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E6 protein of human papillomavirus 16 (HPV16) expressed in *Escherichia coli* sans a stretch of hydrophobic amino acids, enables purification of GST-ΔE6 in the soluble form and retains the binding ability to p53

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ABSTRACT

Recombinant E6 expressed in *Escherichia coli* is known to form recalcitrant inclusion bodies even when fused to the soluble GST protein. This study describes the modification of the HPV genotype-16 oncogenic protein E6 in order to obtain it in the soluble form. The modified protein (ΔE6) was expressed in *E. coli* BL21 as an N-terminal fusion with GST (GST-ΔE6). ΔE6 was constructed by deleting the nucleotide sequences coding for IHDIIL (31–36 a.a), one of the highly hydrophobic peptide stretches, using splicing by overextension polymerase chain reaction (SOE-PCR). The removal of IHDIIL residues rendered the GST-ΔE6 soluble and amenable for purification involving a two step process a preliminary glutathione-GST affinity chromatography followed by gel-filtration chromatography. Evaluation of purified protein fractions by HPLC suggests that GST-ΔE6 exists as a monomer. Further, the ΔE6 in GST-ΔE6 seemed to retain the binding ability to p53 as determined by the glutathione-GST capture ELISA. Purified GST-ΔE6 we reckon, might find use as an essential reagent in immunological assays, in sero-epidemiological studies, and also in studies to delineate the structure and function of HPV16 E6.

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Introduction

HPV infection of the ano-genital tract is the most common sexually transmitted disease in the world [1]. A fall-out of recurrent and persistent infection of high-risk HPVs is cervical carcinoma, the second largest cause of cancer related mortality in women [2]. More than 50% of cervical cancers are attributed to infections by the high-risk HPV of genotype 16 . The role of viral oncoproteins E6 in the degradation of the cell cycle regulatory protein p53 and the E7 interaction with the tumor suppressor protein pRb are critical in the lead up to carcinoma [3–5]. E7 and E6 have also been ascribed several other functions in the host epithelial cells [6–8]. Detailed knowledge of these functions is of prime importance in designing disease management strategies [9–11]. Viral oncogenes E6 and E7 are integrated into the host cell genome of most cancer cells and are expressed constitutively [12]. Logically, hence E6 and E7 are reasoned as ideal cancer specific antigens in the development of therapeutic vaccines [9].

The relative ease in heterologous expression and purification of the E7 has lead to a greater understanding of its physiological roles in the host cells [12,13]. Although the knowledge of the functional roles of 1HPV16 E6 in the host cells has increased over-time [14] the progress has been hindered by the difficulty in obtaining recombinant E6 in the soluble form. Recombinant E6 in heterologous hosts (*Escherichia coli*) is found to fractionate into recalcitrant inclusion bodies non-conducive for further purification [15,16].We sought to obtain purified recombinant E6 in the soluble form, primarily for use in in epidemiological studies based on serology, and in future for the determination of E6 specific immune response in vaccine studies .

Attempts to obtain HPV16 E6 in soluble form despite resorting to the expression of the protein in fusion with soluble tags such as maltose-binding protein (MBP) or glutathione-S-transferase (GST) have only seen modest success [16]. This manuscript details the modification of E6 gene (ΔE6) and its relevance on the solubility of GST-ΔE6 *vis a vis* GST-E6 protein. E6 and MBP fusion protein (MBP-E6) expressed in *E. coli* although found soluble is essentially in a multimeric aggregate hindering its use in structure and functional studies. Therefore, we sought to determine the oligomeric

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[1] *Abbreviations used:* HPV16, human papillomavirus 16; MBP, maltose-binding protein; GST, glutathione-S-transferase; HPLC, high performance liquid chromatography; GnHCl, guanidine hydrochloride; SDS, sodium dodecyl sulphate; SOE-PCR, splicing by overextension polymerase chain reaction; GRAVY, grand average of hydropathicity.

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state of GST-ΔE6 using a simple HPLC approach. Further, a preliminary study in the ELISA format was employed to enquire the binding capability of ΔE6 in the purified GST-ΔE6 to the recombinant p53 protein.

Materials and methods

Genes, bacterial hosts and cloning

The HPV16 E6 full-length gene codon optimized for expression in *E. coli* was obtained as a synthetic construct in a plasmid from GeneArt, Germany. The modified E6 *i.e.*, ΔE6 was generated in the laboratory by SOE-PCR using the full-length E6 gene as the template. Cloning was performed in the cloning host strain, *E. coli* Top 10 cells (Life Technologies™, USA). E6 and ΔE6 gene were subcloned into pGEX4T1 plasmid vector, obtained from GE Healthcare, USA, between *Eco* RI and *Not* I sites. Vector and gene ligations were performed with the Rapid Ligation Kit™ from Roche, USA.

Determination of the hydrophobic regions of E6

The hydrophobicity profile of the linear polypeptide sequence of E6 was analyzed using the ProtScale tool (http://web.expasy.org/protscale/) based on the Kyte and Doolittle hydrophobic scale. The ProtParam tool (http://web.expasy.org/protparam/) was used to determine the hydrophobicity indices as the grand average of hydropathicity (*GRAVY*) values of hydrophobic peak regions.

Construction of HPV16 ΔE6 and cloning

The HPV16 ΔE6 gene was constructed by deleting the nucleotides coding for the P1 region (IHDIIL; amino acid 31–36) from the HPV16 E6 gene using the splicing by overlap extension PCR (SOE-PCR) [14,17]. Briefly, the portion of E6 gene upstream of the DNA coding for the 6 amino acid P1 region and the region downstream were amplified in two separate PCRs. The upstream region was amplified with the primer pair For 1 and Rev 1, and the downstream region with For 2 and Rev 2 respectively. The 3′ region of Rev 1 primer contained complementary sequences (28 bp) to the 5′ of For 2. This ensured overlapping DNA sequences between the 3′ region of the upstream product and the 5′ region of the downstream product. The two regions were spliced together in a second PCR using For 1 and Rev 2 primers and products of the previous PCR, the upstream (fragment 1) and downstream (fragment 2) as templates. The primers used in the PCRs are listed in Table 1. All PCRs were carried out using Hot Star Taq DNA polymerase Kit (Qiagen) according to the manufacturer's instruction. The reactions were performed with the following conditions *viz.*, initial denaturation at 94 °C for 5 min followed by 35 cycles of 60 s each of denaturation (94 °C), annealing (60 °C) and extension (72 °C). The final extension was carried out at 72 °C for 10 min. The amplified DNA of the SOE-PCR was then cloned into pGEX4T1 at *Eco* RI and *Not* I sites and sequence verified as mentioned previously.

Expression and purification of HPV16 E6 and HPV16 ΔE6

E. coli BL21 strain cells were transformed with either pGEX-4T1/HPV16 E6 or pGEX-4T1/HPV16 ΔE6. Recombinant *E. coli* clones were grown in LB broth containing ampicillin (Sigma–Aldrich) (100 μg/ml). Bacteria were induced for the expression of the genes cloned in pGEX4T1 with 1 mM IPTG (Genie, Bangalore) at an OD_{600} of 0.6. After an induction period of 4 h at 22 °C the cell mass were harvested, washed and resuspended in Potassium phosphate buffer (pH 7.2) supplemented with 0.2 M NaCl, 5 mM DTT and 2 mM EDTA. The cells were lysed by sonication and the insoluble fraction along with cell debris was removed by centrifugation at 20,000*g* for 30 min. The supernatant was further subjected to affinity chromatography with the Glutathione Sepharose 4B (GE, USA) according to the manufacturer's instructions for obtaining purified E6 or ΔE6 proteins. Fractions showing the GST-E6 protein were pooled from glutathione Sepharose affinity chromatography step and further purified using pre-packed and calibrated Sephacryl S400 16/60 size exclusion column (GE, USA). Analyses of expression and purification were performed by the immunoblotting procedure on a PVDF membrane. Blotted membrane was probed with goat anti-GST polyclonal antibody (1:10,000) from GE healthcare, USA. Concentration of the purified protein was determined based on BCA method and/or absorbance (A_{280}) at 280 nm using spectrophotometer.

Table 1
List of Primers

For 1-	ATGCGAATTCATGCATCAGAAACGTACCGCCATGTTTCAGG
For 2-	TGCAGACCACCGAATGCGTGTATTGTAAACAACAGCTGTTACG
Rev 1-	TTTACAATACACGCATTCGGTGGTCTGCAGTTCGGTACACAGCTGCGG
Rev 2-	ATGCCTCGAGCAGCTGGGTTTCACGACGGGTACGGCTGC

Determination of the oligomeric state of purified GST-ΔE6

The purity and the state of GST-ΔE6, whether in monomeric or multimeric form was evaluated by subjecting the purified protein to the high performance liquid chromatography (HPLC). A pre-calibrated Yarra SEC-3000 column (Phenomenox Inc., CA) was used in the procedure.

Determination of binding of modified E6 to p53 protein

Maxisorp 96 well ELISA plate (Nunc, Denmark) was coated with 200 ng/well/100 μl of casein-glutathione in 0.5 M carbonate buffer, pH9.6 and was incubated overnight at 4 °C. The plate was blocked with 2% casein in phosphate buffer saline (Sigma–Aldrich). After washing with PBS, the wells were incubated with 200 ng/well of GST-ΔE6 at 37 °C for 1 h. Following which the wells were washed with PBS containing 0.05% Tween-20 (PBST) and recombinant p53 (Sigma–Aldrich, USA) in PBST was added along the rows of wells at a starting concentration of 1200 ng/well until the final concentration of 0.58 ng/well by two-fold serial dilution. After incubation at 37 °C for 1 h, wells were washed using PBST. The p53 bound to HPV16 ΔE6 was detected by anti-p53 monoclonal antibody (Sigma–Aldrich, USA; 1:10,000 dilutions in PBST). Further, the wells were probed with anti-mouse antibodies conjugated with HRPO (Sigma–Aldrich, USA; 1:25,000 dilutions) and developed with 3,3′,5,5′-Tetramethylbenzidine (Sigma–Aldrich, USA) and hydrogen peroxide. The reaction was stopped by adding 1.25N H_2SO_4 and the intensity of colour was measured at 450 nm in an ELISA reader (Beckman coulter DTX 880 multimode detector USA). Adequate control, *viz.*, wells treated with GST alone (antigen control) was incorporated in the assay to delineate E6 specific versus non-E6 specific p53 binding activity. The GST-ΔE6 binding to casein-glutathione conjugate was detected by probing with anti-p53 monoclonal antibody (Sigma–Aldrich, USA).

Results

Cloning of E6

The HPV16 E6 synthetic construct was obtained in a plasmid where the DNA was cloned at *Eco* RI and *Not* I sites. The E6 DNA was therefore sub-cloned between the corresponding sites in the expression vector pGEX-4T1. The pGEX4T1 clones were

subsequently verified for appropriate orientation and error-free sequence by Sanger sequencing.

Modification of HPV16 E6 to HPV16 ΔE6

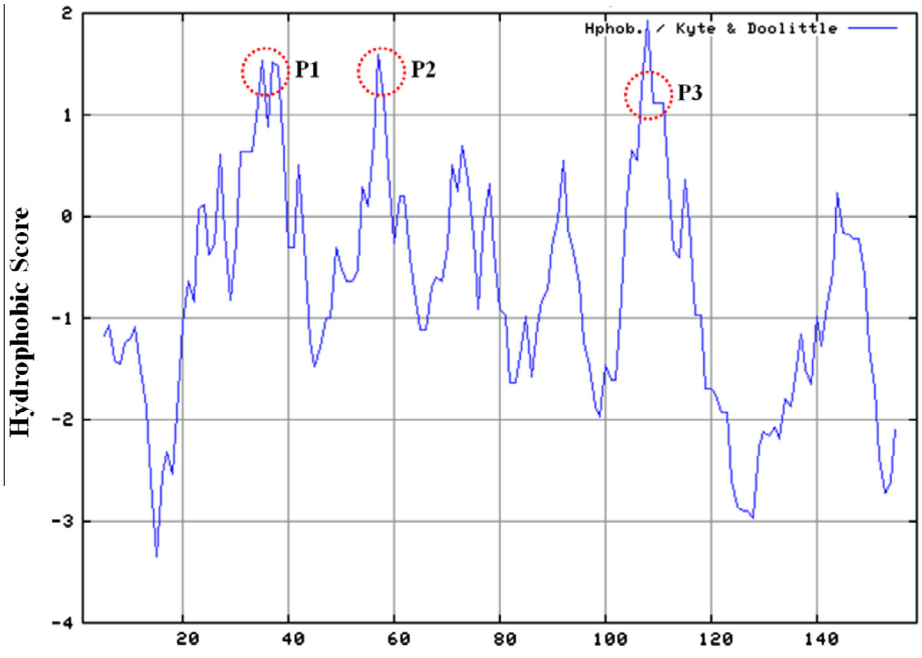
The (http://web.expasy.org/protscale/) ProtScale analysis of theHPV16 E6 protein indicated three peaks above the hydrophobic scale of 1.0 (Fig. 1). The P1 region consisted of the amino acids 31–36 (IHDIIL); the P2 region 58–62 (LCIVY) and the P3 consisted of residues 104–108 (LCDLLI). The GRAVY hyrdropathicity indices of the P1, P2 and P3 determined by the ProtParam analysis (http://web.expasy.org/protparam/) were 1.77, 2.74 and 2.48 respectively.

The nucleotide sequences corresponding to the amino acid residues 31–36 (IHDIIL) of the P1 region were excised from the E6 gene by SOE-PCR in a two step process as illustrated in Fig. 2. The first step PCRs produced amplified products of the size 90 bp and 369 bp (Fig. 2B and C) corresponding to the upstream and downstream regions respectively of the nucleotide sequence coding for amino acids IHDIIL in two separate reactions.

ΔE6 was assembled in the second step SOE-PCR as an amplified a product of size 456 bp (Fig. 2D). The SOE-PCR essentially spliced the 90 bp and 369 bp amplified fragments of the first step PCRs that were used as templates (Fig. 2A). Sequence verification of the pGEX4T1-clone revealed that ΔE6 was identical in its sequence to full-length E6 except for the deletion of nucleotides, 5′–91 to 108–3′ corresponding to IHDIIL.

Expression and purification of proteins

The IPTG induced expression of the modified, GST-ΔE6 was comparable to the full-length HPV16 E6 in fusion with the N-terminal GST (GST-E6) as seen in the coomassie brilliant blue stained SDS–PAGE (Fig. 3A). Although, the coomassie brilliant blue stained gel picture did not suggest a large increase of GST-ΔE6 in the supernatant fraction of the bacterial lysate centrifuged at 20,000*g* *vis a vis* GST-E6 (Fig. 3A) the immunoblot of the same fractions probed with anti-GST polyclonal antibodies indicated appreciable presence of soluble GST-ΔE6 in the supernatant. GST-E6 however was not detected in the supernatant fraction of the corresponding recombinant bacterial lysate (Fig. 3B). Thus, as expected, Glutathione-Sepharose 4B affinity chromatography of the supernatant fraction of HPV16 E6 recombinant bacterial lysate did not yield purified GST-E6 (data not shown). However, the supernatant fraction of HPV16 ΔE6 recombinant *E. coli* BL21 preparation yielded purified GST-ΔE6 that was soluble in PBS (pH 7.2) (Fig. 4). The results were further confirmed by western blotting probed with anti-GST antibodies in which a band of size (~46 KDa) expected for GST-ΔE6 was detected (Fig. 4B). Further,



A

P1; GRAVY=1.77 P2; GRAVY=2.74

MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYA
VCDKCLKFYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIR
GRWTGRCMSCCRSSRTRRETQL

P3; GRAVY=2.48

B

Fig. 1. (A) ProtScale (www.expasy.org) profile of the HPV E6 protein. P1, P2 and P3 correspond to the hydrophobic peak regions based on the Kyte and Doolittle scale. (B) The amino acid sequence of the HPV16 E6 gene. Highlighted in red are the amino acids that constitute the hydrophobic peak regions P1, P2 and P3. The hydropathic values expressed as grand average of hydropathicity (GRAVY) of the respective P region amino acids were analyzed using the ProtParam tool (www.expasy.org). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

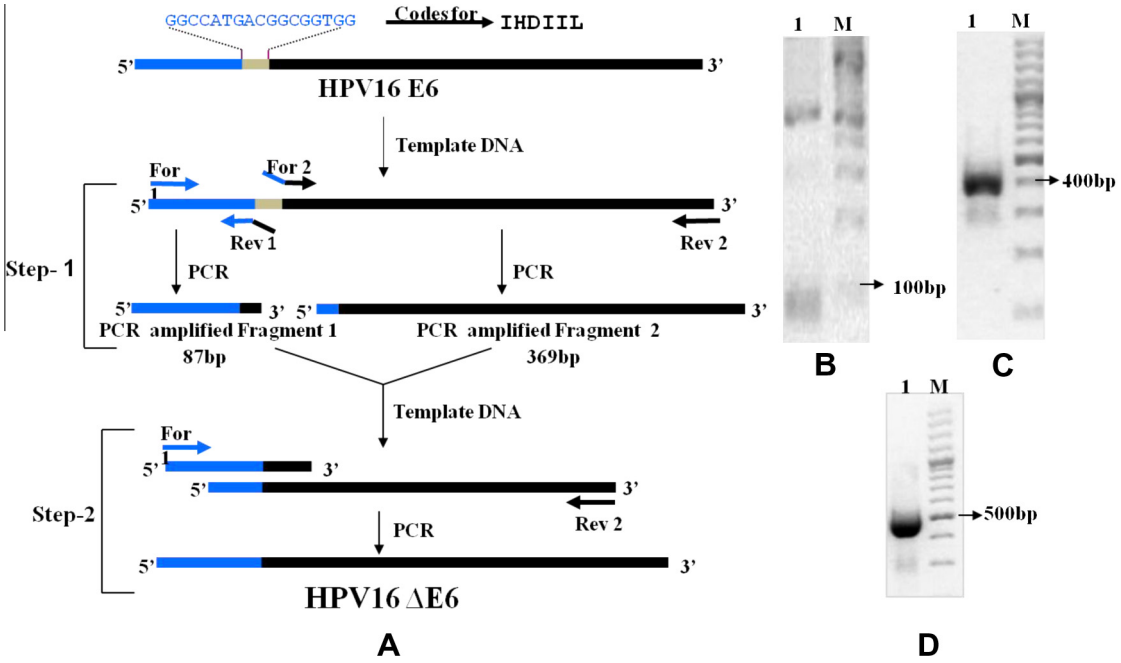


Fig. 2. (A) Schematic illustration of the deletion of hydrophobic core of HPV16 E6 using PCRs and SOE-PCR. (B) The agarose gel picture of the amplified fragment-1 (87 bp) from the PCR in step 1 of Fig. A. (C) The agarose gel picture of the fragment-2 (369 bp) from a separate PCR in step 1. (D) The agarose gel picture of the assembled and amplified ΔE6 product by SOE-PCR in step-2 of Fig. A.

purification using the Sephacryl S400 gel filtration column yielded two prominent protein peaks (Fig. 5A). The chromatographic fractions (68–71) constituted a more homogenous preparation of GST-ΔE6 as indicated by the SDS–PAGE profile (Fig. 5B). The two-step chromatographic purification yielded GST-ΔE6 of 0.1 mg/ml as determined by BCA method and/or absorbance at 280 nm.

HPLC of GST-ΔE6 from the gel filtration chromatography

The HPLC chromatogram (Fig. 6) shows a sharp peak at retention time ~18.2 min for GST-ΔE6 (~46.0 kDa) sample from the gel-filtration chromatography. The BSA standard (~66.5 kDa) analyzed under identical conditions showed a retention time of 12 min. An additional peak at retention time 25.1 min was seen for the free GST in the analyzed samples.

HPV16 ΔE6 binding to p53

Glutathione capture ELISA was used to probe the binding activity of HPV16 ΔE6 to recombinant p53 protein. The ELISA reactivity showed concentration dependent binding with p53. The control lane in which purified GST was used in place of GST-ΔE6 as binding antigen to the casein glutathione coated wells did not show the development of color. The results indicate that p53 binding in the GST-ΔE6 bound well was specific to ΔE6 (Fig. 7).

Discussion

Soluble E6 is an essential reagent in serology and immunological assays. The glutathione-GST capture ELISA is a simple yet specific assay for serological studies to delineate HPV infection [18]. The crucial specificity is imparted by GST in the GST-HPV fusion protein used as the antigen reagent in the assay. The capture ELISA employs coating of the microtitre plate wells with casein-glutathione on to which the HPV proteins in fusion with GST are later bound. The format of the assay (glutathione-GST specific binding) allows the use of even heterogeneous protein preparations; therefore saving extensive downstream process [18].This concept has been also adopted in the high-throughput Luminex assays using glutathione tagged beads [19]. However the crux of the glutathione-GST based assay lay in the GST fusion proteins being soluble. Our primary aim therefore was to obtain the GST-E6 in the soluble form to enable sero-prevalence studies of E6 antibodies in adult women.

E6 in the form of a linear polypeptide is soluble, as determined in the online ProtParam tool (GRAVY index of −0.776). However, recombinant expression of E6 in *E. coli* results in highly recalcitrant inclusion body proteins. An often encountered problem in the overexpression of proteins in *E. coli* is the rapid misfolding of proteins [20]. We therefore hypothesized that the rapidly folding polypeptide of E6 renders exposure of strong hydrophobic amino acid residues on the surface and hence fractionation into inclusion bodies. Therefore, we sought to enquire whether exclusion of a peptide stretch that is predominantly hydrophobic might render the protein soluble. Deletion of some of the hydrophobic residues, we assumed, might impede rapid coiling of the nascent polypeptide and hence render the protein soluble.

As a first step, the protein sequence of E6 was subjected to ProtScale analysis based on the Kyte and Doolittle scale [21] to determine the amino acid specific hydrophobic plot of the linear polypeptide. The analysis showed three prominent hydrophobic peaks P1, P2 and P3 (Fig. 1). The sequence of amino acids residues corresponding to the peaks on analysis with the ProtParam tool showed higher GRAVY indices for P2 and P3 regions than for P1 (Fig. 1). LCIVY stretch that constitute P2 region is a part of a well characterized T cell epitope [7] and hence is an important component of the protein when used in assays for the determination of immune response. The P3 region (a.a residues 104–109) lay in the C-terminal domain of E6 which is reported to play pivotal roles in the PDZ binding domain of the protein [22,23]. The PDZ domain binding property of E6 is implicated in various functions and interactions with the host cell proteins [23]. Therefore, deletion of the amino acid residues of the P1 region, we reasoned, was the most benign option in terms of protein structure, conformation and assay applications. Besides, recent publications suggest that E6

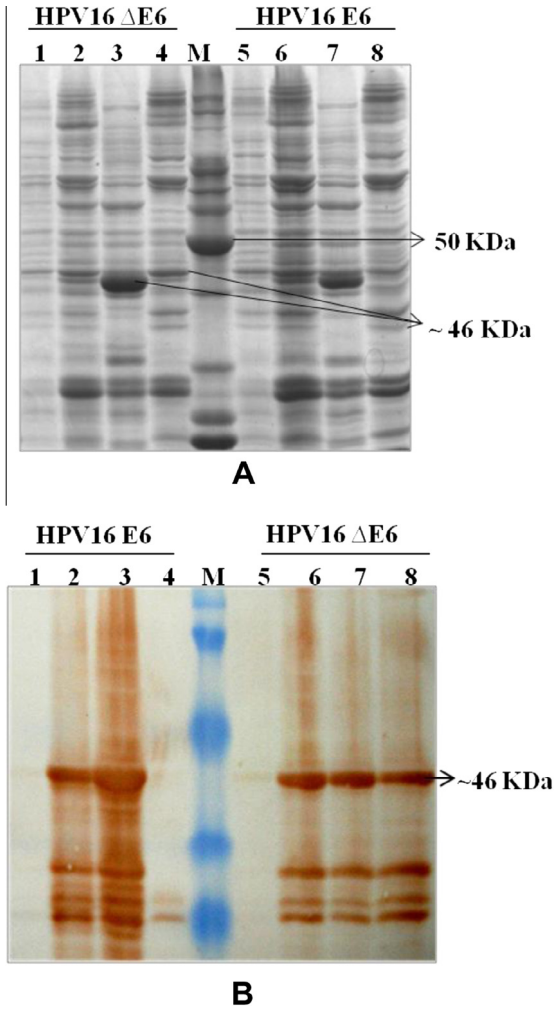


Fig. 3. Expression and solubility of HPV16 Δ E6 and HPV16 E6. (A) SDS–PAGE showing the expression and solubility profile of E6 and ΔE6. Lane 1 – Un-induced ΔE6 recombinant *E. coli* BL21 whole cell lysate; lane 2 – Induced ΔE6 recombinant *E. coli* lysate; lane 3 – 20,000g pellet fraction of induced ΔE6 recombinant lysate; lane 4 – 20,000g supernatant fraction; lane 5 – Un-induced full-length E6 recombinant *E. coli* BL21 whole cell lysate; lane 6 – Induced E6 recombinant *E.coli* lysate; lane 7 – 20,000g pellet fraction of induced lysate; lane 8 – 20,000g supernatant fraction; lane M – Protein molecular weight marker. (B) Western blot probed with goat ant-GST polyclonal antibodies. Lane 1 – Un-induced full-length E6 recombinant *E. coli* BL21 whole cell lysate; lane 2 – Induced E6 recombinant *E. coli* lysate; lane 3 – 20,000g pellet fraction of induced E6 recombinant lysate; lane 4 – 20,000g supernatant fraction; lane 5 – Un-induced ΔE6 recombinant *E. coli* BL21 whole cell lysate; lane 6 – Induced Δ E6 recombinant *E.coli* lysate; lane 7 – 20,000 g pellet fraction of induced lysate; lane 8 – 20,000g supernatant fraction; lane M – Prestained protein molecular weight marker.

oligomerize through interactions in the N-terminal domain (E6N; N-terminal 60 a.a residues) fostering formation of aggregates [15]. The deletion of the IHDIIL (31–36 a.a residues), we assumed, might negatively influence E6 multimerization while improving the solubility.

The splicing by overlap extension is an efficient PCR based DNA manipulation technique for insertions and deletions of nucleotides. PCRs and SOE-PCR in this study enabled the deletion of nucleotides coding for IHDIIL and assembly of ΔE6 respectively (Fig. 2). Cloning ΔE6 in the pGEX-4T1 allowed the protein to be expressed as an N-terminal fusion with GST. As shown in the Fig. 4, the GST-ΔE6 protein could be eluted using reduced glutathione unlike the full-length E6 in fusion with GST (GST-E6). The SDS–PAGE profile of the fractionated samples from bacterial lysates suggest that not all of the expressed GST-ΔE6 protein were soluble. However a significant portion of protein was rendered soluble due to the deletion of the P1 region amino acid residues as evident in the corresponding immunoblot (Fig. 3) .To the best of our knowledge this is the first report where soluble recombinant HPV16 E6, although modified, has been expressed in *E. coli* in fusion with GST.

The difficulty in obtaining soluble E6 and therefore protein preparations of suitable purity and homogeneity has been a significant limitation in the structure and function studies [16]. Majority of the *in vitro* functional studies have been conducted with recombinant E6 expressed in *E. coli* as fusion proteins with the soluble N-terminal protein candidates *viz.*, the maltose binding protein (MBP) or the glutathione S-transferase (GST) [16,24,25]. However, further purification of E6 from MBP or GST fusions has been proven a tall order [16,20].

Till date, elution of glutathione bound GST-E6 from the beads has been reported only by employing denaturing methods with the chaotropic 6 M guanidine hydrochloride (GnHCl) [26] or the cationic detergent, sodium dodecyl sulphate (SDS) [27]. The fractions are subsequently renatured by dialysis against a series of buffers containing diminishing concentrations of GnHCl. However, renaturation has been found to be extremely inefficient and cumbersome [26]. Therefore, most *in vitro* functional studies of E6 have been performed directly with GST-E6 bound to glutathione beads [24,25,28]. Our approach of deleting a stretch of hydrophobic residues not only rendered the protein soluble but also amenable for purification with the standard glutathione-GST affinity chromatography. The GST-Δ6 fractions from the affinity chromatography was further purified to a more homogenous preparation by gel-filtration chromatography (Fig. 5).

It has been previously observed that E6 expressed in fusion with the maltose binding protein (MBP-E6) are soluble [16]. However soluble MBP-E6 protein preparations contain two species – a minuscule fraction of monodisperse, correctly folded E6 and a major fraction containing soluble aggregates with misfolded E6 proteins at the core [16]. The monodisperse MBP-E6 fractions yield soluble E6 on proteolytic cleavage of the fusion protein at the junction while the soluble aggregates produce an insoluble mass of E6 proteins [16]. We sought to examine whether the soluble GST-ΔE6 existed in the desired monodisperse form in view of E6's propensity to form aggregates. This line of enquiry is particularly essential if the purified protein is considered for use in the study of structure and functions.

HPLC of the protein suggest that GST-ΔE6 predominantly exist in the monodisperse form. This can be deduced by the comparison of retention time of GST-ΔE6 (18.2 min) *vis a vis* the standard protein, BSA (12 min) used in the chromatographic evaluation. The relative retention times of the proteins (BSA and GST-ΔE6) conform to their respective size differences (66.5 KDa and ~46.0 KDa respectively). Multimers should logically form aggregates that are multiple times the size of monomeric GST-ΔE6 and hence should have shorter retention times in the SEC-HPLC. No peaks higher than that seen for BSA was observed in the chromatogram. The improved solubility and loss of oligomerization/aggregation may be attributed to the absence of N-terminal IHDIIL residues in ΔE6.

Further a preliminary study was conducted to determine the effect of the loss of N-terminal amino acids on the binding ability of E6 to p53 protein. In the HPV infected cells E6 is known to bind tightly to p53 and thus target the tumor suppressor host protein for degradation. We therefore thought that a binding study might be indicative of the structural integrity of ΔE6. The simple format of the casein–glutathione capture ELISA was employed for this purpose. The ELISA results indicate that p53 bound specifically to ΔE6 in a concentration dependent manner (Fig. 6). However in light of the published reports suggesting alternative binding regions of E6 to p53 [16] the inference on the conformation of ΔE6 need further enquiry.

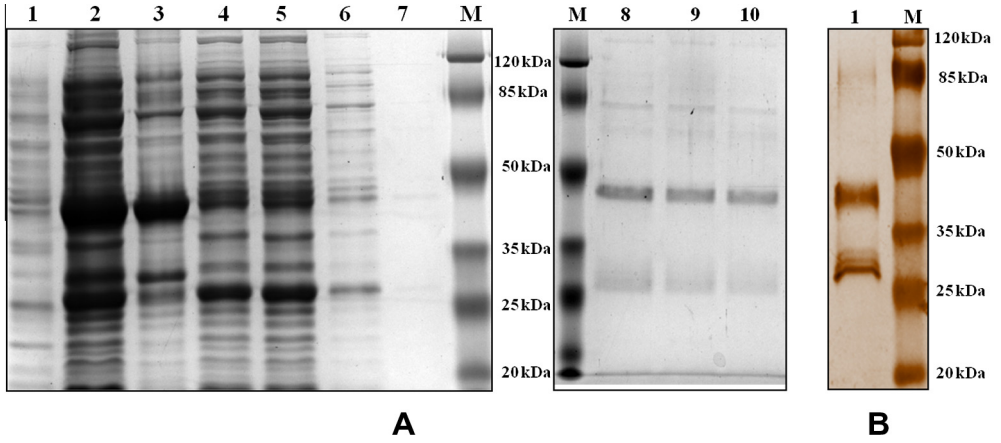


Fig. 4. Glutathione-GST affinity chromatography of GST-ΔE6. (A) SDS–PAGE profile. Lane 1 contains lysate from un-induced *E. coli* BL21 cells; lane 2 – Contains lysate from induced *E. coli* BL21 cells; lane 3 – 20,000g pellet; lane 4 – 20,000g supernatant (load); lane 5 – Flow through; lane 6 – Wash 1; lane 7 – Final wash, lane 8–10 elution fractions showing bands of 46 KDa; lane M – Pre-stained molecular weight protein marker from Fermentas. (B) Western blot analysis probed using anti-GST antibody raised in goat of elution fractions pooled from glutathione-GST affinity chromatography.

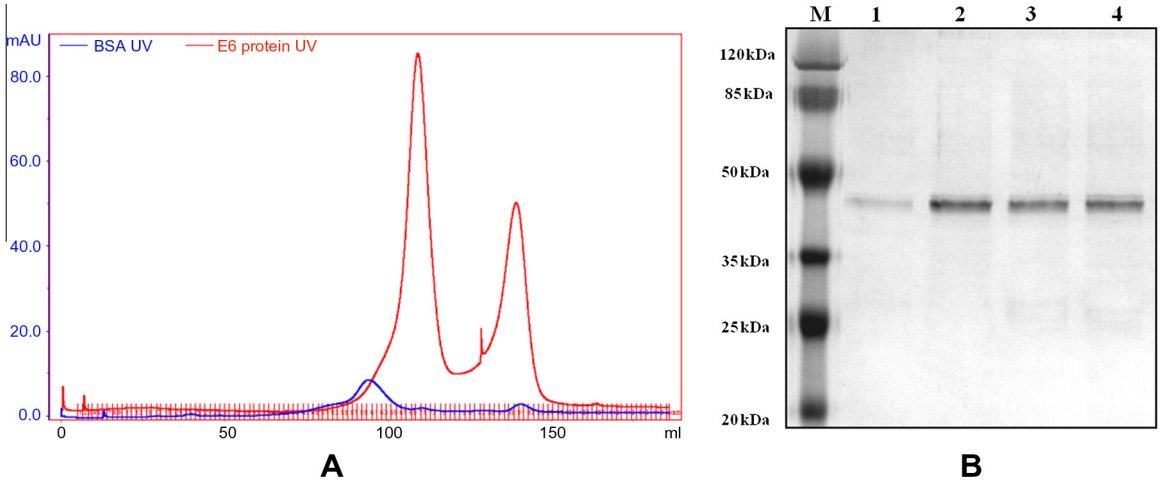


Fig. 5. (A) Chromatogram of the Sephacryl S400 16/60 gel-filtration chromatography. Peak-1 (fractions 68–71) corresponds to GST-ΔE6. The overlaid chromatogram of the standard reference protein (BSA, 66.5 KDa) shows a protein peak eluting at fraction 57–61. (B) SDS–PAGE of the elution fractions corresponding peak-1. Lane M – Protein molecular weight marker, lane 1–4 are elution fractions 68–71. C; Chromatographic profile of BSA (pink), combined fractions GST-ΔE6 (68–71, green), Free GST (blue), and the retention time is mentioned in the chromatogram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

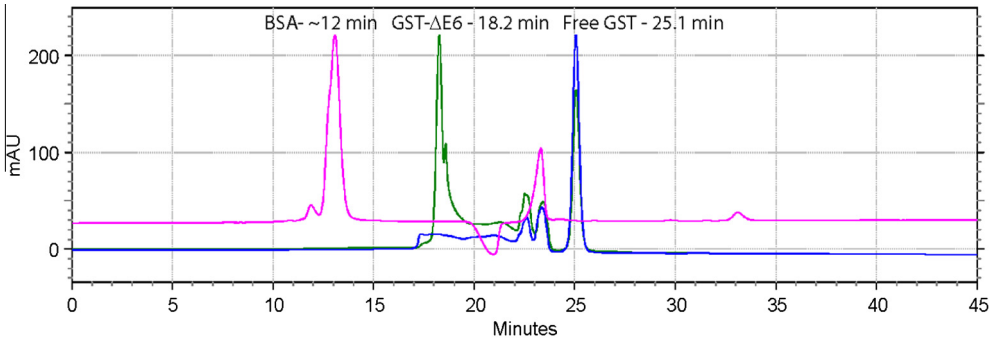


Fig. 6. HPLC-SEC (Yarra SEC-3000 column) profile of BSA (retention time 12 min), combined fractions (68–71, Fig. 5) of GST-ΔE6 from the gel-filtration chromatography (retention time 18.2 min), Free GST (retention time 25.1 min).

Conclusion

The removal of a stretch of predominantly hydrophobic amino acids from E6 rendered expression and purification of GST-ΔE6 in the soluble and predominantly monomeric form. The deletion and assembly of ΔE6 were effectively carried out using SOE-PCR. Preliminary studies indicate that deletion of amino acids do not abrogate the p53 binding activity of the protein. The soluble

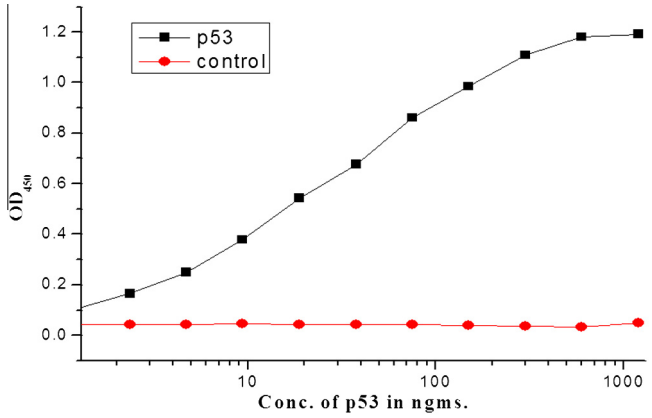


Fig. 7. p53 binding to ΔE6 in the glutathione-GST capture ELISA. The 96-well microtitre plates were coated with casein-glutathione. Purified GST-ΔE6 was then bound to the coated wells. Control wells were bound with purified GST. Later, recombinant p53 was added to the wells in a two-fold serial dilution. Bound p53 was detected using anti-p53 monoclonal antibody.

GST-ΔE6 should find ready application in immunological assays for the evaluation of E6 based vaccines (ELISA, ELIspot assays etc.,) and also in sero-epidemiology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.08.010.

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