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Human papillomavirus type 16 E7 oncoprotein engages but does not abrogate the mitotic spindle assembly checkpoint

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ABSTRACT

The mitotic spindle assembly checkpoint (SAC) ensures faithful chromosome segregation during mitosis by censoring kinetochore–microtubule interactions. It is frequently rendered dysfunctional during carcinogenesis causing chromosome missegregation and genomic instability. There are conflicting reports whether the HPV16 E7 oncoprotein drives chromosomal instability by abolishing the SAC. Here we report that degradation of mitotic cyclins is impaired in cells with HPV16 E7 expression. RNAi-mediated depletion of Mad2 or BubR1 indicated the involvement of the SAC, suggesting that HPV16 E7 expression causes sustained SAC engagement. Mutational analyses revealed that HPV16 E7 sequences that are necessary for retinoblastoma tumor suppressor protein binding as well as sequences previously implicated in binding the nuclear and mitotic apparatus (NuMA) protein and in delocalizing dynein from the mitotic spindle contribute to SAC engagement. Importantly, however, HPV16 E7 does not markedly compromise the SAC response to microtubule poisons.

Introduction

During mitosis, the cell has to equally segregate replicated chromosomes into two daughter cells. Kinetochores, complex protein assemblies on the centromeres of chromosomes, allow for chromosomes to be captured by microtubules emanating from the two spindle poles, which is necessary for chromosome segregation. Capturing of kinetochores by microtubules is by "trial-and-error" and is monitored by the mitotic spindle assembly checkpoint (SAC). The SAC guards genomic integrity by delaying mitotic chromosome segregation until all chromosomes are properly attached by spindle microtubules. Chromosome segregation and mitotic exit require that the mitotic regulators securin and cyclin B be ubiquitinated by the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) before being targeted for degradation by the proteasome. The SAC thus comes into play in prometaphase by sequestering the APC/C coactivator CDC20 and preventing it from activating APC/C (reviewed in Musacchio and Salmon, 2007).

Studies with anti-microtubule drugs in yeast identified genes necessary for the SAC as early as two decades ago. These genes were named *mitotic arrest deficient* (*MAD*) and *budding uninhibited by benzimidazole* (*BUB*) (Hoyt et al., 1991; Li and Murray, 1991). Evolutionarily conserved from yeast to human, the key cytosolic SAC effectors Mad2 (MAD2L1), BubR1 (BUB1B) and BUB3 form a complex with CDC20, known as the mitotic checkpoint complex (MCC). Depletion of checkpoint proteins including Mad2, BubR1, and BUB3 have been shown to abolish the SAC (Meraldi et al., 2004). SAC dysfunction has been linked to development of aneuploidy during tumorigenesis by studies with human cancer cell lines with SAC gene mutations and mouse models of SAC deficiency. Moreover, several tumor suppressors and oncogenes regulate expression and/or activity of SAC proteins (reviewed in Suijkerbuijk and Kops, 2008).

Human papillomaviruses (HPVs) are small DNA viruses that infect squamous epithelia of the skin or mucous membranes. HPVs infect the proliferating basal cell layer through microabrasions or at squamocolumnar transformation zones where basal-like cells are exposed. HPV genomes are maintained as episomes and the productive phase of the viral life cycle occurs exclusively in differentiated cells. Since HPVs are acutely dependent on cellular replication factors, HPVs need to uncouple the proliferation/differentiation switch, an activity that is mainly executed by the E7 protein. Malignant progression of high-risk HPV-associated lesions is a relatively rare event, and is accompanied by accumulation of structural and numerical chromosome aberrations. The high-risk HPV E6 and E7 proteins, which are consistently expressed in HPV-associated lesions and cancers, have been shown to induce genomic instability through a number of different mechanisms (reviewed in Klingelhutz and Roman, 2012; McLaughlin-Drubin and Munger, 2009).

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HPV16 E7 not only overcomes G1/S checkpoint restriction by targeting the retinoblastoma tumor suppressor (pRB) for proteasomal degradation (Huh et al., 2007), but also drives genomic destabilization. HPV16 E7 expression causes synthesis of supernumerary centrosomes, chromosome alignment delays, and persistent presence of double strand DNA breaks (Duensing et al., 2001; Duensing and Munger, 2002; Nguyen and Munger, 2009), each of which is likely to impair the fidelity of mitosis and trigger SAC activation. Several studies have suggested that HPV16 E7 expression abrogates the SAC, with cells escaping prolonged mitotic arrest and developing polyploidy in the presence of microtubule poisons (Patel et al., 2004; Thomas and Laimins, 1998). There is also the opposite view that instead of abrogating the SAC, HPV16 E7 abolishes a postmitotic checkpoint, which becomes active and prevents further cell cycle progression when cells adapt to the SAC, decondense their chromosomes and undergo mitotic slippage to a G1-like state with 4N DNA (Heilman et al., 2009; Khan and Wahl, 1998).

Previous live-cell imaging studies in our lab revealed a prometaphase delay in cells with HPV16 E7 expression (Nguyen and Munger, 2009), suggestive of SAC activation. Here we report that the expression of HPV16 E7 impedes the degradation of mitotic cyclins and that this is in part dependent on E7 mediated SAC engagement. We also demonstrate the functionality of the SAC in response to microtubule poisons in cells with HPV16 E7 expression. We hypothesize that a functional SAC may contribute to the viral life cycle by ensuring viral genome persistence.

Results

HPV16 E7 expression impedes cyclin B degradation during mitosis.

Previous immunofluorescence and live-cell imaging studies in our lab demonstrated a prometaphase delay associated with HPV16 E7 expression (Nguyen and Munger, 2009). Despite earlier reports that linked the expression of HPV16 E7 alone or together with HPV16 E6 to the abrogation of the SAC (Patel et al., 2004; Thomas and Laimins, 1998), we did not observe frequent conversion of delayed chromosome congression in E7-expressing cells during metaphase into lagging chromosomes in anaphase (Nguyen and Munger, 2009). This led us to hypothesize that the SAC is still functional in HPV16 E7-expressing cells. To address this, we examined the degradation of cyclin B in mitosis, which is directly inhibited by the SAC (Musacchio and Salmon, 2007), in HPV16 E7-expressing cells.

Colon carcinoma RKO cells were initially used for these experiments because they have wild type p53 and pRB tumor suppressor activity and an intact SAC (Cahill et al., 1998). Moreover, clonal RKO lines with high-level HPV16 E7 expression have been described (Slebos et al., 1994) (Fig. 1A). To compare cell cycle progression of HPV16 E7-expressing RKO cells (RKO E7) to control vector transfected cells (RKO C), cells were subjected to a double thymidine block, which causes a G1/S arrest. We performed cell cycle analyses by FACS after propidium iodide staining and quantified the percentage of G2/M cells (4N DNA). RKO C and RKO E7 cells responded equally well to double thymidine synchronization, with only ~10% of cells in G2/M at the time of release from double thymidine block (Fig. 1B). After release from the double thymidine block we investigated the mitotic markers phospho histone H3 serine 10 (pH3S10) and cyclin B for a time period of 14 h, during which most RKO C and RKO E7 cells synchronously progressed through G2 and M phase (Fig. 1B). Cyclin B levels peaked at 8 h and 10 h post release in RKO C and RKO E7 cells, respectively, consistent with the high levels of pH3S10 at these time points. By 14 h post release, cyclin B levels decreased by 56% from its peak levels in RKO C cells, while only an 18% decrease was observed in RKO E7 cells, suggesting that cyclin B degradation during mitosis may be inhibited in RKO E7 cells (Fig. 1A).

We decided to use primary human foreskin fibroblasts (HFFs) for more detailed analyses of SAC engagement in response to HPV16 E7 expression. HFFs were used for several reasons: even though RKO cells retain SAC function (Cahill et al., 1998) they are a cancer derived line and may contain mutations that affect the proper functioning of the SAC. In addition, the fact that RKO C and

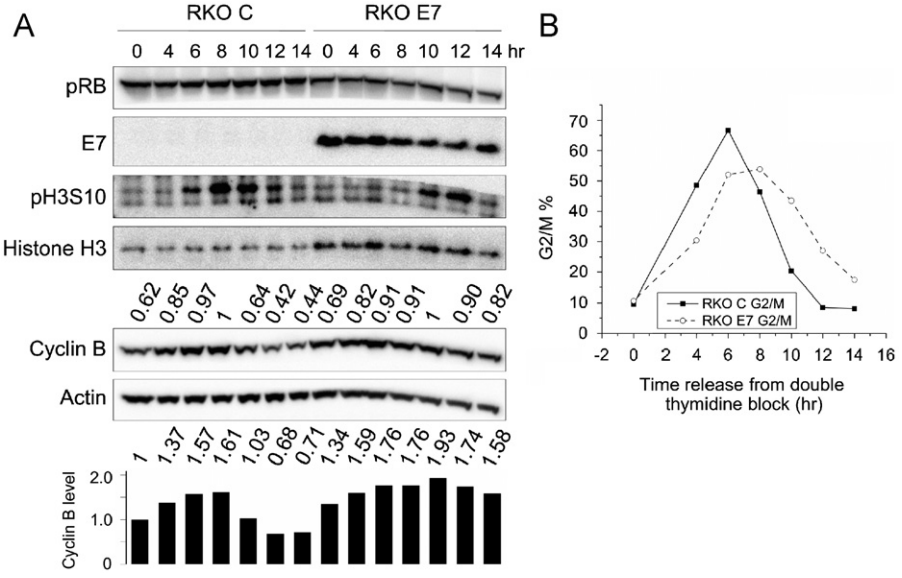

Fig. 1. HPV16 E7 expression impedes cyclin B degradation during mitosis in RKO cells. RKO cells with stable expression of empty vector (RKO C) or HPV16 E7 (RKO E7) were collected at different times after release from a double thymidine block. Shown here is a representative experiment; similar results were obtained in two additional experiments. (A) Western blot analyses of pRB, E7, histone H3 phosphorylated at serine 10 (pH3S10), histone H3, cyclin B and actin are shown. Cyclin B quantifications relative to actin are indicated above the cyclin B blot. Values are presented relative to the highest (1) in either RKO C or RKO E7. The bar graph below the actin blot shows the relative cyclin B levels normalized to actin, providing a basis for comparison of RKO C and RKO E7. (B) FACS analyses of G2/M cell populations are shown for RKO C and RKO E7 at different times after release from a double thymidine block.