FUNCTION OF EC27, A PUTATIVE CYCLIN ENCODED BY BACULOVIRUS, AND COMPOSITION OF THE EC27-ASSOCIATED PROTEIN COMPLEX

A Senior Honors Thesis

By

LUKE JAMES ENGELKING

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2000

Group: Cell Biology 2

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Approval as to style and content by:

Max D. Summers
(Fellows Advisor)

Edward A. Funkhouser
(Executive Director)

April 2000

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ABSTRACT

Function of EC27, a Putative Cyclin Encoded by Baculovirus,

And Composition of the EC27-associated

Protein Complex. (April 2000)

Luke James Engelking
Department of Biochemistry and Biophysics
Texas A&M University

Fellows Advisor: Dr. Max D. Summers
Department of Biochemistry and Biophysics

Baculovirus encodes a unique protein, EC27, that may be multifunctional cyclin. EC27 is a structural component of the virus, has amino acid sequence homology with the cyclin fold region of several cellular cyclins, and is part of immunoprecipitated protein complexes that have cyclin B and cyclin D-like kinase activities. Baculovirus infection apparently alters or overrides at least two checkpoints in the host cell cycle: entry into S-phase and progression through G2/M-phase. We speculate abrogation of these cell cycle checkpoints is accomplished by the function of virus encoded proteins. EC27 potentially interacts with the key regulatory elements of those two cell cycle checkpoints; we know that EC27 is present in active complexes that contain cdc2 (G2/M-phase, cyclin B-like function) and cdk6 (G1/S-phase, cyclin D-like function). EC27 is therefore a likely suspect as a viral protein involved in host cell cycle manipulation. To understand the function of EC27 during infection, we endeavored to identify the proteins that EC27 interacts with in the cell by western blot analysis of EC27 immunoprecipitates. A yeast

two-hybrid screen, performed as part of preliminary studies into EC27 function, identified another baculovirus structural protein, ORF101, as an EC27-interacting protein. We confirmed by immunoprecipitation that that ORF101 can be detected in complexes precipitated by EC27 antisera and vice versa. Using antisera that seem to discriminate between different molecular weight forms of EC27, we demonstrated EC27 may form different complexes with distinct compositions. We showed that ORF101 can associate with the 35 kilodalton form of EC27 but not with the 27 kilodalton form of EC27. To test if EC27 alone can function as a cyclin, we performed a yeast complementation assay in G1-cyclin deficient *Saccharomyces cerevisiae*. We demonstrated that full-length EC27 cannot rescue the G1-cyclin deficient phenotype. This result can be interpreted in two ways: first, since some cellular cyclins also fail the complementation test, this result may be a false negative; second, this result may indicate that the mechanism of EC27's interaction with cdk's is different than cellular cyclins. The latter interpretation supports the idea that EC27's functional state may be as part of a larger protein complex.

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INTRODUCTION

The Biology of Baculoviruses

Autographa californica nucleopolyhedrovirus (AcMNPV), a genetically complex double-stranded DNA virus of the family *Baculoviridae* that is infectious to a wide range of lepidopteran insects, has a long evolutionary history with its host (Miller, 1997). The etymology of the term baculovirus come from the latin word for staff or rod, "baculum", owing to the rod-like shape of the virus particle.

Baculovirus infection is spread between hosts through the ingestion of viral occlusions, or virus particles encapsulated in a protein matrix (see Miller, 1997 for review). In the alkaline pH of the insect midgut, viral occlusions dissolve and release ODV (occlusion derived virus). ODV infect the columnar cells of the midgut epithelium; entry into cells occurs by fusion of the virion envelope and the microvillar membrane. The nucleocapsid then moves into the nucleus and viral DNA replication begins. At approximately 10-12 hours post infection, BV (budded virus) is produced. This type of viral progeny causes secondary infection in other tissues of the host. Budded virus has a different structure than ODV: its envelope is derived from the plasma membrane as it buds from the host cell while ODV's membrane is derived from the nuclear membrane (Braunagel et al., 1994). The principal form of progeny virus in the midgut is BV; tissues that are infected secondarily, such as the fat body, produce enormous titers of ODV. In total, the infection process occurs over 5-10 days and ends

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This thesis conforms to the style and format of *Cell*.

in wilting or liquefaction of the host insect. ODV are released into the environment, protected inside polyhedra (the protein crystals which encapsulate ODV, made of the protein polyhedrin), and await a host.

AcMNPV infection potentially influences at least two cell cycle checkpoints. During primary infection of the migut, the virus must hastily produce BV or risk being fended off by the host. The migut epithelial cell is terminally differentiated, or in the G0 cell cycle phase. Therefore, the midgut cell's DNA replication machinery is inactive. To successfully infect the insect, the virus must have a mechanism to progress the cell into an S-phase like environment so that this first round of viral DNA replication and concomitant BV production can occur.

This promotion of S-phase is seen in the infection of *in vitro* SF9 cell culture. At approximately 8-10 hours post infection, an enrichment in the proportion of cells in S-phase is observed and is accompanied by an increase in viral DNA replication (Braunagel et al., 1998). Abrogation of a second checkpoint is also seen *in vitro*: AcMNPV infection of SF9 cells results in a G2/M phase arrest of approximately 85% of the cell population at 18-24 hours post infection. Viral DNA synthesis continues beyond this point independently of cellular DNA synthesis and "out of phase" since DNA replication occurs in S-phase (Braunagel et al., 1998).

If a cell culture is synchronized in G1/S phase by drug treatment (nocodazole) and infected, the cell population arrests in S phase for approximately 10 hours. If a cell culture is synchronized in G2/M phase by drug treatment (mimosine) and infected, the cells do not progress and remain arrested at G2/M. These results suggest that AcMNPV

regulates two checkpoints: the G1/S and G2/M phase cell cycle checkpoints (Braunagel et al., 1998).

It is thought that the G2/M phase arrest would benefit the virus late in infection, as ODV are being assembled. Our hypothesis is that the ODV envelope is formed from intranuclear microvesicles produced from the nuclear membrane. The nuclear membrane is most fluid during M-phase and a G2/M phase arrest may facilitate the induction of microvesicles.

Cell Cycle Regulation

Control of the eukaryotic cell cycle is accomplished primarily through the activity of cyclin dependent kinases (cdk's) which control the progression from one cell cycle phase to the next by the phosphorylation of various targets (Hutchison et al., 1993). The activity of these kinases is regulated through association with their regulatory proteins, cyclins. In brief, the sequential activation or deactivation of cyclin/cdk complexes occurs through at least three different pathways: 1. Cell cycle phase specific expression and ubiquitin-mediated proteolysis of cyclins (Koepp et al., 1999), 2. Phase specific activating phosphorylation or dephosphorylation events (Nurse, 1990), 3. Phase specific recruitment of cyclins from the cytoplasm into the nucleus (Schafer, 1998). Each cell cycle phase has specific cyclin/cdk complexes which control progression through that phase: cyclin D/cdk(4 or 6) initiates proliferation from G0 to G1; cyclin E/cdk2 promotes progression from G1 to S phase; cyclin A/cdk 2 is active in S and G2; lastly cyclin B/cdc2 promotes transition from G2 though M phase (Schafer, 1998).

AcMNPV seems to manipulate the host cell cycle at checkpoints from G0→S phase and at G2/M phase. The mechanism of cell cycle control at these two checkpoints is well understood in mammalian cells and seems to be highly conserved among animals. Induction of growth in quiescent, or non-dividing, (G0-phase) cells involves the activity of cyclin D/cdk(4 or 6) dimers and the pRb/E2F complex (Weinberg, 1995). Extracellular signaling events, such as activation of a G-protein linked receptor tyrosine kinase by extracellular growth factors, are required for normal entry into the cell cycle from a non-dividing state. The final result of these extracellular signals is progression past the restriction point, at which time normal cell cycle progression depends mostly upon internal signals and will progress through the entire cycle, ending in cytokinesis and re-entry into G1. One downstream effect of mitogenic signal transduction pathways, such as the Ras→MAPK pathway, is the induction of transcription factors that activate the expression of cyclin D (Peeper, et al. 1997). Cyclin D is the regulatory partner of cdk's 4 and 6, whose functions seem to be redundant. Thus, cyclin D levels are induced during mid-G1; once cyclin D binds cdk4/6, the dimer's cyclin kinase activity becomes activated and it can phosphorylate its substrate, pRb. pRb, the retinoblastoma protein, is a tumor-suppressor; its function is to bind and deactivate the transcription factor E2F. E2F-responsive genes are required for entry into S-phase. E2F's activation is accomplished in two steps: first, cyclin D/cdk(4 or 6) phosphorylates pRb's c-terminus, resulting in the release of HDAC (histone deacetylase, which helps to keep DNA in tightly-wound, transcriptionally inactive heterochromatin) and second, cyclin E/cdk2 phosphorylates pRb's pocket domain to release and activate E2F (Harbour et al., 1999).

Although the mechanism is not completely understood, cyclin E/cdk2 activation first requires the activation of cyclin D/cdk(4 or 6); cyclin E activity peaks in late G1 immediately prior to entry into S-phase. Adding another level of complexity to this regulatory process are CKI's (cyclin kinase inhibitors), which bind and inactivate cdk's (the p16 family: p15, p16, p18, and p19) or cyclin/cdk dimers (the p21 family: p21, p27, and p57) (Elledge, 1994). Of central importance to this model is p27, which can bind and inhibit both the cyclin D and cyclin E dimers, and p16, which triggers transcriptional repression by pRb/E2F (Zhang et al., 1999).

Progression through G2/M phase is accomplished by the activity of cyclin B/cdc2 dimers (also known as the MPF, or mitosis promotion factor). Cyclin B/cdc2 activation is controlled by the wee1 kinase/cdc25 phosphatase pair, which works in direct opposition to each other. Dephosphorylation by the cdc25 activates cyclin B/cdc2 while phosphorylation by wee1 deactivates cyclin B/cdc2 (Nurse, 1990). DNA damage pathways prevent entry into M-phase (and the passage of mutated DNA to progeny cells) by inactivating cdc25 (Nurse, 1994). Cyclin B/cdc2 has multiple substrates, including histone H1 and nuclear lamins. It is thought that phosphorylation of lamins by the MPF, which leads to their depolymerization and the breakup of the nuclear lamina, increases nuclear membrane fluidity and facilitates the breakdown of the nuclear membrane into microvesicles (Dessev et al., 1991). Degradation of cyclin B is required for the completion of mitosis. The APC (anaphase promoting complex), a ubiquitin ligase complex, recognizes a region present and highly conserved among cellular cyclins called the "destruction box" near the N-terminus of cyclin B. The APC then polyubiquitinates

cyclin B on lysine residues adjacent to the destruction box, targeting it for proteolysis at the 26S proteasome (Glotzer et al., 1991). The addition of non-degradable cyclin B (i.e. without a functional destruction box) to mitotic cells causes an M-phase arrest.

EC27: a Multifunctional Cyclin?

Viruses use host replication machinery to synthesize and express their genomes. Some viruses directly hijack the host cell cycle regulatory machinery through the activity of virus-encoded proteins (Meijer et al., 1997). For example, the Adenovirus E1A gene product can, among several functions, associate directly with pRb to activate the transcription factor E2F, which can cause quiescent rodent cells to proliferate from G0 phase into active cell cycle progression (Grand et al., 1994). Likewise, human cytomegalovirus induces cyclin E and cyclin B, among other regulatory proteins, to promote proliferation (Salvant et al., 1998). Hepatitis B virus can stimulate cell cycle progression through the action of the viral HBX protein, which increases cdk2 and cdc2 associated kinase activity and can bind and inactivate p53, a vital tumor-suppressor protein, to promote progression through both G1/S and G2/M phases (Meijer et al., 1997). In addition to inducing cell cycle progression, viruses can arrest the cell cycle. Human immunodeficiency virus (HIV) causes a G2/M phase arrest through the activity the viral Vpr protein, which is thought to inactivate cyclin B/cdc2 complexes by activating the cellular phosphatase PP2A, an inhibitor of cdc25 and activator of wee1 (Poon et al., 1998).

Three virus-encoded cyclins were reported in the literature prior to EC27; these cyclins were found in herpesviruses: v-cyclin from herpesvirus saimiri (HVS), k-cyclin from human herpesvirus 8 (HHV8), and m-cyclin from murine gammaherpesvirus-68 (MHV-68) (Laman et al., 2000; Virgin et al., 1997). Both v-cyclin and k-cyclin form active complexes with cdk6, the catalytic partner of cyclin D, and are capable of phosphorylating pRb, the preferred *in vitro* substrate of cdk4/6; in addition, k-cyclin complexes are also capable of phosphorylating histone H1, the preferred *in vitro* substrate of cdc2 (Chang et al., 1996; Jung et al., 1994; Godden-Kent et al., 1997). Both v- and k-cyclin/cdk6 complexes are resistant to inhibition by the CKI's p16, p21 and p27 (Swanton et al., 1997). Ectopic expression of either viral cyclin prevents a CKI-induced G1 arrest in quiescent fibroblast cells. 3-D structure crystallography studies show that the insensitivity of v-cyclin/cdk6 complexes to p21 is due to structural differences between v-cyclin and cellular cyclins that lead to fewer protein-protein contacts between v-cyclin and p21; also, sequence differences in v-cyclin eliminate hydrogen-bonding interactions with p27 (Schulze-Gahmen et al., 1999). Lastly, k-cyclin/cdk6 complexes can phosphorylate p27 its C-terminus, which activates p27 degradation (Mann et al., 1999). These data suggest that herpesvirus cyclins contribute to the activation of cell cycle progression, and even neoplasmic transformation, of infected cells even when high levels of negative cell cycle regulators (e.g. CKI's) are present (Swanton et al., 1999).

As aforementioned, AcMNPV manipulates the host cell cycle at two checkpoints. We believe this abrogation of the host cell cycle is due to the activity of

baculovirus-encoded proteins. Several experiments have led to the speculation that EC27 contributes to host cell cycle manipulation and furthermore that it may be a unique, multifunctional viral cyclin.

Computer-assisted amino acid sequence homology analysis identified a region of sequence similarity between EC27 and the cyclin box, a gene region which is highly conserved among cellular cyclins. EC27 also has sequence homology with helixes 4 and 5 of a larger conserved region called the cyclin fold, which forms contacts with cdk's (Noble et al., 1997). EC27, however, lacks the characteristic destruction box of the cellular cyclins (Belyavskyi et al., 1998). This data suggested that EC27 may have cyclin-like properties. EC27 immunoprecipitates were taken from infected SF9 cell lysates and then assayed for kinase activity. Like k-cyclin, EC27-associated complexes could phosphorylate both histone H1 and pRb. Immunoprecipitates to individual cellular cdk's were taken from infected cells and then probed by western blotting for the presence of EC27. EC27 was shown to associate with cdk6, cdc2, and to a much lesser degree, with cdk7 (Belyavskyi et al., 1998). The EC27 gene region is transcriptionally complex, and codes for three proteins with relative mobilities of 18 kilodaltons, 27 kilodaltons, and 35 kilodaltons (the 35 kilodalton form corresponds is a fusion of the two smaller forms). Internal slippery sequences, or mRNA pseudoknot sites, that can cause ribosomal frameshifting may account for translational readthrough and the multiple forms of EC27 (Braunagel et al., 1996). The 27 kD form of EC27 was detected in these immunoprecipitates. Preliminary studies also have shown that, like the herpesvirus

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cyclins, EC27-associated kinase activity is insensitive to inhibition by CKI's p21 and p15 (personal communication, Belyavskyi).

AcMNPV abrogates the host cell cycle at G1/S, which is controlled by cyclin D/cdk(4 or 6), and at G2/M, which is controlled by cyclin B/cdc2. Curiously, EC27 is present in complexes that contain the key regulatory elements of those two cell cycle checkpoints, which makes EC27 a strong suspect as a virus-encoded protein which may manipulate the host cell cycle.

We speculate that EC27 has both cyclin B and cyclin D–like functions. Like cyclin D, EC27 is present in complexes with cdk6 and PCNA (proliferating nuclear cell antigen, a protein which has a role in the regulation of DNA replication) (Belyavskyi et al., 1998). EC27 is a structural component of both the nucleocapsid and the viral envelope in ODV (Braunagel et al., 1996). Thus, it is introduced into the cell immediately upon infection. The first checkpoint that the virus must exploit is entry into S-phase, as described earlier, when the virus must induce replication of its genome in the G0-phase midgut epithelial cell during primary infection. EC27 could promote an S-phase like environment through one of a few possible models: 1. EC27 could simply mimic cyclin D and dimerize with cdk6, leading to the phosphorylation of pRb. Preliminary studies suggest that EC27/cdk complexes are insensitive to CKI inhibition; these complexes could induce entry into S-phase even in differentiated cells with high levels of inhibitors. 2. EC27 complexes could titrate CKI's such as p27 and p21 from the cell to activate cellular cyclin D or E complexes to promote the activation of E2F. In addition to primary infection, EC27's possible cyclin D-like functions may help to

explain "out of phase" viral DNA replication that can be detected as late as 60 hours post infection, long after the infected cells arrest at G2/M. This viral DNA replication cannot occur without the activation of S-phase specific genes; EC27's cyclin D-like functions could serve to activate these DNA replication genes "out of phase".

Like cyclin B, EC27 is present in complexes with cdc2. This interaction may have an effect in the maintenance of the G2/M phase arrest observed at approximately 18 hours post infection. Cellular cyclin B is degraded at approximately the same time as the initiation of the G2/M phase arrest. Levels of cdc2-associated histone H1 kinase activity are maintained long after cyclin B is no longer detectable (Braunagel et al., 1996). As aforementioned, degradation of cyclin B and its associated kinase activity are the major signal for exit from mitosis; without degradation of cyclin B, cells arrest in metaphase. EC27 may activate cdc2, forming a non-degradable (since it lacks the cyclin destruction box) cyclin-B homologue, which maintains the M-phase arrest as well as nuclear membrane fluidity, a factor which favors maturation of ODV envelopes.

EC27 may be a unique protein. No other reported virus-encoded cyclin is multifunctional. No reported virus-encoded cyclin is a structural component of the virus, immediately introduced into the cell during infection to, perhaps, manipulate the host cell cycle. Studies into EC27 may therefore reveal novel mechanisms of cell cycle regulation.

This report describes a study into three issues in the characterization of EC27. To ascertain if EC27 alone has cyclin-like activities, a yeast complementation assay was performed to determine if EC27 could rescue G1 cyclin deficient *S. cerevisiae*. To

determine the composition of the EC27-associated complex, immunoprecipitations were taken and probed by western blotting for the presence of several putative-EC27 binding partners. Lastly, to resolve differences in the function of the 27 and 35 kilodalton forms of EC27, antisera which seem to be specific for each form were characterized in both immunoprecipitation and kinase assay experiments.

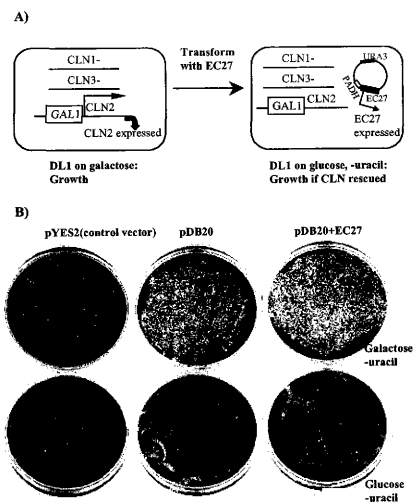


Figure 1. EC27 does Not Rescue CLN- yeast
(A) Schematic of yeast complementation assay. EC27 was cloned into a yeast expression vector (pDB20) and transformed into DL1, a CLN- strain conditionally viable on galactose-based media due to ectopic expression of CLN2. Cells can only grow on glucose-based media if the transformed gene can rescue the CLN- phenotype.
(B) Transformation plates are displayed, with the vector transformed listed atop each column. Growth is only seen on the top row, media containing galactose (to monitor growth with ectopic expression of CLN2) without uracil (to select for the plasmids). No growth is seen on the bottom row, media containing glucose (to monitor growth without native CLN's for rescue by EC27) without uracil.

RESULTS

EC27 Does Not to Rescue G1 Cyclin (CLN) function in *S. cerevisiae*

To determine if EC27 alone has cyclin-like activities, a yeast complementation assay was performed, testing whether or not EC27 could rescue CLN-deficient yeast. Several

cellular cyclins have been cloned by functional complementation in this assay (Leopold et al., 1996; Day et al., 1996). *Saccharomyces cerevisiae* strain DL1 (gift of Steven Reed, Scripps Research Institute) has its three native CLN loci (G1 cyclin) disrupted by insertional mutation and has a copy of CLN2 fused to the GAL1 promoter integrated at the LEU2 chromosomal locus. The GAL1 promoter drives expression of CLN2 when cells are grown on galactose-containing media but represses CLN2 expression when cells are placed on glucose-containing media (Lew et al., 1991). CLN function is required for yeast progression through G1 phase; CLN's dimerize with and activate cdc28, the single cdk in *S. cerevisiae*, which phosphorylates multiple targets to effect cell cycle progression (Forsburg et al., 1991). Therefore, DL1 arrests upon glucose-based media and grows on galactose-based media. Full-length EC27 was generated by PCR from viral genomic DNA and cloned into the yeast expression vector pDB20 (gift of David McNabb, Massachusetts Institute of Technology), which is a high copy plasmid (2μ origin of replication) that drives expression from the strong constitutive alcohol dehydrogenase promoter (PADH1) and contains a URA3 selectable marker (Becker et al., 1991). DL1 was transformed by a high efficiency lithium acetate method with pDB20 + EC27 (Gietz et al., 1992). Transformants were plated on both solid media lacking uracil (to select for the plasmid) and containing glucose (to select for rescue of CLN loss) and media lacking uracil and containing galactose (to monitor growth with ectopic expression of CLN2). Growth was observed on galactose-containing plates (top row, Figure 1) but not on glucose-containing plates (bottom row, Figure 1). Confidence in the success of the transformation can be estimated by the negative control (there was

no growth observed on transformation plates for yeast transformed without a URA3+ plasmid, data not shown), in that the rate of reversion for URA3 is low (<1%), which ensures that the growth on galactose plates is specific for the presence of the URA3 gene on pDB20 or pDB20+EC27.

These data suggest that EC27 cannot rescue CLN loss in yeast. It should be noted that not all cellular cyclins complement CLN loss in DL1 on glucose. For example, Drosophila G2 cyclins fail to rescue DL1 (Leopold, et al., 1991). This assay examines only if a gene can replace the function of CLN2, thus presumably testing if the gene product of interest can dimerize with and activate cdc28. Thus, not all cellular cyclins would be expected to test positive in this assay since different cyclins from higher eukaryotes interact with a wide variety of divergent cdk's, some of which have only a low level of sequence similarity with cdc28 (the yeast homologue of cdc2).

Characterization of the EC27-associated Complex

Prior to the initiation of studies to determine the composition of the EC27-associated complexes, several background studies were done to optimize the conditions for immunoprecipitation. Two working antisera (rabbit polyclonal antibodies) to EC27 were used; both antisera were raised against purified, recombinant EC27. The first studies into EC27 produced and used the first antisera, #7351, which was raised against a GST-EC27 fusion (from here, referred to as EC27-GST or 7351) (Braunagel et al., 1996 and 1998; Belyavskyi et al., 1998). More recent studies by have produced a second antisera (#11209, referred to as EC27-HIS or 11209), which was raised against

affinity purified HIS-tagged EC27 (unpublished data, Braunagel et al., 1999). The studies described here characterize the performance of these antibodies, especially in comparison to each other, in terms of western blotting, kinase assays, and immunoprecipitations.

In the analyses of these results, the buffer conditions used here must be taken into consideration. For the majority of the experiments described, immunoprecipitates were taken in RIPA lysis buffer, a relatively stringent buffer containing 0.1% SDS. Weakly-associated protein complexes may dissociate in this buffer. In latter experiments, immunoprecipitates were taken in a less stringent HEPES-based buffer containing only non-ionic detergents. Thus, experiments using RIPA buffer (figures 2, 3, 4A, and 5) may be too stringent to detect signals resulting from weak protein-protein interactions.

Protein complexes immunoprecipitated with both the EC27-GST and EC27-HIS antisera were taken from whole cell lysates of SF9 cells harvested at 48 hours post infection by AcMNPV strain E2. To optimize the signal to noise ratio and to verify a linear signal response between the amount of antibody used in precipitation and the amount of EC27 detected by western blotting, immunoprecipitates were taken using 5, 10, and 15 ul of either antiserum. These immunoprecipitates were resolved by SDS-PAGE and probed by western blotting with the same antisera. An immunoreactive protein with a relative electrophoretic mobility of 27 kilodaltons was detected by the EC27-GST antiserum in complexes precipitated with the same antiserum. On this form of EC27 (i.e. using the EC27-GST antisera) all previous studies have been performed.

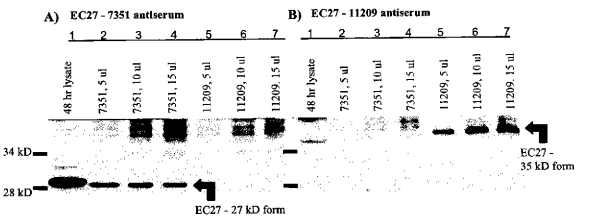


Figure 2. Detection of EC27 in EC27 Immunoprecipitates using Antisera 7351 and 11209
Immunoprecipitates were taken from 48-hr. post infection cell lysates using EC27 antisera 7351 and 11209. The amount of antiserum used to precipitate complexes is indicated. Precipitated protein complexes were separated by SDS-PAGE and EC27 was detected by western blot using both antisera. (A) Western blot of 7351 and 11209-immunoprecipitates and infected cell lysate probed with antiserum 7351. In 7351-immunoprecipitates and infected cell lysate, a 27 kilodalton protein is detected (lanes 1-4). (B) Western blot of 7351 and 11209-immunoprecipitates and infected cell lysate probed with antiserum 11209. In 11209-immunoprecipitates and infected cell lysate, a 35 kilodalton protein is detected (lanes 1,5-7).

Also, an immunoreactive protein with a relative electrophoretic mobility of 35 kilodaltons was detected by the EC27-HIS antiserum in complexes precipitated with the same antiserum. EC27-HIS and EC27-GST antisera do not cross-react with the same target protein band. The 35 kilodalton EC27 form was not detected by the EC27-GST antiserum; the 27 kilodalton EC27 form was not detected by EC27-HIS antiserum. Since these antisera were raised against the same protein (even though in different form: GST fusion versus HIS-tag) and are polyclonal antibodies, they should recognize the same protein. They, however, recognize proteins of two different molecular weights. Assuming that there were no problems with recombinant EC27 proteins used to raise the

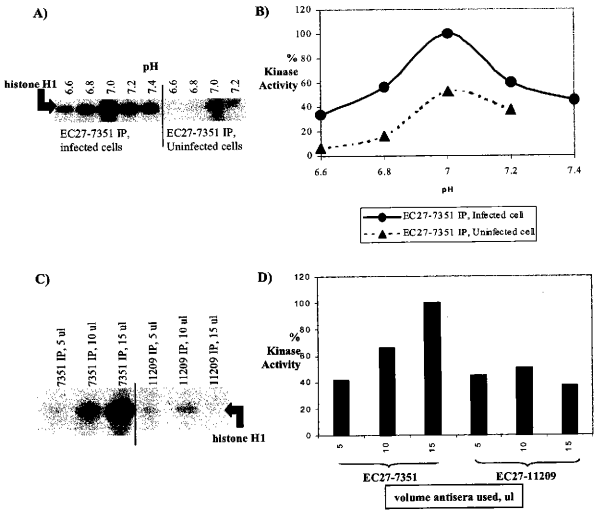


Figure 3. EC27-associated Kinase Activity

(A) To ensure that EC27-associated kinase activity is specific to EC27 and to ensure the maximum activity was being assayed, a pH dependence of EC27-associated kinase activity was determined. Immunoprecipitates were taken using EC27-7351 antiserum from both infected and uninfected SF9 cells, and the kinase activity of the precipitated protein complexes was determined using histone H1 as the *in vitro* substrate in assay buffers of pH 6.8 to 7.4. The phosphorylated histone H1 bands are shown. (B) The histone band is quantitated by Spot Denso function of the Chemimager software package. (C) To compare the kinase activity of complexes precipitated with the EC27-7351 and EC27-11209 antisera, immunoprecipitates were taken with those antibodies and assayed for kinase activity using histone H1 as the in vitro substrate. To verify a linear signal response, a range of 5 to 15 ul of antiserum was used in the immunoprecipitation. Antisera volumes are indicated. (D) The phosphorylated histone H1 band is quantitated by Spot Denso function of the Chemimager software package.

antisera, it appears that the EC27-GST antiserum is specific for the 27 kilodalton form of EC27 and EC27-HIS antisera is specific for the 35 kilodalton form of EC27.

Since most of our interest in EC27 is a result of its presence in precipitated protein complexes with cyclin kinase activity (presence of cdc2 and cdk6, ability to phosphorylate *in vitro* cyclin kinase substrates histone H1 and pRb), we directed our efforts into the optimization of the conditions for the immunoprecipitation and kinase assay procedures.

The kinase assay procedure which had been used previously was optimized for use in mammalian cell system, at a pH of 7.5 to mimic the mammalian intracellular pH. The insect intracellular pH is somewhat lower, closer to 7.0. To ensure that our experimental conditions maximized the signal response, we performed kinase assays in reaction buffers from a range of 6.8 to 7.4.

To establish a "zero-point" activity, or kinase activity in the EC27-associated complexes not specific to EC27, immunoprecipitates were taken from uninfected SF9 cells in addition to infected SF9 cells. The maximum activity in precipitates from infected cells, denoted 100% activity with arbitrary units, is observed at a reaction buffer pH of 7.0 (figure 3A lane 3, figure 3B solid line). Percent activities drop off precipitously in bell curve type dependence at both lower and higher pH's. Thus, the optimal conditions of these kinase assays in insect cells are, as might be expected, closer to the intracellular environment of insect cells at pH 7.0. The maximum activity in precipitates from uninfected cells is 52.5% (based on IDV, or integrated density values from Chemimager software analysis), which is observed at pH 7.0 (figure 3A lane 8, 3B

dashed line). Whether this level of activity is constituted by cellular protein complexes with kinase activity that are precipitated by the EC27-GST antibody (perhaps due to structural homology between EC27 and cellular cyclins) or that this 52.5% activity is the baseline level of activity of this *in vitro* assay (due to autophosphorylation of histone H1, etc.) cannot be determined here.

The kinase activity of protein complexes precipitated with both the EC27-GST and EC27-HIS antisera was assayed. In addition to a directly comparing the protein complexes precipitated by these two antisera, these experiments were performed to optimize the signal to noise ratio and to establish the limits of a linear signal response between the amount of antibody used to precipitate complexes and the detectable kinase activity. A linear increase in kinase activity signal and amount of antiserum used in precipitation is observed with the EC27-GST antiserum but not with the EC27-HIS antiserum (figure 3C,D). 100% activity, based in arbitrary units, is derived from the activity of the EC27-GST 15 ul precipitate (figure 3C lane 3, 3D column 3). The average signal from EC27-HIS precipitates is 44.4% ± 6.6% (figure 3C lane 4-6, 3D columns 4-6). Although it must be kept in mind that the kinase activities measured here reflect stringent buffer conditions, the lack of positive signal response to antiserum concentration and the fact that "baseline" activity of a EC27-GST immunoprecipitate is approximately 50% while the average EC27-HIS activity is 44% may indicate that the EC27-HIS immunoprecipitate's activity is not significant when compared to background.

Composition of the EC27-associated complex

Perhaps the most informative data to be determined about EC27 is the identity other proteins with which it interacts in the cell. Our evidence may suggest the EC27, unlike other viral cyclins, operates in a complex larger than the traditional 1 cyclin : 1 cdk active heterodimer; therefore the strongest indications of EC27's function will be in the protein composition of its complexes.

In preliminary studies, eight clones in a yeast-2-hybrid screen identified another viral nucleocapsid protein, ORF101, as an EC27-interacting protein (unpublished data, Braunagel and Marcano, 1998). To confirm this data biochemically, protein complexes were precipitated using ORF101, EC27-GST, and EC27-HIS antisera from uninfected and infected SF9 cells (60 hr post infection), separated by SDS-PAGE, and probed by western blot with the same set of antisera. First, we probed EC27 (both antisera) immunoprecipitates with ORF101 antibody. ORF101, a protein with a relative electrophoretic mobility of approximately 41 kilodaltons, was detected in the EC27-HIS immunoprecipitate from infected cells as well as the infected cell lysate (figure 4A lanes 6,2) but not in the EC27-GST immunoprecipitates (figure 4A lane 4). Using the EC27-HIS antisera to probe the immunoprecipitates, EC27 (35 kilodalton form) was detected in the ORF101 immunoprecipitate from infected cells as well as the EC27-HIS immunoprecipitate from infected cells and the infected cell lysate (figure 4B lane 3,5,1), but not in the EC27-GST immunoprecipitates (figure 4B lane 7). Using the EC27-GST antisera to probe the immunoprecipitates, EC27 (27 kD) was only detected in the

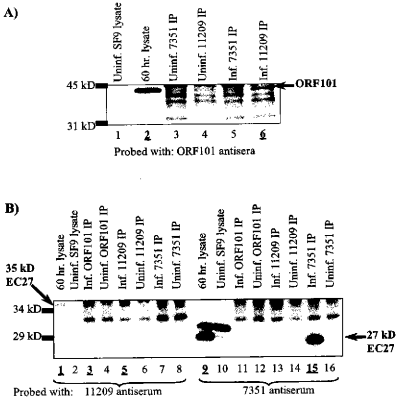


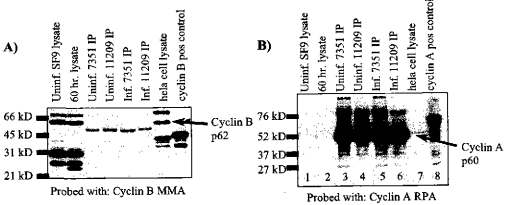
Figure 4. ORF101 is a Component of the EC27-associated Complex
Complexes were precipitated from uninfected and 60 hr post infection SF9 cells using antisera to ORF101, EC27-7351, EC27-11209. Immunoprecipitates were separated by SDS-PAGE and probed by western blotting. (A) Western blot using ORF101 antiserum to probe EC27-7351 and 11209 immunoprecipitates. A 41 kilodalton protein is detected in the infected cell lysate (lane 2) and the 11209-IP from infected cells (lane 5). (B) Western blot using EC27-7351 and 11209 antisera to probe ORF101, EC27-7351, and 11209 immunoprecipitates. For the 11209 probe, a 35 kilodalton protein is detected in infected cell lysate (lane 1), ORF101 IP from infected cells (lane 3), and 11209 IP from infected cells (lane 5). For the 7351 probe, a 27 kilodalton protein is detected in the infected cell lysate (lane 9) and the 7351 IP from infected cells (lane 15).

positive controls, the infected cell lysate and the EC27-GST immunoprecipitate from infected cells (figure 4B lanes 9,16). Thus, ORF101 co-precipitates with the 35 kilodalton form of EC27 and EC27 (same form) co-precipitates with ORF101. This data is strong evidence that ORF101 does associate with the 35 kilodalton and does not associate with the 27 kilodalton form of EC27, especially since the experiments shown in figure 5B were performed in the less stringent HEPES buffer system.

A wide range of other putative EC27-binding partners were analyzed by a similar method: precipitation of protein complexes using EC27 antisera followed by western blot analysis probing with antibodies for the putative EC27 binding partner. Analysis of all major cellular eukaryotic cyclins was performed (cyclins A, B, D, and E) in this manner. These analyses highlighted some major limitations of the technique. EC27 immunoprecipitates were probed for cyclin B using a mouse monoclonal antibody (Santa Cruz Technologies, antibody type GNS1). Cyclin B was detected in both 48 hour infected and uninfected cell lysates, Hela (mammalian cell line) cell lysates, and a commercially available western blotting positive control (figure 5A, lanes 1,2,7,8). No cyclin B was detected in the EC27-GST and EC27-HIS immunoprecipitates (figure 5A, lanes 3-6). Two caveats must be taken into consideration: first, this data is not consistent with previous data, which demonstrated that cyclin B was degraded in infected cells much earlier in the infection process. These studies used a different antibody (Santa Cruz, cyclin B rabbit polyclonal antibody H-432). It unfortunately appears that these data are antibody-dependent for, at least, commercially available antibodies that are almost exclusively raised in mammalian systems. Second, since these experiments were

also performed in the more stringent RIPA buffer system, a weak cyclin B association may not be detected here. With these caveats, it appears that, under the experimental conditions used with these studies, cyclin B does not associate with the EC27-associated complex.

Attempts to analyze EC27 immunoprecipitates for the presence of other cellular cyclins with commercially available antibodies failed. In figure 5B, a western blot of EC27 immunoprecipitates using Cyclin A1 antibodies (Santa Cruz Technologies, cyclin A1 rabbit polyclonal antibody M-20) is shown. This blot typifies other studies using commercially available polyclonal antibodies. No cyclin A can be detected at the exposure used (approximately 10 seconds) in the infected and uninfected cell lysates (figure 5B lanes 1,2). Overexposure conceals any distinct bands in lanes 1 and 2 with background signal and oversaturates the lanes that contain immunoprecipitates (figure 5B lanes 3-6). Thus, the signal generated from background and immunoprecipitate-specific bands is much greater than the signal from the true epitope. This undesirable signal is due to two problems: first, rabbit antibodies are used during both the western blotting and immunoprecipitation steps and thus the rabbit IgG, heavy and light chain, generate signal. Second, under the conditions used, these mammalian antibodies seem to have a low affinity for their insect target proteins. Nonetheless, a band of approximately 60 kilodaltons is detected in HeLa cell lysate, which is the correct electrophoretic mobility of mammalian cyclin A1 (figure 5B lane 7). However, any band of this mobility would be concealed by the heavy chain IgG band in the lanes



Quantitate signal/lane

C)

	IDV	Normalized IDV	Signal/Noise %
mean lanes 3-6	1583712	1130256	n/a
lane 7	604608	151152	15.4378
mean background	453456	0	n/a

Figure 5. Immunoprecipitation & Western Blotting Technique Results Analysis
Immunoprecipitations and western blotting done as previous (see figure 2,4).
A) EC27 immunoprecipitates probed with Cyclin B mouse monoclonal antibodies. A 62 kilodalton protein is detected in the uninfected Sf9, infected SF9, and hela cell lysates (lanes 1,2,7). B) EC27 immunoprecipitates probed with Cyclin A rabbit polyclonal antibodies. A 60 kilodalton protein is detected in hela cell lysate (lane 7). C) Determination of Signal/Noise ratio for a band of the lane 7-cyclin A band intensity buried in immunoprecipitation-specific signal. Total signal intensity per lane is quantified by the Spot Denso function of the Chemimager software package ("IDV", or integrated density value, column). These intensities are normalized for background signal
(total IDV - mean background IDV; "normalized IDV" column). The signal/noise ratio for the normalized signals of a putative cyclin A band ("lane 7", column 2) buried in immunoprecipitation-specific bands ("mean lanes 3-6", column 2)
then is determined (151152 IDV units/[1130256 IDV units - 151152 IDV units]; "Signal/Noise %" column).

containing immunoprecipitate (figure 5B lanes 3-6). In general practice, any band with a mobility of 45-55 kilodaltons would be extremely difficult to resolve from heavy chain IgG in the gel technologies used here. Lastly, we calculated the signal/noise ratio for the cyclin A band in the Hela lysate lane (figure 5B lane 7) if it had been present in an

immunoprecipitate-containing lane (figure 5B lanes 3-6). This calculation assumes a cyclin A band is present but not manifest inside an "average" immunoprecipitate signal. The ratio is approximately 15% (figure 5C). This low signal/noise ratio would make any type of analysis on this data tenuous.

DISCUSSION

EC27 May Not Function Independently as a Cyclin

The failure of EC27 to rescue CLN deficient cells suggests that EC27 alone may not be a functional cyclin. "Alone...functional cyclin" here means strictly that EC27 cannot replace the function of a yeast G1 cyclin, which presumably means that EC27 cannot dimerize with and activate cdc28, the yeast cdk responsible for progression through all cell cycle phases. Some cyclins from higher eukaryotes, for example Drosophila G2 phase cyclins, fail to rescue CLN deficient yeast (Leopold, et al., 1991). Since cyclins are a diverse family of proteins that interact with diverse family of cdk's, the failure of any given cyclin to bind any given cdk may not be significant at face value in determination of the functionality of a cyclin protein. In other words, a positive result in the yeast complementation assay would strongly indicate EC27 is a functional cyclin; however, one cannot conclude from a negative result that EC27 is not a functional cyclin. A negative result could be due to two possible conditions: 1. EC27 can dimerize with and activate cdk's, but not cdc28. 2. EC27 is not able to dimerize with and activate cdk's at all. However, we cannot dismiss this result altogether as the former condition. Of the major animal homologues analyzed, EC27 is most homologous to cyclin B (with an overall sequence similarity of 18%), and EC27 is known to associate with cdc2, the animal homologue of cdc28 (Belyavskyi et al., 1998). Those who developed this technique noted that the majority of clones isolated by functional complementation in DL1 to rescue the CLN- phenotype were cyclin B clones (Lew, et al., 1991). We can

speculate, then, that EC27, a close homologue to a cyclin which complements very strongly in this assay, has a fair probability of successful complementation (in that, through the homology argument, it has a fair possibility of being able to dimerize with cdc28, a homologue of cdc2). Since EC27 fails to complement, this experiment lends credence to the latter condition, that EC27 may not be able to activate any cdk. If that result holds true, we can speculate that EC27 may only be functional as part of a larger complex. We have not yet established whether or not other cellular cyclins are present in the EC27-associated complex and are themselves responsible for the EC27-associated kinase activity. The suggestion from the complementation assay, that EC27 may not be a traditionally functional cyclin, supports in principle a model in which EC27 serves in these protein complexes that have kinase activity not to activate cdk's but to regulate the activities of the pre-existing cyclins.

The EC27-GST and EC27-HIS associated Complexes are Distinct

Although it seems highly unlikely, it appears that the EC27-GST and EC27-HIS antiserum cross-react specifically with different molecular weight forms (EC27-GST:27 kD, EC27-HIS:35 kD) of EC27. We, therefore, have at our disposal a very powerful molecular tool: the ability to resolve different forms of same protein without having to resolve on the basis of mass or electrophoretic mobility.

Of significant import is the observation that as much as half of the maximum kinase activity signal from the EC27-GST immunoprecipitate can be precipitated from uninfected cells. Whether this background signal is due to a baseline rate of

phosphorylation in the assay (autophosphorylation, nonspecific catalysis, etc.) or due to other proteins (cellular kinases, cyclins, or activating factors) which the EC27-GST antisera precipitates cannot be resolved here. However, the same observation was made previously, as EC27-GST immunoprecipitates taken from cells very early in infection, before EC27 protein is detectable inside the cell, have kinase activity against pRb (Belyavskyi et al., 1998).

These kinase assay experiments were done in the more stringent RIPA buffer system, and thus presumably the kinase activities are reduced significantly as compared to activities measured using a less stringent system. We can assert definitively that the EC27-HIS immunoprecipitate from infected cells (44%) has less activity than the EC27-GST immunoprecipitate from infected cells (100%). We can assert that EC27-HIS immunoprecipitates from infected cells have less activity (44%) than the EC27-GST immunoprecipitate from uninfected cells (52.5%). It may be the case that EC27-HIS immunoprecipitates do not have a significant level of kinase background compared to the "zero-point", or baseline activity. The problem is that we cannot necessarily establish the baseline level of activity for the EC27-HIS associated complex with the baseline for the EC27-GST associated complex (we define the baseline to be the activity precipitated from uninfected cells). There are two possible conditions: if the EC27-GST baseline is significantly different than the undetermined EC27-HIS baseline (for whatever reason: EC27-GST precipitates cellular factors that enhance the kinase activity, EC27-HIS simply precipitates less total protein, etc.) then we cannot compare the EC27-

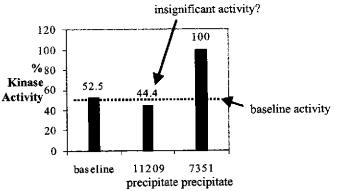


Figure 6: Comparison of 11209- and 7351-associated Kinase Activity
Baseline drawn assuming the 7351 and 11209 baselines do not significantly differ.

HIS associated activity to the EC27-GST baseline. If the EC27-GST baseline is not significantly different from that of the EC27-HIS baseline (i.e., the baseline is due to nonspecific factors) we can compare EC27-HIS associated activity to the EC27-GST baseline. We would then speculate the EC27-HIS associated kinase activity may be insignificant under the experimental conditions used. In plain terms, the EC27-HIS associated activity is less than the EC27-GST associated activity and it may or may not be of significant magnitude under these buffer conditions. Further experimentation is warranted.

What does this result suggest about the function of the two forms of EC27? If future experimentation (under less stringent conditions with a proper measure of the EC27-HIS baseline) demonstrates that the EC27-HIS-associated kinase activity is indeed insignificant, then any models of EC27 must reflect this: the 35 kD EC27-associated

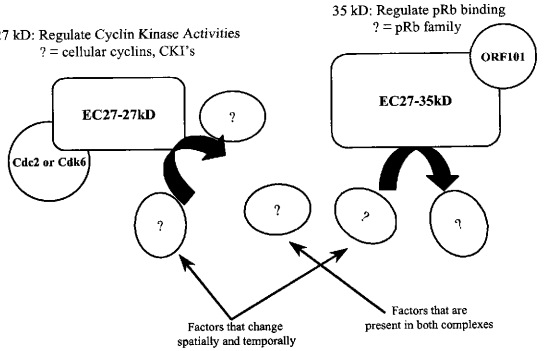


Figure 7: Components of EC27-associated Complexes

complex certainly would not function to activate cdk's; its complex would not even be capable of cyclin kinase activity. Thus, the 27 and 35 kD forms of EC27, which may be isolated spatially and temporally, would also be functionally distinct.

ORF101 is a Member of the (35 kilodalton) EC27-associated Complex

Reciprocal immunoprecipitation experiments demonstrated that ORF101 interacts with 35 kD-EC27. ORF101 is also a structural protein of AcMNPV, meaning that it is introduced into the cell immediately upon infection. Interestingly, like EC27, it has multiple forms in the cell (as recognized by western blot, one form with a relative

mobility of 41 kilodaltons and the other at 28 kilodaltons). ORF101 contains an LXCXE motif; this motif is a pocket-protein or pRb binding consensus motif. ORF101 has sequence similarity to cellular cyclins in the region of the cyclin fold that interacts with p27 (unpublished data, Braunagel et al., 1999).

ORF101 does not associate with 27-kD EC27 under any buffer conditions. This result also supports the hypothesis that the 35-kD EC27 and 27-kD EC27 associated complexes may be distinct in both function and composition. The 27-kD EC27 complex may represent a pool of EC27 involved in the regulation of cyclin kinase activity. The 35-kD EC27 complex may represent a pool of EC27 involved in regulating pRb (especially since EC27 itself has an LXCXE-related motif) that may not contain cyclin kinase activity. We have no reason to assume that the binding partners between the two forms of EC27 are identical. Furthermore, we have no reason to assume that the composition of either complex remains static spatially or temporally.

Limitations in the Western Blotting of Immunoprecipitates

As shown in figure 5A and more dramatically in figure 5B, the percentage of true signal in a western blot of immunoprecipitates using polyclonal antibodies from the same species in both procedures is quite low. As estimated by the signal/noise ratio calculation in figure 5C, the signal specific to the target protein may be as low as 15%. A lower ratio is probably more correct, as the Spot Denso function of Chemimager seems, at least by the naked eye, to be biased towards reading low optical densities as higher than actual. This signal/noise issue is a problem on three accounts:

1. Mammalian antibodies have poor affinity to insect proteins, which exacerbates the amount of noise and often yields unreliable bands for the purported protein target. 2. Signal bands are buried in background bands: the signal of interest may be buried in the very significant IgG bands (again a problem when using same-species antibodies) or other "background" bands, e.g. bands from a cross-reactive protein which is not protein of interest. 3. Multiple banding (not banding due to IgG) yields several bands of approximately the "correct", or reported, mobility of the target protein. These forces generate the temptation to simply test multiple commercially-available antibodies for a target protein and choose the antibody which yields a one band of many bands on the blot with a relative mobility that is closest to the reported mobility. No data based on such an antibody can be deemed reliable. Furthermore, these assays direct energy away from techniques which may be more successful, since they avoid the aforementioned problems, such as N-terminal sequencing and column pull-downs. On a more fundamental level, we have no idea what proteins are present in the EC27 associated complexes, especially as it becomes clearer that we are probably dealing with multiple complexes, and therefore to probe these complexes for suspected proteins is inefficient. Rather, high-throughput screens or techniques, which do not first require a guess at what is present, should be used. Lastly, we currently are faced by inconsistent data (based in results from different antibodies to the same protein) on cyclin B. Figure 5A suggests that it is not a member of the EC27-associated complexes in the buffer system used. Previous data suggests that cyclin B is not present in cells late in infection but Figure 5A detects cyclin B at 48 hours post infection.

Models of EC27 Function

Before we discuss models of EC27 function, a discussion of those models of EC27 function that these data fail to support is warranted. If the negative result of the yeast complementation assay is true, then no model in which EC27 dimerizes with and activates a cdk is valid. Thus, EC27 may not be able to bind cdk6 to mimic cyclin D and phosphorylate pRb directly to activate E2F and induce S-phase. Likewise, EC27 may not be able to bind cdc2 to form a non-degradable cyclin B homologue, phosphorylate nuclear lamins, and create the G2/M phase arrest. However, if we postulate that EC27 performs these tasks, but instead of activating cdk's themselves bind to cellular cyclin/cdk complexes and regulate their activity, then the above models would be possible with the respective modifications. If the result that 35-kD EC27 complexes have no significant kinase activity is confirmed, then the above models are further limited to include only the 27-kD form of EC27. The model of EC27 titrating inhibitors is neither confirmed nor denied by these results; that model requires neither kinase activity nor direct activation of cdk by EC27.

The ORF101/35 kD-EC27 binding may have significant functional implications. Several oncogenes from DNA tumor viruses contain LXCXE motifs. These oncogenes function by displacing pRb from E2F, thus uncoupling E2F activation from pRb phosphorylation. E2F can then induce entry into S-phase without activation of cyclin D or cyclin E complexes and their phosphorylation of pRb. These oncogenes can induce proliferation in quiescent cells by this mechanism (Funk et al., 1998). The EC27/ORF101 model would function identically: EC27/ORF101 (both contain LXCXE

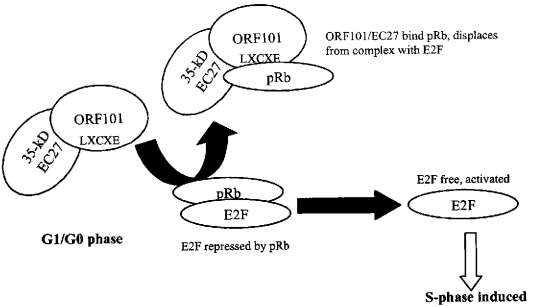


Figure 8. Mechanism for S-phase induction by ORF101/EC27

or related motifs) bind and displace pRb, activating entry into S-phase. This model could account for how ORF101/EC27, both structural proteins introduced into the cell at infection, could induce S-phase entry during primary infection, or how they could induce viral DNA replication (again induction of an S-phase like environment) late in infection.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

DL1 is derived from strain BF264-15Dau, MATα ade1 his2 leu2-3 112trp1-1a ura3 (Lew, et al, 1991). DL1 was disrupted for cln1 (cln1::TRP1), cln2 , and cln2 (unmarked disruption with insertion at XHOI site of cln2). DL1 contains a GAL1::CLN2 fusion integrated at the LEU2 chromosomal locus. DL1 was grown on YEPG (2% galactose, 1% yeast extract, 2% bacto-peptone).

Yeast Transformation

Yeast transformations were done by high-efficiency lithium acetate method (Gietz et al, 1992). 5 mls YPEG was inoculated with a single DL1 colony from YPEG plate and grown overnight at 30°C. 50 mls of YEPG was inoculated with the sufficient overnight culture to give A660 = 0.2 (2 x 10E6 cells/ml). Cells were grown to A660 = 1.0 (2 x 10E7 cells/ml). Cells were pelleted by centrifugation (1000×g), washed in 10 mls sterile water, re-pelleted by centrifugation, and suspended in 1 ml sterile water. Cells were then transferred to 1.5 ml sterile microfuge tube, tap-spun, and resuspended in 1 ml sterile TE / LiOAc (0.01M Tris-HCl, 0.001M EDTA, pH 7.5, 0.1M LiOAc pH 7.5, adjusted with diluted acetic acid). Cells were then were pelleted by centrifugation (1000×g) and resuspended in 0.25 mls TE / LiOAc (4 x 10E9 cells/ml). 50 ul yeast cells were mixed with transforming DNA (.5 ug) and 5 ul single stranded carrier DNA (10 mg/ml, boiled and quick chilled on ice) in a 1.5 ml microfuge tube. 300 ul sterile PEG solution (40%

PEG 4000, 1X TE / LiOAc) was added and then the mixed thoroughly. Cells were incubated at 30°C for 30 minutes and heat shocked at 42°C for 15 minutes. Cells were then pelleted and plated on minimal selective media (Sc-ura galactose or Sc-ura glucose). Approximately 500 transformants/plate were observed.

Recombinant DNA Manipulation

Yeast expression vector pDB20 contains the ADH1 promoter and 2μ origin for high copy, high level complementation analysis (Becker et al., 1991). pDB20 was prepared by maxi-prep (large scale alkaline lysis) from E.coli strain DH5α. pDB20 was prepared for EC27 insertion by HindIII digestion followed by calf intestinal phosphatase (CIP) treatment and electroelution. EC27 was generated by PCR from the eco-B fragment of AcMNPV genomic DNA using primers which generated full length EC27 (~900 bp) with 5' and 3' HindIII sites. EC27 was prepared for ligation with pDB20 by digestion with HindIII (24 hours) and electroelution. Ligation reactions were incubated overnight, float dialyzed, and electroporated into DH5α. Clones were miniprepped and subjected to HindIII and NotI/PstI diagnostic restriction analysis. pDB20+EC27 in the correct orientation was then prepared for yeast transformation by maxi-prep.

Insect Cell Lines and Virus

Spodoptera frugiperda IPLB-Sf21-AE clonal isolate 9 (SF9) cells were cultured in

suspension at 27 C in TNMFH medium and supplemented with 10% fetal bovine serum. Cells are maintained in suspension culture at 1-2X10^6 cells/ml prior to infection. AcMNPV strain E2 was for infection at a m.o.i. of 20.

Immunoprecipitation

Protein complexes precipitated with EC27 antisera were subjected to *in vitro* kinase assay or western blot analysis. SF9 cells were harvested into RIPA lysis buffer (more stringent, 0.1% SDS, 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate) or HEPES lysis buffer (less stringent, 50 mM HEPES pH 7.5, 150 mM NaCl, 0.5% Triton X-100), the protein concentration determined, aliquoted to 200 total protein ug/tube, and stored at -80°C. 200 ug total protein of whole cell lysate was dissolved in 1 ml RIPA or HEPES lysis buffer with 0.01X protease inhibitors cocktail (Sigma). Cells were incubated on ice 30 minutes, passed through a 27 gauge needle twice, and microcentrifuged for 10 minutes at 2.5Krpm. The supernate was transferred into new microfuge tube and pre-cleared by adding 0.20 ug normal animal IgG (Santa Cruz Biotechnolgies), incubating while rotating at 4°C for 1 hr. 25 ul 50% protein A beads slurry (pre-equilibrated in lysis buffer, Sigma) was added and then incubated while rotating at 4°C for 1 hr. The beads were pelleted by microcentrifugation for 3 minutes, 2.5Krpm. The supernate was put into new tubes and immunoprecipitates were taken by adding 1-2 ug purified IgG (Santa Cruz antibodies) or 5-15 ul serum (EC27-7351 and 11209, ORF101) and incubated while rotating at 4° for 1 hr. 25 ul 50% protein A beads slurry (pre-equilibrated in lysis buffer) was added and then incubated while

rotating at 4°C for 1 hr. Precipitates were collected by microcentrifugation at 3 minutes, 2.5Krpm and washed 2 times in Beads Washing Buffer with 0.01X Protease Inhibitor Cocktail (50 mM Tris pH 7.5, 240 mM NaCl, 0.1% NP-40, 5 mM EDTA).

***in vitro* Kinase Assay**

Kinase assays performed as described by Belyavskyi et al., 1998. Following immunoprecipitation, the precipitates were washed 2 times in kinase reaction buffer (50 mM Tris pH 7.0, 10 mM MgCl2, 1 mM DTT). Kinase reactions were initiated by resuspending the immunoprecipitates in a 20 µl reaction mixture containing 1 µg of Histone H1, 100 µm ATP, and 50 µCi of ^{32}P-ATP. Reactions proceeded for 30 minutes and then were terminated with SDS sample loading buffer. Samples were then boiled 3 minutes and loaded on a 15% SDS-PAGE gel. Phosphorylated bands of substrate were quantified by Spot Denso function of the Chemimager Software package.

Western Blot Analysis

Protein samples (immunoprecipitates or cell lysates) were prepared as described above. SDS-sample loading buffer (1.5% SDS, 0.5 mercaptoethanol, 25 mM Tris pH 6.8, 7% glycerol) was added, proteins were separated using a 12% separating gel SDS-PAGE using the method described by Laemmli et al., 1970 and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked in TTBS-Blotto (5% nonfat dry milk, 150 mM NaCl, 10 mM Tris pH 7.5, 0.1% Tween-20) for 1 hr to overnight. Primary antibody was added to TTBS-Blotto at a concentration of 1:1000 (EC27-7351),

1:10000 (EC27-11209), 1:5000 (ORF101), or at various concentrations for Santa Cruz primary antibodies and incubated at 4°C for 2 hours to overnight. Membranes were washed 3 times in TTBS (150 mM NaCl, 10 mM Tris pH 7.5, 0.1% Tween-20) and then incubated in secondary antibody (horseradish peroxidase linked anti-rabbit or anti-mouse IgG, 1:15000 in TTBS-Blotto, Santa Cruz Biotechnology) for 1 hour at room temperature. Membranes were washed 3 times in TTBS and once in TBS (150 mM NaCl, 10 mM Tris pH 7.5) and reacted 1 minute with chemiluminescence reagent (Renaissance, NEN) and exposed to film. Molecular weights were determined using pre-stained or unstained SDS-PAGE low range MW markers (Sigma). Bands were quantitated by Spot Denso function of the Chemimager Software package.

SUMMARY

We have characterized the baculovirus nucleocapsid protein and putative multifunctional viral cyclin EC27 through a series of genetic and biochemical methods. EC27 fails to rescue CLN (G1 cyclin) deficient *S. cerevisiae* as evidenced by a yeast complementation assay. Using antisera that can distinguish between the 35 and 27-kilodalton forms of EC27, we demonstrated that ORF101, a viral nucleocapsid protein that contains an LXCXE motif, can associate with the 35-kD EC27 but not with 27-kD EC27. The 27-kD EC27 associated complex has a higher level of cyclin kinase activity against the *in vitro* substrate histone H1 than 35-kD EC27 associated complex in the buffer system used here; the latter complex may not have a significant level of kinase activity when compared to baseline signal intensity. These studies suggest that EC27 may not function like a traditional, or cellular, cyclin by dimerizing with and activating cdk's and that EC27 may form multiple complexes with different partners that contain different functionalities.

REFERENCES

Belyavskyi, M., Braunagel, S., Summers, M.D. (1998). The Structural Protein ODV-EC27 of *Autographa californica* Nucleopolyhedrovirus is a Multifunctional Viral Cyclin. PNAS USA 95, 11205-11210.

Becker, D.M., Fikes, J.D., Guarente, L. (1991). A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. PNAS USA 88, 1968-1972.

Braunagel, S. C., He, H., Ramamurthy, P., Summers, M. D. (1996). Transcription, Translation, and Cellular Localization of Three *Autographa californica* Nuclear Polyhedrosis Virus Structural Proteins: ODV-El8, ODV-E35, and ODV-EC27. Virology 222, 100-114.

Braunagel, S.C., Parr, I.L., Belyavskyi, M., Summers, M.D. (1998). *Autographa californica* Nucleopolyhedrovirus Infection Results in SF9 Cell Cycle Arrest at G2/M Phase. Virology 244, 195-211.

Braunagel, S.C., Summers, M.D. (1994) *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology 202, 315-328.

Chang, Y., Moore, P.S., Talbot, S.J., Boshoff, C.H., Zarkowska, T., Godden-Kent, P.H., Weiss, R.A., Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus. Nature 382, 410.

Day, I.S., Reddy A.S.N., Golovkin M. (1996). Isolation of of a new mitotic-like cyclin from *Arabidopsis*: complementation of a yeast cyclin mutant with a plant cyclin. Plant Molecular Biology 30, 565-575.

Dessev, G., Iovcheva-Dessev, C., Bischoff, J.R., Beach, D., Goldman, R. (1991). A complex containing p34cdc2 and cyclin B phosphorylates the nuclear lamin and disassembles nuclei of clam oocytes in vitro. J. Bio. Chem. 112, 523-533.

Elledge, S., Harper, J.W. (1994). Cdk inhibitors: on the threshold of checkpoints and development. Current Opinion in Cell Biology 6, 847-852.

Forsburg, S.L., Nurse, P. (1992). Cell cycle regulation in the yeasts S. cerevisiae and S. pombe. Ann. Rev. Cell Biol. 7, 227-256.

Funk, J.O. and Galloway, D.A. (1998). Inhibiting CDK inhibitors: new lessons from DNA tumor viruses. TIBS 23, 337-340.

Gietz, D., St Jean A., Woods R.A., and Schiestl R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 6, 1425.

Glotzer, M., Murray, A.W., Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature 349,132-138.

Godden-Kent, D., Talbot, S.J., Boshoff, C., Chang, Y., Moore, P., Weiss, R.A., Mittnacht, S. (1997). The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J Virol 71, 4193-4198.

Grand, R., Ibrahim, A., Taylor, M., Miler, A., Gregory, C., Gallimore, P., Turnell, A. (1998). Human Cells Arrest in S phase in Response to Adenovirus 12 E1A. Virology 244, 330-342.

Harbour, J.W., Luo, R.X., Dei Santi, A., Postigo, A.A., Dean, D.C. (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98, 859-69

Hutchison C., Glover, D., Ed. (1995). Cell Cycle Control. Oxford University Press: New York.

Jung, J.U., Stager, M., Desrosiers, R.C. (1994). Virus-encoded cyclin. Mol. Cell Biol. 14, 7235-44.

Koepp, D.M., Harper, J.W., Elledge, S.J. (1999). How a Cyclin Became a Cyclin: Regulated Proteolysis in the Cell Cycle. Cell 97, 431-434.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laman, H., Mann, D.J., Jones, N.C., (2000). Viral-encoded cyclins. Curr. Opin. Genet. Dev. 10, 70-74.

Lew, D.J., Vjekoslav, D., and Reed S.I. (1991). Isolation of Three Novel Cyclins by Resuce of G1 Cyclin (Cln) Function in Yeast. Cell 66, 1197-1206.

Leopold, P., and O'Farrell, P.H. (1991). An Evolutionarily Conserved Cyclin Homolog from Drosophila Rescues Yeast Deficient in G1 Cyclins. Cell 66, 1207-1216.

Mann, D,J., Child, E.S., Swanton, C., Laman, H., Jones, N. (1999). Modulation of p27(Kip1) levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. EMBO J. 18, 654-663.

Meijer, L., Guidet, S., Philipe, M. (1997). Viruses and the Cell Cycle. Progress in Cell Cycle Research 3, 1-19.

Miller, Lois K, Ed. (1997). The Baculoviruses. Plenum Press, New York.

Noble, M.E., Endicott, J.A., Brown, N.R., Johnson, L.N. (1997). The cyclin box fold: protein recognition in cell-cycle and transcription control. Trends. Biochem. Sci. 22, 482-487.

Nurse, P. (1994) Ordering S phase and M phase in the cell cycle. Cell 79, 547-50.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature 344, 503-508.

Peeper, D.S., Upton, T.M., Ladha, M.H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J.A., Ewen, M.E. (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 386, 177-181.

Poon, B., Grovit-Ferbas, K., Stewart, S.A., Chen, I.S.Y. (1998). Cell Cycle Arret by Vpr in HIV-1 Virions and Insensitivity to Antiretroviral Agents. Science 281, 266-269.

Salvant, B., Fortunato, E., Specter, D. (1998). Cell Cycle Dysregulation by Human Cytomegalovirus: Influence of the Cell Cycle Phase at Time of Infection and Effects on Cyclin Transcription. Journal of Virology 72, 3729-3741.

Schafer, K.A. The Cell Cycle: A Review. (1998) Vet Pathol 35, 461-478.

Schulze-Gahmen, U., Jung, J.U., Kim, S.H. (1999). Crystal structure of a viral cyclin, a positive regulator of cyclin-dependent kinase 6. Structure Fold. Des. 7, 245-254.

Swanton, C., Mann, D.J., Fleckenstein, B., Neipel, F., Peters, G., Jones, N. (1997). Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature 390, 184-187.

Swanton, C., Card, G.L., Mann, D., McDonald, N., Jones, N. (1999). Overcoming inhibitions: subversion of CKI function by viral cyclins. Trends. Biochem. Sci. 24, 116-120.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell 81, 323-330.

Virgin, H.W., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K.E., Dal Canto, A.J., Speck, S.H. (1997). Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. 71, 5894-5904.

Zhang H.S., Postigo A.A., Dean D.C., (1999) Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell 97, 53-61.

VITA

Luke James Engelking

Academics

Texas A&M University at College Station
Biochemistry & Genetics double major
Cumulative GPA 4.00; Graduation Date: Spring 2000
Honors Distinctions in progress: University Undergraduate Research Fellows Program, Biochemistry Research Scholars, University Honors
Previous schools attended: Houston Community College, GPA 4.0, 12 semester hours, attended 6/97-8/97
University of Texas as Austin, GPA 4.0, 42 semester hours, attended 9/96-5/97

Academic Honors

College of Agriculture & Life Sciences Senior Merit Award 00
Gamma Sigma Delta Senior Merit Award 99
2nd Place, Oral Undergraduate Biological Sciences I, TAMU Resweek Competition 00
Honor Society Memberships: Phi Kappa Phi Honor Society, Gamma Sigma Delta Honor Society, Golden Key Honor Society, Phi Lambda Upsilon Chemical Honor Society
Dean's Honor Role, fall 97, spring 98, fall 98; University Honors, fall 96 and spring 97
Hazelwood Award
Academic Excellence Award 98-99, 99-00
Houston Livestock Show & Rodeo Endowed (98) & Bryce McGee (99) Scholarship Awards
Aid Association for Lutherans All-College Scholar

Research Experience

Associate Member, Sigma Xi
Summer Science for Seniors (S3) Program, Baylor College of Medicine, 6/95-9/95, Mentor: Louis Smith
Summer Undergraduate Research Fellowship (SURF) Program, UT-Southwestern Medical Center, 6/98-8/98, Mentor: Michael White
Summer Medical and Research Training (SMART) Program, Baylor College of Medicine, 6/99-8/99 Mentor: Sharon Plon
Undergraduate Research & University Undergraduate Research Fellows Project, Texas A&M University, 1/98- 5/00, Mentor: Max Summers

Extracurricular Activities

College of Agriculture and Life Sciences Student Council Representative
American Medical Student Association (AMSA) Member
Peer Advisor, Dept. of Biochemistry and Biophysics
Undergraduate Biochemistry Society Member & Officer
Executive Council of Health Organizations (ECHO) Representative
St. Joseph's Regional Hospital Emergency Dept. Volunteer
Zavala Elementary School Young Scientists Program Student Mentor
Austin Diagnostic Medical Center Hospital Emergency Dept. Volunteer
Oakland Manor Assisted Living Center Activities Volunteer

High School

Cypress Creek Senior High School, Houston Texas
Graduated Summa Cum Laude, GPA 4.00; Class Rank: 6 of 530

Military Experience

U.S. Army
Cadet, United States Military Academy at West Point
Honorable Discharge 9/96
Regimental Coin & Platoon E-1 Soldier of Week, Cadet Basic Training (Beast Barracks)

Career & Education Goals

After graduation from Texas A&M, I will matriculate in the Medical Scientist Training Program (MSTP) at Southwestern Medical Center in Dallas, pursuing the MD/PhD degrees.

10527 Green Creek Drive, Houston Texas 77070
engelking@pdq.net