The ATP-binding and ATPase activities of human papillomavirus type 16 E1 are significantly weakened by the absence of prolines in its ATP-binding domain

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The E1 protein of human papillomavirus (HPV) type 16 is the only known papillomavirus E1 which does not contain any proline residues in the phosphate-loop (P-loop) of its ATP-binding site. To ascertain whether this feature influences the activities of HPV-16 E1, we generated a mutant HPV-16 E1 (E1Pro) in which prolines are inserted in place of alanines in this site, making the P-loop identical to its bovine papillomavirus type 1 counterpart. Glutathione S-transferase (GST) fusion proteins (GSTE1wt and GSTE1Pro) were produced, purified and used to assay for ATP-binding ability, ATPase activity and ability to complex with the HPV-16 E2 protein. The results show that the lack of prolines in the P-loop, which is unique to HPV-16 E1, significantly weakens its ATP-binding and ATPase activities without affecting its ability to complex with the HPV-16 E2 protein.

Introduction

In spite of the existence of a large number of papillomavirus subtypes, the amino acid sequence of the two-thirds carboxyl segment of the papillomavirus E1 protein is relatively well conserved among all the known papillomaviruses. Clertant & Seif (1984) first drew attention to a sequence motif within this region of bovine papillomavirus (BPV)-1 E1 which shared significant amino acid homology with the ATP-binding sites of the large T-antigens of simian virus 40 (SV40), polyomavirus and BK virus. This site consists of a motif known as the phosphate-loop (P-loop) which is characteristically present in a group of nucleotide-binding proteins (Saraste et al., 1990). As its name suggests, the P-loop is responsible for the binding of the \( \alpha \)-phosphate of a nucleotide. The consensus sequence of this loop is GXXXXGX(T/S), where X stands for any amino acid. In the BPV-1 E1 protein, the P-loop sequence is GPPNTGKS. The presence of this motif led to the speculation that this protein might also exhibit biochemical activities similar to those of the SV40 and polyomavirus T antigens (Clertant & Seif, 1984), namely ATP-binding, ATPase activity and helicase activity. These activities were initially demonstrated in the E1 protein of BPV-1 (Sun et al., 1990; Seo et al., 1993), followed by the E1 proteins of human papillomavirus (HPV) types 6b (Hughes & Romanos, 1993) and 11 (Bream et al., 1993). To prove that the ATP-binding activity was intrinsic to BPV-1 E1, the first proline within its P-loop (GPPNTGKS, aa 433–438) was mutated to a serine (GSPNTGKS) (Sun et al., 1990). This mutant (434P-S) was defective in binding ATP and the mutant viral genome was defective for DNA replication (Seo et al., 1993), suggesting that the ATP-binding activity of E1 is necessary for its role in viral DNA replication. Besides mutations within the P-loop, mutations of certain amino acids outside the P-loop of BPV-1 E1 can also abrogate ATP-binding, ATPase and viral DNA replication activities (MacPherson et al., 1994). These amino acids are highly conserved in the E1 proteins of most papillomaviruses including that of the HPV-16. MacPherson et al. (1994) further showed that while the wild-type BPV-1 could be detected as a freely replicating plasmid in transformed C127 cells, none of the mutants defective in ATPase activity were able to replicate extrachromosomally; instead, their DNA showed a migration pattern consistent with their having integrated into the host genome. Taken together, it is clear that ATP-binding/ATPase activity of E1 is extremely important for the replication of papillomavirus DNA. Hence, conservation of essential amino acids in the nucleotide binding sites (within and

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papillomaviruses, in having no proline residues. It has instead replaced them with alanines. All other known papillomaviruses have at least one, and many have two prolines in their P-loop.

Although the substitution of proline with alanine is a conservative substitution (both are hydrophobic), the structural difference between proline and alanine is great. The side-chain of proline is unique in that it forms a ring backbone resulting in a change of the polypeptide chain to bend at this position. This bending causes a disruption of the usual organization of the polypeptide backbone resulting in a change of the polypeptide direction. Hence, the absence of prolines in the P-loop of HPV-16 E1 could, in theory, result in a structural difference at this site. This could have a significant effect on its ATP-binding and ATPase activities, which are essential for its helicase (Yang et al., 1993) and DNA replication activities (MacPherson et al., 1994). Even though the consensus sequence of the P-loop shows that, in theory, any amino acid could be placed between the two glycines, the loss or reduction of ATP-binding activity (Sun et al., 1990), ATPase activity (MacPherson et al., 1994), helicase activity (Yang et al., 1993) and DNA replication activity (Sun et al., 1990; Yang et al., 1993; MacPherson et al., 1994) of the 434P-S BPV-1 mutant proves that amino acid alterations within the P-loop can have a significant effect on E1’s activities.

Hence, we decided to analyse the HPV-16 E1 protein to ascertain whether the lack of prolines in its P-loop has any significant effect on its ATP-binding and ATPase activities and also on its ability to complex with the HPV-16 E2 protein (Hibma et al., 1995). As these activities are essential for E1’s function, any effect on them could have an influence on E1’s ability to replicate the viral DNA.

Methods

Verification of the HPV-16 E1 P-loop wild-type sequence. In order to verify that the reported HPV-16 E1 DNA sequence (Seedorf et al., 1985; Matsukura et al., 1986) was truly that of the wild-type virus and not the result of a mutation or a sequencing error, the E1 ORFs (nt 866-2814) of four HPV-16-positive clinical specimens were amplified by PCR and sequenced. The primers used for PCR were E1 Fwd (ATGGCTGATCTCCAGCTAC: nt 866-885) and E1 Rev (TCA-TAACTGCATTT); nt 2814-1996). 32P Dideoxy DNA sequencing with T7 polymerase was carried out according to the manufacturer’s instructions (Pharmacia).

Production and purification of GSTElwt and GSTElPro. The DNA sequence in the P-loop of HPV-16 E1 was mutated by PCR-directed mutagenesis to encode GPPNTGKS. The E1 DNA bearing this mutation (E1Pro) and the wild-type E1 (E1wt) were cloned in-frame with the glutathione S-transferase (GST) gene of the pGEX-2T vector (Pharmacia) at the BamHI site. E. coli TBL1 cells bearing these recombinant vectors, pGSEKE1wt and pGSEKE1Pro, were cultured in 1 M-sorbitol + 2.5 mM-betaine in LB medium and induced with 0.3 mM IPTG when the optical densities of the cultures at 600 nm were 0.8–1.2, to express GSTElwt and GSTElPro respectively. The inductions were carried out at room temperature for 16 h. Bacteria were harvested and the fusion proteins purified according to the method described by Hibma et al. (1995). Essentially, the bacterial pellets were resuspended in 20 ml of lysis buffer (50 mM-Tris–HCl, 10 mM-2-mercaptoethanol, 100 mM-NaCl, 1 mM-EDTA, 20 mM-MgCl2, 1 mM-azide and 10% glycerol), freeze-thawed and sonicated for three rounds of 30 s each. The lysates were centrifuged at 15000 r.p.m. for 20 min in an SS-34 Sorvall rotor, and the supernatant filtered through 0.2 μm filters. Glutathione-Sepharose beads pre-equilibrated with lysis buffer were added (0.5 ml/litre of bacteria) to each of the filtered supernatants and incubated with shaking at 4 °C for 1 h. The supernatants were removed and the beads washed with 7 × 50 ml of lysis buffer each. Fusion proteins were eluted from the beads with 10 mM reduced glutathione in lysis buffer; 10 μl of each sample was analysed on an 8% SDS-polyacrylamide gel and stained with Coomassie blue. An additional purification step was performed by Sphacryl S-200 gel filtration chromatography in lysis buffer. The protein fractions were collected, electrophoretically separated on an SDS–polyacrylamide gel and then silver-stained and Western blotted with a monoclonal antibody against HPV-16 E1 (Raj, 1994).

ATP-binding assay. The ATP-binding assay was performed according to the method described by Clerlant & Cuzin (1981). Essentially, oxidized [α-32P]ATP (1,3-diazoaldehyde ATP), or ox-[α-32P]ATP in short, was prepared by treating 8 μl [α-32P]ATP (80 mCi, 400 Ci/mmol) with 2 μl 24 mM-HCl and 2 μl 24 mM-NaCl at room temperature in the dark for 3 min. The reaction was terminated by the addition of 3 μl 50% glycerol and incubation for 20 min. A 2 μl volume of this ox-ATP was added to the proteins (GST, GSTElwt and GSTElPro) in binding buffer (0.5 mM-KCl, 5 mM-MgCl2, 1 mM-DTT and 1 mM-EGTA) in a final volume of 20 μl. After 30 min incubation at room temperature, 2 μl of NaCNBH3 was added and the mixture was kept on ice overnight. The proteins were boiled in SDS loading buffer and electrophoretically separated through an 8% SDS–polyacrylamide gel, which was then dried and subjected to autoradiography.

ATPase assay. The Sphacryl S-200-purified proteins were subjected to ATPase assay according to the method previously described (Hughes & Romanson, 1993). Essentially, the proteins (GST, GSTElwt and GSTElPro) were incubated with [γ-32P]ATP for 30 min at room temperature in buffer containing 50 mM-Tris–HCl (pH 7.8), 0.1 M-NaCl, 1 mM-DTT and 2% glycerol. A 3 μl volume out of 20 μl for each reaction was spotted onto polyethyleneimine cellulose plates for thin-layer chromatography in 0.75 M-Na2PO4, air-dried and subjected to autoradiography.

Interaction with HPV-16 E2 protein. E2-binding assays were performed as described previously (Hibma et al., 1995). Essentially, the proteins (GST, GSTElwt and GSTElPro) were immobilized on glutathione-Sepharose resin were incubated with bacterially expressed and purified native HPV-16 E2 protein (gift from M. Hibma, University of Otago, New Zealand) in binding buffer (20 mM-Tris–HCl pH 8, 70 mM-KCl, 2 mM-MgCl2, 20 μM-zinc acetate, 0.2 mM-DTT, 6% glycerol, 0.1% Tween 20, 2% BSA, 2 μg calf thymus DNA) for 1 h at 4 °C. The resin was washed extensively with binding buffer and boiled in SDS loading buffer prior to electrophoresis through a 10% SDS–polyacrylamide gel. Western blot analysis was performed with monoclonal antibodies against HPV-16 E2 protein (a kind gift from M. Hibma).
Results

The DNA sequences of the HPV-16 E1 P-loop from four HPV-16-positive clinical samples were found to be identical to the reported sequence, and they all encoded GAANTGKS at their P-loops, confirming that wild-type HPV-16 E1 lacks proline residues in this site (Fig. 1a). DNA sequencing of the E1Pro generated by site-specific mutation revealed that this site was successfully mutated \textit{in vitro} to encode GPPNTGK (Fig. 1b).

GSTE1wt and GSTE1Pro fusion proteins isolated from their respective bacterial lysates with glutathione-Sepharose appeared reasonably pure on a Coomassie-stained SDS-polyacrylamide gel (Fig. 2a), except for some low molecular mass proteins which were probably truncated forms of the fusion proteins. However, silver staining (Fig. 2b, lane 2) revealed that the fusion protein extracts were very heterogeneous. Subjecting these fusion protein extracts to Sephacryl S-200 gel filtration chromatography successfully purified GSTE1wt and GSTE1Pro to homogeneity (Fig. 2b, lane 3, and Fig. 2c). The homogeneity and purity of the fusion proteins were confirmed by Western blot analysis (Fig. 2d) with monoclonal antibody against HPV-16 E1.

Fig. 3(a) shows that purified GSTE1wt bound ox-[\(\alpha^{32}\text{P}\)]ATP weakly (weak band), whereas purified

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\caption{(a) DNA sequence of an HPV-16-positive clinical sample. The P-loop of HPV-16 E1 contains the amino acid sequence GAANTG. (b) DNA sequence of the HPV-16 E1Pro mutant (generated \textit{in vitro}) whose P-loop contains the amino acid sequence GPPNTG.}
\end{figure}
Fig. 2. (a) Coomassie-blue-stained 8% SDS-polyacrylamide gel showing GSTE1Pro and GSTE1wt isolated from bacterial lysate with glutathione–Sepharose beads. (b) Silver-stained 8% SDS-polyacrylamide gel. Lane 1, total lysate of pGEXE1Pro; lane 2, GSTE1Pro purified in a single step with glutathione–Sepharose; lane 3, GSTE1Pro purified in two steps – glutathione–Sepharose followed by Sephacryl S-200 gel filtration chromatography (position indicated by the large arrow); lane 4, GST purified (indicated by small arrow) according to the protocol described for lane 3. (c) Silver-stained 8% SDS-polyacrylamide gel. Lanes 1–3, GSTE1wt purified by glutathione–Sepharose followed by Sephacryl S-200 gel filtration chromatography; lane 4, empty; lane 5, GST purified according to the protocol described for lanes 1–3. In (a)–(c), lanes M contain molecular mass markers. (d) Western blot analysis using monoclonal
GSTE1Pro bound ox-[α-32P]ATP extremely efficiently (strong band). Similar results were obtained with partially purified (prior to Sephacryl S-200 chromatography) GSTE1 and GSTE1Pro (Fig. 3b). Approximately equal amounts of unidentified background bands in both the samples show that the effect was not due to differential loading of protein samples in the wells but to the different ATP-binding capacity of the two proteins.

Even though GSTE1wt was able to hydrolyse [γ-32P]ATP, resulting in the release of the radiolabelled γ-phosphate, its activity was much weaker than that of GSTE1Pro (Fig. 4, lanes 3, 4 and 5, 6): 1.2 μg GSTE1Pro hydrolysed more ATP than 2 μg GSTE1wt, indicating that the ATPase activity (like the ATP-binding activity) of wild-type HPV-16 E1 was significantly weakened by the absence of proline in the P-loop in comparison to the proline-containing E1 mutant.

Western blot analysis with monoclonal antibody against HPV-16 E2 protein detected equal amounts of E2 bound to GSTE1wt and GSTE1Pro but not GST (Fig. 5), demonstrating that the absence or presence of proline in the ATP-binding site of HPV-16 E1 does not affect the ability of E1 to complex with E2.

**Discussion**

The results presented here show that the lack of proline in the P-loop of the ATP-binding site of HPV-16 E1 is not due to analysis of a mutant virus or to a sequencing error, but represents the true sequence of wild-type HPV-16 E1, as revealed by DNA sequences from four HPV-16-positive clinical samples. This feature is indeed unique to HPV-16 as all other papillomaviruses sequenced thus far possess at least one proline residue in their P-loop. A
Fig. 4. ATPase assay of Sephacryl S-200-purified GST, GSTE1wt and GSTE1Pro. [γ-32P]ATP was incubated with these proteins (quantity stated below) for 30 min at room temperature prior to chromatography on cellulose polyethyleneimine and autoradiography. Free 32P migrates ahead of [γ-32P]ATP (both are marked). Lanes 1 and 2, 2000 ng GST; lanes 3 and 4, 2000 ng GSTE1wt; lanes 5 and 6, 2000 ng GSTE1Pro; lanes 7 and 8, 1200 ng GSTE1Pro; lane 9, [γ-32P]ATP as negative control.

Fig. 5. HPV-16 E2 binding assay. Western blot analysis using a monoclonal antibody (TVG261) to HPV-16 E2 to detect E2 proteins that bound to the following proteins: lane 1, GST; lanes 2 and 3, GSTE1wt; lanes 4 and 5, GSTE1Pro.

closer look at the other papillomaviruses revealed that all animal and cutaneous human papillomaviruses possess two prolines in their P-loop (except for HPV-41 which possesses only one proline and a serine in place of the second proline). Most low-risk mucosal papillomaviruses also possess two prolines in their P-loop while a small number possess only one proline. On the other hand, most high-risk mucosal papillomaviruses (HPV types 18, 31, 35 and 33) possess only one proline and the most prevalent high-risk mucosal papillomavirus, HPV-16, does not possess any prolines at all in its P-loop. The possible significance of this observation is discussed below.

To study the effect(s) of this difference on the activity of HPV-16 E1, a mutant HPV-16 E1 was generated whereby the two alanines within the P-loop were substituted with prolines, making it similar to BPV-1 and the cutaneous HPVs. GST fusion proteins of wild-type HPV-16 E1 (E1wt) and mutant HPV-16 E1 (E1Pro) were expressed in bacteria, purified to homogeneity and used to assay for E1 activities. This purification of the GST fusion proteins, as confirmed by silver staining, was crucial because the risk of contamination with bacterial ATPases, especially heat shock proteins, was considerable. Western blot analysis confirmed that the GSTE1wt and GSTE1Pro protein preparations indeed consisted of a homogeneous species as opposed to multiple species before Sephacryl S-200 chromatography. The additional species of proteins were probably a mixture of a small proportion of bacterial proteins and a major proportion
of truncated GSTE1wt and GSTE1Pro whose presence was detected by an E1 monoclonal antibody on a Western blot.

Assay of the purified fusion proteins for ATP-binding activity clearly showed that GSTE1Pro bound ATP more efficiently than GSTE1wt. More importantly, the approximately equal amounts of unidentified background bands in the lanes of the partially purified fusion proteins subjected to this assay further proved that the difference in the signal between the two fusion proteins was not due to a quantitative difference in loading of the two reaction mixes but to a difference in the intrinsic ability of these two proteins to bind ATP. When MBPE1C (maltose-binding protein fused to the last 507 aa of the carboxyl end of the E1 protein) and MBPE1CPro (MBPE1C with two prolines in place of alanines in its P-loop) were assayed for ATP-binding activity (data not shown), MBPE1CPro exhibited greater ability to bind ATP than MBPE1C. This indirectly suggests that the enhancement of the ATP-binding activities of GSTE1Pro and MBPE1CPro was due to changes in the P-loop of E1, as opposed to changes in the general conformation of the two fusion proteins brought about by the substitution of alanines with prolines.

This difference had a significant effect on the ATPase activity, as GSTE1wt was able to exhibit only approximately half the activity of GSTE1Pro. Interestingly, the ATPase activity of GSTE1wt and GSTE1Pro was independent of DNA. This is in contrast to a report by Seo et al. (1993) but in accordance to reports by Bream et al. (1993), Hughes & Romanos (1993), MacPherson et al. (1994) and Santucci et al. (1995). It is not unlikely that papillomavirus E1 is able to hydrolyse ATP in the absence of DNA as this has also been shown for SV40 large T antigen (Tjian & Robbins, 1979). Nevertheless, the results presented here clearly show that highly purified GSTE1wt was able to hydrolyse ATP, but with a reduced activity corresponding, at most, to half that of GSTE1Pro. However, it is not certain at the moment if the change was due to an increase in E1Pro's affinity for ATP or to an increase in E1Pro's catalytic activity.

The ability of HPV-16 E1 to complex with HPV-16 E2 was not affected at all by its ability to bind and hydrolyse ATP. These two sets of activities are clearly independent of each other. This is again not unexpected as recent reports have mapped the E2-interaction site to the N-terminal region of the E1 protein (Hibma et al., 1994; Story et al., 1995), which is physically distant from the P-loop that lies towards the carboxyl end of E1. Nevertheless, this is in conflict with a previous report which showed that a BPV-1 E1 ATPase mutant, 434P-S, was also defective in complexing with the E2 protein (Lusky & Fontane, 1991). However, Benson & Howley (1995) have recently shown that the E2 complexing domain of BPV-1 E1 can function independently of other regions of the E1 protein, indicating that the ATP-binding and ATPase activities are not needed for E1 to complex with E2, hence supporting our observation.

Since helicase and DNA replication activities of E1 have been shown to be dependent on its ATPase activity, it is very likely that weakening of the ATPase activity would have an effect on the former activities (Sun et al., 1990; Seo et al., 1993; Yang et al., 1993; MacPherson et al., 1994).

Since the ability of HPV-16 E1wt to complex with E2 is not affected by the lack of prolines in the P-loop, it can be deduced that the HPV-16 E1wt protein can be brought by E2 to the viral origin of replication efficiently. Hence, the expected effect on DNA replication would be due to biochemical events occurring after the docking of the E1–E2 complex to the viral DNA, namely, the weakened ATP-binding and ATPase activities.

At the moment, it is not possible to know for certain why HPV-16 evolved to possess a suboptimal E1 activity. However, considering the fact that for reasons yet unknown, transfection of cells with replication-defective BPV-1 mutants (including ATP-binding and ATPase defective mutants) results in integration of the mutant BPV-1 genomes (Lusky & Botchan, 1985; Lamb & Howley, 1988; MacPherson et al., 1994), it is possible that the reduced activity of HPV-16 E1 might account for the higher rate of integration of HPV-16 DNA into the cellular genome compared to the wild-type (replication competent) BPV-1 DNA which rarely integrates (Law et al., 1981). Integrated HPV DNA is often detected in a high proportion of cervical cancers and immortalized keratinocyte cell lines. In this light, it is interesting to note that most of the low-risk mucosal human papillomaviruses have two prolines, whereas the more frequently encountered high-risk mucosal papillomaviruses have only a single proline in their P-loops, and HPV-16, the most prevalent HPV subtype found in cervical lesions has no prolines in its P-loop. Nevertheless, it would appear to be a disadvantage for the virus if the lack of prolines in its P-loop indirectly results in the integration of its genome as this event is likely to interrupt the normal viral life cycle.

Alternatively, the weakening of the E1 activity could be the outcome of natural selection exerted by the HPV-16 host cell, the human cervical keratinocyte. For reasons not yet known to us, it might be an advantage for HPV-16 to have a weak E1 activity in order to survive in these cells. This is particularly interesting, as recent observations have shown that the E1 ORF of HPV-16 possesses three more splice donors in addition to the two known donor sites shared by other known papillomavirus E1s, giving HPV-16 E1 a total of at least five splice donors (W. Zhang, K. Raj & M. A. Stanley, unpublished data).
This reflects additional restrictions on the activity of El, but at the transcriptional level. Hence, the activity of HPV-16 E1 in the cervical keratinocyte is modulated not only at the transcriptional level but also at the protein activity level. The presence of abundant splice donors caused great difficulty in attempts to express Elwt and ElPro in transient replication experiments and hence did not permit study of the effect(s) of alanine-proline substitution on HPV-16 DNA replication (Raj, 1994).

The existence of such rigorous measures by the virus to control the expression of E1 must surely be significant to the virus life-cycle. However, at the moment it is not possible to know for certain what the significance is. Nevertheless, recent reports have shown that virus-cell type specificity is not conferred by specialized HPV type-specific receptors (Müller et al., 1995; Volpers et al., 1995). It is therefore possible that HPV DNA replication (and hence E1 activity) might be an important contributing factor which confers such specificity to HPV-16 for its host, the human cervical keratinocyte. Much more must be known about the life-cycle of HPVs, especially HPV-16, before a definitive answer can be found as to why the HPV-16 E1 possesses suboptimal ATP-binding and ATPase activities.

References


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