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Primary cultures of insect midgut cells: a system to study membrane permeability to proteins and specific tools to enhance permeation

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Summary

1. Introduction

1.1 The need of new strategies for pest control

Arthropod pests destroy about 25% of the world's annual crop production (Oerke, 1994), contribute to the loss of nearly 20% of stored food grains (Bergvinson and Garcia-Lara, 2004) and transmit an array of human and veterinary pathogens (Gubler, 1998a-c; Gratz, 1999). Merely considering the cost induced by disease transmission, it should be of primary interest to develop new strategies to control these pests. Moreover, because of the constant increase of pest resistance to chemical pesticides, in the last decades the search of alternative methods has become mandatory.

Since the introduction of DDT in the 1940s, insect pests have been controlled almost exclusively with chemical insecticides (Casida and Quistad, 1998). Fast acting, cheap to produce, relatively easy to deliver and highly potent, chemical insecticides have been viewed with extreme optimism. Some decades after their introduction, these compounds showed all the problems associated with their use, including poor species specificity and development of resistance in target organisms. In response to the environmental hazard that these compounds cause, in 1970s in many countries DDT and many other chlorinated insecticides were banned from agricultural use. In parallel, chemical alternatives were developed and research was focused on biopesticides (Copping and Menn, 2000).

The term biopesticides describes a large number of control techniques, including the application of microbial organisms, entomophagous nematodes, plants-derived pesticides, secondary metabolites from micro-organisms, insect pheromones applied for mating disruption, monitoring or lure-and-kill strategies. Genes also are used to increase the resistance of crops to insect, fungal, viral or herbicide damage (Copping and Menn, 2000). A variety of peptides and proteins have been used to produce biopesticides and a list of these compounds, derived from many different sources, has been recently reviewed by Whetstone and Hammock (2007).

Even if in the last decade it has been produced a large number of new biopesticides, till now these products represent only 1% of the world pesticide market, with the *Bt* products constituting nearly 80% of the total amount (Whalon and Wingerd, 2003). In comparison to chemical counterparts, biopesticides are often slow acting, have narrow host-specificity and poor longevity in the field. Some of these characteristics are positive as well as negative qualities. The rapid environmental degradation of biopesticides obliges to a more frequent application, but also reduces pest-resistance development. The high specificity of biopesticides often requires application of several types of pesticides to control all major pest species involved in a particular agricultural setting, but also eliminates harm infliction to non-target organisms (Whetstone and Hammock, 2007). These characteristics, added to the high production cost and limited applicability of bioinsecticides restrict their employment.

To increase utilisation of bioinsecticides, it is necessary to improve the knowledge and the technologies to overcome the limitations of their slow-action and narrow host specificity, and to increase toxicity and rate of delivery. In most cases, biopesticides are proteins that have haemocoelic targets and must pass undegraded the gut barrier in order to exert their activity. For this reason it is very important to develop basic information on the molecular mechanisms mediating the absorption of macromolecules by the insect midgut, which is a physiological process still poorly understood.

The research work performed during my Ph.D. course fitted in this scenario. We established a culture of midgut cells from *B. mori* larvae in order to: assess in single columnar cells the specific mechanisms involved in albumin endocytosis and the sequence of intracellular events involved in its transcytosis; investigate the possible ability of cell penetrating peptides (CPPs) to deliver into the cell a fused protein; identify the pathway followed by *Jc*DNV, an insect parvovirus lethal for the larval stages of several pest insects species, in crossing the midgut.

1.2 Protein transport in absorptive epithelia

In insect, like in mammals, the intestinal epithelium is one of the main interfaces between the body and the external environment. As a result of this function, epithelial cells have evolved two specialised plasma membrane domains with distinct proteins and lipids composition (Simons and Fuller, 1985). The specificity of these two domains is maintained by the junctions, which are also a fence between neighbouring cells. Ions and solutes can cross this selective barrier following the transcellular or the paracellular pathways (figure 1.1). The passage through the paracellular route, that is the watery channel interposed between two adjacent cells, is finely regulated by the junctions and depends on their permeability. The transcellular pathway of ions and small solutes is sustained by the asymmetric distribution of membrane transporters and channels at the opposite poles of the cell membrane, while proteins can be transported across the intestine by transcytosis, a complex sequence of intracellular events that exploits the membrane traffic involved in internalisation and secretion at the apical and basal poles of the cell (Mostov *et al*., 2000).

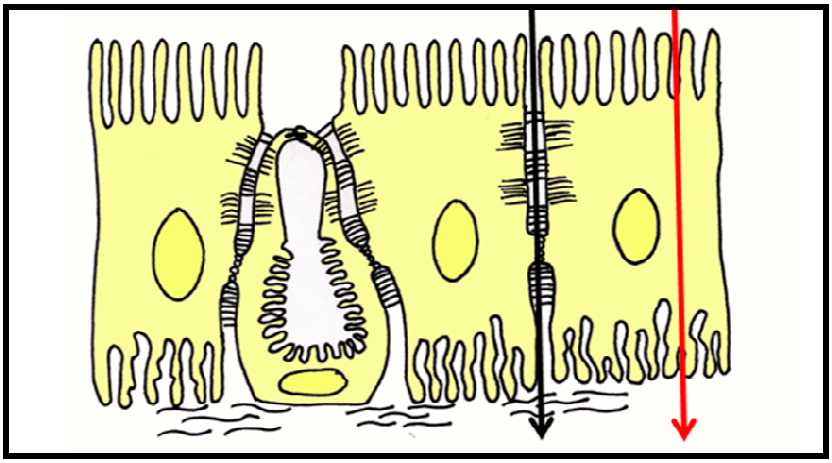

Figure 1.1: Schematic representation of the transcellular (red arrow) and the paracellular (black arrow) pathways.

Transcytosis has been extensively studied in mammals (recently reviewed by Apodaca, 2001; Tuma and Hubbard, 2003). Following the

first suggestions by Palade (1953) on the possible occurrence of transcytosis, several subsequent studies have addressed the functional bases of this transport process. Now, it is known that transcytosis is an active process, regulated by specific internal signals that guide the internalisation and transport of the macromolecules within the cytoplasm, from one pole of the cell plasma membrane to the other one (Apodaca, 2001; Tuma and Hubbard, 2003). It is also evident that, although transcytosis commonly takes place in epithelial tissues (Mostov *et al*., 2000; Apodaca, 2001), it is not restricted to polarised cell types (Tuma and Hubbard, 2003). The molecular mechanism of transcytosis has been studied in detail in mammals, both *in vivo* (Sai *et al*., 1998; Ziv and Bendayan, 2000) and *in vitro* (Heyman *et al*., 1982; Kiliaan *et al*., 1998), or, more frequently, by using model epithelial monolayers (Gekle *et al*., 1995; Gekle *et al*., 1997; Ellinger *et al*., 2001).

1.2.1 Protein endocytosis

The plasma membrane is a dynamic structure that regulates the entry and the exit of all solutes. Particles and macromolecules can be carried into the cell by means of an endocytic process that consists in the formation of vesicles derived from invagination and pinching-off of portions of the cell plasma membrane (Conner and Schmid, 2003). The endocytic processes are classified in two different categories, phagocytosis and pinocytosis, depending on the size of the endocytic vesicles, the nature of the cargo and the mechanism of vesicle formation. Phagocytosis is the uptake of large particles and pinocytosis is the uptake of fluid and solutes (Conner and Schmid, 2003).

Phagocytosis is an active and highly regulated process involving specific receptors and signalling cascades mediated by Rho-family GTPases. It is typical of specialised cells (macrophages, monocytes and neutrophils) and is implicated in many processes among which the activation of inflammatory responses after bacterial infections (Conner and Schmid, 2003). When phagocytosis occurs, the cell creates membrane

protrusions that surround the particle to be internalised, and then fuse forming vesicles that are released into the cytoplasm (figure 1.2).

Pinocytosis is a process well studied in mammals that includes the internalisation of solutes by a non-specific binding to the cell membrane (fluid-phase) or by specific high-affinity receptors concentrated into specialised membrane invaginations. Pinocytosis is divided in four different classes: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003) (figure 1.2).

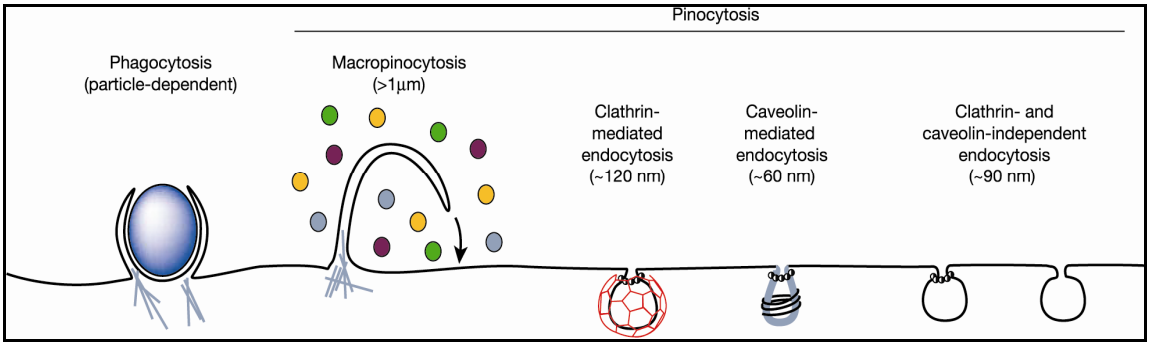

Figure 1.2: Multiple portals of entry into the mammalian cell. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids) and the mechanism of vesicle formation. (Conner and Schmid, 2003)

Macropinocytosis is a process close to phagocytosis and, like the last one, is activated by the signalling cascades mediated by Rho-family GTPases. However, unlike phagocytosis, protrusions named "ruffles" collapse into the plasma membrane and then fuse together generating large vesicles called macropinosomes (diameter > 1 µm). Membrane ruffling is transient and is induced by specific signals like the presence of bacteria and/or growth factors (i.e. Platelet–derived growth factor (PDGF)) (Conner and Schmid, 2003). This is also an endocytic mechanism used by HIV-1 virus to infect macrophages (Marechal *et al*., 2001).

Clathrin-mediated endocytosis (CME) is the most characterised endocytic process and involves the concentration of high-affinity

transmembrane receptors and their bound ligands into "coated pits" on the plasma membrane. Coats act to deform the membrane, to generate a vesicle and to select the cargo (Marsh and McMahon, 1999). Clathrin coats are implicated in many important processes among which the formation of endocytic vesicles at the plasma membrane. Clathrin is composed by a 180-kDa protein called clathrin heavy chain (CHC), which is associated to a 25-kDa protein called clathrin light chain (CLC). This CHC-CLC complex forms the three-legged trimers called triskelions (Kirchhausen *et al*., 1986; Kirchhausen, 2000). The assembly of numerous triskelions generates a basket-like structure that surrounds the pits (Marsh and McMahon, 1999). Clathrin-mediated endocytosis is driven by a cycle of assembly and disassembly of clathrin-coated vesicles (CCVs): the clathrin coat is assembled on the cytoplasmic face of the plasma membrane, where generates pits that invaginate and pinch off, becoming CCVs (Marsh and McMahon, 1999). A subsequent uncoating reaction recycles the coat constituents for the formation of a new CCV (figure 1.3) (Conner and Schmid, 2003). Clathrin-mediated endocytosis is essential for intercellular communication during tissue and organ development (Di Fiore and De Camilli, 2001; Seto *et al*., 2002) and modulates signal transduction by regulating the level of membrane signalling receptors (Conner and Schmid, 2003). Under physiological conditions, clathrin triskelions assembly only in the presence of specialised proteins called assembly proteins (APs) (Conner and Schmid, 2003), which are classified in two categories: the monomeric assembly protein AP180, specifically expressed in neurons (McMahon, 1999), and the four structurally related heterotetrameric adaptor protein complexes (AP1–4). Each mediates vesicle formation at distinct subcellular localisations (Robinson and Bonifacino, 2001). Only the AP2 complex is involved in endocytic CCVs formation. It consists of two large, structurally related subunits called α- and β2-adaptins, a medium subunit μ2, and a small subunit σ2 (figure 1.3) (Conner and Schmid, 2003).

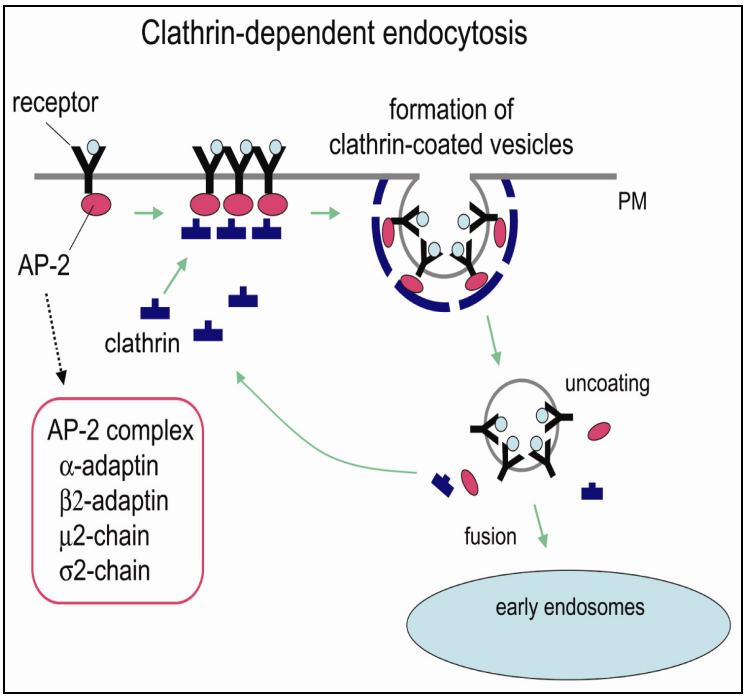

Figure 1.3: **Mechanism of clathrin-dependent endocytosis.** Clathrin and cargo molecules are assembled into clathrin-coated pits on the plasma membrane together with an adaptor complex called AP-2 that links clathrin with the transmembrane receptors, forming mature clathrin-coated vesicles (CCVs). CCVs are then actively uncoated and transported to early/sorting endosomes. (Grant and Sato, 2006)

A fundamental protein for most of the endocytic processes, CME included, is dynamin, an atypically large GTPase, which is thought to self-assemble creating a spiral around the "necks" of the budding coated pits (Conner and Schmid, 2003). Although there are many studies on this protein in different animal models, its exact function is still controversial. One hypothesis suggests that dynamin functions as a mechanochemical enzyme (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998; Stowell *et al*., 1999), the second that it functions, like other GTPase, as a regulatory molecule in the formation of endocytic vesicles (Sever *et al*., 1999). Anyway, there is general agreement that dynamin must undergo GTP-hydrolysis, that generates the conformational changes for its activity (Marks *et al*., 2001).

Caveolae are described as smooth-surfaced flask-shaped pits with a diameter of 55-65 nm (Palade, 1953; Yamada, 1955). They are present in

cholesterol and sphingolipid-rich microdomains of the cell plasma membrane of a large number of different cells (Anderson, 1998). The shape and structural organisation of caveolae are conferred by caveolin, a 22 kDa dimeric protein that binds colesterol, inserts as a loop into the inner leaflet of the plasma membrane and self-associates to form a striated caveolin coat on the surface of the membrane invaginations (Conner and Schmid, 2003). The mammalian caveolin gene family consists of caveolin-1, -2, and -3. Caveolin-1 is present in two alternatively spliced isoforms (α and β). The two isoforms show an overlapping but slightly different distribution in mammalian cells (Scherer *et al*., 1995) but no detailed study concerning their functional diversity has been performed. Caveolin-2 is often coexpressed and associated with caveolin-1 in the same type of cell/tissue, with particularly high levels in adipocytes (Scherer *et al*., 1996; 1997), and caveolin-3 is expressed predominantly in skeletal and cardiac muscles (Way and Parton, 1995). The depletion of the plasma-membrane cholesterol and the knockout of caveolin genes shed light on the important role played by these two molecules in the formation of caveolae (Drab *et al*., 2001; Razani *et al*., 2002).

Small membrane domains rich in cholesterol and glycosphingolipid called lipid rafts are involved in clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003). Recently, these membrane domains have been defined as ‘‘small and highly dynamic membrane microdomains (10–20 nm) that are enriched in cholesterol and sphingolipids that compartmentalise cellular processes. Small rafts can sometimes be stabilised to form larger platforms through protein–protein and protein–lipid interactions’’ (Pike, 2006). This arrangement makes them membrane regions with distinct properties and structural composition. They appear to act as platforms anchoring proteins implicated in processes as diverse as signal transduction, endocytosis and cholesterol trafficking. New evidences suggest that this variety of functions is accompanied by diversity in the composition of lipid rafts (Godoy and Riquelme, 2008). Recent data indicate that lipid rafts are not

the only membrane microdomains involved in clathrin- and caveolae-independent endocytosis, but, till now, these other mechanisms remain poorly understood (Conner and Schmid, 2003).

1.2.2 Intracellular trafficking of endocytic vesicles

Once internalised, the macromolecule inside the endocytic vesicle can follow different fates: it can be delivered at the opposite plasma membrane domain (transcytosis), recycled to the same plasma membrane where the endocytosis had occurred or it can be delivered to lysosomes for intracellular degradation (Apodaca, 2001). Their intracellular pathways are independent of the endocytic mechanism by which they were endocytosed. Protein internalisation by polarised epithelial cells can occur at either the apical or the basolateral membranes and the intracellular pathways involved can be different at one membrane domain or at the other (Apodaca, 2001; Mostov *et al*., 2000). Proteins, whether endocytosed at the apical or at the basolateral membrane, are delivered to early endosomes, from which they will follow a specific route (Apodaca, 2001) (figure 1.4).

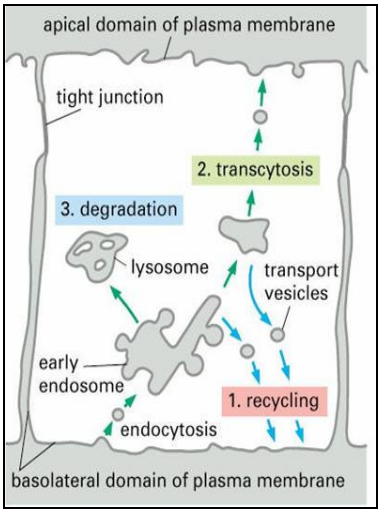

Figure 1.4: schematic representation of the intracellular pathways followed by endocytic vesicles formed at the basolateral side of the epithelial cell (http://mol-biol4masters.org/Co_and_Post_Translational_Events5-ellular_Protein_Traffic.htm)

Proteins endocytosed from the apical or basolateral membrane first reach the apical (AEE) or basolateral early endosomes (BEE), respectively. Most of the apically internalised vesicles are delivered to the apical recycling endosomes (ARE) for the recycling to the apical membrane or are transcytosed to the basolateral membrane; only a small amount is delivered to late endosomes (LE) and then degraded in lysosomes. Apical recycling proteins are delivered from the AEE to the ARE or to the common endosome (CE) before their ultimate release from the apical pole of the cell. Vesicles internalised at the basolateral membrane are primarily delivered to late endosomes and lysosomes or can be destined to the CE. From CE, molecules can be recycled to the basolateral membrane or sent to the ARE and then exocytosed at the apical plasma membrane domain (Apodaca, 2001).

Recent data have shown that epithelial cell specific Rab proteins might be required for polarised sorting. Rabs are a family of over 60 proteins that are characterised by similar structure, function and guanine nucleotide binding properties (Pfeffer and Aivazian, 2004). Like all GTPases, also Rab proteins can bind guanosine triphosphate (GTP) regulating the activation, or guanosine diphosphate (GDP) regulating the inactivation state of the enzyme (Ceresa, 2006). These proteins seem to mediate vesicular trafficking events by regulating the new vesicle formation and their fusion with different intracellular organelles. Recent data support a role of Rab5 in the regulation of trafficking between the plasma membrane (donor membrane) and the early endosome (acceptor membrane) (Ceresa, 2006). Rab11 and Rab 4 seem to be involved in the recycling pathway: Rab 4 regulates trafficking between the early endosome and the recycling endosome and Rab 11 between the recycling endosome and the plasma membrane. Finally, Rab 7 is involved in the degradative pathway since regulates vesicle traffic between the late endosome and lysosomes (Ceresa, 2006) (figure 1.5).

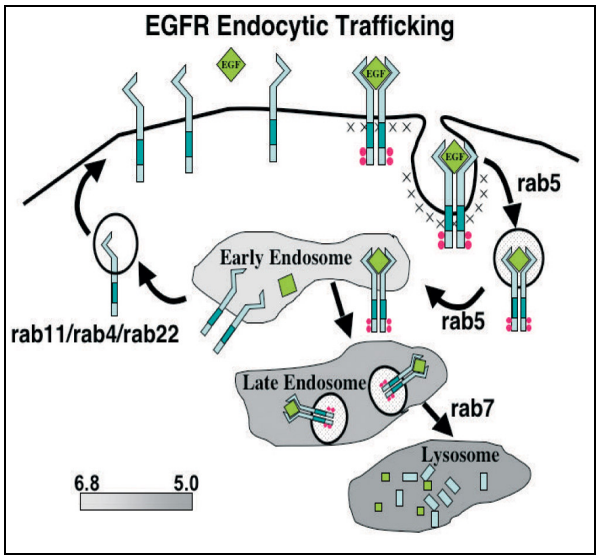

Figure 1.5: Schematic representation of the intracellular trafficking of vesicles regulated by Rab proteins. The pH gradually decreases in the progression from the plasma membrane to the lysosome (indicated by increased shading) (Ceresa, 2006).

Another important player in endocytic trafficking is the cytoskeleton. The cytoskeletal elements involved are microtubules and actin filaments.

Microtubules are ubiquitous cytoskeletal structures formed by the self-assembly of α and β tubulin heterodimers (figure 1.6). The number of protofilaments in a microtubule changes from 9 or fewer to at least 16 (Chrètien and Wade, 1991), which suggests that there is a high degree of flexibility in the lateral association between protofilaments within microtubules. All protofilaments in a microtubule are parallel resulting in one "plus end" and another "minus end" with distinct polarity (Downing, 2000).

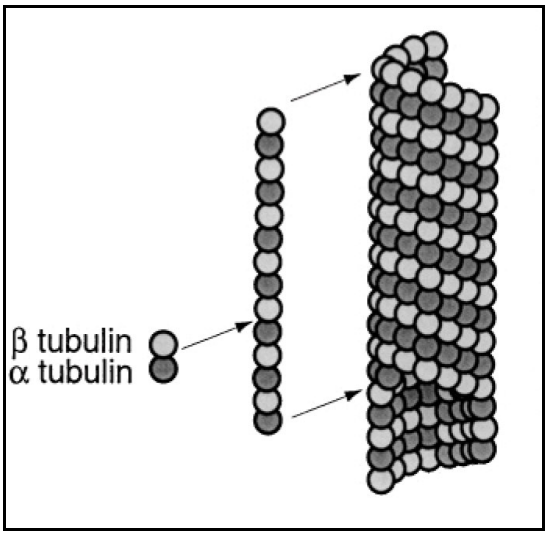

Figure 1.6: Schematic model of the α-β tubulin dimer with the monomers represented as spheres of different shades of grey (Downing, 2000).

Microtubules are dynamic filaments that provide a mechanical basis for cell polarity and for vesicles transport within the cell. These elements of the cytoskeleton are critical players in endocytosis, since drugs that interfere with their organisation reduce receptor-mediated endocytosis in mammals (Elkjaer *et al*., 1995; Hamm-Alvarez *et al*., 1996; Gekle *et al*., 1997; Apodaca, 2001), although a detailed analysis of their role is still lacking. Two types of microtubule motor proteins are implicated in intracellular vesicle transport: kinesin and cytoplasmic dynein. These proteins hydrolyse ATP to power membrane vesicle movements along the microtubule track (Hamm-Alvarez and Sheetz, 1998). Dynein moves toward the minus-end and regulates the traffic to the center of the cell; on the contrary, kinesin moves toward the plus-end and regulates the traffic to the cell surface (Murray and Wolkoff, 2003).

Actin cytoskeleton is composed of both monomeric (globular or G-actin) and polymeric (filamentous or F-actin) actin forms. The two ends of actin microfilaments are biochemically distinct, with different growth rates: the slow growing end, named "minus" or "pointed" end, and the fast growing end, named "plus" or "barbed" end (figure 1.7).

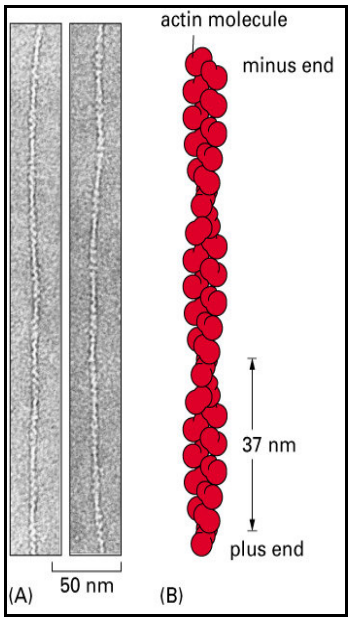

Figure 1.7: (A) electron microscopy image of actin filaments; (B) schematic representation of an actin filament with plus and minus ends (Alberts *et al*., 2004).

Actin polymerisation is the incorporation of monomeric actin into the polymeric form (Fenteany and Zhu, 2003). During the cell's resting state, the ends of actin filaments are bound by specific capping proteins that prevent addition of actin monomers. In the absence of filament-end capping, hydrolysis of the actin-bound ATP is crucial for the insertion of actin monomers at the barbed end and for their dissociation at the pointed end, a phenomenon known as treadmilling (Fenteany and Zhu, 2003).

The microfilaments network is a flexible and dynamic structure involved in cell motility, maintenance of cell shape, cell-cell attachment, interaction with the extracellular matrix and anchorage of cell organelles. The ability of these filaments to assemble and disassemble and the reorganisation in response to internal and external stimuli makes them an important player in endocytosis and exocytosis. The signalling cascade is largely regulated by the Rho subfamily of small GTPases. Over 20 Rho family members have been identified (Ridley, 2001) and are known to play a critical role in the formation and organisation of cortical actin network (Lanzetti *et al*., 2001). In non-polarised cells, microfilaments form cellular stress fibers, lamellipodia, filopodia and cortical actin, a

netlike structure underlying the plasma membrane enriched in spectrin; in polarised epithelial cells, microfilaments form the actin terminal web, the cortical actin and the filamentous core of the microvilli (Da Costa *et al*., 2003).

1.3 Proteins and peptides absorption in the insect midgut

In the last decades, a variety of biocontrol methods employing peptides and proteins derived from microorganisms, animals like predator or parasitoid arthropods, and plants has been examined (Whetstone and Hammock, 2007).

Till recently, the majority of the studies on proteins absorption in insects were performed *in vivo*; they clearly demonstrated that proteins could reach the haemolymph undegraded. One of the first study in this field, published by Wigglesworth in 1943, demonstrated that a small amount of ingested haemoglobin was internalised by the gut of *Rhodnius prolixus* and released into the haemolymph. An interesting research was performed by Sugimura and colleagues (2001). They studied the passage of mulberry leaf urease through the gut wall of the silkworm (*Bombyx mori*) and demonstrated that reach the haemolymph undegraded. They established that the transport of mulberry leaf urease was a selective process with larval-stage dependence and demonstrated that a urease binding molecule(s) was expressed in the brush border membrane of the midgut of 6-day-old fifth instar larvae. In another paper, using confocal laser scanning microscopy and transmission electron microscopy, showed the presence of urease first attached to the surfaces of microvilli, then in the cytoplasm of the cells and, by native gel electrophoresis, detected the presence of the intact protein in the haemolymph (Kurahashi *et al*., 2005). Recently, Jeffers *et al*. (2005) investigated the transport of bovine serum albumin (BSA) and anti-bovine serum albumin (anti-BSA) through the intestine of the tobacco budworm, *Heliothis virescens*. Western blot analyses demonstrated that BSA and anti-BSA both retained their primary

structure and that anti-BSA maintained its antigenic activity in the meal pads and after movement from meal pads into the haemolymph.

Experiments performed on the two sucking insects *Glossina morsitans morsitans* (Modespacher *et al*., 1986) and *Haematobia irritans exigua* (Allingham *et al*., 1992), demonstrated that horseradish peroxidase (HRP) and immunoglobulin respectively, after oral administration, were present undegraded in the haemolymph.

Proteins present in spider and scorpion venoms could also cross the midgut. The venom extract from the cobra *Naya mossambica mossambica* was lethal for the larvae of *Sarcophaga falculata* either orally administered or injected in the haemocoel (Primor and Zlotkin, 1978; 1980). Analogous results were obtained with the oral administration to the flesh fly *Sarcophaga falculata* of an insect selective neurotoxic polypeptide from the venom of the scorpion *Androctonus australis* (AaIT). The data indicated that AaIT was able to cross the gut barrier and to induce paralysis of flies within 1-2 h after oral administration (Zlotkin *et al*., 1992).

The movement of lectins has been examined in a number of insects. In tomato moth larvae, *Lacanobia oleracea*, snowdrop and jackbean lectins were shown to accumulate in the gut, haemolymph and malpighian tubules and the jackbean lectin in the fat body after feeding (Fitches and Gatehouse, 1998; Fitches *et al*., 2001a, b). In the rice brown plant hopper, *Nilaparvata lugens*, snowdrop lectin was shown to accumulate in the haemolymph, fat body and ovarioles (Powell *et al*., 1998).

All the studies reported above demonstrated that *in vivo* proteins are able to cross undegraded the intestinal barrier, but the mechanism involved in this process was not investigated. Recently, functional experiments were performed under *in vitro* controlled experimental conditions, to detect the mechanism of albumin and horseradish peroxidase transport across the intestinal barrier of *Bombyx mori* larvae. The data reported in Casartelli *et al*. (2005) demonstrated in *B. mori* larval midgut isolated and perfused *in vitro*, that albumin can be transported from the lumen to the haemolymph through the transcellular

pathway by transcytosis. The occurrence of this absorption pathway is corroborated by several evidences. First, fluorescein isothiocyanate (FITC)-albumin was localised inside the midgut columnar cells and was not detected in the paracellular space. The flux of the protein is a saturable process, and incubation of the midgut at 4°C caused a drastic inhibition of the flux, a clear indication that an energy-dependent process was involved in the protein transport. In a following study (Casartelli *et al*., 2007), the mechanism of horseradish peroxidase (HRP) transport through *B. mori* larval midgut, isolated and perfused *in vitro*, was identified. The results confirmed that the key mechanism for proteins absorption in *B. mori* midgut was transcytosis. However, the different steps involved in this process could not be elucidated in the isolated midgut. To this end, a powerful approach is represented by midgut cell cultures.

1.4 Differentiation of single mature cells in culture from midgut stem cells of lepidopteran larvae

The lepidopteran midgut is formed by a folded epithelial cell monolayer separated from underlying muscles and tracheae by a basal membrane. It is composed by three main cell types: goblet, columnar, stem cells (Cioffi, 1979; Baldwin and Hakim, 1991), and by some endocrine cells. Goblet cells are typical of lepidopteran larvae and have a peculiar shape, with a basally located nucleus and a cavity lined by an apical plasma membrane forming numerous microvilli, where a V-H^+-ATPase pump (Wieczorek *et al*., 1989) and a $K^+/2H^+$ antiport (Azuma *et al*., 1995) are expressed (figure 1.8). The combined activity of these two transporters generates the high electrical voltage, the active secretion of K^+ and the extreme luminal alkalinisation typical of the lepidopteran midgut epithelium (Wieczorek *et al*., 1989; Azuma *et al*., 1995). Columnar cells have an almost cylindrical shape with a central nucleus, an apical thick brush border and deep infoldings of the basal plasma membrane (Cioffi, 1979; Baldwin and Hakim, 1991). This cell type is involved in

nutrient digestion (Terra and Ferreira, 2005) and absorption (Giordana *et al*., 1982, 1998; figure 1.8).

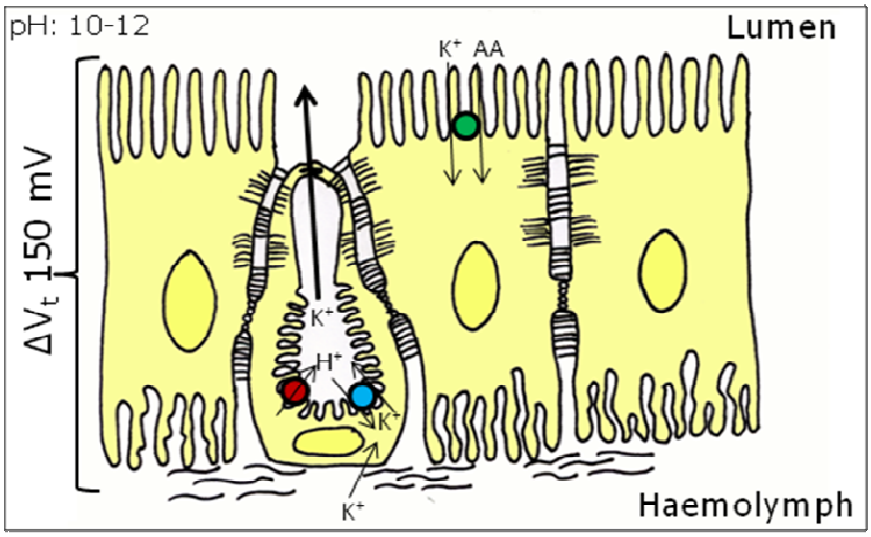

Figure 1.8: Schematic representation of the mechanism involved in amino acid transport in lepidopteran midgut.

The small stem cells, roughly conical- or spindle-shaped with a large nucleus, are located at the base of the epithelium (Turbeck, 1974; Baldwin and Hakim, 1991): they are the only cell type that undergoes mitosis (Loeb and Hakim, 1996) and their proliferation initiates in proximity of the moults (Baldwin and Hakim, 1991).

The method for collecting a highly enriched population of larval midgut stem cells from *Manduca sexta* was achieved by Sadrud-Din *et al*. (1996). Their approach was based on the ultrastructural observation that stem cells are not connected to the epithelium by junctions, in contrast with mature cells that remain anchored to one another by septate junctions (Baldwin and Hakim, 1991). By shaking a suitable insect ringer solution containing newly dissected midgut fragments taken from moulting larvae, the more mobile stem cells migrate away from the tissue mass. This procedure generates a culture enriched in stem cells to approximately 99%. Proliferation of stem cells occurred in the presence of the moulting hormone 20-hydroxyecdysone (20-HE) and of growth factors obtained

from the fat body. The differentiation of stem cells needed also soluble chemical messages released from mature or developing cells of the midgut epithelium. After 3 weeks in culture, up to 20% of the cells were present in various stages of differentiation, including mature columnar and goblet cells. The precursors of mature goblet cells were spherical cells with a neck region and the precursors of mature columnar cells were spherical cells with uniformly distributed microvilli. These evidences clearly indicate that stem cells were able to proliferate and differentiate *in vitro*, and that mature cells presented the typical morphology of the epithelium *in vivo* (Sadrud-Din *et al*., 1996).

The evidence that *in vivo* proliferation and differentiation of midgut cells are regulated (Spies and Spence, 1985; Engelhard *et al*., 1991) and that, *in vitro*, *Manduca sexta* midgut cells required 20-HE and fat body factors for proliferation and intrinsically-produced small soluble factor(s) for differentiation (Sadrud-Din *et al*., 1994, 1996), stimulated the desire to identify these factors. The first finding was that fat body or fat body extract (FBX) from either *Heliothis virescens*, *Manduca sexta*, or *Lymantria dispar* were all comparable in stimulating *in vitro* midgut stem cells proliferation from larvae of either *H. virescens*, *M. sexta* (Loeb and Hakim, 1996) or *L. dispar* (Dougherty *et al*., 2000). These evidences indicated that a common factor(s) able to induce cell proliferation must be present in the FBX of lepidopteran larvae. Recently, Blackburn *et al*. (2004), identified the α-subunit of arylphorin, a protein purified from *M. sexta* fat body extract, as the factor that induces rapid multiplication of midgut stem cells in culture. Other studies identified some of the peptides that induce cell differentiation, acting like the mammalian growth factors (Loeb *et al*., 1999; Goto *et al*., 2001; Loeb and Jaffe, 2002). Their starting point was the fact that the differentiation of stem cells required a medium conditioned by the presence of mature columnar and goblet cells, (Sadrud-Din *et al*., 1996; Loeb and Hakim, 1996). Two factors, the Midgut Differentiation Factor 1 (MDF1) and the Midgut Differentiation Factor 2 (MDF2), were identified (Loeb *et al*., 1999). Midgut Differentiation Factor 1 (MDF1) is a 26 amino acid peptide, modified from a slightly longer

molecule isolated from the conditioned medium, identical to residues 319-351 at the C-terminal portion of bovine fetuin, a high molecular weight glycoprotein constituent of fetal calf serum. Midgut Differentiation Factor 2 (MDF2) is a pentapeptide isolated after chymotrypsin digestion of bovine fetuin. Synthetic MDF2 was biologically active in midgut stem cells bioassays. Two other peptides, MDF3 and MDF4, were identified from the haemolymph of newly moulted *L. dispar* pupae treated with chymotrypsin (Loeb and Jaffe, 2002). The corresponding synthetic peptides were chemically different from MDF1 and MDF2 and were not homologous to other peptides or proteins, or to known insect growth factors. However, MDF3 and MDF4, like MDF1 and MDF2, were able to induce the differentiation of stem cells in culture (Loeb and Jaffe, 2002).

1.5 Cell Penetrating Peptides as a tool for drug delivery

Methods to enhance protein movement across the midgut epithelium can be of primary importance, since insecticidal proteins have to reach their haemocoelic target in sufficient amounts to exert the toxic activity. The group of prof. Gatehouse demonstrated that *Galanthus nivalis agglutinin* (GNA) can be utilised as a delivery system to transport a linked peptide to the haemolymph of lepidopteran larvae. They first demonstrated that a fusion protein GNA-allatostatin was able to reach undegraded the haemolymph of the tomato moth, *Lacanobia oleracea*, at variance to allatostatin alone (Fitches *et al*., 2002). An analogous result was obtained with a fusion protein between GNA and an insecticidal spider venom toxin (*Segestria florentina* toxin 1, SFI1). When this fusion protein was fed to larvae of *Lacanobia oleracea*, 100% mortality was observed 6 days after the administration. The mortality was drastically reduced when larvae were fed with GNA or SFI1 separately (Fitches *et al*., 2004). More recently, it was produced a fusion protein with GNA and a lepidopteran-specific toxin (ButalT) from the red scorpion (*Mesobuthus tamulus*). By western blotting, the intact fusion protein was detected in the haemolymph of tomato moth larvae (*Lacanobia oleracea*) after feeding.

Interestingly, it has been claimed that the scorpion toxin, ButalT, is lepidopteran-specific, but the fusion protein may have a wider range of toxicity. In fact, when the fusion protein was fed to *Nilaparvata lugens*, larval mortality was significantly higher than with GNA alone (Trung *et al*., 2006).

Extensive studies performed in mammalian cells identified a number of peptides, called cell penetrating peptides (CPPs), that posses the intriguing ability to cross the cell membrane entering in the cell. These peptides can act as delivery vectors because they can translocate a variety of cargo molecules attached to them. A wide range of biomolecules such as antigenic peptides, antisense oligonucleotides, full-length proteins, nanoparticles or liposomes have been delivered by this way (Richard *et al*., 2003).

CPPs are also called protein transduction domains (PTDs) or membrane transduction peptides (MTPs). Generally, CPPs are cationic or amphipathic peptides which consist of up to 30 amino acid residues. Cationic CPPs are characterised by the presence in their sequence of clusters of arginine and lysine residues (Patel *et al*., 2007). Amphipathic CPPs, unlike the cationic ones, are characterised by a highly hydrophobic C-terminus and a mainly hydrophilic N-terminus containing lysine and/or arginine residues. These peptides adopt a α-helix conformation in aqueous solution at pH 7 (Fernàndez-Carneado *et al*., 2004).

The presence of the guanidine group in cationic CPPs is thought to mediate their internalisation into the cell through the formation of bidentate hydrogen bonds with the anionic groups on the membrane (Patel *et al*., 2007). On the contrary, it seems to be necessary for amphipathic CPPs internalisation the presence of the hydrophilic and hydrophobic regions inside the α-helical structure of the peptides (Patel *et al*., 2007).

Some examples of CPPs are reported below:

CPP	Source
Penetratin (amino acid 43-58)	*Drosophila* Antennapedia protein.
TAT (amino acid 47-57)	Tat protein, the transactivating factor of the human immunodeficiency virus type 1 (HIV-1)
PEP-1	Synthesised Model Amphipathic Peptide (21 amino acid residues)
$(Arg)_7$	Synthesised CPP (small oligoarginine)
Transportan	Designed CPP (minimal active part of the neuropeptide galanin (1-12) coupled *via* Lys to mastoparan – a peptide toxin from wasp venom -)

In mammals, one of the most studied CPP is still the CPP Tat. The cellular uptake of Tat was first reported by two groups who showed that the exogenously added Tat protein from HIV-1 could enter cells and then trans-activate the HIV-1 LTR promoter (Frankel and Pabo, 1998; Green and Loewenstein, 1998). Subsequently, Frankel hypothesised that Tat might prove a useful vehicle to deliver proteins or peptides into cells (Mann and Frankel, 1991). Later, it was demonstrated that antibodies (Anderson *et al*., 1993) and enzymes (Fawell *et al*., 1994) were transduced into cells when crosslinked to a Tat peptide. The study of several Tat peptides showed that residues 47 to 57, from the basic domain of the Tat protein, were responsible for the functional internalisation into cells (Jeang *et al*., 1999; Schwarze *et al*., 2000; Schwarze and Dowdy, 2000).

The mechanism of CPPs entry into the cell remains controversial. Some studies gave clear evidences that endocytosis was not involved in CPPs internalisation, as indicated by experiments in which the transport was receptor-, energy- and temperature independent (Derossi *et al*., 1996; Vives *et al*., 1997; Futaki *et al*., 2001; Suzuki *et al*., 2002). Other studies, on the contrary, gave a clear indication that some CPPs can enter into the cell using two principal mechanisms: transduction and endocytosis.

Transduction is an energy- and receptor-independent process (Thoren *et al*., 2003) but is inhibited at low temperature (Patel *et al*., 2007). The transduction mechanism has been tentatively explained by

three models: direct membrane penetration, inverted micelle formation and counterion scavenging. The direct penetration model was based on the finding that a Tat-fusion protein can interact electrostatically with the cell surface in the denatured and high-energy form and penetrate directly into the cytosol with subsequent protein refolding mediated by chaperones (Nagahara *et al*., 1998; Schwarze *et al*., 1999). In the inverted micelle model, the peptides bind to the membrane by electrostatic attraction causing a transient formation of inverted micelles that carry the peptides and release them in the cytoplasm (Prochiantz, 1999, 2000). The counterion scavenging model is more recent and regards the guanidinium-rich cationic CPPs. It is based on the hypothesis that attraction of counter anions on the CPP will lead to neutralisation or inversion of the charge of the CPP, causing the alteration to different degrees of the lipophilicity and solubility of the CPP (Sakai *et al*., 2005; Takeuchi *et al*., 2006). The internalisation mechanism according to these models can be summarised in this scheme: membrane interaction, membrane permeation and release of the CPP into the cytosol (Patel *et al*., 2007).

As we have seen, endocytosis is a process energy- and temperature-dependent and is characterised by vesicles formation. It has been demonstrated that all four processes of pinocytosis previously described can be implicated in the internalisation of different CPPs carrying different types of cargos (Drin *et al*., 2003; Ferrari *et al*., 2003; Richard *et al*., 2005; Kaplan *et al*., 2005).

It is important to highlight that the mechanism of internalisation depends on the type of CPP, the type of cargo, the type of linkage between the cargo and the CPP and the cell system used (Patel *et al*., 2007). In fact, just considering only the CPP Tat, there are experimental evidences which indicate that this CPP can follow different mechanisms of internalisation: can be transduced into the cytoplasm (Zaro and Shen, 2003), can enter cells by macropinocytosis (Kaplan *et al*., 2005), or by clathrin-mediated endocytosis (Richard *et al*., 2005) or, when fused to GFP, can enter cells by caveolae-mediated endocytosis (Ferrari *et al*., 2003).

The mechanism of internalisation should govern the intracellular processing and final fate of the CPP and of its cargo. If the CPP is transduced directly into the cytoplasm, it could interact with its cytoplasmic target, be imported to the nucleus, be degraded by cytoplasmic proteases or be redirected out of the cell either intact or after degradation. On the other hand, if it is internalised by endocytosis, it could be targeted for lysosomal degradation, may be able to escape lysosomal degradation and enter the cytoplasm and possibly the nucleus, or be taken to the Golgi apparatus or the endoplasmic reticulum, or transcytosed out of the cell. As for internalisation, the intracellular pathway followed will be highly governed by several factors including the type of CPP, type of cargo attached, the nature of linkage between the cargo and the CPP, and the cell system under investigation (Patel *et al*., 2007).

While a large number of studies have been performed in mammalian cells to investigate the ability of different CPPs to cross the intestinal cell membrane, no information is available regarding insect cells.

1.6 A study of how a virus traverses the insect midgut

During my permanence at the "Laboratoire de Biologie Intégrative et Virologie" INRA-UMII (Institut National de la Recherche Agronomique), Montpellier (France), under the supervision of Dr. Mylene Ogliastro, we studied the interaction between *Junonia coenia* Densovirus (*Jc*DNV) and the permissive host *Spodoptera frugiperda*.

The *Parvoviridae* family is a group that clusters small non enveloped viruses with a linear, single-stranded DNA genome. It has been subdivided into two subfamilies based on their host range: *Parvovirinae* and *Densovirinae*. The *Parvovirinae* subfamily includes all the vertebrate parvoviruses, whereas *Densovirinae* includes arthropods parvoviruses (Bergoin and Tijssen, 2008), that are named Densoviruses (DNVs).

Since DNVs are lethal for several insects at larval stages, including agronomical pests (Lepidoptera) and insect-vector diseases (Diptera),

have been considered as potential bioinsecticide (Belloncik, 1990). Two DNVs, *Sibine fusca* densovirus (*Sf*DNV) and *Casphalia extranea* densovirus (*Ce*DNV) were successfully utilised in Cote d'Ivoire and Columbia, respectively, for the control of their hosts, *C. extranea* and *S. fusca*, two major pests of oil palm industrial plantations. Similarly, a commercial formulation (Viroden) of *Aedes aegypti* densovirus (*Ae*DNV) was used for the control of *A. aegypti* larvae in different areas of the Soviet Union and a *Periplaneta fuliginosa* densovirus (*Pf*DNV) formulation is produced in China for the control of cockroaches (Bergoin and Tijssen, 2008). However, beside these few successful examples, they were never further developed in the past, due at least in part to the poor interest in biocontrol of developed countries. Nowadays, because of the increased insect resistance and a stronger social request to preserve the environment from chemical pesticides, the possibility of their use is reconsidered.

DNVs, as all parvoviruses, are formed by a non enveloped 20-25 nm icosaedric capsid and a single stranded DNA genome encoding structural (VP) and non structural proteins (NS) (Bergoin and Tijssen, 2008). Depending on the virus, the capsid is constituted by two to four viral structural proteins (VP1-4), that share the same common C terminus and vary in the N terminus and are encoded by a unique gene (for a review see Cotmore and Tattersall, 2007). Parvoviral genomes encode two to four NS proteins mostly involved in virus replication. Insect and vertebrate parvoviruses' proteins share little sequence similarity, although they are characterised by conserved regions, as exemplified by the VP1 N-terminal region that carries a phospholipase A2 domain and the NS helicase and ATPase domains (Zadori *et al*., 2001; Cotmore and Tattersall, 2007).

Junonia coenia densovirus (*Jc*DNV) infects the larvae of *Junonia coenia*, the common Buckeye butterfly (Rivers and Longworth, 1972). *Jc*DNV originality is to be ambisense, meaning that separate strands of DNA that encode for NS and VP proteins are packaged separately in equal proportions. Structural analyses of *Jc*DNV showed that this virus has a relatively smooth, spherical capsid with only two small spikes, similar to *Galleria melonella* densovirus (*Gm*DNV) (Bruemmer *et al*., 2005).

However, the fundamental question concerning their mechanism of infection still needs to be addressed.

2. Materials and Methods

2.1 Experimental animals

Bombyx mori eggs and the artificial diet (Cappellozza *et al*., 2005) were provided by CRA-Institute for Sericulture (Padova, Italy). Larvae were reared under controlled conditions (25 ± 1°C, 65-70% RH, 12L:12D photoperiod).

Spodoptera frugiperda eggs were provided by "Laboratoire de Biologie Intégrative et Virologie" in Montpellier (France) and the larvae were reared under controlled conditions (25 ± 1°C, 65-70% RH, 12L:12D photoperiod) on a wheat germ-based artificial diet.

2.2 Histological analysis of IV instar midgut epithelium during pre-moult, moult and V instar feeding period

Silkworms at the above indicated stages of development were anaesthetised with CO_2. The midgut was explanted, deprived of the peritrophic membrane and Malpighian tubules and fixed in pycric acid, formaldehyde and glutaraldehyde (PAFG) at room temperature for 2h and then at 4°C over-night according to Ermak and Eakin (1976). The samples were then washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer for 2h, washed in distilled water and left for 2h in 2% uranyl acetate. After dehydratation in a graded ethanol series, samples were embedded in EPON resin and the polymerisation was performed at 60°C for 48h. Semithin sections were cut with a Reichert Ultracut E microtome and observed at the light microscope (AXIOVERT 200M equipped with AXIOCam HRm, Zeiss, Germany).

2.3 Preparation of midgut cells cultures

Larvae of *B. mori* at the end of the IV instar, just before the IV moult, or larvae of *Spodoptera frugiperda* just before the V moult, were anaesthetised with CO_2 and surface-sterilised by consecutive immersions, lasting approximately 2 min each, in the following solutions: 10% (v/v)

detergent (Pharma Soap Medical); 2% (v/v) p-hydroxybenzoic acid methyl ester (Sigma), prepared from a stock solution of 15% p-hydroxybenzoic acid methyl ester (w/v) in 95% ethanol; 0.1% (v/v) sodium hypochlorite. Silkworms were cut between the second and the third pair of thoracic legs and behind the third pair of abdominal appendages to exclude the foregut and the hindgut, and the peritrophic membrane along with the enclosed intestinal contents were removed. The central part of the larva was transferred to a Petri dish containing an Insect Physiological Solution (IPS) composed of (in mM) 47 KCl, 20.5 $MgCl_2$, 20 $MgSO_4$, 4.3 K_2HPO_4, 1.1 KH_2PO_4, 1 $CaCl_2$, 88 sucrose, pH 7; modified by addition of 0.2% (v/v) gentamicin (50 mg/ml, Sigma), 0.01% (v/v) 1x antibiotic-antimycotic solution (Sigma), 0.003‰ (v/v) sodium hypochlorite. The ventral cuticle was cut longitudinally and the midgut, deprived of muscles and silk glands, was isolated. Dissected midguts were cut along the longitudinal axis and rinsed two times (10 min each) in sterile modified IPS, then two times (10 min each) in the same solution without sodium hypochlorite. Midguts were pooled into a strainer (100 µm mesh size), placed in a Petri dish containing few ml of the latter solution and left under mild agitation for 1h. In these conditions, the loosely attached stem cells migrated away from the tissue. The tissue within the sieve was discarded and the free cells in the filtrate, mostly stem cells (Cermenati *et al*., 2007), were collected and pelletted by gentle centrifugation at 400 g for 5 min. Cells were then resuspended in growth medium, composed by a mixture of 67.4% Grace's insect medium (GIBCO), 11.2% 100 mM KOH, 6.7% Fetal Bovine Serum (GIBCO), 0.5% vitamins mix (composed by, in mg/100ml: 300 riboflavin, 150 pyridoxine hydrochloride, 150 thiamine hydrochloride, 150 folic acid, 600 nicotinic acid, 600 pantothenic acid, 12 biotin, 1.2 vitamin B12), 0.018‰ Antibiotic-Antimycotic Solution 1X (Sigma), 0.1% gentamicine (50 mg/ml, Sigma). Cultured cells were supplemented with $6x10^{-8}$ M 20-hydroxyecdysone (Sigma) and 100 ng/ml α-arylphorin (purified according to Blackburn *et al*., 2004, in Insect Bio-control Laboratory, USDA, Beltsville, MD, USA), kindly donated by Prof. R.S. Hakim, Howard University, Washington, DC, USA. All the solutions used

were routinely sterilised by filtration (Nalgene, 0.2 µm pore size) prior to use. For cells isolated from *Spodoptera frugiperda* larvae, 0.5 µg/ml amphotericin B was added to the medium every three days to prevent culture contaminations. For both, *B. mori* and *S. frugiperda*, three ml of the cell suspension in growth medium were distributed in the wells (35 mm in diameter) of six well plates. Cells, which grow and differentiate in suspension, were incubated at 25°C, and 1 ml of medium from each well was replaced with 1 ml of fresh medium once a week.

2.4 Viability of cells in culture, recognition and count of stem cells, differentiating cells and mature cells along six weeks

Cell viability was checked with the Trypan blue test in the initial stem cell culture from *B. mori* larvae and every seven days: viable cells excluded the dye, whereas dead cells became blue. An aliquot of the cell culture was removed, the cells were centrifuged at 400 g for 5 min and then resuspended in a known amount of the IPS (see above). An aliquot of the suspension was mixed with 0.4% (w/v) trypan blue (Sigma) (2:1). After 2 min, viable and dead cells were counted under the inverted microscope using a haemocytometer slide (Burker). Viable cells were then classified in four different categories (stem, differentiating, columnar and goblet cells) on the basis of their morphological features and counted every seven days for six weeks. Differences between the four categories along this experimental period were tested by Student's *t* test.

2.5 Enzymes assay

Three weeks old midgut cells in culture were pelletted by gentle centrifugation at 400 g for 5 min and resuspended in a small amount of IPS. After three washes, the pellet was resuspended in a buffer solution (100 mM mannitol, 10 mM Hepes-Tris at pH 7.2) and lysated in the eppendorf vial with a motor for pellet pestle (Sigma). Protein concentration in the lysate was determined according to Bradford (1976)

with BSA as standard. All enzymatic assays were conducted under conditions in which products formation depended linearly on enzyme concentration. Aminopeptidase N and alkaline phosphatase activities were determined at 25°C by measuring the release of *p*-nitroaniline from L-leucine-*p*-nitroanilide in 40 mM Tris-HCl at pH 7.5 or of *p*-nitrophenol from *p*-nitrophenylphosphate in 1 M Tris-HCl at pH 8, respectively. Enzymes activities were determined in triplicate or quadruplicate in a Pharmacia Biotech Ultrospec 3000 spectrophotometer.

2.6 Internalisation of FITC-albumin by columnar cells

B. mori cultured cells were pelletted by gentle centrifugation at 400 g for 5 min, and 2.5 ± 0.6 x 10^4 cells were resuspended in 300 µl of the modified IPS, devoid of sodium hypochlorite, for each experimental set up. The incubations, performed at 25°C unless otherwise specified, started when FITC-albumin, in the concentration indicated in figure legends, was added to the cells. At the end of the incubation, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS (Phosphate Buffer Solution (in mM): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4), the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and then examined with a confocal microscope as described in the paragraph "Fluorescence acquisition and analysis".

FITC-albumin kinetics was measured at the following external albumin concentrations (µM): 0.14, 0.4, 0.8, 1.4, 4.0, 8.0 and 14.2. FITC-albumin uptake at the fixed protein concentration of 0.14 µM was measured in the presence of the following increasing unlabelled albumin concentrations (µM): 1.9, 3.8, 7.6, 15.2, 75.8 and 151.5.

When drugs were used, the cells were preincubated for 30 min in the absence (control) or in the presence of the drugs. The effect on FITC-albumin internalisation of the following compounds was tested: 100 µM 2,4-dinitrophenol (DNP), 10 mM sodium azide, 100 µM chlorpromazine, 20 µM phenylarsine oxide, 27 µM nocodazole, 20 µM cytochalasin D. If the

drugs were dissolved in DMSO, control cells were incubated with a corresponding amount of solvent. In all these experiments the cells were then incubated in the presence of 1.4 μM FITC-albumin for 20 min. Afterwards, the cells were fixed and processed for confocal microscopy observations as reported above. To test if the drugs affected cell vitality, midgut cells were incubated for 30 min in the absence (control) and in the presence of either the drug concentrations used for the experiments or the corresponding amount of DMSO. The Trypan blue test showed that none of the experimental conditions induced dye diffusion into the cells (data not shown).

In the experiment in which the ability of 10 mM gentamicin to inhibit 1.4 μM FITC-albumin internalisation was tested, cells were incubated for 20 min at 25°C in the absence (control) or in the presence of the polybasic drug. It was also tested the ability of 14 μM transferrin and 14 μM insulin to inhibit 0.14 μM FITC-albumin internalisation. In these experiments cells were incubated for 30 min at 25°C in the absence (control) or in the presence of the two proteins.

To evaluate the effect of Ca^{2+} on 1.4 μM FITC-albumin internalisation, cells were incubated for 20 min at 25°C in the absence (control) or in the presence of the Ca^{2+} chelator EDTA (20 mM). For these experiments, the incubation medium had the following composition (in mM): 47 KCl, 4.3 K_2HPO_4, 1.1 KH_2PO_4, 179 sucrose, pH 7, and 20 EDTA. At the end of the incubation, cells were rinsed three times in the same solution and fixed as reported above. Control cells were incubated in the modified IPS, devoid of sodium hypochlorite, rinsed, and fixed as reported above.

2.6.1 Colocalisation experiments

B. mori cultured midgut cells were pelletted and resuspended in IPS as described in the paragraph "Internalisation of FITC-albumin by columnar cells". After 20 min of incubation with 1.4 μM FITC-albumin at 25°C, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS, the samples were permeabilised

for 4 min with 0.1% Triton-X 100 in PBS and washed 3 times in PBS. Cells were then incubated for 15 min in PBS containing 1% BSA, and for 1h in the same buffer added with anti-clathrin heavy chain mouse IgG (Affinity BioReagents) diluted 1:400, or with the anti-megalin rabbit IgG A55 (kindly donated by Dr. M. Marinò, Dipartimento di Endocrinologia, Università di Pisa, Italy) diluted to 400 µg/ml, or with the anti-EEA1 goat IgG (kindly donated by Dr. L. Lanzetti, Divisione angiogenesi molecolare, IRCC, Torino) diluted 1:150. The cells were then washed 3 times in PBS containing 1% BSA and incubated for 1h in the same buffer added with either Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Molecular Probes) diluted 1:1000 or with Alexa Fluor 594-conjugated donkey anti-rabbit IgG antibody (Molecular Probes) diluted 1:1000, or with Alexa Fluor 555-conjugated donkey anti-goat (kindly donated by Dr. L. Lanzetti) diluted 1:150, respectively. After 3 rinses in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a confocal microscope (see "Fluorescence acquisition and analysis"). Negative controls were carried out in the same manner, except for omitting the incubation with the primary antibody (data not shown).

In the experiment where lysosomes were stained, cells were pelletted and resuspended in IPS as described in the paragraph "Internalisation of FITC-albumin by columnar cells". After 1h of incubation with 1.4 µM FITC-albumin and 2 µM LysoSensor Blue (Molecular Probes) at 25°C, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a confocal microscope (see "Fluorescence acquisition and analysis").

2.6.2 Immunodetection of microtubules organisation

B. mori cultured midgut cells, pelletted and resuspended in IPS as described in the paragraph "Internalisation of FITC-albumin by columnar cells", were incubated with or without nocodazole (see "Internalisation of

FITC-albumin by columnar cells"). At the end of the incubation, cells were rinsed 3 times in IPS, fixed and permeabilised for 5 min with ice-cold methanol. After 3 rinses in PBS, cells were incubated for 15 min in PBS containing 1% BSA and for 1h with the anti-α-tubulin mouse IgG (Sigma), diluted 1:500 in the same buffer. The cells were then washed 3 times in PBS containing 1% BSA and incubated for 1h with Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (Molecular Probes), diluted 1:1000 in PBS containing 1% BSA. After 3 rinses in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a confocal microscope (see "Fluorescence acquisition and analysis"). Controls were carried out in the same manner, except for omitting the incubation with the primary antibody (data not shown).

2.6.3 Detection of actin filaments

B. mori cultured midgut cells were pelletted, resuspended in IPS as described above and incubated in the absence or in the presence of cytochalasin D (see "Internalisation of FITC-albumin by columnar cells"). At the end of the incubation, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS, the samples were permeabilised for 4 min with 0.1% Triton-X 100 in PBS and washed 3 times in PBS. The cells were then incubated for 20 min with 4.3 µg/ml TRITC-phalloidin (Sigma). After 3 rinses in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and examined with a confocal microscope (see "Fluorescence acquisition and analysis").

2.6.4 Fluorescence acquisition and analysis

Fluorescence was acquired by using a confocal microscope CLSM TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Germany) equipped with an argon ion laser (458, 476, 488, 496 or 514 nm excitation), two

HeNe lasers (543, 594 and 633 nm excitation) and tunable emission wavelength collection. A 63X Leica oil immersion plan apo (NA1, 4) objective and a 2X zoom were used for all the observations. FITC, Alexa Fluor 488, Tat-EGFP and EGFP were excited with the 488 nm laser line and the emitted fluorescence was collected between 500 and 560 nm; Alexa Fluor 594 and 555 was excited with the 594 nm laser line and the emitted fluorescence was collected between 605 and 700 nm; TRITC was excited with the 543 nm laser line and the emitted fluorescence was collected between 555 and 620 nm; LysoSensor Blue was excited with the 373 nm laser line and the emitted fluorescence was collected between 410 and 430 nm. To compare different experimental conditions (i.e. cells incubated with FITC-albumin for different time lapses, with different protein concentrations or in the presence of various drugs), fluorescence acquisitions were always performed with the same hardware settings (laser intensity, sampling, acquisition rate, pinhole, and photomultiplier settings). To evaluate FITC-albumin, Tat-EGFP and EGFP internalisation avoiding the contribution of unspecific binding to the cell membrane, a single optical section in a middle cell focal plane (where the nucleus was clearly evident) was acquired. Regions of interest (ROIs), precisely defining the cell cytoplasm, were drawn and the calculated mean grey values were used. Ten or more cells from at least two independent preparations were analysed for each experimental condition. The data, expressed as arbitrary units of fluorescence intensity (8 bit acquisition), are reported as mean ± standard error. For each set of experiments Student's *t* Test was used for statistical analysis.

2.6.5 Western blot analysis

B. mori midguts isolated from fifth instar larvae were homogenised using a Teflon/glass Potter with 9 strokes at 2000 rev/min (IKA-Labortechnik RE 16 apparatus - Janke & Kukel -) in 100 mM mannitol, 10 mM Hepes-Tris pH 7.2, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM leupeptin, 2 µg/ml aprotinin, 1 µM pepstatin A

(all purchased from Sigma) (10 ml/g tissue). Protein concentration was determined according to Bradford (1976) with bovine serum albumin (BSA) as standard.

Aliquots of midgut homogenates (50 µg of protein) were solubilised in sample buffer and resolved by 7.5% SDS-PAGE, as described by Laemmli (1970). Proteins were transferred to nitrocellulose membranes at 350 mA for 90 min. Membranes were left overnight at 4°C in 150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 5% w/v non fat dry milk, 0.1% v/v Tween 20, and then washed three times for 15 min in 150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 0.1% v/v Tween 20. Incubations with the primary antibody were performed for 1h at room temperature using anti-clathrin heavy chain mouse IgG (Affinity BioReagents) diluted 1:500 in 150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 3% w/v non fat dry milk, 0.1% v/v Tween 20. Membranes were then washed three times (15 min for each wash) and the primary antibody was detected by the enhanced chemioluminescence method (ECL, Amersham Biosciences), with peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at 1:1000 dilution as a secondary antibody.

2.6.6 Analysis of mRNA expression of a putative megalin homologue

Total RNA (0.8 µg) isolated from *B. mori* larval midgut with Tri Reagent (Sigma), following the manufacturer's instructions, was used for RT to generate the cDNA with ImProm-II Reverse Transcription System (Promega). The generated RT-cDNA was used for PCR amplification.

The primers for *B. mori* putative megalin homologue were sense, 5'-TGGACTGGGTGGGCGACAAG-3'; and antisense, 5'-TTCATGTGAGTGCCGTCCATGT-3' (expected size for PCR product 208 bp). The conditions for PCR were: denaturation, 96°C for 30 s; annealing, 61°C for 30 s; extension, 72°C for 1 min (60 cycles). Amplification of *B. mori* cytoplasmic actin A3 (40 cycles) was included as the internal control; the primers, designed according to the sequence available in the database

(GenBank accession number U49854), were sense, 5'-ATGTGCGACGAAGAAGTTGC-3'; and antisense, 5'-CTCACCTGTTGGCCTTGG-3' (expected size for PCR product 331 bp).

The PCR products with or without reverse transcription were separated by electrophoresis on 1.5% agarose gel and visualised under ultraviolet light with ethidium bromide.

2.7 Internalisation of EGFP and Tat-EGFP by columnar cells

B. mori cultured midgut cells were pelletted and resuspended in IPS as described in the paragraph "Internalisation of FITC-albumin by columnar cells". The incubations, performed at 25°C unless otherwise specified, started when 1.5 μM EGFP or 1.5 μM Tat-EGFP were added to the cells. At the end of the incubation, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and then examined with a confocal microscope as described in the paragraph "Fluorescence acquisition and analysis".

When 2,4-dinitrophenol (DNP) or sodium azide were used, the cells were pre-incubated for 30 min in the absence (control) or in the presence of 100 μM DNP or 10 mM sodium azide and then incubated for 30 min with Tat-EGFP. After 3 rinses in PBS, the cells were fixed and processed for confocal microscopy observation as reported above.

2.7.1 Tat-EGFP internalisation in isolated midguts

Silkworms were anaesthetised with CO_2 and cut between the second and the third pair of thoracic legs and behind the third pair of abdominal appendages, to exclude the foregut and the hindgut. Then, the midgut was explanted and deprived of the peritrophic membrane along with enclosed intestinal contents. The removal of the peritrophic membrane and several rinses in a physiological solution (210 mM sucrose, 45 mM KCl, 10 mM Tris–HCl at pH 7), eliminated the proteolytic enzymes present

both in the endo- and ecto-peritrophic spaces. The midgut was then mounted on an apparatus (fig. 2.1), where the shape and orientation *in situ* were maintained. The internal chamber of the apparatus corresponded to the luminal compartment (L in fig. 2.1) and the external chamber to the haemolymphatic compartment (H in fig. 2.1).

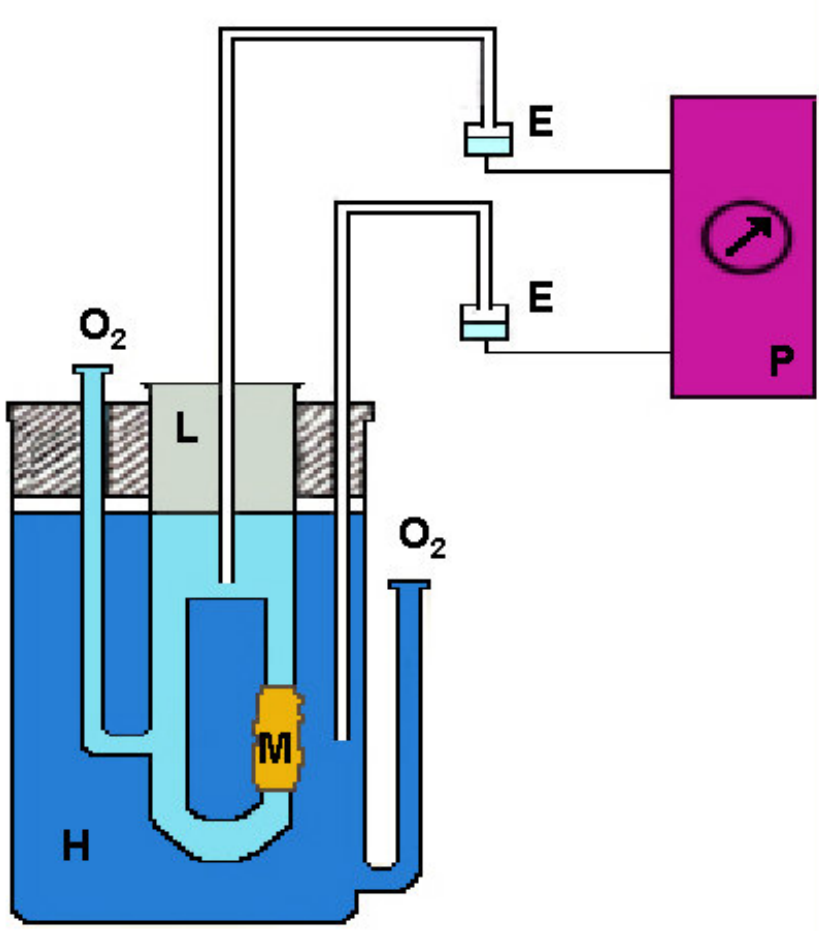

Figure 2.1: Scheme of the apparatus used for transepithelial flux measurements (modified from Nedergaard and Harvey, 1968). M: midgut, L: luminal chamber, H: haemolymphatic chamber, O_2: oxygen inlet; E: electrodes; P: potentiometer.

In the experiments here reported, only the middle region of the midgut was used, because the anterior part is too short and the posterior one too fragile to be mounted on the experimental apparatus. The bathing buffers had the following composition (in mM): 5 K_2SO_4, 4.8 $MgSO_4$, 1 $CaCl_2$, 10 KCitrate, 10 L-alanine, 10 L-glutamine, 10 glucose, 190 sucrose,

5 Tris, pH 8.3 in the luminal compartment and adjusted with HCl to pH 7 in the haemolymphatic one. The buffers present in the luminal and haemolymphatic compartments were continually oxygenated and stirred by bubbling pure O_2. Incubations were carried out at 25°C, in the presence of 1.5 μM Tat-EGFP or 1.5 μM EGFP in the luminal compartment. After 3h of incubation to avoid unspecific binding of Tat-EGFP and EGFP to midgut cell plasma membranes, the midgut was removed from the perfusion apparatus and washed several times with the following rinsing buffer (in mM): 20 K_2SO_4, 14.4 $MgSO_4$, 3 $CaCl_2$, 200 sucrose, 5 Tris–HCl, pH 7. The tissue was fixed for 30 min in 4% paraformaldehyde in rinsing buffer, washed five times, permeabilised with 0.1% Triton-X 100 in rinsing buffer for 4 min and then subjected to five additional washes. To stain cell nuclei, the tissue was incubated for 30 min with 0.2 mg/ml DAPI (40,6-diamidino-2-phenylindole dihydrochloride) (Sigma). After five washes with rinsing buffer, the tissue samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem) and, after coverslip application, were examined with the confocal microscope (CLSM TCS SP2 AOBS (Leica Microsystems Heidelberg GmbH, Germany)).

To monitor the viability of the midgut epithelium, in each experiment the transepithelial electrical potential difference (TEP) was recorded by a Keithley microvoltmeter, with calomel electrodes connected via agar–KCl (3 M) bridges to the solutions bathing both sides of the midgut. TEP decay in healthy midguts during the experiment was 0.25 ± 0.02 mV/min (mean ± SE of 30 experiments), with an initial value of 88 ± 4 mV.

2.8 Enzymatic disaggregation of midgut tissue

8-10 midguts isolated from the 5th instar of *S. frugiperda* larvae, were transferred in a Petri dish containing sterile IPS lacking Ca^{2+} and Mg^{2+} (47 mM KCl, 4.3 mM K_2HPO_4, 1.1 mM KH_2PO_4, 179 mM sucrose, pH 7). After two rinses in the same solution, midguts were transferred in a Petri dish containing sterile IPS lacking Ca^{2+} and Mg^{2+} with 2.5% (w/v) trypsin and left under mild agitation for 30 min. The solution was then filtered through

a strainer (100 µm pore size), the tissue in the sieve was discarded and the free cells in the filtrate, mostly mature midgut cells, were collected and pelletted by gentle centrifugation at 400 g for 5 min. The supernatant was eliminated and the pellet was rinsed twice in IPS. Midgut cells obtained with this procedure were immediately used for the experiments.

2.9 Detection of Cy3-*Jc*DNV in midgut cells

The cultured cells and those obtained by trypsin were pelletted by gentle centrifugation at 400 g for 5 min and resuspended in 100 µl of IPS for each experimental set up. The incubations, performed at 25°C, unless otherwise specified, started when Cy3-*Jc*DNV (at the final concentration of 20 µg/ml) was added to the cells. At the end of the incubation, lasted 10, 30 or 60 min, cells were rinsed 3 times in IPS and fixed for 10 min with 4% paraformaldehyde. After 3 rinses in PBS, the samples were permeabilised for 4 min with 0.1% Triton-X 100 in PBS and washed 3 times in PBS. Cells were then incubated for 30 min with FITC-phalloidin (4.3 µg/ml) (Sigma), rinsed 3 times in PBS and, eventually, incubated for 15 min with DAPI (0.25 µg/ml) (Sigma). The samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and then examined with a fluorescence microscope (AXIOVERT 200M equipped with AXIOCam HRm, Zeiss, Germany) as reported above.

3. Results and Discussion

3.1 Stem cells differentiation in culture and morphology of midgut cells *in vitro*

Recent studies demonstrated that in *B. mori* larval midgut, isolated and mounted in a perfusion apparatus, two selected proteins, albumin and horseradish peroxidase (HRP), crossed unaltered the intestinal barrier by transcytosis (Casartelli *et al*., 2005; 2007). *In vitro* approaches provide a powerful tool to study in a simplified experimental context the pathways involved in the absorption processes, which, however, have always to be interpreted with caution when placed in the more complex physiological scenario occurring *in vivo*. Even though with the isolated midgut it is possible to measure the transepithelial unidirectional fluxes and their modification under controlled experimental conditions, its use appears inadequate to study on a finer-grained scale the absorption process at the cellular level. The internalisation of proteins and the sequence of intracellular steps undertaken by the loaded vesicles can be better analysed in single columnar cells of the larval midgut in culture. Thus, we established a culture of mature midgut cells from *B. mori* larvae, differentiated from stem cells according to Sadrud-Din *et al*. (1994, 1996).

In order to isolate the largest possible number of stem cells from *Bombyx mori* midgut, we performed a histological analysis of the tissue in three different instances of larval development. In figure 3.1 is shown the midgut epithelium dissected from larvae immediately before the IV moult (A), during the IV moult (B) or in the middle of the V instar, during the feeding period (C). In the period just before the last larval-larval moult, stem cells are located in numerous nidi at the base of the epithelium (fig. 3.1A). During the moult (fig. 3.1B), the stem cells proliferate and then differentiate, each of the newly developed cell inserting between the mature cells of the IV instar epithelium. In the V instar, during the feeding period (fig. 3.1C), very few single stem cells are visible, far less numerous than in the pre-moult period. Therefore, the largest number of stem cells can be isolated from *B. mori* larval midguts in the period just preceding the IV moult.

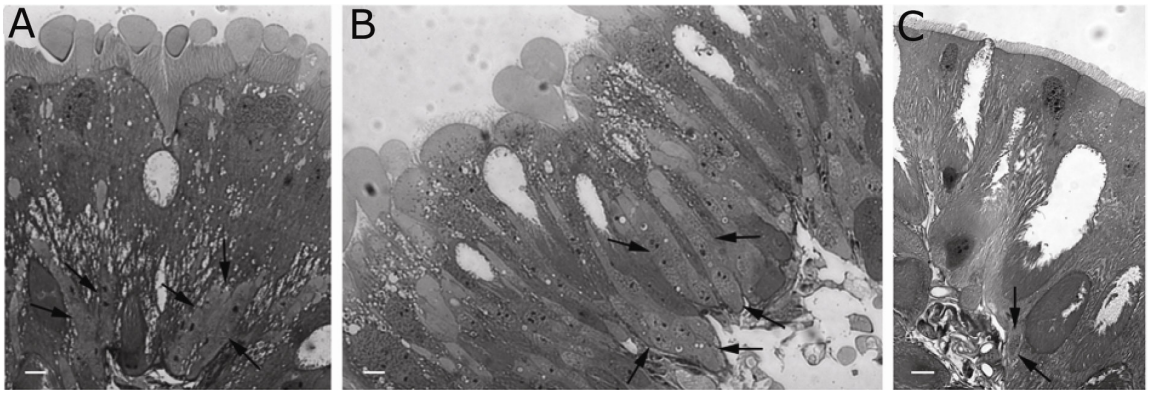

Figure 3.1: Semithin sections of the midgut epithelium of *Bombyx mori* larvae dissected immediately before the IV moult (A), during the moult (B) and in the V instar feeding period (C). Stem cells nidi (A), proliferating cells (B) and a single stem cell (C) are indicated by arrows. Bars: 10 μm.

To monitor the development with time of the stem cells in culture, we followed the proliferation, differentiation and percentage distribution of each cell type for 42 days (figs 3.2, 3.3), by identifying the different cell types every 7 days on the basis of their morphological features. Stem cells were round, with a diameter of 4-8 μm (fig. 3.2A) and some of them could be observed in mitosis (fig. 3.2B). Cells in an early stage of differentiation were round, with long tenuous membrane projections and numerous granules in the cytoplasm (fig. 3.2C). Sadrud-Din *et al*. (1996) suggested that these cells corresponded to the initial phase of differentiation of columnar cells. The cell reported in fig. 3.2D, a representative of columnar cells in a more advanced stage, suggests that differentiation of the basolateral membrane, at least from a morphological point of view, is a delayed process. Young columnar (fig. 3.2E) and goblet (fig. 3.2G) cells had the same shape of the respective mature cells (figs 3.2F, H) but their dimensions were smaller (between 25-30 μm). Mature columnar cells were characterised by a well developed apical membrane with numerous microvilli, a centrally placed nucleus and a cylindrical or cubical shape (fig. 3.2F), while mature goblet cells were flask-like and presented the typical wide cavity, the apical valve and a basally located nucleus (fig. 3.2H).

Both cells showed most of the apparent morphological features seen *in vivo* but they were never as tall as those of the original epithelium (60 to 80 µm, fig. 3.1).

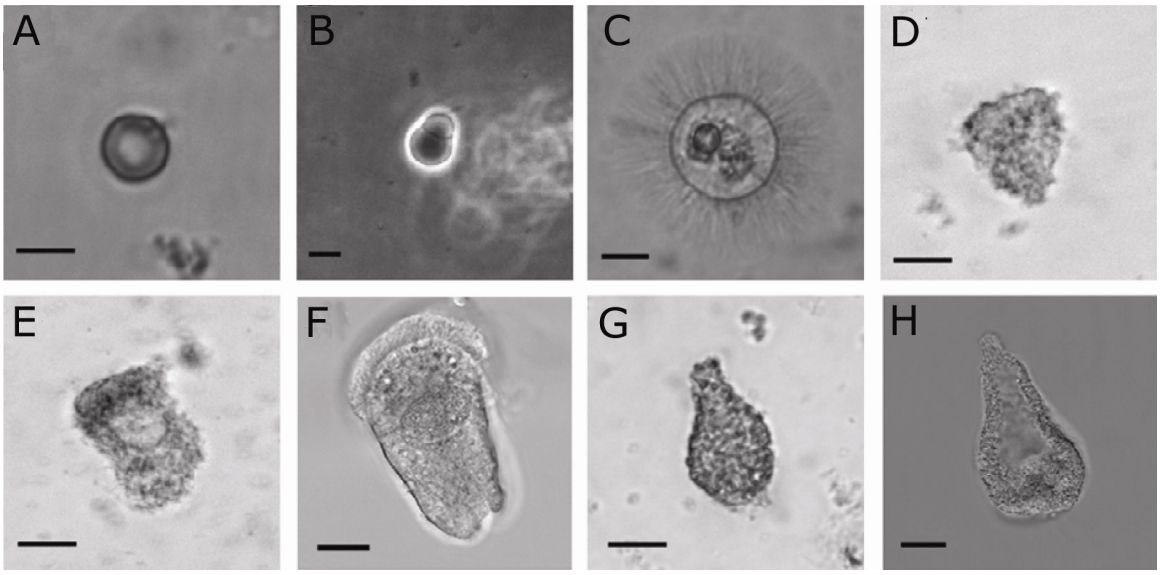

Figure 3.2 Morphology of cultured midgut cells. Brightfield images (acquired with confocal laser scanning microscope) of fixed cells: stem cell (A); stem cell in mitosis (B); differentiating columnar cells (C, D); columnar (E) and goblet (G) cells in an early phase of differentiation; and fully differentiated (F, H). Bars: 5 µm (A, B); 10 µm (C-H).

As shown in fig. 3.3, in the filtrate collected after a mild agitation of the pre-moult midgut tissue, the stem cells represented 87.4 ± 3.6% (3 determinations) of the total viable cells present in the medium. After six days in culture, the reduction of stem cells was accompanied by an increase of differentiating, columnar and goblet cells. At the end of the following 7 days, the percentage of stem cells was further decreased, while differentiating cells were the most represented cell type. From the third week on, less than 10% were stem cells and the remaining cells were represented by percentage values not statistically different (ranging between 27.6 ± 3.4% (3 determinations) and 41.4 ± 5.3% (3 determinations)) of columnar, goblet and differentiating cells.

All along the experimental period here considered, viable cells were 79.2 ± 5.1% (21 determinations) of the total cells present in the culture.

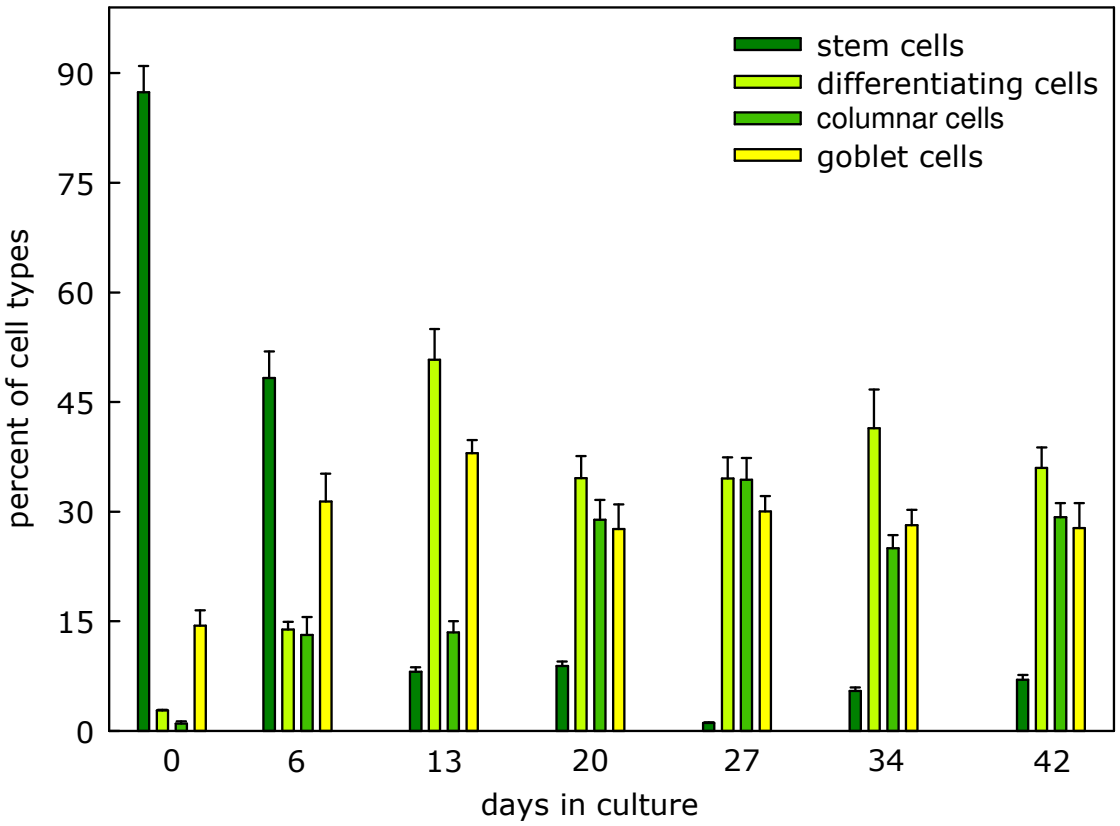

Figure 3.3 Percentage of the various viable cell types in culture in the different days since stem cells isolation. Each bar represents the mean ± SE of three different determinations.

Columnar cells *in vivo* are responsible for the production of the different classes of enzymes involved in the digestion of ingested nutrients (Terra and Ferreira, 2005). We investigated if the activity of two enzymes currently used as marker enzymes of the apical membrane of midgut columnar cells, i.e. leucine-aminopeptidase N (APN) and alkaline phosphatase (AlP), could be detected in the lysate of three weeks old cell cultures. Although after 20 days in culture columnar cells represented only the 28.9 ± 2.7% (3 determinations) of all the cells in culture (fig. 3.3), an activity of both enzymes could be measured: APN and AlP specific activities (mU/mg of protein) were 890 ± 88 (5 determinations) and 131 ± 12 (4 determinations) respectively.

Therefore, *B. mori* columnar cells, differentiated from stem cells *in vitro,* maintained in culture the typical morphological features of the epithelium *in vivo* and were able to perform at least some of the normal digestive functions.

The columnar cells used for all the experiments reported below came from 20 day-old cultures to 42 day-old culture.

A- Endocytosis of albumin and its intracellular movements in insect midgut cells

3.2 Albumin uptake by columnar cells

The different steps involved in albumin transcytosis (Casartelli *et al*., 2005) were investigated in *B. mori* columnar cells in culture.

Fluorescein isothiocyanate (FITC)-albumin uptake was measured at a fixed concentration of 1.4 μM. Figure 3.4, shows the time course of the protein uptake by columnar cells evaluated by confocal laser microscopy. Cells were incubated at 25°C in the presence of FITC-albumin for 5, 10, 20, 30, 45, 60 minutes. After 5 min of incubation, a weak fluorescence was present inside the cell. From 10 to 30 minutes, the fluorescence progressively increased and, after 30 minutes no difference in fluorescence was observed. Fluorescence inside the cell was never uniformly diffused, but was visible as dots into the cytoplasm. This punctuate distribution of FITC-albumin inside the cytoplasm (fig. 3.4) is consistent with a vesicular compartmentalisation, which is expected for a protein that follows intracellular pathways.

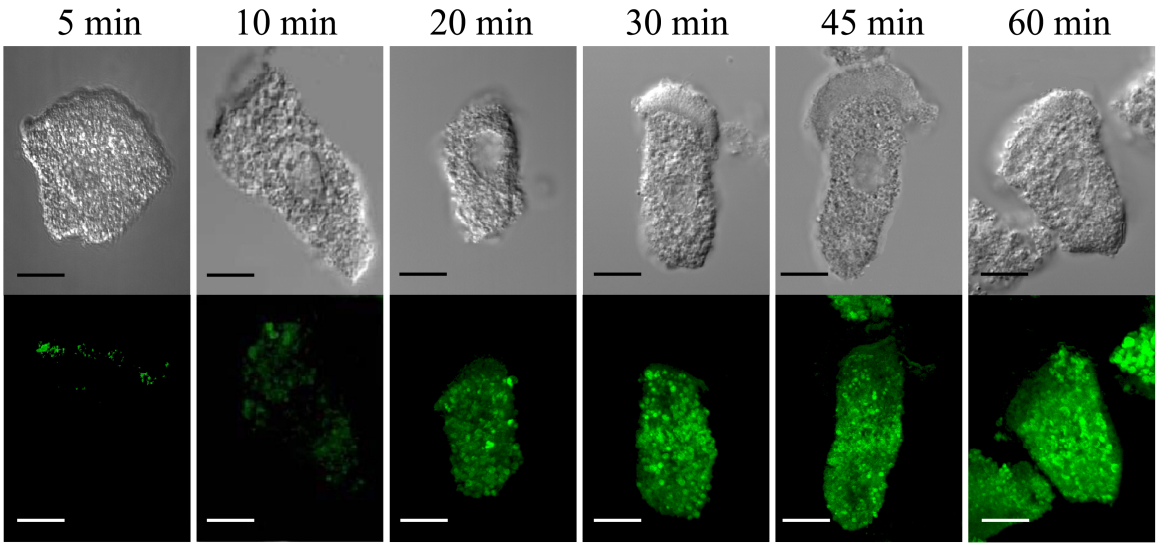

Figure 3.4 Brightfield (row on *top*) and confocal laser-scanning micrographs (maximum projection) (row on *bottom*) of *Bombyx mori* columnar cells cultured *in vitro* and incubated for different time intervals at 25°C in the presence of 1.4 μM fluorescein isothiocyanate (FITC)-albumin. For each time, the image chosen was that best representing the average condition of cells. Bars: 10 μm.

These observations were further corroborated by the analysis of the fluorescence intensity in single optical sections of the cells at the experimental time points considered (fig. 3.5). Each point represents the mean of the fluorescence intensity recorded in single optical sections designed in a middle focal plane of the cell, in which the nucleus was clearly evident. Regions of interest (ROIs), precisely defining the cell cytoplasm, were drawn, and the calculated mean grey values were used. Values are means ± SE of the fluorescence intensity recorded in at least 10 cells for each incubation time. The analysis of the fluorescence intensity clearly indicates that protein uptake reached a steady state in 30 min. This result is explained considering the balance between protein internalisation and its subsequent fate, which can include, in addition to the transepithelial route (Casartelli *et al*., 2005), the recycling to the plasma membrane domain where the vesicle had originated and/or the targeted delivery to lysosomes for intracellular digestion (Apodaca, 2001; Ellinger *et al*., 2001).

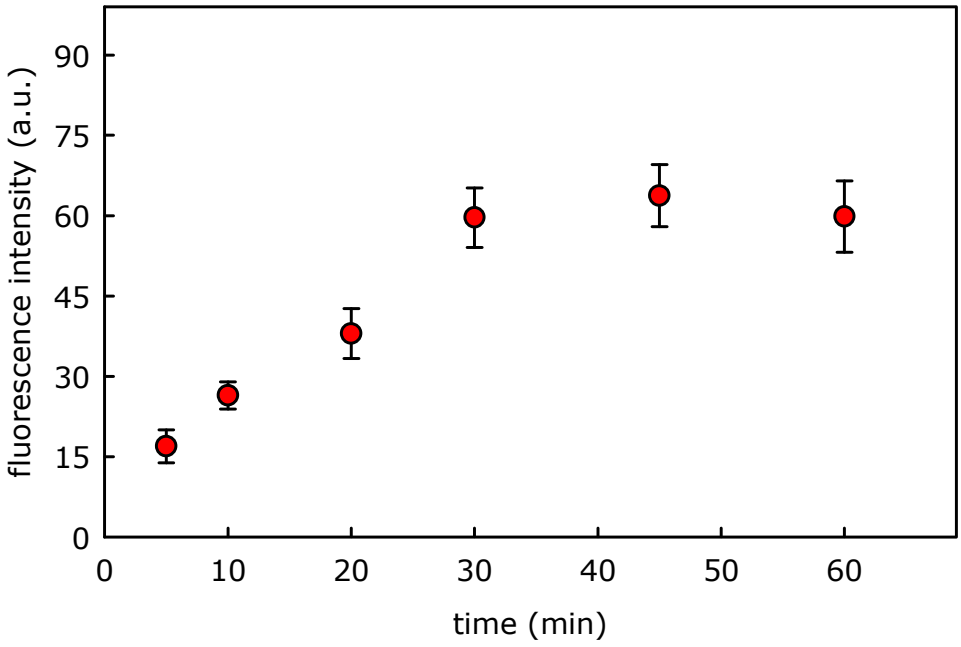

Figure 3.5 Quantification of FITC-albumin internalised with time. Single optical sections in a middle focal plane of the cell, in which the nucleus was clearly evident, were acquired by confocal laser microscope. Regions of interest (ROIs), precisely defining the cell cytoplasm, were drawn, and the calculated mean grey values were used. Values are means ± SE of the fluorescence intensity recorded in at least 10 cells for each incubation time.

To characterise the endocytic process involved in FITC-albumin internalisation, we incubated the cells for 20 min at 4°C. In this condition, protein uptake was drastically reduced with respect to that observed a 25°C (fig 3.6A, B) and the fluorescence intensity recorded in single optical sections of the cells showed a 60% inhibition of the uptake (fig. 3.6C).

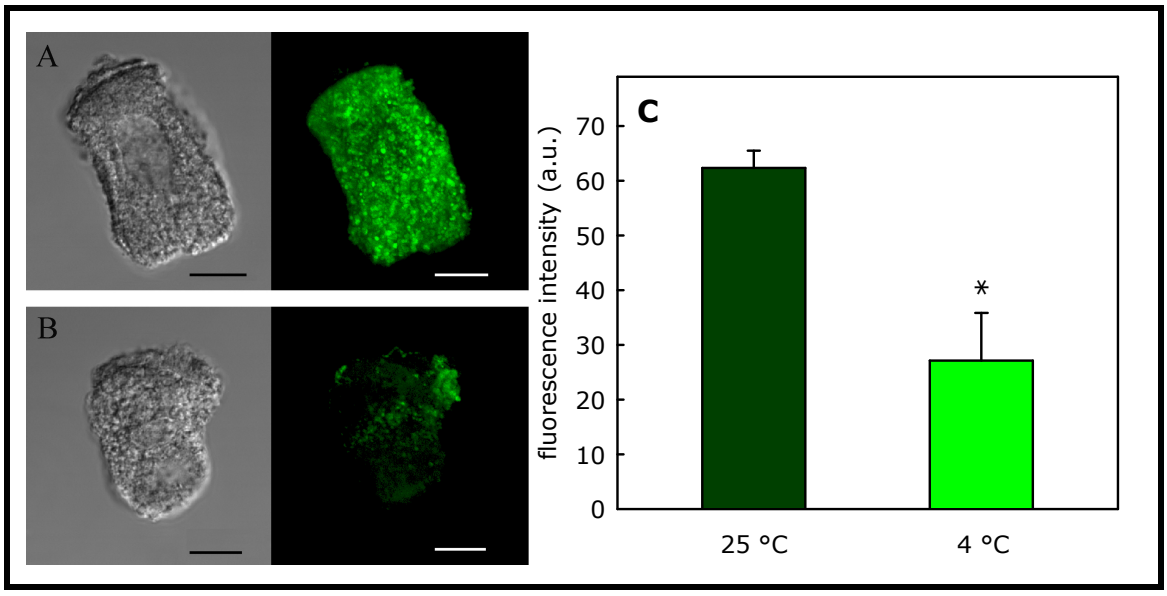

Figure 3.6 Effect of temperature on FITC-albumin internalisation by columnar cells. Brightfield and confocal laser scanning micrographs (maximum projection) of *B. mori* columnar cells incubated for 20 min at 25°C (A) or at 4°C (B) in the presence of 1.4 μM FITC-albumin. The images chosen were those best representing the average condition of cells. Bars: 10 μm. For each experimental condition, the mean ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells is reported in C. Student's *t*-test: $*P < 0.01$.

Then we incubated the cells with the two metabolic inhibitors, 2,4-dinitrophenol (DNP) and sodium azide. Cells were pretreated for 30 minutes with DNP (100 μM) or sodium azide (10 mM) and then incubated with FITC-albumin. As reported in figure 3.7, both drugs induced a significant reduction of albumin transport, with a 50 and 80% decrease of the measured fluorescence intensity, respectively. Together with the inhibition at low temperature, these data confirm that albumin uptake is an energy-dependent process.

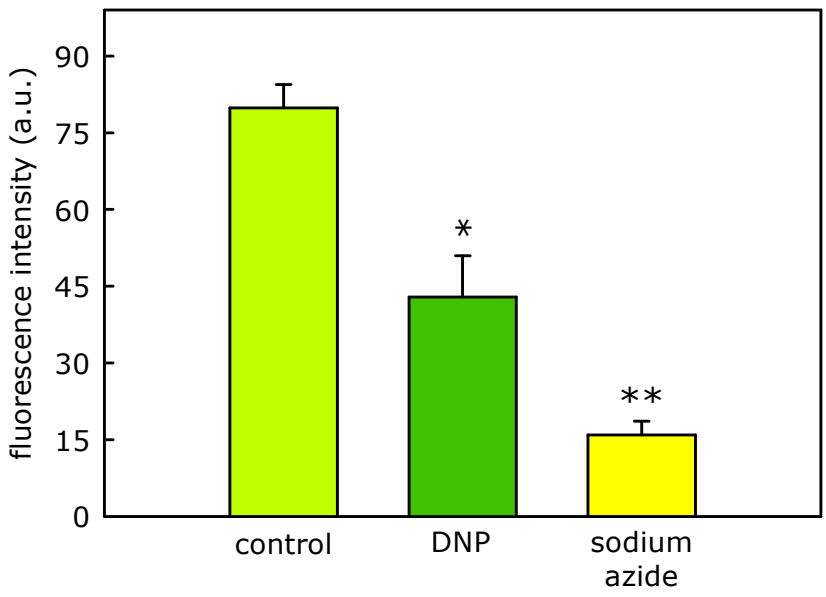

Figure 3.7 Effect of metabolic inhibitors on FITC-albumin internalisation. Cells were pretreated at 25°C for 30 min with 2,4-dinitrophenol (DNP) (100 μM) or sodium azide (10 mM) and then incubated with 1.4 μM FITC-albumin for 20 min. For each experimental condition, values are means ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells. Student's *t*-test vs. control: * $P < 0.01$ and ** $P < 0.001$.

Figure 3.8A shows FITC-albumin uptake as a function of increasing extracellular labeled protein concentration. The experimental values fitted a Michaelis-Menten equation, with a K_m of 2.0 ± 0.6 μM, which evidences that a saturable process is involved in albumin internalisation. As expected, FITC-albumin transport was inhibited by the presence of increasing amounts of unlabeled protein in a concentration-dependent manner (fig. 3.8B). The calculated EC_{50} was 3.6 ± 1.2 μM, a value not significantly different from the K_m value (evaluated with Student's *t*-test). These results suggest that the protein could be taken up by receptor-mediated endocytosis and not by simple fluid-phase endocytosis. In this latter case, as highlighted by Gekle (2005), the molecule, like the classical markers inulin or dextran (Choi *et al*., 1999; Pitterle *et al*., 1999; Takano *et al*., 2002), is not enriched at the plasma membrane surface, and its concentration in the endocytic invagination is the same as that present in

the extracellular fluid. In fluid-phase endocytosis, the molecule uptake is linearly related to its extracellular concentration. Conversely, when a receptor is involved in the process, the ligand concentration in the endocytic invagination exceeds several-fold that of the extracellular fluid and the uptake exhibits saturation kinetics (Gekle *et al*., 1995). Receptor-mediated endocytosis is clearly a more efficient mechanism of transport. The K_m value for albumin, calculated from the kinetic experiments, is in the same range of the apparent affinity constants for albumin endocytosis in mammalian absorptive cells (Gekle *et al*., 1995; 1996; 1998).

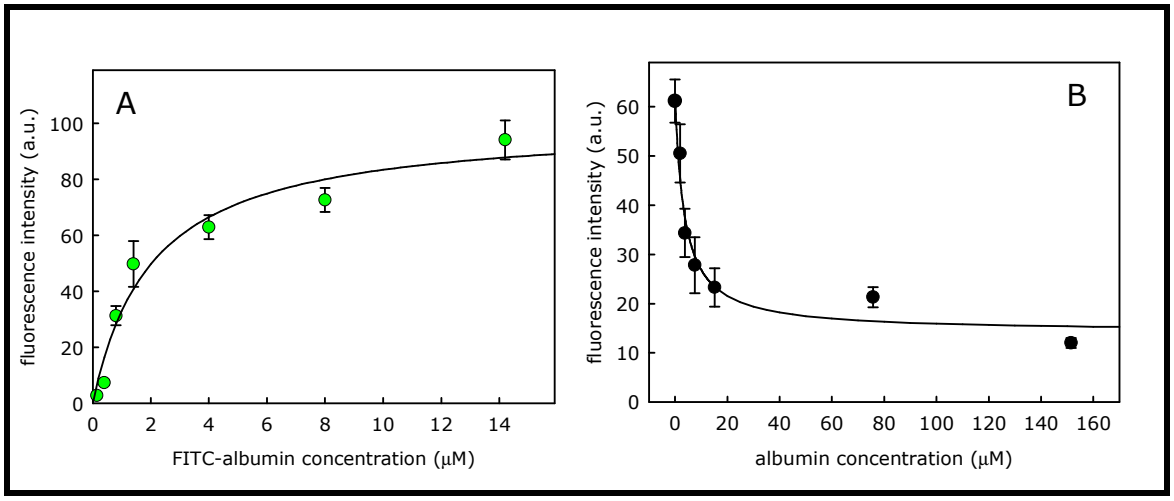

Figure 3.8 A: Kinetics of FITC-albumin uptake with increasing extracellular protein concentrations. Cells were incubated at 25 °C for 20 min with increasing FITC-albumin concentrations (from 0.14 to 14.2 µM). **B**: Effect of increasing extracellular concentrations of unlabeled albumin (from 0 to 152 µM) on 0.14 µM FITC-albumin internalisation. Cells were incubated for 1 h at 25 °C. Values are means ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least ten cells for each concentration.

3.2.1 The endocytic mechanism involved in albumin uptake

Our attention then focused on the elucidation of the type of endocytic process involved in albumin internalisation. First, we examined if clathrin, a protein involved in coated pits formation, and caveolin-1, a protein involved in the formation of caveolae, were expressed in *B. mori* midgut. Western blot analysis of midgut homogenates showed the presence of a band of approximately 180 kDa, corresponding to the clathrin heavy chain molecular weight (fig. 3.9A), while no band corresponding to caveolin 1 molecular weight was detected (data not shown).

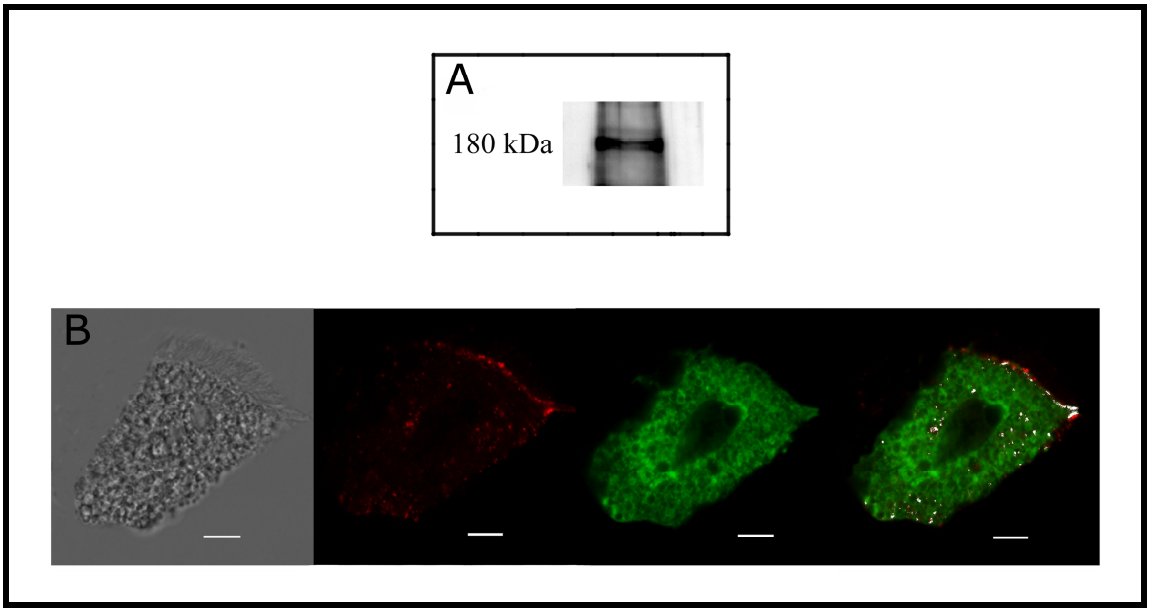

Figure 3.9 A: Western blot analysis of the homogenate of *B. mori* larval midgut with anti-clathrin heavy-chain mouse IgG. **B**: Brightfield and confocal laser scanning micrographs (optical sections) of a typical columnar cell incubated for 20 min at 25°C in the presence of 1.4 μM FITC-albumin and then labeled with the anti-clathrin heavy-chain antibody. Clathrin (red) and FITC-albumin (green) partially colocalised (white), especially in the intermicrovillar regions of the apical plasma membrane. The white pixels are colocalisation pixels with fluorescence intensity values ≥ 60 arbitrary units (a.u.) for both the red and the green channel. Bars: 5 μm.

We verified if clathrin was expressed also in columnar cells cultured *in vitro,* by staining the cells, previously incubated with FITC-albumin, with the antibody used in the immunoblotting experiment. As shown in figure 3.9B, clathrin (red) was present in the intermicrovillar areas of the apical membrane and partially colocalised (white) with albumin (green), a clear indication of the possible involvement of clathrin in albumin internalisation.

We tested the effect on albumin uptake of two inhibitors of clathrin-mediated endocytosis, chlorpromazine and phenylarsine oxide. Pre-treatment of the cells with the two drugs significantly reduced FITC-albumin internalisation (fig. 3.10), with a 75 and 50% inhibition of the uptake, respectively. Chlorpromazine prevents assembly of the coated pit at the cell surface (Wang *et al*., 1993), whereas phenylarsine oxide presumably blocks endocytosis by cross-linking the clathrin coat (Hunyady *et al*., 1991).

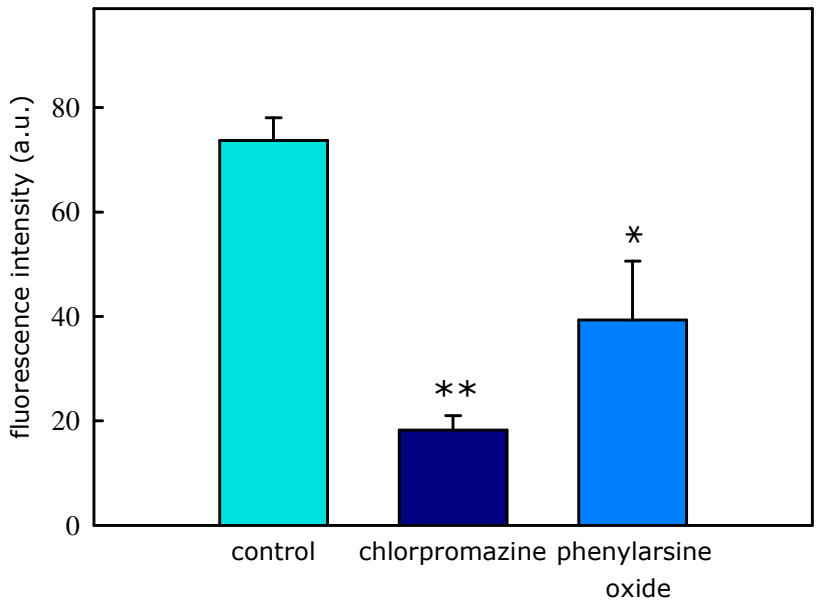

Figure 3.10 Effect of inhibitors of clathrin-mediated endocytosis on FITC-albumin internalisation. Cells were pre-treated at 25°C for 30 min with chlorpromazine (100 μM) or phenylarsine oxide (20 μM) and then incubated with 1.4 μM FITC-albumin for 20 min. For each experimental condition, values are means ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells. Student's *t*-test vs. control: $*P < 0.02$ and $**P < 0.001$.

These results, added to the previous ones, indicate that albumin internalisation is a clathrin-mediated process.

3.2.2 Identification of the receptor involved in FITC-albumin internalisation

Various mammalian epithelial tissues express the multiligand endocytic receptor megalin, a 600-kDa transmembrane protein member of the low-density lipoprotein (LDL) receptor family that recognises molecules of different structure and function, albumin included (Moestrup and Verroust, 2001; Christensen and Birn, 2002). Data reported in literature indicate that megalin is abundant in renal proximal tubule, where it is responsible for the tubular reabsorption of filtered proteins (Gekle, 2005). In the same epithelium, it also acts as a membrane anchor for cubilin, a second multiligand receptor for albumin (Gekle, 2005). In mammals, both megalin and cubilin are expressed in the intestinal brush border, where they are involved in the gastrointestinal uptake and transport of vitamin B12 and folate (Birn *et al*., 1997; Birn *et al*., 2005; Yammani *et al*., 2001).

Because our data show that in *B. mori* columnar cells a receptor is involved in albumin internalisation, which takes place via clathrin mediated endocytosis, we tested the suggestive hypothesis that a megalin-like receptor was responsible for albumin recognition in the insect midgut. Recently a megalin homologue in *Drosophila melanogaster* (Flybase Gene ID CG12139, http://www.ensembl.org) has been identified and we investigated if a putative megalin homologue was expressed also in *B. mori* larval midgut. By BLAST analysis, we identified in an EST database of *B. mori* (http://www.ab.a.u-tokyo.ac.jp/silkbase/), a sequence (clone name NV021862) with a 74% identity with the putative megalin gene of *Drosophila*. The expression of the putative megalin homologue mRNA in *B. mori* larval midgut was examined by RT-PCR analysis, using specific primers that were designed across highly conserved sequence regions. PCR products of the expected size (208 bp)

were observed in samples of the midgut after reverse transcription (fig. 3.11). Positive (actin) and negative (no RT samples of RNA) controls gave the expected amplimer or no amplification products, respectively.

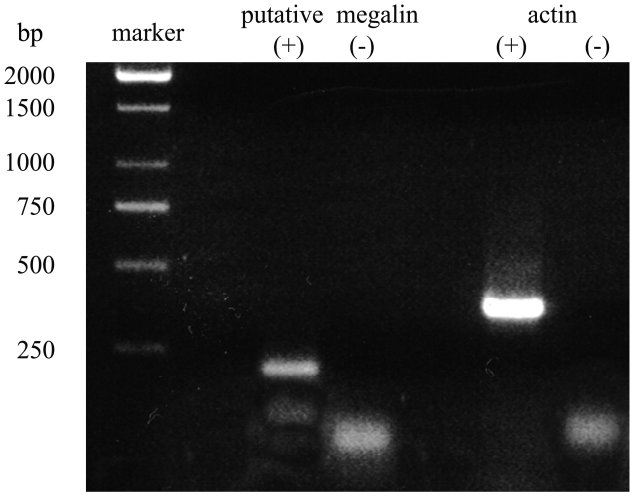

Figure 3.11 Transcriptional analyses by RT-PCR of the megalin gene in the midgut of *B. mori*. The PCR amplification products of RT samples (+) or non-RT samples (-) were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualised under ultraviolet light. Amplification of *B. mori* cytoplasmic actin A3 was included as positive control.

To prove that this receptor was involved in albumin uptake, we incubated cultured columnar cells with FITC-albumin and then stained the cells with an anti-megalin primary antibody. The antibody recognised a protein in the apical region of the cell (red) that colocalised (white) with albumin (green) in the intermicrovillar areas of the plasma membrane (fig. 3.12).

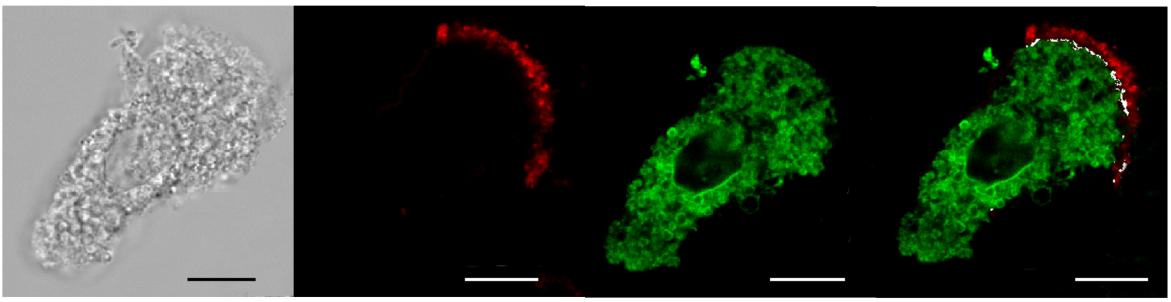

Figure 3.12 Brightfield and confocal laser-scanning micrographs (optical sections) of a typical columnar cell incubated for 20 min at 25°C in the presence of 1.4 μM FITC-albumin and then labeled with anti-megalin antibody. FITC-albumin (green) and megalin-like protein (red) partially colocalised (white) in the intermicrovillar regions of the apical plasma membrane. The white pixels are colocalisation pixels with fluorescence intensity values ≥ 30 a.u. for both the red and the green channel. Bars: 10 μm.

Because binding of ligands to megalin is Ca^{2+} dependent (Christensen *et al*., 1992) we measured the internalisation of FITC-albumin in the presence of the Ca^{2+} chelator EDTA. In this condition, we observed a 62% inhibition of the protein uptake (fig. 3.13).

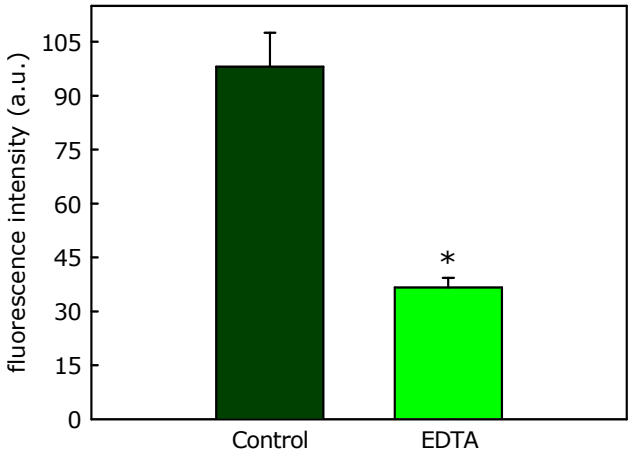

Figure 3.13 Effect of EDTA on 1.4 μM FITC-albumin internalisation. Cells were incubated at 25°C for 20 min with the labeled protein in the presence of the Ca^{2+} chelator (20 mM). For each experimental condition, values are means ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells. Student's *t*-test vs. controls: $*P < 0.001$.

In mammalian polarised epithelia, megalin can bind and internalised insulin (Orlando *et al*., 1998), transferrin (Moestrup and Verroust, 2001; Christensen and Birn, 2002) and several polybasic drugs, including gentamicin (Moestrup *et al*., 1995). To evaluate if this molecules are ligands also for the receptor involved in albumin internalisation, we incubated *B. mori* midgut cells in the presence of FITC-albumin and measured the inhibition exerted by an excess of insulin (fig. 3.14A), transferrin (fig. 3.14B) and gentamicin (fig. 3.14C). We observed a 63%, a 52% and a 40% inhibition, respectively, of the FITC-albumin intake.

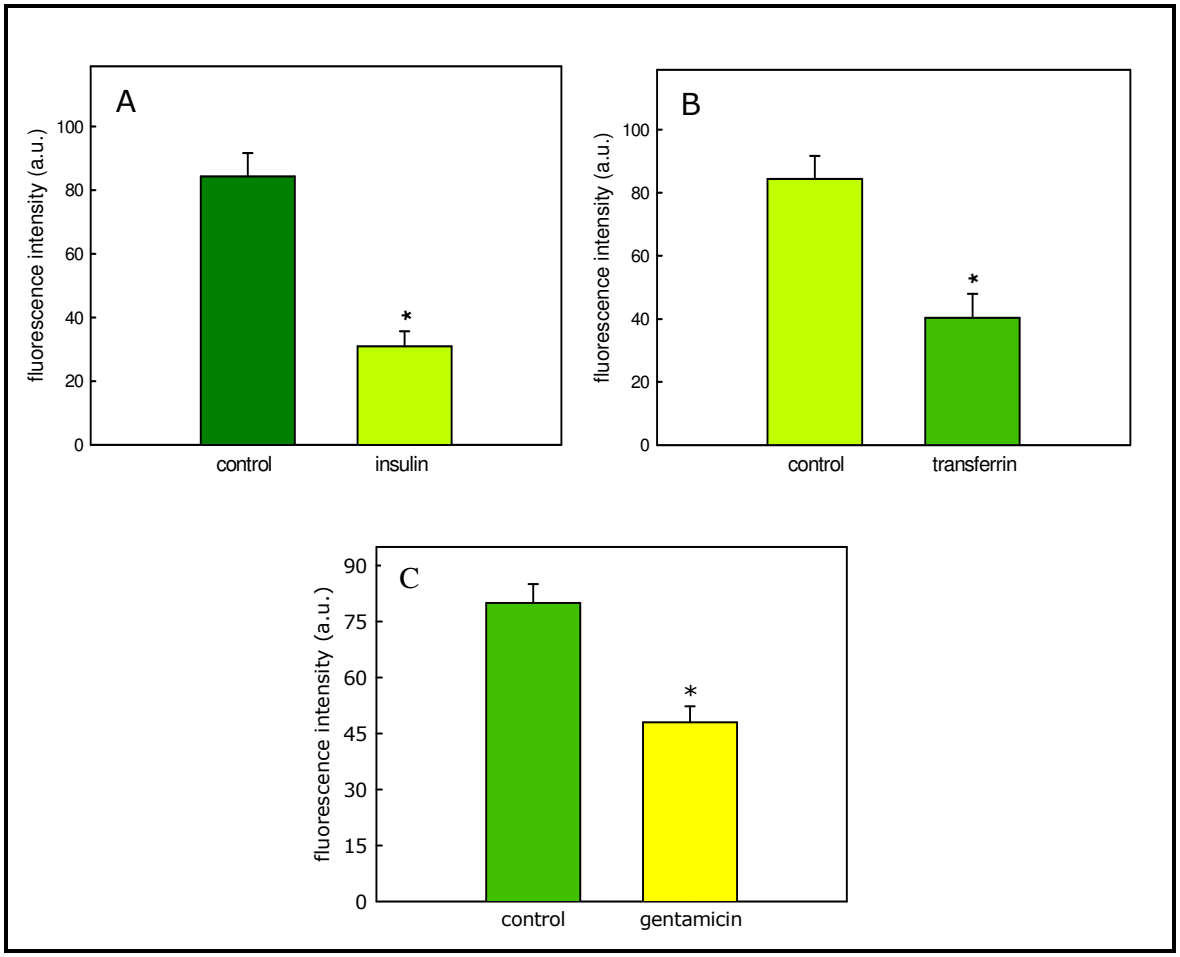

Figure 3.14 Effect of insulin (A) and transferrin (B), on 0.14 µM FITC-albumin internalisation, and of gentamicin (C), on 1.4 µM FITC-albumin internalisation. Cells were incubated for 30 minutes at 25°C with the labelled protein in the presence of insulin (14 µM) (A) or transferrin (14 µM) (B), or 20 minutes at 25°C in the presence of gentamicin (10 mM) (C). Values are means ± SE of the fluorescence intensity recorded in single

optical sections (see legend to fig. 3.5) of at least ten cells for each concentration. Student's *t*-test vs. controls: *$P < 0.001$.

RT-PCR analysis (fig. 3.11) as well as colocalisation (fig. 3.12) and inhibition (fig. 3.13; 3.14) experiments indicate that a putative megalin homologue involved in albumin endocytosis is indeed expressed in *B. mori* columnar cells. As far as we know, this is the first time that a functional description of a putative megalin homologue is reported for an insect.

Megalin expression is not restricted to mammals: it plays a role in the development of the central nervous system in zebrafish (McCarthy *et al*., 2002), and the megalin homologue LRP-1 (Saito *et al*., 1994) is essential for growth and development in the nematode *Caenorhabditis elegans* (Yochem *et al*., 1999; Herz and Bock, 2002). These reports, the recent identification of the putative megalin homologue in *D. melanogaster*, and the data reported here indicate that this protein is highly conserved in evolutionary distant organisms.

It is, of course, logical to question what could be the role *in vivo* of the midgut megalin-like receptor in lepidopteran larvae, especially considering that albumin, a protein so readily recognised and internalised, is absent in the silkworms diet. It can be speculated that the receptor may be responsible for vitamin B or iron absorption, like in mammalian intestine. It may be also involved in the uptake of those proteins present in the diet that escape degradation by digestive enzymes. Actually, in different insect species, dietary (Kurahashi *et al*., 2005) or exogenous proteins orally administered (Wigglesworth, 1943; Primor and Zlotkin, 1978, 1980; Modespacher *et al*., 1986; Allingham *et al*., 1992; Zlotkin *et al*., 1992; Bavoso *et al*., 1995; Zhu *et al*., 2001; Sugimura *et al*., 2001; Fitches *et al*., 2002, 2004; Trung *et al*., 2006; Casartelli *et al*., 2005; 2007) can avoid digestion and enter the haemolymph undegraded. The megalin-like receptor could mediate their transepithelial transport but could also provide substrates for columnar cell metabolism, since proteins, once internalised, can be directed to the intracellular degradative pathway, to supply further free amino acids to midgut cells.

3.2.3 Role of the cytoskeleton in albumin endocytosis

The integrity of the cytoskeleton is fundamental for the maintenance of cell shape and polarity and for the correct localisation of membrane proteins in the apical and basal domains of the cell surface. Efficient targeting of vesicles loaded with the specific proteins, from the Golgi apparatus to the correct membrane domain, requires an intact microtubule organisation (reviewed by Yeaman *et al*., 1999). It has been shown that disruption of microtubule architecture by colchicine or nocodazole leads in enterocytes and other polarised epithelia to a preferential alteration of the delivery to the apical rather than to the basolateral membrane (Waschke and Drenckhahn, 2000).

In rat intestinal epithelium, the microtubules organising center(s), identified with anti γ-tubulin antibodies, is/are located as a band close to the sub-apical space near the terminal web, from which the fast growing positive ends of microtubules grow towards the basolateral membrane, forming bundles of apical-basal filaments along the cell axis, while the negative ends are apically located (Waschke and Drenckhahn, 2000). In the subapical-space, most microtubules run in parallel and only few are oriented obliquely or perpendicularly, but they never cross the terminal web (Waschke and Drenckhahn, 2000). In *B. mori* columnar cells at variance with mammalian cells, microtubules form a well developed felt just under the brush border (fig. 3.15A). Deeper down the cell, numerous bundles are oriented longitudinally along the basal-apical axis of the cell. This particular structure could be related to the lack, in insect columnar intestinal cells, of a terminal web organised as that of mammals (Hull and Staehelin, 1979; Bonfanti *et al*, 1992; Gibson and Perrimon, 2003).

The disposition of actin cytoskeleton in mature columnar cells (figure 3.15B) followed that classically described for polarised cells (Yeaman *et al*., 1999): it was highly organised within the apical microvilli and some filaments, running from the basolateral membrane deep into the cytoplasm, were also visible.

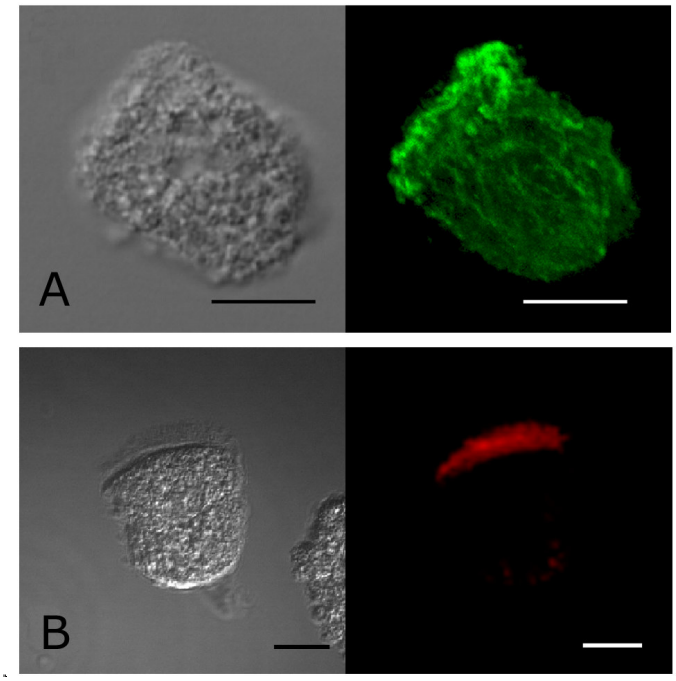

Figure 3.15 A: Brightfield and confocal laser scanning micrographs (maximum projection) of a columnar cell in which is visible microtubules organisation. Immunolocalisation of microtubules was performed with an anti-α-tubulin primary antibody visualised with an Alexa Fluor 488-conjugated secondary antibody. **B:** Brightfield and confocal laser scanning micrographs (maximum projection) of a columnar cell in which is visible microfilaments organisation. Actin filaments were visualised with TRITC-phalloidin. Bars: 10 μm.

To assess if albumin endocytosis was regulated by microtubules and/or by actin filaments, we preincubated columnar cells with either nocodazole, a microtubule depolymerising agent, or cytochalasin D, a drug that cap actin filaments at their barbed end, causing their depolymerisation. In columnar cells pretreated with nocodazole (27 μM) and then incubated with the labeled protein, albumin transport was strongly inhibited (fig. 3.16B) with respect to control cell (fig. 3.16A), indicating that microtubules integrity is essential for the endocytic process. Cells pre-treated with cytochalasin D (20 μM) and then incubated with the labeled protein showed a small decrease of albumin transport (fig. 3.16C). As reported in the histogram (fig. 3.16D), the fluorescence intensity recorded in single optical sections of the cells showed a 70 and

40% inhibition of albumin uptake with nocodazole or cytochalasin D, respectively.

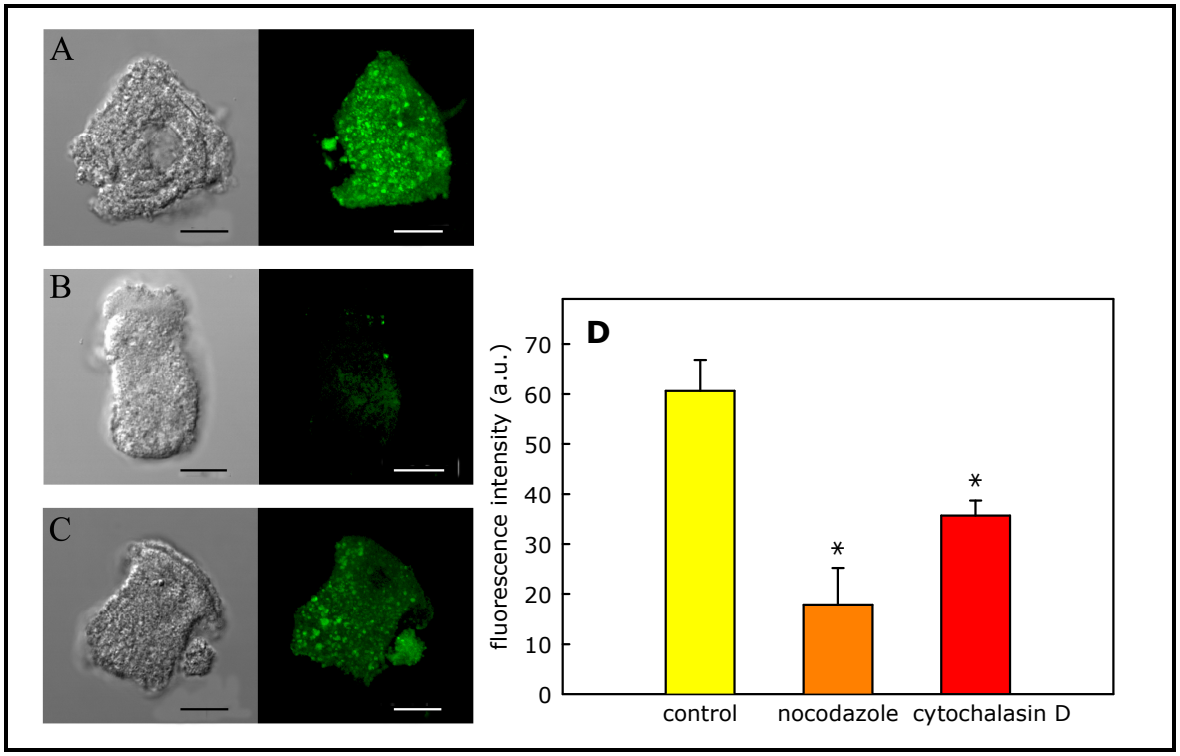

Figure 3.16 Effect of nocodazole and cytochalasin D on FITC-albumin internalisation. Brightfield and confocal laser scanning micrographs (optical sections) of columnar cells preincubated for 30 min with 27 μM nocodazole (B), with 20 μM cytochalasin D (C), or in the absence of the drugs (A, control) and then incubated for 20 min at 25°C in the presence of 1.4 μM FITC-albumin. The images chosen were those best representing the average condition of the cells. Bars: 10 μm. D: means ± SE of the fluorescence intensity recorded in the three conditions in single optical sections (see legend to fig. 3.5) of at least 10 cells. Student's *t*-test vs. control: **P* < 0.01.

Microtubules are dynamic protein filaments that provide a mechanical basis for cell polarity and for transport of organelles and vesicles within the cell. These elements of the cytoskeleton are critical players in endocytosis, since drugs that interfere with their organisation reduce receptor-mediated endocytosis in mammals (Elkjaer *et al*., 1995; Hamm-Alvarez *et al*., 1996; Gekle *et al*., 1997; Apodaca, 2001), although a detailed analysis of their role is still lacking. Actin is a ubiquitous eukaryotic protein that forms dynamic polar microfilaments of the cell

cytoskeleton. Unequivocal data on the involvement of these filaments in endocytosis come from studies on budding yeast, in which actin nucleation at the endocytic site, its role in driving membrane invagination and vesicle scission, as well as the function of actin regulatory proteins have been thoroughly clarified (Smythe and Ayscough, 2006; Kaksonen *et al*., 2006). In mammals, different orthologues of yeast proteins that cap, bundle, and stabilise actin are present (Smythe and Ayscough, 2006) and, like in yeast, membrane invagination and vesicle scission are affected by the depolymerisation of actin filaments (Yarar *et al*., 2005). The localisation and timing of actin polymerisation in yeast and mammals (Kaksonen *et al*., 2003, 2006; Smith *et al*., 2001) are strikingly similar, and it is apparent that the endocytic internalisation processes in these so different organisms are variations of a same ancestral theme. The significant reduction of albumin endocytosis in *B. mori* columnar cells induced by nocodazole and cytochalasin D (fig. 3.16: B, C and D) suggests that microtubules as well as actin filaments are involved in protein intake.

3.2.4 Albumin degradative pathway

Once internalised within endocytic vesicles, molecules can follow different fates: they can be transcytosed, recycled to the same plasma membrane where endocytosis had occurred or they can be delivered to lysosomes for degradation (Apodaca, 2001). To follow albumin pathways within the cell, we performed colocalisation experiment.

Since the first step of the endocytic vesicles is the fusion with early endosomes, we incubated columnar cells with FITC-albumin and then stained the cells with an anti-EEA1 (Early Endosome Antigen 1) primary antibody. The antibody recognised a protein present in vesicles scattered in the cytoplasm (red) that colocalised (white) with albumin (green) (fig. 3.17).

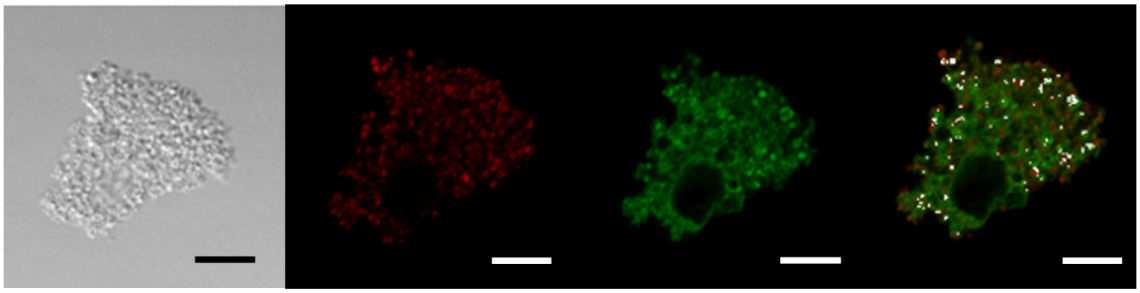

Figure 3.17 Brightfield and confocal laser-scanning micrographs (optical sections) of a typical columnar cell incubated for 1h at 25°C in the presence of 1.4 μM FITC-albumin and then labeled with anti-EEA1 antibody. FITC-albumin (green) and EEA1 (red) partially colocalised (white) in the cytoplasm of the cell. The white pixels are colocalisation pixels with fluorescence intensity values ≥ 40 a.u. for both the red and the green channel. Bars: 10 μm.

To evaluate if albumin followed the degradative pathway, we incubated columnar cells with FITC-albumin and then stained late endosomes and lysosomes with LysoSensor blue, a fluorescent pH indicator that accumulate into acidic organelles. LysoSensor exhibits a pH-dependent increase in fluorescence intensity upon acidification. LysoSensor Blue recognised acid organelles in columnar cells (blue) and it was visible that FITC-albumin (green) was localised in these organelles (white), suggesting that the protein followed also the degradative pathway (fig.3.18).

It has been demonstrated that in *B. mori* larval midgut albumin was readily absorbed by transcytosis (Casartelli *et al*., 2005). Now we have demonstrated that this protein is largely directed also to the degradative pathway since a strong colocalisation with lysosomes was established.

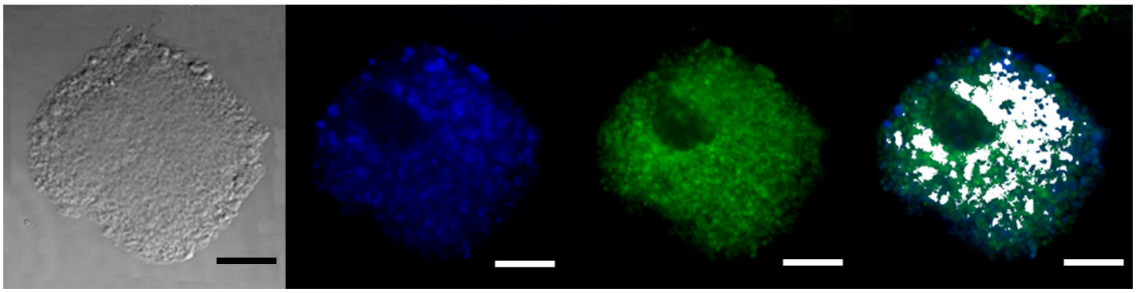

Figure 3.18 Brightfield and confocal laser-scanning micrographs (optical sections) of a typical columnar cell incubated for 1h at 25°C in the presence of 1.4 μM FITC-albumin and then labeled with LysoSensor Blue. FITC-albumin (green) and LysoSensor Blue (blue) partially colocalised (white) in the cytoplasm of the cell. The white pixels are colocalisation pixels with fluorescence intensity values ≥ 80 a.u. for both the blue and the green channel. Bars: 10 μm.

B- Enhancers of protein permeation across the insect midgut

3.3 Tat-EGFP uptake by columnar cells

In the last decades, a variety of peptides and proteins have been identified as biopesticides. The increasing attention on their potential use was coincident with the study *in vivo* and *in vitro* of their delivery across the insect midgut. The main goal for the oral delivery of biological gene products is the identification of potential enhancers of gut permeability. To this end, we investigated in insect midgut cells the efficacy of selected peptide transduction domains, or Cell-Penetrating Peptides (CPPs), known to cross the cell plasma membrane delivering fused model proteins. One of the best known and more often used CPPs is Tat, a peptide containing the membrane translocation region 47-57 that is part of the transactivator protein of the human immunodeficiency virus type 1 (HIV-1).

Tat 47-57 was fused with EGFP in the laboratory of Prof. Rao (University of Naples "Federico II") and used in the experiments. We selected EGFP as a tool for this study because its fluorescence can be observed only if the protein is correctly folded.

To evaluate if EGFP alone, used as control, and Tat-EGFP were able to penetrate into columnar cells, we performed a time course analysis. Figure 3.19 shows the proteins uptake by columnar cells evaluated by confocal laser microscopy. Cells were incubated at 25°C in the presence of Tat-EGFP (1.5 μM) or EGFP (1.5 μM) for 30 min, 1h, 4h, 8h, 24h. Both Tat-EGFP and EGFP were internalised by columnar cells but the fluorescence intensity was higher in the cells incubated with Tat-EGFP than in those incubated with EGFP for all the time points considered. In the latter, a weak fluorescence was present in the cytoplasm (fig 3.19) that did not increase with time. On the contrary, in cells incubated with Tat-EGFP, the fluorescence intensity progressively increased and it is apparent that the fluorescence of the protein inside the cell was diffused uniformly inside the cytoplasm (fig 3.19).

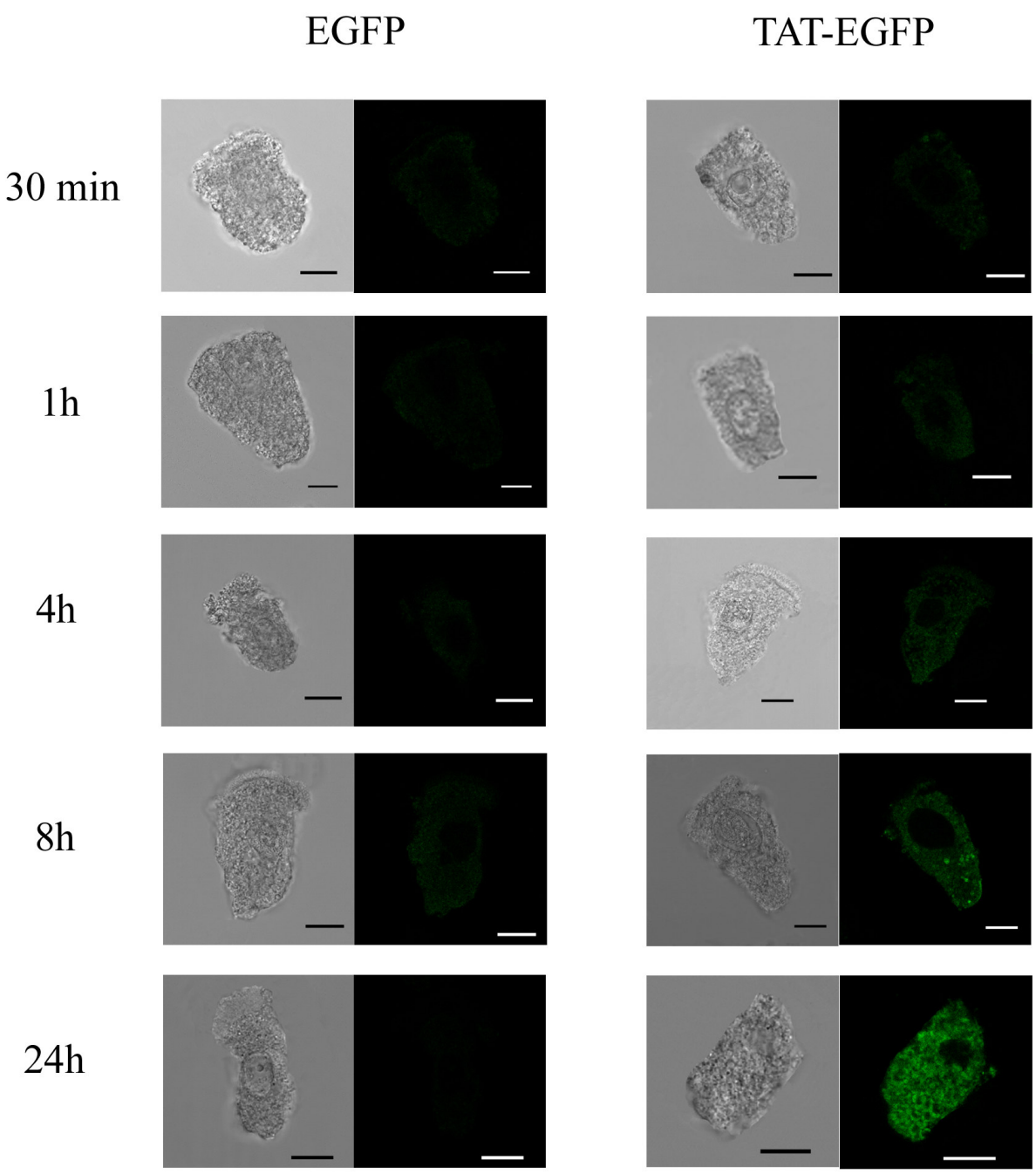

Figure 3.19 Brightfield and confocal laser-scanning micrographs (optical sections) of *Bombyx mori* columnar cells in culture incubated for different time intervals at 25°C in the presence of 1.5 μM Tat-EGFP or 1.5 μM EGFP. For each time, the image best representing the average condition of cells was chosen. Bars: 10 μm.

The fluorescence intensity was quantified in single optical sections of the cells (fig. 3.20). Each point, as described in paragraph 3.2, represents the mean of the fluorescence intensity recorded in single optical sections

acquired in a middle focal plane of the cell in which the nucleus was clearly evident. The figure clearly shows that EGFP uptake was low and did not increase with time, while Tat-EGFP uptake increased progressively between 30 min and 24 h. After 24 h, the fluorescence intensity with Tat-EGFP was 30 fold higher than that with EGFP.

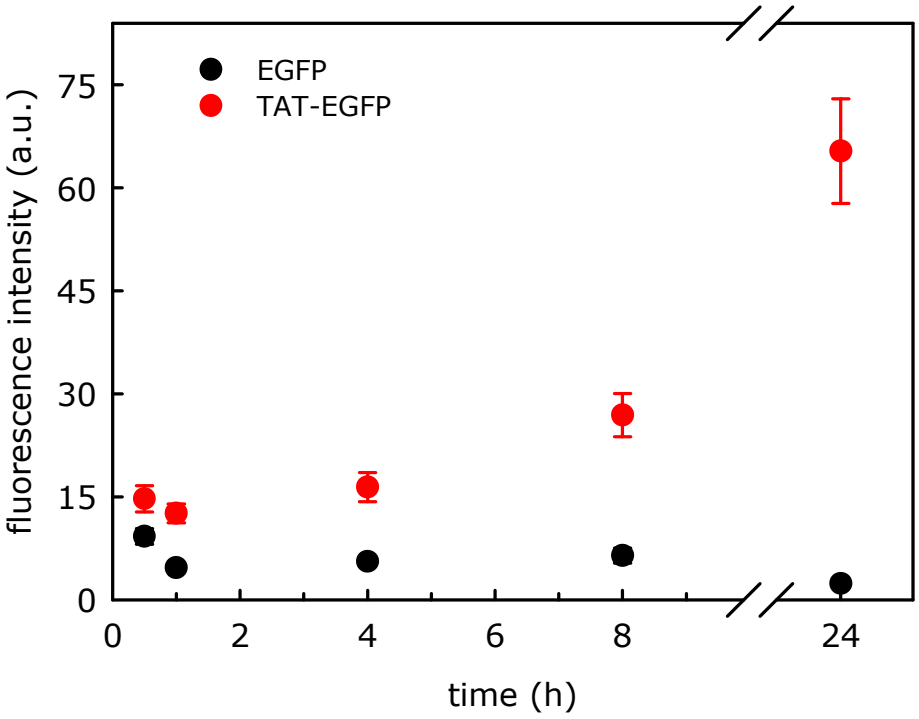

Figure 3.20 Quantification of Tat-EGFP and EGFP internalised with time. Single optical sections in a middle focal plane of the cell, in which the nucleus was clearly evident, were acquired by confocal laser microscope (see legend to fig. 3.5). Values are means ± SE of the fluorescence intensity recorded in at least 10 cells for each incubation time.

To characterise the process involved in Tat-EGFP internalisation, we incubated the cells at 4°C or in the presence of two metabolic inhibitors, sodium azide and dinitrophenol (DNP). In the experiment at low temperature, cells were incubated for 30 minutes at 4°C with the fusion protein and the histogram shows that there was no inhibition of Tat-EGFP internalisation (fig. 3.21).

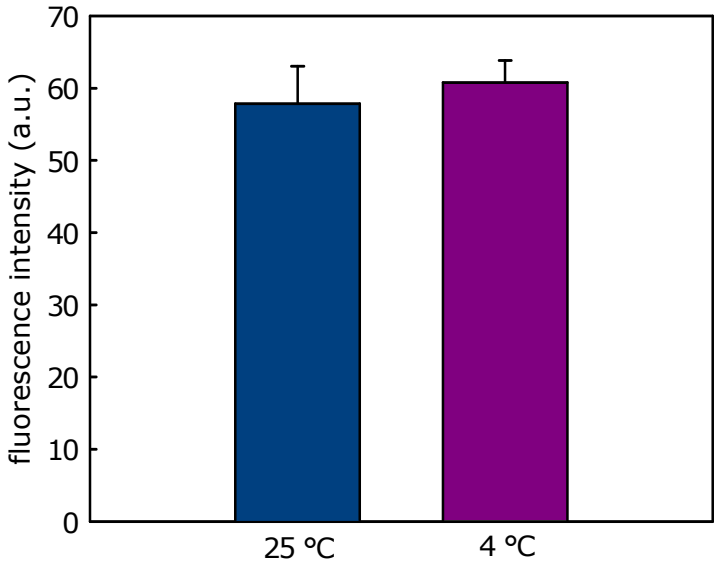

Figure 3.21 Effect of temperature on Tat-EGFP internalisation by columnar cells. *B. mori* columnar cells were incubated for 30 min at 25°C or at 4°C in the presence of 1.5 μM Tat-EGFP. For each experimental condition, the mean ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells is reported in the histogram.

The same result was obtained when the cells were incubated in the presence of sodium azide (10 mM) (fig. 3.22A) or DNP (100 μM) (fig. 3.22B). These data suggest that the internalisation process of Tat-EGFP is energy-independent.

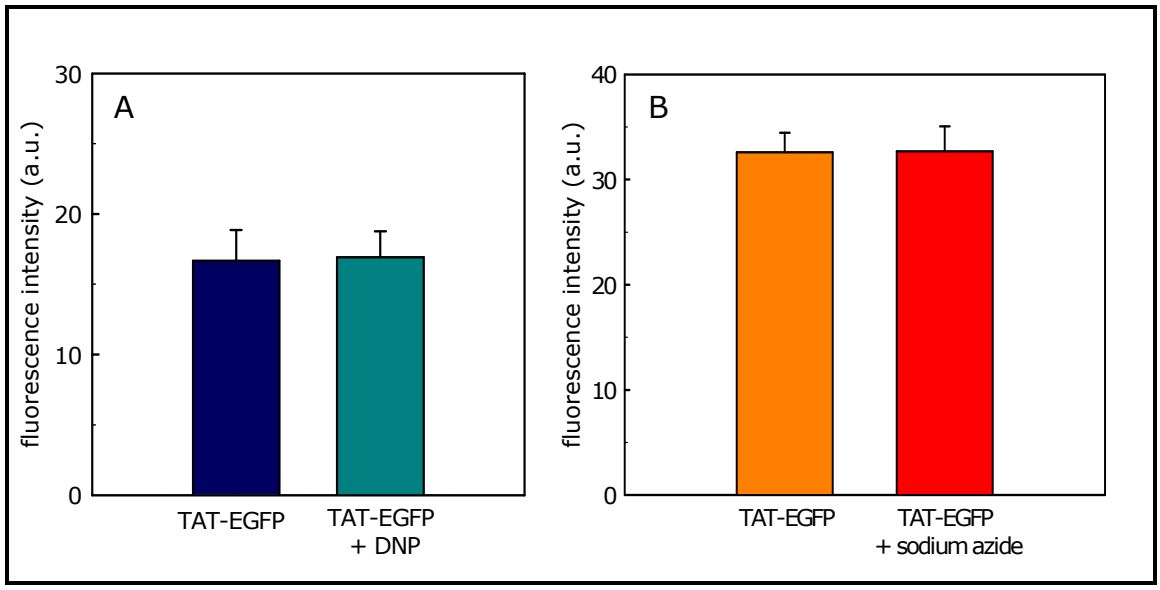

Figure 3.22 Effect of metabolic inhibitors on Tat-EGFP internalisation by columnar cells. Cells were pretreated at 25°C for 30 min with 2,4-dinitrophenol (DNP) (100 μM) or sodium azide (10 mM) and then incubated with 1.5 μM Tat-EGFP for 30 min. In the histograms is reported the mean ± SE of the fluorescence intensity recorded in single optical sections (see legend to fig. 3.5) of at least 10 cells.

Columnar cells in culture grow and differentiate in suspension and expose both the apical and the basolateral membrane to the incubation solution. To investigate specifically the role of the apical membrane in Tat-EGFP internalisation, *B. mori* midguts were isolated and mounted on a perfusion apparatus where their shape and orientation were maintained as *in situ*. Tat-EGFP (1.5 μM) or EGFP (1.5 μM) were added to the physiological solution present in the luminal compartment. After 3h of incubation, the midgut tissues were examined at the confocal microscope. The images in figure 3.23 revealed that the CPP Tat enhanced EGFP internalisation across the apical membrane of midgut cells.

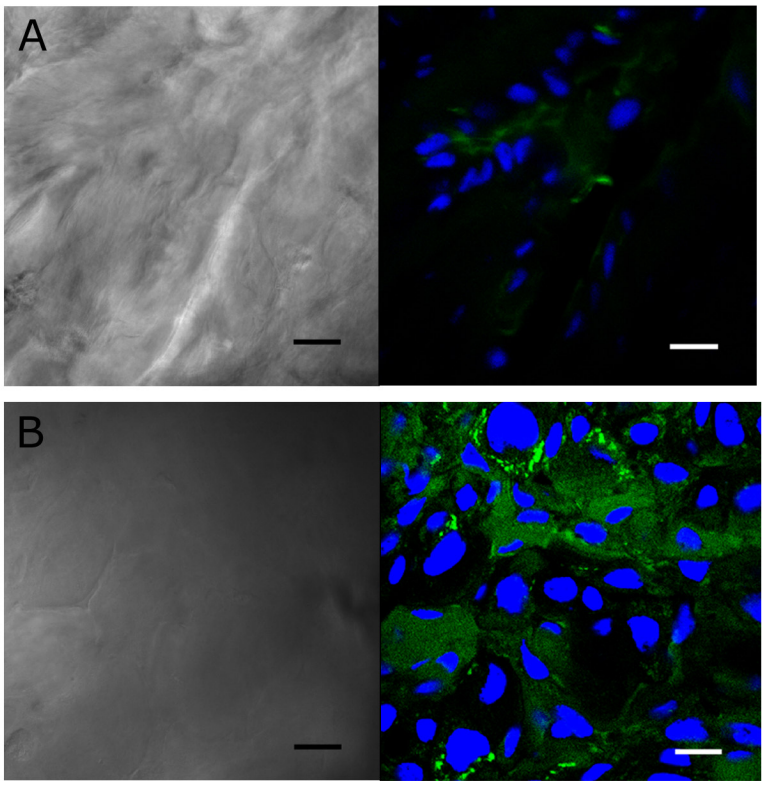

Figure 3.23 Brightfield (left) and confocal laser-scanning micrographs (single optical section) (right) of whole-mount midgut after 3h of incubation in the presence of 1.5 μM EGFP (A) and 1.5 μM Tat-EGFP (B) in the luminal compartment. Nuclei were stained with DAPI (blue). Bars: 20 μm.

Our results indicate that the CPP Tat enhanced the internalisation of EGFP both in columnar cell differentiated in culture and in the midgut tissue, and that the internalisation process is energy-independent, because it is not inhibited by either low temperature or metabolic inhibitors. The last result agrees with a number of studies which indicated an energy-independent process for the uptake of some CPPs (Derossi *et al*., 1996; Vives *et al*., 1997; Futaki *et al*., 2001; Suzuki *et al*., 2002). It has been recently demonstrated that an even mild fixation of cells with formaldehyde or methanol leads to an artifactual redistribution of the membrane-bound fluorescently-labeled peptides into the cytoplasm and the nucleus (Richard *et al*., 2003; Patel *et al*., 2007), although internalisation of penetratin, Tat and transportan was detected in both fixed and live cells from several cell lines (Zorko and Langel, 2005).

In conclusion, we should exclude that endocytosis is the mechanism of internalisation of Tat-EGFP in our cell system, since the protein uptake is not inhibited by metabolic inhibitors and we should, conversely, consider the involvement of transduction, which is an energy- and receptor-independent process (Thoren *et al*., 2003). However, it is evident from the literature that the mechanism of Tat entry is not yet thoroughly understood, since many studies performed *in vitro* to elucidate the process gave contrasting results. In fact, experimental evidences indicate that different mechanisms occur for Tat internalisation in different cell systems. The CPP can be transduced into the cytoplasm (Zaro and Shen, 2003), can enter cells by macropinocytosis (Kaplan *et al*., 2005), by clathrin-mediated endocytosis (Richard *et al*., 2005) or by caveolae-mediated endocytosis (Ferrari *et al*., 2003). These varied results suggest that the mechanism of Tat internalisation, as for other CPPs, depends on the type of cargo, the type of linkage between the cargo and the CPP and the cell system used (Patel *et al*., 2007).

C- Midgut permeation by a densovirus

3.4 *Jc*DNV penetration in midgut cells in culture

Densoviruses (DNVs) are now reconsidered as potential bioinsecticides because they are lethal for several insects at larval stages, including agronomical pests (Lepidoptera) and insects vector diseases (Diptera) (Belloncik, 1990). We studied the interaction of *Junonia coenia* Densovirus (*Jc*DNV) with its host *Spodoptera frugiperda*. After oral ingestion, the virus crosses the intestinal barrier to reach the host haemocoel and replicate in the target tissues, tracheae and visceral muscles (M. Ogliastro, personal communication). We examined if and how the virus can enter midgut epithelial cells in culture, obtained from *S. frugiperda* stem cells. To clarify if the virus penetrated midgut cells, we incubated for 30 minutes the cell suspension with Cy3-*Jc*DNV. Stem and goblet cells were not crossed by the virus, as demonstrated by the

absence of virus particles both on the cells surface and in the cytoplasm (fig. 3.24A and B).

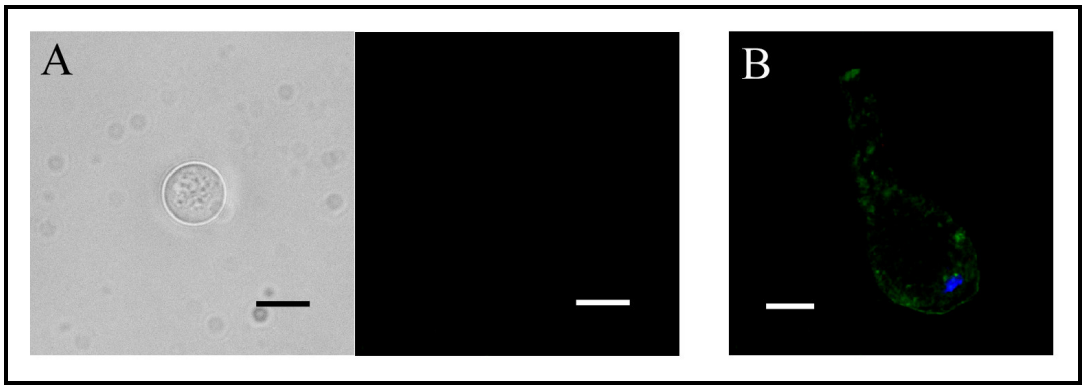

Figure 3.24 A: Brightfield and fluorescent micrographs of a stem cell after incubation with 20 μg/ml Cy3-*Jc*DNV for 30 min. **B:** Fluorescent micrograph of a goblet cell after incubation with the labeled virus (20 μg/ml) for 30 min. Actin filaments were stained with FITC-phalloidin and the nucleus with DAPI. Bars: 10 μm.

In order to understand if the virus penetrated columnar cells, we incubated midgut cells in culture with Cy3-*Jc*DNV for 10 min and observed the cells at different stages of development.

As reported in figure 3.25 A, in columnar cells in an initial stage of differentiation, in which the microvilli are small protrusions, no binding of the virus to the plasma membrane was detectable. When the differentiating cells present well organised long tenuous membrane projections, uniformly distributed all over the surface, some virus particles were visible on the cell surface (fig. 3.25B and C). A strong signal was detectable only when columnar cells had developed the basolateral membrane, reaching the polarised form; the signal was present both at the apical and at the basolateral domain (fig. 3.25 D and E).

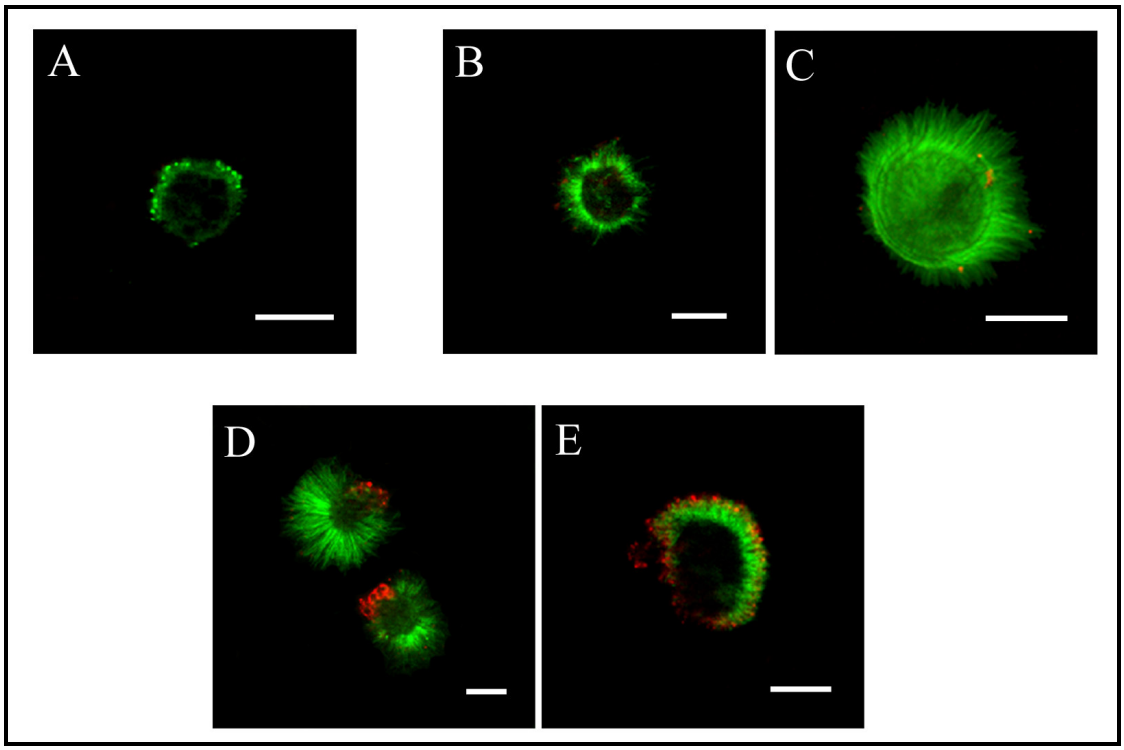

Figure 3.25 Fluorescent micrographs of columnar cells at different stages of development after incubation for 10 min with 20 μg/ml Cy3-*Jc*DNV. **A:** a columnar cell in an initial stage of differentiation; **B and C:** differentiating columnar cells with well organised microvilli uniformly distributed all over the surface; **D and E** columnar cells developing the basolateral membrane. Bars: 10 μm.

The fully developed (mature) columnar cells showed a strong signal both at the apical and at the basolateral membrane (fig. 3.26). With incubations of 10 min, the virus was never observed inside the cytoplasm (fig. 3.26A, B and C), whereas after 30 min of incubation the virus particles were clearly present as spots within the cytoplasm, although never in the nucleus (fig. 3.26D).

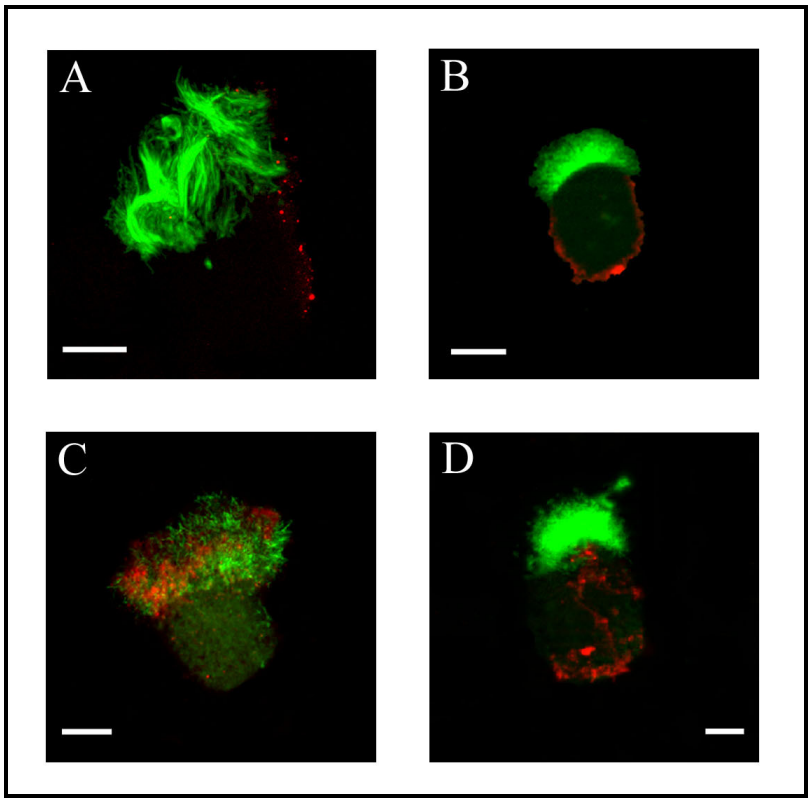

Figure 3.26 Fluorescent micrographs of mature columnar cells differentiated *in vitro*. **A, B and C:** cells incubated with 20 μg/ml Cy3-*Jc*DNV for 10 min; **D:** cells incubated with 20 μg/ml Cy3-*Jc*DNV for 30 min. Bars: 10 μm

The timing of *Jc*DNV penetration in columnar cells differentiated *in vitro* was comparable to that observed in columnar cells obtained by enzymatic disaggregation of the midgut tissue. As shown in figure 3.27, the binding of the virus to the apical or basolateral membrane was visible after 10 min of incubation (fig. 3.27 A, B and C) and virus particles were apparent as spots in the cytoplasm but not in the nucleus after 30 min (fig. 3.27 D and E).

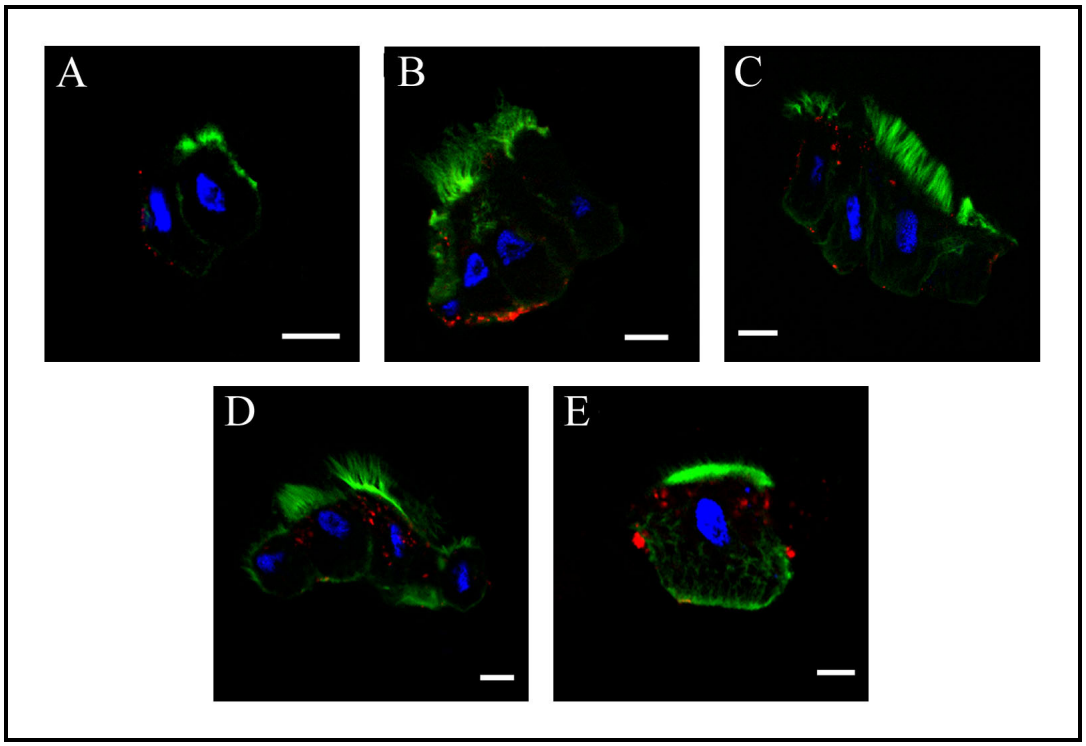

Figure 3.27 Fluorescent micrographs of columnar cells obtained by enzymatic disaggregation of the midgut tissue. **A, B and C** cells incubated with 20 μg/ml Cy3-*Jc*DNV for 10 min; **D and E:** cells incubated with 20 μg/ml Cy3-*Jc*DNV for 30 min. Bars: 10 μm.

The mechanism involved in *Jc*DNV penetration was markedly temperature-dependent. Figure 3.28 shows that inhibition of virus internalisation occurred both in differentiating and in mature columnar cells when the incubation temperature was lowered from 25°C (fig. 3.28 A and C) to 4°C (fig. 3.28 B and D).

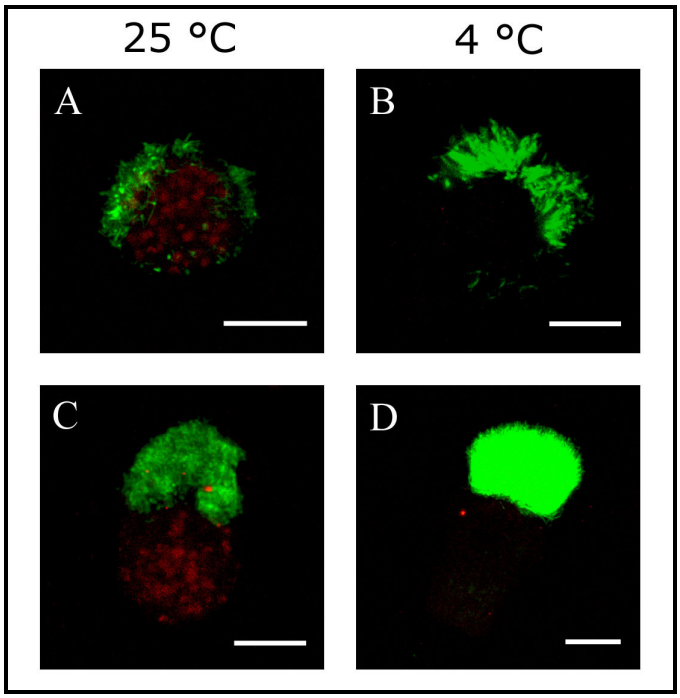

Figure 3.28 Fluorescent micrographs of columnar cells in an advanced stage of differentiation (**A and B**) and fully differentiated (**C and D**) incubated with 20 µg/ml Cy3-*Jc*DNV for 60 min. **A and C:** incubation performed at 25°C; **B and D:** incubation performed at 4°C. Bars: 10 µm.

Our data indicate that the virus is unable to penetrate stem cells and differentiated goblet cells, and that it is internalised exclusively by columnar cells. In these cells, the virus does not penetrate the nucleus, suggesting that they are not the target cells for the replication, and that they only act as a permeation route for haemocoelic infection. Virus internalisation was energy dependent, since it was strongly reduced by low temperature, and the internalised virus particles appeared to be distributed in intracellular compartments since they were apparent as spots inside the cytoplasm.

Studies performed on different densoviruses gave important indications about their structure (Bruemmer *et al*., 2005), but the infection mechanisms have been reported only for the human parvoviruses B19 (Brown *et al*., 1993) and the canine and feline parvosiruses (CPV and FPV) (Palermo *et al*., 2003). Our study is the first

contribution on the mechanism of penetration of *Jc*DNV in *S. frugiperda* larval midgut.

4. Perspectives

During the past decades, numerous new products like peptides and proteins derived from different sources as well as insect pathogenic viruses have emerged from the effort to develop alternative biopesticidal technologies (Whetstone and Hammock, 2007). In most cases, the proteins identified as biopesticides have haemocoelic targets and must pass the gut barrier undegraded to exert their activity. It is therefore of the utmost importance for their successful delivery, to develop basic information on the molecular mechanisms mediating their absorption by the insect midgut, which is a physiological process still poorly understood. The research work presented in this thesis fitted in this scenario even if it represented only the starting point for the full knowledge of these complex processes.

We established a culture of single fully differentiated cells from *Bombyx mori* or *Spodoptera frugiperda* larval midgut, and we utilised this preparation to assess the specific mechanisms implicated in albumin endocytosis and the sequence of intracellular events involved in protein transcytosis; to investigate the ability of the CPP Tat to enhance the delivery of a model protein; to clarify the mechanism of internalisation utilised by a densovirus (*Jc*DNV) to get into the host cells.

In the next future, we intend to investigate further the mechanism of albumin internalisation by the study of the possible expression of a cubilin homologue, a second multiligand receptor for albumin in mammals (Gekle, 2005), that has no transmembrane domain and little structural homology with other known endocytic receptors (Moestrup and Verroust, 2001; Christensen and Birn, 2002). The presence of cubilin in insect cells would further reinforce the emerging notion that absorption mechanisms of intestinal epithelia in insects and mammals share important functional similarities at the cellular and molecular level, as it has been recently demonstrated also for sugar absorption in a parasitic wasp (Caccia *et al*., 2007). In mammals, both megalin and cubilin are expressed in the intestinal brush border, where they are involved in the gastrointestinal uptake and transport of vitamin B12 and folate (Birn *et al*., 1997, 2005;

Yammani *et al*., 2001): the physiological role of megalin and, if present, of cubilin in the insect midgut will also be addressed in our future studies.

CPPs, with their ability to translocate the plasma membrane of eukaryotic cells by a possibly receptor- and endocytosis-independent mechanism, have opened a new avenue in biomedical research (Fischer *et al*., 2001) and could be efficiently used as enhancers of biopesticides delivery in insects, according to the experimental evidences presented in this thesis. In the next future, we intend to clarify the mechanism of internalisation of the fusion protein Tat-EGFP, and to create a fusion protein between the CPP Tat and an insecticidal protein, to test its toxicity *in vivo* on different insect pest.

5. Bibliography

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