Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*

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Abstract

Characterization of the enzymes involved in the chitin biosynthetic pathway in mosquitoes is critical due to the importance of chitin in the formation of the peritrophic matrix [PM] and its potential impact on vector competence. Chitin is the homopolymer of the amino sugar N-acetyl-D glucosamine [GlcNAc]. The final step of incorporation of GlcNAc into the chitin polymer is catalyzed by the enzyme chitin synthase [CS]. CS is a membrane bound enzyme, but the mechanism of its action in the biosynthesis of the PM is not understood. We have isolated and sequenced a CS-encoding cDNA clone from the mosquito *Aedes aegypti*, compared its sequence with CS from other organisms and studied its RNA expression. The cDNA is 3.5 kb in length with an open reading frame of 2.6 kb that encodes a protein of 865 amino acids with a predicted molecular mass of 99.5 kDa. The putative translation product shares 90% similarity to two CS proteins from *Caenorhabditis elegans* and 50% similarity to *Saccharomyces cerevisiae* in the catalytic domain of CS enzymes. Data suggest that CS is a single copy gene. RT-PCR analysis shows CS message in whole non-blood-fed females, whole blood-fed females, non-blood-fed midguts and in midguts dissected at different time points post-blood-feeding. In situ hybridization studies of midgut samples revealed that CS mRNA increases following a bloodmeal and is localized to the periphery of the epithelial cells facing the midgut lumen. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chitin synthase; Peritrophic matrix; *Aedes aegypti*

1. Introduction

All mosquito-borne pathogens initiate infection by being ingested with the blood meal, and the first barrier they face is the midgut. It has been established that the midgut plays a role in influencing vector competence (Ponnudurai et al., 1988; Sutherland et al., 1986; Ramasamy et al., 1997), but there is little information regarding the genetic regulation of critical physiological processes that occur within the midgut following blood feeding. One of these processes is the formation of the peritrophic matrix that surrounds the blood bolus, thereby physically separating it from the midgut epithelium. It functions in mechanical protection of the midgut epithelium by providing a barrier to ingested food (Sudah and Muthu, 1988), protects the insect from insult by toxins and pathogens (Sutherland et al., 1986), and might serve as a retention barrier for keeping protease inhibitors in the lumen (Billingsley, 1990).

The peritrophic matrix is a chito-protein structure that consists of 3–13% chitin in a protein, proteoglycan and glycoprotein mesh (Lehane, 1997), but little information is known regarding peritrophic matrix chitin formation. Chitin is a homopolymer of the amino sugar N-acetyl-D glucosamine [GlcNAc] linked by β-1, 4 glycosidic bonds. Uridine diphosphate-GlcNAc [UDP-GlcNAc] was first demonstrated to be the donor source for GlcNAc in the formation of chitin, but it was Candy and Kilby (1962) who proposed the biosynthetic pathway of chitin in insects. The final step of incorporation of GlcNAc into the chitin polymer is suggested to occur through a stepwise transfer of N-acetylglicosamine groups from the nucleotide to the non-reducing end of the chain of the chitodextrin primer [GlcNAc] by the enzyme chitin synthase [UDP-N-acetyl-D-glucosaminyltransferase, EC 2.4.1.16, CS] (Bulawa, 1993). McCurrough et al. (1971)
provided evidence that chitin of *Mucor rouxii* is the main, if not sole, polymer biosynthesized from UDP-GlcNAc.

Chitin synthase is readily assayed in many of the fungal systems, but the same procedure has met with failure in insect systems. This may be due to the lack of an in vitro CS assay in a cell-free preparation (Vardanis, 1979), owing to the instability of the enzyme. Except for a few enzymatic studies (Cohen and Casida, 1980; Mayer et al., 1980), understanding the biochemistry of CS has not been an easy task, probably due in part to the difficulty inherent in purification (Machida and Saito, 1993). The only characteristic that is common to all chitin-containing organisms is that chitin synthase is membrane-bound; other properties vary extensively among species (Chen, 1987).

It has been suggested that CS forms PM microfibrils from secreted precursors (Richards and Richards, 1977), but available evidence does not support this (Lehane, 1997). The active site of chitin synthase faces the cytoplasm (Sentandreu et al., 1984), and there is no evidence of an active transport system for the chitin precursor UDP-GlcNAc, the belief is that chitin polymers are synthesized from an intracellular pool of precursors and then extruded through the plasma membrane by an unknown mechanism (Cohen, 1991).

Insect CS has been shown, through biochemical studies, to exist in a zymogenic form requiring trypsin proteolysis for activation and chitin biosynthesis (Mayer et al., 1980). Chitin is degraded by hydrolytic action of the enzyme chitinase found in both the mosquito host as well as in various pathogens (Shahabuddin et al., 1996). It has been proposed by De La Vega et al. (1998) that both enzymes work in concert to control cuticle synthesis [CS] and cuticle degradation [chitinases] through alternate phases of enzyme production by epithelial cells. Studies on midgut specific chitinases in *Anopheles gambiae* (Shen and Jacobs-Lorena, 1997) show that, upon feeding, the enzyme is secreted as an inactive proenzyme, and is later activated by trypsin. Therefore, temporal regulation of both CS and chitinase activity are coupled to that of trypsin. When allosamidin, a specific inhibitor of various chitinases, is present in addition to the established activators trypsin and GlcNAc, chitin formation is increased up to 58-fold over the basic synthesis rate (Peter and Schweikart, 1990).

In this paper we report the first molecular cloning of a putative CS from the mosquito *Aedes aegypti*, with sequence comparisons made to known CS proteins from other organisms. Also included are RT-PCR and in situ hybridization studies that assess timing and location of CS-like transcripts in the mosquito following blood feeding.

2. Materials and methods

2.1. Mosquito maintenance

Adult Liverpool strain *Aedes aegypti* were reared according to the methods described by Christensen and Sutherland (1984). Artificial blood meals required for RNA expression analysis were prepared according to Kogan (1990) with minor modifications. The meal contained a final concentration of 15 mg/ml of γ-globulin, 90 mg/ml of albumin and 0.03 ml of 0.2 M ATP in a final volume of 3 ml. Hemoglobin was omitted from the blood meal due to interference with in situ analysis. Mosquitoes were fed through a water-jacketed membrane feeder (Rutledge et al., 1964). All blood meal components were supplied by Sigma Chemical Company (St. Louis, MO, USA).

2.2. Isolation of CS cDNA clone

The *Ae. aegypti* CS cDNA clone was isolated from a λgt10 cDNA library, containing *Ae. aegypti* size selected Poly (A)^+ RNA from 3-day-old females (J. Williams, University of Wisconsin, Madison, WI, USA), using a CS probe generated by PCR amplification. Primers used to amplify the CS cDNA were designed, based on sequence homology of the most highly conserved amino acid residues of the catalytic domain of different forms of yeast and fungal CSs. They are: 5′ CAG AAA TTC GAA TAT GCC 3′ and 5′ CCA GCG GCG GCT TTG 3′. The PCR conditions used were one cycle at 94°C for 4 minutes; 30 cycles at 94°C, 55°C and 72°C for one minute each and one 7 minute extension at 72°C.

Library screening was carried out at moderate stringency (16 h hybridization at 55°C) as described by Maniatis et al. (1982). An *Ae. aegypti* CS-encoding PCR product radioactively labeled by random priming using a Random Primed DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN, USA) was used as probe. Positive phage clones were identified, DNA isolated (Ferdig et al., 1996), subcloned into pBSSK II (Stratagene, La Jolla, CA, USA), sequenced and analyzed.

2.3. Sequence analysis

Sequencing reactions were performed on an ABI 310 automatic sequencer using both the Dye terminator cycle sequencing kit (ABI, Perkin Elmer, Foster City, CA, USA) as well as the ABI prism™ Big dye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems). All reactions were done at least twice from each direction using flanking vector primers T3 and T7 or CS specific primers. Sequence analysis, alignments and contig management were carried out by means of the LASERGENE Sequence Analysis System;
DNA was isolated according to the methods of Severson et al. (1993). Genomic DNA from both individual mosquitoes and bulk preparations were subject to EcoRI restriction digestion and size fractionated on 1% agarose gels, denatured (150 mM NaOH, 3 mM EDTA), neutralized (150 mM NaPO₄, pH 7.8) and transferred onto Hybond-N⁺ Nylon membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) by capillary action in 25 mM Na₄P₂O₇. Southern blots were hybridized with an Ae. aegypti CS-encoding PCR product radioactively labeled by random priming and washed at high stringency (Maniatis et al., 1982).

2.4. Southern blot analyses

DNA was isolated according to the methods of Severson et al. (1993). Genomic DNA from both individual mosquitoes and bulk preparations were subject to EcoRI restriction digestion and size fractionated on 1% agarose gels, denatured (150 mM NaOH, 3 mM EDTA), neutralized (150 mM NaPO₄, pH 7.8) and transferred onto Hybond-N⁺ Nylon membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) by capillary action in 25 mM Na₄P₂O₇. Southern blots were hybridized with an Ae. aegypti CS-encoding PCR product radioactively labeled by random priming and washed at high stringency (Maniatis et al., 1982).

2.5. RNA expression analysis by reverse transcription—PCR (RT-PCR)

Midgut tissues for RNA extraction were dissected in Aedes saline solution (Hayes, 1953) and immediately frozen on dry ice. Total RNA was isolated according to the methods described by Chomczynski and Sacchi (1987). RNA was extracted from all developmental stages of Ae. aegypti, and from whole females and dissected midguts at different time points following blood feeding. Samples were DNase-treated for DNA contamination. RNA was subjected to first strand cDNA synthesis using either an oligo dT primer or a CS specific primer as described by Sambrook et al. (1989). The primers used were: 5’ CAA CGT CGA CCG TGG G 3’ and 5’ CAT GAG GAA GAT CGT TCC 3’ (Fig. 1). The PCR reaction was carried out in a Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA) in a 10 µl final volume. The program profile was: 30 cycles comprised of a denaturation step at 98°C for 10 s, annealing at 50°C for 10 s and extension at 72°C for 35 s. The products of these reactions were size-fractionated on a 1% agarose gel and examined using an EAGLE EYE II (Stratagene, La Jolla, CA, USA).

2.6. Whole mount in situ hybridization using digoxigenin-labeled RNA probes

Probes (sense and anti-sense) were transcribed from the CS cDNA clone using a Digoxigenin labeling kit (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. Full-length digoxigenin-labeled RNA probes were transcribed from T3 (for sense probe) and T7 (for anti-sense probe) promoters, precipitated in ethanol, and resuspended in diethyl pyrocarbonate-treated water. Quantification of the probes was according to the instructions within the labeling kit. Mosquito midguts at different time points after blood feeding were dissected into ice-cold phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (PPF) at 4°C overnight, dehydrated through a methanol series at room temperature, and stored in 100% methanol at −20°C until hybridization was performed. Prior to hybridization, samples were rehydrated through a methanol series, treated with proteinase K, post-fixed with 4% PPF/0.2% glutaraldehyde at room temperature for 20 min, and then pre-hybridized at 55°C for 1 h in hybridization solution (50% deionized Formamide, 5xSSC, 0.02% glycogen, 0.1 mg/ml sheared and denatured salmon sperm DNA, and 0.1% Tween 20). RNA probes then were added to the hybridization solution at a final concentration of 1 ng/µl and allowed to hybridize overnight at 55°C with intermittent agitation. Samples were washed four times for half an hour each in hybridization solution at 55°C, followed by a 1:1 hybridization solution/PBT wash at room temperature and finally washed in PBT.

Samples then were equilibrated with alkaline phosphatase buffer (0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mg/ml BSA). Alkaline phosphatase conjugated goat anti-digoxigenin antibody (Boehringer-Mannheim, Indianapolis, IN) was diluted in alkaline phosphatase buffer according to the manufacturer’s recommendation. Samples were incubated with the diluted antibody at room temperature for 1 h, followed by 10 washes with 0.1% Tween 20 in PBS within a 2 h period. Samples were incubated in staining buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂, and 0.1% Tween 20) containing a 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitro blue tetrazolium chloride tablet (Boehringer Mannheim, Indianapolis, IN). Reaction product was visualized using light microscopy every 10 min until the desired staining intensity was reached. The reaction was terminated by washing the samples 5 times for 10 min each with 0.1 M EDTA in PBT. Whole mount samples then were examined directly or were dehydrated through 70% ethanol and embedded in JB-4 (Electron Microscopy Sciences, Fort Washington, PA). Five-micrometer sections were cut with a glass knife using a Sorvall JB-4 microtome, placed on clean glass slides, counterstained using acidified eosin in ethanol and coverslip mounted using Poly-mount (Polysciences, Warrington, PA). All samples were viewed using bright field illumination on an Olympus Provis AX70 light microscope connected to a DAGE-MTI DC-330 CCD color digital camera. Digital images were captured using Image-Pro Plus® (Media Cybernetics, Silver Spring, MD). Digital images were processed using Adobe Photoshop® (Adobe Systems Inc., San Jose, CA) to enhance brightness and contrast. Experiments were carried out at
Fig. 1. Multiple alignment of *Aedes aegypti* chitin synthase deduced amino acid sequence (*A. aegypti*) with *Caenorhabditis elegans* (*C. elegans1* and *C. elegans2*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). Identical amino acid residues are indicated by shading. The catalytic region of CS is underlined and the catalytic residues are represented by asterisks. Arrows designate primer positions used to amplify CS. The six sites of N-linked glycosylation found in the potential *Ae. aegypti* CS translation product are underlined. Dashes were introduced to maximize the alignment.
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Fig. 1. (continued)
least seven times, simultaneously under identical conditions.

3. Results

3.1. Analysis and comparison of Ae. aegypti C11A2b deduced amino acid sequence with known CS proteins from other organisms

The Ae. aegypti cDNA encodes a putative CS protein of 865 amino acids with a predicted molecular mass of 99.5 kDa. The Ae. aegypti CS deduced amino acid sequence was compared with CS proteins from nematode and yeast (Fig. 1). A conserved region of approximately 130 amino acids between all CSs represents the catalytic site of the enzyme (Nagahashi et al., 1995) (Fig. 1). The Ae. aegypti potential coding region shares 90% similarity to two hypothetical CS proteins from Caenorhabditis elegans (C. elegans1, Accession number T32452, Bradshaw, H.; C. elegans2, Accession number T25284, McMurray, A.) and 50% similarity to CS of Saccharomyces cerevisiae (S. cerevisiae, Accession number AAA34844, CSD2, Bulawa, 1992) in the catalytic domain of CS enzymes. Additionally, sequence comparisons identified conserved residues in the catalytic domains. The N- and C-termini of Ae. aegypti CS-like protein and yeast CS are less conserved. Consistent with the fact that CS is membrane bound, the C-terminus of Aedes CS shows hydrophobic regions that are potential trans-membrane positions include amino acids 473–500, 530–556 and 810–835 (Kihara et al., 1998).

3.2. Southern blot analysis

Southern analyses of EcoRI restriction digests show that the CS PCR fragment hybridized to one or two genomic DNA fragments on an Ae. aegypti single animal blot (Fig. 3(A)). The hybridization pattern on the autoradiograph of a Southern blot of pooled DNA samples from different strains of mosquitoes also shows three or fewer hybridizing bands. This suggests that CS is a single copy gene (Fig. 3(B)). The probe used on the Southern blots was the 300 bp PCR fragment of CS cDNA. This DNA fragment contains an EcoRI site.

3.3. Analysis of CS mRNA by RT-PCR

The cDNAs generated from Ae. aegypti RNA samples by reverse transcription were subjected to PCR analysis using Ae. aegypti CS specific primers. Results showed the presence of mosquito CS message in midguts dissected prior to and at different time points after blood feeding. Message could be detected through 72 h post-feeding with an obvious decrease in message 96 h after feeding (Fig. 4(A)). RT-PCR analysis of CS transcripts in whole female mosquitoes revealed message in non-blood-fed females and through 18 h post-blood-feeding. The carcass sample (3 h blood-fed female with the midgut removed) showed reduced CS mRNA (Fig. 4(B)).

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Fig. 2. Hydrophobicity plot of Aedes aegypti CS obtained by a program developed by Han and Tashjian (1998). Alpha helicity index (Hα): Values above 1.6 are considered hydrophobic, and those below 0.8 are hydrophilic. Horizontal lines indicate stretches of more than 17 hydrophobic residues, denoting potential membrane-spanning domains.
3.4. In situ analysis

To localize CS mRNA, microscopic examination was carried out on midgut tissue sections hybridized with CS ribo-probes (Fig. 5). Midgut samples dissected from 3 h and 6 h post-blood-fed mosquitoes and hybridized with the anti-sense ribo-probe showed localization to the periphery of the epithelial cells facing the midgut lumen (Fig. 5(C) and (E)). Staining in 6 h midguts (Fig. 5(E)) was more intense than in 3 h midguts (Fig. 5(C)). Non-blood-fed midguts showed faint staining (Fig. 5(A)). It is evident that blood feeding increased the amount of CS transcripts. Control samples hybridized with the sense ribo-probe showed no staining (Fig. 5(B), (D) and (F)).

4. Discussion

The PM represents a significant barrier to penetration and subsequent development of Plasmodium sp. (Billingsley and Rudin, 1992) and certain arboviruses (Whitfield et al., 1973). The effectiveness of this barrier depends on the time of its formation (Smartt et al., 1998). Due to the importance of chitin in the formation of the peritrophic matrix and its potential impact on vector competence, chitin synthesis poses interesting and challenging questions. The multi-enzyme system that works to control the biosynthesis [chitin synthase] and degradation [chitinase] of chitin is likely complex. It is evident that both enzymes are zymogenic (Mayer et al., 1980; Shahabuddin et al., 1996) requiring proteolysis by trypsin for activation. Trypsin has been shown to contribute 75% of the proteolytic activity in midguts of Ae. aegypti (Briegel and Lea, 1975). It seems likely that chi-
chitin synthase acts to modulate PM thickness and permeability (Shen and Jacobs-Lorena, 1997). In support of this hypothesis, and in agreement with that of Shahabuddin et al. (1993), Shen and Jacobs-Lorena (1997) found that the PMs are stronger and persist longer in the guts of mosquitoes fed on a chitinase inhibitor. A thicker PM may be a more efficient barrier to parasite development in the mosquito, if induced in a temporally dependent fashion.

In the present study, we isolated the first mosquito chitin synthase encoding cDNA clone. The deduced amino acid translation product shares a region whose sequence is highly conserved in all known chitin synthases. This 130 amino acid region shares 90% similarity to *C. elegans* CS and 50% similarity to *S. cerevisiae* CS. In *S. cerevisiae* (Nagahashi et al., 1995), two domains have been implicated as possible catalytic sites for CS, and in *Ae. aegypti* those correspond to Glu 431 — Asp 432 — Arg 433 and Gln470 — Arg 471 — Arg 472 — Arg 473 — Trp 474. Moreover, because these residues
also are completely conserved in other proteins possessing N-acetylglucosaminyltransferase activity, such as NodC proteins of Rhizobium bacteria, it has been suggested that they are located in the active pocket of the enzyme. Nagahashi et al. (1995) concluded, through mutant analysis, that these two catalytic domains are essential for catalytic activity and that residues corresponding in Ae. aegypti to Asp432, Gln470, Arg473 and Trp474 are the potential catalytic residues of the enzyme.

The presence of potential membrane-spanning domains in the carboxy terminal region of the Ae. aegypti CS protein is consistent with the known membrane localization of the enzyme. Analysis of the Ae. aegypti CS structural hydrophobicity plot (Han and Tashjian, 1998) reveals 3 potential transmembrane regions. Hydrophobicity values calculated according to Kyte–Doolittle analysis in the GCG program (Wisconsin Package Version 10.0, Genetics Computer group (GCG), Madison, WI) also revealed these same three potential membrane-spanning regions (data not shown). The location of CS in the plasma membrane suggests that the enzyme could be a glycoprotein (Duran et al., 1975). The CS reported herein possesses 6 potential glycosylation sites in the coding sequence at amino acids 41 (NAS), 101 (NDT), 186 (NDS), 273 (NTT), 459 (NNS) AND 658 (NFS). Biochemical Con A-sepharose binding experiments in S. cerevisiae negate the possibility that the enzyme is glycosylated (Bulawa et al., 1986); however, whether Ae. aegypti CS is glycosylated remains to be determined.

No structural similarities exist in the amino-termini of known S. cerevisiae CSs. Because this region is dispensable for enzyme activity, it has been suggested (Silverman, 1989) that it may play a role in regulation or localization of respective enzymes. Three CS genes from S. cerevisiae lack a conventional amino terminal signal sequence and have been suggested to have a specific transport system independent of the secretory pathway (Bulawa, 1992). Comparisons also show that the N-terminal region of Ae. aegypti CS is dissimilar to other CSs and seems to lack an N-terminal signal peptide. Membrane topology predictions indicate that the N-terminal region of the Aedes CS is oriented inside, facing the cytoplasm (PSORT prediction program- [Nakai and Horton, 1999]). We suggest that the Ae. aegypti cDNA clone encodes a chitin synthase.

Results of RT-PCR carried out on midguts showed that CS transcript was present in non-blood-fed samples and through 72 h post blood feeding, with a reduction of message seen at 96 h after a blood meal. Both non-blood-fed female, as well as non-blood-fed midgut samples, contained CS message; however female carcasses with the midgut removed showed little CS transcript. CS RNA also could be detected in all developmental stages of the mosquito (data not shown). We propose that Ae. aegypti CS is midgut specific and involved in midgut chitin formation. However, we believe there may be another gene for cuticular chitin synthase. We analyzed transcription of Ae. aegypti CS using RT-PCR because it is a more sensitive technique for detection of messages of low abundance.

In situ CS mRNA localization in midgut samples clearly showed low levels in non-blood-fed midguts and an increase in transcript following blood feeding. CS mRNA in midgut samples was distributed at the periphery of the epithelial cells facing the midgut lumen. Preliminary data suggest that Ae. aegypti chitin synthase is a single gene, however, as with yeast CS, the possibility remains that there may be a number of enzymes each encoded by a separate gene and each having a specific site of localization and chitin deposition (Robbins et al., 1993).

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References


