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Improved immunodetection of human papillomavirus E7

Ju-Hong Jeon[1], Sung-Yup Cho[1], Chai-Wan Kim[1], Dong-Myung Shin[1], Joon-Cheol Kwon[1], Kyung-Ho Choi[1] and In-Gyu Kim[1,2]

[1]Department of Biochemistry and Molecular Biology
Aging Apoptosis Research Center (AARC)
Seoul National University College of Medicine
Seoul 110-799, Korea
[2]Corresponding author: Tel, 82-2-740-8248;
Fax, 82-2-744-4534; E-mail, igkim@plaza.snu.ac.kr

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Abbreviation: HPV, human papillomavirus; Rb, retinoblastoma protein; NC nitrocellulose; TBS, Tris-buffered saline

Abstract

Human papillomavirus E7 (HPV E7) is a viral oncoprotein that plays an important role in cervical carcinogenesis through binding with retinoblastoma protein (Rb). Inactivation of Rb by E7 is necessary but not sufficient for cellular transformation, suggesting other protein-protein interactions are required for E7-mediated cellular transformation aside from the interaction with Rb. However, studies on the oncogenic function of HPV E7 have been limited by its poor immunoreactivity. In this report, we show that the fixation of purified recombinant HPV E7 on blotted nitrocellulose membrane with glutaldehyde markedly enhanced the immunoreactivity of HPV E7 protein. Using HeLa and Caski cell line which are infected with HPV 18 and HPV 16, respectively, we demonstrated that native HPV E7 proteins also could be detected by this method. These results therefore can provide the experimental conditions for detection of HPV E7 proteins with greater sensitivity and may help to analyze E7 functions.

Keywords: glutaral; immunoblotting; immunologic techniques; oncogene proteins, viral, papillomavirus, human

Introduction

Human papillomavirus (HPV) is strongly associated with human cancers including most of cervical cancers (Bosch *et al.*, 1995) and approximately 20% of oral cancers (Gillison *et al.*, 2000). Of more than 25 HPV types known to infect the genital tract, HPV types 16 and 18 have been detected in 50-70% of cervical carcinomas worldwide (Walboomers *et al.*, 1999; zur Hausen, 2000). HPV induces cervical cancer largely through the expression of two viral oncoproteins, E6 and E7. The E6 protein binds and degrades p53 by ubiquitin-dependent proteolysis, which leads to a loss of the cellular protein responsible for maintaining genomic stability (Scheffner *et al.*, 1990). The E7 protein binds to under-phosphorylated retinoblastoma protein (Rb) and inactivates it by causing the release of active E2F, which leads to cell cycle progression regardless of cyclin dependent regulation (Dyson *et al.*, 1989; Munger *et al.*, 1993). Although the identification of cellular targets for HPV E7 has provided a crucial understanding on how HPV causes the disruption of normal cell cycle, there are still unresolved questions on the expression level of HPV E7 during HPV-induced carcinogenesis. However, the investigation of interactions between HPV E7 and its binding partners quantitatively have been hampered by the difficulty in detecting HPV E7 at protein level. Here, we present a sensitive detection method for HPV E7. Our results can provide a valuable tool in exploring cellular function of E7 and HPV-mediated carcinogenesis.

Materials and Methods

Purification of HPV E7

To purify recombinant HPV-16 and -18 E7 proteins, cDNAs of HPV-16 and -18 E7 were cloned into pET-15b vector (Novagen), suitable for N-terminal histidine hexamer tagging. Recombinant proteins were expressed in BL21 (DE3) bacterial cells and purified by Ni-affinity chromatography (Qiagen) according to manufacture's instructions. Protein amount was quantitated by BCA method (Pierce) and purity was evaluated by 15% SDS-PAGE and coomassie staining.

Immunodetection of recombinant HPV E7 protein

The resolved recombinant HPV-16 and -18 E7 proteins in 15% SDS-PAGE were transferred to NC membranes (Schleicher and Schuell, Amersham Biosciences). The NC membranes were washed with distilled water and immediately treated with glutaldehyde (ICN Biomedical Inc.) or formaldehyde (Sigma) at room temperature for varying time period and concentrations. Glutaldehyde or formaldehyde was di-

luted with detergent-free Tris-buffered saline (TBS). After each treatment, glutaldehyde or formaldehyde was removed by washing with distilled water. Subsequently, the membranes were blocked with TBS containing 5% nonfat dry milk and 0.1% Tween 20 at room temperature for 1 hour, and then probed with 1:200 diluted anti-HPV-16 and -18 E7 antibodies (Santa Cruz Biotechnology) and 1:5000 diluted peroxidase-conjugated secondary antibody (Pierce).

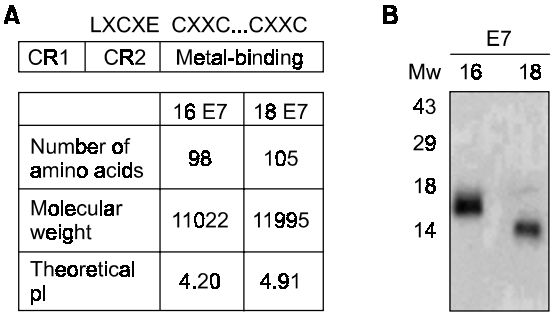
Immunodetection of HPV E7 in cell extracts

To detect HPV-16 and -18 E7 from Caski and HeLa cell lines, cells were plated at 2×10^6 cells per 100 mm-dish in DMEM with 10% FBS. After 16 h of incubation, cells were harvested and resuspended with lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM DTT, 2 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml trypsin inhibitor). The cell lysates were allowed to incubate on ice for 30 min, and centrifuged at 12,000 *g* for 10 min at 4°C. 80 μg of crude extract was boiled for 10 min with 2 sample buffer (120 mM Tris, pH 7.9, 4% SDS, 0.02% Bromophenol blue, 20% glycerol, 2% 2- mercaptoethanol) and resolved in 15% SDS-PAGE. Transferred membranes were treated with 0.5% glutaldehyde at room temperature for 30 min and probed by anti-HPV-16 and -18 E7 antibodies (Santa Cruz Biotechnology). Anti-actin antibody (Santa Cruz Biotechnology) was used as a control.

Results and Discussion

HPV-16 and -18 E7 proteins are small acidic proteins composed of 98 and 105 amino acids with 4.2 and 4.91 of theoretical pI, respectively (Figure 1A). Previous studies revealed that the fixation of NC membrane with glutaldehyde was able to strengthen the retention of small acidic proteins on NC membrane and to enhance the sensitivity of western blotting for these proteins (Van Eldik *et al.*, 1984; Mizzen *et al.*, 1996). Indeed, through the Ponceau S staining, we have observed that transferred recombinant HPV-16 and -18 E7 proteins were rapidly disappeared on NC membrane. Accordingly, we have thought that the fixation of NC membrane may be required to detect HPV E7 proteins.

To test whether the glutaldehyde fixation of blotted membrane could improve the sensitivity of immunodetection, we purified HPV-16 and -18 E7 proteins using Ni-affinity chromatography. The purified proteins were resolved by 15% SDS-PAGE and validated by Coomassie staining (Figure 1B). HPV-18 E7 protein was transferred to NC membrane, and the blotted membrane was treated with glutaldehyde or formaldehyde. The glutaldehyde fixation of NC membrane dramatically enhanced the immunoreactivity for HPV-18 E7, and this effect was proportional to glutaldehyde concentration and treatment time (Figure 2A and C). We found that the treatment of 0.5% glutaldehyde for 30 min gave the optimum result for HPV-18 E7 detection (Figure 2A and C). In contrast to



	16 E7	18 E7
Number of amino acids	98	105
Molecular weight	11022	11995
Theoretical pI	4.20	4.91

Figure 1. (A) The structure and physicochemical properties of HPV-16 and -18 E7. Alignment of protein sequences of HPV E7 show sequence similarity between various HPV types in conserved region 1 (CR1), conserved region 2 (CR2), and C-terminal zinc binding domain. Molecular weight and theoretical pI value of each protein is deduced from amino acids using ProParam tool; http://www.expasy.ch/cgi-bin/protparam. (B) Purification of recombinant HPV-16 and -18 E7 proteins. After nickel affinity chromatography, purified proteins were resolved in 15% SDS-PAGE and visualized by Coomassie staining.

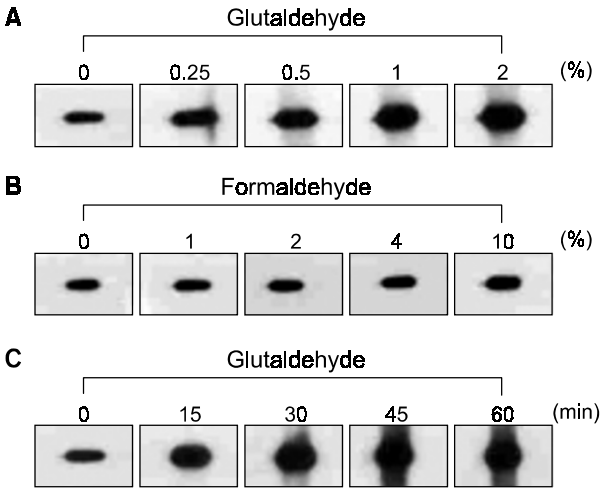


Figure 2. Increased immunoreactivity of recombinant HPV-18 E7 by glutaldehyde fixation of blotted nitrocellulose membrane. The effect of glutaldehyde (A) or formaldehyde (B) fixation on immunoreactivity to HPV-18 E7 was analyzed. Each membrane strip was treated with 0.25, 0.5, 1 and 2% of glutaldehyde or 1, 2, 4 and 10% of formaldehyde. NT, not treated. (C) Membrane strips were treated with 0.5% of glutaldehyde for 15, 30, 45 and 60 min.

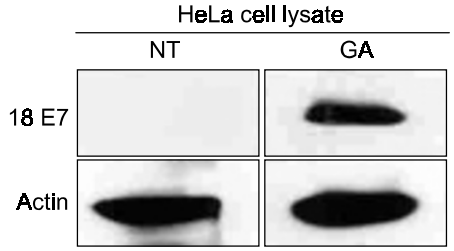


Figure 3. Detection of native HPV-18 E7 from HeLa cell lysate. Membrane strips were treated with 0.5% of glutaldehyde for 30 min and then probed with anti-HPV 18 E7 antibody and anti-actin antibody. NT, not treated; GA, glutaldehyde.

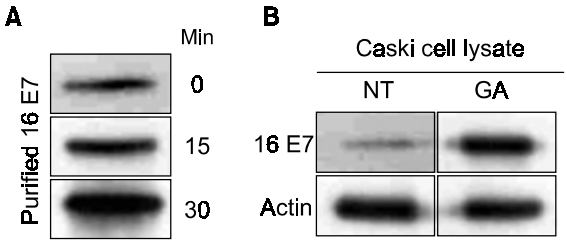


Figure 4. Enhanced immunoreactivity of purified HPV-16 E7 by glutaldehyde fixation (A) and native HPV-16 E7 from Caski cell lysate (B). Membrane strips were treated with 0.5% glutaldehyde for indicated time (A) and for 30 min (B). NT, not treated; GA, glutaraldehyde.

glutaldehyde fixation, formaldehyde failed to show the increased immunoreactivity (Figure 2B), suggesting stronger fixation might be required for improving immunodetection of HPV-18 E7.

Next, we tested whether native HPV-18 E7 could be detected by glutaldehyde fixation method. We found that HPV-18 E7 protein expressed in HeLa cell was able to be detected by the addition of glutaldehyde fixing procedure. When glutaldehyde fixation step was skipped, no HPV-18 E7 band could be detected (Figure 3). However, control immunoblotting with anti-actin antibody did not show any changes in band intensity, indicating that the increased retention of transferred protein by glutaldehyde is not the result of nonspecific fixation of proteins, but specific for HPV-18 E7. To apply this method in detecting E7 from other types of HPV, especially HPV 16 which is found most frequently in cervical cancer, we tested HPV-16 E7 protein using identical conditions. As shown Figure 4, similar to HPV-18 E7, glutaldehyde treatment resulted in the increased sensitivity for recombinant HPV-16 E7 proteins (Figure 4A) as well as native HPV-16 E7 from Caski cells (Figure 4B). These results suggest that glutaldehyde fixation could be helpful to detect all types of HPV E7 proteins.

Although it has been postulated that hydrophobic interactions play a role in binding of proteins to membrane, the exact mechanism of the interactions between protein and NC membrane is not clearly understood (Hoffman *et al.*, 1991). Efficient initial binding and retention of proteins to membrane during western procedures is important to successful detection. Several methods have been known to enhance the initial binding of proteins to NC membrane (Swerdlow *et al.*, 1986), but method of improving the protein retention on membrane is little known. Moreover, milk and nonionic detergent, such as Triton X-100, Nonidet P-40, Tween 20, used during blocking and washing procedure, also remove proteins from membrane (Hauri *et al.*, 1986; DenHollander *et al.*, 1989). Glutaldehyde, which is commonly used as a fixative for immunohistochemical staining and electron microscopic observation, forms extended cross-links of proteins *via* Schiff's base-catalyzed reaction (Johnson *et al.*, 1987). Glutaldehyde may help to maintain NC membrane-protein interaction and thus to increase the detection sensitivity of transferred protein.

We found that the difficulty in detecting HPV E7 is due more to the failure to stable retention on NC membrane of E7 protein than to the poor quality of antibody. Based on our results, glutaldehyde fixation method could help to quantitate protein expression level of HPV E7 in cervical cancer specimens as well as cell lines. Therefore, this method could contribute the investigation of oncogenic roles of HPV E7 at the molceular level.

Acknowledgements

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References

Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995;87:796-802

DenHollander N, Befus D. Loss of antigens from immunoblotting membranes. J Immunol Methods 1989;122:129-35

Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989;243:934-7

Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH,

Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20

Hauri HP, Bucher K. Immunoblotting with monoclonal antibodies: importance of the blocking solution. Anal Biochem 1986;159:386-9

Hoffman WL, Jump AA, Kelly PJ, Ruggles AO. Binding of antibodies and other proteins to nitrocellulose in acidic, basic, and chaotropic buffers. Anal Biochem 1991;198:112-8

Johnson TJ. Glutaraldehyde fixation chemistry: oxygen-consuming reactions. Eur J Cell Biol 1987;45:160-9

Mizzen CA, Cartel NJ, Yu WH, Fraser PE, McLachlan DR. Sensitive detection of metallothioneins-1, -2 and -3 in tissue homogenates by immunoblotting: a method for enhanced membrane transfer and retention. J Biochem Biophys Methods 1996;32:77-83

Munger K, Phelps WC. The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim Biophys Acta 1993;1155:111-23

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990;63:1129-36

Swerdlow PS, Finley D, Varshavsky A. Enhancement of immunoblot sensitivity by heating of hydrated filters. Anal Biochem 1986;156:147-53

Van Eldik LJ, Wolchok SR. Conditions for reproducible detection of calmodulin and S100 beta in immunoblots. Biochem Biophys Res Commun 1984;124:752-9

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12-9

zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690-8