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Research

Thiol-reactive reagents inhibits intracellular trafficking of human papillomavirus type 16 pseudovirions by binding to cysteine residues of major capsid protein L1

Yoshiyuki Ishii[1], Kazunari Kondo[1], Tamae Matsumoto[1], Keiko Tanaka[2], Fumiko Shinkai-Ouchi[3], Ken'ichi Hagiwara[3] and Tadahito Kanda*[1]

Address: [1]Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan, [2]Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan and [3]Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Email: Yoshiyuki Ishii - yishii@nih.go.jp; Kazunari Kondo - k-kondo@nih.go.jp; Tamae Matsumoto - tmmtsmt@nih.go.jp; Keiko Tanaka - keiko5@nih.go.jp; Fumiko Shinkai-Ouchi - fumi-ouc@nih.go.jp; Ken'ichi Hagiwara - hagiwark@nih.go.jp; Tadahito Kanda* - kanda@nih.go.jp

* Corresponding author

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Abstract

Background: A human papillomavirus (HPV) virion is composed of capsid proteins L1 and L2. Several cysteine residues are located on L1 of various HPVs at markedly similar relative positions, suggesting their important functions. Although the authentic virions cannot be studied with cultured cells, surrogate pseudovirions consisting of capsid and reporter plasmid are available for studies dealing with infectivity.

Results: HPV type16-pseudovirions (16PVs) were found to lose their infectivity after incubation with thiol-reactive reagents [biotin polyethyleneoxide iodoacetamide (BPEOIA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), 4-(N-maleimido)benzyl-trimethylammonium iodide (MBTA), and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET)]. A labelled streptavidin was detected to bind to the complex of BPEOIA and L1 of the 16PVs incubated with BPEOIA. The analysis of molecular mass of trypsin-fragments derived from the complex of the BPEOIA and L1 indicated that BPEOIA bound to at least C146, C225, and C229. No appreciable change of the 16PVs carrying DTNB or NEM was detected by sedimentation analysis or electron microscopy. The 16PVs carrying DTNB or NEM were able to bind to and enter HeLa cells but degraded before they reached the perinuclear region.

Conclusion: HPV16 L1 C146, C225, and C229 have free thiol, which are accessible to BPEOIA, DTNB, NEM, MBTA, and MTSET. Binding of DTNB or NEM to the thiols may cause conformational changes that result in the inhibition of the entry and trafficking of the 16PVs.

Background

Human papillomavirus (HPV) is a non-enveloped icosahedral particle (55 nm in diameter) containing an 8-kb double-strand circular DNA [1]. An HPV-capsid is composed of 360 molecules of major capsid protein L1 and 12 molecules of minor capsid protein L2 [2]. To date more

than 100 HPV genotypes, which are classified by DNA homology, have been cloned and are grouped into mucosal and cutaneous types from the tissue tropism [3]. Among mucosal types 15 HPVs detected in cervical cancer, the second most frequent gynaecological malignancy in the world, are called as high-risk types and those detected in benign lesions, such as condyloma, are called as low-risk types [4]. HPV type 16 (HPV16) is believed to account for 50% of cervical cancer [4].

HPVs infect basal cells of the epithelium through microlesions and replicate only in the differentiating cells [5]. These cells are difficult to culture in vitro; hence, no tissue culture system for the large-scale propagation of HPVs is available at present. By using surrogate systems the expression of L1 and L2 in cells harboring episomal copies of expression plasmid results in packaging of the episomal DNA into the HPV capsids to produce infectious pseudovirions (PVs)[6,7]. These PVs are used as a surrogate virus to analyse early steps of HPV infection to cells and to detect neutralizing activity of anti-HPV antibodies [8-13].

An L1 molecule of various HPVs contains several cysteine residues at markedly similar relative positions (Fig. 1), strongly suggesting that these cysteine residues play important roles in the structure and the function of the HPV capsids. Previous studies have shown that cysteine residue at amino acid (aa) 175 (C175) and C428 in HPV16 L1 (505 amino acids long) are involved in the intermolecular disulfide bonding that contributes to the assembly of the capsid [14]. The functions of the other L1 cysteine residues are not known.

In this study we attempted to know whether thiol-reactive reagents affect infectivity of HPV16 PVs (16Pvs) by binding to the L1 cysteine residues.

Results

Infectivity of the 16PVs that have bound to thiol-reactive reagents

The 16PVs was found to lose their infectivity for HeLa cells after binding to thiol-reactive reagents: biotin polyethyleneoxide iodoacetamide (BPEOIA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), 4-(N-maleimido)benzyl-trimethylammonium iodide (MBTA), and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET). 16PVs were incubated with BPEOIA (1 mM), DTNB (2 mM), NEM (2 mM), MBTA (2 mM), or MTSET (2 mM) for 2 h at 37°C. After dilution at 1 to 1,000 the 16PVs were inoculated to the cells. The number of the infected cells, which expressed EGFP, was counted 2 days later. The HeLa cells inoculated with the 16PVs incubated with these thiol-reactive reagents did not express EGFP (Fig. 2). Like HeLa cells, SiHa and 293TT cells inoculated with the 16PVs that had incubated with DTNB did not express EGFP (data not presented). The data indicate that these thiol-reactive reagents inhibited infectivity of the 16PVs.

DTNB did not affect the cellular susceptibility to 16PVs. The normal 16PVs infected HeLa cells that had been cultured in the growth medium containing DTNB (2 mM) for 2 h at 37°C and washed once with fresh growth medium (data not presented). Furthermore HeLa cells cultured with growth medium containing 2 mM of DTNB, MBTA or MTSET for 2 days grew and were maintained

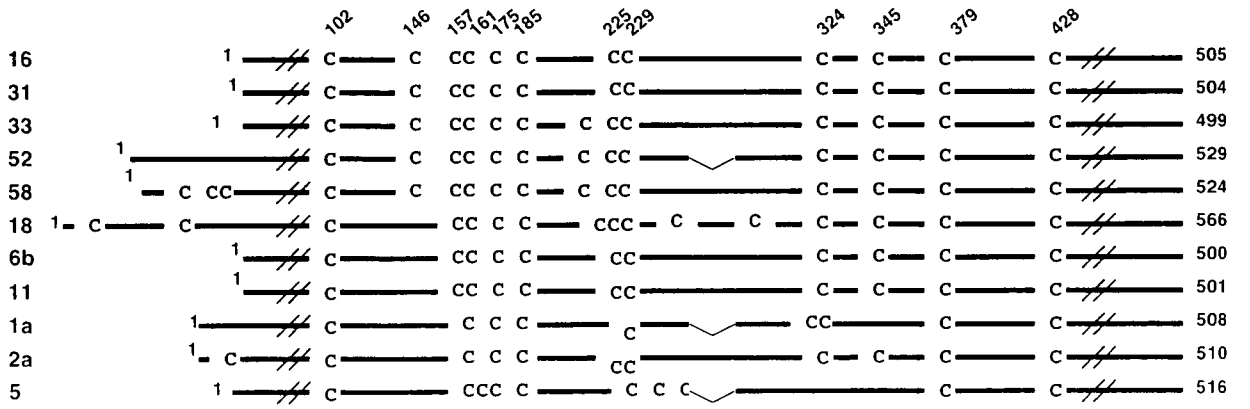

Figure 1
Alignment of L1 amino acid sequences of papillomaviruses. Numbers to the left represent human papillomavirus types. Numbers on the top represent amino acid numbers for cysteines in HPV 16 L1 (positions in L1), starting from the N-terminus. The number of total amino acids constituting each L1 is shown to the right.

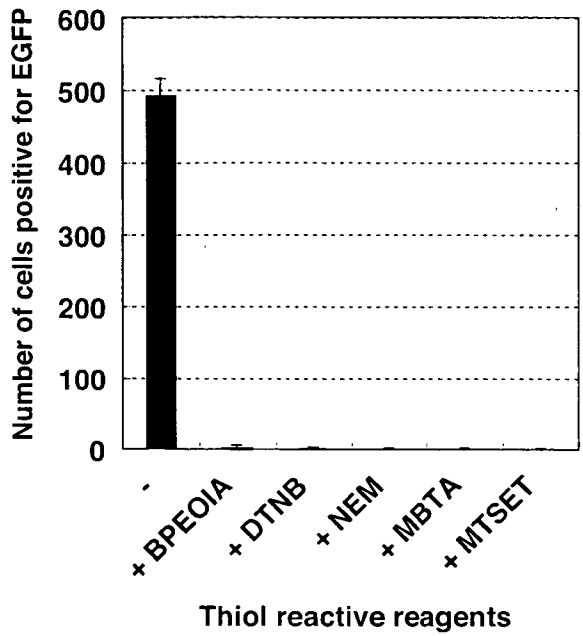

Figure 2
Infectivity of the 16PVs that have bound to BPEOIA, NEM, DTNB, MBTA, or MTSET. The 16PVs were incubated with the thiol-reactive reagent indicated at 37°C for 2 h. The samples were diluted by 1000-fold and added to HeLa cells. The cells were incubated for 2 days and harvested. The cells expressing EGFP were counted by a FACS. BPEOIA: biotin polyethyleneoxide iodoacetamid, NEM: N-ethylmaleimide, DTNB: 5,5'-dithiobis(2-nitrobenzoic acid), MBTA: 4-(N-maleimido)benzyl-trimethylammonium iodide, MTSET: [2-(trimethylammonium)ethyl] methanethiosulfonate bromide.

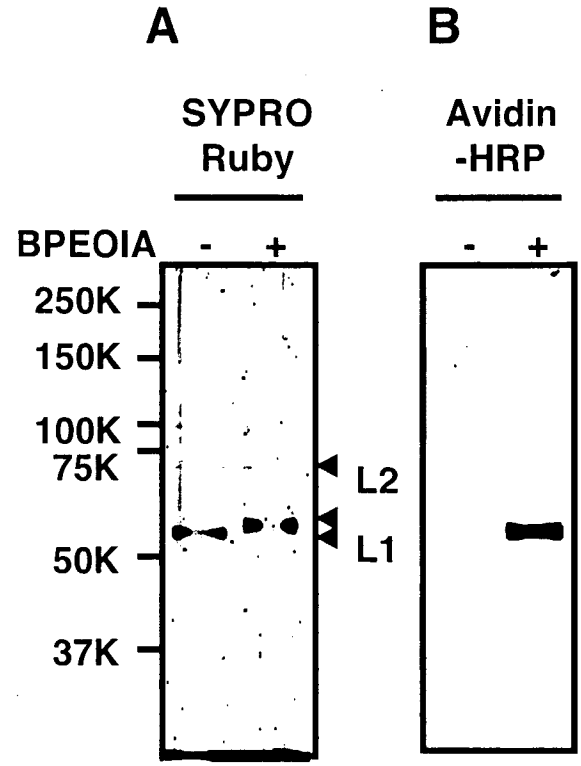

Figure 3
Binding of BPEOIA to L1 of the 16PV. (A) The 16PVs were incubated with 1 mM biotin-PEO-iodoacetamide (1 mM) in DMEM at 37°C for 2 h, electrophoresed on an SDS-polyacrylamide gel, and stained with SYPRO Ruby. (B) The proteins in the gel were transferred to a polyvinylidene difluoride membrane and probed with Streptavidin-HRP.

normally, strongly suggesting the reagents were not harmful to HeLa cells at the concentration of 2 mM.

Binding of BPEOIA to the L1 cysteine residues of 16PV

BPEOIA, capable of making a complex with streptavidin, was found to bind to the free thiol of the cysteine residues of L1 of 16PV, which was produced by packaging of a reporter plasmid into an HPV16 capsid. Purified 16PVs were incubated with 1 mM BPEOIA at 37°C for 2 h. The resultant 16PVs were electrophoresed on an SDS-polyacrylamid gel and the separated proteins were stained by SYPRO Ruby (Fig. 3A) or transferred to a membrane. The membrane was probed by horseradish peroxidase (HRP) conjugated streptavidin (Fig. 3B). After the incubation of 16PVs with BPEOIA, the molecular mass of L1 shifted from 55 kDa to 57 kDa (Fig. 3A). The molecular mass of L2 (68 kDa) was not affected by the incubation. The streptavidin made a complex with only 57 kDa L1 (Fig. 3B). The data indicate that BPEOIA bound to the free thiol of cysteine residue(s) of L1.

The 57 kDa L1/BPEOIA complex was digested with trypsin and the fragments complexing with BPEOIA were selectively obtained by column chromatography with the streptavidin-resin. The molecular mass of the fragments was measured by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Fig. 4). The mass of the three fragments, ECISMDYK, SEVPLDICTSICK, and SEVPLDICTSICK, matched with the calculated mass, indicating that BPEOIA bound to the thiol of C146, C225, and C229. Some of the large tryptic fragments that bound to BPEOIA may not be detected because of their low recovery from LC and/or inefficiency in the ionization.

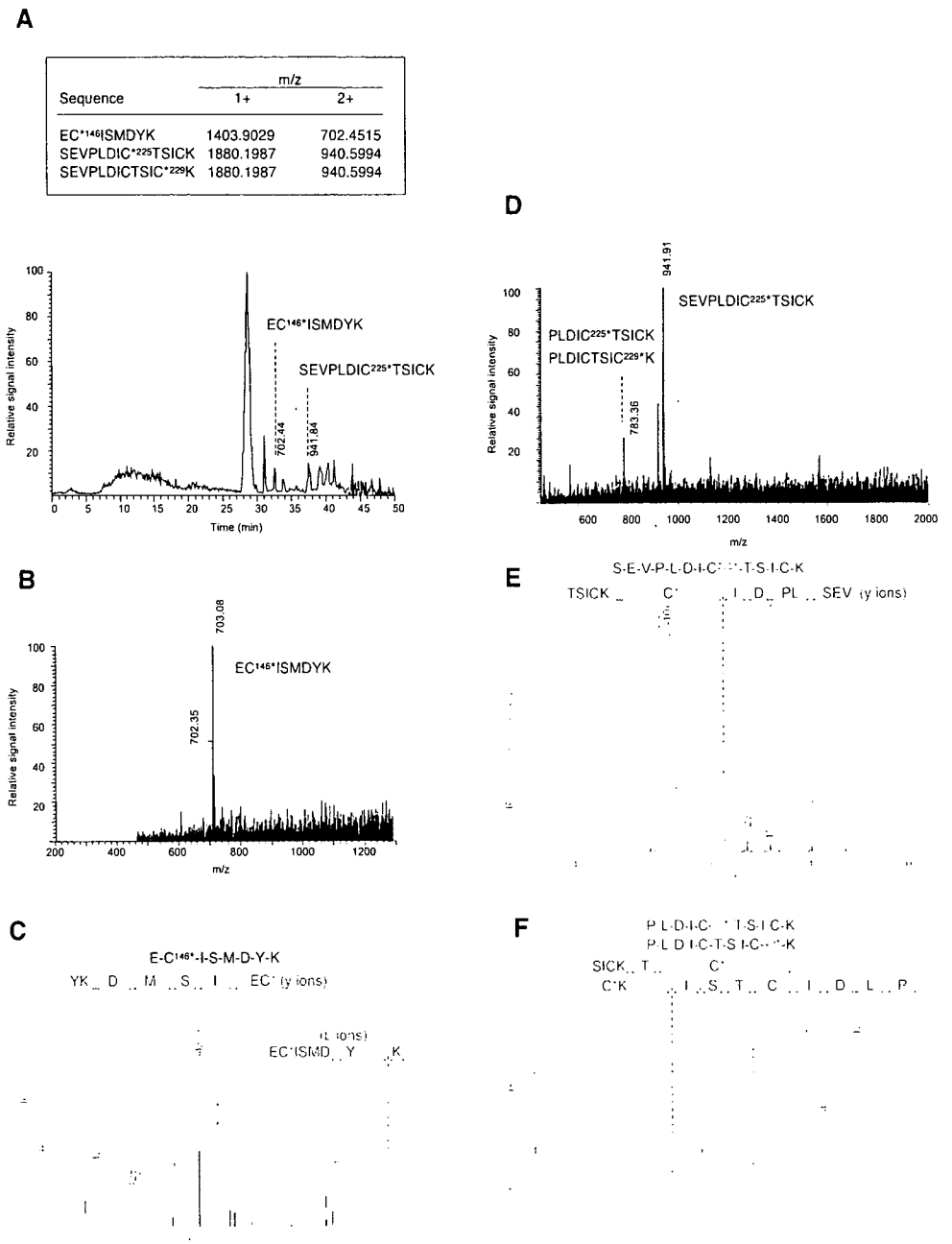

Figure 4

Analysis of L1-cysteine residues that have bound to BPEOIA by mass spectrometry. (A) Chromatogram of the tryptic peptides of L1 that bound to BPEOIA. The upper box shows calculated monoisotopic mass value of mono (1+) and doubly (2+) charged masses of three peptides. The asterisk denotes a cysteine bound with BPEOIA. (B and D) Full scan mass spectra corresponding to the EC^{146}*ISMDYK (B) and SEVPLDIC^{225}*TSICK (D). (C, E and F) MS/MS spectra of corresponding to EC^{146}*ISMDYK (C), SEVPLDIC^{225}*TSICK (E), PLDIC^{225}*TSICK and PLDICTSI C^{229}*K (F).

Sedimentation and morphology of the 16PVs that have bound to DTNB or NEM

The 16PVs that had been incubated with DTNB (2 mM) or NEM (2 mM) sedimented through the sucrose gradient (5–40%) as the normal 16PVs did (Fig. 5A). The 16PVs pre-incubated with MBTA or MTSET sedimented similarly (data not presented). These results strongly suggest that the 16PVs that had bound to these reagents were morphologically similar to the normal 16PVs and did not make aggregates.

The 16PVs that had bound to NEM (NEM-16PVs) were not distinguishable from the normal 16PV by an electron microscopy (Fig. 5B). The 16PVs that had been incubated with NEM (2 mM) at 37°C for 2 h were negatively stained with 4% uranylacetate and examined under a transmission electron microscope. Any morphological abnormalities of the NEM-16PVs were not detectable at a magnification of 1:200,000.

Binding to HeLa cells, internalization, and trafficking of the 16PVs that have bound to DTNB or NEM

The DTNB-16PVs and the NEM-16PVs were found to bind to HeLa cells less efficiently than the normal 16PVs did. The 16Pvs that had been incubated with DTNB (2 mM) or NEM (2 mM) were inoculated to HeLa cells with incubation at 4°C for 1 h. After a wash with cold PBS to remove the unbound 16Pvs, the cells were lysed immediately. Proteins in the lysate were separated by SDS-polyacrylamid gel electrophoresis (PAGE) and transferred to a membrane. L1 on the membrane was detected with mouse anti-HPV16L1 antibody and goat anti-mouse IgG-HRP (Fig. 6A). The levels of L1 from the DTNB-16PVs and the NEM-16PVs were 50–60 % of that of L1 from the normal

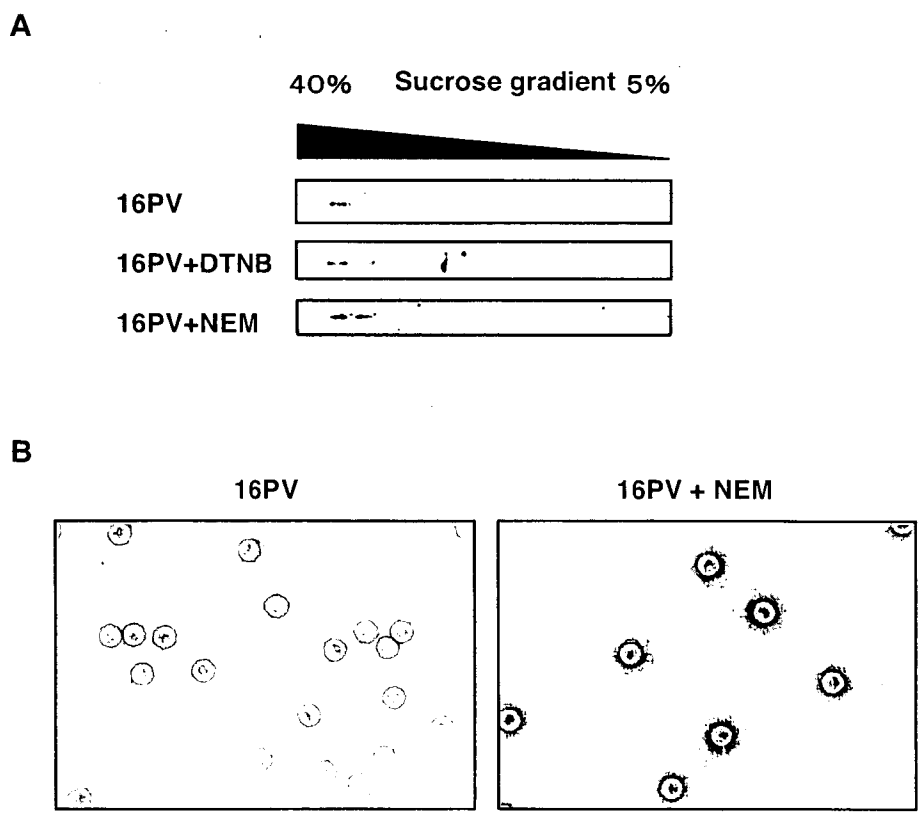

Figure 5
Sedimentation and morphology of the 16PVs that have bound to DTNB or NEM. (A) The 16PVs were incubated with DTNB (2 mM) or NEM (2 mM) at 37°C for 2 h. The sample was loaded on the top of a linear sucrose-density gradient (5 to 40%) and centrifuged. L1 in the fractions obtained by a bottom puncture was detected by immunoblotting with mouse anti-HPV16L1 antibody. (B) The 16PVs were incubated with NEM (2 mM) at 37°C for 2 h and observed under a transmission electron microscope.

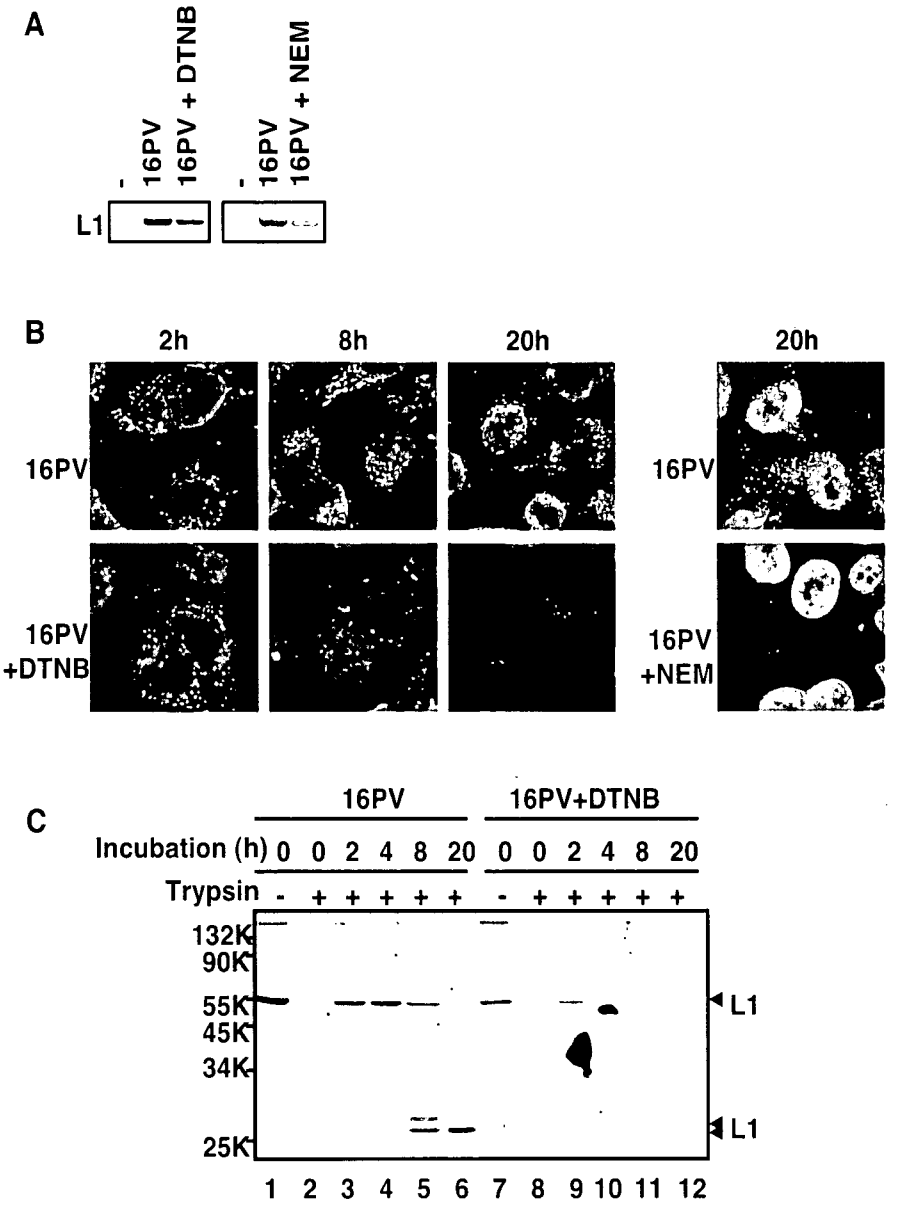

Figure 6

Binding, trafficking, and degradation of the 16PVs that have bound to DTNB or NEM. (A) The 16PVs were incubated with DTNB(2 mM) or NEM (2 mM) at 37°C for 2 h and added to HeLa cells. After incubation at 4°C for 1 h, the cells were washed by PBS and lysed. The lysate was electrophoresed on an SDS-polyacrylamide gel. L1 was detected by immunoblotting with anti-HPV16L1 antibody. (B) The 16PVs incubated with DTNB or NEM were added to HeLa cells and incubated for 1 h at 4°C. The cells were cultured at 37°C for 2, 4, 8 or 20 h and fixed. L1 was detected by rabbit anti-HPV16L1 antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (red). DNA was stained with DAPI (blue). (C) The 16PVs incubated with DTNB were added to HeLa cells and incubated for 1 h at 4°C. The cells were harvested with PBS containing 2.5 mM EDTA (for trypsin – sample at 0 h) or with trypsin (for trypsin + sample at 0 h). The rest of cells were cultured at 37°C for 2, 4, 8 or 20 h and harvested with trypsin. The cells were lysed and the lysates were electrophoresed on an SDS-polyacrylamide gel. L1 was detected by immunoblotting with anti-HPV16L1 antibody.

16 PVs, indicating that DTNB and NEM reduced the cell-binding ability of the 16PVs. But the reduction of the binding efficiency does not fully account for the inhibition of the infectivity of the DTNB-16PVs and the NEM-16PVs.

We found that the DTNB-16PVs and the NEM-16PVs disappeared on their way to the nucleus (Fig. 6B). The 16PVs incubated with DTNB (2 mM) or NEM (2 mM) were inoculated to HeLa cells with incubation at 4 °C for 1 h. After a wash with the growth medium the cells were incubated further with the growth medium for 2, 8 or 20 h, fixed with paraformaldehyde (4 %), and permeated by Triton-X100. L1 was stained with rabbit anti-HPV16-L1 antibody [12] and anti-rabbit-IgG goat antibody conjugated with Alexa Fluor 546. Nuclear DNA was stained with DAPI. The localization of L1 and DNA was observed under a confocal microscope (Fig. 6B). Although the normal 16PVs reached the perinuclear region and accumulated there at 20 h, the DTNB-16PVs and the NEM-16PVs became undetectable at 20 h.

Consistent with the above observation by confocal microscopy, the immunoblotting to detect L1 showed that the DTNB-16PVs were rapidly degraded in the cells (Fig. 6C). HeLa cells were inoculated with the 16PVs pre-incubated with DTNB (2 mM), and the infected cells were incubated at 4 °C for 1 h. After a wash with cold PBS the cells were incubated with trypsin to digest the 16PVs that had not entered the cells. Then, the cells were lysed and subjected to PAGE. The intracellular L1 levels were analysed by immunoblotting with mouse anti-HPV16L1 antibody. The cell-bound 16PVs (lanes 1 and 7) were sensitive to the trypsin digestion (lanes 2 and 8) and became resistant after the incubation at 37 °C for 2 h (lanes 3 and 9). The cells inoculated with the 16PVs were further incubated with the growth medium at 37 °C for 2, 4, 8, and 20 h, digested with trypsin, and analysed. Fig. 6C clearly showed that the DTNB-16PVs were degraded rapidly and became almost undetectable at 8 h (lane 11).

Thus, the DTNB-16PVs had reduced capability of binding to the cells and enhanced sensitivity to the cellular mechanisms controlling the degradation of foreign proteins.

Infectivity of the 16PVs composed of mutant L1s having a replacement of cysteine with alanine

A mutational analysis was unsuccessful to identify one particular cysteine residue having the free thiol essential for the infectivity of the 16PV. We newly constructed the L1 mutants by replacement of the cysteine, except for C175 and C428, with alanine. C161A (C161 was replaced with A) was extremely unstable in 293TT cells and was not available for the analysis. The yields of C157A, C229A, C342A, and C379A were very low. Therefore, the number of HeLa cells infected with the mutant PVs was normalized to the content of L1 (Table 1). The infectivity of the mutant PVs, including C146A, C225A, and C229A, ranged between 58 and 187 % of the infectivity of the normal 16PV.

Discussion

In this study we found that the thiol-reactive reagents bound to C146, C225, and C229 of the 16PVs. In the 3-dimentional-structure model of L1 [15], C146 is involved in forming the DE-loop and C225 and C229 are involved in forming the EF-loop. Because the loops are generally flexible and because C146 and C225 are located at the surface region of the loops and C229 is located near the surface region, it is likely that thiol-reactive reagents easily access the thiols of these cysteine residues. Although it is difficult to examine experimentally whether all or part of the cysteine residues have free thiols, we presume the great majority of C146, C225, and C229 may have free thiols,

Table 1: Infectiviy of mutant 16PVs

	L1 in PV stock		Infectious units		
	ng of L1/μl	%	units/μg of L1	%	SD
WT	82.0	100	1.77.E+05	100	17.5
C102A	29.3	35.7	1.05.E+05	59.3	1.40
C146A	54.9	66.9	1.64.E+05	92.4	3.37
C157A	3.46	4.22	8.60.E+04	48.1	2.04
C161A	ND	-	NT	-	-
C185A	33.8	41.2	1.62.E+05	91.4	3.86
C225A	81.2	99.0	2.82.E+05	159	4.46
C229A	8.53	10.4	9.60.E+04	54.6	5.59
C324A	5.85	7.14	8.50.E+04	50.2	2.47
C345A	44.3	54.0	3.30.E+05	187	4.03
C379A	13.1	16.0	1.62.E+05	91.4	4.43

ND: not detected NT: not tested

because the incubation of the 16PVs with the thiol-reactive reagents induced a large effect on their infectivity. The 16PVs lost their infectivity after binding of the thiol-reactive reagents to the free thiols. The DTNB-16PVs and the NEM-16PVs, whose C146, C225, and C229 carried DTNB and NEM as additional side chains, respectively, bound to HeLa cells less efficiently and were degraded rapidly in the cells. Although the 16PV mutants with two or three Ala substitutions for C146, C225, or C229 were too unstable to be used in the infectivity analysis, we obtained the three mutants with an Ala substitution (C146A, C225A, and C229A) and found that the substitution did not affect the infectivity much. Therefore, it is likely that the steric bulk of DTNB or NEM occludes a neighboring portion of the virion involved in the entry and trafficking processes. But there remains a possibility that the disulfide bonding between an unidentified cellular protein(s) and the two remaining cysteine residues in the mutants plays an additive role in the viral entry and trafficking.

It has been reported that cell-surface protein disulfide isomerase (PDI) is required for the entry process of several viruses including mouse polyomavirus. The siRNA-mediated down regulation of PDI of HeLa cells prevents the cells from being infected with mouse polyomavirus [16]. Inactivation of the cell-surface PDI by adding the thiol-reactive reagents, such as DTNB, which do not permeate the membrane, to the culture medium results in the inhibition of entry of HIV1 and Newcastle disease virus [17,18], suggesting that the modified envelope conformation induced by reforming the disulfide-bonding is required for the membrane fusion, which is the essential step for the virus entry [18,19]. However, pre-incubation of the cells with DTNB did not inhibit infection with the 16PV, indicating that modification of the disulfide-bonding in the capsid by cell-surface PDI is not involved in the early steps of HPV infection. The data are consistent with the recent report that reducing agents, such as DTT and 2-ME, do not inhibit HPV infection [20].

Because the thiol-reactive reagents tested in this study bound to the free thiol of the 16PVs at a concentration not toxic for HeLa cells, these reagents might function as practical inhibitors of HPV infection. It would be necessary to test the efficacy and safety of the reagents in animal models.

Conclusion

HPV16 L1 C146, C225, and C229 have free thiol, which is accessible by the thiol-reactive reagents, such as BPEOIA, DTNB, and NEM. The HPV16 pseudovirions carrying these thiol-reactive reagents lost infectivity by mainly the rapid degradation in the cytoplasm.

Methods

Cells

293TT cells, a human cell line expressing a high level of SV40 T antigen, was a kind gift from J. T. Schiller (National Cancer Institute, USA). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) (No. 21063, Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen Corp.), 1% GlutaMax-I (Invitrogen Corp.), penicillin G potassium (100 units/ml) (Meiji seika Ltd., Tokyo, Japan), kanamycin sulfate (60 μg/ml) (Wako pure chemical industries Ltd. Tokyo, Japan) (growth medium) and hygromycine B (400 μg/ml) (Invitrogen Corp.) in 5% CO2 at 37°C. HeLa cells and SiHa cells were cultured in DMEM supplemented 10% FBS, penicillin G potassium, and kanamycin sulfate.

Plasmids

pYSEAP, ph16L1, and ph16L2 were gift from J. T. Schiller. pEF1a-EGFP was newly constructed by an insertion of EGFP gene derived from pCMS-EGFP (Clonthech laboratories Inc., Mountain View, CA) into the backbone of pYSEAP. Plasmid expressing mutant L1 with a substitution of alanine for cysteine was constructed by overlap extension PCR method [21] using KOD plus polymerase (TOYOBO Corp., Osaka, Japan) and ph16L1 as template. 5'-GCTGGTGTGGGCCGCCGTGGGCGTGGAG-3' and 5'-CCTCCACGCCCACGGCGGCCCACACCAGCC-3' were used as forward (F) and reverse (R) primers to replace C102 with A, respectively. Following oligonucleotides were used as primers to introduce the other mutantions: 5'-CGACAACAGGGAGGCCATCAGCATGGACTACAAG-3' (F for C146A), 5'-GTAGTCCATGCTGATGGCCTCCCT-GTTGTCCAC-3' (R for C146A), 5'-CAAGCAGAC-CCAGCTGGCCCTGATCGGCTGCAAG-3' (F for C157A), 5'-CTTGCAGCCGATCAGGGCCAGCTGGGTCTGCTTG-3' (R for C157A), 5'-CTGTGCCTGATCGGCGCCAAGCCCC CCATCG-3' (F for C161A), 5'-CGATGGGGGGCTTGGCG CCGATCAGGCACAG-3' (R for C161A), 5'-AACCCCG-GCGACGCCCCCCCCCTGGAGCTG-3' (F for C185A), 5'-CAGCTCCAGGGGGGGGCGTCGCCGGGGTTC-3' (R for C185A), 5'-GTGCCCCTGGACATCGCCACCAGCATC TGCAAG-3' (F for C225A), 5'-CTTGCAGATGCTGGTGGC GATGTCCAGGGGCAC-3' (R for C225A), 5'-ACCAG-CATCGCCAAGTACCCCGACTACATC-3' (F for C229A), 5'-ATGTAGTCGGGGTACTTGGCGATGCTGGTGCAGAT G-3' (R for C229A), 5'-CAACAACGGCATCGCCTGGGGC AACCAGCTGTTC-3' (F for C324A), 5'-GAACAGCTGGTT-GCCCCAGGCGATGCCGTTGTTGTG-3' (R for C324A), 5'-CCAACATGAGCCTGGCCGCCGCCATCAGCAC-3' (F for C345A), 5'-GTGCTGATGGCGGCGGCCAGGCTCAT-GTTGGTGCTCC-3' (R for C345A), 5'-CATCTTCCAGCT-GGCCAAGATCACCCTGAC-3' (F for C379A), 5'-GTCAGGGTGATCTTGGCCAGCTGGAAGATGAACTG-3'

(R for C379A). 5'-TGCCTTTACTTCTAGGCCTGTACG-3' and 5'-TGCTCCTGGTGGTGTCCACCACGGTC-3' were used as primers to join the part containing the mutation back to the rest of the entire L1 gene of C146A, C157A, C161A, C185A, C225A, C229A. 5'-AACCTGGCCAGCAG-CAACTACTTCCC-3' and 5'-AACTAGAAGGCACAGTC-GAGGCTG-3' were used similarly to produce C324A, C345A, C379A. The resultant DNA fragments were inserted into ph16L1 after digestion with NotI and ApaI (for C146A, C157A, C161A, C185A, C225A, and C229A) or with ApaI and HindIII (for C324A, C345A, and C379A).

Thiol-reactive reagents

Biotin polyethyleneoxide iodoacetamide (BPEOIA) was purchased from SIGMA-ALDRICH Corp. (Saint Luis, MO). N-ethylmaleimide (NEM) was purchased from Nakarai Tesque. Inc. (Kyoto, Japan). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from SIGMA-ALDRICH Corp (Saint Luis, MO). 4-(N-maleimido)benzyl-trimethylammonium iodide (MBTA), and [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada)

Preparation of 16PV

The 16PV, an HPV16 capsid containing a reporter plasmid expressing EGFP, was produced by the previously described procedure [6,7,12] with minor modification. 293TT cells (40% confluent in 10-cm culture dish) were transfected with mixture of ph16L1 (13.5 μg), ph16L2 (3 μg), and pEF1a-EGFP (13.5 μg) by using Optifect (Invitrogen Corp.) in OPTI-MEMII (Invitrogen Corp.). After incubation for 3 days, the cells were scraped off and suspended in 0.5 ml lysis buffer (PBS containing 9.5 mM $MgCl_2$, 0.35% Brij 58, [Sigma-Aldrich Inc., St. Louis, MO], 0.1% Benzonase [Sigma-Aldrich Inc.], 0.1% Plasmid Safe ATP dependent-DNase [EPICENTRE Corp. Madison, WI], 1 mM ATP) and incubated at 37°C for 20–24 h with slow rotation. The lysate was cooled on ice for 5 min, mixed with 1/4 volume of 5 M NaCl solution, and kept on ice for 10 min, then, centrifuged at 5,000 × g at 4°C for 10 min. The resultant supernatant was laid on an Optiprep gradient composed of 27%, 33%, and 39% in PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.1 mM KCl, and 0.8 M NaCl and centrifuged at 47,900 rpm at 16°C for 3 h with SW50.1 rotor (Beckman Coulter Inc. Fullerton, CA). The fraction containing the purified 16PVs was collected by puncturing the bottom and used as the stock.

Infectivity assay

The 16PV stock was diluted at 10-fold with DMEM and received BPEOIA (1 mM); the 16PV stock was diluted at 10-fold with the growth medium and received DTNB (2 mM), NEM (2 mM), MBTA (2 mM), or MTSET (2 mM). The mixtures were incubated at 37°C for 2 h and diluted with the growth medium at 1,000-fold. HeLa cells (1.5×10^5) in a well of a 24-well culture-plate were inoculated with the sample and cultured for 2 days. The cells were harvested with trypsin. EGFP-positive cells were counted by a fluorescence activated cell sorting (FACS Calibar, Becton Dickinson and Company Ltd., San Joe, CA).

Binding of BPEOIA to 16PVs

BPEOIA was dissolved in H_2O to 18.4 mM. The 16PV stock was diluted at 10-fold with DMEM (No. 21063, Invitrogen Corp.) containing BPEOIA (1 mM) and incubated at 37°C for 2 h. Then, DTT (100 mM), which reacted with remaining excess BPEOIA, was added to the mixture and incubated at 37°C for 30 m (BPEOIA+ sample). The 16PV stock was diluted at 10-fold with DMEM, incubated at 37°C for 2 h, mixed with DTT (100 mM), and further incubated at 37°C for 30 m. Then, BPEOIA (1 mM) was added to the mixture and incubated at 37°C for 30 m (BPEOIA- sample). The 16PVs were concentrated by using a PAGEprep Advance Kit (PIERCE Biotechnology Inc., Rockford, IL) and suspended in the SDS sample buffer (50 mM Tris-HCl pH 6.8, 5% glycerol, 2 % SDS, and bromphenol blue) containing 100 mM DTT. The sample was boiled and electrophorased on an SDS-polyacrylamide gel. The proteins in the gel were stained with SYPRO Ruby (Invitrogen Corp.) or transferred to membrane Hybond-P (GE Healthcare Bio-Science AB, Uppsala, Sweden). The membrane was blocked with skim milk and incubated with the horseradish peroxidase (HRP) conjugated-streptavidin (GE Healthcare Bio-Science AB). The HRP activity was detected by using an ECL plus western blotting detection system (GE Healthcare Bio-Science AB) and Typhoon 9410 (GE Healthcare Bio-Science AB).

Analysis by liquid chromatography electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

The HPV16 pseudovirions that bound to BPEOIA were separated by SDS-PAGE and stained by SYPRO Ruby as described above. The gel pieces containing the L1/BPEOIA complex were excised and the L1 in the gel was digested with trypsin (Trypsin Gold Mass Spectrometry Grade, Promega Corp., Madison WI) as previously described [22]. The digested peptides were extracted from the gel pieces by one change of NH_4HCO_3 (20 mM) and 4 changes of acetonitrile (50 %). The peptides were suspended in PBS by adding equal volume of 2× concentrated PBS and then incubated with monomeric avidin beads (Ultralink Immobilized Monomeric Avidin, PIERCE Biotechnolog Inc.). The beads were washed with PBS twice, with methanol (20 %) in NH_4HCO_3 (50 mM) twice, and with water twice. The peptides that bound to the beads were eluted with acetonitrile (30 %) containing TFA (0.4 %). After volatilization of acetonitrile the peptides were analyzed by a liquid chromatography (MAGIC

2002 system, Michrome Bioresources Inc., Auburn, CA) equipped with C18 column (Inertsil EX-Nano ODS-3, 0.1 mm i.d. × 50 mmL, GL Sciences Inc, Tokyo, Japan) coupled with a nano spray apparatus (AMR Inc. Tokyo, Japan) for electrospray ionization-iontrap mass spectrometry (LC-ESI-IT-MS) (LCQ-decaXP, Thermo electron corp., San Jose, CA). The data were collected by data-dependent mode and the MS/MS sequence were analysed by using software Bioworks (Ver.3.1, Thermo electron corp.) with variable modification option of a mass unit of 414.19 for the biotin polyethleneoxide moiety.

Sedimentation assay

The 16PV stock was diluted at 10-fold with the growth medium, and DTNB (2 mM) was added to the medium. The 16PVs were then incubated for 2 h at 37°C. The sample was loaded on a linear sucrose-density gradient (5 to 40%) in PBS. After centrifugation at 120,000 × g at 4°C for 2.5 h with and SW50.1 rotor, aliquots (400 μl) were collected. Ten μl of the aliquot was mixed with an equal volume of the 2× concentrated SDS-sample buffer containing 100 mM DTT, boiled, and electrophorased on an SDS-polyacrylamide gel. The proteins were transferred to a Hybond-P membrane (GE Healthcare Bio-Science AB). The membrane was blocked with skim milk, incubated with mouse anti-HPV type 16L1 antibody (BD Biosciences Pharmingen Com., San Diego, CA), and then incubated with anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The HRP activity was detected by using an ECL plus western blotting detection system (GE Healthcare Bio-Science AB) and Typhoon 9410 (GE Healthcare Bio-Science AB).

Electron microscopy

The 16PV stock was mixed with NEM (2 mM) and incubated at 37°C for 2 h. The excess NEM was removed by using a Bio-Spin 30 column (Bio-Rad Laboratories Inc., Hercules, CA) equilibrated with phosphate buffer containing 0.5 M NaCl. The 16PVs were concentrated with a Microcon YM-100 (Millipore Corp., Bedford, MA) and then settled on carbon-coated copper grids. The 16PVs were negatively stained with 4% uranylacetate and examined in a transmission electron microscope (Hitachi model H-7650, Hitachi corp., Tokyo, Japan).

Binding assay

The 16PV stock was diluted at 20-fold with the growth medium, and DTNB (2 mM) was added to the medium. The stock was incubated at 37°C for 2 h and used for the binding assay. The 16PV stock was mixed with NEM (2 mM) and incubated at 37°C for 2 h. Then, the excess NEM was removed by using a Bio-Spin 30 column as described above was used for the binding assay. These samples were diluted with the growth medium at 20-fold and added to HeLa cells (1.5×10^5). The cells were incubated at 4°C for 1 h, washed with PBS, harvested with PBS containing 2.5 mM EDTA, and lysed. The lysate was electrophorased on an SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane and L1 was detected by immunoblotting with mouse anti-HPV16 L1 anitbody and anti-mouse IgG-HRP.

Immunofluoresence microscopy

HeLa cells (1.5×10^5) were seeded onto a well of a 4-chamber glass slide (BD Biosciences Falcon, Bedford, MA) with the growth medium. The cells were inoculated with the 16PV samples similarly prepared as the samples for the biding assay and incubated at 4°C for 1 h. The cells were washed with the growth medium and incubated at 37°C for 2, 8 or 20 h. The cells were fixed with PBS containing paraformaldehyde (4%) at room temperature (RT) for 10 m and washed with PBS. The cells were made permeable with PBS containing Triton X-100 (1%) at RT for 10 m and washed with PBS. The cells were incubated with rabbit anti-HPV16 L1 serum [12] in PBS containing BSA (3%) at RT for 1 h, washed with PBS containing Tween-20 (0.2%), incubated with Alexa Fluor 546 goat anti-rabbit IgG (H+L) (Invitrogen Corp.) in PBS containing BSA (3%), and washed with PBS containing Tween-20 (0.2%). The cells were coated with a ProLong Gold antifade reagent with DAPI (Invitrogen Corp.) and imaged in a FLUOVIEW FV1000 confocal microscope (OLYMPUS, Tokyo, Japan).

Internalizaiton assay

HeLa cells (1.5×10^5) in a well of a 24-well culture plate were inoculated with the 16PVs that had been preincubated with DTNB as done for the binding assay. The cells were incubated at 4°C for 1 h. The cells for the samples at 0 h were washed with the growth medium and immediately harvested with PBS containing 2.5 mM EDTA or with PBS containing trypsin. The other cells were incubated with the growth medium at 37°C for 2, 4, 8 and 20 h and harvested with PBS containing trypsin. The cells were lysed and electrophorased on an SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. L1 was detected by immunoblotting with mouse anti-HPV16 L1 anitbody and anti-mouse IgG-HRP.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

YI conceived of the study, carried out the biological experiments, and drafted the manuscript. KK supported the preparation of the 16PV stock. TM constructed the reporter plasmid. KT conducted electron microscopy. FSO

and KH conducted the analysis by LC-ESI-IT-MS. TK supervised the study and helped to draft the manuscript.

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Human papillomavirus type 16 P_{670} promoter is negatively regulated by CCAAT displacement protein

Kaori Sato · Takamasa Takeuchi · Iwao Kukimoto · Seiichiro Mori · Toshiharu Yasugi · Tetsu Yano · Yuji Taketani · Tadahito Kanda

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Abstract HPV16 late gene transcription from P_{670} is suppressed in undifferentiated keratinocytes. To identify DNA sites involved in the negative regulation, we examined the effect of a series of substitutions in the P_{670} promoter region (nucleotide (nt) 106–855) on the transcription, using an expression plasmid having the promoter fragment placed to drive the firefly-luciferase gene. Twenty-base pair-long segments covering the entire promoter region were replaced with a sequence lacking any so far known factor-binding motifs to produce 38 mutants. These plasmids were introduced by transfecton into undifferentiated or partially differentiated human HaCaT and HeLa cells, and transient expression of the reporter was examined with the cell extracts. The reporter expression from the wild-type promoter region was lower, half to one-third, in the undifferentiated cells than in the partially differentiated cells, which expressed hSkn-1a, a keratinocyte specific transcription factor that activates P_{670}, and CCAAT displacement protein (CDP), a transcriptional repressor involved in cell differentiation. Two mutants with substitutions including the putative CDP-binding sites, one from nt 562 to 567 and the other from nt 673 to 678, induced markedly enhanced reporter expression particularly in the partially differentiated cells. Electrophoretic mobility shift analysis demonstrated that bacterially produced GST-CDP bound to the two sites in a sequence-specific manner. The data strongly suggest that CDP acts as a major suppressor for P_{670} transcription by binding to the promoter region in the undifferentiated cells and even in the partially differentiated cells that express the activator hSkn-1a.

Keywords HPV16 · P_{670} · CCAAT displacement protein

K. Sato · T. Takeuchi · I. Kukimoto · S. Mori · T. Kanda (✉)
Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
e-mail: kanda@nih.go.jp

K. Sato · T. Yasugi · T. Yano · Y. Taketani
Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Introduction

Human papillomaviruses (HPVs) are small icosahedral viruses with circular double-stranded DNA genomes of 8 k base pairs (bp) [1]. HPVs infect the keratinocytes of the stratified squamous epithelia and cause proliferative lesions including cervical cancer [1]. More than 100 genotypes of HPVs have been identified to date and classified based on the homology of genomic DNA. HPVs that infect the genital epithelia are divided into two groups: low-risk types such as HPV type 6 (HPV6) and HPV11 found mainly in benign condyloma and high-risk types such as HPV16, HPV18, and HPV31 found in cervical cancer [2, 3].

The life cycle of HPVs is closely associated with epithelial differentiation [1, 2]. HPVs reach and infect the basal cells of the stratified epithelia through small epithelial lesions. In the basal cells the viral DNA is maintained as episomes and very low levels of non-structural proteins are produced. When the host cells initiate terminal differentiation, the HPV genome

starts to replicate and to be transcribed efficiently. Then, HPV virions are generated in the upper layers of the epidermis or mucosa and released from them.

Human papillomavirus genes are present on one strand of the circular DNA genome. The promoter, such as HPV16 P_{97}, for the early genes encoding viral nonstructural proteins is within the non-coding long control region (LCR) and the promoter, such as HPV16 P_{670} or HPV31 P_{742}, for the late genes encoding the L1 and L2 capsid proteins and E1 replication protein is localized in E7 region [4–6]. The activity of the late promoter is suppressed in undifferentiated cells and activated in the terminally differentiating keratinocytes [7–9], yet the molecular mechanisms of the suppression and activation are largely unknown.

Cellular proteins in the differentiating epithelium are reported to bind to the HPV promoter region and regulate the transcriptional activity of the promoters. hSkn-1a, a POU-domain protein playing important regulatory roles in epidermal development and keratinocyte differentiation [10], enhances activities of both P_{97} and P_{670} promoters of HPV16 [11–14]. CCAAT/enhancer binding protein β (C/EBPβ) down-regulates P_{97} and up-regulates P_{670} [15]. CCAAT displacement protein (CDP), a transcriptional repressor [16], down-regulates P_{97} [17] and HPV6 three promoters, P_{90} in LCR, P_{270} in E6 gene, and P_{680} in E7 gene [18].

It is expected that disruption of a yet unidentified binding motif for suppression in the P_{670} promoter region would result in the enhancement of transcriptional activity of P_{670}. In this study we introduced substitutions into 20-base-pair (bp)-long successive segments along the entire promoter region, from 5′ end segment to the 3′ end segment, and examined their effect on the transcription from P_{670} in the previously produced HeLa cells inducible for hSkn-1a expression [19] and undifferentiated and partially differentiated HaCaT cells. The substitutions of the two segments resulted in the efficient transcription in the undifferentiated HeLa and HaCaT cells and further enhancement in the partially differentiated cells. An electrophoretic mobility shift assay (EMSA) revealed that the substitution disrupted binding sites for CDP. The data strongly suggest that CDP is a major suppressor for P_{670} activity.

Materials and methods

Construction of luciferase reporter plasmids

An expression plasmid, p670ΔL-Luc (Fig. 1A), was constructed to monitor transcriptional activity of P_{670} by removing LCR region from the previously constructed p670-Luc [13], which was constructed by insertion of a *Pst*I-fragment of HPV16 (from nt 7003 to 7904 and from nt 1 to 868) into pUC19, followed by insertion of the firefly-luciferase gene at the first ATG of the HPV16 E1 gene. The DNA fragment from nt 7003 (*Pst*I site) to 868 (*Nde*I site) of p670-Luc was replaced with the PCR-produced DNA fragment from nt 103 to 868 to obtain p670ΔL-Luc.

A total of 38 20-bp segments covering along the HPV16 DNA from nt 106 to 855 in p670ΔL-Luc were mutated by replacement of each segment with GAATTCAAGCTTGCGGCCGC (Fig. 1C). The DNA fragment from nt103 to the target region for the mutation was produced by PCR with an antisense-primer having the mutant sequence at 5′ end. Similarly the DNA fragment from the target sequence to nt 868

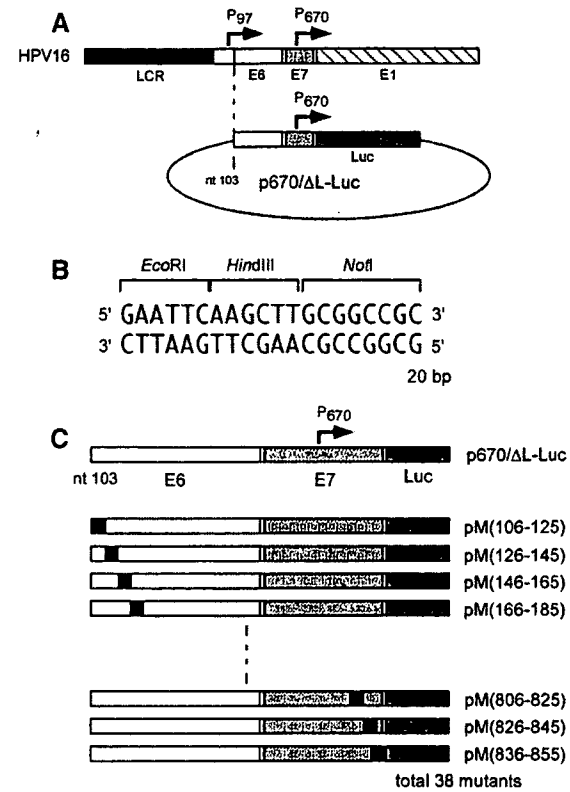

Fig. 1 Schematic representation of the plasmids used in this study **(A)** P_{670} reporter plasmid, p670ΔL-Luc: The firefly-luciferase gene was placed in the position of the E1 gene to monitor transcription from P_{670} by luciferase activity. **(B)** Nucleotides sequence introduced into the mutant plasmids: The 20-nucleotide sequence (EHN) has no known binding motifs for transcription regulators. **(C)** Construction of the mutant plasmids: Twenty-bp-long segments in the promoter region of p670ΔL-Luc were replaced with the sequence shown in Fig. 2B. The mutated segments in the region from nt106 to nt845 were not overlapping each other. The segments, from nt 826 to nt 845 and from nt 836 to nt 855, were partially overlapping

was produced by PCR with a sense primer having the mutant sequence at 5′ end. These two PCR-products were used as the template, which annealed at the mutation regions, to produce the entire HPV region (from nt103 to nt868). The resultant DNA fragment was inserted into the backbone of p670ΔL-Luc.

A plasmid for expression of renilla-luciferase from cyclophilin A promoter, phRG-PPIA, was constructed from phRG-TK (Promega Corp.) by replacement of TK promoter with cyclophilin A promoter (from nt1662 to nt2063, Genbank accession No. AY739283), which was amplified by PCR from HeLa cell DNA.

Cell culture and transfection

HaCaT cells, human immortalized keratinocytes, were grown and passaged in the low-Ca-medium [Ca-free Dulbecco's modified Eagle medium (Invitrogen Life Technologies, Carlsbad, CA) mixed with 1/10 volume of fetal calf serum and 1/10000 volume of 1M $CaCl_2$]. HaCaT cells (1.3×10^3 cells) were transfected with a mixture of 950 ng of p670ΔL-Luc reporter plasmid and 50 ng of phRG-PPIA using the Cell Line Nucleofector Kit V (Amaxa GmbH, Cologne, Germany) by an electoroporator, Nucleofector (Amaxa GmbH). The cells were seeded immediately in 9 wells of a 48-well culture plate with the low-Ca-medium. Sixteen hrs later cells (80% confluent) were lysed to obtain samples for the undifferentiated state. The other cells were grown in the fresh low-Ca-medium to be confluent and then the medium was changed to the high-Ca-medium (Ca-free Dulbecco's modified Eagle medium mixed with 1/10 volume of fetal calf serum and 18/10000 volume of 1 M $CaCl_2$). Twenty-four hrs later cells, which expressed keratin10 (the differentiation marker), were lysed to obtain samples for the partially differentiated state.

HeLa/hSkn-1a cells [19], HeLa S3 cells that can induce expression of hSkn-1a in the culture medium containing doxycycline (4 μg/ml), were grown in the growth medium [Dulbecco's modified Eagle medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum]. HeLa/hSkn-1a cells were seeded in a 24-well culture plate (3×10^4 cells/well) at 24 h before transfection. The cells were transfected with a mixture of 190 ng of p670ΔL-Luc reporter plasmid and 10 ng of phRG-PPIA using the Effectene transfection reagent (Qiagen GmbH). Sixteen hours later medium was changed with the fresh growth medium with doxycycline (4 μg/ml) or without the drug. At 48 h after the medium change, cells were lysed to measure luciferase activity.

Luciferase assay

The luciferase activities of cellular extracts were measured by using the PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink Co., Tokyo, Japan) and a microplate luminometer LB96V (Perkin Elmer Applied Biosystems). Efficiency of transfection was normalized to Renilla-luciferase activity.

Electrophoretic mobility shift assay

The cDNA of human CDP was kindly provided by Dr. Ellis Neufeld [20]. The DNA fragment encoding the CDP DNA-binding domain (cut repeat 3 and the homeodomain, aa positions 3391–3969, GenBank accession No. M74099) was synthesized by PCR with a forward primer (5′-CGG AAT TCA AGA ATT AGT AGC CAT GTC-3′), a reverse primer (5′-CGG AAT TCA CTG AAT TTC CTC AAT GAA C-3′), and the CDP cDNA. The PCR product was digested with EcoRI and inserted into pGEX-2TK (Amersham Bioscience) at the EcoRI site to produce an expression plasmid for GST-fused CDP DNA-binding domain (GST-CDP). GST-YY1 and GST-hSkn-1a were previously described [13]. The GST-CDP, GST-YY1, GST-hSkn-1, and GST were expressed in *Escherichia coli* strain JM109 and purified by GSTrap affinity-column chromatography (Amersham Biosciences) with AKTA*prime* (Amersham Biosciences). A mixture of double-stranded [^{32}P]-labeled oligonucleotides (0.4 pmol), 1 μg of GST-fusion protein, and 1 μg of poly (dI/dC) in a final volume of 10 μl of binding buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 40 μg/ml BSA) was incubated at room temperature for 30 min. Then the samples were loaded on a 5% polyacrylamide gel and electrophoresed in 0.5× Tris-borate/EDTA buffer at room temperature. The gels were dried and visualized by autoradiography on X-ray films. The sense sequences of double-stranded oligonucleotides are as follows:

551-580, 5′-CAG CTG TAA TCA TGC ATG GAG ATA CAC CTA-3′;
m551-580, 5′-CAA GCT TGC GGC CGC ATG GAG ATA CAC CTA-3′
661-690, 5′-GAG GAG GAT GAA ATA GAT GGT CCA GCT GGA-3′;
m661-690, 5′-GAG GAG AAT TCA AGC TTG CGG CCG CCT GGA-3′;
CDP cons, 5′-ACC CAA TGA TTA TTA GCC AAT TTC TGA-3′;
YY1 cons, 5′-CGC TCC GCG GCC ATC TTG GCG GCT GGT-3′.

Numbers in probe names indicate nucleotide numbers of the HPV16 genome (the HPV Sequence Database of Los Alamos National Laboratory) and substitution mutations are underlined.

Immunoblotting

HaCaT cells (10^7 cells), which continuously grew in the low-Ca-medium (undifferentiated sample) or were maintained in the high-Ca-medium for 24, 48, 72, and 96 h after cells became confluent (partially differentiated state), and HeLa/hSkn-1a (10^7 cells), which were cultured with or without induction of hSkn-1a for 48 h, were suspended in the sample buffer of SDS-gel electrophoresis and boiled for 5 min. Then, samples were loaded on a SDS-polyacrylamide gel and electrophoresed. After transfer of proteins to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) the membrane was blocked with 5% skim milk in PBS-0.1% Tween 20 at room temperature and then incubated with anti-CDP (a gift from Dr. Alain Nepveu) [16], anti-YY1 (H-414), anti-Skn-1a (C-20), anti-keratin 10 (DE-K13) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-involucrin (SY5), or anti-alpha-tubulin (B-5-1-2) (Sigma-Aldrich, St. Louis, MO) antibodies for 1 h. The membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies for 1 h. Peroxidase activity was detected with the enhanced chemiluminescence detection method (ECL plus kit, Amersham Biosciences).

Results

Cis-acting elements involved in regulation of transcriptional activity of P_{670}

The effect of substitutions in the promoter region from HPV16 nucleotide (nt) 106 (the end of LCR) to 865 (the first ATG of the E1 gene) on P_{670} transcriptional activities was monitored by a transient reporter expression from p670ΔL-Luc with and without mutations in the undifferentiated and the partially differentiated HaCaT and HeLa cells. The expression cassette of p670ΔL-Luc was composed of an HPV16 DNA fragment from nt 103 to 855 and a DNA encoding the firefly-luciferase gene (Fig. 1A). The sequence from nt 106 to 125 in p670ΔL-Luc were changed into GAATTCAAGCTTGCGGCCGC (EHN), which has no binding motif for any transcription factors so far known, to produce pM (106–125) (Fig. 1B and C). The 20-bp oligonucleotide with the sequence of EHN did not bind proteins in the HaCaT nuclear extract by an electric mobility shift assay (data not shown). Similarly, the serial 20-bp segments covering along the entire promoter region were each replaced with the above non-binding sequence to produce the other 37 mutants. Except for pM (826–845) and pM(836–855) these substitutions were not overlapping.

HaCaT cells, an immortalized human keratinocytes cell line, was used after cultivation in the condition inducing partial differentiation. HeLa/hSkn-1a, which is a HeLa cell line expressing the P_{670}-positive regulator, hSkn-1a [13], in the presence of doxycycline [19], was used to analyze effect of the mutations.

HaCaT cells were transfected with the plasmids by electroporation and cultured in the low-Ca-medium. About 16 h later, part of cells in an 80% confluent culture were lysed to obtain the samples from undifferentiated cells. The rest of the cells were grown in the fresh low-Ca-medium further to form a confluent culture and then maintained in the high-Ca-medium for 1 day to obtain the samples from partially differentiated cells. HeLa/hSkn-1a cells transfected with the plasmids were cultured with or without induction of hSkn-1a for 48 h and lysed. A plasmid (phRG-PPIA) for expression of renilla-luciferase from cyclophilin A promoter, whose transcriptional activity is steady during the early phase of differentiation [21], was used to normalize transfection efficiency. The cells were transfected with one of p670ΔL-Luc plasmids together with phRG-PPIA and the data were presented as a ratio of activities of firefly-luciferase and renilla-luciferase (Fig. 2).

Luciferase expressions from p670ΔL-Luc in the partially differentiated HaCaT cells and hSkn-1a-positive HeLa cells were approximately 2–3 times higher than those in the undifferentiated HaCaT and in hSkn-1a-negative HeLa cells. The data are consistent with our previous findings that hSkn-1a binds to two sites, Skn#1 (from nt 560 to 569) and Skn#2 (from nt 581 to 590) (Fig. 4), and enhances transcription from P_{670} [13].

Luciferase expressions from the two mutants, pM(546–565) and pM(666–685), were significantly higher than those from p670ΔL-Luc in the undifferentiated HaCaT and hSkn-1a-negative HeLa cells, suggesting that the substitutions in these mutants resulted in abrogation of binding sequences for strong negative regulators. The mutants other than these two showed no clear patterns of the substitution-induced enhancement of the activities common to HaCaT and HeLa cells. The significance of minor responses shown by some mutants is unclear at present.

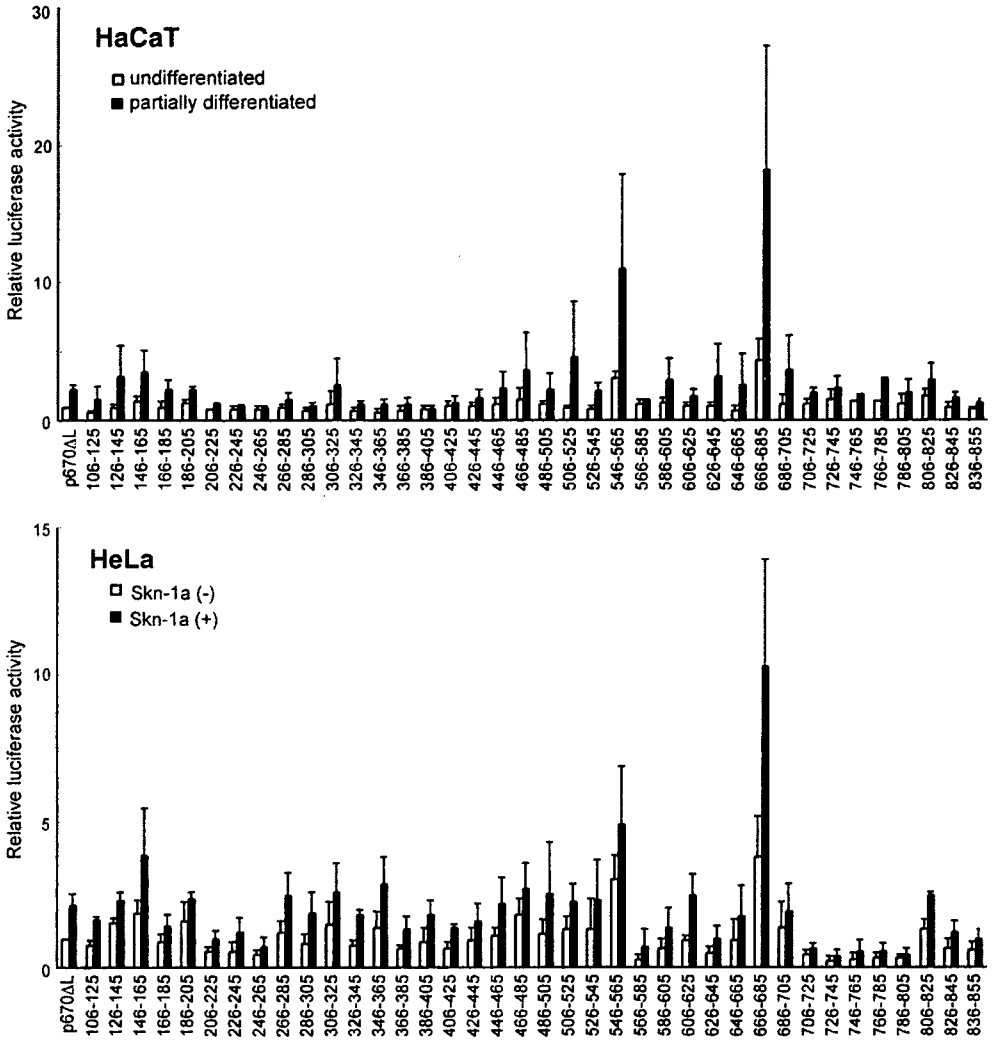

Fig. 2 Relative luciferase activities of extracts from HaCaT and HeLa cells transfected with p670ΔL-Luc and the mutant plasmids. HaCaT cells grown in the low-Ca-medium were transfected with a mixture of p670ΔL-Luc reporter plasmid and phRG-PPIA reference plasmid expressing renilla-luciferase from cyclophilin A promoter. About 16 h later cells were lysed to obtain samples for expression in the undifferentiated cells. The other cells were grown in the fresh low-Ca-medium for 40 h and then maintained with high-Ca-medium for 1 day. The cells were lysed to obtain samples for expression in the partially differentiated cells. HeLa/hSkn-1a cells, a HeLa cell line capable of expressing hSkn-1a in response to doxycycline in the medium, were transfected with a mixture of p670ΔL-Luc reporter plasmid and phRG-PPIA. About 16 h later, the medium was changed with the fresh growth medium with or without doxycycline (4 μg/ml). At 48 h after the medium change, cells were lysed to measure luciferase activity. Efficiency of transfection was normalized to renilla-luciferase activity. Data obtained from five independent experiments were analyzed for Welch's *t*-test. The enhanced transcription from pM (546–565) and pM (666–685) was significant. p670ΔL: p670ΔL-Luc, a plasmid expressing luciferase from P_{670}. 106–125:a a mutated p670ΔL-Luc whose nucleotides from nt 106 to 125 were replaced with GAATTCAAGCTTGCGGCCGC. The other mutated plasmids were indicated similarly

Factors binding to the nt 546–565 and nt 666–685 regions

The substitutions of sequences nt 546–565 and nt 666–685 apparently disrupt the putative binding motif for CDP, ATNNAT [16] (from nt 562 to 567 and from nt 673 to 678) and the binding motif for YY1, CAT (from nt 561 to 563, from nt 564 to 562, and from nt 679 to 677) (Fig. 3). Thus, the bindings of CDP and YY1 with 30-bp-long synthetic DNA probes covering from nt 551 to 580 and from nt 661 to 690 were examined by an EMSA, by using bacterially expressed GST-fusion proteins (Fig. 4). For comparison, probes having typical binding sequences for CDP (CDP cons) and YY1 (YY1 cons) was included. GST-CDP and GST-YY1 bound to the CDP cons and the YY1 cons, respectively, in a sequence-specific manner. GST did not bind any probes used in the assay. Therefore, we

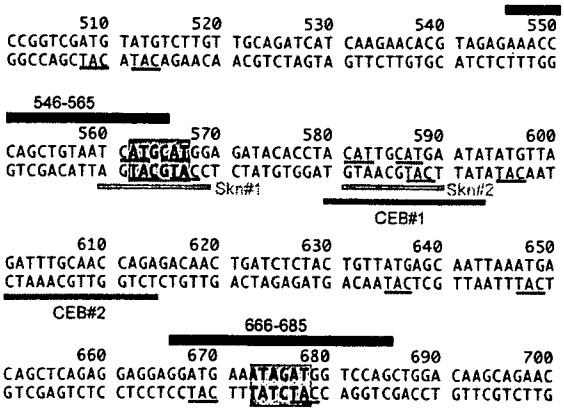

Fig. 3 Nucleotide sequence around P_{670}: The bold bars indicate the segments responsible for the suppression of transcription from P_{670}. Boxed in red is ATNNAT, which is the putative binding motif for CDP. Red, green, and blue lines indicate the putative binding sites for YY1, hSkn-1a, and C/EBPβ, respectively. Sequence is from Los Alamos National Laboratory Database

concluded that the EMSA could detect the sequence-specific bindings of CDP and YY1 with the probes.

GST-CDP bound with the probes 551–580 and 661–690 (Fig. 4A). The level of binding to the probe m551–580, of which nucleotide sequences are identical to those from nt 551 to 580 of pM (546–565), was lowered to approximately 1/2 of the level of binding to the probe without the mutation. The binding of GST-CDP with the probe m661–690, of which nucleotide sequences are identical to those from nt 661 to 690 of pM (666–685), was lowered to the background level. It is not clear why m551–580 retained partial capability of binding with CDP. The levels of binding-reduction of the probes 551–580 and 661–690 to CDP (Fig. 2) by the mutations were parallel to levels of enhancement of the transcription from P_{670} by the mutations.

GST-YY1 bound to the probes 551–580 and m551–580 equally and did not bind to both probes 661–690 and m661–690 (Fig. 4B), indicating that YY1 was not involved in the enhanced transcriptional activity of pM (546–565) and pM (666–685).

GST-hSkn-1a bound to probe 551–580, which contains hSkn-1a binding sequence we previously found [13] (Fig. 4C). GST-hSkn-1a similarly bound to m551–580, the nucleotide substitutions introduced in pM (546–565) did not abrogate the binding of hSkn-1a to the hSkn-1a binding site (Skn#1 in Fig. 3).

Expression of CDP and hSkn-1a in HaCaT and HeLa/hSkn-1a cells

Immunoblotting showed that in HaCaT cells CDP and YY1 were present in the undifferentiated and the partially differentiated state and hSkn-1a, karatin10, and involucrin emerged in the partially differentiated state (Fig. 5A). HaCaT cells were passaged in the low-Ca-medium continuously before the cells became confluent. When the cells became 80% confluent, the cells, which correspond to the undifferentiated cells in Fig. 2, were collected for the immunoblotting sample. The cells were further grown in the low-Ca-medium and were collected when the cells became confluent (sample of confluent cells). Then the medium of the confluent culture was replaced with the high-Ca-medium. The cells were collected at 24 (correspond to the partially differentiated state in Fig. 2), 48, and 72 h after the medium replacement.

In HeLa/hSkn-1a cells CDP was present independently of the induction of hSkn-1a with doxycycline (Fig. 5B).

Discussion

Efficient transcription of the HPV16 late promoter, P_{670}, occurs when the host keratinocytes reach the late stage of terminal differentiation. Our previous studies have indicated that hSkn-1a and C/EBPβ directly bind to the upstream region of P_{670} and activate its transcriptional activity [13, 15]. However, the involvement of yet unidentified factor(s) in the regulation of P_{670} has been postulated because hSkn-1a and C/EBPβ are expressed in the early stage of keratinocyte differentiation, and the level of transcription from P_{670} in the presence of hSkn-1a and C/EBPβ are still rather low. In this study we surveyed cis-acting elements involved in the regulation of P_{670} activity by analyzing effects of substitutions in the promoter region on transcription from P_{670}. With the surveillance we expected to detect all of the binding sites for the putative major repressors including yet unidentified ones.

It was found that disruption of the two CDP-binding motifs in the promoter region, one at 102 bases upstream of P_{670} and the other at the transcriptional starting site, resulted in enhanced transcription from P_{670} in undifferentiated HaCaT and HeLa cells and in further enhanced transcription in the hSkn-1a-positive partially differentiated HaCaT and HeLa cells. EMSA showed that CDP bound to the two sites having the motifs in a sequence-specific manner.

CCAAT displacement protein was present in the partially differentiated HaCaT cells, which expressed hSkn-1a and some of the typical differentiation marker proteins, such as karatin10 and involcurin. The mutations in pM (546–565) and pM (666–685) did not affect