Intracellular Localization of the *Peanut Clump Virus* Replication Complex in Tobacco BY-2 Protoplasts Containing Green Fluorescent Protein-Labeled Endoplasmic Reticulum or Golgi Apparatus

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RNA-1 of *Peanut clump virus* (PCV) encodes the proteins P131 and P191, containing the signature motifs of replication proteins, and P15, which regulates viral RNA accumulation. In PCV-infected protoplasts both P131 and P191 were immunodetected in the perinuclear region. Laser scanning confocal microscopy (LSCM) showed that P131 and P191 colocalized with neosynthesized 5-bromouridine 5'-triphosphate-labeled RNA and double-stranded RNA, demonstrating that they belong to the replication complex. On the contrary, the P15 fused to the enhanced green fluorescent protein (EGFP) never colocalized with the two proteins. In endoplasmic reticulum (ER)-GFP transgenic BY-2 protoplasts, the distribution of the green fluorescent-labeled ER was strongly modified by PCV infection. LSCM showed that both P131 and P191 colocalized with ER green fluorescent bodies accumulating around the nucleus during infection. The replication process was not inhibited by cerulenin and brefeldin A, suggesting that PCV replication does not depend on de novo-synthesized membrane and does not require transport through the Golgi apparatus. Electron microscopy of ultrathin sections of infected protoplasts showed aggregates of broken ER but also visualized vesicles, some of which resembled modified peroxisomes. The results suggest that accumulation of PCV during infection is accompanied by specific association of PCV RNA-1-encoded proteins with membranes of the ER and other organelles. The concomitant extensive rearrangement of these membranous structures leads to the formation of intracellular compartments in which synthesis and accumulation of the viral RNA occur in defined areas.

Replication is a key event of the viral multiplication cycle that ultimately determines the success of the viral infection. For positive-stranded viruses, replication is thought to occur in complexes containing viral RNA template, viral RNA-dependent RNA polymerase (RdRp), cellular factors, and, in some cases, virus-encoded accessory proteins (27, 57). All results obtained so far show that RNA replication occurs in close association with intracellular membranes, which generally undergo extensive reorganization in the virus-infected cell (9).

Depending upon the virus, the membrane component of the replication complex may be of different origins: membranes of the endoplasmic reticulum (ER) are implicated in viral replication of picorna-like viruses such as comoviruses (11) and potyviruses (50) and of alpha-like viruses such as tobamoviruses (42) and bromoviruses (44, 45). Cytoplasmic invaginations of chloroplast membranes are associated with *Tymovirus* replication complexes (18, 41), and multivesicular bodies derived from peroxisomes, mitochondria, or vacuoles are the sites of replication for other viruses: cucumber mosaic and tomato aspermy viruses (21), *Tomato bushy stunt virus* (52), *Cymbidium ringspot virus* and *Carnation Italian ringspot virus* (47), and *Alfalfa mosaic virus* (AMV) (55).

In animal cells, the replication of viruses of the *Togaviridae* and *Coronaviridae* families occurs in modified endosomes or lysosomes (17, 30, 40, 56). Flavivirus RNA synthesis is thought to occur in vesicle packets derived from the trans-Golgi mem-

branes (29, 58), whereas membranes of the ER, of the Golgi apparatus, and of lysosomes have been detected in the vesicles generated by poliovirus replication (51).

In a number of cases (e.g., poliovirus, alphaviruses, and *Brome mosaic virus*), all of the viral replication proteins are localized within the replication complex (2, 3, 5, 6, 17, 39, 45). On the other hand, for *Flavivirus Kunjin*, the replicative proteins and RNA viral synthesis sites coloclate in vesicle packets, while other nonstructural proteins are associated with modified membranes from the intermediate compartment (29). For *Tobacco etch virus*, only the 6-kDa protein and the viral RNA replication complex were associated with vesicles derived from the ER, whereas N1a and N1b, which are also required for replication, predominantly accumulated in the nucleus (43, 50).

To gain insight into the interactions between viral and host factors during *Peanut clump virus* (PCV) RNA replication, we have investigated the in situ localization of the replicase proteins and the RNA synthesis sites. PCV, a type member of the *Pecluvirus* genus, is a positive-strand RNA virus of the alphavirus-like family. The PCV genome is composed of two molecules of RNA. RNA-1 is able to replicate independently of RNA-2 in protoplasts, but both RNAs are indispensable for plant infection (25). Two N-terminally overlapping proteins encoded by RNA-1 (P131 and P191) are essential replication proteins. P131 contains sequence motifs common to methyltransferases and helicases, while P191 contains the signature motifs of RNA-dependent RNA polymerases in its C-terminal extension (20, 24). The RNA-1-encoded protein P15, on the other hand, is not an essential replication factor but is required for efficient viral RNA accumulation. This protein was not

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detected near the sites of viral RNA synthesis (15) and therefore is probably not a component of the replication complex. In the present study, we have further investigated the localization of the viral RNA replication complex and provide evidence that both P131 and P191 are present at the replication sites. Using protoplasts from transgenic lines of tobacco BY-2 cells, we demonstrate that the ER, but not the Golgi apparatus, undergoes extensive reorganization upon PCV infection, and we show that the viral RNA replication complex is associated with the modified ER membranes.

MATERIALS AND METHODS

Plasmids and transcripts. Viral RNA was extracted from purified PCV (isolated from BY-2 33). Plasmid pPC1 and pPC2 are full-length cDNA clones of PCV RNA-1 and RNA-2, respectively, used to produce infectious transcripts. pRep-EG15 is a chimeric RNA molecule consisting of the 5′-terminal sequence of RNA-2, including the CP gene, and the 3′-terminal sequence of RNA-1, including the P15 gene fused to enhanced green fluorescent protein (EGFP). It has been previously shown that this RNA core replicates with RNA-1 and RNA-2 (15). To construct pRep-RP15, the fragment Ncol-EcoRI of pRep-EG15 corresponding to the EGFP gene was replaced by the Neo-Norf fragment excised from pDsRed-N1 (Clontech), corresponding to the DsRed2 red fluorescent protein (RFP) cistron. Capped in vitro transcripts were obtained with a Ribomax transcription kit (Promega) with the modiﬁed ER membranes.

Inoculation of protoplasts and plants. Protoplasts from BY-2 tobacco cells (35) were prepared as previously described (25). ER-GFP protoplasts were obtained from a transgenic ER-GFP BY-2 cell line which constitutively expresses the red fluorescent protein (RFP) cistron. Capped in vitro transcripts were obtained with a Ribomax transcription kit (Promega) according to the manufacturer’s instructions after the plasmids had been linearized with MluI for pPC1 or HinfIII for pPC2.

In vitro translation and Western blots. In vitro translation with wheat germ extract was done as previously described (23) with [35S]methionine in the incubation medium. Proteins extracted from infected plants or obtained by in vitro synthesis were separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis and then electrotransferred for 2 h at 0.8 mA/cm² onto Immobilon-P membranes (Millipore). 35S-labeled proteins were revealed by autoradiography. The membranes containing viral proteins synthesized in vivo were incubated with 5% powdered milk–1% Tween 20 in phosphate-buffered saline (PBS) for 2 h and then overnight with P131 or P191 antisera. The membranes were washed in 1% Tween 20 in PBS and incubated for 2 h with peroxidase-coupled anti-rabbit immunoglobulin G in 5% milk–1% Tween 20 in PBS. After a wash with 0.1% Tween 20 in PBS, bound antibodies were detected by ECL chemiluminescent reaction (Pierce) according to the manufacturer’s instructions.

Northern blots. Total RNA was extracted from protoplasts as previously described (15), and viral RNAs were detected by hybridization with a specific in vitro-transcribed 32P-labeled RNA probe that corresponds to the minus strand of the 3′-terminal 124 nucleotides, which is common to both genomic RNA-2s (33). Radioactive signals were detected by autoradiography.

Immunofluorescent detection of viral proteins. At 24 and 48 h postinfection (hpi), harvested protoplasts were transferred to a fixing solution composed of 1% glutaraldehyde in BY-2 tobacco cell culture medium (MS), washed twice in PBS and once in 0.1% NaBH₄-PBS, and stored at 4°C. After incubation for 1 h in the blocking solution (5% bovine serum albumin [BSA], 5% normal goat serum, and 0.1% Triton X-100) for 1 h, the cells were incubated overnight with the primary P131 or P191 antibodies. After six washes with 0.1% BSA-c (Aurion, Wageningen, The Netherlands) in PBS, protoplasts were further incubated with Alexa 488, 568, or 633 goat anti-rabbit antibodies (Molecular Probes) for 4 h. After six washes in 0.1% BSA-c in PBS, the protoplasts were observed with a Zeiss LSM 510 confocal microscope.

In vivo RNA and dsRNA labeling. At 24 hpi, protoplasts were incubated for 1 h with 10 μg of actinomycin D/ml and then for 6 h with 100 μM 5-bromouridine 5′-triphosphate (BrUTP; Sigma). Incorporation was stopped by addition of the fixation medium (1% glutaraldehyde in MS) with gentle agitation for 30 min. The protoplasts were then processed as for immunofluorescent detection of proteins by using mouse anti-BrUTP primary antibody (Sigma) and Alexa 488- or 568-conjugated secondary antibody to detect BrUTP incorporation or by using guinea pig polyclonal antibody to detect double-stranded RNA (dsRNA; gift from J. Y. Lee, Macfarlane Burnet Center for Medical Research, Fairfield, Australia) and Alexa 568 secondary antibody to detect dsRNA.

Electron microscopy. For transmission electron microscopy, cells were fixed for 1 h with 1% glutaraldehyde in MS and immersed for 15 min at room temperature in 1 ml of a primary fixative containing 2% (vol/vol) glutaraldehyde and 0.1 ml of saturated picric acid in 25 mM potassium phosphate (pH 7.4), before incubation at 4°C for 1 h. After four washes in 25 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid); pH 7.0] at room temperature, the cells were transferred to a secondary fixative containing 2% (wt/vol) osmium tetroxide and 0.5% (wt/vol) potassium ferrocyanide in 25 mM PIPES (pH 7) for 2 h at room temperature. The cells were then washed twice in 25 mM PIPES (pH 7) and twice in distilled water before transfer to 2% (wt/vol) aqueous uranyl acetate for 16 h at 4°C. After the two washes in water, the cells were dehydrated in an acetone series and embedded in Spurr’s resin. Sections were double stained with uranyl acetate and lead citrate before being examined with an electron microscope operating at 80 kV (Hitachi H600).

RESULTS

Detection of the 131K and 191K replication proteins. Polyclonal anti-P131 and anti-P191 sera raised against the C termini of the corresponding proteins were used to detect the two proteins in Western blots of proteins extracted from PCV-infected BY-2 protoplasts at different times postinfection. The longest polypeptide detected by the anti-P131 antibodies (Fig. 1, lanes 2 to 5) and by the anti-P191 antibodies (Fig. 1, lanes 8 to 10) each comigrated with the corresponding 35S-labeled 131K and 191K products translated in vitro from viral PCV RNA (Fig. 1, lane 6). Each of these species accumulated in increasing amounts with time after infection, and neither was detected in proteins extracted from mock-inoculated protoplasts (Fig. 1, lanes 1 and 7). In addition to the full-length P131 and P191 species, the P131- and P191-specific antisera also immunoreacted with a number of shorter polypeptides on the Western blot. However, when the antisera were further purified by affinity chromatography, P131 and P191 were still readily detected, but the immunoreactions with most of the smaller species were eliminated (Fig. 1, lanes 11 and 12). It is important to note that the anti-P131 antibodies did not detectably cross-react with P191 even though P191 contains the entire sequence of P131. This may indicate that the C terminus of P131 is not accessible in P191.

Colocalization of P131 and P191 with replication sites. In a previous study, we used BrUTP-specific antibodies to detect sites of preferential incorporation of BrUTP into neosynthesized PCV RNA in virus-infected BY-2 protoplasts (15). The putative viral RNA replication sites identified in this study consisted of punctate bodies distributed throughout the cytoplasm that were particularly abundant in the perinuclear space. In such experiments, however, it is difficult to eliminate the possibility that neosynthesized BrUTP-containing RNA (Br-
RNA) detaches from the replication complex and delocalizes from authentic replication sites. Therefore, we have also used an anti-dsRNA primary polyclonal antibody (28, 30) to detect the dsRNA replicative form and replicative intermediates produced during viral RNA replication (9), and we have compared the sites of dsRNA and of neosynthesized BrUTP-labeled RNA by dual-label immunofluorescence. In PCV-infected protoplasts, the distribution of Br-RNA (green spots, Fig. 2A) and of dsRNA (red spots, Fig. 2B), observed at 24 hpi, was similar, whereas no labeling was visible in mock-inoculated protoplasts (Fig. 2D and E). Superimposition of the images (Fig. 2C) detected a few green spots without corresponding red spots, but the coincidence of the majority of the Br-RNA foci with those of dsRNA confirmed that, at least for short BrUTP labeling periods, most of the Br-RNA is present at replication sites.

To test whether the two presumed replication proteins, P131 and P191, are associated with active RNA synthesis sites, viral RNA-infected BY-2 protoplasts were analyzed by dual-label immunofluorescence with the purified anti-P131 (Fig. 3A and D) or anti-P191 (Fig. 3G and J) antibodies and antibodies against either Br-RNA (Fig. 3B and H) or dsRNA (Fig. 3E and K). At 24 hpi, P131 and P191 were visualized as irregularly shaped perinuclear spots (Fig. 3A and G). At a later time (48 hpi), the immunofluorescent structures increased in size and formed aggregates which, while remaining essentially perinuclear, also protruded notably into the cytoplasm (Fig. 3D and J). No signal was visible in uninfected cells processed and imaged in parallel (not shown). Staining by anti-BrUTP or by anti-dsRNA antibodies showed the same subcellular localization of viral replication sites, essentially around the nucleus. The labeling appeared in small foci at 24 hpi (Fig. 3B and H) and in large areas which seem to be composed of numerous
small foci at 48 hpi (Fig. 3E and K). Superimposition of images showed extensive coincidence of the Br-RNA- and dsRNA-labeled sites with both P131 and P191 (Fig. 3C, F, I, and L). These results show that both P131 and P191 belong to the replication complex and must therefore be colocalized, although it was not possible to visualize their colocalization by dual labeling, since both the anti-P131 and anti-P191 antibodies originated from rabbits.

P131 and P191 proteins colocalize with ER-derived membranes but not with the Golgi membranes. The perinuclear localization of the replication proteins and of dsRNA suggested a possible association with the ER. To examine more precisely the nature of the membrane compartment involved, protoplasts from transgenic ER-GFP BY-2 cells were used. In these protoplasts, transgenic GFP containing an ER targeting signal is constitutively expressed so that the ER system is

FIG. 4 (Left). Localization of P131 and P191 in transgenic BY-2 protoplasts. Healthy (A and B) or PCV-infected (C to H) ER-GFP protoplasts expressing a GFP targeted to the ER were processed for fluorescence with primary antiserum against P131 at 24 hpi (B and D) or against P191 at 48 hpi (G). Similarly, healthy (I and J) or infected (K to P) Man1::GFP protoplasts expressing a GFP targeted to the Golgi apparatus were processed to visualize the P131 (J and L) or the P191 (O) accumulation sites. The digital superimposition of both fluorescent signals is shown in panels E, H, M, and P. Bar, 10 μm.

FIG. 5 (Right). Localization of P15 compared with that of Br-RNA, P131, and ER. The first row of images shows the same ER-GFP transgenic protoplast infected with T1, T2, and TRep-EG15 processed at 24 hpi. Green fluorescence of EGFP/P15 fusion protein is shown in panel A. Primary antibodies against Br-RNA and P131 were revealed, respectively, by Alexa 568 (red, B) and Alexa 633 (white, C) secondary antibody. Digital superimposition of images from the same 0.45-μm optical section shows Br-RNA and EGFP/P15 (D), P131 and EGFP/P15 (E), and P131 and Br-RNA (F). The third and fourth rows correspond to the same representative ER-GFP transgenic protoplast infected with T1, T2, and TRep-RFP15 (red) in two different optical sections at 48 hpi. Green ER-GFP fluorescence (G and J) and red RFP/P15 fluorescence (H and K) are superimposed in panels I and L. Bar, 10 μm.
visible in ER-GFP protoplasts. Typically, such protoplasts possess a three-dimensional green fluorescent network of continuous tubules and sheets that underlies the plasma membrane, courses through the cytoplasm, and links up with the nuclear envelope (7, 53). This distribution of green fluorescence was not modified when the protoplasts were fixed and processed for immunostaining (Fig. 4A). When PCV RNA-infected ER-GFP protoplasts were observed at 24 hpi, the cytoplasmic ER network largely disappeared and was replaced by fluorescent bodies which accumulated around the nucleus, whereas the nuclear envelope and the cortical ER network remained intact (Fig. 4C). These perinuclear structures, visible as early as 24 hpi, increased in size with the time of infection and invaded the nonperinuclear cytoplasm at 48 hpi (Fig. 4F). Immunostaining of protoplasts at 24 hpi with anti-P131 antibodies (Fig. 4D) or at 48 hpi with anti-P191 antibodies (Fig. 4G) showed aggregates of red fluorescent spots that accumulated around the nucleus. They increased in size and number with time postinfection, forming more diffuse packets, which mainly corresponded to ER fluorescent bodies, as is visible in the image superimposition (Fig. 4E and H). Healthy protoplasts treated similarly showed no fluorescence (Fig. 4B). Careful observation of these images reveals that both types of fluorescence are most concentrated in the center of the aggregates, but red foci corresponding to the proteins are also abundant at the perimeter of the aggregates. When the protoplasts were infected with a transcript of RNA-1 (T1) rather than viral RNA, the same ER aggregates were observed (not shown), demonstrating that the RNA-2-encoded proteins are not involved in the induced modifications of the ER and suggesting that viral RNA-1 replication per se or synthesis of one or more RNA-1 proteins is implicated in the ER modifications.

To further examine the nature of the membranes associated with the viral RNA replication complex, similar experiments were performed with protoplasts prepared from Man1::GFP transgenic BY-2 tobacco cells. These protoplasts constitutively express GFP fused to α,1,2 mannosidase, a protein confined to the cis side of the Golgi stacks (36). As shown (Fig. 4I), such protoplasts contain numerous green fluorescent points corresponding to individual Golgi stacks scattered throughout the cytoplasm and at the cell perimeter. In infected protoplasts (Fig. 4K and N), the number and distribution of the Golgi stacks did not differ from those of noninfected protoplasts. In the infected Man1::GFP protoplasts, the pattern of red immunofluorescence staining obtained with anti-P131 (Fig. 4L) or anti-P191 antibodies (Fig. 4O) rarely, if ever, coincided with Golgi stacks, as shown by the superimposition of images (Fig. 4M and P). Observation of the protoplasts with an increased gain of the confocal microscope photoreceptor often revealed, in addition to the fluorescent Golgi bodies, faint perinuclear Golgi fluorescence (Fig. 4N), which coincides with the perinuclear P191 localization (Fig. 4O) and which therefore probably corresponds to the P191/ER aggregates. This fluorescence may reflect decreased transport of the α,1,2 mannosidase–GFP from the ER to the Golgi apparatus, consequent to the ER perturbations induced by virus infection.

**P15 is not colocalized with P131 and the ER.** We showed previously that EGFP/P15 is not detected at viral RNA replication sites (15). To further explore the localization of P15 relative to the replication complex, we compared its distribution relative to P131 and to the ER. First, the transcript TRep-EG15, expressing an EGFP/P15 fusion protein, was inoculated to wild-type protoplasts together with the transcripts T1 and T2, corresponding to RNA-1 and -2 (15). At 24 hpi the pro-
toproplasts incubated with BrUTP were processed for immunostaining with anti-BrUTP (red, Fig. 5B) and anti-P131 (white, Fig. 5C) antisera. As predicted, the EGFP fluorescence corresponding to P15 (green, Fig. 5A) was associated neither with Br-RNA (Fig. 5D) nor with P131 (Fig. 5E), whereas Br-RNA and P131 colocalized quite well (Fig. 5F).

To visualize the intracellular distribution of P15, ER-GFP protoplasts infected with T1, T2, and TRep-RFP15, which expresses a Discosoma red fluorescent protein fused to P15 (RFP/P15), were observed at 48 hpi. P15 (red) rarely colocalized with the ER aggregates (green) but was present in the same areas and often appeared at the periphery of the condensate ER, as observed on the two optical sections of the same protoplast shown in Fig. 5G to L. When Man1::GFP transgenic protoplasts were similarly infected, the fluorescent punctate bodies of RFP/P15 did not coincide with the Golgi GFP labeling (not shown).

Effect of cerulenin and brefeldin A (BFA) on PCV replication. In the case of poliovirus and cowpea mosaic virus (CPMV), cerulenin, a fungal antibiotic which prevents de novo phospholipid synthesis, was shown to inhibit both viral RNA replication and proliferation of ER, suggesting that de novo membrane synthesis is needed to promote viral RNA replication (11, 19, 38). To test the effect of cerulenin on PCV replication, PCV-infected protoplasts were divided into four aliquots, and the drug was added to the incubation medium at three different concentrations. Northern blot analysis of RNA extracted at 48 hpi showed that the yield of viral RNAs was similar in samples incubated with 15, 30, or 45 μM cerulenin (Fig. 6, lanes 2 to 4) as in the sample incubated without cerulenin (Fig. 6, lane 1). The same result was obtained when the analysis was performed at 12 and 24 hpi (not shown). Since multiplication of grapevine fanleaf virus was found to be affected by a similar cerulenin treatment of BY-2 protoplasts (unpublished data), we conclude that cerulenin penetrates into protoplasts but has no effect on PCV replication. These observations suggest that the ER aggregates observed in PCV-infected protoplasts correspond to a reorganization of preexisting membranes induced by RNA replication rather than to proliferation of de novo-synthesized membranes. Similar findings have been reported for AMV and Tobacco mosaic virus (TMV) (11).

We also investigated the effect on PCV replication of BFA, a fungal metabolite which is currently used to study membrane traffic in eukaryotic cells. BFA inhibits the processing and transport of glycoproteins of enveloped viruses (12, 13, 54, 59), but it has also been shown to inhibit the genome replication of poliovirus, suggesting that the assembly and function of the replication complex require vesicular transport through the Golgi complex (26, 34). BY-2 protoplasts infected with viral RNA were incubated in the absence of BFA or with 10 or 30 μg of BFA/ml added immediately after infection or 6 h later. The accumulation of viral RNA was analyzed by Northern blotting at 24 or 48 hpi. Figure 6 (lanes 6 and 7) shows the results obtained when BFA was added immediately after infection of the protoplasts and when the RNA was extracted at 48 hpi. The amount of viral RNA detected in the presence of BFA was generally two times higher than in the absence of BFA (Fig. 6, lane 5). This suggests that disruption of the Golgi apparatus and its fusion to the ER induced by BFA (22, 46, 49) not only does not inhibit but in fact stimulates PCV RNA accumulation. Similar results were obtained under the other BFA treatment conditions mentioned above (data not shown).

ER modification in infected plants. To investigate whether the ER modifications observed in protoplasts also occur in cells from infected host plants, apical leaves of transgenic N. benthamiana plants expressing GFP targeted to the lumen of the ER were examined by confocal microscopy at 12 days postinfection. In the epidermal cells of mock-inoculated plants (Fig. 7A and B), the typical ER network, the nuclear envelope (Fig. 7A), and the ER tubules traversing the cytoplasmic threads (Fig. 7B) were green fluorescent. In epidermal cells of systemically infected leaves (Fig. 7C and D), perinuclear green fluorescent bodies (Fig. 7C, arrow) were visible in many cells, showing that the infection resulted in the formation of ER membrane aggregates such as those observed in infected protoplasts. Moreover, in most cases, the cortical ER network remained visible (Fig. 7D) and appeared not to have been modified.

Ultrastructural analysis of PCV-infected BY-2 protoplasts. To obtain further information concerning the PCV-induced cytopathic effects visualized by fluorescence microscopy, ultrathin sections of chemically fixed resin-embedded protoplasts harvested at 48 hpi were observed by electron microscopy (EM). At low magnification, infected protoplasts were easy to identify due to the presence of defined regions in which gross ultrastructural alterations of cellular components were detected (Fig. 8). These areas were generally located in the vicinity of the nucleus and clearly contained not only globose bodies (Fig. 8A, black and white arrowheads) but also modified ER (Fig. 8A, black asterisks), as suspected from the laser-scanning confocal microscopy studies. Identification of the ER membranes was based on the presence of ribosomes clearly visible on contiguous membrane regions shown at the higher magnification in Fig. 8B. In this figure, alteration of the ER is also readily apparent; instead of forming a well-organized network of bilayered membranes lamellae and tubules, ER cisternae appeared to be fragmented into pieces (ranging from 0.5 to 1.2 μm in length) due to severe constrictions at defined points that appear to be more electron dense (Fig. 8B and C, black arrows). Vesicles of 80 to 200 nm were frequently associated with the regions containing “pinched” ER frag-
ments and occasionally accumulated into small clusters near the ER fragments (Fig. 8C, D, and E, white arrows). These observations suggest that these vesicles may derive from the ER. The other consistent feature of PCV-infected BY-2 protoplasts was the presence in the cytoplasm of globose multivesicular bodies (MVB) (Fig. 8A, B, D, E, and F, white and black arrowheads) while others contained a central core of granular material surrounded by, at least, one layer of ER. The other consistent feature of PCV-infected BY-2 protoplasts was the presence of clusters of virions (Fig. 8B and F, double arrowheads). None of the other consistent features concerned essentially the ER or from de novo membrane synthesis. In the case of PCV replication differs from TMV and CPMV. We have shown by fluorescence microscopy that P131 or P191 mainly colocalizes in the central region of the green fluorescent bodies formed by the ER, although some spots corresponding to P131 or P191 were also detected at the periphery of these bodies, particularly at 48 hpi. On the other hand, electron microscopic images showed globose bodies containing numerous vesicles at the periphery of the areas of broken ER. As noted in the introduction, viral RNA replication sites frequently colocalize with vesicles that have different membranous origins (1, 4, 14, 17, 28, 29, 37, 48). The MVB associated with tombusvirus infections consist of a main body surrounded by ovoid vesicles measuring 80 to 150 nm in diameter. The presence of dsRNA within these vesicles and their association with proteins of the replicase complex provide evidence that the tombusvirus MVB are the sites of viral replication (1, 14, 48). Among the different cytopathic structures detected in PCV-infected BY-2 protoplasts, the MVB surrounded by a single membrane strongly resemble the MVB induced by cymbidium ringspot virus and tomato bushy stunt virus infections, which have been shown to be modified peroxisomes (10, 16, 47, 48). P15, which is essential for efficient viral RNA accumulation, has recently been shown to act as a suppressor of posttranscriptional gene silencing (P. Dunoyer et al., unpublished data). P15 contains at its C terminus the triplet SKL, which corresponds to the canonical type 1 peroxisomal targeting signal (PTS1). By fluorescence microscopy, GFP-labeled P15 has been detected in punctate structures which are

DISCUSSION

In this study, dual-labeled immunofluorescence was used to demonstrate that, in PCV-infected protoplasts, both P131 and P191 colocalize with dsRNA and neosynthesized RNA and thus are present at active replication sites, in full agreement with their assumed functions in PCV replication. This suggests that, like the brome mosaic virus 1a and 2a proteins (44, 45) and the AMV P1 and P2 proteins (55), the two PCV replication proteins colocalize and are components of the replication complex. On the contrary, P15 and the replication proteins never colocalized, confirming that P15 does not belong to the replication complex (15). Fluorescence microscopy of ER-GFP transgenic BY-2 protoplasts revealed extensive reorganization of the ER upon infection by PCV RNA compared to healthy protoplasts. These modifications concerned essentially the ER surrounding the nucleus and start with the formation of small bodies observed at early stages of infection. The replication proteins always localize with the ER bodies, and their parallel increase in number and size demonstrates that the modifications of the ER are correlated with the replication process. The same cytopathic modifications were produced in protoplasts infected with RNA-1 transcript alone, indicating that they were induced by one or more of the RNA-1-encoded proteins or directly by ongoing replication. Recruitment of the ER membranes for RNA replication has been reported for many plant viruses (8, 11, 32, 45, 50), although the perinuclear aggregates generally observed may originate from different compartments of the ER or from de novo membrane synthesis. In the case of PCV, the formation of large perinuclear ER aggregates in infected protoplasts and in infected N. benthamiana epidermal cells does not occur at the expense of the cortical compartment of the ER, in contrast to what was observed for TMV (42). On the other hand, treatment with cerulein had no effect on PCV replication in protoplasts, suggesting that, in contrast to CPMV, there is no requirement for de novo lipid biosynthesis (11). Furthermore, BFA did not inhibit PCV replication. Treatment of BY-2 tobacco cells with BFA results in the inhibition of the retrograde transport by disruption of COPI vesicles which deliver their cargo to the ER and produces a complete disruption of the secretory system (46). Therefore, COPI vesicles or functional exocytosis pathways are very unlikely to play a role in PCV replication. This is consistent with the fact that the distribution of the Golgi stacks was not significantly modified by PCV, at least at an early stage of infection. Indeed, our results showed that BFA treatment slightly enhanced PCV replication. The fact that, at a late time of infection, we visualized a faint Golgi-specific fluorescence which colocalized with ER aggregates suggests that PCV infection leads to a partial redistribution of the Golgi apparatus in the ER. Thus, BFA treatment may increase viral RNA synthesis because it contributes to membrane modifications induced by PCV or modifies the content of ER cellular factors involved in PCV replication. All of these results thus suggest that recruitment of membranes by PCV replication differs from TMV and CPMV.

From our electron microscopic observations, it appears that the ER near the nucleus is fragmented and vesiculated. This fragmentation probably safeguards the cortical ER and accounts for the condensed appearance of the perinuclear ER in fluorescence observations. A swelling of the ER at the ends of the broken fragments was also frequently observed in the electron micrographs. These may be budding sites and could be at the origin of some of the vesicles and aggregates of vesicles which were observed, although we cannot rule out the possibility that they also arise from modifications of other organelles present in the cytoplasm.
sometimes localized close to the ER aggregates. We have recently demonstrated that these punctate structures correspond to peroxisomes (Dunoyer et al., unpublished). We hypothesize that some of the PCV MVB described here are modified peroxisomes and contain P15. If so, the replication complexes are presumably present in one of the other cytopathic structures.

Our findings suggest that, like the flaviviruses (29, 8), ER modifications and MVB induced by PCV infection correspond to a complex replication factory in which defined areas are assigned special functions. Additional experiments will evidently be necessary to better define the nature of the ER modifications and of the MVB induced by PCV infection, to localize the replication complex more precisely, and to determine the role of the posttranscriptional gene silencing suppressor P15 in the process.

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