Association of Potyvirus Helper Component Protein with Virions and the Cuticle Lining the Maxillary Food Canal and Foregut of an Aphid Vector

E. D. Ammar, U. Järlfors, and T. P. Pirone

Department of Plant Pathology, University of Kentucky, Lexington 40546.

ABSTRACT

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Transmission electron microscopy and immunogold labeling were used to study the role of the helper component protein (HC) that is required for the transmission of potyviruses by aphids. Purified tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV) were fed to the aphid vector, *Myzus persicae*, either with or without purified HC. In sections of aphids that fed on a mixture of purified virions and HC, filamentous viruslike particles (VLP) embedded in a matrix material were associated with the epicuticle, predominantly in the maxillary food canal but also in the precibarium and cibarium of the foregut. No VLP were found at any of these sites in aphids that fed on purified virions without HC. Immunogold labeling demonstrated that the VLP are TEV or TVMV virions and that HC is associated with virions and with the epicuticle lining the food canal and foregut. These results support the hypothesis that HC is directly involved in binding or attachment of virions to the cuticle of the maxillary food canal and foregut of aphid vectors.

Potyviruses constitute the largest group of plant viruses and infect a wide range of economically important crops (14). The majority of potyviruses are transmitted by aphids in a noncircula-tive, nonpersistent manner (8,14). The mechanism of transmission is not yet understood in spite of several investigations to determine the retention sites of potyviruses in their aphid vectors. Using transmission electron microscopy (TEM), Taylor and Robertson (24) detected a few filamentous potyvirus-like particles lining about 20 μm of the distal part of the maxillary food canal in aphids that fed on plants infected with tobacco severe etch poty-virus. Using an indirect immunospecific latex technique with scanning electron microscopy (SEM), Lim et al (11) observed label on the inner surfaces of the mandibular stylets, whereas Jellison (9), using an enzyme-linked antigen distribution assay with SEM, found label attached mainly to the maxillary stylets of aphids that fed on plants infected with the pea seedborne mosaic potyvirus. None of these investigations, however, dealt with the role of the virus-encoded helper component protein (HC), which is essential for transmission of potyviruses by their aphid vectors (20).

Using light microscopy (LM) with autoradiography of frozen sections, Berger and Pirone (4) examined aphids that fed on ^{125}I-labeled virions of potyviruses in the presence of active or inactive HC. They found label associated with the maxillary stylets, foregut, and midgut when aphids were fed on virions mixed with active HC, but label was found only in the midgut when aphids were fed on virions mixed with inactive HC. Although this study suggests a role for HC in virus retention, resolution at the LM level did not allow visualization of virions or their precise retention sites in the aphid stylets and foregut. Further-more, because labeled HC was not used in the study, it could not be determined whether HC plays a direct or indirect role in the putative binding/attachment of virions to retention sites in aphids.

The present study, which utilized TEM and immunogold label-ing, was designed to obtain more detailed information about the interaction between virions, HC, and their cuticular retention sites in the stylets and foregut of an aphid vector.

MATERIALS AND METHODS

Virus and HC preparation. The highly aphid-transmissible strain of tobacco etch virus (TEV-HAT) was purified as described by Pirone and Thornbury (21), as was the type strain of tobacco vein mottling virus (TVMV-AT) (3). Sucrose gradient purified HCs of TVMV and potato virus Y (PVY) were prepared as described by Thornbury et al (25). PVY HC was used with TEV because we are unable to obtain good preparations of TEV HC, and purified TEV is readily transmitted in the presence of PVY HC (19). The activity of the HC preparations was quantified by testing a series of 10-fold dilutions of HC mixed with 100 μg of purified virus in our standard bioassay, which uses 10 aphids per test plant (19). The amount of HC used in the actual experiments was two- to fivefold greater than those required for 100% transmission in this standard bioassay.

The following solutions, all in TSM buffer (100 mM Tris-H$_2$SO$_4$, 20 mM MgSO$_4$, 10% sucrose; pH 7.2) were used for aphid feeding: 1) purified TEV (500 μg/ml) or TVMV (200 μg/ml); 2) a mixture of purified TEV (500 μg/ml) and PVY HC; or 3) a mixture of purified TVMV (200 μg/ml) and TVMV HC.

Acquisition by aphids. *Myzus persicae* (Sulz.) were reared on young tendergreen mustard (*Brassica perviridis* L.) plants in a growth chamber at 22 C under a 16-h light period. Apterae at the late nymphal or early adult stage were starved for 2–3 h and then given a 5- to 10-min acquisition access period through stretched Parafilm membranes to one of the feeding solutions described above. Younger aphids were not used, to reduce the possibility of moulting during starvation or acquisition. Feeding of aphids was monitored through a stereomicroscope, and only aphids probing the solution were processed for TEM. Other groups of aphids given access to the same solutions were assayed individually for transmission of TEV or TVMV on tobacco seed-lings as previously described (19).
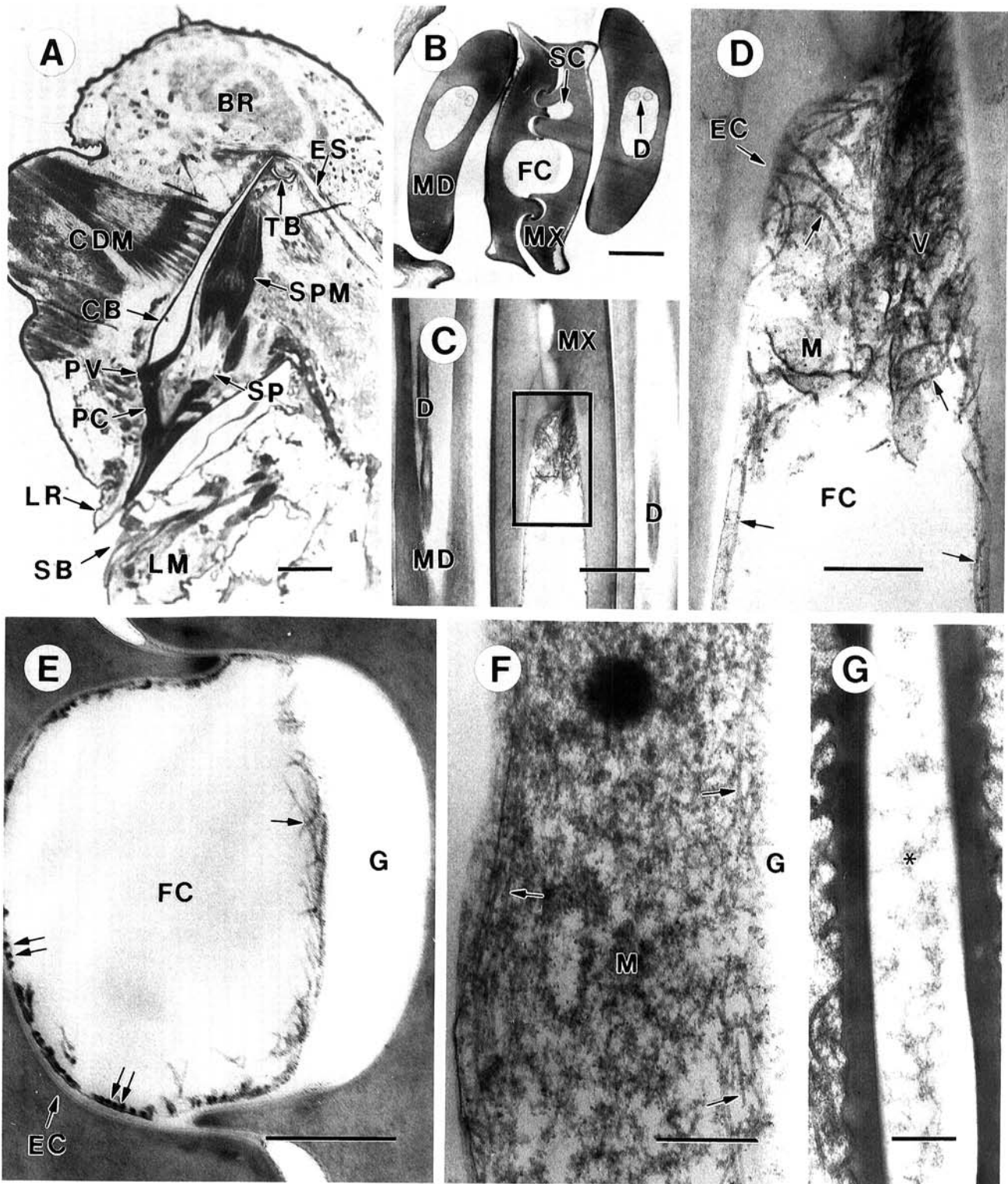
Fig. 1. A, Light micrograph of a sagittal section in the foregut and other structures in the head of *Myzus persicae*. B and C, Electron micrographs of cross- and oblique-sections, respectively, of the stylet bundle in aphids that fed either on tobacco etch virus (TEV) only or on TEV plus helper component (HC) protein, respectively; boxed area in C is shown at higher magnification in D. D–F, Filamentous viruslike particles (V and unlabeled arrows) associated with the epicuticle lining the food canal (D and E) or within matrix material in the cibarium (F); double arrows in E indicate cross-sections of viruslike particles. D and F are from aphids that fed on TEV plus HC; E and G are from an aphid that fed on tobacco vein mottling virus (TVMV) plus HC. G, Noncompact fibrous material (asterisk) in the lumen of the afferent salivary duct; note the absence of label in this section, which was probed with antibodies to TVMV HC. (A–D and F are from osmicated Spurr-embedded specimens, whereas E and G are from nonosmicated LR Gold-embedded specimens). BR = brain; CB = cibarium; CDM = cibarial dilator muscle; D = dendrite; EC = epicuticle; ES = esophagus; FC = food canal; G = gap due to artifactual separation of epicuticle from embedding medium; LM = labium; LR = labrum; M = matrix; MD = mandibular stylet; MX = maxillary stylet; PC = precibarium; PV = precibarial valve; SB = stylet bundle; SC = salivary canal; SP = salivary pump; SPM = salivary pump retractor muscle; TB = tentorial bar. Scale bars: A, 30 μm; B and C, 1 μm; D–G, 300 nm.

Processing of aphids for TEM. Probing aphids were removed from the Parafilm membrane, immediately exposed to CO_2 for a 5-min anesthetization period, and then placed in a drop of one of the fixatives described below. The head-prothorax region of each aphid was severed with a sharp razor blade under a dissecting microscope and processed for TEM by one of four methods. The first method (method 1) gave good ultrastructural preservation of a semipersistent virus in the stylets and foregut of its leafhopper vector (1). The other methods (methods 2–4) represent variations of fixation and embedding procedures used for postembedding immunogold labeling in other systems (18,26,27). To avoid losing the relatively small aphid parts during transfer from one reagent to the next, they were placed in polyethylene specimen-processing holders, 14 mm in diameter and 18 mm high, perforated at the bottom (Electron Microscopy Sciences, Fort Washington, PA). The methods used were basically as follows: 1) Fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, (2 h to overnight at 4 C), postfixation in 1% osmium tetroxide in the same buffer (2 h at 4 C), dehydration in graded ethanol and acetone, embedding in Spurr's resin, and polymerization at 70 C overnight. 2) Fixation in 0.1, 1.0, or 2.5% glutaraldehyde plus 1, 2, or 4% paraformaldehyde in cacodylate buffer for 1–2 h at 4 C or for 15–20 s in a microwave oven (625 W), with temperature rising to 36–40 C. This was followed by dehydration in graded ethanol up to 70%, embedding in LR White resin (Electron Microscopy Sciences), and polymerization at 50–52 C overnight. 3) Fixation in 1.0 or 2.5% glutaraldehyde for 1–2 h at 4 C followed by postfixation in 0.1% osmium tetroxide for 30 min, dehydration in ethanol, and embedding in LR White resin as described in method 2. 4) Fixation as in method 2 and dehydration in graded ethanol up to 90% at 4 C gradually lowered to −10 C. This was followed by low-temperature embedding in LR Gold resin (Polysciences, Warrington, PA), and polymerization with UV light at −15 to −20 C for 24 h.

Embedded specimens were oriented for cutting longitudinal (sagittal) or cross-sections in the aphid's head, prothorax, and stylet bundle. Semithin (1–2 μm thick) serial sections were cut with glass or diamond knives, stained with toluidine blue, and monitored by LM for the desired structures (i.e., the foregut and stylets). When these structures were observed, ultrathin sections were cut for TEM with a diamond knife. Because the foregut and stylets are only a few micrometers wide (Fig. 1A–C), and, thus, are frequently hidden by the grid bars when viewed by TEM, sections were usually mounted on fine-bar hexagonal grids (200 mesh) coated with collodion and carbon, which provided wider viewing areas with reasonable support of the specimen. Thin sections, with or without immunogold labeling, were stained with uranyl acetate and lead citrate and examined in a Philips 400 (Phillips Electronic Instruments, Mahwah, NJ) or a Hitachi 600 (Nissei Sangyo American, Ltd., Mountain View, CA) transmission electron microscope. Usually 40–60 sections, sampled from different parts of the stylets and foregut from each aphid, were examined by TEM at a minimum magnification of ×15,000 on the screen, using a ×10 binocular. In some cases, however, as many as 200 sections in the same stylet bundle were examined to estimate the number of virions in the food canal.

Immunogold labeling. Preliminary experiments indicated that specimens embedded in LR White or LR Gold resin, but not in Spurr's resin, could be used for immunogold labeling. Thin sections in the foregut or stylets of aphids that fed on virions plus HC were immunolabeled with gold essentially as described earlier (2). The primary antibodies used were rabbit polyclonals produced in our laboratory against TEV virions, TVMV coat protein (TVMV CP), or PVY HC, and monoclonals against TVMV HC, provided by R. Jordan (USDA, Beltsville, MD). All antibodies and gold conjugates were diluted in TBS-BSA buffer (0.01% bovine serum albumin plus 0.1% Tween 20 in 0.1 M Tris-HCl, 0.85% NaCl, pH 7.5). Sections were incubated with antiserum to TEV or TVMV CP (diluted 100- to 200-fold) for 1 h or with antiserum to PVY HC (diluted 10- to 20-fold) or monoclonal antibodies to TVMV HC (10–40 μg/ml) for 7 h at room temperature or for 24 h at 4 C. Sections were incubated

for 30–60 min with goat anti-rabbit immunoglobulin G (IgG) (GAR) gold (20 nm in diameter, BioCell, Cardiff, Wales) diluted 25-fold or goat anti-mouse IgG (GAM) gold (10 nm in diameter, Sigma Chemical Co., St. Louis) diluted 10-fold. For double labeling of TVMV virions and HC, sections were incubated first with the monoclonal antibodies to TVMV HC for 7–24 h, followed by the polyclonal antiserum to TVMV CP for 1 h, and then were incubated with a mixture of GAM gold (10 nm) and GAR gold (20 nm) for 30–60 min.

As controls for the immunolabeling, sections from adjacent regions in the foregut or stylets of aphids that fed on virions plus HC were incubated with buffer, preimmune rabbit serum, or monoclonal antibodies produced against a nonrelevant antigen. The same incubation times and concentrations were used as for the primary antibodies, prior to further incubation with gold conjugates as described above.

RESULTS

Definition and structure of the foregut. Definition of the term foregut in aphids and other Homoptera has varied widely between authors; Forbes (7) and Ponsen (22) used this term to include only the esophagus, whereas Lopez-Abella et al (13) and Harris (8) used it to include the esophagus, pharynx (upper part of the cibarium in aphids), cibarium, precibarium, and the maxillary food canal. However, because the food canal is merely part of the modified maxillae in Homoptera (23), we use the term foregut to include the esophagus, cibarium (including the pharynx), and the precibarium. In *M. persicae*, these structures (Fig. 1A), as well as the precibarial valve and associated sensillae, are as described for another aphid species, *Acyrthosiphon pisum* (15). Lumina of the precibarium and cibarium are lined with a thick cuticular intima, whereas the esophagus lumen is lined with a thin cuticular intima (Fig. 1A). The stylets, maxillary food and salivary canals (Fig. 1A and B), and the salivary pump and duct in aphids have been described previously (7,22).

Occurrence of viruslike particles in the stylets and foregut. Filamentous viruslike particles (VLP), 11–13 nm wide with undetermined length due to their oblique orientation in most sections, were found in 10 of 20 aphids that fed on the mixtures of TEV virions plus PVY HC or TVMV virions plus TVMV HC (Table 1). These VLP were closely associated with the inner cuticular surface (epicuticle) lining the food canal in the maxillary stylets (Fig. 1C–E). In osmicated Spurr-embedded sections, the VLP were usually embedded in a variably stained matrix (Fig. 1D). Matrix-embedded VLP also were associated with the cuticular intima lining the precibarium and cibarium (Fig. 1F) in four of the aforementioned 10 aphids. The VLP were found more frequently in the food canal (32.1% of the sections examined) compared to the precibarium (12.1%) or cibarium (9.5%). In most cases, the VLP appeared to be more abundant in sections of the food canal than in those of the precibarium or cibarium (Fig. 1D–F). As many as 80 VLP per section were found in the food canals of some aphids, and at least 1,000–2,000 VLP were estimated to occur in 200 sections (a total depth of 20–30 μm) sampled from the food canal of one aphid. The VLP were found

TABLE 1. Number of aphids in which filamentous viruslike particles (VLP) were found in Spurr-embedded thin sections of the stylets and foregut

Mixture[a]	Acquisition of virus from solution	
	Virus + HC	Virus only
TEV ± PVY HC	5/12[b]	0/10
TVMV ± TVMV HC	5/8	0/10
Total	10/20[c]	0/20

[a]TEV = tobacco etch virus, PVY = potato virus Y, TVMV = tobacco vein mottling virus, and HC = helper component.

[b]Number of aphids in which VLP were found/number of aphids examined.

[c]VLP were found in the food canal of all 10 aphids and in the foregut in only four of these aphids.
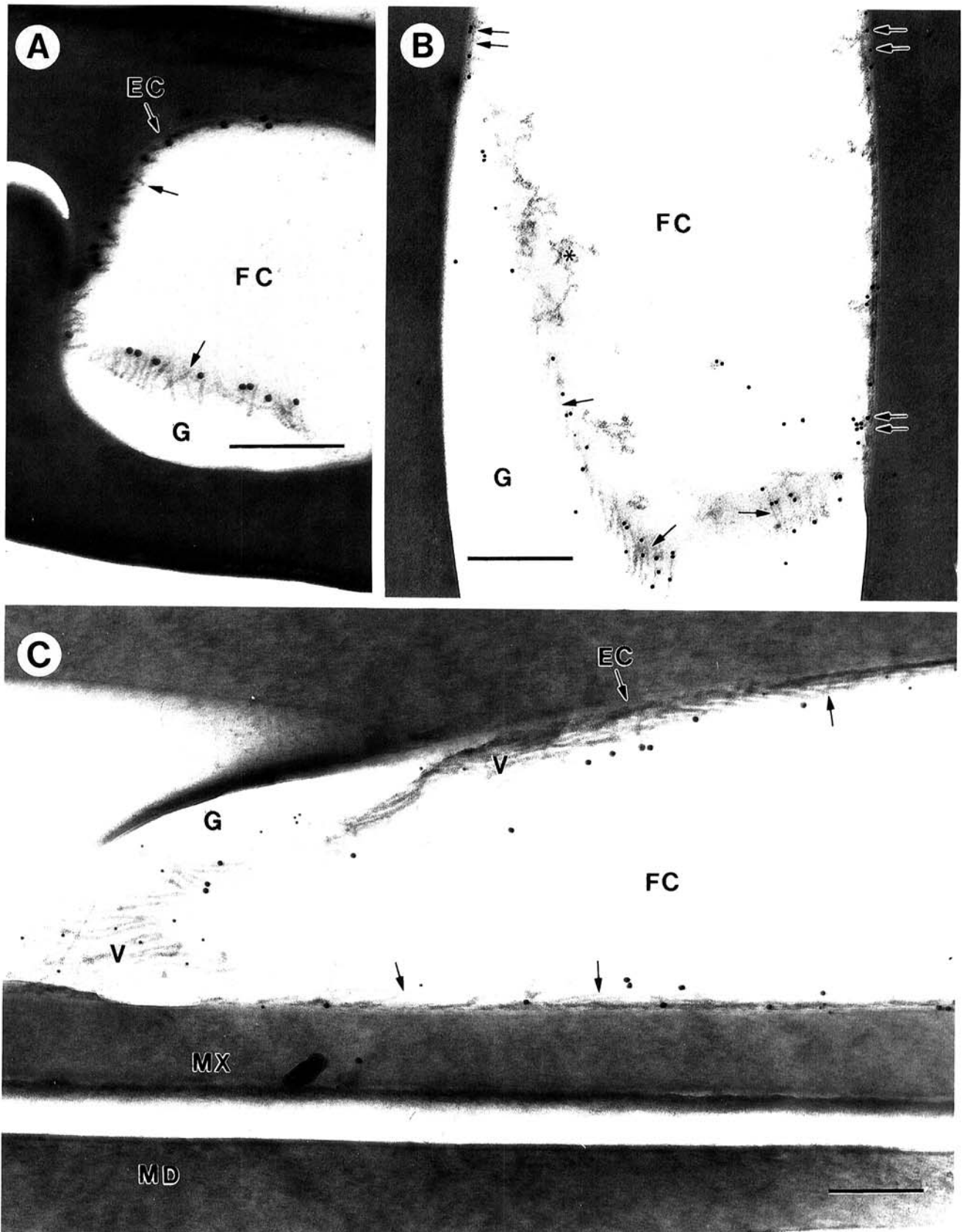
Fig. 2A–C, Electron micrographs of sections in the maxillary food canal (FC) of nonosmicated, LR Gold-embedded aphids that fed on tobacco vein mottling virus (TVMV) plus helper component (HC) protein; G = gap due to artifactual separation of the epicuticle (EC) from the embedding medium. **A,** A section incubated with polyclonal antibodies to TVMV coat protein (CP) followed by 20 nm of goat anti-rabbit immunoglobulin G (IgG) (GAR) gold; cuticle-associated virions (arrows) are gold-labeled. **B,** A section incubated with monoclonal antibodies (MAbs) to TVMV HC followed by 10 nm of goat anti-mouse IgG (GAM) gold; specific labeling is associated with virions (single arrows) and with the epicuticle lining the food canal (double arrows); the noncompact fibrous material (asterisk) in the food canal is not labeled. **C,** A section incubated with TVMV HC MAbs followed by TVMV CP polyclonal antibodies and then probed with a mixture of 10 nm of GAM and 20 nm of GAR gold; HC label (smaller gold particles) and CP label (larger ones) are associated with virions (V and arrows) and the epicuticle; note the lack of labeling on the outer surface of the maxillary stylet (MX) or the inner surface of the mandibular stylet (MD). Scale bars: 300 nm.

in various parts of the food canal, including distal (near the stylet tip), middle, and basal parts (close to the precibarium). Frequently, the VLP appeared to be arranged in one or two layers, oriented longitudinally, more-or-less parallel to the long axis of the food canal (Fig. 1E).

In parallel bioassay tests, aphids fed in a similar manner on virus-HC mixtures transmitted TEV or TVMV at a rate (percentage of infected plants) ranging from 10–30%, using one aphid per each of 20 test plants. No transmission of either virus occurred in the absence of HC, in tests with several hundred aphids.

In Spurr-embedded sections of 20 aphids that fed on purified TEV or TVMV without HC, no matrix or VLP were found in the maxillary food canal or foregut (Fig. 1B; Table 1). However, in these aphids, as well as in aphids fed on virion-HC mixtures, noncompact fibrous material (Fig. 2B) was frequently found in the food canal, salivary canal, and foregut. Material of similar
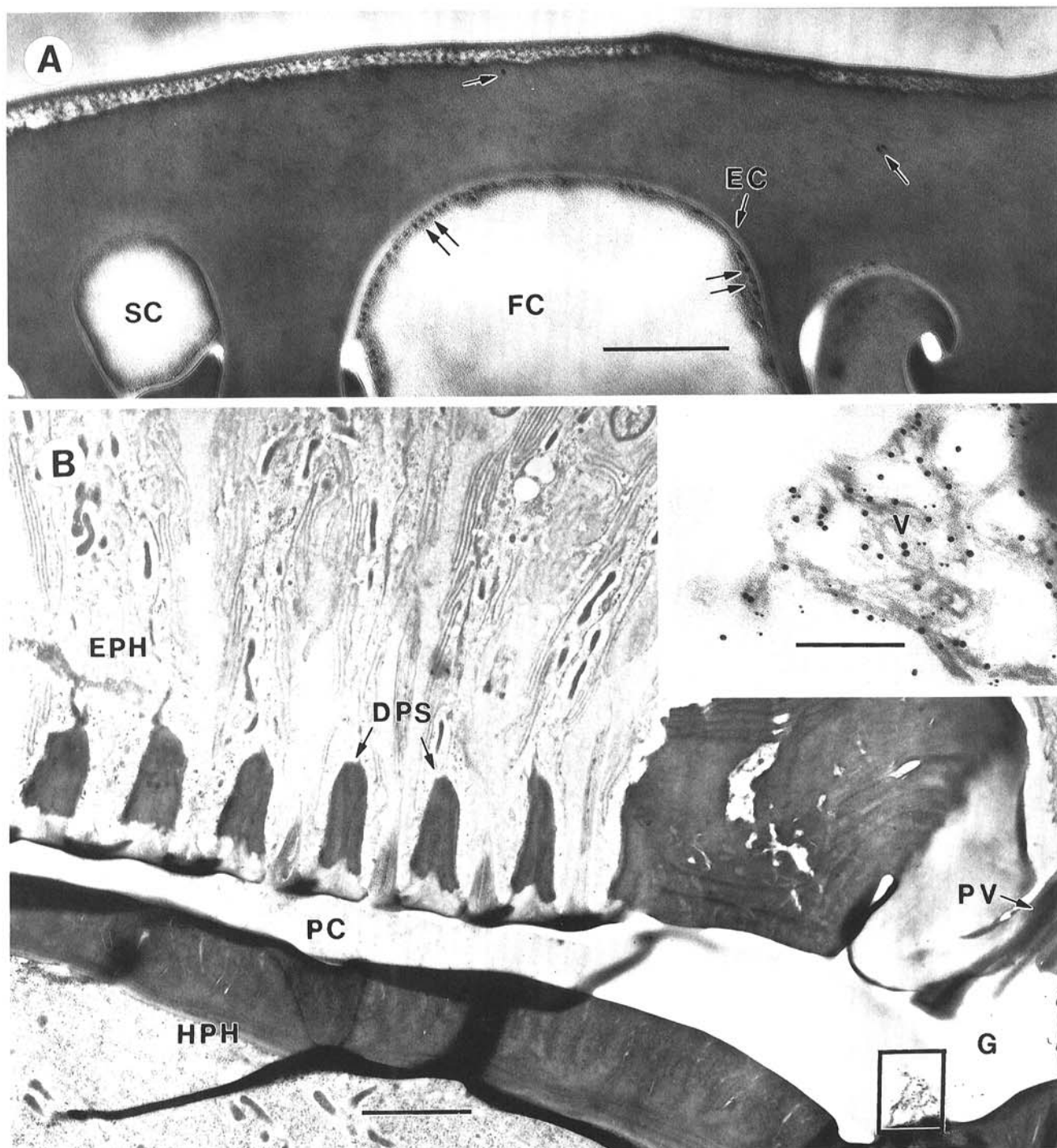

Fig. 3A and B. Electron micrographs of sections from nonosmicated LR Gold-embedded aphids that fed on tobacco vein mottling virus (TVMV) plus helper component (HC) protein. A, A section in the maxillary food and salivary canals incubated with a control monoclonal antibody followed by 10 nm of goat anti-mouse IgG (GAM) gold; only a few anomalous gold particles (single arrows) are seen on the cuticle; double arrows indicate cross-sections of virions associated with the epicuticle (EC) in the food canal (FC) but not in the salivary canal (SC). B, A section in the precibarium (PC), double-labeled for TVMV coat protein (CP) and TVMV HC as described in Figure 2C; the inset shows higher magnification of the boxed area close to the precibarial valve (PV), which contains a group of convoluted virions (V) with specific labeling for both TVMV CP (larger gold particles) and HC (smaller particles). DPS = distal precibarial sensilla; EPH = epipharynx, G = gap due to artifactual separation of cuticle from embedding medium; HPH = hypopharynx. Scale bars: A, 300 nm; B, 2 μm; inset, 300 nm.

appearance also was found in the salivary pump and in the afferent salivary duct (Fig. 1G) that connects this pump to the salivary glands.

Immunogold labeling of virions and HC. With specimens embedded in LR White resin, fixation in lower concentrations of aldehydes (0.1–1% glutaraldehyde plus 1–2% paraformaldehyde) for 1–2 h at 4 C did not preserve the VLP or associated matrix in the food canal or foregut of aphids that fed on TEV plus PVY HC. Higher concentrations of aldehydes or postfixation in 1% osmium tetroxide preserved the above structures but did not allow successful labeling of virions or HC. Microwave fixation for 15 s using 2.5% glutaraldehyde followed by 0.1% osmium tetroxide for 30 min preserved the above structures and allowed reasonable labeling of TEV virions but provided weak labeling of PVY HC (data not shown).

With specimens embedded in LR Gold resin, fixation in 2.5% glutaraldehyde plus 2% paraformaldehyde for 1 h at 4 C led to good preservation and allowed immunolabeling of both virions and HC in the food canal and foregut of aphids that fed on TVMV plus TVMV HC. Polyclonal antibodies to TVMV CP specifically labeled the cuticle-associated VLP (Fig. 2A), demonstrating that they are TVMV virions. Monoclonal antibodies to TVMV HC allowed specific labeling of HC associated with these virions and with the epicuticle lining the food canal and foregut (Fig. 2B). The matrix material associated with VLP in the osmicated Spurr-embedded specimens (Fig. 1D) was apparently more electron lucent in nonosmicated LR Gold specimens and, thus, was not clearly visualized (Figs. 1E, 2A–C, and 3A). In LR Gold sections doubly labeled for TVMV CP and TVMV HC, both virions and HC were clearly associated with each other and with the epicuticle lining the food canal (Fig. 2C), precibarium (Fig. 3B), cibarium, and anterior part of the esophagus. No virions or HC were found on the inner or outer surfaces of the mandibular or maxillary stylets other than in the food canal (Figs. 2C and 3A). The noncompact fibrous material found in the foregut, food and salivary canals, salivary duct, and salivary pump was not labeled either with antibodies to TVMV HC (Figs. 1G and 2B) or TVMV CP.

In control LR White or Gold sections, incubated with buffer, preimmune rabbit serum, or monoclonal antibodies to a nonrelevant antigen prior to incubation with gold conjugates, no gold label was found on or in the vicinity of the cuticle-associated virions in the food canal or foregut of aphids that fed on virions and HC (Fig. 3A).

DISCUSSION

It has been previously postulated that HC may function by binding potyvirus particles to cuticular sites in the food canal or foregut of aphid vectors (4,10). However, the TEM-immunogold labeling study reported here represents the first demonstration of the association of HC, virions, and the epicuticle and, thus, is the first direct evidence supporting this hypothesis. The fact that virions were retained at these sites only when aphids fed on virions plus HC, the combination that also is necessary for transmission (20), indicates that at least some of the virions retained in the food canal and foregut are involved in the transmission process. The association of HC and virions with the epicuticle lining the food canal and foregut also is compatible with the fact that the ability to transmit is lost when the cuticle is shed during moulting (6).

One of the problems often encountered in studying insect cuticular structures by TEM is the separation of the embedding medium at the interface with the epicuticle (12). In our study, this problem was somewhat more acute in LR Gold- than in Spurr-embedded sections (e.g., Fig. 1D and E), particularly with the use of wider mesh fine-bar grids. However, among the three embedding media tested, only LR Gold resin allowed satisfactory labeling of both virions and HC. Another technical problem encountered here was that, unlike the case with circulative or propagative viruses that are located intra- or intercellularly in their vectors (17), the lumenal location of potyviruses in the aphid

makes them likely to be dislodged or washed out during processing. This is probably why fixation without osmium in lower concentrations of aldehydes, which was used satisfactorily for preservation and labeling of TVMV virions and HC in plant cells (2, and E. D. Ammar and T. P. Pirone, *unpublished data*), did not preserve the virions or their associated matrix in the food canal or foregut. This problem was largely overcome by fixation in higher concentrations of aldehydes followed by low-temperature embedding in LR Gold.

The combination of two fixation and embedding procedures allowed the VLP observed in the osmicated Spurr-embedded sections to be positively identified as TVMV virions via immunogold labeling in the nonosmicated LR Gold-embedded sections. The matrix material associated with virions in Spurr-embedded sections (Fig. 1D and F) was not discernible in LR Gold sections probably due to lack of osmium fixation. However, the location of the matrix material appeared to correspond with the location of the HC labeling, which suggests that the matrix is composed of, or at least contains, HC.

Virions were found throughout the maxillary food canal, and none were observed on the outer surfaces of the maxillary stylets or inner surfaces of the mandibular stylets. This contrasts with previous studies with aphids that were fed on infected plants rather than on purified virus; VLP or virus-specific label was found only at the distal part or tip of the maxillae (9,24) or on the mandibular stylets (11). In the present study, virions were found more frequently and abundantly in the food canal compared to the foregut; this was also the case in a previous study in which a large number of aphids was examined by LM, and virus-specific label was more often associated with the maxillary stylets than with the foregut (Table 2 in [4]). It is interesting to note that in the case of semipersistently transmitted viruses, viruslike particles were found mainly in the foregut, rather than the food canal, of aphids (16) or leafhoppers (1,5).

In conclusion, our results indicate that HC is directly involved in the retention of potyviruses on the cuticular lining of the food canal and foregut of aphids. Whereas further studies using non-aphid-transmissible virus isolates and nonfunctional HC as controls are necessary to confirm that this association is of direct relevance to the transmission process, the methodology developed for this study will be useful in future studies with potyviruses and other noncirculatively transmitted viruses with aphid or leafhopper vectors.

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