INTRODUCTION

The family Iridoviridae consists of large cytoplasmic DNA viruses that infect insects and cold-blooded vertebrates (Williams, 1996). In 1954, the first iridovirus was discovered by Smith and Xeros. To date, more than 100 iridoviruses have been isolated. They have been classified into five genera including Iridovirus, Chloriridovirus, Lymphocystivirus, Megalocytivirus and Ranavirus (Williams et al., 2005). Singapore grouper iridovirus (SGIV) is a member of the genus Ranavirus (Chinchar et al., 2005; Williams et al., 2005). SGIV causes significant economic losses in Singapore marine net cage farms (Chua et al., 1994). SGIV was isolated from brown-spotted grouper in 1998 (Qin et al., 2001) and its genome was successfully sequenced with 162 predicted open reading frames (ORFs). Twenty-six SGIV proteins were later identified by proteomics analysis (Song et al., 2004). An additional 25 SGIV viral proteins were reported in 2006 (Song et al., 2006). Recently, Chen and coworkers discovered that 127 ORFs of SGIV were transcriptionally active (Chen et al., 2004). Although a total of 51 SGIV proteins have been identified, the translational products of the remaining 111 ORFs are unknown (Song et al., 2004, 2006). All these earlier studies focused on the identification and structural characterization of the viral proteins.

Isobaric tags for relative and absolute quantification (iTRAQ) is a stable isotope method for protein measurement by using mass spectrometry (Ross et al., 2004). It can be used for the comparison of four or eight different samples at the same time (Ross et al., 2004; Pierce et al., 2008) and can be used to examine the proteomic profiles before and after virus infection. For example, in this study, we applied iTRAQ to quantitatively profile the proteomes of SGIV-infected and non-infected grouper embryonic cells (GEC). This is the first study on the iridovirus and host interactions at the proteomics scale. Forty-nine viral proteins were identified and 11 of them are reported for the first time. Furthermore, 743 protein entries of the host were identified and classified into 218 groups based on protein functions. The host proteins affected by SGIV infection are discussed further. Our work should significantly contribute to the understanding of host–pathogen interactions.

METHODS

Cell and virus infection. GEC, from brown-spotted grouper Epinephelus tauvina (Chew et al., 1994), were cultured in Eagle’s minimum essential medium containing 10% fetal bovine serum, 0.116 M NaCl, 100 IU penicillin G ml⁻¹ and 100 µl streptomycin sulfate ml⁻¹. The culture media were equilibrated with HEPES to a final concentration of 5 mM and adjusted to pH 7.4 with NaHCO₃. GEC were infected with SGIV at an m.o.i. of 5. In this experiment, SGIV-infected GEC were harvested 48 h post-infection to ensure almost all the host cells were infected by the virus.

iTRAQ labelling and two dimensional (2D) LC-MALDI MS. The non-infected and infected cells (48 h post-infection of SGIV) were lysed with lysis buffer (0.5 M triethylammonium bicarbonate, pH 8.5, 1% SDS). One hundred micrograms of total protein from each sample of cell lysate was used for the iTRAQ experiment. Four unique iTRAQ reagents were used: 114, 115, 116 and 117, which are named...
RT-PCR products were then analysed on a 1.2% agarose gel. Table S1 available in JGV Online) and 72

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50

reverse transcription step cDNA was synthesized from total RNA at

protocol using treated RNA and gene-specific primers. Briefly, in the

was conducted as described in the One-Step RT-PCR kit (Qiagen)

extracted RNA was treated with RNase-free DNase I (Qiagen). RT-PCR

stage (48 h post-infection) using an RNeasy mini kit (Qiagen). The

was previously (Li

et al.

, 2007).

The LC fractions were analysed by using an ABI 4700 Proteinomics

Analysyer MALDI TOF/TOF mass spectrometer (Applied Biosystems).

GPS Explorer software version 3.5 (Applied Biosystems) employing

MASCOT search engine (version 2.1; Matrix Science) was used for

peptide and protein identifications and iTRAQ quantification. The

NCBI database was used for the search. Cysteine methane thiolation,

N-terminal iTRAQ labelling and iTRAQ-labelled lysine were selected

as fixed modifications during iTRAQ data analysis (Zieske, 2006).

RT-PCR. Total RNA was extracted from infected cell culture at late

stage (48 h post-infection) using a RNeasy mini kit (Qiagen). The

extracted RNA was treated with RNase-free DNase I (Qiagen). RT-PCR

was conducted as described in the One-Step RT-PCR kit (Qiagen)

protocol using treated RNA and gene-specific primers. Briefly, in the

reverse transcription step cDNA was synthesized from total RNA at

50 ºC for 30 min. Reverse transcriptase was inactivated by a heating step

at 95 ºC for 15 min. The PCR amplification of target genes was started

after activation of the HotStar

Cf o r

Taq

37

C for 30 s (Supplementary

10 of the 11 newly identified proteins, except ORF049L that is

been reported previously (Song

et al.

, 2004, 2006). In addition,

proteins were scored with more than 95 % of total ion score

confidence interval percentage (CI %). Only ORF022L, which

has been reported by Song

et al.

(2006), was identified at

86.351 CI %. Of these 49 proteins, 11 were discovered for the first time (Table 1), the remaining 38 proteins (Table 2) have

been reported previously (Song

et al., 2004, 2006). In addition, 10 of the 11 newly identified proteins, except ORF049L that is

predicted to be a dUTPase-like protein (Song

et al., 2004), are

novel proteins with unknown functions.

RESULTS

Identification of viral proteins

Proteins of SGIV-infected GEC (48 h post-infection) and of

non-infected cells were harvested. The cell lysate was treated

with 5 × SDS loading dye and boiled for 10 min. The proteins were

fractionated by SDS-PAGE on a 15 % gel and blotted onto a

nitrocellulose membrane (Amersham) in blotting buffer (3.205 g Tris

base, 14.25 g glycerine and 200 ml methanol per litre) for 1 h at 70 V.

The blotted membrane was then blocked in 5 % skimmed milk, 1 %

BSA in TBST (20 mM Tris pH 7.4, 154 mM NaCl, 0.1 % Tween 20)

for 1 h and washed three times for 10 min with TBST. The

membranes were incubated with the primary antibodies (different
dilution for each antibodies) for 1 h and washed three times for

10 min with TBST, treated with secondary antibodies for 1 h, then

washed thrice for 10 min. After that, the membranes were

treated with a mixture of Super Signal West Pico Stable Peroxide

Solution and luminol enhancer solution (Pierce) for 5 min and then

exposed to X-ray films for image development.

In addition, antibodies for proteins encoded by SGIV ORF18F,

ORF26R, ORF135L and ORF140L were raised from New Zealand

White female rabbits. The dilution factors for these antibodies for

Western blot analysis were 1:5000, 1:1000, 1:300 and 1:5000,

respectively. Only antibody for SGIV ORF93L protein was raised from

mouse and the dilution factor for Western blot analysis was 1:500.

### Table 1. Eleven SGIV proteins that were newly identified by the iTRAQ experiment

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Gene function</th>
<th>Protein MW</th>
<th>Protein pl</th>
<th>Peptide count</th>
<th>Total ion score</th>
<th>Total ion CI %</th>
<th>Best ion score</th>
<th>Best ion CI %</th>
<th>Gene expression class</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF049L</td>
<td>gi56692686</td>
<td>dUTPase</td>
<td>18678.9</td>
<td>6.51</td>
<td>3</td>
<td>322.16</td>
<td>100</td>
<td>140.84</td>
<td>100</td>
<td>E</td>
<td>Chen et al. (2006)</td>
</tr>
<tr>
<td>ORF099R</td>
<td>gi56692736</td>
<td>dUTPase</td>
<td>10650.5</td>
<td>6.13</td>
<td>3</td>
<td>221.15</td>
<td>100</td>
<td>92.47</td>
<td>99.9996</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ORF107R</td>
<td>gi56692744</td>
<td>dUTPase</td>
<td>41572.6</td>
<td>5.91</td>
<td>1</td>
<td>84</td>
<td>99.9972</td>
<td>84</td>
<td>99.9972</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ORF111R</td>
<td>gi56692748</td>
<td>dUTPase</td>
<td>32503.8</td>
<td>5.47</td>
<td>1</td>
<td>90.83</td>
<td>99.9994</td>
<td>90.83</td>
<td>99.9994</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ORF118R</td>
<td>gi56692755</td>
<td>dUTPase</td>
<td>41293.7</td>
<td>8.76</td>
<td>4</td>
<td>303.46</td>
<td>100</td>
<td>112.84</td>
<td>100</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>ORF127R</td>
<td>gi56692764</td>
<td>dUTPase</td>
<td>21925.3</td>
<td>7.01</td>
<td>1</td>
<td>57.35</td>
<td>98.6845</td>
<td>57.35</td>
<td>98.6845</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>ORF135L</td>
<td>gi56692772</td>
<td>dUTPase</td>
<td>15150.0</td>
<td>5.95</td>
<td>3</td>
<td>248.72</td>
<td>100</td>
<td>94.23</td>
<td>99.9997</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ORF136R</td>
<td>gi56692773</td>
<td>dUTPase</td>
<td>13116.6</td>
<td>7.52</td>
<td>1</td>
<td>80.94</td>
<td>99.9942</td>
<td>80.94</td>
<td>99.9942</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>ORF140R</td>
<td>gi56692777</td>
<td>dUTPase</td>
<td>36630.9</td>
<td>4.85</td>
<td>14</td>
<td>1408.35</td>
<td>100</td>
<td>181.16</td>
<td>100</td>
<td>IE</td>
<td></td>
</tr>
<tr>
<td>ORF155R</td>
<td>gi56692792</td>
<td>dUTPase</td>
<td>68875.1</td>
<td>5.03</td>
<td>13</td>
<td>136.06</td>
<td>100</td>
<td>82.44</td>
<td>99.9823</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>ORF162L</td>
<td>gi56692799</td>
<td>dUTPase</td>
<td>49351.3</td>
<td>6.49</td>
<td>3</td>
<td>158.02</td>
<td>100</td>
<td>61.4</td>
<td>98.4232</td>
<td>E</td>
<td></td>
</tr>
</tbody>
</table>
The transcriptional products of the 11 ORFs, which corresponded to the 11 newly identified proteins, were examined by RT-PCR (primers are listed in Supplementary Table S1 available in JGV Online). These 11 ORFs have full-length transcriptional products as shown in Fig. 1.

In addition, the recombinant proteins of ORF018R, ORF026R, ORF093L, ORF135L and ORF140R were expressed, purified and used to raise antibodies. The
Western blot showed the presence of these proteins in the infected GEC, and as expected, none in the non-infected GEC (Fig. 2).

Identification of differentially expressed host proteins

To investigate the host cell protein responses after SGIV infection, the iTRAQ data of non-infected GEC (as a control) and SGIV-infected cells were searched against the NCBI database. Of the 743 host proteins found, 726 proteins were identified with total ion score CI % ≥95 % (Supplementary Table S2 available in JGV Online). Twelve of them (total ion score CI % ≥95 %) were upregulated more than 1.5-fold upon virus infection (Table 3) and another five were downregulated more than 1.5-fold (Table 4).

Given that two upregulated proteins, matrix protein and putative head-tail adaptor, did not match to any of the 162 ORFs from SGIV genome, they were considered as host proteins.

Upregulation of host histone H3 lysine 79 (K79) methylation upon SGIV infection

This set of iTRAQ data showed that the host histone H3 was downregulated upon SGIV infection but did not indicate the histone modification status (Table 4). In fact, we did not include any variable modifications in the database search; hence, the result only illustrated that non-modified histone H3 was downregulated. The histone H3 peptide identified by iTRAQ was EIAQDFKTDLR, in which K (lysine) is the seventy-ninth amino acid residue. Histone H3 K79 methylation could occur at this position (Peterson & Laniel, 2004). Although the iTRAQ study only showed the non-modified form, a Western blot approach could enable us to examine the expression of total histone H3 and histone H3 K79 methylation. Western blot experiments were conducted for SGIV-infected and non-infected cell lysates using commercial anti-histone H3 antibody and anti-histone H3 K79 methylation antibody. The results demonstrated that histone H3 K79 methylation was upregulated upon virus infection, while total histone H3 remained unchanged (Fig. 3).

DISCUSSION

Identification and validation of viral proteins

In the present investigation, 49 viral proteins were identified using iTRAQ analysis. Some of their encoded ORFs, such as ORF018R, ORF026R and ORF093L, were previously reported as full-length putative genes (Song et al., 2004). In this study, we were able to show using Western blot with specific antibodies that these ORFs encode full-length protein products (Fig. 2). Similarly, the ORFs of the 11 new proteins, first reported in this work, had full-length transcriptional products as evidenced by the RT-PCR results (Fig. 1). In addition, the translational products of ORF140R and 135L were validated by Western blot (Fig. 2), showing that these proteins were expressed in the cell culture.
Among the 49 SGIV viral proteins, only six proteins are predicted to contain conserved domains such as the dUTPase conserved domain (ORF049L), major capsid protein (ORF072R), tyrosine kinase (ORF081L), RNase III (ORF084L), putative immediate-early protein (ORF086R) and ubiquitin/ribosomal-like protein (ORF102L). The remaining 43 viral proteins have no homology to any known proteins. However, the functions of all these viral proteins would need to be determined further.

A total of 51 SGIV proteins was identified by Song et al. (2006); 38 of them were determined in this study. The reasons for the absence of the remaining 13 proteins in these iTRAQ data may be due to the technical differences between the two methods and the amount of samples used in these studies.

### Table 3. Twelve proteins from the host cells that were upregulated more than 1.5-fold upon SGIV infection

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Protein MW</th>
<th>Protein pl</th>
<th>Peptide count</th>
<th>Total ion score</th>
<th>Total ion score CI %</th>
<th>Best ion score</th>
<th>Best ion score CI %</th>
<th>Avg iTRAQ ratio (115/114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP synthase F1, alpha subunit</td>
<td>gi</td>
<td>67920483</td>
<td>57766.36</td>
<td>4.86</td>
<td>1</td>
<td>55.95</td>
<td>98.18407</td>
<td>55.95</td>
<td>98.18407</td>
</tr>
<tr>
<td>Dihydrofolate synthase</td>
<td>gi</td>
<td>76797895</td>
<td>48305.22</td>
<td>5.66</td>
<td>1</td>
<td>53.35</td>
<td>96.69554</td>
<td>53.35</td>
<td>96.69554</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>gi</td>
<td>116207548</td>
<td>26253.87</td>
<td>8.71</td>
<td>1</td>
<td>55.46</td>
<td>97.96717</td>
<td>55.46</td>
<td>97.96717</td>
</tr>
<tr>
<td>Matrix protein</td>
<td>gi</td>
<td>549374</td>
<td>40992.82</td>
<td>8.78</td>
<td>1</td>
<td>63.26004</td>
<td>99.66264</td>
<td>64.68</td>
<td>99.75672</td>
</tr>
<tr>
<td>Mucin-associated surface protein (MASP)</td>
<td>gi</td>
<td>71659996</td>
<td>43040.39</td>
<td>4.89</td>
<td>1</td>
<td>55.46</td>
<td>97.96717</td>
<td>55.46</td>
<td>97.96717</td>
</tr>
<tr>
<td>Predicted: hypothetical protein</td>
<td>gi</td>
<td>118103728</td>
<td>110544.7</td>
<td>8.68</td>
<td>1</td>
<td>53.35</td>
<td>96.69554</td>
<td>53.35</td>
<td>96.69554</td>
</tr>
<tr>
<td>Predicted: similar to alpha-2-macroglobulin isoform 1</td>
<td>gi</td>
<td>91092844</td>
<td>36679.73</td>
<td>9.8</td>
<td>1</td>
<td>51.61</td>
<td>95.06712</td>
<td>51.61</td>
<td>95.06712</td>
</tr>
<tr>
<td>Predicted: similar to CG16944-PA, isoform A</td>
<td>gi</td>
<td>49057575</td>
<td>114733.1</td>
<td>8.68</td>
<td>1</td>
<td>51.78</td>
<td>95.25648</td>
<td>51.78</td>
<td>95.25648</td>
</tr>
<tr>
<td>Procollagen, type IV, alpha 1, isoform CRA_a</td>
<td>gi</td>
<td>15830847</td>
<td>13401.89</td>
<td>9.15</td>
<td>1</td>
<td>53.35</td>
<td>96.69554</td>
<td>53.35</td>
<td>96.69554</td>
</tr>
<tr>
<td>Putative head-tail adaptor</td>
<td>gi</td>
<td>121528555</td>
<td>150867.5</td>
<td>9.15</td>
<td>1</td>
<td>53.98</td>
<td>97.14175</td>
<td>53.98</td>
<td>97.14175</td>
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<tr>
<td>Transposase</td>
<td>gi</td>
<td>84501238</td>
<td>68937.66</td>
<td>9.19</td>
<td>1</td>
<td>53.35</td>
<td>96.69554</td>
<td>53.35</td>
<td>96.69554</td>
</tr>
</tbody>
</table>

### Table 4. Five proteins from the host cells that were downregulated more than 1.5-fold upon SGIV infection

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Protein MW</th>
<th>Protein pl</th>
<th>Peptide count</th>
<th>Total ion score</th>
<th>Total ion score CI %</th>
<th>Best ion score</th>
<th>Best ion score CI %</th>
<th>Avg iTRAQ ratio (115/114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain A, rotamer strain as a determinant of protein structural specificity</td>
<td>gi</td>
<td>5821952</td>
<td>9711.81</td>
<td>6.56</td>
<td>2</td>
<td>137.49</td>
<td>100</td>
<td>69.48</td>
<td>100</td>
</tr>
<tr>
<td>Histone 3</td>
<td>gi</td>
<td>73671832</td>
<td>13666.76</td>
<td>11.02</td>
<td>1</td>
<td>73.3</td>
<td>99.96657</td>
<td>73.3</td>
<td>99.96657</td>
</tr>
<tr>
<td>Histone H3</td>
<td>gi</td>
<td>119690002</td>
<td>15074.91</td>
<td>11.04</td>
<td>1</td>
<td>73.3</td>
<td>99.96657</td>
<td>73.3</td>
<td>99.96657</td>
</tr>
<tr>
<td>Predicted: similar to ribosomal protein S27a</td>
<td>gi</td>
<td>82934837</td>
<td>5322.599</td>
<td>5.05</td>
<td>1</td>
<td>125.604</td>
<td>100</td>
<td>125.604</td>
<td>100</td>
</tr>
<tr>
<td>Rho GTPase</td>
<td>gi</td>
<td>66816373</td>
<td>24570.32</td>
<td>7.56</td>
<td>1</td>
<td>51.65</td>
<td>95.11235</td>
<td>51.65</td>
<td>95.11235</td>
</tr>
</tbody>
</table>
of the responses of the host cell to SGIV infection. Twelve proteins of the host cell were upregulated upon SGIV infection (Table 3). The upregulation of host mucin-associated surface protein, an important factor in host innate immune system (Park et al., 2001; Sheehan et al., 2006), possibly provides the links in the host immune responses to viral infection. However, the downregulation of cellular Rho GTPase, an enzyme involved in complex mechanical processes such as cell motility and phagocytosis (Caron & Hall, 1998; Wherlock & Mellor, 2002), might help the virus to escape the host's macrophages.

Once inside a host cell, viruses hijack the host machinery by controlling or utilizing some of the host cell proteins for their own benefit (Williams et al., 2005). The cellular dihydrofolate synthase, involved in folic acid biosynthesis (Tettelin et al., 2005), was upregulated. Folic acid is one crucial element in DNA replication (Jennings, 1995) and its biosynthesis requires ATP as an energy source (Young, 1986). Interestingly, the ATP synthase F1 alpha subunit, which is involved in ATP synthesis-coupled proton transport (Leyva et al., 2003), showed an increased level of expression in SGIV-infected cells.

On the other hand, several host proteins were downregulated (Table 4). Chain A, rotamer strain as a determinant of protein structural specificity, was identified with the two peptide sequences, IQDKEGIPPDQQR and TLSDYNLQK. Chain A was designed as a variant of ubiquitin in the study of the impact of hydrophobic core packing on protein structure specificity (Lazar et al., 1999).

In cells, ubiquitin is synthesized as precursors of polyubiquitin chains or fusion proteins of monoubiquitin with S27a ribosomal protein (Finley et al., 1989; Redman & Rechsteiner, 1989). The iTRAQ results have shown the steady expression of the host polyubiquitin (Supplementary Table S2) and downregulation of S27a protein. It is possible that the host has two different ubiquitin sequences, one from the polyubiquitin chains and one from monoubiquitin–S27a fusion protein, which is identical to the chain A peptide sequence (Fig. 4). Furthermore, alpha-2-macroglobulin isoform 1, which participates in protease inhibition (Westwood et al., 2001), was upregulated in SGIV-infected GEC. It is likely that the virus can protect its proteins by inhibiting the host proteases and ubiquitin expression.

It has been reported that host cell protein synthesis was rapidly or selectively inhibited by virus infection (Kozak, 1986; Schneider & Shenk, 1987; Sonenberg, 1987). This phenomenon has been shown in cells infected by some iridoviruses such as Chilo iridescent virus (Cerutti & Devauchelle, 1980) and Frog virus 3 (Chinchar & Yu, 1992). In SGIV, the synthesis of host proteins was inhibited upon SGIV infection (data not shown). However, in this iTRAQ study, no host proteins were found to be completely shut off. This phenomenon would require further investigation once the grouper genome sequence is available.

### Viral infection and host histone modification

Another interesting finding about the host protein expression upon SGIV infection is the downregulation of non-methylated histone H3. However, Western blot analysis (Fig. 3) revealed that methylated histone H3 K79 was upregulated and the level of both methylated and non-methylated histone H3 was the same after SGIV infection. As a result, the combination of upregulation of histone H3 K79 methylation and downregulation of non-modified histone H3 would make the total histone H3 expression stable.

Methylation of specific lysine residues in histone H3 was found to be closely related to the control of gene transcription (Tachibana et al., 2005). Recently, histone
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REFERENCES


