Development of a soluble PTD-HPV18E7 fusion protein and its functional characterization in eukaryotic cells

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Though accumulated evidence has demonstrated the transformation capacity of human papillomavirus (HPV) type 18 protein E7, the underlying mechanism is still arguable. Developing a protein transduction domain (PTD)-linked E7 molecule is a suitable strategy for assessing the biological functions of the protein. In the present study, HPV18 E7 protein fused to an N-terminal PTD was expressed in the form of glutathione S-transferase fusion protein in Escherichia coli with pGEX-4T-3 vector. After glutathione-Sepharose 4B bead affinity purification, immunoblot identification and thrombin cleavage, the PTD-18E7 protein showed structural and functional activity in that it potently transduced the cells and localized into their nuclei. The PTD-18E7 protein transduced the NIH3T3 cells in 30 min and remained stable for at least 24 h. In addition, the PTD-18E7 protein interacted with retinoblastoma protein (pRB) and caused pRB degradation in the transduced NIH3T3 cells. In contrast to the pRB level, p27 protein level was elevated in the transduced NIH3T3 cells. The PTD-18E7 protein gives us a new tool to study the biological functions of the HPV E7 protein.

Keywords human papillomavirus; E7; fusion protein; transformation

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Introduction

Human papillomaviruses (HPVs) are small, non-enveloped DNA viruses that induce proliferative lesions of the cutaneous and mucosal epithelium. Epidemiological and molecular genetic studies have demonstrated that infection with high-risk HPV viruses, mainly HPV16 and HPV18, are associated with most cases of cervical cancer [1,2]. The high-risk HPVs exert their transforming ability by constitutively expressing two major oncoproteins, E6 and E7 [3,4]. The interaction of E6 with p53 causes rapid p53 degradation in an ubiquitin-dependent manner, resulting in cell resistance to apoptosis and hence chromosomal instability [5]. The E7 protein exerts its functions in cellular transformation by interacting principally with retinoblastoma protein (pRB) and other cellular targets. E7 proteins resemble adenovirus E1a and SV40 large T antigen both in primary sequences and in trans-activation and transformation properties [6]. When E7 binds to pRB, it causes the release of E2F from the E2F/pRB complex and this subsequently promotes inappropriate cell cycle progression [7,8]. It is believed that the functional inactivation of pRB is responsible for the transforming activity of E7 proteins encoded by high-risk HPV types such as HPV16 and HPV18 [9,10]. In addition to the disrupting of the pRB regulatory pathway, E7 has also been reported to bind and inactivate other known cell cycle regulators, including the cyclin-dependent kinase inhibitors (p21 and p27) [11,12], and the transcription factors (c-Jun, MPP2, and TBP) [13–15]. E7 has been reported to bind to over 20 cellular proteins [16,17], most of which are important cell growth regulators. This binding results in G1/S transition, abnormal centrosome duplication and genomic instability. Glutathione S-transferase (GST) pull-down, co-immunoprecipitation and yeast two-hybrid system are the main methods to study protein–protein interactions. In fact, almost all of the E7 binding proteins are found by using these methods. The major pitfalls of the current approaches are the ignored dynamic interactions of the molecules, and the lack of detailed molecular mechanism of carcinogenesis, which has not reached consensus yet. That is, the interaction of proteins are based on the physical contact regardless of
the protein expression levels in vivo and the functions of E7 protein are often detected in permanent E7 expression construct transfected host cells, which lack the influence of time and dose. Therefore, continuous tracking of E7 protein in the host cells may be able to reveal the physical and functional interaction of E7 with its targets. Using the purified E7 protein fused to the protein transduction domain (PTD) directly targeting the host cells could be a suitable way to investigate the functions of E7 protein.

PTD is a small peptide of approximately 11 amino acids (YGRKKRRQRRR) in length, which can transduce itself as well as the bigger proteins fused with it, into the cells [18–20]. In the present study, we focused on the preparation of the fusion protein of PTD with HPV18 E7 (PTD-18E7) and the assessment of its functional properties.

Materials and Methods

Construction of HPV18 E7 expressing prokaryotic vector

The PTD-18E7 fusion fragment, encoding the whole sequence of HPV18 E7 and the 11-aa PTD peptide was constructed by routine PCR cloning method. First, 5’ primer 5′-cggaattcctagtgcaggaagacgagagacagagagagaattctcatggacctaagg-3′ (EcoRI site in bold and PTD sequence encoding YGRKKRRQRRR underlined), and 3’ primer 5′-cgtcagttatgctgggatgcacacca-3′ (XhoI site in bold) for E7 were synthesized by AuGCT Biotechnology (Beijing, China). HPV18E7 sequence was amplified from pUC18 plasmid (a gift from Dr Schiller, NIH, USA) containing the whole genome of HPV18 using E7 5’ primer and 3’ primer. The PCR products were digested with EcoRI and XhoI. The amplified PTD-18E7 fusion fragment was inserted into a prokaryotic expression vector, pGEX-4T-3 (GE Healthcare, Piscataway, USA), which contained a DNA sequence encoding the GST tag. Plasmid DNA in the ligation mixture was transferred into Escherichia coli DH5α by heat shock and ampicillin-resistant colonies were isolated after overnight culturing on LB plates containing 100 μg/ml ampicillin for selecting the transformed cells. To confirm the presence of the correct PTD-18E7 fusion DNA sequence in E. coli cells, plasmid DNA was isolated from individual transforming colonies and confirmed by PCR analysis, restriction enzyme digestions and DNA sequence analysis (AuGCT Biotechnology). Plasmid with the correct DNA sequence was designated as pGEX-PTD-18E7. pGEX-PTD-18E7cys (HPV18 E7 mutant with Cys24 mutated to be Gly24) does not bind to pRB protein and pGEX-18E7 (a negative control, which can express HPV18 E7 protein without PTD peptide) expression plasmids were constructed using HPV18E7 m24Cys-Gly/pUC18 vector (constructed by Dr Ang Li in our lab) and 5’ primer (5’-cggaattccatggacctaagg-3′), respectively.

Expression of PTD-18E7 in E. coli

Expression vectors encoding the chimeric PTD-18E7 fusion proteins were transformed into E. coli BL21, and the resulting bacterial cells were plated onto LB-agarose containing ampicillin. Single colonies of the PTD-18E7 transformed E. coli were picked out, inoculated into selection medium and grown overnight. The overnight culture was inoculated into 100 ml of fresh 2× YT medium for exponential growth. When the OD600 value reached 0.6, 0.1 mM IPTG was added to the culture to induce protein expression and the cells were incubated for 4 h at 30°C. The induced cells were harvested by centrifugation at 5000 g at 4°C for 5 min and washed twice with PBS. The cell pellet was resuspended in 10 ml of lysis buffer (50 mM Tris, pH 8.0, 1 mg/ml lysozyme). The suspension was sonicated in an ice-bath and Triton X-100 was added to a final concentration of 1%. After mixed gently for 30 min, the lysate was centrifuged at 12,000 g for 30 min. The supernatant was collected and analyzed by 12% SDS-PAGE to test the solubility of the expressed protein.

Purification of the fusion protein

PTD-18E7 fusion protein was purified by using glutathione-Sepharose 4B (GS4B) beads (Amersham, Piscataway, USA) according to the manufacturer’s instructions. Briefly, the obtained supernatant was directly mixed with 50% GS4B slurry pre-equilibrated with 20 mM PBS (pH 7.4). The mixture was rotated for 30 min at room temperature and centrifuged at 500 g for 5 min. The supernatant was discarded and the GS4B pellet was washed three times with 10 volumes of PBS and one time with Tris buffer (20 mM Tris–HCl, pH 8.0, 2.5 mM CaCl2) or HEPES buffer (5 mM HEPES, pH 8.0, 2.5 mM CaCl2, 150 mM NaCl). Tris buffer or HEPES buffer containing thrombin (5 U/mg) was added to the washed GS4B pellet and incubated at room temperature for 1.5 h to remove the GST tag. After centrifuged at 500 g for 5 min, the supernatant was collected, analyzed by SDS-PAGE and immunoblotted with monoclonal mouse anti-HPV18E7 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (1:500). The protein fractions, which contained the high
Proteins were imaged and analyzed by the ECL method. 1:3000 dilution in TBST for 1 h at room temperature.

gated anti-mouse IgG (DAKO, Glostrup, Denmark) at TBST at 4°C overnight and horseradish peroxidase conjugated anti-mouse/rabbit IgG (DAKO). Proteins were analyzed on the FACScanto (Becton Dickinson, Franklin Lakes, USA).

**Intracellular localization analysis by immunocytochemistry**

NIH3T3 cells grown on the cover slips were incubated with PTD-18E7 proteins at a final concentration of 1 μM for different time periods, washed three times with PBS and fixed in 4% paraformaldehyde for 10 min. After washing three times with PBS, the cover slips were permeabilized with 0.3% Triton X-100/PBS for 15 min at 37°C, blocked with 5% normal goat serum for 30 min at 37°C and incubated with goat-anti-HPV18E7 primary antibody (Santa Cruz Biotechnology) (1:100) for 2 h at room temperature. After washed two times with PBS (containing 0.1% Tween 20), the cells were further incubated with donkey anti-goat FITC-IgG (Santa Cruz Biotechnology Inc.) (1:100) for 30 min at room temperature, washed three times with PBS, and stained with DAPI for 3 min. After three times wash with PBS, the slides were mounted with a fluorescent mounting medium (DAKO), and visualized using fluorescence microscope (Nikon, Tokyo, Japan).

**GST pull down assay**

GST pull down assay was performed according to the protocol as described [21]. For GST pull down, NIH3T3 whole cell lysates were produced using ML buffer (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl, 0.5% NP-40, and complete EDTA-free protease inhibitors tablets) and cleared by centrifugation (4°C, 20 min, 16,000g). GST-control, GST-PTD-18E7 fusion protein and GST-PTD-18E7ΔRb mutant protein were expressed and lysed as described in expression and purification parts in ‘Materials and Methods’. The 500 μl supernatant was mixed with 50 μl G54B slurry (pre-equilibrated with PBS) and incubated for 30 min at room temperature. The Sepharose beads were then washed several times with ML buffer and incubated with NIH3T3 whole cell lysates for 4 h at 4°C. Then, beads were washed three times with ML buffer and, when indicated, the proteins were mixed with 2× loading buffer, boiled for 5 min at 99°C and separated in 12% SDS-PAGE. The protein was transferred onto nitrocellulose membrane and blocked in 5% nonfat dry milk/TBST for 3 h at room temperature. The membranes were probed with anti-RB (sc-50; Santa Cruz Biotechnology) (1:500), anti-GST (3818-1; Clontech, Palo Alto, USA) antibodies and horseradish peroxidase conjugated donkey anti-mouse/rabbit IgG (DAKO). Proteins were visualized on the enhanced chemiluminescence system (ECL).
visualized using Western Lightning Chemiluminescence (Cell Signaling Technology, Danvers, USA) methods.

**Functional assays**

Purified E7 proteins were tested for pRB protein degradation in NIH3T3 cells. NIH3T3 cells were incubated with purified 18E7 protein (1 μM). Cells in fresh complete medium were used as control. Cells were harvested at different time intervals and lysed in RIPA buffer. Cell lysates were centrifuged at 4°C for 10 min at 15,000g. The supernatants were collected, and protein yields were determined by Bradford analysis. The same amount of whole cell lysates were separated on 12% SDS-PAGE and transferred to PVDF membranes according to the standard procedures. Membranes were blocked in 5% nonfat dry milk/TBST for 3 h at room temperature and then probed with rabbit anti-RB or anti-p27 primary antibody (Santa Cruz Biotechnology) at 1:1000 dilution in TBST at 4°C overnight and horseradish peroxidase conjugated donkey anti-rabbit IgG (DAKO) at 1:3000 dilution in TBST for 1 h at room temperature. Proteins were imaged and analyzed by the ECL method.

**Results**

**Cloning of the pGEX-PTD-18E7**

The steps involved in the construction of the pGEX-PTD-18E7 fusion expression plasmid were shown in

**Expression and purification of the pGEX-PTD-18E7 recombinant protein**

The pGEX-PTD-18E7 plasmid was transformed into the BL21 strain of E. coli and cultured in 2× YT medium. IPTG was used to induce the expression of the fusion protein (∼37 kDa GST-PTD-18E7). The target protein was detected in the supernatant of lysates [Fig. 2(A)]. The amount of soluble recombinant GST-PTD-18E7 was about 30% of the total proteins in the lysate. The crude lysates were purified by GS4B beads. The purified product was identified by 12% SDS-PAGE [Fig. 2(B)]. Removal of the GST tag is particularly tricky in the case of GST-PTD-18E7 protein because the PTD-18E7 is a relatively small protein as compared with GST. Thrombin was used to cut off the GST tag [Fig. 2(C)]. Two thrombin cleavage buffers were used for the GST tag removal. The resultant proteins were detected by 15% SDS-PAGE analysis [Fig. 2(D)]. It seemed that Tris buffer was better than HEPES buffer, though both of them

![Figure 1](image_url)
containing thrombin could cleave the GST tag from GST-PTD-18E7 fusion protein without non-specific cleavage. The purified GST-PTD-18E7 and cleaved PTD-18E7 proteins were further confirmed by western blot analysis [Fig. 2(E)].

**PTD-18E7 protein transduction**

The 293T and NIH3T3 cells treated with PTD-18E7 proteins were collected and lysed in RIPA buffer at different time intervals. Western blots were performed with anti-HPV18E7 antibody. PTD-18E7 fusion protein effectively transduced the NIH3T3 and 293T cells [Fig. 3(A)]. PTD-18E7 protein was observed in the NIH3T3 cells as early as 15 min after exposure and reached the maximum intracellular concentration within 30 min. The protein remained stable in the NIH3T3 cells for at least 24 h [Fig. 3(B)]. In addition, PTD-18E7 remained stable over long time in the transduced cells. As shown by western blotting [Fig. 3(C)], over 50% of PTD-18E7 fusion protein detected at the 24 h time point can still be found at Day 2. But most of the proteins were degraded at Day 4.

**Figure 2** Expression and purification of the GST-PTD-18E7 recombinant protein in *E. coli* (A) GST-PTD-18E7 recombinant protein expressed in *E. coli*. Lanes 1 and 5, *E. coli* cells containing the pGEX-PTD-18E7 vectors without IPTG induction; lanes 2 and 4, 4 h after IPTG induction in *E. coli* cells containing the pGEX vectors; lane 6, molecular weight standards. (B) Purification of the GST-PTD-18E7 recombinant protein by GS4B beads. Lane 1, molecular weight standards; lane 2, GS4B beads incubated with pGEX-PTD-18E7/BL21 cells lysate without IPTG induction; lanes 3–6, purified GST-PTD-18E7 recombinant protein; lane 7, purified GST. (C) Structure of the GST-PTD-18E7 fusion protein. There is a thrombin cleavage site between GST and PTD domain. (D) Thrombin cleavage of the GST-PTD-18E7 recombinant protein. Lane 1, molecular weight standards; lane 2, cleavage of the GST-PTD-18E7 protein by thrombin in Tris buffer; lane 3, cleavage of the GST-PTD-18E7 protein by thrombin in HEPES buffer. (E) Western blot detection of the GST-PTD-18E7 recombinant protein. Lanes 1 and 2, purified GST-PTD-18E7 recombinant protein; lanes 3 and 4, thrombin-cleaved GST-PTD-18E7 recombinant protein.

**Figure 3** PTD-18E7 protein transduction (A) PTD-18E7 fusion protein transduced into the NIH3T3 and 293T cells. Lane 1, 293T cells with HPV18 E7; lane 2, NIH3T3 cells with HPV18 E7; lane 3, 293T cells with PTD-18E7 protein; lane 4, NIH3T3 cells with PTD-18E7; lane 5, C33A cells as negative control; lane 6, HeLa cells as positive control. (B,C) PTD-18E7 transduction and stability in NIH3T3 cells. NIH3T3 cells were incubated with PTD-18E7 for different time periods.
The transduction efficiency of PTD-18E7 fusion protein into NIH3T3 cells was investigated. At different time of PTD-18E7 addition, the NIH3T3 cells were collected, fixed, and indirectly labeled with immunofluorescence. Flow cytometry was used to analyze the transduction efficiency. As shown in Fig. 4, PTD-18E7 protein transduced into the NIH3T3 cells as early as 10 min after addition. Thirty minutes later, 65.8% of the NIH3T3 cells contained PTD-18E7 protein. When the NIH3T3 cells were treated with PTD-18E7 protein for more than 1 h, almost all the cells were PTD-18E7 positive.

Nuclear localization of PTD-18E7
Nuclear localization is prerequisite for HPV18 E7 protein to exert its function; the functional E7 should possess this property. An expected outcome of the fusion of HPV18 E7 protein with PTD should be its retention in the nucleus of the cell. As shown in Fig. 5, the PTD-18E7 fusion protein was localized in the nucleus after transduction, assessed by indirect immunofluorescence. DAPI counterstaining was used to confirm the nuclear localization of PTD-18E7. In the cells treated with PTD-18E7, the majorities of the PTD-18E7 specific florescent (FITC) signals (green) were detected in the nucleus, and were merged with the nucleus-specific DAPI signals (blue) [Fig. 5(B)]. Whereas, in the NIH3T3 cells treated with HPV18 E7 protein, no HPV18 E7 specific signals were detected. Taken together, our present data implied that the PTD-18E7 recombinant protein was successfully transduced and localized into the nucleus of the NIH3T3 cells.

PTD-18E7 can interact with pRB proteins
To determine whether PTD-18E7 could directly interact with the pRB proteins, we mixed recombinant, purified GST-PTD-18E7, GST-PTD-18E7\textsuperscript{ARb} or GST immobilized onto glutathione-Sepharose beads with whole NIH3T3 cell lysates. After incubation, beads were washed and analyzed by SDS-PAGE followed by western blotting with pRB specific antibodies. The data from these experiments suggested that GST-PTD-18E7 associates with pRB from NIH3T3 derived whole cell lysates whereas GST-PTD-18E7\textsuperscript{ARb} does not (Fig. 6). Hence, the association between GST-PTD-18E7 and pRB is direct and specific, not relying on the PTD peptide.

PTD-18E7 protein caused pRB degradation
The changes of the pRB levels in NIH3T3 cells treated with PTD-18E7 were taken as a measure of functional assay. NIH3T3 cells treated with PTD-18E7 proteins were collected at different time intervals and lysed in

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**Figure 4** Flow cytometry analysis of the PTD-18E7 positive NIH3T3 cells Cells were treated with PTD-18E7 protein for different times. The PTD-18E7 proteins in the NIH3T3 cells were stained by goat anti-HPV18E7 as primary antibody and donkey anti-goat FITC-IgG as second antibody and detected by flow cytometry.
RIPA buffer. Cellular lysates were prepared and western blot analyses were performed with pRB antibody. Compared with the untreated NIH3T3 cell extract, pRB protein was degraded as early as 1 h in PTD-18E7 transduced cell extracts, and after 3 h almost all of the pRB protein was degraded [Fig. 7(A)]. In contrast to pRB protein, p27 protein levels were elevated in PTD-18E7 transduced NIH3T3 cells. The p27 protein level was elevated from 15 min, peaked at 3 h, and maintained till to 10 h [Fig. 7(A)]. These results implied that p27 protein was elevated following pRB degradation. The PTD-18E7ΔRb (m24Cys-Gly) fusion protein, which is incapable of binding to pRB, did not cause the pRB protein degradation and consequently did not cause p27 protein elevation [Fig. 7(B)]. The results implied that PTD-18E7 recombinant protein has the potential to be functional in eukaryotic cells. We then detected the pRB degradation level in the 293T cells and NIH3T3 cells treated for 3 h with different concentrations of PTD-18E7 protein. The results showed that when cells were treated with low concentrations of PTD-18E7, pRB proteins were partially degraded. When the protein level was higher than 0.5 μM, pRB proteins were almost completely degraded. There was no difference in pRB degradation when protein level is higher than 0.5 μM [Fig. 7(C)]. To find whether the E7 protein remains in the cells long enough to degrade cellular pRB when it was withdrawn from the medium, we first treated 293T cells with PTD-18E7 protein for 1 h, then withdrew the PTD-18E7 protein and cultured the cells with complete cell culture medium. The result showed that PTD-18E7 was degraded with the time, whereas pRB level was gradually decreased at first, whereas increased as the E7 protein diminished [Fig. 7(D)].
Discussion

PTDs have emerged as an attractive biological delivery tool and have transduced many proteins into the living cells. PTD of 11 amino acid residues, the basic transduction domain, can transduce itself as well as other bigger proteins fused with it into the cells [22–24]. Despite the fact that the E7 protein is the focus of many studies in cervical carcinogenesis, the detailed molecular mechanism and biological properties that are linked to the malignant transformation of the HPV-infected cells are still unclear. The approach of generating recombinant molecules linked to a PTD is a good method for the assessment of the biological functions of a protein [25]. Until now, there is no single report about applying the PTD-E7 fusion protein directly to the host cells to dissect the functions of E7 protein. In the present study, we cloned, over-expressed and purified the PTD-E7 recombinant protein in E. coli, though Natalie et al. were successful in the expression of the TAT-E7 protein in E. coli, the insolvability of the protein bothered its further application, as the refolding of the functional protein was a tedious procedure [26]. In the present study, we successfully expressed PTD-E7 fusion protein in a soluble form. Perhaps, this was because we used the GST tag whereas they used the His tag. Many proteins fused with GST tag have been expressed in a soluble form in bacterial cell cultures [27,28].

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Our results showed that PTD-E7 protein could transduce more than 99% of the targeted NIH3T3 cells and remain stable in the cells for at least 24 h. The transduction efficiency of the PTD-E7 protein into the NIH3T3 cells was time-dependent. Ten minutes after adding PTD-18E7 protein, there were only 22.1% positive NIH3T3 cells but when the NIH3T3 cells were treated with PTD-18E7 protein for more than 1 h, almost all of the cells had PTD-18E7 protein.

Nuclear localization assay showed that the PTD-E7 recombinant protein could penetrate into the NIH3T3 cells successfully and localized mainly to the nucleus.
GST pull down experiment showed that GST-PTD-18E7 associates with pRB from NIH 3T3 whole cell lysates whereas GST-PTD-18E7/Rb does not associate with pRB. This indicated that the association between GST-PTD-18E7 and pRB is direct and specific, not relying on the PTD peptide. pRB protein was degraded at as early as 1 h after PTD-E7 protein transduced into the NIH3T3 cells and almost all of the pRB protein was degraded within 3 h. In contrast to the pRB, p27 was expressed at increased levels when the pRB pocket proteins were degraded. This result was one of the remarkable observations. In 2009, Philipp Kaldis’s group also reported that depletion of pocket protein by HPV-E7 that enhances p27 levels [29]. This is the same result with us. Because PTD-18E7 and pRB proteins are both localized in the nuclei, this hints that PTD-18E7 associated with pRB and caused its degradation in vivo. These data demonstrated that PTD-E7 proteins expressed in bacterial cells could exert their functions in eukaryotic cells. The purified PTD-E7 protein had biologically active conformation and it can contribute to assess the functional properties of the E7 protein.

In summary, we have succeeded in expressing the PTD-E7 protein in a soluble form in E. coli. The purified protein can be effectively transduced to the living cells and localized to the nucleus. PTD-18E7 protein associated with pRB protein and caused pRB degradation. As the PTD-E7 protein successfully degraded the pRB protein in the transduced cells, we believed that the recombinant protein was similar to that of the HPV protein can be effectively transduced to the living cells and localized to the nucleus. PTD-18E7 protein associated with pRB protein and caused pRB degradation. As the PTD-E7 protein successfully degraded the pRB protein in the transduced cells, we believed that the recombinant protein was similar to that of the HPV produced protein in the infected cells. The PTD-E7 protein can contribute to assess the biological function of the HPV E7 protein further.

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