[Frontiers in Bioscience 11, 1540-1548, May 1, 2006]

Alteration of Rb binding to HPV 18 E7 modified by transglutaminase 2 with different type of polyamines

Ju-Hong Jeon, Sung-Yup Cho, Chai-Wan Kim, Dong-Myung Shin, Gi-Yong Jang, Hye-Jin Lee, Heun-Soo Kang, Sang-Chul Park and In-Gyu Kim

Department of Biochemistry and Molecular Biology/ Aging and Apoptosis Research Center (AARC), Seoul National University College of Medicine, Seoul 110-799, Korea

TABLE OF CONTENTS

1. ABSTRACT

High-risk human papillomavirus (HPV) E7 is a major oncoprotein that plays a crucial role in the development of cervical cancer. A previous study showed that transglutaminase (TGase) 2 catalyzes the incorporation of polyamines into HPV 18 E7 protein, and thereby diminishes its ability to bind Rb. Therefore, TGase 2 activity may be implicated in a suppressive function of host against HPV-induced carcinogenesis. To better understand the nature of polyamination of HPV 18 E7, we investigated the Rb binding of E7 polyaminated *in vitro* with different type of polyamines. The incorporation of spermine diminished the Rb binding of E7 more profoundly compared with that of spermidine, suggesting that either the additional positive charge or a steric effect or both may have altered the chemical or structural properties of the protein. In addition, the treatment of either spermidine or spermine in cultured cell system reduced the ability of E7 to inactivate Rb with a TGase activity-dependent manner. Spermine was more effective in inhibiting E7 activity than spermidine. These results may provide the basis for future investigation aiming at delineating the significance of polyamine metabolism on HPV E7 functions.

2. INTRODUCTION

Among more than 120 HPV types identified, the certain subtypes of human papillomaviruses (HPVs) classified as high-risk types are major etiological factors in the development of cervical cancer (1). Cancer development associated with HPV infection seems to depend on the expression of two viral oncoproteins, E6 and E7, which interact with a number of cellular proteins leading to either abrogation of cell cycle control or induction of chromosomal abnormality (2). However, underlying mechanisms by which high-risk HPVs induce malignant progression have not been clearly understood at molecular levels, even though epidemiological evidence has been accumulated to support a role of HPVs in cancer development (3-6). In addition, the reason why HPV 16 is the most prevalently detected in cervical cancer specimens has been largely unknown.

Despite HPV E7 is a relatively small size of protein composed of approximately 100 amino acids, it has been found to interact with a number of cellular proteins, including cell cycle regulators, glycolytic enzymes and transcriptional modulators, suggesting its pleiotropic roles

in cancer progression (2, 7). The amino-terminal region of E7, termed conserved region 1 and 2 (CR1 and CR2), shares structural and functional homology with adenovirus E1A protein and simian virus 40 large T antigen (2, 7). An LXCXE motif within the CR2 domain of E7 is crucial for binding and inactivating retinoblastoma tumor suppressor (Rb) (2, 7). The carboxyl-terminal zinc-binding domain of E7 is also required for efficient Rb binding and subsequent E2F dissociation (2, 7). Several lines of experimental and clinical evidence indicate that HPV E7 has a crucial role in cervical cancer development (8-10). HPV 58 variants that carry specific polymorphisms of E7 but none of E6 are prone to develop cervical cancer (8). It was also reported that a suppression of E6 expression by means of RNA interference causes the reduction of HPV-infected cervical cancer cells, whereas the suppression of E7 expression induced apoptotic cell death (9). Finally, a progression to microinvasive cervical cancer was observed only in transgenic mice overexpressing E7 gene but not in those overexpressing E6 gene (10). Therefore, understanding the regulation of E7 activity seems to be important to elucidate HPV-induced carcinogenesis.

Transglutaminase 2 (TGase 2) belongs to a family of calcium-dependent enzymes that catalyze the transfer of gamma-acyl moiety of a peptide-bound glutamine residue to the epsilon-amino group of a peptide-bound lysine residue, producing N-epsilon-(L-gamma-glutamyl)-L-lysine isopeptide bond (11). The enzyme also catalyzes the incorporation of the primary amine group of a polyamine into a peptide-bound glutamine residue (11). Naturally occurring polyamines, such as putrescine, spermidine and spermine, are small polycationic molecules that are derived from L-arginine and L-methionine via a series of enzymatic reactions (12). They differ in the number and location of positively charged amine groups joined by methylene groups. Among three types of polyamines, spermidine and spermine are mainly found in human tissues (13). The physicochemical characteristics of polyamines indicate that the extent of positive charge as well as the size of polyamines may alter the molecular function(s) of polyaminated proteins. Although the protein polyamination is less appreciated so far, emerging evidence suggest that this modification contributes to the regulation of biological activity of target proteins (14).

Previously, we identified that HPV 18 E7 is a TGase 2-interacting partner using yeast two-hybrid screen with HeLa cell library (15). HPV 18 E7 protein can be polyaminated by TGase 2 and the polyaminated E7 loses its ability to inactivate Rb. However, HPV 16 E7 is not affected by TGase activity, probably due to lack of glutamine residues at the site where polyamination takes place in HPV 18 E7. These findings lead us to hypothesize that the inability of TGase 2 to inactivate HPV16 E7 may explain the high prevalence of HPV16 in cervical cancer, even though a question remains as to whether the polyamination is a physiological mechanism for host cell to interfere with the function of E7. To further characterize the nature of E7 polyamination by TGase 2, we investigated the binding of Rb to E7 proteins that have been enzymatically modified *in vitro* by different type of polyamines. No significant difference in the polyamination level of E7 was found when either spermine or spermidine was used as a TGase 2 substrate. However, spermine-derivative E7 exhibited a binding to Rb weaker than spermidine-derivative E7 did, suggesting that the polyamine metabolism in cervical epithelia may significantly affect the role of TGase 2 on E7 function.

3. MATERIALS AND METHODS

3.1. Synthesis of biotinylated polyamines

A protocol to synthesize biotinylated spermine (BSPM) was previously described (16). In this study, biotinylated spermidine (BSPD) was synthesized as follows. Di-*tert*-butyl dicarbonate in dry dioxane (3.4 mmol) was added to spermidine 1 solution (1.7 mmol) in dioxane dropwise over a period of 2.5 h. The reaction mixture was stirred for 22 h and the solvent was removed. The resulting oily residue was taken up in H_2O and extracted with methylene chloride. After washing with H_2O, compound 2 was purified by column chromatography (silica gel, 230-400 mesh, Merck) in solvent A, ammonium hydroxide:methanol:dichloromethane (1:5:20). For the amidation, *N*,*N*-di-*tert*-butyl dicarbonate spermidine 2 (0.4 mmol), biotin (0.4 mmol), catalytic amount of 1-hydroxybenzotriazol and 1-(3-dimethylaminopropyl-3-ethyl carbodiimid) chlorohydrate (0.89 mmol) were added to a flame dried round-bottom flask. The reaction was stirred for 24 h at room temperature and extracted with 5% methanol:trichloromethane. After washing with H_2O and drying over Na_2SO_4, pure product 3 was gained by column chromatography in solvent B, methanol:dichloromethane (1:4). Deprotection of compound 3 (52.5 micromol) was performed with 1 M ethereal HCl in methanol in an ice-water bath. By adding HCl, a pale yellow color of the solution gradually thickened, then the reaction was allowed to warm to room temperature with vigorous stirring and its products were analyzed by thin layer chromatography: the analysis showed a total conversion of compound 3 into the lower moving product 4 (R_f 0.12, solvent A). The solvents were removed and pure product 4 was obtained as a white solid by recrystallization with methanol:ethylacetate. Thin layer chromatography was performed using pre-coated silica gel plate (60 mesh, Merck) with fluorescence indicator, and compounds were detected by heating the plates sprayed with 10% phosphomolybdic acid in ethanol. [^{1}H] and [^{13}C] NMR spectra were recorded with a JEOL JNM EX-400 spectrometer. Chemical shifts were referenced to solvent signal of methanol for [^{1}H] and [^{13}C] NMR.

3.2 Preparation of recombinant proteins

Baculovirus Expression System (Pharmingen) was employed to produce recombinant TGase 2 as previously described (17). Briefly, baculovirus expressing full-length human TGase 2 was produced by the co-transfection of pAcHLT-TGase 2 and BaculoGold DNA into Sf21 cells. Recombinant protein was purified with a nickel-nitrilotriacetic acid column according to manufacture's instructions (Qiagen), monitoring TGase 2 activity by [^{14}C] putrescine incorporation assay (8). HPV 18 E7 or Rb was expressed in BL21 *Escherichia coli* cells

as a His-tagged (pET-15b, Novagen) or a glutathione-S-transferase (GST) fusion protein (pGEX-4T-1, Amersham Biosciences), respectively, as previously described (15). Proteins were purified with a nickel-nitrilotriacetic acid column or a glutathione-Sepharose column chromatography (15). The purity of proteins was validated by 8-15% SDS-PAGE with Coomassie staining.

3.3. Microtiter plate transamidation assays

A solid-phase microtiter plate assay was developed in this study to assess the reaction between protein and BSPD, BSPM or biotinylated pentylamine (BP; Pierce). *N,N'*-dimethylcasein (50 microliter, 0.1 mg/ml; Sigma) was added to a 96-well microtiter plate, and incubated with both TGase 2 and BSPD or BSPM in buffer R (50 mM Tris-Acetate, pH 7.5, 10 mM $CaCl_2$, 5 mM DTT, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA) for 1 h at 37°C. After removing the reaction mixture from the well and washing the well three times with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, the extent of biotinylation in *N,N'*-dimethylcasein was assessed by a subsequent reaction with horseradish peroxidase (HRP)-conjugated streptavidin (Zymed). *O*-phenylenediamine dihydrochloride solution (Sigma) was added for color development, which was stopped by 1 N H_2SO_4. The absorbance at 490 nm was measured using a microplate spectrophotometer (Molecular Devices).

3.4. Cytochemical transamidation assays

Human diploid fibroblasts were plated onto glass coverslips placed in the well of a 6-well plate and cultured for 16 h at 37°C. The cells were incubated with 1 mM BSPD or BSPM for 1 h at 37°C in the presence of A23187 calcium ionophore (Calbiochem). Under the experimental condition, cystamine at 0.5 mM was added to culture media to validate TGase activity-dependent incorporation of BSPD or BSPM. The cells were fixed with 4% formaldehyde in PBS for 15 min, and then washed with PBS. The cellular membrane was permeabilized by treating with PBS containing 0.1% Triton X-100 for 5 min at room temperature. The cells were incubated in 3% BSA in PBS at room temperature for 30 min and the extent of BSPD or BSPM incorporated into the cellular proteins was assessed using Texas Red-conjugated streptavidin (Jackson ImmunoResearch Laboratory). Cells were visualized and photographed with a confocal laser scanning microscope (Bio-Rad).

3.5. HPV 18 E7 polyamination assays

His-tagged HPV 18 E7 was incubated with TGase 2 and BSPD or BSPM in buffer R. To ascertain that BSPD or BSPM were indeed incorporated in the protein molecule, SPD or SPM was employed to compete with BSPD or BSPM, respectively. In addition, polyamines (SPD or SPM) incorporation into HPV 18 E7 was verified by competition assay in which BP incorporation into E7 competes with SPD or SPM. The reaction mixtures dialyzed against buffer D (10 mM Tris-Cl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl). The dialysate was subjected to 15% SDS-PAGE analysis, probed with HRP-conjugated streptavidin (Zymed), and subsequently developed following the standard protocol of chemiluminescence-based detection (Pierce). At the same time, the dialysate was coated in a 96-well plate and the extent of BSPD, BSPM or BP incorporated into HPV 18 E7 was probed as described earlier.

3.6. Solid-phase binding assays

The binding of Rb to E7 was evaluated by the solid-phase ELISA as previously described (15). Briefly, E7 protein modified with SPD or SPM by TGase 2 was generated as described earlier. A microtiter plate was coated with E7 or polyaminated E7 (3 microgram/ml), respectively, and overcoated with 5% BSA in PBS. Following incubation with GST-Rb protein (aa 372-787) at various concentrations for 1 h at room temperature, the bound GST-Rb was quantitated by using in sequence anti-GST monoclonal antibodies (Santa Cruz) and HRP-conjugated goat anti-mouse IgG antibody (Pierce).

3.7. Cell culture and transfection experiments

HepG2 (human hepatocarcinoma) cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Hyclone), penicillin (100 Unit/ml), streptomycin sulfate (100 microgram/ml) and glutamine (2 mM). The cells were transfected with E2F-luciferase reporter plasmids and E7 expression constructs in pcDNA3 (Invitrogen) using using LipofectAMINE (Invitrogen) as previously described (15). Empty pcDNA3 vector was used to adjust the equal DNA amount. pSV-beta-galactosidase (Promega) was co-transfected as an internal control to normalize luciferase activity. After 20 h of transfection, SPD or SPM at 0.5-1 mM was treated for 4 h, and luciferase activity was assayed using a commercial kit according to manufacture's instructions (Promega). For inhibiting the activity of TGase 2, the cells were incubated with 0.5 mM cystamine during the treatment with SPD or SPM.

4. RESULTS

4.1. Synthesis of biotinylated polyamines as activity-based probes for TGase 2

We synthesized the biotinylated derivatives of spermidine and spermine that are major polyamines in mammalian cells (12-13). While BSPM was prepared as previously described (16), BSPD was synthesized according to the scheme shown in Figure 1A. Protection of NH_2 with di-*tert*-butyl dicarbonate produced compound 2 with 78% yield. The diagnostic NMR signals for compound 2 was observed at 1.45, 1.42 ppm and 28.6 ppm in the [^{1}H] and [^{13}C] NMR spectrum, respectively. The amidation step was performed by using 1-(3-dimethylaminopropyl-3-ethyl carbodiimid) chlorohydrate and 1-hydroxybenzotriazol. Compound 3 showed a triplet at 4.27 ppm corresponding to ring proton of biotin. In deprotection step, mild conditions had to be used to prevent both molecular deformation and biotin cleavage. Concerning compound 4, the *N*-Boc signals disappeared and a triplet at 2.34 ppm, corresponding to the alpha proton of NH_2, appeared. The molecular structures of BSPD and BSPM are schematically presented in Figure 1B.

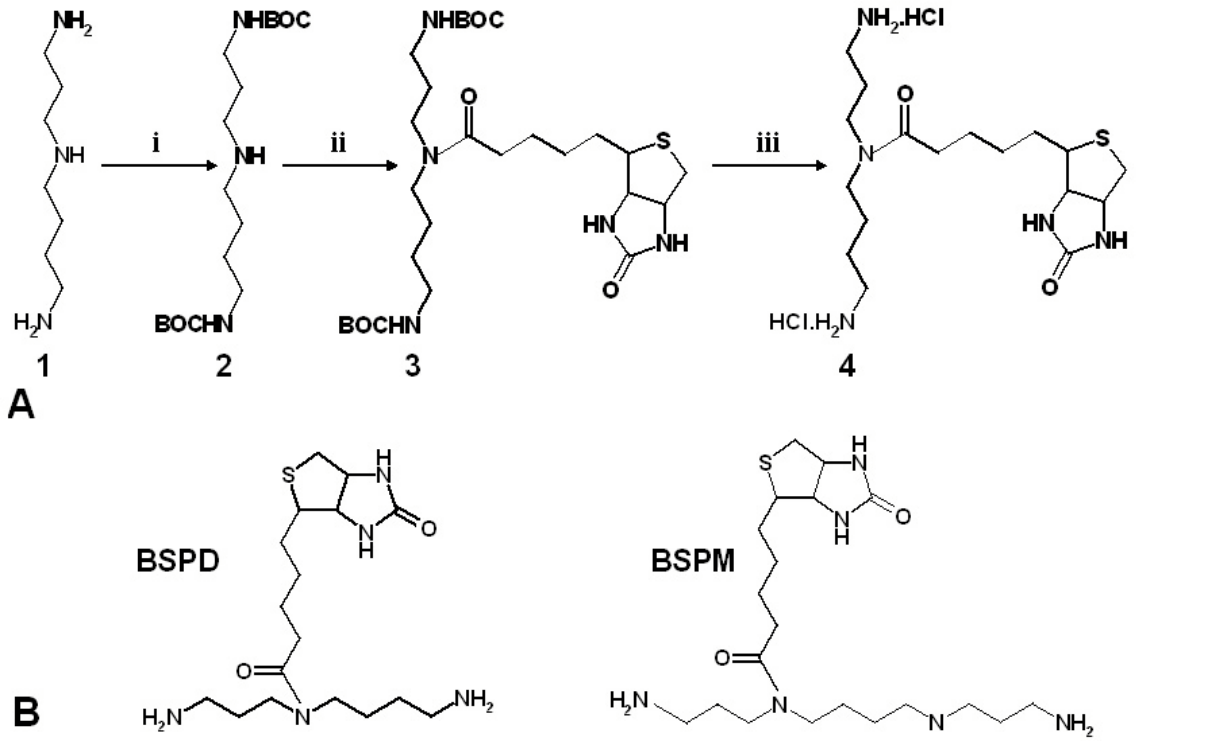

Figure 1. Activity based probes for TGase 2. (A) Schematic representation for the synthesis of biotinylated spermidine. Step **i**, protection of amine groups; reaction of di-*tert*-butyl dicarbonate and 1, 4-dioxane for 22 h at RT. Step **ii**, biotinylation; reaction mixture comprises biotin, 1-(3-dimethylaminopropyl-3-ethyl carbodiimid) chlorohydrate, 1-hydroxybenzotriazol and dimethylformamide for 24 h at room temperature. Step **iii**, deprotection of amine groups; 1M ethereal HCl and methanol for 1 h at 0°C. Each reaction was monitored by thin layer chromatography using pre-coated silica gel plate (60 mesh) with fluorescence indicator, and reaction products were purified by column chromatography using silica gel columns (230-400 mesh). (B) Schematic representations of biotinylated spermidine (BSPD, left) and biotinylated spermine (BSPM, right).

4.2. Properties of biotinylated polyamines as TGase 2 substrates

To prove that TGase 2 specifically catalyzes transamidation reaction with synthetic polyamines, a solid-phase transamidation assay was performed in which BSPD or BSPM were allowed to react with *N,N'*-dimethylcasein in the presence of TGase 2. BP, a sensitive activity-based probe for TGase, was used as a positive control (16). The results shown in Figure 2A indicate that: 1) TGase 2 is able to catalyze the incorporation of BSPD and BSPM into *N,N'*-dimethylcasein; 2) the extent of incorporation depends upon the polyamine concentrations. Under the experimental conditions employed in this study, the extent of polyamination appears to be slightly higher with BSPD than with BSPM (Figure 2A). However, there was no significant difference in incorporation level between BSPD and BSPM. Lineweaver-Burk plot demonstrated that Km values for BP, BSPD or BSPM were calculated to be approximately 10, 96 or 112 microMol, respectively (data not shown). These results demonstrate that both BSPD and BSPM may be used in polyamination studies.

To show that biotinylated polyamines can serve as substrates for intracellular TGase 2 in protein polyamination reactions, cytochemical fluorescence staining experiments were performed. Human diploid fibroblasts were incubated with biotinylated polyamines and polyaminated proteins were probed using Texas Red-conjugated streptavidin. Cystamine was added to the reaction system to inhibit TGase 2 activity, and thus to serve as a negative control. We found no morphological changes of the cells according to the treatment with reagents employed in the experiment. As shown in Figure 2B, endogenous TGase 2 incorporated both biotinylated polyamines into cellular proteins. As expected, the addition of cystamine yielded no detectable polyamination of proteins in human diploid fibroblasts. These results indicate that both BSPD and BSPM can be intracellular substrates for TGase 2.

4.3. Effect of E7 polyamination on Rb binding in vitro

Previously, we observed that polyaminated HPV 18 E7 protein loses partially its ability to bind Rb (15). Since spermidine and spermine are different in size and charge state (Figure 1B), we hypothesized that both BSPD and BSPM may exert different effects following their incorporation into E7. To test this hypothesis, competition tests were carried out in which either BSPD or BSPM was allowed to compete with free spermidine or spermine for the polyamination of HPV 18 E7 in the presence of TGase 2. The incorporation of BSPD or BSPM into HPV18 E7 decreased with increasing concentrations of free spermidine

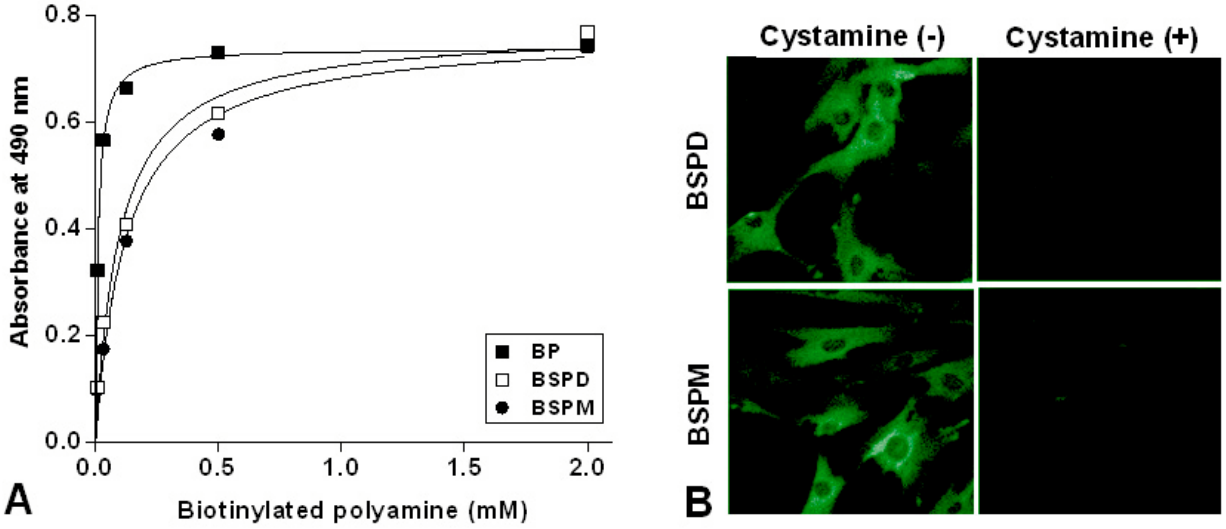

Figure 2. Evaluation of biotinylated polyamines as substrates for polyamination of proteins by TGase 2. (A) Incorporation of BP, BSPD or BSPM into *N,N'*-dimethylcasein catalyzed by TGase 2. A solid-phase microtiter plate transamidation assay was developed to measure the extent of polyamination at various polyamine concentrations. The extent of polyamination was probed by HRP-conjugated streptavidin. *O*-phenylenediamine dihydrochloride solution was added and the absorbance at 490 nm was measured. The figure presents typical data in three independent experiments. (C) Cytochemical staining of BSPD or BSPM incorporated into cellular proteins. Human diploid fibroblast was incubated with either BSPD or BSPM and probed using Texas Red-conjugated streptavidin. Cystamine was used to inhibit TGase 2 activity in the system.

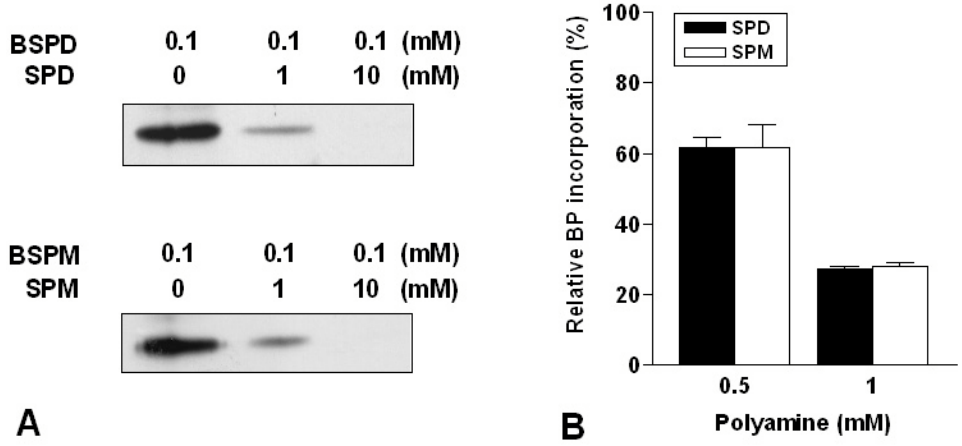

Figure 3. Analysis of HPV 18 E7 polyamination. (A) Visualization of polyaminated HPV 18 E7. E7 was expressed in BL21 cells as a polyhistidine-tagged form and purified with nickel affinity resin. The polyamination of E7 with either BSPD or BSPM (0.1 mM each) was catalyzed by TGase 2. For competition experiments, the above reaction was performed in the presence or in the absence of SPD or SPM at various concentrations, respectively. The reaction mixture was analyzed by 15% SDS-PAGE and the incorporated BSPD or BSPM was probed with HRP-conjugated streptavidin as indicated in Materials and Methods. (B) E7 was subjected to TGase reaction with BP in the presence or in the absence of SPD or SPM. The reaction mixture was analyzed by a microtiter plate assay and the incorporated BP was probed with HRP-conjugated streptavidin as indicated Materials and Methods.

or spermine (Figure 3A). The Western blot analysis of polyaminated E7 did not reveal any significant difference in the extent of polyamination between the two polyamines (Figure 3A). To validate SPD or SPM incorporation into E7 further, SPD or SPM incorporation was assessed by the competition assays in which BP was allowed to compete with SPD or SPM. BP incorporation was significantly reduced in the presence of SPD or SPM with a dose-response manner (Figure 3B). Indeed, the extent of competition did not exhibit any difference between two polyamines, indicating that two natural polyamines are similarly incorporated into HPV 18 E7.

We also investigated by an ELISA system to

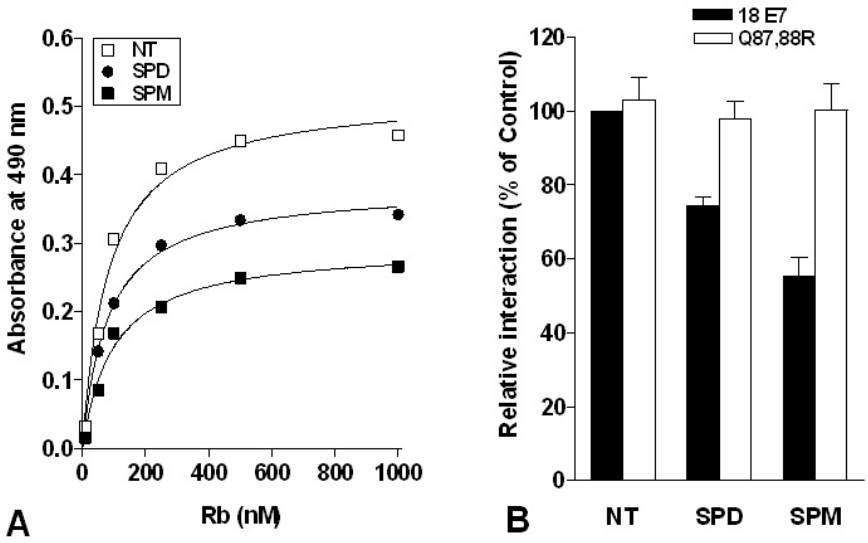

Figure 4. Binding of Rb to polyaminated HPV 18. (A) HPV 18 E7 was incubated with spermidine or spermine in the presence or in the absence of TGase 2. The wells of a microtiter plate were coated with unmodified E7 protein or E7 polyaminated with spermidine or spermine (3 microgram/ml) and incubated with GST-Rb at various concentrations. Bound GST-Rb was detected using anti-GST monoclonal antibodies coupled with HRP-conjugated goat anti-mouse IgG antibodies. The chromogenic substrate *O*-phenylenediamine dihydrochloride developed a color the intensity of which was evaluated by measuring the absorbance at 490 nm. The figure presents typical data in three independent experiments. (B) HPV 18 E7 or its mutant Q87,88R protein was incubated with spermidine or spermine in the presence (SPM or SPD) or in the absence (NT) of TGase 2. Bound GST-Rb was evaluated using a solid-phase ELISA as described in Methods and Materials. Q87,88R represents a mutant of HPV18 E7 which lacks the sites for polyamination. Rb binding to the polyaminated E7 proteins is expressed as a relative value to that of the wild-type. The figure shows the mean values and standard deviation based on three independent experiments.

verify whether the Rb binding of E7 was differentially affected by the type of incorporated polyamine (15). In this test, HPV 18 E7 was polyaminated with either spermidine or spermine in the presence of TGase 2. A microtiter plate was first coated with either E7 or polyaminated E7, GST-Rb protein (aa 372-787) was then added and the plate was incubated. E7 binding to GST-Rb was assayed by anti-GST antibodies. GST-Rb binding to native or polyaminated E7 increased proportionally with the increase of the Rb concentration reaching a plateau at approximately 500 nM Rb (Figure 4A). E7 polyamination diminishes its ability to bind Rb, this effect being stronger with the larger polyamine, spermine, than with the smaller polyamine, spermidine (Figure 4A). Maximal Rb-binding to spermidine- or spermine-modified E7 was, in fact, approximately 75% and 55% of that to E7, respectively (Figure 4B). In order to corroborate the above observations, HPV 18 E7 mutant Q87,88R was also tested in this system (15). Rb binding of Q87,88R E7 was not affected by the presence of spermidine or spermine due to the mutation of polyamination sites (Figure 4B). These results strongly suggest that the charge, size, or both of incorporated polyamines is/are the major factor(s) to determine the extent of functional alteration of polyaminated E7.

4.4. Effect of E7 polyamination on transactivation of E2F activity in vivo

Since the ability of E7 to bind with Rb and subsequently to disrupt Rb-E2F interaction increases E2F transcriptional activity, the biological effect of E7 polyamination was investigated with E2F promoter-luciferase assays in cultured cells. HepG2 cells exhibited high level of TGase 2 expression and intrinsic *in situ* activity (18). The cells expressed with HPV18 E7 exhibited about 3-fold increase in E2F promoter activity (Figure 5A). On the other hand, when the cells overexpressing E7 were treated with SPD or SPM, E2F promoter activity was decreased in proportion to increasing SPD or SPM concentrations (data not shown). Consistent with the results obtained from *in vitro* experiments, SPM more effectively inhibited E7 function than SPD. Under the experimental condition, SPD or SPM did not affect intrinsic reporter activity. To verify these results further, we performed E2F promoter activity assays with cystamine, a TGase activity inhibitor, or Q87,88R E7. As shown Figure 5B, the cells treated with cystamine overcame the suppressive effects of both polyamines on E2F promoter activity. Moreover, the mutant E7 was affected by neither SPD nor SPM (Figure **5B**), indicating that suppressive effects of either SPD or SPM was dependent on TGase 2 activity.

5. DISCUSSION

The ability of TGase 2 to produce cross-linked proteins has been suggested that the enzyme serves as a final mediator of apoptotic pathways, leading to preventing the leakage of cellular constituents from dying cells (19). In addition, the pro-apoptotic roles of TGase 2 have been evidenced by several experimental results. TGase 2 induction was observed in the cells undergoing apoptosis

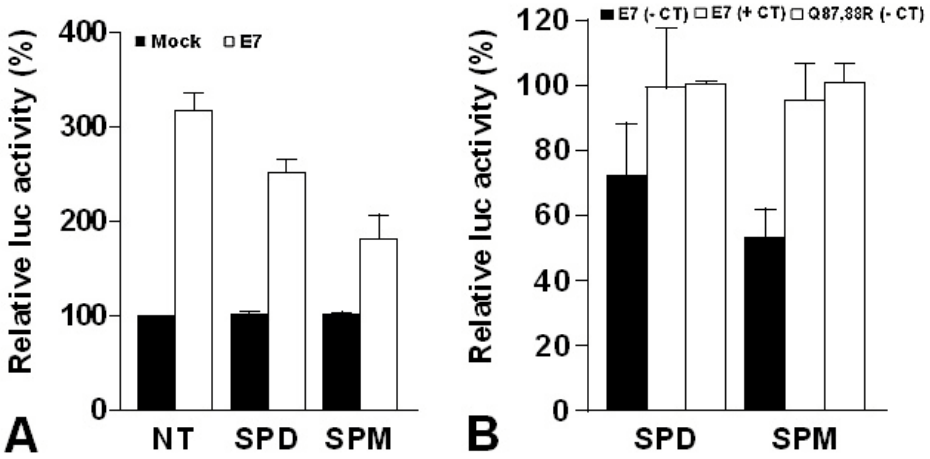

Figure 5. Alteration of HPV 18 E7 activity by treatment with SPD or SPM in HepG2 cells. A plasmid containing six consecutive E2F responsive elements (6×E2F) upstream of a luciferase gene was used as a reporter construct. HepG2 cells were transiently transfected with the reporter construct and pcDNA3-HPV18 E7 plus pCMV-beta-Gal. (A) After 20 h of transfection, the cells were treated with 0.5 mM SPD or SPM for 4 h. NT denotes no treatment with either SPD or SPM. Mock indicates empty pcDNA3 vector. Luciferase activity is normalized by beta-galactosidase activity, and expressed relative to the activity of the reporter construct alone. (B) HPV18 E7-transfected cells were treated 0.5 mM SPD or SPM in the presence or in the absence of 0.5 mM cystamine (CT) for 4 h. Polyamination-defective mutant Q87,88R E7 was used to assess the effect of 0.5 mM SPD or SPM on reporter activity. The figure shows the mean values and standard deviation based on four to six independent experiments.

(11). TGase 2 induction or overexpression enhanced susceptibility to apoptogenic stimuli in several cells (11, 19). However, the role of TGase 2 in apoptosis is still debatable. Mice with null mutations in the TGase 2 gene showed neither overt problems in apoptosis nor developmental abnormalities (20). Moreover, emerging evidence showed that TGase 2 functions as an anti-apoptotic factor under certain conditions (21-22). Accordingly, the precise role of TGase 2 in apoptotic pathway remains to be elucidated.

A covalent incorporation of polyamines into proteins is one of the post-translational modifications of proteins catalyzed by TGases. Current results may provide an insight into the physiological significance of polyamination of proteins (14). In addition, differential biochemical effects of the polyamines incorporated was exemplified by the histone modification catalyzed by TGase 2 (23). In this study, we showed that the type of incorporated polyamines may determine the extent of functional alteration of polyaminated proteins. Polyamination of HPV E7 may disturb the contact surface between Rb and E7 proteins, the incorporation of spermine being more effective than that of spermidine in Rb-polyaminated E7 interaction. Therefore, the extent of disturbance may depend on the charge or size (or both) of incorporated polyamines.

However, a question remains as to whether polyaminated E7 is present in HPV-infected mucosal epithelium of human subjects. Since in the cells a large portion of polyamines are sequestered by association with negatively charged molecules, such as nucleic acids or phospholipids (12-13), only a small portion of free polyamines would be available as amine donors for TGase 2. The data reported in this paper show that TGase 2 catalyzes the incorporation of spermine or spermidine into HPV E7 with similar rates. Thus, the relative intracellular concentration of free spermidine and spermine, which depends on certain physiological or pathological conditions, may be critical to determine the type of incorporated polyamine. However, this is not a simple issue due to the occurrence of a number of complex regulatory mechanisms to maintain polyamine concentration in living cells. Intracellular polyamine concentration is tightly regulated by biosynthesis, degradation and transport pathways (12-13, 24). Two inducible enzymes with extremely short half-life, ornithine decarboxylase and spermidine/spermine N1-acetyltransferase, are key regulatory enzymes for biosynthesis and degradation pathways, respectively (12-13). It was reported that although mice overexpress spermidine/spermine N1-acetyltransferase or ornithine decarboxylase, these animals tend to accumulate putrescine without significant changes in spermidine and spermine levels (25-26). These data suggest that the regulation of polyamine level is extremely complex and that the level of spermidine or spermine is more strictly regulated than that of putrescine. Together, additional studies such as that of establishing a sensitive, convenient method to assess the level of protein polyamination would facilitate future investigation to understand the role of TGase 2 in the modification and the regulation of HPV E7 functions.

Substrate availability may be a critical factor for TGase 2 action. TGase 2 catalyzes two consecutive nucleophilic displacement reactions. Despite of a common acylenzyme intermediate in TGase 2 reaction, reaction

products are divergent depending on nucleophile substrates that act as acyl acceptors. If primary amines are not available for TGase 2 reaction, water as an acyl acceptor can participate in the reaction to convert Gln to Glu residue. Thus, TGase 2 may catalyze the deamidation reaction of HPV 18 E7 depending on intracellular free polyamine level, which may enhance its oncogenic function. In addition, HPVs other than HPV 16 may exploit deamidation to escape from TGase 2 activity. Earlier works established that deamidation spontaneously occurs *in vivo*, and that the rates of deamidation depend on primary or three-dimensional structure, pH, temperature, ionic strength, buffer ions, and other solution properties (27). If the Glu-87 and -88 residues of HPV 18 E7 are vulnerable to spontaneous deamidation, TGase 2 would fail to modify it. Accordingly, the study with HPV 18 E7 at protein level may assist to understand its oncogenic function.

The mechanisms of how intracellular TGase 2 is regulated during viral infection have to be resolved to establish the role of the enzyme in suppressing HPV 18 E7 function. HPV replication in cervix is restricted to the differentiated strata of the mucosal epithelia where cell growth is arrested. Since epithelial cell differentiation is accompanied with intracellular calcium increase (28), TGase 2 seems to be readily activated during differentiation. Hence, the ability of HPVs to uncouple cellular proliferation and differentiation would be important for escaping from TGase 2 activity.

The findings that TGase 2 activity is increased in virus-infected cells suggest the potential role of TGase 2 during viral infection (29). Emerging evidence supports that TGase 2 can modulate viral life cycle and pathogenesis. In addition to the modification of HPV 18 E7, TGase 2 is also able to catalyze the post-translational modification of several viral proteins such as hepatitis C virus core protein (30), human immunodeficiency virus envelope glycoprotein gp41, gp120 and aspartyl protease (31-33). TGase 2 suppressed the ability of hepatitis C virus core protein to bind RNA, suggesting that TGase 2 activity may mediate a host response to suppress viral replication (30). In addition, a recent study provided some evidence that TGase 2 plays a protective role in the initial stages of liver fibrosis in hepatitis C virus-infected patients (34). Interestingly, TGase 2 can catalyze the conversion of hypusine to gamma-glutamyl-omega-hypusine in eIF-5A, a cellular co-factor for human immunodeficiency virus Rev. Because the hypusine of eIF-5A is a critical residue for human immunodeficiency virus RNA trafficking in coordination with human immunodeficiency virus Rev (35), it is probable that TGase 2 may interfere with the functional interaction between eIF-5A and human immunodeficiency virus Rev. It is thus conceivable that TGase 2 can function as an antiviral system against infection of viruses other than HPV.

6. ACKNOWLEDGEMENTS

We thank Dr. Y. D. Kim for critical comments on the manuscript. This work was supported by Korea Science and Engineering Foundation (KOSEF) through the Center for Aging and Apoptosis Research (R11-2002-097-07003-0 and R01-2005-000-10364-0). J.H.J., C.W.K., D.M.S., S.Y.C. and G.Y.J. were supported by the graduate program of BK21 project, administrated by the Ministry of Education and Human Resources Development. Ju-Hong Jeon, Sung-Yup Cho contributed equally to this paper.

7. REFERENCES

1. Clifford G.M., J.S. Smith, M. Plummer, N. Munoz & S. Franceschi: Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer* 88, 63-73 (2003)
2. Munger K. & P.M. Howley: Human papillomavirus immortalization and transformation functions. *Virus Res* 89, 213-228 (2002)
3. Woodman C.B., S. Collins, H. Winter, A. Bailey, J. Ellis, P. Prior, M. Yates, T.P. Rollason & L.S. Young: Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet* 357, 1831-1836 (2001)
4. Munoz N., F.X. Bosch, S. de Sanjose, R. Herrero, X. Castellsague, K.V. Shah, P.J. Snijders, C.J. Meijer & International Agency for Research on Cancer Multicenter Cervical Cancer Study Group: Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 348, 518-527 (2003)
5. Walboomers J.M., M.V. Jacobs, M.M. Manos, F.X. Bosch, J.A. Kummer, K.V. Shah, P.J. Snijders, J. Peto, C.J. Meijer & N. Munoz: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 189, 12-19 (1999)
6. Sherman M.E., A.T. Lorincz, D.R. Scott, S. Wacholder, P.E. Castle, A.G. Glass, I. Mielzynska-Lohnas, B.B. Rush & M. Schiffman: Baseline cytology, human papillomavirus testing, and risk for cervical neoplasia: a 10-year cohort analysis. *J Natl Cancer Inst* 95, 46-52 (2003)
7. Munger K., J.R. Basile, S. Duensing, A. Eichten, S.L. Gonzalez, M. Grace & V.L. Zacny: Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* 20, 7888-7898 (2001)
8. Chan P.K., C.W. Lam, T.H. Cheung, W.W. Li, K.W. Lo, M.Y. Chan, J.L. Cheung & A.F. Cheng: Association of human papillomavirus type 58 variant with the risk of cervical cancer. *J. Natl. Cancer. Inst* 94, 1249-1253 (2002)
9. Jiang M. & J. Milner: Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* 21, 6041-6048 (2002)
10. Riley R.R., S. Duensing, T. Brake, K. Munger, P.F. Lambert & J.M. Arbeit: Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Res* 63, 4862-4871(2003)
11. Lorand L. &, R.M. Graham: Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 4, 140-156 (2003)
12. Wallace H.M., A.V. Fraser & A. Hughes: A perspective of polyamine metabolism. *Biochem J* 376, 1-14 (2003)
13. Igarashi K. & K. Kashiwagi: Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* 271, 559-564 (2000)

14. Jeon J.H. & I.G. Kim: Role of protein modification mediated by transglutaminase 2 in human viral diseases. *Front Biosci* 11, 221-231 (2006)
15. Jeon J.H., K.H. Choi, S.Y. Cho, C.W. Kim, D.M. Shin, J.C. Kwon, K.Y. Song, S.C. Park & I.G. Kim, Transglutaminase 2 inhibits Rb binding of human papillomavirus E7 by incorporating polyamine. *EMBO J* 22, 5273-5282 (2003)
16. Jeon J.H., C.W. Kim, D.M. Shin, K. Kim, S.Y. Cho, J.C. Kwon, K.H. Choi, H.S. Kang & I.G. Kim: Differential incorporation of biotinylated polyamines by transglutaminase 2. *FEBS Lett* 534, 180-184 (2003)
17. Shin D.M., J.H. Jeon, C.W. Kim, S.Y. Cho, J.C. Kwon, H.J. Lee, K.H. Choi, S.C. Park & I.G. Kim: Cell type-specific activation of intracellular transglutaminase 2 by oxidative stress or ultraviolet irradiation: implications of transglutaminase 2 in age-related cataractogenesis. *J Biol Chem* 279, 15032-15039 (2004)
18. Jeon J.H., H.J. Lee, G.Y. Jang, C.W. Kim, D.M. Shin D.M., S.Y. Cho, E.J. Yeo, S.C. Park & I.G. Kim: Different inhibition characteristics of intracellular transglutaminase activity by cystamine and cysteamine. *Exp Mol Med* 36, 576-581 (2004)
19. Fesus L. & Z. Szondy: Transglutaminase 2 in the balance of cell death and survival. *FEBS Lett* 579, 3297-3302 (2005)
20. De LaurenziV. & G. Melino: Gene disruption of tissue transglutaminase. *Mol Cell Biol* 21, 148-155 (2001)
21. Boehm J.E., U. Singh, C. Combs, M.A. Antonyak & R.A. Cerione: Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. *J Biol Chem* 277, 20127-20130 (2002)
22. Antonyak M.A., A.M. Miller, J.M. Jansen, J.E. Boehm, C.E. Balkman, J.J. Wakshlag, R.L. Page & R.A. Cerione. Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. *J Biol Chem* 279, 41461-41467 (2004)
23. Sato N., Y, Ohtake, H. Kato, S. Abe, H. Kohno & Y. Ohkubo. Effects of polyamines on histone polymerization. *J Protein Chem* 22, 303-307 (2003)
24. Pendeville H., N. Carpino, J.C. Marine, Y. Takahashi, M. Muller, J.A. Martial & J.L. Cleveland: The ornithine decarboxylase gene is essential for cell survival during early murine development. *Mol Cell Biol* 21, 6549-6558 (2001)
25. Pietila M., L. Alhonen, M. Halmekyto, P. Kanter, J. Janne & C.W. Porter: Activation of polyamine catabolism profoundly alters tissue polyamine pools and affects hair growth and female fertility in transgenic mice overexpressing spermidine/spermine N1-acetyltransferase. *J Biol Chem* 272, 18746-18751 (1997)
26. Soler A.P., G. Gilliard, L.C. Megosh & T.G. O'Brien: Modulation of murine hair follicle function by alterations in ornithine decarboxylase activity. *J Invest Dermatol* 106, 1108-1113 (1996)
27. Robinson N.E. & A.B. Robinson: Deamidation of human proteins. *Proc Natl Acad Sci USA* 98, 12409-12413 (2001)
28. Candi E., R. Schmidt & G. Melino: The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 6, 328-340 (2005)
29. Amendola A., M.L. Gougeon, F. Poccia, A. Bondurand, L. Fesus & M. Piacentini: Induction of "tissue" transglutaminase in HIV pathogenesis: evidence for high rate of apoptosis of CD4+ T lymphocytes and accessory cells in lymphoid tissues. *Proc Natl Acad Sci USA* 93, 11057-11062 (1996)
30. Lu W., A. Strohecker & J.H. Ou Jh: Post-translational modification of the hepatitis C virus core protein by tissue transglutaminase. *J Biol Chem* 276, 47993-47999 (2001)
31. Mariniello L., C. Esposito, V. Gentile & R. Porta: Transglutaminase covalently incorporates amines into human immunodeficiency virus envelope glycoprotein gp120 in vitro. *Int J Pept Protein Res* 42, 204-206 (1993)
32. Mariniello L., C. Esposito, P. Di Pierro, A. Cozzolino, P. Pucci & R. Porta: Human-immunodeficiency-virus transmembrane glycoprotein gp41 is an amino acceptor and donor substrate for transglutaminase in vitro. *Eur J Biochem* 215, 99-104 (1993)
33. Beninati S. & A.B. Mukherjee: A novel transglutaminase-catalyzed posttranslational modification of HIV-1 aspartyl protease. *Biochem Biophys Res Commun* 187, 1211-1218 (1992)
34. Nardacci R., F. Ciccosanti, L. Falasca, O. Lo Iacono, A. Amendola, G. Antonucci & M. Piacentini: Tissue transglutaminase in HCV infection. *Cell Death Differ* 10, S79-80 (2003)
35. Bevec D., H. Jaksche, M. Oft, T. Wohl, M. Himmelspach, A. Pacher, M. Schebesta, K. Koettnitz, M. Dobrovnik, R. Csonga, F. Lottspeich & J. Hauber: Inhibition of HIV-1 replication in lymphocytes by mutants of the Rev cofactor eIF-5A. *Science* 271, 1858-1860 (1996)

Abbreviations: HPV, human papillomavirus; TGase, transglutaminase; Rb, retinoblastoma tumor suppressor; SPD, spermidine; SPM, spermine; BSPD, biotinylated spermidine; BSPM, biotinylated spermine; BP, biotinylated pentylamine; PBS, phosphate-buffered saline; GST, glutathione-S-transferase; HRP, horseradish peroxidase

Key Words: Human Papillomavirus E7, Transglutaminase 2, Polyamination; Spermine, Spermidine

Send correspondence to: Dr. In-Gyu Kim, Department of Biochemistry and Molecular Biology/AARC, Seoul National University College of Medicine. 28 Yongon Dong, Chongno Gu, Seoul 110-799, Korea, Tel: 822-740-8248, Fax: 822-744-4534/ E-mail: igkim@plaza.snu.ac.kr

http://www.bioscience.org/current/vol11.htm