ORF018R, a highly abundant virion protein from Singapore grouper iridovirus, is involved in serine/threonine phosphorylation and virion assembly

Fan Wang, Xuezhi Bi, Li Ming Chen and Choy-Leong Hew

Department of Biological Sciences, National University of Singapore, Lower Kent Ridge Road, 117543, Singapore

Iridovirus is an important pathogen causing serious diseases among wild, cultured and ornamental fish. Previous studies have shown that Singapore grouper iridovirus (SGIV) contains 162 open reading frames (ORFs) from which 51 viral proteins have been confirmed by proteomics studies. ORF018R, which is conserved among vertebrate iridoviruses, is an abundant virion protein identified from SGIV. Here, immunofluorescence staining showed that ORF018R occurred at high abundance throughout SGIV-infected cells. The function of ORF018R was explored using antisense morpholino oligonucleotides (asMOs). Knockdown of ORF018R expression resulted in a reduction in the expression of viral late genes, distortion of viral particle assembly and inhibition of SGIV infection in grouper embryonic cells. Western blotting with phosphoserine-specific antibody indicated that serine phosphorylation was significantly enhanced for proteins of molecular masses 17–32 kDa by SDS-PAGE when ORF018R expression was eliminated. These proteins were analysed further by two-dimensional gel electrophoresis, and numerous protein spots were found to shift to a lower pl and higher molecular mass as a result of the loss of ORF018R function. Five proteins with enhanced phosphorylation were identified by matrix-assisted laser desorption/ionization time-of-flight (TOF)-TOF mass spectrometry, including three viral proteins: ORF049L (dUTPase), ORF075R and ORF086R, and two host proteins: subunit 12 of eukaryotic translation factor 3 and natural killer enhancing factor. These findings suggest that ORF018R is involved in serine/threonine phosphorylation in SGIV-infected late-stage cells and plays an important role in expression of viral late genes and virion assembly.

INTRODUCTION

Iridoviruses are large DNA viruses that infect invertebrates and poikilothermic vertebrates (Williams, 1996). The iridovirus genome is a linear dsDNA, which is terminally redundant and circularly permuted (Goorha & Murti, 1982). The virion of iridovirus can be separated into three layers: the capsid, lipid layer and a DNA–protein complex (Yan et al., 2000). In recent years, vertebrate iridoviruses have been recognized as key pathogens in wild and farmed fish, reptile and amphibian species (Daszak et al., 1999). Singapore grouper iridovirus (SGIV) has tentatively been assigned to the genus Ranavirus and is unusual among vertebrate iridoviruses in that the genome is not highly methylated due to the lack of a virally encoded methyltransferase (Song et al., 2004). The virus causes major losses in the marine fish-cage agriculture industry (Chua et al., 1994). To explore the infection mechanism of this virus and possibly develop therapeutic agents, our laboratory has sequenced the whole genome of SGIV, which comprises 162 annotated open reading frames (ORFs) (Song et al., 2004), determined its virion protein profiles with different proteomics methods (Song et al., 2006) and demonstrated its transcription profiles using DNA microarrays (Chen et al., 2006).

Previously, the SGIV gene ORF018R has been shown to be conserved in frog virus 3 (FV-3), tiger frog virus, Ambystoma tigrinum virus and grouper iridoviruses (Eaton et al., 2007). BLAST analyses have shown that the encoded protein contained a partial serine/threonine kinase catalytic domain, suggesting that this protein might be involved in phosphorylation processes. Moreover, this protein has been shown to occur at high abundance in mature SGIV virions, indicating an important role in virus infection (Song et al., 2004, 2006).

Protein phosphorylation/dephosphorylation plays a critical role in the regulation of cellular activities. Viruses often hijack cellular kinases, or encode their own kinases, to support numerous viral infection processes including transcription (Nekhai et al., 2007), translation (Mohr, 2006), assembly (Liu & Brown, 1993) and inhibition of apoptosis (Leopardi et al., 1997). Like many other DNA
viruses, iridoviruses also encode their own protein kinases, and bioinformatics analyses have identified two serine/threonine protein kinases as core genes in the family *Iridoviridae* (Eaton et al., 2007).

Here, iridovirus phosphorylation was explored by knock-down of SGIV ORF018R expression using an antisense morpholino oligonucleotide (asMO) technique, using grouper embryonic cells as the host. Immunofluorescence (IF) staining revealed that ORF018R was localized throughout SGIV-infected cells. The delivery of an asMO against ORF018R (asMO<sup>17</sup>) into grouper embryonic cells using Amaza nucleofaction technology resulted in dramatically reduced expression of ORF018R that further led to: (i) partial inhibition of expression of late viral genes and blocking of virus infection, (ii) distortion of viral DNA packaging and virion assembly, and (iii) enhanced serine phosphorylation. Moreover, five proteins with enhanced phosphorylation were identified using two-dimensional gel electrophoresis mass spectrometry (2DE-MS) analyses, of which three were viral proteins: ORF075R, ORF086R and ORF049L (dUTPase), and two were host proteins: natural killer enhancing factor (NKEF) and eukaryotic translation initiation factor 3 (eIF3) subunit 12. Based on these studies, we propose that ORF018R is involved in serine/threonine phosphorylation during SGIV infection.

**METHODS**

**Cell culture and virus infection.** Grouper embryonic (GE) cells from the brown-spotted grouper *Epinephelus tauvina* (Chua et al., 1994) were cultured in Eagle’s minimum essential medium (MEM) containing 10% fetal bovine serum, 0.116 M NaCl, 100 IU penicillin G ml<sup>–1</sup> and 100 μl streptomycin sulfate ml<sup>–1</sup>. Stocks of SGIV were propagated and titres were determined in GE cells (Song et al., 2004). In typical experiments, GE cells were transfected with asMOS, infected with SGIV at an m.o.i. of 3 at 24 h post-transfection (p.t.) and SGIV-infected cells were collected or fixed at 48 h post-infection (p.i.) unless otherwise indicated. To radiolabel proteins, the inoculum was replaced at 24 h p.t. with methionine-free MEM containing 25 μCi (925 kBq) [<sup>35</sup>S]methionine (Perkin-Elmer Life Sciences) ml<sup>–1</sup>.<sup>1</sup>

**Antibodies.** PCR fragments representing ORF018R, ORF093L and ORF140R were amplified as described previously (Song et al., 2004) and digested with restriction enzymes. ORF018R and ORF093L were cloned into pGEX-6p-1, and ORF140R was cloned into pET-15b. Recombinant ORF018R and ORF140R were purified to generate rabbit anti-ORF018R and anti-ORF140R polyclonal sera, respectively; the recombinant glutathione S-transferase (GST)–ORF093L was purified to generate mouse anti-ORF093L polyclonal serum. For the purification of ORF018R, glutathione–Sepharose 4B beads (Amersham) bound to GST–ORF018R were incubated with 10 clavage units PreScission protease mg<sup>–1</sup> (Amersham) at 4 °C for 16 h. The eluted ORF018R was passed through a Superdex 75 column (Pharmacia) to remove the PreScission protease and GST. The purified ORF018R was used to raise antibodies, and the rabbit polyclonal serum was further purified using Affi-Gel (Bio-Rad). The monoclonal antibody against actin was from Chemicon and the monoclonal antibody against phosphoserine was from Qiagen. Horseradish peroxidase-conjugated donkey anti-mouse and donkey anti-rabbit polyclonal antibodies were from Pharmacia.

**AsMO design and transfection.** AsMO design was based on the fully sequenced genome and predicted ORFs (Song et al., 2004). The asMO was designed to be complementary to the first 24 bp of the ORF018R coding sequence and one upstream nucleotide: 5′-GCGTTCAGATAGTTTTACGGACATC-3′. Thus, the target sequence of the SGIV ORF018R gene was: (−1 to +24) 5′-GATGTCC-GTAAAACTATCTGAAGCC-3′. In addition, the sequence was screened using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against the whole database to preclude unintentional gene-silencing effects. The negative-control asMO (asMO<sup>18</sup>) was the standard control oligonucleotide from GeneTools. AsMOS were delivered using a Nucleofector kit (Amaxa) at a concentration of 20 μM. Using an optimized program (T-27) and buffer (T), the efficiency of asMO transfection was found consistently to exceed 90%. All asMOS were synthesized and purified by GenTools.

**Viral protein analysis.** GE cells were transfected with asMOS and infected at 24 h p.t. SGIV-infected cells were collected at 48 h p.i., pelleted, washed twice with ice-cold PBS and lysed in lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 2 mM sodium vanadate and protease inhibitor cocktail (Roche)]. Lysates were clarified by centrifugation at 12,000 r.p.m. (Sigma 1-15 centrifuge with a polypropylene 24 × 1.5/2.2 ml angle rotor 12124) for 10 min at 4 °C. Protein concentrations were determined using a Coomassie Protein Assay kit (Pierce). Expression of viral proteins was analysed by Western blot assays. Similar total amounts of [35S]-labelled proteins were resolved on gels and detected by autoradiography.

For Western blot analysis, proteins were transferred from gels onto nitrocellulose membranes, which were then soaked in Tris-buffered saline/0.2% Tween 20 (TBST) containing 2% BSA and incubated at 25 °C with the indicated primary antibodies in TBST, followed by incubation with secondary, species-specific, horseradish peroxidase-conjugated antibodies. For phosphoserine detection, Western blotting was performed as suggested by Qiagen. The peroxidase activity was developed using Supersignal West Pico Luminol Enhancer Solution and Supersignal West Pico Stable Peroxide Solution (Pierce) according to the manufacturer’s instructions.

**Detection of ORF018R by IF.** GE cells were transfected with asMOS and grown on coverslips. Cells were infected at 24 h p.t. and fixed with 3% paraformaldehyde/0.02% glutaraldehyde at 48 h p.i., permeabilized with chilled methanol and incubated with antibody against ORF018R (diluted 1:200). Anti-rabbit Alexa Fluor 594-conjugated antibody was used as the secondary antibody (Invitrogen). dsDNA was stained with Hoechst 33342 (Invitrogen). Fluorescence was detected using an Olympus Fluoview 500 confocal microscope. Images were processed with Adobe PhotoShop version 7.0.

**Light microscopy and titration of virus yields.** GE cells were transfected with asMOS and infected with virus at 24 h p.t. at an m.o.i. of 0.5. Unadsorbed virions were removed at 2 h p.i. and the cells washed twice with PBS. The monolayer cells were observed under an Axiovert 200M (Zeiss) microscope at different time intervals. For each transfection, four parallel infections were performed. Viral supernatants from five different time intervals were diluted from 10<sup>−2</sup> to 10<sup>−7</sup> and used to infect GE cells with four repetitions per dilution to perform a TCID<sub>50</sub> assay. Viral titres were calculated using the Spearman–Karber method (Hamilton et al., 1977).

**Electron microscopy.** GE cells were transfected with asMOS and infected with SGIV. Cells were fixed in 2.5% glutaraldehyde/2% paraformaldehyde in PBS, post-fixed with 1% osmium tetroxide and embedded using a Spurr kit (Sigma). Ultrathin sections were cut on an electron microscopy microtome, stained with 2% uranyl acetate/1% lead citrate and viewed under a Philips CM10 electron microscope at 100 kV.
GE cells were treated and infected with SGIV. SGIV-infected cells were collected at 48 h p.i., pelleted and resuspended in lysis buffer (2 M thiourea, 7 M urea, 4 % CHAPS, 30 mM Tris base), which was premixed with Halt protease inhibitor mixture (EDTA-free; Pierce) and endonuclease (Sigma). The sample mixture was vortexed and incubated for 30 min at room temperature, followed by centrifugation at 14000 r.p.m. (Sigma 1-15 centrifuge, see above) at 4 °C for 90 min. The protein content of the supernatant was determined using a Coomassie Protein Assay kit using BSA as the standard. 2DE was performed as described previously (Bi et al., 2006).

Protein sample (150 μg) diluted in 300 μl rehydration buffer [7 M urea, 2 M thiourea, 4 % CHAPS, 20 mM DTT, 0.5 % IPG buffer (pH 3–10); Amersham Biosciences] was used for loading onto 17 cm IPG strips, pH 3–10 (Bio-Rad).

Gel staining and image analysis. All gels were treated with a fixation solution (50 % methanol, 10 % acetic acid) for at least 1 h and then stained according to the modified silver staining method of Blum et al. (1987). Gels were scanned in a Bio-Rad GS-710 densitometer using PDQUEST 7.3 software (Bio-Rad). Image analysis was carried out with the PDQUEST 7.32D software package (Bio-Rad) including the quantitative analysis. At least three replicates of each sample pair were compared and several spots were compared using four replicates. Gel images were normalized based on the total spot volume of each gel.

Trypsin digestion and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF MS and MS/MS analysis. Spots of interest were excised and digested using sequencing-grade modified porcine trypsin (Promega) as described previously (Huang et al., 2002). MS and MS/MS spectra were obtained using an ABI 4700 Proteomics Analyser MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The MS and the MS/MS spectra were searched against the whole NCBI database using MASCOT 2.0 (Matrix Science). Searches were performed without the restriction of protein molecular mass or pI, with mandatory carbamidomethylation of cysteines and with variable oxidation of methionine residues.

RESULTS

Inhibition of SGIV ORF018R expression by asMOS and ORF018R distribution in SGIV-infected late-stage cells

To evaluate the effect of asMO18 on the synthesis of the viral protein ORF018R, asMOctrl and asMO18 were transfected into GE cells. Cells were infected with SGIV at 24 h p.i. In order to evaluate the knockdown efficacy,
both control and knockdown GE cells were collected at 48 h p.i. and analysed by autoradiography (Fig. 1a) or Western blotting (Fig. 1b), or cells were fixed at 48 h p.i. for IF staining (Fig. 1c). Both analyses showed that asMO18 was effective in suppressing the expression of ORF018R. Furthermore, accumulated levels of ORF018R were reduced to undetectable levels (Fig. 1). Western blots showed that expression of the two viral late genes ORF093L and ORF140R was also reduced following ORF018R knockdown (Chen et al., 2006). Moreover, autoradiography showed that expression of most other proteins in virus-infected cells was also blocked by ORF018R knockdown. These results suggested that ORF018R might affect expression of viral late genes. IF staining for the control group revealed that ORF018R was localized throughout SGIV-infected late-stage cells (Fig. 1c). All of these results indicated that ORF018R functions primarily throughout SGIV-infected late-stage cells and that asMO18 transfection led to a significant reduction in expression of ORF018R compared with asMOctrl.

**Effect of ORF018R knockdown on particle formation**

In order to explore phenotype changes resulting from ORF018R knockdown, the morphology of virus particles assembled in the presence of asMO18 was examined by transmission electron microscopy (Fig. 2). The iridovirus-infected cell normally contains a viromatrix, which is a viral particle assembly centre (Huang et al., 2006). In this centre, iridoviruses assemble into infectious particles, followed by some of them gaining their envelopes by budding out of the cells (Qin et al., 2001). Our results showed that most virions in ORF018R-knockdown cells were partially packaged, whilst most virions in control cells were fully packaged (Fig. 2). In order to exclude the possibility that the defective viruses shown in Fig. 2(b) were in the assembling stage of virus packaging, budding virions from asMO18-transfected samples were captured (Fig. 2b, lower panel). Comparing these with control virions (Fig. 2a, lower panel) revealed that they retained the capsid integrity accompanied by distortion of the viral DNA–protein complex cores. Most budding virions observed in asMO18-transfected samples had a phenotype similar to those in Fig. 2(b, lower panel). Furthermore, far fewer budding virions were observed in all cells in the asMO18-transfected group compared with the control group. As immature virus did not bud out of the cells, distorted virions in the asMO18-transfected sample represented a special phenotype caused by the ORF018R knockdown.

**Effect of ORF018R knockdown on virus infectivity**

To evaluate the reduction in SGIV infectivity by asMO18 knockdown, an infection of low m.o.i. (0.5) and short duration (2 h) was performed. TCID50 analyses showed that the number of viral progeny in the supernatants of ORF018R-knockdown groups was reduced compared with the supernatants of control groups. Obvious cytopathic effects emerged at 3 days p.i. for non-transfected cells (control 1) and cells transfected with asMOctrl (control 2), and at 6 days p.i. for the ORF018R-knockdown group (Fig. 3), suggesting that knockdown of ORF018R led to a reduction in the production of viral progeny, and thus inhibition of infectivity.

**Enhanced serine phosphorylation in ORF018R-knockdown infected GE cells**

To explore why ORF018R, as a viral late gene, could cause distortion of virion assembly and block expression of other viral late genes, ORF018R was analysed further using bioinformatics analysis. A part of the serine/threonine
kinase conserved domain was identified in the ORF018R sequence. Moreover, ORF018R has been annotated as a putative serine/threonine kinase conserved in several iridoviruses (Eaton et al., 2007). Thus, serine phosphorylation of proteins was evaluated by Western blotting (Fig. 4). Interestingly, compared with the control group, the ORF018R-knockdown group showed enhanced phosphorylation, specifically for proteins in the 17–32 kDa range (Fig. 4), indicating that its predicted role as a kinase may be more complicated.

As an asMO must bind to at least 14–15 contiguous bases to block a gene transcript (Summerton, 2007) and the ORF018R-knockdown target sequence used was unique, i.e. no other continuous stretch of 14 nt was found to match within the SGIV genome, the effect of the asMO18 was specific to ORF018R within the SGIV genome. Furthermore, uninfected GE cells transfected with asMO18 showed no enhanced phosphorylation (Fig. 4), indicating that asMO18 off-target effects on host cells, if any, did not contribute to any of these phenotype changes.

**2DE analysis of proteins with enhanced phosphorylation in ORF018R-knockdown infected GE cells**

In order to clarify further the enhanced phosphorylation of proteins, a 2DE-MS approach was applied. GE cells transfected with asMOctrl or asMO18 were infected and analysed by 2DE (Fig. 5). Specific regions in the 2D gel, corresponding to the enhanced protein phosphorylation region (in the molecular mass range 17–32 kDa) revealed by Western blotting, were analysed by MS. Approximately 40 significantly altered spots were detected, lying mainly in the pI range of 4.5–7.0. However, only 22 spots, representing only seven proteins, could be identified unambiguously by MS (Fig. 5, Table 1). The remaining spots could not be identified unambiguously and may...
represent proteins from host cells; failure to identify these spots was probably due to the unavailability of the genome of the host grouper fish.

Among the seven identified proteins, five were SGIV proteins [ORF018R, ORF049L (dUTPase), ORF075R, ORF086R and ORF156L] and two (NKEF and eIF3 subunit 12) were host proteins, which were identified by sequence homology. The pI and molecular masses of the host protein spots and their isoforms by 2DE were consistent with calculated theoretical values from the protein database.

By comparing the control 2D gels with the ORF018-knockdown group, it could be seen that translation of the target gene was considerably decreased (Fig. 5a, spot 13, and Fig. 5b, black-circled spot), indicating that the knockdown experiment was effective.

Analysis of the 2D gels also showed that translation of another protein, ORF156L (Fig. 5a and b, spots 12a and 12b, respectively), was also partially blocked in the ORF018-knockdown group. This was in agreement with the results of Western blotting, which showed that translation of viral late proteins was partially blocked by ORF018R knockdown (Fig. 1).

Among the other five identified proteins, ORF049 (dUTPase; Fig. 5, spot 6) not only appeared at a reduced level, but was also accompanied by two new isoforms (Fig. 5b, spots 7 and 8) with higher molecular mass and lower pI in the knockdown group. Similarly, the host protein NKEF (Fig. 5, spot 9) showed decreased levels, with the emergence of two new isoforms (Fig. 5b, spots 10 and 11) with lower pI and higher molecular mass.

Both putative intermediate-early (IE) genes ORF086R (Fig. 5b, spot 14) and eIF3 subunit 12 (Fig. 5b, spot 15) were only identified in the ORF018R-knockdown group. The predicted pI for ORF086R is 7.87, so its position on

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**Fig. 4.** Enhanced serine phosphorylation following ORF018R knockdown, detected by Western blotting. GE cells were transfected with asMO ctrl or asMO 18. Uninfected cells were collected at 24 h p.t. and infected cells at 48 h p.i. Cleared cell lysates (30 µg) were loaded into each lane and phosphoserine, ORF018R and actin were detected using specific antibodies as indicated. Proteins with enhanced serine phosphorylation are indicated by arrows.

**Fig. 5.** Detailed 2DE maps of normal SGIV-infected cells (a) and ORF018R-knockdown SGIV-infected cells (b). The identified spots were numbered as indicated in the 2DE maps and identified as listed in Table 1. Arrows indicate spots located in the same area as spot 15 in the ORF018R-knockdown group. Newly emerged spots (white circles), knocked down ORF018R (black circle) and an area of significant change (dashed circle) are indicated.
the 2D gel would lie in the poorly separated alkaline region and may not be detectable. Possible phosphorylation of ORF086R in the knockdown group would shift it to a lower pI, making its identification possible (the observed pI was 6.3). Spot 15 (Fig. 5b) was identified as the host protein eIF3 subunit 12. The control showed the absence of this spot, but instead showed three faint spots in the same location. However, we failed to identify these spots, probably because of the low levels present.

Most interestingly, in the control group, four clustered spots (Fig. 5a, spots 1a–4a) with increasing molecular mass and decreasing pI were identified as ORF075R, indicating that they may represent isoforms. This protein has previously been shown to be a major virus protein (Song et al., 2004, 2006); within the ORF018R knockdown group, it was represented by five clustered spots (Fig. 5b, spots 1b–4b and 5) with similar molecular mass and pI trends (see also Fig. 6). Tryptic digestion of these spots gave very similar peptide mass fingerprints by MALDI-TOF MS (Fig. 6). A 2D map comparison using PDQUEST indicated that spot 5 (Fig. 6a, panel ii) was a newly emerged spot in the ORF018R-knockdown group (Fig. 6). Analysis of this new spot by MALDI-TOF/TOF MS identified it as a variant of ORF075R.

In the control group, 2D gels exhibited a large number of clusters of spots (Fig. 5a, dashed circle). In the ORF018R knockdown group, these same clusters were significantly altered by merging of multiple spots within each cluster and shifting towards a lower pI (Fig. 5a and b).

As previous Western blot analysis with the ORF018R knockdown group had showed significantly enhanced phosphorylation of proteins in the range 17–32 kDa, the observed shifting of protein spots in the same region towards lower pI and higher molecular mass regions is probably indicative of enhanced phosphorylation.

**DISCUSSION**

The conserved iridovirus protein ORF018R occurs at high abundance in mature SGIV virions (Song et al., 2004, 2006). This study showed that loss of ORF018R expression
resulted in inhibition of viral late protein expression, and in virion assembly distortion and enhanced phosphorylation. Furthermore, 2DE revealed that a number of protein spots showed a shift towards lower pI and higher molecular mass regions on the 2DE map. Twenty-two spots were identified by tryptic digestion followed by MALDI-TOF/TOF MS analyses, representing seven proteins. Our results demonstrated that the traditional loss-of-function analysis in combination with proteomics technology is a powerful way of exploring the function of essential viral genes and could be useful in elucidating virus infection mechanisms, thus accelerating antiviral drug discovery.

We tried to understand phenotype alterations using 2DE-MS to identify proteins. Previous one-dimensional SDS-PAGE revealed that ORF075R is one of the most abundant virion proteins (Song et al., 2004, 2006). Our 2DE analyses revealed that four isoforms of ORF075R exist in SGIV-infected cells, and that an additional isoform with a lower pI and higher molecular mass appeared following

**Fig. 6.** (a) ORF075R isoform cluster changes between the control (panel i) and ORF018R-knockdown group (panel ii) on 2DE. This 2DE result was from an independent knockdown experiment, which confirmed that one more isoform of ORF075R emerged (spot 5) and that a lower molecular mass, higher pI isoform (spot 4b) was reduced in the knockdown sample compared with the control. (b) Similar MALDI-TOF MS peptide mass fingerprinting spectra for spots 5 and 4b. (c) MALDI-TOF/TOF MS/MS spectra for the peptide ILDFSLETAATR with peaks of 1449.8303 (spot 5) and 1449.8893 (spot 4b) (indicated by an arrow in b).
ORF018R knockdown. This spot most likely represents enhanced phosphorylation of ORF075R and may be associated with distortion of virion assembly.

Subunit 12 of eIF3 was identified only in the ORF018R-knockdown group and may arise as a result of enhanced phosphorylation. eIF3 is one of the largest initiation factors and assembles on the 40S ribosomal subunit to participate in the different reactions involved in translation (Hinnebusch, 2006). eIF3 plays an important role in protein synthesis of many viruses including herpesviruses (Fontaine-Rodriguez et al., 2004), hepatitis C virus (Fraser & Doudna, 2007) and rabies virus (Komarova et al., 2007). Phosphorylation of eIF3s is regarded as a common method of translational control (Sarre, 1989). Here, ORF018R knockdown resulted in decreased expression levels of viral proteins during the later stages of virus infection; this could be explained by the modification of certain translation elongation factors.

For dUTPase and NKEF, because the extra spots were detected only in the ORF018R-knockdown group, phosphorylation of these two proteins may not contribute to virus infection and these extra spots were probably caused by uncontrolled virus-encoded protein kinases. Similarly, ORF086R is a putative IE gene in SGIV and no obvious phenotype changes were observed following knockdown of its homologue in FV-3, suggesting that this gene may not be an essential gene for SGIV infection (Sample et al., 2007; Willis et al., 1984). The mechanism by which hyperphosphorylated ORF086R contributes to the virus infection process remains to be elucidated further.

A greater than 15-fold increase in the specific activity of protein kinase has been reported previously in FV-3-infected cells, and a virus-specific serine/threonine kinase was identified in the purified virion capable of phosphorylating many FV-3 virion proteins in vitro (Silberstein & August, 1973, 1976). This suggests that the virus-encoded protein kinase probably predetermines in protein phosphorylation in iridovirus-infected late-stage cells. Recently, nine sequenced iridovirus genomes were compared by bioinformatics analysis and two serine/threonine protein kinases were found to be conserved in all nine of them (Eaton et al., 2007). SGIV ORF039L, one of the conserved serine/threonine protein kinases in iridoviruses, has been identified as an abundant virion protein by our previous proteomics studies (Song et al., 2004, 2006). Both SGIV ORF039L and its FV-3 homologue ORF019R contain virus-specific two-cysteine adapter domains followed by a serine/threonine protein kinase catalytic domain. They should function as serine/threonine protein kinases to phosphorylate virion proteins in mature iridovirus virions. Our in vitro studies indicated that the purified recombinant C-terminally truncated ORF039L was able to phosphorylate the recombinant ORF075R (data not shown). In SGIV-infected cells, ORF075R was found to be phosphorylated and loss of ORF018R function led to hyperphosphorylation of ORF075R and distortion of virion particle assembly. Moreover, in vitro experiments showed that the purified recombinant ORF018R had no p-nitrophenyl phosphate hydrolyase activity (data not shown), suggesting that ORF018R is not a traditional phosphatase. Therefore, we postulate that ORF018R functions as a negative regulator of serine/threonine phosphorylation of some proteins. It participates in the regulation of protein serine/threonine phosphorylation and may coordinate with virus-encoded serine/threonine protein kinases such as ORF039L to maintain the phosphorylation/dephosphorylation homeostasis of the viral phosphoprotein targets such as ORF075R for viral protein synthesis and viral genome packaging. Because ORF018R, ORF039L and ORF075R are known to be three abundant proteins in SGIV mature virions (Song et al., 2004, 2006), and both ORF018R and ORF039L are conserved in vertebrate iridoviruses (Eaton et al., 2007), this regulation of phosphorylation/dephosphorylation probably represents a general phenomenon in vertebrate iridoviruses. Its detailed mechanism remains to be elucidated further.

ACKNOWLEDGEMENTS

We thank Shashikant B. Joshi for critical reading of the manuscript. We are grateful to Loy Gek Luan for assistance with transmission electron microscopy and to the staff in the Protein and Proteomics Centre, Department of Biological Sciences, National University of Singapore, for technical support. This work was supported financially by the Academic Research Fund ‘Functional genomic studies of Singapore grouper iridovirus (R-154-000-223-112)’ from the National University of Singapore to C. L. H.

REFERENCES


