A PRELIMINARY REPORT ON AN INTEGRIN-LIKE PROTEIN IN PROTOPLASTS OF THE ENTOMOPATHOGENIC FUNGUS ENTOMOPHAGA AULICAE

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A PRELIMINARY REPORT ON AN INTEGRIN-LIKE PROTEIN IN PROTOPLASTS OF THE ENTOMOPATHOGENIC FUNGUS ENTOMOPHAGA AULICAE

by

BING LI

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This thesis is dedicated to my family, especially to my mother,

Zhenying Yan (1940 – 1997)

and my father,

Zhiming Li (1934 –)

Abstract

Integrins are transmembrane proteins involved in cell adhesion and signal transduction. This study reports findings on a possible integrin-like protein in an entomopathogenic fungus, *Entomophaga aulicae*.

Extracts of *Entomophaga aulicae* protoplasts contained a 71 kDa protein which cross-reacted with antiserum raised against a $ß_1$-chicken-integrin. The inclusion of DTT in the cell extracts had no effect on the mobility of this protein indicating the lack of a significant number of sulfur-sulfur bonds. A combination of eight protease inhibitors was necessary to prevent protein degradation in order to obtain consistent results. Positive and negative controls supported these results.

Using immunofluorescence microscopy, similar patterns of abundant peripheral patches of staining were detected at the periphery of the protoplast when probed with four different anti-integrin or anti-ß-integrin like protein antibodies. Controls showed that staining of the periphery of the nucleus and staining of the nuclear "core" were non-specific. This conclusion is tentative however since we were unable to show characteristic integrin staining in positive controls of monkey kidney fibroblasts.

In cell adhesion assays, there was no evidence to support the hypothesis that *E.aulicae* protoplasts attach to either a host insect cell line, or to the integrin-binding proteins, fibronectin and collagen. Future experiments using newly isolated protoplasts and cell lines derived from insect fat body may be more instructive.

We conclude that while we have preliminary evidence for an integrin-like protein in *E. aulicae*, strong support for integrin in any fungus remains elusive.

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BSA = bovine serum albumin

DTT = DL-Dithiothreitol

FBS = fetal bovine serum

FPMI = Forest Pest Management Institute, Sault Ste. Marie, Canada

GM = Grace's insect cell culture medium

M = high molecular weight

PB = phosphate buffer

PBS = phosphate buffered saline

RT = room temperature

SDS = sodium dodecyl sulfate

TTBS = Tween-20/Tris buffer saline

Single letter abbreviations for amino acids:

A = alanine

D = aspartic acid

E = glutamic acid

F = phenylalanine

G = glycine

K = lysine

P = proline

R = arginine

S = serine

Chapter 1 Introduction

1.0 Fungal attachment

Attachment to the substrate is a critical prelude to a number of important fungal activities including substrate utilization and substrate penetration. It is particularly important during pathogenesis when fungi may form specialized attachment structures to overcome host barriers, such as plant cell walls and insect exoskeletons. Fungi produce specific molecules, usually proteins, glycoproteins or carbohydrates, which may determine the specificity of attachment to ligands on the host. Any of several modes of adhesion may function in the first stages of fungal attachment, and the skillful pathogens may use more than one type of adhesion to start the initial invasion Hostetter (1996). Lectins, on pathogen or host cell, can recognize specific ligands (eg. Hamer *et al.*, 1988). In protein-protein interactions, a surface protein on the pathogen cell can contact a second protein expressed by the host cell, for example, proteins of the extracellular matrix, as in the putative integrin-like molecule of *Candida albicans* (Gale *et al.*, 1996). Other components such as phospholipids or proteoglycans may also be recognized by some other adhesions. Thigmotropic and chemical signals may be important in infection structure formation, as exemplified by the formation of appressoria in *Uromyces* (Hoch *et al.*, 1987).

As a rule fungal attachment to a host involves the physical association of the fungal wall material with the host wall, in the case of plants, or the exoskeleton, in the case of insects. One striking exception to this is the case of the infection of insects by fungi belonging to the order Entomophthorales after they enter the host. After passing

through the barrier of the host exoskeleton, several closely related entomophthoralean species grow inside the host as wall-free protoplast cells and thus a unique dynamic occurs. There is an absence of intervening wall layers between the fungal and insect plasma membranes (Murrin and Nolan, 1987). In the present study, we examined the possible role of the integral membrane protein, integrin, in the pathogenic relationship between protoplasts of *Entomophaga aulicae* and its insect host, the eastern spruce budworm.

1.1 Integrin

Integrins form a large group of transmembrane glycoproteins that are involved in cell adhesion and cell signal transduction. Integrin structure, integrin-mediated adhesion and integrin-mediated signaling have been the subjects of a number of excellent reviews. These include Hynes (1992), Ruoslahti (1991, 1996), Yamada and Miyamoto (1995), Howe *et al.* (1998), and Critchley (2000).

All integrins have two membrane-spanning polypeptide chains, called the α and β subunits (Fig. 1). The α and β subunits are different from each other in terms of molecular weight and construction. Sizes of α subunits fall in the range of 120 to 180 kDa, while the β subunits range from 90 to 110 kDa. The α and β subunits are not homologous to each other, but each of them belongs to their own homologous group of proteins.

The α and β subunits are linked to one another noncovalently, and this is promoted by divalent cations. Both integrin subunits have a small cytoplasmic domain, a single, short membrane-spanning segment, and a large extracellular portion.

On the inside of the cell, the small cytoplasmic domain of integrin interacts with a variety of cytoplasmic proteins, including those that connect the integrin to the actin cytoskeleton, such as talin, vinculin, α-actinin and filamin. This interaction links integrins to the contractile apparatus necessary for cell migration, and also supplies a surface for molecules that are involved in integrin signaling (Critchley, 2000). The interactions between the cytoplasmic domains of integrin and the components of the cytoskeleton may be regulated by phosphorylation of the β subunit on the cytoplasmic side of the membrane (Tapley *et al.*, 1989; Buck and Horwita, 1987).

On the outside of the cell, the large extracellular chains of the integrin have binding sites for components of extracellular materials, such as extracellular matrix proteins, substrates or other cells. The ligand-binding sites for extracellular materials require the sequences from both the α and β subunits in order to function (Loftus *et al.*, 1990; Vogel *et al.*, 1990). Thus, integrins have the ability to act as a link between the substances on both sides of the plasma membrane (Hynes, 1987). There is evidence that integrin is colocalized with actin filaments as shown by immunofluorescence microscopy. Integrin has a dispersed distribution pattern in vertebrate cells on the plasma membrane, at scattered, discrete sites called focal adhesions (Karp, 1999), and these are required for cells to adhere to their substratum.

Integrins are expressed on a wide variety of vertebrate cells and most express several integrins (Hynes, 1992). There are 17 different α subunits and 8 β subunits reported (Karp, 1999). Most of them have been sequenced at the cDNA level (Hynes, 1992). More than 20 different integrins have been identified on the surface of cells in

many different combinations of the α and β subunits, and the number is still rising. Because of the great diversity of integrins, different cells can recognize many different adhesive substrates and extracellular matrices (Ruoslahti, 1991).

Integrin-ligand interactions and adhesive specificities of individual integrins have been studied by different methods, including cell adhesion assays, monoclonal antibody binding, and affinity chromatography. Usually, individual integrins can bind to more than one ligand, and individual ligands can be recognized by more than one integrin (Hynes, 1992). Most ligands are extracellular matrix proteins involved in cell-substrate adhesion. Those include fibronectin, laminin, various collagens, vitronectin, entactin, tenascin, thrombospondin and von Willebrand factor (Ruoslahti and Pierschbacher, 1987; Hemler, 1990).

The integrins bind to extracellular matrix proteins at specialized cell attachment sites. The target sequence for the integrin binding is often the specific amino acid sequence, arg-gly-asp (RGD). Thus, most of these molecules have the RGD sequence that can recognize and bind to the RGD binding site on the large extracellular portion of the integrin molecule. RGD peptides that compete for the attachment site on the integrin molecule may inhibit this binding and this experimental approach has been frequently used to identify integrin activity (Pottratz *et al.*, 1991).

As reviewed by Ruoslahti (1991) and Hynes (1992), some integrins bind to cell membrane proteins on the surface of another cell to promote cell-cell adhesion, and other integrins promote cell-cell aggregation through soluble adhesion proteins such as fibrinogen and von Willebrand factor.

In addition to the multiple functions of integrin-mediated cell adhesion, integrin is also important in cell signaling events (Hynes, 1992; Yamada and Miyamoto, 1995; Burridge and Chrzanowska-Wodnicka, 1996; Yamada and Geiger, 1997; Giancotti, 1997; Howe *et al.*, 1998). Its physical position linking cytoskeletal structures and the extracellular matrix is crucial in this regard. Integrin-mediated cell signaling often affects integrin-mediated cell adhesion and *vice versa* (Yamada and Geiger, 1997; Giancotti, 1997; Howe *et al.*, 1998). Integrins transduce signals either into or out of cells (Hynes, 1992). Many cellular events can be influenced by integrin-mediated signaling, including motility, cell division, differentiation, cell survival and proliferation, migration and programmed cell death (Giancotti, 1997; Howe *et al.*, 1998).

As discussed by Shyy and Chien (1997), experiments also have shown that mechanical stress of cells and cell adhesion processes have many common characteristics, and that integrins may act as mechanotransducers in cellular events.

Most of the work on integrins has been done with vertebrate cells, particularly human tissue culture cells, such as platelets, leukocytes, neutrophils and lymphocytes. However, there is evidence that integrins arose at an early point in evolutionary history (Hynes, 1992). Integrin homologs have been reported from *Drosophila* (MacKrell *et al.*, 1988), *Xenopus* (Ransom *et al.*, 1993), crayfish (Holmblad *et al.*, 1997), sea urchins (Marsden and Burke, 1997), *Caenorhabditis elegans* (Gettner *et al.*, 1995), *Entamoeba histolytica* (Talamas-Rohana *et al.*, 1998), and cyanobacteria (May and Ponting, 1999). Thus, while the interpretation of some of these is not unambiguous, there is reason to

believe that the search for the origin of integrin should not be restricted to vertebrate cells (Hostetter, 1999). This has been supported by the continued progress made in the study of integrin-like proteins in some fungal organisms.

1.2 Integrin-Like Proteins in Fungi

For approximately 15 years, integrins have been documented as a commonly expressed family of cell surface adhesion receptors that mediate cell-matrix and cell-cell interactions. Because of their great diversity, scientists from many different fields have studied integrins (Hynes, 1987). Although most of the work on integrin has been done with animal cells and human tissue culture cells, there are several reports of integrin-like molecules in true fungi: *Candida* species (Marcantonio and Hynes, 1988; Santoni *et al.*, 1994; Gale *et al.*, 1996; Gale *et al.*, 1998), *Pneumocystis carinii* (Pottratz and Martin II, 1990; Pottratz *et al.*, 1991), *Uromyces appendiculatus* (Corrêa *et al.*, 1996), and *Neurospora crassa* (Degousée *et al.*, 2000). With the exception of *Neurospora*, all of these are important pathogens. An integrin homologue has also been reported from the oomycete, *Saprolegnia ferax* (Kaminskyj and Heath, 1995).

1.2.1 *Candida* species

Among the studies of integrin-like protein in fungal pathogens, species of *Candida*, especially *Candida albicans*, have been most thoroughly studied. *C. albicans* causes candidiasis, more commonly called "thrush". It is the leading opportunistic pathogen of invasive fungal disease in premature infants, diabetics, surgical patients, cancer patients and people with AIDS or other immunosuppressed conditions (Gale *et al.*, 1996). It can be found in healthy humans on the mucosal surface, and thus *Candida* is

one of several commensal eukaryotes found in the gastrointestinal tract of humans (Klotz, 1994). The ability of *C. albicans* to adhere to the endothelial basement membrane and/or subendothelial extracellular matrix of its host plays a crucial role in the development of candidiasis (Klotz, 1992).

There are several lines of evidence supporting the presence of proteins related to integrin in *C. albicans*. A 95 kDa protein in lysates of *C. albicans* was detected using anti-β_1-chicken integrin antiserum on immunoblots (Marcantonio and Hynes, 1988). A 165 kDa protein in the lysates of *C. albicans* membrane and cytosol was identified on immunoblots by a monoclonal antibody, raised against human complement receptor type 3, and later identified as an integrin (Hostetter *et al.*, 1990). Another single protein of 185 kDa also was detected by the anti-αX (integrin) monoclonal antibody BU15. Thus, both an α and β integrin homologue appear to present in *C. albicans* (Hostetter, 1999).

Some other investigators also reported integrin-like proteins in *C. albicans* (Santoni *et al.*, 1994). When *C. albicans* yeast and germ tube phases were incubated with several monoclonal antibodies directed against human α_5 or β_1 integrin subunits, or two different antisera to the fibronectin receptor, and analyzed by immunofluorescence microscopy and fluorescence activated cell sorting, positive results were obtained (Santoni *et al.*, 1994). An intensified immunoreactivity was found during transition from the yeast to germ tube. Also, the binding of *C. albicans* yeast and germ tubes to fibronectin containing the RGD sequence was noticeably (~60%) inhibited by GRGDSP, but not GRGESP peptides. Furthermore, anti-integrin antibodies or anti-fibronectin receptor antisera significantly blocked the binding of both *C. albicans* phases to

fibronectin. All these results suggested that a receptor antigenically related to $\alpha_5\beta_1$ integrin was expressed by *C. albicans* yeast and germ tube phases, and this receptor might mediate their adhesion to fibronectin.

By screening with a cDNA probe from the transmembrane domain of human leukocyte integrin αM, the gene *αINT1* was isolated from a library of *C. albicans* genomic DNA (Gale *et al.*, 1996). It was found that the predicted polypeptide of 188 kDa had several motifs common to αM and αX integrins. An internal peptide that had a RGD sequence was also found. Although no immunofluorescent micrographs have been presented, the surface localization of αInt1p in *C. albicans* blastospores was shown by the positive results in the binding experiments of polyclonal antibodies prepared against the potential extracellular domains of αInt1p as analyzed by fluorescent activated cell sorting. Among yeast tested, *C. albicans*, *C. tropicalis* and *Saccharomyces cerevisiae*, *αINT1* was found to be unique to *C. albicans* by Southern blotting.

In haploid *S. cerevisiae* yeast cells and a *ste12* mutant (which lacks a yeast transcription factor necessary for morphologic change in response to mating pheromones and nutrient limitations), the expression of *αINT1* under control of a galactose-inducible promoter resulted in the formation of germ tubes (Gale *et al.*, 1996). Further study found that *αINT1* expression in *S. cerevisiae*, which is normally a non-adherent yeast, resulted in its adhesion to human epithelial cells. Furthermore, interruption of *αINT1* in *C. albicans* led to the inhibition of three characteristics important in pathogenicity: hyphal growth, adhesion to epithelial cells and virulence (Gale *et al.*, 1998).

Results from another study (Bendel and Hostetter, 1993) also showed that there were integrin-like proteins in *C. albicans* and *C. tropicalis*, and showed species-specific and functional differences between them.

There is also evidence for a β_1 integrin in *C. tropicalis* (DeMuri and Hostetter, 1996). It was found that the binding of soluble fibronectin to *C. tropicalis* blastospores was saturated at a concentration of 1.8×10^{-9} M, and this binding could be inhibited significantly by membrane extracts from *C. tropicalis*. A membrane protein of 125 ± 25 kDa was recognized by purified fibronectin, antibodies to integrin $\alpha_5\beta_1$ (the fibronectin receptor on human placenta), and antibodies specific for the integrin β_1 subunit on immunoblots. A protein of 105 ± 15 kDa was detected by immunoprecipitation of radiolabeled proteins from *C. tropicalis* with purified human fibronectin. All these results support the idea that a protein with antigenic and functional properties common to the vertebrate β_1 integrin fibronectin receptor is expressed by *C. tropicalis* (DeMuri and Hostetter, 1996).

1.2.2 *Pneumocystis carinii*

Pneumocystis carinii causes pneumonia in humans and is another main reason of morbidity and death in immunocompromised hosts, especially in those with AIDS. It is an extracellular pathogen that binds tightly to alveolar epithelium.

It was found that fibronectin binding to *P. carinii* was inhibited by the addition of RGDS, a tetrapeptide of arginine, glycine, aspartic acid and serine, containing the active site of the cell-binding domain of fibronectin (Pottratz and Martin II, 1990). Also,

attachment was decreased by the addition of an anti-fibronectin antibody. It was concluded that the binding of *P. carinii* to fibronectin was an important initial step in the attachment of the organism to cultured lung cells and *P. carinii* recognized and bound to the RGD sequence of fibronectin (Pottratz and Martin II, 1990).

Further study (Pottratz *et al.*, 1991) showed that a 110-120 kDa membrane glycoprotein (gp120) on *P. carinii* promoted the attachment of the organism to cultured lung cells. This protein possessed the binding site for fibronectin. The gp120 isolated from the whole *P. carinii* cells significantly reduced the percentage of attachment of *P. carinii* to alveolar epithelial cells, from 44% to 22%. Pre-incubation of *P. carinii* with a polyclonal antibody to gp120 also led to an obvious decrease in attachment of *P. carinii* to lung cells, from 47 % to 21%. In addition, using free gp120 significantly reduced the specific binding of ^{125}I-fibronectin to *P. carinii*, and anti-gp-120 antibody also lessened the fibronectin binding. Cell lysates of *P. carinii* separated by gel electrophoresis and blotted with ^{125}I-fibronectin displayed specific binding of the ^{125}I-fibronectin to gp120. This gp120 protein could cross-react with a specific anti-$ß_1$-integrin antiserum. All of the results suggested that gp120 served as a fibronectin binding protein and was necessary for optimal *P. carinii* attachment to alveolar epithelial cells, an essential initial step in the development of *P. carinii* infection (Pottratz *et al.*, 1991).

1.2.3 *Uromyces appendiculatus*

The plant pathogenic fungus, *U. appendiculatus*, is the cause of "bean rust", a disease of bean that usually can be recognized by the rusty-red powdery masses on leaves and other plant surfaces. Germlings of urediospores of this fungus react to topographical

signals on the leaf surface by going through a cell differentiation process that leads to the formation of a structure called an appressorium (Hoch *et al.*, 1987). The appressorium produces an infection hypha, which grows through the stomatal aperture after which infection progresses (Hoch *et al.*, 1987).

Corrêa *et al.* (1996) found that germlings, grown on substrata which normally induce the formation of appressoria, were inhibited from developing appressoria by incubating with several synthetic peptides containing the amino acid sequence RGD. But two non-RGD peptides and two RGD peptides (GRGDS and RGDSPASSKP) did not affect appressorium formation. Normally, 0.5 μm diameter micropipettes are inductive for appressorium formation when put between the germling apex and the substratum, but when coated with the RGD peptide they were not. Those findings led to the hypothesis that an integrin-like protein might be associated with the process of signaling which initiates appressorium formation in *Uromyces* germlings. Additionally, one protein of 95 kDa isolated from *Uromyces* germlings by using an RGDSPC-affinity column was shown to cross-react with two different antibodies of $ß_1$-integrin on immunoblots (Corrêa *et al.*, 1996).

1.2.4 *Saprolegnia ferax*

Although the water mold, *S. ferax*, is recognized as phylogenetically distinct from the true fungi (Kendrick, 1996), it exhibits hyphal growth and has been shown to be an excellent model to study the hyphal cytoskeleton and tip growth (Heath, 1990; 1995).

In hyphae of *Saprolegnia*, a homologue of integrin was identified by an

anti-β_1-integrin antiserum on immunoblots (Kaminskyj and Heath, 1995). There was a 178 kDa integrin in non-reduced samples, and a conversion from 178 kDa to 120 kDa in reduced samples as well. Distributions of plasma membrane-associated patches of integrin on *Saprolegnia* were illustrated by immunofluorescence images, and were abundant at the hyphal tip. By differential resistance to plasmolysis-induced separation of the plasma membrane from the cell wall, it was found that this putative integrin homologue of *Saprolegnia* might be instrumental in the attachment of the plasma membrane to the cell wall.

1.2.5 *Neurospora crassa*

Neurospora crassa is one of the most studied fungi and is used widely in cell biology studies. This includes the study of fungal tip growth (Degousée *et al.*, 2000). By cell fractionation and immunocytochemistry, these investigators found that one protein cross-reacted with an anti-β_1-integrin antibody at ~ 63 kDa in the reduced sample and at ~ 120 kDa in an unboiled plasma membrane extract. The anti-β_1-integrin antibody showed a tip-high concentration of fine plasma-associated spots. The authors suggested that this putative integrin might play a role in attaching the membrane skeleton to the membrane, in forming cell wall at the hyphal apex and in retaining cytoskeleton-membrane-cell wall attachments subapically (Degousée *et al.*, 2000).

1.3 Experimental Organisms

1.3.1 *Entomophaga aulicae*: an insect pathogenic fungus

The purpose of the present study was to determine whether integrin is involved in disease progression during the infection of host larvae by the zygomycete, *E. aulicae*. *E. aulicae* is an aggressive pathogen of the eastern spruce budworm, *Choristoneura fumiferana*, and the eastern hemlock looper, *Lambdina fiscellaria*, both of which cause serious economic loss due to softwood forest destruction in eastern North America, including Newfoundland and Labrador. *E. aulicae* can cause the decline of natural outbreaks of these forest pests (Otvos *et al.*, 1973). The development of this fungus as a biocontrol agent offers an alternative to chemical pesticides against eastern spruce budworm and hemlock looper (Nolan, 1985).

Infection is initiated when a germinated conidium penetrates the larval cuticle (Fig. 2). When a conidium lands on the cuticle of a potential host, a germ tube is produced. Then an appressorium is produced at the germ tube tip, which enhances the strength of the attachment of the fungus to the host cuticle. From the base of this appressorium is produced a narrow infection hypha, which penetrates the host cuticle and releases a protoplast into the host hemolymph. The protoplast lacks a cell wall. In the host hemolymph, the protoplast absorbs nutrients and divides rapidly. When the nutrients are depleted, it lays down a cell wall and forms a thick-walled hyphal body. This hyphal body produces conidiophores, which penetrate outwards through the host cuticle to produce another round of infective conidia (Murrin and Nolan, 1987; Murrin, 1996).

The protoplast stage of *E. aulicae* is a unique cell type. It has a characteristic spindle shape with two tapering terminal extensions and rounded nucleate regions with internuclear constrictions. It exhibits undulating movement. The absence of a cell wall allows the fungus to go undetected by the host insect's immune system, and to rapidly colonize in the hemolymph of host insects (Dunphy and Nolan, 1980; 1982a). During infection, all internal organs of the insect are eventually destroyed.

Entomophaga aulicae has been identified as a potential biocontrol agent for use against several serious forest insect pests (Otvos *et al.*, 1973). Previous studies of this pathogen have investigated its morphological development *in vitro* by light microscopy, features of its physiology and biochemistry, and interaction with host immune systems (reviewed in Nolan, 1985). Also, cytological work has been completed on its nuclear ultrastructure (Murrin *et al.*, 1984), genome characteristics (Murrin *et al.*, 1986), and ultrastructure of the infection process in the host (Murrin and Nolan, 1987). Microtubules, but not actin filaments, play a key role in cell shape and movement in protoplasts (Murrin *et al.*, unpublished data). Microtubules are present throughout the protoplast cytoplasm but are concentrated in the terminal extensions and internuclear constructions (Taylor, 1992). Actin has been identified in extracts of *E. aulicae* protoplasts by immunoblotting; using rhodamine conjugated phalloidin, this actin appears to be diffusely distributed throughout the cell (Murrin *et al.*, unpublished data). In germ tubes, microfilaments but not microtubules are important in growth and shape of the tip (Borden, 1998; Pike, 1999).

There is evidence to support the hypothesis that entomophthoralean protoplasts attach to insect host cells. Microscopy was used to show evidence of the attachment of protoplasts of the closely related fungus, *Entomophaga grylli,* to the insect fat body at all stages of infection in its grasshhopper host (Funk *et al.*, 1993). In a study of the attachment of protoplasts of *E. aulicae*, collagen was one of the attachment factors which gave a high percentage of protoplast attachment (Lake, 1994). The attachment of protoplasts to collagen-coated slides started initially at their terminal extensions, then the whole cells attached under favorable conditions. Such an interaction might be mediated by integrin or an integrin-like molecule.

1.3.2 *Choristoneura fumiferana*, the eastern spruce budworm

The eastern spruce budworm is one of the most devastating defoliators of forest trees in northeastern North America (Blais, 1984). A native lepidopteran species, it feeds primarily on balsam fir and white spruce in boreal forests. Its appearance at epidemic levels is cyclic, occurring at 25 to 30 year intervals, with five to ten years at maximum population size. The spruce budworm normally produces one generation per year. The early instar larvae feed on new leaves and buds, burrowing into them. Budworm damage can range from a browning of infected foliage to loss of entire tree stands.

Chemical pesticides for control of budworm outbreaks have to some extent been augmented by biological control agents, relying on naturally occurring predators and disease-causing organisms of the larval stages (Sanders *et al.*, 1984). Most of the natural microbes causing disease in this species, including several viruses, bacteria and a number of protists, infect during insect feeding and thus gain access to the digestive tract. Unlike

the foregut and hindgut, which are ectodermal in origin, the midgut is of endodermal origin and as such lacks the protective cuticular lining of other parts of the digestive tract. Despite the harsh environment of the midgut, and its protective peritropic membrane and mucins, the midgut is the main site of action of many microbes associated with the insect gut (Wang and Granados, 1997). While there is a great deal known about the occurrence and distribution of such microbes, there has been little work done on the mechanisms of interaction and attachment (Douglas and Beard, 1996) with the clear exception of the bacterium, *Bacillus thuringiensis*.

Bacillus thuringiensis, or *Bt*, is the most widely used biological insecticide in the world (Piertrantonio and Gill, 1996) and it is often used to control outbreaks of eastern spruce budworm. It produces a variety of related crystalline proteins associated with its spore which are lethal to lepidopterans, dipterans, and coleopterans. The crystal is solubilized and activated by proteases in the insect gut. The protein binds to receptors in the membrane of the brush border of the midgut. The receptors are clusters of animopeptidases associated with specific gycolipids (Adang *et al.*, 1997). The receptor-toxin complex undergoes conformational changes, and inserts itself into the membrane, causing the formation of ion channels. This leads to osmolysis and massive destruction of the midgut cells. Insect death follows within hours or days. This mechanism of action of *Bt* toxins, while extremely well studied, is quite complex: the exact modifications at the cellular level are unknown and activity may vary depending on the specific insect and toxins involved.

While the majority of budworm pathogens infect via the mouth, entomopathogenic fungi are an exception to this rule and are the only potential biological control agents which do not depend on the insect actively feeding. Fungi infect by attaching to the exoskeleton, forming infection structures and penetrating the cuticle and underlying tissue layers to gain access to the nutrient rich hemolymph. Attachment of fungi to their hosts at the initial stages of infection may be via a variety of methods (see above) but once entomopathogenic fungi enter the insect hemolymph a different interaction occurs. As a part of the non-specific insect immune response, host granulocytes are stimulated to release components of the prophenoloxidase system, which coats foreign material triggering melanization and encapsulation by hemocytes (Gotz, 1991). The beta-glucans in the walls of invading fungi trigger this response. The success of some of the most aggressive of the entomopathogens, including *Beauvaria bassiana*, is based on their ability to out-grow the encapsulation process. On the other hand, some entomophthoralean fungi, including *E. aulicae*, exhibit a different strategy. As they grow into the hemolymph, there is little or no activity of the enzymes for beta-glucan and chitin production (Beauvais and Latgé, 1989). This results in the formation of the wall-free protoplast (as described above), which is not recognized by the host and which grows unimpeded in the nutrient-rich hemolymph (Dunphy and Nolan, 1982a).

Eastern spruce budworm was one of the earliest insects from which continuous insect cell lines were developed (Sohi, 1973). The cell line IPRI-CT-124 originated from neonate larvae and was subsequently developed to attach to tissue culture flasks (Bilimoria and Sohi, 1977). The development of insect-cell lines has been crucial to

recent advances in our understanding of microbe-insect interactions, in the mass production of viruses for biological control purposes, and is becoming increasingly important in basic molecular biology of viral gene expression (eg. Castro *et al.*, 1999).

Integrins and their ligands would be expected to occur in a broad spectrum of insect cells, including those of the spruce budworm cell lines. Early progress on the study of insect extracellular matrix components was hampered by the inability to access sufficient material and the low solubility of some of its components compared to mammalian sources (Ashhurst, 1984). Recent progress has been greatly facilitated by the use of the fruit fly, *Drosophila melanogaster*, a genetically tractable model organism with a long history of study. *Drosophila* shares with crayfish and mammalian cells at least two integrins. Integrin adhesion in *Drosophila* is required for muscle-exoskeleton linkages that translate into movement, and for cell migration during morphogenesis (Brown, 2000). *Drosophila* imaginal disc cell lines, which possess one of these intergrins, was recently shown to attach to human fibronectin (Miller *et al.*, 2000). While progress is rapid in this area, knowledge of the suite of interacting molecules in this system remains unknown, and its regulation is still under investigation. Interestingly, cell-cell adhesion via integrin is critical in the cellular immune response in invertebrates. In crayfish, a peroxidase of the prophenoloxidase system interacts with integrin molecules to mediate hemocyte-hemocyte adhesion during encapsulation, and this interaction appears to be widely conserved (Johansson, 1999).

1.4 Objectives and Approaches

Three sets of experiments were designed to determine whether integrin is present in protoplasts of *E. aulicae* and whether it is important in pathogenesis during infection of host larvae: immunoblotting, immunofluorescence localization and attachment assays.

1.4.1 Immunoblotting

Immunoblotting was used to identify proteins that cross-react with anti-integrin antibodies in extracts of *E. aulicae* protoplasts and to help determine the specificity of these antibodies. Immunoblotting, also known as Western blotting, is an immunochemical technique that is used to detect a protein immobilized on a matrix (Towbin *et al.*, 1979).

Before applying this procedure, an antibody and a solution containing the protein of interest are necessary. The antibody should be able to specifically recognize the protein, and this antibody can be either monoclonal or polyclonal antibody. A polyclonal antibody is a mixture of antibodies produced by an organism exposed to an antigen, each of which recognizes a different epitope on the antigen. A monoclonal antibody is produced from a hybridoma and is not a mixture; it recognizes only one epitope. Thus, while the specificity of the monoclonal antibody may be higher than that of the polyclonal antibody, the polyclonal antibody may give a higher signal. The solution containing the protein of interest can be either a crude cell extract/lysate or a more purified preparation. Protease inhibitors may be necessary to prevent proteolysis and improve the yields of protein activity (Bollag *et al.*, 1996). Immunoblotting is a very effective technique for identifying a single protein when separated from a composite

mixture by different methods, such as SDS-PAGE, nondenaturing gel electrophoresis, isoelectric focusing or two-dimensional gel electrophoresis (Bollag *et al.*, 1996). Differences in mobilities between reduced and non-reduced proteins due to the presence of intrachain disulfide bonds may be detected by the use of the reducing agent DDT.

Immunoblotting can be divided into two steps: transfer of proteins from the gel to a solid support and detection by probing with the specific antibody. Blocking or quenching agents are necessary in order to block all unoccupied binding sites of the filter before the blot has been probed with selected antibodies. Sufficient washing is indispensable to prevent nonspecific adsorption of the probe to unrelated areas of the blot (Gershoni, 1987). Also, proper experiment design including adequate negative and positive controls are crucial for interpreting the experiment results. Positive controls can indicate whether the system works or not, and the negative controls can help eliminating the nonspecific results from the related ones.

Immunoblotting was conducted in this study in order to determine if integrin is present in *E. aulicae*. More than 30 immunoblots were completed. Five different anti-integrin antibodies and three different cell types were used, including *E. aulicae* protoplasts (hereafter referred to as protoplasts), monkey kidney fibroblasts (hereafter referred to as fibroblasts) and the yeast, *C. albicans* (hereafter referred to as yeast). Fibroblasts and yeast were used as controls.

1.4.2 Immunofluorescence Experiments

The sub-cellular distribution of integrin in the protoplast was investigated by immunofluorescence microscopy. Immunocytochemistry is a sensitive method for

locating an antigen to a particular structure or subcellular compartment when an antibody specific for the protein of interest is available (Spector *et al*., 1998).

Usually the indirect technique is used since it is less costly and results in higher signal. In this technique, the primary antibody is unlabeled and binds to the antigen. Then a second antibody, made against IgGs of the species in which the first antibody was raised, is added. The secondary antibody is conjugated to a fluorochrome, such as fluoroscein isothiocyanate, and the distribution of the antigen can then be viewed in a microscope equipped with the appropriate filters. Blocking is again important in order to occupy all non-specific binding sites in the cell prior to application of antibodies: dry milk powder is one of the most useful blocking agents, as well as bovine serum albumin (BSA), and fetal calf serum. Proper negative and positive controls in the experimental design are necessary so that the results can be interpreted correctly.

Immunofluorescence microscopy was conducted in this study in order to determine the sub-cellular distribution of integrin in *E. aulicae* protoplasts. Four different anti-integrin antibodies were used. One hundred and thirty eight semi-permanent slides were viewed, including positive and negative controls.

1.4.3 Attachment Experiments

Attachment experiments were set up in order to determine the involvement of integrin in the adhesion of *E. aulicae* protoplasts to host cells *in vitro* and if protoplasts attach to surfaces coated with integrin-binding proteins. There are a wide variety of ways to demonstrate and measure cell attachment/adhesion. For example, cell adhesion can be measured directly by measuring shear flow or by micromanipulation (Curtis and Lackie,

1991). Precise measurements are particularly useful when cell adhesion/attachment has already been determined. In other cases, different methods are chosen or designed for the special needs of specific studies. Fluorescence activated cell sorting and adhesion assays were performed in the study of a fibronectin receptor in *C. albicans* (Santoni *et al.*, 1994). Flow chambers were used in the study of an integrin-like protein in *U. appendiculatus* (Terhune and Hoch, 1993).

Previous experiments in our laboratory were conducted in an attempt to promote attachment of protoplasts of *E. aulicae* to glass slides by supplying exogenous attachment factors and by varying the medium in which protoplasts were resuspended (Lake, 1994). Different tests were applied to test adhesive strength. These tests, with increasing force, included tapping the slide, removal of medium by wicking, rinsing the slide by carefully pipetting off the medium and adding a drop of the same resuspension medium as that being tested, and dipping the slide in the same resuspension medium as that being tested. The results showed that attachment of protoplasts was influenced by both the medium and attachment factor, and that these interact in a complex manner. Attachment levels ranging from zero to 100% were distinguished using the wicking and rinsing procedures (Lake, 1994), and thus these protocols were judged to be well suited for the examination of protoplast attachment.

The attachment experiments were conducted in this study in order to determine if *E. aulicae* protoplasts attach to host insect cells or to the integrin-binding proteins fibronectin and collagen. To determine if adhesion occurs, we adjusted the attachment protocols developed by Lake (1994) and used three different peptides in inhibition

assays. We also examined the effect of fetal bovine serum and different isolates on attachment.

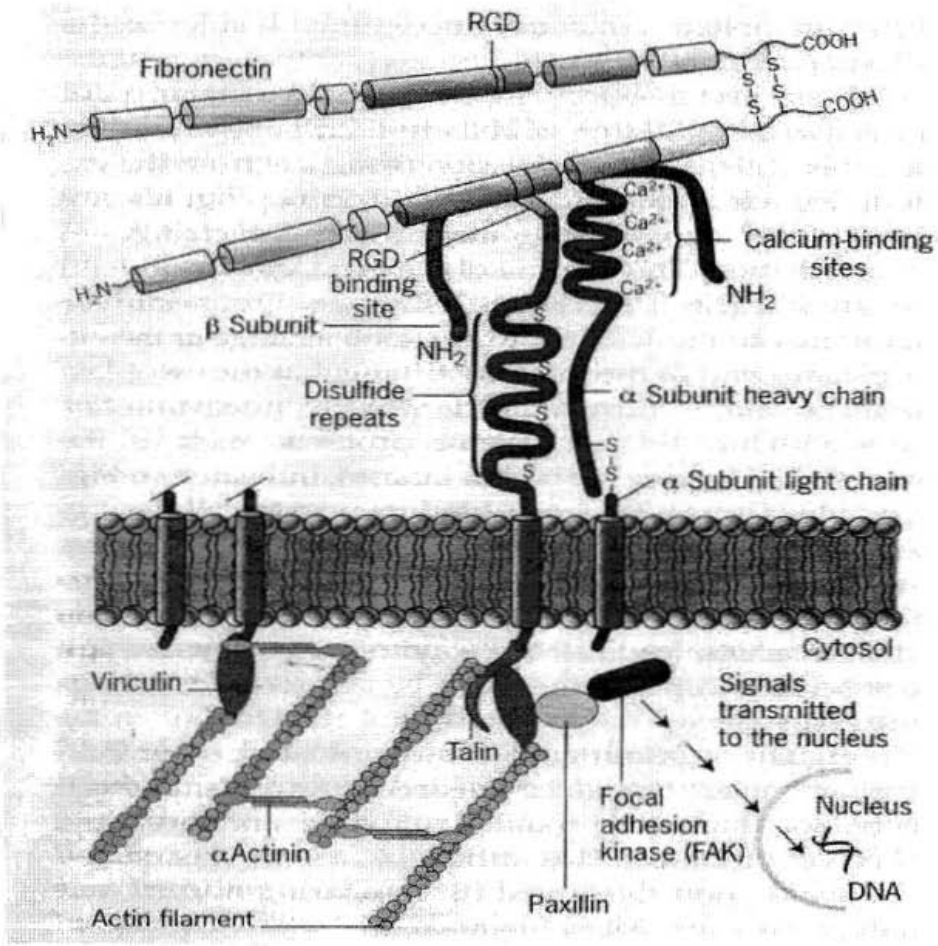


Figure 1. Structure and interactions of integrin (green) with fibronectin and other cell components (from Karp, 1999).

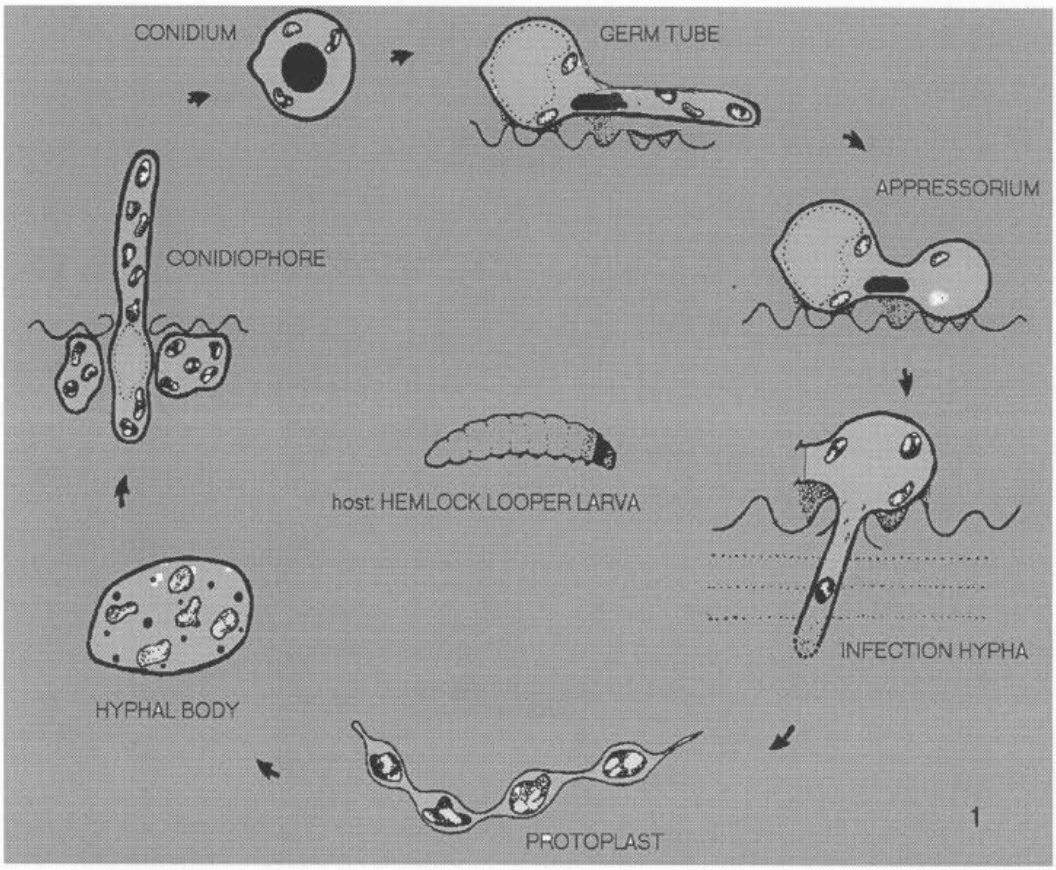


Figure 2. Asexual stages in the life cycle of *E. aulicae* (Entomophthorales, Zygomycetes) (Modified by Murrin from Murrin, 1996)

Chapter 2 Materials and Methods

2.1 Cell Lines and Culture Maintenance

Protoplasts of *Entomophaga aulicae*, isolates FPMI-521, and FPMI-646, were obtained from the Forest Pest Management Institute, Sault Ste. Marie, Canada. Stock cultures were maintained in Grace's insect cell culture medium (GM, Canadian Life Technologies Inc., Burlington, Canada) supplemented with 5% fetal bovine serum (FBS, GIBCO), and incubated in an incubator (Psycrotherm, New Brunswick Scientific, N. J., U.S.A.) at 19 °C in the dark. Logarithmic cultures were used in all experiments.

Monkey kidney fibroblasts, cell line CV-1, were kindly supplied by Dr. M. Grant, Faculty of Medicine, Memorial University of Newfoundland. Fibroblasts were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 2% bicarbonate, 1% amino acids (GIBCO), 1% penicillin/streptomycin (GIBCO) and 10% fetal calf serum (GIBCO). Cells were harvested by scraping from the flask surface and centrifuged at 150 x *g*.

The yeast *Candida albicans* was obtained from the American Type Culture Collection (ATCC# 10261). *C. albicans* culture was maintained on yeast malt agar (DIFCO) plates at 4 °C.

The spruce budworm insect cell line, IPRI-CF-124T (hereafter referred to as insect cells) was obtained from Dr. S. Sohi, Great Lakes Forestry Centre, Sault Ste. Marie. This cell line originated from neonate larvae of the spruce budworm *Choristoneura fumiferana* (Sohi, 1995). Insect cell line stock culture was maintained in GM supplemented with 0.25% (w/v) Bacto Tryptose broth (DIFCO) and 10% FBS, and

incubated at 25 °C in the dark. Before sub-culturing, overall cell condition was examined under an inverted microscope. The medium was removed and the cell monolayer was rinsed with 3-4 ml of room temperature 0.05% trypsin solution. The trypsin solution was removed, and 3-4 ml of trypsin solution was added again and left for 2-3 minutes at RT. The second trypsin solution was removed, and 5 ml of growth medium was added. The flask was shaken to triturate the monolayer until all the monolayer had come loose. From this cell suspension 0.5-0.8 ml was transferred into a new flask already containing approximately 4.5 ml growth media. They were mixed gently and incubated at RT in the dark.

2.2 Antibodies and Sources

Rabbit polyclonal anti-β_1-chicken integrin antiserum was kindly donated by Dr. R. Hynes, Massachusetts Institute of Technology. Freeze-dried antiserum was resuspended at a concentration of 3 mg/ml. This stock concentration is 22 times less than the suggested concentration, and thus our working dilutions are also comparatively weaker than those reported in the literature.

Three different rabbit polyclonal antibodies, UMN12, UMN13 and αINT1-600, which recognize different domains of the integrin-like protein αIntp in *C. albicans*, were obtained from Dr. M. K. Hostetter, Yale University. UMN12 was raised against a peptide containing the second divalent cation-binding motif of αInt1p; UMN13 was raised against αInt1p amino acids 1143 to 1157 (a RGD region of the αInt1p predicted to be

extracellular); and αInt1-600 was raised against the first 600 amino acids of αInt1p in *C. albicans* (Gale *et al.*, 1996; Gale *et al.*, 1998).

Goat anti-rabbit IgG-alkaline phosphatase (Sigma, A-3687) was used as the secondary antibody in immunoblots. The secondary antibody, ALEXA 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon) was used for immunofluorescence microscopy.

Since all primary antibodies were raised in rabbits, non-immune rabbit serum (Sigma, R-9133) and used in controls, replacing the primary antibody. The original concentration of protein in the serum was 40-70 mg/ml, and thus was diluted as indicated for the experiments. As a result, the protein concentration in the non-immune serum was high relative to the concentration in the immune serum (see above).

All antibody stock solutions were divided into 20 µl aliquots and stored at –70 °C. For immunoblots they were diluted in Tween-20 wash solution (see below) before use. For immunofluorescence microscopy, antibody dilution buffer was made by adding 1% BSA (Sigma) and 1% sodium azide (Sigma) to 0.01 M PBS (0.15 M NaCl, pH 6.8).

2.3 Chemicals and Buffers

The following solutions were used in for immunoblotting: 0.2 M phosphate buffer (PB; 39% 0.2 M NaH_2PO_4 and 61% 0.2 M Na_2HPO_4, adjusted to pH 7.0); Sample buffer 1 {65 mM Tris-HCl, 1.3% (w/v) SDS, 13% (v/v) glycerol, 0.02% (w/v) sodium azide and a small amount of Bromophenol Blue (Schwarz-Mann Biotech), with or without DTT (DL-Dithiothreitol, Promega Corporation) in distilled deionized water (hereafter referred to as water), and pH adjusted to 6.8}; Sample buffer 2 {as sample

buffer 1 but using 0.5% Triton X-100 (Sigma) instead of SDS}; Transfer buffer {10 mM CAPS (3-[Cyclohexyamino]-1-propanesulfonic acid; Sigma) and 10% methanol in water, pH adjusted to 11}; Tween-20 wash solution {TTBS, Tween-20/Tri-buffered saline, 20 mM Tris, 500 mM NaCl and 0.05% Tween-20 (Bio-RAD Laboratories) in water; pH adjusted to 7.5}.

Stock solutions of eight different protease inhibitors (hereafter referred to as inhibitors) were purchased from Sigma and prepared as follows. 1) AEBSF {12.5 mg/ml (50 mM) in water}, stored at –70 °C and used at a final concentration of 1 mM. 2)Aprotinin (1 mg/ml in water), stored at 4 °C, and used at a final concentration of 5μg/ml. 3) Leupeptin (1 mg/ml in water), stored at –70 °C, and used at a final concentration of 2 μg/ml. 4)TLCK {3.7 mg/ml (10 mM) in 1 mM HCl (pH 3.0), stored at –70 °C, and used at a final concentration of 0.1 mM. 5) TPCK {3.5 mg/ml (10 mM) in 100% methanol}, stored at –70 °C, and used at a final concentration 0.1 mM. 6) PMSF {1.7 mg/ml (1 M) in 100% methanol}, stored at –20 °C, and used at a final concentration 1 mM. 7) EDTA (0.5 M in water), stored at RT, and used at a final concentration of 1 mM. 8) Pepstatin (1 mg/ml in 100% methanol), stored at –20 °C, and used at a final concentration 1 μg/ml.)

The following solutions were used in the immunofluorescence experiments: 0.1M phosphate buffered saline (PBS) was prepared by adding 51% 0.2 M NaH_2PO_4 with 49% 0.2 M Na_2HPO_4, diluted 1:1 with water, 15 M NaCl was added, and it was filtered through a 0.22 μm Nalgene filtration unit; pH adjusted to 6.8; TritonX-100 solution {0.5% TritonX-100 (Sigma) in PBS; Blocking buffer {10% FBS (Sigma), 3% BSA

(Sigma), 0.02% sodium azide (Sigma) in PBS; milk-blocking buffer (2% non-fat instant skim milk powder in water); Tween-20 buffer {0.1% Tween-20 (Bio-RAD Laboratories) in PBS; mounting solution {90% glycerol, 10% Citifluor antifade solution (Marivac Ltd, Halifax, Canada); protease inhibitors was used at the same concentration as used in immunoblotting (above); formalin (37%) was diluted to a final concentration of 3.7%.

Fibronectin stock solution (Sigma, F-1141) was added to Tris buffer (0.5 M NaCl, 0.05 M Tris, pH 7.5) to make 100 and 25 μg/ml fibronectin solutions. Collagen solution (Sigma, C-8919; type 1, from calf skin) was used at a concentration of 1% collagen in 0.1N acetic acid.

The following solutions were used in attachment experiments: Trypsin solution {0.5g trypsin powder (Anachemia) in Rinaldini's salt solution (Rinaldini, 1959), filtered through a 0.22 μm Nalgene filtration unit, and stored at 4 °C}; Tris buffer (0.05 M Tris, 0.5 M NaCl in water) pH adjusted to 7.5, filtered, and stored at 4 °C; fibronectin and collagen solutions as described above. Each of the following peptides was dissolved separately in water to make a stock solution of 20 mM, stored at 4 °C and used at a final concentration of 1mM: 1) the RGD-containing linear peptide ARG-GLY-ASP (Sigma, A-8052) hereafter called linear RGD), 2) the RGD-containing circular peptide 1-adamantaneacetyl-CYS-GLY-ARG-GLY-ASP-SEP-PRO-CYS (disulfide bridge 1-8; Sigma A-1430; hereafter called circular RGD), and 3) the control non-RGD peptide ARG-PHE-ASP-SER (Sigma, A-1675) ; hereafter called non-RGD or RFD).

2.4 Immunoblotting Experiments

2.4.1 Cell Cultures and Cell Extract Preparation

Protoplasts: For culturing larger volumes of protoplasts, l0 µl stock culture was transferred to a 50-ml tissue culture flask containing 5 ml of GM supplemented with 5% FBS, incubated for two days, and 0.8 ml of this culture transferred to each of 6 flasks containing 10 ml GM supplemented with 5% FBS, and incubated for 1-2 days.

Protoplasts were harvested by centrifugation at 150 x *g* for 5 minutes in a DYNAC centrifuge (Dickinson and company, N.J.). Cell pellets were combined and rinsed with PB containing all eight protease inhibitors (as above). Cells were centrifuged in a microcentrifuge at 13,000 x g for 20 seconds. During this procedure, cells and PBS were kept on ice except when being centrifuged.

To prepare cell lysates, an aliquot (~50-70 µl) of cells was mixed with ~300 µl of sample buffer 1 containing the eight inhibitors, and then briefly vortexed. This cell extract was divided into ~50 µl aliquots and stored at –70 °C or used immediately.

Prior to electrophoresis and gel loading, an aliquot of cell extract was placed in a boiling water bath for 5 minutes and then clarified by centrifugation in a microcentrifuge at 14,000 x g for 1 minute.

In the early trials, the procedures were the same as described above, except 1) no inhibitors were used, or 2) only three inhibitors were used: Pepstatin, PMSF and EDTA.

Fibroblasts: The same procedure was conducted to make fibroblast lysates as described for protoplasts, but no protease inhibitors were used. Prior to electrophoresis

and gel loading, the cell extract was placed in a boiling water bath for 5 minutes and then clarified by centrifugation in a microcentrifuge at 14,000 x g for 1 minute.

Yeast: Yeast was grown in a 250 ml Erlenmeyer flask containing 100 ml sterile Sabouraud's Dextrose broth (DIFCO) on a rotary shaker (Gyrotory Shaker Model G2, New Brunswick Scientific Co. Inc, Edism, N. J., U.S.A.) at 25 °C for 2 days. The 100 ml-inoculum was used to inoculate a 2 L Erlenmeyer flask containing 1 L sterile Sabouraud's Dextrose broth. The culture was then incubated in a rotary shaker (Psycrotherm Controlled Environment Incubator Shaker, New Brunswick Scientific, Inc., N. J., U.S.A.) at 25 °C at 150 x g for 5 days.

Cells were harvested by centrifugation in a Sorval RC 5C Plus Centrifuge (Dupont Sorvall Instruments, Delaware, U.S.A.) at 12,000 x g for 15 minutes at 4° C. Pelletted cells were collected and frozen at –50 °C, and then lyophilized and kept in the refrigerator at 4 °C until use.

A thick slurry of freeze-dried yeast cells (15.4 g) in 30 ml PB containing eight inhibitors, was broken by three passages through a French Pressure Cell (SLM Instruments, Inc., Urbana, IL. U.S.A.) operated at 32,000 PSI. Broken cells were mixed well with 15 ml PB containing inhibitors and centrifuged in a Sorval RC 5C Plus Centrifuge (Dupont Sorvall Instruments, Delaware, and U.S.A.) at 16,500 x g at 4 °C for 30 minutes. Both the supernatant and the cell pellet were collected and stored at –70 °C.

To prepare the cell extract, ~100 µl cell pellet was mixed with 100 µl PB containing eight inhibitors, and ~200 µl sample buffer 2 containing eight inhibitors was added. The mixture was vortexed well and kept on ice for about 20 minutes. This cell

extract was divided into ~80 µl aliquots and stored at –70 °C. Prior to electrophoresis and gel loading, an aliquot of cell extract was placed in a boiling water bath for 5 minutes and then clarified by centrifugation in a microcentrifuge at 14,000 x g for 1 minute.

2.4.2 Immunobotting (Western Blot)

Electrophoresis was performed on a Bio-Rad mini-Protein II apparatus (Bio-Rad, Richmond, CA). All gels were 0.75 mm thick and consisted of an acrylamide/N,N-methylene-bis-acrylamide (w/v) ratio of 37.5:1. SDS-PAGE was carried out by the method of Laemmli (1970) on 8% polyacrylamide slabs, with a 3% stacking gel at 180v constant voltage. Protein bands were visualized by staining in a shaking bath containing 0.2% (w/v) Coomassie Brilliant Blue R-250 (Kodak) in 50% (v/v) ethanol, 10% (v/v) acetic acid and then destained in 20% (v/v) ethanol, 10% (v/v) acetic acid.

Western transfer from SDS polyacrylamide gels to polyvinylidene difluoride membrane was conducted in a Bio-Rad mini Trans Blot Electrophoresis Transfer Cell. The blotting conditions were 60 V for 4 hours in 10 mM CAPS, 10% (v/v) methanol. Immobilized proteins were probed with four different anti-integrin antibodies separately (see above). All primary antibodies were used at a dilution of 1:100-500. Reactive proteins were detected with the secondary antibody (see above) at a dilution of 1:2000, and a solution of 0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$ (pH 9.8), 0.15 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.3 mg/ml 4-nitroble tetrazolium chloride (Boehringer Mannheim Germany) for color reaction. Molecular weights of cross-reacting bands were estimated from immunoblots by linear regression of log-transformed data.

For negative controls, all the procedures were the same except 1) non-immune rabbit serum was used at a dilution of 1:100 to replace the primary anti-integrin antibody; or 2) the primary antibody was omitted and only the secondary antibody was used to incubate the blotted membrane. Fibroblast and yeast cell extracts were used as positive controls, and all working conditions of immunoblots were the same as described above.

Images of immunoblots were digitised using a Hewlett Packer Scan Jet 5100C and printed without enhancement of contrast.

2.5 Immunofluorescence Experiments

2.5.1 Cell Culture Preparation

A culture of protoplasts of *E. aulicae*, isolate FPMI-521, was centrifuged in a DYNAC centrifuge at 150 x g for 3 minutes, and suspended in 0.1 M PBS for 5-10 minutes to allow protoplasts to regain shape. Fibroblasts were grown on autoclaved cover slips (Baxter, Canlab, #1) squares, made by breaking glass cover slips into ~1 cm x 1 cm squares, or in chamber slides (Lab-Tek) with or without fibronectin (100 μg/ml) coating.

2.5.2 Immunofluorescence Microscopy

Protoplasts: Approximately 18 μl of culture was gently pipetted onto glass cover slip squares and incubated at 19 °C for ~30 minutes in a moist container in the dark until normal spindle cell shape returned. Approximately 2 μl of 37% formalin solution was gently added to each culture droplet to give a final concentration of 3.7% formalin, and left for 10 minutes, then checked under the microscope for the maintenance of cell shape. Protoplasts were processed immediately, without storage.

Protoplasts were rinsed in PBS three times for 10 minutes each, incubated in 0.5% TritonX-100 buffer for 10 minutes, blocked in blocking buffer twice for 5-7 minutes each, and blocked in 2% milk-blocking buffer for 5 minutes. Finally, protoplasts were rinsed in blocking buffer twice for 5-7 minutes each, and then left in the blocking buffer for 2 hours in a moist chamber at RT.

For the primary antibody incubation, 20 µl of antibody solution was added to the fixed protoplasts, and incubated overnight in a moist chamber at 4 °C. The protoplasts were rinsed in 0.1% Tween-20 buffer three times over 30 minutes, ~20 µl of the secondary antibody solution was added, and protoplasts were incubated in a light-tight box for 2 hours at RT.

The protoplasts were rinsed in 0.1% Tween-20 buffer two times over 20 minutes, and then stained in 20 µl DAPI for 2-3 minutes (optional), and rinsed in 0.1% Tween-20 buffer two times over 20 minutes (optional). Protoplasts were rinsed in Citifluor for 5 minutes, and then mounted in antifade solution. Cover slips were sealed with nail polish.

Controls: Fibroblasts were fixed in 3.7% formalin for 10 minutes and rinsed in 0.1 M PBS three times over 30 minutes, and then stored in PBS up to one month. Other procedures were the same as the methods as described for protoplasts above.

For negative controls, all the procedures were the same as the method as described above except 1) rabbit serum was used at different dilutions to replace the anti-integrin antibodies and 2) the primary antibody was omitted.

Many controls were performed in order to determine the source of non-specific fluorescence in protoplasts. Slides of the protoplasts were observed by fluorescence

microscopy before fixation, and after fixation for different periods of time (2, 5, 10, 15, and 30 minutes). Also, slides were observed after each step in the procedure, including rinsing in PBS, incubating in 0.5% TritonX-100, blocking in the blocking buffer and 2% milk-blocking buffer, incubating in the primary antibody solution, rinsing in 0.1% Tween-20 buffer, and after the antifade solution. These slides were observed immediately after being prepared.

Cells were observed at 400x using a Nikon Eclipse (E600) microscope equipped with a 40x Plan Fluor lens (numerical aperture 0.75). The filter for fluorescence imaging was B-2A (Ex 450-490; DM 505; BA 520). All pictures were taken using a Nikon FDX-35 camera and T-MAX 400 film, and developed with T-MAX developer diluted 1: 4, for 7 minutes. Pictures exposure times were in the range of 8 to 25 sec except where longer exposures are indicated in order to emphasize the low signal. Pictures were printed on Kodak Polycontrast III paper. Immunofluorescence pictures were digitised using a Hewlett Packer Scan Jet 5100C and printed without enhancement of contrast.

2.6 Attachment Experiments

2.6.1 Attachment of Protoplasts to Coated Surfaces

Collagen and Fibronectin Coated Surfaces

Collagen (1000 μg/ml) and fibronectin (25, 100, and 1000 μg/ml) were tested for their ability to promote protoplast attachment to the surface of multiwell glass microscope slides (CN Biomedicals, Inc., Ohio).

Slides were washed briefly in detergent, rinsed in water and air-dried. To coat the slides, 20 µl of the appropriate solution was added to each well. Slides were air-dried and sterilized by UV light overnight. Wells without coatings were used as controls.

Logarithmic protoplast cultures were centrifuged at 150 x *g* for 5 minutes, and the supernatant was discarded. Protoplasts were rinsed with GM once and centrifuged again. After discarding the supernatant, GM was added and protoplasts were gently resuspended. Protoplast concentration was adjusted to give a final concentration of 20-50 cells per field of view at 150x magnification.

The slides were rinsed twice with water. For each treatment, 20 µl of protoplast suspension was added to each of eight wells and slides were kept in a moist chamber at 19 °C in the dark for 30 minutes, 3 hours or 9 hours. Cell numbers in each well were counted and then treatments to test protoplast attachment were applied. The treatments included 1) wicking: gently drawing medium from the well, using a 1/8 of Ahlstorm filter paper (Grade 601, size 9 cm; Rose Scientific Ltd., Alberta) and 2) rinsing: gently rinsing with medium by pipetting medium from the well and gently adding new medium to the well surface. All observations were made using an inverted microscope (Zeiss, West Germany) at 150x magnification. The protoplasts were observed through the microscope while the treatments were being performed.

Peptide Inhibition

The three peptides were tested separately for their ability to inhibit protoplast attachment to multiwell slides coated with fibronectin (25 µg/ml): the linear RGD peptide, the circular RGD peptide and the non-RGD peptide. All procedures were as

described above, except as follows. Protoplasts were pre-incubated for 1 hour at 19 °C in the dark in medium containing 1 mM peptide before adding them to the multiwell slides. Peptide was also omitted as a control. Incubation was for three or nine hours.

Effects of FBS and Different Isolates

Experiments were carried out to determine if effects were isolate specific, and to determine if FBS affected attachment. The same procedure was followed as described above but using two isolates, FPMI-521 and FPMI-646, and protoplasts were resuspended in GM to which 5% FBS was added or were again left without FBS.

2.6.2 Attachment of Protoplasts to Insect Cells

Growth Curves

In order to determine conditions of growth compatible to both protoplasts and fibroblasts, growth curves experiments were performed on the cell lines separately and then together.

Protoplasts: A logarithmic culture of spindle-shaped protoplasts of *E. aulicae* isolate FPMI-521 was centrifuged at 150 x *g* for 5 minutes, the supernatant was discarded and GM with 2.5% FBS was added to resuspend the protoplasts. An aliquot of 0.1 ml of protoplast suspension was added to each of three flasks containing 10 ml GM with 2.5% FBS. Protoplasts were incubated at 19 °C in the dark and counted at 12-hour intervals. The concentration of protoplasts was calculated using a hemocytometer.

Insect cells: Insect cells were cultured, as described previously, in tissue culture flasks until attached but non-confluent. The medium was discarded, the cells were rinsed with 3-4 ml GM (with 2.5% FBS) and this medium was removed; then 10 ml GM (with

2.5% FBS) was added to each flask. Four randomly chosen fields in each of the three flasks were marked and the initial insect cell numbers per field of view (viewed at 300x) were counted under an inverted microscope (Leitz, Wetzlar, Germany). Insect cells were incubated at 19 °C in the dark and cell numbers were counted at 12-hour intervals.

Protoplasts and insect cells: The insect cell medium in three flasks of an attached but non-confluent culture of insect cells was replaced with 10 ml GM with 2.5% FBS as described above. An aliquot of 0.1 ml of protoplast suspension was added to each of three flasks containing 10 ml GM with 2.5% FBS. Flasks were incubated at 19 °C in the dark. Protoplasts and insect cell numbers were counted, separately, at 12-hour intervals as described above.

Cell-Cell Attachment Experiments

Logarithmic spindle-shaped protoplast cultures were centrifuged at 150 x *g* for 5 minutes, and the supernatant was discarded. Protoplasts were rinsed with GM once, centrifuged again, and the supernatant was discarded. GM was added to resuspend protoplasts and the concentration of protoplasts was adjusted to ensure the cell number was countable. All counts were performed using an inverted microscope, at 300x magnification.

Insect cells were cultured in a 96-well tissue culture plate (Falcon) by adding 300 µl of a suspension of insect cells into each well in a total of 25 wells on one 96-multiwell plate, and they were cultured at 25 °C in the dark until confluent. Then, the insect cell medium was removed and insect cells were rinsed with GM once. A 300 µl aliquot of protoplasts was added to each of the 25 wells. Plates were incubated at

19 °C in the dark for 4 hours.

After incubation, the number of protoplasts in each well was counted. Then, the medium from each well was gently pipetted and removed to another well. The original well was rinsed with 300 µl of GM twice and the rinsing medium was again pipetted to new wells each time. The number of protoplasts remaining in the original well with the insect cells after rinsing was counted, and these were considered to be "attached". The number of protoplasts removed by the initial pipetting and by each of the two rinsings were also counted and considered as "not attached". A 96-multiwell plate without insect cells was set up to control for the non-specific attachment of protoplasts to the plate itself and the same procedure was performed as described above.

2.7 Statistical Analysis

All statistical tests were performed using Minitab Statistical Software, Standard Version, Release 9.1.

Chapter 3 Results

3.1 Immunoblotting Experiments

Anti-$ß_1$-chicken integrin antiserum (Plate One) :

Positive controls: Fibroblast and yeast extracts were used as positive controls. The extracts of fibroblasts probed with anti-$ß_1$-chicken integrin antiserum gave consistent positive results: two bands were observed at apparent molecular masses of 106 and 92 kDa, respectively (Fig. 3). These two bands could be seen clearly only in the blots of freshly prepared cell extracts, but not using stored extracts (data not shown). Lower molecular weight bands were also evident as commonly seen in other studies (eg. Marcantonio and Hynes, 1988).

Yeast extracts probed with anti- $ß_1$-chicken integrin antiserum gave poor results, and non-specific background staining was a persistent problem (data not shown).

Protoplasts: A ~71 kDa protein was recognized in freshly prepared extracts of protoplasts containing eight inhibitors when probed with anti-$ß_1$-chicken integrin antiserum at a dilution of 1:200 (Fig. 4). The inclusion of DTT in the cell extract had no effect on the mobility of the 71 kDa band (Fig. 4).

A combination of eight inhibitors and freshly prepared cell extracts were necessary to obtain consistent results. Protein degradation in protoplast extracts was a problem in preliminaries trials and multiple bands were observed with zero or three inhibitors, or using cell extracts that had been frozen (data not shown).

Negative controls: No staining was observed when the primary antibody was omitted and the blotted membrane was incubated only with the secondary antibody

(Fig. 5A). Little or no staining was observed when using non-immune rabbit serum at a dilution of 1:100 to replace the primary antibody (data not shown).

***C. albican's* antibodies:**

Results using the anti-*Candida* antibodies were variable and not reproducible. They resulted in no bands, high background or multiple bands in both protoplasts and yeast cell extracts (data not shown). There was no clear labeling of the expected 185 kDa integrin-like protein in yeast extracts. There was no consistent labeling of a 71 kDa fragment in protoplast extracts as observed using the anti-β_1-chicken-integrin antiserum as described above (data not shown).

3.2 Immunofluorescence Experiments

Positive Control (Plate Two): For fibroblasts, omission of the primary antibody or storage in PBS for one month resulted in no staining (Fig. 6A,B). When probed with the anti-β_1-chicken integrin antiserum, fairly bright fluorescence staining was noticeable throughout the cell (Fig. 6C). A similar pattern of staining was found when the anti-integrin antibody was replaced with the non-immune rabbit serum (Fig. 6D). The inclusion of fibronectin coating did not make a marked difference on fibroblast immunofluorescence images (data not shown).

Experimental (Plate Three): Three areas of staining were observed in protoplasts 1) patches at the cell periphery, 2) nuclear surface staining, and 3) nuclear "core" staining.

The staining pattern of peripheral patches was best seen on the thin terminal cytoplasmic extensions of the protoplasts (Fig. 7A), and internuclear constrictions (not

illustrated). Patches were also seen on the periphery of the cell body (Fig. 7A). Similar patterns of staining on the periphery of the cell could be observed when probed with the three yeast antibodies, UMN12 (Fig. 7), αINT1-600, UMN13 (not illustrated), and the anti-chicken integrin antibody (Fig. 8,9).

A distinct staining of a "core" in the nucleus was common (Fig. 7-9) and staining around the nucleus was also evident (Fig. 9). Both types of nuclear staining were also observed in some of the controls and are explainable by non-specific staining (as described below). At no time was the patchy staining of the cell periphery observed in controls .

Controls (Plates Four and Five): Protoplasts fixed for 10 minutes without further processing showed little or no fluorescence (Fig. 10, long exposure time to emphasize lack of signal), but those fixed for relatively longer periods (30 minutes) showed staining around the nuclei (Fig. 11). Omission of the primary antibody from the staining procedure resulted in no fluorescence (Fig. 12, long exposure to emphasize lack of signal).

Replacing the primary antibody with non-immune rabbit serum revealed staining of the periphery of the nucleus (Fig. 13) as well as staining of the nuclear "core" (Fig. 14) with only the nuclear core staining at high dilutions of the serum (data not shown). Using the primary antibodies at dilutions sufficiently high to preclude staining of the cell periphery also gave staining of the core of the nucleus (Fig. 15). At no time was the patchy staining at the cell periphery, in terminal extensions, or internuclear constrictions observed in controls.

3.3 Attachment Experiments

3.3.1 Attachment of Protoplasts to Coated Surfaces

Collagen and Fibronectin Coated Surfaces

Surface coating significantly influenced percentage attachment; time did not affect attachment and there was no interaction between these two factors (ANOVA surface: $F = 648$; $P = 0.000$; time: $F = 0.19$; $P = 0.826$; no interaction).

The attachment of protoplasts to the uncoated glass surface was high, up to 100% regardless of incubation time or treatment (Table 1). Protoplasts showed little to no attachment to collagen (1000 μg/ml) or to fibronectin at concentrations of 1000 μg/ml or 100 μg/ml, regardless of incubation time or treatment. However, at a concentration of fibronectin of 25 μg/ml, the percentage of attachment was relatively high with 61– 92% remaining after wicking; this was reduced only somewhat by rinsing.

Peptide Inhibition

Inhibition experiments were designed to test whether or not the attachment of protoplasts to fibronectin at 25 μg/ml was dependent upon RGD binding sites, a reflection of integrin involvement. Again surface significantly influenced percentage attachment; time did not affect attachment and there was no interaction between these two factors (ANOVA surface: $F = 22.15$; $P = 0.000$; time: $F = 0.34$; $P = 0.563$; no interaction).

Inclusion of linear or circular RGD peptides did not substantially influence percentage of attachment of protoplasts to slides coated with fibronectin at 25 μg/ml

regardless of time (Table 2, Trial One). Surprisingly, inclusion of the RFD control peptide resulted in higher percentage attachment (Table 2, Trial One).

There was significant variation between replicates of this experiment. In Trial One, attachment of protoplasts was fairly low in both experimental treatments and controls at both three and nine hours (Table 2). However, when the 3-hour incubation was repeated (Table 2, Trial Two), attachment of protoplasts was high, up to 96.2% in controls, and this result was close to the result in earlier experiments (Table 1). These results suggested that the attachment was non-specific.

Effects of FBS and Different Isolates

To determine if other factors might be interfering with the attachment we looked at 1) possible isolate differences and 2) the effects of FBS on attachment. Neither isolate FPMI-521 nor FPMI-646 attached to any of the surfaces including uncoated glass when FBS was added to medium (Table 3). As in the previous experiment, without FBS, both FPMI-521 and FPMI-646 protoplasts attached to glass surface at high percentage, but not to collagen or fibronectin coated surfaces at these concentrations. There was no difference between isolates.

3.3.2 Attachment of Protoplasts to Insect Cells

Growth Curves

We first determined conditions that would allow growth of both protoplasts and insect cells for the cell-cell attachment experiments. The results showed that when protoplasts and insect cells were grown separately in GM with 2.5% FBS, their numbers

increased exponentially. Protoplasts increased over 72 hours before decreasing (Fig. 16, filled circles). Insect cells continued to increase up to 144 hours (Fig. 17, filled circles).

When these two cell types were grown together, protoplasts grew faster with insect cells (Fig. 16, open circles) than without them, but insect cells grew better without protoplasts than with them (Fig. 17, open circles). Insect cells grown with protoplasts increased in number over the first 24 hours, but then declined and none were detected at 144 hours. Thus, it was not possible to have lengthy experiments with both cell types combined because of the decline in insect cells over time. However, within a period of 20 hours insect cell number did increase (Fig. 17, open circles), and so attachment experiments were kept well with this time frame.

Cell-Cell Attachment Experiments

Protoplasts did not attach to either the bottom of the 96-well plate (control) or the host insect cells after four hours incubation (Table 4). Few protoplasts (~2%) remained in control wells, and no protoplasts were found in wells with the insect cells. A second experiment and further direct microscopic observations also revealed no evidence of interaction between protoplasts and this host insect cell line.

Plate One: Immunoblots of protoplasts of *Entomophaga aulicae* and monkey kidney fibroblasts. (M, high molecular weight marker; LM, low molecular weight marker; F, fibroblast; P, protoplast)

Figure 3. Immunoblot of fibroblast extracts probed with rabbit polyclonal anti- $ß_1$chicken integrin antiserum at a dilution of 1:100 (duplicate lanes) .

Figure 4. Immunoblot of freshly prepared protoplast extracts extracted with eight protease inhibitors and probed with rabbit polyclonal anti-chicken integrin antiserum at a dilution of 1:200. Lane 1, high molecular weight marker (M); lane 2 with DTT; lane 3, without DTT.

Figure 5. Negative control of immunoblots. Fibroblast and protoplast extracts without the primary antibody and incubated with only the secondary antibody, goat anti-rabbit IgG-alkaline phosphatase antibody.

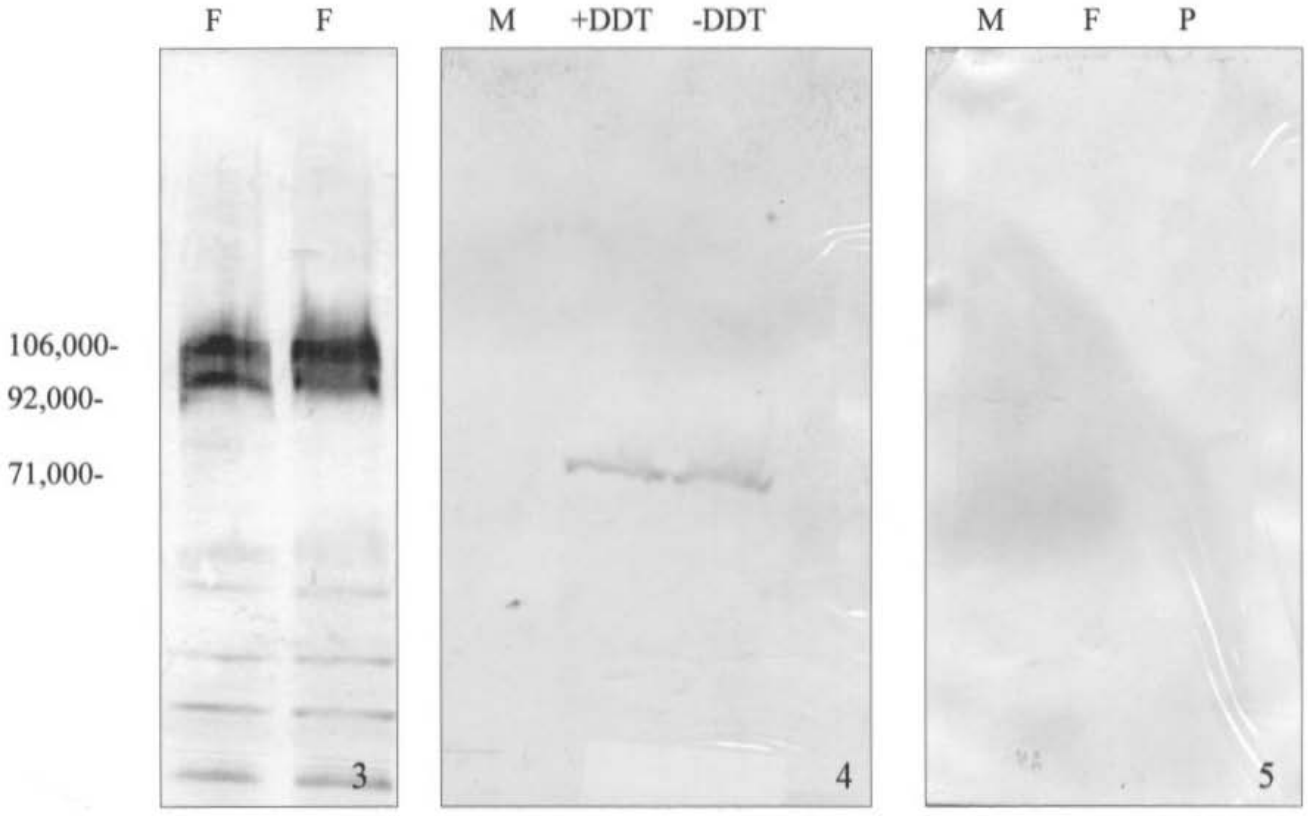

F
F
106,000-
92,000-
71,000-
3
M
+DDT
-DDT
4
M
F
P
5

Plate Two: Immunofluorescence images of monkey kidney fibroblasts. All photographs printed at 1000X.

Figure 6. Immunofluorescence images of monkey kidney fibroblasts. A, a short fixation time without any antibody incubation after storage in PBS for one month. B, omission of the primary antibody (the secondary antibody at a dilution of 1: 200). C, probed with rabbit polyclonal anti-β_1-chicken integrin antibody at a dilution of 1: 5. D, fibroblasts probed with pre-immune rabbit serum at a dilution of 1: 25.

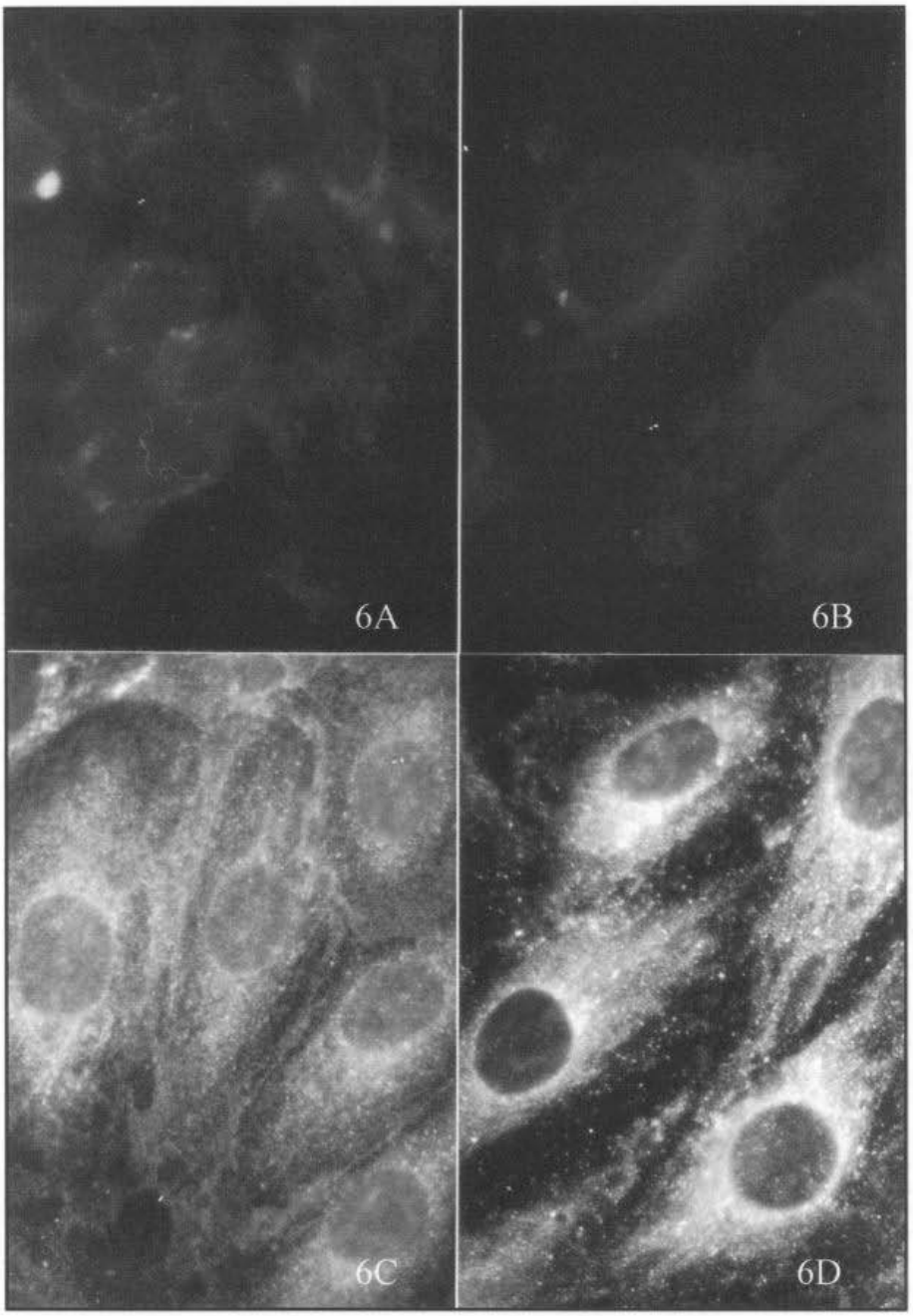

6A
6B
6C
6D

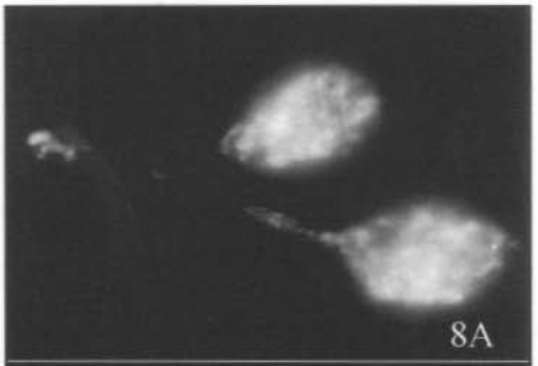
Plate Three: Protoplasts of *Entomophaga aulicae* probed with anti-integrin antibodies. All photographs printed at 1000X.

Figure 7. Protoplasts probed with rabbit polyclonal anti-*Candida albicans* integrin antibody (UMN12) at a dilution of 1:100. A, peripheral immunofluorescence image. B, near-medial immunofluorescence image. C, DIC image. Note staining of patches in the upper extension of the cell and the periphery of the cell, and staining of a core in the nucleus.

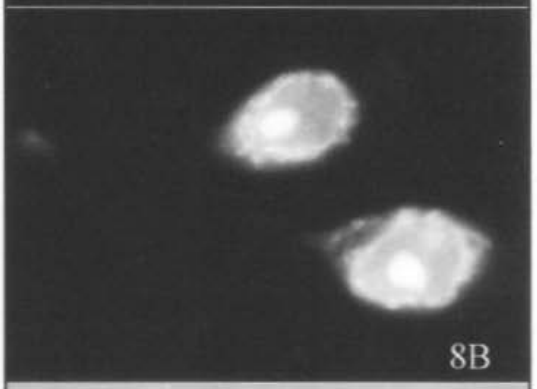
Figure 8. Protoplasts of probed with rabbit polyclonal anti-β_1-chicken integrin antibody at a dilution of 1:5. A, peripheral immunofluorescence image. B, near-medial immunofluorescence image. C, DIC phase image. Note staining of patches in the extension and the periphery of the cell, and around the nucleus, also staining of a core in the nucleus.

Figure 9. Protoplasts probed with rabbit polyclonal anti-β_1-chicken integrin antibody at a dilution of 1: 5 . A and C, peripheral immunofluorescence images. B and D, near-medial immunofluorescence images. Note staining of patches in the periphery of the cell and around the nucleus, also staining of a core in the nucleus.


7A

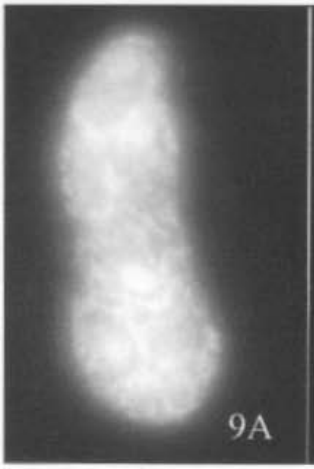

8A


7B


8B


7C


8C


9A

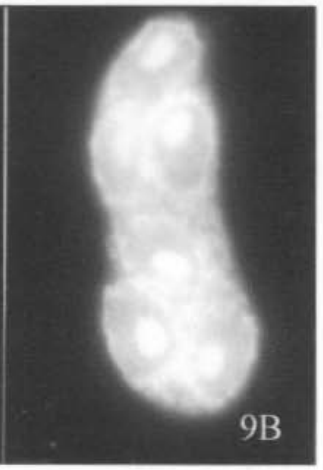

9B

Plate Four: Controls for immunofluoresence staining of protoplasts of *Entomophaga aulicae*. All photographs printed at 1000X.

Figure 10. A, B: Control for fixation (short 10 min fixation, as experimental, with no antibody incubation, and long photographic exposure time to emphasize low signal: 809 seconds). (A. DIC image of B).

Figure 11: Demonstration of fluorescence around nuclei, an artifact caused by long fixation time (30 minutes), without any antibody incubation .

Figure 12: Control omitting the primary antibody; the secondary antibody was used at a dilution of 1: 200 and the exposure time is long to emphasize low signal (143 seconds).

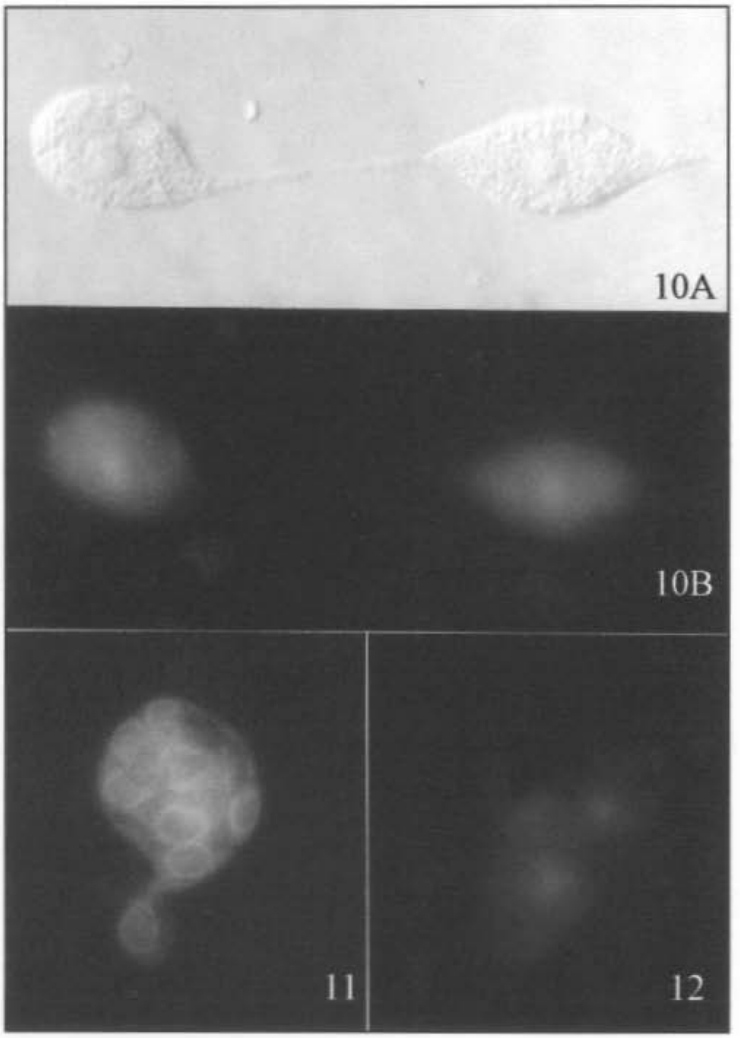

10A
10B
11
12

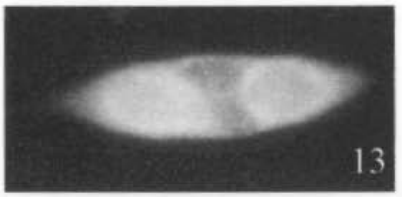
Plate Five: Controls for immunofluoresence staining of protoplasts of *Entomophaga aulicae*: staining with non-immune rabbit serum and highly diluted antibody. All photographs printed at 1000X.

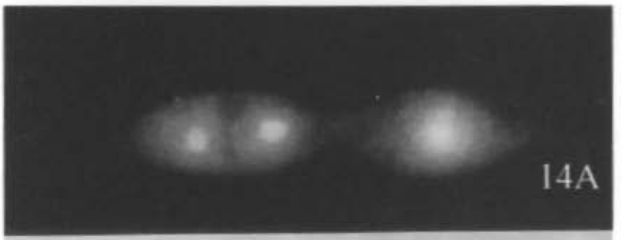
Figure 13. Protoplasts showing very strong non-specific fluorescence around the nucleus caused by pre-immune rabbit serum at a dilution of 1: 25.

Figure 14. Protoplasts showing strong non-specific fluorescence of "cores" within the nuclei caused by pre-immune rabbit serum at a dilution of 1:500. (B, DIC phase image of A).

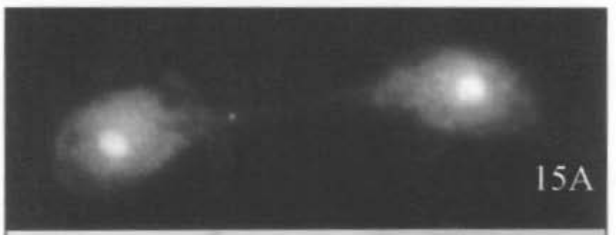
Figure 15. Protoplasts probed with rabbit polyclonal anti-β_1-chicken integrin antibody at a relatively high dilution of 1:500. Note staining of "cores" in the nuclei, but no peripheral staining at this relatively high dilution[1].

[1] Note higher dilution of stock solution (3 mg/ml) used in this study.


13


14A


14B


15A


15B

Table 1. Percentage attachment of *Entomophaga aulicae* FMPI-521 protoplasts in GM to surfaces coated with collagen (CN) or fibronectin (FN) after different incubation times and treatments (Eight wells, with 20-50 cells per well, were counted per treatment).

	Glass		**CN (1000 μg/ml)**		**FN (1000 μg/ml)**		**FN (100 μg/ml)**		**FN (25 μg/ml)**	
	W [a]	**R**	**W**	**R**	**W**	**R**	**W**	**R**	**W**	**R**
30min.	99.6±1.1	99.6±1.1	4.2±4.1	0.7±1.3	1.8±5.2	0.0±0.0	2.3±2.1	0.0±0.0	61.0±12.9	10.2±10.7
3hrs	100.0±0.0	99.7±0.9	4.4±6.6	0.2±0.6	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	92.8±8.5	51.2±32.0
9hrs	100.0±0.0	100.0±0.0	1.4±3.9	0.0±0.0	0.0±0.0	0.0±0.0	1.4±2.6	0.0±0.0	70.4±20.4	29.5±26.9

(ANOVA surface: F=648, P=0.000; time: F=0.19, P=0.826; no interaction)

[a] Treatments: wicking (W) and rinsing (R) protoplasts while observing the attachment of them to surfaces.

Table 2. Effect of peptides on percentage attachment of *E. aulicae* FMPI-521 protoplasts to surfaces coated with fibronectin (25 μg/ml) after 3 or 9 hours incubation and treatments (Eight wells, with 20-50 cells per well, were counted per treatment).

	Control		**RGD-l**		**RGD-c**		**RFD**	
	W [a]	**R**	**W**	**R**	**W**	**R**	**W**	**R**
Trial One 3 hrs	3.5±2.6	1.1±2.0	4.3±4.9	0.5±0.8	5.0±6.0	0.4±1.1	45.7±12.6	10.3±11.1
Trial One 9 hrs	11.6±16.0	2.6±4.3	3.6±2.8	0.3±0.8	9.9±12.3	1.6±2.4	23.3±15.9	5.3±5.5
Trial Two 3 hrs	96.2±7.1	88.9±11.1	99.5±2.2	95.8±5.7	67.1±27.6	43.6±21.3	61.1±21.6	50.6±19.3

(ANOVA surface: F=22.15, P=0.000; time F=0.34, P=0.563)

[a] As see Table 1.

Table 3. Percentage attachment of protoplasts of *E. aulicae* FPMI-521 and FPMI-646 in GM with or without FBS to surfaces coated with collagen or fibronectin after 3 hours incubation and treatments (Eight wells, with 20-50 cells per well, were counted per treatment).

	Glass		**Collagen (1000 μg/ml)**		**Fibronectin (100 μg/ml)**	
	W	**R**	**W**	**R**	**W**	**R**
FPMI-521 With FBS	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
FPMI-521 Without FBS	100.0±0.0	99.7±0.9	4.4±6.6	0.2±0.6	0.0±0.0	0.0±0.0
FPMI-646 With FBS	0.0±0.0	0.0±0.0	0.8±0.2.1	0.8±2.1	0.0±0.0	0.0±0.0
FPMI-646 Without FBS	100.0±0.0	100.0±0.0	0.9±2.0	0.9±2.0	0.0±0.0	0.0±0.0

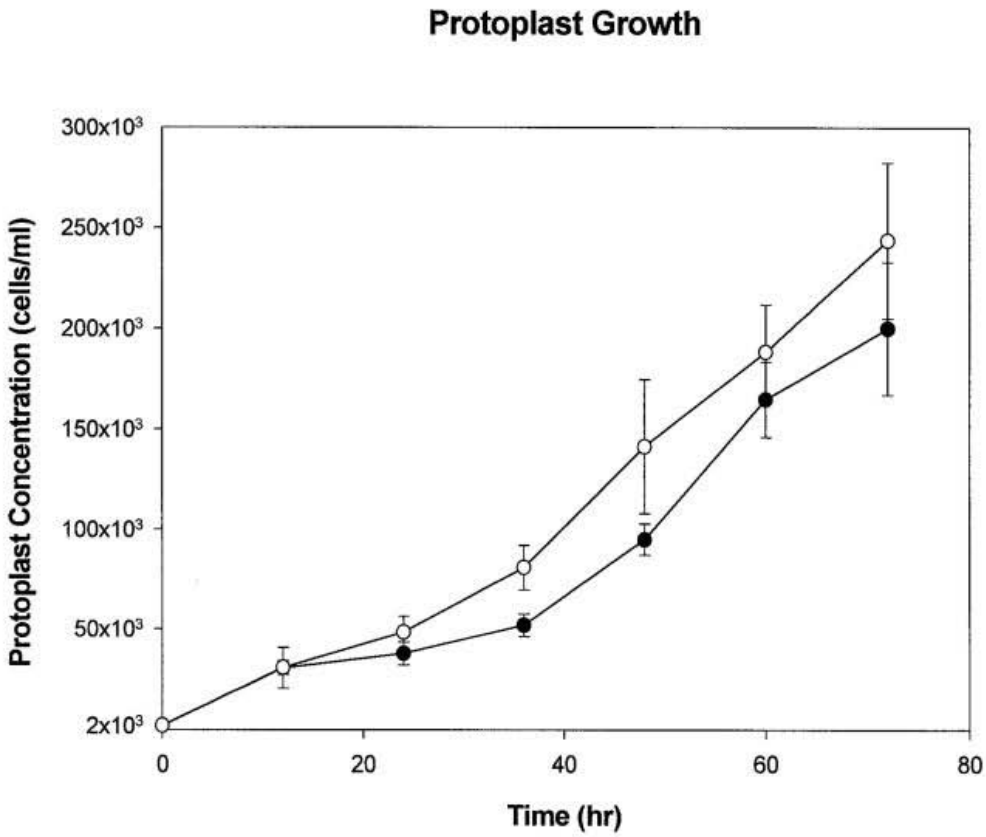


Figure 16. The growth of *Entomophaga aulicae* protoplasts in GM (2.5% FBS) alone (filled circles) or with insect cells (open circles) (Mean ± SD).

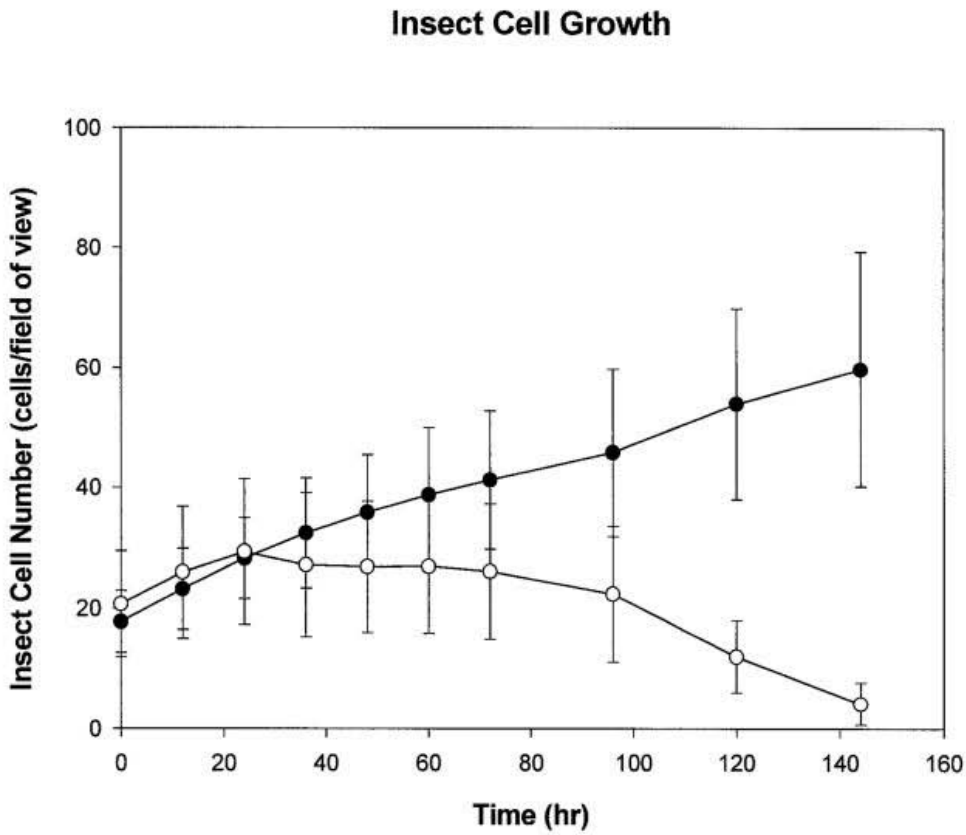


Figure 17. The growth of insect cells CF-124T in GM (2.5% FBS) alone (filled circles) or with *E. aulicae* protoplasts (open circles) (Mean ± SD).

Table 4. Attachment of protoplasts of *Entomophaga aulicae* to host insect cells grown to confluence in GM medium in multiwell plates (Expressed as the X±SD of attached protoplasts per well; n = number of wells)

	Control (without insect cells) n=22	**Experimental (with insect cells) n=25**
Initial # of protoplasts (%)	55±14 (100%)	46±10 (100%)
# of attached protoplasts (%)	1±1 (2%)	0±0 (0%)
# of unattached protoplasts (%)	~99%	49±8 (~106%)

Chapter 4 Discussion

This is the first study to test the hypothesis that an integrin-like protein is present in an insect pathogenic fungus, and that it has a role in attachment to host insect cells. From this study there is some preliminary evidence for the presence of an integrin-like protein in protoplasts of *E. aulicae*. We have identified a 71 kDa protein in protoplasts of *E. aulicae* that cross-reacts with an antibody against vertebrate integrin on immunoblots; and we have shown with immunofluorescence that the protein appears to be distributed at the plasma membrane in a non-uniform pattern. However caution is warranted. In immunofluorescent localizations we were unable to localize integrin satisfactorily in positive fibroblast controls. Further, we were unable to illustrate specific protoplast attachment to the host insect cells, or to surfaces coated with integrin-binding proteins.

4.1 Immunoblotting

In protoplast extracts, a single band of protein was detected on immunoblots by the anti-β_1-integrin antiserum. The molecular weight of this protein was approximately 71 kDa. The specificity of the reaction was supported by the positive and negative controls.

The putative integrin homologue in *E. aulicae* falls within the range of sizes reported for integrins and integrin-like proteins from various sources, which range from 72 to 240 kDa (Schindler *et al.*, 1989; Hemler, 1990, 1991). Published sizes of the β_1-integrins show considerable variation, from 76 to 130 kDa (Hynes, 1987; Marcantonio and Hynes, 1988; Quatrano *et al.*, 1991). There is even a predicted 43 kDa protein from an *Arabidopsis thaliana* cDNA (Nagpal and Quatrano, 1999). In fungi the anti-β_1-integrin

antiserum used in this study also recognized a 95 kDa protein in *C. albicans* (Marcantonio and Hynes, 1988), a 95 kDa protein (reduced) from *U. appendiculatus* (Corrêa *et al.*, 1996) and a 125±15 kDa protein (reduced) in *C. tropicalis* (DeMuri and Hostetter, 1996), a ~63kD protein in *Neurospora* (Degousée *et al.*, 2000), and a 110-120 kDa protein in *P. carinii* (Pottratz *et al.*, 1991). Therefore, the molecular weight of the 71 kDa protein detected in *E. aulicae* protoplast extracts falls within the previously reported range of sizes of integrin-like proteins detected in fungi using the same antibody.

There may be slightly different mobilities between reduced and non-reduced integrins after separation by SDS-PAGE. Such changes in apparent molecular weight in β_1-integrins are often due to the presence of intrachain disulphide bonds (Marcantonio and Hynes, 1988). Alternatively, interchain disulphide bonds may result in higher mobility after reduction (Hynes, 1987; Kaminskyj and Heath, 1995). The fact that DTT, which breaks disulfide bonds, had no effect on the mobility of the 71 kDa protein detected in protoplasts suggests that neither intrachain nor interchain disulfide bonds are present.

The introduction of the combination of the eight protease inhibitors was indispensable for reducing multiple bands in protoplast extracts and obtaining consistent results. Fungi in general, and fungal pathogens of insects in particular, would be expected to possess a variety of proteases. For example, fungal proteases are instrumental in breaking down components of the insect cuticle during host penetration (Charnley and Leger, 1991).

The positive control for the immunoblots, monkey kidney fibroblasts probed with anti-β_1-integrin antiserum, consistently showed two positive bands at ~ 106 and 92 kDa. These sizes fall well within the range of sizes for integrin β_1 protein. Since we know of no other study of integrin in this cell line, it may be that this cell line does indeed possess two closely related integrins. Other bands at lower molecular weight positions showed up in fibroblast extracts. These bands may represent proteolytic products of the detected protein or non-specific recognition by the polyclonal antiserum and are commonly seen in fibroblast extracts (Marcantonio and Hynes, 1988). Our results probing yeast extracts with the β_1 integrin antibody were inconsistent, with overstaining and multiple banding patterns. The use of isolated plasma membrane cell fractions instead of the whole cell extracts may give more consistent results (Marcantonio and Hynes, 1988).

Three anti-*C. albicans*-integrin antibodies were also used in this study to probe cell extracts of protoplasts, fibroblasts and yeast. These three polyclonal antibodies were raised in rabbits against different domains of the integrin-like protein αInt1p in *C. albicans* (Gale *et al.*, 1996; Gale *et al.*, 1998). Immunoblotting experiments using these three antibodies was not satisfying, with multiple bands detected due likely to a combination of protein degradation and non-specific staining. Therefore, no definite conclusion and comparison could be made from these results.

4.2 Immunofluorescence

Two types of non-specific staining were observed in *E. aulicae* protoplasts in this study. Long fixation times gave rise to the annular fluorescence around the nuclei, but this could be avoided easily by using ten minute fixations. The non-immune rabbit serum

also stained the periphery of the nucleus and a core in the nucleus. Thus non-specific staining by the secondary antibody was problematic and further purification might significantly improve the clarity of these results.

We observed staining specific to the experimental protoplast treatments not seen in any of the controls. This staining was patch-like and especially noticeable on the thin terminal cytoplasmic extensions, the internuclear constrictions and the periphery of the protoplast. It is thus tentatively concluded that the peripheral patches seen in protoplasts represents staining of an integrin homologue on the protoplast plasma membrane.

This uneven pattern is consistent with what might be expected for the distribution of a fungal integrin-like protein. In hyphal apices of both *S. ferax* and *N. crassa*, patches associated with the plasma membrane were stained by this anti-β_1-chicken integrin antiserum (Kaminskyj and Heath, 1995; Degousée *et al.*, 2000). Due to the very low percentage of attachment of protoplasts to the fibronectin-coated surface (see Table 1) no fibronectin coating was used in the immunofluorescence experiment. Thus, the surface was without fibronectin coating or any other integrin-binding protein that might induce integrin aggregation. Based on these observations, we conclude that the distribution of the putative integrin-like protein in protoplasts may be non-diffuse and patchy without induction by an integrin-ligand and may not be associated with focal adhesion-like structure in protoplasts of *E. aulicae*.

Our conclusion that we have localized a homologue of vertebrate integrin is tentative however because we were unable to show characteristic integrin staining in the positive fibroblast control. Typically, immunofluorescence localization studies of integrin

are conducted with vertebrate cell cultures grown on a fibronectin-coated surface in order to trigger the formation of focal contacts and the conjugation of integrin from the diffused form. This distribution pattern of vertebrate integrin serves as a prototype for determining the distribution of integrin-like proteins in other eukaryotes such as fungi. In the present study, although fibroblast extracts contained integrin on immunoblots probed with anti-$ß_1$-integrin antiserum, no clear immunofluorescence patterns of integrin patches or focal adhesions were observed at the plasma membrane of fibroblasts. Several explanations might be offered for this including the transient nature of focal adhesions which can be quickly disassembled when the adherent cell is triggered to move or starts mitosis (Karp, 1999), too low a concentration of the fibronectin for the coating, (Burridge and Chrzanowska-Wodnicka, 1996), or the possible lack of specificity of the integrin of monkey kidney fibroblast integrin for the bovine fibronectin used here. Not withstanding these possible explanations, a high level of confidence for the experimental results with the protoplasts requires a successful positive control for the localization of vertebrate integrin in the fibroblasts in this study. An alternate positive control would be staining of yeast cells with the antibodies against αInt1. Unfortunately to date, we know of no published immunofluorescence micrographs of the distribution of the integrin-like protein αInt1 in *C. albicans* cells, nor of its distribution in other cells using any of the three anti-αInt1 antibodies used here.

4.3 Attachment Experiments

There was a low percentage attachment of protoplasts to collagen-coated surfaces, and this was in disagreement with Lake (1994) who found that protoplasts firmly attached to collagen-coated surface. We hypothesize that loss of attachment sites has occurred during subculturing of the protoplast cultures in the intervening eight years. Further experiments with protoplast cultures newly isolated from infected hosts might prove more useful.

Protoplasts did not adhere to the fibronectin coatings at concentrations of 1000 μg/ml and 100 μg/ml, but attached to the surface coated at a concentration of 25 μg/ml. However, the results from two 3-hour experiments at 25 μg/ml fibronectin were very different from one another. This suggested that these attachments were artifacts. The likely explanation for this is that the fibronectin coating was uneven at this low concentration with protoplasts attaching to uncoated areas of the glass as they did in controls. If this was true, then the high percentage attachment of protoplasts to 25 μg/ml fibronectin coating was a non-specific artifact. The peptide inhibition experiments are therefore also inconclusive but support the hypothesis that non-specific attachment to the glass slide is a likely explanation for the attachment observed.

Consistently, protoplasts attached firmly to glass surfaces in this study. Usually, when a solution is added to a pH-sensitive glass surface or membrane, the charge of the glass surface will be changed to positive, and this is due to an ion-exchange reaction between singly charged cations in the glass lattice and protons from the solution (Skoog

et al., 1998). The positive charge on the glass surface might have been electrostatically conducive to the adhesion of protoplasts (Dunphy and Nolan, 1980).

Interestingly, it was found that protoplasts incubated in the non-RGD peptide solution incubation did not maintain their normal spindle shape and became round, while protoplasts in all other groups retained their spindle shape (data not shown). Also, there were fewer protoplasts after incubation in presence of non-RGD peptide than in RGD peptides and control. The original report on the RFD peptide identified it as a highly conserved sequence of the major histocompatibility complex proteins of the human immune system of unknown function (Auffray and Novotny, 1986). More recently it has been suggested that RFD acts to modulate cell attachment (Homandberg and Hui, 1994). Thus, while this may be an interesting subject for future research, this non-RGD peptide was not a good choice as a control for attachment studies. GRGESP peptides would be better candidates for controls in future experiments (Santoni *et al.*, 1994; Corrêa *et al.*, 1996).

In vertebrate cells, integrins provide the adhesive strength for attachment, but the activation steps provide the specificity, and integrins can lose their activity during development but still persist on the surface (Hynes, 1992). If this applies to the present research, then there may be another interpretation for the obtained results. If this integrin-like protein needs to be activated by ligands or other stimuli, which is absent at the protoplast stage or lost during the process of the attachment experiments, protoplasts would not have the ability to attach. This might be the answer to why protoplasts did not attach to either insect host cells or integrin-binding protein coatings. The activated

integrin form may be important at other stages of *E. aulicae*, such as conidia, appressorium, hyphal body or germ tubes. This hypothesis needs to be tested in the future. For example, there is evidence that integrin-like proteins are present and function in hyphal tips (Kaminskyj and Heath, 1995; Degousée *et al.*, 2000). Conidial germ tubes of *E. aulicae* could be used to determine if integrin is involved in the adhesion of germ tube plasma membrane to cell wall. Alternatively, integrin-like protein could be involved in formation of the membrane skeleton in *E. aulicae* protoplasts as suggested for *Neurospora* (Degousée *et al.*, 2000).

Serum in culture medium may affect attachment of vertebrate cells, and its components may inhibit or promote attachment depending on the cell type and conditions. The presence or absence of FBS did not affect protoplast attachment to collagen or fibronectin coated slides, although the culture medium without fetal bovine serum favored the attachment of protoplasts to the glass surface. This last result was consistent with the reports of Dunphy and Nolan (1980) and Lake (1994).

Preliminary work for attachment experiments showed that the GM was suitable for growth of protoplasts and insect cells, when they were grown separately. Protoplasts grew faster when they were cultured in the presence of insect cells than without insect cells. On the other hand when grown with protoplasts, the insect cells did not grow well. Insect cells increased for the first 24 hours, but then started declining after 24 hours, and eventually disappeared or were destroyed. This phenomenon might be due to competition between these two cell lines. Alternatively, toxins reportedly produced by *E. aulicae* in response to its host might be involved (Dunphy and Nolan, 1982b; Milne *et al.*, 1994).

Entomophaga aulicae protoplasts did not attach to the insect cells after 4-6 hours incubation under conditions suitable for their growth together. One of the possible reasons might be due to the source of the insect cell line used in this trial (IPRI-CF-124T). Fat body is the tissue to which protoplasts may likely attach *in vivo* (Funk *et al.*, 1993). The insect cell line used by us was isolated from neonate larvae of *C. fumiferana*, and thus the exact tissue of origin is unknown. It is not certain, and indeed unlikely, that this cell line is from the fat body (Sohi, personal communication). If this cell line originated from hemocytes, a more probable source, then the lack of interaction may be expected since *in vivo* hemocytes do not recognize and bind to protoplasts, and this is an important mechanism by which protoplasts avoid immune detection.

A fat body cell line from *C. fumiferana* has not yet been developed. However, there is one available from the gypsy moth, *Lymantria dispar,* a host of *Entomophaga maimaiga*, which is very closely related to *E. aulicae*. Attachment experiments could be designed using *E. maimaiga*, and a fat body cell line from its host.

The protocol we developed and used in our attachment experiment using wicking and rising is relatively simple, but is sensitive and good for preliminary studies. However, the question of whether protoplasts attach to insect host cell and/or integrin-binding protein remains open and more investigation is necessary.

4.4 Conclusions

Entomophaga aulicae protoplasts extracts contained a protein which cross-reacted on immunoblots with the antiserum to $ß_1$-integrin, raised against a highly conserved

cytoplasmic domain in vertebrates, and a range of other organisms. In immunofluorescence analysis, abundant peripheral patches of staining were detected on the thin terminal cytoplasmic extension, internuclear constriction and the periphery of the protoplast incubated with different anti-integrin antisera. Our study did not reveal evidence to support the idea that *E. aulicae* protoplasts attach to either host insect cells or integrin-binding protein coated surface, fibronectin and collagen.

The question of whether or not fungi possess molecules structurally and functionally homologous to vertebrate integrins remains a tantalizing one. While suggestive evidence continues to accumulate from studies such as this one, definitive examples are lacking. Perhaps the most convincing and exciting example has been that of the 288 kDa functional homologue designated αInt1 from *C. albicans* (Gale *et al.*, 1996). This protein was shown to be necessary for attachment and pathogenicity and it remains the only one for which gene sequence data are available (Gale *et al.*, 1996). However, attempts in our lab to identify a transmembrane domain in the αInt1 protein using hydrophobicity plotting was unsuccessful (Murrin, personal communication), and also it has been suggested that the αInt1 protein is not related to integrin at all but is a homologue of a known regulatory protein (May and Ponting, 1999). If true, this may help explain our unsuccessful attempts to use antibodies raised against αInt1 in immunoblot experiments; it also calls into question our interpretation of the immunolocalizations as nuclear trapping might be expected in that event. More recently a shift from the vertebrate integrin paradigm for fungal attachment to an alternative one, focused on adhesion molecules which themselves possess the RGD sequence, has been articulated

(Hostetter, 2000). Whether this shift will occur and facilitate our understanding of fungal attachment, or whether a structural and functional integrin homologue, based on gene sequence data, will be discovered, remains an exciting unknown in fungal cell biology.

4.5 Future work

Further investigations into protoplast attachment might be instructive. As discussed above, newly isolated protoplasts of the gypsy moth pathogen, *E. maimaiga*, and cell lines from the gypsy moth are available would be excellent candidates. In addition, a variety of possible interactions between the integrin-like proteins and ligands should be considered. Once cell-cell interactions are identified, characterization of attachment using a liquid flow chamber (Terhune and Hoch, 1993) would allow quantification of forces. Other adhesive proteins on the protoplasts and other receptors may also be investigated. To search for integrin-like proteins at other stages in the fungal life cycle might be fruitful, particularly in the attachment of plasma membrane to the wall of germ tubes, or of the germling to the host surface.

Non-specific staining by the secondary antibody was problematic in our immunocytochemical experiments. Further purification of the antibodies might be attempted using affinity chromatography. Alternatively, directly labeling the primary antibody with the flurochrome, might eliminate the cross-reactivity encountered here.

At the molecular level, isolation and sequencing of the putative integrin-like protein using RT-PCR (reverse transcriptase PCR) and primers against *C. albicans* integrin-like protein and others is in progress in our laboratory and should allow

comparison with other integrins and suggest possible functions of the molecule. Isolation and purification of the protein could be done to determine the amino acid sequence and characterize this protein's interactions and activity, especially with extracellular matrix proteins found in insect cells.

In the applied area, if an adhesive molecule is identified which has a role in pathogenicity, it may be a valuable candidate for bioengineering or isolate selection for enhancement of attachment and disease progression in forest insect pest biocontrol.

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