Association of Sf9 cell proliferating cell nuclear antigen with the DNA replication site of Autographa californica multicapsid nucleopolyhedrovirus

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The accumulation of cellular proliferating cell nuclear antigen (PCNA) in the nucleus of Sf9 cells has been shown to increase upon infection with Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV). Here, analysis by DNase I treatment and chromatin immunoprecipitation revealed that cellular PCNA in the nucleus of Sf9 cells bound AcMNPV DNA. Immunocytochemical analysis showed colocalization of Sf9 cell PCNA and AcMNPV DNA replication sites. Similar colocalization was also observed in BmN-4 cells infected with Bombyx mori NPV, which is inherently missing the pcna gene. The amount of cellular PCNA associated with viral DNA replication sites was greater in cells infected with pcna-defective AcMNPV mutants than in cells infected with wild-type AcMNPV. These results suggest that both cellular and viral PCNAs are involved in AcMNPV DNA replication and that pcna-defective AcMNPV mutants are able to substitute cellular PCNA for viral PCNA.

In this study, we have shown that Sf9 cell PCNA in non-ionic detergent-extracted nuclei binds AcMNPV genomic DNA and colocalizes with viral DNA replication sites. By confocal imaging, we have also shown that the amount of cellular PCNA in the nucleus of cells infected with pcna-defective AcMNPV mutants was greater than that of cells infected with wt AcMNPV. These results suggest that both cellular and viral PCNAs are involved in AcMNPV DNA replication and that cellular PCNA substitutes for viral PCNA in pcna-defective AcMNPV mutants.

Sf9 cells from the fall armyworm, Spodoptera frugiperda, were maintained in Grace’s medium (Gibco-BRL) containing 10% fetal bovine serum (FBS), and BmN-4 cells from the silkworm, Bombyx mori (Maeda, 1989), were maintained in TC100 medium (JRH Biosciences) containing 10% FBS. Wild-type AcMNPV (AcMNPV L1; Lee & Miller, 1989) and three pcna-defective AcMNPV mutants, vAcpcna73–252, vAcpcna262–645, and vAcpcna262–265, were generated recombinant AcMNPVs, vAcpcna73–252 and vAcpcna262–645, comprising PCNA with deletions in the N (C73–G252) and C (G262–G645) termini, respectively (Iwahori et al., 2002b), and confirmed that Ac-PCNA is a non-essential factor for viral DNA replication. It was also found that the amount of cellular PCNA in the nuclear fraction after extraction with non-ionic detergent was greater in cells infected with pcna-defective AcMNPV mutants than in cells infected with wt AcMNPV (Iwahori et al., 2002b), suggesting that cellular and viral PCNAs are responsible for viral DNA replication.
vAcpcna\textsuperscript{262–645} (Iwahori et al., 2002b) and vETL/gal (Crawford & Miller, 1988), were each propagated in Sf9 cells. Bombyx mori nuclearpolyhedrosisvirus (BmNPV) N9 (Nagamine et al., 1989) was propagated in BmN-4 cells. Viruses were used for infection at an m.o.i. of 20.

Infected and mock-infected cells were lysed with modified CSK buffer (10 mM PIPES, pH 6-8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl\(_2\), 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 10 μg aprotinin ml\(^{-1}\) ) containing 0-5% Triton X-100 (TX-100) on ice for 10 min. The TX-100-extracted nuclei were pelleted by centrifugation at 1000 × g for 3 min and the resultant pellet was referred to as P1 (Fig. 1a). The P1 fraction was separated by SDS-PAGE and transferred on to an Immobilon transfer membrane (Millipore). Cellular PCNA was detected using anti-PCNA antibody (PC10; Santa Cruz Biotechnology; Waseem & Lane, 1990) and high-salt NSB buffer fractionation and DNase I digestion of virus-infected Sf9 cells. (a) Schematic diagram showing the procedure for fractionation and DNase I digestion of virus-infected Sf9 cells. (b) Sf9 cells infected with wt AcMNPV (W), vAcpcna\textsuperscript{73–252} (N), vAcpcna\textsuperscript{262–645} (C) or vETL/gal (E) were collected at 0 and 10 h p.i. and fractionated into P1 and S1. P1 fractions (equivalent to 7-5 × 10\(^5\) cells) were separated by SDS-PAGE and subjected to immunoblot analysis with anti-PCNA antibody (PC10). (c) P1 fraction was prepared from Sf9 cells infected with wt AcMNPV and pcna-defective AcMNPV mutants at 11 h p.i. and incubated with (+) or without (−) DNase I. After centrifugation, P2 and S2 fractions (equivalent to 1-3 × 10\(^5\) cells for mock and pcna-defective AcMNPV mutants and 1-5 × 10\(^6\) cells for wt AcMNPV) were subjected to immunoblot analysis and cellular PCNA was visualized. M, Mock-infected cells.

When analysed at 10 h post-infection (p.i.), the amount of cellular PCNA in the P1 fraction increased in Sf9 cells infected with pcna-defective AcMNPV mutants, while only a small increase was observed in cells infected with wt AcMNPV (Fig. 1b). To examine whether the cellular PCNA bound genomic DNAs, the P1 fraction was resuspended in modified CSK buffer containing 0-5% Triton-100 and 10 mM MgCl\(_2\) and incubated with DNase I (1000 U ml\(^{-1}\); Roche) at 4°C for 60 min, as described previously (Fujita et al., 1997). Supernatant (S2) and pellet (P2) fractions were obtained by centrifugation at 1000 × g for 3 min (Fig. 1a).

As shown in Fig. 1(c, row W), almost all of the cellular PCNA in the P1 fraction from wt AcMNPV-infected Sf9 cells was detected in the P2 fraction without DNase I treatment. In contrast, when the P1 fraction was incubated with DNase I, most of the cellular PCNA was released and recovered in the S2 fraction. Similar release of the cellular PCNA by DNase I digestion was observed in the P1 fractions from mock-infected and pcna-defective AcMNPV mutant-infected cells (Fig. 1c, rows M, N, C and E). These results indicated that cellular PCNA in the TX-100-extracted nuclei of Sf9 cells was bound to genomic DNA.

To investigate whether the cellular PCNA bound viral genomic DNA, a chromatin immunoprecipitation assay was performed, as described previously (Ladenburger et al., 2002; Schepers et al., 2001; Ohzeki et al., 2002) with some modifications (Fig. 2a). After infection with wt AcMNPV or pcna-defective AcMNPV mutants, infected cells were cross-linked with 1% formaldehyde. The cross-linked cells were washed sequentially with RSB buffer (10 mM Tris/HCl, pH 8-0, 10 mM NaCl, 3 mM MgCl\(_2\) and complete protease inhibitor (Roche)) and high-salt NSB buffer [10 mM Tris/HCl, pH 8-0, 1 M NaCl, 0.1% IGEPIAL CA-630 (Sigma), 1 mM EDTA and complete protease inhibitor] and sonicated in TE (10 mM Tris/HCl, pH 8-0, 1 mM EDTA). The DNA was digested with micrococcal nuclease to yield DNA fragments with a mean size of about 500 bp. For immunoprecipitation, the samples were suspended in W buffer (2 mM EDTA, 20 mM Tris/HCl, pH 8-0, 1% Triton X-100, 1% BSA) containing 0.1% SDS and 150 mM NaCl and incubated for 4 h with agarose conjugated to anti-PCNA antibody or to normal mouse IgG as a control. The immunocomplexes were washed with W buffer...
containing 500 mM NaCl and either 0-6 or 0-65 % SDS. The washed precipitates were eluted sequentially with TE containing 1 % SDS for 30 min at 65 °C and TE containing 2-5 % SDS for 10 min at room temperature and the eluates were incubated in TE containing 1 % SDS for 15 h at 65 °C.

The DNA was isolated according to a standard protocol using proteinase K treatment and phenol/chloroform extraction, followed by RNase treatment. The recovered DNA was used as a template for PCR. A region of polh (polyhedrin gene) (207 bp, nt 4360–4566; nucleotide numbers of AcMNPV genome) or gp37 (199 bp, nt 51839–52037) was amplified by Taq polymerase (Takara) using either 5'–GCCATTGTAATGAGACGCAC–3' and 5'–TACACGTAGGTACGCCCGAT–3' or 5'–TCGTTTGAACCGGCACCGCA–3' and 5'–GCGTGCCGCAATGCTTACAA–3' as the paired primers, respectively. PCR products were collected after 35, 38 or 41 cycles and resolved on agarose gels. As shown in Fig. 2(b), both polh and gp37 were amplified from DNA templates that were co-precipitated with anti-PCNA antibody (P) from virus-infected cells. In contrast, little amplification of polh and gp37 was observed from DNA templates obtained by immunoprecipitation with normal mouse IgG (N) as a control. This result indicated that cellular PCNA bound viral DNA.

To obtain additional information about the functional role of cellular PCNA in viral DNA replication, localization of the sites of DNA replication and cellular PCNA in the infected nucleus was undertaken. Viral DNA replication sites were visualized by 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA. BrdU (10 μM) was added to the medium and cells were incubated for 1 h. Labelled cells were seeded on to coverslips and extracted with modified CSK buffer containing 0-5 % TX-100. Cells were treated with 2 M HCl for 30 min at 37 °C, followed by sequential incubation with anti-BrdU antibody (Oxford Biotechnology) and Cy3-conjugated anti-rat IgG antibody (Jackson ImmunoResearch Laboratories). The stained cells were observed under a confocal laser-scanning microscope (LSM510; Zeiss). Aphidicolin (50 μg ml⁻¹) was used to block cellular and viral DNA synthesis.

In mock-infected SF9 cells, BrdU staining was observed as a dotted pattern throughout the nucleus (Fig. 3a, first row) and was completely blocked by the addition of aphidicolin (Fig. 3a, second row), indicating that the staining was at the sites of cellular DNA synthesis. In wt AcMNPV-infected cells, BrdU staining was observed as several intense spots within the nucleus at 5 h p.i. (Fig. 3b, first row) and the spots were enlarged by 10 h p.i. (Fig. 3b, second row). In addition, it was found that BrdU incorporation was completely blocked by the addition of aphidicolin (Fig. 3b, third row). These results, together with the fact that viral DNA replication had started by 8 h p.i. (Iwahori et al., 2002b), indicate that the increase in intensity and size of these spots was due to viral DNA synthesis. A similar pattern of BrdU staining has been reported in AcMNPV-infected TN-368 cells (Mainz et al., 2002) and BmNPV-infected BmN-4 cells (Okano et al., 1999).
Sf9 cells

(a) Mock

(b) wt AcMNPV

(c) vAcpcna

(d) vAcpcna

(e) vETLβgal

BmN-4 cells

(f) Mock

(g) BmNPV
Upon staining with anti-PCNA antibody, Sf9 cell PCNA was observed throughout the nucleus in mock-infected cells (Fig. 3a, first row). In wt AcMNPV-infected Sf9 cells, the staining of cellular PCNA formed foci within the nucleus at 5 h p.i. and the foci were enlarged at 10 h p.i., overlapping strongly with the BrdU staining (Fig. 3b, first and second rows). Formation of these foci was blocked when viral DNA synthesis was decreased by aphidicolin treatment (Fig. 3b, third row). These results suggested that the formation of cellular PCNA foci depends on viral DNA replication. Localization of the sites of viral DNA replication and cellular PCNA were examined in BmN-4 cells infected with BmNPV, which is inherently missing the pcna gene (Gomi et al., 1999). Similar to the AcMNPV-infected Sf9 cells, BmNPV DNA replication foci were observed as large foci and staining of BmN-4 cell PCNA overlapped with these viral DNA replication sites (Fig. 3g).

This is the first report that demonstrates colocalization of cellular DNA replication factors and NPV DNA replication sites. Although further studies are required to reveal the functional role of cellular PCNA in NPV DNA replication, it has been suggested that cellular PCNA functions as a DNA sliding clamp in viral DNA replication. In contrast to wt AcMNPV-infected cells, Sf9 cells infected with pcna-defective AcMNPV mutants showed stronger PCNA staining (Fig. 3b, second row versus Fig. 3c, d and e, second rows). This result was consistent with previous findings that the amount of Sf9 cell PCNA in the nucleus is greater in cells infected with pcna-defective AcMNPV mutants than in cells infected with wt AcMNPV (Fig. 1b; Iwahori et al., 2002b). These results suggest that wt AcMNPV utilizes both cellular and viral PCNAs in its DNA replication, whereas pcna-defective AcMNPV mutants substitute cellular PCNA for viral PCNA. Previous results indicating that Ac-PCNA is not essential for viral DNA replication are probably as a result of cellular PCNA functioning instead of Ac-PCNA. Although localization of Ac-PCNA has not yet been visualized, it is probable that Ac-PCNA associates with the replicating virus DNA and is involved in virus DNA replication. Recent studies in our laboratory have shown that the infectivity of pcna-defective AcMNPV mutants is lower than that of wt AcMNPV in NSC-HyCu15B (Iwahori et al., 2002a) and FRI-Splm-1229 cells (Mitsushashi & Inoue, 1988) derived from Hyphantria cunea and Spilosoma imparilis, respectively (data not shown). It is likely that Ac-PCNA is responsible for the stimulation of viral DNA synthesis in these two insect cells, which can influence the host range of AcMNPV. Further studies are required to elucidate the molecular mechanism of Ac-PCNA function in AcMNPV DNA replication.

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References


