The unique N terminus of herpes simplex virus type 1 ribonucleotide reductase large subunit is phosphorylated by casein kinase 2, which may have a homologue in Escherichia coli

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Studies were performed to determine if the unique N-terminal domain of the R1 subunit from herpes simplex virus (HSV) type 1 ribonucleotide reductase is a substrate for casein kinase 2 (CK2). Transphosphorylation assays demonstrated that R1 was highly phosphorylated by this enzyme with multiple phosphorylation sites mapped to the N terminus between residues 1 and 245. Immunoprecipitation pull-down assays using R1-specific antisera failed to demonstrate a stable interaction between R1 and CK2 but residual amounts of CK2 present after immunoprecipitation efficiently transphosphorylated R1. Activity assays with a peptide substrate identified CK2 in R1 immunoprecipitated from infected-cell extracts but did not detect activity in R1 proteins immunoprecipitated from bacterial extracts. However, Western blotting identified potential E. coli homologues of the CK2 alpha and beta subunits. These results support conclusions that the N-terminal domain of HSV R1 is not a protein kinase and that all previous results can be explained by contaminating kinases, principally CK2.

Conversion of ribonucleotides to the corresponding deoxy-ribonucleotides by ribonucleotide reductase (RR) is essential for the de novo synthesis of DNA in all living organisms (Reichard, 1993). Many herpesviruses encode their own RR and the active form is a tetramer of homodimeric R1 and R2 subunits (reviewed in Conner et al., 1994a). RR activity is a target for antiviral chemotherapy and has provided a paradigm to study peptides which disrupt protein–protein interactions as a route to the development of antiviral drugs (Dutia et al., 1986; Cohen et al., 1986; Marcello et al., 1994; Liuzzi et al., 1994). In addition to their involvement in RR activity, the R1 subunits of herpes simplex virus (HSV) types 1 and 2 have been reported to possess a serine/threonine phosphokinase (PK) activity (R1PK) (Chung et al., 1989; Conner et al., 1992a; Paradis et al., 1991; Cooper et al., 1995) that is distinct from conventional eukaryotic kinases. The novel kinase activity is thought to be located within the N-terminal 310 amino acids, a domain which is unique to HSV R1 and is not required for ribonucleotide reduction (Conner et al., 1992b, 1993, 1994b; Lankinen et al., 1993); also, its role in HSV replication/pathogenesis is not yet established. Protein R1 is essential for virus pathogenicity as evidenced by the avirulence and failure to reactivate from latency of deletion mutants (Cameron et al., 1988; Jacobson et al., 1989; Yamada et al., 1991; Heineman & Cohen, 1994; deWind et al., 1993) which could reflect either the lack of RR or N-terminal activity or of both.

The HSV R1 subunits are synthesized in infected tissue culture cells with immediate-early kinetics, in contrast to R2 which is an early protein (Clements et al., 1977; Wymer et al., 1989; Conner et al., 1995). During infection the R1 promoter is uniquely regulated: expression is induced by the immediate-early transactivator Vmw65 (VP16) and, intriguingly, by Vmw110 (ICP0) (Desai et al., 1993), a viral transactivator required for efficient reactivation from latency (Clements & Stow, 1989; Leib et al., 1989; Russell et al., 1987). Interestingly, the R1 promoter contains functional response elements for cellular activator protein 1 (AP-1) (Wymer et al., 1992; Zhu & Aurelian, 1997), a bipartite complex of two cellular immediate-early proteins, c-Fos and c-Jun. HSV-1 R1 is twice as abundant as R2 in infected cells and the intracellular localization of the free R1 is distinct from that of the active enzyme (Conner et al., 1995). These data have been used to propose a role for R1 additional to its involvement in RR that requires the phosphorylation of viral/cellular proteins during immediate-early times by the putative N-terminal protein kinase (R1PK).

However, there are a number of discrepancies relating to the putative R1PK (see Peng et al., 1996) and these have culminated in a recent proposal by Langelier et al. (1998) that
the R1 subunit is not a PK but is a good substrate for host cell PKs, particularly casein kinase 2 (CK2). The results presented here support this observation and strengthen the conclusion that the N terminus of HSV R1 is not a protein kinase.

Analysis by SDS–PAGE and autoradiography of bacterially expressed HSV-1 R1 (1R1) immunoprecipitated by antiserum 106 from ammonium sulphate fractions as described in Cooper et al. (1995) in the presence of increasing amounts of purified Drosophila CK2 indicated that R1 was a substrate for this kinase (Fig. 1, lanes 1–6). CK2 was added before immunoprecipitation and, after extensive washing, phosphorylation was determined by addition of 30 µl of CK2 assay buffer (50 mM Tris–HCl buffer, pH 8.2, 20 mM MgCl₂) with 1 µl [32P]ATP to the Protein A–Sepharose pellet. After incubation at 25 °C for 30 min, the pellet was washed once with CK2 assay buffer and 32P incorporation was observed by SDS–PAGE and autoradiography. The levels of 1R1 phosphorylation increased with increasing amounts of CK2; Fig. 1, lane 1 shows an immunoprecipitated control bacterial extract and lanes 2–6 show phosphorylation of 1R1 after immunoprecipitation in the absence (lane 2) or presence of 0.5 µg CK2; lanes 9 and 10, 0.9 ng CK2; lanes 11 and 12, 1.8 ng CK2; lanes 13 and 14, 3.6 ng CK2; lanes 15 and 16, 7.2 ng CK2. CK2 phosphorylations of bacterially expressed dN245R1, dC449R1, in112R1 and in343R1 immunoprecipitated in the presence of 4 µg CK2 using antisera 106 are shown in lanes 17–20 respectively.

![Fig. 1. Autoradiographs which demonstrate CK2 transphosphorylation of protein 1R1. Bacterially expressed 1R1 was immunoprecipitated with antisera 106 in the absence (lane 2) or presence of 0.5 (lane 3), 1 (lane 4), 2 (lane 5) or 4 (lane 6) µg CK2. Lane 1 is an immunoprecipitated control bacterial extract that contains no 1R1-related proteins. Lanes 7–16 demonstrate the effects of increasing amounts of CK2 on phosphorylation of bacterially expressed and purified 1R1. Samples are in duplicate: lanes 7 and 8, 0 ng CK2; lanes 9 and 10, 0.9 ng CK2; lanes 11 and 12, 1.8 ng CK2; lanes 13 and 14, 3.6 ng CK2; lanes 15 and 16, 7.2 ng CK2. CK2 phosphorylations of bacterially expressed dN245R1, dC449R1, in112R1 and in343R1 immunoprecipitated in the presence of 4 µg CK2 using antisera 106 are shown in lanes 17–20 respectively.](image-url)
Table 1. Amounts of CK2 pulled down by 1R1 measured using the peptide phosphorylation assay

<table>
<thead>
<tr>
<th>Protein</th>
<th>Immunoprecipitating antibody*</th>
<th>CK2 added (µg) †</th>
<th>CK2 ‘pulled down’ (µg) ‡</th>
<th>% CK2 ‘pulled down’</th>
<th>Molar ratio R1:CK2§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1R1</td>
<td>106</td>
<td>8</td>
<td>0.037</td>
<td>0.46</td>
<td>13:1</td>
</tr>
<tr>
<td>1R1</td>
<td>7689</td>
<td>8</td>
<td>0.044</td>
<td>0.55</td>
<td>11:1</td>
</tr>
</tbody>
</table>

* 1 µg 1R1 is immunoprecipitated from bacterial extracts by these antibodies (Cooper et al., 1995).
† CK2 was added to the 1R1 ammonium sulphate fraction prior to immunoprecipitation.
‡ CK2 pulled down was calculated from the levels of peptide phosphorylation using a calibration curve.
§ Molecular masses for molar ratio determinations were as follows: 1R1, 140000 kDa; CK2, 65000 kDa.

Fig. 2. (a) Chart demonstrating levels of CK2 peptide phosphorylation activity present in 1R1, 2R1 and dN245R1 proteins immunoprecipitated by antiserum 106 from bacterial extracts or 1R1 (17) immunoprecipitated from infected-cell extracts and assayed in the presence (17) or absence (17 – pep) of peptide. Con is a control bacterial extract lacking any 1R1-related proteins. No exogenous CK2 was added to any of these samples. (b) Western blots of ammonium sulphate fractions from bacterial (lanes 2 and 3) or infected-cell (lanes 1 and 4) extracts probed with antisera specific for CK2 alpha (lanes 3 and 4) or beta (lanes 1 and 2). The positions of molecular mass markers are shown on the right-hand side.

Phosphorylation of HSV R1 by CK2

determined by peptide phosphorylation and the results are presented in Table 1. Less than 1% of the available CK2 was pulled down suggesting that there is no strong interaction between R1 and CK2. Molar ratios of R1:CK2 were calculated from estimates of the amounts of 1R1 and CK2 in immunoprecipitates and, although the resultant values must be treated with some caution, the expected 1:1 ratio for a stable R1–CK2 complex was not obtained; 8 µg CK2 is a 15-fold molar excess over immunoprecipitated R1. Despite the lack of interaction, the residual amounts of CK2 present after immunoprecipitation transphosphorylated 1R1 (see Fig. 1, lanes 3–6).

R1 proteins immunoprecipitated from infected cell and bacterial extracts were investigated for CK2 contamination using peptide phosphorylation assays (Fig. 2a). Levels of peptide phosphorylation were determined in duplicate samples of 1R1, HSV-2 R1 (2R1) and dN245R1 immunoprecipitated by antiserum 106 from bacterial extracts and in an immunoprecipitated control extract but no CK2 activity was evident; the levels of $^32$P detected were consistent with background for this assay. Background levels of phosphorylation were higher in 1R1 immunoprecipitated from infected cell extracts but this higher level of $^32$P incorporation was augmented in the presence of the CK2 peptide indicating that this sample contained contaminant CK2 activity; a similar level of phosphorylation was obtained with 1 ng CK2. The ammonium sulphate fractions from bacterial and infected-cell extracts used for 1R1 immunoprecipitation were analysed by Western blotting with antisera specific for either CK2 alpha or beta (Fig. 2b) and both alpha (lane 4) and beta (lane 1) subunits were detected in infected cell extracts. Surprisingly, a band cor-
substrates and may act to regulate the activity, stability and/or intracellular location of many cell growth control proteins. The beta subunit is involved in protein–protein interactions with substrates and has been shown to form a stable complex with two of these, p53 (Filhol et al., 1992) and DNA topoisomerase II (Bojanowski et al., 1993).

Initial experiments demonstrated that 1R1 was an excellent substrate for CK2 and the majority of 1R1 phosphorylation sites were mapped to the N-terminal domain between residues 1 and 245. CK2 can phosphorylate either serines or threonines at a variety of motifs with a broad consensus of S/TXXD/EX, although, in the majority of sites, there are three consecutive acidic residues following the phosphate acceptor (Pearson & Kemp, 1991). Located within the N termini of both 1R1 and 2R1 is a conserved block of residues (amino acids 190–237) consisting mostly of serines and aspartic acids and this probably provides CK2 phosphorylation sites. The highly conserved region could be functionally important and CK2 phosphorylation of serines may regulate an activity of the N-terminal domain in vivo.

Langelier et al. (1998) proposed that R1 and CK2 may interact but results presented here consistently provided no evidence for a stable interaction between R1 and CK2. Strikingly, they indicated that 1R1 was readily phosphorylated by trace amounts of CK2 contaminating the immunoprecipitates and suggest an alternative explanation for the mutagenesis studies by Cooper et al. (1995) in which four amino acid inserts at residues 22 and 112 abrogated R1PK activity whereas similar insertions at residues 257, 262, 292 and 343 had no effect. Immunoprecipitated in112R1, the polypeptide with a four amino acid insert at residue 112, was a poor substrate for CK2, possibly because the insert affected its structural integrity resulting in the inefficient display of phosphate acceptor sites. The same insertion at residue 343 caused no structural alterations that affected CK2 phosphorylation.

Peptide phosphorylation assays identified CK2 contamination of 1R1 immunoprecipitated from infected cell extracts but failed to detect any CK2 activity in R1 proteins immunoprecipitated from bacterial extracts. However, Western blotting indicated the presence of potential CK2 alpha and beta subunits in bacterial extracts suggesting the existence of a prokaryotic CK2 homologue, although further studies will be needed to confirm this. The peptide phosphorylation assay was optimized for the eukaryotic enzyme and the putative E. coli kinase may not be fully active under these conditions; its phosphorylation motifs and/or cation and pH requirements may differ. Such differences would explain the low levels of activity consistently detected with bacterially expressed R1 proteins (Conner et al., 1992; Cooper et al., 1995; Langelier et al., 1998), since R1 would not be efficiently phosphorylated by E. coli CK2.

The author is grateful to Dr Odile Filhol (CNRS, Grenoble, France) for the kind gifts of purified casein kinase 2 and casein kinase 2 antisera and...
to Dr Howard Marsden (Institute of Virology, Glasgow) for synthesis of the peptide substrate. This study was funded in part by project grant support from The Wellcome Trust.

References


Received 14 September 1998; Accepted 24 February 1999