EJ072200000009

J Microbiol Immunol Infect
2000;33:9-13

Identification of virus-specific vesicles in Giardiavirus-infected *Giardia lamblia*

Shiou-Jeng Ong, Jung-Hsiang Tai[1]

Department of Parasitology, College of Medicine, National Taiwan University, and [1]Division of Infectious Diseases, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, ROC

Received: September 9, 1999 Revised: October 6, 1999 Accepted: October 14, 1999

Giardiavirus (GLV), which infects the parasitic protozoan *Giardia lamblia*, is a nonsegmented double-stranded (ds) ribonucleic acid (RNA) virus. We previously purified two distinct types of related GLV from infected *G. lamblia*, and showed differential export of one of the viruses from infected cells. In the present study, fractionation of cell lysate was performed, revealing the presence of viruses in the membranous fraction. Distribution of viral antigens in the infected cells was examined by immunocytochemistry. The signal was enriched in certain regions of the cytoplasm, suggesting that a portion of GLV is confined to certain cellular compartments. A significantly reduced signal was also detected in the nuclei. We directly observed the viruses in the infected cells by electron microscopy. Consistent with previous observations, virus-like particles were clearly observed in some membranous vesicles in the cytoplasm at 48 h postinfection, and virus-like particles were again seen in the cytoplasm and then in the nuclei toward the late phase of virus infection. The virus-associated vesicles and some electron-dense nuclear structures were only observed in virus-infected cells, suggesting that virus infection may induce ultrastructural alteration of *G. lamblia*.

Key words: Giardiavirus (GLV), *Giardia lamblia*, virus-specific vesicle, electron microscopy

Giardiavirus (GLV) is a nonsegmented double-stranded (ds) ribonucleic acid (RNA)virus that infects the intestinal parasite *Giardia lamblia* [2]. GLV's 6.2-kb double-stranded (ds) RNA genome is composed of an overlapping capsid protein gene *(cap)* and an RNA-dependent RNA polymerase gene *(pol)* [3]. GLV is the only member of Totiviridae that can infect virus-free susceptible cells [4]. GLV-infected cells produce a full-length single-stranded viral RNA, which is sufficient for the virus to complete its replication cycle upon transfection into virus-free host cells [5,6], indicating that the single stranded viral RNA serves as a messenger RNA as well as the replication intermediate of the virus. Exploiting this feature, an RNA transfection system for *G. lamblia* was established using *in vitro* transcribed recombinant viral RNA [7,8].

The time course of GLV infection in *G. lamblia* reveals that the virus enters susceptible host cells via endocytosis, in which the lysosomal-like peripheral vacuoles serve as translocation ports for the virus to reach the cytoplasm [9]. In a previous study, virus-like particles were observed in peripheral vacuoles, and a few of the particles reached the cytoplasm as early as 2 h postinfection. Replication of viral RNA was detected in the cytoplasm as early as 4 h postinfection and accumulated there rapidly. Viral RNA was detected in both nuclei at 72 h post-transfection [10]. In another study, GLV was actively released into the medium to initiate a second round of infection. It was suggested that release of GLV from infected *G. lamblia* trophozoites might be mediated by exocytosis [1].

Virus infection in *G. lamblia* does not cause apparent growth inhibition in infected cells. However, the subtle morphological and cellular changes evident within the host cells during virus infection have not been characterized. In the present study, we used purified GLV to infect virus-free *G. lamblia* trophozoites and examined the cellular appearance of the virus in infected cells by electron microscopy. We found that virus-like particles are confined to certain vesicles by a double-layered membrane in the cytoplasm during early infection, and spread to the cytoplasm in heavily infected cells later. GLV enters the nuclei and produces profound changes in the nuclear structure in the late phase of infection. Our observations provide a detailed insight into virus replication and transportation

Corresponding author: Dr. Jung-Hsiang Tai, Division of Infectious Diseases, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, ROC.

at the cellular level.

Materials and Methods

Culture

Virus-free and virus-infected *G. lamblia* WB strains were maintained in axenic cultures as previously described [2].

Virus purification

Virus was purified from virus-infected *G. lamblia* WB cells as previously described [1]. RNA was extracted from purified virus as previously described [11], and analyzed by agarose gel electrophoresis as described elsewhere [12]. Proteins from viral samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described [12].

Immunomicroscopy

Anti-GLV serum used in this experiment was obtained from our previous study [1]. Subcellular localization of the viral antigens was examined by immuno microscopy as previously described [9], except that goat-anti-mouse immunoglobulin conjugated to 1 nm gold particles (Auroprobe one GAM) was used as secondary antibody, and the signal was enhanced by silver reagent according to the supplier's protocol (Amersham).

Electron microscopy

Purified viral samples were negatively stained by 0.5% uranyl acetate and examined by a transmission electron microscope (Joel 2000). Virus-infected cells were fixed and processed for electron microscopy as previously described [9].

Results

We previously demonstrated the presence of two distinct types of GLV, GLV[p100] and GLV[p95], in *G. lamblia* WB trophozoites [1]. GLV[p100], which consists of a major capsid protein p100, was selectively secreted into medium. GLV[p95], which consists of a major capsid protein p95, was retained in the infected cells. In the present study, viruses were first purified from the soluble cytosolic fraction and the membranous fraction of *G. lamblia* WB cells by CsCl gradient centrifugation. Each gradient was divided into seven density fractions from top to bottom. An aliquot of viral sample from each density fraction was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and agarose gel electrophoresis (Fig. 1). Very little viral RNA was found in samples purified from

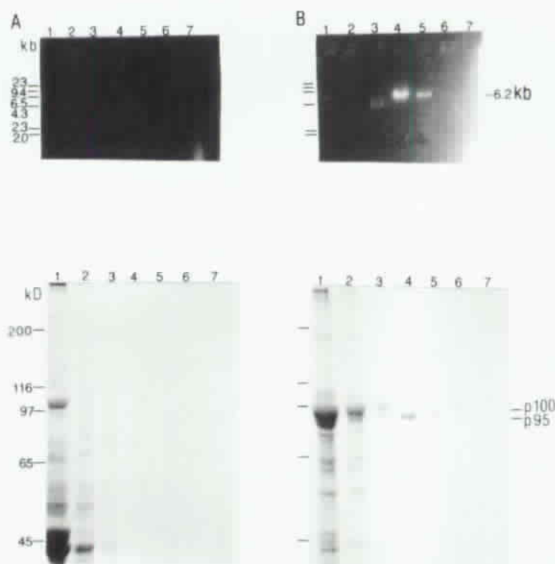

Fig. 1. Subcellular fractionation of GLV. Lysate from virus-infected *G. lamblia* WB trophozoites was separated into soluble cytosolic fraction (**A**) and membranous fraction (**B**) by centrifugation. Viruses were then purified separately from these fractions by CsCl gradient centrifugation. An aliquot of each gradient was taken from top to bottom (lanes 1 to 7) for RNA (top) and protein (bottom) analysis. The capsid proteins of GLV[p100] and GLV[p95] are indicated by p100 and p95, respectively. The 6.2-kb viral genomic ds RNA is also indicated.

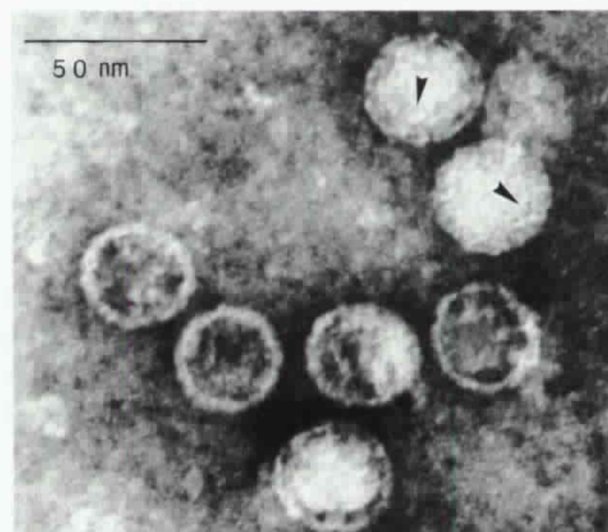

Fig. 2. Electron micrograph of GLV. Virus-like particles were stained by 0.1% uranyl acetate. Bar represents 50 nm. Capsomere structures are indicated by arrows.

the cytosolic fraction of the cell lysate (Fig. 1A, top panel, lanes 1-7). Protein analysis of these samples revealed abundant p100 in fraction one, the lowest density fraction (Fig. 1A, bottom panel, lane 1), but

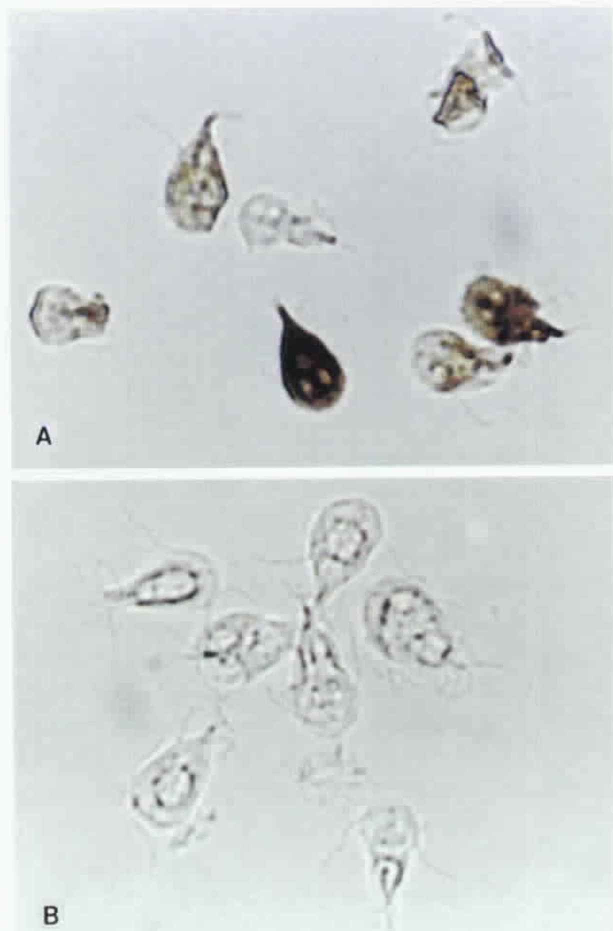

Fig. 3. Subcellular localization of GLV by immunocy-tochemistry. *G. lamblia* WB cells were fixed prior to virus infection (**B**) or at 48 h postinfection (**A**), and sequentially reacted with anti-GLV serum (1,000 fold dilution) and secondary antibody conjugated with 1 nm immunogold particles (1,000 fold dilution). The signal was amplified by silver enhancement.

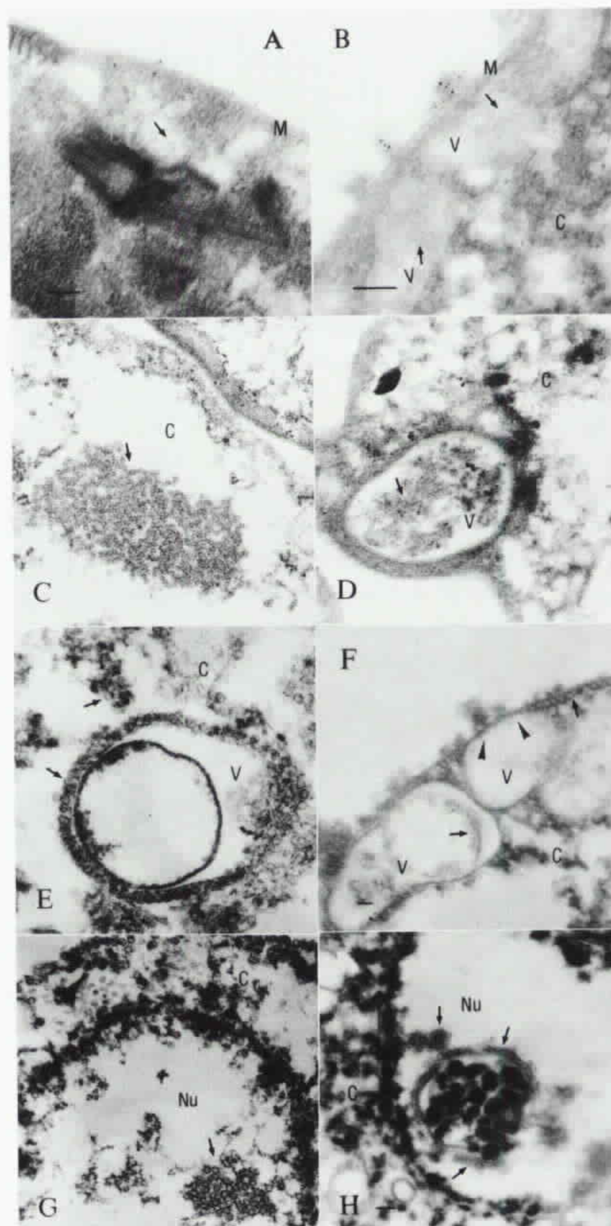

Fig. 4. Electron micrographs of virus-infected *G. lamblia* WB cells. Virus-infected *G. lamblia* WB cells were fixed at 24 h (**A**), 48 h (**B**), 72 h (**C-F**) and 96 h (**G-H**) postinfection for electron microscopic observations. The sites of virus-like particles are indicated by arrows. Abbreviations are shown as: C = cytoplasm; M = plasma membrane; V = vesicle; Nu = nucleus. Arrowheads in F indicate the site of fusion between the plasma membrane and a virus-associated vesicle. The bar in each micrograph represents 100 nm.

not in other density fractions (Fig. 1A, bottom panel, lanes 2-7). Virus-like particles were observed in negatively stained samples obtained from this fraction using electron microscopy (data not shown), suggesting that most of the virus-like particles obtained from soluble cytosolic fraction are empty capsids. By contrast, the 6.2-kb viral ds RNA was enriched in fractions four and five in viral samples from the membranous fraction of the cell lysate (Fig. 1B, top panel, lanes 4 and 5). A broader but fainter 3-kb to 4-kb RNA band, which probably consists of several deletion mutants of viral genomic ds RNA [1], was also detected in the viral sample from fraction three (Fig. 1B, top panel, lane 3). A much smaller amount of the 6.2-kb ds RNA was seen in fraction one (Fig. 1B, top panel, lane 1). Protein analysis of these viral fractions revealed abundant p100 and a minor amount of p95 in fractions one to three (Fig. 1B, bottom panel, lanes 1-3). By contrast, p95 was more abundant than p100 in fractions four and five (Fig. 1B, bottom panel, lanes 4 and 5). These observations suggest that most of the complete virus-like particles are associated with the membranous fraction, whereas most of the virus-like particles

associated with the cytosolic fraction are empty capsids.

The morphologies of the membrane-bound viruses purified by multiple rounds of CsCl gradient centrifugation were observed by electron microscopy after negative staining of the viral sample (Fig. 2). Two intact icosahedral-shaped virions with a diameter of 33 nm were observed. Several other incomplete or partially-damaged virus-like particles were also observed. Although virus-like particles from purified GLV were previously reported by us and by others [1-3], this electron micrograph revealed capsomere structures on the surface of the virions that were not clearly seen before.

We next performed immunocytochemistry using anti-GLV serum to localize the viral antigens in infected cells. Viral antigens were detectable to various extents in most cells at 48 h postinfection (Fig. 3A). The staining intensity in individual cells probably reflects the degree of virus infection. More even staining was observed in a heavily infected cell that exhibited a much stronger signal in its cytoplasm and a relatively lighter signal in both nuclei. In some other cells, the staining in the cytoplasm was reduced and unevenly distributed, and the nuclei were not stained. The signal was apparently more enriched in certain areas of the cytoplasm. The specificity of the viral signals in infected cells was confirmed by immuno staining of cells fixed prior to virus infection, which exhibited no staining in all cells examined (Fig. 3B). Similarly, no staining was observed in virus-infected cells when normal mouse serum was used in the staining process (data not shown). In conjunction with an earlier observation [10], these results suggest that assembly and replication of GLV may take place in the cytoplasm, and a fraction of the virus translocates into the nuclear compartment during the late phase of infection.

During the same infection course, virus-like particles were first detected in virus-infected cells at 24 h by electron microscopic observation (Fig. 4A). At this time point, a few virus-like particles were barely seen in very few cells in the cytoplasm. The cellular morphology of infected cells and uninfected cells was very similar, and it was very difficult to find virus-like particles due to the low contrast in cellular morphology. At 48 h postinfection (Fig. 4B), virus-like particles were more frequently seen in infected cells where they were confined within certain vesicles by a double-layered membrane. These vesicles were usually found near the plasma membrane. At 72 h postinfection (Figs. 4C-F), the cellular structure was severely damaged in heavily infected cells. Numerous aggregated virus-like particles were seen in the cytoplasm, and some of these virus-like particles were seen beneath the plasma membrane (Fig. 4C). The cellular contents were largely lost in these heavily infected cells, suggesting that the cells were disintegrated by virus infection. By contrast, in less severely-infected cells, in which more cellular contents were preserved, numerous membrane bound vesicles were seen in the cytoplasm, and virus-like particles were confined within some of these vesicles (Fig. 4D). Occasionally, virus-like particles were associated with a second type of vesicle that contained an outer double-layered membrane and an inner double-layered membrane. Virus-like particles were seen between the two layers of the outer membrane of the vesicle (Fig. 4E). Fusion of the virus-related vesicles with the plasma membrane was also observed (Fig. 4F), suggesting that the virus may utilize this type of vesicle for export.

At 96 h, most of the cells were heavily infected and virus-like particles were seen in both nuclei (Fig. 4G,H). The nuclear structures were largely destroyed in these cells. Virus-like particles in aggregated form were also seen in the nuclei (Fig. 4G). Interestingly, numerous membrane-bound electron dense structures were also seen in the nuclei (Fig. 4H). Virus-like particles were found surrounding these structures. These structures were not seen in noninfected cells. Our observations suggest that virus infection in *G. lamblia* WB cells induces an ultrastructural change of cellular morphology, and eventually leads to the disintegration of infected cells.

Discussion

The aim of the present study was to reveal the subcellular localization of GLV and ultrastructural alterations of virus-infected *G. lamblia* WB cells. We found that a large portion of the viruses with complete ds RNA genomes was associated with the membranous fraction of the cell lysate, and that a major component in this category was GLV[p95]. By contrast, a majority of the viruses associated with the soluble cytosolic fraction of the cell lysate consisted of empty capsids of GLV[p100]. Since virions of GLV[p100] are more fragile than those of GLV[p95] [1], we were not able to exclude the possibility that virions of GLV[p100] were more prone to open up and lose genomic ds RNA during virus purification.

Consistent with cellular fractionation of the viruses, subcellular localization of the viral antigens in virus-infected *G. lamblia* WB cells by immunocytochemistry also suggests that a fraction of the GLV is confined to certain cellular compartments (Fig. 3). Using immuno electron microscopy, we also found membranous and cytosolic forms of virus-like particles. Our subcellular

fractionation studies suggest that the cytosolic virus-like particles observed in infected cells are most likely GLV[p100]. Whether they are empty capsids or complete virions cannot be determined by morphological observation. On the other hand, the virus-like particles found within membranous structures are most likely mature virions of GLV[p95], with only a small portion being GLV[p100]. Interestingly, two distinct types of virus-associated vesicles were observed in virus-infected cells (Figs. 4D,E). Since GLV[p100] is actively released into the medium and GLV[p95] is retained in the infected cells, it is tempting to speculate that they are likely confined to different vesicles. This hypothesis cannot be verified at this moment because of the cells' indistinguishable morphology and the lack of a species-specific antibody. Although direct comparison of the *in vitro* and *in vivo* distributions of GLV[p100] and GLV[p95] cannot be made by current studies, these parallel observations provide valuable insight into the life cycle of GLV.

In general, RNA viruses usually do not need to enter the nucleus or other cellular compartments to fulfill the viruses' replication cycle, with the exception of the influenza virus during the synthesis of viral messenger RNA [13,14]. Elegant studies by Wickner and his colleagues elucidated the replication cycle of *Saccharomyces cerevisiae* L-A virus, which is the best understood virus in Totiviridae [15]. Replication and transcription of viral RNA take place within this virus' capsids in the cytoplasm. The plus strand viral RNA synthesized in the capsids is then extruded into the cytoplasm for protein translation. Virus assembly and packaging of the plus strand viral RNA occur in the cytoplasm. Therefore, it was unusual that we found virus-like particles confined within the vesicles of virus-infected *G. lamblia* WB cells. Since membrane-bound virus-like particles were observed earlier than cytoplasmic virus-like particles, it is tempting to speculate that some steps of the virus replication take place in the vesicles. These vesicles are also likely of the intracellular transport form that travels along some cellular routes for secretory products. These possibilities remain to be studied. It will be equally intriguing to see how and why GLV translocates to the nuclear compartment during the late phase of infection. However, it is clear that virus infection eventually and inevitably leads to disintegration of the cells.

In conclusion, our observations reveal the presence of GLV within some membranous structures, and suggest that the virus-associated vesicles are induced by virus infection and may be important for replication and translocation of GLV.

Acknowledgments

This work was supported by Academia Sinica. Grateful acknowledgment is made to Ms. Shu-Chen Chang for technical assistance in virus purification. Electron microscopy was performed with the help of Hsiu-Jung Ma of the EM core facility at the Institute of Biomedical Sciences. We appreciate the help of Mr. Douglas Platt in reading and correcting the manuscript.

References

1. Tai JH, Chang SH, Chou CF, Ong SJ. Separation and characterization of two related Giardiaviruses in the parasitic protozoan *Giardia lamblia*. Virology 1996;216:124-32.
2. Wang AL, Wang CC. Discovery of a double-stranded RNA virus in *Giardia lamblia*. Mol Biochem Parasitol 1986;21:269-76.
3. Wang AL, Yang HM, Shen KA, Wang CC. Giardiavirus double-stranded RNA genome encodes a capsid polypeptide and a *gag-pol*-like fusion protein by a translation frameshift. Proc Natl Acad Sci USA 1993;90:8595-9.
4. Wang AL, Wang CC. Viruses of the protozoa. Ann Rev Microbiol 1991;45:251-63.
5. Furfine ES, White TC, Wang AL, Wang CC. A single-stranded RNA copy of the *Giardia lamblia* virus double-stranded RNA genome is present in the infected *Giardia lamblia*. Nucleic Acids Res 1989;17:7453-6.
6. Furfine ES, Wang CC. Transfection of the *Giardia lamblia* double-stranded RNA virus into *Giardia lamblia* by electroporation of a single-stranded RNA copy of the viral genome. Mol Cell Biol 1990;10:3659-63.
7. Yu DC, Wang AL, Wu CH, Wang CC. Virus-mediated expression of firefly luciferase in the parasitic protozoan *Giardia lamblia*. Mol Cell Biol 1995;15:4867-72.
8. Yu DC, Wang AL, Wang CC. Stable coexpression of a drug-resistance gene and a heterologous gene in an ancient parasitic protozoan *Giardia lamblia*. Mol Biochem Parasitol 1996;83: 81-9.
9. Tai JH, Ong SJ, Chang SC, Su, HM. Giardiavirus enters *Giardia lamblia* WB trophozoites via endocytosis. Exp Parasitol 1993; 76:165-74.
10. Tai JH, Wang AL, Ong SJ, Lai KS, Lo C, Wang CC. The course of Giardiavirus infection in the *Giardia lamblia* trophozoites. Exp Parasitol 1991;73:413-23.
11. Scherrer K, Darnell JE. Sedimentation characteristics of rapidly labeled RNA from Hela cells. Biochem Biophys Res Commun 1962;7:486-90.
12. Sambrook J, Fritsch EF, Maniatis T, eds. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
13. Joklik WK. The virus multiplication cycle. In: Joklik WK, ed. Virology, 3rd ed. New Jersey: Prentice Hall, 1988:48.
14. Shapiro GI, Gurney T Jr, Krug RM. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J Virol 1987;61:764-73.
15. Wickner RB. Double-stranded RNA viruses of *Saccharomyces cerevisiae*. Microbiol Rev 1996;6:250-65.