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REVIEW ARTICLE

Host and Virus Protein Interaction Studies in Understanding Shrimp Virus Gene Function

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Abstract Protein–protein interaction studies have been widely used in several fields to characterize an unknown protein. This in turn helps to find out several pathways to understand a complex mechanism or discover a drug for treatment. Among the methods, yeast two-hybrid has widely been used in human, animal and plant research studies. This aspect of research has also been found useful in understanding the shrimp virus gene function. With respect to *White spot syndrome virus*, interaction studies have been applied to elucidate virus structure, understand the mode of entry of the virus, mechanism of virus replication and also to discover some of the host anti-viral proteins. Interaction studies on other shrimp viruses are scanty and only few reports available on Yellow head virus and Taura syndrome virus. All these findings are still in preliminary stage and lot more studies are necessary to have the clear picture. Protein interaction research on other shrimp viruses are still lacking. Considering all these, it appears that this field of research has a wide scope to understand the virulence mechanism of shrimp viruses where very little information is available till date.

Keywords Penaeid shrimp · Viral disease · WSSV · Protein–protein interaction · Yeast two-hybrid · Gene function

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Introduction

The high export value has made the shrimp culture practice popular throughout the world. Added to this, the generation of high returns within a very short span of time has eventually attracted attention from a wide range of business community to venture into this industry. There was rapid conversion of lands for aquaculture practice and evolutionary modifications for the intensification and other practices which subsequently led to a sharp increase in production of cultured shrimps between early 1980's to early 1990's. Disease is a main constraint on the way to success of any aquaculture industry and shrimp aquaculture in no way an exception. The rapid expansion and intensification of this industry created imbalance of the ecosystem. Shrimps were affected by several viruses. The under developed immune system of these species which force them to solely depend upon innate immune system [21] was another added factor, as a result of which there were outbreaks of viral diseases. A global shrimp viral pandemic and rapid decline in production was observed during 1992–2001.

All the early attempts, and to a major extent at present too, have been diverted just for the diagnosis in shrimp virus research [25, 26, 33, 34]. Several conventional and molecular methods have been applied for shrimp disease diagnosis that have been modified from time to time to make it more sensitive, less time consuming and economic. Attempts have also been made to develop suitable preventive methods against several shrimp viruses. Some of the research works have proved that the immune system of shrimp could be induced using inactivated pathogens or recombinant proteins against virus [13, 31, 47, 56]. However, variations in results have been seen while using the recombinant proteins from different expression systems

[10, 46]. Differential opinions have also been made regarding the efficacy of several vaccines or immunostimulants and so far no success was claimed for a product to give 100 % protection. Moreover, the delivery of any vaccine or immunostimulant offers a stiff challenge from the aquatic system to prove its efficacy. This is the reason for which viral diseases of shrimp still persists as a major concern for shrimp farmers throughout the world.

While lot of progress has been made in the case of human and animal virus research, in developing a suitable treatment method, this has not been that encouraging in case of shrimp virus. This is due to lack of knowledge of the basic biology and mechanism of shrimp virus infection and also to the lack of a suitable shrimp cell line for proper shrimp virus research [6]. Although to some extent the primary shrimp cell cultures have been used [20], it has been limited only to the study of virus multiplication. This clearly indicates the need for alternative approaches to better understand the virus and host interaction mechanisms. The basic question as to how the virus gets entry into the host system, uses the host machinery to suppress the host immune system for multiplying and thereby affecting the shrimp health are the subjects of research to be investigated. Therefore, protein–protein interaction studies such as yeast two-hybrid screening, viral overlay protein binding assay, phase display method and other immunological methods (pull down assay, co-immunoprecipitation) look promising for a detail investigation of shrimp virus virulence mechanism and will subsequently shed light on possible ways for the development of treatment strategies. Among all the methods, yeast two-hybrid assay is more popular and widely used for protein–protein interaction. In this review, some of these research findings on shrimp virus have been discussed.

Yeast Two-Hybrid System

Originally developed by Fields and Song [11], the technique has subsequently undergone several modifications. The basic principle has been derived from the function of a transcription factor containing two separate domains—DNA binding domain and a transcription activation domain. It was possible to dissect out these two domains and make them functionless and also revert the functional ability by bringing them closer. These two domains are inserted separately into two vectors. One of the vectors contains the DNA binding domain fused with the protein of interest designated as “bait” where the other vector contains the activation domain usually fused with a library and designated as “prey”. Both the bait and the library are then transformed to genetically modified yeast which makes it suitable for screening in an amino acid deficient medium. If the bait protein interacts with any of the proteins in a library (prey), it results in both the domains coming in close proximity and the transcription of the reporter gene taking place (Fig. 1). Some of the basic steps in two-hybrid technique should be followed carefully in order to avoid false reaction. The bait should be cloned to the Binding Domain (BD) vector, so that it should be in frame and correct fusion with the GAL4-BD. This should be confirmed by sequencing the right clone that is obtained after screening. The next step is to check if the bait is getting expressed inside the host and satisfying the autoactivation reaction (should not be able to express the reporter by itself). Subsequently, the false reactions should be minimized by following the most stringent screening with the library. Any positive interactions should be re-verified by immuno-precipitation before proceeding further to characterise the clones.

The popular yeast two-hybrid screening is based on the GAL4 transcriptional activator of yeast, *Saccharomyces cerevisiae*, which is involved in the transcription of a

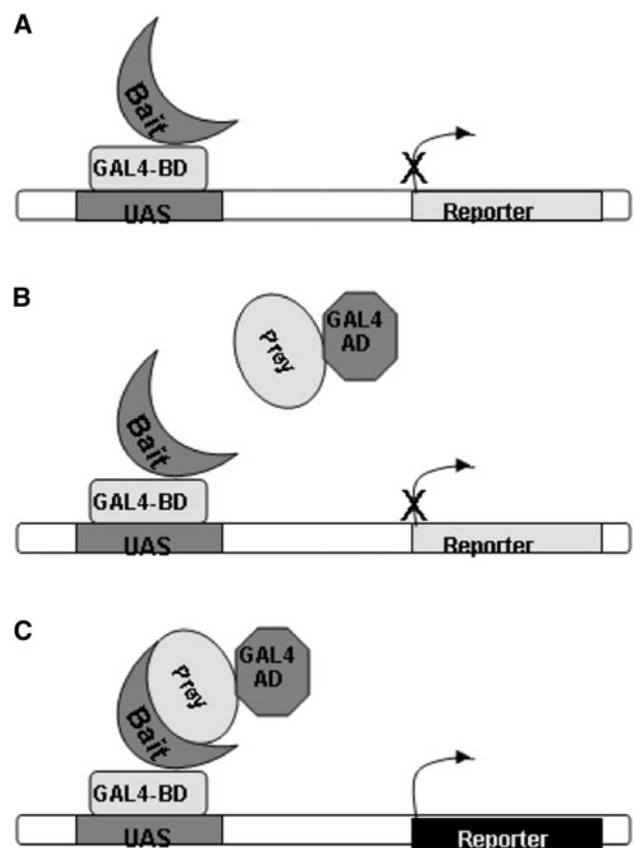

Fig. 1 Basic principles of yeast two-hybrid system. **a** The bait protein alone, with the GAL4 DNA binding domain not sufficient to activate the expression of the reporter gene. **b** The only presence of prey protein with the activation domain, without the interaction, can not activate the reporter gene expression. **c** The reporter gene is expressed only when the bait and the prey interact and both binding domain and activation domain comes together

protein for galactose utilization. Subsequently, the bacterial "LexA" system was also used [15] with an intention to reduce the false positive reactions. Latter utilizing the zinc finger protein, a complete bacterial two-hybrid system was also developed [19]. In this way, the protein–protein interaction studies have been made popular by the use of several of these described systems or with modification of the existing systems. Many of these two-hybrid systems are available commercially. While the original GAL4 system in different forms are available from Invitrogen (Invitrogen, CA, USA) and Clontech (Clontech, CA, USA), the LexA based system is marketed by Origin Technologies (Origin, MD, USA). Similarly, the Bacteriomatch II two-hybrid system at present is being marketed by Agilent Technologies (Agilent, CA, USA). The availability of many ready to use libraries (Human, animal and plant science research) has made this approach even more technical friendly.

Since from its invention, the two-hybrid system has been extensively used for protein interaction studies in all fields of research. All these studies provide new insights on systems biology, drug discovery and disease research. A lot of research on this line still continues for normal human organ studies [45], cancer research [35] for human pathogens such as virus [30], bacteria [52] and parasites [37]. It took quite some time for this technique to get introduced into fisheries. However, as early as 2000 [14], yeast two-hybrid has been proved useful in fin fish research. This technique was further introduced for shrimp virus research in 2004 by Lu and Quang [29]. Since then, it has been used by many labs for different shrimp viruses.

Protein Interaction Studies with *White spot syndrome virus* (WSSV)

To elucidate WSSV structure

To elucidate the role of viral proteins in WSSV structure formation, several interaction studies based on envelop and capsid proteins have been carried out. Viral Overlay Protein Binding Assay (VOPBA) showed that VP26 interacts with the most abundant WSSV protein, VP28 [50] and the viral capsid protein VP51 [44]. Therefore, VP26 was thought to be a matrix-like linker between the envelop and nucleocapsid protein of WSSV. This linker property of VP26 was further described by Chang et al. [3] who reported that the WSSV structural protein VP51A interacts directly with VP26 and indirectly with VP28. Glutathione S-transferase (GST) fused VP38 was expressed and it was able to pull down VP24 [18]. Mutational studies carried out to characterize the exact binding position revealed that VP38 of WSSV interacts with the C-terminal region of VP24. Since VP24 is a major viral protein and also interacts with other structural proteins, it has been speculated that groups of these proteins form a basic frame work for the virus upon which the other proteins build up [18]. Liu et al. [27] attempted to study the morphology of WSSV by interaction study. The overlay assay and the subsequent His-pull down assay confirmed the interaction of VP37 with VP26 and VP28. The authors speculate that the study will provide a clue as to how the different envelop and nucleocapsid proteins interact to determine the virus morphology. Using the four major envelop proteins of WSSV (VP19, VP24, VP26 and VP28) and employing Co-immunoprecipitation, Zhou et al. [54] observed that these four proteins interact with each other to form a complex. While VP24 was able to interact with itself and also with VP19 and VP26, VP28 was able to interact with VP19. In an extension to these studies, Chang et al. [4] predicted a 3-D structure taking different WSSV proteins and proposed a model regarding the virion assembly. All these show that the proteins bind to each other in different ways which probably helps to reassemble the virus structure inside the host.

To understand WSSV entry into the host

WSSV infection mechanism still remains a mystery as far the entry of virus into the host is considered. Several protein interaction studies provide initial clues regarding this. The structural protein, VP53A of WSSV was used as bait in a yeast two-hybrid screening and found that it specifically interacts with the chitin binding protein of *P. monodon* (PmCBP) [7]. This was an important observation as PmCBP was thought to act as a receptor for WSSV entry into the host. Further, studies based on immunofluorescence assay revealed PmCBP to be located at the cell surface and had the ability to interact with multiple envelop proteins [5]. Except VP28, PmCBP interacted with 11 other structural proteins of WSSV and therefore, the authors speculate that the entry of WSSV into host cells involves the interaction with PmCBP. Whether WSSV directly binds to PmCBP for entry or PmCBP binds to one of the envelop proteins, then helps the other proteins to enter the host, requires further investigation.

In a phase display assay, Xu et al. [51] found that WSSV VP28 protein interacted with the heat shock cognate protein 70 (Hsc70). This interaction was ATP dependent as it required both the ATPase and peptide domain of Hsc70. Both VP28 and Hsc70 were co-localized in the cytoplasm and this was also ATP dependent. As it has already been seen in case of other viruses, that Hsc70 is necessary for cell entry, virion assembly and disassembly, the present interaction was thought to determine the role of VP28 during the course of virus infection. Phase display method was used to screen a cDNA library from WSSV infected

shrimp against the WSSV protein. A novel protein of 171 bp was obtained which was interacting with WSSV protein and to be more specific with VP26 of WSSV [53]. When WSSV was neutralized by the novel protein, it provided 89 % protection compared to 100 % mortality in control. It was clear from the experiment that the novel protein was able to prevent the invasion of the virus.

Interaction study with the phase display library found that WSSV envelop protein VP187 binds with the beta integrin of shrimp [23]. Use of integrin specific antibody or knock down of integrin by dsRNA could prevent the WSSV infection. This clearly proves the role of beta-integrin as a receptor for WSSV infection. Similarly, a 53 kDa protein called BP53 and homolog to F1-ATP synthase beta unit, interacted with WSSV protein by VOPBA [24]. BP53 was found to present in cell surface of gill and hemocytes cells and therefore thought to be a receptor for WSSV infection. The recombinant product BP53 was also able to attenuate the WSSV infection.

To elucidate WSSV replication and multiplication

The virulence of the virus depends on its life stage, particularly the replication and multiplication mechanism. The little information available on WSSV virulence todate is mainly from protein interaction studies. In a first kind of yeast two-hybrid interaction study with shrimp virus, Lu and Kwang [29] identified a novel shrimp protein phospatase that interacted with the latency associated WSSV protein, ORF427. This protein phosphatase had high homology with the human protein phosphatase 1 and also exhibited phosphatase activity. The authors speculated that the protein phosphatase might be having a role in controlling the life cycle of the virus in association with ORF427. Later it was found that the same phosphatase also interacts with another protein, WSSV403 [16]. WSSV403 is characterized as a E3 ubiquitin ligase involved in virus latency. The ring-H2-type protein of WSSV (WSSV222) is also involved in ubiquitination. This protein was used as bait in yeast two-hybrid screening with a shrimp cDNA library [17] and found to interact with shrimp tumor suppressor like protein (TSL). Expression of TSL in cell line resulted apoptosis which could be rescued by WSSV222 as it brought ubiquitin mediated degradation of TSL thus showing that the anti-apoptotic role of WSSV222 is necessary for virus survival.

The suggestive role of WSSV VP26 was found out by the protein interaction study. Initially Xi and Yang [49] used co-immunoprecipitation to show that VP26 interacts with actin. Latter using the recombinant VP26 and following the viral overlay protein binding assay with the hemocyte membrane fraction of *Feropenaeus chinensis*, Liu et al. [28] further confirmed that VP26 in fact interacts with the beta-actin fraction of the hemocytic membrane protein. This finding was interesting to speculate the nuclecapsid movement of the virus. The authors suggested that the movement of WSSV nucleocapsid after virus penetration might be taking place by the interaction of VP26 of WSSV and beta-actin of the host.

Taking a 210 bp fragment of the WSSV homolog region 2 (hr2) as bait, Zhu et al. [55], conducted a phage display screening to find out its interacting partner. They found that WSSV021 specifically interacted with WSSV hr2. WSSV021 was transcribed at the early stage of WSSV infection and therefore this gene was thought to be functional gene involved in WSSV replication and transcriptional regulation. An EST screening identified a receptor protein for activated protein kinase C1 of *P. monodon* infected with WSSV (PmRACK1). Up regulation of PmRACK1 during WSSV infection was observed in several organs. Both yeast two-hybrid screening and GST-pull down assay proved that PmRACK1 interacts with a non-structural protein VP9 [43]. This interaction is perhaps required for regulating the intracellular function of VP9.

Liu et al. [48] based on immunoprecipitation and GST-pull down assay showed that the immediate early gene 1 (IE1) of WSSV interacts with *P. monodon* TATA box binding protein (PmTBP). By further refining the binding sites and using dsRNA experiments, the authors concluded that PmTBP is an important target for WSSV IE1's transactivation which is essential for virus gene expression and virus replication. Considering the suggestive role of WSSV VP15 protein in genome packaging, Sangsuriya et al. [38] tried to elucidate the exact mechanism through a yeast two-hybrid screening. VP15 interacted with a 46 kDa immunophilin protein (PmFKBP46) having DNA binding activity. It was therefore suggested that PmFKBP46 might be involved in genome packaging of WSSV during virion assembly.

To Discover Anti-Viral Proteins

Using gene expression studies, Bangrak et al. [1] showed that a protein named syntenin was up regulated during the acute phase of WSSV infection. Further experiments to elucidate the exact mechanism of up regulation using a yeast two-hybrid screening taking syntenin as bait and the WSSV infected shrimp as library showed that the eukaryotic translation initiation factor 5A (elf5A) was an interactor for syntenin [36]. Real time PCR analysis for both the proteins showed that they have differential pattern in the expression during the course of WSSV infection. In humans, interaction of syntenin with elf5A was suggested to prevent apoptosis [22] whereas in shrimp, apoptosis was proposed to be the cause of shrimp death by WSSV infection [12]. Therefore, this finding is significant as far as

WSSV infection is concerned. Syntenin was also found to interact with alpha-2 macroglobulin (alpha-2 M) from a WSSV infected hemocyte library [41] and both syntenin and alpha-2 M were over expressed during the acute phase of WSSV infection. It was therefore postulated that both syntenin and alpha-2 M play important roles during the viral infection.

Deachamag et al. [9] immunized the shrimp with various agents and observed that phagocytosis activating protein (PAP) was highly expressed in the hemolymph of shrimp. The same protein when injected to shrimp also provided better protection against WSSV. In order to characterize this protein, Chotigeat et al. [8] conducted a two-hybrid screening and found that alpha-2-macroglobulin interacts with PAP. When the shrimp was infected with WSSV, both PAP and alpha-2 M were highly expressed in hemolymph. Hemocytes incubated with PAP provided better phagocytic activity. It was thought that alpha-2 M acts as a receptor and internalize PAP into hemocytes and thereby increase the protective activity of hemocytes.

The anti-apoptotic protein fortilin from *P. monodon* (PmFortilin) was shown high level of expression during the initial stage of WSSV infection. Therefore, this protein was interesting to study about the mechanism of its action. The rFortilin was found to interact with a novel polypeptide (93 amino acids) having XPPX motif and therefore suggesting of antiviral action [42]. In order to further characterize the function of PmFortilin, Nupan et al. [32] used a cell culture system and observed that PmFortilin was able to suppress the activity of several early and late genes of WSSV indicating the interaction of this host protein with the viral proteins to control the activity.

Other Shrimp Viruses and Protein Interaction Studies

When the metal ion-binding domain of yellow head virus (YHV) was used as bait and screened against a hemocyte library, it interacted with a serine protein homolog (SPH) cDNA, namely SPH516 [40]. SPH are well known to play role in the defense mechanism of arthropods against bacteria and fungi as they activate the phenol oxidase system. However, its role against virus was not known. The experiment also showed the down regulation of SPH after YHV infection and specific expression of SPH in hemolymph. Therefore, it was thought that SPH also plays a role in the defense of host against virus and probably interacted with the virus (YHV) to restrict its multiplication [40].

Taking three viral capsid proteins of Taura syndrome virus (TSV), Senapin and Phongdara [39] conducted yeast two-hybrid screening to find out their interacting partners. While VP1 protein interacted with beta actin, elongation factor 1 alpha (E1alpha), lysozyme and lamin receptor, VP interacted only with beta actin and EF1 alpha. Similarly, VP3 interacted with all the proteins like that of VP1 except lamin. In mammals, the lamin has been proved to be a receptor for a number of arthropod borne viruses and therefore, this result was found to be interesting regarding the entry of virus into the shrimp host. Later it was found that lamin receptors are kind of specific to RNA virus as it also interacted with the capsid proteins of YHV and infectious myonecrosis virus (IMNV) but not with *Macrobrachium rosenbergii* nodavirus (MrNV). Lamin also did not interact with the capsid/envelop proteins of two other DNA viruses [2].

Conclusion

As in other fields, protein interaction studies with shrimp viruses have proved to be very useful to understand the virulence mechanism. Though lots of initial information is available, still it is a long way to go in order to get a clear picture. An intensive research in this regard is necessary for an early solution to this serious problem. While several attempts on interaction aspects have been tried for WSSV, research on other shrimp viruses are still scanty. Considering the economic importance of shrimp aquaculture, protein–protein interaction research on shrimp viruses looks very promising.

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