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Full Length Research Paper

Virus infection disturbs cyclin expression, leading to cell cycle arrest in the unicellular marine algae *Emiliania huxleyi* and *Chrysochromulina ericina*

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To investigate the influences of viral infection on cell cycle of marine algae, we examined the changes of expression and activity of G $_2$/M-phase cell cycle regulators in two unicellular marine algae *Emiliania huxleyi* and *Chrysochromulina ericina* after viral infection by immunoblot. The results revealed that the expression of cyclins could be altered by viral infection, thus inhibiting the progression of cell cycle. *E. huxleyi* virus (EhV) repressed the activity of p34cdc2/cyclinB complex by inactivating p34cdc2 kinase through phosphorylation of Cdc2, and block the life cycle of host cells at G_2/M checkpoint. *C. ericina* virus (CeV) interfere with the activity of p34cdc2/cyclin B complex by the downregulation of cyclinB combined with the overexpression of kinase-active p34cdc2, resulting in a G_2 cell cycle. The G $_2$/M cell cycle arrest caused by EhV infection indicated that EhV may transcribe its own genes without relying on the host cell cycle control. *C. ericina* cells blocked in a delayed G_2 phase may partly turn into the second S phase to synthesize proteins useful for viral nucleic acid metabolism, suggesting that the replication of CeV viruses was dependent on cell cycle control. Our results indicated that algal virus infection selectively activates/inactivates certain components of the cell cycle with the aim to establish a more efficient environment for their gene expression and DNA replication.

Key words: Marine algal virus, cyclins expression, cell cycle arrest.

INTRODUCTION

Viruses are the most abundant biological entities in marine environments that manipulate the marine environment (Bergh et al., 1989; Fuhrman, 1999; Suttle, 2005; Rohwer and Thurber, 2009). Viral infection may be important in regulating succession and composition of phytoplankton communities (Bratbak et al., 1993; Brussaard, 2004). Phytoplanktonic host-virus systems brought into culture have been used to investigate the environmental factors presumed to affect the host virus interaction, for example, nutrient supply, light conditions, UV radiation, etc. However, the biochemistry of viral infection in phytoplankton and the cellular mechanisms regulating the host- virus interaction, including virus proliferation, cell lysis and death, is not well studied and understood.

Phytoplankton is unicellular organisms. Their population growth results directly from the completion of a cell cycle. During viral infection both the host and the virus will try to modify cellular processes, that is, the host try to hinder viral replication in an attempt to protect itself, and the virus will also try to facilitate its own replication. Expression and replication of several viruses infecting eukaryotic cells, including marine phytoplankton, are known to depend on the cell cycle of their host, whereas other viruses are independent of their host's cell cycle (Lewis and Emerman, 1994; Thyrhaug et al., 2002; Wolf et al., 2004). Understanding how viral infections affect cell-cycle progression in phytoplankton will therefore help to explain the host-virus interactions and how viral activity

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is regulated in natural ecosystems.

In eukaryotic cells, there are two major control points in the cell cycle, the G_1/S and G_2/M transitions, respectively. Traversals of the two control points are mediated by cyclins and the cell cycle dependent kinases (CDKs). Progression from G_2 to M is controlled by activation of the mitosis - promoting factor (MPF), a complex of the catalytic kinase subunit, Cdc2 (p34cdc2), and its positive regulatory subunit, cyclin B (Heuvel and Harlow, 1993). The activation of the Cdc2/cyclin B complex is required for entry into M phase. Cdc2 kinase activity is regulated both by the nature of the newly synthesized cyclin B and by phosphorylation (Heuvel and Harlow, 1993). Two of the well-known Cdc2 regulators are Cdc25C phosphatase and Wee1 kinase. Wee1 is active when dephosphosry-lated and Cdc2 phosphosrylated are kept and deactivated while Cdc25C is activated by phosphor-srylation and dephosphosrylate and activate Cdc2 **(**Perry and Kornbluth, 2007). The key regulating points of the cell cycle of algae are the initiation of DNA synthesis (S phase) and initiation of mitosis (M phase) (Lin et al., 1996; Carpenter et al., 1998). In phytoplankton, cell cycle associated proteins cyclinB, Cdc2 kinase and PCNA have been studied extensively. Their coding regions are similar to their counterparts in other organisms, both of which contain highly conserved functional domains (Lin et al., 1996; Lin and Corstjens, 2002).

Emiliania huxleyi (Haptophyceae) has a worldwide distribution and they can form massive blooms in both oceanic and coastal waters (Holligan et al., 1983; Balch et al., 1992). Blooms of *E. huxleyi* are frequently observed to be terminated by viral infection (Bratbak et al., 1993; Brussaard et al., 1996; Jacquet et al., 2002) and numerous large dsDNA viruses specific to *E*. *huxleyi* have been isolated (Schroeder et al., 2002; Wilson et al., 2002). *Chrysochromulina ericina* (Haptophyta, Prymnesiophyceae) occurs most often in low numbers but has also been observed to form blooms together with other *Chrysochromulina* species (Simonsen and Moestrup, 1997). Large dsDNA viruses infecting *C. ericina* have been isolated successively over a period of years from the western coast of Norway (Sandaa et al., 2001).

The aim of this work was to explore how *E. huxleyi* virus (EhV) and *C. ericina* virus (CeV) infection affects the cyclin expression and cell cycle of their hosts. To achieve this, we investigated the occurrence of cell cycle-associated proteins and activity of protein kinase complexes as the infections progressed. The results of this study will contribute to our understanding on how host-virus interactions and viral activity are regulated in natural ecosystems.

MATERIALS AND METHODS

Algal culture

The algal species and their respective viruses used in this study were *E. huxleyi*, *C. ericina*, *E. huxleyi* virus (EhV- 99B1) (Castberg et al., 2002) and *C. ericina* virus (CeV-01B) (Sandaa et al., 2001). The algae were grown in f/2-Si medium (Guillard, 1975) based on aged and autoclaved seawater at 15°C and with a light:dark cycle of 14:10. The light intensity was about 40 mol quanta $m^{-2} \cdot s^{-1}$.

Infection experiments

Viral inoculums for the infection experiments were prepared by filtering 1.5 L lysed cultures through 0.45 m pore size. Then the filtrate was concentrated to a final volume of 30 ml using a 50 K MW cut-off Minimate tangential flow ultrafiltration system (Millipore). The virus concentrates were then passed through a 0.2 m axenic syringe filter (Millipore) and stored at 4°C.

Exponentially growing cultures were split into subcultures of 3 L (experiment groups and control groups), diluted 1:1 in fresh medium and infected with concentrated EhV and CeV lysate on the 7th hour of light period (T = 0h). The virus was added with an initial virus-to-host ratio of ca 1.5 for Eh-EhV and *ca.* 8 for Ce-CeV in amount. With this experimental design we tried to control the cultures to go through one lytic cycle before the majority of the cells are infected and lysed at the end of the second lytic cycle. Control cultures received heat-killed viral additions but were otherwise treated as the experimental cultures.

Sample collection

Samples for algal and viral counts were fixed with 1.0 and 0.5% glutaraldehyde (final concentration) respectively, frozen in liquid N_2 and stored at -70°C until analysis. At ca 20, 30 and 40 h post inoculation, that is, early, mid and late in the second lytic cycle when the majority of the cells were infected, 1 L of the cultures were harvested by centrifugation at 2800 g for 8 min. The cell pellets were stored at -70°C for protein extraction.

Flow cytomety counting of algae and viruses

The samples analysis were performed with an Epics Attra II flow cytometer (Beckman–Dickinson) equipped with an external quantitative sample injector (Harvard Apparatus PHD 2000) and a water-cooled laser providing 0.100 ± 0.01 W at 488 nm. For enumeration of algal cells, frozen samples were quickly thawed and run with an injection flow rate of 50 to 100 events s^{-1} and with the discriminator set on red fluorescence. Virus enumerations were performed on frozen samples that were quickly thawed, diluted from 1:10 to 1:1000 in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and stained for 10 min at 80°C with SYBRGreen- 1 (Molecular probes, Invitrogen, USA,) at a final concentration of 10^{-4} of the commercial solution. The samples were analyzed at a flow rate of 50 to 100 events s^{-1} with the discriminator set on green fluorescence.

Preparation of cell lysates and western blot analysis

Total crude protein extraction and western blot analysis followed the method of Liu et al. (2005). In brief, crude protein extracts were prepared by sonicating cells in 50 mM Tris–HCl extraction buffer (pH 7.4). The extracts were centrifuged at 13500 g for 1 h at 4°C to remove cell debris and the proteins in the supernatant concentrated by ultrafiltration using a Microcon YM-10 (Millipore Corp., Bedford, Mass). An equal amount of protein was separated on 12% SDS – PAGE (Laemmli) and electrophoretically transferred to nitrocellulose or PVDF membranes (Amersham) by electric current (Bio-Rad-Mini-Protein and Mini Trans-Blot). The membranes were blocked in TTBS (Tris-buffer saline with Tween-20, pH 7.5)

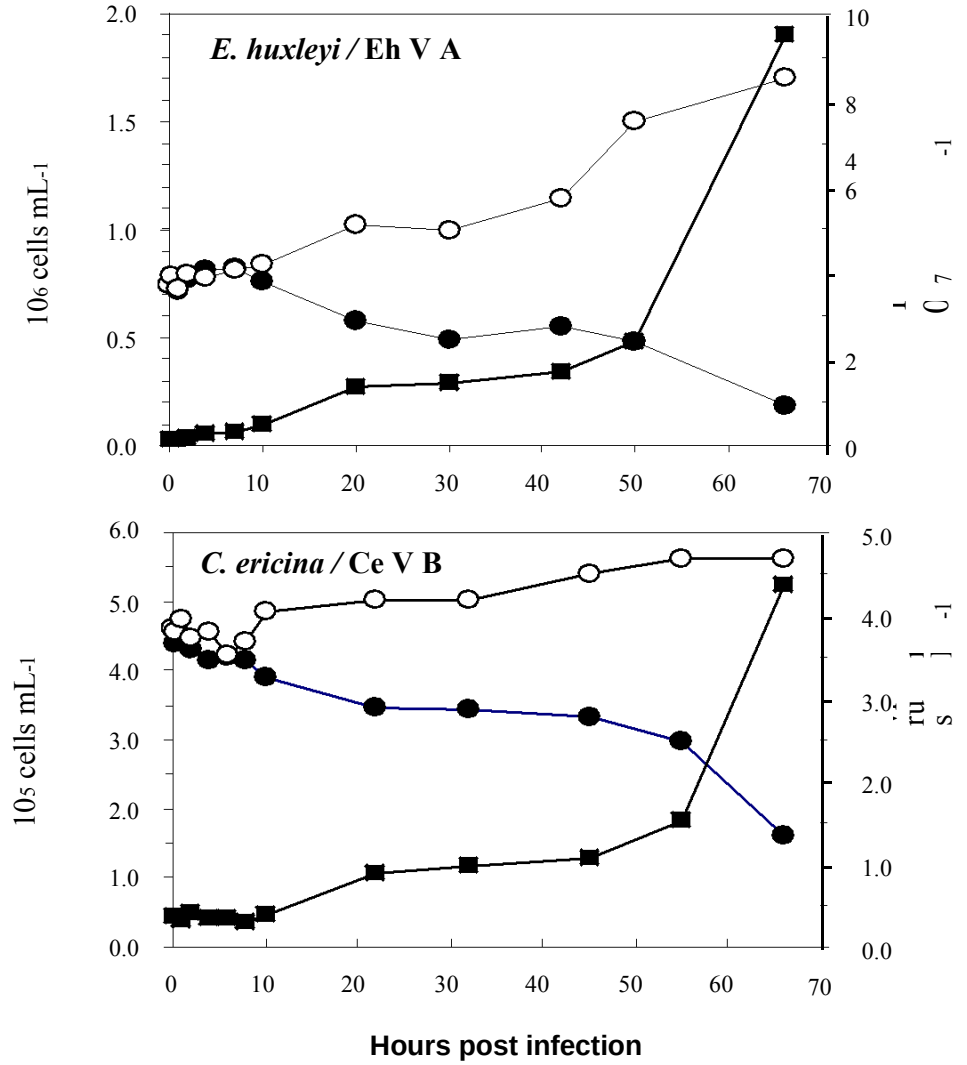

Figure 1. Abundance of algae in the uninfected control ( ) cultures, and algae ( ) and virus ( ) in the infected cultures. A: *E. huxleyi* / EhV; B: *C. ericina*/ CeV. Vertical bars on the x- axis mark sampling of proteins early, mid and late in the second lytic cycle. Light:dark cycles are marked on the x-axis (thick line = dark period).

containing 3% BSA. The protein blots were incubated overnight at 4°C with the rabbit polyclonal antibodies Anti-p34Cdc2 (H-81: sc-5616), Anti-Cdc25C (H- 150: sc-5620), Anti-Wee1 (C-20: sc-325) and Anti- -tubulin (E-19: sc-12462) (Santa Cruz Biotechnoloty, CA, USA) in 1:200 dilution and Anti-cyclinB1 (No. C 8831, Sigma-Aldrich, Inc.) in 1:300 dilution. Protein bands were visualized using horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (1:200 dilution) and a chemiluminescence detection system (Invitrogen immunodetection, Zymed Laboratories).

RESULTS

Virus infection suppresses cell growth and induces host cells lysis

The samples for protein analysis were, as intended, collected during the second lytic cycle in the cultures, that is, 20 to 45 h post inoculation when the abundance of cells in the infected cultures were relatively stable between the first and the second lysis period. The length of the second lytic cycle seems to be a slightly longer than what is observed under optimal conditions which is 12 to 14 h for EhV (Castberg et al., 2002) and 14 to 19 h for CeV (Sandaa et al., 2001) (Figure 1).

EhV inhibits Cdc2/cyclinB complex activity by inhibiting the activity of Cdc2 kinase in a phosphorylation -related manner, blocking G_2/M phase transition

We used commercially available antibodies to examine the presence of G_2/M-phase regulatory proteins homologs

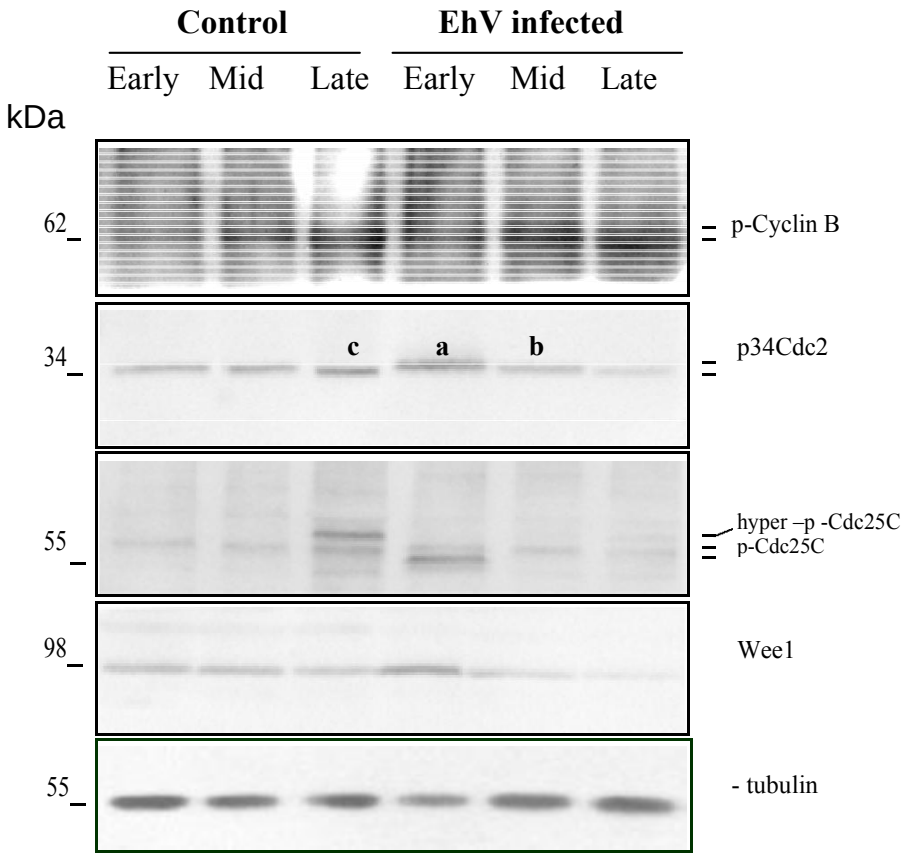

Figure 2. Change in expressed and phosphorylated status of G_2/M regulators in EhV infected *E. huxleyi* cells and in uninfected *E. huxleyi* control cells. Cells were harvested at the indicated times, and subjected to immunoblot analysis with antibodies against cyclin B1, p34Cdc2, Cdc25C, Wee1 or -tublin (control). Bands marked a, b and c denotes species with altered electrophoretic mobility due to different degree of phosphorylation.

in two marine phytoplankton species. The expression level of cyclin B-like proteins increased and was higher in the infected cells, in the middle of the infection period, compared to the uninfected control (Figure 2). The mobility of the p34Cdc2 homolog was not constant, suggesting different levels of phosphorylation. According to Liu et al. (1999), the slowest electrophoretic form of Cdc2 (species a in Figure 2) is phosphorylated on both Thr-14 and Tyr-15, while the intermediate form (species b) is phosphorylated on Thr-14 or Tyr-15, but not both. The fastest electrophoretic form of Cdc2 (species c) is unphosphorylated and represents the active form of the kinase. Thus, the Cdc2 present early in the EhV infected cells appear to be phosphorylated and inactive, while the active form of Cdc2 was found to accumulate late in control culture (Figure 2).

Phosphorylated Cdc25C were detected both in the infected and control cells (Figure 2). The slowest migrating form of Cdc25C is due to hyperphosphorylated. The fastest electrophoretic form of Cdc25C is dephosphorylated. Hyperphosphorylated and active Cdc25C was only detected late in the control cells while dephosphorylated and inactive Cdc25C was only present early in the infected cells (Figure 2). Increased concentration of Wee1 like proteins early in the EhV infected cells, may together with the dephosphorylated Cdc25C, account for the phosphorylation and inactivation of Cdc2 in these cells (Figure 2). Our results suggest that EhV infection repress Cdc2/cyclinB complex activity by inhibiting the activity of Cdc2 and thereby blocking host cells at the G_2/M checkpoint.

CeV decreases the activation of the p34cdc2/cyclin B complex by reducing cyclinB combined with overexpression of kinase-active p34cdc2, resulting in a G_2 cell cycle delay

In the CeV infected cells, the level of Cyclin B proteins were reduced early during the infection and almost undetectable in the late stage as compared to the uninfected control (Figure 3). Cdc2 seems to be dephosphorylated and active only in the late sample of the control cells, while in the infected cells the dephosphorylated form dominates throughout (Figure 3). The level of active Cdc2 was significantly higher in

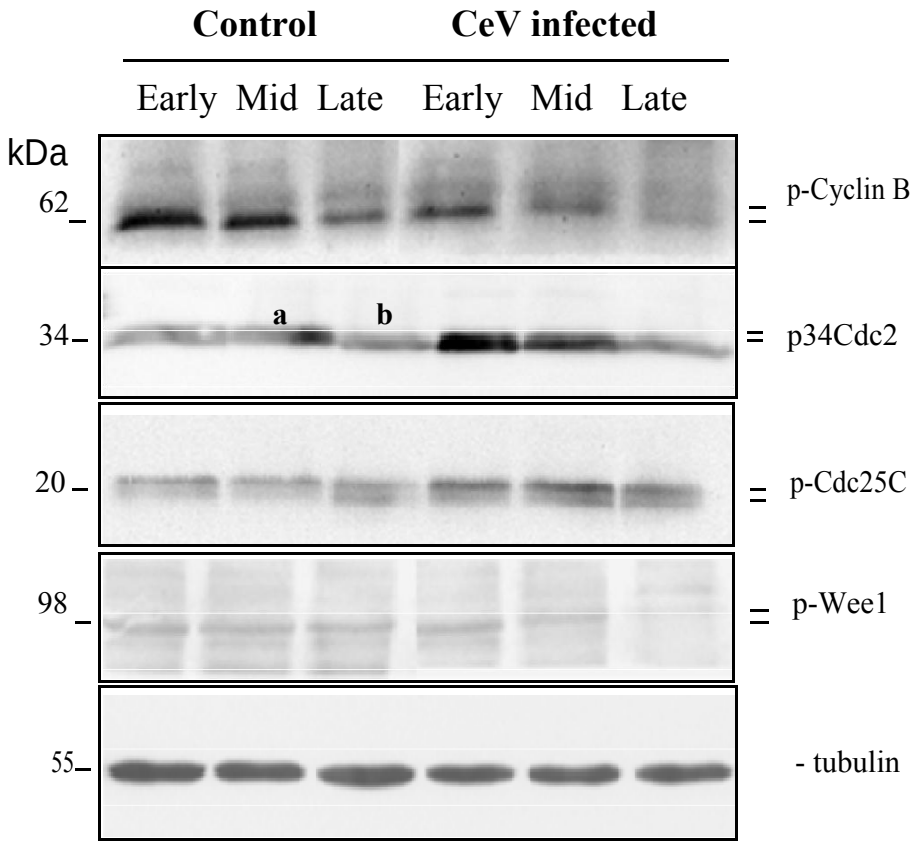

Figure 3. Change in expressed and phosphorylated status of G_2/M regulators in CeV infected *C. ericina* cells and in uninfected *C. ericina* control cells. Cells were harvested at the indicated times, and subjected to immunoblot analysis with antibodies against cyclin B1, p34Cdc2, Cdc25C, Wee1 or -tublin (control). Bands marked a and b denotes species with altered electrophoretic mobility due to different degree of phosphorylation.

infected cell lysates than those of corresponding proteins present in lysates of control cells at early infection and the more significant observation was that CeV infected cells exhibit a considerably high level of Cdc2 activity in the absence of detectable levels of cyclin B (Figure 3).

The Cdc25C phosphatase had a molecular weight of about 20 kDa that is much less than the 55 kDa reported for most other organisms. There is a double bands of Cdc25C which was detected and the upper phosphorylation /activation of the Cdc25C bands dominated in both the infected and control cells, and increased in CeV infection cells (Figure 3). The Wee1 proteins appear dephosporylated and active throughout in the uninfected cells while as it becomes phosporylated / inactivated and decline in concentration in the infected cells (Figure 3). The reduced inhibitory kinase Wee1 and the increased activating phosphatase Cdc25C in CeV infection farther suggest that Cdc2 was active (species b). These results suggest that CeV infection leads to the decreased activation of the Cdc2/cyclin B complex by reducing and early phosphorylation cyclinB, but also to an overexpression of kinase-active Cdc2, resulting in a G_2 cell cycle delay.

DISCUSSION

Viral infection and G_2/M transition regulation in *E. huxleyi* and *C. ericina*

Using commercially available antibodies, G_2/M-phase regulatory proteins homologs in two marine phytoplankton species have been detected, which indicate that cell cycle regulatory mechanisms in phytoplankton may be similar to that of other higher eukaryotes. *E. huxleyi* 1516 genome is publicly available from the Joint Genome Institute website (http://genome.jgi psf.org/Emihu1/Emihu1.home.html) (Wahlund et al., 2005) and it is confirmed that all the cell cycle regulators mentioned previously do exist in *E. huxleyi* (Table 1). The phosphorylation status of Cdc2 can be assessed by its electrophoretic mobility. Meanwhile, since phosphorylated Cdc2 accumulate in S or G_2 but not M, it can also be used as an indicator of cell cycle stage (O'connell et al., 2000; Yuan et al., 2004). Hence, if the G_2/M transition is blocked, it can be expected that the Cdc2 is phosphorylated (Advani et al., 2000). The infected *E. huxleyi* cells showed loss of unphosphorylated

Table 1. Cell cycle regulator factors of G_2/M phase transition in *E. huxleyi* CCMP1516 EST evidence *.

Relevance	Type	Assigned name	Protein ID	Detail
1.0	Transcript	CycB	466575	Core cell cycle gene cyclinB
1.0	Transcript	Cdk2	436308	Core cell cycle gene cyclin dependant kinase C2
1.0	Transcript	Fgenesh_NewkGS_KG.43_57_2686716:1	356004	Ras1 guanine nucleotide exchange factor cdc25
1.0	Transcript	Fgenesheh_PG.58_53	104255	Cyclin-dependent kinase Wee1

*E-value <1.0 e^{-20}

Databse: Emiliania huxleyi CCMP1516 main genome assembly v1.0

Cdc2 (species c) and accumulation of phosphorylated Cdc2 (species a) early after infection, suggesting that Cdc2 was inhibited and Cdc2/cyclin B1 activation was impaired, thus the EhV infection delayed the entry of host cells into mitosis. Dephosphorylation and inactivation of Cdc25C combined with up-regulation of Wee1 expression early during the infection appears to be the important mechanisms by which EhV represses Cdc2/cyclinB complex activity.

A number of possibilities can be envisioned for the role that G_2/M accumulation plays in EhV replication. By prolonging the time that a host cell spends in the activated state, the virus may maximize the output of progeny virus. The discovery of a functional RNA polymerase in EhV (Wilson et al., 2005; Allen et al., 2006) suggests that the virus may have the capacity to transcribe its own genes during infection without relying on the control from host cell cycle (Allen et al., 2006). To exert its own control it may be necessary for the virus to interfere with and block the host cell cycle control mechanism.

Declined cyclin B in CeV infected cells can be explained by the relatively short half-life of this protein combined with total shutoff of protein synthesis, active degradation through the ubiquitin pathway or virus directed degradation. In the normal cell cycle Cdc2 kinase activity peaks at the G_2/M interphase and is then shut off precipitously as the result of cyclosome activation by Cdc2 and ubiquitin-dependent degradation of cyclin B (King et al., 1994; Advani et al., 2000) . The loss of cdc2 activity serves as a signal for mitosis to proceed. It seems that CeV infected cells do not cross the G_2/M interface since cdc2 is in a higher accumulation of active form and the infected cell does not receive the signal for mitosis. The reason for CeV infected cells to exhibit a high level of Cdc2 activity in the absence of detectable cyclin B is not immediately obvious, but may suggest that Cdc2 or one of its aberrantly modified forms had acquired a new partner, possibly a viral protein. The observations described earlier indicate that CeV infection induced the overproduction and early activation of Cdc2 kinase, combined with the decreased activation of the Cdc2/cyclin B complex by early deactivation and reduced concentration of cyclin B and thus a G_2 cell cycle delay.

The selective advantage provided to the virus by G_2 cell cycle delay is unknown, but there may be several possibilities. Blocked cells may produce more viruses if transcription factors required for expression of the viral genome are more abundant in this phase of the cell cycle. A preferred explanation is that cells blocked in a delayed G_2 phase partly will turn into the second S phase to acquire or preserve proteins useful for viral nucleic acid metabolism. The requirement for S phase suggests that the replication of CeV may depend on cellular factors that are active for cellular DNA replication and that CeV virus scavenge such factors to replicate its own DNA. From these results, we suppose that CeV has adopted a strategy of early cellular activation which facilitates its own replication at the expense of host cellular DNA replication.

Ecological implications

In order to cope with hostile intracellular host environments, many viruses have developed strategies to perturb the cellular machinery to suit their replication needs. EhV propagation strategy is to transcribe its own genes without relying on the host cell cycle control, while CeV seem to require an S phase suggesting that it is dependent on host cell cycle control. *Pyramimonas orientalis* virus is dependent on the phase of the cell cycle for optimal viral replication, whereas, *Phaeocystis pouchetii* virus is independent on the phase of the cell cycle for viral replication and the differences in virus production may be attributed to cell cycle dependent regulation of host infection, metabolism, or burst size (Thyrhaug et al*.,* 2002). Interestingly, *E. huxleyi and P. pouchetii* are typical bloom-forming species, whereas C. *ericina* and *P. orientalis* usually occur in low abundance in seawater. Expression and replication of algal viruses dependent or independent on the cell cycle might have

implications on the interaction between algal virus infection and cell cycle progression of their host, and the way this interaction is regulated, may determine the host species abundance in field. Viruses infecting algae have been found to be important for regulating growth dynamics of the primary producers and several examples of viruses causing algal blooms to terminate are known (Bratbak et al., 1993; Brussaard, 2004). The different cell cycle progression response to viral infection may be related to differences in the ecological strategies of the hosts and their ability to form blooms. It seems reasonable to assume that there are some connection between the ecology of the host and the mode of host -virus interaction as revealed by cell cycle events during infection. At present, it is still controversial about the opinion that whether or not it is the host ecology, for example, abundance, growth rate, bloom formation ability etc. that dictates the interaction, or vice versa, the virus dictates the interaction and determine the host ecology. Our results suggest that the interactions between host and viruses are optimized to maintain sustainable populations of both and to avoid extinction.

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