Expression of PDZ domain 1 of the Scribble Polarity Complex

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Abstract

Infection by high-risk human papillomavirus (HPV) is the leading cause of cervical cancers. The E6 proteins produced by HPV have been implicated as determining factors in the development of tumors following infection of an individual. E6 has been shown to bind proteins with PDZ domains through PDZ binding motifs, or PBMs. This interaction between viral PBMs and host-cell protein PDZ domains has been found to play a significant role in viral pathogenesis. More specifically, E6 binds to PDZ domains on the Scribble Polarity Complex protein (SAP-97). This binding event promotes degradation of the scribble complex which contributes to tumorigenesis by interfering with the regulation of cell growth and polarity. This study establishes a protocol for the expression of a peptide containing the PDZ domain 1 (PDZ-1) of SAP-97 by means of a recombinant expression vector. Potential methods for purification of the PBM peptide are posed as well. Recombinant expression would provide sufficient levels of isotopically labeled proteins so that these interactions may be studied by NMR spectroscopy. The results presented here, therefore, provide a basis for future analysis of small viral PBM's with their host cell PDZ domains.

Introduction

Infection by low-risk human papillomavirus (HPV) results in the development of benign skin lesions in epidermal and mucosal tissues. However, infection by high-risk strains of HPV can lead to the development of cervical cancer, the second most common cancer for women globally (17). Vaccines against four of the high-risk HPV strains exist, however, infection by these strains account for only about 70% of cervical cancers. As such, 30% of HPV-related cervical cancers are the result of infection by a high-risk strain of HPV not protected against by current vaccinations (4). Furthermore, vaccinations act only in a preventative manner and do not treat already infected individuals. Understanding the cellular mechanisms of HPV and its ability to cause cancer is meaningful for the development of therapies for infected individuals at risk of developing cancer as a result of infection by HPV (16).

In general, when a virus infects a cell, the cellular machinery of the host cell is hijacked to produce virally encoded proteins. By these means, the virus uses the host cell to produce new virions that infect other cells (3). Of the virally encoded HPV proteins are the oncogenic E6 and E7 proteins. E6 and E7 work in tandem to support tumorigenesis by promoting abnormal cellular proliferation and amplification of the viral genome (12, 14). In previous studies, cell lines derived from cancerous cervical cells entered apoptosis or senescence following depletion of E6 and/or E7. These findings indicate E6 and E7 as "major oncogenic determinants of high-risk HPVs" (11).

The E6 protein promotes and sustains cervical tumor formation through multiple actions. For example, E6 promotes the degradation of a key tumor suppressor protein, p53. Loss of p53 prevents the induction of cellular apoptosis that is normally triggered in response to viral infection (20). Additionally, E6 upregulates the activity of the enzyme telomerase, a hallmark of tumorigenesis (7). Finally, high-risk HPVs utilize PDZ binding motifs, or PBMs to modulate binding interactions with multiple host-cell PDZ proteins to promote tumorigenesis (18).

PDZ domains are highly conserved protein interaction domains commonly found within larger scaffolding proteins. Scaffolding proteins organize and coordinate cellular signals by localizing appropriate components. More than 150 human proteins are known to contain PDZ domains (8). PDZ domains are generally 90 amino acids in length with six beta-strands and two alpha-helices (14). PDZ domains have been identified as common targeting sites for viruses. PDZ domains typically interact with other proteins by binding to a specific amino acid sequence on the interacting protein. This binding recognition sequence is commonly referred to as a PDZ-binding motif or PBM. PBMs generally

lie at the far C-terminal of the viral protein primary sequence. (8,10).

HPV was one of the first viruses found to utilize PBMs to interact with host-cell PDZ proteins (12). Since this discovery, there have been eight virus families identified to produce proteins with PBMs. The effect of viral PBM binding varies, causing a loss, gain, or modulation of the cellular protein's normal functioning. The resulting change in function incited by viral PBM binding is suspected to contribute to the pathogenicity of a given virus by promoting amplification of the viral genome, diffusion in the host, or transmission to new hosts (11).

The E6 protein of high-risk HPV has a c-terminal PBM while low-risk HPV E6 proteins do not (12, 13). The E6 PBM is the only protein-interaction domain unique to high-risk HPVs, implicating it as a key factor in determining the carcinogenicity of a given HPV strain (23). The binding interaction between E6 PBM is of particular interest as cells infected with HPV-31 E6 with deletion of its PBM displayed reduced growth rates and decreased viral genetic material (13). The Scribble Polarity Complex (SAP97) protein is one of the PDZ proteins targets by HPV E6 (5).

The Scribble Polarity Complex protein is composed of the Scribble, Lethal giant larvae (Lgl), and human homologue discs large (hDlg) proteins (22). The hDlg protein component contains 3, internal PDZ domains. The E6 PBM has the strongest binding affinity for PDZ-2 of SAP97, while PDZ-1 shows a slightly weaker affinity, and PDZ-3, the weakest (12, 14).

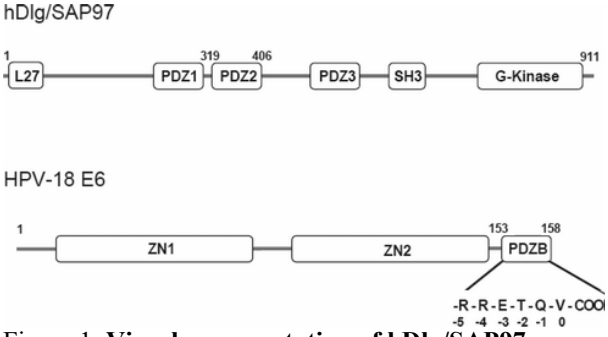

Figure 1. **Visual representation of hDlg/SAP97 (top) and the HPV-18 E6 (bottom) protein domains.** SAP-97 has three internal PDZ domains. Relative locations of these domains are shown as labeled rectangles along the protein sequence. The extreme C-terminal of the E6 protein is shown with the PDZ binding motif (PBM or PDZB) occupying the 9 terminal residues of the protein sequence (15).

The SAP97 protein organizes the basolateral membrane domain of epithelial cells to maintain proper intracellular cell-signaling pathways. The complex also regulates cell proliferation and modulates the formation and preservation of tight junction and adherens junctions. SAP97 also functions as a tumor suppressor. (22). The binding of SAP97 by the E6 PBM promotes ubiquitin-mediated degradation of SAP97 (18). Protein expression of SAP97 is reduced or entirely absent in cervical cancer tissues. This finding further suggests that binding of the E6 PBM to the complex may play a determining role in carcinogenesis of high-risk HPV (1).

Similar to HPV E6, the NS5 protein encoded by Tick-Borne encephalitis virus (TBEV) utilizes PBM-mediated binding to interact with Zonulaoccluden-1(ZO-1) (2). Nuclear Magnetic Resonance (NMR) spectroscopy can be used to gain insight into the interaction between viral PBM and host-cell protein PDZ such as that between TBEV NS5 and ZO-1 (16). Isotopically labeled peptides containing the host-cell PDZ domain and unlabeled peptides containing the viral PBM can be expressed using recombinant bacterial expression systems. A ^{1}H-^{15}N-HSQC spectrum of the labeled PDZ peptide alone has been overlayed with a spectrum collected of the complexed PDZ and PBM peptides. Shifts in the peaks between the PDZ only and PDZ-PBM complex spectra can reveal which amino acids of the PDZ domain are interacting with the PBM.

With this previous research as a launching point, this project pivots its focus to the PDZ-PBM interaction of HPV E6 and SAP-97 PDZ-1. We anticipate the previously used experimental protocols for expression and purification to be largely transferable to other PBM and PDZ domains of interest. However, these protocols must be verified and, if necessary, modified for the HPV E6 PBM and PDZ-1 of SAP-97 before NMR studies can be completed. As such, this project aims to verify a previously used expression technique for the expression of PDZ-1 of SAP-97.

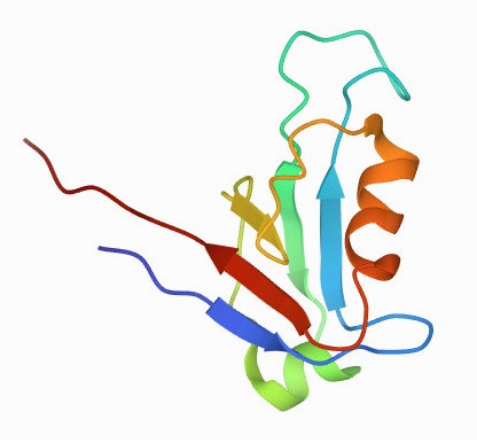

Figure 2. **The structure of PDZ-1 of SAP-97 as determined by NMR spectroscopy (19).** PDZ-1 of SAP-97 has two alpha-helices, and five beta-strands.

Materials and Methods

Expression

pGEX-2tk vectors with DNA coding for the PDZ-1 domain of SAP97 were transformed into BL21 competent *E.coli* cells and plated on LB media with carbenicillin antibiotic and left overnight at 37 ºC. A colony was selected from the plate and inoculated into 2.5 mL of LB with carbenicillin and left in a shaking incubator at 37 ºC and 225 rpm for about 3 hours. After the 3-hour growth period, the entire growth was added to 100 mL of LB with carbenicillin and left shaking once again at 225 rpm and 37 ºC. 1 mL aliquots of the growth were periodically collected and measured for absorbance until the sample reached an optical density (OD) of ~1 at 600 nm (actual OD_{600}: 1.137). Once the growth reached the appropriate OD, 22.5 mL were transferred into 1 L of prepared M9 medium (0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 g/L $(NH_4)_2SO_4$, 4 g/L glucose, vitamins, and carbenicillin). The liter growth was left shaking at 37 ºC and 225 rpm until the OD_{600} reached ~0.5 at 600 nm (actual OD_{600}: 0.570). Protein expression was induced with IPTG. The growth was left incubating overnight at 20 ºC. The absorbance was measured (OD_{600}: 2.107) the following day. The cells were centrifuged at 5500 rpm and 4 ºC for 15 minutes to form a pellet. The cell pellet was collected and stored for future use at -80 ºC.

Cell Lysis and Protein Harvest

The cell pellet was resuspended in lysis buffer (150 mM NaCl, 10% glycerol, 1X triton, 5 mM dithiohreitol, 25 mM Tris HCl). The cells were lysed via sonication at 3 seconds on, 3 seconds off, ten times, repeated twice. The cell lysate was separated by centrifugation at 1400 rpm for 25 minutes, and the supernatant was collected. The lysis and centrifugation process were repeated a second time, and the supernatant was collected.

Gel electrophoresis

The supernatant samples were prepared for gel electrophoresis with 2X Tricine SDS sample buffer, and 10X Bolt Sample Reducing Agent. The samples were loaded into a Novex 10-20% Tricine gel. Lane 1 was loaded with SeeBlue Plus2 Prestained Protein ladder standard. Lanes 2 and 3 were loaded with the supernatant collections from the first and second cell lysis, respectively. The gel was run at 110 V for approximately 1 hr. and 10 minutes. The gel was then stained for 2 hours with SimplyBlue stain, shaking gently. The stain was removed and placed in milliQ water overnight to de-stain for visualization the following day.

Results and Discussion

Gel electrophoresis of the supernatant from the lysed BL21 competent cells revealed a large, dark protein band just below the 38 kDa protein standard band. A second band was visualized at approximately the same molecular weight in lane 3.

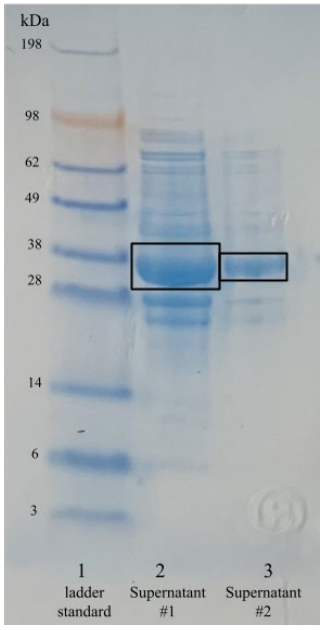

Figure 3. **Expression of PDZ-1 of SAP-97 results in a concentrated band on tricine gel at ~36 kDa.** The collected supernatant samples from the first (lane 2) and second (lane 3) lysis collections were loaded onto a tricine gel and separated according to molecular weight by gel electrophoresis. Well 1 was loaded with a protein ladder standard.

This study validated a previously established protocol for the expression of PDZ-1 of SAP-97 through recombinant bacterial expression. The structure weight of the PDZ-1 domain of SAP-97 is 10.09 kDa (24). The GST protein tag is 25.5 kDa (19). Thus, tagged PDZ-1 of Sap-97 is expected to have a total structural mass of ~36 kDa. Therefore, the presence of a large dark band in lane 2 below the 38 kDa standard suggests the successful expression of PDZ-1 of SAP-97 tagged with GST. A second, slightly lighter band appeared in lane 3 as well. This indicates that the PDZ-1 protein was still present in the second lysis collection from the cell pellet. In light of this, multiple cell lyses should be repeated to optimize the protein yield.

Due to the small size of the PDZ-1 peptide (93 amino acids), it is ideal to express the peptide fused to a larger protein for purification purposes. The pGEX-2tk vector used in this study code for the expression of the protein glutathione s-transferase (GST) fused to the protein of interest. The gene for this protein is inserted into the multiple cloning site of the vector. pGEX-2TK vectors have a thrombin cleavage site located between the GST protein tag and the target protein. As a result, thrombin, a protease enzyme can be used to cleave the GST from the PDZ-1, isolating the protein. We propose that, similar to previously optimized systems, thrombin cleavage along with FPLC can be used following this cleavage to further purify the PDZ-1 peptide for use in NMR analysis.

The results of this project offer foundational information for future research of HPV E6 and its interaction with SAP97 PDZ-1. An optimized expression and purification protocol for HPV E6 is still needed. Once this is established, the purified peptides can be used in NMR analysis. An HSQC baseline spectrum of isotopically labeled SAP97 PDZ-1 can be overlayed with a spectrum of labeled PDZ-1 SAP97. Any shifts in peaks between the baseline and complexed spectra will reveal which amino acid residues of SAP97 PDZ-1 are bound by HPV E6. As previously mentioned, this information will reveal potential therapeutic targets to inhibit the interaction between HPV E6 and SAP97 PDZ-1. These therapeutics would have the potential to slow or stop tumorigenesis caused by high-risk HPV strains.

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