AN INTEGRIN REQUIRED FOR THE ENCAPSULATION IMMUNE RESPONSE IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* L. (LEPIDOPTERA: SPHINGIDAE)

by

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B.S., University of Maryland, 1987
M.S., University of Illinois, 1993

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

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Abstract

Cellular encapsulation is the immune response in which insects protect themselves from multicellular parasites such as nematodes or parasitoids. During an encapsulation episode, certain insect hemocytes become attracted to a foreign invader and aggregate on its surface. In short order, the invading entity will become entrapped within a capsule comprised of thousands of hemocytes, thus rendering the parasite harmless to the insect host. Although the process of cellular encapsulation has been known for a great many years, very little knowledge yet exists regarding the biochemistry underlying capsule formation. It would seem likely that cell surface adhesion proteins mediate this immune response.

In a series of in vivo encapsulation assays in the tobacco hornworm, *Manduca sexta*, a collection of anti-hemocyte monoclonal antibodies (mAbs) was screened for their ability to inhibit cellular encapsulation. Two of the mAbs that inhibited this immune response and incidentally specifically bind plasmatocytes, MS13 and MS34, were used to isolate a ≈ 90 kDa protein. Several short peptide sequences contained within this protein were acquired via Edman degradation. Degenerate primers based on two of these peptide sequences and total RNA from *M. sexta* hemocytes were used to perform RT-PCR and 5′ and 3′ RACE. This resulted in a full-length cDNA sequence of 2426 bp. A 2301 bp open reading frame within this cDNA sequence codes for a protein of 767 residues. This protein, denominated βMs1, exhibits significant sequence homology to the β-subunits of integrins, which are a family of transmembrane, heterodimeric glycoproteins that possess adhesive properties. Analysis of recombinant segments of βMs1 showed that the protein

produced from the PCR product is the antigen to MS13 and MS34 and that these mAbs bind to the region of the integrin that contains the extracellular binding site. Northern blot analysis of various *M. sexta* tissues together with immunofluorescence labeling with MS13 and MS34 shows that βMs1 is solely expressed in plasmatocytes. The totality of these experiments demonstrates that integrins are essential for the cellular immune response of encapsulation.

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Dedication

I like to dedicate this thesis first and foremost to my two children, **Samuel** and **Acacia**, for all the love and understanding they have shown towards me while daddy was so far away working on the "Incredible Project." I would also like to include my fiancée **Sharon** who also showed great understanding and stayed with me while I had to keep running back to Kansas. And finally to my great uncle **Sonny** who only wanted two things from me, to finish my thesis and get a haircut. I'll give you the former, but neither you nor anybody else will get the latter.

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Abbreviations

AC-saline: Anticoagulant saline

AD: Adipohemocyte

AFP: antifungal protein

ANP: atrial natriuretic peptide

βMs1: integrin β subunit found on *Manduca sexta* plasmatocytes that are essential for cellular encapsulation

BSA: bovine serum albumin

CHO cells: chinese hamster ovary cells

CRR: cysteine-rich [pseudo]-repeat region

DEAE: diethylaminoethyl

DOC: deoxycholate

ECM: extracellular matrix

EDTA: ethylenediamine tetra-acetic acid; $(CH_2COOH)_2NCH_2CH_2N(CH_2COOH)_2$

EGF: epidermal growth factor

ERK: extracellular signal–regulated kinase

FAK: focal adhesion kinase

GBP: growth-blocking peptide

GR: granular cells

ICAP: integrin cytoplasmic domain-associated protein

ILK: integrin linked kinase

JNK: Jun-N-terminal kinase

LIBS: ligand-induced binding sites

mAb: monoclonal antibody

MAP: mitogen-activated protein

MAPK: mitogen-activated protein kinase

MSB: *Manduca* saline buffer

OE: Oenocytoid

PAGE: polyacrylamide gel electrophoresis

PAMP: pathogen-associated molecular pattern

PBS: phosphate buffered saline

PL: plasmatocyte

PMSF: phenylmethylsulfonyl fluoride

PO: phenoloxidase

PR: prohemocyte

pro-PO: pro-phenoloxidase

PRP: pattern recognition protein

PRR: pattern recognition receptors

PSP: plasmatocyte spreading peptide

PTU: phenyl thiourea

Q: quaternary amines

RACE: rapid amplification of cDNA ends

Rack1: receptor for activated protein kinase C

RGD: arginine-glycine-aspartic acid

RGDS: arginine-glycine-aspartic acid-serine

RGES: arginine-glycine-glutamic acid-serine

(RT)-PCR: (reverse transcription)-polymerase chain reaction

S: sulfonic

SH2: Src homology 2

SDS: sodium dodecyl sulphate

SP: spherulocyte

TEA: triethanolamine

TEM: transmission electron microscopy

TLCK: Na-*p*-tosyl-L-lysine chloromethyl ketone

TM: transmembrane domain

TPCK: N-tosyl-L-phenylalanine chloromethyl ketone

Organisms

(*= non-insect)

Acalolepta luxuriosa (Coleoptera: Cerambycidae; Udo longicorn beetle)

**Acropora millepora* (Cnidaria: Anthozoa: Scleractinia: Acroporidae; coral)

Aedes aegypti (Diptera: Culicidae; yellow fever mosquito)

Anastrepha obliqua (Diptera, Tephritidae; West Indian fruit fly)

Anopheles gambiae (Diptera: Culicidae; malaria mosquito)

Antheraea pernyi (Lepidoptera: Saturniinae; Chinese oak silk moth)

Apidae (Hymenoptera: bees)

Apis mellifera (Hymenoptera: Apidae: domestic honey bee)

Armigeres subalbatus (Diptera: Culicidae; mosquito)

Bacillus rossius (Phasmatodea: Bacillidae; stick insect)

Bactrocera cucurbitae (Diptera: Tephritidae; melon fly)

Bactrocera dorsalis (Diptera: Tephritidae; Oriental fruit fly)

Blattella germanica (Blattaria: Blattellidae; German cockroach)

Bombyx mori (Lepidoptera: Bombycidae; domestic silkworm moth)

**Caenorhabditis elegans* (Nematoda)

Calliphora vicina (Diptera: Calliphoridae; European bluebottle fly)

Candida albicans (Fungi: Ascomycota; yeast infection pathogen)

Ceratitis capitata (Diptera: Tephritidae; Mediterranean fruit fly)

Cetonischema aeruginosa (Coleoptera: Scarabaeidae; scarab beetle)

Coccus hesperidum (Homoptera: Coccidae; the brown soft scale)

**Crassostrea gigas* (Mollusca: Bivalvia; Ostreidae; Pacific oyster)

**Dictyostelium amoebae* (Amoebozoa: Mycetozoa: plasmodial slime molds)

Drosophila melanogaster (Diptera: Drosophilidae; fruit fly)

Encyrtus infelix (Hymenoptera: Encyrtidae; chalcid wasp)

Encyrtus lecaniorum (Hymenoptera: Encyrtidae; chalcid wasp)

**Entamoeba dispar* (Amoebozoa: Archamoebae; amoeba)

**Entamoeba histolytica* (Amoebozoa: Archamoebae; amebiasis amoeba)

**Escherichia coli* (Proteobacteria; Gram-negative rods)

Fopius arisanus (Hymenoptera: Braconidae; parasitoid wasp)

Galleria mellonella (Lepidoptera: Pyralidae; greater wax moth)

Glossina morsitans morsitans (Diptera: Glossinidae; tsetse fly)

Gromphadorhina portentosa (Blattaria: Blaberidae; giant Madagascar hissing cockroach)

Helicoverpa armigera (Lepidoptera: Noctuidae; cotton bollworm)

Heliothis virescens (Lepidoptera: Noctuidae; tobacco budworm)

Holotrichia diomphalia (Coleoptera: Scarabaeidae; chafer beetle)

Hyalophora cecropia (Lepidoptera: Saturniidae; cecropia moth)

Hyalophora gloveri (Lepidoptera: Saturniidae; Glover's silk moth)

**Hymenolepis diminuta* (Cestoda: Cyclophyllidea; rat tapeworm)

Hypera brunneipennis (Coleoptera: Curculionidae; Egyptian alfalfa weevil)

Hypera postica (Coleoptera: Curculionidae; alfalfa weevil)

Hyphantria cunea (Lepidoptera: Arctiidae; fall webworm moth)

Ichneumonidae (Hymenoptera: ichneumonid wasps)

Lacanobia oleracea (Lepidoptera: Noctuidae; tomato moth)

Leptopilina boulardi (Hymenoptera: Figitidae; cynipoid wasp)

Lygus spp. (Hemiptera: Miridae; lygus bug)

Lymantria dispar (Lepidoptera: Lymantriidae; gypsy moth)

Manduca sexta (Lepidoptera: Sphingidae; tobacco hornworm/ Carolina sphinx moth)

Metaphycus helvolus (Hymenoptera: Encyrtidae; chalcid wasp)

Metaphycus swirskii (Hymenoptera: Encyrtidae; chalcid wasp)

**Metarhizium anisopliae* (Fungi: Ascomycota; entomopathogenic fungus)

**Micrococcus luteus* (Actinobacteria: Gram-positive cocci)

Mythimna (=Pseudaletia) separata (Lepidoptera: Noctuidae; northern armyworm)

**Mytilus galloprovincialis* (Mollusca: Bivalvia; Mediterranean mussel)

**Pacifastacus leniusculus* (Crustacea: Decapoda: Astacidae; signal crayfish)

**Penaeus japonicus* (Crustacea: Decapoda: Penaeidae; kuruma prawn)

Periplaneta americana (Blattaria: Blattidae; American cockroach)

Phormia terranovae (Diptera: Calliphoridae; blow fly)

Pieris brassicae (Lepidoptera: Pieridae; white butterfly [adult], cabbage looper [larvae])

Pieris rapae (Lepidoptera: Papilionidae; cabbage white butterfly)

**Plasmodium berghei* (Apicomplexa; Haemosporida; murine rodent malaria parasite)

*_Plasmodium gallinaceum_ (Apicomplexa; Haemosporida; avain malaria parasite)

Podisus maculiventris (Hemiptera: Pentatomidae; spined soldier [stink] bug)

*_Podocoryne carnea_ (Cnidaria: Hydrozoa: Hydroida: Hydractiniidae; marine jellyfish)

Protopulvinaria pyriformis (Homoptera: Coccidae; pyriform scale)

Pseudacanthotermes spiniger (Isoptera: Termitidae; termite)

Pseudoplusia includens (Lepidoptera: Noctuidae; soybean looper)

*_Saccharomyces cerevisiae_ (Fungi: Ascomycota; Saccharomycetes; baker's yeast)

Saissetia coffeae (Homoptera: Coccidae; hemispherical scale)

Samia cynthia ricini (Lepidoptera: Saturniidae; Indian eri silkmoth)

Sarcophaga peregrina (Diptera: Sarcophagidae; flesh fly)

Schistocerca gregaria (Orthoptera: Acrididae; desert locust)

*_Schizosaccharomyces pombe_ (Fungi: Ascomycota; Schizosaccharomycetes; fission yeast)

Simulium damnosum (Diptera: Simuliidae; black fly)

Sphecidae (Hymenoptera: digger [including thread-waisted] wasps)

Spodoptera exigua (Lepidoptera: Noctuidae; beet armyworm)

Spodoptera frugiperda (Lepidoptera: Noctuidae; fall armyworm)

Triatoma infestans (Hemiptera: Reduviidae; kissing bug)

Trichoplusia ni (Lepidoptera: Noctuidae; cabbage looper)

Vespidae (Hymenoptera: vespid wasps)

***** _Xenopus laevis_ (Anura: Pipidae; African clawed toad)

Zophobas atratus (Coleoptera: Tenebrionidae; "King" mealworm beetle)

Objective

In most insects, when a large foreign body invades the hemocoel, certain hemocyte types from the host will recognize the intruding entity and adhere to it. Within a short period, thousands of host hemocytes will form a multilayered capsule around the alien mass, thus rendering it harmless. This process is known as cellular encapsulation. The objective of this thesis is to determine the molecular basis for hemocyte adhesion that allows for the formation of cellular capsules using the tobacco hornworm, *Manduca sexta*, as the experimental insect.

Introduction

1) Insect Immune System:

Parasitism has likely existed at least since the first eukaryotic cell evolved about 2.5 billion years ago. The serial endosymbiosis hypothesis maintains that the first eukaryotic cell came into being when an anaerobic protoeukaryote host cell was invaded by a prokaryotic aerobic symbiont (Margulis, 1981). From that point to the time the Hexapoda arose 2.1 billion years later (Borror et al., 1989) to the present, the immune responses of extant insects to the onslaught of parasitic challenges have undergone great evolutionary advances and sophistication.

Insects have well-developed innate immune systems that utilize genetically predetermined proteins that coordinate the engulfment and breakdown of infectious organisms (Fearon, 1997; Gillespie et al., 1997; Lavine and Strand, 2002). These germline-encoded immune proteins, both soluble in the hemolymph and membrane bound on cells, provide a rapid defense against pathogens by being either habitually present in the host or quickly induced upon infection. Although the "hard-wired" nature of innate immunity imparts a degree of genetic inflexibility, hundreds of millions of years of evolution has honed this system to recognize and respond to a great assortment of molecular classes common only to pathogens, such as the peptidoglycans and lipopolysaccharides that are found on bacteria (see below "pathogen-associated molecular pattern (PAMP) molecules"). Innate immunity thus functions by detecting entities that possess characteristics of infectious organisms, not by distinguishing every extant pathogen individually (Fearon and Locksley, 1996; Fearon, 1997).

As of yet, there exists no evidence that insects possess an acquired immune

system, a trait known among vertebrates (Wallace et al. 1991, Lavine and Strand, 2002). In contrast to innate immunity, acquired immunity is characterized by a targeted attack upon a unique invader via specific antigen receptors produced by somatic gene rearrangement in the host. In other words, pathogens are recognized by their unique surface chemical distinctiveness. Through somatic gene rearrangement, evolution is compressed to a few weeks, allowing the host to quickly adapt to the selection of variant pathogens. In addition, cells of the acquired immune system that express distinct antigen receptors to a particular pathogen will remain in the host after the infection is cleared. The continued presence of these "memory cells" allows for a rapid response to any foreign entity that is multiply encountered (Fearon, 1997).

The complexity of interactions between the host insect and the particular invading parasite or pathogen are great and vary enormously even among closely related species (Beerntsten et al., 1989; Hillyer et al., 2003). Differences are known to exist even among the various strains within a species (Collins et al., 1986). Within this complexity, the insect immune system can be broken down into three integrated and well-developed lines of defense: physical, humoral and cellular (Gillespie et al., 1997; Lavine and Strand, 2002).

1.1) Physical Defense:

The first line of protection is the barrier of the integument and gut. All insects are covered with a layer of cuticle. The cuticle also lines the ectodermal invaginations of the foregut and hindgut as well as the tracheal system. This protective shield can reach thicknesses up to 200 μm and is comprised of an endocuticle, an exocuticle and an epicuticle. The characteristic component of the endocuticle and exocuticle is the

polysaccharide chitin. Each chitin molecule attaches to neighboring chitin molecules via hydrogen bonds to form microfibrils that are fixed in a protein matrix. In exocuticle, cross-linking among the protein molecules facilitated by highly reactive catecholamines causes this part of the integument to harden in a process called sclerotization. Covering the exocuticle is the epicuticle. This layer contains extracellular phenoloxidase, but no chitin. The presence of this enzyme in the epicuticle facilitates the sclerotization of this layer during repair after damage. The outer covering of the epicuticle is a wax layer (Chapman, 1998).

These cuticular layers make for a very formidable four-fold barrier to pathogens and parasites. First, they act as a physical obstruction. Second, the lipids that compose the wax layer are hydrophobic and thus repel agents of disease that are mostly hydrophilic. Third, the products phenoloxidase activity within the cuticle can be cytotoxic to pathogens. And fourth, upon disturbance of the integument, the underlining epidermal cells can secrete antibiotic peptides and initiate a signalling mechanism to which hemocytes respond (Blomquist and Dillwith, 1985; Dimarcq et al., 1990; Lee, and Brey, 1994. Gillespie et al., 1997; Chapman, 1998).

1.2) Humoral Defense:

Pathogenic microorganisms (i.e. bacteria, fungi, and protozoa) that elude the physical barriers must contend with a collection of cytotoxic peptides and proteins in the hemolymph (Hoffmann et al., 1994; Gillespie et al., 1997; Hetru et al., 1998; Bulet et al., 1999; García-Lara et al., 2005). Recognition by the host of the presence of microorganisms within its body will induce the production of these cytotoxins. Each insect species produces its own specific assortment of cytotoxic peptides and proteins that

will act in synergy against invading pathogens (Bulet et al., 1999). The cytotoxic molecules are primarily produced in the fat body, but are also known to be synthesized by cells of the epidermis (Brey, et al., 1993; Lee and Brey, 1995), midgut (Dunn, et al. 1994; Russell and Dunn, 1996), nephrocytes, (Russell and Dunn, 1990; Chapman, 1998) Malpighian tubules, and hemocytes (Mulnix and Dunn, 1994; Gillespie et al., 1997). Of the more than 170 cytotoxic peptides and proteins found to date, most share in the features of a molecular weight less than 5 kDa (larger proteins include lysozyme and attacin) and a structure containing either amphiphilic α-helices or hairpin-like β-sheets or a combination of the two (Bulet et al., 1999).

Antimicrobial peptides and proteins can be grouped into gene families based on similar amino acid sequences and three-dimensional structures (Gillespie et al., 1997). The Hoffmann research group at Le Institut de Biologie Moléculaire et Cellulaire has further categorized these antibacterial biomolecules into four groups: (i) cecropins, (ii) defensins and other cysteine-containing peptides, (iii) proline-rich peptides, and (iv) glycine-rich peptides/polypeptides (Hetru et al. 1994; Hetru et al. 1998; Bulet et al, 1999; Lamberty et al. 1999).

1.3) Cellular Immunity:

Upon invasion by either micro- or macroorganisms, in addition to a humoral immune response, insects launch a hemocyte-mediated immune reaction (Chapman, 1998; Lavine and Strand, 2002), in which the hemocytes undergo both morphological and behavioral changes (Gillespie, et al, 1997; Chapman, 1998). Cellular and humoral immunity do not operate independently from one another, but in a well-coordinated fashion in which hemocytes and humoral biomolecules influence each other's actions.

For example, recognition of a foreign entity by hemocytes is accomplished either by direct contact or through humoral factors that are produced by the host and bind to the invader to opsonize its surface. And, as mentioned above, a number of cytotoxic peptides are produced by hemocytes. The specific cellular response is dependent on the type and number of invading organisms. Cellular responses come in three forms: phagocytosis, nodule formation and encapsulation.

1.3.1) Insect Hemocyte Types:

Prior to a discussion of the insect cellular immune response, it is essential to characterize the hemocyte types involved.

Hemocytes have been described from many orders throughout the Hexapoda including Collembola (Ksiazkiewicz-Ilijewa, 1979), Blattaria (Baerwald and Bousch, 1970; Moran, 1971; Chain et al. 1992; Fenoglio et al. 1993), Orthoptera (Costin, 1975), Hemiptera (Barracco et al. 1987; Barracco and Loch, 1989), Hymenoptera (Ahmad, 1988), Coleoptera (Ahmad, 1992; Giulianini, 2003), Diptera (Luckhart et al., 1992; Hillyer and Christensen, 2002; Silva et al., 2002; Meister and Lagueux, 2003) and Lepidoptera (Akai and Sato, 1973; Beaulaton, 1979; Chain and Anderson, 1983; Butt and Shields, 1996; de Andrade et al, 2003; Falleiros et al. 2003; Ling et al. 2003).

Historically, the various types of hemocytes were distinguished from each other by functional, morphological and histological characteristics (Gupta, 1985; 1986; 1991; Brehelin and Zachary, 1986). These methods for categorizing hemocytes have proved to be insufficient due to many hemocytes having overlapping characteristics (e.g. both plasmatocytes and granular cells are known to be phagocytic; the presence of granules, while a defining characteristic of granular cells, is in some species of insects a

characteristic of other hemocyte types too). To add to the confusion, within a species many hemocyte types consist of subtypes. In addition, functional, morphological and histological characteristics of a specific hemocyte type can vary con- and interspecifically (Gupta, 1991).

During the 1990s, monoclonal antibody (mAb) markers were created to antigens that are expressed on hemocytes from several insect species (Chain et al., 1992; Mullett et al., 1993; Willott et al., 1994; Scapigliati et al., 1996; Strand and Johnson, 1996; Hori et al., 1997; Gardiner and Strand, 1999). This greatly improved the ability to identify specific types and subtypes of hemocytes within the particular species for which the antibodies were developed, however, many of these antibodies do not cross-react with hemocytes from different species (Willott et al., 1994).

The result of this ambiguity is a body of literature muddled with numerous names for all the variant forms of hemocytes and the likelihood of single hemocyte types being given multiple names. This has led to the situation in which it is very difficult to perform interspecific comparisons of hemocytes (Brehelin and Zachary, 1986, Gupta, 1991). In spite of these difficulties, some general hemocyte types can be described. It is important to note that not all insects possess all hemocyte types.

Prohemocytes (PR): Prohemocytes are small cells that are round or oval (4 to 10 µm wide by 4 to 22 µm long) (Gupta, 1985; 1991; Brehelin and Zachary 1986; Butt and Shields, 1996, Chapman, 1998; Silva et al., 2002; Giulianini et al. 2003). These hemocytes are characterized by possessing a relatively large nucleus that nearly fills the cytoplasmic space. Other organelles (e.g. smooth and rough endoplasmic reticulum, mitochondria, Golgi bodies) are generally low in number. Prohemocytes have been

observed containing centrioles and microtubules, thus suggesting a mitotic nature. This last observation has led some to speculate that prohemocytes are the precursors to some or all the other hemocytes. In *in vitro* experiments with *B. mori* hemocytes, Yamashita and Iwabuchi (2001) observed that isolated non-dividing prohemocytes differentiate into either granular cells or plasmatocytes and that some of these granular cells subsequently transition into spherulocytes. Yamashita and Iwabuchi also observed other prohemocytes divide directly into granular cells, plasmatocytes, spherulocytes or new prohemocytes. During embryonic development of *Drosophila* hemocytes, Tepass et al. (1994) observed procephalic mesoderm differentiate into prohemocytes, which then underwent a second differentiation into plasmatocytes.

Prohemocytes are often found in small groups and constitute 1% to 7% of the population of hemocytes (Gupta, 1985; 1991; Brehelin and Zachary 1986; Chapman, 1998).

Plasmatocytes (PL): Plasmatocytes are polymorphic cells of variable size (3 μm to 40 μm) (Gupta, 1985; 1991; Götz and Boman, 1985; Brehelin and Zachary 1986; Butt and Shields, 1996; Chapman, 1998; Silva et al., 2002). While free-floating in the hemolymph, plasmatocytes are round or oval. Upon contact with certain surfaces (e.g. glass or a foreign body undergoing encapsulation), plasmatocytes attach, spread by sending out filopodia, and become very flat (thus the pseudonym lammellocytes; other pseudonyms include: vermiform cells, podocytes and thrombocytoids). The cytoplasmic space contains many organelles, including a well-developed rough endoplasmic reticulum, Golgi bodies, and numerous mitochondria. The nucleus is most often positioned centrally. A recent examination of plasmatocytes of *M. sexta* larvae revealed

that the cells of this species are endomitotic and are thus polyploid (Nardi et al. 2003). Silva et al. (2002) observed binucleated plasmatocytes from the larvae of the tephritid fruit fly *Anastrepha obliqua*.

Plasmatocytes may or may not contain granules. Both Gupta (1991) and Brehelin and Zachary (1986) designate all granulated plasmatocytes as granular cells (probably Brehelin and Zachary's granular hemocyte 1).

Plasmatocytes are among the most numerous of hemocytes and generally comprise between 30% to 60% of the total hemocyte count. These hemocytes possess the ability to adhere to foreign surfaces and play an important role in wound healing and in the immune responses of phagocytosis, nodule formation and encapsulation (Gupta, 1985; 1991; Götz and Boman, 1985; Brehelin and Zachary 1986; Chapman, 1998; Lavine and Strand, 2002). Plasmatocytes also have the important task of phagocytosing cells that have undergone apoptosis during embryogenesis and metamorphosis (Tepass et al. 1994; Franc et al. 1996; 1999; Chapman, 1998).

Granular cells (GR): Granular cells are either spherical or oval with a diameter range between 4 and 45 μm. The main distinguishing characteristic of these cells is their great number of membrane-bound granules. Granules appear to originate from the Golgi bodies (Gupta, 1985) and come in various forms which Gupta (1985; 1986; 1991) has combined into three groups: 1) structureless electron-dense granules (amorphous blobs that appear dark in transmission electron microscopy (TEM)), 2) structureless electron-lucent granules (amorphous blobs that appear light in TEM) and 3) structured granules (granules with defined shape). Based on the types of granules present, Brehelin and Zachary (1986) developed four categories of granular cells; the granular hemocytes (GH)

1 through 4.

Other characteristics of granular cells include the possession of numerous lysosomes, free ribosomes and well developed ER and RER, but only a few mitochondria (Gupta, 1985; Chapman, 1998). The nucleus is round or elongate and located centrally. Nardi et al. (2003) showed that these cells are diploid. Granular cells may or may not have micropapillae or filopodia (Gupta, 1985) (although some of what have been described as filopodiated granular cells may be what others have referred to as granulated plasmatocytes).

Like plasmatocytes, granular cells can adhere to foreign surfaces and play an important role in wound healing and in the immune response, including nodule formation and encapsulation (Gupta, 1985; 1986; 1991; Götz and Boman, 1995; Brehelin and Zachary 1986; Chapman, 1998). Upon injury or invasion by a foreign body, granular cells will readily degranulate. Degranulation is often followed by recruitment of other immunocytes (i.e. plasmatocytes and granular cells) and localized coagulation (the coagulocytes of some insects may in fact be granular cells) (Gupta, 1991; Pech and Strand, 1996). Granular cells in some species are also known to be phagocytic (Wago, 1991; Gillespie, 1997; Silva et al., 2002).

Granular cells comprise over 30% of the hemocyte count and, along with plasmatocytes, are together the two most numerous of hemocytes (Chapman, 1998).

Spherulocytes (SP): Spherulocytes are round or oval and variable in size with diameters from 5 μm to 25 μm (Gupta, 1985; 1991; Butt and Shields, 1996; Chapman, 1998). The defining characteristic of these hemocytes is the membrane-bound spherules in the cytoplasmic space. The number of spherules varies and range in diameter from 1

μm to 5 μm. These spherules appear to enclose some sort of granulated material. Histochemical analysis of the spherules has shown that they contain mucopolysaccharide and glucomucoproteins (Gupta, 1985; 1991). The cytoplasm also contains ribosomes, Golgi bodies, lysosomes, mitochondria and rough endoplasmic reticulum. Although many have speculated as to the function of these hemocytes (e.g. silk production, melanization, phagocytosis, regulation of clotting and cell adhesion), a definitive answer to this question remains elusive (Gupta, 1985; 1991; Brehelin and Zachary 1986; Chapman, 1998).

Oenocytoids (OE): Oenocytoids are opaque hemocytes that range in size from 16 μm to over 54 μm and have been observed in oval, spherical, elongated and crescent shape. They comprise over 5% of the hemocyte count. In the Lepidoptera, oenocytoids are the largest in size of all hemocytes (Gupta, 1985; 1991; Brehelin and Zachary, 1986; Butt and Shields, 1996; Chapman, 1998). The plasma membrane is generally without irregular processes, although SEM of oenocytoids of the German cockroach *Blattella germanica* did show micropapillae (Chiang et al., 1988). The nucleus is small and located eccentrically. Gupta (1991) observed mitotic nuclei in the oenocytoids of the hissing cockroach *Gromphadorhina portentosa.* Sometimes oenocytoids possess two nuclei. This condition may be the result of nuclear division without or prior to cellular division. The cytoplasm appears homogeneous. In oenocytoids from most insect species observed, the organelles (e.g. mitochondria, Golgi bodies, RER) are generally underdeveloped, however, the cytoplasm is known to contain numerous free ribosomes. A few oenocytoids have inclusions that are rod, filament, needle-like, or crystal in shape. In *D. melanogaster*, the oenocytoids are referred to as crystal cells due to the shape of the

inclusions (Gupta, 1985; Götz and Boman, 1995; Tepass et al. 1994).

Oenocytoids tend to lyse very easily, thus spilling the content of their cytoplasm into the hemolymph (Gupta, 1985; 1991; Da Silveira et al. 2003). The cytoplasm of oenocytoids contains a number of proteins and peptides that are important to the host insect's physiology. Recent studies of the lysate of oenocytoids from the noctuid moth *Mythimna* (also known as *Pseudaletia*) *separata* have shown that it contains growth-blocking peptide (GBP)-binding protein (Matsumoto et al., 2003). GBP-binding protein has a specific affinity for growth-blocking peptide (GBP), a molecule involved in stimulation of plasmatocytes, cell proliferation, and larval growth regulation. GBP also causes oenocytoids to lyse. It is believed that the release of GBP-binding protein from lysed oenocytoids acts as an inhibitor of GBP.

Oenocytoids have also been shown to contain pro-phenoloxidase (pro-PO) which, when released into the hemolymph and activated to phenoloxidase, plays an important role in the melanization response associated with wound healing, protein cross-linking and immunity (Jiang et al., 1997; Gillespie, 1997; Hillyer and Christensen, 2002; Hillyer et.al., 2003; Da Silveira et al., 2003; Kanost et al., 2004).

Recently, there have been two observations of phagocytic oenocytoids; one from the scarab beetle *Cetonischema aeruginosa* in which latex beads were phagocytosed by means of a large number of short filopodia (Giulianini et al., 2003), and the second from the mosquito *Armigeres subalbatus* wherein individual bacteria, capsules of melanized bacteria, as well as latex particles, were phagocytosed (Hillyer et al., 2003).

Adipohemocytes (AD): Adipohemocytes are usually spherical or oval (Gupta, 1985; 1991), but have been observed in the tephritid fruit fly *Anastrepha obliqua* to be

polymorphic (Silva et al., 2002). These hemocytes can vary in diameter from 7 μm to 45 μm and are characterized by lipid droplets located in the cytoplasm (Gupta, 1985; 1991; Butt and Shields, 1996; Chapman, 1998; Silva et al., 2002). The cytoplasm often contains non-lipid granules and well-developed mitochondria, Golgi bodies, rough endoplasmic reticulum and polyribosomes. The nucleus is small and may be located anywhere in the cell.

The number of adipohemocytes in the hemolymph is generally low and normally represents only 1% to 4% of the total hemocyte population (Gupta, 1991), however adipohemocytes are known to reach levels as high as 39% in the pupae of the gypsy moth, *Lymantria dispar* (Butt and Shields, 1996), and are the second most abundant hemocyte type in the adult female mosquito *A. aegypti* (Hillyer and Christensen, 2002).

Adipohemocytes are believed to function in storage of energy in the form of glycogen and lipid (Hillyer and Christensen, 2002).

1.3.2) Cellular immune responses:

The three principle immune responses involving hemocytes are phagocytosis, nodule formation and encapsulation (Chapman, 1998; Lavine and Strand, 2002). For most species studied, the primary hemocytes involved in the cellular immune responses are granular cells and plasmatocytes. As mentioned above, oenocytoids can play a role in any melanization events that may occur during a cell immune response.

1.3.2.1) Phagocytosis: Phagocytosis is the process in which individual cells, referred to collectively as phagocytes, engulf small particulates. This behavior of cells is believed to be among the most ancient. It probably first served as a means of obtaining nutrition, but has evolved within the Animalia to aid in the defense against invading

organisms and to remove cells that have undergone apoptosis (Bayne, 1990; Tepass et al. 1994; Götz and Boman, 1995).

Phagocytosis is the primary cellular response against an invasion of a low number of small-sized pathogens (e.g. bacteria, fungal spores or protists). The primary phagocytes of insects are plasmatocytes and granular cells (Gillespie, et al, 1997; Chapman, 1998; Tojo et al. 2000).

Phagocytosis initiates with a phagocyte identifying a particle as foreign (Bayne, 1990, Götz and Boman, 1995; Gillespie, et al, 1997). Such recognition may be achieved directly by the detection of unique surface molecules on the particle by the phagocyte. In the case of microbes, these surface molecules, called pathogen-associated molecular pattern (PAMP) molecules (Lavine and Strand, 2002), include peptidoglycans, lipopolysaccharides, lipoteichoic acid, and β-1,3-glucans. Recognition may be indirect by the tagging of foreign particles with soluble hemolymph factors called pattern recognition proteins (PRPs), or more generally opsonins, which mark the particle for cellular attack. Such PRPs include hemolin, peptidoglycan recognition proteins, β-1,3-glucan recognition proteins, and C-type lectins (Bayne, 1990; Fabrick, 2003; Kanost et al., 2004). In either recognition situation, direct or opsonin-dependent, immunocytes require pattern recognition receptors (PRRs) on their surfaces to facilitate the identification of alien particles for immune attack (Bayne, 1990, Lavine and Strand, 2001; 2002).

Following recognition, the stages of phagocytosis consist of attachment to the particle, outside-in signal transduction to initiate engulfment behavior, formation of pseudopodia, ingestion of the particle, and internal sequestering of the particle in a vesicle known as a phagosome (Bayne, 1990, Götz and Boman, 1995; Gillespie, et al,

1997). The mechanics of engulfment are driven by cytoskeletal actin (Castellano et al., 2001). As the extrinsic particle is internalized, cytoplasmic organelles, called lysosomes, that contain digestive enzymes move towards and fuse with the phagosome, resulting in the disintegration of the engulfed particle (Cheng et al. 1975; Bayne, 1990). Individual phagocytes are known to be capable of ingesting over 100 bacteria (Hillyer et al., 2003). If a phagocyte engulfs particles of indigestible material, the entire cell will migrate to a location within the body where it becomes dormant and there adopts permanent residence (Bayne, 1990).

The degree of phagocytic response is dependent upon the nature of the target particle and may be influenced by factors in the plasma. In the mosquito *Armigeres subalbatus*, "primary response towards the Gram-negative bacterium *E. coli* is phagocytosis" while the "primary response towards the Gram-positive bacterium *Micrococcus luteus* is melanization" (Hillyer et al., 2003).

In the greater wax moth, *G. mellonella*, phagocytic behavior by granular cells was reduced by "p-NPGB, a serine proteinase inhibitor…known to inhibit the activation of the prophenoloxidase cascade," and to a lesser extent by the phenoloxidase inhibitor phenyl thiourea (PTU), thus suggesting that phenoloxidase plays a role in phagocytosis by this hemocyte (Tojo et al. 2000).

1.3.2.2) Nodule Formation: Nodules are aggregates of hemocytes and extracellular coagulum that have entrapped large numbers of small-sized foreign entities. The formation of nodules is the immune response to high concentrations of non-living particles or microbes such as bacteria, fungal spores, yeast cells or protozoa (Götz and Boman, 1985; Guzo and Stoltz, 1987; Gillespie, et al, 1997; Chapman, 1998).

An ultrastructural examination of nodule formation in larval *G. mellonella* by Ratcliffe and Gagen (1977) appears to be typical for most Lepidoptera. Within 1 minute after injection of heat-killed *Bacillus cereus*, the granules within the granular cells that had randomly encountered the bacteria began to swell. These granules migrated out towards the granular cells' periphery and expelled an adhesive flocculent substance into the surrounding hemolymph, which then entrapped the bacteria. This resulted in an aggregation of granular cells and bacteria embedded in an extracellular matrix. Little to no phagocytosis of bacteria by granular cells was observed. Occasionally, a random non-granular cell blood cell was caught in the forming nodule. By 5 minutes, the forming nodules were 50-100 μm in diameter. Granular cells were still undergoing degranulation as the nodule continued to enlarge. The aggregation became more compact and depositions of melanin appeared near entrapped bacteria. The edges of granular cells lost their integrity, followed by the disintegration of the granular cells' nuclei and other organelles.

Two to four hours after initiation of nodule formation, plasmatocytes began to attach to the periphery and flatten in a manner similar to encapsulation (See below, section 1.3.2.3).

Nodule formation was complete by 24 hr and nodules ranged in diameter between 100-150 μm. The completed nodules were comprised of a central flocculent mass consisting of bacteria and the remnants of granular cells and any other hemocytes that had become entrapped, all embedded in a melanized matrix and surrounded by a multilayer of flattened plasmatocytes (≈10 cells thick). Granular cells and plasmatocytes were the only hemocytes observed to play a role in nodule formation, however one can

speculate that oenocytoids play an indirect role since this hemocyte type contains pro-PO, essential for melanization (Ratcliffe and Gagen, 1977; Götz and Boman, 1985; Gillespie, et al, 1997; Chapman, 1998).

1.3.2.3) Cellular Encapsulation: Cellular encapsulation is the immune response in which thousands of hemocytes become attracted and attach to a foreign object, thus forming a multilayered capsule of cells around an intrusive entity. Targets of encapsulation are objects that have found their way into the body of an insect and are too large to undergo phagocytosis or nodulation (Götz, 1986; Bayne, 1990; Tanada and Kaya 1993; Gillespie, et al, 1997; Chapman, 1998). Targets of encapsulation include both biotic objects (e.g. trematodes, cestodes, nematodes, parasitoids, eggs of parasites and parasitoids, fungi and interspecific tissue transplants) and inanimate experimental objects (e.g. Sephadex beads, nylon beads, cotton thread, glass, nylon, latex) (George et al. 1984; Götz and Boman, 1985; Götz, 1986). Cellular encapsulation is also known to occur in a number of other non-insect protostomes including crustaceans (Bauchau, 1981), annelids (Cooper and Stein, 1981), diplopods (Ravindranath, 1981) and molluscs (Sminia, 1981; Cheng, 1981; Cowden and Curtis, 1981), thus indicating that this immune response is very ancient and most probably predates the hexapods.

Physical description of cellular capsules: Nearly all insects so far investigated are known to possess the immune response of cellular encapsulation, with resulting capsules exhibiting broadly similar morphology (Götz and Boman, 1985; Götz, 1986). The major exceptions are the many Diptera that possess so few hemocytes that only humoral (or melanotic) encapsulation occurs. Melanotic encapsulation is an enzymatic process whereby melanin polymers and proteins cross-link to form a melanotic coat

around a parasite, thus killing the invading entity (Paskewitz et al., 1988; Zhao et al., 1995).

The generalized cellular capsule consists of three regions. The innermost region, approximately 10 cells thick, is made up of granular cells. These cells are not flattened, but show signs of autolysis. The middle layer is approximately 20-40 cells thick and is solely composed of flattened plasmatocytes. The intercellular spaces between the plasmatocytes of this middle layer are filled with electron-dense material. The outer region is a covering of granular cells, one to ten layers thick. Often the capsule undergoes some degree of melanization (Götz and Boman, 1985; Götz, 1986; Tanada and Kaya, 1993; Peck and Strand, 1996; Gillespie, et al, 1997; Chapman, 1998). While granular cells and plasmatocytes are the only hemocytes generally observed in capsules, a few studies have mentioned the presence of other hemocyte types including thrombocytoids (which may be misclassified granular cells), lamellocytes (which may be misclassified plasmatocytes), and oenocytoids. Some of these observations may be fallacious and a result of the inconsistencies in hemocyte identification and classification in the years prior to immunolabeling (Götz and Boman, 1985). Oenocytoids may play an indirect role in encapsulation by providing the pro-phenoloxidase used for capsule melanization.

Encapsulation sequence of events: There is a great similarity in the actual process of cellular encapsulation throughout much of the Hexapoda. Based on observations from a number of insects including the American cockroach, *Periplaneta americana* (Lackie, 1981), the desert locust, *Schistocerca gregaria* (Lackie, 1981), the greater wax moth, *G. mellonella* (Gagen and Ratcliffe, 1976; Ratcliffe and Gagen, 1977), the cabbage white butterfly, *Pieris brassicae* (Gagen and Ratcliffe, 1976; Ratcliffe and

Gagen, 1977), the soybean looper, *Pseudoplusia includens* (Peck and Strand, 1996; Lavine and Strand, 2001) and the tobacco hornworm, *M. sexta* (Wiegand et al., 2000), the following generalized sequence of events can be constructed:

1) Hemocytes contact a foreign object via random movement or directed chemotaxis.
2) Granular cells that contact the foreign object adhere and degranulate.
3) Material discharged from granular cells binds to foreign surfaces and to hemocytes.
4) Substances released from granular cells attract other granular cells and plasmatocytes.
5) Plasmatocytes attach to the capsule, spread and flatten, forming a multilayer sheath.
6) Intercellular spaces between attached immunocytes fill with electron-dense material.
7) Granular cells attached to the target object begin to disintegrate.
8) A second thin layer of granular cells covers the capsule.
9) The capsule melanizes.

The very first events of cellular encapsulation occur within minutes of an intrusion of a foreign entity. Granular cell attachment occurs within the first minute and by 5 minutes the intruding object is coated with the first layer of cells. The entire encapsulation process can be completed in as little as 2 hr, but on average the encapsulation process takes between 12 to 24 hr (Götz and Boman, 1985; Peck and Strand, 1996). Studies of hemocytes from *P. includens* have shown that both granular cells and plasmatocytes are required to properly form cellular capsules, with granular cells being the first cell type to attach to the target (Pech and Strand, 1996; Lavine and Strand, 2001). In *in vitro* experiments using isolated granular cells and with Dowex 1X-2 beads as the target, the granular cells initially attached to the beads as they would in the

initial phase of encapsulation, but did not form overlapping cell layers. In a similar experiment in which only plasmatocytes were present, only 0.4 percent of the Dowex beads became encapsulated after 24 hr as compared to about 43 percent in assays involving unsegregated hemocytes. However, when the Dowex beads were preincubated in a medium conditioned by granular cells, the number of beads encapsulated in plasmatocyte-only assays increased to 24 percent. This suggests that granular cells possess a signal that recruits and activates plasmatocytes into capsule formation (Pech and Strand, 1996).

This observation from Pech and Strand agrees well with the proposed mechanism of cell recruitment into the encapsulation process put forward by Götz and Boman (1985). They noted that of all the hemocytes that compose the capsule, only the first layer has contact with the target surface, thus the signal that recruits the cells of the first layer must be different from the signal that recruits the cells that form the subsequent layers. Furthermore, because the capsule does not grow indefinitely, the signal to recruit new hemocytes for encapsulation must decrease with each increased capsule size.

Studies have shown that there exist many factors that affect the frequency and degree to which an object is encapsulated. One factor is host age. The frequency of parasitoid egg encapsulation in nymph soft scales (Homoptera: Coccidae, various spp.) is relatively low as compared to ovipositing female soft scales, which often encapsulate all eggs and thus thwart parasitism (Blumberg, 1997). Enhanced encapsulation of parasitoid eggs in older staged insects has also been observed in other insects including the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae) (Berberet 1982), Egyptian alfalfa weevil, *Hypera brunneipennis* (Coleoptera: Curculionidae) (Van den Bosch and Dietrick,

1959), various lygus bugs, *Lygus* spp. (Hemiptera: Miridae) (Debolt, 1991), noctuid moths (Lepidoptera: Noctuidae) (Lynn and Vinson 1977), fall webworm moth, *Hyphantria cunea* (Lepidoptera: Arctiidae) (Morris, 1976), and cabbage white butterfly, *Pieris rapae* (Lepidoptera: Papilionidae) (Van Driesche, 1988).

A second factor affecting encapsulation is host strain. The same insect host species of differing geographic location are known to differentially encapsulate the eggs from a single parasitoid stain. The brown soft scale, *Coccus hesperidum*, from California will encapsulate 93% to 100% of the eggs from the parasitoid *Metaphycus helvolus* (Hymenoptera: Encyrtidae), but brown soft scale from Israel encapsulates only 71% to 74% of *M. helvolus* eggs, and brown soft scale from the Netherlands encapsulate only 40% of the parasitoid eggs (Blumberg, 1997). In another example, the parasitoid *Encyrtus lecaniorum* (Hymenoptera: Encyrtidae) originally obtained from the Mediterranean area successfully produced offspring from 98% of brown soft scale from Texas, but in only 2% of the brown soft scale from California (Bartlett and Ball, 1966).

Host stress can also cause a reduction in the rate of parasite encapsulation. Exposure of three species soft scale insects (the brown soft scale, *Coccus hesperidum*; the hemispherical scale, *Saissetia coffeae*; and the pyriform scale, *Protopulvinaria pyriformis*) to the extreme temperature of 40º C for 24 h prior to being parasitized by either *Metaphycus swirskii* (Hymenoptera: Encyrtidae) or *Encyrtus infelix* (Hymenoptera: Encyrtidae) significantly decreases the encapsulation response (Blumberg, 1976; Blumberg and Goldenberg, 1992). [On the other hand, increased temperatures within a biologically natural range for scale insects, 20° C to 33° C, will produce a corresponding increase in the number of parasitoid eggs encapsulated (Blumberg, 1997).] Other stresses

known to decrease the level of encapsulation within scale insects include increased number of parasitoids per host (Blumberg, 1997). This has also been observed in caterpillars of the tomato moth (*Lacanobia oleracea*) (Salt, 1959) and in the Egyptian alfalfa weevil (Van den Bosch and Dietrick, 1959). Also removal of a scale insect from its host plant or detachment of a leaf upon which a scale is feeding will induce increased stress on the insect and result in a lower level of encapsulated parasitoid eggs (Blumberg, 1982).

Recognition of targets of encapsulation: In order for the innate immune systems of insects to function, the physical surface properties of non-self bodies must be recognized by a set of genetically predetermined receptors in the host. The principles and mechanisms of target recognition for encapsulation are the same as those mentioned above for phagocytosis and involve the presence of PRRs on the surfaces of the hemocytes that possess an affinity to certain PAMPs on biotic targets or some other molecular characteristic on abiotic targets (Lavine and Strand, 2002). An examination of the *Drosophila* genome revealed that it contains several genes that code proteins that possess both a sequence homology similar to peptidoglycan recognition proteins and a transmembrane domain. Northern blot analyses showed that one of these genes is expressed in hemocytes, thus suggesting the presence of membrane-spanning PRRs on these cells (Werner et al., 2000).

As will be mentioned below and throughout the rest of this compendium, there exists strong evidence that integrins, a large family of (α,β)-heterodimeric transmembrane proteins, play an important role as PRRs on immunocytes.

Properties of substratum that elicit encapsulation: Within a single insect

species, different materials evoke different degrees of encapsulation (Götz, 1986). Obligate parasitoids and parasites as well as conspecific transplants of organs or tissue are most often not encapsulated, whereas accidental parasites and interspecific transplants will produce an immune response (Tanada and Kaya 1993). Of those biotic entities that do not normally generate an immune response, most will become encapsulated if they are wounded or if their surfaces are altered in some other way (Tanada and Kaya 1993; Lavine and Strand, 2002). Likewise, different insect species can exhibit different encapsulation responses to the same target. For example, newly hatched oncospheres of the Cestoda *Hymenolepis diminuta* are encapsulated within the American cockroach, *P. americana*, but are not by the locust *S. gregaria* (Lackie, 1981).

The physical surface properties of different materials can play a role in the degree an object becomes encapsulated. Hemocytes of *P. americana* adhere more readily to polystyrene of greater hydrophilicity and negative charge. In addition, charged Sepharose beads, either positive or negative, received a thicker encapsulating coat of cells than Sepharose beads of neutral charge. These results appear to be species specific. In the same study, hemocytes of *S. gregaria* adhered to polystyrene surfaces to the same minimal degree regardless of charge or wettability, and did not encapsulate negatively charged Sepharose beads (Lackie, 1983). In a set of *in vitro* experiments with the soybean looper, *P. includens*, chromatography beads of various matrices tended to be encapsulated more readily if they possessed a positive charge (Lavine and Strand, 2001). *In vivo* assays comparing the encapsulation of chromatography beads with four different noctuid moth species, CM-Sephadex and CM-Sepharose (both negatively charged) did not encapsulate well in *P. includens* and *T. ni*, yet did so easily in *H. virescens* and

Spodoptera frugiperda (Lavine and Strand, 2001). It has been speculated that this interspecific variation in the encapsulation response may be a result of the variation of the surface charges on the hemocytes themselves. *S. gregaria* hemocytes possess a charge significantly more negative than hemocytes of *P. americana* This electrostatic mechanism could explain why hemocytes of *S. gregaria* are seemingly not attracted to negative substrata (Takle and Lackie**,** 1985).

The various molecular units attached to a foreign entity's surface also play a major role in the ability of hemocytes to recognize the entity as a target of encapsulation. *In vitro* assays with *P. includens* hemocytes demonstrated that chromatography beads with differing functional groups exhibited significant variations in their ability to be encapsulated. Chromatography beads with the functional groups diethylaminoethyl (DEAE), quaternary amines (Q) or sulfonic (S) were normally encapsulated more readily than other bead types examined. The effects of the functional group have shown to be greater than that of other recognition properties including charge and the matrix of the chromatography bead (Lavine and Strand, 2001).

Opsonizing targets of encapsulation: Some targets require opsonization in order for hemocytes to recognize them for encapsulation. In *in vitro* assays, the hemocytes of *P. includens* do not encapsulate the chromatography beads SP-Sephadex, CM-Sephadex, DEAE-MacroPrep and Q-MacroPrep unless the beads are first preincubated in the host plasma. As mentioned above, *in vitro* experiments with *P. includens* hemocytes, plasmatocytes alone cannot encapsulate chromatography beads. However, plasmatocytes by themselves will encapsulate certain bead types (e.g. SP-, QAE-, CM- and DEAE-Sephadex) if these beads are preincubated in the insect's plasma. Preincubating CM-

Sephadex beads in plasma prior to injecting them into the hemocoel of larval *P. includens* significantly reduced the time required for the beads to become encapsulated (Lavine and Strand, 2001). These experiments indicate that there exist factors in the plasma of insects that help target foreign entities for cellular immune attack.

In the lepidopteran species *P. includens* and *M. sexta*, a peptide has been identified that facilitates the adhesion and spreading of plasmatocytes (Clark et al., 1997; Yu et al., 2001). This 23 amino acid plasmatocyte spreading peptide (PSP) is expressed as a 142 propeptide by fat body, nervous tissue and granular cells (Clark et al., 1998; Strand et al., 2000). The mature PSP binds to a 190 kDa protein from hemocytes, the identity of which has yet to be determined (Clark et al., 2004).

Encapsulation involves an RGD-dependent mechanism for cellular adhesion: In order for encapsulation to occur, it is necessary that the immunocytes involved possess adhesion molecules that can perform cell-substratum and/or cell-cell bonding (Johansson, 1999). Thus, to fully understand the workings of the encapsulation process, it is important to discern the molecular basis of immunocyte adhesion. An insight to how this mechanism functions in insects came through the discovery that peptides containing the amino acid sequence arginine-glycine-aspartic acid (RGD) can inhibit cellular encapsulation and have other profound effects on immunocyte behavior (Pech and Strand, 1995). Using hemocytes from *P. includens*, Pech and Strand (1995) demonstrated that soluble Arg-Gly-Asp-Ser (RGDS), but not Arg-Gly-Glu-Ser (RGES), inhibits plasmatocytes and granular cells from spreading on the plastic surfaces of culture plates. This suggests that the RGDS peptide may specifically bind to a receptor on these hemocytes that is directly involved with cellular attachment and spreading on surfaces.

The importance of RGD in hemocyte attachment and spreading was further demonstrated in *in vitro* experiments in which RGDS-Sepharose beads, but not RGES-Sepharose beads, were encapsulated when placed in a solution containing free-floating *P. includens* blood cells. Moreover, in assays with purified populations of hemocytes, RGDS-Sepharose beads, but not RGES-Sepharose beads, could be encapsulated solely with plasmatocytes without the presents of granular cells, while granular cells alone could only form the initial layer of attached cells. The *in vitro* encapsulation of RGDS-Sepharose beads was inhibited if the assay was performed in a solution containing soluble RGDS (Pech and Strand, 1995).

In vivo studies showed that RGDS-Sepharose beads, when injected into the hemocoel of larval *P. includens*, were readily encapsulated within 3 hr and solely with plasmatocytes, while RGES-Sepharose beads were never encapsulated within this period of time and could take as long as 24 hr to be encapsulated (Pech and Strand, 1995).

The totality of the above evidence suggests that a mechanism of cell adhesion mediating hemocyte attachment and spreading upon a foreign target involves adhesion molecules on the immunocytes that contained an RGD recognition sequence. Pech and Strand (1995) speculate that granular cells, as the first hemocyte type to recognize and attach to foreign entities, release during degranulation a protein possessing an RGD sequence that opsonizes the intruding body that results in capsule formation by plasmatocytes.

The significance of RGD as a recognition sequence to which hemocyte adhesion proteins bind is that this peptide motif is known to be bound by adhesion proteins of the integrin family (Hynes, 1987; 1992; Haas, and Plow, 1994; Johansson, 1999; Plow et al,

2000). The role of RGD as a ligand of integrins has been mostly studied in humans and other mammals (Hynes, 1987; 1992; Ruoslahti, 1996), but has also been studied in several invertebrates (Johansson, 1999).

2) Integrins:

Integrins are a major family of animal transmembrane adhesion proteins that physically link the cytoskeleton to extracellular entities (Hynes, 1987, 1992; Hemler, 1990; Ruoslahti, 1991; Calderwood et al., 2000; McDonald, 2000; van der Flier and Sonnenberg, 2001; Alberts et al., 2002). Present throughout the Animalia, integrins are expressed on all cells that are adhesive or are capable of rapidly become adhesive in response to stimuli (Takagi et al., 2001). These molecules constitute the primary receptors on the plasma membrane that mediate cell attachment to the extracellular matrix (ECM). The ECM, composed of an intricate network of proteins (e.g. collagen, fibronectin, laminin) and polysaccharides, is the scaffolding to which the body's cells attach for support (Alberts et al., 2002). In addition, integrins participate in adhering cells to other cells or to other extracellular entities such as pathogens, parasites or abiotic objects (Hynes, 1987, 1992, Ruoslahti, 1991; Nelson and Cox, 2000; van der Flier and Sonnenberg, 2001; Alberts et al., 2002). Specifically, integrins serve as connector molecules that link extracellular ligands to a cell's cytoskeleton and thus function as conduits of mechanical force across the plasma membrane (Tamkun et al., 1986; Grinblat et al., 1994; Burridge and Chrzanowska-Wodnicka, 1996; Calderwood et al., 2000, 2002; Alberts et al., 2002; Foletti et al., 2005). The name "integrin" was coined for both the integral membrane nature of these proteins and for integrating extracellular ligands with the cytoskeleton (Tamkun et al., 1986).

Integrins not only function as adhesive molecules, but are also involved in signal transduction. The adhesive behavior of integrins is regulated by the bi-directional signaling that is transmitted through these molecules (Hynes, 1992; Schwartz, 1995; Burridge and Chrzanowska-Wodnicka, 1996; Defilippi et al., 1997; Yamada, 1997; Takada, 1997; O'Toole, 1997; Giancotti and Ruoslahti, 1999, Qin et al., 2004). Interactions between the integrin cytoplasmic regions with various cytoplasmic proteins can induce an inside-out signal transduction event that will travel the length of the protein to mediate the adhesive nature of the extracellular ligand binding site. Likewise, the binding of ligand to the extracellular binding site can induce an outside-in signal through to the integrin cytoplasmic regions, which in turn interacts with various cytoplasmic proteins resulting in numerous intracellular changes (see below for more detail) (Sastry, and Horowitz, 1993; Calderwood et al., 2000; Calderwood, 2004; Qin et al., 2004; Zhu et al., 2007).

Integrins are involved in nearly all animal biological processes that require cellular adhesiveness. These receptors mediate myriad cellular functions throughout the Animalia including cellular response to mechanical stress, skin integrity, clot retraction, hemostasis, tissue repair, inflammation and immune response, muscle attachment and function, bone resorption, leukocyte function (including activation, homing, diapedesis and phagocytosis), cellular migration, apoptosis, embryogenesis (including cell differentiation, gastrulation, neural crest migration, lymphangiogenesis, vasculogenesis, neuronal path-finding, morphogenesis), proper development of wing epithelia, and tumor cell growth and metastasis (Newgreen et al., 1982; Leptin et al., 1989; Wilcox et al., 1989; Duband et al., 1996; Springer, 1990; Wilcox, 1990; Balzac et al. 1993; DeSimone,

1994; Grinblat et al. 1994; Brower et al., 1995; Clark and Brugge, 1995; Bunch et al., 1998; Hogg and Bates, 2000; Gasque, 2004; Bennett, 2005; Sackstein, 2005; Morozevich et al., 2006; Pylayeva and Giancotti, 2006; Wiedemann et al., 2006; Takada et al., 2007).

Integrins are heterodimeric proteins consisting of two noncovalently linked type-I glycopeptide subunits; an α-subunit ranging in size from 120-180 kDa and a β-subunit whose size ranges from 90-110 kDa (Hynes, 1992, Humphries et al., 2006). Electron microscopy and crystallography reveal that these two chains assemble into a single globule headpiece with two stalk regions that protrude through the plasma membrane and anchor in the cytoplasm (Carrell et al., 1985; Kelly et al., 1987; Nermut et al., 1988; Xiong et al., 2001; Adair and Yeager, 2002; Arnaout, 2002).

In mammals, 18 α- and 8 β-subunits combine to form the 24 receptors thus far discovered; note that not all 144 possible α/β subunit combinations exist (van der Flier and Sonnenberg, 2001; Humphries et al., 2006). α-subunits are homologous to all other α-subunits (sequence homology among the mammalian α-subunits ≈ 30 percent) and are believed to have evolved from a single ancestral α-subunit gene. The same is true for the β-subunits (sequence homology among mammalian β-subunits ≈ 45 percent) (see section on "Integrin origins"). However, α- and β-subunits are not homologous to each other and most likely evolved from distinct genes (Hynes, 1987; Eble, 1997; Burke, 1999; Hughes, 2001; Huhtala et al., 2005; Takada et al., 2007).

Both subunit chains participate in ligand binding (D'Souza et al., 1988; Smith and Cheresh, 1988). In electron micrographs, integrins appear as globular heads with two rod-like stalks, one for the extracellular C-terminal regions of each subunit, extending into the cell plasma membrane. It is the globular head region that binds ligand (Carrell, et al.,

1985; Kelly et al., 1987; Nermut et al., 1988; Weisel et al., 1992; Adair and Yeager, 2002).

Ligand specificity is determined by the various combinations the α- and β-subunits form. Not all α/β combinations bind all ligands of integrins; however single α/β combinations may possess multiple ligands (e.g. $\alpha_V\beta_3$ is known to bind tenascin, LAP-TGF-β, fibrinogen, fibronectin, thrombospondin, vWF and fibrillin) and multiple α/β combinations are known to bind an identical ligand (e.g. $\alpha_V\beta_3$, $\alpha_9\beta_1$ and $\alpha_8\beta_1$ can all bind tenascin) (Delwel et al., 1994; Niessen et al., 1994; van der Flier and Sonnenberg, 2001; Humphries et al., 2006). Redundancy in ligand specificity may have evolved to protect against phenotypic mutations in cases where a genotypic mutation rendered a particular integrin subunit nonfunctional. This role for redundancy is suggested in studies observing apical ectodermal ridge formation and organogenesis using single and double knockout mice for the α_3 and α_6 subunits (De Arcangelis et al. 1999). However, numerous other studies in mice exhibited distinct phenotypic changes for each integrin subunit knocked-out, suggesting that many integrins, while possessing redundancy in ligand specificity, have specific non-redundant function (Hynes, 2002). This also leads to the hypothesis that integrin function may be greatly influenced by which type of cell hosts the receptor.

The specificity of which subunits heterodimerize to form whole integrins and which ligands bind to those integrins is further refined through the alternative splicing of the subunit mRNA (Altruda et al., 1990; Languino and Ruoslahti, 1992; Balzac et al., 1993; 1994; Zhidkova et al., 1995) and by posttranslational modification including the glycosylation of some α or β subunits (van der Flier and Sonnenberg, 2001; Gu and Taniguchi, 2004).

The extracellular ligands with which integrins interact include (Plow et al., 2000; Humphries et al., 2006):

• **Laminin** (ECM protein that forms sheet-like networks in the basal laminae.)

• **Thrombospondin** (adhesive ECM protein synthesized, secreted by various cells including platelets - involved in cell adhesion, platelet aggregation, cell proliferation, angiogenesis, tumor metastasis, vascular smooth muscle growth, and tissue repair.)

• **Fibronectin** (ECM protein involved in adhesion of cells to matrix and help direct migrating during development.)

• **Collagen** (ECM protein; major component of ECM and connective tissue.)

• **Osteopontin** (ECM protein; regulates bone metabolism.)

• **BSP** (bone sialoprotein: a significant protein component of the bone ECM.)

• **Del-1** (developmental endothelial locus-1: ECM protein; mediates attachment of endothelial cells to ECM – involved in vascular development.)

• **Vitronectin (**protein found in blood plasma and the extracellular matrix – regulates coagulation, fibrinolytic, and complement cascades, also involved in tissue remodeling.)

• **vWF** (von Willebrand factor: a blood protein involved in coagulation - important in platelet adhesion to wound sites.)

• **Tenascin** (ECM protein; regulates cell-matrix interactions including states of matrix attachment that promotes cell motility.)

• **PECAM-1** (platelet endothelial cell adhesion molecule-1 [also CD31]: protein secreted by endothelial cells and by platelets following thrombin activation - involved in platelet aggregation, tumor metastasis, vascular smooth muscle growth, and skeletal muscle repair. Also the receptor that allows *Plasmodium falciparum* infected erythrocytes to

attach to the vascular endothelium.)

• **LAP-TGF-β** (latency associated peptide-transforming growth factor β: TGF-β are a family of cytokines that are involved in cell proliferation, ECM synthesis, integrin expression, immune function, and development . In their secreted latent form, TGF-β is noncovalently associated with LAP. This LAP-TGF-β association is also referred to as the small latent complex (SLC).)

• **Fibrillin** (the protein that is the main component of extracellular microfibrils.)

• **Fibrinogen** (blood plasma protein: major substrate for blood coagulation. It is converted into the fibrin by the coagulation cascade protease thrombin.)

• **Factor X** (also prothrombinase and thrombokinase: a serine endopeptidase involved in the coagulation cascade; converts prothrombin to thrombin.)

• **C3bi**, (a complement protein formed from the cleavage of C3 into C3a and C3b. C3bi is an opsonin [an enhancer of phagocytosis] that attaches to the surfaces of pathogens and apoptotic cells, thus marking these particles for ingestion by leukocytes.)

• **ICAM** (intercellular cell adhesion molecule: adhesion molecules that possesses either five immunoglobulin-like domains [ICAM-1] or two [ICAM-2] immunoglobulin-like domains. These molecules are expressed on B and T lymphocytes, fibroblasts, keratinocytes, and endothelial cells and function as the ligand for the leukocyte integrin "leukocyte function–associated antigen 1 [LFA-1].")

• **VCAM-1** (vascular cell adhesion molecule-1: a molecule expressed by endothelial cells that promotes the adhesion of the following leukocytes; lymphocytes, monocytes, eosinophil granulocytes, and basophil granulocyte. VCAM-1 is the ligand for the integrin "Very Late Antigen-4 [VLA-4].

• **E-cadherin** (epithelial-cadherin: an epithelial transmembrane protein; Ca^{2+}-dependent adhesion molecules that bind together epithelial cells.)

Integrins are also metalloproteins whose ability to recognize and bind extracellular ligand is regulated by the presence of the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+}. Ca^{2+} in high concentrations is inhibitory and $Mg^{2+,}$ and Mn^{2+} are activators (D'Souza et al., 1988; Gailit and Ruoslahti, 1988; Smith and Cheresh, 1988; Shimaoka et al., 2002). Both the α and β integrin chains possess highly conserved low affinity cation binding sites called metal ion-dependent adhesion sites (MIDAS) (see more on MIDAS below) (Shimaoka et al., 2002). Studies show that the MIDAS on both the integrin chains are among the sites in which the divalent cations interact (see LIMBS and ADMIDAS in the section below on β-subunit structure for other cation binding sites) (Lee et al., 1995; Shimaoka et al. 2002).

Electron microscopy has revealed three conformations for integrin extracellular domains; (1) a bent (or closed) conformation corresponding to a state of low-affinity (unactivated) (2) a protracted conformation, but with a 'closed' headpiece corresponding to a state of intermediate-affinity (partially activated), and (3) a protracted conformation with an 'open' headpiece corresponding to a state of high-affinity (activated) (Beglova et al., 2002; Xiao et al., 2004). An example of the biological importance of the three conformational-affinity states would be leukocyte integrin ($\alpha_4\beta_7$) function; the low-affinity conformation for integrins occurs on circulating leukocytes, intermediate-affinity (rolling adhesion) on leukocytes rolling on endothelium that are detecting inflammation stimuli, and high-affinity (firm adhesion) for diapedesis (the migration of leukocytes through intact capillary walls) and localization at infection site (Chen et al., 2003).

Broadly, when in the inactive state, integrins exist in a highly bent structure (Fig. 1A). Upon activation, the integrin will open in a switchblade-like motion into its adhesive conformation (Fig. 1B) (Beglova et al., 2002).

2.1) Integrin domain structure:

2.1.1) <u>α-subunit domain structure:</u> Integrin α-subunits range in size from ≈950 to ≈1140 amino acids (Eble, 1997). The typical mature α-subunit consists of an extracellular region of ≈940 amino acids, a single transmembrane-spanning region ≈25 amino acids in length, and a short cytoplasmic region 20 to 50 amino acids in length.

The β-propeller domain: The α-subunit N-terminal region contains seven 60-residue repeats that have weak homology to one another (Tuckwell et al., 1994; Springer, 1997; Shimaoka et al., 2002). Each repeat is predicted to fold into a four-stranded β-sheet. Together, the seven β-sheets are configured in a torus, thus forming a ≈440 residue seven-bladed β-propeller (Springer, 1997, Xiong et al., 2001). Repeats 5, 6, and 7 of all α-subunits and repeat 4 on some α-subunits possess a Ca^{2+} binding site between strands 1 and 2 of the β-sheet (Chothia and Jones, 1997; Oxvig and Springer, 1998). [The β-strands of each repeat are numbered 1 through 4 going in the amino-terminal to carboxyl-terminal direction and arranged with β-strand 1 closest to the propeller axis and β-strands 2, 3 and 4 aligned in antiparallel successional order out towards the periphery (Chothia and Jones, 1997; Springer, 1997).] There is also a putative Mg^{2+} binding site on the upper face of the propeller at its axis where the ion interacts with amino acids from repeats 2 and 3 (Chothia and Jones, 1997; Springer, 1997).

The β-propeller domain interfaces with the I-like domain of the β-subunit (see β-subunit section below) to form the ligand binding site (Chothia and Jones, 1997; Springer, 1997; Zang et al., 2000; Xiong et al., 2002; Takagi et al., 2003).

I-domain: Nine of the eighteen mammalian α chains (mammalian subunits α_1, α_2, α_{10}, α_{11}, α_D, α_E, α_L, α_M and α_X) have an additional domain of ≈200 amino acids inserted between β-propellers 2 and 3. This domain is thus called the inserted (I) domain (Larson et al., 1989; Shimaoka et al., 2002). Crystal structures have shown that the I domain adopts a "Rossmann" fold, which consists of a central β-sheet of five parallel and one antiparallel β-strands that is surrounded by seven α helices (Lee et al., 1995; Qu, and Leahy, 1995). I domains also possess the highly conserved DxSxS divalent cation binding sequence known as a metal ion-dependent adhesion site (MIDAS) (Lee et al., 1995). The MIDAS is predicted to be located on the surface of the I domain and is known to bind such cations as Mg^{2+} and Mn^{2+} (Humphries, 2000; Plow et al., 2000).

Studies have demonstrated that the I domain also forms a site to which ligands bind (Randi and Hogg, 1994). The I domain exhibits two conformations, an open high-affinity conformation and a closed low affinity conformation. Experiments that locked the I domain in one of the two conformations via disulfide bonding by introduced cysteines showed that locking this domain in the open position activates ligand binding and locking it closed prevents ligand binding (Lu et al., 2001b).

It is the β-propeller together with the I domain (for those α chains that have it) that comprise the α-subunit's contribution to the integrin's globular head (Xiong et al., 2001;Adair and Yeager, 2002; Arnaout, 2002).

Stalk region: Extending out from the carboxyl-terminus from the β-propeller is the α-subunit stalk region, which connects the α-subunit head region to the plasma membrane (Xiong et al., 2001;Adair and Yeager, 2002; Arnaout, 2002). The stalk is formed by an amino-terminal thigh domain and two sequential carboxyl-terminal regions designated calf-1 and calf-2 (Arnaout, 2002). The thigh domain interfaces with the β-propeller and is nearly perpendicular to the β-propeller's central axis (Arnaout, 2002).

Connecting the thigh to calf-1 is the genu. This site exhibits great flexibility and is the location at which the α-subunit bends back and forth between the bent inactive position and the extended adhesive position (Arnaout, 2002, Xie et al., 2004; Nishida et al., 2006).

The calf-1 connection to calf-2 is largely hydrophobic and lacks an amino acid linker. This prevents any interdomain bending between these two regions (Arnaout, 2002). Modeling by Xiong et al. (2001) and Adair and Yeager (2002) suggests that there could be twisting between calf-1 and calf-2 as well as at the interface of the thigh with the β-propeller.

In α-subunits that lack I domains (mammalian subunits α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_v, and α_{IIb}), post-translational proteolytic cleavage occurs, resulting in a heavy and light chain that are linked via a disulfide bond. The heavy chain (M_r ~105 to 125) forms the extracellular region of the subunit while the light chain (M_r ~23 to 25) contains the membrane-spanning region. (Delwel et al.,1993; Du and Ginsberg, 1997; Defilippi et al., 1997; Eble, 1997; Berthet et al., 2000; van der Flier and Sonnenberg, 2001). This cleavage site is located within calf-2 (Arnaout, 2002). The two mammalian exceptions are α_4, which lacks an I domain but is cleaved at an unusual position near the middle of

the pro-peptide (Teixido et al., 1992) and α_E, which possesses an I domain, yet is cleaved near the N-terminous of the stalk (Shaw et al., 1994).

Transmembrane (TM) domain: The transmembrane (TM) domain of the α-subunit spans the plasma membrane once. The amino acids that comprise this domain are mainly hydrophobic and fold into an α-helix (Adair and Yeager, 2002). The TM functions mainly as a conduit for signal transduction between the extracellular and cytoplasmic regions of the α-subunit [see β-subunit TM section below for greater detail] (Qin et al., 2004; Schneider and Engelman, 2004).

Cytoplasmic tail: The cytoplasmic tail of the α-subunit is relatively short, ranging from 20 to 50 residues in length (Eble, 1997). The exception is α_7 with a 77 amino acid tail (Sastry and Horwitz, 1993). While there is a high degree of amino acid sequence conservation of the cytoplasmic tail among the vertebrates within an α-subunit group (i.e. there is >90% identity among the tails of α_3 from murine, human and bird sources), there seems to be very little similarity among the tails of the different α-subunit groups. This suggests a unique role for each cytoplasmic tail type (Sastry and Horwitz, 1993; Eble, 1997).

There does exist one motif that is shared among all α-tails, the consensus sequence GFFKR located adjacent to the TM domain (Sastry and Horwitz, 1993, Calderwood, 2004). Removal of or mutations to the GFFKR motif will constitutively activate integrins, thus the role of this motif is to maintain the integrin in a state of low affinity (O'Toole et al., 1994; Calderwood, 2004).

2.1.2) β-subunit domain structure: Integrin β-subunits range in size from around 730 to 800 amino acids (90–110 kDa) (Argraves et al., 1987; Fitzgerald et al.,

1987; Kishimoto et al., 1987; Law et al., 1987; Rosa et al., 1988; Zimrin et al,. 1988; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki, et al., 1990; Yuan et al. 1990; Erle et al., 1991; Moyle et al., 1991; Hynes, 1992; Eble, 1997). The exception is the mammalian β_4, which can contain as many as 1778 residues (205 kDa) due to its extraordinarily large cytoplasmic domain of over 1000 residues (variant lengths of cytoplasmic region occur due to alternative splicing) (Hemler et al., 1989; Kajiji et al., 1998; Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990).

The typical mature β-subunit consists of an extracellular region of ≈660 to 725 amino acid residues, a single transmembrane-spanning region ≈25 residues in length, and a short cytoplasmic region ≈53 residues in length with the exception of β_4 (Zimrin et al,. 1988; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki, et al., 1990; Yuan et al. 1990; Erle et al., 1991; Moyle et al., 1991; Hynes, 1992). The various domains of the β-subunit are as follows:

The PSI Domain: The first ≈50 N-terminal residues of the β-subunit comprise the PSI domain (Bork et al., 1999; Zang and Springer, 2001; Shimaoka et al., 2002; Xiong et al., 2004). This domain of integrin β-subunits shares sequence homology with the extracellular parts of over 500 signaling proteins primarily in the plexin and semaphorin families, thus giving rise to its name; **P**lexin, **S**emaphorin, **I**ntegrin domain (Bork et al., 1999). PSI containing proteins tend to be glycoproteins that mediate cell growth, differentiation and migration (Xiong et al., 2004). The PSI domain of β-subunits contain seven cysteines of which six are shared among all PSI domains (Bork et al., 1999; Shimaoka et al., 2002; Xiong et al., 2004). This region folds into a two-stranded antiparallel β-sheet that is flanked by two short helices (Xiong et al., 2004). The first

cysteine of the PSI domain pairs with a cysteine immediately N-terminal to the Cys-rich pseudo repeat region [see below] (Calvete et al. 1991; Zang and Springer, 2001), However, Xiong et al. (2004) claim that it is the second cysteine that pairs with the Cys-rich pseudo repeat region. A highly conserved tryptophan positioned immediately prior to the fifth cysteine is found in all PSI domains and forms a small hydrophobic core within this domain (Xiong et al., 2004).

The PSI domain is involved in integrin activation (Xiong et al., 2004). Evidence suggests that the long-range disulfide bond between the PSI domain and the cysteine immediately prior to the Cys-rich pseudo repeat region (see below) holds the integrin in the inactive position (Zang and Springer, 2001). Studies that disrupted this long-range disulfide bond, either by amino acid substitution (Zang and Springer, 2001; Sun et al., 2002) or by the binding of monoclonal antibodies (mAbs) to the PSI domain (Honda et al. 1995), results in an integrin that is constitutively active. It is believed that the disruption of this long-range disulfide bond allows the integrin to become more flexible, thus exposing the ligand-binding site of the protein (Xiong et al., 2004).

The I-like domain: About 60 amino acids carboxyl-terminal from the PSI domain is a highly conserved region known as the inserted (I)-like domain (Huang et al., 2000;Shimaoka et al. 2002). This name refers to the slight but discernible sequence homology to the I-domain of the integrin α-subunit (Huang et al., 2000; Shimaoka et al. 2002). Among the mammalian β-subunits, the I-like domain spans roughly 240 amino acids (Huang et al., 2000; Shimaoka et al. 2002). This domain has been implicated in the ligand binding function for the integrin (D'Souza et al., 1988; Smith and Cheresh, 1988; Loftus et al., 1990; Bajt et al., 1992; Lanza et al., 1992; Takada et al., 1992; Bajt and

Loftus, 1994; Bajt et al., 1995; Huang et al., 1995; Puzon-McLaughlin and Takada, 1996; Huang et al., 2000). Monoclonal antibodies (mAbs) specific to this region have been shown to obstruct the binding of ligands (Andrieux et al., 1991; Calvete et al., 1991; Huang et al., 2000).

Nine residues in from the amino-terminal start of the I-like domain is an absolutely conserved metal-binding DxSxS sequence motif present in all β-subunits known as a metal ion-dependent adhesion site (MIDAS) (Lee et al., 1995; Tozer et al., 1996). [A MIDAS is also present in the I-domain of the α-subunit (see section on α-subunits above).] Point mutations in the aspartic acid or either serine of this motif abolishes the ability of integrins to bind to ligands, thus implicating the MIDAS of the β-subunit as being integral to the function of ligand binding (Loftus et al., 1990; Takada et al., 1992; Bajt and Loftus, 1994; Bajt et al., 1995; Huang et al., 1995). Ligand binding by the β-subunit requires the presence of divalent cations (e.g. Ca^{2+}, Mg^{2+}, and Mn^{2+}) (Smith and Cheresh, 1988).

A second site within the I-like domain ≈90 amino acids to the carboxyl-terminal side of the MIDAS has also been linked to ligand binding function. The binding of polyclonal antibodies to this site blocked the integrin from binding to its ligand (Charo, et al., 1991). In addition, site-directed mutagenesis of amino acids at this location also resulted in the inhibition of ligand binding (Puzon-McLaughlin and Takada, 1996). It has also been shown that some cases of thrombasthenia, a rare autosomal recessive disease in which platelets fail to aggregate, are a result of a single nucleotide change that switches an arginine located in this second site to either a glutamine (Bajt et al., 1992), or to a tryptophan (Lanza et al., 1992) in β_3 subunits. This single mutation disrupts the adhesive

nature of platelet integrins.

The region between the MIDAS and this second binding site plays a role in integrin ligand specificity. Experiments that swapped a 39-residue segment from this region from one β-subunit into another resulted in a corresponding switch in ligand selection (Lin et al., 1997). Other experiments have shown that for some ligands the β-subunit binding site is the area immediately carboxyl-terminal to the DxSxS motif, thus suggesting that this site contributes to the integrin ligand-binding pocket (Pasqualini et al., 1995; Chen et al., 1998). (Although a study that substituted alanine for the various residues within this site on β_3 had no effect in inhibiting integrin $\alpha_{IIb}\beta_3$ from binding of fibrinogen (Bajt and Loftus, 1994).)

Crystal structures of integrins show that the β I-like domain folds into a cluster of three aligned divalent cation-binding sites. At the center of the cluster is the MIDAS. Flanking the MIDAS to one side is the ligand-induced metal-binding site (LIMBS) and on the other is the adjacent to metal ion-dependent adhesion site (ADMIDAS) (Xiong et al., 2001; Shimaoka et al. 2002; Xiao et al., 2004; Chen et al., 2006).

If Ca^{2+} is the only ion present or is present at high concentrations [10 mM], the ion occupies the ADMIDAS and has an inhibitory effect. Mn^{2+} competes with Ca^{2+} for the ADMIDAS site. When Mn^{2+} is in the ADMIDAS, the cation-binding cluster takes on a differing conformation that promotes ligand binding. At low Ca^{2+} concentrations [50 μM] in the presence of Mg^{2+}, Ca^{2+} occupies the LIMBS and has a positive effect. Mg^{2+} is the preferred cation for binding to the MIDAS. The MIDAS can take on two different geometries and it is the presence of Mg^{2+} at this site that stabilizes the conformation. If the MIDAS and LIMBS are occupied by Mg^{2+} and Ca^{2+} respectively, the MIDAS

stabilizes the cation-binding cluster in a high-affinity (firm adhesion) conformation. If the MIDAS and ADMIDAS are occupied by Mg^{2+} and Ca^{2+} respectively, the presence of Mg^{2+} offsets the inhibitory effect of Ca^{2+} and the cluster stabilizes in the intermediate-affinity (rolling adhesion) conformation (Chen et al., 2003).

ADMIDAS also appears to play a role in signal transduction through the integrin. In integrins with mutant ADMIDAS, signal transduction is disrupted. One result is, in spite of the mutations positive adhesive effect, cells possessing this type of mutant integrin fail to spread on adhesive substrates. Spreading is a dynamic process requiring cooperation between the integrin cytoplasmic domain/cytoskeleton and the extracellular region. Thus if a non-functional ADMIDAS prohibits transmembrane signaling, no spreading can occur (Chen et al., 2006).

The I-like domain associates with the β-propeller of the α-subunit I domain to form the integrin ligand-binding headpiece (Zang et al., 2000). Current understanding of integrin function is that in integrins with α-subunits containing an I domain, the I-like domain of the β-subunit acts in a regulatory role. This is based on evidence that shows that isolated α_L subunits locked in the open (i.e. binding-receptive) position by disulfide bonds will bind ligand equally well as wild-type $\alpha_L\beta_2$ integrin. Additionally, mAb that bind to the I-like domain of β_2 and inhibit ligand binding in wild-type $\alpha_L\beta_2$ did not inhibit ligand binding when bound to β_2 in $\alpha_L\beta_2$ integrins that were locked in the open position (Lu et al., 2001b; Shimaoka et al., 2002). Studies on integrins with α-subunits lacking I domains (specifically α4β7 and α5β1) show that the LIMBS and ADMIDAS of the β-subunit I-like domain mediates the binding of ligand to the β chain too (Chen et al., 2006).

The stalk and the cysteine-rich pseudo-repeat (or EFG-like) region: The remaining portion of the extracellular region, that which is carboxyl-terminal to the I-like domain, produces the stalk region for the β chain upon which the β-subunit globular headpiece sits (Adair and Yeager, 2002; Arnaout, 2002; Shimaoka et al., 2002).

Much of the stalk is composed of four consecutive cysteine-rich pseudo-repeats. These repeats show statistically significant sequence homology to epidermal growth factor (EGF) and EGF-like domains of other proteins (Yuan et al., 1990; Takagi et al., 2001; Tan et al., 2001). Because of this homology, the cysteine-rich pseudo-repeat region (CRR) is also referred to as the EGF-like domain with each individual repeat designated EGF-1 through EGF-4 (Adair and Yeager, 2002; Arnaout, 2002; Shimaoka et al., 2002). The repeats also show statistically significant sequence homology to one another, thus are most likely themselves to be evolutionarily related (Takagi et al., 2001).

Each repeat is ≈40 residues in length and generally contains 8 cysteines. The exception is EGF-1, which possess just six cysteines (Argraves et al., 1987; Fitzgerald et al., 1987; Law et al., 1987; Rosa et al., 1988; Zimrin et al., 1988; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki et al 1990; Tan et al., 2001; Takagi et al., 2001). The disulfide bonds formed by the cysteines of a repeat are to the other cysteines within the same repeat. The pairings of the cysteines within a repeat are predicted to be as follows; first-fifth, second-fourth, third-sixth, seventh-eighth (Takagi et al., 2001). Each repeat is predicted to fold in the manner typical of EGF motifs; that is of two antiparallel β-strands (Takagi et al., 2001).

As mention above (see PSI-domain section), the first cysteine N-terminal to EGF-1 forms a long-range disulfide bond with the very first (some say second) cysteine of the

mature β chain (Calvete et al. 1991; Zang and Springer, 2001; Xiong et al., 2004). This long-range disulfide bond causes the β-subunit to fold in a manner in which the PSI domain is proximal to EGF-1 (Xiong et al., 2001; Adair and Yeager, 2002; Arnaout, 2002).

One major function of the CRR is to be a conduit for signal transduction between the β chain cytoplasmic tail and the ligand-binding headpiece (Lu et al., 2001a; Shimaoka et al., 2002). Compared to other signaling proteins, the distance signals must travel through the β-subunit is relatively large (Du et al., 1993; Lu et al., 2001a; Takagi et al., 2001). Conformational changes to specific sites in the CRR can induce ligand binding in the headpiece. This has been deduced from experiments involving mAbs that bind to specific sites of the CCR and subsequently induce integrin activation. Evidence suggests that the binding of antibody creates a change in the conformation of the CRR that results in activating the receptor (Andrew et al., 1993; Du et al., 1993; Stephens et al., 1995; Faull et al., 1996). Thus, the current model of integrin function is that conformational changes at one end of the β-subunit (i.e. either in the I-like domain or cytoplasmic tail) will send a signal to the opposite end of the protein via conformational changes in the CRR.

Transmembrane (TM) domain: The β-subunit spans the plasma membrane once. This region contains ≈22 to 26 amino acids and spans ≈30 Å (Adair and Yeager, 2002). The amino acids that comprise the TM segment are mainly hydrophobic and fold into an α-helix as the case is for the TM region of the α-subunit (Adair and Yeager, 2002).

The TM segments of both the α and β chains are the conduit for propagating

signals back and forth between the extracellular and cytoplasmic regions of the integrin receptor (Qin et al., 2004; Schneider and Engelman, 2004).

In resting integrins, the β chain TM domain associates with the TM domain of the α chain in a parallel crossed α-helical structure (Fig. 1A) (Adair and Yeager, 2002; Qin et al., 2004). Upon the activation, these domains undergo dissociation and separation (Fig. 1B) (Lou et al., 2004; Qin et al., 2004; Yin et al., 2006). This TM separation is necessary for transmembrane signaling leading to integrin activation (Lou et al., 2004; 2005; Zhu et al., 2007). The activated integrins cluster into focal adhesions. It is believed that in these clusters the separated TM domains associate with TM domains from other integrins, but of the same chain type (i.e. α-subunit TM domains associate with other α-subunit TM domains and β-subunit TM domains associate with other β-subunit TM domains) in interaction called homotypic oligomerization of the transmembrane domains (Li et al. 2003; Qin et al., 2004).

Cytoplasmic tail: The carboxyl-terminal most region of the β-subunit is the cytoplasmic tail, usually consisting of 20 to 60 residues (Argraves et al., 1987; Fitzgerald et al., 1987; Kishimoto et al., 1987; Law et al., 1987; Rosa et al., 1988; Zimrin et al,. 1988; Ramaswamy and Hemler, 1990; Sheppard et al., 1990; Suzuki, et al., 1990; Yuan et al. 1990; Erle et al., 1991; Moyle et al., 1991; Hynes, 2002; Calderwood et al., 2000, 2003) The exception is the mammalian subunit β_4 whose tail possesses over 1000 residues (Hemler et al., 1989; Kajiji et al., 1998; Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). Due to its relative shortness and intrinsic lack of enzymatic activity, the cytoplasmic tail is believed to be a mediator of protein–protein interactions which anchor the receptor to the cytoskeleton and of signal transduction

through the receptor and signaling pathways within the cytoplasm (Fitzgerald, 2001; Chen et al., 2006).

In spite of the shortness of the cytoplasmic tail compared to the rest of the β chain, the specific amino acid sequence that comprises this domain profoundly influences the behavior of the entire integrin (Burridge and Chrzanowska-Wodnicka, 1996). This is readily demonstrated by the four naturally occurring isoforms for the human β_1 subunit; β_{1A}, β_{1B}, β_{1C} and β_{1D} (Altruda et al., 1990; Languino et al., 1992; Balzac et al., 1993; 1994; van der Flier et al., 1995; Belkin et al., 1996; 1997). Generated by alternative splicing, the amino acid sequences for all β_1 isoforms are homologous save for the unique alternatively spliced domain located at the very distal end of the COOH-terminal cytoplasmic tail; all β_1 isoforms even share the same first 26 membrane proximal amino acids in the cytoplasmic tail. Yet this small change at the terminal end of the β_1 subunit differentially affects cell adhesion, cell migration, cell proliferation, integrin localization in focal adhesions, and interactions with intracellular proteins that influence the signal transduction pathway and integrin attachment to the cytoskeleton (Languino and Ruoslahti, 1992; Balzac et al., 1993, 1994; Meredith et al., 1995, 1997, 1999; Belkin et al., 1996, 1997; Fornaro and Languino, 1997; Pfaff et al., 1998; Fornaro et al., 2000).

The cytoplasmic tails of most β subunits possess three conserved clusters of amino acids. Reszka et al. (1992) labeled these 3 clusters as cyto-1 (≈10 residues in length beginning ≈7 amino acids carboxyl-terminal from of the TM domain; β_4 lacks a cyto-1), cyto-2 (the first NPxY motif beginning ≈10 residues from the carboxyl-terminus of cyto-1), and cyto-3 (the second NPxY motif beginning ≈8 residues from the carboxyl-terminus of cyto-2).

Cyto-1 appears to play an inhibitory regulation role. Deletions in this region will result in an integrin constitutively in the high-affinity ligand binding state that is independent of all other cellular signals (Hughes et al., 1995; Lu et al., 2001c; Calderwood, 2004). Cyto-2 and –3 appear to mediate integrin activation. Deletions or mutations at these NPxY motifs will block integrins from switching from a low affinity state to a state of high affinity (Calderwood, 2004). The NPxY motifs also control the recruitment of integrins into focal adhesions (Vignoud et al., 1997).

Interactions between these three conserved clusters and various cytoplasmic proteins are important for integrin signal transduction (O'Toole et al., 1994, Clark and Brugge, 1995; Leisner et al., 1999), intracellular signaling (Fitzgerald, 2001), the localizations of integrins in focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Vignoud et al., 1997), and the anchoring the integrin receptor to the actin cytoskeleton (Grinblat et al., 1994; Burridge and Chrzanowska-Wodnicka, 1996; Maniotis et al., 1997; Calderwood et al., 2000; 2003).

The structural proteins that are known to anchor the β chain cytoplasmic tail to the actin cytoskeleton include F-actin, myosin, skelemin, talin, vinculin, α-actinin, filamin, tensin and paxillin (Otey et al., 1993; Burridge and Chrzanowska-Wodnicka, 1996; Calderwood et al., 1999; 2000; 2002; Lu et al., 2000; Liddington, and Ginsberg, 2002; Tadokoro et al., 2003).

The β tail also interacts with a number of intracellular signaling proteins including cytohesin-1, cytohesin-3, focal adhesion kinase (FAK), integrin linked kinase (ILK), integrin cytoplasmic domain-associated protein-1 (ICAP-1), and receptor for activated protein kinase C (Rack1) (Hemler, 1998; Liu et al., 2000). An outside-in signaling event

can result in an interaction between the cytoplasmic tail and these signaling proteins, leading to an intracellular cascade that can result in such cellular responses as clustering of integrins on the plasma membrane and the formation of focal adhesions, altered gene expression, apoptosis, cellular differentiation or cellular proliferation. An interaction with the cytoplasmic tail initiated by a signaling protein often leads to an inside-out signal transduction event (Burridge and Chrzanowska-Wodnicka, 1996; Liu et al., 2000).

In addition, the β cytoplasmic tail associates with calnexin, a chaperone protein that it is involved in assembling the α and β chains into a complete integrin protein (Lenter and Vestweber, 1994).

NPxY motifs are known to bind to certain proteins that possess phosphotyrosine-binding (PTB) domains. Some PTB domains require the NPxY tyrosine to be phosphorylated to bind, while others bind to this motif via a hydrophobic interaction with nonphosphorylated tyrosine or phenylalanine (Uhlik et al., 2005). In a set of *in vivo* experiments with integrin β_1, replacement of the two NPxY motif tyrosines with alanines made all PTB interaction with the cytoplasmic tail defunct and resulted in a complete loss of integrin function. This indicates that this motif is critical for integrin function. The replacement of the tyrosines with phenylalanines, however, resulted in a reduced loss of function. This would suggest that these NPxY motifs interact with a variety of proteins, some requiring tyrosine phosphorylation to bind, and some that do not (Chen et al., 2006).

The different phosphorylation states of the NPxY motif play an important role in regulating which cytoplasmic proteins interact with the integrin. For example, phosphorylated NPxY disrupts talin binding, but not tensin binding. This leads to the

speculation that tyrosine phosphorylation could be a regulatory switch for integrin function (McCleverty et al., 2007).

Experimental evidence indicates that the binding of talin to the NPxY motif of cyto-2 is necessary for inside-out integrin activation (Calderwood et al., 1999, 2000; 2002; Liddington, and Ginsberg, 2002; Tadokoro et al., 2003). It is believed that this binding event disrupts a salt bridge that links the α- and β-subunits, thus holding the integrin in the inactive state (Vinogradova et al., 2002).

Through outside-in signaling, interactions of the extracellular domain of the integrin modulate the ability of the β chain cytoplasmic tail to bind with the various cytoplasmic proteins. An unoccupied binding site in the extracellular domain suppresses the integrin from locating into a focal adhesion. Attachment of ligand to the integrin extracellular binding site creates conformational changes to occur along the length of the receptor. This action exposes the ligand-induced binding sites (LIBS) in the cytoplasmic tail, thus allowing the integrin to interact with the appropriate cytoplasmic proteins (LaFlamme et al., 1992; Burridge and Chrzanowska-Wodnicka, 1996). This event often initiates a protein kinase pathway resulting in the attachment of the integrin receptor to the cytoskeleton and to localization within focal adhesions (Fornaro and Languino, 1997; Fornaro et al., 2000; Fitzgerald, 2001).

2.2) Integrins throughout the Animalia:

Integrins have been exceedingly well studied in mammals, particularly humans. A PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed) search in April 2007 for the term "integrin" resulted in retrieving 35,000-plus citations with roughly 70 percent dealing with integrins from humans and another 23 percent were studies from

mice. Although only 2 percent of citations from the search pertained to integrins from invertebrates (with only 0.8% from insects), this protein has been found in every organism throughout the Animalia for which integrins where searched (see Burke, 1999 for early review of invertebrate integrins). The following is a near-complete survey of invertebrate taxa in which integrins have been described at the time of this writing:

• **Proifera**: the sponges (Brower et al., 1997; Wimmer et al., 1999; Kuhns et al., 2001).

• **Cnidaria**: represented by the coral *Acropora millepora* (Brower et al., 1997), and the jellyfish *Podocoryne carnea* (Reber-Muller et al., 2001).

• **Nematoda**: specifically *Caenorhabditis elegans* (Gettner et al. 1995; Lee et al. 2001; Cox and Hardin, 2004).

• **Mollusca**: including snails (Davids, et al., 1999; Plows et al. 2006) and the oyster *Crassostrea gigas* (Terahara et al., 2006).

• **Arthropoda-Crustacea**: represented by the signal crayfish, *Pacifastacus leniusculus* (Holmblad et al., 1997) and the kuruma prawn, *Penaeus japonicus* (Rojtinnakorn et al., 2002).

• **Arthropoda-Insecta**: represented in the Diptera by the fruit fly, *Drosophila melanogaster* (Wilcox, 1990; Bunch et al., 1992; Burke, 1999), the African malaria mosquito, *Anopheles gambiae* (Mahairaki et al. 2001; Moita et al., 2006) and the black fly, *Simulium damnosum* (Hagen and Klager, 2001); and in the Lepidoptera by the soybean looper, *Pseudoplusia includens* (Lavine and Strand, 2003), the tobacco budworm, *Heliothis virescens*, (Loeb et al., 2001; Loeb, 2006) and the tobacco hornworm, *Manduca sexta* (Levin et al., 2005).

• **Echinodermata**: represented by the purple sea urchin, *Strongylocentrotus purpuratus*

(Marsden and Burke, 1997) and the green sea urchin, *Lytechinus variegates* (Hertzler and McClay, 1999).

• **Urochordata**: represented by the ascidians/sea squirts, *Halocynthia roretzi* (Miyazawa et al., 2001; Miyazawa and Nonaka, 2004), *Ciona intestinalis* (Ewan et al., 2005) and *Polyandrocarpa misakiensis* (Huhtala et al. 2005).

When the research described in this dissertation commenced in the mid-1990s, practically nothing was known about integrins in invertebrates. At the time, the best characterized invertebrate integrins were from *Drosophila melanogaster*, a result of the extensive genetic research performed on this organism. The first reported mutation that later was shown to be in an integrin gene was *inflated*, which exhibited the phenotype of blisters on the wings (Weinstein, 1918; Wilcox et al., 1989). Other mutations of integrins include *mys* (*myospheroid*: embryonic lethal; muscles detach from site of attachment after first contraction and body subsequently rounds-up) (Wright, 1960; MacKrell et al., 1988), *mew* (*multiple edematous wings*: mostly larval lethal with abnormal gut morphogenesis. If adulthood is reached, wings display blisters.) (Brower et al., 1995), and *scb* (*scab*: embryonic lethal due to a failure of dorsal closure) (Stark et al., 1997). *D. melanogaster* is now known to possess five integrin α-subunits (α_{PS1} through α_{PS5}) and two β-subunits (β_{PS} and β_{ν} [beta-nu]) (Burke, 1999; Yee and Hynes, 1993; Stark et al., 1997; Humphries, 2000; Narasimha and Brown, 2006).

2.3) Integrin-like proteins in fungi and amoeboid protozoa: Integrins have long believed to be a protein found solely in Animalia, however integrin-like molecules have been identified from several amoeboid protozoa and yeast. A β_2 integrin-like protein was reported from the internal-primate protozoans *Entamoeba histolytica* (responsible for

amoebic dysentery in humans) and *Entamoeba dispar* (commensal in humans) (Pillai and Kain, 2005). This β_2 integrin-like protein is believed to be involved in allowing these protists to adhere to the host's intestinal and vascular endothelium. In the slime mold *Dictyostelium amoebae*, a transmembrane adhesion molecule with a number of features similar to integrin β-subunits, (i.e. domains with similar amino acid sequence and structure, similarity of overall protein function and association with the anchor protein talin), has been identified (Cornillon et al., 2006). Both the *Dictyostelium* and the *Entamoeba* are taxonomically related and are placed within the same phylum, Amoebozoa.

The parasitic yeast *Candida albicans*, the main causative agent of candidiasis, possesses a protein called fungal cell adhesion molecule (F-CAM), which is involved in allowing the yeast to adhere to host tissue. This protein exhibits similarities to integrins based on antigenic, structural and functional homologies (Tronchin et al., 1991). In addition, Gale et al. (1996) isolated a gene from *C. albicans* that codes for a protein, designated αInt1p, that shares numerous motifs with integrin α-subunits including "a putative I domain, two EF-hand divalent cation-binding sites, a transmembrane domain, and a cytoplasmic tail with a single tyrosine residue."

2.4) Integrin origins: The presence of integrins in all taxa of the kingdom Animalia leads to the likelihood that integrins evolved prior to the diversification of the animals. With the discovery of integrin-like proteins in Amoebozoa and fungi, Cornillon et al. (2006) speculate that the animal, protistan and fungal integrin and integrin-like molecules could have stemmed from the same ancestral protein. Genomic analysis of the Amoebozoa indicates that this group diverged from the fungus/animal clade after the

plant - fungus/animal split (1000 mya), but prior to the fungus - animal split (965 mya) (Bapteste et al., 2002; Eichinger et al., 2005). As integrin-like molecules are not believed to be present in plants, the hypothesis of Cornillon et al. implies that the first ancestral integrin originated between 1000 and 965 mya. (Note: Hughes [2001] phylogenetic analysis of just the animal integrin genes estimates the origin of the α-subunit occurred about 993mya [β-subunit estimate was unresolved]. This estimate is based on the phylogenetic analysis by Wang et al. [1999] of 571 sequences across 75 genes who place the plant-fungi-animal split at 1576 ± 88 mya and the Porifera-Cnidaria-Ctenophora) divergence between 1200-1500 mya. With integrin-like proteins now known from the Amoebozoa and fungi, the Wang et al. estimate would place the integrin origins between 1200-1664 mya.)

In the following set of experiments, I describe an integrin β-subunit found exclusively on plasmatocytes from *M. sexta* and demonstrate that this integrin β-subunit is involved in the process of cellular encapsulation.

Materials and Methods

Insects: Fourth or fifth instar larvae of *Manduca sexta* were used in all of the following experiments. The laboratory colony was originally started from eggs purchased from Carolina Biological Supply. Larvae were raised in incubators at 26°C under a photoperiod of 16 L: 8 D and fed on artificial diet as described by Dunn and Drake (1983).

Hemocyte Buffers: *Manduca* saline buffer (MSB) and anticoagulant saline (AC-saline) were prepared as according to Willott et al. (1994). MSB: pH 6.8, consisting of 4 mM NaCl, 40 mM KCl, 1.7 mM PIPES, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 146 mM sucrose, and 1 g polyvinylpyrrolidone per liter. AC-saline: pH 6.8, consisting of 4 mM NaCl, 40 mM KCl, 1.7 mM PIPES, 8 mM EDTA, 9.5 mM citric acid, 27 mM sodium citrate, 146 mM sucrose, and 1 g polyvinylpyrrolidone per liter.

Monoclonal Antibodies: The monoclonal antibodies (mAbs) specific for *M. sexta* hemocytes were produced by Willott et al. (1994). Table 1 lists all mAbs used in the following experiments, their immunoglobulin class, and hemocyte type to which they bind. Antibody-producing hybridoma cells were grown in RPMI medium with 1-5% fetal bovine serum. Antibody MS2, MS9 and MS13 were purified by ammonium sulfate precipitation followed by dialysis against phosphate buffer solution (PBS) and then MSB. MS2 and MS13 were further purified by protein A affinity chromatography (Pierce: AffinityPak™Prepacked Columns of ImmunoPure® Plus Immobilized Protein A).

Hemocyte spreading assay: Three fifth instar day 4 *M. sexta* larvae were chilled for several minutes until lethargic then surfaced sterilized with 70 percent ethanol. Hemolymph was extracted by dripping out through a distally amputated proleg and individually collected into three separate sterile polyproylene test tubes, one for each insect, each containing 2 ml ice cold sterile anti-coagulant (AC) saline. The hemolymph/AC saline mixture was mixed thoroughly with a polyethyene transfer pipette, followed by centrifugation at 300xg for 20 min at 4°C in order to pellet hemocytes. The supernatant was removed and replaced with fresh ice cold AC saline. The hemocyte pellet was then re-suspended in the AC saline with a transfer pipette and centrifuged again (300xg, 20 min, 4°C). After the second wash, the supernatant was removed, and the pellet was re-suspended in 3 ml ice cold sterile Manduca saline buffer (MSB). The cell count in each test tube was approximately $3.0x10^6$ cells/ml.

Each of the three hemocyte preparations was divided in half; one experimental and the other control. The experimental samples for each insect were further divided into three 50 µl aliquots with each receiving a different concentration of purified mAb MS13 (final concentrations were: 1.0 µg/ml, 0.1 µg/ml, or 0.01 µg/ml). Two separate controls for each insect were also prepared; a 50 µl hemocyte sample with the final concentration of 1.0 mg MS2/ml (MS2 is an IgG1 mAb that binds to granular cells) and a 50 µl hemocyte sample in MSB alone. All mixtures were incubated for 1 h at 4°C.

After incubation, 20 µl from each mixture was pipetted onto individual wells of a 12 well glass slide. The slides were left at room temperature (24°C) for 1 h. The cells were then fixed with a 4% solution of paraformaldehyde. Indirect immunofluorescence using MS9 (IgM mAb against plasmatocytes) was used to visualize plasmatocyte

spreading. Immunofluorescence photographs were taken of 3 to 4 microscope fields in each well, meaning that approximately 450 plasmatocytes were examined for each treatment (range 350–634). Each photograph was scored for the percentage of plasmatocytes that spread.

***In vivo* Encapsulation Assay:** Fourth instar larvae were chilled on ice for several minutes until they became lethargic. The larvae were then surface sterilized with 70% ethanol and injected with a solution containing a specific anti-hemocyte mAb or a control solution lacking antibodies. For assays involving MS16 (n=3), MS34 (n=5), X11G9 (n=3), Z7E8 (n=4), X2F2 (n=4) and X7C4 (n=4), larvae were injected with 100 µl of 2.5 to 10 times concentrated supernatant from mAb-producing hybridoma cell cultures. For assays involving mAbs MS2 (n=24), MS9 (n=3) and MS13 (n=6), larvae were injected with 100 µg purified mAb and 100 µg bovine serum albumin (BSA) dissolved in 50 µl MSB. Two controls were conducted. Control A (n=5) insects were injected with 100 µl of 10 times concentrated mAb-free hybridoma cell culture media. Control B (n=48) insects were injected with 300 µg BSA dissolved in 50 µl MSB.

Following this first injection, the larvae rested at room temperature for 1 h, were chilled again on ice, and then injected with approximately 100 DEAE-Sephadex beads stained with Congo red in 100 µl MSB (Lavine and Beckage, 1996). The larvae were place on diet at room temperature for approximately 24 h. Insects were then dissected, and all beads that could be found were removed and placed in a solution of 4 percent paraformaldehyde. Beads were then observed by phase contrast microscopy and scored for encapsulation. Beads were judged fully encapsulated if there was a minimum of one

complete layer of hemocytes attached with no surface of the bead visible. Beads were scored as partially encapsulated if more than 15 hemocytes were attached, but the surface of the bead was not completely covered with cells. Beads with fewer than 15 hemocytes attached were scored as not encapsulated (Fig. 2).

Statistical differences among the mean number of encapsulated beads from the various treatments were calculated by the Student-Newman-Keuls multiple comparison test.

Purification of a membrane protein recognized by MS13 and MS34 (performed by Dr. James Nardi, University of Illinios): Developing wings from insects 3 to 6 days post pupation, which are abundant in plasmatocytes, were removed and immediately frozen on dry ice prior to storage at -80°C. Wings were thawed and washed in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF; a protease inhibitor), 1 mM EDTA, and 80 μM phenylthiourea (for inhibition of melanization). Wing samples were then centrifuged at 10,000xg for 10 minutes.

To solubilize cell membrane proteins in the resulting pellet, the pellet was homogenized using a glass dounce homogenizer in a lysis buffer containing 10 mM triethanolamine (TEA), 0.15 M NaCl, 2% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate (DOC), 0.5 mM sodium EDTA, 1 mM PMSF, and the following six protease inhibitors each at a concentration of 1 μg/ml: leupeptin, pepstatin, antipain, chymostatin, Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). The homogenate was stirred for 1 h in the

cold and then centrifuged at 13,000xg for 30 min. The supernatant was re-centrifuged at 100,000xg for 1 h to remove any extra-fine cellular debris.

The supernatant was applied to a 2 ml affinity column containing mAb MS13 or MS34 coupled to agarose beads. To prepare the column, antibody was affixed to the beads in a solution of 0.1 M phosphate buffer (pH 7.0) containing 4-6 mg of purified MS13 or MS34 using sodium cyanoborohydride as a reducing agent. A flow rate of 5-6 column volumes (10-12 ml) per hour was maintained. After application of the wing lysate samples, the column was washed at 10-12 ml per h with 40 ml each in sequence of the following wash buffers (pH 8.2): (a) 10 mM TEA, 0.15 M NaCl, 1% NP-40; (b) 10 mM TEA, 0.15 M NaCl; (c) 10 mM TEA, 0.15M NaCl, 0.5% DOC; (d) 10 mM TEA, 1.0 M NaCl, 1% NP-40. All four wash buffers contained the protease inhibitors listed above for the lysis buffer, each at 1 μg/ml. Antigens to MS13 or MS34 bound to the affinity column were eluted with 12 ml of 50 mM triethylamine, 0.15 M NaCl, 1% NP-40 (pH 11.5). The elution buffer also contained all the protease inhibitors listed above for the lysis buffer, each at 1 μg/ml. Each 1.0 ml eluted fraction was neutralized with 30 μl of 1.0 M sodium phosphate (pH 4.25). Proteins eluted from the column were concentrated with Amicon microconcentrators and then precipitated by the addition of four volumes of 1:1 acetone:methanol followed by storage at –25ºC for at least 24 hours. The protein precipitate was collected by centrifugation at 10,000 rpm for 30 min.

Amino acid sequencing: The proteins isolated from MS13 and MS34 columns were dissolved in nonreducing sample buffer and resolved by SDS-PAGE (8% acrylamide). Gels were visualized by silver staining followed by Western blot analysis using MS13

and MS34 as primary antibody. A band ~90 kDa recognized by MS13 and MS34 from each preparation was excised from the gels. Samples of excised protein, approximately 10 μg each, were sent to the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University. At Yale, the samples were subjected to trypsin digestion. The resulting peptides were isolated by reverse phase HPLC and sequenced via Edman degradation.

cDNA cloning: Three degenerate primers based on the peptide sequences obtained from immunoaffinity purification were used for reverse transcription (RT)-PCR. The single forward primer MS34-5F (5′-CARTTYAARCCICARGTIAT-3′) was based on the peptide sequence QFKPQVM. The two reverse primers MS34-4BR (5′-ACRAAIGAIGGDATRTCRTARTC-3′) and MS34-4CR (5′-ACRAARCTIGGDATRTCRTARTC-3′) were based on the reverse complement for the sequence DYDIPSFV. RT-PCR was performed using total RNA from fifth instar *M. sexta* larval hemocytes as template, isolated as described by Yu et al. (1999). RT-PCR was carried out using Superscript II/Platinum Taq (SuperScript One-Step RT-PCR with Platinum Taq; Invitrogen) under the thermocycler conditions of 50°C, 30 min; 94°C, 2 min; followed by 35 cycles of 94°C, 15 s; 45°C, 30 s; and 72°C, 1 min, with a final incubation at 72°C, 10 min. The RT-PCR product was re-amplified using PCR Master Mix (Promega) under condition of 94°C, 30 s; 45°C, 30 s; and 72°C, 1 min for 35 cycles. PCR product was isolated by electrophoresis on a low melting point (LMP) agarose gel. DNA bands of expected size (≈ 800 bp) were excised and purified via Wizard Preps kit (Promega). This purified DNA was ligated with pGem-T vector (Promega) and used to

transform *E. coli* XL1 blue strain. Plasmids were isolated using Wizard Plus Minipreps (Promega) and sequenced by Iowa State University DNA sequencing facility.

To obtain a full length cDNA sequence, 3′ and 5′ rapid amplification of cDNA ends (RACE) was employed using the GeneRacer Kit version E (Invitrogen) and the following three gene specific primers based upon the partial DNA sequence obtained: FOR34R-1 (5′-AGTCGGAGCTGAGTACGCCGAACTGAA-3′) for 3′ RACE and REV34-3 (5′-TCCAGTTCGGCGTACTCAGCTCCGACTA-3′) and REV34-4 (5′-GCCTTGAGCCAGGCCTCCACCCTATTTC-3′) for 5′ RACE. The template used was hemocyte total RNA collected from day three fifth instar *M. sexta* larvae. The resulting DNA products were isolated by electrophoresis on a LMP agarose gel. DNA bands of expected size (≈ 1500 bp for 3′ RACE and ≈1175 bp [from use of primer REV34-3] and ≈800 bp [from use of primer REV34-4] for 5′ RACE) were excised and purified using Wizard Preps (Promega). Purified DNA was ligated into pGem-T vector (Promega), which was used to transform *E. coli* strain TOP10 (Invitrogen). Plasmids were purified using QIAprep spin miniprep kit (250) (Qiagen) and sequenced at the Kansas State University DNA sequencing facility.

Computer analysis of sequence data: Multiple sequence alignment was carried out using the ClustalW program: http://www.ebi.ac.uk/clustalw/ (Thompson et al., 1994). Potential O-glycosylation sites were predicted using NetOGlyc 3.0 (www.cbs.dtu.dk/services/NetOGlyc-3.0).

Northern blot analysis: Total RNA from hemocytes or fat body from naïve fifth instar larvae and from larvae 24 h after injection of *E. coli* was prepared as described earlier (Yu et al., 1999). Total RNA from Malpighian tubules, epidermis and midgut were prepared as described by Dittmer et al. (2004). RNA samples (20 μg) were resolved by electrophoresis in agarose gels containing formaldehyde, transferred to uncharged nylon membranes (GeneScreen Plus; DuPont) and probed with ^{32}P-labeled *M. sexta* β-integrin cDNA using ULTRAhyb (Ambion) as the hybridization solution. The radioactive probe was made with the Prime-a-Gene Labeling System (Promega), using the original 810 bp partial β-integrin cDNA as the template. To confirm equal RNA sample loading, membranes were re-probed with a ribosomal protein S3 (rpS3) cDNA (Jiang et al., 1996).

Expression and purification of β-integrin protein fragments and western blot analysis: Segments of the β-integrin cDNA were generated by RT-PCR using the SuperScript One-Step RT-PCR kit (Invitrogen). The three segments created and the primers used for their creation were: segment (a) (forward primer X34-30F [5′-CCGGAATTCAATCACTTAGGGACATGCGGG-3′] and reverse primer X34-270R [5′-CGGGGTACCTCATCACATTTTGCCGTCACCAGCGCT-3′]; 720 bp), segment (b) (forward primer X34-130F [5′-CCGGAATTCGCCGAGCACTTTCTTTTAGAT-3′] and reverse primer X34-270R; 420 bp) and segment (c) (forward primer X34-270F [5′-CCGGAATTCATGATAGGCATTATCAAACCC-3′] and reverse primer X34-700R [5′-CGGGGTACCTCATCAATTTCCTCTTGGGGGTGCCTC-3′]; 1290 bp). All forward primers possessed an Eco RI site for ligation into the transformation vector. All reverse primers contained two consecutive stop codons, followed by a Kpn I site to facilitate the

ligation into the transformation vector. Thermocycler conditions for RT-PCR were as follows: reverse transcription at 50°C, 30 min; 94°C, 2 min; followed by 40 amplification cycles of 94°C, 15 s; 55°C, 30 s; 72°C, 1 min; with a final incubation at 72°C, 10 min. The cDNA products were resolved by low melting point agarose gel electrophoresis. The bands of expected size were excised and purified with QIAquick Gel Extraction Kit (Qiagen). Following digestion with EcoRI/KpnI, the purified cDNA segments were ligated into the plasmid vector pPROEX HTa (LifeTechnologies), and then used to transform *E. coli* strain DH5α (Subcloning Efficiency DH5α Competent Cells (LifeTechnologies)). To express β-integrin segments, two 3 ml cultures of LB with 0.01% ampicillin were inoculated with 200 µl from an overnight culture and incubated with shaking at 37°C. When cell density reached an OD_{600} between 0.5 and 0.7, 30 µl of IPTG (100 mM) was added to one of the tubes for each sample pair to induce recombinant protein production. Bacteria were then incubated with shaking for 6 h to 8.5 h at 37°C. Cultures were centrifuged and pellet was frozen at –20°C. To lyse bacteria, each pellet was resuspended in 25 ml CelLytic B (Sigma) with 125 µg DNase I followed by centrifugation at 27000xg for 25 min at 4°C. The pellet was resuspended with 5 ml CelLytic B, 6 mg lysozyme and 25 ml Tris (20mM, pH 7.5). After centrifugation at 27000xg for 25 min at 4°C, the protein pellet was suspended in 20 ml equilibration buffer (0.1M sodium phosphate pH 8, 8.0 M urea) and then centrifuged at 12000xg to remove any remaining debris. The expressed protein was then purified via his-affinity chromatography by applying all 20 ml of the supernatant to a HIS-Select Cartridge (Sigma). The cartridge was washed once with 10 ml equilibration buffer and eluted with

10 ml elution buffer (0.1M sodium phosphate pH 8, 8.0 M urea, 250 mM imidazol). Eluted protein was collected in 1 ml fractions.

Prior to SDS-PAGE, the protein samples were prepared by mixing 1.7 µl protein sample with 0.8 µl 1.0 M sodium phosphate buffer (pH 8), 1.7 µl 6x SDS-PAGE buffer containing β-mercaptoethanol, and 5.8 µl H_2O. The prepared samples were then heated at 95° C for 5 min. Immediately following the heating step, the protein samples were then loaded into a 12.5% acrylamide Criterion Precast Gel (Bio-Rad) and underwent electrophoresis at 200 V for 50 minutes. Gels were either stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis. Primary antibodies used for Western blot analysis were anti-6 histidine (1:2000 dilution), MS13 (1:250 dilution of hybridoma cell supernatant) and MS34 (1:125 dilution of hybridoma cell supernatant). Secondary antibody was goat anti-mouse AP conjugate (1:3000 dilution).

Results

Monoclonal antibodies against insect hemocyte antigens are a potentially valuable tool for understanding the functioning of these cells. Insects from which anti-hemocyte mAbs were created include the American cockroach *Periplaneta americana* (Chain et al., 1992), the stick insect *Bacillus rossius* (Scapigliati et al., 1996), the soybean looper *Pseudoplusia includens* (Strand and Johnson, 1996), the flesh fly *Sarcophaga peregrina* (Hori et al., 1997), and the fruit fly *Drosophila melanogaster* (Kurucz et al, 2003). In the early 1990's, mAbs against the hemocytes of the tobacco hornworm, *Manduca sexta*, were generated to distinguish among the various blood cell types and (Willott et al., 1994) to probe the functions of hemocyte proteins. Using this group of *M. sexta* mAbs, I isolated and identified an adhesive protein on plasmatocytes that is essential to the encapsulation immune response.

Effect of mAb MS13 on plasmatocyte spreading: Wiegand et al. (2000) showed that plasmatocytes were inhibited from spreading on glass microscope slides after incubation with mAb MS13. To further characterize this effect, a dose response experiment was performed to determine the concentration of MS13 needed to inhibit encapsulation. Over the entire 100-fold range of antibody concentrations from 1.0 µg/ml to 0.01 µg/ml, MS13 inhibited the spreading of plasmatocytes as compared to the two controls of Manduca saline buffer (MSB) alone and a 1.0 µg/ml concentration of anti-granulocyte mAb MS2 (Fig. 3). The percentage of plasmatocytes that displayed spreading was not statistically different among the MS13 treated samples (Mann-Whitney Test); i.e. no dose response was observed. However, hemocytes at all three MS13 concentrations exhibited

significantly less spreading than either control. The two controls were not significantly different from one another. This result can be accounted for if all three MS13 concentrations tested exceeded a threshold required for binding sufficient antigen to block spreading of plasmatocytes.

***In vivo* assay analysis:** A group of mAbs previously shown to label the surface of plasmatocytes was screened for their ability to inhibit the encapsulation of DEAE-Sephadex beads injected into the larvae of *M. sexta*. Fourth instar larvae were injected with one of ten mAbs that bind to hemocyte surfaces (Table 1) or with one of the two control substances followed by an injection of DEAE-Sephadex beads. After 24 h, the beads were dissected out of the larvae and scored for the degree to which they were encapsulated (Fig. 4).

Treating larvae with MS13 or MS34 significantly lowered the number of beads encapsulated as compared to the controls or to the other mAbs. Both of these mAbs specifically label plasmatocytes, but not other *M. sexta* hemocyte types (Fig. 5). In earlier experiments, MS13 had been shown to inhibit the intrinsic ability of plasmatocytes to spread (i.e. produce pseudopodia) on glass microscope slides (Wiegand et al., 2000). The remaining mAbs did not significantly lower the number of beads encapsulated.

Immunoaffinity purification and analysis of the membrane protein recognized by MS13 and MS34: Previous experiments have shown that the antigens recognized by MS13 and MS34 are associated with the plasma membrane of plasmatocytes and that they can be extracted with detergents (Jeremy Gillespie, Sherry Anderson, Michael

Kanost; personal communication). This suggests that these antigens are integral membrane proteins.

In order to isolate and identify the corresponding antigens on plasmatocyte surfaces to which MS13 and MS34 bind, thus causing the inhibition of encapsulation, two immunoaffinity columns, one for each of these mAbs, were created. A lysate from the developing wings of pupating *M. sexta* was passed through these columns. This tissue was chosen for isolating the antigen due to the abundance of hemocytes that accumulate in the hemocoel between the monolayers of the developing wings. Proteins eluted from the column were separated by SDS-PAGE and visualized by silver staining and by immunoblotting with MS13 or MS34 as the primary antibody. Both MS13 and MS34 recognized a single band at approximately 90 kDa (Fig. 6A and 6B).

To identify the 90 kDa protein antigens recognized by the mAbs MS13 and MS34, the purified proteins eluted from each column were subjected to trypsin digestion followed by Edman degradation of purified peptides. This procedure revealed for the antigen recognized by MS13 the two peptide sequences LDYDIPSFVR and THELVINPVSLNDK (Fig. 7, yellow highlighted fragments C and D) , and for MS34 the three peptide sequences, IQFKPQVMR, EALISQANQIYK and LDYDIPSFVR (Fig. 7, yellow highlighted fragments A, B and C). It is of interest to note that the peptide sequence LDYDIPSFVR is common to both antigens, thus suggesting that MS13 and the MS34 recognized related or identical proteins.

cDNA cloning and analysis: The peptide sequence data from the Edman degradation were used to design degenerate oligonucleotide primers that were used for reverse

transcription-PCR (RT-PCR) using hemocyte RNA as template. Primers based on the MS34 antigen sequences QFKPQVM and DYDIPSFV (Fig. 7, fragments A and C) yielded an 803 bp product that encoded a protein fragment with high similarity to the β-subunit of integrins. 5′ and 3′ RACE was employed to obtain a full-length sequence of 2426 bp (Genbank accession AY630342). This complete cDNA sequence possesses a 2301 bp open reading frame that encodes a deduced amino acid sequence of 767 residues. Searches of amino acid sequence databases revealed that the protein has significant similarity with integrin β-subunits (Fig. 7), including 35% identity to a *Drosophila melanogaster* β-integrin known as βPS (Position Specific) or myospheroid (MacKrell et al., 1988), 34% identity to a β-integrin from the mosquito, *Anopheles gambiae* (accession XP_311354), and 31% identity to integrin βPi-1 from *Pseudoplusia includens* (Lavine and Strand 2003). The *M. sexta* protein also had 33% identity with β-integrins from hemocytes of a crustacean (the crayfish, *Pacifastacus leniusculus*) (Holmblad, 1997) and a mollusk (the Pacific oyster, *Crassostrea gigas*) (Terehara, 2003).

The deduced protein possesses a predicted 19 amino acid signal sequence (Levin et al. 2005), a 675 amino acid extracellular region (Gln20-Lys695), a 22 residue hydrophobic membrane-spanning domain (Trp_{696}-Trp_{721}), and a 46 residue cytoplasmic tail. All four peptide sequences obtained by the Edman degradation from both the MS13 and MS34 antigens are also present in the deduced sequence. The sequence includes 10 potential N-glycosolation sites, one potential O-glycosylation site at Thr_{391}, and 52 cysteines, 42 of which are conserved among all known integrin β-subunits from other arthropods. The predicted protein exhibited the following sequence characteristics common to nearly all β-subunits of integrins (refer to Fig. 1B; Fig. 7; and Fig 8A):

1) An amino-terminal PSI domain consisting of the first 50 residues of the mature protein. Consistent with PSI domains from all other β-subunits, the predicted protein contains seven cysteines and a tryptophan immediately prior to the fifth cysteine, all located in highly conserved positions. This domain type is shared with plexins and semaphorins, thus giving rise to its name; Plexin, Semaphorin, Integrin.
2) An I-like domain spanning the 95 residues from Pro_{128} through Asp_{223}. This domain acts together with the β-propeller (and the I-domain if present) of the integrin α-subunit to form the extracellular ligand-binding site (Diamond et al., 1993; Michishita et al., 1993; Bajt et al., 1995; Plow et al., 2000; Zang et al., 2000; Shimaoka et al., 2002).
3) A metal ion-dependent adhesion site (MIDAS; residues Asp_{136}-Thr_{140}) within the I-like domain. The MIDAS of this *Manduca* integrin exhibits the distinctive DXSXS motif; however, it does possess a conserved substitution in the fifth position of threonine for the serine. Occupation of the MIDAS by Mg^{2+} or Mn^{2+} results in a conformational change in integrins that stimulates ligand binding (Bajt et al., 1995; Goodman and Bajt, 1996; Harris et al, 2000; Liddington and Ginsberg, 2002; Shimaoka et al., 2002).
4) A region with four consecutive cysteine-rich pseudo-repeats (Cys_{433}-Asp_{596}). This region shows similarity to motifs in epidermal growth factor. An unusual feature of the predicted protein is that the third repeat has only six cysteines instead of the typical eight. This atypical number of cysteines in the third

repeat is also a characteristic of mammalian subunits β_4 and β_8 (Moyle et al., 1991; Tan et al., 2001; Takagi et al., 2001). In mammalian integrins, the cysteine-rich pseudo-repeat region acts as a conduit for signal transduction between cytoplasmic tail and the ligand-binding headpiece (Lu et al., 2001a; Shimaoka et al., 2002).

5) An extremely conserved lysine (K_{722}) twenty-three amino acid residues downstream from the predicted start of the membrane-spanning domain.

6) Three conserved sequences on the cytoplasmic tail; the first corresponding to the residues His_{728}-Glu_{739} and the two NPXY motifs, Asn_{750}-Tyr_{753} and Asn_{762}-Tyr_{765}. The amino acids at these sites are required for proper localization of integrins in focal adhesions (Reszka et al., 1992; Ylänne et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Vignoud et al., 1997) and are known to interact with various cytosolic proteins including talin (Horwitz et al., 1986; Vignoud et al., 1997; Hemler, 1998; Pfaff, 1998; Yan, 2001; Calderwood et al., 1999; 2000; 2003), filamin (Hemler, 1998; Pfaff, 1998), α-actinin (Otey et al., 1990, 1993; Burridge and Chrzanowska-Wodnicka, 1996), and integrin cytoplasmic domain-associated protein (ICAP)-1 (Hemler, 1998; Calderwood et al., 2002; 2003; Chang, 2002).

In the published article describing the integrin β subunit from *M. sexta* (Levin et al. 2005), this protein was christened "*M. sexta* integrin β1." Upon later reflection, I believe that this designation may prove to be confusing with the nomenclature established for mammalian β subunits (i.e. β_1 through β_8), particularly since the *M. sexta* β subunit has the greatest degree of similarity with mammalian subunit β_3. Therefore, following the

nomenclature establish by Lavine and Strand (2003) for hemocyte integrin subunits found in the soybean looper, *P. includens*, the integrin β subunit characterized in this thesis will be referred to hereafter as βMs1.

Analysis of recombinant segments of integrin β-subunit: Immunoblot analysis was employed to identify the regions of βMs1 recognized by MS13 and MS34. To accomplish this, three truncated recombinant forms of the βMs1 were created: peptide-fragment (1) corresponds to amino acids 24 to 264 of the full length β subunit, which represents the region of the protein from the mature amino-terminus through just past the predicted I-like domain; peptide-fragment (2) corresponds to amino acids 124 to 264, which represents the region containing the predicted I-like domain; and peptide-fragment (3), corresponding to amino acids 264 to 694, which represents the region from just after the I-like domain to the predicted start of the membrane-spanning sequence (Fig. 8A). Analysis by SDS-PAGE confirmed that all three purified recombinant protein sequences were of expected size (Fig. 8B; Coomassie stain). Immunoblot analysis showed that both MS13 and MS34 recognize fragments 1 and 2, representing the amino-terminal end of βMs1. Neither mAb recognized the carboxyl-terminal fragment 3 representing the remainder of the extracellular portion of the β subunit (Fig. 7B; MS13 and MS34 blot). Recognition of peptide-fragment 2 indicates both mAbs bind to an epitope within the I-like domain.

This experiment establishes that the mAbs used to block encapsulation *in vivo* also bind to the integrin β-subunit fragments encoded by the cloned cDNA. Since it has been demonstrated that MS13 and MS34 bind to ligands on no other cell in *M. sexta*

other than those on plasmatocytes (Willott et al., 1994; Wiegand et al., 2000), this result further confirms that the antigen blocked by these mAbs *in vivo* and required for encapsulation is a β subunit of integrins.

Analysis of the *M. sexta* integrin β-subunit gene expression: Northern blot analysis was employed to investigate tissue-specific expression of βMs1. The βMs1 cDNA probe hybridized with a 2.3 kb RNA from hemocytes, a size consistent with that expected from the cDNA sequence (Fig. 9A). No transcript was recognized from total RNA samples from midgut, epidermis, Malpighian tubules, or fat body. Northern blotting was also performed to investigate if βMs1 is inducible by a bacterial challenge. Injection of larvae with *E. coli* did not significantly affect the expression of integrin β-subunit in hemocytes and did not result in detectable β-subunit mRNA in fat body (Fig. 9B).

These results in combination with fluorescent immunolabeling studies with MS13 and MS34 (Willott et al., 1994; Wiegand et al., 2000) indicate that the integrin β-subunit discovered in the above experiments are present solely on plasmatocytes. In addition, βMs1 appears to be constitutively present on plasmatocytes and its induction is not stimulated by a bacterial challenge from *E. coli.*

Discussion

The research presented here definitively demonstrates that an integrin located on the surface of plasmatocytes plays an important role in the encapsulation immune response in the tobacco hornworm, *Manduca sexta*. This investigation was accomplished through a series of experiments that first assayed mouse monoclonal antibodies (mAb) generated against *M. sexta* hemocytes (Willott et al., 1994) to determine which of these antibodies, if any, could inhibit the encapsulation of DEAE-Sephadex chromatography beads *in vivo*. This test was conducted under the assumption that if a mAb did inhibit encapsulation, it may be doing so by directly binding to a protein necessary for this immune response and physically or sterically hindering it from performing its function. If such a case did occur, then the inhibiting mAb could then be used for isolating its antigen. As reported in the Results section, two inhibiting mAbs were attained; MS13 and MS34 (Fig. 4). It is of interest to note that both of these antibodies bind to antigens solely found on the surface of plasmatocytes and not to any other hemocyte nor any other tissue in *M. sexta*. MS13 and MS34 were subsequently shown to prevent plasmatocytes from spreading on glass surfaces, a natural behavior of untreated plasmatocytes (Wiegand et al., 2000).

The mAbs MS13 and MS34 were chosen for the isolation their plasmatocytic antigens via immunoaffinity purification. This purification step was performed by Dr. James Nardi of the Department of Entomology, University of Illinois at Champaign-Urbana and made use of a lysate from the developing wings of pupating *M. sexta*, a tissue profuse with hemocytes. Immunoaffinity purification of the lysate with both MS13 and MS34 each resulted in the isolation of a single protein antigen approximately 90 kDa in

size (Fig. 6). This is in congruence with a previously performed Western blot analysis of hemocyte lysate with MS13, which recognized a protein of the same size (Wiegand et al., 2000). To further analyze these purified protein products, Dr. Nardi treated the MS13 and MS34 antigens with trypsin, which then underwent Edman degradation. The Edman degradation produced two peptide sequences for the MS13 antigen and three peptide sequences for the MS34 sequence. As noted earlier, one of the MS13 sequences was identical to one of the MS34 sequences, which led us to believe at this point of the investigation that both antigens were either related or identical proteins.

To fully resolve the identity of the isolated antigens, it next was necessary to obtain the DNA sequence that encodes these proteins. This was accomplished by reverse transcription-PCR (RT-PCR) using hemocyte RNA as the template and degenerate oligonucleotide primers based on two of the MS34 antigen amino acid sequences acquired from the Edman degradation. The results of the RT-PCR produced an 803 bp product representing an internal segment of the complete gene. The full-length 2426 bp cDNA sequence (Fig. 6), here designated βMs1 (Genbank accession AY630342) was obtained via the technique of rapid amplification of cDNA ends (RACE). An examination of the 767-length amino acid sequence shows that it possesses all the peptide sequences from the Edman degradation, further suggesting that both MS13 and MS34 recognize the same protein antigen. A survey of amino acid sequence databases indicated that the deduced amino acid sequence from the above set of experiments possesses a high degree of similarity to the β subunits of proteins known as integrins.

Before progressing further, it was necessary to establish that the βMs1 gene sequence obtained did in fact code for the plasmatocyte protein recognized by MS13 and

MS34. By producing three truncated recombinant forms of the βMs1 representing different segments of the complete protein and performing an immunoblot analysis on these three peptides not only would I be able to demonstrate that βMs1 is the antigen to MS13 and MS34, but also which segment of protein contains the epitope for these antibodies. The results of this experiment revealed that both antibodies recognized an epitope between amino acids 124 to 264 of the full-length protein (Fig. 7). The I-like domain of the β-subunit is located within this peptide segment. The I-like domain of the β-subunit, together with the β-propeller (and I-domain if present) of the α-subunit, forms the extracellular ligand-binding site of integrins (Diamond et al., 1993; Michishita et al., 1993; Bajt et al., 1995; Plow et al., 2000; Zang et al., 2000; Shimaoka et al. 2002). Accordingly, it is reasonable to believe that the binding of MS13 or MS34 to the I-like domain would interfere with the proper function of βMs1, thus offering a highly probable explanation why these mAbs inhibit the encapsulation response. Given that both MS13 and MS34 recognize only one antigen in *M. sexta*, these results offer further proof that βMs1 is in fact the antigen in question that is involved in immune encapsulation and that the epitope is located near the amino-terminal end of the protein.

Northern blot analysis was employed to investigate tissue-specific expression of βMs1. The probe hybridized solely to a 2.3 kb RNA from the hemocyte sample and not to RNA from other tissues. This is consistent with results of antibody labeling with MS13 and MS34, indicating that the protein is present only in hemocytes, specifically plasmatocytes (Wiegand et al., 2000). These results are in partial agreement with the experiments of Foukas et al. (1998). As mentioned in the Results section, the mammalian integrin subunit most similar to βMs1 is β_3. Using antibodies against β_3, Foukas et al.

found a protein from the Mediterranean fruit fly (*Ceratitis capitata*) that is the same size as β_3 from hemocytes and, unlike my results, from fat body too. The treatment of *C. capitata* hemocytes with anti-β_3 antibody or RGD peptide was shown to inhibit phagocytosis (see below for discussion on integrin mediated phagocytosis), further supporting the notion that the protein of Foukas et al. is an integrin.

A second northern blot analysis tested whether βMs1 gene expression is affected by bacterial challenge. The results of this analysis show that the βMs1 transcript is constitutively present in hemocytes and that introduction of *E. coli* into the hemocoel of *M. sexta* does not alter this gene's expression (Fig. 9B). Integrins are known to mediate phagocytosis in vertebrates (Arnaout, 1990; Ylänne et al., 1995; Hill et al., 1998; Xia and Ross, 1999; Plow et al., 2000; Castellano et al., 2001) and evidence from the Mediterranean fruit fly, *Ceratitis capitata* (Foukas et al., 1998; Metheniti et al, 2001) and *Anopheles gambiae* (Moita et al., 2006) suggest that insect hemocytes possess integrins that are also involved in the phagocytosis of bacteria. It is also known that some integrins under certain conditions are inducible; i.e. the fibronectin receptor $\alpha_5\beta_1$ and the fibronectin and tenascin receptor $\alpha_v\beta_6$ of mammalian keratinocytes (= epidermal cells that produces keratin) are upregulated during wound healing (Breuss et al. 1995; Häkkinen et al., 2000; Watt, 2002). In light of such facts, one can speculate that the presence of bacteria, which are phagocytized by *M. sexta* plasmatocytes, could promote an upregulation of integrins in these hemocytes too. This, however, appears not to be the case for the βMs1 subunit. Although this inquiry needs further examination, these early results suggest that plasmatocytes possess a physiologically predetermined complement of βMs1 regardless of the presence of foreign entities. This does not address the

possibility of bacterial induced production of other integrins that may exist on plasmatocytes or even granulocytes, which are hemocytes also known to phagocytize bacteria in insects (Lavine and Strand, 2002).

Comprehensive studies of the total insect immune response show that the hemocyte response, together with the action of constitutively present antibacterial peptides, occurs early and is then followed by an induced humoral response (see introduction for details). Thus, there is an early period in an infection while the insect immune system gears-up to fight against an assault by invading pathogens. This means that circulating pre-infection hemocytes must be instantaneously ready to act against a bacterial challenge to protect the host from pathogen invasion while antibiotic peptides are being synthesized. Circumstantial evidence does exist for rapid immune response by hemocytes. When *M. sexta* is injected with bacteria or bacterial cell walls, plasmatocytes become rapidly removed from circulation (Geng and Dunn, 1989). This is likely a result of these hemocytes becoming adhesive and forming nodules or adhering to internal structures. Furthermore, insect plasmatocytes, along with other hemocytes, also play an important role in plugging wounds (Chapman, 1998; Lai, 2001; Lavine and Strand, 2002). It is likely that integrins are involved in this process too, as is the case for hemocyte integrins in mammals (Shattil, et al., 1998; Shimaoka et al., 2002; Grüner et al., 2003). Sealing a wound must occur expeditiously in order to prevent seepage of bodily fluids and the invasion of pathogens. Being that rapid hemocyte action in insects is critically important in both immune response and wound repair, if integrins are crucial in these processes, it would be reasonable to assume that insects evolved in a manner in

which hemocytes constantly express the necessary complement of integrin in order to respond to these situations quickly.

Subsequent to the completion of my research, an RNA interference (RNAi) experiment was performed by fellow graduate student Lisha Breuer using small interfering double stranded RNA (siRNA) based on the βMs1 gene (Levin et al., 2005). In RNAi, the presence of siRNA suppresses the expression of genes with complementary nucleotide sequences. Injecting fourth instar *M. sexta* larvae with βMs1-siRNA resulted in a gradual decrease in βMs1 levels on plasmatocytes starting 3 days post-inoculation. This was determined by (a) visual analysis via immunofluorescent labeling of plasmatocytes using anti-βMs1 mAb MS34 as the primary antibody (plasmatocytes from βMs1-siRNA treated larvae did not fluoresce nearly as well as plasmatocytes from control larvae), and (b) measuring the βMs1-mRNA levels in hemolymph samples through RT-PCR assays. Additionally, the encapsulation of DEAE Sephadex beads in βMs1-siRNA treated larvae was severely impaired. These three observations further the evidence that plasmatocytes possess integrin β-subunit βMs1 and that this subunit is necessary for encapsulation.

Additional evidence for the involvement of integrins in the lepidopterous encapsulation response had been previously presented for the soybean looper, *Pseudoplusia includens*. This evidence showed that: (a) encapsulation involves the recognition of an RGD amino acid motif; the same motif recognized by many integrins to prompt adhesion (Pech and Strand, 1995) and (b) integrins are expressed on the hemocytes of *P. includens* (Lavine and Strand, 2003). This role for integrins was further supported by a study by Irving et al. (2005) demonstrating that mutations in the

Drosophila integrin β-subunit βPS can disrupt the encapsulation of the larval stage of the parisitoid wasp *Leptopilina boulardi* by lamellocytes, a *Drosophila* hemocyte type that is adhesive and involved in encapsulation (Russo et al., 1996; Lavine and Strand, 2002). The Irving et al. (2005) experiment utilized two thermosensitive alleles for the βPS gene. At the permissive temperature of 18° C, flies carrying the thermosensitive alleles encapsulated the same percentage of wasp larvae as the wild-type flies. At the restrictive temperature of 29° C, however, flies carrying the thermosensitive alleles encapsulated significantly lower percentages of parasitoids than that of the wild-type flies. The encapsulation percentage of the wild-type flies at the restrictive temperature was not significantly different than those of any of the fly groups at the permissive temperature.

Potential applications of this research:

There are known instances of indigenous or introduced parasitoids attempting to utilize an exotic insect pest as a host, but fail due to the ability of the host to encapsulate the eggs of the parasitoid. For example, in Hawaii, the generalized parasitoid *Fopius arisanus* (Hymenoptera: Braconidae), chooses as a host the larvae of three pest tephritid flies; the Mediterranean fruit fly (*Ceratitis capitata*), the oriental fruit fly (*Bactrocera dorsalis*), and the melon fly (*Bactrocera cucurbitae*). The wasp can successfully parasitize both the Mediterranean and oriental fruit flies, but not the melon fly. In the melon fly, the eggs of *F. arisanus* become encapsulated and subsequently fail to develop (Neil Miller, personal correspondence). Now that it is known that integrins play an essential role in encapsulation, perhaps future researchers can use this knowledge to help *F. arisanus* overcome the cellular immune response of the melon fly.

The promotion of encapsulation in instances where it does not occur can be of importance too. Insects vector numerous diseases of humans and livestock. With many of these diseases, the etiological agent is potentially suitable for encapsulation (i.e. large in size and spends a part of its life cycle in the hemocoel of the insect host), yet is able to avoid inducing the cellular immune response in the insect host. Such diseases include malaria, filariasis, onchocerciasis (or river blindness), trypanosomiasis (specifically sleeping sickness), cestodiasis (i.e. tapeworm) and loiasis (Harwood and James, 1979; Lane and Crosskey, 1993; Service, 2004). Future control of these maladies may possibly be achieved by either the manipulation of host hemocyte integrins in a manner that will allow for integrin recognition of surface antigens on the parasite or by the manipulation of parasite surface antigens in a manner that will allow for recognition by host integrins. If either is achieved, an encapsulation response by the host against the parasite may result, thus preventing the spread of these ailments.

Future research:

Finding αMs1: As βMs1 has been sequenced and shown to be the β-subunit of an integrin essential for encapsulation, an important next step would be to characterize the corresponding α-subunit and demonstrate that it too mediates encapsulation. Based on my findings described in this dissertation, fellow laboratory associate Shufei Zhuang purified a putative integrin from larval hemocytes via immunoaffinity to MS13 (Levin et al. 2005). SDS-PAGE separated this protein into two bands, one at ~90 kDa and the other at ~105 kDa (Fig. 6C). Amino-terminal sequencing of the 90 kDa band together with immunoblot analysis using rabbit antiserum generated against a recombinant protein

produced from βMs1 cDNA (Fig. 6D) confirmed the 90 kDa band to be βMs1. The 105 kDa band associated with βMs1 is a size consistent with the heavy chain of the integrin α_{IIb} subunit (Du and Ginsberg 1997; Adair and Yeager, 2002). (It is interesting to note that α_{IIb} associates with β_3 to form an integrin on platelets that mediates thrombosis and β_3 is the subunit from mammals that is the most similar to βMs1.) From this purified presumed α subunit heavy chain, a full DNA sequence needs to be deduced and examined to make certain that its sequence is that of an integrin α-subunit. If this protein is in fact an α-subunit, to verify that it is involved in encapsulation, antibodies to a recombinant protein based on the presumed α-subunit DNA sequence should be produced and injected into larval *M. sexta* to see if it inhibits encapsulation.

Finding ligands to plasmatocyte integrin: It will also be important to determine the ligands to which the *M. sexta* plasmatocyte integrin binds. Little is known about such ligands in insects, however there are a few speculative candidates.

A 23 amino acid peptide from *M. sexta* designated paralytic peptide 1 (PP1) promotes plasmatocyte aggregation and spreading behaviors, along with inducing rapid paralysis and an inhibition of growth and development (Wang et al., 1999). PP1 has a high sequence identity (>70%) with plasmatocyte-spreading peptide 1 (PSP1) from *P. includens*, growth-blocking peptide (GBP) of *Mythimna* (= *Pseudaletia*) *separata*, and the paralytic peptides from *Heliothis virescens* and *Spodoptera exigua*. (Volkman et al., 1999; Strand et al., 2000). Among these peptides, PSP1 has been best studied. *In vitro* experiments demonstrated that increasing concentrations of this peptide results in a corresponding increase in the number of plasmatocytes that spread (Clark et al., 1997). Northern blot analysis showed that PSP1 mRNA is expressed in fat body, nervous tissue

and granulocytes (Clark et al. 1998). PSP1 seems to stimulate plasmatocyte adhesion by promoting surface changes on this hemocyte (Strand and Clark, 1999). In receptor labeling experiments, a photoaffinity analog of PSP1 crosslinked to a 190 kDa protein from hemocytes (Clark et al., 2004). To date, the receptor to PSP1 or any of its naturally occurring analogs has yet to be characterized in the literature, but the 190 kDa size is consistent with that of an integrin β-subunit together with the heavy chain of an α-subunit. It is thus possible that the peptides of the PSP1/PP1/GBP family could function as an opsonizing agent for plasmatocyte integrins.

Regulation of plasmatocyte integrin: In response to an invading foreign entity, plasmatocyte integrins must shift from being passive non-adherent receptors into active adherent ones. While there is an overabundance of knowledge on how integrins are activated and regulated in mammalian and other vertebrate systems (Calderwood et al., 1999; 2000; 2002; Woods and Couchman, 2000; Fitzgerald, 2001; Liddington and Ginsberg, 2002; Takagi and Springer, 2002; Calderwood, 2004), next to nothing is known about the regulation of invertebrate integrins. In *M. sexta*, in response to an immune challenge, granular cells release the proteins lacunin and a ligand for the lectin peanut agglutinin (PNA ligand). Both lacunin and PNA ligand co-localize on plasmatocyte surfaces with the integrin to which βMs1 is a subunit and neuroglian, another adhesion receptor and a member of the immunoglobulin superfamily (i.e. it contains 6 Ig-like domains). This results in the clustering of neuroglian and integrin on plasmatocyte plasma membranes (Nardi et al., 2005; 2006; 2007). Studies of mammalian systems have demonstrated that such clustering of adhesive receptors on cell surfaces enhances avidity (Burridge and Chrzanowska-Wodnicka, 1996; Alberts et al., 2002;

Sastry and Burridge, 2000; Lo, 2006). The mechanisms behind the receptor clustering and how neuroglian and integrin interact has yet to be elucidated and an area for future inquiry.

Intracellularly, a couple of studies have been published showing that the Ras/mitogen-activated protein kinase signal transduction pathway and the FAK/Src complex plays a role in regulating the putative hemocyte integrins that mediate phagocytosis in Mediterranean fruit fly, *C. capitata* (Foukas et al., 1998; Metheniti et al., 2001). Another study with *Drosophila* has shown that the Jun-N-terminal kinase (JNK) pathway coordinates *myospheroid* and *scab* integrin function during the embryogenic process of dorsal closure (see introduction on *Drosophila* integrins) (Homsy et al., 2006). Sorting out the homologous pathway for *M. sexta* plasmatocyte integrins would be important for gaining a full understanding of the encapsulation process.

Search for undiscovered *M. sexta* integrins: βMs1 is to date the only integrin subunit isolated and sequenced from the tobacco hornworm and, as mentioned above, a presumptive α-subunit to βMs1 has been isolated. Knowing that integrins are involved in a multitude of physiological functions and essential for animal life and that multiple α- and β-subunits have been found in *Drosophila* (Wilcox, 1990; Bunch et al., 1992; Burke, 1999) and on *P. includens* hemocytes (Lavine and Strand, 2003), there exists most certainly a number of undiscovered integrin subunits in *M. sexta*. A search for *M. sexta* integrins and an elucidation of their function will greatly increase the capacity to gain an in-depth and accurate understanding of the role of these cell adhesion proteins in insect physiology.

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Table

mAb	Ig class	hemocyte type recognized
MS2	IgG	GR
MS9	IgM	SP, PL
MS13	IgG	PL
MS16	IgM	most cells
MS34	IgG	PL
X11G9	?	GR, PL
Z7E8	IgM	OE
X2F2	IgG	GR, OE, PL
X7C4	?	GR, PL

Table 1. Monoclonal antibodies used in the experiments described in this paper. GR= granular cell, OE= oenocytoid, PL= plasmatocytes, SP= spherulocyte.

Figures

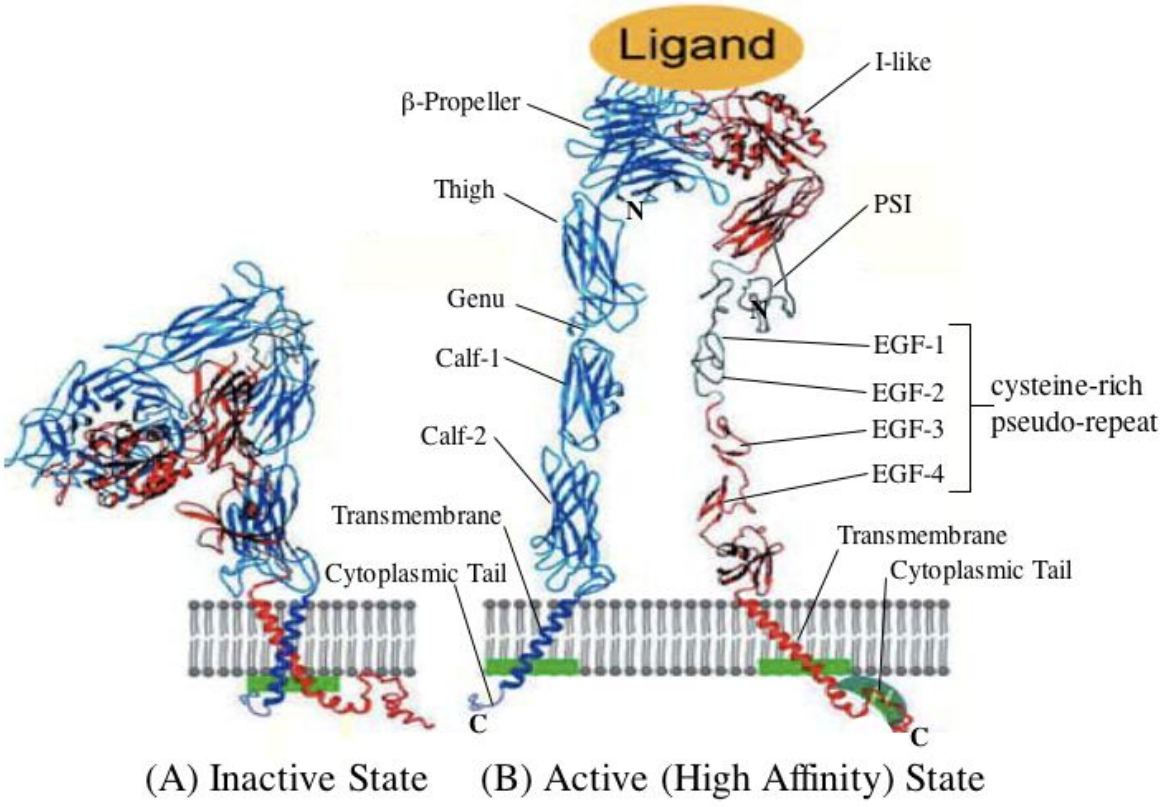
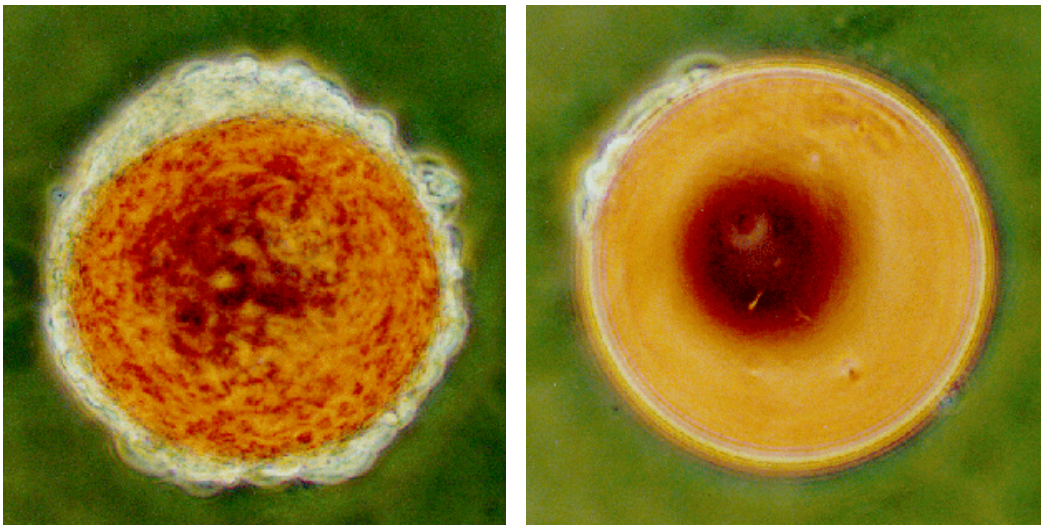


FIG. 1. Ribbon models depicting the shape and domain structure of an integrin in the inactive (A) and active states (B). The α -subunit is colored blue and the β-subunit is colored red. (A) In the inactive state, the transmembrane domains (TMs) of the two chains associate with one another in a crossed parallel structure. This maintains the extracellular domains in a bent configuration. (B) Upon activation, the TMs unclasp and separate. This permits the integrin to spring open into an extended conformation. It is in this elongated shape that ligand can bind to the integrin at the globular headpiece. This headpiece is comprised of the I-like domain of the β chain and the β-propeller domain and if present the I domain of the α chain. The various integrin domains of the heterodimer are labeled. The extracellular amino-termini of each chain are labeled N and the cytoplasmic carboxyl-termini are labeled C. (Modified from Qin et al., 2004. with permission.)



(A) (B)

FIG. 2. Examples of *in vivo* encapsulation of DEAE-Sephadex beads. (A) a bead exhibiting complete encapsulation. (B) a bead exhibiting no encapsulation.

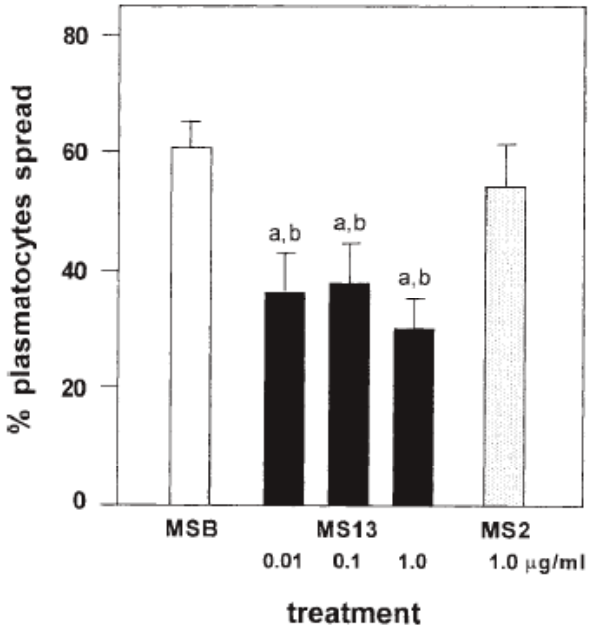


Fig. 3. Inhibition of plasmatocyte spreading by MS13. Washed hemocytes were preincubated in different concentrations of mAb MS13 in MSB. MSB and 1.0 mg/ml of mAb MS2 in MSB were used as controls. Data are expressed as mean values ± S.E.M. for three insects; Mann-Whitney Test of significance: (a) significantly different from MSB treatment, $P << 0.005$; (b) significantly different from treatment with mAb MS2, $P << 0.02$ to 0.07.

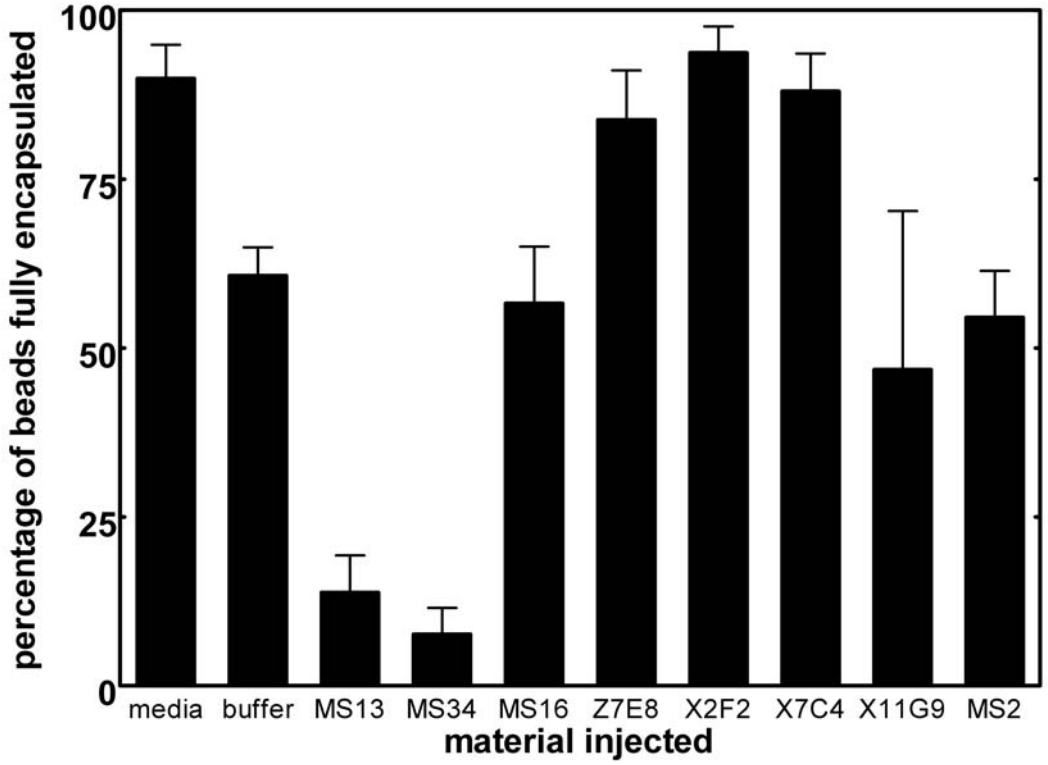


Fig. 4. Inhibition of encapsulation by monoclonal antibodies to hemocyte surface antigens. Experimental larvae (n=3 to 6) were injected with 100 µl of concentrated supernatant from mAb- producing cell cultures. For MS13 (n=6) and MS2 (n=24), larvae were injected with 50 µl (100 µg) of purified mAb. Control A larvae (n=5) were injected with 100 µl of 10x concentrated cell culture media. Control B larvae (n=48) were injected with 50 µl of MSB containing 300 µg BSA. After 1 h, larvae were injected with DEAE-Sephadex beads suspended in 100 µl MSB. Beads were dissected out 24 h later and examined for degree of encapsulation.

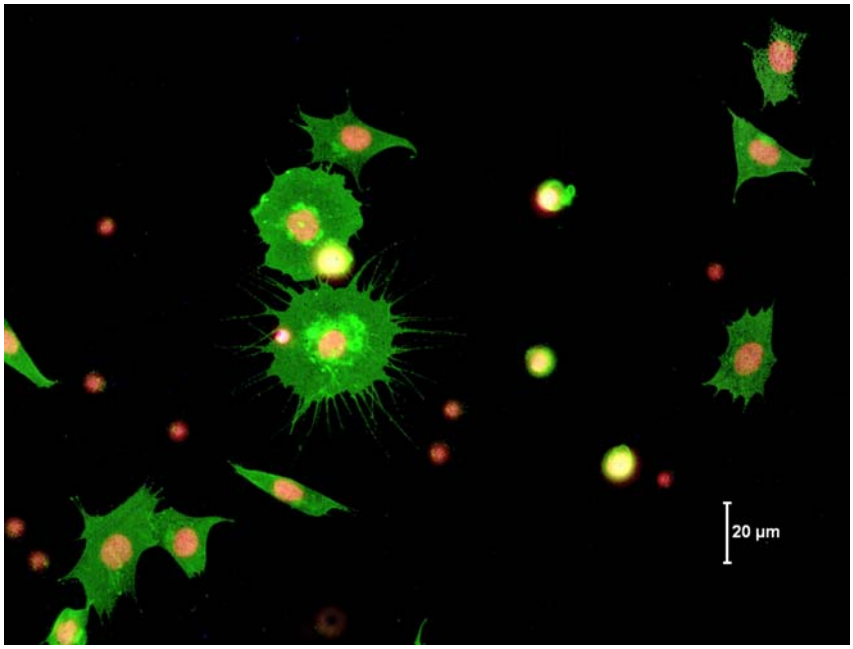


Fig. 5. Specific labeling of plasmatocytes by monoclonal antibody MS34. Hemocytes were allowed to settle on a glass coverslip submerged in Grace's medium (Invitrogen) for 60 min before being fixed with 4% paraformaldehyde in PBS. Cells were double labeled with MS34-FITC (green) and with propidium iodide (red), which binds to nuclei (granular cell nuclei are small and diploid; plasmatocyte nuclei are larger and polyploid). Plasmatocytes, displaying varying degrees of spreading, are labeled with MS34-FITC. Granular cells, which are not labeled with MS34 under these conditions, appear as smaller propidium iodide-labeled nuclei.

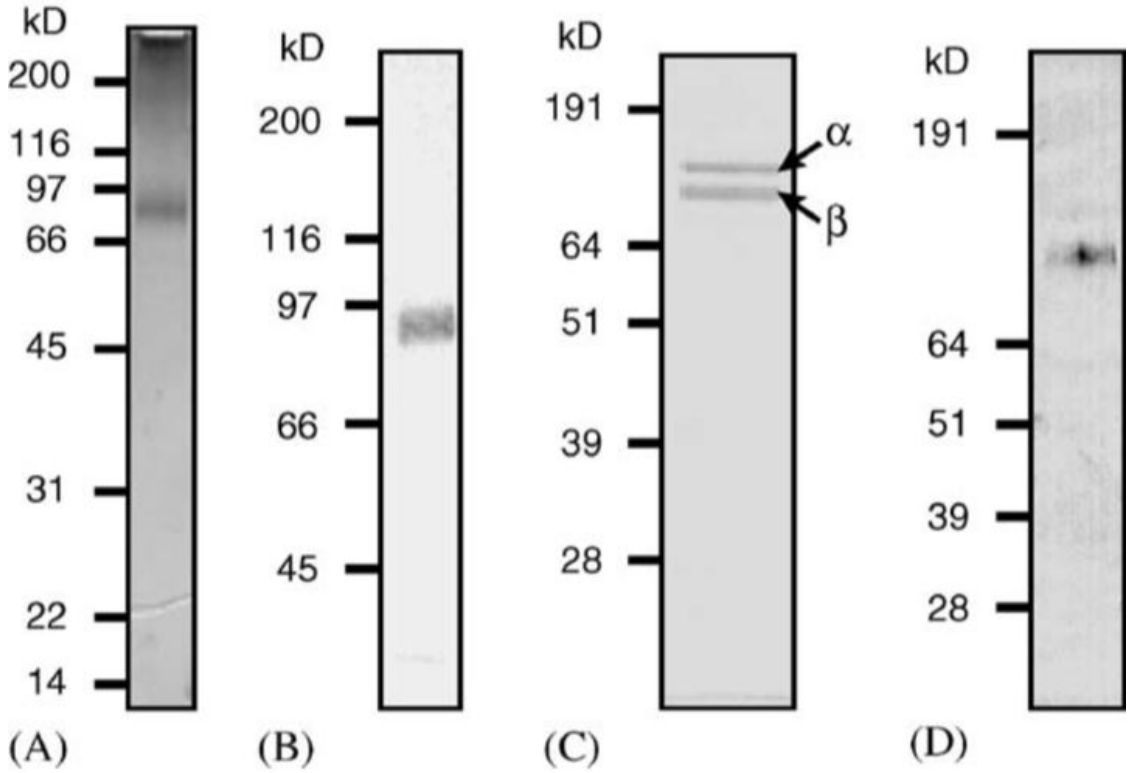


Fig. 6. Hemocyte encapsulation-facilitating antigen purified by immunoaffinity chromatography. (A) SDS-PAGE analysis (silver stain) of protein isolated from a lysate of developing wings by affinity to monoclonal antibody MS34. (B) Immunoblot analysis of the protein shown in A, using monoclonal antibody MS34 and goat anti-mouse IgG second antibody. A and B represent separation on 8% acrylamide gels. (C) SDS-PAGE analysis (Coomassie stain, 10% acrylamide bis-Tris gel) of integrin isolated from larval hemocytes. The band marked β was shown by amino-terminal sequencing to match the predicted amino-terminus of *Manduca sexta* integrin β-subunit βMs1 predicted from its cDNA sequence. This band was recognized by antibody to recombinant βMs1 (see panel D). The band marked α is the expected size for an integrin α-subunit of the purified integrin heterodimer. (D) Immunoblot analysis of the protein shown in C, using rabbit polyclonal antiserum to the recombinant amino-terminal fragment of βMs1 (construct 1 in Fig. 8A) and goat anti-rabbit IgG as secondary antibody. (A) and (B) preformed by Dr. James Nardi. (C) and (D) preformed by Dr. Shufei Zhuang.

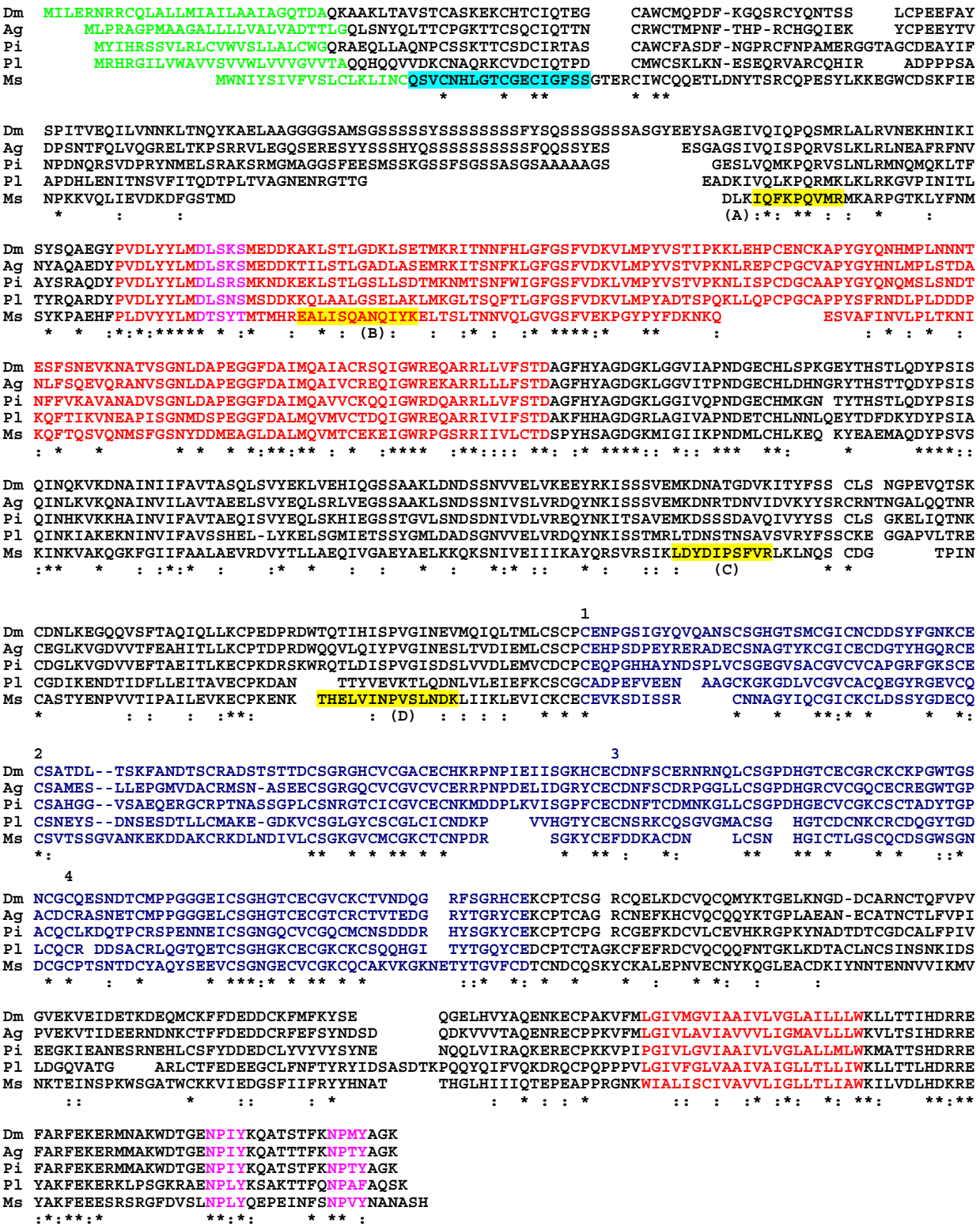


Fig. 7. Alignment of the deduced amino acid sequence of *M. sexta* integrin βMs1 with β integrins from *Drosophila melanogaster* (myospheriod protein, accession number A30889) (Dm), *Anopheles gambiae* (Ag) (XP_311354), *Pseudoplusia includens* (Pi) (AAO85806), and *Pacifastacus leniusculus* (Pl) (CAA67357). The "*****" marks positions with identical residues, and the "**:**" marks positions with conservative substitutions. The predicted secretion signal peptide sequences are highlighted in green. Residues in the I-like domain are in red. The MIDAS motif within the I-like domain is in purple. The region containing the four cysteine-rich pseudo-repeats is colored blue, with each repeat designated by a number above the sequence. The predicted transmembrane region is orange. The motifs in pink near the carboxyl terminus are two conserved intracellular NPXY motifs. The sequences highlighted in yellow correspond to sequences obtained by Edman degradation analysis of tryptic peptides from protein purified by immunoaffinity from wing lysate using monoclonal antibody MS34 (A, B, C) or MS13 (C, D). The sequence highlighted in turquoise corresponds to the amino-terminal sequence obtained by Edman degradation analysis of β integrin isolated from larval hemocytes using MS13.

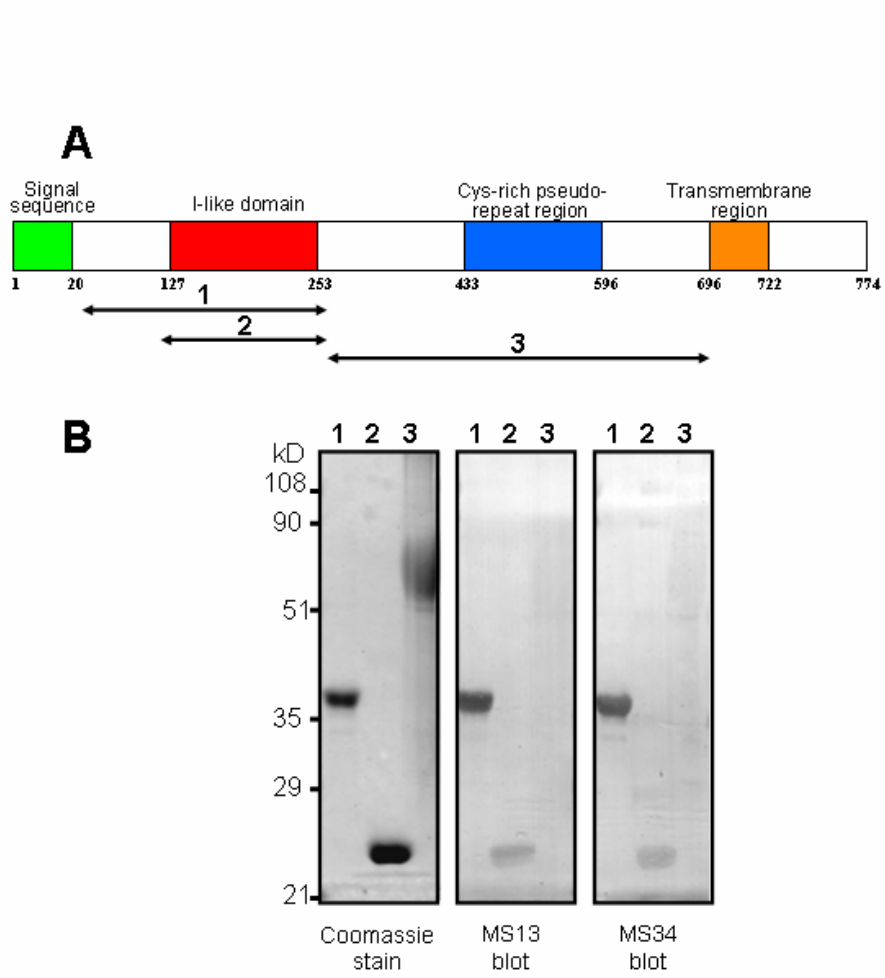


Fig. 8. Mapping of the region recognized by MS13 and MS34: (A) Schematic map of integrin βMs1 and constructs expressed as recombinant proteins. Regions labeled 1, 2, and 3 were expressed in E. coli, purified, and analyzed by immunoblotting. (B) SDS-PAGE and immunoblot analysis of recombinant integrin βMs1 protein segments. Lanes are labeled with the number of the recombinant integrin βMs1 fragment that was loaded. In panels labeled ‘‘MS13 blot’’ and ‘‘MS34 blot’’ proteins were detected using the indicated mAb, with goat anti-mouse IgG-alkaline phosphatase conjugate as secondary antibody.

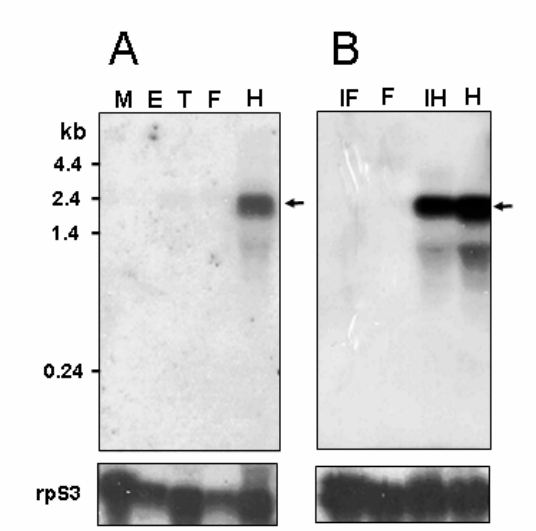


Fig. 9. Integrin βMs1 mRNA expression. (A) Tissue specificity of β integrin expression. Samples of total RNA (20 mg) from larval midgut (M), epidermis (E), Malpighian tubules (T), fat body (F) and hemocytes (H) were separated by agarose gel electrophoresis. The RNA was then transferred to uncharged nylon membranes and probed with ^{32}P-labeled integrin βMs1 cDNA. The arrow points to the 2.3 kb integrin βMs1 mRNA observed solely in hemocytes. The membrane was re-probed with ribosomal protein S3 (rpS3) cDNA to assess mRNA loading in each lane. (B) Exposure to bacteria does not affect integrin βMs1 expression. Samples of total RNA (20 mg) from fat body of larvae injected with saline (F) or with *E. coli* (IF), and hemocytes from βMs1 mRNA observed solely in hemocytes. The membrane was re-probed with ribosomal protein S3 (rpS3) cDNA to assess mRNA loading in each lane. (B) Exposure to bacteria does not affect integrin βMs1 expression. Samples of total RNA (20 mg) from fat body of larvae injected with saline (F) or with E. coli (IF), and hemocytes from uninjected larvae (H) or larvae injected with E. coli (IH) were analyzed as described for part A.