Cloning, Characterization, and Phylogenetic Analysis of a Shrimp White Spot Syndrome Virus Gene That Encodes a Protein Kinase

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INTRODUCTION

Shrimp white spot syndrome (WSS) is one of the most serious diseases faced by the shrimp farming industry all over the world (Chou et al., 1995; Fiegel, 1997; Lo et al., 1999). White spot syndrome virus (WSSV), the causative agent of WSS, is a large, double-stranded DNA virus (Wang et al., 1995) that primarily attacks tissues originating from the ectoderm and mesoderm (Wongteerasupaya et al., 1995; Lo et al., 1997). There is little genetic variation among WSSV isolates from around the world (Lo et al., 1999; Chang et al., 2001).

Several WSSV genes have been identified, including genes that encode the ribonucleotide reductase large and small subunits (van Hulten et al., 2000a; Tsai et al., 2000a); nucleocapsid protein VP22 and envelope protein VP25 (van Hulten et al., 2000b);* and a novel chimeric protein of thymidine kinase and thymidylate kinase (Tsai et al., 2000b). These studies as well as morphological studies (Wang et al., 1995, 2000a; Wongteerasupaya et al., 1995) have provided evidence that WSSV is a newly isolated virus. However, genome analysis is needed to conclusively establish WSSV's taxonomic position. As part of our continuing work to identify and define the genetic structure of the virus, we report here on a protein kinase gene (the pk gene) of WSSV.

The eukaryotic protein kinases (PK) make up one of the largest protein superfamilies, and they all feature a homologous catalytic domain (for a review, see Hanks and Hunter, 1995). These enzymes usually use the γ phosphate of ATP (or GTP) to generate phosphate moieties, and they use the protein alcohol groups on serine/threonine or the protein phenolic groups on tyrosine as phosphate acceptors (Hunter, 1987; Hanks and Hunter, 1995). They are thus classified into two broad groups, the protein serine/threonine kinases (PSK) and protein tyrosine kinases (PTK).

The protein kinases and the phosphoprotein phosphatases control reversible protein phosphorylation, which is a common mechanism in eukaryotes for regulation of normal cell function such as intracellular protein sorting (Herman et al., 1991), growth, proliferation, differentiation, apoptosis, and stress responses (reviewed in Widmann et al., 1999; Hunter, 2000). Phosphorylation of numerous cellular and viral proteins is also observed in virally infected cells, which suggests that the protein kinases may have a role in regulating a wide variety of viral functions.

An open reading frame (ORF) that encodes a 715-amino-acid polypeptide was found in an 8421-bp EcoRI fragment of the shrimp white spot syndrome virus (WSSV) genome. The polypeptide shows significant homology to eukaryotic serine/threonine protein kinase (PK) and contains the major conserved subdomains for eukaryotic protein kinases. Coupled in vitro transcription and translation generated a protein having an apparent molecular mass of about 87 kDa according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For transcriptional analysis of the pk gene, total RNA was isolated from WSSV-infected shrimp at different times after infection. Northern blot analysis with pk-specific riboprobe found a major and a minor transcript of 2.7 and 5.7 kb, respectively. Rapid amplification of the 5′ cDNA ends of the major 2.7-kb pk transcript showed that there were two transcriptional initiation sites located at nucleotide residues -38(G) and -39(G) relative to the ATG translational start codon. Temporal expression analysis by RT-PCR indicated that the transcription of the pk gene started 2 h after infection and continued for at least 60 h. Phylogenetic analysis showed that WSSV protein kinase does not have any close relatives and does not fall into any of the major protein kinase groups.
infections (Leader and Katan, 1988; Burma et al., 1994; Guarino et al., 1992; Kann et al., 1999). Protein phosphorylation in virally infected cells may depend on cellular protein kinases (Prives, 1990) or virally encoded protein kinases (for examples, see Smith and Smith, 1989; Wu et al., 1990; Zhang et al., 1990; Barik and Banerjee, 1992; Lin et al., 1992; Baylis et al., 1993; Bischoff and Slavicek, 1994; Reilly and Guarino, 1994; Li and Miller, 1995). These virally encoded protein kinases possess most, if not all, of the conserved motifs (subdomains I–IX as defined by Hanks et al., 1988) of the catalytic domains found in eukaryotic protein kinases.

Previous sequencing (Liu et al., unpublished data) of a 3.4-kb HindIII genome fragment from WSSV revealed a 1020-bp open reading frame (ORF) that encodes a 339-amino-acid polypeptide with a high level of homology to the eukaryotic protein kinase catalytic domain, but this protein contained only a portion of the conserved kinase domain and was thus suspected to be a partial WSSV PK. However, a recently published paper (van Hulten and Vlak, 2001) suggested that this 1020-bp ORF encodes the entire PK catalytic domain and therefore may have misinterpreted a number of critical features of this gene. Specifically, less than half of the WSSV PK sequence is shown [although van Hulten and Vlak (2001) correctly give the ORF as 2193 bp/730 aa, they provide sequence data for only 339 amino acid residues out of 730 amino acid residues in total], the sequence of the catalytic domain is incomplete, and its location is incorrect. Further, van Hulten and Vlak (2001) locate subdomains I to V within a region that, as we show in the present paper, is in fact a large (and unique) insertion between subdomains VI and VII. They also constructed a phylogenetic tree based on this misinterpretation of the PK catalytic domain sequence data. In this paper, we describe the cloning and characterization of the full-length pk gene, the first WSSV-encoded protein kinase gene to be identified. We also investigated and discuss the phylogenetic position of this pk gene.

RESULTS AND DISCUSSION

Location and structure of the WSSV pk gene

The virus used in this study was isolated from a batch of WSSV-infected Penaeus monodon collected in Taiwan in 1994 (Wang et al., 1995) and which is now known as WSSV Taiwan isolate (Lo et al., 1999). From this virus, a plasmid library (referred to as the pmh library, where pm indicates Penaeus monodon and h indicates HindIII) of WSSV HindIII genomic fragments was constructed (Wang et al., 1995). During the sequencing of a 3.4-kb HindIII genomic fragment (pmh12), a 1020-nt ORF located at nucleotides 151–1170 of the pmh12 was found. When the deduced amino acid sequence of this 1020-nt ORF was compared with other sequences in GenBank at the National Center of Biotechnology Information by using the BLAST network service (Altschul et al., 1990), it was found to contain only the C-terminal region catalytic domains (subdomains VI to XI) of the eukaryotic protein kinases and therefore did not appear to correspond to the complete coding region of the WSSV pk gene. Subsequently, from a plasmid library of EcoRI genomic fragments (pme library), a 1020-nt ORF-specific probe was used to identify a plasmid clone with an 8421-nt WSSV genomic fragment (pme902) containing an ORF encoding the full-length WSSV PK (Fig. 1). The putative WSSV PK ORF has two potential in-frame ATG initiation codons at nt 1617 and nt 1662. Thus the presumed PK-coding region was either located between positions nt 1617 and 3809 (2193 nt in length, encoding 730 amino acid residues in total) or nt 1662 and 3809 (2148 nt; 715 aa residues) of the pme902 DNA fragment. The pk gene (GenBank Accession No. AF272979) coding for a WSSV major envelope protein is adjacent to the pk gene and arranged in a tail-to-tail configuration (Fig. 1).

Coupled in vitro transcription and translation

To confirm that the entire length of the putative WSSV PK ORF was not interrupted by any overlooked stop codons, nt 1617 to nt 3809 of the pme902 DNA fragment
were inserted into the expression vector, which was then used for coupled *in vitro* transcription and eukaryotic *in vitro* translation with rabbit reticulocyte lysate. The translated protein was expected to be a hemagglutinin epitope (MCYPYDVPDYASLA)-tagged polypeptide (HA-tagged PK). As shown in Fig. 2, the synthesized HA-tagged PK had an apparent molecular mass of about 87 kDa according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It was also recognizable with a commercial anti-HA antibody (data not shown). At 87 kDa, the apparent molecular mass was in fairly good agreement with the size predicted from the full length PK sequence (i.e., ~80 kDa; this predicted size does not include the HA sequence at the N-terminus).

Major transcripts of the putative WSSV *pk* gene in WSSV-infected shrimp

To determine whether the putative WSSV *pk* gene was expressed during viral infection, total RNAs extracted from WSSV-infected shrimp *P. monodon* at 0, 6, 12, 18, 60 h p.i. (hours postinfection) were analyzed by Northern blot hybridization using a WSSV *pk*-specific riboprobe. A minor transcript of approximately 1.9 kb was present throughout the 60 h of the experiment, but since it was also present at 0 h, it could not have been a WSSV-specific transcript. Two WSSV-specific transcripts were detected: a major transcript of approximately 2.7 kb and a minor transcript of approximately 5.7 kb (Fig. 3). Neither transcript was found until 12 h p.i. The size of the major WSSV *pk* transcript was consistent with the predicted size of the *pk* mRNA after allowing for the presumed PK-coding region (2148 nt) plus stretches of 5′/3′ UTRs (38 nt and 25 nt, respectively; see below) and a poly(A) tail (~500 nt).

**Determination of the 5′ terminus of the 2.7-kb *pk* major transcript**

The 5′ region of the *pk* transcript was obtained by rapid amplification of the cDNA 5′ ends (5′ RACE) (Frohman *et al.*, 1988) using a 5′/3′ RACE kit (Roche) in which oligo(dT)-anchor primer, anchor primer, and other key reagents were included. The locations of the primers used in this study are shown in Fig. 4. For the first step of 5′ RACE, the appropriate gene-specific primer (5′ RACE-SP6 primer; Fig. 4a) was used for first-strand cDNA synthesis from the total RNA isolated from the shrimp 60 h after artificial infection with WSSV by using an AMV (avian myeloblastosis virus) reverse transcriptase. After the poly(A) head was added to the cDNA products, these cDNAs were used as templates for the first PCR amplification with the 5′ RACE-SP4/oligo(dT)-anchor primer set (Fig. 4a). The first amplification products were then used as a template for the second PCR amplification with the A93R11/anchor primer set. The PCR products from the second amplification formed a single band in an agarose gel at ~166 bp (Fig. 4c). Analysis of 5′ RACE products cloned in pGEM-T Easy vector (Fig. 4d) revealed that all the 5′ termini of the first 14 randomly picked clones were located downstream of the first predicted ATG initiation codon. Contrary to the...
conclusion reported in van Hulten and Vlak (2001), we therefore conclude that the first predicted ATG initiation codon did not play a role in translation initiation for the WSSV pk gene and that the second predicted ATG initiation codon is the translational initiation site. This conclusion is further supported by the fact that the sequences surrounding the putative second translation initiation codon (GTGATGG) conform reasonably well to the eukaryotic translation consensus sequence (Kozak, 1987, 1997).

Of the 14 clones from the 5' RACE products that were subjected to sequencing, 4 had their 5' termini at nucleotide residue −38(G) and 7 at −39(G) [relative to the A in the second potential translation initiation codon, which is defined as +1(A)]. The remaining 3 clones were apparently early termination clones (examples: clones 1 and 7 in Fig. 4d). These results suggest that for the WSSV pk gene, there are two transcriptional initiation sites, located at nucleotide residues −38(G) and −39(G). Upstream of the transcriptional initiation sites, two putative TATA boxes were found at nt 59 to 64 and at nt 120 to 125.

In our previous study (Tsai et al., 2000a), we found that, for the genes encoding the WSSV ribonucleotide reductase large and small subunits (rr1 and rr2 genes), the transcriptional start points were located within a consensus motif (TCAc/tTC). Recently, we found that the same consensus was also present in the WSSV tk-tmk gene encoding the novel chimeric protein of thymidine kinase and thymidylate kinase (Tsai et al., 2000b). However, this consensus was not present in the transcriptional start site of the WSSV pk gene. Further work is needed to confirm whether there are other WSSV genes that use the same consensus sequence for their transcription initiation sites as the WSSV pk gene.

Determination of the 3' terminus of the 2.7-kb pk major transcript

To determine the 3' terminus of the major WSSV pk transcript, 3' RACE was performed. The first-strand cDNA was synthesized using the oligo(dT)-anchor primer

FIG. 4. Determination of the termini of the 2.7-kb major pk transcript. The locations of primers used for 5' RACE (A93R11, 5' RACE-SP4, 5' RACE-SP6) and for 3' RACE (A93F3) are indicated in a and b, respectively. The bent arrows indicate the 5' termini (transcriptional start points) revealed by sequencing of 11 5' RACE clones. The first and second ATG initiation codons in the 2193-nt ORF are boxed. (c–f) Agarose gel analysis of RACE products (c, e) and the same RACE products cloned in the pGEM-T Easy vectors (d, f) (arrows). The predicted TATA box and polyadenylation signal (AATAAA) are in boldface type. The poly(A) addition site is indicated by the straight arrow. M in c and e represents a 100-bp DNA marker ladder (Promega).
and AMV reverse transcriptase. The amplification of the 3′ region of the resulting cDNA was carried out by PCR using the A93F3/anchor primer set (Fig. 4b), which yielded a PCR product of 362 bp (Fig. 4e). Sequence analysis of the cloned 3′ RACE products (Fig. 4f) revealed that poly(A) was added at a site 16 nt downstream of the AATAAA polyadenylation signal (nt 2152 to 2157), which was found 3 nucleotides downstream of the translation stop codon (Fig. 4b).

Based on the above results, we conclude that the WSSV pk gene consists of 2148 nt with the potential to encode a polypeptide of 715 amino acids with a theoretical size of 80 kDa and a pI of 9.36. The WSSV pk gene sequence has been deposited with GenBank under Accession No. AF335541.

**WSSV pk transcription analysis in WSSV-infected shrimp**

RT-PCR analysis was used to detect the pk-specific transcript in DNase-treated total RNAs from shrimp specimens before infection (0 h) and at 2, 4, 6, 8, 18, 24, 36, and 60 h after artificial infection with WSSV. As a control, PCR was also used to monitor the presence of the viral DNA in the infected shrimp. For the control, the total DNAs were extracted from pleopod tissues (200 mg) from each of the artificially infected shrimp. An aliquot (0.5 μg) of the total DNA of each specimen was subjected to WSSV DNA-specific PCR using a WSSV pk-specific primer set. As shown in Fig. 5, the viral DNA was first detected at 2 h and then continued to be found through to 60 h p.i. The relatively small amounts of WSSV DNA observed at 2 h p.i. are likely the result of some of the injected virions reaching the pleopod tissues that were used for the analysis, while the marked increase in intensity at 8 h p.i. probably indicates replication of the viral DNA. This interpretation of the data is only provisional, but it is consistent with what is already known about the WSSV replication cycle, which, in the cuticular epidermis of artificially infected shrimp, has been estimated at 22 h (Chang et al., 1996). This question is further discussed below.

For the RT-PCR analysis, total RNA extracted from the same pleopod tissues (500 mg) of each shrimp specimen was treated with DNase to eliminate any viral genomic DNA contamination in the preparations. An aliquot (10 μg) of total RNA was used to synthesize the first-strand cDNA by using Superscript reverse transcriptase (Life Technologies) and oligo(dT) primer in a 20-μl reaction mixture. An aliquot (2 μl) of the reaction product containing about 1 μg cDNAs was then subjected to PCR amplification with the pk-specific primer set and other appropriate primer sets (i.e., a primer set for the adjacent vp25 gene for comparison and a β-actin primer set for control). The pk transcript was first detected at 2 h p.i. and continued to be found through to 60 h p.i. (Fig. 6a). The vp25 transcript was first detected at 4 h p.i. and also continued to be expressed through to 60 h (Fig. 6b). Unlike the pk gene, the intensity of the vp25 RT-PCR product band increased significantly over time, especially after 12 h p.i., and during this advanced infection period, the amount of vp25 transcript was much higher than that of pk.

As a quality control, the first-strand cDNAs were subjected to PCR with a WSSV genomic DNA-specific IC-F2/IC-R3 primer set derived from an intergenic region of the WSSV genome. The region delimited by this primer set should not appear in the cDNA, and no RT-PCR products specific primer set. As shown in Fig. 5, the viral DNA was first detected at 2 h and then continued to be found through to 60 h p.i. The relatively small amounts of WSSV DNA observed at 2 h p.i. are likely the result of some of the injected virions reaching the pleopod tissues that were used for the analysis, while the marked increase in intensity at 8 h p.i. probably indicates replication of the viral DNA. This interpretation of the data is only provisional, but it is consistent with what is already known about the WSSV replication cycle, which, in the cuticular epidermis of artificially infected shrimp, has been estimated at 22 h (Chang et al., 1996). This question is further discussed below.

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were in fact yielded (data not shown), thus confirming that no viral genomic DNA was left in the prepared RNA for WSSV pk transcription analysis.

Due to the lack of a suitable shrimp cell line, it is impossible to do a temporal analysis of WSSV gene transcription in cells synchronously infected with the virus. However, WSSV, which systematically targets cells originating from the mesoderm and ectoderm (Wong-teerasupaya et al., 1995; Lo et al., 1997), is extremely virulent (this is true not only for the Taiwan isolate that we have consistently used since 1994, but also applies to isolates from other laboratories) and in infected individual shrimp it spreads rapidly (Chou et al., 1995) and infects both cuticular epithelial and connective tissue cells at a similar rate. In our previous study (Chang et al., 1996) of WSSV-specific in situ hybridization of the tissue sections of experimentally WSSV-infected shrimp, WSSV-positive cells were initially observed at 16 h postinfection in the cuticular epidermis, and by 22 h p.i., WSSV-positive cuticular epidermal cells with hypertrophied nuclei and other obvious cytopathological changes were readily observed in almost all the tissues examined. The sequential progression of the disease is also very similar across different individual shrimp. Thus, when pleopod tissues, which most often showed the highest prevalence of the virus in early infection (Lo et al., 1997; Kou et al., 1998), are used as the source of total RNA in temporal gene expression experiments, the results are generally consistent and reproducible (see, e.g., Tsai et al., 2000a,b). Although this may not be an ideal assay system for temporal WSSV gene transcription analysis, the consistency of the results, whether using the same gene in different sets of RNA samples or different genes in the same set of RNA samples, suggests that this method can provide good estimates of the relative time and expression patterns for WSSV genes.

Screening for WSSV pk homologues in the WSSV genome by Southern blot hybridization

Using Southern blot hybridization analysis, the WSSV pk-specific probe hybridized only with a 3.4-kb HindIII fragment and an 8.4-kb EcoRI WSSV genomic DNA fragment (presumably pmh12 and pme902, respectively) (Fig. 7). These data suggest that the WSSV genome does not contain other sequences encoding WSSV PK homologues.

Amino acid sequence alignment of WSSV PK

When the deduced amino acid sequence of WSSV PK was compared with other sequences in GenBank at the National Center of Biotechnology Information by using the Blast network service (Altschul et al., 1990), its carboxyl-terminal region (residues 283–715; see Fig. 8) showed homology to the catalytic domain [subdomains I to IX (Hanks et al., 1988)] from a variety of eukaryotic organisms and their viruses. In particular, multiple alignments of the highly conserved regions of each subdomain show that the important residues with assigned functions for subdomains I and II (protein kinase ATP-binding site), subdomain VI (serine/threonine protein kinase active-site site), subdomain VII (Mg$^{2+}$-binding loop), and subdomain VIII (recognition of peptide substrate) are all conserved in WSSV PK. To take each of the subdomains in turn:

![Figure 7](image1)

**FIG. 7.** Hybridization of a DIG-labeled WSSV pk-specific probe to Southern blots of WSSV DNA digested with HindIII (lane 1) and EcoRI (lane 2) restriction endonucleases. Size standards are from a Lambda HindIII DNA marker (Promega).

![Figure 8](image2)

**FIG. 8.** WSSV PK. Locations of the conserved motifs in catalytic subdomains I to XI.
At the N-terminal extremity of the catalytic domain of WSSV PK (Fig. 9), there is a region (subdomain I; residues 283–295) containing a glycine (G)-rich stretch of residues (284GFGSKN289), and nearby (17 residues downstream) there is the lysine residue (306K) of subdomain II (residues 303–306). The region from subdomains I to II has been shown to be involved in ATP binding (Hanks and Hunter, 1995). In the PROSITE database, this ATP-binding region is one of only two regions that have been selected to build a protein kinase signature. The WSSV PK sequence in this region \[283VGFGSKNLSVLDT-x(7)-RLCK306\] matches well (although not exactly) the protein kinase signature consensus pattern

\[
\text{I: \[LIV\]-G-{P}-G-{P}-[FYWMGSTNH]-[SGA]-{PW}-[LIVCAT]-[PD]-x-[GSTACLIVMFY]-x-(5, 18)-[LIVMFYWCSTAR]-[AIVP]-[LIVMFAGCKR]-K}
\]

where K is the amino acid residue that binds ATP. (The convention used throughout this paper for consensus patterns is to list the acceptable alternative amino acids for a given position between square brackets "[" and to place between a pair of curly brackets \{"\}" those amino acids that are not acceptable at a given position.)

The sequences of 334ESIL337, 372ASQVVMI378, 389VGVYYMLETGKVIKFM404, and 551IVNIVTRLS559 show homology to subdomains III, IV, V, and VIA, respectively. In subdomain VIB, which is highly conserved

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**FIG. 9.** The nucleotide sequence and deduced protein sequence of the coding region of the WSSV pk gene. The sites of the conserved catalytic subdomains are numbered I to XI and underlined.
among protein kinases and is used to build the protein kinase signature consensus pattern II, the WSSV PK sequence 563LVNPDPDKSNDIVI575 matches well with the protein kinase signature consensus pattern II, [LIVMF-YC]-x-[HY]-x-D-[LIVMFY]-K-x(2)-N-[LIVMFYCT](3), where the aspartic acid residue (567D) is probably the residue important for the catalytic activity of the enzyme (Knighton et al., 1991). The residue of WSSV PK at position 569 is K, indicating that it is a serine/threonine-specific protein kinase.

Subdomain VII includes a Mg$^{2+}$-binding loop that contains a highly conserved Asp-Phe-Gly (DFG) triplet (Hanks and Hunter, 1995). In WSSV PK subdomain VII, the corresponding residues are found in a highly conserved region, 584MIDFGL589. Subdomain VIII plays a major role in the recognition of peptide substrates and contains a highly conserved Ala-Pro-Glu (APE) motif. In WSSV PK, this APE motif is located in a highly conserved region, 610SNHPHTAPE618. In WSSV PK, subdomains IX to XI are not so clearly defined, but three sites were found (amino acid residues 629 to 641, 642 to 646, and 666 to 678) that show significant homology to the corresponding regions of subdomains IX to XI in some of the selected protein kinases (Table 1). The WSSV PK subdomain sequence data presented here, however, should be considered only as preliminary evidence, and further experiments on mutations should be made to confirm the validity of these sites, to precisely determine the location of each subdomain, and to identify which residues are functionally important.

The alignments also clearly demonstrate the overall similarity of the WSSV PK catalytic domain to the other selected protein kinases in terms of the order and spacing of the subdomains (Fig. 8). However, a very large insert (146 residues) occurs in WSSV PK between subdomains V and VI, and this causes the WSSV PK catalytic domain (433 residues) to be considerably larger than most of the other protein kinase catalytic domains, which usually range from 250 to 300 amino acid residues (Hanks and Hunter, 1995). It should also be noted that while the location of the catalytic domain within the protein kinase is not fixed, in most single subunit enzymes it lies near the carboxyl terminus, the amino terminus being devoted to a regulatory role, whereas in protein kinases having a multiple subunit structure, subunit polypeptides consisting almost entirely of catalytic domain are common (Hanks et al., 1988). WSSV PK resembles the single subunit enzymes in that its catalytic domain lies near the carboxyl terminus. The sequence of its long amino terminus (282 residues) upstream of the catalytic domain is unique, however, and more work will be needed to elucidate its function. To date, binding sites for regulatory elements such as Ca$^{2+}$, cAMP, or phorbol esters/diacylglycerol have not been identified in this region.

Pairwise comparisons

For pairwise comparisons and phylogenetic analysis of the WSSV PK catalytic domain to the PK catalytic domains of other protein kinases, 29 viral PK (including WSSV) and 55 PK from fungi, invertebrates, vertebrates, a bacterium, and a plant were selected. Of the 55 nonviral protein kinases, 21 are representative members of the four major groups of protein kinases designated by Hanks and Hunter (1995), that is, (1) the AGC group, which includes the cyclic-nucleotide-dependent family (PKA and PKG), the protein kinase C (PKC) family, the ribosomal S6 kinase family, and other relatives; (2) the CaMK group, which includes the family of protein kinases regulated by calcium/calmodulin, the Snf1/AMPK (kinase essential for release from glucose repression/AMP-activated protein kinase) family, and other close relatives; (3) the C-M-G-C group, which includes the family of cyclin-dependent kinases, the Erk (MAP) kinase (extracellular signal-regulated kinase) family, the glycogen synthase 3 (GSK3) family, the casein kinase II family, the Clk (Cdk-like kinase) family, and other close relatives; and (4) the "conventional" PTK group. The other 34 nonviral protein kinases fall outside these four major groups (Table 2). As for the 29 viral protein kinases, in addition to WSSV, 9 were from the Poxviridae, 8 from the Baculoviridae, 11 from the Herpesviridae, 2 from the Phycodnaviridae, and 1 each from the Iridoviridae and Asfarviridae (Table 3).

The pairwise identity and similarity (BLOSUM 35) of the WSSV PK catalytic domain to the PK catalytic domains of the selected PKs are summarized in Table 1. The overall homology of the WSSV PK and the protein kinases is not high: approximately 4 to 11% identity and 11 to 26% similarity was shown over the entire length of the catalytic domains. Within the subdomains, however, conservation of amino acid sequences is much higher, with the highest levels of homology [identity(%)/similarity(%)] for the conserved sequence in subdomains I, II, III, IV, V, VI, VII, VIII, IX, X, and XI being 46/61, 50/50, 42/71, 25/56, 33/66, 61/92, 83/100, 44/66, 42/71, 60/80, and 38/69, respectively.

Phylogenetic analysis

The sequences of the PK catalytic domains of all 84 of the proteins listed in Tables 2 and 3 were included in our original analysis, but it was found that WSSV PK did not cluster with any of the eukaryotic PK or with any of the viral PK (phylogenetic tree not shown). To focus on the relationships among viral PK, most of the eukaryotic PSK were therefore omitted and the phylogenetic tree was reconstructed. In addition, since all of the selected viral PK are PSK, the eukaryotic PTK group was also omitted. Thus in total 38 sequences (29 viral PK and 9 eukaryotic PK) from three of the major groups were used to construct the final phylogenetic trees (Fig. 10). [Note that
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**Note:** Refer to Fig. 9 for the location of each region and to the text for the abbreviated names.
### AGC group

1. PKA-Cα (cyclic AMP-dependent protein kinase catalytic subunit, α-form)  
   **Source**: H. sapiens  
   **Accession No.**: CAA04753

2. PKG-1 (cyclic GMP-dependent protein kinase, type I)  
   **Source**: Bos taurus  
   **Accession No.**: CKB0G

3. cPKCa (Ca<sup>2+</sup>-dependent protein kinase C, α-form)  
   **Source**: Rattus norvegicus  
   **Accession No.**: P06966

4. βARK1 (β-adrenergic receptor kinase, type 1)  
   **Source**: H. sapiens  
   **Accession No.**: A53791

5. S6K (70-kDa S6 kinase with single catalytic domain)  
   **Source**: H. sapiens  
   **Accession No.**: NM_003952

6. RSK1 (Nt) (90-kDa S6 kinase, type 1)  
   **Source**: H. sapiens  
   **Accession No.**: NM_002953

7. DMPK (myotonic dystrophy protein kinase)  
   **Source**: H. sapiens  
   **Accession No.**: NP_004400

### CaMK group

1. CaMKIIα (Ca<sup>2+</sup>-calmodulin kinase type II, α subunit)  
   **Source**: R. norvegicus  
   **Accession No.**: A30355

2. PhK (skeletal muscle phosphorylase kinase catalytic subunit)  
   **Source**: Oryctolagus cuniculus  
   **Accession No.**: NP_001883

3. skMLCK (skeletal muscle myosin light chain kinase)  
   **Source**: H. sapiens  
   **Accession No.**: A35021

4. Mre4 (protein required for meiotic recombination)  
   **Source**: S. cerevisiae  
   **Accession No.**: AAA35058

5. Snf1 (kinase essential for release from glucose repression)  
   **Source**: S. cerevisiae  
   **Accession No.**: S62887

### C-M-G-C group

1. Cdk2 (type 2 cyclin-dependent kinase)  
   **Source**: Mus musculus  
   **Accession No.**: NP_058036

2. Erk2 (extracellular signal-regulated kinase, type 2)  
   **Source**: B. taurus  
   **Accession No.**: P46196

3. GSK3α (glycogen synthase kinase 3, α-form)  
   **Source**: R. norvegicus  
   **Accession No.**: P18265

4. CK2α (casein kinase II, α subunit)  
   **Source**: M. musculus  
   **Accession No.**: CAA04753

5. Clk (Cdc-like kinase)  
   **Source**: H. sapiens  
   **Accession No.**: NP_004062

### PTK group

1. Src (cellular homologue of Rous sarcoma virus oncprotein)  
   **Source**: H. sapiens  
   **Accession No.**: NP_005408

2. EGFR (epidermal growth factor receptor)  
   **Source**: H. sapiens  
   **Accession No.**: NP_005219

3. PDGFRβ (platelet-derived growth factor receptor, type β)  
   **Source**: M. musculus  
   **Accession No.**: P05622

### Other protein kinase families

1. Polo (protein kinase homologue required for mitosis)  
   **Source**: Drosophila melanogaster  
   **Accession No.**: PS2304

2. Cdc5 (product of gene required for cell cycle progression)  
   **Source**: S. cerevisiae  
   **Accession No.**: M84220

3. MEK1 (MAP ERK kinase, type 1)  
   **Source**: H. sapiens  
   **Accession No.**: Q02750

4. Ste7 (kinase required for haploid-specific gene expression)  
   **Source**: S. cerevisiae  
   **Accession No.**: P06784

5. Ste11 (protein required for cell-type-specific transcription)  
   **Source**: S. cerevisiae  
   **Accession No.**: S51380

   **Source**: S. cerevisiae  
   **Accession No.**: S28394

7. Nek1 (NimA-related kinase)  
   **Source**: M. musculus  
   **Accession No.**: P51954

8. NIMA (cell cycle control protein kinase)  
   **Source**: Aspergillus nidulans  
   **Accession No.**: P11837

9. Fused (product of gene required for segment polarity)  
   **Source**: D. melanogaster  
   **Accession No.**: JC4243

10. Weel (Hs) (gene product able to complement S. pombe weel mutant)  
    **Source**: H. sapiens  
    **Accession No.**: CAA43979

11. SpWeel ("Wee" size at division kinase; Cdc2 negative regulator)  
    **Source**: Schizosaccharomyces pombe  
    **Accession No.**: AAA35354

12. PKR (double-stranded RNA-dependent kinase)  
    **Source**: H. sapiens  
    **Accession No.**: JC5225

13. Gcn2 (protein required for translational derepression)  
    **Source**: S. cerevisiae  
    **Accession No.**: AAA34881

14. ACTRII (type II receptor for activin)  
    **Source**: M. musculus  
    **Accession No.**: P27038

15. TGFßRII (type II receptor TGF-ß)  
    **Source**: H. sapiens  
    **Accession No.**: P37173

16. ZmPK1 (putative receptor protein-serine kinase)  
    **Source**: Z. mays  
    **Accession No.**: P17801

17. CK1α (casein kinase I, type α)  
    **Source**: H. sapiens  
    **Accession No.**: NP_001883

18. PKN1 (protein kinase homologous to eukaryotic kinases)  
    **Source**: Myxococcus xanthus  
    **Accession No.**: A41090

### Other protein kinase families (each with no known close relatives)

1. Mos (cellular homologue of retroviral oncogene product)  
   **Source**: H. sapiens  
   **Accession No.**: TVHUMS

2. Pim1 (proto-oncogene activated by murine leukemia virus)  
   **Source**: M. musculus  
   **Accession No.**: TVMSP1

3. Cot (product of oncogene expressed in human thyroid carcinoma)  
   **Source**: H. sapiens  
   **Accession No.**: P41279

4. Esk (*embryonal carcinoma STY kinase*; dual specificity)  
   **Source**: M. musculus  
   **Accession No.**: AAA37578

5. NinaC (product of gene essential for photoreceptor function)  
   **Source**: D. melanogaster  
   **Accession No.**: B29813

6. Pelle (product of gene required for dorsoventral polarity)  
   **Source**: D. melanogaster  
   **Accession No.**: AAA28750

7. Sp1A (spore lysis A protein kinase)  
   **Source**: Dictyostelium discoideum  
   **Accession No.**: P18160

8. Cdc7 (*cell-division-cycle* control gene product)  
   **Source**: S. cerevisiae  
   **Accession No.**: AAA34458

9. Cdc6 (cell-division-cycle control gene product)  
   **Source**: S. cerevisiae  
   **Accession No.**: S15038

10. Npr1 (product of gene required for activity of ammonia-sensitive amino acid)  
    **Source**: S. cerevisiae  
    **Accession No.**: AAA02892

11. Elm1 (product of gene required for yeast-like cell morphology)  
    **Source**: S. cerevisiae  
    **Accession No.**: AAA7541

12. Ykl516 (putative protein kinase gene on chromosome XI)  
    **Source**: S. cerevisiae  
    **Accession No.**: AAB21999

13. Rna1 (product of gene required for normal meiotic function)  
    **Source**: S. pombe  
    **Accession No.**: A25685

14. YpkA (enterobacterial protein kinase essential for virulence)  
    **Source**: Yersinia pseudotuberculosis  
    **Accession No.**: S30060
since both the neighbor-joining (NJ) and parsimony trees generated similar results, and since the NJ tree also revealed finer structures within major phylogenetic clades, only the NJ tree (Saitou and Nei, 1987) is shown here. On the tree, the three major PK groups (i.e., AGC, CaMk, and C-M-G-C groups), as expected, form three major clades while the viral PK reflect their current phylogenetic grouping. There are two well-supported (bootstrap value greater than 90%) clades within the Poxviridae. There may have been two different sources for the PK genes. The baculoviruses form a well-supported clade with high bootstrap value, while WSSV, one of the Iridoviridae (IV6), the two Phycodnaviridae (FSv and PBCV1), and the type species of the newly defined family Asfarviridae (ASFV) (van Regenmortel et al., 2000) all form distinct viral groups of their own. However, the relationships among the major clades, including the eukaryotic PK, constitute an unresolved polytomy. This star phylogeny and the relatively long branch length both suggest that WSSV is a distinct virus (likely at the family level) that does not belong to any of the virus families that are currently recognized.

**MATERIALS AND METHODS**

**Virus, plasmid clone, and sequence analysis**

The virus (WSSV Taiwan isolate) was isolated from a batch of WSSV-infected *P. monodon* collected in Taiwan in 1994 (Wang et al., 1995; Lo et al., 1999). From the HindIII and EcoRI libraries of genomic fragments, plasmid clones (pmh12; pme902) carrying the *pk* gene were sequenced on both DNA strands by using universal M13 forward and reverse primers. The internal sequences of the cloned fragments were obtained by automatic sequencing using universal M13 forward and reverse primers. The internal sequences of the cloned fragments were obtained by automatic sequencing using universal M13 forward and reverse primers.
pared with the latest GenBank/EMBL, SWISSPROT, and PIR databases using FASTA and BLAST. Alignments of amino acid sequences were made in CLUSTAL_X (Thompson et al., 1997) and edited in GeneDoc (Nicholas et al., 1997).

Coupled in vitro transcription and translation

An expression vector with the full-length WSSV pk gene was constructed for in vitro transcription and translation. The WSSV pk gene with additional EcoRI and NotI restriction endonuclease sites at both ends was amplified from pme902 by PCR with pk1-EcoRI/pk1-NotI primer sets (5'-CCGAATTCATGGAGGGTGGGGACCAACGGCA-3'/5'-GGGCGGCCGCCTACTTAACCTT-3'; where the underlined bases indicate the restriction sites) and then cloned into pGEM-T Easy (Promega). The resultant plasmid (pPK) with the correct sequence of insertion was cleaved with EcoRI and NotI and introduced into a modified form of the

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FIG. 10. Unrooted neighbor-joining phylogenetic tree of the WSSV PK catalytic domains of 29 viral PK (listed in Table 3) and 9 eukaryotic PK from three of the major PSK groups (i.e., AGC, CaMk, and C-M-G-C groups; refer to Table 2). Numbers indicate bootstrap values.

WSSV pk GENE ENCODES A PROTEIN KINASE

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0.1 changes
pcDNA3 (Invitrogen) based vector. To create this modified form (pcDNA3PK) a hemagglutinin (HA) epitope tag excised from a HA epitope-tagged mammalian expression vector (Clontech) was inserted at the HindIII and BamHI cloning sites. TNT (Promega) Quick Master Mix (20 μl) was mixed with 1 μl [35S]methionine (1000 Ci/mmol, 10 μCi/ml) and pcDNA3PK (1 μg) in 5 μl nuclease-free water). The reaction mixture (25 μl) was incubated at 30°C for 90 min, and an aliquot of the translation product (4.5 μl) was analyzed by 12.5% SDS–PAGE. After electrophoresis, the gel was stained, destained, dried, and exposed to Fuji medical film at room temperature for 17 h. The same reaction but with 0.5 mM methionine instead of [35S]methionine was also carried out for Western blot analysis with PK antibodies.

WSSV pk transcription analysis

WSSV-infected shrimp. Since to date no WSSV-susceptible shrimp cell lines have become available, all the RNA for the transcriptional analysis was taken from WSSV-infected shrimp at different times after infection. Healthy (that is, two-step WSSV diagnostic PCR negative; Lo et al., 1996) subadult P. monodon (15–20 g) were infected with WSSV by injection using the method described previously by Tsai et al. (1999). At various times over the course of the next 60 h, two or three specimens were selected at random and their pleopods were excised. The collected pleopods were immediately frozen and stored in liquid nitrogen until used for RNA isolation.

RNA isolation. For the isolation of total RNA, the frozen pleopods (500 mg) from WSSV-infected P. monodon were homogenized in 5 ml TRIzol reagent (Life Technologies) and then subjected to ethanol precipitation according to the manufacturer’s recommendations. The total RNA was stored in 75% ethanol at −20°C. Following procedures described in Lo et al. (1996), total DNA was extracted from the same tissues and used to check the viral DNA loading in the tissues using PCR with a pk-specific primer set pk-F2/pk-R7 (5’-TTTAGTCAGGTCTTTGAGGG-3’/5’-GCCAGTAGCTTTGAAGCATCC-3’).

Detection of WSSV pk transcripts in WSSV-infected shrimp by Northern blot hybridization analysis with a pk gene-specific riboprobe. A WSSV pk-specific [α-32P]CTP-labeled riboprobe was used for Northern blot analysis. To generate the riboprobe, the RNA polymerase promoter addition kit Lig’N’Scribe (Ambion) was used in accordance with the manufacturer’s instructions to produce templates from WSSV pk-specific PCR products for the in vitro transcription. Briefly, the WSSV pk-specific fragment was amplified from WSSV genomic DNA by PCR with the primer set pk-F2 and pk-R4 (5’-TTTAGTCAGGTCTTTGAGGG-3’ and 5’-ACATGCACCATTACAGCGGC-3’, respectively). An aliquot (25 ng) of the WSSV pk-specific PCR product was then ligated with T7 promoter adapter (supplied with the kit) using T4 DNA ligase. To generate WSSV pk-specific fragments that contained the T7 RNA polymerase promoter, an aliquot (2 μl) of the reaction mixture (10 μl) was used as a template in PCR with a primer set consisting of the PCR adapter primer 1 (supplied with the kit) and pk-F2 (5’-GCTTCCGGCTCGATGTTGTTGAGG-3’ and 5’-TTTAGTCAGGTCTTTGAGGG-3’, respectively). An aliquot (3.6 μl) of PCR product (50 μl) was then used to generate the WSSV pk-specific [α-32P]CTP-labeled riboprobe by in vitro transcription (Sambrook et al., 1989) in a 20-μl reaction mixture containing 40 U of T7 RNA polymerase (Roche) and 0.02 mCi [α-32P]CTP for 2 h at 37°C. The reaction mixture was then treated with 200 U RNase-free DNase I for 30 min at room temperature, terminated at 68°C for 15 min, and filtered through a Sephadex G50 column.

For Northern blot analysis, 10 μg total RNA was separated on 1% formaldehyde-containing agarose gel and transferred to a Hybond-N+ membrane (Amersham). The membrane was prehybridized for 1 h at 65°C in a prehybridization buffer (0.25 M phosphate buffer, 1 mM EDTA, 1% BSA, and 7% SDS) and then hybridized to the WSSV pk-specific [α-32P]CTP-labeled riboprobe for 16 h at 65°C. After washing, the membrane was exposed to Kodak BioMax MR film via an intensifying screen for several days at −70°C and then developed.

Temporal analysis of WSSV pk transcription by RT-PCR. Total RNA in 75% ethanol was centrifuged at 14,000g for 30 min at 4°C. The pellet was resuspended in DEPC-water and quantified by spectrophotometry at 260 nm. An aliquot of 10 μg RNA was treated with 200 U of RNase-free DNase I at 37°C for 30 min to remove any residual DNA and then extracted with phenol–chloroform. The DNase-treated total RNA (−10 μg) was denatured by heating at 85°C for 10 min in 10 μl DEPC-water containing 100 pmol oligo(dT) primer (Roche). The first-strand cDNA was synthesized by the addition of 4 μl Superscript II 5× buffer, 1 μl of 100 mM DTT, 1 μl of 10 mM dNTPs, 10 U RNasin (Promega), and 100 U Superscript II reverse transcriptase (Life Technologies). DEPC-water was added to make a final volume of 20 μl. The reverse transcription proceeded at 37°C for 1 h, followed by heating at 95°C for 5 min to stop the reaction. One-tenth of the products of the cDNA reaction (2 μl; ~1 μg) was subjected to RT-PCR in a 50-μl reaction buffer containing 10 mM Tris–HCl, pH 8.8, 1.5 mM MgCl2, 150 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 100 pmol each primer [the primer sets were pk-F2/pk-R7 (5’-TTTAGTCAGGTCTTTGAGGG-3’/5’-GCCAGTAGCTTTGAAGCATCC-3’) for pk and vp25-F1/vp25-R1 (5’-CAGTGGCCAGTAGGTACGAGG-3’/5’-ATGAAGGAAGAGAGTGCCG-3’) for vp25, where vp25 is an envelope gene adjacent to the pk gene; it is used here for comparison], and 2 U DyNAzyme II DNA polymerase (Finnzymes). The PCR cycles were as follows: 94°C for 2 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by an elongation at 72°C for 30 min. A β-actin transcript was amplified with
the actin-F1/actin-R1 primer set (5′-GAGGATGAGAAA-
GATCTGG-3′/5′-CCRGGTCATGGGGTRCC-3′) and
used as an internal control for RNA quality and amplifi-
cation efficiency. A WSSV genomic DNA-specific primer
set IC-F2/IC-R3 (5′-CAGACTATTAATGCAAGTGCG-3′/
5′-GAATGATGGTGCTGGTGAAC-3′) derived from an
intergenic region of the WSSV genome was used to
confirm that the RNA was not contaminated by any viral
DNA.

Southern blot analysis

Southern blot analysis (Southern, 1975) was used to
screen the WSSV genome for possible ORFs that poten-
tially encode eukaryotic protein kinase homologues.
Since the 1020-nt ORF contains the conserved se-
quences of the eukaryotic protein kinase subdomains VI
to XI, the PCR-generated, DIG-labeled 1020-nt ORF-spe-
cific probe was used for this analysis. WSSV DNA ex-
tracted from virions purified from WSSV-infected P. mon-
don (Wang et al., 1995) was digested with HindIII or
EcoRI restriction enzymes, separated by electrophoresis
on a 0.7% agarose gel, transferred to Hybond-N+ mem-
brane (Amersham), and then hybridized with the DIG-
labeled probe. DIG-labeled nucleotides in the blots were
detected as described previously (Lo et al., 1999).

Amino acid sequence comparison and phylogenetic
construction

Selected protein kinases (29 viral PKs and 56 other
PKs from fungi, invertebrates, vertebrates, a plant, and a
bacterium) from GenBank were used in the alignment
and phylogenetic analyses (Tables 2 and 3). The multiple
sequence alignments were done by the multiple se-
quence alignments program CLUSTAL_X (Thompson
et al., 1997). Phylogenetic analysis based on PK sequences
was performed using NJ and parsimony methods with the
PAUP 4.0b1 program (Swofford, 1998), using
CLUSTAL_X (Thompson et al., 1997) to produce input
files of aligned protein sequences. One thousand boot-
strap replicates were generated to test the robustness of
the trees.

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