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Cloning and characterization of a Dim1-like mitosis gene of *Spodoptera frugiperda* cells (Sf9) induced by *Autographa californica* multiple nucleopolyhedrovirus

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ABSTRACT

Dim1 proteins are evolutionarily highly conserved throughout the eukaryotes and are present in numerous species. These proteins are essential for mitosis and pre-mRNA splicing. In this study, the full-length cDNA of Dim1-like gene from *Spodoptera frugiperda* cells (Sf9) was obtained. *S. frugiperda* Dim1 (SfDim1) cDNA is comprised of 975 bp including a 429 bp open reading frame (ORF), 225 bp 5′ untranslated region (5′ UTR), and 321 bp 3′ untranslated region (3′ UTR) with a poly A tail. The predicted polypeptide encoded by this gene is 142 aa with a molecular weight of 16.76 kDa and a PI of 5.53. Sequence alignment and phylogenetic analysis showed high similarities with Dim1 of other species. The evolutionary conserved site of Dim1 proteins (^{35}Asp–Pro–Thr–Cys^{38}) is also present in SfDim1. Silencing of *SfDim1* gene decreased cell proliferation at 72 h post-treatment in comparison to mock and control transfected cells. Using RT-PCR, we found relatively higher *SfDim1* transcript levels following *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) infection compared to mock-infected cells from 4 h post-infection (hpi) up until 24 hpi. The expression level diminished dramatically at 36 hpi up to 120 hpi with no expression detected at 144 hpi. Silencing of *SfDim1* resulted in lower levels of virus DNA production in comparison to mock-infected cells, which suggested that SfDim1 might benefit the virus and facilitate viral replication. Overall, the results showed that SfDim1 protein is involved in cell proliferation as well as cell–virus interaction.

1. Introduction

Dim1 (defective entry into mitosis) is a small protein of 15 kDa that was initially identified in *Schizosaccharomyces pombe* and shown to be essential both for cell cycle progression as well as for chromosome segregation in mitosis (Berry and Gould, 1997). Dim1 proteins are evolutionarily highly conserved throughout the eukaryotes and are present in numerous species with about 79% sequence identity throughout the entire length of 142 amino acids (Reuter et al., 1999; Zhang et al., 1999). Dim1 deletion from *S. pombe* genome led to a lethal G2 arrest phenotype which was rescued by overexpression of the homologous mouse Dim1. In contrast, a Dim1-35 temperature sensitive mutant entered mitosis but showed a loss of viability during this process (Berry and Gould, 1997). Taken together, these findings clearly showed the vital function of Dim1 for entry into mitosis and cell cycle progression.

In an effort to characterize the protein composition of *Saccharomyces cerevisiae* (U4/U6.U5) tri-snRNP, an orthologue of Dim1 (Dib1) was found to be associated with pre-mRNA splicing suggesting that Dib1 might function in pre-mRNA splicing and that its role in cell cycle progression could be indirect (Gottschalk et al., 1999; Stevens and Abelson, 1999). In subsequent studies for characterization of Dim1 function, Dim1 protein was co-purified with the U4/U6.U5 tri-snRNP component Prp1 and was shown to be required for efficient splicing of Lid1 pre-mRNA since *S. pombe* cells lacking Dim1 protein or *S. cerevisiae* cells lacking Dib1 (orthologue of Dim1) failed to splice pre-mRNA in vivo (Carnahan et al., 2005). These findings indicated that Dim1 is implicated in not only cell cycle progression but also in a more specific molecular process such as pre-mRNA splicing.

Baculoviruses are a family of large rod-shaped viruses that infect arthropods (specially insects) and have double-stranded DNA circular genomes ranging from 80 to 180 kb in size. The most studied baculovirus is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), the type species of *Nucleopolyhedrovirus* (NPV) of *Baculoviridae* family, which was originally isolated from a lepidopteran insect and contains a 134-kbp genome with 154 open reading frames (ORFs) (Ayres et al., 1994; Jehle et al., 2006). Comparison of different genomes of baculoviruses has revealed the

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presence of common genes in these viruses which can be categorized into RNA transcription, DNA replication, structural proteins, auxiliary proteins, and proteins of unknown function (Herniou et al., 2003). Expression of baculovirus genes is regulated at the level of transcription and based on the timing of expression they are categorized into early, late, and very late genes (Herniou et al., 2003). Early genes are transcribed by host RNA polymerase II; however, late and very late genes are transcribed by a viral RNA polymerase (Fuchs et al., 1983; Guarino et al., 1998). Several studies have revealed differential expression of host genes in response to baculovirus infection either at the level of transcriptome (Breitenbach et al., 2011; Nobiron et al., 2003; Salem et al., 2011) or proteome (Carinhas et al., 2011; Popham et al., 2010). According to the studies, most host genes are down-regulated during the infection, although a small number are up-regulated. It has been suggested that down-regulation of host protein synthesis is a host immune response to AcMNPV infection (Du and Thiem, 1997), however, it is now accepted that viruses mainly suppress the host translation machinery (known as "shut-off") by hijacking or modifying certain genes to ensure an efficient translation of viral mRNAs and the simultaneous decline of host translation (Bushell and Sarnow, 2002; Schneider and Mohr, 2003; Thompson and Sarnow, 2000; Toribio and Ventoso, 2010).

Both virus and host cell genes and their proteins are involved in the interactions. Several baculovirus genes have been reported to confer advantage to the virus during infection of hosts by modifying host defense reactions (Thiem, 2009). Here, we report cloning, characterization, and expression analysis of a Dim1-like gene from *Spodoptera frugiperda* cells (Sf9), a widely used model insect cell. Dim1 proteins remain mainly uncharacterized and their functions are not well known in insects. *S. frugiperda* Dim1-like protein (SfDim1) shows high similarities to other reported Dim1 proteins and contains the evolutionary conserved sites found in Dim1 proteins. We also show that *SfDim1* expression is highly induced in response to AcMNPV infection and thereby may facilitate AcMNPV replication in the cells.

2. Materials and methods

2.1. Cell culture and virus infection

S. frugiperda cell line (Sf9) was maintained in SF900-II serum free medium (Invitrogen) as monolayers in cell culture flasks at 27 °C.

AcMNPV was amplified in Sf9 cells and budded viruses accumulated in the medium were used for inoculations. For AcMNPV infection, 2×10^6 cells were infected at a multiplicity of infection (MOI) of 5 as described (King and Possee, 1992) diluted in Sf900-II medium. An hour after incubation at 27 °C, fresh medium was added to the cells and incubated further at 27 °C.

2.2. Electrophoresis and Western blotting

Protein samples were run on a denaturing 12% SDS–PAGE gel and Western blotting was carried out as previously described (Green and Sambrook, 2012). The blot was blocked in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween20) containing 5% non-fat dry milk for 1 h, washed three times in TBST and incubated in TBST-1% non-fat dry milk containing a gp64 monoclonal antibody (1:10,000) followed by an anti-mouse IgG antibody conjugated with alkaline phosphatase (1:10,000) overnight at room temperature. The blot was washed and developed using nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) reagents.

2.3. RNA extraction and gene expression analysis

The in vitro temporal expression patterns of SfDim1 were determined by reverse transcription polymerase chain reaction (RT-PCR). Sf9 cell monolayers were incubated in 20 mm petri dishes at 27 °C and infected with 300 µl of AcMNPV inoculum (MOI of 5) as described above. Cells from a single petri-dish were resuspended by pipetting, centrifuged (1 min at 16,000*g*), and the cell pellet was frozen at −20 °C at 4, 8, 24, 36, 48, 72, 96, 120, and 144 h post-infection. RNA was extracted from cells using Tri-reagent according to the manufacturer's instructions (Molecular Research Center). All samples were then treated with DNase I (Promega) to remove possible DNA contamination. Reverse transcription was performed at 42 °C for 1 h with avian myeloblastosis virus reverse transcriptase (AMV-RT, Promega) using both SfDim1-specific and actin-specific reverse primers (Table 1). Samples were also run without the addition of AMV-RT to ensure that detection by PCR was not due to amplification of viral genomic DNA. The samples were run on 1% agarose gel (for SfDim1) and 2.5% agarose gel (for actin) containing ethidium bromide and viewed under UV light.

2.4. Genomic DNA extraction and southern hybridization

Total genomic DNA was isolated from cells using a method described previously (Glatz et al., 2003). DNA samples (2 µg) were digested with EcoRI for 3 h, run on a 1% agarose gel and transferred onto a nylon membrane overnight using 0.4 M NaOH. The membrane was probed with a DNA fragment coding for AcMNPV capsid protein gene and washed four times under stringent conditions before exposure to a phosphorimager screen and scanning.

2.5. Quantitative PCR

Total genomic DNA was extracted from cells and subjected to qPCR using specific primers to *ie*-1 from AcMNPV genome. DNA concentrations were measured with Nanodrop and 10 ng total genomic DNA was used for each qPCR reaction using SYBR Green (Invitrogen) with a Rotor-Gene 6000. Primers used for qPCR are shown in Table 1. Real-Time PCR conditions were 50 °C for 2 min and 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 20 s, and final extension of 72 °C for 20 s. Actin was used for normalizing the data. Relative DNA levels from each sample were compared in Qgene template program. Reactions from three biological replicates were repeated three times.

2.6. RNAi silencing

DNA fragments of ~500 bp in size were amplified by PCR from both *SfDim1* gene and green fluorescent protein (GFP). Forward and reverse primers contained T7 promoter sequence (Table 1) at their 5′ end for in vitro RNA synthesis. Double stranded RNA (dsRNA) was then produced and purified for each fragment using the MEGAScript kit according to the manufacturer's instructions (Ambion). Synthesis was confirmed by running dsRNA on an agarose gel and the concentration of RNA was determined by measuring absorbance at 260 nm.

To induce RNA silencing in vitro, Sf9 cells were resuspended and $\sim 1 \times 10^3$ cells added to individual wells of a 12-well plate. Once the monolayers had formed (~1 h) the medium was removed and a transfection medium was added. This medium consisted of 0.5 ml SF-900II, 8 µl Cellfectin (Invitrogen), and 2 µg dsRNA either for *SfDim1* gene or *GFP*. 24 h after, each well was infected with 200 µl of AcMNPV inoculum (MOI of 5). The plate was then incubated at 27 °C for 48 h for analyses.