

cdc2 Cyclin-Dependent Kinase Binds and Phosphorylates Herpes Simplex Virus 1 U_L42 DNA Synthesis Processivity Factor

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Earlier studies have shown that cdc2 kinase is activated during herpes simplex virus 1 infection and that its activity is enhanced late in infection even though the levels of cyclin A and B are decreased below levels of detection. Furthermore, activation of cdc2 requires the presence of infected cell protein no. 22 and the U_L13 protein kinase, the same gene products required for optimal expression of a subset of late genes exemplified by U_S11, U_L38, and U_L41. The possibility that the activation of cdc2 and expression of this subset may be connected emerged from the observation that dominant negative cdc2 specifically blocked the expression of U_S11 protein in cells infected and expressing dominant negative cdc2. Here we report that in the course of searching for a putative cognate partner for cdc2 that may have replaced cyclins A and B, we noted that the DNA polymerase processivity factor encoded by the U_L42 gene contains a degenerate cyclin box and has been reported to be structurally related to proliferating cell nuclear antigen, which also binds cdk2. Consistent with this finding, we report that (i) U_L42 is able to physically interact with cdc2 at both the amino-terminal and carboxyl-terminal domains, (ii) the carboxyl-terminal domain of U_L42 can be phosphorylated by cdc2, (iii) immunoprecipitates obtained with anti U_L42 antibody contained a roscovitine-sensitive kinase activity, (iv) kinase activity associated with U_L42 could be immunodepleted by antibody to cdc2, and (v) U_L42 transfected into cells associates with a nocodazole-enhanced kinase. We conclude that U_L42 can associate with cdc2 and that the kinase activity has the characteristic traits of cdc2 kinase.

In this report we show that the mitotic cyclin-dependent kinase interacts with the herpes simplex virus 1 (HSV-1) protein product of the U_L42 open reading frame. The viral protein functions as a DNA polymerase-associated processivity factor. We have identified this protein as a potential virally encoded partner for cdc2 on the basis of its cyclin-like characteristics, and in this report we show that it interacts physically with cdc2. Relevant to this report are the following.

(i) The roots of this investigation rest on the observation that infected-cell protein 0 (ICP0), a promiscuous transactivator encoded by the α 0 gene, binds to and stabilizes cyclin D3 (13, 31). In the ensuing investigation it became apparent that the stabilization of cyclin D3 was not associated with the transition from G₁ to S phase of the cell cycle. Specifically, cdk2 was inactive and members of the E2F family of proteins required for transcriptional activation of the S phase genes were either sequestered in the cytoplasm or rendered inactive (2, 8, 23, 32). It also became apparent that while HSV stabilized cyclin D3, at least two other herpesviruses, herpesvirus thymus virus and human herpesvirus 8, encoded cyclin D homologs (17, 21). The obvious conclusion was that herpesviruses require D cyclins although, at least in the case of HSV, for other purposes than those used by the host cell. Ultimately, cyclin D3 was shown to play a role in the translocation of ICP0 from the nucleus to the cytoplasm in HSV-1-infected cells (32).

(ii) The evolving studies on the interaction of cyclin D3 with viral proteins led us to investigate the mitotic cyclins and their kinase. In these studies we found that while cyclins A and B turned over by 8 h after infection, their partner, cdc2, was stabilized and actively phosphorylated histone H1 (1). Moreover, the stabilization of cdc2 required the expression of two viral genes, a regulatory protein, ICP22, encoded by the α 22 gene and a viral protein kinase encoded by the U_L13 open reading frame.

(iii) The requirement for ICP22 and U_L13 protein kinase for the stabilization of cdc2 was investigated in two series of experiments. First, HSV-1 genes form several groups whose expression is coordinately regulated and sequentially ordered (26). The expression of α genes, the first set to be expressed, does not require prior synthesis of viral proteins. The expression of β genes requires α gene products but does not require viral DNA synthesis. The γ ₁ genes are expressed in the absence of viral DNA synthesis, but their expression is significantly enhanced by the onset of synthesis of viral DNA. Lastly, the γ ₂ genes require viral DNA for their expression. Of the γ ₂ genes, a small subset exemplified by U_S11, U_L38, and U_L41, require the presence of ICP22 and U_L13 protein kinase, the same proteins required for the stabilization of cdc2 (22, 25, 28). To test the connection, cells were transfected with a dominant negative form of cdc2 (cdc-dn) and then infected with wild-type HSV-1. The results were that infected cells expressing cdc2-dn also expressed representative α , β , and γ ₁ proteins but not the U_S11 protein (3).

The second series of experiments centered on the pathway by which cdc2 becomes activated. cdc2, like other cyclin-de-

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pendent kinases, is present throughout the cell cycle, but its activity is tightly regulated (14). In the case of *cdc2*, the kinase activity is turned off by phosphorylation by *wee-1* and *myt-1* and activated by dephosphorylation by activated (phosphorylated) *cdc25C* and, at a subsequent stage, by phosphorylation by cyclin-dependent activating kinase. These studies indicated that in wild-type virus-infected cells *wee-1* is modified and *cdc25C* is hyperphosphorylated (1). The posttranslational modification of *cdc25C* phosphatase required the presence of ICP22 and U_L13 protein kinase.

These studies led to the conclusion that *cdc2* is specifically activated by virally induced modification of *cdc2* regulators.

(iv) In uninfected cells, activated *cdc2* forms a heterodimer with its cognate partner, cyclin A or cyclin B. Inasmuch as cyclins A and B are no longer detectable at the time after infection when *cdc2* is fully active, it could be expected that *cdc2* acquired a new cognate partner. The members of the cyclin family members have a characteristic cyclin box motif described as a 32-amino-acid pattern that is also shared by the cyclin homologs encoded by herpesvirus saimiri, human herpesvirus 8, and *Autographa californica* nucleopolyhedrovirus (6, 17, 21). The consensus phosphorylation site of activated *cdc2* is S/T-P-X-K/R/H (11, 18). Earlier we have shown that the sequence encoded by exon 2 of ICP0 and ICP4 containing the consensus site are indeed phosphorylated by *cdc2*, and other herpesvirus proteins carrying this consensus sequence have been shown to serve as substrates of *cdc2* (3, 4). However, these substrates do not appear to have the properties of a putative cognate partner inasmuch as they do not have cyclin box motif. In an attempt to determine whether any HSV protein matched the predicted structure of a cyclin, the HSV-1 open reading frames were scanned for the presence of this motif. The results were that although no HSV-1 protein contained a perfect cyclin box consensus pattern, a degenerate version of the consensus pattern was identified in the U_L42 open reading frame. Starting at amino acid 51, the sequence of U_L42 is RTSLLDLLVMGDRGILIHNTIFGEQVF-LPLEH, which resembles a cyclin box motif. The hypothesis that U_L42 protein could serve as a partner for *cdc2* emerged from the report that the crystal structure of U_L42 (34) contains elements similar to those of proliferating-cell nuclear antigen (PCNA). PCNA has been previously reported to interact with *cdk2* (16). The objectives of this study were to test this hypothesis. We report that U_L42 is able to physically associate with *cdc2*. The carboxy half of U_L42 can be phosphorylated by *cdc2* kinase. Immunoprecipitation with U_L42 antibody pulls down a kinase activity with properties similar to those of *cdc2* in that it is inhibited by roscovitine, immunodepleted by *cdc2* antibody, and enhanced by nocodazole.

MATERIALS AND METHODS

Cells and viruses. HEP-2 cells were initially obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NBCS). HSV-1(F) is the prototype HSV-1 wild-type strain used in this laboratory (9).

Plasmids. The U_L42 gene was cloned into PGEX4T-1 and pCDNA3.1(+) and *cdc2*-dn was cloned into PGEX4T-1 by PCR. The oligonucleotides listed below containing terminal *EcoRI* or *XhoI* restriction sites were used in PCR to generate full-length U_L42 or N- and C-terminal portions of U_L42 from the *BamHI* I fragment of HSV-1(F) within pRB130. Full-length *cdc2*-dn was generated from a plasmid kindly provided by Sander van den Heuvel (30). The oligonucleotides

used for this purpose were oligonucleotide 1 (5' CC GAA TTC ATG ACG GAT TCC CCT GGC GG), oligonucleotide 2 (5' CCG CTC GAG G TCA GGG GAA TCC AAA ACC), oligonucleotide 3 (5' CC GAA TTC ATG GTG TCG TCC AGC ACC AGC), oligonucleotide 4 (5' CCG CTC GAG G TCA CTT GGT GAG CGC GTT G), oligonucleotide 5 (5' CC GAA TTC ATG GAA GAT TAT ACC AAA ATA G), and oligonucleotide 6 (5' CCG CTC GAG G CTA CAT CTT CTT AAT CTG ATT G). Full-length U_L42 (amino acids 1 to 488) was generated with oligonucleotides 1 and 2, N-terminal U_L42 (amino acids 1 to 244) was generated with oligonucleotides 1 and 4, and C-terminal U_L42 (amino acids 226 to 488) was generated with oligonucleotides 2 and 3. The PCR products were ligated into *EcoRI-XhoI*-digested pGEX4T-1 or pCDNA3.1(+). Plasmids with U_L42 inserts were sequence verified at the ligation juncture. Full-length *cdc2*-dn was generated by PCR with oligonucleotides 5 and 6. The PCR product and PGEX4T-1 were digested with *EcoRI-XhoI* and ligated. The sequence of plasmid PGEX4T-1/*cdc2*-dn was verified.

Production and purification of GST fusion proteins. Glutathione *S*-transferase (GST) fusion proteins containing GST alone or GST fused to full-length U_L42, N-terminal U_L42, C-terminal U_L42, or full-length *cdc2*-dn were produced as previously described (3). Briefly, *Escherichia coli* BL21 cells were transformed with the above-mentioned GST-encoding plasmids (PGEX4T-1 based). Fusion protein production was induced with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were lysed, GST fusion proteins were absorbed to glutathione-agarose beads (Sigma), and fusion proteins were eluted with 10 mM glutathione in 50 mM Tris (pH 8.0). The eluted protein solution was dialyzed against phosphate-buffered saline. Protein production was assessed by polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining, and protein concentrations were measured by the Bradford assay (Bio-Rad).

In vitro transcription-translation. In vitro-coupled transcription-translation of U_L42 was carried out with the TNT T7 Quick Coupled Transcription/Translation System as described by the manufacturer (Promega). Briefly, 40 μl of reticulocyte lysate master mix was combined with 25 μCi of [³⁵S]methionine and 1 μg of plasmid DNA [pCDNA3.1(+)] with full-length, amino-terminal, or carboxyl-terminal U_L42. The mixture was reacted at 30°C for 90 min. An aliquot was taken out, and the remainder of the reaction mixture was subjected to GST pull down as follows: 300 μl of IP buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% NP-40, 200 mM NaCl, 0.1 mM sodium orthovanadate, 10 mM NaF, 2 mM dithiothreitol [DTT], 100 μg each of phenylmethylsulfonyl fluoride and tolylsulfonyl phenylalanyl chloromethyl ketone per ml, 2 μg each of aprotinin and leupeptin per ml) was added to the reaction mixture, and samples were processed as described below for the GST pull down assay. Blots were analyzed by autoradiography and PhosphorImager (Storm 860; Molecular Dynamics) analysis.

GST pull down assay. HEP-2 cells were lysed in high-salt lysis buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% NP-40, 400 mM NaCl, 0.1 mM sodium orthovanadate, 10 mM NaF, 2 mM dithiothreitol, 100 μg each of phenylmethylsulfonyl fluoride and tolylsulfonyl phenylalanyl chloromethyl ketone per ml, 2 μg each of aprotinin and leupeptin per ml) on ice for 1 h. Insoluble material was pelleted by centrifugation. The supernatant was brought up in an equal volume of high-salt lysis buffer without NaCl (final concentration, 200 mM NaCl). Samples were precleared with 50 μl of a 50% slurry of glutathione beads for 2 h at 4°C. The precleared supernatant was incubated for 3 h at 4°C with either 2, 5, or 10 μl of a 50% slurry of glutathione beads bound to GST alone or GST fused to the above-mentioned U_L42 protein constructs. The beads were pelleted by centrifugation and washed three times with IP buffer. Then 40 μl of SDS gel-loading buffer (2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 50 mM Tris [pH 6.8], 2.75% sucrose) was added to the beads. The samples were heated to 95°C for 5 min, resolved by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with antibody to *cdc2* (SC-54; Santa Cruz Biotechnology). The blots were developed by peroxidase-conjugated secondary antibody and reacted with chemoluminescent substrate (Super Signal; Pierce).

Immunoprecipitation. HEP-2 cells were infected with 10 PFU of HSV-1(F) per cell. The cells were harvested 12 h after infection and lysed in high-salt lysis buffer. Samples were kept on ice for 1 h, and insoluble material was pelleted by centrifugation. The supernatant was brought up in an equal volume of lysis buffer without NaCl (final concentration, 200 mM NaCl). Lysates were precleared with preimmune serum for 2 h, and 50 μl of a 50% slurry of protein A-conjugated agarose was added. Samples were centrifuged, and the supernatant was transferred to new tubes. Antibody to U_L42 was added to the samples, and immunocomplexes were collected by the addition of 40 μl of 50% protein-A slurry (29).

Immunoprecipitation-linked in vitro kinase assay. Mock- or HSV-1-infected HEP-2 cells were harvested and immunoprecipitated with antibodies to *cdc2* or U_L42 as above. Immunocomplexes were collected with 20 μl of a 50% protein A slurry. The samples were washed twice with IP buffer, twice with low-salt buffer

(20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% NP-40, 1 mM NaCl, 2 mM dithiothreitol), and twice with incomplete kinase buffer (50 mM Tris [pH 7.4], 10 mM MgCl₂, 5 mM dithiothreitol). The beads were then resuspended in 20 μ l of incomplete kinase buffer with 0, 2, 10, or 40 μ M roscovitine (Calbiochem); all samples contained 0.4% dimethyl sulfoxide. The samples were incubated for 5 min at 30°C, and 20 μ l of complete kinase buffer (50 mM Tris [pH 7.4], 10 mM MgCl₂, 5 mM dithiothreitol, 10 μ M ATP, 20 μ Ci of [γ -³²P]ATP, 2 μ g of histone H1) was added, with final concentrations of roscovitine of 0, 1, 5, or 20 μ M. Samples were incubated for an additional 20 min at 30°C. Reactions were terminated by addition of SDS gel-loading buffer and heated to 95°C for 5 min. The samples were resolved by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by autoradiography. Quantification of ³²P phosphorylation of the substrates was done with the aid of a PhosphorImager (Storm 860).

Purified cdc2 Kinase Assay. GST fusion proteins were reacted with purified recombinant cdc2 kinase (New England Biolabs). A 25 U portion of cdc2 kinase was combined with either 2 μ l of a 50% slurry of glutathione beads attached to GST or GST-U_L42 fusion proteins in 30 μ l of reaction buffer supplemented with 100 μ M ATP and 20 μ Ci of [γ -³²P]ATP. The samples were reacted at 30°C for 30 min, and the reactions were terminated by the addition of SDS gel-loading buffer and heated to 95°C for 5 min. The samples were resolved by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and analyzed by autoradiography. Quantification of ³²P phosphorylation of the substrates was done with the aid of a PhosphorImager (Storm 860).

Immunodepletion kinase assay. HSV-1(F)-infected HEP-2 cell lysates were harvested, lysed, and precleared as above. Samples were then immunodepleted of cdc2 or U_L42 by incubation with the respective antibody for 2 h at 4°C, 50 μ l of a 50% protein A slurry was added for 1 h at 4°C, and the samples were pelleted by centrifugation. The supernatant was then transferred to a new tube, antibody to cdc2 or U_L42 was added for 4 h at 4°C, and immunocomplexes were collected by the addition of 20 ml of a 50% protein A slurry for 1 h at 4°C. Samples were washed as above (i.e., twice in IP buffer, twice in low-salt buffer, and twice in incomplete kinase buffer) and incubated in 40 μ l of complete kinase buffer for 20 min at 30°C. The samples were then processed as above by bis-polyacrylamide gel electrophoresis.

Transient expression following plasmid transfection. pCDNA3.1(+) encoding full-length U_L42 was transfected into cells using Lipofectamine Plus (Gibco BRL). HEP-2 cells were seeded in T-25-cm² flasks the day prior to transfection in DMEM containing 10% NBCS. The following day, 2 μ g of plasmid was mixed with 10 μ l of Plus reagent in 375 μ l of DMEM (no serum and no antibiotics) for 15 min. Then 375 μ l of DMEM containing 15 μ l of Lipofectamine was added to the plasmid DNA. The mixture was incubated for an additional 15 min and added to HEP-2 cells in 2 ml of DMEM without serum or antibiotics. At 4 h later, 2.8 ml of DMEM (20% NBCS and 2 \times antibiotics) was added to the cells. The cells were harvested as above in high-salt lysis buffer 36 h after transfection. For nocodazole-treated samples, 24 h after transfection the cell culture medium was replaced with medium containing 5 μ g of nocodazole (Sigma) per ml. Nocodazole-treated flasks were harvested 12 h after the addition of nocodazole. Kinase assays were performed as above.

RESULTS

HSV-1 U_L42 and cdc2 physically interact. Two studies were done to determine if U_L42 and cdc2 associate with each other. In the first study, pcDNA3.1(+) containing full-length, N-terminal, or C-terminal U_L42 was in vitro transcribed and translated in the presence of [³⁵S]methionine. The products of the in vitro transcription-translation of the U_L42-encoding plasmids are shown in Fig. 1 (lanes 1 to 6). GST or GST-cdc2-dn fusion protein were generated in *E. coli* and captured on glutathione-agarose beads. GST or GST-cdc2-dn was then mixed and allowed to react with the radiolabeled in vitro-translated U_L42 to determine if GST-cdc2-dn could specifically pull down U_L42 (Fig. 1). GST-cdc2-dn pulled down full-length U_L42 whereas GST did not (lanes 7 and 8). Next, we determined whether either the amino terminus or carboxyl terminus of U_L42 interacted with cdc2. Interestingly, polypeptides containing both termini of the full-length U_L42 protein were also

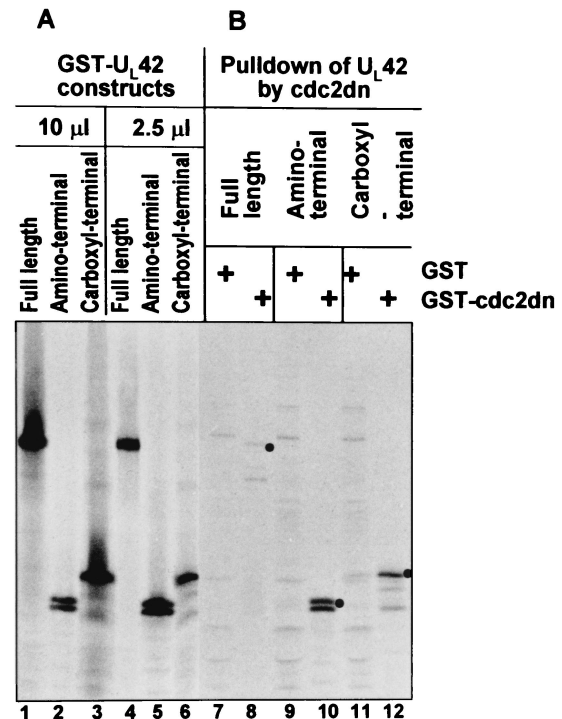


FIG. 1. Autoradiographic image of in vitro-transcribed/translated U_L42 followed by GST-cdc2dn pull down. Full-length (FL) or amino (N') or carboxyl (C') halves of U_L42 protein were in vitro transcribed and translated (lanes 1 to 6). The in vitro-translated U_L42 proteins were then reacted with GST or GST-cdc2-dn for pull down assays (lanes 7 to 12), electrophoretically separated, and developed by autoradiography.

specifically pulled down by GST-cdc2-dn but not by GST (lanes 9 to 12).

The above study was done with a dominant negative mutant of cdc2. The dominant negative mutant has a single-amino-acid substitution of Asp146Asn, such that it cannot coordinate ATP to the catalytic domain of cdc2 (30). To determine if U_L42 could interact with wild-type cdc2, the following study was done. The above U_L42 constructs (FL, N', or C') were produced as GST fusion proteins in *E. coli* and collected on glutathione-agarose beads. HEP-2 whole-cell lysates were then incubated with 2, 5, or 10 μ l of glutathione-agarose beads bound to GST or GST fused to full-length, amino-terminal, or carboxyl-terminal U_L42. Following extensive washing, the beads were solubilized and the proteins bound to the beads were subjected to electrophoresis in denaturing gels, transferred to a nitrocellulose sheet and reacted with antibody against cdc2. As shown in Fig. 2 GST-U_L42 pulled down cdc2 from whole-cell lysates in a concentration-dependent manner (Figure 2, lanes 3 to 8). GST fused to the amino or carboxyl terminus of U_L42 also pulled down cdc2 from cell lysates (lanes 9 to 14), whereas GST did not. cdc2 from whole-cell lysates is shown in lanes 1 and 2.

We conclude from these studies that cdc2 and U_L42 can physically associate with each other and that the sites of interaction reside at both the amino and carboxyl termini of U_L42.

The carboxyl terminus of U_L42 is phosphorylated by cdc2 kinase. Since both the amino and carboxy termini of U_L42

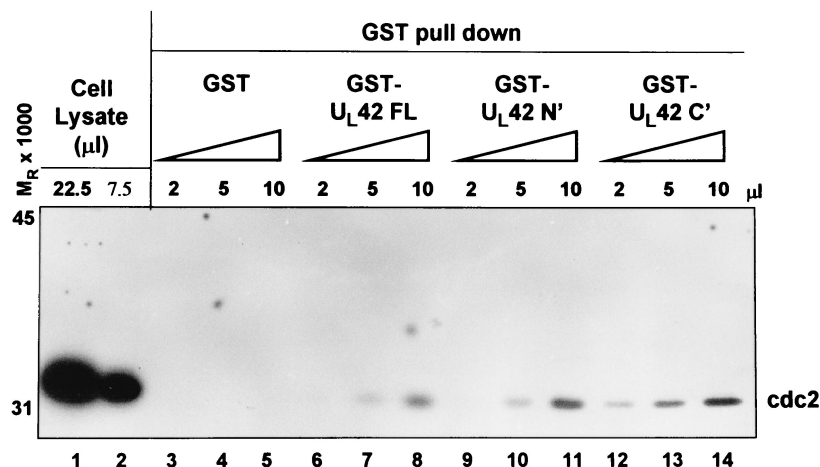


FIG. 2. *cdc2* immunoblot of HEP-2 cell lysates reacted with GST or GST fusion proteins expressing full-length (FL) or amino (N') or carboxyl (C') halves of U_L42 protein. Immunoreactivity of *cdc2* from whole-cell lysates is shown in lanes 1 and 2. Whole-cell lysates were then incubated with 2, 5, or 10 μ l of GST (lanes 3 to 5), GST-UL42 FL (lanes 6 to 8), GST-UL42 N' (lanes 9 to 11), or GST-UL42 C' (lanes 12 to 14), and GST beads were collected, electrophoretically separated, and immunoblotted for *cdc2*.

could interact with *cdc2*, we next determined if U_L42 could be phosphorylated by *cdc2* kinase. *cdc2* is a proline-directed serine/threonine kinase, and its substrate is located 3 amino acids downstream from a basic amino acid (11, 18). The minimally defined consensus phosphorylation site for *cdc2* kinase is S/T-P-x-K/R/H. U_L42 does not have a serine or threonine that fits the consensus phosphorylation site for *cdc2* kinase. However, U_L42 does have a putative *cdc2* phosphorylation site that resembles that found on cyclins. In *in vitro* kinase assays, *cdc2* can phosphorylate the cyclin associated with it (15). This site is loosely S/T-P-x-P. Amino acids 294 to 297 of U_L42 are T-P-V-P.

GST fusion proteins of full-length (1 to 488), amino-terminal (1 to 244), or carboxyl-terminal (226 to 488) U_L42 were generated and used as substrate for the purified *cdc2* kinase as described in Materials and Methods. As shown in Fig. 3, GST fused to full-length U_L42 or to the carboxyl-terminal domain of U_L42 was phosphorylated by *cdc2* whereas the amino-terminal domain of U_L42 was not phosphorylated. These results are consistent with the prediction of a cyclin-like *cdc2* phosphorylation site in the carboxyl-terminal domain of U_L42.

Anti-U_L42 antibody precipitates a roscovitine-sensitive kinase activity from HSV-1-infected cells. The purpose of the series of experiments in this section was to determine whether anti-U_L42 antibody precipitated a kinase from infected cells and whether this kinase had the characteristics of *cdc2*.

Roscovitine has been reported to be a highly selective inhibitor of *cdc2*, *cdk2*, and *cdk5* cyclin-dependent kinases (19). Mock- or HSV-1-infected cell lysates were reacted with antibodies to either *cdc2* or U_L42. The immunoprecipitates were mock treated or reacted with roscovitine and then assayed for their ability to phosphorylate histone H1. To determine the sensitivity of *cdc2* kinase to roscovitine, the *cdc2* immunoprecipitated from uninfected HEP-2 lysates served as a positive control. The results shown in Fig. 4 were as follows. Both the anti-*cdc2* and the anti-U_L42 antibodies precipitated a kinase activity that phosphorylated histone H1. The precipitated kinase activities were sensitive to roscovitine (50% inhibitory

concentration between 1 and 5 μ M). The sensitivity of the kinase activities precipitated from infected cells was similar to that of the kinase activity precipitated by the *cdc2* antibody from mock-infected cells. Moreover, as we have previously shown, the *cdc2* immunoprecipitated with the anti-*cdc2* antibody from HSV-1-infected cell lysates showed elevated kinase activity compared to that from uninfected cell lysates (1). The

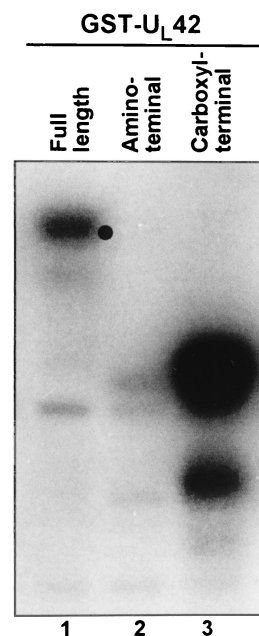


FIG. 3. Autoradiographic image of U_L42 phosphorylation by *cdc2* kinase. Full-length (lane 1), N-terminal (lane 2), C-terminal (lane 3) U_L42 GST fusion proteins were incubated with purified *cdc2* kinase in the presence of [γ -³²P]ATP, separated by gel electrophoresis, and developed by autoradiography. Dots indicate the molecular weight of the respective GST fusion proteins.

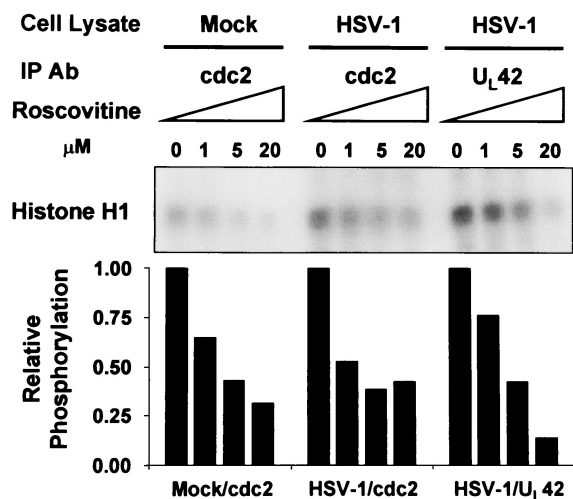


FIG. 4. Autoradiographic image of roscovitine-sensitive histone H1 phosphorylation by cdc2 or U_L42 immunocomplexes. Mock- or HSV-1-infected HEp-2 cell lysates were subjected to immunoprecipitation (IP) by antibodies (Ab) to cdc2 or U_L42. Immunocomplexes were preincubated with 0, 1, 5, or 20 μM roscovitine and subjected to *in vitro* kinase assays using histone H1 as the substrate. Phosphorylated histone 1 from the reactions was resolved by polyacrylamide gel electrophoresis, and the blots were developed by autoradiography. Histone phosphorylation was quantified by PhosphorImager analysis. Samples not treated roscovitine were assigned a value of 1.

U_L42 antibody did not immunoprecipitate appreciable histone H1 kinase activity from uninfected cells (data not shown).

The kinase activity coprecipitated by the anti-U_L42 antibody can be immunodepleted by the anti-cdc2 antibody. To determine if cdc2 was immunoprecipitated with U_L42 antibody, the following experiment was done. Lysates from HSV-1-infected HEp-2 cell lysates were reacted with preimmune serum, cdc2 antibody, or U_L42 antibody. The immune complexes were then removed, and the immunodepleted lysates were reacted with antibody to U_L42. The immune complexes were then harvested and assessed for their ability to phosphorylate histone H1 kinase. The results indicate that the anti-U_L42 antibody depleted 63% of the U_L42 associated histone H1 kinase activity compared to lysates immunodepleted with preimmune sera (Fig. 5, lanes 1 and 3) and that lysates immunodepleted with antibody to cdc2 resulted in a 48% reduction of U_L42-associated histone H1 kinase activity compared to that of control lysates immunodepleted with preimmune sera (lanes 1 and 2).

The results therefore indicate that the histone H1 kinase activity associated with U_L42 antibody could be attenuated by immunodepleting the infected-cell lysate with cdc2 antibody.

U_L42 made in cells transfected with a plasmid encoding the protein is associated with histone H1 kinase activity. The purpose of the series of experiments in this section was to determine whether the association of the U_L42 protein with kinase activity for histone H1 required the presence of other viral proteins. HEp-2 cells were transiently transfected with pcDNA3.1 encoding U_L42 and were mock treated or incubated in medium containing nocodazole. U_L42 protein production from transfection was verified by immunoblotting cell lysates with antibody to U_L42 (data not shown). The cells were

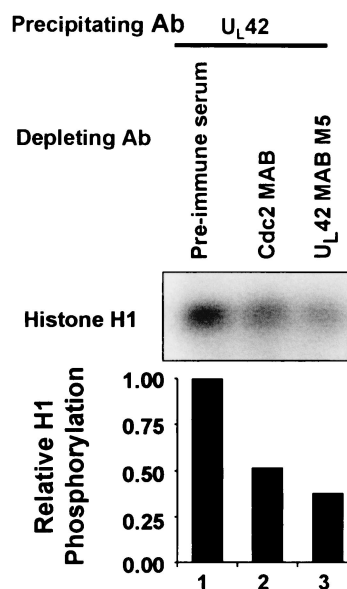


FIG. 5. Autoradiographic image of histone H1 kinase activity associated with U_L42 immunocomplexes following immunodepletion. HSV-1-infected HEp2 cell lysates were immunodepleted with preimmune serum (lane 1), antibody (Ab) to cdc2 (lane 2), or antibody to U_L42 (lane 3). The immunodepleted lysates were then immunoprecipitated with antibody to U_L42. Immunocomplexes were assayed for histone H1 kinase activity. Phosphorylated histone H1 was resolved by polyacrylamide gel electrophoresis, and the blots were developed by autoradiography. Histone phosphorylation was quantified by PhosphorImager analysis. The sample immunodepleted with preimmune sera was given a value of 1.

harvested 36 h after transfection, and the lysates prepared as described in Materials and Methods were reacted with anti U_L42 antibody. The precipitates were tested for histone H1 phosphorylation. The results were as follows. (i) The baseline histone H1 phosphorylation obtained by immune complexes obtained from mock-transfected cells was not enhanced by nocodazole treatment of cells and most probably represented nonspecific binding of cellular kinases during immunoprecipitation (Fig. 6, lanes 1 and 3). (ii) The U_L42 immunoprecipitate from transfected cells exhibited a twofold increase in relative histone H1 phosphorylation compared to that from nontransfected cells (lanes 1 and 2). (iii) Nocodazole treatment of U_L42-transfected cells resulted in a further appreciable enhancement of histone H1 kinase activity (lanes 2 and 4). These results indicate that the association of U_L42 with a nocodazole-enhanced cellular kinase activity for histone H1 does not require the presence of other viral gene products.

DISCUSSION

The studies conducted in the past several years have shown that HSV gene products scavenge the cell for cellular regulatory proteins which may be stabilized and translocated to specific compartments. In some instances, the purpose of the diversion seems clear. For example, ICP34.5 binds and redirects cellular protein phosphatase 1 to dephosphorylate the α subunit of elongation initiator factor 2 that has been phosphorylated by the activated protein kinase R (7, 10). In the case of the association of cyclin D3 and ICP0, the accumulated evi-

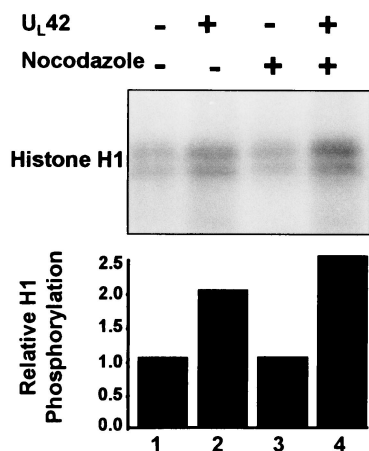


FIG. 6. Autoradiographic image of histone H1 kinase activity from U_L42 transfected cells. HEP-2 cells were transfected with pCDNA3.1 encoding U_L42 (lanes 2 and 4). Cells were also treated with nocodazole following transfection (lanes 3 and 4). Cell lysates were then immunoprecipitated with antibody to U_L42 and subjected to histone H1 kinase assays. Phosphorylated histone H1 was electrophoretically separated in a denaturing polyacrylamide gel and subjected to autoradiography. Histone phosphorylation was quantified by PhosphorImager analysis. Untransfected lysate without nocodazole treatment was assigned a value of 1.

dence suggests that the ultimate effect of the new function of cyclin D3 is to expedite the translocation of ICP0 to the cytoplasm (32). In other instances, either the purpose of the diversion or the mechanism by which the diverted cell protein performs its function remain unclear. The objectives of the studies reported here were to solve a mystery related to the stabilization and activation of cdc2 late in infection.

Specifically, earlier studies indicated that HSV-1 was specifically activating cdc2 kinase by specific alterations in protein levels, activity, and posttranslational modifications of both cdc2 and regulators of cdc2 activation (1). Thus, between 8 and 12 h after infection, cdc2 kinase activity was significantly enhanced, the levels of cyclins A and B were dramatically reduced, and cdc25C and wee-1 were posttranslationally modified. HSV-1-induced hyperphosphorylation of cdc25C is especially striking, since it appears to enhance the phosphatase activity that is required to relieve inhibitory phosphorylations within the kinase domain of cdc2. Both the modifications of cdc25C phosphatase and the activation of cdc2 kinase activities require the presence of ICP22 and of the U_L13 protein kinase. The problem we wished to address was that cdc2 plays a role in the expression of a subset of late (γ_2) proteins exemplified by U_S11 but that if cdc2 behaved like all other cyclin-dependent kinases, it should have acquired a surrogate cyclin to replace the missing cyclins A and B.

The studies described in this report attempt to reconcile this apparent paradox. Analyses of the open reading frame of HSV-1 genome led to the identification of the U_L42 protein as a viral gene product with properties similar to those of bona fide cyclins. In this report we show that cdc2 interacts physically and is phosphorylated by cdc2 in vitro and that a kinase activity characteristic of cdc2 can be precipitated from infected cells or cells transfected with U_L42 protein by the anti-U_L42 antibody. Curiously, the association of cdc2 with a factor in-

involved in DNA synthesis has a precedent. Thus, U_L42 protein is structurally related to PCNA and the association of PCNA with cdk2 has been reported (16, 34).

Irrespective of whether U_L42 is the surrogate cyclin partner of cdc2, the interaction of cdc2 with the U_L42 DNA synthesis processivity factor may be significant and related to the expression of the subset of γ_2 proteins exemplified by U_S11. As noted above, cdc2 kinase is activated between 8 and 12 h after infection, and functional cdc2 kinase activity was necessary for the accumulation of U_S11 protein, a member of a unique subset of γ_2 proteins that also requires ICP22 and the U_L13 protein kinase for maximum accumulation (1, 3, 22, 25, 28). Since γ_2 gene expression is intimately linked to viral DNA replication, it could be predicted that a viral protein involved in DNA synthesis facilitated the function of cdc2. U_L42, expressed with β kinetics, appears to satisfy this requirement. The kinetics of expression of U_L42 coincides with the activation cdc2 kinase in infected cells, and the known function of U_L42 is to act as a processivity factor for viral DNA polymerase links it to γ_2 gene expression.

The present studies begin to further clarify a pathway for the production of HSV-1 late proteins. A summary model of the results obtained to date is presented in Fig. 7. ICP22 and U_L13 are involved in "priming" cdc2 to become active, most probably by modifying the proteins that regulate the function of cdc2. The data presented here indicate that U_L42 can associate with cdc2. U_L42 appears to have at least two binding sites for cdc2. While both the amino and carboxy halves of U_L42 bind cdc2, only the carboxy half is efficiently phosphorylated by cdc2 kinase. This would imply that a portion of the carboxyl-terminal half of U_L42 protein binds in proximity to the kinase domain of cdc2. Immunoprecipitation with U_L42 antibody also brings down a cdc2-like kinase activity. Taken together, these data indicate that the association of U_L42 with cdc2 occurs in the context of a functional cdc2 kinase activity.

A common theme emerging from the studies on HSV-1 proteins is their multifunctionality. With coding capacity at a premium in viruses, one method to achieve greater versatility is for viral proteins to play multiple roles. The studies presented here begin to assess a novel function for the U_L42 viral protein. Studies to date on U_L42 have focused on its ability to associate with viral DNA polymerase and act as a processivity factor for DNA replication (26). U_L42 is an unorthodox processivity factor since it also binds to DNA. Recently, the viral origin binding protein U_L9 was reported to interact with U_L42 (20). Interestingly, U_L9 appears to be phosphorylated by a cellular kinase (12). Also, other viral proteins associated with DNA replication machinery contain consensus phosphorylation sites for cdc2 kinase, including DNA polymerase (U_L30), helicase/primase (U_L52), and single-stranded DNA binding protein (ICP8/U_L29) (3). Recent studies also indicated that ICP0 continued to undergo phosphorylation between 6 and 10 h after infection and that this phosphorylation was blocked by the cdc2 inhibitor roscovitine (4). Thus, one hypothesis that could be envisioned is that U_L42 acts as an adapter or bridge protein to bring in substrates that are subsequently phosphorylated by cdc2 kinase. This scenario is akin to one recently reported for PCNA (16). The interaction of PCNA with cdk2/cyclin A results in the phosphorylation of PCNA-interacting proteins such as replication factor C and DNA ligase I. In this example, cdk2

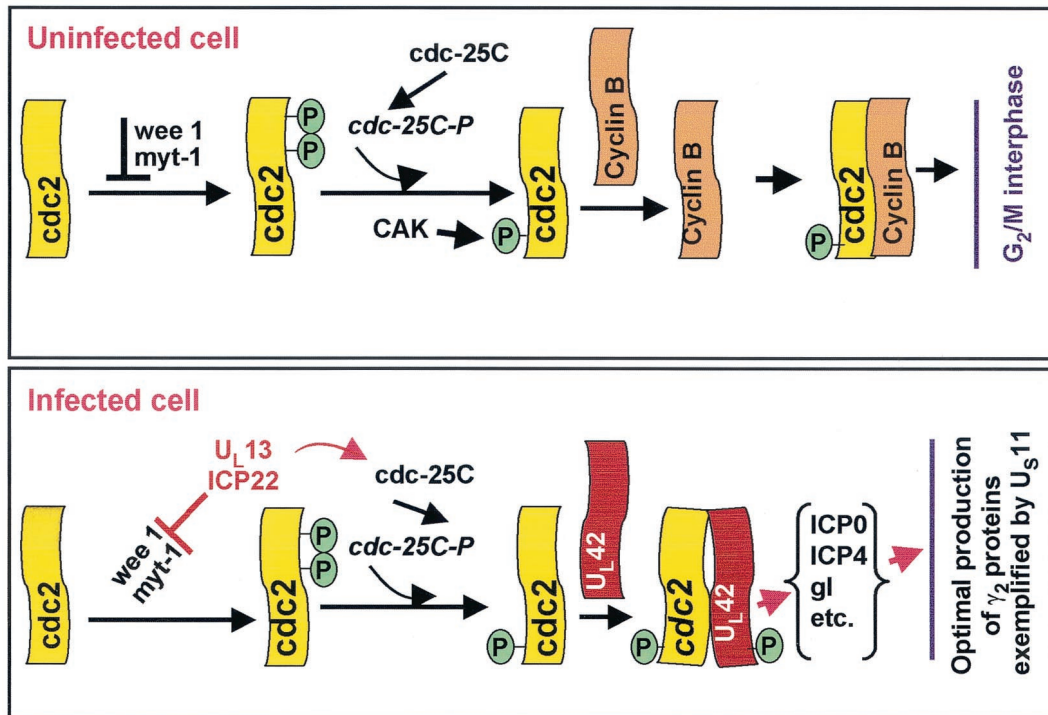


FIG. 7. Schematic diagram of *cdc2* regulation and activity in the uninfected (top) and HSV-1-infected (bottom) cell. In uninfected cells, *cdc2* is inactivated by *wee-1* and *myt-1* kinases and activated by *cdc-25C* phosphatase, cyclin-dependent activating kinase (CAK), and association with cyclin B at the G_2/M interphase. HSV-1 infection targets *wee-1* and *cdc-25C* through the actions of ICP22 and U_L13 , and U_L42 associates with *cdc2*. The substrates for *cdc2* in infected cells include ICP0, ICP4, and *gl*. The activation of *cdc2* in the infected cell is associated with optimal accumulation of a subset of γ_2 proteins exemplified by U_S11 protein.

is also associated with cyclin A, and PCNA serves as a substrate specificity protein.

The intermingling of cell cycle proteins with the virus life cycle appears to be critical for viral replication. Certain members of the herpesvirus family encode functional homologs of cyclins to induce cdk activity. While cyclins tend to have short half-lives and are expressed only during certain phases of the cell cycle, the cyclin-dependent kinase subunits are relatively stable. By encoding the cyclin component, viruses overcome a rate-limiting step in cdk activation. A key question that still remains unresolved is what are the critical substrates for cyclin-dependent kinases activated in the infected cell. Viral proteins from many herpesvirus family members have been reported to be phosphorylated by these kinases (3, 4, 24, 33). HSV-1 infection of roscovitine-treated cells has been reported to grossly interfere with viral replication and gene expression (27). Also, a consensus *cdc2* phosphorylation site in a core protein of hepadnavirus has been reported to be involved in capsid assembly and disassembly (5). The identification of key substrates for cyclin-dependent kinases within cells has been a difficult road. Hopefully, the elucidation of these substrates as well as functional consequences in the virally infected cell may also point to important paradigms within the cell.

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