Grasshopper (Melanoplus differentialis) lectin genes : southern analysis and polymerase chain reaction by Tanya Gedik

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University
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Abstract:
A component of an invertebrate's innate immune response to pathogens includes lectin proteins. Lectins have the ability to discriminate self from non-self by recognizing specific carbohydrates that are present on the surface of microorganisms. Lectins bind these carbohydrates and target them for humoral or cellular defensive reactions. Hemolymph of grasshopper, Melanoplus differentialis, contains a lectin with two carbohydrate recognition domains (CRDs) with specificity toward galactosidic and glucosidic carbohydrates (Stebbins and Hapner 1985). The protein, GHA, is a C-type lectin in light of its dependence on calcium for sugar binding activity. GHA is known to associate with fungal blastospores and aid in their removal from the hemolymph by hemocytes (Wheeler et al. 1993). GHA protein has been isolated, as have two related grasshopper lectin cDNA clones (Hapner K.D., Rognlie M.C. and Radke J.R. Unpublished results). These clones, Clone 3 and 4, show 80% sequence identity. Partial amino acid sequence of the GHA protein revealed that it was not encoded by Clone 3 or 4. This fact suggested that the grasshopper may contain multiple C-type lectins and may have multiple lectin genes encoding these proteins.

The objectives of this study are to confirm that grasshopper genomic DNA contains multiple C-type lectin genes and to determine the intron character of genes 3 and 4 coding for Clones 3 and 4, respectively. Primary methodology includes Southern analyses, polymerase chain reaction (PCR), endonuclease restriction and random primed probe preparation.

Restricted grasshopper genomic DNA gives multiple bands on autoradiographs hybridized with 32P-labeled grasshopper C-type lectin cDNA probes. Interpretation of the results indicates the presence of at least four C-type lectin genes in the grasshopper genome. PCR amplification was performed on grasshopper genomic DNA with primer sets that anneal to either Clone 3 or 4. Restriction analyses of the PCR products indicated gene 3 and 4 to be the amplification products. Southern analysis, with grasshopper C-type lectin cDNA probe, proved the PCR producst were amplified from C-type lectin sequences. The results strongly suggested that both CRD-coding regions of gene 4, and the carboxyl CRD-coding region of gene 3, lack introns. The intronless character of the CRD-coding regions of C-type lectin genes indicates possible evolutionary relationship with intron-lacking CRDs of lectins from other organisms.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

A component of an invertebrate's innate immune response to pathogens includes lectin proteins. Lectins have the ability to discriminate self from non-self by recognizing specific carbohydrates that are present on the surface of microorganisms. Lectins bind these carbohydrates and target them for humoral or cellular defensive reactions. Hemolymph of grasshopper, *Melanoplus differentialis*, contains a lectin with two carbohydrate recognition domains (CRDs) with specificity toward galactosidic and glucosidic carbohydrates (Stebbins and Hapner 1985). The protein, GHA, is a C-type lectin in light of its dependence on calcium for sugar binding activity. GHA is known to associate with fungal blastospores and aid in their removal from the hemolymph by hemocytes (Wheeler et al. 1993). GHA protein has been isolated, as have two related grasshopper lectin cDNA clones (Hapner K.D., Rognlie M.C. and Radke J.R. Unpublished results). These clones, Clone 3 and 4, show 80% sequence identity. Partial amino acid sequence of the GHA protein revealed that it was not encoded by Clone 3 or 4. This fact suggested that the grasshopper may contain multiple C-type lectins and may have multiple lectin genes encoding these proteins.

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INTRODUCTION

Insect Immunity

Insects have been remarkably successful in evolution. Current estimates are that they make up 90% of all extant animal species and colonise all terrestrial ecological niches (Hoffmann 1995). Consequently, they are confronted by an extremely large variety of potentially harmful microorganisms. Insects are able to build up an efficient defense system that has both a physical and an innate facet. The hard external skeleton functions as a physical barrier to pathogen invasion. A current view (Hoffmann et al. 1996) describes the innate response of insects as three interconnected reactions. The first is the induction of proteolytic cascades by wounding, even when potentially harmful microorganisms are absent. The proteolytic coagulation cascade leads to localized blood clotting that may immobilize the foreign invader and allow other processes to destroy the pathogen, as well as restricting blood loss (Muta and Iwanaga 1996). The prophenoloxidase cascade leading to melanization of large invaders is another example of a proteolytic cascade. Potentially cytotoxic quinoid intermediates of melanin generated in the prophenoloxidase cascade are thought to have bactericidal and fungicidal activity (Vass and Nappi 1996). The second innate response includes a variety of cellular defense reactions, that consist predominantly of phagocytosis or encapsulation of invading

microorganisms. Phagocytosis involves endocytosis of pathogens, mainly by plasmatocytes and granular cells, with lysosomal breakdown. Encapsulation is a multicellular process in which foreign objects too large for phagocytosis are surrounded by hemocytes recruited from the circulation (Ratcliffe 1993). The cells lyse and flatten, forming a layer of cells around the foreign organism. Melanotic compounds may be deposited in the inner layers. This capsule may stop the growth and development of the invader or kill it directly. The third innate response is the induction of the transient synthesis of a battery of peptides by the fat body that are secreted into the hemolymph. Close to 100 antimicrobial peptides and proteins have now been characterized. They include defensins, magainins, cecropins, proline-rich and glycine-rich polypeptides. Understanding the mode of action of these peptides remains unsatisfactory due to their only recent discovery, although it has been proposed that cecropins could act as detergents thereby causing lysis of bacterial cells through the disintegration of their cytoplasmic membranes (Hoffmann et al. 1996). Another strongly held idea is that an insect's innate immune response includes a forth component. This component involves lectin proteins that are thought to protect the insect from parasitic invasions by having the ability to discriminate self from non-self (Arason 1996). Lectins bind avidly and reversibly to carbohydrates. Carbohydrates are present on cell surfaces and carry, per unit weight, more information than can amino acids or proteins (Sharon and Liz 1995). Lectins can detect subtle differences in carbohydrate structures, a characterisic useful and important in biological recognition and differentiation.

Animal Lectins

Lectins are ubiquitous proteins that function in fertilization, development, leukocyte migration and self/non-self distinction (Arason 1996). The latter role originates from their ability to discriminate, through hydrogen bonding and hydrophobic interactions, between endogenous carbohydrates or those that are presented by microbial invaders. Animal lectins have enormous structural diversity but carbohydrate binding activity can often be ascribed to a limited polypeptide segment of each lectin, designated the carbohydrate-recognition domain (CRD) (Drikamer 1993). Several types of CRD have been discerned, each of which shares a pattern of invariant and highly conserved residues over a 115-140 amino acid region. Three major groups of animal lectins; P, S and C-types, contain CRDs with distinct sequence motifs. Proteins of the major lectin groups share properties beyond similarity of primary structure (Drickamer and Taylor 1993). For example, S-type lectins often are dependent on reducing agents, such as thiols, for full activity and they all bind β-galactosides. P-type CRDs bind mannose-6-phosphate as their primary ligand. The animal C-type lectins are characterized by a dependence on calcium for sugar binding activity (Drickamer 1994). They occur in serum, extracellular matrix, and membranes (Drickamer and Taylor 1993).

The C-type lectin family includes among others the hepatic asialoglycoprotein receptor (Lodish 1991), macrophage mannose receptor (Sharon and Liz 1995), selectins (Lasky 1992), and soluble collectins (Hoppe and Reid 1994). The hepatic asialoglycoprotein receptor is a mambrane-bound lectin found on the surface of

hepatocytes. This receptor binds certain glycoproteins that have lost terminal sialic acid residues, and the receptor-ligand complexes are then internalized. The macrophage mannose receptor may participate in antimicrobial defense by mediating phagocytosis of infectious organisms that expose mannose-containing glycans on their surface (Sharon and Liz 1995). The collectins include the rat mannose binding protein (MBP) that mediates humoral defense either via complement fixation or by direct opsonization of potential pathogens (Drickamer 1993). The three dimensional structure of the CRD of rat MBP has been determined by X-ray crystallography (Weis et al. 1991). The structure appears divided by two transverse β-strands that separate a compact scaffold of two helices and two β-sheets from an extended loop. The loop creates a pocket for two calcium ions and a binding site for the carbohydrate ligand. The carbohydrate binding site is thus exposed at the surface of the CRD, allowing for binding to sugars contained within complex oligosaccharide chains. The selectin family members play a crucial role in leucocyte trafficking to sites of inflammation, and in the migration of lymphocytes to specific lymphoid organs (Lasky 1992). The X-ray crystal structure of E-selectin provided a second example of a C-type lectin CRD (Graves et al. 1994). The three dimensional structures of the CRDs of rat MBP and E-selectin are very similar, although loop regions flanking the carbohydrate binding site differ significantly. The difference leads to altered directionality of carbohydrate-binding residues as well as the complete lack of a pocket around the Ca^{2+} site. The changes in structure enable MBP to bind mannose while E-selectin recognizes a sialic acid analogue.

Classification of C-type Lectins

Most C-type lectins have alternative functional domains in addition to their CRDs. These additional functional regions can be classified into several groups (Bezouska 1991). Group I C-type lectins have hyaluronic acid-binding regions while group II lectins are joined to N-terminal membrane anchors to form type II transmembrane proteins. All of the CRDs of collectins are associated with collagenous domains and are classed as group III C-type lectins. Selectins, group IV, have epidermal growth factor-like domains, while group V proteins consist of a type I transmembrane domain. Group VI consists of merely one protein, the macrophage mannose receptor. The protein contains a fibronectin type-II repeat domain but, unlike the C-type lectins mentioned thus far, has multiple CRD domains. Some C-type lectins consist simply of isolated CRDs and form group VII. Such proteins occur in snake venom (Hirabayashi et al. 1991) and in some invertebrate body fluids such as BRA-2 and BRA-3 lectins from acorn barnacle (Takamatsu et al. 1994 and Takamatsu et al. 1993). Insect soluble C-type lectins also belong in group VII.

A particularly striking observation was made by Drickamer et al. (1991) when the gene structures of members of these C-type lectin groups were compared. The CRDs, from group I or II lectins, are encoded by three exons. The two introns within the CRD-coding regions of group II genes are found at exactly corresponding positions. Similarly, the CRDs in group I are found at nearly these same positions. Collectins and selectins, groups III and IV respectively, lack introns within their CRD-coding regions. Group VI,

the macrophage mannose receptor, is one C-type lectin that does not fall into the gene structure classification. Introns are found in all of its eight CRDs but their number varies. Evolutionary relationships of C-type lectins have been considered based on occurence of introns in the CRD-coding regions (Bezouska et al. 1991).

C-type Lectin Evolution

Drickamer and collaborators (Bezouska et al. 1991) have proposed an order of events that may have occured during the evolution of lectins containing the C-type CRDs. Divergence of intron-containing and intron-lacking CRDs preceded shuffling events in which the other functional domains were associated with the CRDs. For example, during evolution a CRD-encoding gene segment became juxtaposed to a collagenous domain and all of the group III C-type lectins derive from this single precursor. Similar arguments are made for the group I, II and IV proteins. Therefore, from the long-term evolutionary point of view, it appears useful to classify C-type lectins on the basis of their genetic organization rather than domain shuffling (Arason 1996).

It is not known how group VI, the macrophage mannose receptor, evolved (Drickamer 1993) but it is thought that duplication of CRDs that led to its generation must have been an early event, occuring at roughly the same period as the duplications that led to the progenitor CRDs for each of the other groups of C-type lectins.

Insect Lectins

Lectins from several insects have been isolated and characterized and have been proposed as defense molecules. It is thought that the *Sarcophaga* lectin, produced by the flesh fly *Sarcophaga peregrina*, has dual functions in defense and in development (Natori 1990). During ontogenesis of developing *S. peregrina* only certain cells proliferate to form body structures, while unwanted cells are eliminated. *Sarcophaga* lectin is essential in removal of the unnecessary cells and foreign pathogens by mediating cell lysis. Recently, a C-type lectin was found in *Drosophila melanogaster* that has similar functions in defense and development as does *Sarcophaga* lectin (Haq et al. 1996), although the two lectins are assumed not to be structurally related. In the silkworm, *Bombyx mori*, it was reported that the hemagglutinating activity increased significantly in the hemolymph after infection with cytoplasmic polyhedrosis virus (Mori et al. 1992). The *Bombyx* lectin protein is induced concomitantly with infection thereby suggesting the lectin's involvement in the silkworm's defense system. The beet armyworm, *Spodoptera exigua*, also has a lectin that has been characterized as a defense molecule (Pendland et al. 1988). A galactose-specific agglutinin purified from *S. exigua* sera opsonizes fungal cells having exposed galactose residues. These fungal cells are rapidly cleared from the *S. exigua* hemolymph in *in vivo* studies. The hemolymph of the American cockroach, *Periplaneta americana*, contains *Periplaneta* lectin that acts as an opsonin to facilitate phagocytosis of injected bacteria by hemocytes (Kawasaki et al. 1993). The fat body of the cockroach has recently been shown to contain a family of lectins with similar

sequence to *Periplaneta* lectin (Kawasaki et al. 1993). Kawasaki et al. claims their find is the first published demonstration of the presence of a lectin-related protein family in an insect. Unconfirmed data has indicated grasshopper, *Melanoplus differentialis*, as having a family of hemolymph lectin proteins. One of these proteins, named GHA for 'grasshopper hemagglutinin', is thought to have a role in defense through its pathogen agglutinating activity (Wheeler et al. 1993). Research on these lectins is the focus of work in Dr. Hapner's laboratory.

Published GHA Work

GHA, a C-type lectin found in the hemolymph of grasshopper *M. differentialis*, was purified by affinity chromatography on a column of Sepharose-galactose followed by elution with EDTA. The agglutinin has binding specificity toward galactosidic and glucosidic carbohydrates (Stebbins and Hapner 1985). Hemagglutination activity was destroyed by treatment of the hemoagglutinin with heat, trypsin or EDTA. The mature GHA, is a glycoprotein and was measured to be approximately 70kDa by non-reducing electrophoresis. The protein was shown to contain two disulfide-linked polypeptide chains. The hemagglutinin is released from fat body, ovary and testes tissues as demonstrated by metabolic incorporation of ^{35}S-methionine into the relevant tissue cultures (Stiles et al. 1988). The lectin does not opsonize asialo human erythocytes, *Bacillus thuringiensis* bacteria nor spores of *Nosema locustae* (Bradley et al. 1989). The lectin does associate with blastospores from *Beauvaria bassiana* (Wheeler et al. 1993).

Insects injected with *B. bassiana* blastospores treated with agglutinin have the fungal cells cleared more than twice as rapidly as those not treated. It is suggested that the grasshopper hemagglutinin has a role in immune recognition of this fungus and functions in its removal from the hemolymph.

Current GHA Work

Two clones, Clones 3 and 4, have been isolated and sequenced from a grasshopper fat body cDNA library (Radke J.R. Unpublished work). These clones are 80% identical with only one segment where they show significant differences. Clone 3 is 1221bp and includes a 972bp open reading frame (ORF) coding for 324 amino acids. The initiating codon, stop codon and polyA tail are represented. There is no sequence available to complete the 5' end of Clone 4's ORF. A 5' Rapid Amplification of cDNA Ends (RACE) procedure is underway to obtain the putative 120bp of missing 5' ORF. The first 19 amino acid residues of the ORF of Clone 3 are uncharged and mostly hydrophobic. These residues most probably represent a signal peptide. The coded amino acid sequence includes two glycosylation consensus sequences, at least one of which is glycosylated (Wenzlick D.L. Unpublished work). The amino acid sequence also includes two C-type lectin CRDs that are approximately 30% identical to one another and to other invertebrate C-type lectins. GHA has been HPLC-purified and subsequently undergone amino acid analysis, molecular mass determination and cyanogen bromide fragmentation with Edman sequencing (Hapner K.D. and Wenzlick D.L. Unpublished work). Accurate

molecular mass determination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF MS) has demonstrated that the grasshopper lectin is a disulfide-bond stabilized dimeric molecule consisting of two glycosylated monomers of identical size. The dimeric GHA molecule has been measured to approximately 72kDa while the monomers have the equivalent mass of 36.1kDa. Edman protein sequencing of two cyanogen bromide fragments has produced sequences that differ from sequences found in Clones 3 or 4. Therefore, three different C-type lectin sequences have been documented in the lab. One hypothesis from these observations is that the gasshopper contains a family of C-type lectins.

Information of three dimensional structure was gained through computer modeling of the GHA CRDs (Radke J.R. Unpublished work). X-ray crystal structures of rat MBP (Weis et al. 1991) and E-selectin (Graves et al. 1994) were used as reference proteins. The sites of GHA expression are being determined through Northern analysis and reverse transcription PCR amplification (Gedik L. Unpublished work).

Research Rationale and Approaches

Knowledge of homologous Clone 3 and 4 cDNA sequences and the amino acid sequences from fragments of GHA lectin protein has suggested the presence of a family of grasshopper lectin genes. Southern analysis can confirm that a family of genes may encode multiple grasshopper lectins. Southern analyses use either radioactive or non-radioactive isotopes for DNA detection. The techniques may have differing

sensitivities. Comparison of the two techniques may determine which is more appropriate for genomic Southern analyses. Knowledge of intron occurence in C-type lectin genes is useful in lectin classification and evolutionary relationships (Arason 1996). Gene structure can be investigated without the availability of a grasshopper genomic library. An indirect approach using PCR amplification may be used to determine the size of the lectin gene. A gene larger than the mRNA it encodes will suggest the gene contains introns. Southern analysis and PCR amplification techniques are briefly discussed below.

Southern Analysis

Southern analysis involves the detection of a specific fragment of DNA. The DNA of interest is immobilized onto a nylon membrane. Subsequently, a 'probe' is required. A probe is a DNA fragment of complementary sequence to the immobilized DNA. The probe is modified to allow for its detection. This modification involves the incorporation of biotinylated or radioactive nucleotides into the probe DNA. When the probe is added to immobilized DNA, complementary sequences anneal, and the target bands are visualized by autoradiography or chemiluminescence. One chemiluminescent detection method involves a complex of biotin and a streptavidin alkaline phosphatase conjugate. A phenylphosphate-substituted 1,2-dioxetane substrate is cleaved by the alkaline phosphate and this triggers the decomposition of the 1,2-dioxetane with the simultaneous production of light. The light emission is detected using X-ray film.

PCR Amplification

PCR is a method for the amplification of DNA sequences *in vitro*. PCR is based on a series of incubation steps at different temperatures. One set of these steps, referred to as a PCR cycle, allows the annealing and extention of two primers, usually 17- to 20-mers, complementary to the target. The temperature is then raised to denature the DNA. The PCR process is a repetition of the cycle. The target is copied with each cycle, resulting in an exponential amplification. With PCR, DNA sequences can be amplified by at least 10^5 fold and potentially as high as 10^9 fold (Saiki et al. 1988). Reaction setup at room temperature may allow for non-specific primer annealing and extension (Chou et al. 1992). Undesirable non-specific constructs that begin this way are amplified throughout the remaining PCR cycles, resulting in misprimed products. Hot start is a technique that ensures that the polymerase enzyme is unable to function during PCR set up at room temperature. Perkin-Elmer AmpliTaq Gold™ (Roche Molecular Systems Inc., Branchburg, NJ) was one of the thermostable DNA polymerases used in the PCR amplification. The enzyme is provided in an inactive state and high temperatures are required to activate the enzyme. Using a pre-PCR heat step provides a PCR hot start, since primer extension cannot occur during PCR set up when the enzyme is inactive. Another hot start method used recombinant Taq DNA polymerase (Life Technologies, Grand Island, NY) and Mg^{2+}-free PCR buffer. Mg^{2+} was provided in a wax bead and the Mg^{2+} released only once the bead melted at higher temperatures.

Research Objectives

The overall objective of this research is to gain more understanding of the genes encoding C-type lectins in grasshoppers. The specific goals are listed below:

1. Confirm that the grasshopper genome contains a family of C-type lectin genes. Determine this through genomic Southern analyses. Subobjectives include comparison of radioactive and non-radioactive Southern analyses.

2. Determine if genes representing Clone 3 and 4 are continuous or discontinuous and contain introns. Sub-objectives involve strategic primer design, PCR optimization and confimatory differential endonuclease restriction analysis.

MATERIALS AND METHODS

Primers and Probes

Two probes were utilized in Southern analyses. One probe was obtained by cleaving out the 879bp grasshopper cDNA insert, from pGem 3.0 recombinant plasmid, using EcoRI and Acc I restriction enzymes (figure 1). EcoRI alone cleaves out the 879bp grasshopper insert but also generates a fragment of phage and plasmid DNA that is 920bp in length. This latter fragment may not be resolved on an agarose gel, making isolation of the grasshopper insert difficult. Thereby, the plasmid was cleaved with Acc I to cut the 920bp fragment into smaller sizes. The cDNA fragment, refered to as '879bp' probe, represents 72% of the total sequence of Clone 3. The second probe, named '580bp' probe, was PCR amplified from pGem 3.0 template with the primers 5'B and 3'D (figure 3). The regions of Clones 3 and 4 where the probes anneal are shown in figure 2.

Oligomer primers, required for PCR experimentation, were purchased from NBI (National Biosciences Inc., Plymouth, MN). Primers were required that were either specific to individual grasshopper cDNA clones or annealed to both clones. Primer design is an important part of PCR optimization. Rules in the design of efficient primers include length between 17-25bp, 50-60% GC composition, above 55°C Td, non-complementarity at the 3' ends of primer pairs and non-complementarity to self. All these factors were considered when the primers were designed from grasshopper Clone 3 and 4

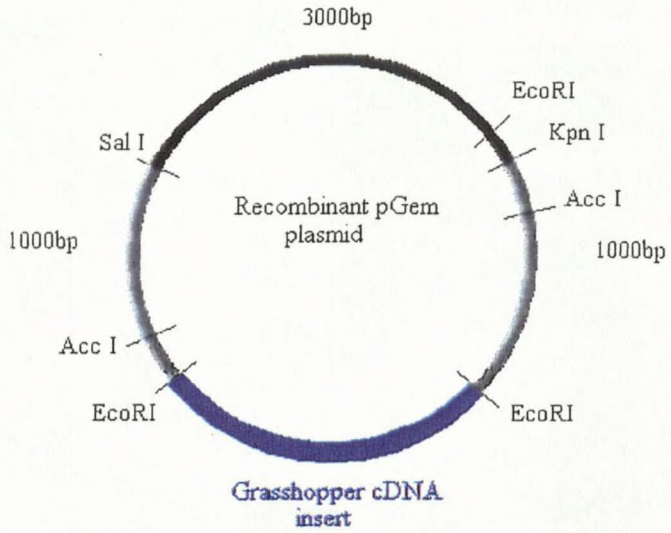

Figure 1. Map of Recombinant PGem Plasmid. Black portion indicates pGem-7Zf(+) plasmid. Grey regions indicate λgt11 DNA. The blue region represents inserted 879bp grasshopper Clone 3 cDNA. The plasmid was utilized in probe DNA preparation, determination of restriction enzyme activities and creation of a standard ladder for Southern analyses.