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Interaction of HPV E6 oncoproteins with specific proteasomal subunits

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ABSTRACT

The Human Papillomavirus E6 oncoproteins have the capacity to target several of their cellular interacting partners for proteasome mediated degradation, and recent proteomic analyses suggest a close involvement of E6 with the cellular proteasome machinery. In this study we have performed an extensive analysis of the capacity of different E6 oncoproteins to interact with specific proteasome components. We demonstrate that multiple subunits of the proteasome can be bound by different HPV E6 oncoproteins. Furthermore, whilst most of these interactions appear independent of the E6AP ubiquitin ligase, the association of E6 with the major ubiquitin-accepting proteasome subunit, S5a, does require the presence of E6AP. One consequence of the interaction between E6/E6AP and S5a is enhanced ubiquitination of this proteasome subunit. These results suggest a complex interplay between E6 and the proteasome, only some aspects of which are dependent upon the E6AP ubiquitin ligase.

Introduction

Human Papillomaviruses (HPVs) are the causative agents of cervical cancer. They are also responsible for a large number of other anogenital cancers, plus an increasing number of head and neck cancers (Bouvard et al., 2009). Currently over 150 different HPV types have been described, but only a small subset of these, the so-called high-risk types are cancer-causing and these are exemplified by types HPV-16 and HPV-18 (Doorbar et al., 2012). The HPV E6 and E7 oncoproteins are essential for the ability of the virus to induce cervical cancer. They cooperate in the immortalisation of primary human keratinocytes (Hawley-Nelson et al., 1989; Barbosa and Schlegel, 1989), the natural target cell of the virus *in vivo*, and also cooperate in the induction of cervical cancer in transgenic animals (Riley et al., 2003). Furthermore, both E6 and E7 are retained and expressed in the tumours many years after the initial immortalising events, suggesting a continuing role in tumour development and maintenance (Smotkin and Wettstein, 1986; Androphy et al., 1987; Banks, et al., 1987). Indeed a large number of studies have shown that inhibition of their expression or function has deleterious effects upon continued tumour cell proliferation, resulting in the induction of apoptotic and senescence phenotypes (Yoshinouchi et al., 2003; Butz et al., 2003). Thus both of these viral oncoproteins are prime targets for therapeutic intervention in HPV-associated diseases.

The high-risk HPV E6 and E7 oncoproteins function by perturbing key elements of cellular homoeostasis (Doorbar et al., 2012). HPV E7 is primarily responsible for inducing an environment favourable for viral DNA replication. This occurs through perturbation of key cell cycle regulators such as the pRb family of tumour suppressors and the cyclin dependent kinase inhibitors (Dyson et al., 1989; Funk et al., 1997). HPV E6 on the other hand is largely responsible for blocking the cell's normal pro-apoptotic responses that are induced as a result of E7 activity. This is achieved in part through blocking the normal pro-apoptotic activities of p53 and Bak (Kessis et al., 1993; Thomas and Banks, 1998). Intriguing features of both E6 and E7 are their abilities to direct many of their cellular substrates for proteasome-mediated degradation (Banks et al., 2003). In the case of E7, this is seen for pRb, p130 and p107, and in the case of pRb, involves recruitment of the cellular cullin-2 ubiquitin ligase complex to induce poly-ubiquitination of the substrate, which is then directed for subsequent degradation at the 26S proteasome (Gonzalez et al., 2001; Huh et al., 2007). In the case of E6, major degradation targets include p53 (Scheffner et al., 1990) and a number of different PDZ domain-containing proteins (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000). For many of these substrates, this degradatory activity relies upon the ability of E6 to recruit the cellular ubiquitin

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ligase E6AP to the E6-substrate complex, which in turn results in substrate poly-ubiquitination and degradation at the proteasome (Scheffner et al., 1993). Thus, whereas much is known about the ubiquitin ligases that are recruited by the viral oncoproteins to direct their substrates for degradation, much less is known about the capacity of these oncoproteins to associate directly with the proteasome itself.

The 26S proteasome is a multi-subunit macromolecular machine, composed of multiple protein components, which captures ubiquitin-conjugated proteins via the 19S regulatory cap that sits above the catalytic 20S proteolytic core (Kish-Tier and Hill, 2013). Bound to this is the S5a subunit, which interacts with polyubiquitinated chains and is believed to either tether or direct such polyubiquitinated proteins directly to the proteasome for subsequent degradation (Wang et al., 2005; Isasa et al., 2010). Interestingly, significant amounts of S5a protein are not directly bound to the proteasome complex at any given time, suggesting that it may also have additional regulatory roles within the cell. In the case of HPV, recent proteomic analyses have identified multiple proteasome subunits, including S5a, as potential interacting partners of the HPV E6 oncoproteins (Tomaić et al., 2011; Rozenblatt-Rosen et al., 2012) and its associated ubiquitin ligase E6AP (Martinez-Noel et al., 2012), with previous studies also showing that E6AP could directly ubiquitinate the S5a subunit (Uchiki et al., 2009). Whether individual components of the proteasome can be bound by E6 and whether this simply reflects an indirect association that is mediated via E6AP, however, remains to be determined. We were therefore interested in investigating whether the HPV E6 oncoproteins can directly interact with the isolated ubiquitin subunits and, furthermore, in determining the potential role for E6AP in this recognition process. We now show that multiple subunits can be directly bound by E6, but that the gatekeeper subunit, S5a, is bound and polyubiquitinated by E6 in an E6AP-dependent manner.

Results

HPV E6 interacts with multiple proteasome subunits

Recent proteomic analyses have identified multiple proteasome subunits as potential interacting partners of the HPV E6 oncoproteins and of the associated E6AP ubiquitin ligase (Tomaić et al., 2011; Rozenblatt-Rosen et al., 2012; Martinez-Noel et al., 2012). This raises the question of which, if any, of these proteasome subunits is bound directly by the HPV E6 oncoproteins. To investigate this, we first performed a series of GST pull-down assays using either full length GST.18E6 or the alternatively spliced GST.18E6*, which lacks most of the carboxy terminal half of the protein. These were incubated with a large panel of [^{35}S]-methionine radiolabeled proteasome subunits that were translated in a wheat germ system (WGE), so as to avoid any possible contamination with E6AP. The results obtained are shown in Fig. 1, where it can be seen that both the full length HPV-18 E6 (Fig. 1A) and the alternatively spliced HPV-18 E6* (Fig. 1B) are capable of interacting with multiple proteasome subunits, with particularly strong associations seen between E6 and the S4 and S8 subunits. Interestingly, there is remarkable similarity between the E6* and the full-length HPV-18 E6 oncoprotein, both in terms of the pattern of subunits recognised and in the relative strengths of interaction. This indicates that the capacity of HPV-18 E6 to interact with the individual proteasome subunits is contained within sequences lying primarily within the N-terminal half of the protein. It is also noteworthy that whilst the S5a subunit was identified in the proteomic analyses as a potential interacting partner of HPV-18 E6, this subunit does not seem to be bound by E6 under these *in vitro* conditions.

Having found that HPV-18 E6 has the potential to interact with multiple proteasome subunits we wanted to determine how the strength of this interaction compared with some of E6's strongest bound partners, the PDZ domain containing substrates Dlg and Scribble. To do this GST pull down assays were performed using GST.18E6 and *in vitro* translated [^{35}S]-methionine radiolabeled Dlg, Scribble and for comparison, the S4 subunit. The results in Fig. 1C show a very strong association between E6 and the PDZ domain containing substrates (approximately 46% of input bound) in agreement with previous studies (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000), with a somewhat weaker but nonetheless highly significant interaction (approximately 12% of input bound) with the S4 subunit.

We next proceeded to compare the relative abilities of the low risk HPV-11 E6 and the high risk HPV-16 E6 to interact with selected proteasome subunits. In this case we used purified GST fusion proteins of the S2 and S4 subunits, as examples of weak and strong interacting partners of HPV-18 E6, and performed interaction assays with [^{35}S]-cysteine radiolabeled E6 proteins, which were translated in the wheat germ system. The results in Fig. 2 demonstrate that HPV-16 E6 interacts most strongly with the proteasome subunits, and with a very similar affinity for both S2 and S4. HPV-18 E6, recognises both subunits, but again binds to S4 somewhat more strongly than it does to the S2 subunit. In contrast, HPV-11 E6 can also interact with both proteasome subunits, but in this case binds S2 and S4 to a similar weak degree. These results demonstrate that the capacity of the HPV E6 oncoproteins to interact with individual subunits of the proteasome is conserved across different HPV types, but that there are significant differences in the levels to which different E6 oncoproteins can recognise specific proteasome subunits.

The above assays were all performed in the absence of E6AP, however we wanted to determine if the pattern of proteasome subunits recognised by HPV-18 E6 might differ if E6AP was present. To do this we repeated the interaction assays using a panel of different GST fusion proteins of the proteasome subunits, incubated with [^{35}S]-cysteine radiolabeled E6 that had been *in vitro* translated in rabbit reticulocyte lysates (RRL). The results in Fig. 3A generally show a pattern of proteasome subunit recognition by the HPV-18 E6 protein that is very similar to that seen using the wheat germ expression system. However, there is a marked increase in the capacity of HPV-18 E6 to interact with the S5a subunit. Interestingly, when this interaction assay was done with E6*, there was still no interaction with the S5a subunit (data not shown). This suggests that the capacity of E6 to recognises the S5a subunit of the proteasome requires the full length E6 protein and furthermore, is most likely indirect and potentially mediated via the E6AP ubiquitin ligase.

To investigate this possibility further, we transfected HA-tagged HPV-18 E6 into 293 cells where E6AP expression was either wild type or stably knocked down following transfection with an shRNA targeting vector (Fig. 3B iii). After 24 h the cells were extracted and interaction assays performed using a GST fusion protein of the S5a subunit. Bound E6 was then detected by anti-HA Western blotting. The results in Fig. 3B demonstrate a very efficient pull-down of HPV-18 E6 from the transfected 293 cells with the GST S5a fusion protein, but only when E6AP is present. These results confirm that HPV-18 E6 can recognise the S5a proteasome subunit but that this is dependent upon the presence of E6AP.

HPV E6 induces polyubiquitination of S5a.

The S5a subunit is one of the proteasome's major acceptors of polyubiquitin chains and is potentially a critical controlling factor

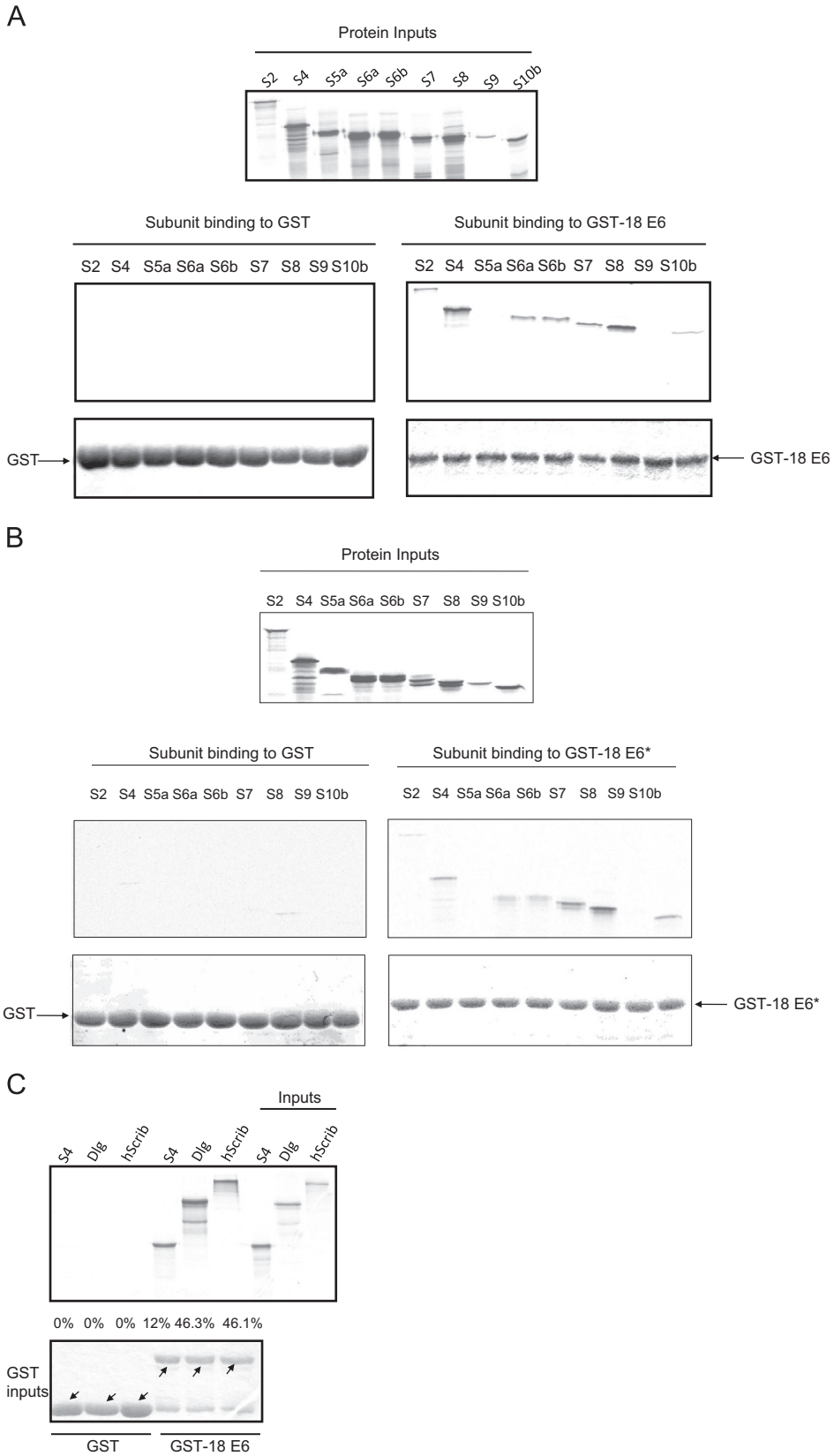

Fig. 1. HPV-18 E6 can interact with multiple proteasome subunits. The indicated proteasome subunits were in vitro translated and radiolabelled using wheat germ extracts (WGE) and then incubated with purified GST, GST.18E6 (Panel A) or GST.18E6* (Panel B) as indicated. After extensive washing the bound proteins were detected by SDS-PAGE and autoradiography. The upper panels show 20% of the *in vitro* translated protein inputs used in the pull down assays. The two lower panels show the autoradiogram and the Coomassie stain of the gel confirming equal levels of protein loading. For comparison interaction (Panel C) assays were performed with purified GST or GST.18E6 with *in vitro* translated and radiolabelled S4 subunit, Dlg and Scribble (hScrib). The upper panel shows the amount of *in vitro* translated protein bound by the GST and GST.18E6, with the numbers showing the percentage of input protein bound and the lower panel showing the Coomassie stain of the gel confirming equal levels of protein loading.

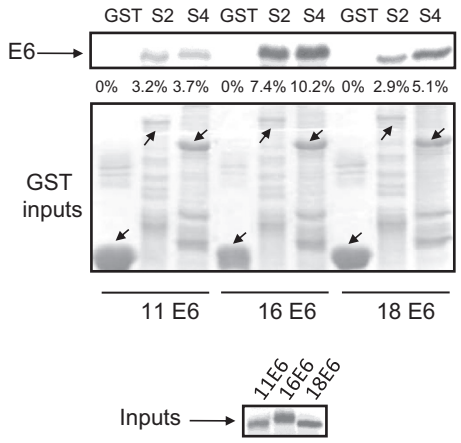

Fig. 2. High- and low-risk HPV E6 proteins recognise proteasome subunits with similar efficiency. The indicated proteasome subunits were expressed and purified as GST fusion proteins and incubated with *in vitro* translated and radiolabelled HPV-11, HPV-16 and HPV-18 E6 proteins in WGE. After extensive washing the bound proteins were detected following SDS-PAGE and autoradiography. The upper panel shows the autoradiograph of bound E6. The GST inputs are also shown as a Coomassie stained gel, with arrows indicating the full-length GST proteins. The bottom panel shows 20% of the input E6 proteins used in the pull down assays and the numbers represent the mean percentage of input protein bound from at least 3 independent assays.

in regulating protein degradation at the proteasome (Elangovan et al., 2009; Wang et al., 2005; Isasa et al., 2010). The above results suggest that E6 can potentially recognise multiple proteasome subunits, but that recognition of the S5a subunit is dependent upon E6AP. Since the E6-E6AP complex functions as unique ubiquitin ligase, where E6 provides novel substrate specificity for the E6AP ubiqutin ligase, we next wanted to determine whether E6 might have any influence on S5a stability. To do this, the HPV-16 and HPV-18 E6 proteins, together with the S5a subunit, were *in vitro* translated and radiolabeled in rabbit reticulocyte lysate. The translates were then mixed and incubated together for different periods of time, and the pattern of S5a expression ascertained following SDS-PAGE and autoradiography. The results obtained are shown in Fig. 4A. As can be seen, there is very little change in the pattern of S5a expression alone over the period of the assay. However, in the presence of HPV-16 E6 there is a slight reduction in the levels of S5a protein, with a corresponding dramatic increase in the appearance of higher molecular weight slower migrating forms of the protein. This laddering of S5a is also seen in the presence of HPV-18 E6, but is much weaker.

The appearance of the slower migrating forms of the S5a protein is apparent within 30–60 min of incubation with HPV-16 E6, and we wanted to determine how quickly these modifications could be induced. To do this the assay was repeated, but the pattern of expression was monitored from 5 mins onwards. As can be seen from Fig. 4B, HPV-16 E6 induces a readily detectable change in the pattern of S5a protein expression after 15–30 min of incubation, with higher molecular weight forms appearing by 1–3 h. By 5 h these higher molecular weight forms of S5a begin to disappear. These results indicate that the HPV-16 E6 induced modification to S5a is quite rapid, whilst E6-induced degradation of S5a is only very weak when compared to the rate of degradation observed with the p53 control.

The S5a proteasome subunit is a major acceptor of ubiquitin chains, and the pattern of laddering seen in the *in vitro* assays is very reminiscent of ubiquitination. Therefore we were interested in determining whether E6 could modulate the levels to which S5a is ubiquitinated *in vivo*. To do this, 293 cells were transfected with HPV-16 E6 or HPV-18 E6, plus S5a and HA-tagged ubiquitin expression plasmids. After 24 h proteasome inhibitors were added

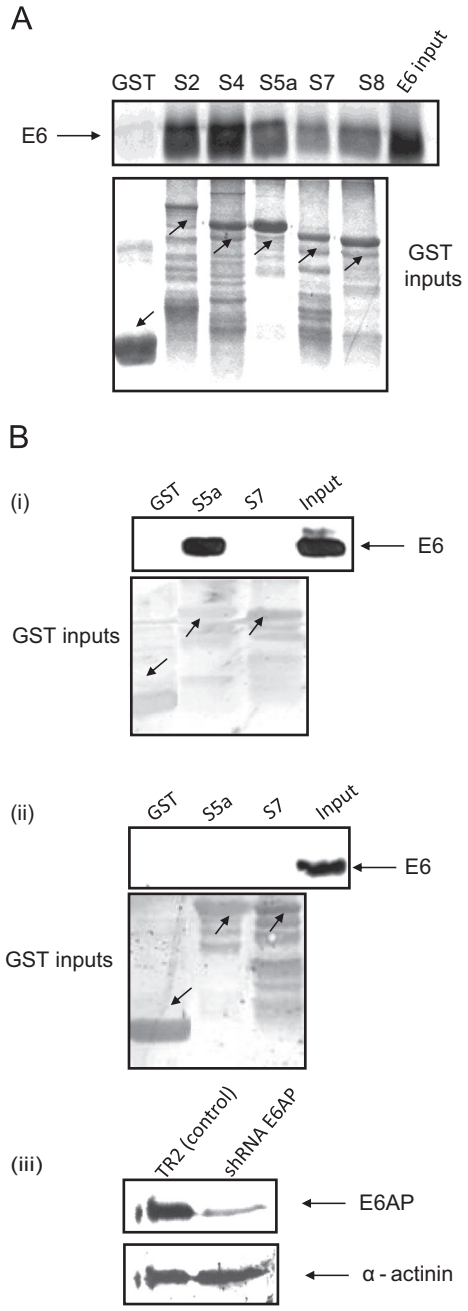

Fig. 3. HPV-18 E6 interacts with the S5a proteasome subunit in an E6AP dependent manner. Panel A. The indicated proteasome subunits were expressed as GST fusion proteins and purified. These were then incubated with *in vitro* translated and radiolabelled HPV-18 E6 in rabbit reticulocyte lysate. After extensive washing the bound protein was detected by SDS-PAGE and autoradiography. The upper panel shows the autoradiograph of bound E6 together with 20% of the E6 input. The bottom panel shows the Coomassie stain of the gel showing the levels of GST inputs used in the pull down assays. Panel B. 293 control TR2 scrambled shRNA (i) and 293 shRNA E6AP, (ii) cells were transfected with HA-tagged HPV-18 E6 expression plasmid and after 24 h cells, were harvested and extracts incubated with the indicated purified GST fusion proteins. After extensive washing, bound E6 was detected by Western blotting using the anti-HA antibody and is compared with the amount of E6 present in 10% of the input. The lower panels in each case show the Ponceau stains of the nitrocellulose membranes showing the levels of GST proteins used in the pull downs with the arrows indicating the position of the GST and GST fusion proteins. The lower panel (iii) shows the Western blot of E6AP expression levels in the control 293 cells transfected with scrambled shRNA (TR2) and cells transfected with shRNA E6AP. Also shown is the α-actinin loading control.

and the cells were incubated for a further 3 h. Ubiquitinated proteins were then immunoprecipitated from the cell extracts using anti-HA antibodies, and the pattern of S5a expression was

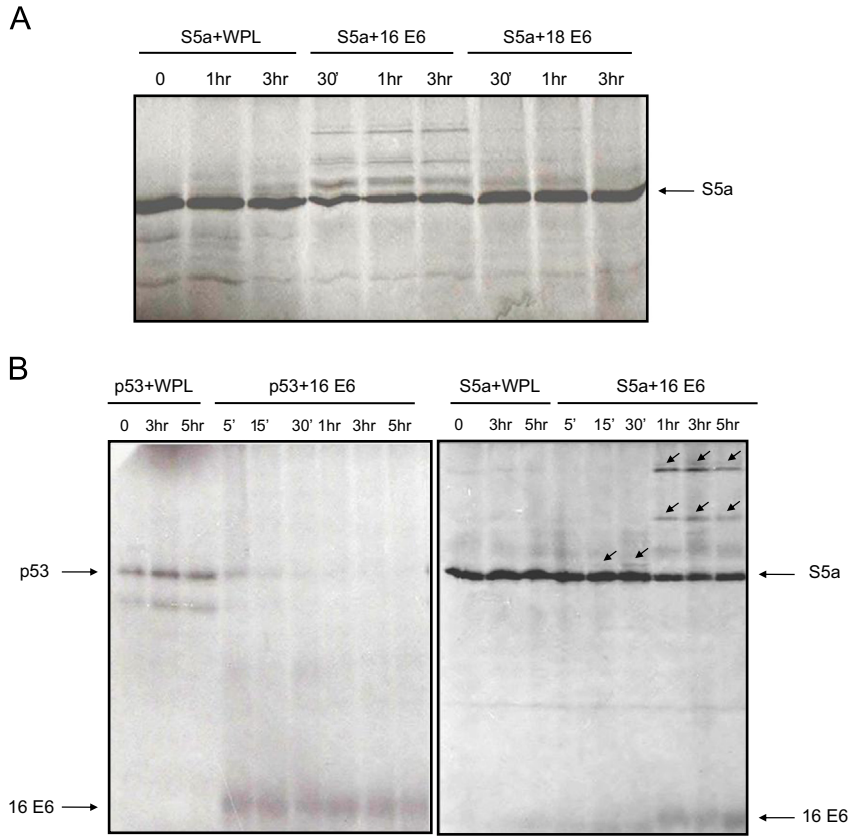

Fig. 4. HPV-16 E6 induces S5a polyubiquitination *in vitro*. Panel A. HPV-16 E6, HPV-18 E6 and the S5a proteasome subunit were *in vitro* translated and radiolabelled in RRL. These were then mixed as indicated and incubated at 30 °C for the 30 min, 1 h and 3 h. Remaining protein was then visualised by SDS-PAGE and autoradiography. Note the appearance of slower migrating forms of S5a in the presence of HPV-16 E6. Panel B. HPV-16 E6 and the S5a proteasome subunit were *in vitro* translated as in Panel A, and mixed and incubated for the indicated times. Note the appearance of slower migrating forms of S5a 15–30 min post incubation with E6 as indicated by the arrows and the progressive loss of the higher molecular weight slower migrating forms over time. The left hand panel shows a parallel assay performed with p53 where E6 induces rapid degradation of the protein after as little as 5 mins incubation.

analysed by Western blotting with anti-S5a antibody. The results obtained are shown in Fig. 5A. In the absence of E6 there are significant amounts of ubiquitin-conjugated S5a protein, which increase following proteasome inhibition. In the presence of HPV-16 E6, the levels of ubiquitinated S5a are slightly higher and these increase dramatically upon proteasome inhibition. However in the presence of HPV-18 E6 there is minimal change in the level of ubiquitinated S5a. These results reflect the patterns of modification seen *in vitro*, and demonstrate that HPV-16 E6 can modulate the levels of S5a ubiquitination *in vivo*.

We then wanted to determine whether E6AP might also modulate the levels to which S5a is ubiquitinated. To do this, 293 cells were transfected with S5a and HA-tagged ubiquitin together with combinations of HPV-16 E6 or E6AP. The pattern of S5a expression was then ascertained following anti-HA immunoprecipitation and Western blotting. The results in Fig. 5B again demonstrate that HPV-16 induces a strong increase in the levels of S5a ubiquitination, and this is even more apparent when E6AP is overexpressed, demonstrating that it can also directly affect the levels of S5a ubiquitination *in vivo*. When both E6 and E6AP are co-expressed there is a marked reduction in total S5a levels, and indications of higher molecular weight forms of ubiquitinated protein. Interestingly, these ubiquitinated forms of S5a appear to be extremely susceptible to rescue by MG132 treatment. These results suggest that E6-induced ubiquitination of S5a renders it susceptible to degradation at the proteasome, whereas E6AP-induced ubiquitination does not. When E6 and E6AP are co-expressed, then S5a is ubiquitinated, but appears to be directed more efficiently for degradation. These results suggest that both E6 and E6AP can modulate the levels of S5a ubiquitination, but that the consequences of such ubiquitination are somewhat different.

We were also interested in determining whether E6AP was in complex with S5a in the immunoprecipitations. To do this, the Western blots were re-probed for E6AP and the results are also shown in Fig. 5B. As can be seen, a small amount of E6AP appears to be co-precipitated with ubiquitin and S5a, and this is in agreement with previous studies (Uchiki et al., 2009). The amount of E6AP co-precipitated also increases significantly in the presence of HPV-16 E6 when MG132 is also present, and interestingly, the amount is directly proportional to the amount of ubiquitinated S5a that is immunoprecipitated. This suggests that the E6AP in complex with ubiquitinated S5a is also subject to proteasome mediated degradation.

Discussion

The ability of the HPV E6 oncoproteins to direct the degradation of their substrates for proteasome-mediated degradation appears to be a central facet of their ability to support the virus life cycle, and also to ultimately induce cancer (Doorbar et al., 2012). Much of this activity is linked to their capacity to act as a bridge between the E6AP ubiquitin ligase and the respective target proteins, thereby resulting in polyubiquitination of the substrate and its subsequent degradation at the 26S proteasome (Scheffner et al., 1993). We now show that E6 also has the potential to

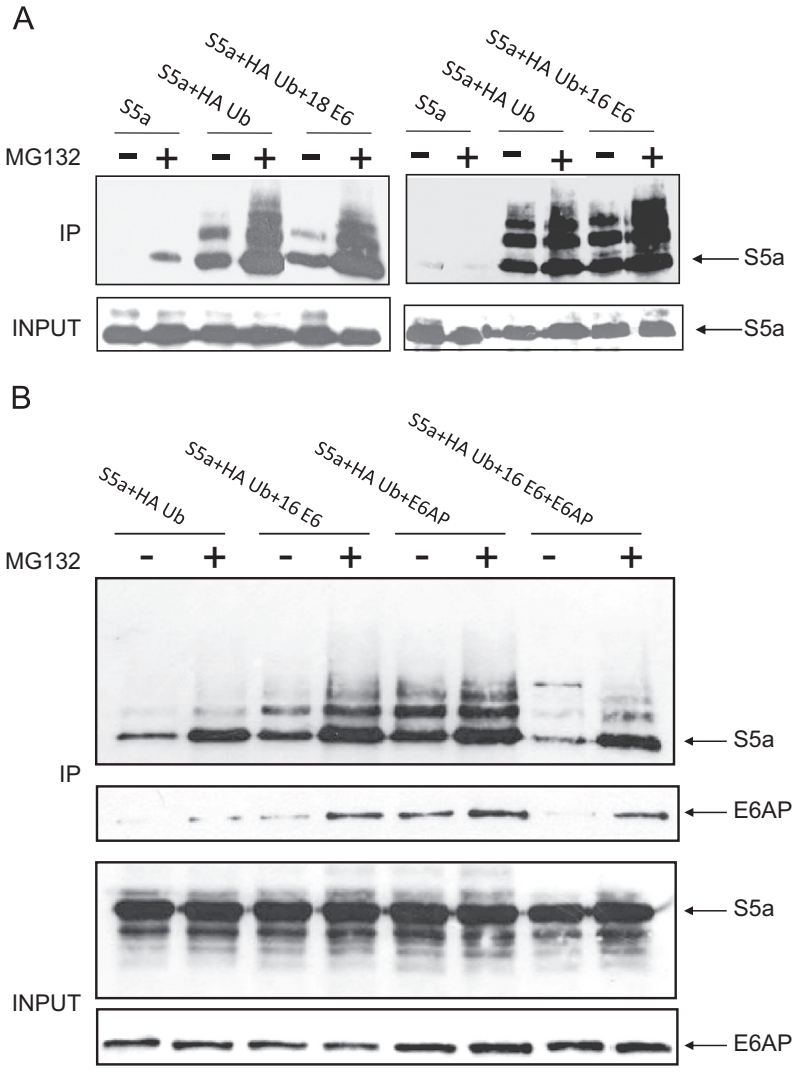

Fig. 5. HPV-16 E6 induces S5a polyubiquitination *in vivo*. Panel A. 293 cells were transfected with S5a, S5a plus HA.ubiquitin (HA-Ub) expressing plasmid together with HPV-16 or HPV-18 E6 expression plasmids as indicated. After 24 h the cells were incubated with MG132 for 3 h (+), as indicated, and then extracted and immunoprecipitated using anti-HA antibody conjugated agarose beads. Ubiquitin bound S5a was then detected by Western blotting with anti S5a antibody. The lower panel shows the input levels of S5a protein used in the immunoprecipitations. Panel B. 293 cells were transfected with S5a plus HA-Ub expression plasmids together with HPV-16 E6 and E6AP as indicated. After 24 h the cells were incubated with MG132 for 3 h (+) and ubiquitin bound S5a was detected by Western blotting with anti-S5a antibody. The co-immunoprecipitating E6AP was detected by Western blotting using anti E6AP antibody. The lower panels show the input levels of S5a and E6AP used in the immunoprecipitations.

interact directly with a number of specific proteasome subunits. Furthermore, these studies highlight the S5a subunit as a particularly intriguing novel target of the E6 oncoprotein. It is bound strongly by E6 in an E6AP-dependent manner and, in addition, appears to be subjected to increased levels of ubiquitination in the presence of E6, both *in vitro* and *in vivo*.

A number of proteomic studies have demonstrated that the HPV E6 oncoproteins interact closely with the ubiquitin proteasome machinery, with many different proteasome subunits being identified in these analyses (Tomaić et al., 2011; Rozenblatt-Rosen et al., 2012). These studies used immunoprecipitations coupled with mass spectrometry to identify E6 protein partners, and indicated a close proximity of E6 to the proteasome machinery. However these studies did not verify these associations with the proteasome, nor did they analyse the potential of individual proteasome subunits to interact with E6. To address these issues we performed an extensive series of *in vitro* interaction assays between E6 and a panel of isolated proteasome subunits. We have identified multiple subunits as potentially direct interacting partners of all the HPV E6 oncoproteins analysed, with the S4 and S8 being consistently the strongest bound subunits in these in vitro assays. Weak interactions were obtained with S2, S6a, S6b and S7, but no interaction was observed with S5a or S9 *in vitro*. Obviously we cannot exclude the possibility of indirect associations with some of these subunits, possibly via a primary interacting target such as S4 or S8. In addition, in the majority of cases these assays were performed in wheat germ expression systems, thereby excluding the E6AP ubiquitin ligase from having a role in these interactions. Finally, differences in the strengths of association between E6 and the different subunits and the fact that efficient interactions were obtained regardless of the manner in which the E6 and proteasome subunits are expressed (GSTs or *in vitro* translated) also tends to preclude a major involvement of indirect protein interactions in most cases. Interestingly, the full length HPV-18 E6 recognised a proteasome subunit profile very similar to that seen with the alternatively spliced HPV-18 E6* protein. This

suggests that the capacity of E6 to associate with these proteasome subunits is mediated by amino acid residues that reside largely within the N-terminal region of the E6 protein. In addition, both HPV-11 and HPV-16 E6 were also found capable of interacting with the isolated proteasome components, although HPV-16 E6 bound these subunits consistently more strongly than either the HPV-11 or HPV-18 E6 proteins. These results therefore confirm specific proteasome subunits as *bona fide* interacting partners of E6. It is also intriguing to note that the apparent strength of association with the proteasome subunits does reflect the degree of association with cancer development, suggesting that this series of interactions may be directly relevant to the ability of E6 to contribute towards tumour development.

As noted above the S5a subunit was absent in the *in vitro* pull down assays when E6AP was not present. This was surprising, since S5a had been detected previously in a number of the proteomic analyses, and the protein appears to play a central role in the ubiquitin proteasome pathway. However, when these interaction assays were performed in the presence of E6AP, a robust interaction was obtained with HPV-18 E6. This suggested that S5a fitted into the class of E6 substrates that are part of the E6-E6AP complex, raising the possibility that the S5a subunit might be subject to proteolytic degradation by the E6 oncoprotein. Surprisingly our in vitro assays indicate only weak levels of proteasome-mediated degradation by E6, but a marked increase in the presence of slower migrating higher molecular weight forms of the protein, indicative of increased rates of ubiquitination. This ability of E6 to stimulate ubiqutination of S5a correlates directly with its ability to interact with E6AP, with HPV-16 E6 being much more efficient at binding S5a and interacting with E6AP (Scheffner et al., 1993) when compared with HPV-18 E6. Previous studies had indeed shown that the E6AP ubiquitin ligase could interact with the S5a subunit (Uchiki et al., 2009; Martinez-Noel et al., 2012), and our studies demonstrate that overexpressed E6AP can significantly increase the levels of S5a ubiquitination *in vivo*, but without significantly affecting its susceptibility to proteasome mediated degradation. In contrast, HPV-16 E6 also increases the levels of S5a ubiquitination, which appears to be dependent upon the presence of E6AP. However, in this case these ubiquitinated forms of S5a appear significantly more susceptible to proteasome turnover. When E6 and E6AP are both co-expressed with S5a, then the ubiquitinated S5a appears highly susceptible to proteasome mediated degradation. These results indicate that whilst E6 has no major effect on the total levels of S5a protein expression, the E6-induced ubiquitinated S5a is more susceptible to proteasome-mediated degradation than the E6AP-induced ubiquitinated forms of S5a. This suggests that E6 redirects either the patterns or the levels of E6AP induced ubiquitination of the S5a subunit. Whether this is directly related to the ability of E6 to redirect many of its protein partners to the proteasome remains to be determined. However it is possible that this activity may have a broader consequence for the overall functioning of the proteasome, possibly by increasing its overall activity. In addition, whilst we can detect E6 interaction and induced polybiqutination of S5a, it is important to remember that considerable amounts of S5a are not directly associated with the proteasome, and currently we do not know which pools of S5a are being directly targeted by E6. Therefore, whether these non-proteasome bound forms of S5a are also relevant for E6 function is an open question and requires further investigation.

Taken together, these results demonstrate a complex interplay between the HPV E6 oncoproteins and the ubiquitin proteasome pathway. Further studies are now required to elucidate the biological relevance of the ability of E6 to increase ubiquitination of the S5a proteasome subunit, and to determine how this might affect the overall functioning of the proteasome pathway within the cell.

Materials and methods

Cells and transfections

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS). Transfections were done using calcium phosphate precipitations (Matlashewski et al., 1987). HEK293 cells stably ablated for E6AP expression were generated by transfecting cells with expression vectors containing targeting sequences that were scrambled (TR2) or directed against human E6AP (shRNA E6AP) that were purchased from OriGene. Cells were placed under selection for 3 weeks and after this time clones were isolated and analysed for E6AP expression levels by western blotting. One such clone stably ablated for E6AP was used in the study.

Plasmids

The HPV-11 E6, HPV-16 E6, HPV-18 E6 and p53 expression plasmids have been described previously (Gardiol et al., 1999; Pim et al., 1994; Pim et al., 1997; Thomas and Banks, 1999), as have the different proteasome subunit expression plasmids (Jin et al., 2008). The GST-fusion proteins HPV-18 E6 and HPV-18 E6* have been described previously (Pim et al., 1997; Thomas et al., 1996).

Antibodies

The following antibodies were used: anti-HA monoclonal antibody 12CA5 (Roche), anti-S5a polyclonal antibody (Proteintech) and anti-E6AP monoclonal antibody (BD, Transduction Laboratories).

Fusion protein purification and in vitro binding assays

GST-tagged fusion proteins were expressed and purified as described previously (Thomas et al., 1996). Proteins were translated *in vitro* using a Promega TNT kit and radiolabeled with [^{35}S] cysteine or [^{35}S]methionine (Perkin Elmer). Equal amounts of *in vitro* translated proteins were added to GST fusion proteins bound to glutathione agarose (Sigma) and incubated for 1 h at 4 °C. After extensive washing with phosphate-buffered saline (PBS) containing 0.25% NP-40, the bound proteins were analysed by SDS-PAGE and autoradiography.

GST pulldowns using cellular extracts were performed by incubating GST fusion proteins immobilised on glutathione agarose with cells extracted in E1A buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% NP-40, plus protease inhibitor cocktail set I [Calbiochem]) for 1 h at 4 °C on a rotating wheel. After extensive washing, the bound proteins were detected using SDS-PAGE and Western blotting. Protein detections were done as described previously (Tomaić et al., 2009).

Immunoprecipitations and western blotting

Cells were transfected with the indicated expression plasmids and after 24 h were incubated in the presence of 50 μM MG132 or DMSO control for 3 h as indicated. The cells were then harvested and extracted with E1a extraction buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% NP-40). Cell extracts were then incubated with anti-HA conjugated agarose beads (Sigma) for 2 h on a rotating wheel at 4 °C. The beads were then extensively washed, and immunoprecipitates were analysed for S5a using anti S5a antibody by SDS-PAGE and Western blotting. Protein detections were done as described previously (Tomaić et al., 2009).

In vitro degradation assays

Proteins were transcribed and translated *in vitro* in rabbit reticulocyte lysate or wheat germ extracts using the Promega TNT system according to the manufacture's instruction. The HPV-18 E6 and HPV-16 E6 were radiolabelled with [^{35}S]-cysteine (Perkin Elmer) while S5a and p53 were radiolabelled with [^{35}S]-methionine (Perkin Elmer). Degradation assays were performed as previously described (Thomas et al., 2001). Briefly, radiolabelled proteins were mixed and incubated for the indicated times at 30 °C. Volumes were adjusted using water-primed lysate. The remaining S5a or p53 proteins were analysed by SDS-PAGE and autoradiography.

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