Accumulation of Substrates of the Anaphase-Promoting Complex (APC) during Human Cytomegalovirus Infection Is Associated with the Phosphorylation of Cdh1 and the Dissociation and Relocalization of APC Subunits[∇]

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Cell cycle dysregulation upon human cytomegalovirus (HCMV) infection of human fibroblasts is associated with the inactivation of the anaphase-promoting complex (APC), a multisubunit E3 ubiquitin ligase, and accumulation of its substrates. Here, we have further elucidated the mechanism(s) by which HCMV-induced inactivation of the APC occurs. Our results show that Cdh1 accumulates in a phosphorylated form that may prevent its association with and activation of the APC. The accumulation of Cdh1, but not its phosphorylation, appears to be cyclin-dependent kinase dependent. The lack of an association of exogenously added Cdh1 with the APC from infected cells indicates that the core APC also may be impaired. This is further supported by an examination of the localization and composition of the APC. Coimmunoprecipitation studies show that both Cdh1 and the subunit APC1 become dissociated from the complex. In addition, immunofluorescence analysis demonstrates that as the infection progresses, several subunits redistribute to the cytoplasm, while APC1 remains nuclear. Dissociation of the core complex itself would account for not only the observed inactivity but also its inability to bind to Cdh1. Taken together, these results illustrate that HCMV has adopted multiple mechanisms to inactivate the APC, which underscores its importance for a productive infection.

Human cytomegalovirus (HCMV) infection is the leading viral cause of birth defects and results in severe disease in immunocompromised individuals (for a review, see reference 26). The replication of this virus is temporally regulated and involves an intricate set of interactions between the virus and the host cell machinery that optimize the cellular environment for viral replication and assembly. Similar to DNA tumor viruses that can infect quiescent cells, HCMV induces cells towards S phase such that the cellular DNA machinery is activated and available for viral DNA replication. Subsequent dysregulation of multiple cellular factors involved in the cell cycle causes the infected cell to arrest in a pseudo-G₁/S state (1, 2, 6, 16, 21, 29, 30, 43–45). The Rb family pocket proteins that regulate transcription in complex with E2Fs in a cell-cycle-dependent manner become phosphorylated and accumulate, while the tumor suppressor protein p53 is stabilized (9, 16, 23, 25). Cyclin A mRNA synthesis is blocked, and only low levels of cyclin A protein and its associated kinase activity can be detected (16, 29). In contrast, cyclins E and B, as well as their associated kinase activities, are upregulated (16, 23, 29).

Expression of cyclin, along with other cell cycle proteins, is partially regulated by the ubiquitin-proteasome pathway, in which a protein becomes ubiquitinated and then degraded by the proteasome (11, 12). Ubiquitination occurs through a multistep mechanism involving the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) enzymes. Target specificity is determined at the level of E3s, where each E3 interacts with specific E2s and protein substrates. The main E3s involved in cell cycle regulation are the SCF (Skp1-cullin-F-box) complex and the APC (anaphase-promoting complex).

The APC, also known as the cyclosome, is a large multisubunit complex that is evolutionarily conserved from yeasts to plants to mammals (for reviews, see references 4 and 28). It is active from mitosis through G₁ to ensure proper cell cycle progression, particularly for anaphase entry and exit from mitosis. Cryo-negative staining electron microscopy, biochemical reconstitution assays, and labeling experiments have been used to delineate the architecture of the APC (7, 10, 27, 38). Vertebrate APC contains at least 12 subunits, which can be further divided into two separable subcomplexes (38). Subunits APC2 and APC11 (catalytic core), along with APC10, form the platform that binds the E2 enzyme (UbcH5 or UbcH10) and allows the transfer of ubiquitin. APC3 (Cdc27), APC6, APC7, and APC8, all of which contain tetratricopeptide repeats (TPR), form the arc lamp that functions mainly in binding the activator proteins. APC1, APC4, and APC5 serve as a scaffold

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connecting the two subcomplexes. APC activation and regulation is achieved through interactions with its coactivator protein Cdc20 or Cdh1, which binds to APC3. APC2 and APC7 also have been shown to facilitate the interaction between Cdh1 and the APC (39, 40). Phosphorylation of the APC upon entry into mitosis mediates binding of Cdc20, thus forming an active complex that initiates mitotic cyclin degradation (19, 34, 46). During late anaphase, inactivation of cyclin-dependent kinases (CDKs) relieves the inhibitory phosphorylation of Cdh1, which now is able to bind and activate the APC. APC^{Cdh1} remains active through G₁ and prevents the premature accumulation of cyclin A, cyclin B, and S-phase regulators (e.g., Cdc6 and geminin). As cells enter S phase, rising cyclin A/Cdk2 activity results in the phosphorylation of Cdh1, which blocks the binding of Cdh1 to the APC and shuts off ubiquitylation of the APC substrates. Cell cycle-specific expression of *trans*-acting factors such as Emi1, RASSF1A, and the mitotic spindle checkpoint proteins also modulates APC activity.

Initial studies from our laboratory and others showing that several substrates of the APC (e.g., cyclin B, Cdc6, and geminin) abnormally accumulate early in the HCMV infection led to the hypothesis that APC activity is downregulated during the infection (1, 16, 30, 42, 45). Subsequently, Wiebusch et al. (42) reported that the APC isolated from HCMV-infected cells had significantly reduced to no activity, as measured by *in vitro* ubiquitination assays. This decrease in APC activity did not appear to be due to an overexpression of the APC inhibitor Emi1 or a lack of E2 expression (i.e., UbcH5 or UbcH10). It also was noted that Cdh1 protein expression was significantly upregulated during the infection, while mRNA levels remained unchanged. However, immunoprecipitation (IP) assays using an antibody to APC3 indicated that little to no Cdh1 was associated with this subunit as the infection progressed, although it was not determined whether other APC subunits remained in a complex with APC3. Based on these results, it was proposed that the decreased APC activity during HCMV infection is due to the lack of Cdh1 binding and activation of the complex. However, questions regarding the mechanism by which this occurs were not addressed.

In this report, we have further investigated the mechanism(s) by which the APC becomes inactivated during the HCMV infection. Importantly, we show that Cdh1 is phosphorylated during the infection in a CDK-independent manner and that the APC becomes destabilized, as evidenced by the dissociation of not only Cdh1 but also APC1, the largest subunit of the APC. In contrast, subunits that contain the TPR motif (APC3, APC7, and APC8) remain in a complex. We also show that this dissociation coincides with the retention of APC1 in the nucleus and redistribution of the TPR subunits to the cytoplasm. Thus, it appears that multiple mechanisms are involved in mediating the inhibition of APC activity during the infection.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts (HFFs) were obtained from the Medical Center of the University of California, San Diego, and were cultured in minimum essential medium with Earle's salts supplemented with 10% heat-inactivated fetal bovine serum, 1.5 µg/ml amphotericin B, 2 mM L-glutamine, 200 U/ml penicillin, and 200 µg/ml streptomycin. All cell culture media were from Gibco-BRL. Cells were kept in incubators maintained at 37°C and 7% CO₂. The

Towne strain of HCMV was obtained from the American Type Culture Collection (VR 977) and propagated as previously described (36).

Cell synchronization and infection. All experiments were performed under G₀ synchronization conditions (29). Cells were trypsinized 3 days after the monolayer became confluent and were replated at a lower density to induce progression into the cell cycle. At the time of replating, cells either were infected with HCMV at a multiplicity of infection of 5 or were mock infected with tissue culture supernatants as described previously (29). Stock solutions of MG132 (Calbiochem) and roscovitine (Calbiochem) were made in dimethylsulfoxide (DMSO). Cell cultures were incubated with 2.5 µM MG132, 20 µM roscovitine, or an equivalent volume of DMSO as a control at the times shown. Cells were harvested at the indicated times postinfection (p.i.) and processed as described for each experiment. All experiments were performed at least twice.

Western blot analysis. For Western blot analysis, cells were lysed in Laemmli reducing sample buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol) supplemented with a protease inhibitor cocktail (Roche) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate). The lysate was sonicated, boiled for 5 min, and clarified by centrifugation for 10 min at 16,000 × g. Equal amounts of lysate (i.e., by cell number) were loaded onto sodium dodecyl sulfate-polyacrylamide gels unless otherwise stated. Following electrophoresis, the proteins were transferred to nitrocellulose (Schleicher & Schuell), and then Western blot analyses were performed using the appropriate antibodies. The Supersignal West pico and West femto chemiluminescent detection methods (Pierce) were used to visualize the proteins according to the manufacturer's instructions.

Phosphatase assay. For phosphatase assays, cell samples were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM MgCl₂, 10% glycerol, 300 mM NaCl, 0.1% NP-40, protease inhibitor cocktail) or in buffer B (buffer A plus the following phosphatase inhibitors: 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate). After incubation on ice for 5 min, cells were subjected to three freeze-thaw cycles. The lysate then was centrifuged at 16,000 × g for 10 min; the supernatant was collected and analyzed for protein concentration using the Bio-Rad protein assay. For λ-protein phosphatase (λpp) treatment, buffer A lysates were incubated with 1 × λpp buffer (New England Biolabs), 2 mM MnCl₂, and λpp (New England Biolabs) at 5 U/µg protein for 30 min at 30°C. Buffer B lysates were incubated in parallel without λpp. Reactions were terminated with the addition of 2 × Laemmli reducing sample buffer. Samples then were boiled and analyzed by Western blotting.

IPs. Cell pellets were lysed in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.2% NP-40, 10% glycerol, 1 mM dithiothreitol; supplemented with 1 × protease inhibitor cocktail, 50 mM sodium fluoride, 10 mM β-glycerophosphate, and 1 mM ATP) using an end-over-end rotator at 4°C. Lysates were centrifuged at 16,000 × g for 10 min, and supernatants were collected. For APC3 coimmunoprecipitation assays, lysates first were pre-cleared by protein G beads (Santa Cruz Biotechnology) coupled to mouse immunoglobulin G (IgG) (Jackson ImmunoResearch) and then incubated with protein G beads coupled to an anti-APC3 monoclonal antibody (BD Biosciences). Beads were washed with TBS-T (Tris-buffered saline with 0.01% Tween 20) between incubations and eluted in Laemmli reducing sample buffer by being boiled for 5 min. Samples also were collected pre- and post-IP and were boiled in reducing sample buffer. Samples were analyzed by Western blotting. Pre-IP and post-IP lanes were loaded with the same cell equivalents, whereas IP lanes were loaded with 5 to 10 times more. All incubations and washes were performed at 4°C.

In vitro binding assay. Rabbit reticulocyte lysate (T7-Quick Couple TNT kit; Promega) first was immunodepleted of APC with an anti-APC3 antibody before being used to generate ³⁵S-labeled Cdh1 via an *in vitro* transcription/translation (TNT) reaction. Human Cdh1 (a gift from Jan-Michael Peters) was cloned into pcDNA3 (Invitrogen) under the T7 promoter. TNT reactions using pcDNA3 vector alone were used as a negative control. Mock- or HCMV-infected cells were harvested at 8 and 16 h p.i. and were lysed in extraction buffer. ³⁵S-Cdh1 or ³⁵S-pcDNA3 was preincubated with cell lysates at room temperature for 1 h. The preincubation mixture then was immunoprecipitated for APC3. IPs using mouse IgG-coupled beads were performed in parallel as a negative control. Following SDS-polyacrylamide gel electrophoresis, the gel was divided in half such that the upper portion was used to detect APC3 by Western blotting, and ³⁵S-Cdh1 was detected by autoradiography using the lower portion. IP lanes were loaded with cell equivalents 20 times larger than those of pre- and post-IP lanes.

IFA. For immunofluorescence assays (IFA), cells were seeded onto glass coverslips at the time of infection. At the indicated times p.i., cells were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20

min. Cells then were permeabilized with 0.2% Triton X-100 for 5 min and washed in PBS prior to immunofluorescence staining. Normal goat serum (10% in PBS) (Jackson ImmunoResearch) was used as a blocking solution and to dilute the primary and secondary antibodies. A mouse monoclonal antibody to APC3 and rabbit antibodies against APC1, APC7, APC8, and APC10 were used. Mouse or rabbit IgG (Jackson ImmunoResearch) served as a negative control. Following primary antibody incubation and subsequent washes in PBS, coverslips were incubated with appropriate fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch) plus Hoechst stain. Coverslips were treated with SlowFade Gold, an antiphotobleaching reagent (Molecular Probes), and were mounted onto a slide for imaging. Costained samples were analyzed by a DeltaVision deconvolution microscopy system (Applied Precision) using a 100 $\times$ oil immersion objective lens with SoftWoRx software (Applied Precision) on a Silicon Graphics O2 workstation. Images were taken at 0.2- μ m increments along the z axis, with pixel intensities maintained in the linear range, by a Photometrics charge-coupled-device camera mounted on a fluorescence/differential interference contrast microscope. The fluorescence data sets were deconvolved and analyzed by DeltaVision SoftWoRx programs. Adobe Photoshop 7.0 was used to prepare images for the figures.

Antibodies. The antibodies (and sources) used are the following: Cdh1 (Ab-2; Calbiochem); Rb (Ab-1, IF8; Neomarkers); Cdc6 (180.2; Santa Cruz Biotechnology); geminin (FL-209; Santa Cruz Biotechnology); actin (AC-15; Sigma); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6c5; Fitzgerald); IE1/IE2 (Ch16.0; Virusys). APC antibodies were the following: APC1 (a gift from Michael Green [15, 37]); APC3 (clone 35 [BD Biosciences] and AF3.1 [Santa Cruz Biotechnology]); APC7 (poly6113 [Biolegend] and H-300 [Santa Cruz Biotechnology]); APC8 (poly6114 [Biolegend] and H-300 [Santa Cruz Biotechnology]); APC10 (poly6115; Biolegend); and APC11 (poly6116; Biolegend).

RESULTS

Cdh1 becomes phosphorylated during infection. A previous report by Wiebusch et al. (42) suggested that APC inactivity during HCMV infection is due to the lack of Cdh1 binding. In accord with their study, we also have observed the dissociation of Cdh1 from the APC, despite a significant upregulation in expression beginning 8 to 12 h p.i. (data not shown). However, by Western blot analysis, the Cdh1 from infected cells appeared to migrate slower on the gel, suggesting that the association of Cdh1 with the APC is inhibited due to a modification of Cdh1 during the infection. Because Cdh1 phosphorylation normally prevents its association with the APC (20, 48), phosphatase assays were used to determine whether Cdh1 becomes phosphorylated. Lysates from mock-infected or HCMV-infected HFFs harvested at 24 h p.i. were left untreated or were treated with λ pp and analyzed by Western blotting. As a control, samples were blotted for Rb, which becomes hyperphosphorylated upon HCMV infection (16). As shown in Fig. 1, more hyperphosphorylated Rb was present in the infected cells. Subsequent treatment with phosphatase reduced the hyperphosphorylated Rb in both the mock- and virus-infected samples. The lysates also were tested for viral IE1-72 and IE2-86 expression and phosphorylation status. Consistent with previous studies (13), IE2-86 is phosphorylated, as treatment with phosphatase resulted in a lower-molecular-weight form, whereas IE1-72 was unaffected. In the case of Cdh1, no mobility changes were observed with the mock-infected cell samples upon phosphatase treatment, indicating that Cdh1 is not phosphorylated at this time point. Phosphatase treatment of the infected sample, however, resulted in a mobility shift such that the Cdh1 band comigrated with the unphosphorylated form found in the unin-

FIG. 1. Cdh1 becomes phosphorylated during HCMV infection. HFFs infected with HCMV Towne at a multiplicity of infection of 5 (V) or incubated with conditioned medium (M) were harvested at 24 h p.i. Cell lysates were incubated with or without (–) λ pp and were analyzed by Western blotting with antibodies to Rb, IE2, IE1, and Cdh1. Actin served as a control for protein loading. For Rb, IE2, and Cdh1, the phosphorylated forms are indicated with an asterisk.

fected cell. These results illustrate that Cdh1 is phosphorylated upon HCMV infection.

Cdh1 normally is phosphorylated by CDKs in late G₁ phase to inactivate the APC. To assess whether the observed Cdh1 phosphorylation is due to the activation of the several CDKs upon HCMV infection, HFFs either were infected with virus or were mock infected in the presence or absence of roscovitine, an inhibitor of CDK1, CDK2, CDK5, CDK7, and CDK9 (5, 8, 24, 33, 41). Three different periods of drug treatment were used, from 0 to 24, 4 to 24, and 8 to 24 h p.i. The choice of these time intervals was based on results of our previous studies that showed that the addition of roscovitine at the beginning of the infection altered viral immediate-early (IE) and early gene expression, while a delay in the addition of the drug until 4 to 8 h p.i. allowed the infection to progress normally until late times (31). Cells were harvested at 24 h p.i. and processed for Western blot analysis. Again, Rb and IE expression were used as controls. roscovitine should inhibit CDK-mediated phosphorylation of Rb. As expected, significantly more phosphorylated Rb was present in the virus-infected cells than in the mock-infected cells, and treatment with the drug reduced the amount of hyperphosphorylated Rb in all cases (Fig. 2A). The incomplete inhibition in the infected cells likely is due to the phosphorylation of Rb by kinases that are not affected by roscovitine. The addition of the drug from 0 to 24 h p.i. favored IE2 expression over that of IE1, an effect of the drug that has been characterized previously (31). For Cdh1, the roscovitine treatment from 0 to 24 h p.i. was most effective in inhibiting its accumulation in the virus-infected cells, whereas no significant differences were observed in the mock-infected cells. Interestingly, increasingly more phosphorylated Cdh1 accumulated as the drug was administered later during the infection. To further confirm that Cdh1 is phosphorylated, phosphatase assays were performed as previously described. As shown in Fig. 2B, the subsequent mobility shift in the infected sample upon phosphatase treatment indicates that Cdh1 was phosphorylated in the presence of roscovitine. Similar to the case for Rb, the lack of inhibition of Cdh1 phosphorylation by roscovitine suggests that other kinases may be responsible for its phosphorylation in the infected cell.

FIG. 2. Roscovitine treatment at early times after infection inhibits Cdh1 accumulation but not phosphorylation. (A) DMSO (–) or 20 μ M roscovitine (+) was added at 0, 4, or 8 h p.i. to mock-infected (M) or HCMV-infected (V) cells. Cells were harvested at 24 h p.i. for Western blot analysis with antibodies to Rb, IE2, IE1, Cdh1, Cdc6, and geminin. Actin served as a loading control. For Rb and Cdh1, the phosphorylated forms are indicated with an asterisk. The Cdh1 blot for the 0- to 24-h-p.i. samples is slightly overexposed to show the presence of phosphorylated Cdh1 in the infected cells that were treated with roscovitine. (B) Mock-infected (M) or HCMV-infected (V) cells were treated with 20 μ M roscovitine from 8 to 24 h p.i., harvested at 24 h p.i., incubated with or without (–) λ pp, and analyzed by Western blotting as previously described.

Since Cdh1 remained phosphorylated in the infected cells treated with roscovitine, we also assessed the accumulation of the APC substrates Cdc6 and geminin by Western blotting to help determine whether the APC still remained inactive. In general, treatment of both the mock- and HCMV-infected cells with roscovitine resulted in lower levels of Cdc6 and geminin relative to those of the corresponding untreated cells at all time periods (Fig. 2A). In the infected cells, the difference was most notable for both proteins when the drug was administered at the beginning of the infection, whereas the effect was greater for geminin than for Cdc6 when the drug was added at later times. However, significantly more Cdc6 and geminin accumulated in the infected cells when the inhibitor was added after 4 h p.i. than in the uninfected cells in the absence of the inhibitor, suggesting that APC activity was downregulated. Taken together, these results suggest that the increased accumulation of the APC substrates and Cdh1, but not Cdh1 phosphorylation, is at least partly CDK dependent during the infection.

The APC becomes destabilized upon HCMV infection. Although CDK-mediated phosphorylation of Cdh1 during late G_1 has been shown to inhibit its association with the APC (18, 22), we could not conclude that the induced phosphorylated state of Cdh1 upon HCMV infection impeded its association, since the phosphorylation of other sites on Cdh1 or other modifications may affect its physical structure differently. On the other hand, an alteration in the APC itself might also

inhibit binding to Cdh1. To this end, *in vitro* binding assays were used to determine whether the ability of the APC to bind exogenous Cdh1 also is affected during the infection. 35 S-labeled Cdh1 was synthesized in an *in vitro* TNT reaction using rabbit reticulocyte lysate. The reticulocyte lysate was first immunodepleted of endogenous APC (35, 47) and assayed for endogenous Cdh1 expression, which was not detected by Western blotting, before being used for the TNT reactions. Excess 35 S-Cdh1 was incubated with cell lysate from mock- or virus-infected HFFs harvested at 8 and 16 h p.i., and the complexes then were immunoprecipitated with antibody against APC3. Negative controls included a vector-alone TNT reaction (p3) and IP using beads coupled with mouse IgG. Samples were analyzed for immunoprecipitated APC3 by Western blotting and for coprecipitated 35 S-Cdh1 by autoradiography. 35 S-Cdh1 was detected as a doublet (Fig. 3), which may be due to an alternative start site within the coding region or a small amount of degradation. It also should be noted that the proteins in the IP lanes appeared to migrate slightly slower, a phenomenon we have often observed with our IP gels. As shown in the APC3 Western blot (short exposure), the amount of precipitated APC3 was comparable between the samples. APC3 also was immunodepleted in these samples, as evidenced by its absence in the post-IP lanes (longer exposure). 35 S-Cdh1 coprecipitated with APC3 in the lysate from the HCMV-infected cells at 8 h p.i., although the amount was less than that from the mock-infected cells. In contrast, at 16 h p.i., very little 35 S-Cdh1

was found in the coprecipitate from the infected cells, whereas the level of ^{35}S -Cdh1 in the coprecipitate from the mock samples was comparable to that observed at the 8-h time point. These results suggest that the APC binding capacity for exogenous Cdh1 also is affected during the infection, which could contribute to the loss of endogenous Cdh1 binding.

To further investigate whether the APC core complex is altered upon HCMV infection, Western blotting first was used to assess the steady-state levels of the APC subunits during the infection. Individual subunits could be degraded or modified such that the complex is not properly formed. Protein levels for APC3, APC7, and APC11 remained comparable between virus-infected and mock-infected samples through 48 h p.i. (Fig. 4), which is consistent with a previous report that APC2 and APC3 levels remained unchanged (42). A modest increase in protein expression was observed for APC1 beginning at 24 h p.i. and for APC8 and APC10 beginning at 36 h p.i. Although not all APC subunits have been tested, the loss of activity does not appear to be due to decreased subunit expression.

Coimmunoprecipitation experiments using an antibody to APC3 coupled with Western blot analysis of the coprecipitated proteins then were utilized to determine complex stability in infected cells between 8 and 16 h p.i. We first assessed whether APC7 and APC8, which are TPR subunits normally in a subcomplex with APC3, were present (38). GAPDH was used as a negative control. As shown in Fig. 5, the amount of APC7 and APC8 that coimmunoprecipitated with APC3 was comparable

between virus-infected and mock-infected cells throughout the time course. Both subunits also remained in complex with APC3, as evidenced by the depletion of the proteins post-IP, although a small amount of APC7 was present after IP of the lysate from infected cells at 16 h p.i. In striking contrast, a significant decrease in associated APC1, a scaffolding protein, was observed in the infected cells. While APC1 levels in the complex were similar for both mock-infected and virus-infected cells at 8 h p.i., a slight decrease was observed at 12 h p.i. in the infected cells, and no APC1 was detected in the complex by 16 h p.i. In the mock-infected cells, the amount of APC1 coprecipitated with APC3 remained comparable at all time points, although not all APC1 was in complex, since some protein still was seen in the post-IP sample. The presence of Cdh1 also was checked as a positive control for association with APC3. As expected, Cdh1 remained in complex with APC3 throughout the time course in the uninfected samples but was lost from the APC as the infection progressed. Taken together, these results suggest that the APC becomes destabilized upon HCMV infection, as evidenced by the dissociation of at least two subunits.

APC subunits relocate to the cytoplasm of HCMV-infected cells. Given that APC1 protein levels are not affected between 8 and 16 h p.i., the loss of APC1 from the complex may be associated with the altered localization of either APC1 or other APC subunits. IFA were used to further examine the localization pattern of various APC subunits during an infection time course. Samples were costained for APC3 (mouse monoclonal antibody) along with APC1, APC7, APC8, or APC10 (rabbit polyclonal antibodies) and were analyzed by deconvolution microscopy. Hoechst dye was added to visualize the nuclei of the cells. Mouse and rabbit IgG, which were used as respective negative controls, gave minimal background staining (Fig. 6). As expected, the APC subunits all showed a predominantly nuclear staining pattern in mock-infected cells, which remained unchanged between 8 and 16 h p.i. At 8 h p.i., both APC1 and APC7 remained relatively colocalized with APC3 in the nuclei of the infected cells. However, as the infection progressed, APC3 and APC7 showed a more cytoplasmic staining pattern, whereas APC1 remained primarily nuclear. A similar cytoplasmic redistribution in HCMV-infected cells also was observed for APC8 and APC10 (data not shown). These results provide additional evidence that APC1 becomes dissociated from the complex during the infection. We could not determine if the other APC subunits showed a similar cytoplasmic

FIG. 4. Levels of some APC core subunits increase during HCMV infection. Mock-infected (M) or HCMV-infected (V) HFFs were harvested at the times indicated and were processed by Western blot analysis with antibodies to APC3, APC7, APC8, APC10, APC11, and APC1. Actin was used as a loading control.

FIG. 5. APC becomes destabilized during the course of HCMV infection. Mock-infected (M) or HCMV-infected (V) HFFs were harvested at the times shown and were immunoprecipitated for APC3. Western blots were used to identify coprecipitating APC subunits with antibodies to APC1, APC3, APC8, APC7, and Cdh1. The Cdh1 blot for the 8-h time point is slightly overexposed to show the presence of coprecipitating Cdh1 in the infected cells. GAPDH is shown as a negative control. Pre, input lysate; PC, IgG IP; IP, APC3 IP; PIP, post-IP.

pattern of localization due to a lack of antibodies that were suitable for immunostaining. These studies suggest that both destabilization of the complex and altered localization could account for the loss in APC activity and accumulation of its substrates, which are primarily nuclear proteins.

DISCUSSION

The APC plays a key role in cell cycle regulation by targeting the degradation of specific cell cycle proteins in a timely manner. Not surprisingly, viruses target the APC as they manipu-

FIG. 6. Several APC subunits are relocalized to the cytoplasm of HCMV-infected cells. Mock- or HCMV-infected HFFs at 8, 12, and 16 h p.i. were fixed and assayed by immunostaining for APC1, APC3, and APC7. Mouse and rabbit IgG were used as negative controls on HCMV-infected cells from the 8-h-p.i. time point. Samples were analyzed by deconvolution microscopy using a 100 $\times$ oil immersion lens, with pictures taken at 0.2- μ m sections along the z axis. A midsectional plane of representative cells is shown. Images are separated to show the staining of each individual subunit; merged images also are shown with Hoechst staining in blue.

FIG. 7. APC is inactivated through multiple mechanisms upon HCMV infection. (A) Schematic diagram of activated APC. Subunits are numbered accordingly. Unphosphorylated Cdh1 associates with and activates the complex, allowing the ubiquitination of the recruited substrate in concert with E1 and E2 ubiquitination enzymes. The relative location of the subunits is based on the model presented by Thornton et al. (38). (B) Model of APC upon HCMV infection. Cdh1 is phosphorylated (P) and no longer associates with the complex. APC1 becomes dissociated from the complex. It is unclear whether the subunits other than APC3, APC7, and APC8 (shaded) remain in complex together.

late the host cell cycle to facilitate their own replication (14). Adenovirus E4orf4 has been implicated in targeting phosphatase PP2A to APC6 to inactivate the complex through dephosphorylation (17). The chicken anemia virus apoptin protein also has been shown to target, and perhaps sequester, APC1, causing complex destabilization and G₂/M arrest (15, 37). Similarly, HCMV appears to specifically inhibit the APC in the process of creating a cellular environment more conducive to viral replication. Previous work indicated that Cdh1 no longer binds to APC3 as the infection progresses, suggesting that the loss in APC function is due to the lack of activation by Cdh1 (42). Our studies show that this is only one of the multiple effects of the infection on the APC that could be responsible for its inactivation.

We find that Cdh1 becomes phosphorylated early after HCMV infection. In normal uninfected cells, Cdh1 phosphorylation and its subsequent dissociation from the complex are key mechanisms in mediating APC inactivation as the cells transition from G₀/G₁ to S phase. The phosphorylation of Cdh1 in the infected cells would not be surprising given the heightened state of CDK activity during the infection. However, treatment with the CDK inhibitor roscovitine did not inhibit Cdh1 phosphorylation, although it did affect its accumulation. This implies that other kinases are involved in phosphorylating Cdh1 in infected cells. Alternatively, these results could be attributed to the indirect effects the drug has on the infection. We also noted that the addition of roscovitine at the beginning of the infection prevented the accumulation of not only Cdh1 but also two other APC substrates, geminin and Cdc6. Roscovitine had less effect on the accumulation of these proteins when administered at 4 or 8 h p.i. There are several explanations for this result, and they are not mutually exclusive. As our laboratory and others have shown, roscovitine severely reduces viral replication (3, 31, 32). Addition of the drug at the time of infection alters IE gene expression such that IE2-86 expression is enhanced while that of IE1-72 is reduced. Early viral gene expression and viral DNA replication also are inhibited. However, if the drug is added at 6 h p.i., it no longer affects IE1-72 expression, and early gene expression along with viral DNA replication is restored (31). Thus, some

viral early gene expression may be necessary for inactivation of the APC. The kinetics of stabilization of the APC substrates (beginning around 8 h p.i.) provides support for this (1, 16, 30, 42, 45). Alternatively, CDK activity may be required for the accumulation of some APC substrates due to direct effects on phosphorylation of other APC subunits or other proteins involved in the ubiquitin-proteasome degradation pathway. It also is possible that the drug affects the levels of RNA. The latter two possibilities may apply to both infected and uninfected cells, as the levels of geminin also were lower in the uninfected cells treated with the drug during all of the intervals. A small decrease in Cdc6 also was observed in the treated uninfected cells, although the levels were at the limit of detection in both the treated and untreated cells. Taken together, the results show that the phosphorylation of Cdh1 in infected cells is not CDK dependent, but the accumulation of the APC substrates may be partially affected, either directly or indirectly, by the inhibition of CDK activity.

While phosphorylation of Cdh1 in infected cells may contribute to its lack of association with the APC, we also found that exogenous TNT-synthesized Cdh1 had decreased binding affinity for APC3 in lysates obtained from infected cells as the infection progressed from 8 to 16 h p.i.; however, there was little change in binding to APC3 in uninfected cell lysates at any time point. APC3 from the uninfected cell lysates still was able to bind more ³⁵S-Cdh1 despite potentially competing cellular Cdh1, whereas this would not have been a factor in the infected cells at 16 h p.i. based on the APC3 coimmunoprecipitation data. These results indicate that the core APC in infected cells is no longer capable of associating with the activator as the infection progresses. While unlikely, we cannot exclude the possibility that ³⁵S-Cdh1 was modified by a factor in the infected-cell lysate.

In accord with the *in vitro* binding experiments using exogenous Cdh1, we demonstrate by coimmunoprecipitation assays that APC1 becomes dissociated from the TPR subunits with similar kinetics. Recent studies have further defined the intricate architecture of the APC (7, 10, 27, 38). The complex is composed of two main subcomplexes, one containing the catalytic core (i.e., APC2 and APC11) and the other containing the TPR

subunits (i.e., APC3, APC6, APC7, and APC8), that are bridged by APC1, APC4, and APC5 (39) (Fig. 7A). The binding between APC1, APC4, APC5, and the TPR subunit APC8 also is interdependent, in that each subunit is required for the association of the other three (38). Without APC1, the overall structure of the APC would be greatly affected, as the two subcomplexes likely would be separated. Since full binding of Cdh1 to the APC is dependent on both APC3 and APC2 (38), the dissociation of the core complex also could account for the inability of Cdh1 to bind the complex and for the lack of APC activity. Interestingly, previous studies have suggested that the APC contains multiple copies of the TPR subunits (7, 27) and that these TPR subunits remain assembled even in the absence of APC1 (38). This correlates with our finding that APC3, APC7, and APC8 still remained in complex together despite the dissociation of APC1 upon HCMV infection.

We further showed by IFA that several APC subunits relocalized to the cytoplasm as the infection progressed, while APC1 remained nuclear. It is unclear whether the dissociation of APC1 causes the other subunits to disperse into the cytoplasm or whether it is the TPR subcomplex that is dissociating from the rest of the APC.

An important question raised by these studies is the following: why does HCMV destabilize the APC? There are at least three different possibilities. First, to inhibit host cell functions or promote viral replication, the virus may require high levels of cellular proteins that normally would be ubiquitinated by the APC and degraded by the proteasome. An example might be the premature accumulation of geminin, which inhibits the licensing of cellular origins of DNA replication. A second possibility is that there may be essential viral proteins that would be targeted for degradation by a functional APC. Finally, one or more of the individual APC subunits may need to be recruited for a specific role in the viral infection. Studies are currently in progress to address this question and to further elucidate the molecular mechanisms by which the APC is destabilized.

In summary, multiple mechanisms appear to be involved in inactivating the APC upon HCMV infection, including dissociation of the core APC and the relocalization of some subunits to the cytoplasm of the infected cells, beginning 8 to 12 h p.i. This time frame also correlates with the observed accumulation of APC substrates (1, 16, 30, 42, 45) and loss of APC activity (42). Although it is unknown at this time whether these events are interdependent or represent redundant pathways, they underscore the importance of disabling the APC during the infection.

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