# CHAPTER I
# INTRODUCTION

## 1.1 IHHNV infection in penaeid shrimp

Infectious hypodermal and haematopoietic necrosis (IHHN) disease is one of the most important viral diseases of cultured shrimps which are infected by Infectious hypodermal and haematopoietic necrosis virus (IHHNV). The postlarvae and juveniles of *Penaeus stylirostris* is highly susceptible to IHHNV causing mortalities up to 90% in 1983 (1). However, IHHNV does not cause lethal infections in *Penaeus monodon* and *Litopenaeus vannamei*. The virus usually causes runt-deformity syndrome (RDS) and physical abnormalities in *L. vannamei.* Infection by IHHNV in *P. monodon* has no clinical sign of IHHN although IHHNV-PCR products can be detected in IHHNV-infected shrimps. Histopathology results show that Cowdry Type A inclusion (CAI) is rarely seen in *P. monodon* but it is common in *L. vannamei*. By transmission electron microscopy, necrosis cells are found in the tissue of IHHNV-infected *L. vannamei*, while apoptotic cells are found more than necrosis cells in the tissue of IHHNV-infected *P. monodon*. So, it is possible that more CAI and apoptosis in *P. monodon* may involve in the absence of the clinical symptom from IHHNV infection (2).

## 1.2 IHHNV genome

IHHNV, recently called *Penaeus stylirostris* densovirus (*Pst*DNV), is an icosahedral non-enveloped particle, 22 nm in diameter. The genome is a single-stranded linear DNA, approximately 4.1 kb in length by measurement in transmission electron microscopy (3). It is a member of the *Parvoviridae* family. Analysis of the IHHNV genome revealed three large open reading frames (ORFs) on the plus strand; left ORF (ORF1), mid ORF (ORF2) and right ORF (ORF3) (4) (Figure 1-1)

The left ORF starts at the nucleotide 313 and terminates with a TAA codon at the nucleotide 2596. This region encodes a product of 666 amino acids corresponding to a molecular mass of 75.77 kDa. The 666-amino acid gene product reveals the highest homology with nonstructural protein NS-1 of two mosquito brevidensoviruses; *Aedes* densovirus and *Aedes albopictus* parvovirus. The mid ORF starts at the nucleotide 543 and terminates with a TAG codon at the nucleotide 1631. So, the mid ORF is located within left ORF but in a different reading frame. The mid ORF encodes a protein of 363 amino acid with a molecular mass of 42.11 kDa. The predicted gene product can not be detected in the protein databases. This region may encode the nonstructural protein, NS-2, whose function is unknown. The right ORF starts at the nucleotide 2535 and terminates with the TAA codon at the nucleotide 3527. Thus, the 3' end of left ORF overlaps with the 5' end of right ORF. The right ORF encodes a protein of 329 amino acid with a molecular mass of 37.48 kDa which is structural polypeptides (VPs), as in other parvoviruses. Moreover, two putative promoters are presumed to regulate expression of NS and VP gene of the left and right ORFs, respectively (4).

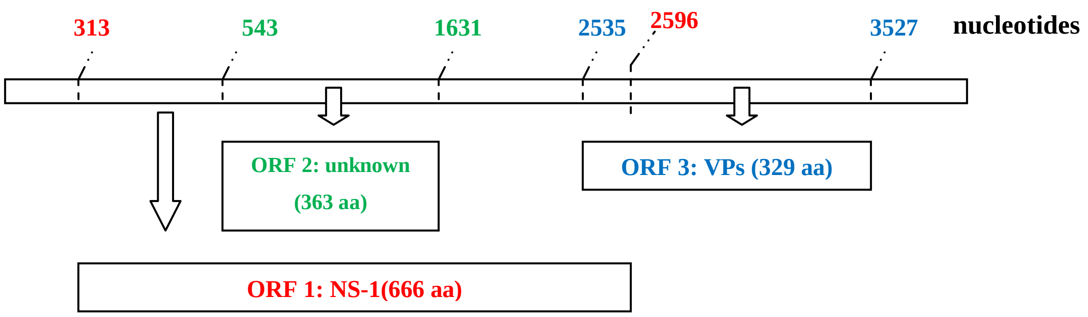


**Figure 1-1 Diagram of IHHNV genome.**

The three open reading frames (ORFs) located on the same plus strand: left ORF (ORF1), mid ORF (ORF2) and right ORF (ORF3).

## 1.3 Prevention and reduction of IHHNV infection

Previously, the vertical transmission of IHHNV through female *L. vannamei* was confirmed by analyzing embryos and larvae produced, respectively. The embryos and larvae produced from IHHNV-infected females and IHHNV-free males were also found to be infected through the nested-PCR analysis. Thus, the verification of broodstock can prevent the disease from spreading through vertical transmission by detection IHHNV-free males and females of broodstock (5). Furthermore, previous studies have indicated that warm-water culture conditions inhibited the replication rate of shrimp viruses such as white spot syndrome virus (WSSV), taura syndrome virus (TSV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV) in *L. vannamei*. The shrimp injected with WSSV had a zero percent survival rate when held in cool water temperature (26±1°C) while shrimp injected with WSSV held in warm-water condition (32±1°C) had a 93% survival rate. For TSV, the TSV-infected shrimp held in cool-water condition (27±1.5°C) showed a 30% survival rate, while the TSV-infected shrimp held in warm-water condition (30±1°C) showed a survival rate of 85%. The IHHNV-infected shrimp demonstrated the same results as the previous two shrimp viruses. These results support that warm-water condition has reduced replication of IHHNV in *L. vannamei* (6).

Furthermore, the recent report presented the inhibition of IHHNV replication by dsRNA corresponding to a viral gene. IHHNV genome has 3 ORFs. According to the previous study, the dsRNAs were designed corresponding to ORF1-2 (encode a nonstructural gene) and ORF3 (encode a structural gene). To investigate the preventive effect, *L. vannamei* were injected with 150 mM NaCl, dsRNA-ORF1-2 and dsRNA-ORF3. After 12 hours of the first injection, shrimp were challenged with IHHNV. The pleopods were collected to detect IHHNV replication by PCR on day 5, 8 and 10. The result showed that dsRNA-ORF1-2 was more effective than dsRNA ORF3 on inhibition of IHHNV replication. The expression levels of IHHNV in shrimp injected with dsRNA-ORF1-2 was almost 100% decreased at every time point compared with the control shrimp. To study of therapeutic the effect of dsRNA-targeting ORF of IHHNV on IHHNV replication, injection of dsRNAs was performed twice every 3 days after 12, 24 or 48 hours IHHNV challenge. The pleopods were collected on day 3 and 5 after IHHNV challenge. The result showed a completely inhibition of IHHNV on day 5 in shrimp injected with dsRNA within 24 hours after IHHNV challenge (7).

## 1.4 Rab GTPases and Rab7 protein

The Rab proteins (Ras related in Brain) is small GTPase proteins that belong to the Ras superfamily of small GTPases. Firstly, *rab gene* (YPT1) was identified by Gallwitz and colleagues (1983) (8) in *Saccharomyces cerevisiae*. The Rab proteins attached reversibly to the cytoplasmic side at specific region of different vesicular and organellar membranes (9). Rab GTPase proteins act as molecular switches cycling between GTP-bound (active form) and GDP-bound (inactive form) (10). The conformational changes were regulated by guanine nucleotide exchange factor (GEF) when its inactivation through hydrolysis of bound GTP into GDP is catalyzed by GTPase activation protein (GAP).

Rab GTPases were found in all eukaryotes including *Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster*. These showed the important role in eukaryotic cell biology. To date, more than 60 different Rab GTPases were discovered in human (11). The alternative splicing of Rab genes generate different isoforms of Rab GTPases and caused in an individual function of different Rab proteins (12). The most variable region of Rab GTPases proteins located in the carboxyl terminal domain that was implicated in subcellular targeting (13). Rab proteins play important roles to regulate several steps in endocytosis and exocytosis trafficking pathways including vesicle docking, vesicle formation, vesicle motility, membrane remodeling, membrane fusion and are involved in multiple stages of intracellular transport processes (14-16).

There are several Rab GTPases which are involved in the endocytic and exocytic pathways (Figure 1-2). The two Rab GTPases, Rab1 and Rab2 involved in mediating endoplasmic reticulum-to-Golgi transport (17-19). Three Rab proteins which localize to early endosomes are Rab4, Rab5 and Rab11 (20-23). Moreover, Rab4 and Rab11 were found on perinuclear recycling endosomes (23-25). Rab7 and Rab9 were found to localize at late endosomes and lysosomes (26). The four isoforms of Rab3 are largely localized to cell lineages, such as neurons, endocrine and exocrine cells, with regulated secretory pathways (27,28). In addition, Rab11a, Rab17 and Rab25 located to recycling endosomes system (25,28,29).

Rab7, one of the small GTPase proteins, is involved in endosomal trafficking pathway from early to late endosome (30).The recent data provide evidence that Rab7 is required for the cargo transport from the late endosome/multivesicular body (LE/MVB) to the lysosome and for maintenance of endocytic organelle (31, 32). Moreover, Rab7b controls trafficking process between endosomes and the *trans*-Golgi network (TGN) after the suppression of TLR9-triggered production of proinflammatory cytokines (33). Inhibition of Rab7 function by using dominant-negative mutant of Rab7 resulted in inhibition of influenza virus and other enveloped viruses. Overexpression of Rab7-T22N (the mutant Rab7) caused an accumulation of semliki forest virus in the early endosome (34). Furthermore, the suppression of both Rab5 and Rab7 showed an inhibition of hepatitis C virus (HCV) replication. It indicated that HCV required Rab proteins for its replication.

Rab7 interacts with protein interaction cascades, which are HOPs, RILP, ORP1L, FYCO1 and Mon1/Sand1-CCZ1 complex, to regulate their role in the cascades of endo-lysosomal trafficking process. Previously, homotypic fusion and protein sorting complex (HOPs) and TBC1D15 (Gyp7p in yeast) were characterized and found to serve as GEF and GAP, respectively. For Rab7, HOPs may play dual roles as an upstream GEF of Rab7 to facilitate endosomal membrane fusion and endo-lysosomal transport. Rab7-interacting lysosomal protein (RILP) interacts with oxysterol-binding protein (OSBP) related protein (ORP1L) to regulate endo-lysosomal morphogenesis and microtubule minus-end directed transport. Rab7-FYCO1 interaction regulates plus-end directed transport. The cooperation of HOPs and Sand1/Mon1 regulates Rab5–Rab7 transition on the endosomal membrane and endosomal maturation. The binding of Rab7 and retromer complex (VPS26–VPS29–VPS35) regulates endosome-TGN transport. Rab7-interacting ring-finger protein (Rabring7) may associate with cell migration (35).

In shrimp, *Penaeus monodon* Rab7 (PmRab7) was identified as one of the WSSV-VP28 binding protein on the hemocyte membrane protein by using viral overlay protein binding assay (VOPBA). The full-length PmRab7 cDNA is 1,357 bp encoding 205 amino acids. Sequence analysis showed sequence homology to small-

GTP binding Rab7 protien . The PmRab7 protein size is 21,930 Da. There are five conserved region involved with GTP-binding, GTPase activity and an isoprenylation (26).

Inhibition of viral entry by using dsRNAs which target to the viral binding proteins such as the putative YHV receptor or WSSV-VP28 binding protein (PmRab7) demonstrated inhibition of YHV or WSSV replication, respectively (26-28). Recently, prevention of shrimp viruses by dsRNA-PmRab7 can inhibit both LSNV and TSV (29, 30). Therefore, the results suggested that viral particle may accumulate in early endosome and blocking viral trafficking when PmRab7 was suppressed.

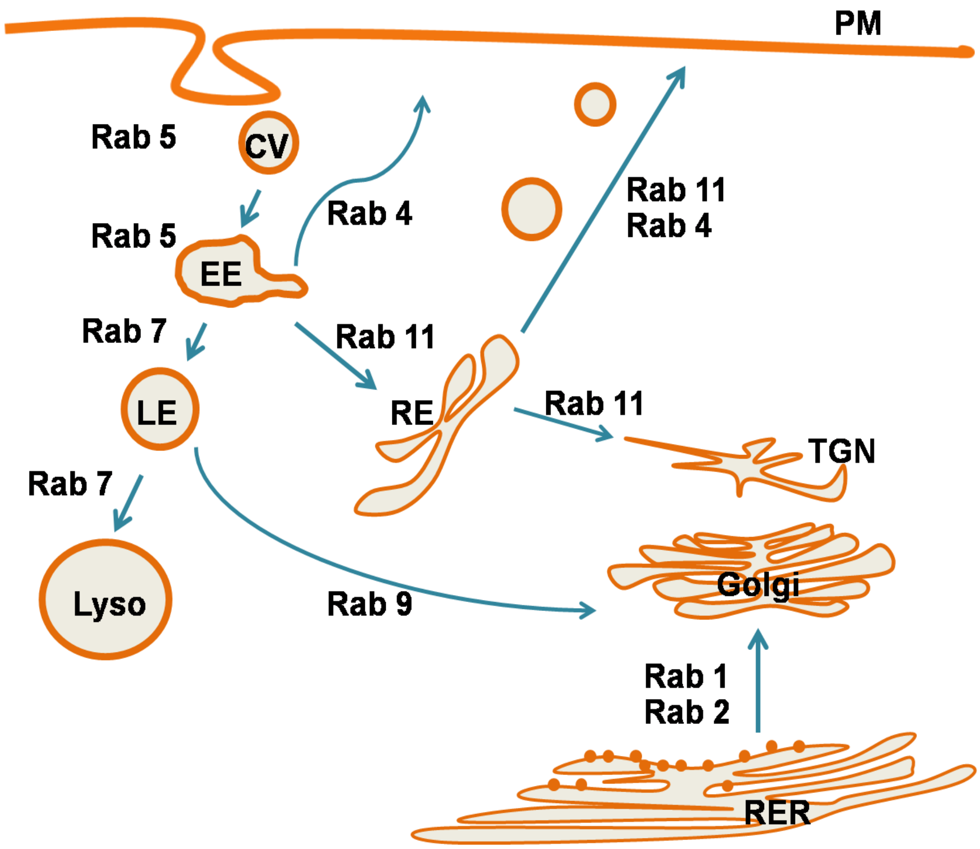


**Figure 1-2 Map of the intracellular localization of various Rab GTPases**.

The Rab GTPase family is involved in the endocytic and exocytic pathways. Arrows is transport between compartments. CV is clathrin-coated vesicles. EE is early endosomes. LE is late endosomes. Lyso is lysosome. RE is recycling endosomes.  PM is plasma membrane. TGN is *trans*-Golgi network. Golgi is golgi apparatus. RER is rough endoplasmic reticulum (modified from Seachrist JL et al, 2003 (41).

## 1.5 RNA interference

RNA interference or RNAi is a process by which double-stranded RNA (dsRNA) yielding small interfering RNA (siRNA) and inducing degradation of target mRNA that homolog to siRNA at the post-transcriptional level (42). RNAi existed in plant, nematode, insect and mammal. Microinjection of dsRNA was more effective in inhibition of gene expression than injection of sense or antisense single-stranded RNA alone(43). So, RNAi is the powerful strategy to investigate gene function or to combat several virus infections as well as an innate antiviral response in plants, fungi and animals. The RNAi mechanism occurred when dsRNA is cleaved by ribonuclease III (RNase III) – like enzyme, dicer into 21 – 23 nucleotides small interfering RNA (siRNA) with 2 – nucleotides overhang at the 3´ end and 5´ phosphate. SiRNA is loaded into RNA-induced silencing complex (RISC) and unwound into sense and antisense form. The antisense-RISC complex targeted to the homolog mRNA at complimentary sequence leading to mRNA cleavage (42).

For virus infected shrimp, RNAi was used to inhibit the replication of various viruses (Table 1-1). DsRNAs designed corresponding to viral gene (s) can inhibit WSSV, YHV, PstDNV (IHHNV) and PmDNV (HPV) replication. In addition, suppression of dsRNA corresponding to an endogenous gene which is required for viral transport or viral replication can inhibit WSSV, YHV, LSNV and TSV. These evidences proved that Rab7 may involve in an endosomal trafficking process for virus replication in the shrimp. Therefore, dsRNA corresponding to an endogenous gene, Rab7, was used to study the inhibitory effect through RNAi pathway.

**Table 1-1 RNAi study in shrimp**

| Viruses | Shrimp | Genes | dsRNA/ siRNA | P * | C * | References |
|---|---|---|---|---|---|---|
| YHV | *P. monodon* | protease, polymerase and helicase | dsRNA | √ | | Tirasophon et al, 2005 (44) |
| | *P. monodon* | Protease | dsRNA | √ | | Yodmuang et al, 2006 (45) |
| | *P. monodon* | Protease | dsRNA | | √ | Tirasophon et al, 2007 (46) |
| | *P. monodon* | PmRab7 | dsRNA | √ | | Ongvarrasopone et al., 2008 (38) |
| | *L. vannamei* | RdRp | dsRNA | √ | | Saksmerprome et al,2009 (47) |
| WSSV | *P. monodon* | vp15 , vp28 | siRNA | √ | | Westenberg et al, 2005 (48) |
| | *P. chinensis* | VP28, VP281 and protein kinase | dsRNA | √ | | Kim et al, 2007 (49) |
| | *L. vannamei* | DNApol, rr2, tk-tmk, vp24 and vp28 | siRNA | | √ | Wu et al, 2007 (50) |
| | *P. japonicus* | vp28 | siRNA | √ | | Xu et al, 2007 (51) |
| | *L. vannamei* | caspase-3 | dsRNA | √ | | Rijiravanich et al, 2008 (52) |
| | *P. monodon* | PmRab7 | dsRNA | √ | | Ongvarrasopone et al., 2008 (38) |
| | *P. monodon* | ie1, ie3, pol, rr2, vp26 and vp28 | dsRNA | √ | | Attasart et al, 2009 (53) |
| | *M. japonicus* | WssvORF285 and WssvORF332 | dsRNA | √ | | Dang et al, 2010 (54) |
| | *L. vannamei* | vp28 and vp26 | dsRNA | √ | | Mejía-Ruíz et al,2011 (55) |
| TSV | *L. vannamei* | PmRab7 | dsRNA | √ | | Ongvarrasopone et al., 2011 (40) |
| LSNV | *P. monodon* | PmRab7 | dsRNA | √ | √ | Ongvarrasopone et al, 2010 (39) |
| IHHNV | *L. vannamei* | ORF1-2 and ORF3 | dsRNA | √ | √ | Ho et al, 2011 (7) |

* : P and C represent the dsRNAs or siRNA were used in the preventive or curative experiments, respectively. Number in parentheses ( ) represents the reference number.