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DISCUSSION

Part I. Identification of Pm-syntenin binding proteins from yeast two-hybrid screening

Pm-syntenin was identified from subtractive cDNA library of the heamolymph of *P. monodon* infected with WSSV. The transcription of Pm-syntenin was up-regulated in WSSV infected shrimp (Bangrak et al., 2002). In an attempt to identify Pm-syntenin binding proteins that could identify a role for Pm-syntenin in the shrimp response to WSSV infection, we performed a yeast two-hybrid screen using full length Pm-syntenin as bait to screen the cDNA library from WSSV infected shrimp and subtractive cDNA library from the haemocytes of WSSV infected and non infected shrimp. The sequences of the positive clones were analyzed using BLAST search and the sequencing data showed similarity to six proteins including elongation factor-1-alpha (EF1α), elongation factor-2 (EF2), β-actin, lysozyme, proteasome subunit alpha 6 and alpha-2-macroglobulin (α_2M).

In order to identify the role of Pm-syntenin in WSSV infected shrimp, the functions of candidate Pm-syntenin binding proteins have been reviewed. EF1α and EF2 both play essential roles in translation of protein synthesis within cells (Moldave, 1985). EF1α and EF2 have been reported to interact with cytoskeleton proteins, such as tubulin and actin, in several organisms (Nakazawa et al., 1999; Ohta et al., 1990; Yang et al., 1990; Kurasawa et al., 1996; Numata et al., 1991). Nevertheless, the functions of EF1α and EF2 in shrimp immune response have not been identified. β-actin, is involved in various types of cell motility and is ubiquitously expressed in all eukaryotic cells (Percipalle and Visa, 2006). Lysozyme has been described in invertebrates as a component of the innate immune system, functioning as an antibacterial protein (Sotelo-Mundo et al., 2003). In addition, Hikima et al. (2003) isolated lysozyme from kuruma shrimp and identified it as displaying lytic activities against several *Vibrio* species and fish pathogens. Proteasome subunit alpha 6, identified from the yeast two-hybrid screening, is a subunit of 20S proteasome complex; a multicatalytic enzyme complex involved in many biological processes including the removal of abnormal, misfolded or miss-assembled proteins, the stress response, cell-cycle control, cell differentiation and metabolic adaptation and cellular

immune response (Adam, 2003 ; Kisselev et al., 2001; Groll et al., 1997). The three major activities of 20S proteasomes correlate directly to the three subunits β subunits (β1, β2, β5). In constrast, α subunits serve as mediators for the interaction with the regulatory subunits and may play unknown roles in protein translocation or substrate selection (Voges et al., 1999; Nandi et al., 2006). α_2M plays an important role in immune system and the regulation of proteolysis in invertebrates which has been explored previously (Hergennahn et al., 1988; Aspan et al., 1990; Bender et al., 1992; Melchior et al., 1995; Kanost, 1999; Armstrong, 2001) but also known as a binding proteins for many growth factors and cytokines, including growth hormone and members of the transforming growth factor (TGF)-β superfamily; TGF-β1, TGF-β2, platelet-derived growth factor BB, nerve growth factor and interleukin-1β (Chu and Pizzo, 1994; Armstrong and Quigley, 1999). Inhibin and activin, other members of the TGF-β superfamily, as well as follistatin bind preferentially to transformed species of α_2M (Phillips et al., 1997). The exact role of the interactions with these non-proteolytic proteins remains unknown. Recently, a purified α_2M from white shrimp (*P. vannamei*) was shown to have proteinase inhibitory properties (Gollas-Galván et al., 2003). In 2004, Rattanachai et al. cloned α_2M from *M. japonicus* and demonstrated its high expression in shrimp fed the immune stimulant, peptidoglycan. Their work concluded that shrimp α_2M plays an important role in the immune system. Among the six Pm-syntenin binding proteins, we interested in α_2M and performed additional experiment to confirm their interaction. However, further study is needed to examine the roles of Pm-syntenin and its partner.

Part II. Pm-syntenin and Pm-α_2M interaction

In this study, the interaction of Pm-syntenin and α_2M was confirmed using GST-pull down. The result showed that GST-α_2M, but not GST alone, was capable of co-precipitating syntenin and another GST pull-down assay showed that GST-syntenin, but not GST alone, was also capable of co-precipitating the C-terminal part of ^{35}S-α_2M. The receptor binding domain located in the C-terminal region of α_2M functions in the binding to a cell surface receptor for receptor-mediated endocytosis (Armstrong and Quigley, 1999). Although homologues of human α_2M have been identified in numerous species, including primitive invertebrates (Sottrup-Jensen, 1989a; Starkey and Barrett, 1982; Quigley and Armstrong, 1983; Hoffmann et al., 1999; Gudderra et al., 2002), the function of this

protein remains incompletely defined especially in invertebrate. The vertebrate α_2M has been defined as a ubiquitous high molecular weight proteinase inhibitor. The mechanism of proteinase inhibition is initiated by the protease cleaving at a unique N-terminal part of α_2M and this induces a conformational change in the α_2M, which physically traps the proteinase within an interior cavity (Sottrup-Jensen et al., 1989b). Consequent cleavage of the α_2M-protease molecule at the internal thiol ester results in the exposure of the carboxy terminal receptor region and the clearance of the proteinase-α_2M complex (Tapon-Bretaudierre et al., 1985; Sottrup-Jensen et al., 1986; Sottrup-Jensen, 2002). The binding of the receptor region to a cell-surface receptor is followed by endocytosis (Maxfield et al., 1981; Hanover et al., 1983; Yamashiro et al., 1989; Huang et al., 1998. Théry et al. (2001) isolated proteins from exosomes that were purified from a growth factor-dependent dendritic cell line (DC cell line). The proteins were loaded on 10 or 15% SDS gels. All bands obtained were subjected to trypsin digestion and peptide mass mapping by MALDI-TOF mass spectrometry. This systematic proteomic approach allowed them to identify 21 new exosomal proteins that included α_2M and syntenin together in dendritic cell-derived exosomes. In humans, dendritic cells (DC) are potent antigen presenting cells (APCs) that act as initiators and modulators of the immune response against virus, microbes, tumors and self-antigens. In addition to cytokines, dendritic cells produce a specific population of small membrane vesicles: exosomes with a unique molecular composition that are involved in the initiation of T-cell immunity (Théry et al., 2001; Banchereau and Steinman, 1998; Mellman et al., 1998; Lanzavecchia et al., 1992; Stockinger, 1992; Nelson et al., 1994). Results from Théry's work indicate that although α_2M has been described as an extracellular protein and syntenin as an intracellular protein, under a certain condition both can be present in the same intracellular space. These results taken together with our results provide the first evidence of a Pm-syntenin and Pm-α_2M interaction both *in vivo* and *in vitro*. We suggest that the binding could be involved in downstream activation of the signaling pathway. The physiological significance of these interactions for the invertebrate immune response remains to be determined.

Part III. Pm-α_2M binds the N-terminus domain of Pm-syntenin

Syntenin has been identified as an adaptor protein that couples various types of molecules in a signal transduction pathway. The binding can occur through the N-

terminal or the PDZ domains of the molecule. The majority of the known partners of syntenin, PDZ domain are usually the site for their interaction. However, some partners, for the example Sox4 transcription factor, bind directed to the N-terminal of syntenin not to PDZ domain (Geijen et al., 2001), which is similar to our result as well as the recent report that the N-terminal domain of syntenin was necessary for its interaction with eIF5A (Li et al., 2004). Pm-syntenin is a 322 amino acid residues long protein, it was predicted to contain a tandem of PDZ domains (PDZ1 and PDZ2) proceeded by an N-terminal fragment of 134 amino acids. Two putative PDZ domains were identified by an 83 residue stretch of PDZ1 amino acids followed by a stretch of another 81 PDZ2 amino acids. The crystal structure of Pm-syntenin has been reported to contain a protein similar to human and mosquito syntenin (Kang et al., 2003b). The yeast two-hybrid assay between Pm-syntenin that was separately cloned in a BD-vector and an AD-vector demonstrated the self interaction of the proteins through the PDZ domain (data not shown). To investigate the binding domain of Pm-syntenin to Pm-α_2M, several plasmids with different domains of Pm-syntenin were constructed and used in the yeast two-hybrid assay. We determined that the N-terminal sequence spanning amino acids 1-131 of Pm-syntenin binds to the 181 amino acid residue and the 286 amino acid residues of the C-terminal domain or the receptor binding domain of Pm-α_2M. Receptor binding domain of Pm-α_2M is composed of positively charged residues from lysine that presented on the side of α-helix which expected to bind with the negative charged residues of cell receptor (Huang et al., 1998). In addition, negative charged residues were shown in the N-terminal of Pm-syntenin that would be appropriate surface to interact with the basic residues of Pm-α_2M. Now that the binding sites have been located at the N-terminal of Pm-syntenin and the C-terminal of the Pm-α_2M, future studies may employ mutants to map these binding site precisely.

Part IV. Expression of Pm-α_2M transcripts in WSSV infected shrimp

In our previous study, using semi-quantitative RT-PCR, we determined that the mRNA transcript of Pm-syntenin was greatly upregulated in the acute phase of the viral infection *P.monodon*. In order to determine the expression level of syntenin binding protein at the same condition in shrimp, we performed semi-quantitative RT-PCR to analyze the expression level of Pm-α_2M. The result indicated that Pm-α_2M is expressed constitutively in uninfected shrimp and inducible in WSSV infected shrimp after 48 hrs post

injection. Recently, Rattanachai et al. (2004) found that the expression of α_2M in haemocytes of kuruma shrimp also was significantly induced by administration of peptidoglycan (PG), which is well known as an immune stimulant. The highest expression of α_2M mRNA was observed 7 days after feeding with PG. The results strongly suggested that kuruma α_2M function is involved in immune system (Rattanachai et al., 2004). In 1996, Iwaki et al. determined that the α_2M expression is also found in haemocytes of the horseshoe crab. These indicated that α_2M may be mainly synthesized in the haemocyte. Taken together with our result, indicates a correlation between the expression level of Pm-α_2M and Pm-syntenin, to the viral infection process in the haemolymph of the black tiger shrimp. Therefore, Pm-α_2M interact with Pm-syntenin may serve as an adaptor for Pm-α_2M, allowing further activation of the immune system signaling pathway(s) involved in defense of the host haemolymph from the foreign invader.