Induction of apoptosis in *Saccharomyces cerevisiae* results in the spontaneous maturation of tetravirus procapsids *in vivo*

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The *Tetraviridae* are a family of small, non-enveloped, insect RNA viruses consisting of one or two single-stranded, positive-sense genomic RNAs encapsidated in an icosahedral capsid with $T=4$ symmetry. Tetravirus procapsids undergo maturation when exposed to a low pH environment *in vitro*. While the structural biology of the conformational changes that mediate acid-dependent maturation is well understood, little is known about the significance of acid-dependent maturation *in vivo*. To address this question, the capsid-coding sequence of the tetravirus *Helicoverpa armigera stunt virus* was expressed in *Saccharomyces cerevisiae* cells. Virus-like particles were shown to assemble as procapsids that matured spontaneously *in vivo* as the cells began to age. Growth in the presence of hydrogen peroxide or acetic acid, which induced apoptosis or programmed cell death in the yeast cells, resulted in virus-like particle maturation. The results demonstrate that assembly-dependent maturation of tetravirus procapsids *in vivo* is linked to the onset of apoptosis in yeast cells. We propose that the reduction in pH required for tetraviral maturation may be the result of cytosolic acidification, which is associated with the early onset of programmed cell death in infected cells.

INTRODUCTION

*Helicoverpa armigera stunt virus* (HaSV) belongs to the genus *Omegetetravirus* of the family *Tetraviridae*, which exclusively infect the midgut cells of lepidopteran insects (van Regenmortel et al., 2000). The best-studied member of the omegatetraviruses is *Nudaurelia capensis* o virus (NoV), the type virus for the genus (van Regenmortel et al., 2000). The availability of an X-ray crystal structure (at 2.4 Å resolution) of the NoV capsid has led to a wealth of information about the structural changes that mediate assembly and maturation of omegatetravirus capsids (Munshi et al., 1996; Taylor et al., 2002; Helgstrand et al., 2004). These processes are most likely very similar for NoV and HaSV because (i) there is a high level of sequence identity (67%) and an even higher level of sequence similarity (76%) between the coat protein sequences of these two viruses (Agrawal & Johnson, 1992; Hanzlik et al., 1995), (ii) the X-ray crystallographic coordinates of the HaSV capsid can, to a large extent, be superimposed on to those of the NoV capsid (Taylor, 2003) and (iii) the maturation of HaSV and NoV virus-like particles (VLPs) occurs in a similar fashion *in vitro* (Taylor, 2003).

The omegatetravirus genome is bipartite, consisting of two single-stranded, positive sense RNAs. HaSV RNA1 (5.3 kb) and RNA2 (2.5 kb) encode the viral replicase (187 kDa) and capsid protein precursor (71 kDa), respectively (Gordon et al., 1995; Hanzlik et al., 1995). During virus assembly, 240 copies of capsid protein precursor (designated p71 for HaSV and z for NoV) and genomic RNA assemble into a round, porous assembly intermediate (procapsid) with a mean diameter of 45 nm (Agrawal & Johnson, 1995; Canady et al., 2000). It has been shown *in vitro* for baculovirus-expressed VLPs that omegatetravirus procapsids undergo a biphasic maturation process to produce smaller mature capsids with a mean diameter of 41 nm (Canady et al., 2001). The first phase of maturation is triggered by a reduction in pH and involves large-scale structural rearrangements that alter the geometry of the procapsid to produce an intermediate with a similar size and shape to that of the mature capsid (Canady et al., 2000; Taylor et al., 2002). These structural rearrangements occur very quickly (less than 100 ms) and result in the shrinking of the capsid shell (Canady et al., 2001). The onset of the second phase is dependent on the first and involves the relatively slow ($t_{1/2}$ of several hours) autoproteolytic

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cleavage of the capsid precursor protein subunits at their carboxyl termini (Canady et al., 2001). This leads to the production of the mature capsid protein (designated p64 for HaSV and β for NoV) and a small peptide (designated p7 for HaSV and γ for NoV).

Viruses from a variety of different families, including the Nodaviridae and Picornaviridae, undergo a similar autoproteolytic cleavage event during maturation (Friesen & Rueckert, 1981; Dasgupta et al., 1984; Fricks & Hogle, 1990; Yuan et al., 2003), but the pH-dependent, large-scale structural rearrangements that occur during the first phase of tetravirus maturation have not been observed for any other positive-strand RNA viruses. At present, the maturation of tetravirus procapsids has only been triggered in vitro via a reduction in pH (Canady et al., 2000; Taylor et al., 2002). How maturation is triggered during the assembly of provirions in vivo and how this relates to the infectious life cycle of tetraviruses in their host cells is unknown.

The biology of the tetraviruses and their interaction with host cells is poorly understood. This is due to the lack of experimental systems as a result of their exceptionally narrow host range (Bawden et al., 1999) with only Providence virus (PrV), which was discovered in a Helicoverpa zea midgut cell line, being able to replicate in tissue culture (Pringle et al., 2003). As a consequence, insight into tetravirus biology has only been possible through studies on infected insects and the non-host production of VLPs by way of baculovirus expression or expression in transfected plant protoplasts (Agrawal & Johnson, 1995; Gordon et al., 2001). There is evidence in the literature that apoptosis or programmed cell death (PCD) plays a role in the infectious life cycle of tetraviruses, as studies on the pathology of HaSV in its insect host have shown a strong correlation between tetravirus infection and apoptosis in larval midgut cells (Brooks et al., 2002). Apoptosis is important in virus life cycles, with many viruses from different families inducing apoptosis in infected cells. Host cells in turn may induce apoptosis as a defence mechanism against virus infection. Thus, viruses have evolved strategies to combat the host immune response and apoptosis, including suppression of apoptosis to maintain virus latency and the regulated induction of apoptosis to ensure the production of infectious progeny (reviewed by Hardwick, 1998; Roulston et al., 1999). Members of the Picornaviridae, including avian encephalomyelitis-like virus, Poliovirus and coxsackieviruses, have been shown to induce apoptosis in a wide variety of cells (Carty et al., 1998; Barco et al., 2000; Calandría et al., 2004; Liu et al., 2004). The induction of apoptosis has also been observed in cells infected with the betanodavirus greasy grouper nervous necrosis virus (Guo et al., 2003).

In this study, the HaSV capsid protein was expressed in Saccharomyces cerevisiae and it was discovered that VLPs spontaneously underwent maturation as the cells began to age. Further investigation linked VLP maturation to the onset of PCD by showing that induction of apoptosis in S. cerevisiae resulted in autoproteolytic cleavage of p71 to p64. We propose that the reduction in pH required to trigger VLP maturation is brought about by cytosolic acidification, which is associated with the early stages of PCD.

**METHODS**

**Micro-organisms and growth conditions.** All recombinant plasmids were maintained in Escherichia coli DH5α grown in Luria–Bertani (LB) broth or on LB agar plates containing the relevant antibiotics for plasmid selection (Sambrook et al., 1989). The yeast strain INVS1c (MATα, trpl, his3, leu2, ura3) was used for expression of HaSV capsid precursor proteins. Yeast cells were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose) or supplemented minimal medium (SMM) (Kaiser et al., 1994). Competent yeast cells were prepared and transformed using a Frozen-EZ Yeast Transformation II kit (Zymo Research).

**Construction of the yeast expression vector.** The yeast expression vector pY2Tg (Venekesi et al., 1996) containing the alcohol dehydrogenase 2/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAPDH) (P<sub>ADH2</sub>) hybrid promoter (Barr et al., 1987; US Patent no. 0608994) used in the course of this work was obtained from László Szilagyi (Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary). The expression vector pMT9 carries the HaSV capsid coding sequence p71 fused at its 5’ end with the 3’ end of the ADH2 promoter incorporating the translation signal of P<sub>GAPDH</sub> and translational start of p71. Plasmid pMT9 is a 2µ-based multicopy expression vector containing a uracil auxotrophic marker, a T<sub>CYE</sub>1 transcriptional terminator and P<sub>GAPDH</sub> for high-level expression of p71.

**p71 expression in S. cerevisiae.** Freshly transformed yeast colonies were patched on to selective SMM agar plates, grown for 2 days and inoculated at an OD<sub>600</sub> of 0.05 into SMM with 0.1% glucose or 1% glycerol as carbon source. Expression of p71 was monitored by removing two OD<sub>600</sub> units of cells, which were pelleted and resuspended in 30 µl EB [50 mM Tris/HCl (pH 7.4), 250 mM NaCl, 1 mM DTt, 1 mM PMSF, 1 µM pepstatin A] and 0.1 g acid-washed glass beads (Sigma). Cells were lysed by homogenization in 30 s bursts on ice for a total time of 10 min. Protein concentrations were determined using the Bradford method (Bradford, 1976) to ensure that equivalent protein concentrations were used for SDS-PAGE.

**VLP isolation.** Yeast cells induced for p71 expression were harvested at the appropriate time by centrifugation and the pellet was resuspended in 2 ml EB (g wet cell mass) with 100 µg/ml lysozyme and 200 mM MgCl<sub>2</sub>. Yeast cells were disrupted with glass beads as above, followed by centrifugation at 12 000 r.p.m. in a Beckman JA20 rotor for 15 min at 4°C. The VLPs were purified by centrifugation through a 30% sucrose cushion at 25 000 r.p.m. in a Beckman SW28 rotor for 5.5 h followed by sedimentation through a 10–40% sucrose gradient in a Beckman SW41 rotor at 40 000 r.p.m. for 1.25 h at 11°C as described by Taylor et al. (2002). The VLP band was collected by extraction with a syringe from the side of the tube.

**Induction of apoptosis.** Cells containing pMT9 were grown at 28°C for 24–28 h after which apoptosis was induced by the addition of acetic acid to 80 mM or H<sub>2</sub>O<sub>2</sub> at concentrations varying from 0 to 180 mM and further incubation at 28°C for 200 min, as described previously (Ludovico et al., 2001, 2002). TdT-mediated dUTP nick end-labelling (TUNEL) was used to test for apoptosis and the DNA fragments were labelled using the In situ Cell Death Detection kit, POD (Boehringer Mannheim). Yeast cells were fixed, digested with lyticase and applied to a polylysine-coated slide, as described previously (Adams & Pringle, 1984). Cellular

**Cell Death Detection kit, POD** (Boehringer Mannheim) was used for apoptosis induction in vitro via a reduction in pH (Canady et al., 2000; Taylor et al., 2002). How maturation is triggered during the assembly of provirions in vivo and how this relates to the infectious life cycle of tetraviruses in their host cells is unknown. The biology of the tetraviruses and their interaction with host cells is poorly understood. This is due to the lack of experimental systems as a result of their exceptionally narrow host range (Bawden et al., 1999) with only Providence virus (PrV), which was discovered in a Helicoverpa zea midgut cell line, being able to replicate in tissue culture (Pringle et al., 2003). As a consequence, insight into tetravirus biology has only been possible through studies on infected insects and the non-host production of VLPs by way of baculovirus expression or expression in transfected plant protoplasts (Agrawal & Johnson, 1995; Gordon et al., 2001). There is evidence in the literature that apoptosis or programmed cell death (PCD) plays a role in the infectious life cycle of tetraviruses, as studies on the pathology of HaSV in its insect host have shown a strong correlation between tetravirus infection and apoptosis in larval midgut cells (Brooks et al., 2002). Apoptosis is important in virus life cycles, with many viruses from different families inducing apoptosis in infected cells. Host cells in turn may induce apoptosis as a defence mechanism against virus infection. Thus, viruses have evolved strategies to combat the host immune response and apoptosis, including suppression of apoptosis to maintain virus latency and the regulated induction of apoptosis to ensure the production of infectious progeny (reviewed by Hardwick, 1998; Roulston et al., 1999). Members of the Picornaviridae, including avian encephalomyelitis-like virus, Poliovirus and coxsackieviruses, have been shown to induce apoptosis in a wide variety of cells (Carty et al., 1998; Barco et al., 2000; Calandría et al., 2004; Liu et al., 2004). The induction of apoptosis has also been observed in cells infected with the betanodavirus greasy grouper nervous necrosis virus (Guo et al., 2003).

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permeabilization, treatment of the cells with the TUNEL mixture and detection of peroxidase were carried out as described previously (Madeo et al., 1999). Cells visualized using an Olympus BX-60 reflected-light microscope.

**Immunoblot analysis.** Crude protein extracts and HaSV VLP preparations were subjected to immunoblot analysis using anti-HaSV antibodies (obtained from Andrew Dinsmore, Syngenta, UK). A Roche Chemiluminescence Western blotting kit was used for immunodetection.

**Electron microscopy.** HaSV virions and VLPs were negatively stained using 2% uranyl acetate (Dong et al., 1998) and viewed using a JOEL JEM-1210 transmission electron microscope.

**RESULTS**

**Assembly and maturation of HaSV VLPs in *S. cerevisiae***

The yeast expression vector pMT9 with the p71 ORF inserted downstream of the hybrid \( P_{\text{GADH}} \) was used for the expression of this protein in *S. cerevisiae*. \( P_{\text{GADH}} \) is repressed by glucose and activated under glucose starvation or carbon-limiting conditions (Denis et al., 1983). To characterize \( P_{\text{GADH}} \)-dependent p71 expression, pMT9-transformed yeast cells were grown in either glucose-starvation medium (containing 0.1% glucose) or carbon-limiting medium (containing 0.1% glucose supplemented with 5% glycerol). Western blot analysis of crude protein extracts from these cells showed that p71 was detectable up to 50h post-induction in both types of growth medium (Fig. 1, lanes 12 and 13). However, a band co-migrating with mature capsid protein (p64) was observed after 32 h in cells incubated in glucose-starvation medium (Fig. 1, lane 9) but only after 45 h in cells grown under carbon-limiting conditions (Fig. 1, lane 10). The detection of the p64 cleavage product in cellular lysates suggested that p71 underwent maturation cleavage within *S. cerevisiae* cells. It also suggested that yeast cells supported the assembly of HaSV VLPs. However, the continued presence of p71, even after 55 h, showed that not all capsid protein precursor was cleaved and that assembly of procapsids may not be efficient in these cells.

![Fig. 1. \( P_{\text{GADH}} \)-regulated induction of yeast cells expressing HaSV p71. Western blot analysis of total protein extracts from \( P_{\text{GADH}} \)-derived expression of p71. Cells were incubated at 30°C in SMM containing glucose (–) or glucose plus glycerol (+). Arrows indicate the positions of p64 and p71. Wild-type HaSV (1 ng) was used as a positive control (wt; lanes 7 and 14).](image)

To confirm that HaSV VLPs were assembling in *S. cerevisiae* cells, VLPs were prepared from cells after 32 h growth under glucose-starvation or carbon-limiting conditions. Western blot analysis of selected fractions collected from sucrose gradients showed, in addition to p71, the presence of a major band corresponding to p64 in glucose-starved cells (Fig. 2a, lane 1). Transmission electron microscopy (TEM) examination of these VLPs showed the presence of particles that closely resembled wild-type particles (compare Fig. 2b and c with Fig. 2e). The sample also contained rounded particles of approximately 40 nm in diameter with dark centres, indicating penetration of the stain, consistent with the presence of HaSV procapsids (Fig. 2b, c). The major band in VLPs prepared from cells grown under carbon-limiting conditions was p71 although a small amount of p64 could also be observed on the Western blot (Fig. 2a, lane 2). TEM examination confirmed the presence of mostly procapsids with a small number of mature particles in this sample (Fig. 2d). The ratio of procapsids to mature capsids observed by TEM in both samples was approximately equivalent to the ratio of p71 to p64 detected on the Western blot (Fig. 2a). These results confirmed that the detection of p64 in crude cell extracts and VLP preparations indicated the presence of mature VLPs.

**Fig. 2.** Purification of HaSV VLPs. (a) Western blot analysis of VLPs purified from cells containing pMT9 incubated in SMM under conditions of glucose starvation (–) or carbon limitation (+). Lane 3 contains wild-type HaSV virus. (b–d) Transmission electron micrographs of VLPs purified from cells expressing p71, incubated in SMM under conditions of glucose starvation (b, c) or carbon limitation (d). (e) Mature HaSV capsids. Bars, 50 μm.
Correlation between the onset of PCD and VLP maturation

The most likely explanation for VLP maturation occurring earlier in cells grown under glucose-starvation conditions compared with carbon-limiting conditions (i.e. 32 h compared with 45 h) is the delay in p71 translation in cells grown in medium supplemented with glycerol (Fig. 1). However, the data might also support a link between cell ageing and VLP maturation, as the supplementation of growth medium with glycerol would extend the active growth period of the yeast cells and consequently postpone ageing. It has been shown that ageing yeast cells undergo PCD (Herker et al., 2004), exhibiting the characteristic markers of apoptosis, including the presence of reactive oxygen species (ROS), caspase induction and chromosomal fragmentation typical of PCD in multicellular eukaryotes (Herker et al., 2004).

In order to test whether VLP maturation coincided with the onset of PCD, yeast cells expressing p71 were grown in carbon-limiting medium for 28 h to ensure the exclusive production of procapsids (Fig. 1). Apoptosis was then induced by the addition of either 80 mM acetic acid or various concentrations of H2O2. Crude protein extracts of cells treated with acetic acid contained both p64 and p71, while the control (without acetic acid) contained only p71 (Fig. 3, compared lanes 1 and 2). The addition of H2O2 at concentrations of between 20 and 180 mM also resulted in the appearance of p64 (Fig. 3, lanes 9–13 and 7), with p71 alone only present in untreated cells or in those treated with an H2O2 concentration of 15 mM or lower (Fig. 3, lanes 3–6). These data suggested a strong link between maturation of VLPs and the onset of PCD in the yeast cells.

TUNEL tests were carried out to determine whether cells treated with acetic acid and H2O2 were indeed undergoing apoptosis. Accordingly, cells that were treated with either acetic acid or H2O2 and showed the presence of p64 (Fig. 4, lanes 2 and 3) were treated with TUNEL reagent, examined by light microscopy and the TUNEL-positive cells were counted. Eighty per cent of cells treated with either acetic acid or H2O2 were TUNEL-positive (Fig. 4b), while only 5% of control cells exhibited a positive reaction (Fig. 4b). These results were confirmed by staining with 4,6-diamidino-2-phenylindole (DAPI) to detect chromosomal condensation, together with cell viability tests (data not shown). Taken together, these results confirmed that PCD was induced by acetic acid and H2O2 and that this correlated with maturation of HaSV VLPs.

As exposure to low pH (acetic acid) induces maturation of VLPs in vitro (Canady et al., 2000; Taylor et al., 2002), it was necessary to ensure that this was not also the case for treatment with H2O2. To test this, crude extracts containing p71 from cells harvested after 28 h were incubated in vitro with either 80 mM acetic acid or 40 mM H2O2. Western blot analysis revealed that, as with the controls, exposure of procapsids to H2O2 in vitro did not induce maturation, even after 19 h (Fig. 5, compare lanes 1, 4 and 7 with lanes 3, 6 and 9). However, as expected, procapsids incubated with acetic acid had matured after 3.5 h (Fig. 5, lanes 2, 5 and 8). It was therefore not possible to discern whether acetic acid-triggered maturation was brought about directly (due to a lowering of cytoplasmic pH by acetic acid) or indirectly (due to decreased levels of growth factors or increased levels of ROS).
to the induction of apoptosis by acetic acid). In conclusion, at least in the case of cells treated with H$_2$O$_2$, VLP maturation in vivo was dependent on the onset of PCD.

**DISCUSSION**

This is the first report that maturation of a small insect RNA virus procapsid correlates with the onset of apoptosis in its host cell. Apoptosis was triggered in yeast cells by H$_2$O$_2$, which has been shown to induce apoptosis by increasing the levels of intracellular ROS that induce the subsequent cascade of events resulting in PCD in yeast cells (Madeo et al., 1999). We also successfully triggered apoptosis by exposing the yeast cells to acetic acid, which has been shown to result in the activation of a mitochondrion-dependent apoptotic pathway (Ludovico et al., 2001). However, as tetravirus maturation has been shown to be pH dependent (Canady et al., 2000; Taylor et al., 2002), it was unclear whether the VLP maturation in acetic acid-treated cells was reduced by the low pH of the acetic acid-containing growth medium or by acetic acid-triggered PCD. The incomplete cleavage of p71 to p64 in vivo (Fig. 1), even after 55 h, may have been due to the fact that not all of the yeast cells had undergone PCD. However, the observation of incomplete cleavage during in vitro acid maturation (Fig. 5) indicated inefficient assembly of VLPs, as reported by Canady et al. (2000) for acid maturation of NoV VLPs in vitro. Assembly of omegatetravirus VLPs is thus not as efficient as the replication-dependent assembly of virus particles in infected cells. The proposal that maturation of HaSV VLPs is dependent on cell ageing and progression through PCD is supported by the observations of Brooks et al. (2002) who demonstrated a clear association between apoptosis and HaSV infection of *H. armigera* midgut cells. They showed that HaSV infection resulted in increased cell sloughing, which might be responsible for the stunting observed in infected early-instar larvae. Whether apoptosis is the result of virus infection or a host response to the infection in order to regenerate the midgut is unknown. However, our data suggest that, either way, apoptosis in the host cells may play an important part in the life cycle of HaSV by providing the conditions required for maturation and consequently stabilization of the virion. While this hypothesis might hold for the omegatetraviruses, the same may not be true for the betatetraviruses, as a number of lines of evidence suggest that the mechanism of assembly and maturation of betatetraviruses might be different. First, the capsid precursor proteins of the betatetraviruses PrV and *Thosea asigna virus* (TaV) undergo additional cleavage at their amino-terminal ends prior to assembly and maturation, while such a cleavage does not occur for omegatetraviruses. Secondly, PrV and TaV VLPs are not readily assembled in baculovirus-infected Sf21 cells, while high yields of omegatetravirus VLPs are obtained from these cells (Pringle et al., 1999; Taylor et al., 2005).

A number of virus capsids have been shown to undergo large-scale conformational changes following a reduction in pH. The incidence of such changes during virus life cycles is, however, limited to transitions that occur during virus entry where acidification of endosomes is required for fusion with the endosomal membrane (Lescar et al., 2001; Skehel & Wiley, 2002). Omegatetraviruses are distinct from other viruses in their requirement for pH reduction during the assembly stage of the virus life cycle. The major question arising from this work is how apoptosis could be involved in reduction of the pH of a cellular compartment to trigger the maturation of HaSV provirions. There are two main apoptotic pathways: the first is mitochondrion-dependent (intrinsic pathway) and results in the release of cytochrome c and subsequent activation of the caspase pathway. The second (extrinsic pathway) bypasses the mitochondria and results in direct activation of the caspase pathway (Adams, 2003). Experiments using pH-sensitive green fluorescent protein have shown a change in the regulation of cellular pH during induction of PCD via the intrinsic pathway (Matsuyama et al., 2000). Induction of this pathway is accompanied by alkalization of the mitochondrial matrix and acidification of the cytoplasm. These changes are thought to be required for efficient cytochrome c-mediated activation of the caspase pathway at an optimum pH of 6.3–6.8 in vitro (Matsuyama & Reed, 2000; Matsuyama et al., 2000). Recently, Nilsson et al. (2006) demonstrated that cytosolic acidification of U937 cells (from pH 7.2 to 5.7) occurred during the early stages of apoptosis as a result of the release of protons from the lysosomes. We propose that this drop in cytoplasmic pH may be the trigger for maturation of provirions in infected cells.

It is possible that tetraviral factors could contribute to cytoplasmic acidification over and above their potential role in triggering apoptosis. It has been shown that the replication complexes of the nodavirus *Flock house virus* localize to the outer mitochondrial membrane and that this results in extensive mitochondrial degeneration (Garzon et al., 1990; Miller et al., 2001). Furthermore, it has been proposed that the assembly of this small, insect RNA virus, which is structurally analogous to tetraviruses, occurs in the vicinity of the mitochondria (Venter et al., 2005). It is not unreasonable to suggest that tetravirus replication may also be associated with the mitochondria or, alternatively, the lysosomes and that this leads to membrane permeabilization and the consequent enhancement of cytosolic acidification. We are currently investigating the subcellular localization of tetravirus replication.

The provirion and mature virion forms of tetraviruses have structural characteristics that enable them to fulfils different functions during a tetravirus infection. A proposed function for the provirion assembly intermediate is that it facilitates assembly by allowing newly assembled capsid protein subunits to be associated as nearly equivalent units in the context of a virus particle (Canady et al., 2000). The procapsid should also function as the predominant form of
this virus during the cellular phase of the virus life cycle. On the other hand, mature virions are sufficiently stable to effectively withstand extracellular environments and are capable of infecting new host cells. The discovery that maturation might be triggered by the onset of cell death therefore fits well into the tetravirus life cycle when the biological functions of provirions and virions are considered.

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