Cell cycle arrest mediated by hepatitis delta antigen

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Abstract Hepatitis delta antigen (HDAg) is the only viral-encoded protein of the hepatitis delta virus (HDV). This protein has been extensively characterized with respect to its biochemical and functional properties. However, the molecular mechanism responsible for persistent HDV infection is not yet clear. Previously, we reported that overexpression of HDAg protects insect cells from baculovirus-induced cytolysis [Hwang, S.B. Park, K.-J. and Kim, Y.S. (1998) Biochem. Biophys. Res. Commun. 244, 652–658]. Here we report that HDAg mediates cell cycle arrest when overexpressed in recombinant baculovirus-infected insect cells. Flow cytometry analysis has shown that HDAg expression in *Spodoptera frugiperda* cells causes an accumulation of substantial amounts of polyploid DNA in the absence of cell division. This phenomenon may be partly responsible for the persistent infection of chronic HDV patients.

Key words: Cell cycle arrest; Flow cytometry; Hepatitis, delta antigen; Baculovirus; Hepatitis delta virus; *Spodoptera frugiperda*

1. Introduction

Hepatitis delta virus (HDV) is an RNA virus which contains a 1.7 kb single stranded circular genome [1–3]. The RNA forms a rod-like structure, of which approximately 70% of the bases are self-complementary. The genome encodes only one viral protein, the hepatitis delta antigen (HDAg), from the antigenomic sense RNA and two isoforms of HDAg are found in the liver and sera of infected patients and animals [4]. The small form of HDAg (SHDAg) consists of 195 amino acids and the large form (LHDAg) is 214 amino acids long. LHDAg is generated as a result of an RNA editing event on the UAG termination codon of SHDAg during viral replication [5]. These two proteins are identical in sequence except that LHDAg contains an additional 19 amino acids at its C-terminus [6]. Both HDAgs have similar functional domains. The N-terminal domain contains a coiled-coil sequence responsible for protein oligomerization [7] and nuclear localization signals [8]. The middle one-third is a bipartite arginine-rich motif responsible for RNA binding [9,10]. The C-terminal domain of the SHDAg contains the unique 9E4 epitope [11] while the C-terminus of the LHDAg contains an isoprenylation signal [12,13].

Both HDAgs are phosphoproteins and localize to the nuclei of infected cells [9,12], each having a distinct function. SHDAg *trans*-activates HDV RNA replication [14], while LHDAg suppresses genome replication [15]. It has also been shown that LHDAg activates gene expression *in trans* [16]. Furthermore, LHDAg interacts with the hepatitis B virus surface antigen through an isoprenylated cysteine residue [17] and is necessary for virion assembly [18–20].

Our previous findings demonstrated that overexpressed HDAg protected insect cells from wild-type baculovirus-induced cytolysis [21]. In the present study we have further shown that HDAg mediates cell cycle arrest in baculovirus-infected insect cells. This result may partially explain persistent infection of HDV patients.

2. Materials and methods

2.1. Cell culture and viruses

Spodoptera frugiperda (Sf9) insect cells were maintained in Grace's insect tissue culture medium (Gibco BRL) supplemented with 10% fetal calf serum and 100 U/ml penicillin-streptomycin as described previously [22]. Both wild-type and recombinant baculoviruses were cultured by infecting insect cells at a multiplicity of infection (m.o.i.) of 10 and by incubation at 27°C.

2.2. Viability assays

Sf9 insect cells infected with either wild-type or recombinant viruses were removed from the culture flask for determination of cell viability at indicated time points. Cells were stained with 0.2% trypan blue (Gibco BRL) in a culture medium and the total viable cell number was determined in a hemocytometer.

2.3. Immunofluorescence assay

Sf9 insect cells grown on chamber slides were infected with either wild-type or recombinant virus expressing HDAg at a m.o.i. of 10. At 2 days postinfection, slides were washed in phosphate-buffered saline (PBS) and fixed in acetone for 5 min at room temperature. After washing twice in PBS, cells were incubated with rabbit anti-HDAg antibody for 1 h at 37°C. Slides were then rinsed in PBS and incubated with rhodamine-conjugated goat-rabbit IgG (American Qualex) as a secondary antibody. Finally, cells were washed in PBS several times and then examined using a fluorescent microscope.

2.4. FACS analysis

Sf9 cells were either mock-infected or infected with either wild-type or recombinant viruses and harvested at 24 h intervals, washed twice in PBS, and fixed with 70% ethanol. Fixed cells were washed twice in PBS and incubated with RNase A (1 mg/ml) for 30 min at 37°C. Cells were further incubated with propidium iodide (50 μg/ml in hypotonic citrate (2.80 mM trisodium citrate, 9.92 mM NaCl; Sigma) for 30 min at 37°C. After washing in PBS, 10^4 cells were measured for their total DNA content using a FACStar Plus (Becton Dickinson, Mountain View, CA) as described previously [23].

3. Results and discussion

Our previous study showed that overexpressed HDAg protected insect cells from wild-type baculovirus-induced cytolysis [21]. Both isoforms of HDAg showed the same effect on cytolytic protection and thus SHDAg was used throughout this study for convenience. To examine the effect of overexpressed HDAg on cell viability, we first compared the protein expression level of recombinant virus-infected insect cells expressing SHDAg with those of either the wild-type baculovirus expressing polyhedrin protein or the recombinant baculo-

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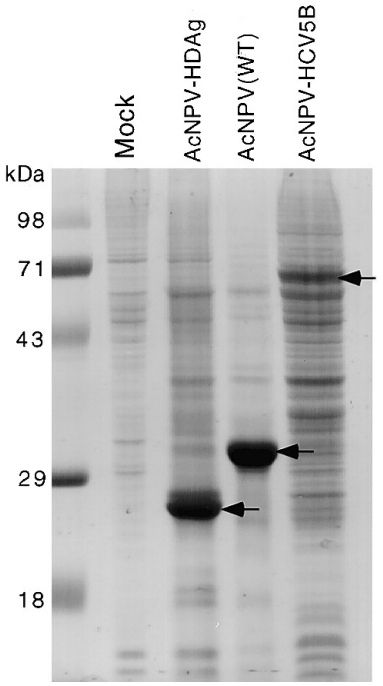

Fig. 1. Comparison of protein expression in insect cells. Sf9 cells were infected with either wild-type or recombinant baculoviruses using the same m.o.i. (10^{10}) and harvested 3 days postinfection. Proteins were separated by electrophoresis on a 12.5% polyacrylamide gel containing SDS and stained with Coomassie brillant blue. The positions of the SHDAg (24 kDa), polyhedrin (32 kDa), and HCV NS5B (64 kDa) are indicated by arrows.

virus expressing hepatitis C virus NS5B (RNA polymerase) protein [22]. Fig. 1 shows that the expression levels of polyhedrin and HCV RNA polymerase are comparable to that of SHDAg in baculovirus-infected insect cells. Next we infected Sf9 cells using the same m.o.i. (10^{10}) of these viruses and total viable cell number was determined for 100 h. As shown in Fig. 2, the total viable cell number increased slightly at 20 h postinfection in all cases. However, the cells gradually died in both wild-type virus-infected and recombinant virus expressing HCV NS5B-infected Sf9 cells and less than one tenth of the cell population remained alive at 100 h postinfection. In contrast, the majority of SHDAg-expressing (AcNPV-HDAg) cells were viable throughout the experimental period. Since there was no increase in total cell number, it seemed plausible that these cells were arrested at a certain stage of the cell cycle.

To further investigate whether HDAg can alter the cell cycle progression of host cells, we examined the total cellular DNA profile of both wild-type and recombinant virus-infected cells for 100 h by flow cytometry analysis. We used the high m.o.i. (10^{10}) to infect cells and confirmed by the indirect immunofluorescence assay that all cells were infected (data not shown). Cells were harvested at 20 h intervals, fixed, and stained with propidium iodide. Subsequently, the total cellular DNA profile was determined by flow cytometry. As shown in Fig. 3, the host cell cycle was perturbed by baculovirus infection. In wild-type baculovirus-infected cells, no cells were in the S phase as early as 20 h postinfection and only a small proportion of the cells showed polyploid levels of DNA (Fig. 3). At 40 h postinfection, these cells finished one round of cell division and the entire cell population in wild-type virus-infected cells gradually shifted to the G_0/G_1 phase of the cell cycle. At 100 h postinfection, cells started dying and most of the cells were in an apoptotic phase probably due to virus-induced DNA cleavage. Likewise, Sf9 insect cells infected with recombinant viruses expressing HCV NS5B were shifted to the G_0/G_1 phase of the cell cycle with no increase in total cell number (data not shown). We also performed these experiments using recombinant viruses expressing HCV core protein and hepatitis B virus surface antigen, and obtained similar results (data not shown). However, cells infected with recombinant viruses expressing SHDAg gradually moved to G_2 and mitotic phases. At 60 h postinfection, a small proportion of these cells accumulated polyploid levels of DNA. Thus, overexpressed HDAg mediates continuous accumulation of DNA in baculovirus-infected insect cells. The total viable cell number had not increased in these cells (Fig. 2), suggesting that mitosis was blocked. It is noteworthy that the G_1 cell population decreased gradually and almost no cells were in G_1 phase at 100 h postinfection in SHDAg-expressing cells (Fig. 4). On the other hand, the majority of wild-type infected cells were at the sub-G_1 phase of the cell cycle 80 h postinfection. In mock-infected cells, cell number increased constantly and no alteration of the cell cycle occurred (data not shown).

Some viruses are known to encode proteins that alter the cell cycle progression of the host cells. The Vpr protein encoded by human immunodeficiency virus type 1 (HIV-1) causes T cells to accumulate in G_2+M phases of the cell cycle [24–26]. Stable cells expressing bovine papillomavirus E1 protein also altered the duration of the host cell cycle [27], probably promoting a host cell environment suitable for viral persistence. Likewise, insect cells infected with recombinant viruses expressing HDAg accumulated polyploid levels of DNA with no increase in cell number, suggesting that these cells were arrested at the mitotic phase of the cell cycle.

Viral infection also affected cell cycle progression. The growth of HF cells was inhibited by infection with human cytomegalovirus (HCMV) [28]. HCMV infection caused cell cycle arrest at multiple phases including both the G_1 and G_2/M phases.

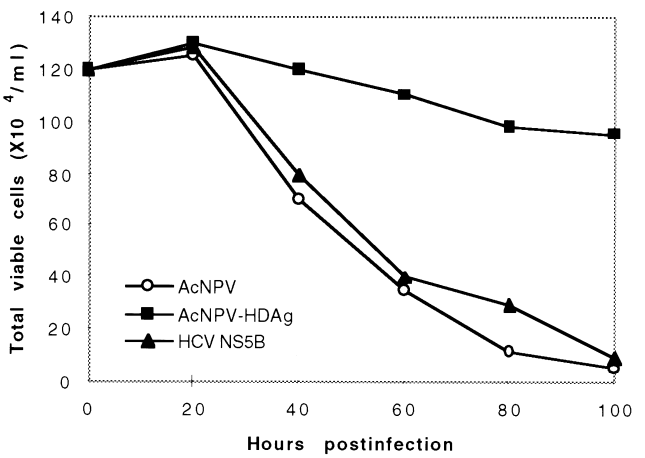

Fig. 2. Cell viability. Sf9 insect cells infected with either wild-type (AcNPV) or recombinant baculoviruses expressing either SHDAg (AcNPV-SHDAg) or HCV NS5B were stained with trypan blue at the indicated time points (hours postinfection) and total viable cell number was determined. The data presented are the mean value obtained from three individual experiments.

Cell cycle arrest would be beneficial for viruses because they can infect more cells by preventing cells from finishing the cycle. This is particularly true in insect cells because normally recombinant baculovirus-infected cells undergo cytolysis within 5 days postinfection. Cell cycle arrest would be an advantage for HDV because it uses host RNA polymerase for replication [29] and thus they can maximize production of viral progenies.

To examine whether HDAg can cause cell cycle arrest in a mammalian cell system, we performed cell cycle analysis using stably transformed mammalian cells expressing SHDAg [30] or LHDAg (unpublished data) and found that cell cycle progression was not altered (data not shown). Since HDAg expression in these cells was low and only detectable by Western blot analysis (data not shown), we propose that a certain threshold level of HDAg may be required to block cell cycle progression. However, further studies are necessary to verify this mechanism in mammalian cells. To date, it is not known how HDAg levels are regulated in HDV patients.

Recently, we reported that HDAg in recombinant baculovirus-infected cells inhibited wild-type virus induced cytolysis and thus prolonged the survival of these cells [21]. In patients, the inhibition of cytolysis may facilitate the development of a persistent infection and may also interfere with host antiviral functions. Likewise, Ray et al. [31] demonstrated that the hepatitis C virus core protein inhibited tumor necrosis factor-mediated apoptosis in a human breast carcinoma cell line. We proposed the possible interaction between p35 and HDAg in preventing cell death [21]. Currently, we are examining the effect of HDAg overexpression on the p35 gene of baculoviruses. Alternatively, the possible involvement of p35-linked cellular proteins activated by HDAg is also under investigation.

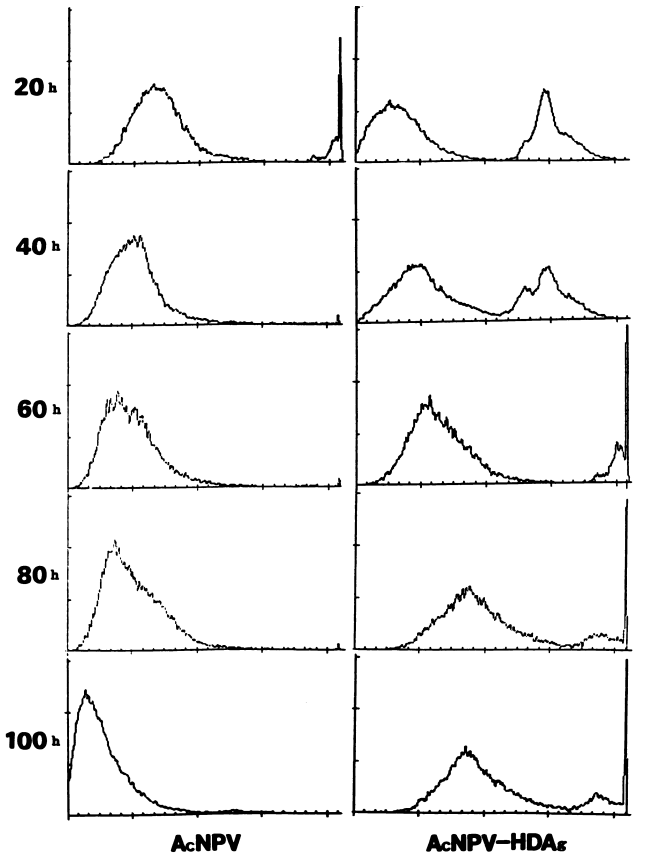

Fig. 3. Flow cytometry profiles. Sf9 insect cells infected with either wild-type (AcNPV) or recombinant viruses expressing small hepatitis delta antigen (AcNPV-HDAg) were harvested at the times shown, fixed, stained with propidium iodide, and analyzed by flow cytometry. Abscissa: DNA content. Ordinate: relative cell number.

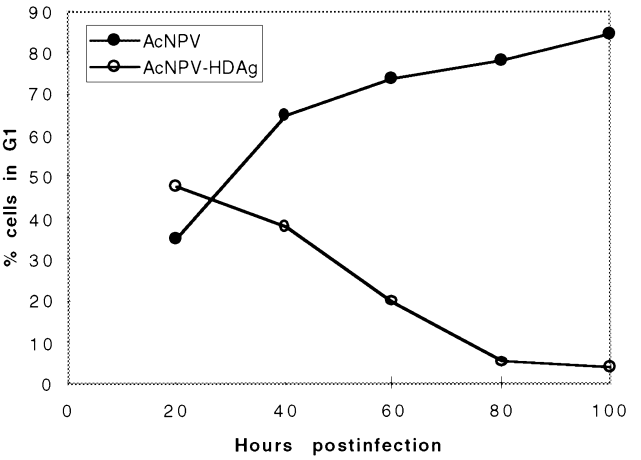

Fig. 4. The percentage of cells in the G_1 phase of the cell cycle. Data obtained from flow cytometry were analyzed by using CellQuest software. For each analysis, 10 000 gated events were collected to permit cell cycle analysis of both AcNPV and AcNPV-HDAg cell populations.

Our current studies further show that HDAg mediates cell cycle arrest in recombinant baculovirus-infected cells. Although the exact mechanism of cell cycle arrest was not elucidated, this property together with cytolytic inhibition of HDAg may confer insights into the establishment of HDV persistent infection. Since the biochemical properties of HDAgs expressed in insect cells were identical to those in mammalian cells [12], the data presented in this report may be applicable to the mammalian cell system.

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