Identification and Characterization of a *Penaeus monodon* Lymphoid Cell-Expressed Receptor for the Yellow Head Virus

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The yellow head virus is a positive-sense, single-stranded RNA virus that causes significant mortality in farmed penaeid shrimp. This study sought to isolate and characterize the receptor protein used by the virus to gain entry into *Penaeus monodon* Oka (lymphoid) organ cells, a primary target of yellow head virus infections. Virus overlay protein binding assay on Oka organ membrane preparations identified a 65-kDa protein, and antibodies raised against this protein inhibited virus entry in primary Oka cell cultures by approximately 80%. N-terminal sequence analysis of the 65-kDa protein generated a 17-amino acid peptide fragment which was used to design degenerate primers that amplified a 1.5-kbp product from Oka organ total RNA, which was cloned and sequenced. Northern analysis and PCR were used to confirm a single RNA transcript that was expressed in most tissues. Subsequently, the mature cDNA was recloned and the expressed protein shown to cross-react with the antibody raised against the original virus binding band. Down regulation of the message through double-stranded RNA-mediated RNA interference silencing resulted in the complete inhibition of virus entry. While the identity of the clone remains unknown, it nevertheless represents the first invertebrate *Nidovirus* receptor isolated to date.

The yellow head virus (YHV) is a shrimp virus that causes significant damage to farmed penaeid shrimp throughout Asia through mass pond fatalities, which results in heavy production losses and consequent severe economic damage (13, 22). Susceptible species include *P. duorarum*, *P. stylirostris*, and *P. vannamei*, although in the latter two species, the characteristic light yellowing coloration of the cephalothorax is not observed (23). Infected animals frequently swim erratically near the surface of the pond, and death of an infected shrimp normally occurs within 2 to 3 days from the onset of symptoms. YHV infects cells of both ectodermal and mesodermal origins (6), and gill and lymphoid organ (Oka) tissues are primary targets for YHV replication (24). Cellular necrosis has been reported to be widespread in connective tissues, hematopoietic organs, and the lymphoid organ, and massive necrosis of circulating hemocytes is characteristic of YHV infection (6).

Originally classified as a coronavirus or rhabdovirus based upon virus morphology and the presence of a single-stranded RNA genome (44), the subsequent elucidation of the RNA genome as a positive-sense RNA molecule (34) as well as considerations of sequence identity, genome organization, and gene expression have indicated that YHV and the closely related gill-associated virus from Australia (10) belong to a new genus *Okavirus* within the family *Roniviridae* and order *Nidovirales* (11, 12, 19, 31).

Physically, the YHV is an enveloped, rod-shaped virus of approximately 40 nm by 170 nm with peplomers of approximately 11 nm and a tubular helical nucleocapsid containing a positive-sense single-stranded RNA of approximately 26 kb (19, 31). Analysis of the structural proteins of the virus by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) shows three major structural proteins of molecular masses 116 kDa (gp116), 64 kDa (gp64), and 20 kDa (p20). Structural proteins gp116 and gp64 are glycosylated (19, 42) and are believed to be the proteolytic products of a predicted membrane polyprotein containing six hydrophobic transmembrane domains encoded by open reading frame 3 (ORF3) (19).

The process of viral entry into a permissive cell is mediated by both cellular and viral factors. We have previously shown that antibodies directed against yellow head virus protein gp116, but not gp64, show virus-neutralizing ability (3), suggesting that this protein is involved in the binding of the yellow head virus to cellular receptor proteins, but to date, there is no information as to the nature of any possible cellular mediators of yellow head virus entry into cells.

**MATERIALS AND METHODS**

*Virus propagation and purification.* YHV, a generous gift from Boonsrim Wittayachummanrkul, Faculty of Science, Mahidol University, Thailand, was propagated in *P. monodon* shrimp and purified using urograffin gradient ultra-centrifugation as previously described (2, 44).

*Primary lymphoid organ cell culture from *P. monodon*. Primary cell cultures were prepared from the lymphoid (Oka) organ of *P. monodon* as previously described (2). Briefly, Oka organs collected from *P. monodon* shrimp were washed with 2× Leibovitz’s L-15 medium (Gibco BRL, Gaitherburg, MD) containing 15% fetal bovine serum, 5% lactalbumin, 100 U/ml penicillin, and 100 μg/ml streptomycin and then minced into small pieces in the same medium. The cell suspension was seeded onto 96-well tissue culture plates in the same medium supplemented with 15% shrimp meat extract. The cells were grown to monolayers at 28°C.

*Cell membrane preparation.* Cell membranes were prepared according to the protocol of Martinez-Barragan and del Angel (26), with minor adaptations.
Briefly, suspensions of lymphoid cells prepared as described above were centrifuged at 300 × g for 3 min to pellet the cells. The cell pellet was lysed by the addition of 0.2% Triton X-100 in buffer M (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 2 mM MgCl₂, 1 mM EDTA [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride and subsequently centrifuged at 600 × g for 5 min to remove nuclei and cell debris. The supernatant was collected and centrifuged at 6,000 × g and subsequently at 20,000 × g for 20 min. The crude membrane pellet was resuspended in 0.2% Triton X-100 in buffer M, pH 7.5. The protein concentration of the crude membrane preparation was determined by the Bradford assay (7).

**Western blots and virus overlay protein binding assay (VOPBA).** Approximately 80 to 100 μg of the crude membrane preparation was separated by electrophoresis through SDS–8% polyacrylamide gels, following which the gels were soaked in transfer buffer for 15 min to remove electrophoresis buffer and detergent, and proteins were transferred to polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA). Membranes were prewetted in absolute methanol for 10 s and then equilibrated in Tris-glycine transfer buffer for 5 min prior to transfer at 100 V, 4°C for 150 min. After transfer, the membranes were blocked to prevent nonspecific binding by immersion in blocking solution (5% [wt/vol] skim milk in 1× TBS [20 mM Tris base and 140 mM NaCl, pH 7.6]) at room temperature for 3 h with gentle agitation or at 4°C overnight.

For Western blot analysis, the membranes were incubated with rat polyclonal anti-virus overlay protein (VOP)–antiserum (VOPBA) (1:4,000). The antigen-antibody complex was visualized using the ECL Plus Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

**Polycanal antisera preparation.** Crude membrane proteins from the lymphoid organ of *P. monodon* shrimp were electrophoresed on a parallel (to the VOPBA) SDS–8% PAGE gel as described above, and the gel was stained with Coomassie brilliant blue R-250. The region of the gel corresponding to the p65 VOPBA binding band was cut out and used to raise rat polyclonal antibod- ies. The polyclonal antibody production was performed by the Antibody Production Laboratories, Beverly, MA., and an appropriate transformant (pL980-1218) was used for riboprobe synthesis according to the manufacturer's recommendation using [α-32P]UTP (Amersham Biosciences). The p65 VOPBA binding band was cut out and used to raise rat polyclonal antibod-ies. The polyclonal antibody production was performed by the Antibody Production Laboratories, Beverly, MA., and an appropriate transformant (pL980-1218) was used for riboprobe synthesis according to the manufacturer's recommendation using [α-32P]UTP (Amersham Biosciences). The p65 VOPBA binding band was cut out and used to raise rat polyclonal antibod-ies. The polyclonal antibody production was performed by the Antibody Production Laboratories, Beverly, MA., and an appropriate transformant (pL980-1218) was used for riboprobe synthesis according to the manufacturer's recommendation using [α-32P]UTP (Amersham Biosciences). The p65 VOPBA binding band was cut out and used to raise rat polyclonal antibod-

**Identification of a YHV receptor protein**

Reverse transcription (RT)-PCR, PCR, cloning, and sequencing. Total RNA from the lymphoid organ of *P. monodon* was extracted using TRI reagent (Sigma Chemical Co.) and was used as a template to generate first-strand cDNA with primer PRT (38). Briefly, total RNA (1 to 5 μg) was mixed with 0.5 μg PRT primer in a final volume of 5 μl RNase-free water. The mixture was heated to 70°C for 5 min and quickly cooled on ice, following which the volume was adjusted to 20 μl containing 1× ImPromII reaction buffer, 3 mM MgCl₂, 0.5 μM each deoxynucleoside triphosphates (dNTPs), 20 U of RNasin RNase inhibitor, 3 U of ImPromII reverse transcriptase (Promega, Madison, WI), and RNase-free water. The reaction was mixed by pipetting and then incubated at 25°C for 5 min to allow the PRT primer to bind to the mRNA poly(A) tail. First-strand cDNA was synthesized at 42°C for 60 min, followed with 70°C for 15 min to inactivate the reverse transcriptase. This first-strand cDNA was directly used as the template for PCR amplification using a degenerate N-terminal primer, YRP1, based upon the amino acid sequence generated from the N-terminal amino acid sequencing of p65 (above) and PM1 primer (38). The PCR was composed of 3 μl first-strand cDNA, 1× thermophilic DNA polymerase buffer, 0.4 μM (each) primer, 0.2 μM (each) dNTPs, 5 mM MgCl₂, and 1.25 U of Taq DNA polymerase (Promega). Cycle conditions are shown in Table 1. The PCR product was cloned into pGEM-T Easy vector (Promega) and fully se- quenced on both strands.

**Northern analysis and tissue distribution of pmYRP65.** To assess the tissue distribution of pmYRP65, total RNA was prepared from gill and lymphoid organ as described above, and approximately 30 μg of total RNA was fractionated on a 1% agarose gel containing 3.5% formaldehyde. After gel electrophoresis, RNA was transferred to solid matrix (Hybond N+; Amersham Biosciences, Little Chalfont, United Kingdom) and subsequently prehybridized in 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)-5× SSC–0.5% SDS–50% formamide–100 μg/ml salmon sperm DNA for 3 h at 67°C, followed by hybridization with a 32P-labeled CRNA riboprobe specific to nucleotides 980 to 1218 of pmYRP65 for 18 h at 67°C. The blot was subsequently washed at 60°C in successive washes of 1× SSC–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.2× SSC–0.1 SDS for 20 min each and then exposed to an X-ray film (AgFA, Brussels, Belgium). The riboprobe was constructed by amplifying a unique region of pmYRP65 (nucleotides 980 to 1218) using primers Sense-U-P65 and anti-U-67

**Materials and Methods.** The PCR was composed of 1 μl of first-strand cDNA, 1× Thermophilic DNA polymerase buffer, 0.4 μM (each) primer, 0.2 μM (each) dNTPs, 5 mM MgCl₂, and 1.25 U of Taq DNA polymerase (Promega). Cycle conditions are shown in Table 1. The PCR product was digested with BglII and Stu restriction enzymes and cloned into plasmid Litus286 (New England Bio- labs, Beverly, MA), and an appropriate transformant (pL990-1218) was used for riboprobe synthesis according to the manufacturer's recommendation using [α-32P]UTP (Amersham Biosciences).

For tissue distribution, total RNA was prepared from brain and nerve cord, gill, ovary, heart, abdomen, and lymphoid (Oka) organ and used to produce first-strand cDNA with primer PRT as described above. The first-strand cDNA was used directly as a template in a subsequent PCR using the forward primer Ntr-Ex and reverse primer Anti-Ntr-Ex (Table 1), primers designed to amplify the full-length mature RNA with additional cloning sites (see next section in Materials and Methods). The PCR was composed of 1 μl of first-strand cDNA, 1× Thermophilic DNA polymerase buffer, 0.4 μM (each) primer, 0.2 μM (each) dNTPs, 5 mM MgCl₂, and 1.25 U of Taq DNA polymerase (Promega), and cycle conditions are as shown in Table 1. A control reaction using primers specific for actin, Actin F and Actin R (Table 1), was performed under similar conditions, although extension time was decreased to 1 min. All PCR products were subse- quently analyzed by agarose gel electrophoresis.

**Recombinant GST fusion protein expression in Escherichia coli.** The cDNA encoding mature p65 was generated by PCR using the forward primer Ntr-Ex and reverse primer Anti-Ntr-Ex (Table 1). The forward primer was designed from residues P1 to Q7 and included the EcoRI and Notel restriction sites. The reverse primer was designed from residues ES08 to DS512 and included a stop codon and a SalI restriction site. PCR was performed as described above. The insert was digested with EcoRI and SalI and cloned into the multiple cloning site of pGEX-5X-1. The recombinant plasmid was then transformed into *E. coli* strain BL21(DE3) pLysS (Novagen, Madison, WI). An overnight culture of *E. coli* BL21(DE3) pLysS was diluted 1:20 with fresh Luria-Bertani broth and grown at 30°C to an optical density at 600 nm (OD600) of 0.4. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. At an
OD$_{600}$ of 0.5, the culture was pelleted, suspended in protein loading buffer, heated at 100°C for 10 min, and analyzed by SDS-PAGE. The recombinant protein expressed in E. coli was confirmed by immunoblot analysis using an anti-glutathione S-transferase (GST) monoclonal antibody (Amersham Pharmacia).

After induction of the fusion protein, cells were pelleted by centrifugation at 8,000 $\times$ g for 10 min at 4°C and the pellet was washed with 30 ml of STE (150 mM NaCl, 25 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer followed by centrifugation at 8,000 $\times$ g. The washed pellet was resuspended in 3.5 ml of STE buffer with protease inhibitors (apotinin, leupeptin, and phenylmethylsulfonyl fluoride) and Triton X-100 and then centrifuged at 12,000 $\times$ g for 10 min to remove cell debris. The supernatant was mixed with a 50% glutathione-Sepharose suspension to a final concentration of 10%, and samples were incubated overnight at 4°C before centrifugation at 2,000 $\times$ g for 5 min to pellet the beads. The bead pellet was washed several times with phosphate-buffered saline buffer and the fusion protein subsequently released with 20 mM reduced glutathione. Following centrifugation at 2,000 $\times$ g for 5 min, the supernatant was kept at −80°C.

**dsRNA production, pmYRP65 silencing, and YHV infection.** Recombinant plasmid pL980-1218 (from above) was purified using a QIAGEN DNA purification column and linearized by restriction digestion with either BglII or Stul. Sense and antisense RNAs were produced by in vitro transcription using the Ribomax kit (Promega) according to the manufacturer’s recommendations. Equal amounts of sense and antisense RNA were annealed to produce double-stranded RNA (dsRNA) according to the methodology of Worby et al. (45) and quantitated by UV spectroscopy. Irrelevant dsRNA was produced from a clone of green fluorescent protein (GFP) as described previously (36). Double-stranded RNAs were encapsulated in liposomes using the Transmessenger RNA transfection kit (QIAGEN) according to the manufacturer’s instructions. Silencing was undertaken essentially as described previously (36). Briefly, primary cultures of Oka cells at 70% confluence in 24-well tissue culture plates were incubated with 2 $\mu$g formulated dsRNA for 3 h, followed by media replacement. Cells were allowed to recover for 80 h at 28°C. Cells were then challenged with 250 $\mu$l of virus stock containing 10$^{3}$ TCID$_{50}$/250 $\mu$l of purified YHV prepared in culture medium for 1.5 h. Cultures were then extensively washed to remove excess viruses, fresh complete medium was added, and cultures were subsequently maintained at 28°C until 30 h postinfection, at which point the cells were harvested for further analysis. Control cells were neither transfected nor infected, while mock cells were transfected without dsRNA and subsequently infected with YHV. Total RNA from silencing and control cells was prepared using Trizol reagent (Gibco-BRL). The presence of YHV RNA was assessed in a duplex PCR using primers directed against the YHV helicase gene together with primers for actin as previously described (36). Detection of pmYRP65 expression was undertaken with primers Sense-U-P65 and Anti-Nter-Ex (Table 1). PCR products were analyzed by agarose gel electrophoresis.

**Nucleotide sequence accession number.** The nucleotide sequence and deduced amino acid sequence of pmYRP65 was deposited in GenBank under accession number DQ073920.

### RESULTS

**Virus overlay protein binding assay.** To identify yellow head virus binding proteins expressed on the surfaces of *P. monodon* lymphoid (Oka) cells, 80 to 100 $\mu$g of cell membrane proteins was separated on a denaturing (sodium dodecyl sulfate)–8% polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated with purified yellow head virus and, to visualize the position of binding, subsequently with an anti-yellow head virus mouse monoclonal antibody and a secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody. As shown in Fig. 1A (lane iv), a single yellow head virus binding protein of approximately 65 kDa was observed.

The 65-kDa yellow head virus binding band was extracted from a parallel SDS–8% polyacrylamide gel and used to produce anti-p65 rat polyclonal antibodies. The rat polyclonal serum was used in a Western analysis and detected the expected 65-kDa band, while no band was seen with the nonimmunized control serum (Fig. 1B, lane iii). To assess the role of the 65-kDa yellow head virus binding protein in the entry of the yellow head virus into Oka cells and to determine if the binding protein acts as a receptor, the polyclonal serum was used in an infection inhibition analysis.

**Inhibition of infection.** Confluent primary cultures of *P. monodon* Oka organ cells were cultured as described previously (2) in 96-well plates and incubated independently four times with either a 1:2 dilution of an anti-65-kDa yellow head binding protein rat polyclonal antisera or a 1:2 dilution of control (nonimmunized) serum for 3 h before being incubated with 50 $\mu$l of 10$^{3}$ TCID$_{50}$/50 $\mu$l purified yellow head virus. High serum antibody concentrations were employed given the apparent abundance of the protein in membrane fractions to ensure the complete blocking of all potential receptor sites. To prevent multiple rounds of cell reinflection by de novo virus, samples were incubated for 30 h, a time selected based on our previous propagation profile of yellow head virus in Oka cells (2), following which levels of infectious yellow head virus in the growth medium were determined by TCID$_{50}$ analysis. Results
(Fig. 1C) show an approximately 80% reduction in yellow head virus production as a result of preincubation with the rat polyclonal anti-65-kDa yellow head binding protein, suggesting that this protein acts as a receptor protein for the yellow head virus.

Characterization of the 65-kDa yellow head virus binding protein. To determine the identity of the 65-kDa protein, a combination of N-terminal sequence analysis and RT-PCR was employed. The 65-kDa band was excised from a parallel 8% polyacrylamide gel as described above for rat polyclonal antibody production. In this instance, however, the band was subjected to Edmans N-terminal sequencing. Results of N-terminal sequencing were a peptide sequence of (N-terminal) PED KPA PQKKAKGAXSA EE. A BLASTP search (1) against the GenBank database (http://www.ncbi.nlm.nih.gov) resulted in no significant matches.

To isolate a full-length cDNA clone for the yellow head virus binding protein, a degenerate primer, YRP1 (Table 1), was designed based upon the N-terminal amino acid sequence obtained. First-strand cDNA was generated with reverse transcriptase treatment of total RNA prepared from the Oka (lymphoid) organ of \textit{P. monodon}, primed with primer PRT (38). First-strand cDNA was used as template for PCR product generation using degenerate primer YRP1 (Table 1) and primer PM1 (38). A product of approximately 1.5 kbp was seen after electrophoresis of the PCR mix. The PCR product was cloned into pGEM-T Easy vector, and transformants were subjected to DNA sequence analysis on both strands. DNA sequencing revealed that the PCR insert consisted of a long open reading frame of 1,536 nucleotides with a stop codon approximately 10 to 12 bases upstream from a canonical polyadenylation signal sequence (AA TAAA). A BLASTN (1) search of the nucleotide sequence against the GenBank database resulted in no significant matches (http://www.ncbi.nlm.nih.gov; last search, September 2005), while a BLASTP search (1) of the deduced amino acid sequence of pmYRP65 against the GenBank database (http://www.ncbi.nlm.nih.gov; last search, September 2005) revealed significant similarity (90.4%) between the C-terminal 20% of pmYRP65 and the L22 ribosomal protein conserved domain (25) as shown in Fig. 2, but no further similarity with other characterized proteins. The sequence was then deposited at GenBank (accession number DQ073920).
Tissue distribution of pmYRP65. To investigate the nature of the pmYRP65 transcript, total RNA was prepared from both the Oka (lymphoid) organ and gill and transferred to solid matrix after electrophoresis through a 1% agarose gel containing 3.5% formaldehyde. The matrix was probed with a 32P-labeled riboprobe complementary to the sense strand of pmYRP65. After hybridization and washing, a single transcript of approximately 1.5 kb was seen in both Oka and gill RNA samples (Fig. 3). To further investigate the tissue distribution of pmYRP65, an RT-PCR approach was employed, and total RNAs from brain and nerve cord, hepatopancreas, gill, ovary, heart, abdomen, and lymphoid (Oka) organs were prepared and used for first-strand cDNA synthesis. PCR was undertaken using primers designed to amplify sequences corresponding to the entire mature protein, and the results were analyzed by agarose gel electrophoresis. Parallel reactions were undertaken to amplify a portion of the actin transcript as control reactions. Results (Fig. 3) show that transcripts for pmYRP65 were detectable in all tissues examined.

Expression of pmYRP65 in E. coli. To confirm that the cDNA for pmYRP65 that was cloned and sequenced was recognized by the rat polyclonal antibody antibody used in the inhibition of infection experiment, the mature full-length sequence for pmYRP65 was amplified by PCR using primers Nter-Ex and Anti-Nter-Ex and subcloned into pGEX-5X-1. The forward primer spanned sequences corresponding to amino acids P1 to Q7 and was designed to read in frame with the GST tag of the pGEX-5x-1 expression vector, while the reverse primer spanned sequences corresponding to amino acids E508 to D512 and included the endogenous translation termination codon. Subsequent to cloning, the identity of the clone was confirmed by DNA sequencing and expression of the fused pmYRP65-GST protein induced with IPTG. Upon induction, a band of approximately 82 kDa was observed (Fig. 1D, lane i), which reacted with both an anti-GST monoclonal antibody, albeit weakly, possibly due to the low abundance compared with the control lane (Fig. 1D, lanes i and ii, Anti-GST), and the rat anti-p65 polyclonal antibody used in the inhibition of infection experiment (Fig. 1D, lane i, Anti-p65), confirming that the clone isolated corresponded to the identified receptor protein. Attempts to bind the virus to the bacterially expressed protein in an overlay assay were unsuccessful, suggesting that a degree of posttranslational modification may be required for virus binding.

Inhibition of YHV infection by dsRNA-mediated RNA interference of the pmYRP65 message. To conclusively prove the role of pmYRP65 as a receptor protein for the yellow head virus protein, expression of pmYRP65 was down regulated through dsRNA-induced RNA interference, a process recently shown to be functional in penaeid shrimp (36). Double-stranded RNAs corresponding to nucleotides 980 to 1218 of the pmYRP65 cDNA were produced and incubated with primary cultures of Oka cells for 3 h in parallel with dsRNA of an unrelated protein, GFP (36). After incubation and recovery for 80 h, cells were incubated with 250 μl YHV stock virus containing 105 TCID50/250 μl for 1.5 h. Parallel primary cultures of nontransfected/noninfected and mock transfected (no dsRNA)/YHV-infected Oka cells were also established. After a further 30 h of incubation, total RNA was subsequently extracted and the presence of YHV, actin, and pmYRP65 RNAs was established by RT-PCR. Cellular morphology of all cultures was examined immediately after transfection and after 80 h of recovery, with no differences noted (data not shown). Results (Fig. 4) show that dsRNA treatment was able to effectively silence pmYRP65 expression when the dsRNA was specific for the pmYRP65 message. No silencing of pmYRP65 was seen with either the mock-transfected (no dsRNA)/YHV-infected Oka cells were also established. After a further 30 h of incubation, total RNA was subsequently extracted and the presence of YHV, actin, and pmYRP65 RNAs was established by RT-PCR. Cellular morphology of all cultures was examined immediately after transfection and after 80 h of recovery, with no differences noted (data not shown). Results (Fig. 4) show that dsRNA treatment was able to effectively silence pmYRP65 expression when the dsRNA was specific for the pmYRP65 message. No silencing of pmYRP65 was seen with either the mock-transfected (no dsRNA) or irrelevant dsRNA (GFP) silencing experiments. YHV RNA was clearly detectable in the mock-transfected and GFP silencing experiments but was completely absent from the pmYRP65 silenced cultures, confirming that entry of YHV into Oka cells requires the expression of pmYRP65.

DISCUSSION

The initial interaction between a cell and a virus is a critical determinant of viral tropism and thus of pathogenicity, and as such, considerable interest lies in determining the nature of the proteins used by viruses to enter into cells. The yellow head virus has emerged over the last decade as a significant mediator of farmed penaeid shrimp mortality, and thus, considerable
interest lies in understanding the pathobiology of the virus-host interaction. While several receptor proteins for viruses of the order Nidovirales have been identified, including angiotensin-converting enzyme 2 for severe acute respiratory syndrome (17, 21), aminopeptidase N or CD13 for human coronavirus 229E (5, 27, 43, 47), N-acetyl-9-O-acetylneuraminic acid for bovine coronavirus and human coronavirus OC43 (29, 41), and porcine sialoadhesin (p210) for porcine reproductive and respiratory syndrome virus (39), no invertebrate nidovirus receptor has been identified to date, and as such, this report represents the first identification of an invertebrate nidovirus receptor.

The virus overlay technique used here has been previously employed to identify a number of putative receptor proteins (18, 20, 33, 35, 46). While the technique is normally undertaken with reduced and denatured proteins separated by SDS-polyacrylamide gel electrophoresis, the successful identification of a number of receptors would suggest that a degree of protein renaturation occurs during the overlay process and using this approach, we have identified and characterized an approximately 65-kDa protein expressed by Penaeus monodon Oka organ (lymphoid) cells. Both antibodies against this protein and down regulation of the message through dsRNA interference are able to specifically inhibit the entry of the yellow head virus into Oka cells, suggesting that the protein identified is indeed a yellow head virus receptor protein. The cDNA sequence isolated for this protein encodes a polypeptide of 512 amino acids with a predicted molecular mass of approximately

![FIG. 3.](image-url)

**FIG. 3.** (A) Northern blot analysis of pmYRP65. Total RNAs from lymphoid organ (Lym) and gill (G) were separated on a 1.2% denaturing agarose gel, transferred, and hybridized sequentially with a riboprobe derived from a unique region of pmYRP65. A single transcript in both lymphoid and gill tissues was observed. M, RNA marker (Promega). (B) RT-PCR detection of pmYRP65. BN, brain and nerve cord; Hep, hepatopancreas; Gv, ovary; Hea, heart; G, gill; Ad, abdomen; Lym, lymphoid organ; -ve, negative PCR control, +ve, positive control of pmYRP65; -RT, negative control of reverse transcriptase reaction.

![FIG. 4.](image-url)

**FIG. 4.** RT-PCR analysis for actin, YHV, and pmYRP65 expression after dsRNA-mediated gene silencing. Control, primary cultures of Oka cells, not transfected, not infected with YHV; Mock, primary cultures of Oka cells mock transfected (no dsRNA) and infected with YHV; GFP, primary cultures of Oka cells transfected with GFP dsRNA; pmYRP65, primary cultures of Oka cells transfected with pmYRP65 dsRNA.
52,800 Da. Alignment of the peptide sequence generated by N-terminal sequencing of the 65-kDa VOPBA band with the predicted N-terminal sequence of the cloned cDNA resulted in perfect identity between amino acids 11 and 17 of the N-terminal sequence and amino acids from K11 to E17 of the translated cDNA, corresponding to the region immediately after the amino acids used to design the degenerate primers were used in the original isolation.

The molecular mass of pmYRP65 calculated from the predicted amino acid sequence is smaller (~12 kDa) than that estimated by SDS-PAGE for the mature form of the protein, suggesting a degree of posttranslational modification, and the protein contains both putative O-linked glycosylation sites (at threonine residues 39, 59, 151, 331, 339, and 345) and three putative N-myristoylation sites. Multiple potential phosphorylation sites are present in the protein, including four putative protein kinase C phosphorylation sites, two putative casein kinase II phosphorylation sites, and one putative tyrosine kinase phosphorylation site. A hydropathy plot of the pmYRP65 polypeptide sequence predicted no hydrophobic transmembrane helices, suggesting that membrane anchorage occurs through a different mechanism.

The deduced amino acid also shows that the C-terminal 20% of this protein has a high identity with ribosomal protein L22, a protein known to be an intrinsic component of the ribosome, and as such, is synthesized in the cytoplasm and then passes through the nuclear pores before reaching the nucleolus for the completion of ribosome assembly. The movement of ribosomal protein L22 is believed to be controlled by two primary sequences, a cluster of three or four lysine residues which direct nuclear localization and a further sequence of KYLKXX that controls nucleolus localization (30). Interestingly neither of these two sequences is found intact in pmYRP65, and in particular, the nucleolus localization signal shows a specific disruption (from KYLKXX to KYKLO at positions 471 to 476 of pmYRP65), which probably precludes translocation of pmYRP65 to the nucleolus. It has further been shown that L22 possesses heparin binding activity (15), and an initial interaction between a virus and heparin sulfate or glycosaminoglycans has been proposed for viruses as divergent as adenovirus (40), genogroup II noroviruses (32), Sindbis virus (8), and the dengue virus (9). As such, similar to models for the dengue virus and Sindbis virus (8, 35), it is possible that YHV undergoes a low-affinity interaction with heparin sulfate before complexing with a specific receptor with affinity for both heparin sulfate and the virus. Outside of the L22e domain, no significant matches to characterized proteins were found, although further searches of the nucleotide sequence against the GenBank expressed sequence tag database (4) resulted in several positive matches of high identity. In particular, the sequence showed high identity to clone HC-W-SO1-0639-LF isolated from a *P. monodon* white spot syndrome virus-infected hemocyte-cDNA library as well as clone AIMS-P.mon25 isolated from a *P. monodon* eyestalk cDNA library, suggesting that the mRNA may be relatively abundant and possibly pointing to a ubiquitous expression profile. This was partially confirmed by RT-PCR Northern analysis, which showed that the message for pmYRP65 was expressed in all tissues examined. This result is also consistent with the known pathobiology of the yellow head virus, which infects cells of both ectodermal and mesodermal (6) origins as well as the gill and lymphoid organ (Oka) as primary targets (24), and as such, it provides further confirmatory evidence of the proposed role of pm65YRP as a yellow head virus receptor protein.

RNA interference (RNAi) is the process by which a gene is posttranscriptionally suppressed through the action of dsRNAs in a sequence-specific manner (16, 37). RNAi-mediated gene silencing has recently shown to be operative in shrimp cells (36), and here, RNAi was employed to specifically down regulate the pmYRP65 message. In the absence of the pmYRP65 message, Oka cells were shown to be refractory to infection with the yellow head virus, providing conclusive evidence that pmYRP65 acts as a receptor protein for the yellow head virus.

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