Degradation of Nucleosome-associated Centromeric Histone H3-like Protein CENP-A Induced by Herpes Simplex Virus Type 1 Protein ICP0*

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Cells infected by herpes simplex virus type 1 in the G2 phase of the cell cycle become stalled at an unusual stage of mitosis defined as pseudoprometaphase. This block correlates with the viral immediate-early protein ICP0-induced degradation of the centromere protein CENP-C. However, the observed pseudoprometaphase phenotype of infected mitotic cells suggests that the stability of other centromere proteins may also be affected. Here, we demonstrate that ICP0 also induces the proteasome-dependent degradation of the centromere protein CENP-A. By a series of Western blot and immunofluorescence experiments we show that the endogenous 17-kDa CENP-A and an exogenous tagged version of CENP-A are lost from centromeres and degraded in infected and transfected cells as a result of ICP0 expression. CENP-A is a histone H3-like protein associated with nucleosome structures in the inner plate of the kinetochore. Unlike fully transcribed lytic viral DNA, the transcriptionally repressed latent herpes simplex virus type 1 genome has been reported to have a nucleosomal structure similar to that of cellular chromatin. Because ICP0 plays an essential part in controlling the balance between the lytic and latent outcomes of infection, the ICP0-induced degradation of CENP-A is an intriguing feature connecting different aspects of viral and/or cellular genome regulation.

Herpes simplex virus type 1 (HSV-1) is a major human pathogen whose lifestyle is based on a long-term hide-and-seek interaction with the infected host. After initial infection at the periphery, the virus enters sensory neurons and establishes a lifelong latent infection (for review, see Ref. 1). Periodic reactivation from latency usually results in mild illness symptoms such as cold sores. These episodes of reactivation have enabled the virus to evolve optimally in parallel with the host. During lytic infection, the HSV-1 152-kilobase pair double-stranded DNA genome expresses at least 74 temporarily regulated genes. These genes are classified as immediate-early (IE), early, and late, depending on the time course of their synthesis and requirement for prior viral gene expression and DNA replication. During latency, the viral genome undergoes dramatic changes resulting in an almost complete silencing of transcription (for review, see Ref. 2). After successful penetration of the cell and release of viral DNA in the nucleus, the first step of a productive infection is the synthesis of IE proteins. Four of the five IE proteins encoded by HSV-1 regulate gene expression. ICP4 and ICP27 are essential for virus replication (3, 4), whereas ICP22 is dispensable for virus viability in most cell types (5). The requirement for ICP0 (also called Vmw110) for the onset of lytic infection is not absolute. Viruses either deficient for its expression or expressing an inactivated form of the protein, rather than being noninfectious in cultured cells, show a cell type- and multiplicity-dependent growth defect (6). ICP0 appears to increase the probability of the initiation of productive infection. This feature assumes greater significance during reactivation of the virus from latency, because it has been shown that in the absence of ICP0 the probability for mutant viruses to reactivate is dramatically decreased both in cultured cells and in mouse models (7, 8). This defect can be overcome in cultured cells by providing exogenous ICP0 (9–12).

ICP0 is a RING finger zinc-binding protein that was initially studied because of its transactivation activity in transfection assays (reviewed in Ref. 13). The RING finger domain has been shown to be essential for biological activities of ICP0 outlined above (for review, see Ref. 14). New fields of interests arose after the discovery that ICP0 localizes to, and then disrupts, nuclear domains called ND10, nuclear dots, PML nuclear bodies, or promyelocytic oncogenic domains (15–17). More recently, it was observed that centromeres are also targeted by ICP0 early in infection. Centromeres contain a number of specific proteins (reviewed in Ref. 18), several of which are recognized by anti-centromere autoantibodies in the sera of patients with a variety of conditions (19). CENP-B (80 kDa) is a sequence-specific α-satellite DNA-binding protein that localizes throughout the centromeric heterochromatin located beneath the kinetochore (20, 21). CENP-C (140 kDa) is an essential component of the inner kinetochore plate of active centromeres (22, 23) that is required for maintaining proper kinetochore size and has been implicated in the metaphase-anaphase transition in mitotic cells by stabilizing microtubule attachments (24). Because the 17-kDa CENP-A protein localizes in centromeres and copurifies with nucleosome core particles, it was suggested to function as a centromere-specific core
treated with appropriate secondary antibodies. After a further 30-min wash, coverslips were washed at least three times with PBS plus 1% newborn calf serum, and then incubated at room temperature for 1 h. Coverslips were washed at least three times with PBS containing 1% newborn calf serum. After incubation at room temperature for 1 h, coverslips were washed at least three times with PBS containing 0.5% Nonidet P-40 and 10% sucrose. Primary antibodies were diluted in PBS containing 1% newborn calf serum.

After incubation at room temperature for 1 h, coverslips were washed at least three times with PBS containing 0.5% Nonidet P-40, 0.5 mM dithiothreitol) and incubated on ice for 30 min. After centrifugation at 6000 × g for 4 min, supernatants were removed, and pellets containing nuclei were resuspended in 70 µl of Laemmli buffer and boiled for 5 min. SDS-polyacrylamide gels (12.5%) were prepared and run in the Bio-Rad MiniProtein II apparatus, and then proteins were electrotransfered to nitrocellulose membranes (BA85, Schleicher & Schuell) according to the manufacturer’s recommendations. After blocking in PBS containing 0.1% Tween 20, 5% dried milk overnight at 4 °C, filters were soaked in improved bioluminescent reagent in PBST, 2% dried milk at room temperature and then washed in PBST at least three times before incubation with horseradish peroxidase-conjugated secondary antibody in PBST, 2% dried milk at room temperature for 1 h. After extensive washing, filters were soaked in improved bioluminescent reagent (PerkinElmer Life Sciences) and exposed to film. Primary antibodies were as follows: huACA serum (dilution 1:10,000), mAb 12CA5 (Roche Molecular Biochemicals) against HA-1 epitope (dilution 1 µg/ml).

Western Blotting—HFL cells were seeded at 1 × 10⁵ cells per well in 24-well Linbro multwell plates and infected 48 h later. HeLa TTA-CAHA cells were seeded at 0.5 × 10⁵ cells per well in medium not containing tetracycline to induce synthesis of CENP-A-HA. Forty-eight hours later, medium was replaced by fresh medium containing 0.5 µg/ml tetracycline to repress synthesis of CENP-A-HA for 24 h. Cells were then infected and harvested for Western blotting. For the preparation of nuclear fractions, HFL cells were harvested after infection in 100 µl of a hypotonic buffer (20 mM HEPES (pH 7.4), 1 mM MgCl₂, 10 mM KCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol) and incubated on ice for 30 min. After centrifugation at 6000 × g for 4 min, supernatants were removed, and pellets containing nuclei were resuspended in 70 µl of Laemmli buffer and boiled for 5 min. SDS-polyacrylamide gels (12.5%) were prepared and run in the Bio-Rad MiniProtein II apparatus, and then proteins were electrotransfered to nitrocellulose membranes (BA85, Schleicher & Schuell) according to the manufacturer’s recommendations. After blocking in PBS containing 0.1% Tween 20, 5% dried milk overnight at 4 °C, filters were soaked in improved bioluminescent reagent in PBST, 2% dried milk at room temperature and then washed in PBST at least three times before incubation with horseradish peroxidase-conjugated secondary antibody in PBST, 2% dried milk at room temperature for 1 h. After extensive washing, filters were soaked in improved bioluminescent reagent (PerkinElmer Life Sciences) and exposed to film. Primary antibodies were as follows: huACA serum (dilution 1:10,000), mAb 12CA5 (Roche Molecular Biochemicals) against HA-1 epitope (dilution 1 µg/ml).

Cotransfection Experiments—Hep2 cells were seeded at 1 × 10⁵ cells per well in 24-well Linbro multwell plates. The following day, plasmid pcDL CA-HA (40 ng) and plasmid pCIneo, pC1110, or pCIFIXE (350 ng) were cotransfected according to the manufacturer’s recommendations (LipofectAMINE PLUS, Life Technologies, Inc.). Twenty-four h later, cells were harvested for Western blotting. Alternatively, the medium was changed and replaced by fresh complete medium containing the proteasome inhibitor MG132 at a concentration of 5 µM. Cells were then harvested 0, 2, 4, and 8 h post-addition of MG132 for Western blotting.

RESULTS

A 17-kDa Centromere Protein Previously Reported as Being CENP-A Is Degraded as a Result of Infection with a Virus Expressing Functional ICP6, Provided That the Proteasome Pathway Is Active.—To analyze the putative degradation of centromere proteins other than CENP-C during infection, we performed Western blotting of HSV-1-infected cell extracts using a huACA serum that can recognize centromere proteins CENP-A, -B, and -C having molecular masses of 17, 80, and 140 kDa, respectively (19). However, it is not unusual that only one or two of these proteins are predominantly detected by Western blotting, with CENP-A seemingly being the most antigenic. A complex signal was detected at the high molecular weight range because of the recognition of multiple viral proteins by anti-herpesvirus protein antibodies often present in human sera (data not shown). We thus restricted the number of viral proteins synthesized in infected cells using the temperature-sensitive virus tsK and its ICP0-deficient derivative in1330,
which express nonfunctional ICP4 at 38.5 °C and therefore synthesize only the viral IE proteins (3). Coupled with the use of nuclear extracts, this approach limited the number of viral antigens detected by the huACA serum. HFL cells were infected at a multiplicity of infection of 20 plaque-forming units per cell and harvested 1.5, 4, and 6 h post-infection. Analysis by Western blotting demonstrated that a 17-kDa (hereafter referred to as 17K/CENP-A) protein was noticeably diminished by 4 h post-infection in cells infected by tsK (see Fig. 1B). The detection of this protein by the huACA serum and its electrophoretic mobility were consistent with it being CENP-A. 17K/CENP-A was not affected in cells infected by in1330, suggesting that ICP0 was implicated in the effect. The expression of ICP0 in cells infected by tsK but not in1330 was confirmed by stripping and reprobing the membrane with mAb 11060 against ICP0 (data not shown). Parallel analysis demonstrated that, as expected, the ICP0 target proteins CENP-C and PML (32, 41) were also degraded in tsK- but not in1330-infected cells (data not shown). A 15-kDa protein detected beneath 17K/CENP-A by huACA was also detected by rabbit 554 and rabbit-L sera against CENP-C and CENP-B, respectively (21, 24), making it a protein nonspecifically recognized by at least three different sera directed against centromeric proteins (data not shown).

Because we did not expect to detect too many viral antigens at the low molecular weight range that would interfere with the detection of 17K/CENP-A, we then performed Western blotting on HFL cells infected by HSV-1 viruses expressing the wild type (17+) or the RING finger mutant (FXE) version of ICP0—ICP4ts or in1330 (ICP0–ICP4ts) in the presence (+) or absence (−) of the proteasome inhibitor MG132. Proteins from cell nuclear extracts were harvested 1.5, 4, and 6 (B) or 8 (A) h post-infection and analyzed by Western blotting using huACA serum. hpi, hours post-infection. MW corresponds to molecular mass markers 220, 97.4, 66, 46, 30, 21.5, and 14.3 kDa indicated by dashes down the left-hand side of the panels.

Recently several cellular proteins have been reported to undergo degradation in an ICP0- and proteasome-dependent manner during HSV-1 infection. We thus checked whether 17K/CENP-A was degraded via the same pathway. We performed infections of HFL cells in the presence or absence of proteasome inhibitor MG132 under conditions that do not interfere with viral gene expression (41). Fig. 1B shows that, as expected, the 17K/CENP-A degradation was sensitive to the proteasome inhibitor MG132. These results show that a 17-kDa protein recognized by huACA antibodies, which is most likely CENP-A, is degraded in infected cells with an efficiency correlating with the amount of ICP0 synthesized, and this effect is dependent on the presence of ICP0 and its RING finger domain and on an active proteasome pathway.

CENP-A Is Lost from Centromeres in Wild Type but Not FXE-or dl1403-infected Cells—To visualize the effects of infection on the nuclear distribution of CENP-A, immunofluorescence was performed on Hep2 cells infected for 2 or 8 h with viruses 17+, dl1403, or FXE. CENP-A, centromeres, and infected cells were detected using a monospecific rabbit serum, huACA, and monoclonal antibodies against ICP0 or ICP4, respectively. Two h post-infection, CENP-A was still present at centromeres of infected cells (data not shown). Eight h post-infection, CENP-A was no longer detected at centromeres of cells infected by wild type virus (Fig. 2A, panels A–D), whereas those infected by dl1403 (Fig. 2A, panels E–H) or FXE (Fig. 2A, panels I–L) still retained CENP-A in centromeres. Of note is the decrease in centromere labeling by the huACA serum in wild type virus-infected cells, which is probably because CENP-A is one of the major antigens recognized by this serum (19). The addition of MG132 at the start of the infection abrogated the disappear-
The loss of the protein CENP-A from centromeres of infected cells is dependent on the expression of functional ICP0. Subconfluent Hep2 cells were infected by 17+, dl1403, or FXE viruses. Eight h post-infection, cells were fixed and treated for immunofluorescence using mAbs 11060 against ICP0 (red in A, green in B) and 58S against ICP4 (red), monospecific anti-CENP-A rabbit serum (green in A, red in B), and huACA serum (blue). A, panels A–D, cells infected with the wild type 17+ virus. The upper right cell is not infected and shows the colocalization of CENP-A with centromeres. Panels E–H, cells infected with the ICP0-null mutant dl1403 detected by the expression of ICP4 (the upper left cell is not infected). Panels I–L, cells infected with the ICP0 RING finger mutant FXE. The presence of CENP-A in centromeres is shown by the light blue dots in the merge figures resulting from the colocalization of CENP-A and centromere proteins. B, panel A, a wild type virus-infected cell in pseudoprometaphase showing the loss of CENP-A from centromeres in cells with this phenotype. Panel B, a rare wild type virus-infected cell in metaphase, with CENP-A still present at centromeres. Panel C, a dl1403 infected cell in the common anaphase-telophase phenotype, with CENP-A still present at centromeres. The presence of CENP-A at centromeres is shown by the pink dots resulting from the colocalization of CENP-A and centromere proteins. The bars represent 5 μm. Centr, centromeres.
likely that CENP-A is required at centromeres for a mitotic cell to reach metaphase, because in its absence the phenotype most commonly observed in wild type HSV-1-infected mitotic cells is pseudoprometaphase.

ICP0 Specifically Induces the Loss of CENP-A from Centromeres—ICP0 alone was able to induce the loss of CENP-A from centromeres, because Hep2 cells transfected with plasmids expressing wild type ICP0 or its RING finger mutant FXE gave results similar to those observed in infected cells (Fig. 3). These results confirm the essential role played by ICP0 and rule out any requirement for other viral proteins. As expected, the RING finger mutant protein FXE (which does not localize in centromeres) had no effect on the distribution of CENP-A.

ICP0 Specifically Induces the Degradation of CENP-A—The results obtained so far have shown that HSV-1 is able to induce the degradation of the 17K/CENP-A protein recognized by huACA serum in an ICP0- and proteasome-dependent manner. These results correlate with the loss of CENP-A from centromeres both in infected and transfected cells. The rabbit anti-CENP-A serum used in the immunofluorescence studies gave poor results in our Western blotting experiments; so to demonstrate unequivocally that CENP-A is degraded during HSV-1 infection and to show that ICP0 alone was responsible for that effect, we used either HeLa tTA-CAHA cells, which constitutively express an influenza hemagglutinin (HA) epitope-tagged derivative of CENP-A (CENP-A-HA), or cells transfected with plasmid pcDLCA-HA, which expresses the same protein (26, 27). Prior to infection, HeLa tTA-CAHA cells were treated as described under “Experimental Procedures” to induce CENP-A-HA expression. Fig. 4A shows a significant reduction of the amount of CENP-A-HA protein in cells infected by the tsK virus expressing wild type ICP0. This effect was abrogated in the presence of MG132 (data not shown) and did not occur in in1330-infected cells. The relatively less efficient degradation of CENP-A-HA compared with 17K/CENP-A could be explained by the somewhat exogenous nature of CENP-A-HA, whose synthesis from a constitutive promoter could increase its amount compared with endogenous 17K/CENP-A.

Immunofluorescence experiments confirmed the loss of CENP-A-HA from centromeres in tsK- and wild type-infected cells but not in in1330-, dl1403-, or FXE-infected cells, results identical to those observed for endogenous CENP-A (data not shown, but see Fig. 2A). These results perfectly match those observed for the degradation of 17K/CENP-A and demonstrate that CENP-A is effectively degraded during infection in an ICP0- and proteasome-dependent manner.

To demonstrate that ICP0 could induce the degradation of CENP-A by itself, Hep2 cells were cotransfected with plasmids pcDLCA-HA and pCIneo, pCI110, or pCIFXE and harvested for Western blotting 24 h later. Fig. 4B shows the decrease in the intensity of the CENP-A-HA band in cells cotransfected with pCI110 but not pCIFXE, which express wild type ICP0 and the RING finger mutant protein FXE, respectively. To confirm that the CENP-A-HA ICP0-induced degradation occurred via the active proteasome pathway, MG132 was added to the samples 24 h post-transfection for a period of time between 0 and 8 h. The idea was that inhibition of the proteasome activity with MG132 should stabilize CENP-A-HA, resulting in a re-increase of the CENP-A-HA signal even in the presence of ICP0. Fig. 4C shows that the amount of CENP-A-HA in cells coexpressing ICP0 increases as early as 2 h post-addition of MG132 to reach a maximum after 8 h. The amount of CENP-A-HA never returned to the level observed in cells cotransfected with pCIneo, but this might be expected considering the time period of the experiment. MG132 had no effect on the expression of CENP-A in cells cotransfected with pCIneo. These results confirm the essential role of ICP0 and its RING finger domain in the induced degradation of CENP-A and correlate with the immunofluorescence data showing that ICP0 alone is able to induce the loss of CENP-A from centromeres.

**DISCUSSION**

We recently observed that ICP0 blocks the progression of cells through mitosis, and we suggested that this effect correlates with the ICP0-induced degradation of at least one centromere protein, CENP-C (33). However, based on previous data (24), we also suggested that the observed pseudoprometaphase phenotype indicated that effects on other centromere functions were also occurring. The present study demonstrates that the 17-kDa centromere protein CENP-A is also degraded in an ICP0- and proteasome-dependent manner both in infected and transfected cells, and this correlates with the loss of CENP-A from centromeres. These new data confirm the previous interpretations about the effects of ICP0 on the stability of several cellular proteins and give new insights into the possible cellular mechanisms acting on viral chromatin structure during virus infection.

ICP0: From Regulator of Gene Transcription to Regulator of Cellular Protein Stability—ICP0 was initially characterized as a broad transactivator of viral and cellular genes and was found to regulate the balance between latent and lytic infection by promoting the onset of lytic gene expression. In its absence, low multiplicity infection of some cultured cell lines leads to viral genomes preferentially maintained in a quiescent or latent state. Early in the infection ICP0 localizes to subnuclear domains called ND10 and then induces their destruction (39). Interestingly, parental HSV-1 genomes (as well as those of several other DNA viruses) preferentially migrate to the periphery of ND10 at the earliest stages of infection (40), which suggests a possible link between the ICP0-induced loss of ND10
and activation of transcription from the viral genome. The destruction of ND10 following infection occurs because PML and Sp100 (two of the major ND10 components), and their SUMO-1 modified isoforms, are degraded in response to ICP0 activity (41–44). ICP0 thus controls the stability of at least two major ND10 proteins via the ubiquitin degradation pathway, an idea consistent with the induction by ICP0 of colocalizing conjugated ubiquitin (45).

More recently, ICP0 has been shown to disrupt centromeres and induce the degradation of the essential centromere protein CENP-C in a similar manner (32). The presence of ICP0 in both ND10 and centromeres could be related to the presence of certain proteins in both structures. These include the heterochromatin-associated protein HP1, which binds to Sp100 (46, 47), and hDaxx, which interacts with both CENP-C and SUMO-1-modified PML (48, 49); both these proteins are dynamically associated with both ND10 and centromeres (50). Because Sp100, HP1, and hDaxx have all been implicated in the repression of gene expression (47, 51, 52), the association of parental viral genomes in the vicinity of structures rich in these proteins assumes considerable significance, especially because they or their binding partners are degraded in response to ICP0 expression. The idea that these structures and proteins may in some way be involved in repression of viral gene expression and that ICP0 therefore relieves this repression is very compelling.

New Implications from the ICP0-induced Degradation of CENP-A—Our previous study describing the ICP0-induced degradation of CENP-C raised a number of questions concerning the mechanism and implications of this effect. Because two of the first ICP0 target proteins to be identified were both modified by SUMO-1, we previously suggested that SUMO-1-modified proteins may in some way be targeted by ICP0. Because of previous evidence linking proteins related to CENP-C and SUMO-1 in yeast, we considered it possible that CENP-C may be modified by SUMO-1 and thus be an ICP0 target. However, given that the CENP-A calculated molecular mass is about 16 kDa (26) and that a monomer of SUMO-1 has a gel mobility of about 22 kDa (53), it is highly unlikely that the 17K/CENP-A band is modified by SUMO-1. Therefore SUMO-1 modification is unnecessary for a protein to be degraded in response to ICP0 expression, a conclusion consistent with the lack of evidence that the catalytic subunit of DNA-PK, another ICP0 target protein, is modified by SUMO-1 (54).

We also suggested that the ICP0-induced loss of CENP-C would inevitably have serious consequences for kinetochore structure and function during mitosis. The additional degradation of CENP-A underlines this previous conclusion and confirms the prediction that the stability of centromere components other than CENP-C would also be affected by HSV-1 infection. Our results are consistent with the suggestion of Tomkiel et al. (24) that the pseudoprometaphase arrest phenotype could be due to the inactivation of CENP-A. The observations of Fig. 2B provide additional support for this hypothesis, because they show that CENP-A is absent from centromeres of HSV-1-infected cells in pseudoprometaphase, whereas in the very rare infected cells in metaphase, CENP-A was still present.

The Significance of CENP-A Degradation—CENP-A is a particularly intriguing target for ICP0-induced degradation. It is highly unlikely that CENP-A (or CENP-C) is an “innocent bystander” that happens to be degraded because it is present in the wrong place at the wrong time. Indeed, whereas CENP-A (or CENP-C; 32) is removed from centromeres of HSV-1-infected cells, previous data showed that CENP-B seems not to be affected (32), a result consistent with the detection of centromere antigens by huACA serum. On the other hand, it has been shown that whereas most of the viral DNA found in the nervous system of acutely infected mice is not assembled into a regular nucleosomal structure (55), during latency the viral genome is closely associated with nucleosome structures (56). These studies suggested that this association could be part of the mechanism by which viral genomes are repressed during latency. It is an exciting prospect to determine whether CENP-A could be a component of these latent viral genome-
associated nucleosomal structures. This is particularly pertinent because, firstly, CENP-A can associate not only with α-satellite DNA independently of its sequence (22, 25, 26, 28), but also with other chromosomal loci that are not normally used for centromere assembly (for reviews, see Ref. 57), and secondly, the association of CENP-A with heterochromatic DNA is not restricted to human DNA (58). These recent observations suggest at least the possibility that the chromatin structure of latent viral DNA could include CENP-A-containing nucleosomes correlating with a heterochromatic state that would silence viral gene expression.

This study shows for the first time that a nucleosome-associated heterochromatin protein is targeted for degradation by a viral protein known for its key role in the reactivation of the virus from latency. In addition to the potential use of ICP0 for a better understanding of the role of CENP-A in the cell, this study might be of considerable significance with respect to several aspects of viral and/or cellular gene expression, including association of DNA with alternative nucleosome-like structures, repression of gene transcription, heterochromatin assembly, mechanisms of cellular defense against viral infection, and latency.

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