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Rubella Virus Replication Complexes Are Virus-Modified Lysosomes

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Replication complexes are membrane-bound cytoplasmic vacuoles involved in rubella virus (RV) replication. These structures can be identified by their characteristic morphology at the electron microscopy (EM) level and by their association with double-stranded (ds) RNA in immunogold labeling EM studies. Although these virus-induced structures bear some resemblance to lysosomes, their exact nature and origin are unknown. In this study, the localization of two lysosomal markers, lysosomal-associated membrane protein (Lamp-1) and acid phosphatase, relative to the replication complexes was examined by light and electron microscopy. Confocal microscopy using antibodies to dsRNA and Lamp-1 showed colocalization of these two markers in the cytoplasm of RV-infected cells. Immunogold labeling EM studies using antibodies to Lamp-1 confirmed that Lamp-1 was associated with RV replication complexes. EM histochemical studies demonstrated the presence of acid phosphatase in the vacuoles of RV replication complexes. Taken together, these studies show that RV replication complexes are virus-modified lysosomes.

INTRODUCTION

Rubella virus (RV) is the only member of the genus *Rubivirus* within the family *Togaviridae;* the genus *Alphavirus,* of which Sindbis virus (SIN) and Semliki Forest virus (SFV) are well-characterized members, is the only other genus within this family. RV is a single-stranded positive polarity RNA virus which shares a similar replication strategy with the alphaviruses. The RV 40S genomic RNA is 9757 nucleotides in length and contains two open reading frames (ORF)(Dominguez *et al.,* 1990). The 5′ proximal ORF encodes the nonstructural proteins while the 3′ ORF encodes the RV structural proteins, the capsid C, and envelope proteins, E1 and E2; the RV structural proteins are translated from a 24S subgenomic RNA (Oker-Blom *et al.,* 1984; Dominguez *et al.,* 1990). During RV infection, the 40S genomic RNA provides the template for the synthesis of a complementary RNA which in turn acts as a template for the production of both the 40S genomic and 24S subgenomic RNA. This transcription process also produces a fully double-stranded (ds) replicative form (RF) of 19–20S and a partially dsRNA replicative intermediate (RI) of 21S (Wong *et al.,* 1969; Sedwick and Sokol, 1970). The 40S genomic and 24S subgenomic RNAs can be detected as early as 12 h postinfection (p.i.) with peak synthesis reported at 26 h p.i. (Hemphill *et al.,* 1988).

A striking feature of RV infection is the presence of cytoplasmic membrane-bound structures (Lee *et al.,* 1992). These structures have been termed replication complexes because they are morphologically similar to the replication complexes previously described in alphavirus-infected cells (Grimley *et al.,* 1968; Friedman *et al.,* 1972). In RV-infected cells, these structures comprise vacuoles which are lined internally with membrane-bound vesicles measuring approximately 60 nm in diameter. These vesicles contain thread-like inclusions and are usually attached to the surrounding vacuole membrane via a membranous neck. Furthermore, RV replication complexes are consistently associated with the rough endoplasmic reticulum (RER) which tends to follow the contours of the replication complex vacuole and are observed in RV-infected cells as early as 8 h p.i. Peak numbers of RV replication complexes occur at 24 h p.i., coinciding with maximum viral titers (Lee *et al.,* 1992).

Immunogold labeling electron microscopy (EM) using antibodies to dsRNA have shown localization of dsRNA in the membrane-bound vesicles which line the RV replication complexes (Lee *et al.,* 1994). The detection of dsRNA within the vesicles indicated the presence of RV RI and RF RNA and demonstrated that these structures are sites of viral replication. Parallel studies using SFV-infected cells also showed the presence of dsRNA in alphavirus replication complexes, suggesting that they share similar functions to RV replication complexes (Lee *et al.,* 1994).

While it has been shown that replication complexes in RV-infected cells are sites of viral replication, the biogenesis of these virus-induced structures is unknown. The presence of degenerating material within the vacuole of the complex suggests that it is lysosomal in nature (Lee *et al.,* 1992). For the alphaviruses, cytochemistry studies

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using endocytic tracers and lysosomal markers have demonstrated the endosomal and lysosomal origin of alphavirus replication complexes (Froshauer *et al.,* 1988). As RV and alphavirus replication complexes are similar in morphology and function, it is most likely that they share a similar biogenesis. To examine this question, we used immunofluorescence and EM techniques to study the location of the lysosomal markers, lysosomal-associated membrane protein (Lamp-1) (Hunziker and Gueze, 1996) and acid phosphatase (Griffiths, 1979), relative to the RV replication complex.

MATERIALS AND METHODS

Cells and virus

Vero cells were grown in medium 199 (M199) supplemented with 5% (v/v) fetal bovine serum (FBS) (Flow Laboratories, Australia) and were infected with the Putnam strain of RV as described previously (Lee *et al.,* 1992). Cells inoculated with RV were maintained in M199 supplemented with 0.1% (w/v) bovine serum albumin (BSA).

Antibodies

Mouse monoclonal and rabbit polyclonal antibodies against human Lamp-1 were kind gifts of Professor M. Fukuda (Cancer Research Center, La Jolla, CA). Guinea pig polyclonal antibody to dsRNA produced by Lee *et al.,* (1994) was used as a marker for RV replication complexes. Fluorescein isothiocyanate (FITC)-conjugated swine anti-guinea pig antibodies and FITC-conjugated sheep anti-mouse antibodies were from Dako (Copenhagen, Denmark) and Silenus (Melbourne, Australia), respectively. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antibodies against mouse or rabbit were obtained from Sigma Chemical Company (St. Louis, MO). Protein A–gold (10 nm) was from Amersham (UK).

Immunofluorescence assay

Vero cells grown on glass coverslips were infected with RV at a multiplicity of infection (m.o.i.) of 10. At 24 h p.i., cells were fixed with chilled acetone:methanol (1:1) for 10 min. Alternatively, cells were fixed with 4% (w/v) paraformaldehyde for 20 min and then permeablized with 0.05% (v/v) Triton X-100 for 20 min. The fixed cells were incubated in blocking buffer (phosphate buffered saline (PBS)/0.1% (w/v) BSA) for 30 min at room temperature and then reacted with anti-dsRNA (1/20 dilution in blocking buffer) or anti-Lamp-1 (1/25 dilution in blocking buffer) for 1 h at room temperature. The cells were washed several times in PBS and the appropriate secondary antibodies then added. After 1 h at room temperature, the cells were washed in PBS, mounted, and viewed using a Axiovert Microscope MC 500 (Zeiss, Germany).

For double-labeled immunofluorescence studies, two sequential immunofluorescence procedures were applied. Mounted coverslips were viewed with a Bio-Rad MRC-500 confocal scanning laser microscope using an argon ion laser with excitation wavelength at 488 nm. A red/green double detector filter system was used to detect emitted fluorescence at the fluorescein/rhodamine optimal ranges ($\lambda = 527–565$ and $\lambda > 600$ nm). The two fluorochromes used were TRITC and FITC. Double labeling with two fluorochromes enabled colocalization studies. Images were stored in a digital form and photographed using Mitsubishi CK 100L film.

Conventional thin section electron microscopy

RV- and mock-infected Vero cells were harvested at 24 h p.i. and embedded for EM as described previously (Lee *et al.,* 1992). Briefly, harvested cells were pelleted, fixed in 2% (w/v) glutaraldehyde/Sorensen phosphate buffer, pH 7.3 (SPB), followed by fixation in 1% (w/v) osmium tetroxide (OsO_4)/SPB prior to embedding in LX-112 resin (Ladd Research Industries Inc., U.S.A). Ultrathin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips CM12 electron microscope.

Postembedding immunogold labeling

RV- and mock-infected Vero cells were harvested at 48 h p.i. using 0.125% (w/v) trypsin/0.02% (w/v) versene as described previously (Lee *et al.,* 1992). The cells were centrifuged for 5 min at 2000*g,* washed in SPB, and fixed in 0.25% (w/v) glutaraldehyde/ 2% (w/v) paraformaldehyde/SPB for 1 h at 4°C. After washing several times in SPB, the cells were partly dehydrated in 70% (v/v) ethanol and infiltrated with a mix of LR White resin and 70% (v/v) ethanol (2:1). The cells were then infiltrated with several changes of 100% (v/v) LR White before being embedded in gelatin capsules for 24 h at 60°C. Ultrathin sections with silver to gold interference color were mounted on uncoated 200-mesh gold grids. For immunogold labeling, the grids were incubated in blocking diluent (SPB / 1% (w/v) BSA) for 15 min before being transferred to drops of rabbit polyclonal antibody to Lamp-1 (1/50 dilution in PBS/ 0.5% (w/v) BSA). After 1 h at 37°C, grids were washed several times and then incubated with protein A–gold (10 nm) (1/20 dilution in SPB/ 0.5% (w/v) BSA) for 1 h at room temperature. Excess protein A–gold was removed by several washes in SPB followed by washes in distilled water. Sections were then stained with uranyl acetate and lead citrate and viewed as described above.

Acid phosphatase assay

A modified Gomori acid phosphatase assay as described by Griffiths (1979) was used to demonstrate acid phosphatase activity in lysosomes. RV- and mock-infected Vero cells were harvested at 24 h p.i. and fixed in 0.5% (w/v) glutaraldehyde in PIPES (0.12 M 1,4-pipera-

zinediethanesulfonic acid, pH 7.3) buffer for 30 min at 4°C and then rinsed several times in the PIPES buffer before preincubation in acetate buffer (0.05 M acetic acid, 0.05 M sodium acetate, pH 5.0) for 15 min at 37°C. The cells were then incubated in acetate buffer containing 0.004 M lead nitrate and the substrate sodium β-glycerophosphate (0.3% w/v) for 45 min at 37°C. To determine the specificity of the assay, 0.001 M sodium fluoride (NaF) was included in the acetate buffer in parallel experiments as NaF is known to quench the activity of acid phosphatase (Griffiths, 1979). Samples were then pelleted and refixed in 2.5% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde for 1 h at 4°C. After several rinses in SPB, the samples underwent secondary fixation in 1% (w/v) OsO_4. Fixed cell pellets were processed for thin section EM as described above prior to embedding in Spurr resin (Ladd Research Industries Inc.).

RESULTS

Confocal microscopy studies of Lamp-1 and dsRNA localization

Anti-Lamp-1 was initially used in an immunofluorescence assay to determine the intracellular localization of Lamp-1 in Vero cells. Examination of both mock- and RV-infected cells revealed cytoplasmic fluorescent staining with anti-Lamp-1 (results not shown). No significant difference was observed in the pattern of fluorescent staining between the mock- and RV-infected preparations, showing that the distribution of Lamp-1 had not been affected by RV replication.

We have previously shown by immunofluorescence assay and immunogold labeling EM using anti-dsRNA that RV replication complexes are sites of RNA synthesis. Thus, the anti-dsRNA can be used as a convenient marker for RV replication complexes. When this marker is used in conjunction with anti-Lamp-1 in double labeling immunofluorescence studies, colocalization of the two markers would indicate that RV replication complexes and lysosomes represent the same organelles.

Mock- and RV-infected cells were double labeled with anti-dsRNA and anti-Lamp-1 followed by staining with FITC- and TRITC-conjugated secondary antibodies, respectively (Figs. 1A and 1B). When anti-dsRNA was used, a distinct cytoplasmic fluorescent pattern was observed in RV-infected preparations (Fig. 1A) similar to that reported previously (Lee *et al.,* 1994). No specific fluorescence was detected in mock-infected cells (results not shown). When digital superposition of the colorized dsRNA and Lamp-1 was performed, colocalization of green (Fig. 1A) and red (Fig. 1B) signal in a single pixel produced yellow (Figs. 1C and 1D). Digital superpositioning of mock-infected cells revealed only the red signal representing anti-Lamp-1 staining (results not shown). The colocalization of dsRNA and Lamp-1 in the cytoplasm of RV-infected cells indicated that RV replication complexes are modified lysosomes. Interestingly, following superpositioning of RV-infected cells, some red signal was still observed indicating that not all lysosomes are modified to be RV replication complexes.

EM localization of Lamp-1

To confirm that RV replication complexes are lysosomal in nature, postembedding immunogold labeling EM using anti-Lamp-1 was employed. We have previously shown by conventional thin section EM studies that RV replication complexes can easily be recognized by such characteristic features as vesicles which line the inner vacuole membrane, the intimate association with the RER, and the presence of degenerating material (Lee *et al.,* 1992) (Fig. 2A). For postembedding immunogold labeling EM, membrane features within the cell are not well defined due to the omission of osmium tetroxide in the cell preparation. Nevertheless, RV replication complexes can still be identified with confidence by their size, shape, and characteristic close association with the RER (Figs. 2B and 2C). After immunogold labeling, gold particles were observed in the cytoplasmic vacuoles of the RV replication complexes (Figs. 2B and 2C). Gold particles also labeled lysosomes in RV- and mock-infected cells but these organelles do not appear to be replication complexes because no close association with the RER was observed (results not shown).

To investigate whether replication complexes retain some lysosomal function, RV replication complexes were assayed for the presence of acid phosphatase, another lysosomal marker (Griffiths, 1979). Mock-infected and RV-infected Vero cells were harvested at 24 h p.i. and assayed for acid phosphatase using the modified Gomori method (Griffiths, 1979). Lead phosphate deposits, the electron dense marker of acid phosphatase, were detected in the vacuoles of RV replication complexes (Fig. 3A). The replication complexes can be identified by size, shape, and characteristic association with the RER. Mock-infected cells show similar electron dense deposits in lysosome-like vacuoles (results not shown). To demonstrate specificity of acid phosphatase activity, RV- and mock-infected cells were pretreated with NaF in parallel studies. NaF quenches the acid phosphatase activity, resulting in the absence of lead deposits within the vacuoles of RV replication complexes (Fig. 3B) and the vacuoles of lysosomes in both RV-infected and mock-infected cells (results not shown).

DISCUSSION

RV infection of Vero cells induces the formation of replication complexes which are characterized as membrane-bound vacuoles lined internally with vesicles (Lee *et al.,* 1992). While RV replication complexes have been shown to be sites of viral replication, the origin and nature of these structures have not been

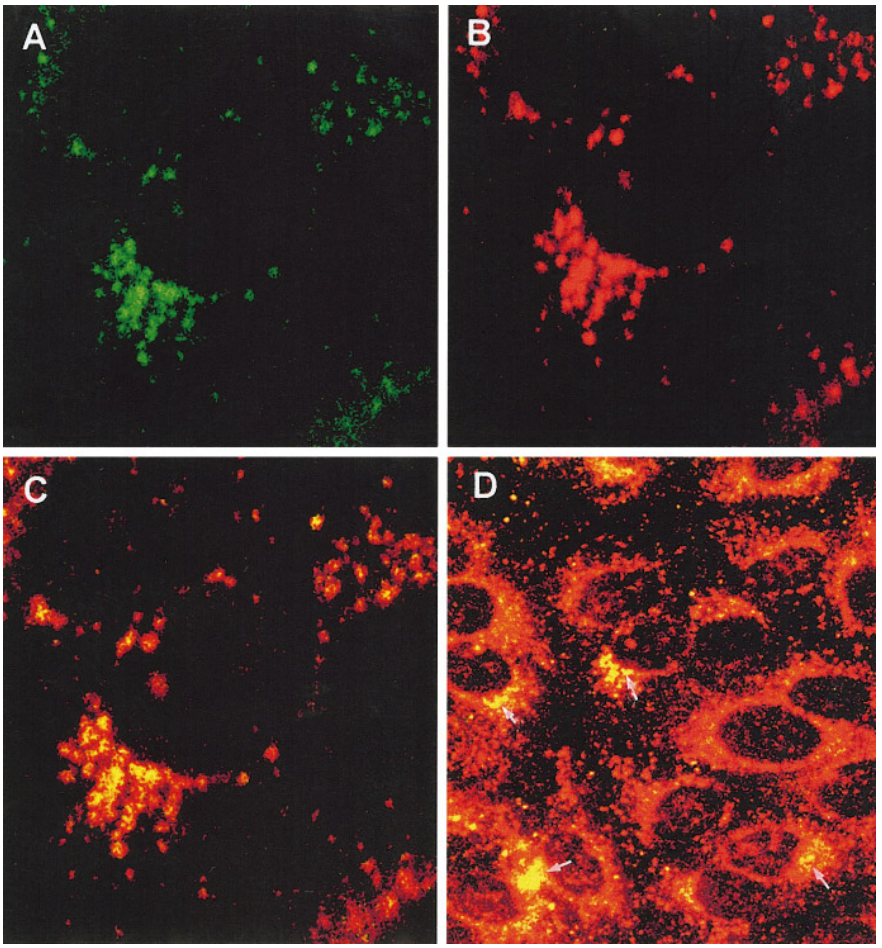

FIG. 1 Colocalization of dsRNA and Lamp-1 in RV-infected cells by confocal microscopy. Vero cells were infected with RV at a m.o.i. of 1–10 and at 24 h p.i. cells were fixed in acetone:methanol (1:1) and processed for double-labeled immunofluorescence using (A) polyclonal guinea pig antibodies to dsRNA and (B) monoclonal antibodies to Lamp-1 (C) Colocalization of Lamp-1 and dsRNA was observed following digital superposition of the two fluoroprobes. (D) Lower magnification of the digital superposition of the two fluoroprobes; note other areas (arrows) where dsRNA and Lamp-1 are colocalized.

elucidated. RV replication complexes appeared to share some lysosomal features as they were similar in size and shape to lysosomes and frequently contained degenerating material (Lee *et al.,* 1992). In this study, we employed confocal microscopy to show colocalization of markers for RV replication complexes and lysosomes. When anti-Lamp-1 and anti-dsRNA, a marker for RV replication complexes, were used in dual label immunofluorescence microscopy, both markers were found to be colocalized. Furthermore, immunogold labeling EM studies demonstrated the presence of Lamp-1 in RV replication complexes. Finally, histochemical EM studies revealed the presence of acid phosphatase, another lysosomal marker, in the vacuole of RV replication complexes. It appears that these virus-induced structures preserved at least some of the functions of lysosomes. The fact that two distinct lysosomal markers are directly associated with RV replication complexes clearly shows that RV replication complexes are modified lysosomes.

Immunofluorescence studies using anti-Lamp-1 revealed that RV infection did not alter the distribution of Lamp-1 in infected cells with no difference in the cytoplasmic staining observed between mock and infected preparations. This suggests that RV replication does not alter the biogenesis of cellular lysosomes. It is likely that RV replication complexes are derived from existing lysosomes rather than involving the production of new lysosomes following infection. The confocal microscopy studies demonstrated that not all lysosomes are utilized by RV as sites of replication.

While EM studies using anti-dsRNA have indicated the presence of RV RI and RF RNA in replication complexes (Lee *et al.,* 1994), any association of RV-specified proteins with these structures has yet to be determined. For the alphaviruses, studies have shown the presence of nucleocapsids in close proximity to alphavirus replication complexes (Froshauer *et al.,* 1988; Lee *et al.,* 1992). Moreover, the nonstructural proteins (nsP1–nsP4) have been detected in replication complexes of alphavirus-infected cells (Froshauer *et al.,* 1988; Peränen and Kääriäinen, 1991). Interest-

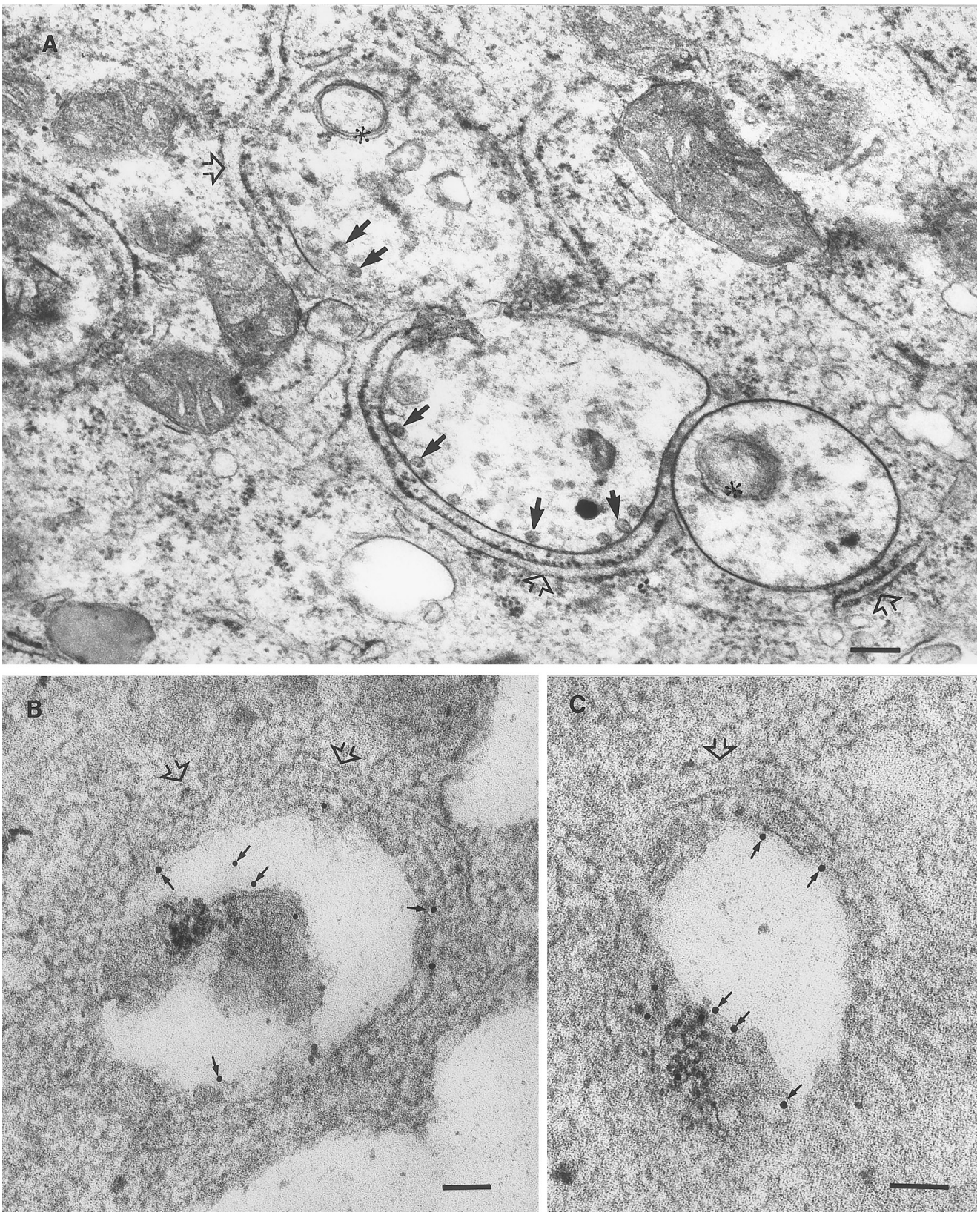

FIG. 2. Characterization of RV replication complexes. Vero cells infected with RV at a m.o.i. of 10 were harvested and processed for (A) conventional thin section EM or for (B, C) postembedding immunogold labeling EM using polyclonal rabbit antibodies to Lamp-1 as described under Materials and Methods. (A) Typical RV replication complexes. Note the characteristic vesicles (solid arrow), the presence of degenerating material (asterisk), and the intimate association of RER (open arrow) with the surrounding membrane of the replication complex. Bar, 200 nm. (B, C) Gold particles (solid arrow) can be seen in the vacuole of the replication complex. Note the characteristic close association of the RER (open arrow) with the membrane-bound vacuole and the presence of degenerating material within the vacuole. Bars, 100 nm.

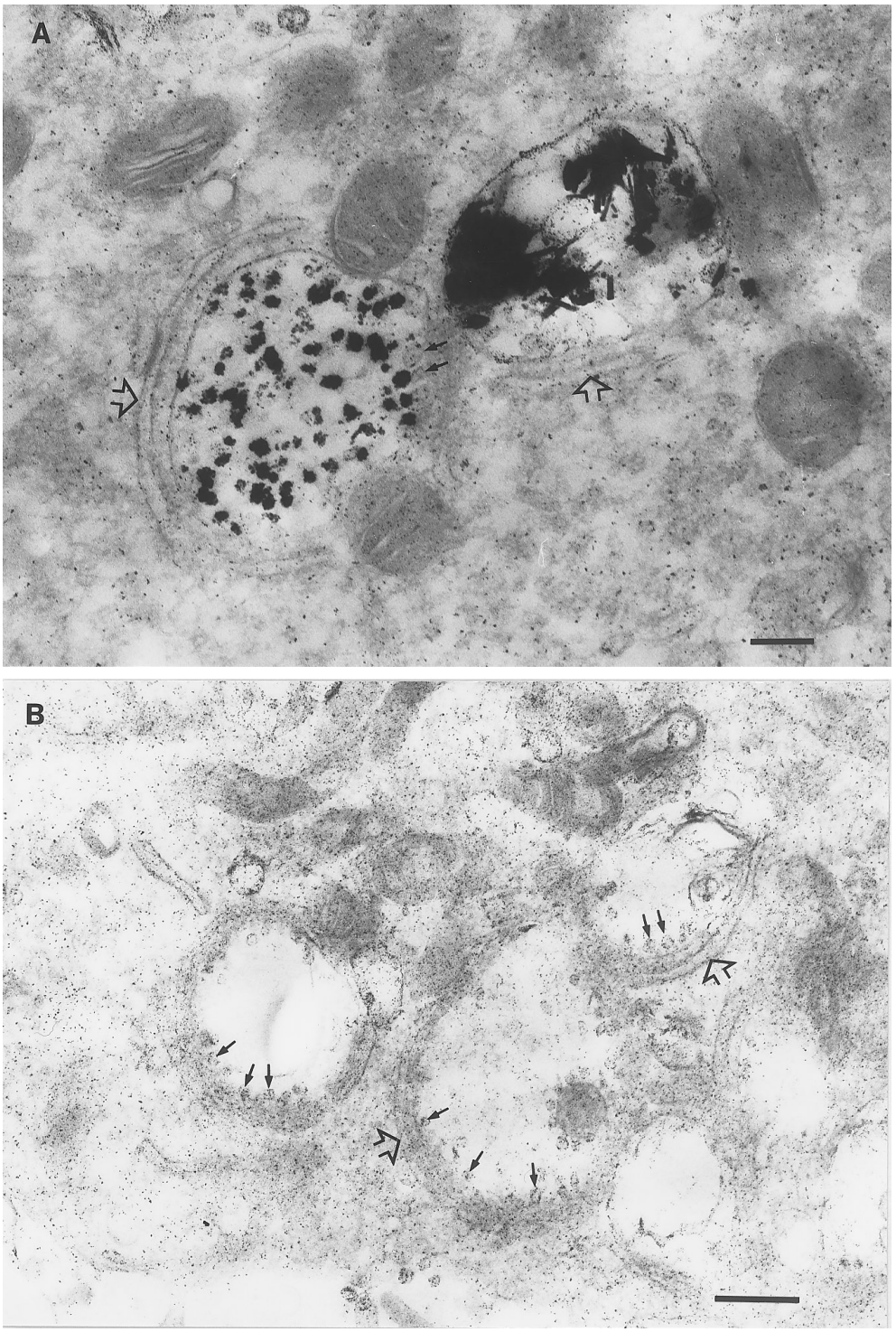

FIG. 3. Acid phosphatase assay of RV-infected Vero cells. (A) Electron micrograph of unstained RV-infected cell preparation showing dense black deposits of lead phosphate, the marker of acid phosphatase, within the vacuoles of RV replication complexes. The replication complex can be identified by their shape, size, and characteristic association with the RER (open arrows). Note the vesicles (small solid arrows) within the vacuole of the replication complex. Bar, 200 nm. (B) Electron micrograph of unstained RV-infected cell preparation treated with NaF, a known inhibitor of acid phosphatase activity, showing that the NaF has prevented formation of dense black deposits of lead phosphate within the vacuoles of RV replication complexes. Note the vesicles (solid arrows) within the vacuoles of the replication complexes and the close association of the RER (open arrows) to the complexes. Bar, 400 nm.

ingly, SFV transfection studies by Peränen *et al.,* (1995) using nsP1 cDNA found colocalization of nsP1 with endosomes and lysosomes. For RV, the role the nonstructural proteins play in the biogenesis of replication complexes is unknown.

The findings from our studies demonstrated that RV rep-

lication complexes appear to share similar biogenesis to their alphavirus counterparts. For the alphaviruses, replication complexes have been found to be endosomal and lysosomal in origin (Froshauer *et al.,* 1988; Peränen *et al.,* 1995). Endosomes and lysosomes play an important role in the endocytic pathway and have been shown to be involved in the entry and uncoating of many enveloped viruses (Helenius *et al.,* 1989). However, the use of endosomes and lysosomes as sites of viral replication appears to be unique to the togaviruses. The viral-induced double membrane-bound vesicles that line the vacuoles of RV and alphavirus replication complexes most likely represent the precise sites of viral RNA replication (Lee *et al.,* 1994). Similar virus-induced vesicles have been reported as replication complexes for the flaviviruses and picornaviruses (Ng *et al.,* 1983; Bienz *et al.,* 1990). However, the replication complexes of these viruses appear to be derived from or accumulate in the endoplasmic reticulum rather than in endosomes or lysosomes.

Our studies confirmed that RV replication complexes are virus-modified lysosomes indicating that lysosomes provide part of an important matrix for viral replication. Our confocal microscopy studies using anti-Lamp-1 and anti-dsRNA also revealed that not all RV replication complexes are lysosomal in nature (results not shown). It is most likely that some RV replication complexes may be endosomal in origin similar to that observed for the alphaviruses. Endosomal markers are currently being used to further investigate the biogenesis of RV replication complexes.

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