R1 were comparable with those of intact 1R1; purification of these truncations and structural studies by X-ray crystallography are under way.

The production of 1R1 and 2R1 under immediate-early conditions suggests a role for the kinase activity at this time postinfection such as phosphorylation of cellular or viral proteins involved in subsequent stages of the replication cycle. We have recently shown that during HSV-1 infection R1 is present in a twofold excess over R2 and in contrast to the active enzyme which localizes to discrete cytoplasmic foci, the uncomplexed R1 is found throughout the cytoplasm (13).

The unique N termini of 1R1 and 2R1 share 38% overall amino acid homology (38) which is sufficient for conservation of motifs involved in protein kinase activity. The poorly conserved regions may be responsible for reported differences in the activities of the proteins, such as a consensus SH3 binding motif between residues 150 and 159 in 2R1 which is absent from 1R1 (43), and Chung et al. (4) have reported a potential transmembrane domain between residues 86 and 105 present only in 2R1. Studies have reported the association of 2R1 but not 1R1 with cell membranes (4, 5, 30) and activation of transformation-related signalling pathways (43). The precise role of the unique N terminus of HSV R1 in viral pathogenesis is unknown and will require the analysis of virus mutants in which the protein kinase activity has been inactivated. We have identified regions of 1R1 which will be used in the production of these mutants.

ACKNOWLEDGMENTS

We are grateful for the excellent technical assistance of Jill Murray. Project grant support for J. Cooper and J. B. Clements from the Medical Research Council, UK, is gratefully acknowledged.

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