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Centromeric protein CENP-B proteasomal degradation induced by the viral protein ICP0

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Abstract **The ICP0 protein of herpes simplex virus type 1 (HSV-1) is a nuclear protein that possesses a well-characterized E3 ubiquitin ligase activity. This activity is responsible for the proteasomal-dependent degradation of several cellular proteins. This study shows that ICP0 induces the proteasomal-dependent degradation of the centromeric protein CENP-B in infected as well as ICP0-expressing cells. It is also shown that the ICP0-induced CENP-B degradation occurs as efficiently in human and mouse cells. CENP-B is one of the major proteins of centromeres and its degradation is likely to contribute to the severe damage induced to centromeres by ICP0.**

Keywords: Nucleus; Centromeres; CENP-B; ICP0; HSV-1

1. Introduction

Centromeres are specialized chromosomal regions whose structure is the support for the building of the kinetochore, which is the attachment site of spindle microtubules during meiosis and mitosis. They are made of both specific AT-rich alpha-satellite (or alphoid) tandem DNA repeats and constitutive proteins present throughout the cell cycle. Human centromeres are specified by two regions on the basis of DNA and protein content, (i) the central core region, which is exclusively formed by type I alpha-satellite 171-bp repeated DNA associated with several specific centromeric proteins (CENPs), (ii) the peri-centromere which is composed of a mix of type I alpha-satellite DNA as well as other divergent alphoid sequences associated with classical histones in a heterochromatic conformation. One of the major features of the central core centromere, at the chromatin structure level, is the presence of nucleosomes containing the histone H3-like protein CENP-A, in addition to classical histone H3-containing nucleosomes. Moreover, type I alpha-satellite DNA includes a specific 17-nt sequence, called the "CENP-B box", serving as an anchor platform for the DNA-binding protein CENP-B. CENP-B could be considered as the historical centromere protein as it was the first described constitutive CENP. Its precise role in the centromere structure has not yet been determined but some data obtained in vitro highly suggests that it is implicated in the positioning of centromeric nucleosomes [1,2].

The herpes simplex virus type 1 (HSV-1) is a common human pathogen (for a review see [3]). ICP0 is an important nuclear protein synthesized during infection by HSV-1 (for reviews see [4,5]). It contains a RING finger zinc-binding domain, which confers to ICP0 an E3 ubiquitin ligase activity implicated in the proteasomal degradation of a series of cellular proteins [4–6]. As soon as ICP0 enters the nucleus it specifically and temporarily localizes in two nuclear domains, i.e., the PML bodies (also called ND10), and the centromeres. Immediately, ICP0 provokes the proteasomal degradation of the PML protein and of the major centromeric proteins, CENP-A and -C. The consequences being the disappearance of the PML bodies, and the destabilization of the centromeres, provoking mitotic defects [7–10]. In this study we describe, in human and mouse cells, the ICP0-induced proteasomal degradation of a third CENP, the CENP-B protein.

2. Materials and methods

Cell lines, plasmids and viruses. HeLa cells were cultivated in BHK-21 medium, NIH3T3 in DMEM medium; HeLa T-REX (Invitrogen) and TREX-ICP0 (TR-ICP0.39) in MEM, all media complemented with at least 10% fetal calf serum, L-Glutamin (1% v/v), 10 U/ml of penicillin, 100 μg/ml of streptomycin; for T-REX, blasticidin was added to the medium (5 μg/ml); for TR-ICP0.39 blasticidin and zeocin (100 μg/ml) were added. The wild type strain HSV-1 17syn+ (HSV-1wt) is the parental strain used in this study. The viruses dl1403, deleted of ICP0 [11], and vFXE, expressing an ICP0 isoform mutated in its RING finger domain [12] were also used.

TR-ICP0.39 cell line, stably and inducibly expressing ICP0, was constructed by transfecting T-REX cells with a pcDNA 4/TO plasmid (Invitrogen) designed to express the ICP0 gene. Cell clones were then isolated under zeocin selection. Expression of ICP0 was induced by addition of tetracycline (1 μg/ml) to the medium, and checked in several clones by Western blotting and immunocytochemistry.

Immunofluorescence. TR-ICP0.39 and NIH3T3 cells were seeded at 2.5×10^5 and 7.5×10^4 cells per well, respectively, in 24 well plates containing a round coverslip prior immunofluorescence (IF) assay. Cells were treated for IF as described in [9].

Western blotting. HeLa (5×10^5 cells) or NIH3T3 (3×10^5 cells) in 35 mm Petri dish were infected for 6 h at a multiplicity of infection of 10 (all cells are infected) by the virus HSV-1wt, vFXE or dl1403, in the presence or not of proteasome inhibitor MG132 (2.5 μM). For TR-ICP0 cells, cells were seeded at 1×10^6 cells per 60 mm Petri dish then the following day tetracycline was added or not to the medium for 24 h. Forty micrograms of total proteins were loaded per well of a SDS–polyacrylamide gel, before running, transfer and detection as described in [13].

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Antibodies. Antibodies used for immunofluorescence (IF) and Western blotting (WB) experiments are the following: monoclonal antibodies (mAbs) [11 060] anti-ICP0 (1/1000, IF; 1/10 000, WB), [3–19] anti-CENP-A (1 μg/ml, WB; Abacm), [5E6C1] anti-CENP-B (1/1000, IF; 1 μg/ml WB), [5E10] anti-PML (1/50, IF); rabbit polyclonal antibodies R190 anti-ICP0 (1/200, IF), anti-CENP-A (1/200, IF; Upstate), r554 anti-CENP-C (1/1000, IF and WB), anti-actin (1 μg/ml, WB; Sigma); a human autoimmune serum huACA against centromeric proteins was also used (1/3000, IF in human cells; 1/500, IF in mouse cells). For IF secondary antibodies used were goat anti-rabbit, anti-mouse, and anti-human coupled to AlexaFluor 488, 555, or 647 (1/200; Molecular probes).

3. Results and discussion

In a previous study describing the ICP0 and proteasomal-induced degradation of the centromeric CENP-C protein [7], we suggested that CENP-B was not destabilized by ICP0 because, by immunocytochemistry, a CENP-B signal was still present at the centromeres in ICP0-expressing cells. Because we know that CENP-A stability is also affected by ICP0 [9], we wanted to have a clear mind concerning the truthfulness of the existing stability discrepancy among different CENPs with regard to ICP0 activity. Western blotting was then performed on infected HeLa cells to analyze the CENP-B stability (Fig. 1). The CENP-B signal greatly diminished in HSV-lwt infected cells in the absence of the proteasome inhibitor MG132 (compare lanes 3 and 4 in Fig. 1). Moreover infection by the ICP0 mutant viruses, dll403 and vFXE, had no effect on CENP-B stability. As a positive control, CENP-C was degraded in HSV-lwt infected cells in the absence of MG132, as previously shown. This result suggests that unexpectedly, and unlike our previous suggestion, CENP-B is degraded in infected cells. We then decided to go further in our investigations.

By immunocytochemistry, the disappearance of CENP-A and CENP-C signals are clearly visible at a single cell level, in transfected cells expressing ICP0. However, it has never been possible to attest by Western blotting the effect of ICP0 on endogenous CENPs stability in transfected cells, because of the low amount of cells (less than 30%) expressing ICP0 by transfection. To avoid this problem we decided to construct a cell line stably and, inducibly expressing ICP0 (see Section 2). One clone named TR-ICP0.39 was selected on the basis of three criteria: (i) ICP0 expression was null unless tetracycline was added to the medium, (ii) ICP0 was expressed in 100% of the cells, (iii) ICP0 expression was low, but enough to confer its biological activities, especially on the induction of degradation of cellular proteins. In the absence of tetracycline no ICP0 was detectable by immunofluorescence (data not shown). Addition of tetracycline set off the expression of ICP0 (Fig. 2Aii, iv, vi; green labelling). As expected, CENP-A, -C, and PML signals disappeared in the presence, but not in the absence, of tetracycline (compare Fig. 2Ai, iii, v and 2Aii, iv, vi). It was verified that tetracycline on its own did not affect the stability of these proteins (data not shown). We then performed Western blotting to detect CENP-A, -B, and -C degradation. In the absence of tetracycline no ICP0 was detectable, which matched with the observations made by immunofluorescence. In these conditions all three centromeric proteins were easily detectable (Fig. 2B, lane 1). When tetracycline was added, ICP0 became detectable and, as expected, CENP-A and -C amounts were significantly decreased (Fig. 2B, lane 2). The CENP-B signal also decreased, which supported our observations made in infected cells about an ICP0-induced degradation of this protein. The addition of MG132 restored the corresponding signals in accordance with ICP0 acting through the ubiquitin-proteasome pathway (Fig. 2B, lane 3). These results are important for two reasons. Firstly, because this is the first time that we are able to formally show that ICP0 on its own (outside of an infection) targets endogenous CENPs for proteasomal degradation. Secondly, because it highly suggests that CENP-B stability is affected by ICP0, similarly to CENP-A and -C.

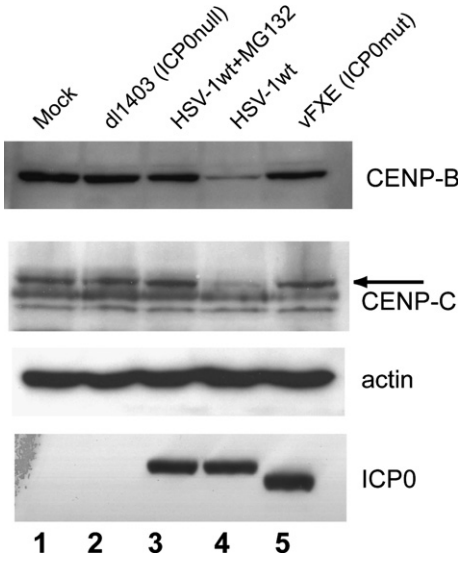

Fig. 1. The CENP-B signal decreases in human cells infected with HSV-1wt. HeLa cells were all infected (multiplicity of infection of 10) with viruses HSV-1wt (in the presence or not of proteasome inhibitor MG132), dl1403 (ICP0 null), or vFXE (ICP0 mut) for 6 h. Western blotting was performed for the detection of CENP-B, and -C (arrow), ICP0, and actin as loading control.

However it remains true that, using an immunocytochemistry approach, the human cells expressing ICP0 (infected, transfected or tetracycline-induced TR-ICP0.39 cells) do not show a clear disappearance of the CENP-B signal, unlike that of CENP-A and -C (data not shown, but see Fig. 2C and H in [7]). The reasons for such a discrepancy might be of two orders. Firstly, for unknown reasons ICP0 could induce CENP-B degradation less efficiently than CENP-A and -C. Secondly, ICP0 could target CENP-B for proteasomal degradation as efficiently as CENP-A and -C, but the different CENP-B localization in the centromere compared to CENP-A and -C, suggests that part of the pool of CENP-B is not destabilized by ICP0. Indeed, type I alpha-satellite sequences containing the "CENP-B box" are part of the central core centromere but also extend beyond to flanking regions. This suggests that, unlike for CENP-A and -C, the CENP-B localization on the centromere might be a lot wider then the region covered by ICP0. According to this, CENP-B was found, by electron microscopy, to localize also beneath and beside the central core centromere region [14]. Our different studies describing centromeric ICP0 localization tend to show a localization of ICP0 restricted to the central core centromere and not to the adjacent peri-centromeric regions. If we consider that ICP0 only ubiquitinates efficiently proteins being in its immediate vicinity, it is reasonable to anticipate a partial degradation of the CENP-B protein present on centromeres.

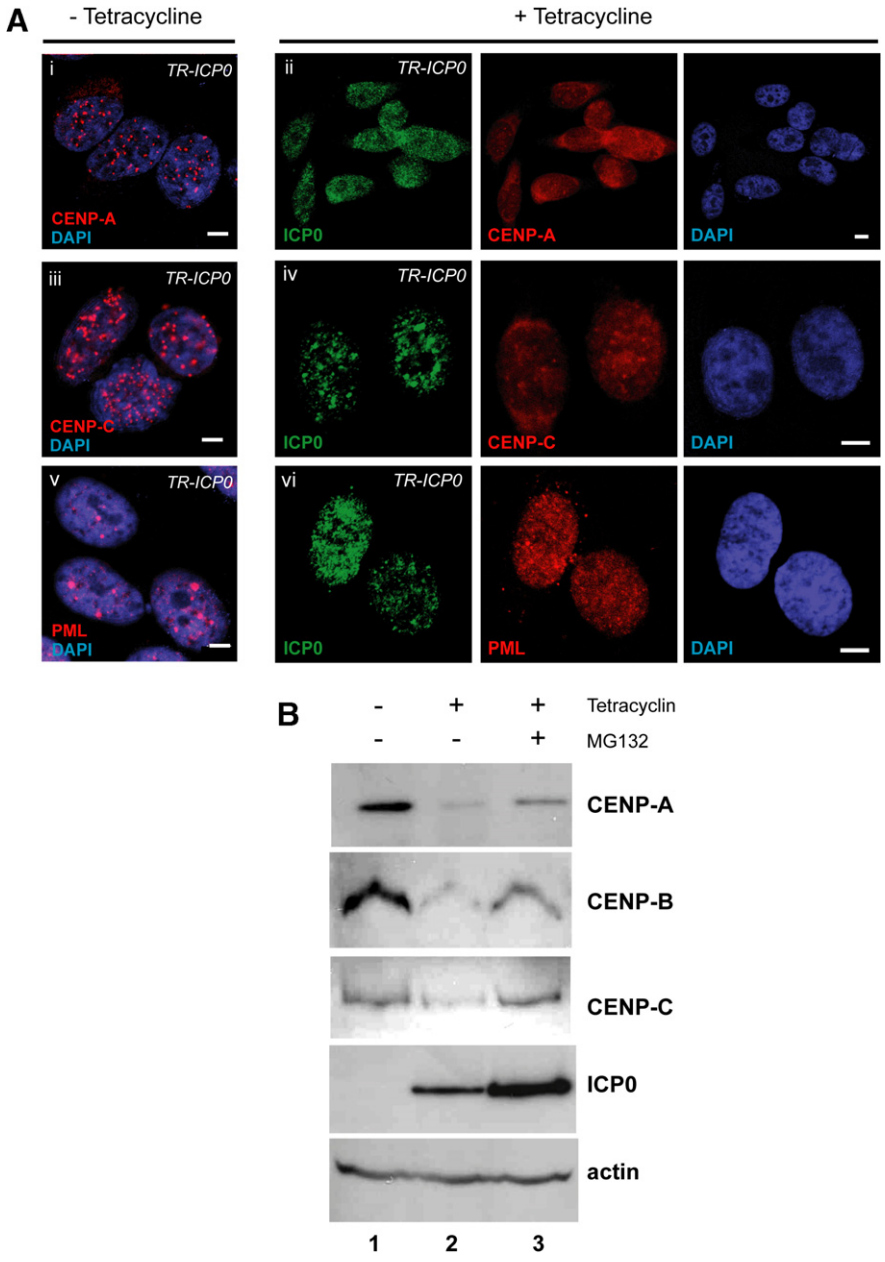

Fig. 2. CENP-B is degraded in the TR-ICP0.39 cell line. (A) Immunofluorescence study showing the correct ICP0 expression and activity in the TR-ICP0.39 (TR-ICP0) cell line. In the absence of tetracycline, the CENP-A, -C and PML signals are not affected (i, iii, v). Addition of tetracycline for 24 h induces the expression of ICP0 (ii, iv, vi; green signal), and the disappearance of the CENP-A, -C and PML dots (red signal). Nuclei are shown by the DAPI staining (blue). Bars represent 5 μm. (B) TR-ICP0.39 cells were induced or not by tetracycline for 24 h, in the presence or not of proteasome inhibitor MG132. Western blotting was performed for the detection of CENP-A, -B, and -C, ICP0, and actin as loading control.

Mouse cell centromeres are composed of two types of repeats called minor and major satellite repeats [15]. The minor satellite units are exclusively part of the central core centromere and the major satellite repeats are exclusive of the pericentromeric regions [16]. Another major difference is that the "CENP-B box" is only present in the minor satellite repeats [17]. CENP-B is thus likely to exclusively associate with this region. Therefore, we decided to repeat infection experiments in mouse cells to verify if the ICP0-induced CENP-B degradation was more obviously detectable in this species. Infected NIH3T3 mouse cells were treated for immunofluorescence using a human autoimmune serum (huACA) recognizing several human CENPs [18], and also working in mouse cells [16]. Additionally, a monoclonal antibody (mAb) raised against human CENP-B, which also recognises mouse cenpB was used. As expected, in ICP0-expressing cells the centromeric signal significantly decreased when using the huACA serum, and more importantly, the cenpB signal completely disappeared when the mAb anti-CENP-B was used (Fig. 3Ai and ii). Nothing comparable was observed in cells expressing a non-functional ICP0 protein mutated in its RING finger domain (data not shown). To finalise this study, NIH3T3 cells were infected to perform Western blotting to detect mouse cenpB stability. Human CENP-B is an 80 kDa protein, and gi-

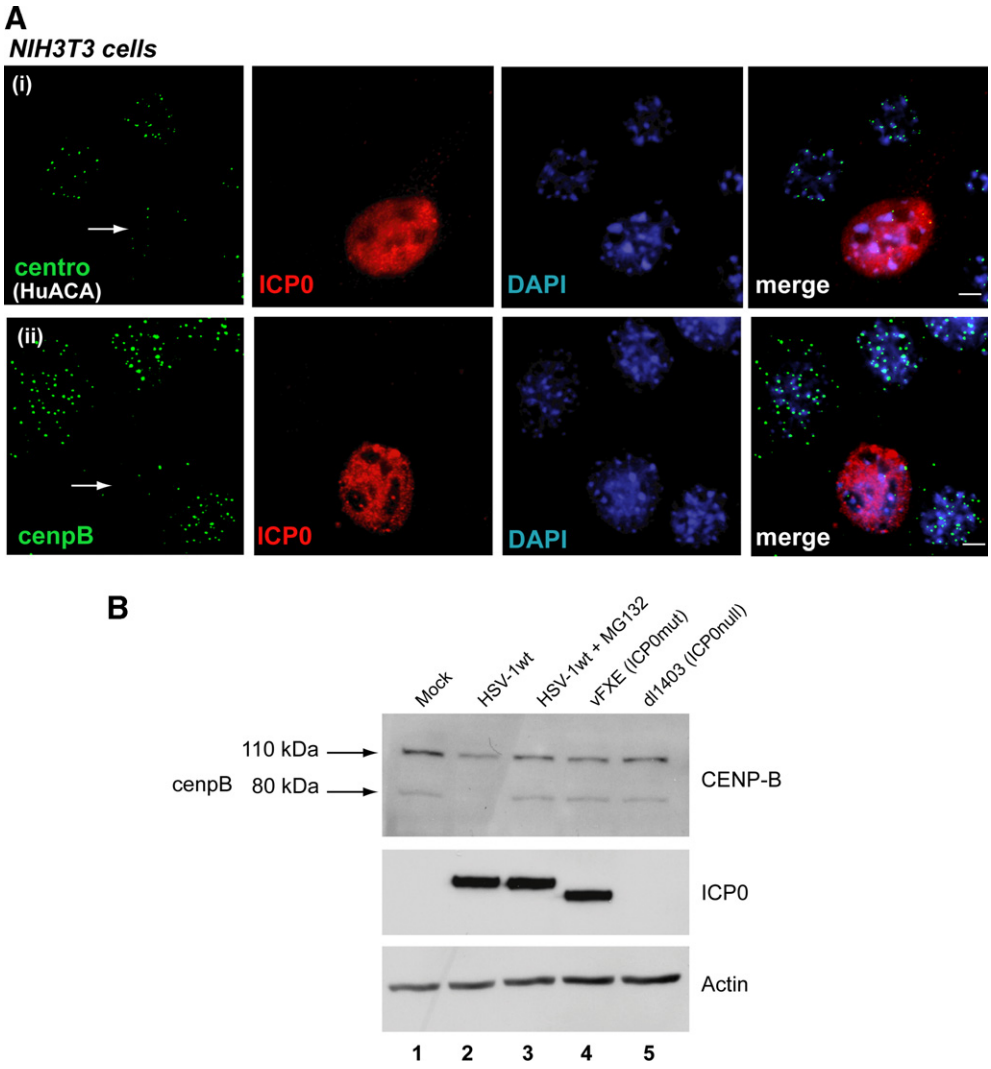

Fig. 3. ICP0 induces the degradation of cenpB in mouse cells. (A) NIH3T3 mouse cells were infected by HSV-1wt for 4 h to perform immunofluorescence assays, (i) HuACA serum was used to detect centromere proteins (green), (ii) [5E6C1] mAb anti-CENP-B was used to specifically detect mouse cenpB (green). Infected cells are detected by the ICP0 positive signal (red). Nuclei are labelled by DAPI staining (blue). Merge images show the co-localization of green dots (central core centromeres) with DAPI spots (peri-centromeric heterochromatin) in non-infected cells. Arrows point out infected cells. Bars represent 5 μm. (B) NIH3T3 mouse cells were all infected (multiplicity of infection of 10) by viruses HSV-1wt (in the presence or not of proteasome inhibitor MG132), dl1403, or vFXE for 6 h prior to performing Western blotting for the detection of cenpB (80 kDa), ICP0, and actin as loading control. 110 kDa indicates an extra band specifically detected with the [5E6C1] mAb anti-CENP-B only in mouse cells.

ven the high degree of identity in amino acids between both human and mouse CENP-B it is expected for mouse cenpB to possess a similar molecular mass. Looking at the protein migrating at this size it is clear that cenpB disappeared in HSV-1wt infected cells in the absence but not in the presence of MG132 (Fig. 3B, compare lanes 2 and 3). In samples coming from mutant virus-infected cells, cenpB was still detectable (Fig. 3B, lanes 4 and 5). These data undoubtedly confirm the ICP0- and proteasome-induced degradation of the CENP-B protein. Furthermore they indicate that ICP0 keeps its activity in mouse cells. However, another protein was detected at a size of about 110 kDa. The amount of this protein slightly diminished in HSV-lwt infected cells but did not completely disappear. We would like to propose two hypotheses for the detection of this 110 kDa protein. The first one is a putative cross reactivity of the anti-CENP-B mAb against an undetermined protein. However, the stability of this protein is also partially affected by ICP0. It is excluded that this could be an unspecific effect of the infection because this partial degradation is absent in the presence of MG132 and in cells infected with ICP0-mutant viruses. It is thus striking and highly coincidental that the stability of a protein unspecifically recognized by the anti-CENP-B antibody would also be affected by ICP0. Therefore, a second hypothesis is that at least part of the pool of the 110 kDa protein would be present in the centromeric region targeted by ICP0. In this case, the 110 kDa protein could well be an undetermined post-translationally modified form of cenpB that only partially localizes to the central core region of centromeres.

In this study we show that in addition of CENP-A and -C, the stability of a third centromeric protein, CENP-B, is affected by ICP0. Recent publications described on the basis of tandem affinity purification techniques, as many as 11 new CENPs, directly or indirectly interacting with the CENP-H-I

complex and/or the CENP-A-associated nucleosomes [19,20]. In light of these data, we can reasonably speculate that ICP0 would affect the stability of a lot more of these CENPs provided that they are localized in the immediate vicinity of ICP0 during the time it resides on the centromere. This gives ICP0 a major importance as an exceptional tool for studying the consequences of centromeric destabilization on the cell physiology. Indeed, no other proteins to date have been found which possess such an activity on centromere proteins. Moreover, siRNA technique, although helpful to target one protein or another, is unlikely to be as efficient as ICP0, if multiple centromeric protein depletion is required.

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