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Phosphorylation of bovine papillomavirus E1 by the protein kinase CK2 near the nuclear localization signal does not influence subcellular distribution of the protein in dividing cells

Michael R. Lentz* and Tess Shideler¹

Department of Biology, University of North Florida, 1 UNF Drive, Jacksonville, FL 32224

Abstract

The bovine papillomavirus E1 helicase is essential for viral replication. In dividing cells, DNA replication maintains, but does not increase, the viral genome copy number. Replication is limited by low E1 expression and an E1 nucleocytoplasmic shuttling mechanism. Shuttling is controlled in part by phosphorylation of E1 by cellular kinases. Here we investigate conserved sites for phosphorylation by kinase CK2 within the E1 nuclear localization signal. When these CK2 sites are mutated to either alanine or aspartic acid, no change in replication phenotype is observed, and there is no effect on the subcellular distribution of E1, which remains primarily nuclear. This demonstrates that phosphorylation of E1 by CK2 at these sites is not a factor in regulating viral DNA replication in dividing cells.

Keywords

papillomavirus; E1 protein; kinase CK2; DNA replication; protein phosphorylation

Papillomaviruses infect stratified squamous epithelial tissue in a variety of vertebrate hosts. They have a complex life cycle that is intimately linked to the differentiation state of the host cell. The primary site of infection is the basal epithelial cell layer, which is accessed following minor trauma of the tissue. The virus remains latent in dividing basal epithelial cells with minimal gene expression [14]. Viral gene expression changes as the infected cells divide, migrate outward, and differentiate. In differentiated cells, viral genomes are amplified to high copy number and capsid genes are expressed so that progeny virions are assembled only in superficial layers of the epithelium [18, 29]. These viruses therefore undergo three distinct modes of replication during a productive infection: transient amplification in newly infected cells, maintenance replication in dividing cells, and vegetative replication in differentiated cells [24]. The latent stage of viral infection is characterized by maintenance of a constant copy number of 50–200 viral genomes per infected cell nucleus. In order to maintain this copy number, DNA molecules replicate an average of once per cell cycle in synchrony with host cell DNA [5, 31]. This low viral DNA copy number maintenance in dividing cells is a unique feature of papillomaviruses.

*Corresponding author: 904-620-1064 (phone), 904-620-3885 (fax), mlentz@unf.edu.

¹Current Address: Department of Pathology, University of New Mexico, Albuquerque, NM 87131

Viral DNA replication is dependent on the viral E1 protein. E1 recognizes and binds to the viral replication origin, aided by the E2 protein. E1 unwinds the viral DNA and functions as the replication fork helicase. E1 also recruits and modifies the host cell replication machinery through direct protein-protein interaction with DNA polymerase alpha-primase, replication protein A, and topoisomerase I [3, 7, 8, 13, 23, 30, 34, 35, 38]. Three distinct functional domains of the protein have been mapped. The carboxy-terminal region contains conserved helicase and ATPase domains and is required for oligomerization. The central portion contains origin-DNA recognition activity. Both of these domains are required for replication activity *in vivo* and *in vitro*. The amino-terminal region is required *in vivo* but is dispensable *in vitro*, suggesting a regulatory function. This region includes nuclear localization and nuclear export signals as well as the majority of known phosphorylation sites [3, 22, 37].

Research from several labs has led to a model for regulated latent viral DNA replication. This model proposes that replication is controlled by nucleo-cytoplasmic shuttling of the E1 protein. Shuttling reduces E1 access to viral DNA in the nucleus, thereby limiting opportunities for viral DNA replication [3, 4, 10, 12, 15, 32, 40]. This “nucleocytoplasmic shuttling” model is a feature common to bovine papillomavirus (BPV) and strains of human papillomavirus (HPV) that have been analyzed, but the details vary from virus to virus [3]. The phosphorylation state of E1, and possibly other protein modifications such as sumoylation, determines whether E1 nuclear export or nuclear import signals are functional. Subcellular localization of BPV E1 is specifically determined by cyclin E/A-Cdk2 phosphorylation at positions 102 and 283, and by PKC at serine 109. Phosphorylation at these positions promotes nuclear export of E1 and downregulates viral DNA replication [4, 15].

E1 is phosphorylated by several kinases, including Cdk2, CK2, MAPK, and PKC [4, 12, 15, 19–22, 25, 33, 40, 41]. Three BPV E1 phosphorylation sites (serines 94, 95, and 100) are within the bipartite nuclear localization signal (NLS), and all three are phosphorylated by CK2 [22, 33]. CK2 is a ubiquitous serine/threonine kinase implicated in a wide range of essential cell functions including cell growth, apoptosis, gene expression and others. It has been demonstrated to control the subcellular location of numerous proteins, including viral replication proteins, through nucleocytoplasmic shuttling [1, 6, 11, 27, 28, 36, 39]. This paper addresses the potential role of CK2 in regulating the subcellular localization of BPV E1 and activity in latently transformed dividing cells.

We previously identified BPV E1 serines 94, 95, and 100 (S94, S95, S100, respectively) as phosphorylation targets by mass spectrometry analysis of E1 protein expressed in insect cells [22]. These amino acids fall within the bipartite NLS of E1, and in the vicinity of other known regulatory phosphorylation sites [4, 20]. Algorithms that analyze protein phosphorylation predicted S94 and S95 as potential CK2 sites, which was recently confirmed [33]. If threonine 102 (T102, a Cdk2 phosphorylation site in E1) is phosphorylated, then S100 becomes a strongly predicted CK2 site [26]. Potential CK2 sites in the vicinity of the NLS of other papillomavirus E1 sequences were identified by manual sequence analysis. At least one and up to three CK2 sites are present in the NLS region of most papillomavirus E1 proteins examined. Representative examples are shown in Figure 1.

To determine if serines 94, 95, and 100 are substrates for CK2, FLAG-tagged wild-type E1 protein and E1 with alanine substitutions at all three sites (E1-AAA) were expressed in insect cells from recombinant baculovirus and purified by immunoprecipitation as described previously [41]. E1 proteins were reacted with purified CK2 enzyme and radiolabeled ATP. Reaction products were separated by electrophoresis and visualized by western transfer and autoradiography. The wild-type E1 protein was readily phosphorylated by CK2. The triple alanine mutant had reduced radioactivity compared to the wild-type protein (data not shown). Residual activity may be accounted for by phosphorylation of S48 and S584, two other known targets for CK2 [21, 25]. BPV E1 is therefore an *in vitro* substrate for purified CK2, and serines 94, 95, and 100 are likely target sites for this enzyme. This confirms similar data published recently [33].

Since serines 94, 95, and 100 are known to be phosphorylated, lie within the NLS of E1, and are targets of the cellular kinase CK2, we investigated a possible role for these sites in modification of E1 function in BPV replication. E1 replication function was investigated by introducing viral genomes or expression plasmids into suitable cells, followed by Southern blot analysis of replicated target DNA. Cells expressed either the wild-type E1 protein or E1 carrying alanine or aspartic acid triple substitution mutants. Full-length viral genomes carrying the E1-AAA and E1-DDD mutations, as well as wild-type E1, were introduced into mouse C127 cells by electroporation. Viral DNA replication was assessed by Southern blot analysis of DNA harvested periodically following electroporation as described previously [20, 41]. This assay relies on proper expression of viral replication proteins to replicate the full-length genome. Input (bacterial) DNA is sensitive to DpnI, and methylation in mammalian cells renders the replicated DNA DpnI resistant. Therefore, accumulation of DpnI-resistant viral genomes over several days of cell growth indicates successful replication. As seen in Figure 2, each of the viral genomes carrying an E1 mutation replicated to a similar extent as the wild-type genome. Three replicate experiments yielded similar results. There was no difference in focus-forming ability between the wild-type and mutant genomes (data not shown). To support the conclusions of the genome replication assay, a three-plasmid replication analysis was performed. In this assay, E1 and E2 proteins were expressed from heterologous vectors, and E1-dependent replication was assessed by observing accumulation of DpnI-resistant plasmids carrying the BPV origin of replication. The origin plasmid was replicated by wild-type E1 and both mutant E1 proteins in this assay, but not in the absence of the E1 protein. Replication was origin-specific. Three replicates were carried out with similar results (data not shown). We conclude that low-level E1-dependent replication of the wild-type origin sequence occurred in these cells and was not affected by the CK2 mutations in the E1 protein. Mutation of these three amino acids therefore does not alter the replication phenotype of the E1 protein in two distinct replication assays. This supports published replication data using a slightly different panel of E1 CK2 mutants [33].

Serines 94, 95, and 100 were chosen for study for three reasons: they are known to be phosphorylated by CK2, they are within the region of the E1 NLS, and phosphorylation is known to contribute to E1 subcellular localization. We hypothesized that these amino acids may contribute to this subcellular distribution phenotype. Since proper E1 transport is essential for viral DNA replication *in vivo*, the above replication data suggest that these

amino acids do not contribute significantly to the subcellular localization of E1. To confirm this prediction, COS-7 cells grown on glass coverslips were transfected with expression plasmid constructs expressing wild-type or mutant E1 proteins and analyzed for E1 subcellular distribution as described previously [20]. As seen in Figure 3, wild-type and mutant E1 proteins were all primarily localized to the nuclear compartment. This is the expected behavior of the wild-type protein. There were no observable differences in the cellular location between wild-type and either of the mutant E1 proteins, and there was no observable cytoplasmic staining by any of the E1 proteins. These three CK2 target amino acids therefore do not contribute to the E1 nucleocytoplasmic shuttling mechanism in dividing cells.

Since latent viral DNA replication and subcellular distribution of E1 are not affected by the mutations described, we conclude that CK2 phosphorylation at these sites is not a controlling factor for E1 activities in dividing cells. Several other possibilities warrant consideration. First, the identification of S94, S95, and S100 as sites of phosphorylation in an insect cell system may not accurately reflect the situation in mammalian cells. S94, S95, and S100 can serve as CK2 substrates, as demonstrated by the *in vitro* phosphorylation experiment described above. Stenlund et al. also demonstrated phosphorylation of sites 94 and 95 (and others) by CK2 *in vitro* [33]. The conservation of one or more CK2 sites near the NLS of most other papillomavirus E1 proteins further supports a role for CK2 phosphorylation in papillomavirus biology. Mutation of all three serines to either alanine or aspartic acid has no observable effect on *in vivo* transient replication, arguably the most stringent E1 assay available. An intriguing possibility is a role for E1 CK2 phosphorylation in vegetative replication or the switch to vegetative replication. Recent work by others demonstrated a role for CK2 phosphorylation of E1 in origin DNA binding *in vitro*, but again, there was no effect on transient DNA replication in dividing cells [33]. CK2 has been shown to play a role in keratinocyte cell cycle control and differentiation. These effects are mediated by CK2 interactions with the p38MAPK pathway [16, 17]. Increased papillomavirus DNA replication late in infection is hypothesized to result from increased E1 and E2 expression from the viral late promoter, and increased E1 expression correlates with the onset of vegetative DNA replication [2, 9]. While increased late gene expression likely plays a significant role in viral DNA amplification, no experiments to date have addressed E1 protein modification in differentiated epithelial tissue. The lack of a phenotype with our CK2 mutant E1 proteins together with these other observations may suggest a role for CK2 phosphorylation of E1 in late-stage viral infection.

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BPV1	84	KRKVLGS-----SQNSSGSEASETPV-----KRRK
HPV6	83	KRKYLGSFY-VSPINTIAEA-VESEISPRLDIAIKLTRQPKKVK
HPV16	87	KRKYLG-----SPLSDISE-CVDNNISPRLLKAICIEKQSRAAK
HPV18	86	KRKFAAGSTENSPLGERLE--VDTELSPRLQEISLNSGQKKAK
HPV31	86	KRKYVG-----SPLSDISS-CVDYNISPRLLKAICIENNSKTAK

Fig. 1.

CK2 phosphorylation sites near the NLS of representative papillomavirus strains. Sequences were first aligned to the bipartite nuclear localization signal and then adjusted for optimal sequence alignment. Sequences were manually screened for CK2 sites (inverted; S/TX/D/E/Y_p/T_p/S_pXD/E/Y_p/T_p/S_p). The basic clusters of the bipartite NLS are shaded. BPV, bovine papillomavirus; HPV, human papillomavirus

Fig. 2.

Transient viral genome replication assay. Low-molecular-weight DNA was harvested from cells on days two, four, and six post-electroporation (d.p.e.). DNA was treated with DpnI and EcoRI prior to electrophoresis and blotting. The blot was probed with random-primed BPV DNA. Full-length, DpnI-resistant linear viral DNA migrates to the position shown in the marker (M) lane. DpnI digestion products of input DNA can be seen at the bottom of the blot (DpnI prod.).

Fig. 3. Subcellular localization of wild-type and mutant E1 proteins. Plasmids expressing FLAG-tagged wild-type or mutant E1 proteins were introduced into COS-7 cells and fixed 48 hours later. Cells were stained with anti-FLAG antibody followed by FITC-conjugated anti-mouse secondary antibody and photographed using epi-fluorescence. A, no E1 protein (negative control); B, wild-type E1; C, E1-AAA; D, E1-DDD. Bar = 20 μ m