A megalin-like receptor is involved in protein endocytosis in the midgut of an insect (*Bombyx mori*, Lepidoptera)

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Albumin endocytosis by insect midgut cells in culture

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ABSTRACT

The mechanism responsible for FITC-albumin (fluorescein isothiocyanate-albumin) internalisation by columnar cells in culture obtained from the midgut of *Bombyx mori* larvae was examined by confocal laser scanning microscopy. Protein uptake changed over time and it appeared to be energy-dependent, as it was strongly reduced by both low temperatures and metabolic inhibitors. Labelled albumin uptake as a function of increasing protein concentration showed a saturation kinetics with a $K_m$ value of $2.0 \pm 0.6 \, \mu M$. These data are compatible with the occurrence of receptor-mediated endocytosis. RT-PCR analysis and colocalisation experiments with an anti-megalin primary antibody indicated that the receptor involved was a putative homologue of megalin, the multiligand endocytic receptor belonging to the low-density lipoprotein (LDL)-receptor family, responsible for the uptake of various molecules, albumin included, in many epithelial cells of mammals. This insect receptor, like the mammalian counterpart, required $Ca^{2+}$ for albumin internalization and was inhibited by gentamicin. FITC-albumin internalisation was clathrin-mediated, since two inhibitors of this process caused a significant reduction of the uptake and clathrin and albumin colocalised in the intermicrovillar areas of the apical plasma membrane. The integrity of actin and microtubules organisation was essential for the correct functioning of the endocytic machinery.

**Key words:** lepidopteran larval midgut, columnar cells in culture, albumin endocytosis, clathrin-mediated endocytosis, megalin.
INTRODUCTION

The active absorption of proteins across the intestinal wall in mammals has been extensively studied (53). Transcytosis, the route followed by proteins to cross epithelial barriers, implies their internalisation by endocytosis at one pole of the cell plasma membrane and, after vesicle-mediated internal transport, their release by exocytosis in the extracellular milieu of the opposite plasma membrane domain. The intracellular pathway followed by the vesicles containing the protein depends on the plasma membrane domain where endocytosis had occurred and implies their fusion with specific cell compartments (2).

Protein absorption by the insect midgut currently attracts increasing research efforts, in part fostered by the need of developing efficient strategies for oral delivery of bioinsecticides targeting haemocoelic receptors. To date, even though gut absorption of undegraded proteins has been unequivocally demonstrated in a number of insect species in vivo (1, 3, 18, 21, 22, 23, 29, 34, 37, 39, 46), it is still unclear which cellular pathway is involved in this process. We recently demonstrated in vitro that the isolated midgut of Bombyx mori larvae is able to perform transepithelial translocation of FITC-albumin (9) and horseradish peroxidase (10) by transcytosis. In vitro approaches provide a powerful tool to study in a simplified experimental context the pathways involved in the absorption process, 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 cellular level. The internalisation of proteins and the sequence of the intracellular steps undertaken by the loaded vesicles can be better analysed in single columnar cells of the larval midgut in culture. Mature columnar and goblet cells, the two main cell types present in the Lepidoptera midgut epithelium, can be obtained from the proliferation and differentiation of stem cells detached from the insect midgut epithelium and can survive in culture.
up to 6 months (6, 30, 40, 47, 48). Thus, to define at cellular level the steps involved in the transcytosis of macromolecules in the insect midgut, we recently established a culture of midgut cells from *B. mori* larvae (12), which is here used to investigate albumin endocytosis.

The uptake by endocytosis of macromolecules starts with the formation of vesicles derived from the invagination and pinching-off of plasma membrane portions. This phenomenon is a multifaceted mixture of multiple mechanisms, which fall into two broad categories (16): phagocytosis (i.e. the uptake of large particles) and pinocytosis (i.e the uptake of fluid and solutes). While phagocytosis occurs in specialised cells, pinocytosis is common to all cells and occurs by four different modality: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin-and caveolae-independent endocytosis (16). The best studied cell uptake mechanism is clathrin-mediated endocytosis, which ensures an efficient internalisation of diluted solutes by high-affinity receptors concentrated in pits coated on their cytosolic side by clathrin and other associated proteins. These coated pits invaginate and pinch off to form the endocytic vesicles, that are delivered to early endosomes, from which ligands and receptors will be addressed to their proper destination. A large number of regulatory proteins are involved in this process (16) and the cytoskeleton is implicated both in the formation of the endocytic vesicles and in their release from the plasma membrane (44, 51).

In the framework of a larger research effort, aiming at characterising the transepithelial transport of proteins in the insect midgut, here we report experimental data on the endocytic mechanism responsible for albumin uptake *in vitro* by cultured columnar midgut cells from *B. mori* larvae. FITC-albumin was used as a model protein and we examined in detail the general features of its uptake by columnar cells, the mechanism responsible for its internalisation, the possible receptor(s) involved and the role of the cytoskeleton.

**MATERIALS AND METHODS**
Isolated midgut cells in culture

A detailed description of the protocol followed to obtain isolated midgut cells in culture from *B. mori* larvae is reported in Cermenati et al. (12), where all the modifications from the original procedure (48) are specified. Briefly, larvae of *B. mori* just before the 4th molt were anaesthetised with CO₂ and surface-sterilised. 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. Then the midgut was deprived of the peritrophic membrane along with the enclosed intestinal contents. The central part of the larva was transferred to a Petri dish containing a sterile Insect Physiological Solution (IPS) (47 mM KCl, 20.5 mM MgCl₂, 20 mM MgSO₄, 4.3 mM K₂HPO₄, 1.1 mM KH₂PO₄, 1 mM CaCl₂, 88 mM 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 cuticle of the ventral side was longitudinally cut and the midgut was isolated. Midguts dissected from 8-10 animals were cut along the longitudinal axis and rinsed twice (10 min each) in sterile modified IPS, then twice (10 min each) in the same solution without sodium hypochlorite. Midguts were pooled into a strainer (100 µm pore size), placed in a Petri dish containing few ml of the latter solution and left under mild agitation for 1 h. In these conditions, the loosely attached stem cells migrated away from the tissue. The tissue in the sieve was discarded and the free cells in the filtrate, mostly stem cells (12), were collected and pelleted by gentle centrifugation at 400 g for 5 min. Cells were then resuspended in a growth medium (12) supplemented with 6x10⁻⁸ M 20-hydroxyecdisone (Sigma) and 100 ng/ml α-arylphorin (purified according to Blackburn et al. (6) in the Insect Bio-Control Laboratory, USDA, Beltsville, MD, USA) kindly donated by Prof R.S. Hakim, Howard University, Washington DC, USA. All the solutions were sterilised by filtration (Nalgene, 0.2 µm pore size) prior to use. Three ml of the cell suspension were distributed in the wells (35 mm in diameter) of six well-plates. Cells, that 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. The cells to be used for the experiments were
harvested from 3-weeks-old cultures, in which differentiated cells considerably exceeded stem cells: of total viable cells, as determined in three different experiments, 8.9 ± 0.6 % were stem cells, 34.6 ± 3 % were differentiating cells, 27.6 ± 3.4 % were goblet cells and 28.9 ± 2.7 % were columnar cells (12). These last ones were characterised in their ability to internalise albumin since in vivo the completely differentiated columnar cells are responsible for digestion and absorption of molecules. For the confocal analysis (see Fluorescence acquisition and analysis), only those cells that could be considered fully mature columnar cells on the basis of their morphological features (12) were chosen.

Internalisation of FITC-albumin by columnar cells

The cultured cells were pelleted 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 hypochloride, 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: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄), the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem), covered with a cover-slip and then examined with a confocal microscope as described in Fluorescence acquisition and analysis.

When drugs were used, the cells were preincubated for 30 min in the absence (control) or in the presence of the drug. 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 experiments 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.

In order to evaluate the effect of calcium 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 Ca²⁺- chelator EDTA (20 mM). For these experiments, the incubation medium had the following composition: 47 mM KCl, 4.3 mM K₂HPO₄, 1.1 mM KH₂PO₄, 179 mM sucrose, pH 7, 20 mM EDTA. At the end of the incubation, cells were rinsed 3 times in the same solution and fixed as reported above. Control cells were incubated in the modified IPS, devoid of sodium hypochloride, rinsed and fixed as reported above.

Colocalisation experiments

Midgut cells were pelletted and resuspended in IPS as described in 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-X100 in PBS and washed 3 times in PBS. Cells were then incubated for 15 min in PBS containing 1 % BSA, and for 1 h 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. The cells were then washed 3 times in PBS containing 1 % BSA and incubated for 1 h 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, 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). Controls were carried out in the same manner, except for omitting the incubation with the primary antibody (data not shown).

**Immunodetection of microtubules organisation**

Cultured midgut cells, pelleted and resuspended in IPS as described in Internalisation of FITC-albumin by columnar cells, were incubated with or without 27 µM nocodazole. 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 1 h 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 1 h 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).

**Detection of actin filaments**

Cultured midgut cells were pelleted, resuspended in IPS as described above and incubated in the absence or in the presence of 20 µM cytochalasin D. 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-X100 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*).

**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 and Alexa Fluor 488 were excited with the 488 nm laser line and the emitted fluorescence was collected between 500 and 560 nm; Alexa Fluor 594 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. To compare different experimental conditions (i.e. cells incubated with FITC-albumin for different time intervals, 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 internalization 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. In all cases, *P* value ranged between *P* < 0.001 and *P* < 0.02.

**Western blot analysis**
B. mori midguts isolated from fifth instar larvae were homogenised using a glass/Teflon 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 (7) 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 (38). 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 1 h 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.

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) (see Results, Identification of the receptor involved in FITC-albumin internalisation). The condition 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 internal control; the primers, designed according to the sequence available in the database (GenBank accession number U49854), were sense, 5’-ATGTGCAGCGAGAAGTGC-3’; and antisense, 5’-CTCAGCCGGCGCTTGG-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.

**RESULTS**

*Albumin uptake by columnar cells*

FITC-albumin uptake was measured at a fixed concentration of 1.4 µM. Figure 1 A, shows the time course of the protein uptake by columnar cells evaluated by confocal laser microscopy. After 5 minutes of incubation, a weak fluorescence was present inside the cell, which progressively increased up to 30 minutes and showed no detectable changes for the remaining experimental time points considered. This was further corroborated by the time course analysis of fluorescence intensity in single optical sections (Fig. 1 B), which clearly indicated that protein uptake reached a steady state in 30 minutes.

FITC-albumin transport was then characterised in cells incubated for 20 minutes. Albumin uptake was significantly inhibited when the incubation temperature was lowered from 25 °C to 4 °C (Fig. 2 A and B, respectively). From the fluorescence intensity recorded in single optical sections, a 60 % inhibition of the uptake at low temperature was calculated (Fig. 2 C). Moreover, in cells pretreated for 30 min with the two metabolic inhibitors 2,4-dinitrophenol and sodium azide, a significant reduction of FITC-albumin transport was measured, with a 50 % and 80 % decrease of the measured fluorescence intensity, respectively (Fig. 3).
Figure 4 shows FITC-albumin uptake as a function of increasing extracellular protein concentration. The experimental values fitted a Michaelis-Menten equation, with a $K_m$ of $2.0 \pm 0.6 \mu M$.

These data confirm that albumin uptake by columnar cells is mediated by an active process and indicate that a receptor is involved in the internalisation process.

The endocytic mechanism involved in albumin uptake

We examined if clathrin, a protein involved in coated pits formation, was expressed in *B. mori* midgut. Immunoblotting experiments, performed with an anti-clathrin heavy chain primary antibody on midgut tissue homogenates, revealed a band of approximately 180 kDa, corresponding to the molecular weight of clathrin heavy chain (Fig. 5 A).

We verified the actual expression of clathrin also in columnar cells cultured *in vitro* by staining the cells, previously incubated for 20 min with FITC-albumin, with the antibody used in the immunoblotting experiment. As shown in Figure 5 B, clathrin (red) was effectively 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. Therefore, we tested the effect on albumin uptake of two inhibitors of clathrin-mediated endocytosis, chlorpromazine and phenylarsine oxide. Pretreatment of the cells with the two drugs significantly reduced FITC-albumin transport (Fig. 6), with a 75 % and 50 % inhibition of the uptake, respectively.

These data support a clathrin-mediated mechanism for albumin internalisation.

Identification of the receptor involved in FITC-albumin internalisation

Various mammalian epithelial tissues express the multiligand endocytic receptor megalin, a member of the low-density lipoprotein (LDL) receptor family that recognises molecules of different structure and function, albumin included (14, 43). A gene has been recently identified as a megalin
homologue in *Drosophila melanogaster* (Flybase Gene ID CG12139, http://www.ensembl.org). Thus, we investigated if a putative megalin homologue was expressed also in *B. mori* larval midgut. By BLAST analysis, we identified in a 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, which were designed across sequence regions highly conserved. PCR products of the expected size (208 bp) were observed in samples of the midgut after reverse transcription (Fig. 7). Positive (actin) and negative (no RT samples of RNA) controls gave the expected amplimer or no amplification products, respectively.

To prove that this receptor was involved in albumin uptake, we incubated 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. 8). Since binding of ligands to megalin is calcium dependent (13) and megalin can bind and internalise several polybasic drugs, including gentamicin, in renal proximal tubule (42), we measured the internalisation of FITC-albumin in the presence of the Ca$^{2+}$-chelator EDTA or of the polybasic drug gentamicin. In these conditions we observed a 62 % and 40 % inhibition of the protein uptake, respectively (Fig. 9A and B). These experiments, together with the result obtained with RT-PCR, strongly support the conclusion that the receptor involved in albumin internalisation is a putative megalin homologue.

*Role of the cytoskeleton in albumin endocytosis*

The cytoskeleton is involved in a wide variety of cellular activities including endocytosis and vesicles transport inside the cytoplasm. To assess if albumin endocytosis was regulated by microtubules and/or by actin filaments, we preincubated columnar cells with either nocodazole or cytochalasin D and then measured FITC-albumin uptake. As shown in Figure 10, albumin uptake
was significantly reduced by both drugs, with a 70% and 40% reduction of the measured fluorescence intensity with nocodazole and cytochalasin D, respectively (Fig. 10 D).

To prove that the reduction of the uptake with the two drugs was due to their specific action on the cell cytoskeleton, columnar cells were incubated in the presence or in the absence (control) of nocodazole or cytochalasin D and then stained with an anti-α-tubulin antibody to disclose microtubules or with TRITC-phalloidin to detect microfilaments. Figure 11 A shows the organisation of microtubules in a control columnar cell and Figure 11 B its dramatic disorganisation, especially at the apical pole, in nocodazole treated cells. Therefore, the effect of the drug on albumin endocytosis was effectively associated with the disruption of microtubules organisation. Figure 11 C reports a typical columnar cell showing the arrangement of actin filaments: phalloidin distinctly stained microfilaments within the brush border microvilli. Treatments with cytochalasin D had no visible effects on actin scaffolding (Fig. 11 D), in spite of the fact that this drug clearly altered albumin endocytosis (Figs. 10 C and D).

**DISCUSSION**

In this study we examined *in vitro* the mechanism involved in FITC-albumin uptake in isolated columnar cells, obtained from the midgut of *B. mori* larvae. These cells in culture proved to be a powerful tool to disclose the mechanism responsible for the internalisation of FITC-albumin, a model protein transported by transcytosis in the larval midgut of *B. mori* (9).

Albumin uptake linearly increased over time and reached a steady state after 30 min (Fig. 1), as a result of the balance between protein internalisation and its subsequent fate, which can include, in addition to the transepithelial route (9), the recycling to the plasma membrane domain where the vesicle had originated, and/or the targeted delivery to lysosomes for intracellular digestion (2, 20). The punctuate distribution of FITC-albumin inside the cytoplasm (Fig. 1 A) is consistent with a vesicular compartmentalisation, which is expected for a protein that follows these intracellular pathways.
Albumin uptake showed a marked temperature-dependence (Fig. 2) and was inhibited by the two metabolic inhibitors DNP and sodium azide (Fig. 3). This indicates that the protein uptake is, as expected, energy-dependent. Albumin internalisation was a saturable process (Fig. 4) with a $K_m$ value of $2.0 \pm 0.6 \mu M$, suggesting 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 (28), the molecule, like the classical markers inulin or dextran (15, 45, 52), 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. Thus, molecule uptake is linearly correlated with its extracellular concentrations. Conversely, when a receptor is involved in the process, the ligand concentration in the endocytic invagination exceeds several folds that of the extracellular fluid and the uptake exhibits a saturation kinetics (24). Receptor-mediated endocytosis is clearly a more efficient mechanism of transport. The $K_m$ value for albumin, calculated in the kinetics experiments performed with B. mori columnar cells in culture, is in the same range of the apparent affinity constants for albumin endocytosis in mammalian absorptive cells (24, 25, 27).

The expression of clathrin in B. mori midgut epithelium (Fig. 5 A) as well as in columnar cells cultured in vitro (Fig. 5 B), and the colocalisation of FITC-albumin with clathrin in the intermicrovillar areas of the apical plasma membrane of these cells (Fig. 5 B), strongly indicate that clathrin is involved in albumin internalisation. This conclusion is reinforced by the significant inhibition of albumin uptake observed in columnar cells preincubated with two inhibitors of clathrin-mediated endocytosis (Fig. 6). Chlorpromazine prevents assembly of the coated pit at the cell surface (54), while phenylarsine oxide presumably blocks endocytosis by cross-linking the clathrin coat (33).

No information is currently available regarding the molecular mechanisms of protein endocytosis in the insect midgut. In mammalian absorptive epithelia megalin, a multiligand receptor, plays a fundamental role in clathrin-mediated endocytosis of different ligand. Megalin is a 600 kDa transmembrane protein belonging to the LDL receptor family, acting in mammalian
polarised epithelia as a receptor for hormones, vitamin binding proteins, enzymes, enzyme inhibitors and proteins like albumin and lactoferrin (14, 43). It is abundant in renal proximal tubule, where it is responsible for the tubular reabsorption of filtered proteins (28). In the same epithelium, it acts also as a membrane anchor for cubilin, a second multiligand receptor for albumin (28), which has no transmembrane domain and little structural homology with other known endocytic receptors (14, 43). 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 (4, 5, 57).

Since our data show that in B. mori columnar cells a receptor is involved in albumin internalisation via clathrin-mediated endocytosis, we tested the suggestive hypothesis that a megalin-like receptor was responsible for albumin recognition in the insect midgut. This was stimulated and made possible by the presence of a megalin homologue in the genome of both Drosophila melanogaster and Bombyx mori. RT-PCR analysis (Fig. 7) as well as colocalisation (Fig. 8) and inhibition (Fig. 9) 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 central nervous system in zebrafish (41) and the megalin homologue LRP-1 (49) is essential for growth and development in the nematode Caenorhabditis elegans (32, 59). These reports, the recent identification of the putative megalin homologue in D. melanogaster and the data here reported 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 megalin-like receptor in the midgut of lepidopteran larvae, especially if we consider that albumin, a protein so readily recognised and internalised, is absent in the silkworms diet. We can speculate that the receptor may be responsible for vitamin B absorption like in mammalian intestine and that it may be involved in the uptake of those proteins present in the diet that escape degradation by digestive enzymes. Actually, in different insect species dietary (37) or exogenous proteins added to the diet can avoid
digestion and enter the haemolymph undegraded (1, 3, 18, 21, 22, 23, 29, 34, 39, 46). The mechanism involved in the absorption of at least two proteins in *B. mori* larvae was trancytosis (9, 10). Therefore, 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.

Microtubules are dynamic protein filaments that provide a mechanical basis for cell polarity as well as 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 (2, 19, 26, 31), although a detailed analysis of their role is still lacking. As discussed in detail in Cermenati et al. (12), in *B. mori* cultured columnar cells (Fig. 11 A) microtubules are arranged in bundles oriented longitudinally from the apical to the basal pole of the cell as in other absorptive epithelia (55), whereas, at odds with the latter, numerous microtubules run parallel to the apical cell surface just below the microvilli, forming a dense network. The cells treated with nocodazole displayed a remarkable disorganisation of microtubules (Fig. 11 B) and a strong inhibition of FITC-albumin internalisation (Figs. 10 B and D), proving that also in the insect midgut these cytoskeletal elements are critical for the endocytic process.

Actin is an 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 (recently reviewed by: Smythe and Ayscough (51); Kaksonen et al.(36)). In mammals different orthologues of yeast proteins that cap, bundle and stabilise actin are present (51) and, like in yeast, membrane invagination and vesicle scission are affected by the depolymerisation of actin filaments (58). The localisation and timing of actin polymerisation in yeast and mammal (35, 36, 50) are strikingly similar and it is apparent that the endocytic internalisation processes in these so different organisms are variations of a same ancestral theme. In
B. mori columnar cells, cytochalasin D induced a significant reduction of FITC-albumin endocytosis (Figs 10 C and D), suggesting that actin structures involved in the formation of the endocytic vesicles in these insect cells follow the same pattern described for yeast and mammalian cells. However, a clear-cut disorganisation of actin filaments was not apparent (Fig. 11 D), so we assume that cytochalasin D concentration was adequate to alter actin filaments involved in the endocytic process but insufficient to cause a significant alteration of actin cytoskeleton in the microvilli and in the submicrovillar area, where microfilaments in insects are present in considerable density (17).

PERSPECTIVES AND SIGNIFICANCE

Gut absorption of intact proteins in insects had been unequivocally demonstrated in vivo (1, 3, 18, 21, 22, 23, 29, 34, 37, 39, 46), but the cellular pathway involved in the process was unknown and only speculations on the mechanism were advanced. We showed recently that albumin, a protein absent from the insect diet and chosen as a model molecule, was readily absorbed by the isolated B. mori larval midgut by transcytosis (9) and now we have characterised, for the first time in an insect, the mechanism of its internalisation by midgut cells. Unexpectedly, the receptor and the endocytic mechanism involved resemble those described in the kidney epithelium for the reabsorption of plasma proteins, albumin included. To understand how conserved is this physiological process further studies are undoubtedly necessary. We intend than to define the possible co-expression of a cubilin homologue, which, if present, would further reinforce the emerging notion that absorption mechanisms of intestinal epithelia in insects and mammals share important functional similarities at cellular and molecular level, as we recently demonstrated also for sugar absorption in a parasitic wasp (8). We want to detect how similar is the specificity of the lepidopteran receptor(s) for the large number of megalin and, possibly, cubilin ligands of the mammalian counterpart(s) (14). Finally, we want to disclose the effective functional role of the
receptor for the insect physiology. We are confident that the future directions of our research will lead to a more complete knowledge of the physiological role of these multiligand endocytic receptors from an evolutionary point of view.

Moreover, our study contributes to a current innovative research effort aiming at defining new strategies for insect control, including agronomical pests and insects vectors for a multitude of human pathogens. Since 1940s, with the introduction of DDT, insect pest control has been performed essentially with chemical insecticides (11). The well documented environmental and health impact of these chemicals, their poor species specificity and the rapid development of insect resistance have stimulated the search for new insecticidal compounds, novel molecular targets and alternative control methods. In the last decades a variety of biocontrol methods employing peptides and proteins derived from microorganisms, animals, like predator or parasitoids artropods, and plants has been examined (recently reviewed by Whetstone and Hammock (56)). In most cases, these proteins have haemocoelic targets and must pass undegraded the gut barrier in order to exert their activity. For their successful delivery, it is essential 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.

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FIGURE LEGENDS

**Figure 1.** A: brightfield (upper row) and confocal laser scanning micrographs (maximum projection) (lower row) of *Bombyx mori* columnar cells cultured *in vitro* and incubated for different time intervals at 25 °C, in presence of 1.4 µM FITC-albumin. For each time, the picture best representing the average condition of cells was chosen. Bars: 10 µm. B: 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 ± s.e. of the fluorescence intensity recorded in at least ten cells for each incubation time.

**Figure 2.** 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 pictures chosen were those best representing the average condition of cells. Bars: 10 µm. For each experimental condition, the mean ± s.e. of the fluorescence intensity recorded in single optical sections (see legend to figure 1) of at least ten cells is reported in C. Student’s *t*-test *P* < 0.01.

**Figure 3.** Effect of metabolic inhibitors on FITC-albumin internalisation by columnar cells. Cells were pretreated at 25 °C for 30 min with 2,4-dinitrophenol (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 ± s.e. of the fluorescence intensity recorded in single optical sections (see legend to figure 1) of at least ten cells. Student’s *t*-test vs control: *P* < 0.01, **P** < 0.001.
**Figure 4.** Kinetics of FITC-albumin uptake with increasing extracellular protein concentrations. Cells were incubated at 25 °C for 20 min with the following FITC-albumin concentrations (in µM): 0.14, 0.4, 0.8, 1.4, 4.0, 8.0, 14.2. Values are means ± s.e. of the fluorescence intensity recorded in single optical sections (see legend to figure 1) of at least ten cells for each concentration.

**Figure 5.** A: western blot analysis with anti-clathrin heavy chain mouse IgG of the homogenate of *B. mori* larval midgut. 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 labelled with 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 colocalization pixels with fluorescence intensity values ≥ 60 a.u. for both the red and the green channel. Bars: 5 µm.

**Figure 6.** Effect of inhibitors of clathrin-mediated endocytosis on FITC-albumin internalisation. Cells were pretreated 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 ± s.e. of the fluorescence intensity recorded in single optical sections (see legend to figure 1) of at least ten cells. Student’s *t*-test vs control * P < 0.02, ** P < 0.001.

**Figure 7.** Transcriptional analysis by RT-PCR of the megalin gene in the midgut cells 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.
Figure 8. 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 labelled 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 colocalization pixels with fluorescence intensity values ≥ 30 a.u. for both the red and the green channel. Bars: 10 µm

Figure 9. Effect of EDTA (A) and gentamicin (B) on 1.4 µM FITC-albumin internalisation. Cells were incubated at 25 °C for 20 min with the labelled protein in the presence of the calcium chelator (20 mM) or the polybasic drug (10 mM). For each experimental condition, values are means ± s.e. of the fluorescence intensity recorded in single optical sections (see legend to figure 1) of at least ten cells. Student’s t-test vs controls * P < 0.001.

Figure 10. 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 pictures chosen were those best representing the average conditions of cells. Bars: 10 µm. The mean ± s.e. of the fluorescence intensity recorded in the three conditions in single optical sections (see legend to figure 1) of at least ten cells are reported in D. Student’s t-test vs control * P < 0.01.

Figure 11. Brightfield and confocal laser scanning micrographs (maximum projection) of the cytoskeletal organisation in columnar cells. A: microtubule distribution visualised with an anti-α-tubulin primary antibody and an Alexa Fluor 488-conjugated secondary antibody in a control cell. B: a columnar cell incubated in the presence of nocodazole (27 µM) for 30 min and then labelled
with the same antibodies used in A. C: organisation of actin filaments in a control cell visualised with TRITC-phalloidin. D: a columnar cell incubated in the presence of cytochalasin D (20 µM) for 30 min and then labelled with TRITC-phalloidin. A typical cell is reported for each experimental condition. Bars: 10 µm.
Figure 2

170x120mm (600 x 600 DPI)
Figure 3

90x99mm (600 x 600 DPI)
Figure 4
Figure 5

170x56mm (600 x 600 DPI)
Figure 6
Figure 7
Figure 8
Figure 9
Figure 11

170x120mm (600 x 600 DPI)