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Molecular cloning of a gene encoding matrix metalloproteinase-like protein from *Gnathostoma spinigerum*

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Abstract The advanced third-stage larvae (aL3) of *Gnathostoma spinigerum* contain a 24 kDa glycoprotein with diagnostic potential. Immunoscreening with the monoclonal antibody to the 24-kDa protein (mAb GN6/24) has identified a cDNA clone with an insert of 932 base pairs (bp). The insert contains a full-length gene of 732 bp encoding a protein that is 33–39% similar to matrix metalloproteinases (MMPs) of *Caenorhabditis elegans* and several lower and higher vertebrates. The MMP-like protein of *G. spinigerum* possesses the catalytic domain, but lacks the propeptide and hemopexin-like domains found in other MMPs. A signal peptide of 23 amino acids at its amino terminus indicates that it is a secretory protein, which is confirmed by Western blot analysis showing the presence of the 24 kDa protein in the excretory–secretory products of aL3.

Introduction

Gnathostomiasis is a disease caused by nematodes of the genus *Gnathostoma*. The disease is mainly encountered in Asian countries, especially Thailand and Japan. The species most frequently responsible for the disease is *Gnathostoma spinigerum*. Recently, gnathostomiasis due to *G. spinigerum* has become an emerging public health problem in Ecuador and Mexico (Feinstein and Rodriguez-Valdes 1984; Ollague et al. 1984; Diaz Camacho et al. 1998; Ogata et al. 1998). In Japan, *Gnathostoma hispidum*, Gnathostoma doloresi and *Gnathostoma nipponicum* also cause human gnathostomiasis (Miyazaki 1991).

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Adult *G. spinigerum* residing in a tumor of the stomach wall of the definitive hosts (usually cats and dogs) discharge eggs into the environment in faeces. First- and second-stage larvae develop in eggs and hatch in water (Miyazaki 1991; Ando et al. 1992). The free-swimming second-stage larvae are ingested by copepods, the first intermediate host, and develop into the early third-stage larvae. These larvae mature into the advanced third-stage larvae (aL3) in the visceral organs and muscles of the second intermediate host, such as fish, frogs, snakes, chickens, and ducks that have ingested infected copepods. The aL3 may be found in other animals that have ingested another second intermediate host. When a definitive host ingests the second intermediate or paratenic host, the larvae eventually develop into mature male and female worms in the gastric wall. Consuming raw or inadequately cooked meat of the second intermediate or paratenic host leads to infection in man. In humans, the third-stage larvae migrate to various organs throughout the body and some appear to develop into immature or mature worms (Daengsavang 1980). The clinical diseases caused by migrating larvae and their severity depend on the organ invaded (Daengsavang 1980; Rusnak and Lucey 1993). The skin and subcutaneous tissue are affected frequently, infection being characterized by intermittent migratory swelling. The disease becomes serious when the larva invades vital organs, such as the central nervous system and the eye, causing permanent damage or even death (Boongird et al. 1977; Teekhasaenee et al. 1986; Punyagupta et al. 1990).

The diagnosis of gnathostomiasis depends largely on the clinical presentation of patients, supported by serological tests. Among the serological tests, immunoglobulin enzyme-linked immunosorbent assay (IgG ELISA) has been most widely studied and shown to be highly sensitive. However, the test also shows varying degrees of specificity – cross-reaction being as high as 33% with angiostrongyliasis, and 23% with other parasitic infections (Suntharasamai et al. 1985). Studies by Western blot analysis have shown that sera from

confirmed cases of gnathostomiasis reacted with several larval proteins, including a 24-kDa protein that is not recognized by sera from patients with other parasitic infections (Tapchaisri et al. 1991). The partially purified 24 kDa protein obtained by column chromatography was evaluated for its diagnostic potential and showed 100% sensitivity and specificity to ELISA (Nopparatana et al. 1991). Production of the monoclonal antibody (mAb) to the 24-kDa protein (mAb GN6/24) has been reported previously (Chaicumpa et al. 1991). We have shown that the mAb GN6/24 recognizes the deglycosylated form of the 24-kDa protein, implying that this mAb could be used to screen the cDNA library constructed in *Escherichia coli* (Uparanukraw et al. 1999). The present study attempts to identify and express the gene encoding the 24-kDa protein from the cDNA library of *G. spinigerum* aL3.

Materials and methods

The parasite

Cysts of *G. spinigerum* aL3 were dissected from the livers of naturally infected eels and rinsed in 70% ethanol. aL3 were aseptically excised from the cysts and transferred to an RPMI-1640 medium, supplemented with 10% FCS, 5 μg/ml gentamicin and 10 μg/ml amphotericin B, and maintained at 37 °C in an atmosphere of 5% CO_2 (Maleewong et al. 1995). The culture medium was changed every 3 days. The living larvae were later used for isolation of total RNA.

Isolation of total RNA and mRNA

For each preparation, approximately 200 aL3 were removed from culture, washed, pulverized using liquid nitrogen, and homogenized in 4.0 M guanidinium thiocyanate solution. Total RNA was isolated from the homogenate by centrifugation through a cesium chloride step-gradient, as described previously (Sambrook et al. 1989). Polyadenylated mRNA was purified from total RNA using an Oligotex mRNA Mini Kit (QIAGEN), according to the manufacturer's instructions. The yields of mRNA were 1–2% of total RNA input in several experiments.

Construction of cDNA library and immunoscreening

The ZAP-cDNA Synthesis Kit (Stratagene) was used to construct a cDNA library from 5 μg of polyadenylated mRNA of *G. spinigerum* aL3 according to the manufacturer's instructions. The primary cDNA library obtained was further amplified and aliquots of the amplified library were stored in 7% DMSO at –80 °C. The amplified library was later used for immunoscreening. Approximately 45,000 pfu of phage library were transferred onto a sheet of 132 mm diameter nitrocellulose (NC) membrane (NitroPure, 0.45 μm, Micron Separations Inc.) and probed with the mAb GN6/24 (culture supernatant). Plaques that reacted with the mAb were identified by incubation with peroxidase-conjugated goat anti-mouse IgG (1:20,000 dilution), and subsequently with luminol/enhancer/stable peroxide solution (SuperSignal substrate, Pierce). Positive signals were visualized by exposing the filters of plaque lifts to X-ray films. Selected positive plaques were subjected to secondary screening to obtain isolated positive clones.

DNA sequence analysis

cDNA inserts were amplified from bacteriophage of positive clones by polymerase chain reaction (PCR) using T3 and T7 primers. Five microliters of bacteriophage suspension was used as the DNA template for amplification. PCR conditions consisted of 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The amplified cDNA inserts were purified prior to sequence analysis, using either Centricon-100 (Amicon) or the Gel Purification kit (Bio-Rad). The DNA sequence was determined by cycle sequencing using dRhodamine Cycle Sequencing Kits (Perkin-Elmer) and an automated DNA sequencer (ABI Prism 310). The complete sequences were obtained by T3, GS1 (5′-GGTCATTTCGGCTGCCCATG-3′, corresponding to bases 373–392 in clone 8.7, Fig. 1) and GS2 (5′-CTTTGTCCACGTTCCCAACG-3′, complementary to bases 287–306 in clone 8.7, Fig. 1) primers. Deduced amino-acid sequences were submitted for similarity searches against nonredundant protein-sequence databases at the National Center for Biotechnology Information (NCBI) using BLASTP 2.0.10 (Altschul et al. 1997) or with swall protein databases at the European Bioinformatics Institute (EBI) using FASTA3 (Pearson and Lipman 1988; Pearson 1990). Multiple-sequence alignments were performed by ClustalW 1.8 through the Baylor College of Medicine Search Launcher and consensus alignments were shaded by BoxShade 3.21 through the European Molecular Biology Network (EMBnet) server.

Rapid amplification of cDNA at the 5′ end

Rapid amplification of cDNA at the 5′ end (5′ RACE) was performed to obtain the sequence at the 5′ end of the cloned gene (Frohman et al. 1988). Briefly, 1 μg of aL3 total RNA was reverse transcribed by SuperScript II reverse transcriptase (Gibco BRL) using GS4 primer (5′-*GCGC*AAGCTTACGGCATTGGAG-3′, complementary to bases 779–792 in clone 8.7, italicized sequence is introduced, *Hin*dIII site is underlined, Fig. 1) followed by dG tailing of the first-strand cDNA using terminal deoxynucleotidyl transferase (Boehringer Mannheim) according to the protocol provided with the enzyme. The dG-tailed cDNA was used as a template for PCR using ESdC15 (5′-TGTGAATTCGTCGACTGCCCCCCCCCCCCCCC-3′, *Eco*RI and *Sal*I sites are underlined) and GS2 primers. The PCR product was blunted with T4 DNA polymerase, digested with *Eco*RI, and ligated to pBluescript II SK previously digested with *Eco*RI and *Eco*RV. *E. coli* XL1-Blue MRF′ were transformed by the ligation product using an electroporator (Gene Pulser II Plus, Bio-Rad). Positive colonies were identified by colony hybridization with a DNA probe labeled by primer extension method using a DIG DNA labeling kit (Boehringer Mannheim). Plasmid DNA from positive colonies was prepared and sequenced as described above.

Expression of the *Gs24* gene

The expression vector used in this study is *pMAL-c2X* (New England Biolabs). The *Gs24* gene spanning from the putative initiation codon to the stop codon was created by PCR using the GS3 (5′-ATGAAACTACAGAGTGTGATTTGG-3′, corresponding to bases 58–81 in clone 8.7, Fig. 1) and GS4 primers. The amplified gene was blunted by T4 DNA polymerase and digested with *Hin*dIII. The blunted and *Hin*dIII-digested DNA was then ligated to *Xmn*I-, *Hin*dIII-cleaved *pMAL-c2X*. Transformation of *E. coli* and identification of positive colonies were carried out as described above. To check for expression of the fusion protein, transformed *E. coli* cells were grown in LB broth at 37 °C to log phase, after which they were induced by 0.3 mM IPTG for 1, 2, and 3 h. ninduced and induced samples of bacteria were analyzed by SDS-PAGE for Coomassie blue staining or by Western blotting. Solubility of the fusion protein was determined by suspending the induced *E. coli* cells in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). The bacterial-cell suspension was frozen at –70 °C, thawed, and sonicated on ice using a Branson sonifier. The conditions of sonication were 12–10 s bursts with

1 min rest between bursts. The lysate was centrifuged at 9,000 *g*, 4 °C for 20 min. The supernatant was collected and the pellet (insoluble material) resuspended in the same volume of column buffer. Samples of the supernatant and the insoluble material were analyzed by SDS-PAGE, along with samples of uninduced and induced cells.

SDS-PAGE and Western blot analysis

SDS-PAGE and electrotransfer of proteins onto NC membrane were performed as previously described (Laemmli 1970; Towbin et al. 1979). The NC membrane was blocked with PBS containing 0.3% Tween 20 for 30 min at room temperature. The membrane was allowed to react with the mAb GN6/24 at 4 °C overnight. The membrane was washed three times with PBS containing 0.05% Tween 20 (PBS-T), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (1:1,000 dilution) for 60 min at room temperature. After another three washes with PBS-T and one wash with PBS, the membrane was finally developed in the substrate solution (0.6 mg/ml 3,3′-diaminobenzidine, 0.03% H_2O_2 in PBS). The reaction was stopped by washing the membrane in distilled water.

Results

Isolation of the *Gs24* gene

Screening of 45,000 pfu of the cDNA library with the mAb GN6/24 resulted in approximately 200 positive plaques. Following secondary screening, 24 positive clones were selected for further analyses. cDNA insert from each clone was amplified by PCR using T3 and T7 primers (T3 and T7 primers are 98 bp and 49 bp from the 5′ and 3′ ends of the cDNA insert, respectively). The sizes of the PCR products ranged from 0.7 to 2.5 kb and most of them (18 of 24) were approximately 1.0 kb in length (data not shown). The DNA sequences of two of the PCR products, designated clones 8.6 and 8.7, were determined by cycle sequencing. Both clones had identical DNA sequences. The complete sequence data showed that the PCR product of clone 8.7 was 1,079 bp

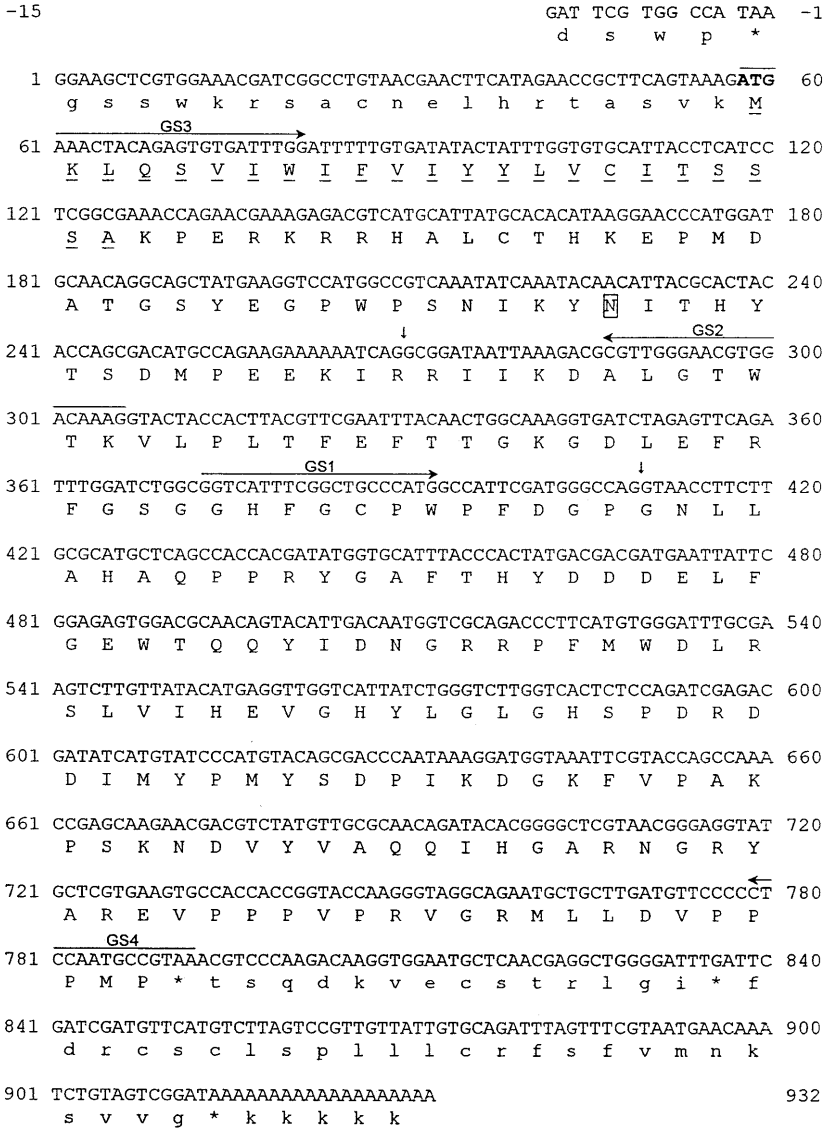

Fig. 1 DNA sequence of *Gs24* gene and its deduced amino acid sequence. Sequence of *Gs24* cDNA insert derived from clone 8.7 (bases 1–932, *upper line*) and its deduced amino acid sequence (*lower line*). The ATG in *boldface* is the putative initiation codon. The amino acids in the signal sequence are *underlined*. Bases –15 to –1 are obtained by 5′ RACE. A potential N-linked glycosylation site is *boxed*

long. The length of the cDNA insert in clone 8.7 was actually 932 bp (Fig. 1). Clone 8.6 was 51 bp shorter than clone 8.7 at the 5′ end. Analysis of the sequences revealed that cDNA inserts of both clones had an open reading frame (ORF) continuing from the *lacZ* gene of the vector to the base position 789 of the cDNA insert of clone 8.7 (Fig. 1). The gene represented in clones 8.6 and 8.7 was designated *Gs24*.

Identification of the 5′ end of *Gs24* cDNA

The deduced amino acid sequence of the cDNA insert of clone 8.7 showed that it might represent a full-length gene, with the putative initiation codon at bases 58–60 (Fig. 1). However, the ORF preceding this initiation codon indicated that there might be a full-length gene longer than the cDNA insert of clone 8.7. One of the approaches used to identify the 5′ end of the mRNA of the *Gs24* gene was reverse transcriptase (RT)-PCR. The approach takes advantage of the conserved spliced leader (SL) of 22 nucleotides found at the 5′ end of most nematode mRNA (Blaxter and Liu 1996). Assuming that the mRNA of the *Gs24* gene might have the SL sequence, RT was performed on total RNA of aL3 using oligo dT primer, followed by PCR using a primer designed to contain the SL sequence and the GS2 primer. However, only nonspecific PCR products were obtained (data not shown), indicating that the SL sequence was absent from the mRNA of the *Gs24* gene. This finding was also supported by the fact that PCR using the same set of primers performed directly on the cDNA library did not give any amplified product (data not shown).

The 5′ RACE technique was then applied to identify the 5′ end of the *Gs24* cDNA, as described above in the Materials and methods. Two of the plasmid DNA samples prepared from *E. coli* transformants contained an insert of approximately 0.35 kb in size. One of the inserts showed a sequence that was 23 bases shorter than that of clone 8.7. However, the DNA sequence of the other insert revealed 15 additional bases at the 5′ end of the cDNA insert in clone 8.7 (Fig. 1). A stop codon was found in the additional sequence immediately before the existing ORF, indicating that it is already the longest ORF (bases 1–789 in clone 8.7). From all the sequence data obtained, it is therefore suggested that the translation of the *Gs24* gene should be initiated at the first ATG found at base position 58. This would make the size of the translation product 27 kDa, which is very close to that of the native protein.

Analysis of deduced amino acid sequence of *Gs24* gene

The amino-acid sequence deduced from the putative initiation codon to the stop codon was submitted for similarity searches against those in nonredundant protein databases at the NCBI. It was found that the amino-acid sequence of this gene was 33 to 39% similar to those of various matrix metalloproteinases (MMPs) or matrixins of many species. Examples of these MMPs are MMP of *Caenorhabditis elegans*; collagenase 3 precursor (MMP-13) of newt, frog, horse, human, mouse and rabbit; stromylesin-2 precursor (MMP-10) of mouse, rat and human; and matrilysin precursor (MMP-7) of human, cat and pig. Similar results were obtained when the deduced amino-acid sequence was queried against swall protein databases at the European Bioinformatics Institute (EBI). The lengths of these MMPs range from 267 to 608 amino acids, and most of them are in the range of 470–480 amino acids. These proteins belong to various subfamilies of MMPs. When the amino-acid sequence of Gs24 protein was aligned with those of selected MMPs, it was found that Gs24 protein was similar to the central part of the MMPs (Fig. 2). Generally, most members of MMP family are organized into three basic, distinctive, and well-conserved domains: an amino-terminal propeptide, a catalytic domain, and a hemopexin-like domain at the carboxy terminus (Massova et al. 1998). Most MMPs, except for MMP-7, which lacks the hemopexin-like domain, contain all three domains. A hydrophobic stretch of approximately 25 amino acids, representing a transmembrane domain, is found at the carboxy terminus of the membrane-type MMPs (MT-MMPs). The catalytic domain contains a matrixin sequence and a Zn-protease sequence with a characteristic active-site glutamic acid and two histidines in its vicinity serving as zinc ligands (Fig. 2). It is apparent from the sequence alignment that Gs24 protein contains the catalytic domain typical of MMP. The amino-acid sequence of the Gs24 protein was submitted for the prediction of signal peptide by SignalP V2.0.b2 (Nielsen et al. 1997) at the World Wide Web Prediction Server, Center for Biological Sequence Analysis, Technical University of Denmark and it was found that there was a signal peptide of 23 amino acids at the amino terminus of the protein (Fig. 1). However, it lacks the propeptide and hemopexin-like domains. Removal of the signal peptide from the Gs24 protein would result in a secreted protein of approximately 24 kDa.

Expression of the *Gs24* gene

Initially, expression of the recombinant *pMAL-c2X* (*pMAL8.7*) was performed using TB1 *E. coli* as the host bacteria, as suggested by the manufacturer. However, the expression in TB1 could not be observed by Coomassie blue-stained gel, but could only be detected by the mAb GN6/24 on a Western blot (data not shown). Expression of *pMAL8.7* in XL 1-Blue MRF′ showed a much better yield of the fusion protein (Fig. 3). The size of the fusion protein is approximately 69 kDa (42 kDa of maltose-binding protein and 27 kDa of *Gs24* gene). The mAb GN6/24 could react with the fusion protein on a Western blot, confirming that the protein fused to MBP was the product of the *Gs24* gene (Fig. 3). Further analysis showed that the fusion protein

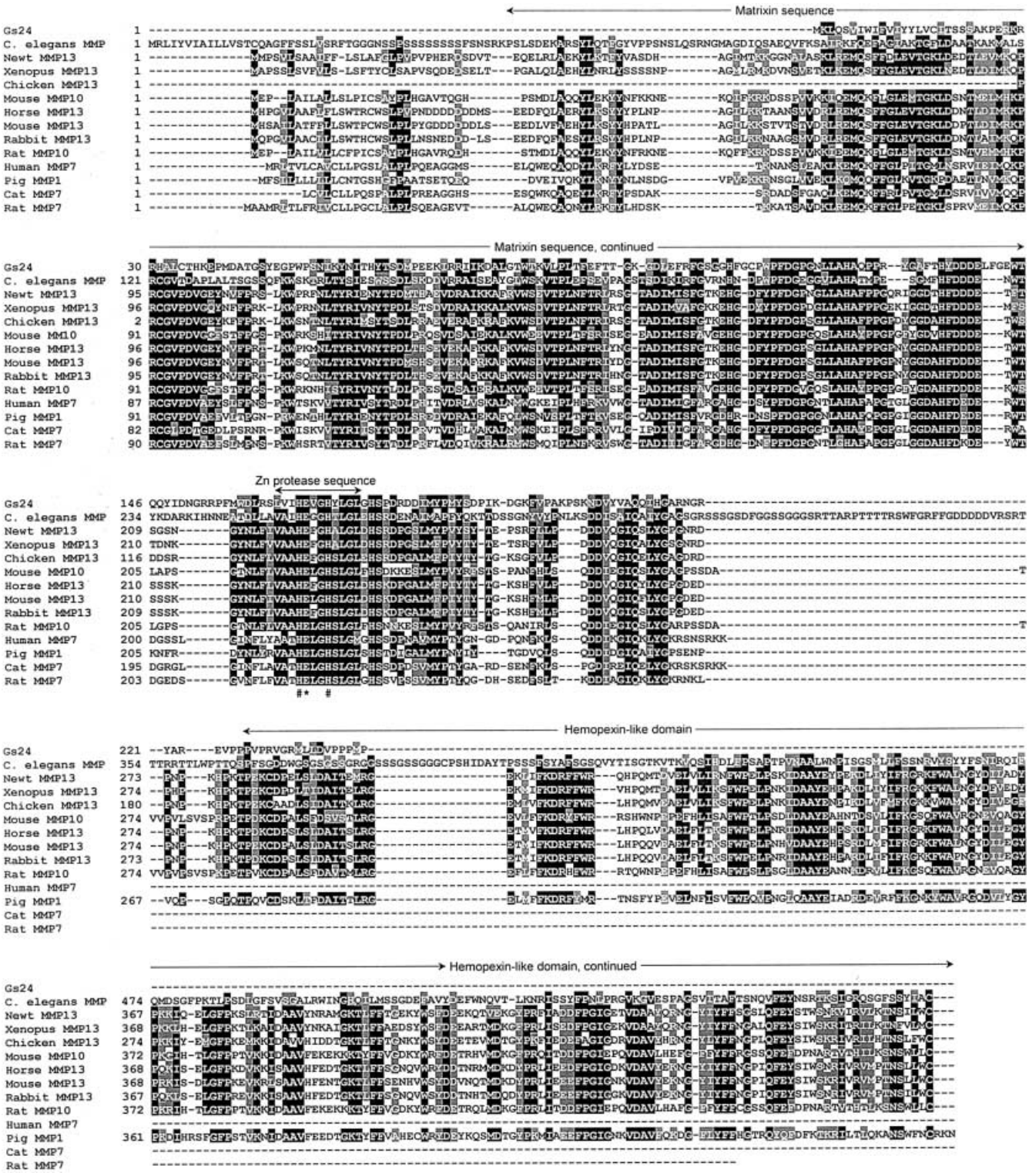

Fig. 2 Alignments of amino acid sequence of Gs24 protein with those of selected matrix metalloproteinases (MMPs). Multiple sequence alignments were performed by ClustalW 1.8 through the Baylor College of Medicine Search Launcher and the consensus alignments were shaded by BoxShade 3.21 through the European Molecular Biology Network (EMBnet) server. The *asterisk* denotes the active site residue. The *pound sign* marks the residues serving as Zn ligands

almost exclusively resided in the insoluble fraction (inclusion bodies) of the lysate of induced bacteria (Fig. 3).

Discussion

Screening of the cDNA library by the mAb GN6/24 identified ~200 positive clones out of 45,000 clones

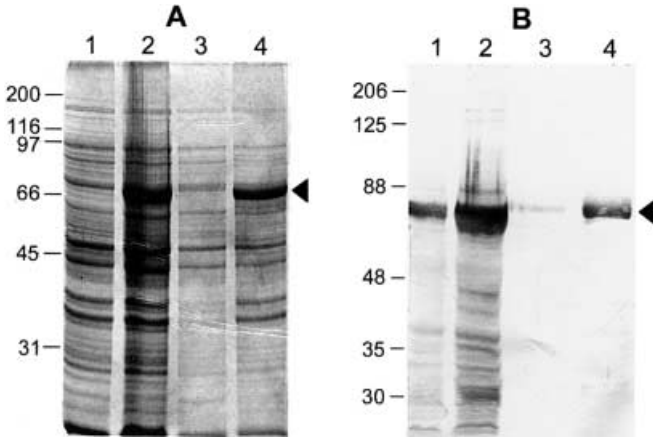

Fig. 3A, B Expression of the *Gs24* gene in *Escherichia coli*. A Coomassie blue-stained SDS-PAGE (**A**) and a Western blot probed by the monoclonal antibody GN6/24 (**B**) of uninduced (*lane1*) and induced transformant (*lane 2*) and soluble (*lane 3*) and insoluble (*lane 4*) fractions of induced transformant. The markers (in kilodaltons) on the left margins are unstained (**A**) and prestained (**B**) broad range SDS-PAGE standards (Bio-Rad). *Arrowhead* indicates the 69 kDa fusion protein

examined, indicating the relatively abundant representation (~0.5%) of this gene in the library. Twenty-four clones selected for analyses showed varying lengths of the cDNA inserts. These cDNA inserts represent the same *Gs24* gene since they show common patterns of restriction fragments (data not shown). Most inserts were in the range of 1 kb, suggesting that they might represent the longest cDNA. The inserts larger than 1 kb could be artifacts of cloning caused by joining of the *Gs24* cDNA with other unrelated DNA fragments. Clone 8.7 contains the *Gs24* cDNA insert of 932 bp, with an ORF from the beginning of the insert to base 789. The translation product of this ORF would be 263 amino acids, or approximately 29 kDa in molecular weight. Although the calculated size already exceeds the size of native protein, we may not have reached the 5′ end of the gene yet, because the migration of the native protein in polyacrylamide gel may not agree with its calculated size. We chose to identify the sequence further upstream from the 5′ end of clone 8.7 by various approaches. The 5′ RACE proved to be very useful, and we concluded that the additional 5′ sequence did not contain an ORF extending from the existing one in clone 8.7. Further interpretation of the data indicated that translation of the native Gs24 protein should be initiated at the first ATG, found 58 bases downstream from the beginning of the insert (Fig. 1). The 57 bases before the putative initiation codon in clone 8.7 happen to contain an ORF that continues from the translation frame of the vector's *lacZ* gene and is followed by the ORF of the *Gs24* gene.

Analysis of the deduced amino-acid sequence of the Gs24 protein, starting from the putative initiation codon, has shown that it is very similar to various extracellular MMPs. MMPs, also known as matrixins, belong to the family M10B of metalloproteases (Rawlings and Barrett 1995). They are zinc-dependent enzymes synthesized as inactive precursors (zymogens) that differ from the mature enzymes by the presence of an N-terminal propeptide. All matrixins possess three domains: an amino-terminal propeptide domain, a zinc-binding active site domain and a hemopexin-like domain. The amino-terminal propeptide, cleaved during the activation step, includes a conserved PRCGVPDV octapeptide, known as the cysteine switch, whose cysteine residue chelates the active-site zinc atom, rendering the enzyme inactive. The active enzyme degrades components of the extracellular matrix, playing a role in the initial steps of tissue remodeling during morphogenesis, wound healing, angiogenesis and tumor invasion (Wilhelm et al. 1989; Lepage and Gache 1990). Extrapolating from its similarity to known MMPs, the MMP-like protein of *G. spinigerum* aL3 we have identified might have a similar function, of degrading extracellular matrix macromolecules of host tissues. It is not surprising that *G. spinigerum* aL3 synthesize an MMP, since they are well known for their ability to migrate through tissues of intermediate and definitive hosts. The Gs24 protein contains two signature regions characteristic of MMP: the matrixin and Zn protease sequences in the active site domain. However, it lacks the propeptide and hemopexin-like domains found in most members of the MMP family. The hemopexin-like domain is also missing in MMP-7 (matrilysin) (Massova et al. 1998). The Gs24 protein is much shorter than other MMPs. Only members of MMP-7 have comparable lengths of amino-acid sequences with the Gs24 protein. A signal peptide of 23 amino acids is found at the amino terminus of the deduced amino-acid sequence, supporting the assumption that the ATG at base 58 is the initiation codon. This also indicates that Gs24 is a secreted MMP-like protein. This is also confirmed by Western blot analysis showing the presence of the 24-kDa protein recognized by the mAb GN6/24 in the excretory–secretory proteins of aL3 (data not shown). The molecular weight of the mature Gs24 protein (221 amino acids excluding the signal peptide) would be approximately 24 kDa, which agrees with the apparent size of the native protein observed by Western blot analysis (Chaicumpa et al. 1991). The amino-acid sequence data showing a potential N-linked glycosylation site also support the findings from deglycosylation and immunoprecipitation experiments, that the Gs24 protein is a glycoprotein with N-linked sugars (Uparanukraw et al. 1999).

Only two nematode MMPs have so far been identified. One of them is the *C. elegans* MMP, with 579 amino acids (Wada et al. 1998). This MMP is most similar (39.6%) to the Gs24 protein, by pairwise comparison. The size difference and lack of some domains in the Gs24 protein indicate that they are related only because they belong to the MMP family. The other nematode MMP identified was also from *C. elegans*, and was described as being similar to hatching-enzyme precursor (Wilson et al. 1994). This protein, comprising 253 amino acids, is 30% similar to the Gs24 protein in the central portion, encompassing the matrixin and Zn protease regions. Similar to the Gs24 protein, it also lacks the propeptide and

hemopexin-like domains. The functional descriptions of these two *C. elegans* proteins were inferred only by sequence similarity to their known counterparts. As suggested by its amino-acid sequence, the Gs24 protein is most likely a Zn-protease enzyme belonging to the MMP family. It will be interesting to find out whether the Gs24 protein actually has a protease activity. One possible approach to prove this would be the production of functional Gs24 protein in a eukaryotic expression system and direct assessment of its protease activity.

Expression of the *Gs24* gene in *E. coli* using *pMAL-c2X* has consistently resulted in very good yields of the fusion protein, as judged by Coomassie blue-stained gels. However, the fusion protein resides exclusively in the inclusion bodies. This is not unexpected, because the expression of any eukaryotic protein in prokaryotic expression systems will usually give unpredictable results. The insolubility of the fusion protein prevented us from evaluating it as a diagnostic antigen. To overcome this, we plan to express the *Gs24* gene in the histidine tag expression system, in which solubilization of the inclusion bodies by denaturing agent will not affect the purification of the fusion protein by affinity chromatography.

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