iTRAQ analysis of Singapore grouper iridovirus infection in a grouper embryonic cell line

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We report, here, the first proteomics study of a grouper embryonic cell line (GEC) infected by Singapore grouper iridovirus (SGIV). The differential proteomes of GEC with and without viral infection were studied and quantified with iTRAQ labelling followed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Forty-nine viral proteins were identified, of which 11 were identified for the first time. Moreover, 743 host proteins were revealed and classified into 218 unique protein groups. Fourteen host proteins were upregulated and five host proteins were downregulated upon viral infection. The iTRAQ analysis of SGIV infection in GEC provides an insight to viral and host gene products at the protein level. This should facilitate further study and the understanding of virus–host interactions, molecular mechanisms of viral infection and pathogenesis.

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 are transcriptionally active (Chen et al., 2006). 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 a 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 microliters 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. Total RNA was extracted from infected cell culture at late stage (48 h post-infection) using an RNeasy mini kit (Qiagen). The amplification reactions were carried out for 30 cycles under conditions of 95 °C for 1.5 min per cycle. The PCR amplification of target genes was started after activation of the HotStar Taq DNA polymerase. The amplification reactions were carried out for 30 cycles under conditions of 95 °C for 30 s, annealing temperature of 55 or 58 °C for 30 s, and 72 °C for 1 min per cycle. The RT-PCR products were then analysed on a 1.2 % agarose gel.

After drying, the iTRAQ-labelled peptides were resuspended in 20 µl 5 mM KH2PO4 buffer containing 5 % acetonitrile, pH 3.0, and separated by 2D-Liquid Chromatography (2D-LC) using an Ultimate dual-gradient LC system (Dionex-LC Packings), as described previously (Li et al., 2007).

The LC fractions were analysed by using an ABI 4700 Proteinics Analyser 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).

### RESULTS

#### Identification of viral proteins

Proteins of SGIV-infected GEC (48 h post-infection) and of the non-infected cells were extracted and prepared for iTRAQ. A total of 49 viral proteins were identified, in which 48 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.

#### 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</th>
<th>Total ion score Cl %</th>
<th>Best ion score Cl %</th>
<th>Gene expression class [Chen et al. (2006)]</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>
</tr>
<tr>
<td>ORF099R</td>
<td>gi56692736</td>
<td>Unknown</td>
<td>10650.5</td>
<td>5.13</td>
<td>3</td>
<td>221.15</td>
<td>100</td>
<td>92.47</td>
<td>99.9996</td>
</tr>
<tr>
<td>ORF107R</td>
<td>gi56692744</td>
<td>Unknown</td>
<td>41572.6</td>
<td>3.91</td>
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<td>84</td>
<td>99.9972</td>
<td>84</td>
<td>99.9972</td>
</tr>
<tr>
<td>ORF111R</td>
<td>gi56692748</td>
<td>Unknown</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>
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<tr>
<td>ORF118R</td>
<td>gi56692755</td>
<td>Unknown</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>
</tr>
<tr>
<td>ORF127R</td>
<td>gi56692764</td>
<td>Unknown</td>
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<td>7.01</td>
<td>1</td>
<td>57.35</td>
<td>98.6845</td>
<td>57.35</td>
<td>98.6845</td>
</tr>
<tr>
<td>ORF135L</td>
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<td>Unknown</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>
</tr>
<tr>
<td>ORF136R</td>
<td>gi56692773</td>
<td>Unknown</td>
<td>13116.6</td>
<td>7.52</td>
<td>1</td>
<td>80.94</td>
<td>99.9942</td>
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<tr>
<td>ORF140R</td>
<td>gi56692777</td>
<td>Unknown</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>
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<tr>
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<td>gi56692792</td>
<td>Unknown</td>
<td>68835.1</td>
<td>5.03</td>
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<td>136.06</td>
<td>100</td>
<td>82.44</td>
<td>99.9959</td>
</tr>
<tr>
<td>ORF162L</td>
<td>gi56692799</td>
<td>Unknown</td>
<td>49351.3</td>
<td>6.49</td>
<td>3</td>
<td>158.02</td>
<td>100</td>
<td>61.4</td>
<td>99.4823</td>
</tr>
</tbody>
</table>
RT-PCR and Western blot analysis of the viral proteins

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.

Fig. 1. RT-PCR products of 11 SGIV genes encoding 11 newly identified proteins by iTRAQ. Total RNA (harvested after 48 h of infection) was isolated using the RNeasy mini kit and amplified by using the OneStep RT-PCR kit. Full-length sequences of these 11 genes were amplified. Lane M, 100 bp DNA ladder (Axygen); lane C, control, in which the template RNA was added into the RT-PCR after the inactivation of reverse transcriptase (15 min at 95 °C).

Fig. 2. Western blot analysis of proteins expressed by SGIV ORF18R, ORF026R, ORF093L, ORF135L and ORF140R. These proteins were detected in infected GEC but absent in non-infected GEC (lanes labelled C).

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 pI</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>
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<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>76979895</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 CHGG_03067</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>6.86</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-macroglubulin 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>
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<tr>
<td>Procollagen, type IV, alpha 1, isoform CRA_a</td>
<td>gi</td>
<td>15830847</td>
<td>13401.89</td>
<td>9.99</td>
<td>1</td>
<td>53.35</td>
<td>96.69554</td>
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<td>96.69554</td>
</tr>
<tr>
<td>Putative signal peptide protein</td>
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<td>150867.5</td>
<td>9.15</td>
<td>1</td>
<td>53.98</td>
<td>97.14175</td>
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<td>Transposase</td>
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<td>68937.66</td>
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<td>1</td>
<td>53.35</td>
<td>96.69554</td>
<td>53.35</td>
<td>96.69554</td>
</tr>
</tbody>
</table>

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.

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### 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 pI</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>99.91944</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>
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<tr>
<td>Histone H3</td>
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<td>15074.91</td>
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<td>125.64</td>
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<td>100</td>
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<tr>
<td>Rho GTPase</td>
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http://vir.sgmjournals.org 2873
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
H3 K79 methylation was postulated as an important factor in DNA repair (Huyen et al., 2004) and leukaemic transformation (Okada et al., 2005). In SGIV-infected cells, histone H3 K79 methylation was found to be upregulated. However, the consequences of this event on gene activation or DNA repair are still not clear.

To our knowledge, this is the first study of SGIV and host cell relations at the proteomics scale. Eleven new viral proteins were identified. In addition, our data showed that expression levels of some host proteins were clearly regulated by the viral infection. These observations provide important information on SGIV–GEC interactions that could help to understand the pathogenesis of iridoviruses.

ACKNOWLEDGEMENTS

This work was financially supported by the Academic Research Fund ‘Functional genomic studies of Singapore grouper iridovirus (T207B3106-RS)’ from the National University of Singapore to C. L. H.

REFERENCES


