6. DISCUSSION

Part I. Expression of Pm-fortilin/TCTP

1. Expression of Pm-fortilin/TCTP transcripts in infected shrimp

WSSV is the most catastrophic pathogens of cultured shrimp (Lightner, 1996). The evidence that survivors of WSSV outbreak survived the next infection by WSSV (Flegel and Pasharawipas, 1998; Venegas et al., 2000) has suggested the occurrence of protective immune response in shrimp. This reason drives many scientists to concentrate their work on the identification of the protective components produced by this animal. In our group, we used the cDNA subtraction technology to differentially screen the cDNA libraries from normal and white spot syndrome virus (WSSV) infected shrimp. Of 808 clones obtained in the library, 45 positive clones were selected for partial sequence analysis (Bangrak et al., 2002). One of the clones showed a statistically significant similarity with fortilin/TCTP. In the subtraction cDNA library, this clone was found in high frequency (4%, unpublished data) (Bangrak, 2004). Similar result has been reported by Rojtinnakorn et al. (2002) in WSSV-infected *P. japonicus* by EST approach that fortilin/TCTP was found abundantly in the library. Recently, He et al. (2005) used the technique of suppression subtractive hybridization (SSH) and differential hybridization (DH) to identify the genes differentially expressed along with their expression profiles in the hemocytes of the virus-resistance shrimp. This interested us very much and confirmed our finding of the high expression of fortilin/TCTP in the virus-resistant shrimp, *P. Japonicus*. Results from these three different labs imply the same conclusion that fortilin/TCTP

plays a critical role in the defense process during viral infection. The importance of this component for the survival of shrimp is under intensive investigation, including this work.

Here, we used RT-PCR technique to determine fortilin/TCTP level in WSSV-infected shrimp and compared it with moribund shrimp. The results showed that the severe systematic illness of the WSSV-infected shrimp correlates with the loss of fortilin/TCTP message. Moreover, a higher amount of fortilin/TCTP protein was also detected by Western blot analysis in an infected shrimp than in an uninfected one (data not shown). These results strongly indicated that fortilin/TCTP is a special molecule for viral protection.

We propose that fortilin/TCTP may be the key factor to allow WSSV-infected haemocytes to survive. It is intriguing that message of fortilin/TCTP is severely diminished in shrimp showing severe systemic signs of viral infection when considering that recent reports suggest fortilin/TCTP is an antiapoptotic protein. First, it is possible that viral proteins negatively regulate the transcription of fortilin/TCTP. The loss of fortilin/TCTP from haemocytes, in turn, would cause the death of haemocytes and the loss of host defense. This would allow further propagation of the virus and eventually result in the death of the shrimp. Secondly, it is possible that viral infection in haemocytes would prompt the programmed cell death pathway, leading to the down regulation of survival genes, including fortilin/TCTP. The work of Flegel and Pasharawipas (1998) and Wongprasert et al. (2003) supports the link between viral infection and the activation of cell death pathway or apoptosis. Finally, it is possible that fortilin/TCTP protects virally infected haemocytes and prevents more severe illness in shrimp. The final possibility is likely because the presence of virus in

haemocyte alone did not make shrimp ill (Result, Figure 21) while the presence of virus, when combined with the loss of fortilin/TCTP, made them severely ill.

2. Human-fortilin/TCTP and Pm-fortilin/TCTP prevent 5-FU-induced cell death

In previous study, Bangrak and coworker (2004) have shown that shrimp fortilin/TCTP binds to Ca^{2+}. This result is consistent with Kim et al. (2000), who reported that rat-TCTP consisting of 172 amino acids is a Ca^{2+} binding protein. Taken together, the binding of TCTP to Ca^{2+} strongly suggest that TCTP functions as an antiapoptotic protein can be through Ca^{2+} scavenging in the cells. Furthermore, because of the similarity between the Pm-fortilin/TCTP and human-fortilin/TCTP, to investigate the molecular function of fortilin/TCTP in the cell, we then raise the question whether this protein holds some functions the same as human-fortilin/TCTP. The Pm-fortilin/TCTP and human-fortilin/TCTP cDNA encode a 168 and 172 amino acid polypeptide, respectively. The calculated size of both shrimp and human fortilin/TCTP is about 19 kDa. Sequence analysis with a clustal X alignment of Pm-fortilin/TCTP showed a similarity to human-fortilin/TCTP at 64% with the similar tertiary structure as predicted by computational program, Gen3D (http://geno3d-pbil.ibcp.fr) (Figure 38). Because of the lack of shrimp culture and to address the above question, we believe that this highly conserve protein should show the same function in a different system (in this case U2OS cell) and different organism (in this case human-fortilin/TCTP, see Part II). We determined Pm-fortilin/TCTP function by challenge U2OS overexpressing either Pm-fortilin/TCTP or human-fortilin/TCTP with various cell death stimuli reagents. The result shows that Pm-fortilin/TCTP prevents cells from undergoing apoptosis, the same as human-fortilin/TCTP when compared

with $U2OS_{empty}$. These data demonstrated for the first time that Pm-fortilin/TCTP can be expressed in human cell culture and the Pm-fortilin/TCTP can function as an anti-apoptotic protein. The result can also benefit the investigation of functions of other genes isolated from shrimp by using human cell culture system. The anti-apoptotic property of Pm-fortilin/TCTP will support the current hypothesis that apoptosis induced by WSSV may be a part of the pathophysiology leading to shrimp death (Flegel and Pasharawipas, 1998; Flegel, 2001) and Pm-fortilin/TCTP protects virus-infected haemocytes from dying, and keeps shrimp healthy, in spite of the infection (Bangrak et al., 2004).

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                    *        20         *        40         *        60
Shrimp : MKVFKDMLTGDEMFTDTYKYEEVDDAFYMVIG-KNITVTEDNIE--LEGANPSAEEAD-EGTDT :  60
Human  : MIIYRDLISHDEMFSDIYKIREIADGLCLEVEGKMVSRTEGNIDDSLIGGNASAEGPEGEGTES :  64
         M 654D663 DEMF3D YK  E6 D   6 6  K 63 TE NI   L G N SAE    EGT 3

                *        80         *       100         *       120
Shrimp : TSQSGVDVVIYMRLQETGFQVKKDYLAYMKEYLRNVKAKLEG-TPEASK--LTSIQKPLTDLLK : 121
Human  : TVITGVDIVMNHHLQETSFT-KEAYKKYIKDYMKSIKGKLEEQRPERVKPFMTGAAEQIKHILA : 127
         T  3GVD6V6   LQET F  K  Y  Y6K Y64 6K KLE    PE  K  6T     6  6L

          *       140         *       160         *
Shrimp : KFKDLQFFTGESMDPDGMVVLMDYKDIDGEERPVLYFPKYGLTEEKL : 168
Human  : NFKNYQFFIGENMNPDGMVALLDYREDG--VTPYMIFFKDGLEMEKC : 172
          FK1 QFF GE M1PDGMV L6DY4       P 6 F K GL  EK
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Figure 38. Comparison of the amino acid sequences from Pm-fortilin/TCTP and human-fortilin/TCTP.

The identical amino acid residues between the two sequences are highlighted.

Part II. siRNA system and Apoptosis assay

Fortilin/TCTP is a 172 amino acid polypeptide that was originally identified by yeast two-hybrid library screening as a molecule that specifically interacted with MCL1, a protein of the antiapoptotic Bcl-2 family (Li et al., 2001). Fortilin/TCTP was found to interact only with MCL1, but not with other Bcl-2 family proteins. The fortilin/TCTP-MCL1 interaction stabilizes and increases the half-life of fortilin/TCTP, but not necessarily of MCL1 (Zhang et al., 2002a). It is not known to what extent each protein depends on the other for its apoptotic activity. Therefore, we used the siRNA system to investigate the relation of each protein in apoptotic activity.

1. Development of siRNA system to knockdown MCL1 and human-fortilin/TCTP.

The siRNA system (Elbashir et al., 2001a; Elbashir et al., 2001b) we used in this work was a powerful tool for investigating the functional dependence between human-fortilin/TCTP and MCL1 proteins. In the siRNA system that we have developed, current system, human-fortilin/TCTP and MCL1 messages are targeted by multiple kinds of siRNA duplexes. This knockdown strategy, while requiring much lower concentrations, results in much longer silencing of MCL1 and human-fortilin/TCTP genes. Consequently, the strategy has allowed us to attain higher cell viability after transfection without compromising the silencing efficiency.

By using this strategy of siRNA-mediated gene silencing to test the independence of MCL1 and human-fortilin/TCTP anti-apoptotic function, we obtained more biologically relevant data than we would have obtained using mutants of MCL1 and human-fortilin/TCTP. For example, Zhang et al. (2002a) attempted to generate

multiple monoclonal cell lines of $U2OS_{Huamn-Fortilin/TCTP-R21A}$, and U2OS cells overexpressing human-fortilin/TCTP point mutant R21A, in which the 21st amino acid of human-fortilin/TCTP protein had been altered from arginine to alanine. Human-fortilin/$TCTP_{R21A}$ does not interact with MCL1. This approach proved to be inadequate, because the expression levels of human-fortilin/$TCTP_{R21A}$ were always low, most likely because human-fortilin/$TCTP_{R21A}$ could not be stabilized by MCL1. Because most cell lines express human-fortilin/TCTP, the low level expression of human-fortilin/$TCTP_{R21A}$ could prevent its biological activities from being revealed in U2OS cells and other cell lines. Western blot analyses have shown that U2OS cells express relatively low level of human-fortilin/TCTP in comparison with other cell line cells (Li et al., 2001).

2. The depletion of human-fortilin/TCTP and MCL1 does not change the intracellular localization of partner's protein, MCL1 and human-fortilin/TCTP, respectively.

In a previous study using siRNA system, Zhang et al. (2002a) found that MCL1 depletion by siRNA targeting MCL1 drastically reduced the intracellular concentration of human-fortilin/TCTP; whereas, the siRNA targeting human-fortilin/TCTP-mediated depletion of human-fortilin/TCTP did not affect the intracellular concentration of MCL1. Further investigation revealed that $siRNA_{MCL1}$ did not affect the amount of human-fortilin/TCTP transcripts in the cell in a real-time quantitative reverse transcription-PCR assay and that $siRNA_{MCL1}$-induced MCL1 silencing drastically shortened the half-life of human-fortilin protein in a pulse-chase assay. Considering that human-fortilin/TCTP and MCL1 specifically interact with

each other, we evaluated whether the depletion of intracellular human-fortilin/TCTP and MCL1 would change the intracellular localization of MCL1 and human-fortili/TCTP, respectively.

In immunocytochemical and cell fractionation experiments using siRNA system, we showed that the intracellular localization of human-fortilin/TCTP and MCL1 remained the same in the absence of MCL1 and human-fortilin/TCTP, respectively. First, regardless of the presence of human-fortilin/TCTP, MCL1 was present to a greater extent in the cytosol and to a lesser extent in the nucleus. Second, regardless of the presence of MCL1, human-fortilin/TCTP was shown by immunostaining to predominate in the nucleus, but shown by subcellular fractionation to predominate in the cytosol. Despite this apparent discrepancy, the facts are true that the patterns of subcellular localizations of human-fortilin/TCTP and MCL1 did not differ depending on the presence of their protein partners. Taken together, these data suggest that it is unlikely that (human-fortilin/TCTP)-MCL1 interaction regulates the subcellular localization of human-fortilin/TCTP and MCL1. The fact that human-fortilin/TCTP and MCL1 can be located in both the nucleus and the cytosol suggests that human-fortilin/TCTP and MCL1 are shuttle molecules, like p53 (Zhang and Xiong, 2001), mdm2 (Mayo and Donner, 2001), β-catenin (Mulholland et al., 2002), and many components of the phosphatidylinositol 3-kinase pathway such as the insulin receptor (Podlecki et al., 1987), insulin receptor substrates (Wu et al., 2003), phosphatidylinositol 3-kinase (Kim, 1998), and protein kinase B (Meier, 1997), move back and forth from the nucleus in response to changes in cellular microenvironment.

3. Human-fortilin/TCTP or MCL1 prevents cells from undergoing cell death in the absence of its protein partner MCL1 or human-fortilin/TCTP.

In this study, we have shown that the protein partner human-fortilin/TCTP and MCL1 functioned as anti-apoptotic proteins, even in the absence of the other. Human-fortilin/TCTP and MCL1 are independently antiapoptotic. The independency in anti-apoptotic function by MCL1 and human-fortilin/TCTP of their protein partner human-fortilin/TCTP and MCL1, respectively, has not been reported in literature. Taken together with a previous report that MCL1 stabilizes human-fortilin/TCTP (Zhang et al., 2002a), the current data suggest that MCL1 (a) functions as an antiapoptotic protein by itself and (b) stabilizes human-fortilin/TCTP which by itself functions as an antiapoptotic molecule. It is likely that (human-fortilin/TCTP)-MCL1 interaction represents a mechanism that cells use to quickly generate anti-apoptotic microenvironment against certain extracellular conditions. Heterodimerization of anti-apoptotic proteins has clearly been demonstrated for Bcl-2 family member proteins (Sato et al., 1994; Yin et al., 1994; Sedlak et al., 1995; Zha et al., 1996; Sattler et al., 1997; Zha et al., 1997; Hirotani et al., 1999). However, heterodimerization between a Bcl-2 family member protein (in this case MCL1) and a non-Bcl-2 family member protein (in this case human-fortilin/TCTP) has not been reported. Taken together with the fact that MCL1 is an inducible molecule (Kozopas et al., 1993; Zhan et al., 1997), it is likely that (human-fortilin/TCTP)-MCL1 interaction represents a unique cellular mechanism for quickly creating an antiapoptotic microenvironment protective against certain noxious extracellular conditions.

In the previous report, Zhang and coworker (2002a) used a standard pulse-chase assay to show that MCL1 stabilizes human-fortilin/TCTP and that the lack of

MCL1 leads to the destabilization of human-fortilin/TCTP, which is sometimes shown by Western blot analysis. In this study, this destabilization of human-fortilin/TCTP in response to the silencing of MCL1 was again seen in Figure 28 (lanes 1 and 3), Figure 31 (lanes 1 and 3), and Figure 36 (lanes 6 and 7). In addition, 25 nM $siRNA_{human-fortilin/TCTP}$ was capable of knocking down human-fortilin/TCTP within 24 h in the absence of MCL1 (Figure 27), whereas $siRNA_{human-fortilin/TCTP}$ took more than 24 h to silence human-fortilin/TCTP in the presence of MCL1 (Figure 25, bottom panel). It is not entirely clear why human-fortilin/TCTP signals occasionally persisted upon the silencing of MCL1, as seen in the Western blot analyses (Figures 25 and 26). It is possible that human-fortilin/TCTP expression was upregulated by unknown transcriptional factors, thus masking the destabilization of human-fortilin/TCTP attributable to the lack of MCL1. The transcriptional regulation of human-fortilin/TCTP appears to be very complex (Thiele et al., 1998), and further investigation is needed to evaluate the role of various pathways in it.

This work places human-fortilin/TCTP in a new class of antiapoptotic molecules. Human-fortilin/TCTP is not a co-factor of antiapoptotic MCL1, augmenting its function. However, this does not rule out the possible presence of an apoptosis executioner protein that is inhibited by human-fortilin/TCTP or an antiapoptotic molecule other than MCL1 that is stimulated by human-fortilin/TCTP. The amino acid sequence of human-fortilin/TCTP does not resemble that of either the Bcl-2 family or of inhibitor of apoptosis proteins (IAPs) (Li et al., 2001). The exact mechanism of action of human-fortilin/TCTP as an anti-apoptotic molecule is unknown. Thaw et al. (2001) have uncovered a structural similarity between fortilin/TCTP and Mss4 (mammalian suppressor of Sec4). Mss4 is a guanyl nucleotide

exchange factor, which facilitates GDP release from and the subsequent GTP binding to a subset of the Rab GTPases (Burton, 1994). GTP-bound Rab GTPases function as active forms and recruit effector molecules, such as coiled-coil proteins involved in membrane tethering and docking, enzymes, or cytoskeleton-associated proteins (Stenmark and Olkkonen, 2001). Recently, Cans et al. (2003) reported that fortilin/TCTP interacts with translation elongation factor eEF1A and with its guanyl nucleotide exchange factor, eEF1Bβ. Intriguingly, despite its homology to guanyl nucleotide exchange factor, fortilin/TCTP exhibited guanine nucleotide dissociation inhibitor activity, and stabilized the GDP (inactive) form of eEF1A (Cans et al., 2003). The up-regulation of eEF1A is reported to be associated with oxidative stress-induced apoptosis (Chen et al., 2000). Although it is possible that fortilin/TCTP binds to and keeps eEF1A inactive when eEF1A is up-regulated by apoptotic stimuli. Further investigation is needed to define the role of fortilin/TCTP-eEF1A interaction in the regulation of apoptosis.

The role of fortilin/TCTP in tumorigenesis has been established. Tuynder et al. (2002) performed differential gene expression analyses in which they compared aggressive cancer cell lines. Human-fortilin/TCTP showed the most striking upregulation (up to 124 fold on the transcriptional level) in aggressive cell lines. Human-fortilin/TCTP overexpression also reduced the sensitivity of cancer cell lines to chemotherapeutic agents, such as etoposide (Li et al., 2001) and 5-FU (Figures 28, 29, 32 and 33). On the contrary, the depletion of intracellular human-fortilin/TCTP was associated with spontaneous death of MCF7 cells (Li et al., 2001), poly (ADP-ribose) polymerase cleavage in U937 cells (Tuynder et al., 2002), and increased susceptibility to 5-FU in U2OS cells (Figures 28, 29, 32, 33, 34 and 35). Further

dissection of the mechanism of antiapoptotic of fortilin/TCTP will be important if fortilin/TCTP is to be developed as a novel target of cancer therapy. The siRNA systems we have described will be a highly useful tool for such investigations. In addition, this knowledge can be use to study shrimp-fortilin/TCTP molecule in apoptosis and lead to the strategies to keep shrimp healthy; even, when they are infected with WSSV or other pathogen.