Development of screening systems for drugs against human papillomavirus-associated cervical cancer: based on E6-E6AP binding

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Abstract

Human papillomavirus (HPV) E6 protein forms a ternary complex with the cell-cycle regulator p53 and the E6-associated protein (E6AP) known as an E3 ubiquitin protein ligase, leading to the degradation of p53 via the ubiquitination pathway. As an attempt to employ interaction between HPV viral oncogene E6 and a cellular protein E6AP for in vitro screening system of drugs against HPV infection, we primarily investigated the E6AP-E6 binding through pull down assay and enzyme-linked immunosorbent assay (ELISA). E6AP immobilized on the resin produced specifically complexes with bacterially expressed E6 in a dose-dependent manner, as determined by immunoblot analysis. This result was collinear with that shown in ELISA, which is a useful system for mass-screening potential drugs with rapidity and cheapness. Screening system based on the interaction between E6AP and E6 may be a promising system in the development of drugs against cervical cancer caused by HPV infection. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: HPV; E6AP; ELISA

1. Introduction

Human papillomaviruses (HPVs) have been recognized as the primary cause of cervical cancer

because of detection of HPV genomes in about 90% of all cervical cancers and transforming properties of their encoding proteins in cell culture and in situ (zur Hausen and de Villiers, 1994; Howley, 1996). HPV 16 is the most common HPV types in malignant neoplasia and is found in about 60% of all cervical carcinoma, while other HPV types account for another 30% of these malignancies and are often associated with benign neoplasia, such as genital warts (zur Hausen and de Villiers, 1994; Howley, 1996). More than ten

million American women are infected with high-risk HPVs, such as HPV-16 and HPV-18, and an estimated 15 000 American women are diagnosed with cervical cancer each year (zur Hausen and de Villiers, 1994; zur Hausen and Schneider, 1987). While routine Papanicolaou (Pap) screening has significantly reduced the rate of cervical cancer in developed countries, this cancer remains a leading cause of death in many other parts of the world. There are approximately 500 000 cases of cervical cancer worldwide each year; about one-third of them are fatal (de Villiers, 1989). HPVs have circular, double-stranded DNA genomes that are approximately 8 kb in size and encode eight genes, of which E5, E6 and E7 have transforming properties. These proteins have pleiotropic function, such as transmembrane signaling, regulation of the cell cycle, transformation of established cell lines, immortalization of primary cell lines, and chromosomal stability (Filatov et al., 1998; Crusius et al., 1999; Syrjanen and Syrjanen, 1999; Zwerschke and Jansen-Durr, 2000). Specifically, E6 has been reported to activate or repress transcription, to stimulate telomerase, to immortalize primary cell cultures, and to interfere with the differentiation of human keratinocytes (Sherman et al., 1997). Although limited success is achieved with immune modulator like interferon against HPV 11- and HPV 6-associated lesions, current treatment for HPV 16-associated lesions is surgery (Stellato et al., 1997; Trobs et al., 1998). Prevention of HPV infection by vaccination and immune therapy is under investigation but not yet established. Therefore, prevention through early detection and ablation of dysplastic tissues is the best management of cervical cancer.

E6 oncoprotein can form a ternary complex with the cell-cycle regulator p53 and the E6-associated protein (E6AP), leading to the degradation of p53 via the ubiquitination pathway (zur Hausen and de Villiers, 1994; Howley, 1996). E6AP is a member of a family of proteins, known as E3 ubiquitin-protein ligases, which have been proposed to play a role in defining the substrate specificity of the ubiquitin-proteasome degradation system. The schematic functional domains of E6AP are depicted in Fig. 1 as reported previously (Huibregtse et al., 1993). The 100-kDa

E6AP protein contains an 18-amino acid region (amino acids 391–408) that is sufficient for binding to E6. The E6AP-dependent binding of p53 involves amino acids 280–781, a domain encompassing the E6-binding region. Finally, an intact COOH terminus hect (homologous to E6AP C-terminal) domain is necessary for E6-mediated p53 ubiquitination. E6 can also bind E6-binding protein (E6-BP, also known as ERC-55), a calcium-binding protein localized in the endoplasmic reticulum, with possible consequences for intracellular signaling (Chen et al., 1995). E6 can change cellular morphology by interacting with paxillin and disrupting the actin cytoskeleton (Tong and Howley, 1997).

Recently, with extensive knowledge of molecular mechanism by HPV oncoproteins, a new drug has been designed to suppress infections by the HPV so as to prevent the development of cervical cancer (Beerheide et al., 1999). Therefore, an in vitro screening system for development of drugs to treat HPV infection is primarily required to search compounds with desirable properties. In our report, we established in vitro screening system, using interaction between E6 and E6AP, and its application for the ELISA system could be available for the development of potential drugs against HPV infection.

2. Materials and methods

2.1. Construction and overexpression of E6 and E6AP

E6 and E6AP were prepared with the pGEX system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or pET express system (Novagen, Madison, WI). E6 was obtained using following primer sets by PCR amplification from total RNA isolated from CaSki cell lines. The primers for E6 amplification are 5′-GCG GCC GCC ACC ATG TTT CAG GAC CAC AG-3′ (sense) and 5′-CTG CGG CCG CGA TTA CAG CTG GGT TTT CTC T-3′ (antisense). The PCR product was inserted into T-vector prepared as described using pBluescript II KS (+) (Stratagene, La Jolla, CA; Marchuk et al., 1990). E6 insert was excised from

digestion of pBluescript/E6 with *Bam*HI/*Sal*I and subcloned into corresponding expression vectors, pET28a (Novagen) and pGEX4T-1 which were prepared by digesting with *Bam*HI/*Sal*I, respectively. Transformation of pGEX/E6 and pET28a/E6 into DH5α or BL21 (DE3 pLys) was performed to express GST-tagged and $6 \times$ His-Tagged E6, respectively. To prepare E6AP cDNA, two halves of cDNAs with overlapping region in the middle of full ORF were amplified using two different primer sets by RT-PCR from total RNA isolated from CaSki, cervical carcinoma cell line. The primers of the former half in E6AP (E6APa) are 5′-*AGA TCT* ATG AAG CGA GCA GCT GCA AAG CAT CTA ATA-3′ (sense) (nt 1–30) and 5′-CTC TAG CCG GAC

AAG TGC ATC ATC TAT GAT-3′ (antisense) (nt 1524–1553), and the latter half primers (E6APb) are 5′-AGC GAG CTG ACA CTT CAG GAA CTT TTG GGA-3′ (sense) (nt 1143–1172) and 5′-TTA CAG CAT GCC AAA TCC TTT GGC ATA CGT-3′ (antisense) (nt 2529–2558). The respective PCR products, E6APa and E6APb were ligated to PCR®2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA) and then designated as PCR®2.1-TOPO/E6APa and PCR®2.1-TOPO/E6APb, respectively. To construct bacterial expression vector for wild type E6AP, E6APa was excised from PCR®2.1-TOPO/E6APa by digestion with *Bgl*II/*Hin*dIII, and ligated into pET28a predigested with *Bam*HI/*Hin*dIII, resulting in pET28a/E6APa. E6AP was finally con-
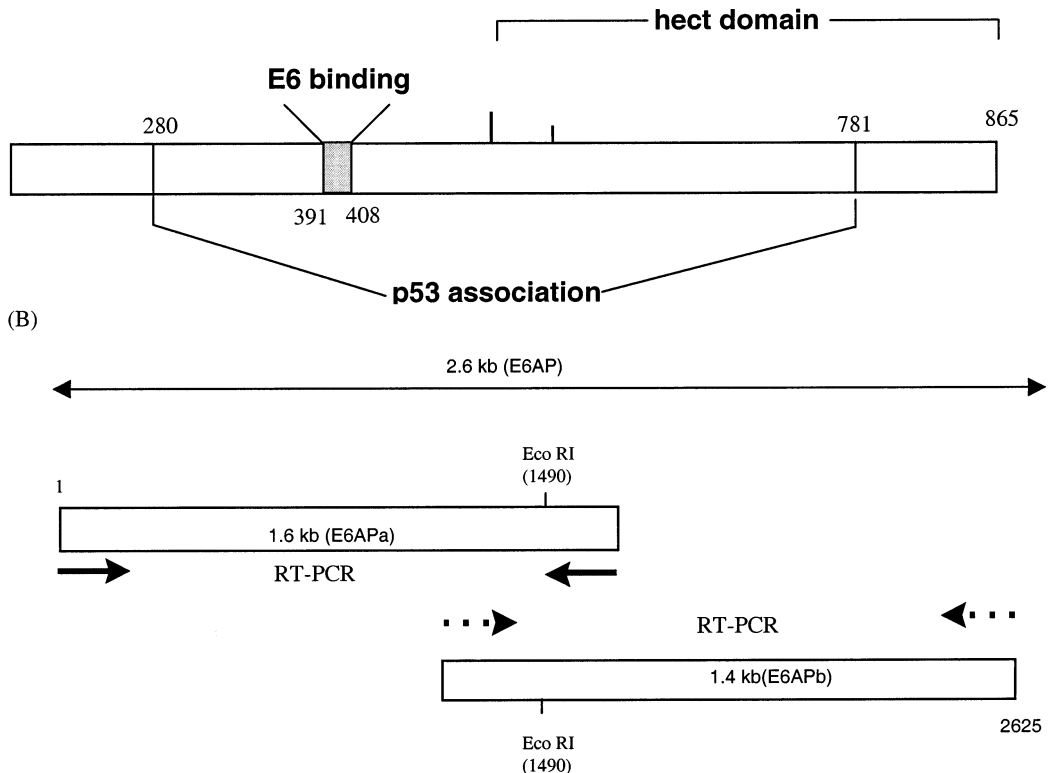

Fig. 1. The schematic representation of the functional domains of E6AP that direct E6 and p53 binding (A) and a strategy for construction of E6AP (B). E6AP binding regions were dissected into 3 domains responsible for E6 binding (amino acids 391–408), E6-dependent association with p53 (amino acids 280–781), and E6-dependent ubiquitination following ternary complex formation requiring the hect catalytic domain. To obtain E6AP cDNA RT-PCR was performed using total RNA extracted from CaSki cell line and two oligonucleotide primers sets overlapped in the middle of E6AP. The complete cDNA was joined in a single fragment by taking advantage of unique restriction site within partial cDNA sequence as described in Section 2.

structed by ligation of E6APb insert, excised by digestion of PCR®2.1-TOPO/E6APb with *Eco*RI, into pET28/E6APa which was predigested with *Eco*RI. For glutathione *S*-transferase (GST)-fused E6AP, E6APa prepared from PCR®2.1-TOPO/E6APa by predigestion with *Bgl*II/*Eco*RI was subcloned into pGEX4T-1 vector digested with *Bam*HI/*Eco*RI to construct pGEX/E6APa. The resulting product pGEX/E6APa was digested with *Eco*RI and subsequently combined with E6APb insert obtained from digestion of PCR®2.1-TOPO/E6APb with same restriction enzyme *Eco*RI. In addition, to examine the specificity of E6AP-E6 binding, lysates derived from *Escherichia coli* expressing E7 or vector were prepared with pET28 system (Novagen, Madison, WI). E7 insert was amplified from total RNA extracted from CaSki cell lines by RT-PCR using primer set comprising 5′-CACCATGGCATG-GCATGGAGATACACCT-3′ (sense) and 5′-TTATGGTTTCTGAGAACA-3′ (antisense) and inserted into T-vector prepared from pBluscript KS. E7 fragment was cloned into pET28 vector to construct 6 × His-fused E7 as an insert *Bam*HI/*Sal*I. GST-fused proteins were expressed in *E. coli* DH5α by induction with 1 mM isoprophyl-β-D-thiogalactopyranoside (IPTG), Cells were harvested and lysed in phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluride (PMSF) and 10 μg/ml of aprotinin, and followed by sonication. Proteins expressed in pET28 vector system were prepared in BL21 (DE3 pLys) through same procedures as described above, except for using lysis buffer, pH 8.0, containing 50 mM NaH$_2$PO$_4$ and 150 mM NaCl. The resulting mixture was centrifuged for 30 min at 12 000 rpm to remove pellet containing cell debris. Supernatants were used as cell lysate for binding assay. For immobilization of GST-E6AP on a resin, the supernatants were incubated with glutathione (GSH)-Sepharose (Glutathion Sepharose™4B, Phamacia Biotech, Sweden) for 1 h at 4°C. His-tagged E6AP proteins were purified by incubating supernatant with Ni-NTA Agarose (Peptron Co., Taejon, South Korea) and following extensive washing with buffer (20 mM imidazole in lysis buffer). The beads were collected and washed

three times with PBST buffer for utilizing as a bait of binding assay. Also, E7 lysates as histidine tagging protein were prepared in the same procedure as described above and their concentrations of lysates, GST-E6, His-E6 and His-E7, were approximately 9.7, 8.2 and 7.5 mg/ml, respectively, as determined by Bradford reagents (Bio-Rad Lab., Hercules, CA).

2.2. E6AP and E6 binding assay

E6 binding assays were performed by combining 6 × His-E6AP or GST-E6AP fusion protein immobilized on nickel- or GSH-Sepharose beads, respectively, with various amounts of bacterially expressed GST-E6 or 6 × His-E6 supernatants (50, 100 and 200 μl). The mixtures were rotated in microcentrifuge tubes at 4°C for 1 h. The Sepharose beads were then collected by centrifugation, washed three times with 1 ml of lysis buffer, and then boiled for 5 min in sodium dodecyl sulfate (SDS) gel loading buffer. The amounts of E6 protein that bound to the beads were determined by SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) and Western blot analysis. Gels were transferred to Immobilon-P membranes (Millipore, Bedford, MA) at 50 V for 1.5 h at room temperature and were blocked by soaking into methanol for 5 min and drying at room temperature. The membrane was probed with goat anti-E6 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in 3% skimmed milk, followed by an alkaline phosphatase-conjugated anti-goat antibody (Sigma), and visualization was achieved with NBT/BCIP substrate kit (Bio-Rad Lab.).

2.3. Optimization of ELISA

Ten milliliters of supernatants of bacterially expressed 6 × His-E6AP was bound to Ni-NTA Agarose beads pre-equilibrated with 10 mM imidazole buffer containing 0.5 N NaCl and 20 mM Tris–HCl, pH 8.0, and then eluted by addition of 200 mM imidazole buffer after washing with 50 mM imidazole. Maxisorb 96-well plate (Nunc, The Netherlands) was coated with eluted proteins at a dose of 4 μg/ml and blocked with PBS
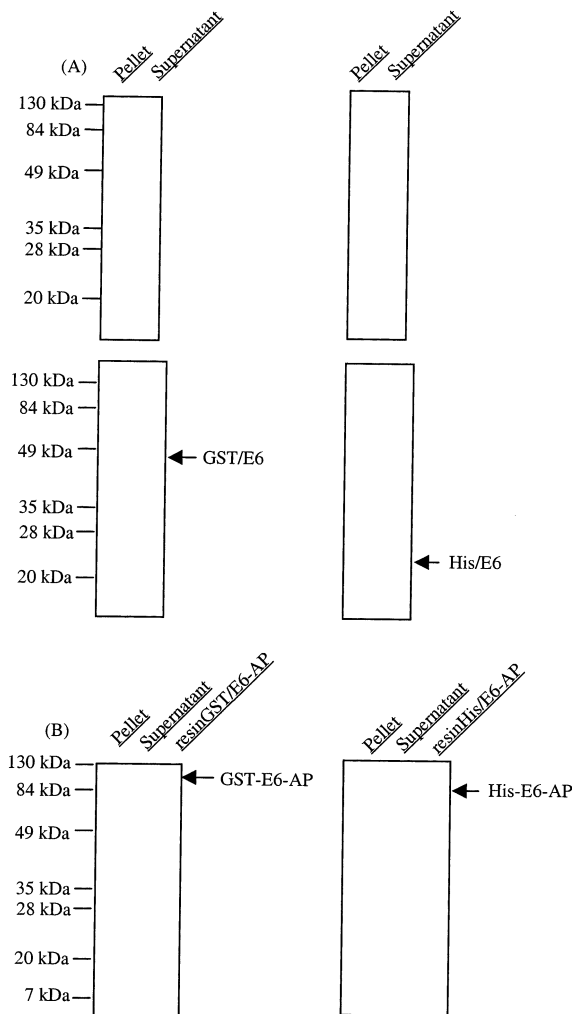
Fig. 2. Overexpression of recombinant E6 and E6AP proteins. (A) GST-tagged E6 and 6 × His-tagged E6, (B) GST-tagged E6AP and 6 × His-tagged E6AP. The bacterial cell lysates after sonication were separated into pellets and supernatants by centrifugation at 12 000 rpm for 30 min. Diluted aliquots (1:10) of each fraction were analyzed on SDS-PAGE. The upper and lower panels of (A) represent the Coomassie blue staining and immunoblotting, respectively. For fused E6, E6 protein was identified with goat anti-E6 (N-17) antibody followed by incubation of alkaline phosphatase-conjugated anti-goat IgG. After washing, the bound enzyme was visualized using alkaline phosphatase conjugate substrate kit.

containing 3% skimmed milk. Serial dilutions of GST-E6 or His-E6 lysates were added to the plate pre-coated with E6AP and then allowed to incubate for 1 h at room temperature. After exhaus-

tive washing with PBS containing 0.5% Tween 20 (PBST) to remove unbound E6, goat anti-E6 was dispensed to plate at a concentration of 0.1 µg/ml followed by 1-h incubation. After washing, horseradish peroxidase (HRP)-conjugated anti-goat antibody (Sigma) was applied to the plate for immunoassay. After washing twice with PBST, 100 µl of substrate (4 mg o-phenylenediamine, 5 µl 37% H_2O_2 per 10 ml of 0.1M citrate buffer, pH 5.1) was added, and the enzyme activity was stopped 15 min later with 2.5 N sulfuric acid and detected at 490 nm using an ELISA reader (Molecular Devices, Hercules, CA).

3. Results

As a strategy for amplification of E6AP cDNA, two pairs of primers which are overlapped at nucleotides (nt) 1524–1553 were designed as depicted in Fig. 1(B). Both E6 and E6AP cDNA were fused to pGEX or pET28 vector to prepare the GST- or 6 × His-fused proteins appropriate for screening systems. Overexpression and immobilization of fusion proteins on resins for pull down assays are shown in Fig. 2. Expression levels of E6 proteins present in both supernatant and pellet after sonication were detected by Coomassie-blue staining and Western blot analysis. Immunoblot analysis showed that apparent molecular weights of expressed His-E6 and GST-E6 proteins were approximate 24 and 46 kD, respectively. The migration of His-tagged E6 was faster than the 20-kDa molecular weight marker as predicted, which likely to be due to the additional nucleotides generated on subclone and positive charges of six histidine residues linked to N-terminus of E6 (Fig. 2(A)). Also, expression plasmids for E6AP were transformed into *E. coli* BL21 and DH5α, and proteins present in the bacterial lysates resolved by SDS-PAGE and Coomassie staining, proteins migrating at the predicted molecular weights for E6AP were clearly visible. His-tagged E6AP and GST-tagged E6AP were over than 100 kD, molecular weight corresponding to authentic E6AP alone, and were immobilized on GSH- or Ni-NTA Sepharose, respectively, to utilize as a bait in pull down

assays. E6AP was purified to be homogeneous as bead bound form of either GST- or His-tagged (Fig. 2(B)). E6AP immobilized on resins was incubated with E6 fusion proteins derived from different vector systems, respectively, to avoid bindings to the same ligands on resins (Fig. 3). E6 fusion proteins were bound to His-E6AP and GST-E6AP in a dose dependent manner, respectively, whereas E6 proteins had binding to neither GST immobilized on GSH- nor Ni-NTA-resin alone, indicating that binding systems using E6AP-E6 interaction may be useful for drug screening. For practical application, pull down assays between E6AP and E6 were extended to ELISA systems (Fig. 4). However, His-E6AP was readily eluted from Ni-NTA Agarose resin by addition of 200 mM immidazole, compared with elution of GST-

E6AP from GSH-Sepharose resins by elution buffer (10 mM glutathione in PBS). His-E6AP was coated to plate at a concentration of 4 μg/ml and then mixed with various dilutions of E6 lysates. E6 proteins bound to E6AP were assayed with antibody, which recognize specifically E6. As shown in Fig. 4, GST-E6 or His-E6 fusion proteins were specifically bound to E6AP in a dose dependent manner while any other non-relevant lysates derived from E. coli harboring pET28-E7 and vector alone pET28a, were not. For mass-screening potential drugs against cervical cancer caused by HPV infection, we determined 16× dilution of GST-E6 or His-E6 lysates as optimum dose in the ELISA based on the interaction between E6AP and E6.
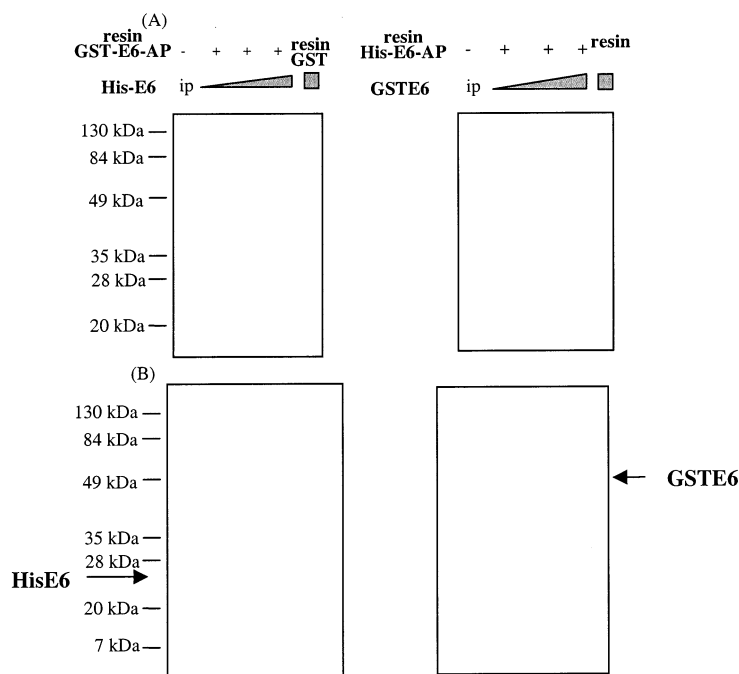

Fig. 3. Pull-down assays of bead following binding between bead bound E6AP and E6 lysates. E6AP immobilized on resins was combined with 50–200 μl of E6 crude lysates in either GST- or His-fused form. As a control, E6AP was incubated with GST alone bound to GSH-Sepharose beads. E6AP-bound E6 was analyzed by SDS-PAGE on a 12% gel followed by Western blot analysis using antibody, which specifically recognize E6. Input (ip) was loaded directly into the well and represents approximately 0.6% of E6 lysates at the highest levels used in each binding reactions. Staining with Coomassie blue staining, (B) immunoblotting against HPV E6.
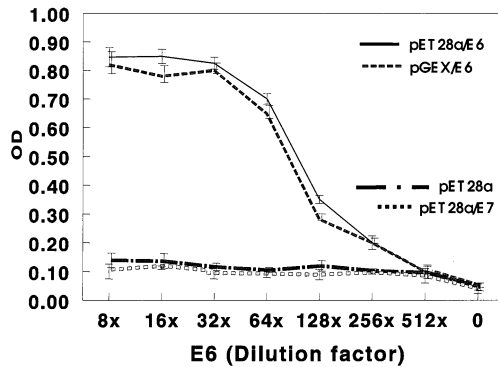
Fig. 4. Optimization of ELISA for screening drug targeting to HPV E6. Eluted E6AP was coated onto Maxisorb 96-well plate for overnight and blocked with PBS containing 3% skimmed milk for 2 h at room temperature. After washing with PBST, various doses of E6 supernatants derived from pET28a/E6 and pGEX/E6 were incubated, respectively, for 1 h. The E6 bound to E6AP was detected with goat anti-E6 (N-17) antibody followed by incubation of horseradish phosphatase-conjugated anti-goat IgG. After washing, the bound enzyme activity was read at 490 nm by using ELISA reader. As non-specific controls, non-relevant agents such as E7 and supernatant of vector, pET28a, were incubated with E6AP. Results are the mean ± standard deviation and are representative of three experiments.

4. Discussion

E6AP is a 100-kDa cellular protein which mediates the stable association of the high-risk HPV E6 protein with p53. The region of E6AP required for stable association with HPV 16E6 has been mapped to 18 amino acids in the central portion of the molecule (Huibregtse et al., 1993). The 18-amino acid region contains five glutamic acids and two arginine residues, suggesting that the overall acidic character of this region may be important for the interaction with the basic E6 proteins. A peptide composed of 18 amino acid sequences is able to compete with E6AP binding to E6 at a high concentration (5000-fold molar excess of peptide over E6AP for 50% inhibition; Huibregtse et al., 1993). It might not be possible to utilize this 18-amino acid peptide as an antiviral drug because high levels of peptide are required for competition. Therefore, the experiments described here were designed to apply direct binding between E6AP and E6 to ELISA

system for screening antiviral drugs. Screening system based on interaction between E6AP and E6 has been recently reported (Beerheide et al., 1999). In previous report, the zinc-ejecting inhibitor of E6 binding to E6-associated protein (E6AP) and E6-binding protein (E6-BP) was investigated using BIACORE assays. Among compounds ejecting zinc from E6 protein, some inhibit the interaction of E6 with E6AP and E6-BP. The E6 protein of HPV 16 consists of 158-amino acid residues and contains two hypothetical Cys-X2-Cys-X29-Cys-X2-Cys zinc fingers. The conservation of the zinc fingers in E6 and E7 among distantly related HPV types suggests that this zinc-binding motif is strictly required for the function of the E6 and E7 oncoproteins. However, BIACORE system is very expensive and laborious compared to the ELISA system. In this study, we first adopted ELISA system for screening system for anti-HPV drugs. First, we constructed the GST- or His-fused E6AP and E6 to investigate the direct binding between E6AP and E6. Western blotting showed that His-E6 was bound to GST-E6AP immobilized on resins but not to GST. Similar results were obtained with the His resin pull-down assay, which showed that GST-fused E6 was efficiently retained by His-E6AP but not by resin alone. Second, we applied in vitro binding of E6 and E6AP into the development of ELISA. Elution of His-tagged E6AP by addition of imidazole was more readily achieved than that of GST-fused E6AP. Hence, E6AP protein, eluted from His-E6AP bound to resin, was coated onto the plate to which various doses of E6 derived from GST-E6 or His-E6 were added. Either GST-E6 or His-E6 lysates were bound to E6AP in a dose dependent manner to 32:1 dilution. E6AP-E6 binding was saturated at concentrations more than 32-fold diluted E6 lysates. These ELISA systems were first established and may be useful in the screening of drugs against cervical cancer caused by HPV infection. Moreover, the establishment of these screening systems may provide a basis for the further analysis of the normal cellular functions as well as development of anti-cancer drugs.

Acknowledgements

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