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Human papillomavirus E1 and E2 mediated DNA replication is not arrested by DNA damage signalling

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ABSTRACT

Integration of human papillomaviruses into that of the host promotes genomic instability and progression to cancer; factors that promote integration remain to be fully identified. DNA damage agents can promote double strand breaks during DNA replication providing substrates for integration and we investigated the ability of DNA damage to regulate HPV E1 and E2 mediated DNA replication. Results demonstrate that HPV E1 and E2 replication is not arrested following DNA damage, both *in vivo* and *in vitro*, while replication by SV40 Large T antigen is arrested and ATR is the candidate kinase for mediating the arrest. LTAg is a target for PIKK DNA damage signalling kinases, while E1 is not. We propose that the failure of E1 to be targeted by PIKKs allows HPV replication in the presence of DNA damaging agents. Such replication will result in double strand breaks in the viral genome ultimately promoting viral integration and cervical cancer.

Introduction

Human papillomaviruses (HPV) are double stranded DNA viruses that are causative agents in a number of human diseases including cervical cancer (zur Hausen, 2009). Throughout the viral life cycle two viral proteins, the replicative helicase E1 and the DNA binding protein E2, are required for viral genome replication (Wilson et al., 2002). E2 is a DNA binding protein that binds to target sequences around the viral origin and recruits the E1 helicase which forms a di-hexameric complex that replicates the viral genome via interaction with host proteins (Steger et al., 1996). In addition to its role in the initiation of viral DNA replication, the E2 protein regulates transcription of the E6 and E7 encoding sequences via an adjacent promoter. In most circumstances E2 represses this promoter and therefore controls the levels of E6 and E7 protein present in the cell (Thierry, 2009).

High risk human papillomaviruses (HPV) are causative agents in cervical cancer and are increasingly implicated in the development of head and neck cancer (Psyrri et al., 2009). One of the hallmarks of cervical cancer is viral genome integration into that of the host resulting in disruption of the HPV E2 open reading frame (Pett and Coleman, 2007). This integration allows for increased expression of E6 and E7 promoting cell growth, genomic instability and carcinogenesis. In addition to the increased expression of the transforming proteins viral integration may also promote genome damage via partial replication of the integrated genomes by E1 and E2 proteins (Kadaja et al., 2007; Kadaja et al., 2009a; Kadaja et al., 2009b). This partial replication results in dsDNA breaks (DSBs) generating substrates for non-homologous end joining that could be mutagenic and therefore contribute to carcinogenesis.

It is clear that viral integration contributes towards the transformed phenotype from studies carried out in cervical tumour samples and also using *in vitro* model systems (Wentzensen et al., 2004; Dall et al., 2008). Most tumours have integrated HPV genomes, while some have a mixture of episomal and integrated viral genomes (Vinokurova et al., 2008). The factors that promote viral integration have been less well studied. For integration to occur there must be a double strand break in the circular viral genome and this broken genome can then act as a substrate for viral integration into the host genome via non-homologous end joining. One way for this breakage to occur is for viral replication to occur in the presence of DNA damaging agents, promoting double

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strand DNA breaks. Cellular replication is controlled by the DNA damage response pathways mediated by the ATR/ATM pathways following DNA damage. These pathways arrest ongoing DNA replication, prevent new initiation, maintain stalled DNA replication forks, and activate DNA damage repair pathways (Jackson and Bartek, 2009). Following DNA repair, DNA replication can restart from the stalled DNA replication forks and new origins are fired to complete genome replication. In this way, the cell can ensure that damaged genomes are not replicated therefore maintaining genomic integrity. The precise targets of the DNA damage pathways that allow the immediate stalling of DNA replication forks are not completely clear, although several of the MCM proteins in the replicative helicase complex are phosphorylated by ATR/ATM. It is proposed that this phosphorylation of the helicase contributes to the stalling and maintenance of DNA replication forks in the presence of DNA damaging agents (Cortez et al., 2004; Ishimi et al., 2003a, b).

This report demonstrates that etoposide, a Topoisomerase IIa inhibitor that generates DSBs resulting in DNA damage signalling that arrests DNA replication and the cell cycle (Minocha and Long, 1984), does not result in inhibition of E1–E2 DNA replication. E1–E2 DNA replication is not inhibited by the presence of etoposide even though the ATR pathway is activated. As a control we investigated the regulation of SV40 Large T antigen (LT) mediated DNA replication, which uses a very similar DNA replication strategy and uses the same cellular replication factors as SV40 (Muller et al., 1994, Melendy et al., 1995). Unlike PV E1 DNA replication, LT DNA replication is arrested following etoposide treatment. We confirm these results *in vitro*: LT DNA replication is arrested by addition of DSBs, while HPV DNA replication is not. Further, we show this arrest to be sensitive to the DNA damage PIKK inhibitor, wortmannin, and *in vivo* and *in vitro* studies with kinase specific inhibitors suggest that ATR is responsible for replication arrest. We demonstrate that LT is phosphorylated by the ATR/ATM kinases while E1 is not. We propose a model in which the failure of E1 to be phosphorylated by ATR/ATM allows E1–E2 mediated DNA replication in the presence of DNA damage therefore promoting viral genome double strand breaks.

Results

E1–E2 mediated DNA replication is not arrested by etoposide

To investigate whether E1–E2 mediated DNA replication is arrested by DNA damage, we carried out transient DNA replication assays in 293T cells using the real-time PCR protocol developed in our lab (Taylor and Morgan, 2003; Morgan and Taylor, 2005). We used etoposide as the DNA damaging agent. Etoposide inhibits Topoisomerase IIa, resulting in double strand DNA breaks (DSBs) during the S phase of the cell cycle. As a control for inhibition of DNA replication, we also used the replicative DNA polymerase (alpha/delta/epsilon) inhibitor aphidicolin (Ikegami et al, 1978). This drug arrests both cellular and viral (HPV and SV40) DNA replication. For comparison, we investigated the ability of these reagents to interfere with SV40 Large T antigen (LT)-dependent DNA replication. Fig. 1a details the results obtained with HPV11 E1 and E2; identical results were obtained with HPV16 E1 and E2 proteins (data not shown). No DNA replication signal is detected in the non-transfected cells, nor when a plasmid containing the HPV origin (pOriM) is transfected with or without an E1 expression plasmid alone are co-transfected (lanes 1–3). As expected, DNA replication is only detected when an E2 expression plasmid is also co-transfected (lane 4). When the cells are treated with aphidicolin following transfection there is a minimal level of DNA replication that presumably occurs prior to application of the drug (lane 6). When etoposide is added to the cells, there is no reduction in DNA replication and if anything there is a small increase in E1–E2 mediated DNA replication (lane 5). As this was a surprising result, we wanted to confirm that this was not due to 293T cells not receiving or responding to a DNA replication arrest signal. To investigate this LT-mediated DNA replication was also evaluated (Fig. 1b). 293T cells express SV40 LT constitutively. Without any plasmid transfected there is no signal (lane 1) and when a plasmid containing the luciferase gene but lacking the SV40 origin is added there remains no detectable signal (lane 2, p−SV40 ori). But when a plasmid containing the SV40 origin is added and replication is evaluated using a luciferase probe and primer set there is clear replication detected (lane 3, p+SV40 ori). As with E1–E2 mediated DNA replication, aphidicolin substantially reduces the DNA replication signal as expected (lane 5). When etoposide is added the LT-dependent DNA replication is also substantially reduced, in striking contrast to the results obtained with E1–E2 dependent replication. These results were reproducible with camptothecin (not shown), another DNA damaging agent that induces DNA double strand breaks during S phase.

In vitro results show that LT replication arrest is inhibited via a checkpoint response, while E1–E2 replication is not

To demonstrate that this difference in DNA replication sensitivity to etoposide treatment is due to an authentic DNA damage checkpoint response, dependence on checkpoint kinases needs to be demonstrated. However, etoposide is highly toxic to cells deficient in checkpoint kinase function or in the presence of checkpoint kinase inhibitors, therefore experiments such as those in Fig. 1 were not feasible (data not shown). Hence, *in vitro* viral DNA replication assays were utilized to address this question. *In vitro* DNA replication assays were carried out using established protocols for both LT and E1 mediated replication. To adapt this protocol to address checkpoint arrest, plasmid DNA digested with a restriction enzyme (to generate DSBs) was added to the cell extract to determine whether DSBs produce a DNA damage signal that can regulate LT or E1 mediated *in vitro* DNA replication. Circular undigested plasmid DNA provided a control demonstrating that addition of the same amount of DNA without DSB ends would not alter the DNA replication activity of LT or E1. Fig. 2a shows that addition of undigested supercoiled DNA had no affect on LT replication (compare lanes 1 and 2) while addition of linearised DNA resulted in a significant reduction in LT replication (compare lanes 1 and 4). To determine whether this arrest in LT replication was due to a checkpoint response, wortmannin (an inhibitor of the DSB-triggered PI3 kinase-related protein kinases (PIKKs), ATM, ATR and DNA-PK) was added (Wang et al., 2005). Addition of wortmannin did not affect replication in the presence of the supercoiled DNA (compare lanes 1 and 3) while it relieved the inhibition of LT replication by the linearised DNA (compare lanes 4 and 5). These results demonstrate two things: firstly that DNA ends can induce an arrest of LT-mediated DNA replication; and secondly, that wortmannin can relieve this inhibition. To determine whether the DSB-induced wortmannin-sensitive signal could interfere with E1 mediated DNA replication *in vitro*, identical experiments were carried out with E1. E1 mediated DNA replication was not affected by linearised DNA fragments (Fig. 4b), nor by treatment with wortmannin (data not shown). Therefore, these *in vitro* results mimic what is observed *in vivo* as shown in Fig. 1; LT replication is sensitive to DNA damage signalling while E1 mediated replication is not. Further, they show that this response is dependent on the PIKKs, the kinases that initiate the cellular DNA damage checkpoint pathways. To investigate which of the PIKKs was responsible for the inhibition of LT induced DNA replication these experiments were carried out in the presence of specific DNA damage kinase inhibitors that target DNA-PK and ATM; there is no specific inhibitor of ATR. Fig. 2c is a summary of four individual experiments quantified as described. Addition of either a DNA-PK-specific inhibitor (NU7026), a ATM-specific inhibitor (KU55933), or both kinase inhibitors, prevented replication arrest by addition of DNA fragments (such as that seen with wortmannin). These results suggest that it is the

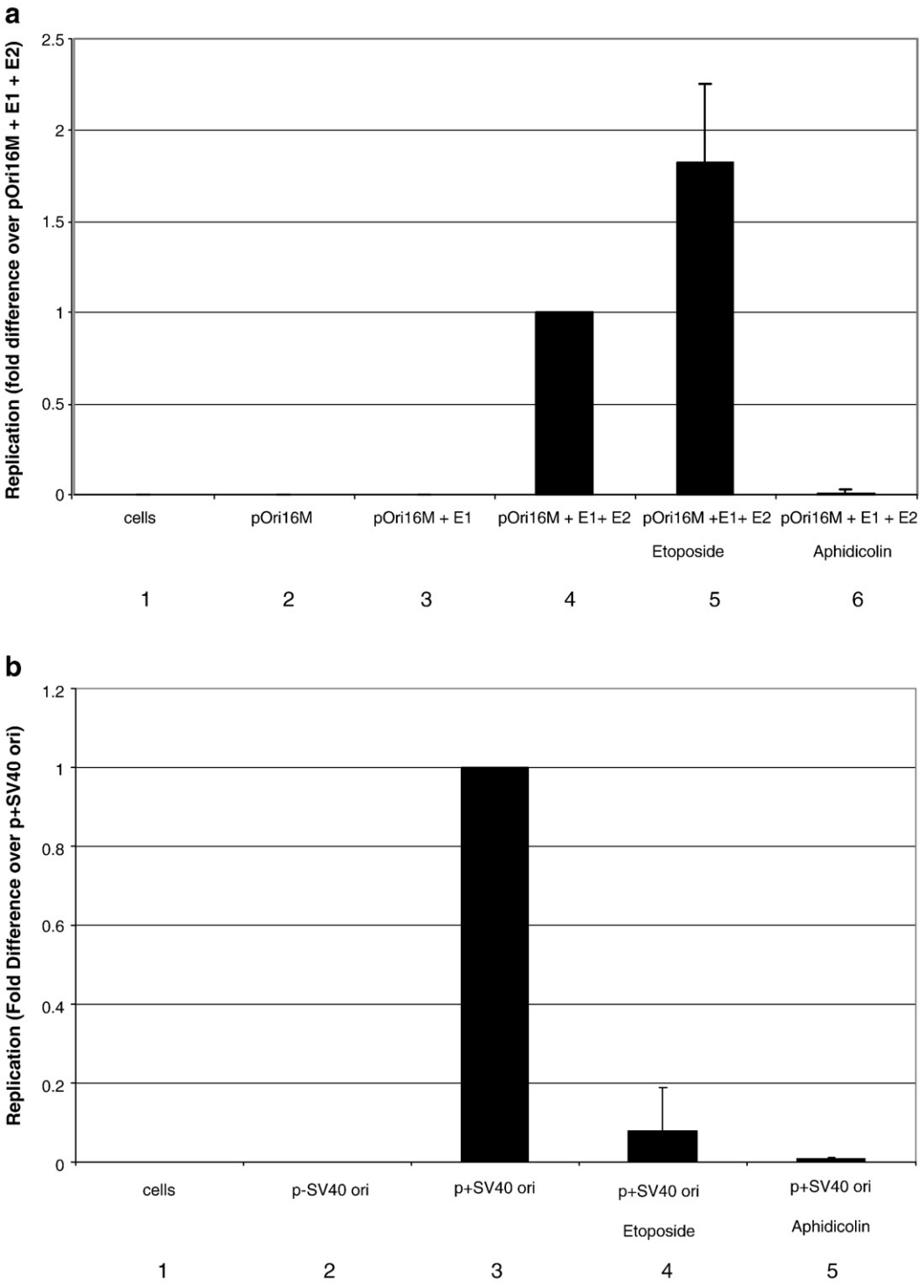

Fig. 1. HPV E1 and E2 mediated DNA replication is not arrested by DNA damage agents, while SV40 LTAg replication is. a) 293T cells were transfected with 100 pg of pOriM (lanes 2–6), 5 μg of pCMV-E1 (lanes 3–6) and 2 μg of pCMV-E2 (lanes 4–6). The day after transfection, cells were left untreated (lanes 1–4) or treated with 50 mM of etoposide (lane 5) or 2.5 mg/ml of aphidicolin (lane 6). Forty-eight hours later low molecular weight DNA was harvested from the cells for replication assays. The results shown represent the summary of three experiments and the results are standardised to E1 + E2 without treatment (lane 4) equalling 1. b) 293T cells were transfected with 1 ng of luciferase gene containing plasmid DNA (lanes 2–5). In lane 2, the plasmid contained no SV40 origin of replication, in lanes 3–5 the plasmid contained an SV40 origin of replication. The day after transfection, cells were left untreated (lanes 1–3) or treated with 50 μM of etoposide (lane 4) or 2.5 mg/ml of aphidicolin (lane 5). Forty-eight hours later low molecular weight DNA was harvested from the cells for replication assays. The results shown represent the summary of three experiments and the results are standardised to LTAg without treatment (lane 3) equalling 1.

ATR kinase that mediates this checkpoint response. (Inhibition of DNA-PK was verified using the Promega DNA-PK assay kit; inhibition of ATM was confirmed by evaluation of the hyper-phosphorylation of NBS1, confirming that these inhibitors were functional in these assays.)

E1 is not a substrate for DNA damage signalling PIK kinases in vivo

In order to arrest DNA replication following DNA damage, an enzymatic activity associated with DNA replication must be blocked. As both of these viral replication complexes use the same cellular DNA

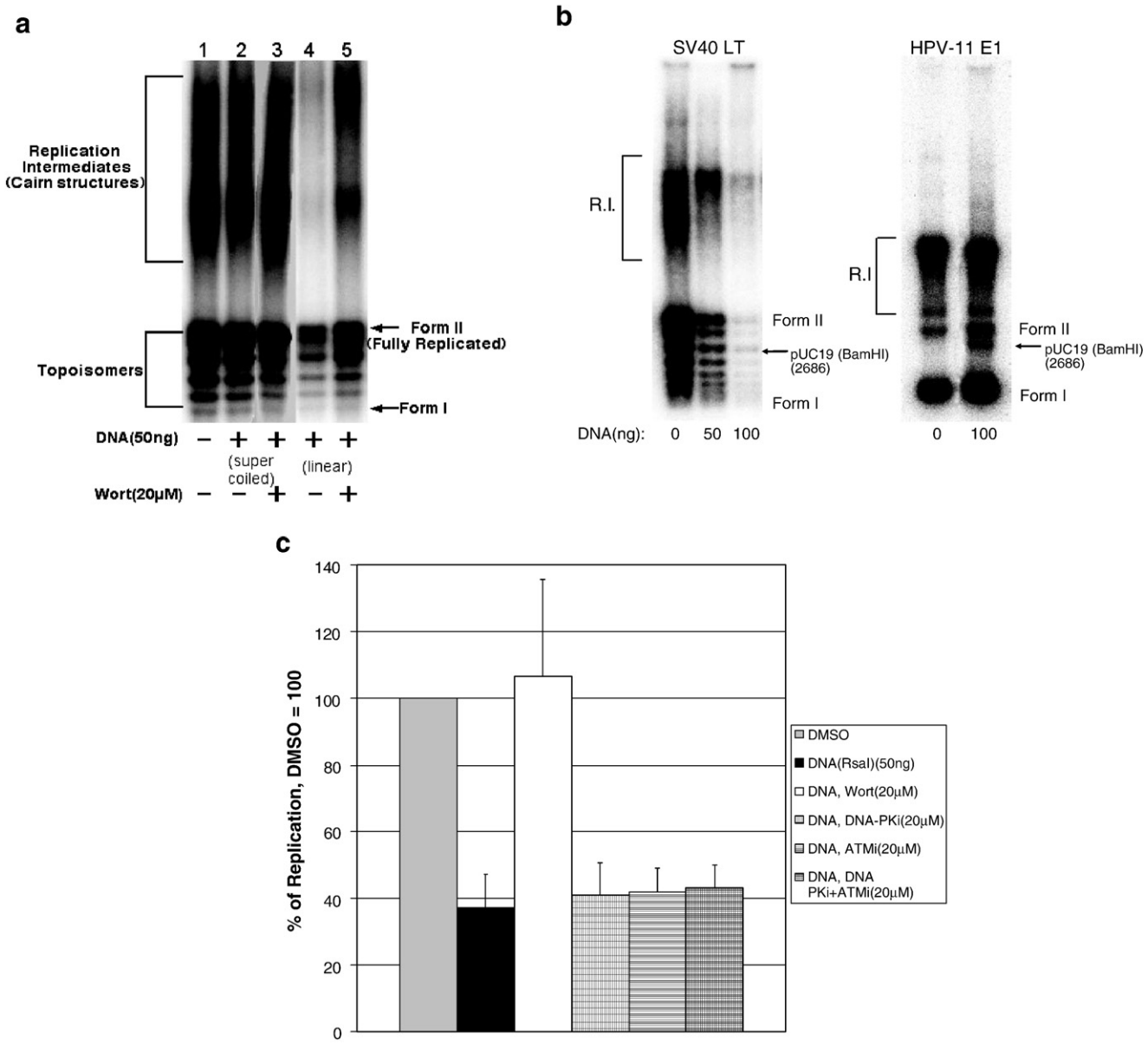

Fig. 2. HPV E1 and E2 mediated DNA replication is not arrested by DNA damage checkpoints *in vitro* while SV40 DNA replication is. a) *In vitro* SV40 DNA replication as described in Materials and methods was carried out in the presence of 0.1% DMSO (lane 1). Undigested pUC19 DNA (50 ng) was added to HEK 293 cell extract prior to assembly of replication reaction, either in the absence (lane 2) or presence of 20 μM wortmannin (lane 3). Restriction-enzyme digested pUC19 (50 ng) was added to the HEK 293 cell extract prior to the assembly of SV40 replication reaction either in the absence (lane 4) or presence of 20 μM wortmannin (lane 5). b) Left panel — SV40 replication reactions were carried out as in panel A lane 1 (except with no DMSO). Zero, 50 or 100 ng of restriction enzyme digested pUC19 DNA was added as indicated. Right panel — HPV DNA replication reactions were carried out as described in Materials and methods using the same extract and reaction buffer as in the left panel, varying only the replication template DNA and using HPV11 E1 in place of SV40 large T antigen. Either zero or 100 ng of restriction enzyme digested pUC19 DNA was added as indicated. c) Testing whether specific inhibitors can alleviate DNA replication inhibition. The inhibitors indicated (DNA-PKi = NU7026; ATMi = KU55933) were added to the extracts on ice 5 min prior to addition of template and replication reaction components (concentrations of the inhibitors given are final concentration in the reaction). Replication levels were quantified as described in Materials and methods. Results shown represent four independent experiments; error bars indicate the standard deviation.

polymerases to replicate their genomes (Bourre et al., 1989; MacGregor et al., 1987; Taylor et al, 2003) and PV DNA replication has been shown to be dependent on the same cellular replication proteins as SV40 DNA replication (Muller et al., 1994; Melendy et al., 1995), the most obvious difference between the enzyme activities used by E1–E2 versus LT-mediated DNA replication is the actual helicase itself. In addition, studies have demonstrated that MCM proteins are targets for DNA damage signalling pathways and it has been hypothesised that this phosphorylation could contribute towards the arrest of DNA replication in the presence of DNA damaging agents (Cortez et al., 2004; Ishimi et al., 2003a, b). Studies have shown that DNA damage induced phosphorylation of LT by ATM at Ser120 and we postulated that etoposide treatment resulted in phosphorylation of LT (Shi et al., 2005). There have been no corresponding studies carried out with E1 but we hypothesise that E1 is not a substrate for the ATM/ATR signalling pathways allowing replication in the presence of DNA damage. To investigate this 293T cells, 293T cells expressing an HA-tagged E1, were treated with etoposide and protein extracts immunoprecipitated with a phospho-(Ser/Thr)-glutamine ATR/ATM substrate antibody (pS/Q antibody). These immunoprecipitations were subjected to immunoblotting for E1 or LT. The pS/Q antibody is known to immunoprecipitate phosphorylated ATR/ATM substrates. In Fig. 3a the failure of the pS/Q antibody to immunoprecipitate an HA-tagged HPV11 E1 is shown. The input E1 levels used in these experiments are shown in lanes 5–8 and the cells were treated with 50 mM of etoposide for 30 min prior to cell harvest and protein preparation. Treatment with etoposide does not result in

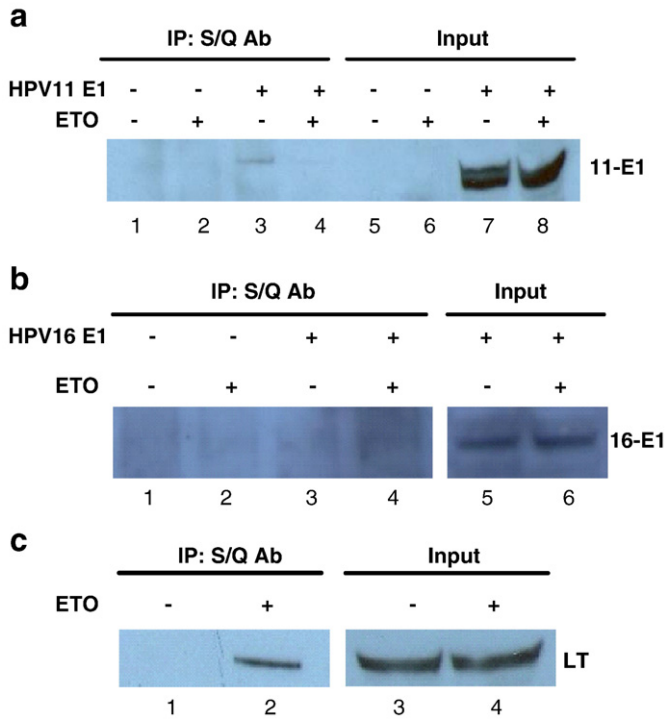

Fig. 3. LT Ag is a substrate for DNA damage kinases, while E1 is not. a) 293T cells were transfected with 1 μg of HPV11 HA-E1 (lanes 3, 4 and 7, 8) or 1 μg of empty control vector (lanes 1, 2 and 5, 6). Twenty-four hours later the cells were treated with 50 μM of etoposide for 30 min (+) or left untreated (−) as indicated and protein extracts prepared. A fraction of the protein extract was then immunoprecipitated with an S/Q antibody (lanes 1–4) and the resultant precipitate probed with an HA antibody. It is clear that no substantial amounts of HA-E1 are immunoprecipitated with the S/Q antibody even though there are detectable levels of this protein in the input (lanes 7, 8). b) 293T cells were transfected with 1 μg of HPV16 HA-E1 (lanes 3, 4 and 5, 6) or 1 μg of empty control vector (lanes 1, 2). Twenty-four hours later the cells were treated with 50 μM of etoposide for 30 min (+) or left untreated (−) as indicated and protein extracts prepared. A fraction of the protein extract was then immunoprecipitated with an S/Q antibody (lanes 1–4) and the resultant precipitate probed with an HA antibody. It is clear that no substantial amount of HA-E1 is immunoprecipitated with the S/Q antibody even though there are detectable levels of this protein in the input (lanes 5, 6). c) 293T cells were treated with 50 μM of etoposide for 0.5 h (+) or left untreated (−). After treatment protein extracts were prepared and a fraction of this was then immunoprecipitated with the S/Q antibody (lanes 1 and 2). The immunoprecipitates were then immunoblotted for LTAg using pAb101. Lanes 3 and 4 show the input levels of LTAg in the immunoprecipitation inputs.

degradation of the E1 protein (compare lanes 7 and 8) and it is also clear that there is very little E1 protein co-immunoprecipitated with the pS/Q antibody, with no increase in precipitated E1 following etoposide treatment (compare lanes 3 and 4). Fig. 3b details the results obtained with HA-tagged HPV16 E1 where identical results are obtained, suggesting that this protein is not a substrate for ATM/ATR. To evaluate whether LT is an ATM/ATR substrate the same experiment was carried out and the presence of the LT protein in the pS/Q immunoprecipitate was monitored (Fig. 3c). In the input lanes (3 and 4) it is again clear that following 30 min of etoposide treatment there is no degradation of LT. However, in contrast to E1, following treatment with etoposide the pS/Q antibody immunoprecipitates a significant amount of LT demonstrating that following etoposide treatment, this protein is targeted by the ATM/ATR kinases.

LT protein levels are reduced following extended exposure to etoposide

The DNA replication assay results in Fig. 1 are extended over 48 h following etoposide treatment, not the 30 min used in Fig. 3. To investigate the levels of the LT protein following extended etoposide treatment an extended time course of etoposide treatment followed by LT western blotting was carried out and the results are shown in Fig. 4a. The level of LT protein begins to drop after 8 h of exposure to etoposide and after 24 h there is a significant reduction in the level of LT. To confirm that etoposide induced a DNA damage response, a phospho-Chk1 antibody was used and etoposide clearly induces Chk1 phosphorylation, a substrate for activated ATR (Zhao and Piwnica-Worms, 2001). We also confirmed that Chk2 was phosphorylated on threonine 68 demonstrating that ATM is also activated (not shown). This phosphorylation persists over the 24 hour period of the experiment. Gamma-tubulin levels are shown as a loading control. Fig. 4b shows that the level of the HPV16 E1 protein is not decreased following 24 h of etoposide treatment, while phosphorylation of Chk1 demonstrates that a DNA damage signal was generated in the presence of E1 and that it persisted throughout the 24 hour time point. If anything the levels of E1 increase following 24 h of treatment with etoposide. This demonstrates that E1 does not block activation of ATR as around 90% of the cells express E1 following transfection (not shown). The level of HPV16 E2 protein is also not decreased over a 24 hour time period following etoposide treatment (Fig. 4c), and again, like E1, there is an increase in E2 protein levels suggesting a stabilisation following DNA damage. The level of LT RNA in 293T cells is reduced by two thirds following 24 h of etoposide treatment (not shown). However, the level of LT protein is reduced to a greater extent, suggesting that the decrease in LT levels may be due to more than just the reduction in mRNA levels. In addition, the *in vitro* experiments described in Fig. 2 demonstrate that the regulation of LT-mediated DNA replication by DNA damaging agents is due to a direct regulation of the LT protein. Therefore although there is a reduction in LT RNA levels following etoposide treatment, this is unlikely to completely explain the inhibition of DNA replication function. To determine whether the reduction of LT is due to a proteasome-mediated degradation, the proteasomal inhibitor MG132 was used in the etoposide experiments. The result of this experiment is shown in Fig. 4d where it is clear that blocking of proteasomal degradation by treatment with MG132 blocks reduction of LT protein levels following etoposide treatment.

Discussion

HPV are causative agents in cervical cancer and in the majority of cases, the viral genome is integrated into that of the host. This integration disrupts the E2 open reading frame resulting in relief of transcriptional repression of the HPV oncogenes E6 and E7. Their increased expression is proposed to promote genomic instability and carcinogenesis (Pett and Coleman, 2007). In this report we have investigated factors that could contribute towards viral genome integration, specifically focusing on the role that DNA damage may play in this process. The results show that E1–E2 mediated DNA replication is not arrested in the presence of DNA damage, even though an intact DNA damage response is mounted in the E1–E2 expressing cells. This failure to arrest results in replication in the presence of DNA damaging agents increasing the risk of aberrant DNA replication and promotion of DSBs generating substrates for viral integration.

Previous studies on SV40 infection have demonstrated that the DNA damage response is activated in the infected cells and that this activation is required for a productive infection, phosphorylation of LT on S120 results in a stimulation of replication (Shi et al, 2005). In addition, SV40 infected cells alter the host DNA damage response by targeting the MRN complex for degradation via the proteasome (Digweed et al., 2002; Zhao et al., 2008). Therefore the host cell DNA damage response is re-programmed by SV40 infection presumably to promote viral DNA replication. More specifically it has also been shown that expression of the LT protein by itself can promote genomic instability by binding to Bub1 and interfering with the function of this protein in mitosis (Cotsiki et al., 2004; Hein et al., 2009) as well as

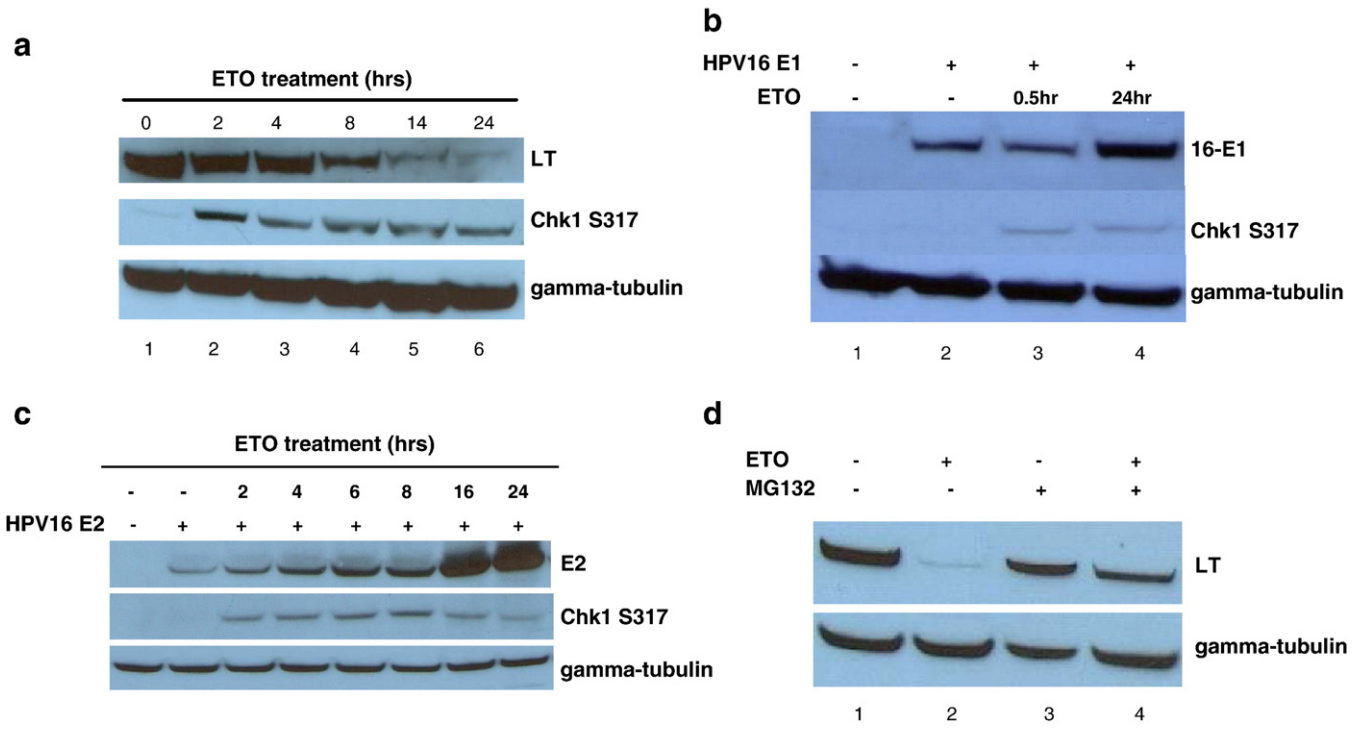

Fig. 4. LTAg protein levels are reduced via the proteasome following etoposide treatment. a) 293T cells were treated with 50 μM of etoposide for the time periods shown and then protein extracts prepared. These extracts were then western blotted for the proteins indicated. Chk1 S317 serves as a control to confirm activation of the DNA damage response and gamma-tubulin as a loading control. b) 293T cells were transfected with 1 μg of HPV16 HA-E1 plasmid and the following day cells left untreated (−) or treated with 50 μM of etoposide (+) for the time periods shown. Protein extracts were then prepared and western blotted for the proteins indicated. Chk1 S317 serves as a control to confirm activation of the DNA damage response and gamma-tubulin as a loading control. c) 293T cells were transfected with 2 μg of HPV16 E2 plasmid and the following day cells left untreated (−) or treated with 50 μM of etoposide (+) for the time periods shown. Protein extracts were then prepared and western blotted for the proteins indicated. Chk1 S317 serves as a control to confirm activation of the DNA damage response and gamma-tubulin as a loading control. d) 293T cells were treated with 50 mM etoposide (lanes 2 and 4) and/or MG132 (lanes 3 and 4) as indicated and protein extracts prepared 24 h later. Western blotting was then carried out for LTAg and gamma-tubulin as a loading control.

inducing genomic instability by binding to and inactivating p53 and pRb (Cheng et al., 2009). Paradoxically, DNA damage signals can suppress SV40 DNA replication in mammalian cells via activation of the DNA damage signalling kinases (Miao et al., 2003). 293T cells retain the ability to signal via the DNA damage signalling kinases despite stably expressing LT (Shirata et al., 2005). Clearly the results describe here agree with these previous studies demonstrating arrest of LT-mediated DNA replication following DNA damage. The reason for the different responses of SV40 LT-mediated DNA replication following either viral infection (activation of damage signalling pathways and stimulation of replication) or treatment with DNA damaging agents (which activate damage signalling pathways but suppress replication) may be related to differential modification of LT in different circumstances. Phosphorylation of S120 results in stimulation of LT replication function following viral infection and it is possible that following exposure to DNA damaging agents LT is differentially modified resulting in replication repression. Proteomic and mutational studies will be required to determine whether this is the case.

Significantly, this report demonstrates that neither HPV11 nor HPV16 E1 is a substrate for DNA damage kinases. Like SV40, recent work has demonstrated that HPV can activate DNA damage signalling kinases during their life cycle (Moody and Laimins, 2009) but unlike SV40, DNA damage per se does not arrest E1/E2 mediated DNA replication (this report). Therefore the difference in the DNA replication response of LT and E1–E2 is likely related to either the differential phosphorylation of LT and E1 following DNA damage, or to differential interactions of these two viral proteins with cellular factors phosphorylated by DNA damage response pathways. This is of interest when considering cellular DNA replication and the response to DNA damage. Previous work has demonstrated that following DNA damage SV40 DNA replication control mimics that of cellular DNA replication (Miao et al. 2003). Therefore the results presented here strongly support the hypothesis that the replicative helicase is a direct target for DNA damaging signalling kinases and that phosphorylation by these kinases plays a key role in arresting DNA replication. Previous studies have demonstrated phosphorylation of MCM proteins following DNA damage (Cortez et al., 2004; Ishimi et al., 2003a, b) and it seems clear that phosphorylation of these proteins is integral to the arrest of DNA replication following DNA damage.

The apparent stabilisation of E1 and E2 following etoposide treatment (Fig. 4) may be due to the arrest of the cells in S phase where it has recently been shown that E2 is stabilised (Johansson et al., 2009) and the ongoing replication by E1 and E2 due to non-targeting of E1 by DNA damage response kinases. This would provide an ideal environment to promote double stranded DNA breaks in the replicating HPV genome promoting viral integration and progression to cancer. Further investigations are merited to determine whether agents such as estrogen, which can regulate the DNA damage response (Pedram et al, 2009), promote double strand breaks in HPV genomes therefore contributing to progression to cancer.

Materials and methods

Plasmids

pCMV HPV16 E2 plasmid (HPV16 E2) expresses a wild type HPV16 E2 protein from a CMV promoter. pCMV HPV16 E1 (HPV16 E2) expresses a wild type HPV16 E1 protein from a CMV promoter. pHPV16 E1HA (expresses the wild type HPV16 E1 protein) and

pHPV11 E1HA (expresses the wild type HPV11 E1) both contain a HA tag, and were a kind gift from Mart Ustav. pOri16M (pOriM) contains the HPV16 origin of replication cloned into pSKII (−) (Taylor and Morgan, 2003). pGL3-control vector (p+SV40 ori) contains a SV40 promoter and luciferase gene. pGL3-basic vector (p−SV40 ori) contains a luciferase gene but no SV40 promoter. pcDNA 3.1 (Invitrogen) was used as a carrier plasmid. The HPV11 E2 and E1 expression vectors were a kind gift from Dr Jacques Archambault, University of Montreal. The DNA-PK and ATM inhibitors, NU7026 and KU55933, were gifts from Dr. Graeme Smith, KuDOS Pharmaceuticals, Division of Astra Zeneca. The HA-tagged E1 expression vectors were a kind gift from Dr Mart Ustav, Estonian Biocentre, Tartu.

Cell growth and transfection

HEK-293T cells were cultured in DMEM (Dulbecco's modified Eagles medium) supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin mixture (Invitrogen) at 37 °C in a 5% CO_2/95% air atmosphere.

Cells were plated out on 100 mm^2 plates so as to achieve around 80% confluence upon harvest. Cells were transiently transfected using a standard calcium phosphate precipitation technique.

DNA replication assay

Transient DNA replication assays were carried out in a similar method as described previously (Taylor & Morgan, 2003). Briefly 3×10^5 293T cells were plated out and were $CaPO_4$ transfected the following day with the HPV replicative plasmids (pOriM, pCMV-E1 and pCMV-E2) or the SV40 replicative plasmids (p−SV40 ori and p+SV40 ori). The following morning cells were washed twice in PBS and left untreated, treated with etoposide (Sigma) or aphidicolin (Sigma) then harvested 48 h later in Hirt solution (10 mM EDTA, 0.5% SDS). DNA was extracted and purified as described. Dam methylated DNA was digested using Dpn1 (New England Biolabs) and ExoIII (New England Biolabs) to allow freshly replicated pOriM/p+SV40 DNA to be measured. For real-time PCR assays, probe and primers were designed to amplify the HPV and LT origin plasmids, pOriM and p+/−SV40 respectively. pOriM: forward primer 5′ ATCGGTTGAACCGAAACCG 3′, reverse primer 5′ TAACTT-CTGGGTCGCTCCTG 3′ and probe 5′ 6-FAM-ACCAAAAGAGAACTGCAA-TGTTTCAGGATCC-TAMRA 3′ (Eurogentec). p+/−SV40: forward primer 5′ TCCTTCGATAGGGACAAGACAATT 3′, reverse primer 5′GGCAGAGCGA-CACCTTTAGG 3′ and probe 5′ 6-FAM-CACTGATCATGAACTCCTCTGGATC-TACTGGCT-TAMRA 3′ (Eurogentec). Replication was determined by the quantity of signal detected compared to a standard curve of the plasmid.

Western blotting and immunoprecipitation

Cell lysates were harvested in lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris pH 8.0) and protein levels were standardised using a Bradford Assay.

For western blotting equal amounts of protein were boiled in 2 μl of 10× Sample Reducing Agent (Invitrogen) and 5 μl 4× LDS Buffer (Invitrogen). The lysates were loaded onto a 4–12% gradient gel (Invitrogen), ran at 200 V for 1 h and transferred onto nitrocellulose membranes using an iBlot system (Invitrogen). After blocking in 5% milk PBS-T at room temperature for an hour the membranes were incubated with primary antibodies and their relevant secondary antibody according to manufacturer's instructions. The antibodies used were pAb101 (a monoclonal antibody against Large T Antigen), HA.11 (Covance) anti-HA epitope to detect HPV16 E1 and HPV11 E1, phospho-Chk1 (Ser 317) antibody (Cell Signalling Technology #9931), GTU-88 to detect gamma-tubulin (Abcam), anti-rabbit IgG peroxidise (Sigma) and anti-mouse IgG peroxidise (GE Healthcare) for development. For immunoprecipitation the lysates were incubated on a spinning rotor overnight at 4 °C with phospho-(Ser/Thr) ATM/ATR substrate antibody (Cell Signalling Technology #2851) according to the manufacturer's instructions. The following morning protein G sepharose beads (Sigma) were added to the lysate and incubated for 1 h at 4 °C on the rotor. The samples were spun and washed several times in lysis buffer then boiled in 5 μl 10× Sample Reducing Agent and 15 μl 4× LDS Buffer. Samples were prepared as described above.

MG132 treatment

Cells were treated with 50 μM etoposide, 30 μM MG132 (Calbiochem) or a combination of both for 24 h. Cells were harvested for western blot as described.

In vitro DNA replication assays

Viral *in vitro* DNA replication assays were performed as previously detailed (Melendy et al., 1995; Narahari et al, 2006). Thirty nanograms of template DNA containing the SV40 or HPV origins of replication (pSV011 or p7974-99) was incubated with 200 ng of SV40 TAg or 40 ng of HPV11 E1 and 20 ng of HPV11 E2, replication buffer (30 mM HEPES(pH 7.5), 40 mM creatine phosphate, 7 mM $MgCl_2$ 0.5 mM DTT, 4 mM ATP, 200 μM CTP, 200 μM UTP, 200 μM GTP, 100 μM dCTP, 100 μM dGTP, 100 μM dTTP, 25 μM dATP), 0.1 mg/ml acetylated BSA, 0.625 μg creatine phosphokinase (Sigma), 1 μCi αP^{32} dATP, and 30–50 ng of HEK 293 cell cyto/nucleosolic extract in 10 μl reactions. Reactions were carried out for one hour at 37 °C and were terminated by addition of stop buffer (20 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 0.1%SDS, 1 μg/μl proteinase K) for twenty minutes at 37 °C. Replication was evaluated by resolution of replication products on 0.8% agarose gels followed by phosphorimager autoradiography. To determine the effect of the kinase inhibitors wortmannin, NU7026, and KU55933, either dimethylsulfoxide (DMSO) or the inhibitor dissolved in DMSO, was first incubated with cellular extracts for 5 min on ice prior to assembly of the replication reaction. Non-template plasmid added to the reactions (pUC19) was isolated using standard protocols, and then either added directly or first digested with the restriction enzyme RsaI to completion, and then phenol:chloroform extracted and precipitated prior to addition. Several restrictions enzymes, with varying termini (3′ overhand, 5′ overhang, and blunt ended) were also used and all types of ends produced very similar results (data not shown). Quantification of DNA replication activity was by phosphorimager analysis; incorporation of isotope from the top of the Replication Intermediates all the way through Form I was quantified, background from an unused lane was subtracted, and the results were compared to the DMSO only positive control lane (set to 100%).

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