Interactions between a luteovirus and the GroEL chaperonin protein of the symbiotic bacterium *Buchnera aphidicola* of aphids

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Luteoviruses and poleroviruses are important plant viruses transmitted exclusively by aphids in a circulative manner via the aphid haemolymph. A chaperonin protein, GroEL, synthesized in aphids by a symbiotic bacterium, *Buchnera aphidicola*, is hypothesized to bind to virus particles in the haemolymph, thereby promoting transmission. To investigate this hypothesis, the GroEL-binding site for barley yellow dwarf virus (BYDV) was determined *in vitro*, and the abundance of GroEL protein in different aphid tissues was investigated. Virus binding to a peptide library representing the full GroEL molecule revealed a single binding site that coincides with the site that anchors two GroEL rings to form the native GroEL tetradecamer. In the functional form of the GroEL protein, virus binding would compete with the formation of the two GroEL rings. Using a mAb raised against a *Buchnera*-specific GroEL epitope, GroEL was detected in *Buchnera* cells by immunoblotting and immunocytochemistry, but not in the aphid haemolymph, fat body or gut. From the prediction here that GroEL–virus interactions are probably severely limited by competition with other GroEL molecules, and the evidence that GroEL is not available to interact with virus particles *in vivo*, it is concluded that GroEL–virus interactions are unlikely to contribute to virus transmission by aphids.

**INTRODUCTION**

Luteoviruses and poleroviruses (family *Luteoviridae*) are ecologically and economically important viruses of plants, including major crops (D’Arcy & Burnett, 1995). Their prevalence in the field depends exclusively on transmission by aphids feeding on the plants. Luteoviruses and poleroviruses are transmitted in a circulative manner that involves uptake of the virus particles with the plant phloem sap into the aphid gut, transport across the gut into the haemocoel and subsequent transfer to the accessory salivary glands, from which they are expelled into the plant sieve elements with the aphid saliva (Gildow, 1999). Transport of the luteovirus particles across the hindgut epithelial cells into the haemocoel and through the accessory salivary gland epithelial cells occurs by an endocytosis–exocytosis process that is suggested to be receptor-mediated (Gildow, 1993). It has been proposed that, as the virus particles transit the haemolymph from gut to salivary gland, they become bound to a chaperonin protein, GroEL, which protects them from degradation.

GroEL is a very abundant protein produced by *Buchnera aphidicola*, the Gram-negative symbiotic bacterium of aphids (Ishikawa *et al.*, 1985; Baumann *et al.*, 1996; Humphreys & Douglas, 1997). *Buchnera* resides in large, specialized cells, known as bacteriocytes, in the aphid body cavity (Douglas, 1989). It has been argued that GroEL is released in large quantities from the bacteriocytes to the insect haemolymph (van den Heuvel *et al.*, 1994, 1997). GroEL has also been reported to be associated with the brush border membrane of the aphid gut, and it has been identified as a candidate ligand of a garlic lectin that is toxic for aphids (Banerjee *et al.*, 2004). Conceivably, GroEL associated with the gut could also affect luteovirus acquisition.

The GroEL protein of *Buchnera* shares >80% sequence identity with the GroEL of the related bacterium *Escherichia coli* (Hara & Ishikawa, 1990); understanding of the *E. coli* GroEL can, therefore, provide insight into the likely function of the *Buchnera* GroEL. In *E. coli*, GroEL, along with its GroES cofactor, assists the refolding and assembly of newly translated proteins in an ATP-dependent manner (Hartl, 1996). The functional form of GroEL is a tetradecamer made of two rings of seven subunits, each ring forming a cavity that is stacked back to back with the
opposing ring. Each GroEL subunit consists of three structural domains: the equatorial (residues 5–132 and 408–522), apical (residues 190–375) and intermediate (residues 133–189 and 376–407) domains. Unfolded cytoplasmic peptides are recognized by the apical domain of the protein and are encapsulated in one of the cavities, where they are refolded (Roseman et al., 1996). The Buchnera GroEL is functional as a chaperone in vitro and rescues a GroEL-null mutant strain of E. coli (Ohtaka et al., 1992), indicating that it also functions as a chaperone in vivo. The mechanism by which GroEL interacts with the virus particles presumably differs from its chaperone function, because the virus particles are 25–30 nm in diameter (Miller & Rasochová, 1997) and are too large to enter the 4.5 Å diameter GroEL cavity (Braig et al., 1994).

The chief evidence for the role of GroEL in luteovirus transmission is that it binds to the readthrough domain of the polerovirus potato leaffroll virus (PLRV) and the luteovirus barley yellow dwarf virus (BYDV) in vitro (Filichkin et al., 1997; van den Heuvel et al., 1997). The putative PLRV-binding sites are two regions of the GroEL protein (residues 9–19 and 426–456) that lie in close proximity in the three-dimensional structure of the protein (Hogenhout et al., 2000). These in vitro observations, taken together with evidence that haemolymph extracts of the aphid vector of PLRV, Myzus persicae, contain GroEL (van den Heuvel et al., 1994), have led to the suggestion that the Buchnera GroEL protein protects virus in the haemolymph against proteolytic breakdown.

This study focused on the role of Buchnera GroEL in aphid transmission of BYDV. Its specific purpose was to investigate two key predictions of the proposed GroEL–BYDV interaction: (i) that BYDV binds to specific residues of the GroEL molecule, and (ii) that GroEL is released from Buchnera cells into the haemolymph, where it can interact with BYDV. A key reagent for the experiments was a diagnostic mAb that was developed in this study (see Methods) and reacts specifically to a linear epitope unique to Buchnera GroEL, giving assurance that the GroEL detected was of Buchnera origin. The recombinant GroEL for mAb production and some other experiments were conducted on pea aphid Acrithosiphon pisum, making use of its large body size and extensive genomic resources (International Aphid Genomics Consortium, 2010). Complementary experiments were conducted on the bird cherry-oat aphid Rhopalosiphum padi, which is a vector of BYDV.

**RESULTS**

**Production of anti-GroEL mAbs**

mAbs were raised against recombinant Buchnera GroEL that had been expressed in E. coli. From three fusions, 17 mAbs were generated. We selected monoclonal DO65, with a linear epitope located between residues 478 and 501 (as identified by using an A. pisum–Buchnera GroEL peptide library) of the Buchnera GroEL because comparisons of many bacterial GroEL sequences available in GenBank showed that the sequence of this region is unique to Buchnera (data not shown). According to this epitope, the mAb is expected to be specific to Buchnera GroEL. Monoclonal anti-GroEL DO65 is, therefore, a diagnostic antibody for Buchnera GroEL and can be used as a reagent for detecting and quantifying Buchnera GroEL without cross-reactivity to GroEL molecules from other bacteria that may contaminate the aphid sample.

**In vitro and in silico analysis of the Buchnera GroEL–BYDV interaction**

To investigate the predicted binding site of GroEL for BYDV, the luteoviruses BYDV-PAV and BYDV-MAV were administered in vitro to a 77-peptide library (see Methods) representing the full GroEL sequence. Both virus species reacted significantly with only one peptide (#64) (Fig. 1) corresponding to the amino acid sequence VALRAMEA-PLRQIVSN, located between aa 442 and 460 of A. pisum–Buchnera GroEL. This sequence is conserved among all Buchnera GroEL sequences publicly available in GenBank (sequences obtained from GenBank on 30 September 2010) (see Supplementary Fig. S1, available in JGV Online).

The three-dimensional structure of the A. pisum–Buchnera GroEL protein was generated by using structure homology modelling, with the E. coli GroEL PDB file 1mnfG (Wang & Chen, 2003) as a modelling template. The resulting modelled structure showed no structural differences from the E. coli template (Fig. 2a). The virus-binding region (identified by peptide #64) is located in the equatorial domain of the protein monomer (Fig. 2b, c). When Buchnera GroEL is represented as a tetradecamer, the known functional form of the E. coli GroEL, the virus-binding site appears at the junction of the trans and cis rings of the protein and includes Arg 452, the amino acid residue that participates in the formation of salt bridges linking the two rings of GroEL (Braig et al., 1994).

**Localization of the GroEL protein in the aphid body**

For the two aphid species A. pisum and R. padi, GroEL was detected readily in the protein extracted from whole aphids, by Western blotting using the anti-GroEL mAb DO65 (Fig. 3a–c). Aphids contain bacteriocytes (the cells containing Buchnera) in two locations: free in the haemocoel (body cavity) and in embryos, and GroEL was detected in both dissected bacteriocytes and embryos. The aphid guts and haemolymph samples contained no detectable GroEL. When homogenate of the dissected bacteriocytes was separated by centrifugation into supernatant fraction, which lacks Buchnera cells detectable by light microscopy, and pellet, which contains intact
**Fig. 1.** Reactivity of BYDV-PAV- and BYDV-MAV-infected plant homogenates against the 77 peptides of the *Buchnera* GroEL peptide library by ELISA test.

**Fig. 2.** (a) Three-dimensional homology protein modelling of the *Buchnera* GroEL monomer protein. The *E. coli* GroEL modelling template is represented in black and the *Buchnera* GroEL in red. (b, c) Localization of the virus-binding site (b) on the *Buchnera* GroEL protein monomer and (c) on the protein superstructure.
Buchnera cells, GroEL was detected in both fractions (Fig. 3d). The detection limit of the assay is 15 ng per sample, as determined by immunoblotting a dilution series of recombinant GroEL (data not shown).

Localization of GroEL was also investigated by immunocytochemistry (Fig. 4). GroEL was detected in Buchnera cells in the bacteriocytes located in both the haemocoel and the embryos (Fig. 4a), but not in the surrounding cell contents of the bacteriocyte (Fig. 4b). GroEL was not detectable in any aphid structure other than Buchnera cells, including the gut and haemolymph. No signal was obtained for the negative controls (see Methods).

Subsequent experiments addressed two apparent contradictions. First, we hypothesized that the report of GroEL in aphid haemolymph by van den Heuvel et al. (1994, 1997), but not in this study (Fig. 3b, c), could be explained by the different sampling methods. To test this, haemolymph was collected from parallel aphids by cornicle amputation (van den Heuvel et al., 1994) and leg amputation (this study). GroEL was detected only in the cornicle-derived haemolymph (Fig. 3e).

Second, we hypothesized that the detection of GroEL by Western blotting in the Buchnera-free fraction of bacteriocytes (Fig. 3d), but not by immunocytochemistry of intact bacteriocytes (Fig. 4d), could be explained by breakage of a minority of Buchnera cells during preparation. To test for Buchnera breakage, a homogenate of 10 A. pisum aphids was supplemented by either purified Buchnera or buffer, rehomogenized and separated into Buchnera and Buchnera-free supernatant fractions by centrifugation. If this procedure did not disrupt any Buchnera cells, the GroEL

Fig. 3. Localization of GroEL by immunoblotting. (a) SDS-PAGE and Coomassie staining of A. pisum dissected body parts. (b, c) Western blots of (b) A. pisum extracts and (c) R. padi extracts using the anti-GroEL mAb DO65. (d) Western blot of dissected bacteriocyte homogenate (aphid cells containing Buchnera bacteria) and homogenate fractionated by centrifugation into pellet (containing Buchnera) and Buchnera-free supernatant. (e) Western blot of A. pisum haemolymph extracts obtained from excised leg and excised cornicles. (f) Dot immunoblot of protein extract from aphids that were homogenized with supplementary Buchnera cells in buffer (+Bu) or buffer only (−Bu). Bact, Bacteriocytes; haem, haemolymph; rGroEL, recombinant GroEL. Size markers are shown in kDa.
content of the supernatant in the *Buchnera*-supplemented treatment and control (buffer-supplemented) homogenates should be comparable; however, if some *Buchnera* cells are broken by the homogenization, then the GroEL of the supernatant of the *Buchnera*-supplemented treatment should be elevated. The latter result was obtained (Fig. 3f), suggesting that recovery of GroEL in the aphid fraction of bacteriocytes is an experimental artefact.

**DISCUSSION**

It has been proposed that viruses of the genera *Luteovirus* and *Polerovirus* are protected from aphid immune attack in the aphid haemolymph by binding to the protein GroEL, released from cells of the aphid symbiont, *Buchnera* (Filichkin et al., 1997; van den Heuvel et al., 1997; Hogenhout et al., 1998). This study investigated two elements of this hypothesis: virus–GroEL binding and the localization of GroEL in the aphid body.

We have demonstrated binding *in vitro* of BYDV to the GroEL protein at a single epitope that has also been identified as a PLRV-binding site (Hogenhout et al., 2000). However, the second epitope identified for PLRV binding, located in the N terminus (peptide #2) of the protein, did not display binding to BYDV (Fig. 1). Analysis of the three-dimensional superstructure of GroEL suggests that virus binding would compete with the formation of the GroEL tetradecamer. Specifically, the amino acid Arg 452 in the virus-binding site is involved in the formation of salt bridges linking the two rings of GroEL. It has been demonstrated that *Buchnera* GroEL exists as a tetradecamer in the aphid body (van den Heuvel et al., 1997). This raises the important implication that the incidence of BYDV–GroEL binding depends on the relative affinity of the homologous interactions among GroEL subunits versus the heterologous BYDV–GroEL interaction. Although no relevant data are currently available, these considerations demonstrate that virus binding to recombinant GroEL or its constituent peptides *in vitro* may not be sufficient evidence for binding *in vivo*.

The suggestion that GroEL is released in large quantities from the intracellular *Buchnera* bacteria to the bacteriocyte and thence the haemolymph is central to the proposed involvement of the GroEL protein in the polerovirus and luteovirus transmission process. Evidence from both Western blotting and immunocytochemistry in this study refutes the published claims to *Buchnera* contamination of haemolymph collected from amputated cornicles (van den Heuvel et al., 1994). Between one and several bacteriocytes tend to be apposed closely to the cornicle base and therefore vulnerable to breakage during cornicle amputation, leading to bacteriocyte cell contents, including *Buchnera*, contaminating the cornicle secretions. Amputation of the anterior leg, in comparison, yields pure haemolymph without contamination by bacteriocytes (or other structures, such as the fat body). The absence of credible evidence that GroEL is released from *Buchnera* cells makes the proposed GroEL binding to virus particles in the aphid haemolymph implausible.

*Buchnera* GroEL has additionally been proposed to be transferred to the gut of the mustard aphid *Lipaphis erysimi*, in which it has been identified as a gut receptor of the garlic leaf lectin, the interaction between the two proteins being mediated by a mannose residue of the glycosylated GroEL molecule (Banerjee et al., 2004). Subsequent analysis, however, could not confirm GroEL as the candidate receptor for garlic lectin (Fitches et al., 2008). Furthermore, the GroEL sequence of most aphids,
including \textit{A. pism} and \textit{R. padi}, lacks the putative glycosylation site. Even so, the study of Banerjee \textit{et al.} (2004) raises the possibility that GroEL could interact with the virus at the gut interface, prior to virus transfer to the haemolymph. The demonstration here that GroEL was undetectable by both immunoblotting and immunocytochemistry refutes this potential interaction.

An intriguing alternative scenario is that the virus interacts with GroEL derived not from \textit{Buchnera} but from secondary symbionts, which are bacteria present in some aphids (Oliver \textit{et al.}, 2010). This arises from the recent indication that GroEL protein derived from a secondary symbiont, ‘\textit{Candidatus Hamiltonella defensa}’ of the whitely \textit{Bemisia tabaci} and tomato yellow leaf curl virus (Gottlieb \textit{et al.}, 2010), ‘\textit{Candidatus H. defensa}’ is also a secondary symbiont of some aphids (Haynes \textit{et al.}, 2003; Moran \textit{et al.}, 2005). The vector competence of aphids containing and lacking ‘\textit{Candidatus H. defensa}’ has not been studied systematically, but circumstantial evidence suggests that this interaction is not generally important. Specifically, all 20 aphid lines in a panel of \textit{Schizaphis graminum} that varies widely in vector competence lacked any known secondary symbiont (Gray \textit{et al.}, 2002), and a great many \textit{M. persicae} clones are vector-competent for PLRV, even though this species very rarely bears any secondary symbiont (Bouvaine, 2010).

The conclusion from this study that \textit{Buchnera} GroEL is most unlikely to contribute to virus transmission of BYDV is congruent with the analysis of Liu \textit{et al.} (2009), who demonstrated that mutants of pea enation mosaic virus lacking the readthrough domain are as persistent as wild-type viruses.

\textbf{METHODS}

\textbf{Aphids and virus isolates.} The aphids used were the pea aphid \textit{Acyrthosiphon pism} (Harris) clone LLO1, collected in 1988 from alfalfa in Lusignan, France, and maintained on \textit{Vicia faba} cv. Windsor; and the bird cherry-oat aphid \textit{Rhopalosiphum padi} (L.) clone RpG1, collected in 2006 from barley plants in York, UK, and maintained on \textit{Hordeum vulgare} cv. Avena sativa cv. Coast Black.

\textbf{Virus overlay assay.} A set of 77 peptides representing the full sequence of the \textit{A. pism} GroEL protein (GenBank accession no. NP_239860) was obtained from Mimotopes (www.mimotopes.com). Each peptide was 18 aa long and overlapped with other peptides by 11 aa (i.e. 7 aa were unique to each peptide). Each peptide was linked to biotin by a spacer sequence of 4 aa (SGSG). Each peptide (100–300 ng) was then added to a well of microtitre plates, coated previously with 1 μg streptavidin and blocked with 1% sodium caseinate diluted in PBS containing 0.05% Tween 20 (PBS-T).

\textbf{Production of anti-GroEL mAb.} The GroEL gene of \textit{Buchnera} from \textit{A. pism} was amplified using PCR in a mix containing 1× Taq polymerase optimbuffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.25 μM each primer (5’-GGATTTCCATGCGCGCTTTAAGGTG-3’ and 5’-CCGCGGCGGCGCCATCATTCACTCCCATG-3’), 1 μl template DNA and 2.5 U Taq polymerase. Mixtures were incubated for 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final incubation of 10 min at 72 °C. The identity of the PCR product was confirmed by Sanger sequencing and the product was then inserted into a pET-21b vector (Novagen) next to a sequence encoding a His-tag peptide. The vectors were then transformed in \textit{E. coli} Rosetta competent cells (Invitrogen). Protein production was induced with 1 mM IPTG and the recombinant GroEL protein was purified by using a nickel column. The anti-GroEL mAb was produced in mice by using the purified recombinant \textit{Buchnera} GroEL as antigen. The splenocytes were fused with SP2/0-Ag14 myeloma cells (European Collection of Cell Cultures). After cloning by limiting dilutions, the cell-culture supernatants were tested by using plate-trapped antigen ELISA tests (Crook & Payne, 1980) for their reactivity against recombinant GroEL protein extracts of \textit{R. padi} and \textit{A. pism}, and a negative control comprising His-tagged recombinant prephenate dehydratase (PheA) of \textit{Buchnera}. The mAb DO65 was selected based on the criterion that it reacted with the aphid extract, but did not react with the His-tagged PheA (data not shown).

\textbf{Detection of the GroEL protein by gel electrophoresis and immunoblotting.} Aperterous 7-day-old larvae were dissected in sterile PBS, comprising 2 mM KH₂PO₄, 8 mM NaH₂PO₄, 140 mM NaCl and 2 mM MgCl₂. Embryos and the gut were separated carefully from the rest of the body, washed in sterile PBS and transferred to 100 μl ice-cold PBS. Bacteriocytes and fat body were then aspirated into capillary pipettes and transferred to clean tubes. To extract haemolymph, the aphid was immersed in water-saturated paraffin oil, one anterior leg of each aphid was removed and a drop of haemolymph was collected into a microcapillary tube. The aphid tissues were homogenized using a plastic pestle in 500 μl buffer containing 35 mM Tris, 25 mM KCl and 10 mM MgCl₂, pH 7.4. The homogenate was centrifuged at 12 000 g for 10 min, and the protein content of the supernatant was assayed by using an RC/DC protein assay kit (Bio-Rad) following the manufacturer’s instructions. SDS-PAGE was performed as described by Laemmli (1970) by loading 10 μg protein on a polyacrylamide gel consisting of 10 % resolving gel and 4 % stacking gel containing 0.1 % SDS. For Western blots, 1 μg of each protein was loaded on an SDS-PAGE gel and then transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) at 350 mA for 60 min in Tris glycine buffer. After blocking with 5 % milk powder, the membrane was incubated successively with the anti-GroEL mAb DO65 diluted 1/2000 in PBS-T and anti-mouse IgG conjugated to peroxidase (Sigma) diluted 1/4000 in PBS-T. The membrane was incubated in ECL substrate (Amersham Biosciences) and visualized by using a Molecular Imager ChemiDoc XRS (Bio-Rad).

\textbf{Detection of the GroEL protein by immunocytochemistry.} The legs and head were removed from aperterous 7-day-old larvae, and the body was fixed overnight in 4 % paraformaldehyde at 4 °C. Fixed
insects were washed in 70% ethanol, dehydrated through an ethanol series and embedded in paraffin wax. The sections (4 μm thick) were deparaffinized with xylene and ethanol, blocked in 0.5% H₂O₂/methanol and then in 10% horse serum/2× casein. The anti-GroEL mAb, DO65, was applied diluted 1/1000 in Tris-buffered saline (TBS: 20 mM Tris/HCl pH 7.4, 150 mM NaCl)/1× casein for 1.5 h at 37°C. Samples were then washed twice in TBS and incubated with biotinylated horse anti-mouse IgG (H&L) (Vector Laboratories) diluted 1/100 for 20 min at room temperature. After washing in TBS, the samples were incubated in streptavidin–HRP (Zymed) for 20 min, counterstained with Gill’s #2 haematoxylin (Fisher Scientific) and mounted with aqueous mounting medium (Fluoromount G; Southern Biotech). All experiments included four negative controls: incubations with either the primary or secondary antibody replaced by PBS, with pre-immune serum, and with primary antibody previously titrated with excess recombinant GroEL.

Detection of GroEL by dot-blot assay. Ten aperterous last-instar A. pism larvae were ground by using a plastic pestle in 400 μl buffer containing 35 mM Tris pH 7.5 and 0.2 M sucrose. The fraction was separated into two tubes of equal volume and supplemented with 50 μl of either the same buffer or a suspension of pure Buchnera cells. The samples were ground again before being centrifuged at 1480 g for 5 min. One microlitre of the supernatant of each sample was applied to a nitrocellulose membrane pre-wetted in TBS (100 mM Tris, 150 mM NaCl, pH 7.4) using a 48-well Bio-Dot Microfiltration Apparatus (Bio-Rad). The membrane was rinsed three times in TBS. Blocking and blotting were done as described above for immunoblotting.

Isolation of Buchnera cells from aphids. Buchnera cells were isolated from bacteriocytes that had been dissected from aphids (as described above). About 500 bacteriocytes were hand-homogenized lightly in 500 μl buffer A (25 mM KCl, 10 mM MgCl₂, 35 mM Tris/HCl, 0.25 M sucrose). The homogenate was centrifuged at 1300 g for 10 min, yielding a supernatant that lacked any Buchnera cells by light microscopy and a pellet comprising intact Buchnera cells and contaminating aphid-cell debris. To obtain purified Buchnera from whole aphids, 200 mg A. pism aphids were ground in 3 ml ice-cold buffer A. The suspension was passed through muslin and the filtrate was centrifuged at 1750 g for 5 min at 4°C. The pellet was resuspended in buffer A+0.008% Igepal Ca-360 detergent, and centrifuged at 1300 g for 5 min at 4°C. The pellet was washed three times in buffer A and resuspended in 0.25 M sucrose. The Buchnera cells were then purified by using a step gradient of 45, 27, 18 and 9% Percoll by centrifugation for 20 min at 52 g and 5 min at 85 g. The fraction containing Buchnera cells was collected and checked by light microscopy at ×1000 magnification.

Three-dimensional modelling. A structural model of Buchnera GroEL was generated by using the automated homology modelling server SWISS-MODEL (Guex & Peitsch, 1997) running at Glaxo Wellcome Experimental Research (Geneva, Switzerland). The predicted three-dimensional template was displayed and the regions of interest were highlighted by using the program DS Visualizer (version 1.7; Accelrys Software Inc.).

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