The Human Papillomavirus E7 Oncoprotein Functionally Interacts with the S4 Subunit of the 26 S Proteasome

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Human papillomaviruses (HPV) have been etiologically linked to human cervical cancer. More than 90% of cervical cancer tissues express two HPV-encoded oncoproteins E6 and E7. Both E6 and E7 proteins possess transformation activity, and together they cooperate to transform primary human keratinocytes, fibroblasts, and epithelial cells. The transforming activity of E7 is associated with its ability to bind the retinoblastoma tumor suppressor protein (Rb). However, the carboxyl-terminal mutants of E7 are also defective for transformation, suggesting that other cellular targets for E7 might exist. We screened a human placenta cDNA library by yeast two-hybrid assay using HPV 16 E7 as a bait and identified the subunit 4 (S4) ATPase of the 26 S proteasome as a novel E7-binding protein. E7 binds to S4 through the carboxyl-terminal zinc binding motif, and the binding is independent of E7 sequences involved in binding to Rb. The interaction between S4 and E7 can be easily detected by in vitro protein binding assays. Moreover, we found that E7 increases the ATPase activity of S4. A recent study has shown that, in epithelial cells, E7 degrades Rb through the 26 S proteasome pathway. We hypothesize that E7 might target Rb for degradation by 26 S proteasome through its interaction with the subunit 4 of the proteasome.

Human papillomaviruses (HPV) have been etiologically linked to human cervical cancer. The HPVs are broadly subdivided into two groups: 1) “low risk” HPVs (HPV 6 and HPV 11), which cause benign squamous epithelial tumors (warts and papillomas); and 2) “high risk” HPVs (HPV 16, HPV 18, HPV 31, and HPV 54), which are associated with malignant tumors. Two high risk HPV early genes, E6 and E7, are selectively retained and expressed at high levels in more than 90% of cervical cancer tissues (reviewed in Refs. 1–3). Both E6 and E7 proteins encoded by the high risk HPVs have transformation activities. The E7 oncoprotein by itself can transform established rodent cells such as NIH 3T3 and cooperates with activated Ras oncoprotein or HPV 16 E6 oncoprotein to immortalize primary human keratinocytes, fibroblasts, or epithelial cells in culture (4–10).

HPV 16 E7 shares sequence homology with other DNA tumor virus oncoproteins such as the adenovirus E1A and SV40 large T antigen. These homologous regions are referred to as conserved region 1 (CR1) and conserved region 2 (CR2). All these oncoproteins including E7 bind the cellular tumor suppressor protein retinoblastoma (Rb) through the CR2 homology region (11–13). The carboxyl-terminal region of E7 does not share sequence homology with either E1A or T antigen but is highly conserved among all E7 proteins from different HPV serotypes (1–3). The carboxyl-terminal region of E7 contains two “Cys-X-X-Cys” motifs that can form a zinc finger. The HPV E6 oncoprotein has two zinc fingers and shares partial amino acid sequence homology with E7 (1, 2). However, a recent study revealed that E6 and E7 zinc binding motifs can form different secondary structures (14). The carboxyl-terminal sequences of E7 are also involved in dimerization of the protein (15).

The interaction with Rb is essential for the transformation function of E7 (1, 2, 6, 15, 16). Mutations in the Rb-binding region severely impair the transformation activity of E7. The HPV E7 protein of low risk HPV is less efficient in binding to Rb and has very weak transformation activity. However, detailed mutagenesis of E7 has revealed that Rb/E7 binding alone is not sufficient because mutations in the carboxyl-terminal sequences of E7, outside the Rb-binding site, also severely impair the transformation function (15, 16). These results suggest that, aside from binding to Rb, other protein-protein interaction(s) are also necessary for E7-mediated transformation.

The 26 S proteasome is a large multimeric protein complex, which catalyzes ATP- and ubiquitin-dependent protein degradation in eukaryotic cells (reviewed in Ref. 17). The 26 S proteasome controls programmed degradation of many critical cell cycle regulatory proteins. For example, programmed degradation of mitotic cyclins (18), maturation-promoting factor (19), and the inhibitor of cyclin-dependent kinase p27 (20) by 26 S proteasome are crucial for cell cycle progression. In addition, the 26 S proteasome is responsible for signal-dependent maturation of an inactive precursor of the transcription factor NF-κB and proteolysis of its negative regulator I-κB (21, 22). The levels of the tumor suppressor proteins Rb (23) and p53 (24), proto-oncoproteins c-Jun and c-Fos (25, 26), and the regulatory subunit of protein kinase A (27) are also regulated by the 26 S proteasome.

The 26 S proteasome contains multiple ATPase subunits (19). ATP hydrolysis is crucial for protein degradation by 26 S proteasome and is also required for the assembly of the proteasome enzymes CF1, CF2, and CF3. The S4-ATPase is known to be important for the 26 S proteasome function because fission yeasts showed defective proteolysis due to mutation in the mts2 gene, which codes for the yeast homologue of human S4 (28). Interestingly, the human S4-ATPase can rescue these mts2 null alleles (28). The S4-ATPase, together with TBP-1, Mss1, and Sug1, belongs to a recently described ATPase family (re-
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viewed in Ref. 29. All these ATPases have an evolutionary conserved ATPase module and partially conserved carboxy-terminal domains. In contrast, these ATPases have only limited sequence homology in the amino-terminal domains, and it is believed that the variable NH2-terminal regions of these ATPases are involved in regulation of the ATPase activity. Consistent with this idea, it has been shown that deletion of the variable NH2-terminal region of the budding yeast protein Yhs4p, a homologue of human S4, resulted in 4-fold increase of its ATPase activity (30).

Our present studies show that human S4-ATPase interacts with the HPV-16 E7 oncoprotein, but not with the HPV-16 E6 oncoprotein. Mutational analysis revealed that E7 interacts with S4 through its carboxy-terminal sequences. Most interestingly, our results demonstrate that the binding of recombinant E7 to S4 results in significant stimulation of the S4-ATPase activity. We hypothesize that the E7-mediated increase in S4-ATPase activity is important for the cell cycle regulatory function of E7.

MATERIALS AND METHODS

Cells and Cell Culture—The yeast strains, HF7c (31) and SPY526 (32), used in this study were grown either in rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in synthetic medium (32) with appropriate supplements at 30 °C.

Plasmids and Oligonucleotides—The HPV 16 E7-expressing plasmids and the various mutants have been described previously (33). Polymerase chain reaction (PCR) fragments from the wild type and various E7 mutants were subcloned into EcoRI/PstI sites of the yeast vector pGBT9 (34) in frame with GAL4 DNA-binding domain. For the PCR reactions, 5'-GCG GAA TTC CAT ATG GAT GAC ACA CC-3' was used as the upstream primer and 5'-GGCG CTG CAG TTA TGG TTT TTA AGA ACA ACA GAT-3' as the downstream primer. For subcloning of the PTHLH mutant, 5'-GGCG GAA TTC CAT ATG GAT GAC ACA CC-3' was used as upstream primer. The carboxy-terminal mutant Q91S was created by site-directed mutagenesis using the same upstream primer as for E7 wild type construct, but a different downstream primer 5'-GGCG GAA TTC ATT CTT ATG GTT TCT GAC AAG TGA TGG GGG ACA CAA TTC CTA GGA GAT ACA CC-3' was used for cloning into EcoRI site of pGBT9. The HPV 16 E6 was subcloned into EcoRI/PstI sites of pGBT9. To construct the GAL4-AC-Rb fusion protein, a 1.6-kilobase pair PCR fragment of the Rb cDNA containing sequence between base pairs 1146 and 2793 was subcloned into EcoRI/PstI sites of the yeast vector pGAD424 (34) in frame with GAL4 transcription activation domain. For this PCR reaction, 5'-GGCG GAA TTC CAT ATC CAA TTA ATG ATG ATT-3' was used as the upstream primer and 5'-GGCG CTG CAG TCG TAT TCA TCA TCA TTC CTC CCT TTT G-3' was used as the downstream primer. Cloning of all the above constructs were verified by DNA sequence analysis. Construction was confirmed by subcloning a fragment of S4-cDNA corresponding to amino acids 72–440 into the EcoRI site of pGEX-1AT vector (Pharmacia Biotech Inc.) in frame with GST.

Library Screening, β-Galactosidase Assay, and Sequence Analysis—We screened a human placenta cDNA library (CLONTECH, Palo Alto, CA) representing clones of hybrid proteins between the GAL4 activation domain and random cDNA fragments in yeast PAG10 vector. An estimated 5 x 10^7 transformants from the library were introduced into the Saccharomyces cerevisiae reporter strain HF7c, with the pGBT-E7 plasmid, by lithium acetate (LiAc) transformation method, as described previously (35). The transformants were plated on the selective medium lacking histidine, tryptophan, and leucine and containing 40 mM 3-aminotriazole (3-AT, Sigma) to isolate clones with histidine prototrophy. The transformants were grown for 6 days at 30 °C. The growing clones were replated on the same selective medium and were assayed for another marker, the β-galactosidase activity. In the β-galactosidase assay, the transformants were replica-blotted on Whatman filter papers, immersed into liquid nitrogen for 20 s, and incubated for 40 min to 6 h at 30 °C in buffer (16.1 g/liter Na2HPO4, 5.5 g/liter NaH2PO4·H2O, 0.75 g/liter KCl, 0.246 g/liter MgSO4·7H2O, and 2.7 ml of freshly added β-mercaptoethanol) containing 0.34 mg/ml X-Gal (Sigma). Thirty-eight clones that developed blue color within the first hour were examined further. These clones were grown on synthetic medium containing tryptophan but lacking leucine to separate the cDNAs from the bait, pGAL4-DB-E7 plasmid. Purified plasmid DNAs were analyzed for the size of inserts by agarose gel electrophoresis followed by partial DNA sequencing and data base analysis using the Blast program.

ATPase Assay—GST, GST-E7, GST-S4, GST-E7 C918, and GST-E6 fusion proteins were purified as described previously (33). The quality of GST fusion proteins was analyzed by SDS-polyacrylamide (10%) gel electrophoresis, followed by Coomassie Blue staining. Concentrations of the fusion proteins were measured by Bio-Rad protein assay. The GST-S4 used in the ATPase assay was dialyzed against the ATPase reaction buffer described below. ATPase activity of GST-S4 was assayed in the reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 5 mM MgCl2, and 50 μM ATP, γ-32P]ATP (1,000–2,000 cpm/μl) at 37 °C for 30 min. Reactions were stopped by addition of 10 volumes of 10% trichloroacetic acid, and 1/100 of the reaction mixture (5 μl) was analyzed by thin layer chromatography on polyethyleneimine-cellulose (J.T. Baker, Inc.) in 0.4 M LiCl. 1 μM acetic acid buffer to separate the released [32P]P phosphate from the substrate γ-32P]ATP (36). After developing the chromatogram, the spots representing the 32P-labeled released phosphate were cut out from the chromatogram and quantitated by scintillation counting. The ATPase activities were calculated as picomoles of ATP degraded/μg of S4 protein/min. All these ATPase assays were performed in the linear range of assays. Every ATPase assay were repeated at least three times with each point in duplicate, and standard deviations were calculated.

Western Blot Analysis—Yeast extracts for Western blot analysis were prepared by lysing cells in radiolabeled precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate) with three volumes of acid-washed glass beads (425–600 μm) for 30 min at 4 °C (34). For Western blot analysis, cell lysates (200 μg) were resolved by SDS-polyacrylamide (12.5%) gel electrophoresis. The proteins were then electrochemically transferred to a nitrocellulose membrane, probed with monoclonal E7 antibody (Triton Diagnostics), and developed by ECL (Amersham).

GST Pull-down Assay—The GST pull-down assays were performed according to previously described procedures (37). Briefly, 32S-labeled S4 protein was synthesized by in vitro transcription/translation reactions in rabbit reticulocyte lysates. The labeled S4 proteins (5 μl) were incubated with the才会hion-Sepharose beads containing GST-E7 fusion proteins (25 μg each). After extensive washing, the bound proteins were released from the beads by boiling for 3 min at 95 °C in 1 x Laemmli buffer and analyzed by SDS-polyacrylamide (10%) gel electrophoresis.

RESULTS

The Subunit 4 (S4) ATPase of 26 S Proteasome Is a Target of the HPV 16 E7 Oncoprotein—We used the yeast two-hybrid screening assay to identify novel E7-binding proteins. The assay is based on the reconstitution of the yeast transcriptional activator GAL4 from two separate fusion proteins: one containing the DNA-binding domain (GAL4-DB) and another containing the transcription activation (GAL4-AC) domain (34). When the two fusion proteins interact, the GAL4 activator is reconstituted and can initiate transcription from reporter promoters dependent on GAL4 binding. The yeast strain HF7c used in our experiments has two such reporter genes: 1) histidine synthase and 2) β-galactosidase. Expression of β-galactosidase allows screening for blue colored colonies, whereas the expression of histidine synthase allows the growth of these clones in medium lacking histidine (histidine prototrophy). The His3 reporter has a low level of transcription in absence of any GAL4 function; therefore, to eliminate yeast growth as a result of His3 reporter leakage, low level of an inhibitor of histidine synthase, 3-AT, was used in all interaction experiments.

We used the GAL4-DB-E7 fusion protein as a “bait” to screen a human placenta cDNA library. The expression of the fusion protein was assayed in the transformed yeast cells (HF7c) by Western blot analysis. A 34-kDa protein of the predicted molecular mass was detected with the E7 monoclonal antibody in the extracts of GAL4-DB-E7-transformed yeast cells, but not in the extracts of the untransformed control (Fig. 1A). The Gal4-DB-E7 construct by itself showed no histidine prototrophy or β-galactosidase activity in the transformed HF7c yeast cells (data not shown) and was considered as a reasonable bait for the library screening. To screen for the E7-binding proteins, we
isolated and further selected for expression of the selected for histidine prototropy. The growing colonies were the bait Gal4-DB-E7 construct into the yeast strain HF7c, and further tailed analysis of the interaction between E7 and S4.

We analyzed the ability of S4 to interact with the HPV 16 E7 oncoprotein, as well as with another unrelated protein, lamin, by yeast two-hybrid interaction assays (Fig. 1). Thirty-eight clones that developed color within 1 h of GAL4-DB-E7 expression plasmid (lane 1) were separated by 12.5% SDS-PAGE, and subjected to Western blot analysis with E7 monoclonal antibody. The S4 subunit of 26 S proteasome specifically interacts with E7 in yeast two-hybrid assay. The yeast (strain HF7c) were transformed with different E7 mutant-expressing plasmids and either S4 (panels S4) or Rb (panels Rb) expression plasmid. The cells were grown on -Leu/-Trp media selective for transformation only (upper panel), or in -Leu/-Trp/-His/+25 m M 3-AT medium, selective for histidine prototrophy (lower panel).

To determine the specificity of the interaction between S4 and E7, we analyzed the ability of S4 to interact with the HPV 16 E6 oncoprotein, as well as with another unrelated protein, lamin, by yeast two-hybrid interaction assays (Fig. 1B). The transformants were allowed to grow in medium (-Leu/-Trp) to determine the transformation efficiencies or in the medium (-Leu/-Trp/-His/+25 m M 3-AT) to analyze for histidine prototrophy. Results from these experiments showed that HPV 16 E7 interacted efficiently with S4, whereas neither HPV 16 E6 nor lamin showed any interaction with S4 (Fig. 1B).

**E7/S4 Interaction Requires the Carboxyl-terminal Cys-X-X-Cys Motif of E7** —To identify the domain of E7 involved in the yeast two-hybrid interaction assays with either S4 or Rb. In agreement with results from previous biochemical analysis, the Rb/E7 interaction in yeast two-hybrid assay showed dependence on the sequences in the CR2 homology region of the HPV 16 E7 (Fig. 2B). The deletion mutant DLYC or the substitution mutant E26Q in CR2 region were defective in Rb binding. The E7 deletion mutant, EDE, in the CR2 region outside the Rb-binding domain interacted with Rb as efficiently as wild type E7. The E7 mutant with deletion in CR1 homology region PTLHE and a substitution mutant in the carboxyl-terminal region C91S interacted efficiently with the Rb protein.

When the same series of E7 mutants were used in a parallel assay to characterize the E7/S4 interaction, the carboxyl-terminal E7 mutant C91S with mutation in the Cys-X-X-Cys motif was found to be severely impaired in interaction with S4 (Fig. 2B). Interestingly, this COOH-terminal E7 mutant showed strong interaction with Rb. The other E7 mutants harboring changes in the CR1 and CR2 homology regions interacted efficiently with S4 (Fig. 2B). The E7 mutants used in this assay were expressed in equivalent amounts in yeast cells (Ref. 40 and data not shown). Therefore, these results suggest that the carboxyl-terminal Cys-X-X-Cys motif of E7 is important for the E7/S4 interaction and the motif involved in Rb/E7 interaction is different from the motif required for S4/E7 interaction.
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**Recombinant E7specifically binds to S4 in vitro through the carboxyl-terminal Cys-X-X-Cys motif.** A, a Coomassie-stained SDS gel shows the relative level of GST-E7 and the different GST mutant E7 proteins used in the S4-binding assay in B. B, binding of S4 to GST-E7. [35S]Metionine-labeled S4 was incubated with GST or the indicated GST-E7 fusion protein immobilized onto GHS beads. The bound S4 was analyzed by SDS-polyacrylamide (10%) gel electrophoresis and autoradiography. Load represents [35S]metionine-labeled S4 protein alone.

**Recombinant E7 Binds S4 in Vitro in a Specific Manner—**The results from the two-hybrid assays demonstrated an interaction between the carboxyl-terminal sequences of E7 and S4 in yeast cells. To establish an interaction between S4 and E7 without possible participation of yeast proteins, we performed an in vitro binding assay between the [35S]-labeled S4 protein made in rabbit reticulocyte lysate and the GST-E7 fusion proteins. The fusion protein GST-HPV 16 E7 and various mutants including GST-E7 E26Q, GST-E7 EDE, GST-E7 DLYC, GST-E7 PTLHE, and GST-E7 C91S were expressed in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography. An SDS gel analysis of the purified GST fusion proteins is shown in Fig. 3A. Equal amounts (25 µg each) of these fusion proteins immobilized onto GSH-Sepharose beads were incubated with 5 µl of reticulocyte lysate containing [35S]methionine-labeled S4. After extensive washing, the bound proteins were eluted from the beads and were separated by SDS-polyacrylamide (10%) gel electrophoresis (Fig. 3B). Interestingly, in agreement with the specificity observed in the yeast two-hybrid assay, S4 interacted efficiently with the wild type GST-E7 but not with the GST-E7 C91S fusion protein (Fig. 3B, lanes 3 and 8). Moreover, S4 interacted efficiently with other GST-E7 fusion proteins, including GST-E7 E26Q, GST-E7 EDE, GST-E7 DLYC, and GST-E7 PTLHE (Fig. 3B, lanes 4–7). These results showed that E7 can bind to S4 through its carboxyl-terminal sequences independent of any yeast protein.

**HPV E7 Oncoprotein Stimulates the ATPase Activity of the S4 Subunit of 26 S Proteasome—**The S4 subunit of 26 S proteasome is an ATPase; studies with yeast homologue of S4-ATPase, Yhs4p, demonstrated that the recombinant protein GST-Yhs4p had a similar ATPase activity with a $K_m$ of 5.0 µM and a $V_{max}$ of 6.7 pmol of ATP/min/µg of protein (Fig. 4C). Interestingly, addition of GST-E7 did not alter the $K_m$ value (5.0 µM) for the S4-ATPase, but the $V_{max}$ was increased to 35.7 pmol of ATP/min/µg of protein (Fig. 4C). This finding suggests that E7 binding leads to induction of the catalytic activity of S4 in vitro.

The induction of S4-ATPase activity was a specific effect of E7, because in a parallel assay, the GST-E6 fusion protein showed no detectable effect on the S4-ATPase activity (Fig. 5). Furthermore, the GST-E7 C91S fusion protein (carrying a substitution in amino acid 91 from cysteine to serine) showed no stimulation of S4-ATPase activity (Fig. 5). The E7 C91S protein was impaired in interaction with S4 in both in vitro binding assay and in vivo yeast two-hybrid interaction assays; therefore, the lack of induction of the S4-ATPase activity by this mutant E7 protein strongly suggests a specific functional interaction between S4 and E7. A previous study with the yeast homologue of S4-ATPase, Yhs4p, demonstrated that the recombinant protein GST-Yhs4p had a similar ATPase activity with a $K_m$ for ATP of 5.0 µM and a $V_{max}$ of 7 pmol of ATP/min/µg of protein (30). The activity of the S4-ATPase is essential for the function of the 26 S proteasome; therefore, an increase in S4-ATPase activity by E7 is likely to have an important regulatory effect on the function of the 26 S proteasome.

**DISCUSSION**

HPV 16 E7 protein has been shown to interact with the Rb family proteins Rb, p107, and p130, and cyclins such as cyclin A and cyclin E through the sequences in the CR2 region. However, these interactions only partially account for the oncogenic functions of E7. For example, the carboxy-terminal and the amino-terminal regions of E7 protein are critical for its immortalization function, but no cellular targets for either of these two domains have been identified (5, 15, 18, 41, 42). In this study, using the yeast two-hybrid screening assays, we identified the S4-ATPase of 26 S proteasome as a novel E7-binding protein. The S4/E7 interaction was specific because another HPV oncoprotein, E6, with partial sequence homology with E7 was unable to interact with S4. Furthermore, we provided evidence that S4 interacts through the carboxy-terminal domain of E7. The S4 is the only known cellular target for the carboxy-terminal region of E7, and further studies with S4 are likely to provide insights in the functions of this highly conserved domain of E7. The carboxy-terminal domain is involved in dimerization of E7, and it is possible that S4 binding might interfere with the dimerization of E7 (15). Although the effect of dimerization on the biological functions of E7 is not clear, it will be interesting to determine how the dimerization is affected by binding of the S4 subunit of 26 S proteasome.

The papillomavirus oncoprotein E6 binds and degrades the tumor suppressor p53 through the 26 S proteasome pathway. E6 binds the ubiquitin ligase E6-AP and p53, which leads to ubiquitination and subsequent degradation of p53 by 26 S proteasome (43). The papillomavirus E7 oncoprotein has not been shown to participate in this p53 degradation process. Our conditions for the linear range of assay were established. Using these optimum conditions, the GST-S4 fusion protein exhibited an ATPase activity in a concentration-dependent manner, whereas at the same level of assay, the fusion partner GST showed no detectable ATPase activity (Fig. 4A). To analyze the effect of E7 binding on the S4 ATPase activity, different concentrations of affinity-purified GST-E7 were incubated with GST-S4 before the ATPase assays. The results, as represented in Fig. 4B, showed that addition of E7 fusion protein significantly increased the ATPase activity of GST-S4. GST-E7 alone showed no ATPase activity (Fig. 4B). Quantitation of ATPase activity of GST-S4 revealed that it has a $K_m$ for ATP of 5.3 µM and a $V_{max}$ of 6.7 pmol of ATP/min/µg of protein (Fig. 4C). Interestingly, addition of GST-E7 did not alter the $K_m$ value (5.0 µM) for the S4-ATPase, but the $V_{max}$ was increased to 35.7 pmol of ATP/min/µg of protein (Fig. 4C). This finding suggests that E7 binding leads to induction of the catalytic activity of S4 in vitro.
present studies revealed that E7 binds S4 and induces the ATPase activity of S4. Analysis of the kinetic parameters of the recombinant S4 revealed that E7 interaction does not change the $K_m$ value of the S4-ATPase reaction. This result suggests that E7 does not interact with the catalytic site of S4. The 4-fold increase in the $V_{\text{max}}$ of S4-ATPase reaction by E7 suggests that E7 can increase the rate of S4 enzyme activity without significantly altering the substrate binding. The ATPases associated with the 26 S proteasome are involved in the assembly of the 26 S proteasome enzymes and proteolysis through the 26 S proteasome. At present, it is not clear how E7 binding might affect the function of the 26 S proteasome; future studies will be necessary to determine that.

26 S proteasome has multiple ATPase subunits, and they have been suggested to be involved in targeted degradation of specific substrates. A recent study has shown that the ATPase SUG1 brings c-Fos protein for degradation to the 26 S proteasome (26). By analogy, S4/E7 interaction might commit specific E7-binding protein(s) for targeted proteolysis by the 26 S proteasome. Among them, the most important target for E7 protein is the retinoblastoma protein Rb. Several studies have shown that the level of the E7-binding tumor suppressor Rb protein is reduced in E7-expressing cell lines (9, 10). Most importantly, a recent study has shown that, in transformed breast epithelial cells, E7 induces degradation of Rb through 26 S proteasome pathway (23). Because E7 binds Rb and S4 through independent domains, it is possible that a E7/Rb/S4 ternary complex is an intermediate leading to the proteolytic degradation of Rb. The tumor suppressor Rb has been implicated as a regulator of several cellular processes such as cell cycle progression, differentiation, and apoptosis. It will be important to determine whether the S4/E7 interaction is a crucial step in E7-induced degradation of Rb through the 26 S proteasome pathway.

![Figure 4: E7 stimulates S4-ATPase activity.](image)

**A**, human GST-S4 fusion protein exhibits ATPase activity. The ATPase reactions were performed with different concentrations of affinity-purified GST and GST-S4 protein as described under “Materials and Methods.” Aliquots (5 μl) of the reaction mixtures were subjected to thin layer chromatography on polyethyleneimine-cellulose to separate the released 32P-labeled inorganic phosphate from the substrate. The representative chromatograms show the released 32P-labeled inorganic phosphate in GST and GST-S4 ATPase reactions. **B**, recombinant E7 increases the ATPase activity of S4. The ATPase activity of GST-S4 (26 ng) was determined in presence of increasing amounts of the GST-E7 fusion protein (0.2–4 μg). The released 32P-labeled inorganic phosphate in ATPase reactions containing GST-S4 with different concentrations of GST-E7 and GST-E7 alone is shown in two chromatograms. The ATPase assay was performed following the procedure described under “Materials and Methods.” **C**, kinetic parameters of the S4-ATPase activity in the presence or absence of E7. The ATPase activities of GST-S4 (20 ng) and GST-S4 with GST-E7 (200 ng) were measured for 10 min using different concentrations of ATP (between 2.5 and 100 μM) as described under “Materials and Methods.” The $V_{\text{max}}$ and the $K_m$ values were calculated by Lineweaver-Burk plot. Each point on the graph represents three independent assays in duplicate. The $V_{\text{max}}$ and $K_m$ values are shown in the bottom.

**Figure 5.** The carboxyl-terminal E7 mutant E7 C91S or HPV 16 E6 protein has no effect on the ATPase activity of S4. Affinity-purified GST, GST-E6, GST-E7, or GST-E7 carboxyl-terminal mutant (E7 C91S) proteins (150 ng each) were incubated with GST-S4 fusion protein (20 ng). The reaction mixtures were analyzed for ATPase activity as described under “Materials and Methods.” The ATPase activities in different reaction mixtures were calculated as picomoles of ATP degraded/min/μg of protein, and the result shows -fold stimulation of ATPase activity after addition of different recombinant proteins. Each bar represents the average of three assays with standard deviation.
The papillomavirus oncoproteins E6 and E7 disrupt the functions of the tumor suppressors p53 and Rb, respectively. More than 90% of the human cervical cancer tissues express high levels of these two oncoproteins but wild type Rb and p53 proteins. However, both p53 and Rb are mutated in a majority of HPV-negative human cervical carcinomas. E6 degrades p53 through the 26 S proteasome pathway. It is now reported that E7 can also target Rb degradation through the 26 S pathway (23). These results together imply that human papillomaviruses have evolved a novel mechanism for degradation of crucial tumor suppressor proteins through 26 S proteasome to induce tumor formation.

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