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Degradation of cyclins D in pseudorabies virus (PRV) infected proliferating cells

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Abstract

The pseudorabies virus code for an ICP0 protein which is half the size of the HSV1 ICP0 protein. In this work, we made the assumption that some function might have been lost in the ICP0 from PRV. One function attributed to the ICP0 from HSV1 was the stabilization of cyclins D. We then looked at the stability of these cyclins during the lytic infection with the PRV. Our results show that cyclins D are not stabilized during infection with the PRV. These results are in accord with recent data from the literature.

Keywords: Pseudorabies virus; Cyclin D; Cyclin B; ICP0

1. Introduction

Herpesviruses have developed a replication strategy, which does not require an S phase environment (they code for their own DNA metabolizing enzymes) and conversely a cell cycle block strategy, which prevents entry of the cell into S phase. This strategy has been developed by the three herpes subfamilies (alpha, beta and gamma herpesviruses) (Flemington, 2001). For the alpha herpes subfamily, most work on cell cycle control has been done with the herpes simplex virus type 1 (HSV1) with the pioneering study being de Bruyn Kops and Knipe (1988). Ten years later, the involvement of the herpes simplex protein ICP0 in the cell cycle arrest was demonstrated (Hobbs and DeLuca, 1999; Lomonte and Everett, 1999) but others viral proteins are also implicated in this process. Since then, ICP0 has been widely studied, and it now appears to be a multifunctional protein and a major player in the control of the host cell (Hagglund and Roizman, 2004). Amongst its multiple functions, ICP0 has been shown to bind and stabilize cyclin D3 (Kawaguchi et al., 1997) and also to stabilize cyclin D1 (Van Sant et al., 2001). However, the stabilization of these cyclins is controversial (Ehmann et al., 2000; Everett, 2004; Song et al., 2000).

Except for a RING domain, the ICP0 protein family is poorly conserved (Hagglund and Roizman, 2004) The ICP0 homologue from pseudorabies virus (PRV), called EP0 (Cheung, 1989) is a 409 amino acids protein whereas ICP0 is 775 amino acids long. The best identity observed between these two proteins is only 35% within an 87 amino acid fragment, which

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contains the RING domain. There are no other significant similarities between these two proteins. So the question is whether all the functions fulfilled by ICP0 are also fulfilled by its PRV homologue EP0. To answer this question we looked at the cyclin D stabilizing property, which might be attributed to the ''ICP0 related protein family'', during the lytic replication of PRV in vitro.

2. Material and methods

2.1. Cells and viruses

Human cervical carcinoma (HeLA), human embryonic kidney cells (HEK-293) and porcine kidney (PK15) were grown in EMEM containing 5% foetal calf serum and 100 U penicillin/ml and 100 U streptomycin sulphate/ml. The stock of PRV (NIA-3 strain) used in this study was prepared as follows: confluent PK15 was infected with PRV overnight. When 80% of the infected cells presented cytopathogenic effects (CPE), cell culture was stopped by freezing at −80 °C. After two cycles of freeze-thawing, virus-containing media were centrifuged (1800 × *g*, 30 min, 4 °C) and the supernatant aliquoted and stored at −80 C. UV virus inactivation was performed by exposure of a viral solution to the UV lamp of a laminar flow hood for 15 min. This UV treatment resulted in a 5-log drop of viral titer.

For infection, the cells were seeded at a density of 2.10^5 cm^{-2} two days before infection (one day for the PK15), then infected with 6 MOI PRV (or an equivalent volume of UV-irradiated viral solution) under gentle agitation for 1 h at 37 °C, medium was then added and culture continued until the cells were harvested.

2.2. Flow cytometry

Cells were fixed with the Cytofix/Cytoperm kit (BD Bioscience, San Diego) following the manufacturer's instructions. Immunolabelling was performed following the instructions from the same kit. The antibodies used for the immunolabelling were mouse anti-human cyclin D, mouse anti-human cyclin B. A mouse IgG was used for isotype control and goat anti-mouse FITC labelled antibodies was used as secondary antibody. All the antibodies used throughout this study were from BD Bioscience. DNA labelling was performed by incubation of the cells in a propidium iodine solution (40 μg PI/ml, 100 μg RNAse A/ml in PBS) for 1 h before the flow cytometry analysis. A minimum of 10,000 events were counted per sample.

2.3. Western blotting

The medium was removed, and the cells were rinsed in phosphate-buffered saline (PBS), scraped into PBS, pelleted by centrifugation and solubilized in 150 μl of disruption buffer (2% sodium dodecyl sulphate [SDS], 50 mM Tris [pH 7.2], 2.75% sucrose, 5% β-mercaptoethanol and bromophenol blue) for 10^6 cells. The extract was centrifuged (15,000 × *g*, 15 min), sonicated, boiled for 5 min, subjected to electrophoresis on 12% bisacrylamide gels, transferred to nitrocellulose membranes, blocked for 2 h (5% dry milk, 1% BSA, TBS 1×, 0,1% Tween 20) and reacted with the appropriate antibody. Antibodies against cyclins D and B were the same as for the flow cytometry analysis, antibody against actin (Sigma) was used as internal control for some blots. All the primary antibodies were diluted 1:1000 in PBS and reacted overnight at 4 °C with blots. Secondary antibody diluted 1:5000 (sheep anti-mouse antibody conjugated to peroxidase; Sigma) was applied for 1 h. Blots were developed by enhanced chemiluminescence (ECL) following the instructions supplied by the manufacturer (Amersham).

3. Results

3.1. The level of cyclins D decreases in PRV infected cells compared to those of mock infected cells or cells infected with UV-inactivated virus

The purpose of this study was to determine the cyclin D accumulation, in a proliferating cell line, during the course of an infection by the PRV. A stabilization of cyclins D1 and D3 has been described in HSV1 infected cells (Kawaguchi et al., 1997; Van Sant et al., 2001). Fig. 1 shows the flow cytometric profiles of cyclins D accumulation in HEK 293 infected cells with native PRV (Fig. 1a) or UV-irradiated PRV (Fig. 1b) during the first 7 h of infection. For the cells infected with the native PRV (Fig. 1a) by 1 h PI, we had observed an early decrease for the cyclin D labelling (dash dotted line). By