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Development of a Screening System for Drugs Against Human Papillomavirus-Associated Cervical Cancer: Based On E7-Rb Binding

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The human papillomavirus E7 protein can form a specific complex with a retinoblastoma tumor suppressor gene product (p105-Rb) that results in the release of the E2F transcription factor, which is critical for the growth-deregulation and transforming properties of the viral E7 oncoprotein. In an attempt to apply interaction between the E7 oncoprotein and a target cellular protein Rb for an *in vitro* screening system for drugs against human papillomavirus infection, we primarily investigated the E7-Rb binding through a pull down assay and enzyme-linked immunosorbent assay. The pull down assay showed that both glutathione S-transferase-tagged E7 and His-tagged E7 immobilized on resins specifically produced complexes with bacterially expressed Rb in a dose-dependent manner, as determined by immunoblot analyses. This result coincided with that of an enzyme-linked immunosorbent assay, which is a useful system for the mass screening of potential drugs. Taken together, this screening system (based on the interaction between E7 and Rb) can be a promising system in the development of drugs against cervical cancers caused by human papillomavirus infection.

Keywords: ELISA, E7, HPV, Rb

Introduction

Human papillomaviruses (HPVs) have been recognized as the primary cause of cervical cancer. Among them, specific types of HPV (16, 18 and several others) have been identified as causative agents of at least 90% of cervical cancers. They are also linked to more than 50% of other anogenital cancers (zur Hausen and de Villiers, 1994). 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). More than 10 million American women are infected with high-risk HPVs, and an estimated 15,000 American women are diagnosed with cervical cancer each year (zur Hausen and Schneider, 1987; zur Hausen and de Villiers, 1994). While routine Papanicolaou (Pap) screening has significantly reduced the rate of cervical cancer in developed countries, it remains a leading cause of cancer death in many other parts of the world. There are approximately 500,000 cases of cervical cancer worldwide each year; of which one-third are fatal. HPVs have circular, double-stranded DNA genomes that are approximately 8 kilobases in size and encode eight genes, of which E5, E6 and E7 have transforming properties. These proteins have a pleiotropic function, such as transmembrane signaling, regulation of the cell cycle, transformation of established cell lines, immortalization of primary cell lines, and chromosomal stability. Especially, the E7 gene of the high-risk HPV genotypes encodes a 98-amino acid zinc-binding phosphoprotein, which contains two CysXXCys motifs (Barbosa *et al.,* 1989) and is sufficient for the induction of a focus formation of established rodent fibroblasts (Kanda *et al.,* 1988; Bedell *et al.,* 1989). It is functionally similar to the adenovirus E1A proteins (AdE1A) in that it can transactivate the adenovirus E2 promoter and cooperate with an activated *ras* oncogene to transform primary rat kidney cells (Phelps *et al.,* 1988). Like AdE1A, the HPV 16 E7 protein can form a specific complex with the retinoblastoma tumor suppressor gene product (p105-Rb) (Clark *et al.,* 1998). This results in the release of the E2F transcription factor, which is critical for the deregulation of growth and transforming properties of the

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viral E7 oncoprotein. Thus, a complex formation between the products of oncogenes and the tumor suppressor gene is believed to be important in the cellular transformation that leads to the disruption of the normal physiological functions of the specific tumor suppressor gene products. The pocket domain of the retinoblastoma (Rb) tumor suppressor is central to its function; and it is frequently inactivated by the binding of the HPV E7 oncoprotein in cervical cancer. A nine-residue E7 peptide, containing the LxCxE motif, binds to the pocket domain of the Rb tumour suppressor and is shared by other Rb-binding viral and cellular proteins (Lee *et al.*, 1998).

Current treatment for HPV 16-associated lesions is surgery, though there is some limited success achieved with an immune modulator like interferon against HPV 11- and HPV 6-associated lesions. Also prevention of HPV infection by vaccination and immune therapy is under investigation, but has not yet been established. Recently, a new drug to suppress infections by the HPV has been designed (Beerheide *et al.*, 1999), as a result of the extensive knowledge gained of the molecular mechanism by HPV oncoproteins. Therefore, an *in vitro* screening system for development of drugs to treat HPV infection is primarily required to develop compounds with desirable properties. In this report, we established an *in vitro* screening system based on the interaction between E7 and Rb, and its application for enzyme linked immunosorbant assay (ELISA). This system could be available for the development of potential drugs against HPV infection.

Materials and Methods

Recombinant plasmid DNAs and Overexpression E7 was prepared with the pGEX system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or pET28 system (Novagen, Madison, USA). Total RNA was extracted from CaSki cell lines using a TriReagent (Molecular Research Center, Cincinnati, USA). An E7 insert was amplified by RT-PCR, as described (Lee *et al.*, 2000), using primer sets comprising 5'-CAC CAT GGC ATG GCA TGG AGA TACACCT-3' (sense) and 5'-TTA TGG TTT CTG AGA ACA-3' (antisense) and inserted into T-vector prepared from pBluscript KS (+). An E7 fragment was cloned into the pGEX, or pET28 vector, to construct GST- or 6xHis-fused E7 as an insert *BamH* I/*Sal* I. Transformations of pGEX/E7 and pET28a/E7 into DH5α and BL21 (DE3) *E. coli* (Pharmacia) were performed to express GST- and 6xHis-fused E7, respectively. *E. coli* harboring pGEX-E7 was grown, induced for protein expression overnight with 1 mM isoprophyl-β-D-thiogalactopyranoside (IPTG), harvested and lysed in a phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml of apportion, by sonication. The resulting mixture was centrifuged for 30 min at 12,000 rpm to remove pellets containing cell debris. Supernatants were used as a cell lysate for the binding assay. Proteins expressed in pET28 vectors was prepared in *E. coli* BL21 (DE3) using the same procedure as described above, except for using a lysis buffer containing 50 mM NaH_2PO_4 and 150 mM NaCl, pH 8.0. The recombinant E7 protein, which contained six histidine residues at the N terminus, was purified by Ni^{+2} affinity chromatography under native conditions (Kim *et al.*, 1999; Park *et al.*, 2000). After removal of cell debris by centrifugation, the resulting cell extract was then loaded onto the Ni^{+2} NTA column (Peptron, Taejon, Korea). The column was washed twice with a wash buffer (20 mM imidazole, 50 mM $Na_2H_2PO_4$, 300 mM NaCl, PH 8.0) followed by elution with an elution buffer (200 mM imidazole, 50 mM $Na_2H_2PO_4$, 300 mM NaCl, PH 8.0). The purified His-E7 was then aliquoted and stored at -80°C until use. The pET3-Rb plasmid for Rb expression was kindly offered by Dr. Cho YJ (Korea Institute of Science and Technology, Seoul, Korea). The Rb protein used in this experiment was constructed for improvement of solubility in a supernatant fraction, resulting in the production of truncated protein composed of N-terminal and pocket domain (AA 373-928). E6, used as a control in the binding experiment, was obtained using the following primer sets by PCR amplification from total RNA isolated from CaSki cell lines. The primers for the E6 amplification are as follow: 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 the T-vector prepared as described using pBluescript II KS (+) (Stratagene, La Jolla, CA) (Marchuk, *et al.*). The E6 insert was excised from digestion of pBluescript/E6 with *BamH* I/*Sal* I, and subcloned into a corresponding expression vector, pET28a (Novagen), which was prepared by digesting with *BamH* I/*Sal* I. Transformation of pET28a/E6 into BL21 (DE3/pLys) was performed to express 6xHis-Tagged E6 as described (Cho, *et al.*, 2000). The lysates of E6 and Rb were prepared using the same procedure as described previously, and concentrations of the total proteins in lysates were, respectively, 8.2 and 7 mg/ml as determined by Bradford reagent (Bio-Rad Lab., Hercules, USA).

E7 and Rb binding assay E7-Rb binding assays were performed by combining either 6xHis-E7 or GST-E7 fusion protein, which were immobilized on Ni^{+2}-NTA Agarose or glutathione (GSH)-Sepharose with increasing amounts of bacterially expressed Rb supernatant (20, 50, 100 and 200 μl). The mixtures were rotated in microcentrifuge tubes at 4°C for 1 h. The beads were then collected by centrifugation, washed three times with a 1.5 ml of washing buffer (PBS containing 0.5% Triton X-100 for the pGEX system and 20 mM immidazole-containing lysis buffer for the pET system) and then boiled for 5 min in a sodium dodecyl sulfate (SDS) gel loading buffer. The amounts of Rb protein that bound to the beads were determined by SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) and Western blot assay. Gels were transferred to Immobilon-P membranes (Millipore, Bedford, USA) at 50V for 1.5 h at room temperature and the membranes were blocked by soaking methanol for 5 min and dried at room temperature. The membrane was probed with a mouse monoclonal anti-Rb antibody (Ab-6) (Oncogene, Cambridge, USA) diluted 1 : 1,000 in 3% skim milk, followed by an alkaline phosphatase-conjugated anti goat antibody (Sigma, St. Louis, USA). Visualization was achieved with a NBT/BCIP substrate kit (Bio-Rad Lab., Hercules, USA).

Optimization of ELISA The 6xHis-E7 recombinant proteins

that are bound to beads were dissociated with an elution buffer (50 mM sodium phosphate containing 300 mM NaCl, 200 mM NaCl, pH 8.0). A Maxisorb 96-well plate (Nunc, Netherlands) was coated with eluted proteins at a dose of 10 μg/ml. Serial dilutions of Rb lysates were added to an E7-precoated plate and then allowed to incubate for 1 h at room temperature. After exhaustive washing with PBS containing 0.5% Tween-20 (PBST) to remove unbound Rb, mouse anti-Rb antibody (Ab-6) (Oncogene) at a concentration of 0.1 μg/ml was dispensed to the plate followed by 1 h incubation. After washing, horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Sigma) was applied to the plate for immunoassay. After washing with PBST, 100 μl of substrate (4 mg *o*-phenylenediamine, 5 μl 37% H_2O_2 per 10 ml of 0.1 M citrate buffer, pH 5.1) was added, and the enzyme activity was detected at 490 nm using an ELISA reader (Molecular Devices, Hercules, USA) after stopping the enzyme reaction with 2.5 N sulfuric acid.

Results

In an attempt to apply the physical interaction of E7 with the Rb protein for the screening system, the E7 cDNA was primarily cloned into the bacterial expression vector pET28a and pGEX, and then prepared as GST- and 6xHis-tagged forms. Both recombinant vectors were transformed into *E. coli* BL21 (DE3) and DH5α competent cells for the induction of corresponding fused proteins. Overexpression of the two different kinds of fused-E7 proteins (used for immobilization on the beads) is shown in Fig. 1. The proteins present in both the pellet and supernatant fraction after lysis were resolved by SDS-PAGE (Fig. 1A) and tested for E7 by Western blotting (Fig. 1B). Both GST-E7 and His-E7 proteins were clearly visible at the predicted molecular weights, migrating at approximately 24 and 46 kD, respectively. A considerable amount of E7-fusion proteins were present in the supernatant, as determined by Western blot and then immobilized on GSH- or Ni^{2+}-NTA Sepharose, respectively, in order to utilize them as baits in pull down assays. E7 proteins were purified to homogeneous as a bead bound form of either GST- or His-tagged (Fig. 1). The full length Rb protein, was badly insoluble when expressed in *E. coli*. Therefore, the truncated Rb in the pET vector was expressed in *E. coli* BL21 (DE3), and its expression level in both supernatant and pellet fraction was analyzed by SDS-PAGE and immunoblot assay. Immunoblot analysis showed that a large number of Rb proteins that migrated at approximately 60 kDa were present as a soluble form in the supernatant fraction as expected (Fig. 2). As a preliminary experiment for the E7-Rb interaction, E7 proteins immobilized on resins were incubated with an increasing number of lysates that contained Rb. After extensive washing, E7-bound Rb was analyzed by immunoblotting using an anti-Rb antibody (Fig. 3). As controls, immobilized GST, or His-tagged E6, was incubated

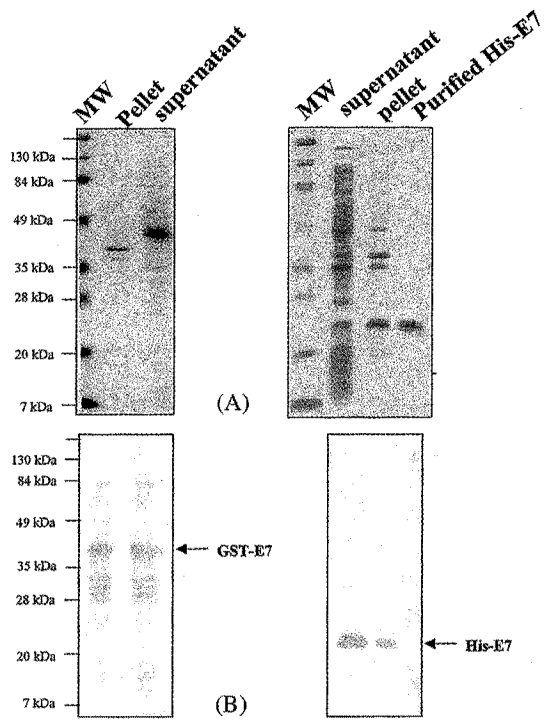

Fig. 1. Overexpression of GST-tagged- and His-tagged E7 proteins. The bacterial cell lysates after sonication were separated into pellet and supernatant by centrifugation at 12,000 rpm for 30 min. Diluted aliquots (1/10) of each fraction were analyzed on SDS-PAGE (A). The left and right panels indicate electrophoregram of fractionated lysates prepared from *E. coli* overexpressing GST- and His-E7 fusion proteins, respectively. Their expression and solubility were confirmed by Western blot analysis using a mouse monoclonal anti-E7 antibody, diluted 1 : 1,000 in 3% skim milk, followed by an alkaline phosphatase-conjugated anti-mouse IgG. After washing, the bound enzyme was developed with an NBT/BCIP substrate kit (B). (A) Staining with Coomassie blue, (B) Immunoblotting against E7 protein.

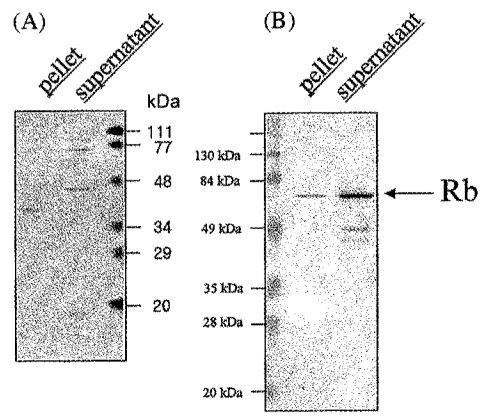

Fig. 2. Overexpression of recombinant Rb protein. The bacterial cell lysates after sonication were separated into pellet and supernatant by centrifugation at 12,000 rpm for 30 min. Diluted aliquots (1/10) of each fraction were analyzed on SDS-PAGE (A) and the Rb protein was identified by Western blot analysis using a mouse monoclonal anti-Rb antibody (Ab-6), followed by an alkaline phosphatase-conjugated anti-mouse IgG. After washing, the bound enzyme was developed with an NBT/BCIP substrate (B). (A) Staining with Coomassie blue, (B) Immunoblotting against Rb protein.

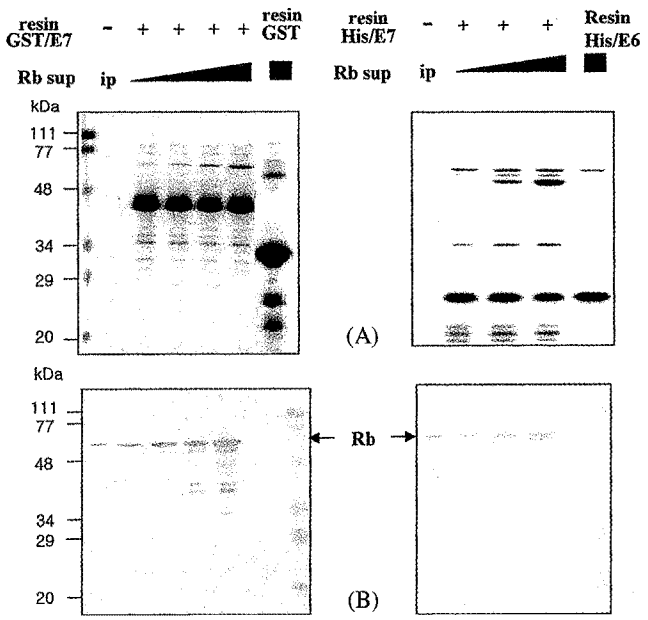

Fig. 3. Pull-down assays of binding between bead bound E7 and Rb lysates. E7 immobilized on either GSH-Sepharose (left panel), or nickel-Agarose beads (right panel), was combined with 20 (omitted for the latter), 50, 100 and 200 μl of bacterial lysates containing Rb. For Western blotting, binding mixtures were separated on 12% SDS-PAGE (A) and blotted on the Immobilon-P membrane (B). E7-bound Rb was analyzed by SDS-PAGE on a 12% gel followed by Western blot analysis using a mouse monoclonal anti-Rb antibody (Ab-6), followed by alkaline phosphatase-conjugated anti-mouse IgG. After washing, the bound enzyme was developed with an NBT/BCIP substrate kit. Input (ip) was loaded directly into the well and represented approximately 0.6% of the Rb lysates at the maximum level used in the binding reaction. (A) Staining with Coomassie blue, (B) Immunoblotting against Rb protein.

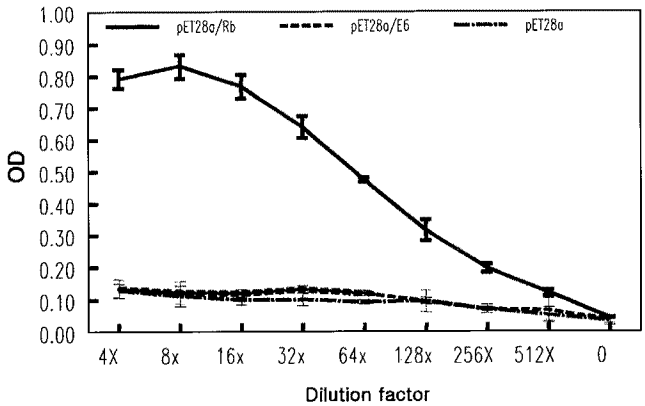

Fig. 4. Optimization of ELISA for screening drugs targeted to HPV E7. The purified E7 protein was coated onto a Maxisorb 96-well plate overnight and blocked with PBS containing 3% skimmed milk for 2 h at room temperature. After washing, serial dilutions of lysates containing Rb derived from pET/Rb were incubated for 1 h. The Rb bound to E7 was detected with a mouse monoclonal anti-Rb antibody followed by incubation of a horseradish phosphatase-conjugated anti-mouse IgG. After washing, the bound enzyme activity was detected at 490 nm by using an ELISA reader. As nonspecific controls, non-relevant agents, such as His-tagged E6 and lysates prepared from *E.coli* harboring the pET28a vector alone, were incubated with Rb lysates. Results are the mean± standard deviation and are representative of the three experiments.

with Rb lysates. Immunoblotting (using an anti-Rb antibody) showed that there was no interaction between Rb and either GST or His-E6 (Fig. 3B). On the other hand, both GST-tagged and His-tagged E7 immobilized on resins could bind Rb as a function of the Rb dose used. In addition, the binding of Rb to His-E7 was more effective than that of Rb to GST-E7, judging from the stained gels that the Rb proteins were recovered with His-E7 a little more than with GST-E7. For practical application, pull down assays between E7 and Rb were extended to an ELISA system (Fig. 4). However, His-E7 was readily eluted from Ni^{+2}-NTA beads by the addition of immidazole at 200 mM, compared to the elution of GST-fusion proteins with the elution buffer (10 mM GSH containing 50 mM Tris, pH 8.0). Hence, His-E7 was coated to the plate at a dose of 10 μg/ml and then incubated with serial dilutions of Rb bacterial lysate. As shown in Fig. 4, the Rb protein was bound to E7 pre-coated on the plate in a dose dependent manner to a 1 : 8 dilution, while other non-relevant agents, such as E6 and supernatants of vector control pET28a, were not. This indicates that the E7-Rb binding system is useful for the screening of potential drugs against HPV.

Discussion

The development of antiviral agents that interfere with the replication of HPVs, which are responsible for anogenital warts and cervical carcinomas, is being pursued. However, the discovery of antiviral compounds against HPV has been hindered by the difficulties in culturing virus *in vitro*, or assaying stable HPV DNA replication. Recently, an assay system for inhibitors of HPV DNA replication was developed (Plumpton *et al.*, 1995; Clark *et al.*, 1998). In addition, using an NMR-based screen, compounds for binding to the DNA-binding domain of the HPV-E2 protein, which is required for the replication of HPVs, were tested (Hajduk *et al.*, 1997). With the extensive knowledge on the structure of the E6 oncoprotein encoded by HPV, the approach targeting the oncoprotein E6 for inactivation by displacing the bound Zn^{2+} was investigated with potential compounds to facilitate their participation in sulfhydryl residue-specific redox reactions (Beerheide *et al.*, 1999). The E6 and E7 genes from high-risk HPVs have been major viral oncogenes by inactivating two tumor suppressors, p53 and Rb, respectively. E7 is the main transforming protein of HPV type 16, which is implicated in the formation of cervical cancer. The transforming activity of E7 has been attributed to its interaction with the Rb tumor suppressor. However, additional cellular proteins been have recently identified (Brehm *et al.*, 1999; Luscher-Firzlaff *et al.*,

1999). These interact with E7 and may play a role in mediating its transforming function.

Accordingly, in this report, an experimental assay system for monitoring the E7-Rb interaction was primarily optimized for application as a screening tool of anti-drugs against cervical cancer. For the *in vitro* interaction assay, E7 was expressed as a GST-fused protein, or a His-tagged protein in a bacterial expression system, and then immobilized on GSH-Sepharose beads or on nickel beads, respectively. Both E7 fusion proteins were functionally active with respect to Rb binding, while there were no nonspecific interactions between either GST or His-tagged E6 and Rb (Fig. 3B). For ELISA, His-tagged E7 was coated onto the plate because it was readily eluted from immobilized beads compared to GST-tagged E7, and then allowed to incubate with increasing doses of Rb. Similarly, a significant increasing level of Rb interaction with E7 was observed, whereas there was no interaction between E7 and E6, or lysates prepared from the host harboring vector alone instead of Rb as negative controls. These data suggest the basis for an *in vitro* binding assay evaluating the inhibitory activity of potential chemotherapeutic agents directed on E7-Rb interaction.

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