Role of Cellular Phosphatase cdc25C in Herpes Simplex Virus 1 Replication[▽]

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Earlier studies have shown that in herpes simplex virus 1-infected cells, ICP22 upregulates the accumulation of a subset of γ_2 proteins exemplified by the products of the U_L38, U_L41, and U_S11 genes. The ICP22-dependent process involves degradation of cyclins A and B1, the stabilization and activation of cdc2, physical interaction of activated cdc2 with the U_L42 DNA synthesis processivity factor, and recruitment and phosphorylation of topoisomerase II α by the cdc2/U_L42 complex. Activation of cdc2, the first step in the process, is a key function of the mitotic phosphatase cdc25C. To define the role of cdc25C, we probed some features of the ICP22-dependent pathway of upregulation of γ_2 genes in cdc25C^{-/-} cells and in cdc25C^{+/+} cells derived from sibling mice. We report that cyclin B1 turned over in cdc25C^{+/+} or cdc25C^{-/-} cells at the same rate, that cdc2 increased in amount, and that U_S11 and U_L38 proteins and infectious virus accumulated in smaller amounts than in wild-type infected cells. The reduction in U_L38 protein accumulation and virus was greater in cdc25C^{-/-} cells infected with virus lacking ICP22 than in cells infected with wild-type virus. We conclude that cdc25C phosphatase plays a role in viral replication and that this role extends beyond its function of activating cdc2 for initiation of the ICP22-dependent cascade for upregulation of γ_2 gene expression.

The studies reported here stemmed from the discovery that herpes simplex virus 1 (HSV-1) activates and diverts the mitotic kinase cdc2 (also known as cdk1) to enhance the expression of a subset of late genes exemplified by U_L38, U_L41, and U_S11. Specifically, activation of cdc2 during viral replication requires the regulatory protein ICP22, a product of the α 22 gene, and the viral protein kinase encoded by the U_L13 gene. In the process, the natural partners of cdc2, cyclins A and B1, are degraded. Activated cdc2 physically interacts with U_L42, an HSV protein known primarily as a DNA synthesis processivity factor (12). U_L42-bound cdc2 kinase is redirected to phosphorylate U_L42 and to recruit and phosphorylate topoisomerase II α for post-DNA synthesis expression of the subset of γ_2 late genes listed above (1, 3–5).

In uninfected interphase cells, cdc2 is inactive, in part as a consequence of phosphorylation of threonine 14 and tyrosine 15 (6). Activation of cdc2 requires phosphorylation of threonine 161 and removal of phosphates from threonine 14 and tyrosine 15 by cdc25C. In this process, the role of cdc25C is critical. cdc25C has been characterized as the “mitotic trigger” for its ability to rapidly activate cdc2 (13); consequently, the activation of cdc25C is tightly regulated by the cell. The growth suppressor p53 inhibits transcription of cdc25C mRNA and directly binds to the cdc25C protein to prevent entry into mitosis (20).

In infected cells, activation of cdc2 during infection takes place concurrently with the down-regulation of the cdc2 inhibitor, wee1 kinase, and increased activity of the cdc2 activator, cdc25C phosphatase, as measured by a generic phosphatase

assay (1; S. Advani and B. Roizman, unpublished observations).

The sequence of events described above raises the question as to the role of cdc25C phosphatase in the course of the HSV-1 replicative cycle. To address this question, we took advantage of the availability of murine cdc25C^{-/-} cells and cdc25C^{+/+} sibling cells (10). The cdc25C^{-/-} cells were derived by targeted disruption of exon 3 of endogenous cdc25C, resulting in viable mouse and murine embryonic fibroblasts that, surprisingly, had no apparent defect in growth or cell cycle checkpoint (10).

In this report, we examine the role of the cdc2 activator cdc25C in the replication of HSV-1. We show that in cdc25C^{-/-} cells, the rate of degradation of cyclin B1 remained similar to that of wild-type cells, whereas the amounts of cdc2 actually increased. Concurrently, the accumulation of viral DNA, the U_S11 and U_L38 proteins, and infectious virus was reduced. However, cdc25C was not required for the virally mediated increase in cdc2 kinase activity and phosphorylation of topoisomerase II α .

MATERIALS AND METHODS

Cells and viruses. The HEp-2 and Vero cell lines were initially obtained from the American Type Culture Collection. cdc25C^{-/-} and cdc25C^{+/+} murine embryonic fibroblast (MEF) cell lines (10) were a kind gift of H. Piwnicka-Worms (Washington University, St. Louis, MO). Telomerase-transformed human embryonic lung fibroblasts (HEL cells) were a gift of T. E. Shenk (Princeton University, Princeton, NJ). Cell lines were grown in Dulbecco's modified Eagle medium supplemented with 5% newborn calf serum (HEp-2 and Vero), 10% fetal bovine serum (HEL cells), or 10% fetal bovine serum, 1% L-glutamine, and 1% nonessential amino acids (cdc25C^{-/-} and cdc25C^{+/+} MEF cells). HSV-1(F) is the prototype HSV-1 wild-type strain used in this laboratory (11). R325 (in which α 22 does not encode the C-terminal domain [CTD] of ICP22 [α 22 Δ CTD]), R7356 (U_L13⁻), and R7802 (α 22⁻ U_S1.5⁻) have been previously described (16, 18).

Plasmids. pRB143, containing the terminal BamHI S fragment of the HSV-1 genome, has been described previously (15) and was used in Southern blotting as described below.

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Cell infection. Cultures of the indicated cell type were infected with the appropriate virus at an appropriate multiplicity of infection in medium 199V (mixture 199 supplemented with 1% calf serum) on a rotary shaker at 37°C. After 2 h, the inoculums were replaced with fresh growth medium and culture flasks were incubated at 37°C until cells were harvested. For infection times delineated in the figures, time zero indicates when infection was initiated. Cells were harvested by scraping into their own medium, pelleted by low-speed centrifugation, washed twice in phosphate-buffered saline (PBS) A (0.14 M NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄), and then lysed in the appropriate buffer.

One-dimensional electrophoretic separation and immunoblotting. Cell pellets were lysed and denatured in disruption buffer (50 mM Tris [pH 7.0], 2.75% sucrose, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate). Protein samples were boiled for 5 min and then were electrophoretically separated in a 10% denaturing polyacrylamide gel and electrically transferred to a nitrocellulose sheet. The membrane was then blocked with 5% nonfat milk and reacted with primary antibody followed by an appropriate secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories) or horseradish peroxidase (Sigma). Immunoblots were developed either with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Sigma) or through enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to film or by ECL Plus (Amersham Biosciences) followed by quantification with the aid of a Molecular Dynamics Storm imaging system.

Two-dimensional electrophoretic analysis of topoisomerase IIα. Two-dimensional electrophoresis was performed using an immobilized pH gradient for first-dimension isoelectric focusing (9). A protocol previously described (8) was modified. Briefly, 150-cm² flasks of *cdc25C*^{+/+} or *cdc25C*^{-/-} MEF cells, mock or HSV-1(F) infected, were harvested for two-dimensional gel electrophoresis as previously described (2). After electrophoretic separation, proteins were transferred and immunoblotted as described above.

Antibodies. The antibodies to cellular proteins used in these studies were antiactin (catalog no. A4700; Sigma), anti-*cdc2* (catalog no. sc-54; Santa Cruz), anti-cyclin B1 (catalog no. sc-245; Santa Cruz), anti-TopoII (catalog no. Ab-1; Oncogene), all monoclonal, and polyclonal anti-TopoII alpha (catalog no. A300-054A; Bethyl Labs). Additionally, the polyclonal anti-*cdc2* (catalog no. 9112; Cell Signaling) antibody was used at a dilution of 1:1,000 and the monoclonal anti-cyclin B1 (catalog no. 4135; Cell Signaling) antibody was used at a dilution of 1:2,000. To detect viral proteins, we used the monoclonal antibodies anti-ICP0, anti-ICP4, and anti-US11 and polyclonal antibodies anti-ICP22 and anti-UL38 (Goodwin Cancer Research Institute.). Anti-mouse immunoglobulin G (IgG)-peroxidase (catalog no. A4416; Sigma), anti-rabbit IgG-peroxidase (catalog no. A0545; Sigma), anti-mouse IgG-alkaline phosphatase conjugate (catalog no. 170-6520; Bio-Rad), and anti-rabbit IgG-AP conjugate (catalog no. 170-6518; Bio-Rad) were used as secondary antibodies for immunoblotting.

Histone H1 kinase assay. The histone H1 kinase assay was performed as previously described (1).

Southern blotting. For analysis of DNA synthesis, cultures in 25-cm² flasks of Vero cells or *cdc25C*^{-/-} or *cdc25C*^{+/+} MEF cells were infected with 5 PFU of HSV-1(F) per cell and harvested at 20 h after infection. Total viral DNA was isolated as previously described (7, 17), digested with BamHI restriction endonuclease, subjected to electrophoresis on 0.7% agarose gels, transferred to zeta-probe membranes, and hybridized with the radiolabeled plasmid pRB143, which contains the terminal BamHI S fragment (15). The bound probe was visualized by autoradiography. The probe hybridized with both the junction fragment (BamHI SP) and the terminal fragment (BamHI S).

Infectious virus yield in MEF cells. Replicate *cdc25C*^{-/-} or *cdc25C*^{+/+} MEF cells were exposed to 0.01 PFU per cell of R7356, R7802, R325, or wild-type HSV-1(F) virus in 1 ml medium 199V while on a rotating shaker at 37°C. After 2 h, the inoculum was removed, cells were rinsed three times, and 2.5 ml medium 199V was added. Cells were harvested at 2 and 24 h after infection by scraping directly into the medium. The sample was frozen and thawed three times and then sonicated before being titrated in Vero cells.

RESULTS

HSV-1(F)-mediated changes in the levels of *cdc2* in MEFs.

The purpose of this series of experiments was to determine whether *cdc25C* affects the levels of *cdc2* and cyclin B in infected cells. Figure 1 shows immunoblots of cyclin B1 and of *cdc2* in mock-infected and infected *cdc25C*^{+/+} or *cdc25C*^{-/-} MEFs. The cells were infected with wild-type HSV-1(F) or mock infected and harvested at 6, 12, and 24 h after infection.

FIG. 1. The metabolism of *cdc2* and cyclin B1 proteins in MEFs infected with wild-type HSV-1(F). *cdc25C*^{+/+} or *cdc25C*^{-/-} MEF cells were mock or HSV-1(F) infected for the indicated amount of time, and cell lysates were electrophoretically separated and immunoblotted. The blots were scanned with the aid of a Molecular Dynamics PhosphorImager and normalized with respect to the amounts detected in corresponding mock-infected cells. (A) Immunoblot and results of scanning of cyclin B1. (B) Immunoblot and results of scanning of *cdc2*.

Electrophoretically separated proteins from cellular lysates were immunoblotted for cyclin B1 and *cdc2*. As reported earlier for HSV-1(F)-infected HeLa cells (1), the cyclin B1 protein largely disappeared from HSV-1(F)-infected *cdc25C*^{+/+} MEFs (Fig. 1A, lanes 1, 3, 5, and 7). Cyclin B1 also decreased in *cdc25C*^{-/-} cells but at a rate similar to that of wild-type sibling cells (Fig. 1A, lanes 2, 4, 6, and 8). *cdc2* levels tended to decrease at least during the first 12 h after infection of *cdc25C*^{+/+} cells (Fig. 1B, lanes 1, 3, 5, and 7). In contrast, there was a consistent net increase in the amount of *cdc2* in infected *cdc25C*^{-/-} cells (Fig. 1B, lanes 2, 4, 6, and 8). The results of this and other experiments in this series suggest that *cdc25C* expression was necessary for the maintenance of or actual increase in the levels of the *cdc2* protein in HSV-1(F)-infected

FIG. 2. *cdc25C* is not required for increase in *cdc2* kinase activity after HSV-1(F) infection. *cdc25C*^{+/+} or *cdc25C*^{-/-} MEF cells were mock or HSV-1(F) infected for 16 h. *cdc2* kinase complex immunoprecipitated using antibody against cyclin B1 (lanes 1 to 4) or against *cdc2* (lanes 5 to 8) was incubated with purified histone H1 in a [γ -³²P]ATP-labeled kinase assay. (A) Light exposure of autoradiogram. (B) Dark exposure of same autoradiogram.

cells. The absence of *cdc25C* played no role in the disappearance of cyclin B1.

HSV-1(F)-mediated increase in *cdc2* activity does not require presence of *cdc25C*. To determine the functional relevance of *cdc25C* for the *cdc2* kinase activity in mock-infected and infected MEFs, *cdc25C*^{+/+} and *cdc25C*^{-/-} MEFs were infected and maintained for 16 h and then harvested and lysed. *cdc2* complex immunoprecipitated with antibody against cyclin B1 or *cdc2* was reacted with purified histone H1 in a [γ -³²P]ATP-labeled kinase assay. As previously reported for HeLa cells (1), in *cdc25C*^{+/+} MEFs the kinase activity of the complex immunoprecipitated by antibody against cyclin B1 decreased after infection (Fig. 2, lanes 1 and 3) whereas the kinase activity of the complex immunoprecipitated by antibody against *cdc2* was increased after infection (Fig. 2, lanes 5 and 7). The same trends were observed in *cdc25C*^{-/-} MEFs.

We conclude that the activation of *cdc2* is not significantly affected by the absence of *cdc25C* in MEFs.

Topoisomerase II α is posttranslationally modified in infected *cdc25C*^{-/-} and *cdc25C*^{+/+} cells. As reported earlier, *cdc2* binds the U_L42 protein and this complex recruits, phosphorylates, and activates topoisomerase II α (4). To define the role of *cdc25C* in the regulation of topoisomerase II α , *cdc25C*^{+/+} or *cdc25C*^{-/-} MEF or HEL cells were mock infected or infected with R325 (α 22 deletion mutant) or HSV-1(F). The cells were harvested, lysed, subjected to electrophoresis, and reacted with antibody to topoisomerase II α . Human topoisomerase II α migrates more slowly in the presence of HSV-1(F) infection than with mock infection or infection with R325 virus (data not shown), which is consistent with the earlier report (4) that intact, functional ICP22 is required for the posttranslational modification of topoisomerase II α . In

FIG. 3. *cdc25C* is not required for modification of TopoII α to negatively charged forms. *cdc25C*^{+/+} and *cdc25C*^{-/-} MEF cells were mock infected or infected with 10 PFU/cell HSV-1(F) for 12 h. Cell lysates were separated electrophoretically for linear analysis or separated by charge followed by electrophoresis for two-dimensional analysis. (A) Linear separation, immunoblot for topoisomerase II α . (B) Two-dimensional separation, immunoblot for topoisomerase II α . Linear migration is marked with an arrowhead; charge distribution is indicated with a line.

contrast, there was no detectable shift in electrophoretic mobility of murine topoisomerase II α in mock-infected, R325-infected, or HSV-1(F)-infected *cdc25C*^{+/+} or *cdc25C*^{-/-} MEF cells (data not shown).

In order to detect a modification of topoisomerase II α that did not affect its linear gel migration, the lysates of *cdc25C*^{-/-} or *cdc25C*^{+/+} MEFs harvested 12 h after mock infection or infection with HSV-1(F) were subjected to both one-dimensional separation (Fig. 3A) and two-dimensional separation (Fig. 3B). The one-dimensional separation (Fig. 3A, lanes 1 to 4) revealed an increase in the amounts of topoisomerase II α in infected cells over those in uninfected *cdc25C*^{+/+} or *cdc25C*^{-/-} MEFs. The two-dimensional gel analysis showed that following infection, most of the topoisomerase II α shifted to the right, indicating an increasing negative charge (Fig. 3B).

FIG. 4. *cdc25C* is required for maximal production of HSV-1(F) DNA. Total viral and cellular DNA was purified from Vero and MEF cells 20 h after mock or HSV-1(F) infection. DNA was digested with BamHI restriction endonuclease, electrophoretically separated in a 0.7% agarose gel, transferred to a membrane, and labeled with a probe specific for the genomic junction region designated BamHI fragment S. Digested genomic concatemers and circles migrate more slowly and are identified as BamHI SP. Digested genomic terminal fragments migrate more quickly and are identified as BamHI S.

This is in contrast to results for mock-infected cells, in which topoisomerase II α varied more extensively with respect to charge. The results obtained from infected *cdc25C*^{+/+} and *cdc25C*^{-/-} cell lines indicate that topoisomerase II α is posttranslationally modified after HSV-1 infection and that the absence of *cdc25C* does not affect the apparent level of modification of topoisomerase II α . We should note, however, that although the data are consistent with the earlier report that in HSV-1-infected cells topoisomerase II α is posttranslationally modified, we present no evidence to support the hypothesis that the modification of topoisomerase II α in HSV-1-infected murine cells is similar to or as extensive as that seen in infected human cells (4). Topoisomerase II α is highly conserved among humans and mice, although several sites of phosphorylation have been found in the human protein while the mouse protein has not been extensively mapped (14).

The production of viral DNA during HSV-1(F) infection was lower in *cdc25C*^{-/-} cells than in *cdc25C*^{+/+} MEF cells. Advani et al. (4) suggested that *cdc2* may play a role in HSV-1 DNA synthesis. To test the hypothesis that *cdc25C* plays a role in viral DNA accumulation, total DNA was purified from HSV-1(F)-infected Vero cells or MEFs, digested with BamHI restriction endonuclease, electrophoretically separated in a 0.7% agarose gel, transferred to a membrane, and hybridized with a labeled BamHI S DNA fragment. This fragment is derived from the terminus of the L component of linearized DNA and is also present in the BamHI SP fragment present at the junction between the L and S components in both linear and circular or concatemeric DNA. DNA extracted from mock-infected or HSV-1(F)-infected Vero cells served as negative and positive controls for the assay (Fig. 4, lanes 1 and 2).

As shown in Fig. 4, lanes 3 and 4, the amounts of BamHI SP and BamHI S DNA fragments, reflecting linear and concatemeric DNAs and ends of linearized DNA, respectively, that accumulated in *cdc25C*^{-/-} MEFs were smaller than those present in *cdc25C*^{+/+} MEFs. These results suggest that *cdc25C* plays a role in viral DNA accumulation in infected cells.

Accumulation of a subset of γ_2 proteins is dependent on presence of *cdc25C*. A subset of γ_2 proteins depend on *cdc2* kinase activity and ICP22 for their accumulation in infected cells (5, 18). In order to evaluate the role of *cdc25C* in the accumulation of these γ_2 proteins, *cdc25C*^{+/+} and *cdc25C*^{-/-} MEFs were mock infected or infected with HSV-1(F) (Fig. 5A and B) or R325 (Fig. 5B) and harvested at times shown in Fig. 5. The electrophoretically separated proteins were reacted with antibodies to ICP4 (panel A), ICP0 (panels A and B), ICP22 (panel A), U_L38 (panels A and B), U_S11 (panels A and B), and actin (panel B). The results were as follows. (i) The accumulation of ICP22 and ICP0 was not significantly affected by the absence of *cdc25C*. (ii) In the absence of *cdc25C*, there was a decrease in the accumulation of ICP4, U_S11, and U_L38. (iii) As expected, MEFs infected with R325 mutant virus accumulated less of the U_L38 or U_S11 protein than corresponding cells infected with wild-type virus (compare Fig. 5B, lanes 15 and 16, with lanes 17 and 18). In addition, *cdc25C*^{-/-} MEFs infected with the mutant virus accumulated less of the U_L38 protein than infected *cdc25C*^{+/+} cells (compare lanes 15 and 16 in panel B). The accumulation of U_S11 was less affected by the absence of *cdc25C* than by that of the U_L38 protein.

We conclude that *cdc25C* is required for optimal accumulation of at least a subset of HSV-1 proteins and that the combined absence of both intact ICP22 and *cdc25C* has a more drastic effect on the accumulation of U_L38, one member of the subset of γ_2 proteins regulated by ICP22.

HSV-1 accumulates at lower levels in absence of *cdc25C*. In this series of experiments, replicate cultures of *cdc25C*^{+/+} and *cdc25C*^{-/-} MEFs were exposed to 0.1 PFU/cell of HSV-1(F), R325, R7802 ($\Delta\alpha 22$), or R7356 ($\Delta U_L 13$). The cells were harvested at 24 h after infection, and titers of virus were determined on Vero cells. The results, depicted in Fig. 6, were as follows. (i) As predicted, the R325 mutant, lacking the carboxyl-terminal 220 residues of ICP22, replicated less well than the wild-type virus. The R7802 mutant, lacking the entire ICP22 open reading frame, barely replicated in wild-type murine cells. (ii) For each virus that replicated in murine cells, the yields in infected *cdc25C*^{-/-} MEF cells were at least 10-fold lower than those obtained from infected *cdc25C*^{+/+} MEFs. We conclude that *cdc25C* plays a role in viral replication.

DISCUSSION

In earlier studies, this laboratory reported that after infection, cyclins A and B1 are degraded, the residual *cdc2* kinase binds U_L42, and this complex recruits and phosphorylates topoisomerase II α (1, 3, 4). This chain of events is mediated by ICP22 and is essential for optimal expression of a subset of γ_2 genes exemplified by U_S11, U_L38, and U_L41 and production of infectious progeny (5, 18). A key step in the activation of *cdc2* is the removal of inhibitory phosphates by the *cdc25C* phosphatase. To clarify the partial stabilization and activation of *cdc2*, we examined the role of *cdc25C* in key aspects of the

FIG. 5. Accumulation of a subset of γ_2 proteins is depends on *cdc25C* and functional ICP22. *cdc25C*^{+/+} and *cdc25C*^{-/-} MEF cells were mock infected or infected with HSV-1(F) (A and B) or R325 (B) and harvested at the times indicated. Electrophoretically separated proteins were immunoblotted for ICP4 (A), ICP0 (A and B), ICP22 (A), UL38 (A and B), US11 (A and B), or actin (B).

pathway leading to the expression of the ICP22-regulated genes by taking advantage of the availability of *cdc25C*^{-/-} MEFs and wild-type *cdc25C*^{+/+} MEFs derived from a sibling mouse. A priori we should note that the phenotype of *cdc25C*^{-/-} mice was not significantly different from that of wild-type mice, suggesting that the functions of *cdc25C* were taken over by other genes or metabolic pathways (10).

Our results may be summarized as follows. (i) In the cell lines tested, cyclin B1 was degraded at the same rate in both *cdc25C*^{-/-} and in the wild-type sibling *cdc25C*^{+/+} MEFs. Of

particular significance is the observation that the *cdc2* protein was actually upregulated in the course of infection of *cdc25C*^{-/-} cells compared to results with *cdc25C*^{+/+} MEFs. (ii) *cdc2* kinase was more active in infected *cdc25C*^{+/+} and *cdc25C*^{-/-} MEFs than in uninfected cells. (iii) Topoisomerase II α was posttranslationally modified in both *cdc25C*^{+/+} and *cdc25C*^{-/-} MEFs infected with wild-type virus. As noted in the results, however, we have no data on the nature of the modification or its identity to that observed in human cells. (iv) The accumulation of representative γ_2 proteins regulated by ICP22 and virus yields were reduced in *cdc25C*^{-/-} MEFs from those in *cdc25C*^{+/+} MEFs. Equally significantly, the decrease in virus yields and in the accumulation of at least the UL38 protein observed in *cdc25C*^{-/-} MEFs infected with wild-type virus was also observed in cells infected with a virus mutant lacking the CTD of ICP22 and which yields less virus and accumulates smaller amounts of ICP22-dependent γ_2 proteins. This suggests a role for *cdc25C* that is not initiated solely by ICP22.

One strategy of HSV takeover of the infected cells is to sequester and redirect cellular proteins to perform functions similar to or very different from those performed by the same protein in uninfected cells (19). For heuristic reasons, it seems appropriate to consider two hypotheses. The first is that *cdc25C* plays a role consistent with its normal function in uninfected cells. The alternative hypothesis is that the functions of *cdc25C* are not related to activation of *cdc2*.

The scenario consistent with both hypotheses is that the fraction of active *cdc2* corresponding to the protein band that remains in infected *cdc25C*^{-/-} MEFs binds to UL42 even though cyclin B1 is not degraded and topoisomerase II α is

FIG. 6. *cdc25C* and $\alpha 22$ are each important for virus growth at a low multiplicity of infection. *cdc25C*^{+/+} and *cdc25C*^{-/-} MEF cells were infected with 0.01 PFU of R7356 (Δ UL13), R7802 (Δ α 22), R325 (α 22 Δ CTD), or wild-type HSV-1(F) per cell. At 24 h after infection, the cells were harvested, the virus was extracted by repeated freeze thawing, and titers were determined on Vero cells.

modified by the cdc2/U_L42 complex. The discriminating datum is the observation that cdc25C plays a role even in MEFs infected with an ICP22 mutant incapable of upregulating the accumulation of the representative γ_2 proteins. Since all available data support the conclusion that ICP22 regulates the accumulation of the γ_2 proteins, exemplified by U_L38, U_L41, and U_S11, loss of any component of the pathway regulated by ICP22 should have the same effect as loss of ICP22. This is clearly not the case. By necessity, we conclude that cdc25C phosphatase acts in addition to its role in activating cdc2. The precise mechanism remains to be elucidated.

Of note, a mild defect in accumulation of the immediate-early proteins ICP4 and ICP22 was observed in cdc25C^{-/-} MEFs. Can this explain the defects seen in infection of this cell line—DNA replication, late gene expression, and viral yields? It is critical to note that the defect seen in infection of these cells is not merely a delay in the actions of the immediate-early proteins. If that were the case, the ICP22 mutant should have the same phenotype, due to a functional overlap, in the presence or absence of cdc25C. In contrast, the ICP22 mutant in combination with the absence of cdc25C demonstrated a cumulative decrease in the accrual of the γ_2 genes U_L38 and U_S11 and in the replication of the virus.

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