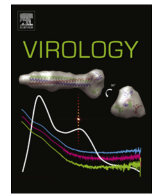
Virology 446 (2013) 251–259

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Human papillomavirus type 16 E7 oncoprotein inhibits the anaphase promoting complex/cyclosome activity by dysregulating EMI1 expression in mitosis

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A R T I C L E I N F O

Article history:
Received 30 May 2013
Returned to author for revisions 20 June 2013
Accepted 14 August 2013
Available online 5 September 2013

Keywords:
Human papillomaviruses
E7 oncoprotein
Mitosis
Anaphase Promoting Complex/Cyclosome (APC/C)
EMI1
Genomic instability
Cervical cancer

A B S T R A C T

The anaphase promoting complex/cyclosome (APC/C) is a ubiquitin ligase complex that orchestrates mitotic progression by targeting key mitotic regulators for proteasomal degradation. APC/C dysfunction is a frequent event during cancer development and can give rise to genomic instability. Here we report that the HPV16 E7 oncoprotein interferes with the degradation of APC/C substrates and that the APC/C inhibitor, EMI1, is expressed at higher levels in HPV16 E7-expressing mitotic cells. HPV16 E7 expression causes increased EMI1 mRNA expression and also inhibits EMI1 degradation. The resulting abnormally high EMI1 levels in HPV16 E7-expressing mitotic cells may inhibit degradation of APC/C substrates and cause the prometaphase delay that we have previously observed in such cells.

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Introduction

Ubiquitin-mediated degradation of cellular proteins constitutes an essential mechanism to tightly regulate steady state levels of proteins and their biological activities in a cell. The selectivity of ubiquitylation is achieved by ubiquitin-protein ligases (E3s), among which the anaphase promoting complex/cyclosome (APC/C) serves as a master regulator of mitotic progression as well as DNA replication licensing in G1. The APC/C complex is composed of over a dozen subunits and the CDC20 and CDH1 co-activators confer activation and some degree of substrate specificity during the somatic cell cycle (Peters, 2006). The APC/C^{CDC20} complex becomes active in early mitosis and targets securin and cyclin B for degradation, which is required for sister chromatid separation and mitotic exit. From late mitosis to G1, the APC/C^{CDH1} complex forms and replaces APC/C^{CDC20} by ubiquitylating CDC20. APC/C^{CDH1} also targets other mitotic regulators including Polo-like kinase 1 (PLK1) and Aurora kinases for degradation. These sequential waves of APC/C substrate degradation drive many events during mitotic progression including sister chromatid separation and cytokinesis (Pines, 2011).

Since premature degradation of APC/C substrates such as securin and cyclin B increases the risk of chromosome segregation errors, there are several mechanisms that control APC/C activity. The mitotic spindle assembly checkpoint (SAC) inhibits APC/C^{CDC20} activity until chromosomes are attached to microtubules and aligned at the metaphase plate (Musacchio and Salmon, 2007). However, APC/C^{CDC20} targets cyclin A and NEK2A for degradation right after nuclear envelope breakdown despite the activation of the SAC (van Zon and Wolthuis, 2010). There are also SAC-independent inhibitors of the APC/C. The Ras association domain-containing family 1 isoform A (RASSF1A) protein and RAE1-NUP98 are thought to inhibit the early mitotic APC/C, whereas early mitotic inhibitor 1 (EMI1) has been reported to inhibit both the mitotic APC/C^{CDC20} and the interphase APC/C^{CDH1} complexes (Di Fiore and Pines, 2007; Hsu et al., 2002; Jeganathan et al., 2005; Margottin-Goguet et al., 2003; Reimann et al., 2001a; Song et al., 2004).

Given its essential roles in regulation of chromosome segregation and DNA replication, dysregulation of the APC/C activity has been linked to genomic instability and cancer development in many ways (Lipkowitz and Weissman, 2011; Nakayama and Nakayama, 2006). Studies with mouse models, for example, have established a clear link between SAC dysfunction and aneuploidy

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0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.08.013

(Suijkerbuijk and Kops, 2008). CDH1 functions as a haploinsufficient tumor suppressor and reduced CDH1 expression impairs genetic stability (Engelbert et al., 2008; Garcia-Higuera et al., 2008). Overexpression of APC/C substrates such as cyclin A, cyclin B, Aurora A, PLK1, TPX2, CDC6, or SKP2 are also associated with chromosomal instability and are predictors of poor prognosis in human cancers (Carter et al., 2006).

High-risk mucosal human papillomaviruses (HPVs) are small DNA viruses that are causative of most cervical cancers and a significant portion of other anogenital tract and oral carcinoma (McLaughlin-Drubin et al., 2012). They infect squamous mucosal epithelia and maintain their genomes as episomes in the basal cell layer. The productive phase of the viral life cycle occurs exclusively in differentiated cells. E6 and E7 are two low molecular size viral proteins that are consistently expressed in HPV-associated cancers. They lack intrinsic enzymatic activities and contribute to the induction and maintenance of the transformed phenotype through protein–protein interactions (Klingelhutz and Roman, 2012; Moody and Laimins, 2010; Munger et al., 2004). Interestingly, the major transforming activities of the high-risk HPV16 E6 and E7 proteins have been linked to the proteolytic inactivation of tumor suppressors p53 and pRB by reprogramming the cellular ubiquitin ligase E6AP and the CUL2-containing cullin RING ligase complex, respectively (Huh et al., 2007; Scheffner et al., 1993; White et al., 2012).

In addition to uncoupling the host cell differentiation program from proliferation by dysregulating the pRB/E2F axis, HPV16 E7 drives genomic destabilization (McLaughlin-Drubin and Munger, 2009a, b). Double stranded DNA breaks and anaphase bridges are observed at a higher frequency in HPV16 E7-expressing cells than in control cells (Duensing and Munger, 2002). Expression of HPV16 E7 also induces centrosome overduplication and increases the risk for multipolar mitoses and chromosome missegregation (Duensing et al., 2000). In fact, multipolar mitoses are histopathological hallmarks of high-risk HPV-associated premalignant lesions and cancers (Winkler et al., 1984). Furthermore, HPV16 E7-expressing cells progress more slowly through prometaphase as originally detected by live cell videomicroscopy (Nguyen and Munger, 2009). More recent studies revealed that degradation of APC/C substrates including cyclin A and cyclin B is inhibited in HPV16 E7-expressing cells (Yu and Munger, 2012). Although HPV16 E7 expression triggering the SAC may account for inhibition of cyclin B degradation, this mechanism cannot account for the inhibition of cyclin A degradation (Yu and Munger, 2012). Hence, HPV16 E7 may also negatively regulate APC/C activity through SAC-independent mechanisms.

In this report, we examine the degradation of a range of different APC/C substrates during mitotic progression and find that HPV16 E7 can be added to an expanding list of viral proteins that manipulate the APC/C (Fehr and Yu, 2013; Heilman et al., 2005; Mo et al., 2012). The SAC-independent APC/C inhibitor, EMI1, is an E2F transcriptional target (Hsu et al., 2002) and is not only transcriptionally upregulated by HPV16 E7, but the EMI1 protein is also stabilized during mitosis. Furthermore, EMI1 depletion partially reverses the inhibition of APC/C substrate degradation in HPV16 E7-expressing cells. The discovery that HPV16 E7 targets the APC/C inhibitor, EMI1, suggests that the inhibition of APC/C activity may play important roles in E7-induced mitotic abnormalities.

Results

HPV16 E7 expression affects APC/C substrate degradation during mitosis

We have previously reported that HPV16 E7 expression impedes the degradation of the mitotic APC/C substrates cyclin A and cyclin B (Yu and Munger, 2012). While inhibition of the SAC partially restores cyclin B degradation in HPV16 E7-expressing cells, the inhibition of degradation of cyclin A, an APC/C^{CDC20} substrate not regulated by the SAC, remains unaffected. Thus we decided to pursue the possibility that HPV16 E7 may inhibit APC/C activity independent of the SAC and hence may impede the degradation of other APC/C substrates as well. To address this, we examined three distinct categories of APC/C substrates: (a) Cyclin A and NEK2A are degraded after nuclear envelope breakdown by the APC/C^{CDC20} complex and therefore evade control by the SAC (van Zon and Wolthuis, 2010); (b) Cyclin B and securin are the best-characterized APC/C^{CDC20} substrates that are degraded only after the SAC is satisfied by proper microtubule attachment to the kinetochores; (c) CDC20, PLK1, and Aurora B are ubiquitylated by the APC/C^{CDH1} complex after anaphase (Pines, 2011).

Primary human foreskin fibroblasts (HFFs) were used for these experiments as, unlike keratinocytes, they are amenable to the double thymidine synchronization scheme. We generated donor and passage matched primary HFF populations retrovirally transduced with empty vector (HFF C) or HPV16 E7 (HFF E7) and assessed E7 expression by Western blotting (data not shown). HFF C and HFF E7 populations were arrested at the G1/S boundary by a double thymidine block and then released and followed through S and G2 phases and mitosis. We harvested cells and measured mitotic indices by two-dimensional FACS analyses to assess DNA content with propidium iodide staining and expression of the mitotic marker MPM-2. A mitotic peak was observed between 8 h and 10 h after release in both HFF C and HFF E7 (Fig. 1A) and most of the cells passed through mitosis between 8 h and 14 h after release. We consistently observed a noticeably smaller decrease between 8 h and 14 h after release in levels of all three categories of APC/C substrates in HFF E7 than in HFF C. For the APC/C^{CDC20} substrates that are degraded independent of SAC activation, cyclin A levels decreased by 93% in HFF C but only by 56% in HFF E7, and NEK2A levels decreased by 85% in HFF C and only by 45% in HFF E7. For the APC/C^{CDC20} substrates that are only degraded after the SAC is turned off, cyclin B levels decreased by 98% in HFF C and by 75% in HFF E7, and securin levels decreased by 76% in HFF C and by 35% in HFF E7 (Fig. 1A and B). Similar differences were also observed with APC/C^{CDH1} substrates. CDC20 levels declined by 52% in HFF C and by 16% in HFF E7, PLK1 levels declined by 36% in HFF C and by 21% in HFF E7, and Aurora B levels declined by 80% in HFF C and by 58% in HFF E7.

To determine whether some of these observed differences may be caused by differences in the proliferative activity between the two cell populations, we performed immunofluorescence analyses of the proliferation marker Ki67 in HFF C and HFF E7. 85% of HFF C and 86% of HFF E7 were Ki67-positive ($n=200$) (Fig. 1C). Hence the proliferative activity of HFF E7 is similar to that of HFF C. Thus, the observed differences in APC/C substrate levels during mitosis do not reflect differences in proliferative activity but are consistent with the model that E7 may inhibit APC/C activity during mitosis.

HPV16 E7-expressing cells maintain high EMI1 levels during mitosis

Increased steady state levels of all three categories of APC/C substrates in mitotic HFF E7 populations led us to search for an SAC-independent APC/C inhibitor that may account for these differences. EMI1 was an attractive candidate as it has been shown to inhibit the mitotic APC/C^{CDC20} and unlike the SAC component MAD2, it can stabilize cyclin A in *Xenopus* egg extracts (Reimann et al., 2001b). The mechanisms by which EMI1 inhibits APC/C involve inhibition of both substrate binding and ubiquitin chain elongation as suggested by biochemical and structural studies (Frye et al., 2013; Miller et al., 2006; Wang and Kirschner, 2013).

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