Human Papillomavirus 16 E6 Oncoprotein Interferences with Insulin Signaling Pathway by Binding to Tuberin*

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Tuberous sclerosis complex (TSC) is a genetic disorder caused by mutations in either TSC1 or TSC2 tumor suppressor gene. TSC1 and TSC2 products, Hamartin and Tuberin, form the functional complex to serve as the negative regulator for insulin-induced phosphorylation of S6 kinase and elf4E-binding protein 1. High-risk human papillomavirus (HPV) infection is the necessary cause for cervical cancer. E6 oncoprotein encoded by HPV plays a pivotal role in carcinogenesis by interference with the host intracellular protein functions. In this study, we show that HPV16 E6 interacts with tumor suppressor gene TSC2 product, Tuberin, and results in the phosphorylation of S6 kinase and S6 even in the absence of insulin. The overexpression of Tuberin overcomes the effect of E6 on S6 kinase phosphorylation. Binding with HPV16 E6 causes the proteasome-mediated degradation of Tuberin. A DILG motif and an ELVG motif located in the carboxyl-terminal of Tuberin are required for E6 binding. In addition, the Tuberin interaction region in E6 has been mapped in the amino-terminal portion of HPV16 E6, which is different from the binding domain with p53. These results provide a possible link between E6-induced oncogenesis and the insulin-stimulated cell proliferation signaling pathway.

Human papillomaviruses (HPVs)3 infect epithelial cells and induce epithelial tumors or benign lesions (1). Based on their clinical outcomes, HPVs are grouped into high-risk and low-risk types. The high-risk types, such as HPV16, HPV18, HPV31, HPV33, have been identified as the cause of cervical carcinoma (2). Two oncoproteins encoded by HPVs E6 and E7 are able to immortalize human epithelial cells in vitro (3). The transgenic mice carrying the E6 gene under the control of the keratin promoter developed skin cancer (4). Moreover, E6 and E7 oncoproteins were found in HPV-infected malignant tumors (5), indicating that E6 and E7 functions are required for tumorigenesis.

E6 interacts with a variety of host cell proteins and interferes with their functions. The high-risk HPV E6 proteins bind to tumor suppressor protein p53 for degradation (6). This observation provides an important functional clue in which E6 contributes to cell transformation. Several studies showed that additional E6 activities are required to reach full transformation potential (7, 8). A growing number of E6 targets involved in the broad spectrum of cellular functions have been identified (9) including the transcriptional coactivator p300/CREB-binding protein (10), hMcm7 (12, 13), IRF-3 (14), and ElFp1 (15) and others. Thus, E6 interferes with multiple cellular pathways leading to malignant transformation. However, the mechanism that coordinates E6 targeting cellular activities and leads to tumorigenesis currently remains unclear.

Tuberous sclerosis complex (TSC) is an inheritable genetic disorder characterized by the formation of benign tumors in multiple organs (16). Genetic studies show that TSC is caused by mutations in either the TSC1 or TSC2 tumor suppressor gene (17, 18). TSC1 encodes a 130-kDa protein, Hamartin, whereas TSC2 encodes Tuberin, which is ~200 kDa with a carboxyl-terminal homology to the Rap GTPase-activating protein (19). Studies in both mammalian (20, 21) and Drosophila (22) indicate that Hamartin and Tuberin form a functional complex in vivo and negatively control cell growth and proliferation (23, 24). The Hamartin-Tuberin complex plays a negative regulator role in the insulin signaling pathway downstream of Akt (25–27). Akt phosphorylates Tuberin and disrupts the formation of the Hamartin-Tuberin complex, leading to the relief of its inhibition of S6 kinase (S6K) and to the activation of the elf4E-binding protein 1 (4EBP1) (26, 28, 29). The direct downstream target of Tuberin is the Ras family GTP-binding protein Rheb (30–33). The GTP-bound Rheb promotes the phosphorylation of mammalian target of rapamycin and ultimately stimulates S6 kinase and 4EBP1 phosphorylation (22, 34, 35). Tuberin suppresses the Rheb activity by reducing the GTP bound Rheb level in vivo (31).

In this study, we identified Tuberin as an intracellular target for HPV16 E6 protein. E6 binds and induces the degradation of Tuberin, leading to the destruction of its inhibitory function in regulation of S6 kinase activation in response to insulin stimulation.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—The different HPV GST-E6s were constructed by PCR amplification and insertion of the full-length E6s into pcEX-4T-2 (Amerham Biosciences) at EcoRI/XhoI sites. The pCMV-Myc-16E6 was constructed by the insertion of PCR-amplified full-length HVP16 E6 into pCMV-Myc vector (Clontech). The resulting construct is in-frame with a Myc tag placed at the amino terminus. The GST-HPV16 E6 deletion mutants illustrated in Fig. 3A were generated by PCR and subcloned into pGEX-4D-2. The resulting clones were confirmed by DNA sequencing. The TSC2-C-Gal4-AD contains the amino acid residues 1315–1807 of TSC2 and was acquired from a HeLa cDNA two-hybrid library (Clontech) by screening with the 16E6-Gal4-BD bait. The pCMV-HA-TSC2-C was generated by insertion of the
results

HPV16 E6 Interacts with Tuberin—During a yeast two-hybrid screen using HPV16 E6 open reading frame fused to the Gal4 DNA-binding domain as the bait, we identified several previous known E6-interacting proteins including IRF-3 (14) and E6TP1 (15). Interestingly, another positive clone turned out from the carboxyl-terminal 492 amino acids of Tuberin.

To confirm the interaction between Tuberin and HPV16 E6 and to determine whether this association is HPV type-specific, we performed a GST-E6 pull-down experiment to precipitate Tuberin from NIH 3T3 and BGC823 (a gastric carcinoma cell line) (for review see Ref. 36) cell extracts. GST-E6 fusion proteins from HPV6, HPV11, HPV16, and HPV18 were incubated with cell extracts, respectively (Fig. 1A). Tuberin was expressed in both NIH 3T3 and BGC823 cells (Fig. 1A, input lines). GST-E6 from HPV16 was strongly associated with the endogenous Tuberin from both NIH 3T3 and BGC823 cells. In contrast, GST-E6 proteins from HPV6, HPV11, HPV16, and GST alone did not bind to Tuberin. These results suggest that HPV16 E6 specifically interacts with Tuberin. Two bands between 170- and 200-kDa molecular mass markers were detected by anti-Tuberin antibody in NIH 3T3 cells. This result is consistent with a previous report suggesting that the upper
The Expression of HPV16 E6 Promotes the Degradation of Tuberin in HEK293 Cells—HPV E6 interferes with p53 function by degradation of the protein via ubiquitin-mediated pathway (6). To investigate the mechanism showing that HPV16 E6 interrupts Tuberin function, we determined whether the expression of E6 affects the stability of Tuberin. HEK293 cells were transfected with an increased amount of the HPV16 E6 construct. The expressions of the endogenous Tuberin and p53 were determined by Western blot. The levels of Tuberin and p53 were decreased, whereas the E6 expression level was increased (Fig. 3A). Interestingly, the phosphorylation but not the expression of endogenous S6 was increased simultaneously with the decreased Tuberin expression, suggesting that the phosphorylation of S6 might result from the degradation of Tuberin. To determine that the decreased expression of Tuberin upon HPV16 E6 expression occurred after transcription, a Northern blot was performed. The expressions with different amounts of E6 did not affect the TSC2 transcription (Fig. 3B).

The half-life of the Tuberin in the cells expressing E6 was compared with that in the cells without E6. HEK293 cells were transfected with either Xpress-tagged TSC2 or TSC2 plus E6. 48 h after transfection, cells were treated with cycloheximide for the indicated hours. The Tuberin level was detected at different time points. The result is shown in Fig. 3C. The amount of Tuberin significantly reduced 4 h after cycloheximide treatment and completely vanished 6 h after treatment in the cells with E6. In contrast, without E6 expression, Tuberin

Expression of E6 Interferences with S6 Kinase Phosphorylation Induced by Insulin—We next sought to determine the biological significance of the association of Tuberin with HPV16 E6. Recent studies (29, 38, 39) demonstrate that Tuberin negatively controls the activation of the p70 ribosomal protein S6 kinase 1 in response to insulin stimulation. We examined whether the activation of S6 kinase was affected by the expression of HPV16 E6. Previous studies demonstrate (40) that S6 kinase is activated by phosphorylation on several residues in which the anti-phosphospecific antibody was used to analyze S6 kinase activity. COS-7 cells were transfected with TSC2 or E6 alone, or TSC2 plus E6. 24 h after transfection the cells were cultured in 0.5% serum overnight. Insulin was added to stimulate the phosphorylation of S6 kinase and S6. The phosphorylation of S6 kinase and S6 was determined by anti-phospho-S6 antibodies. The addition of insulin stimulated the phosphorylation of S6 kinase and S6 in COS-7 cells transfected with the vector (Fig. 2A, lanes 1 and 2). Overexpression of TSC2 inhibited insulin stimulated S6 kinase and S6 phosphorylation (Fig. 2A, lanes 3 and 4). Interestingly, the expression of HPV16 E6 alone resulted in the phosphorylation of both S6 kinase and S6 even in the absence of insulin (Fig. 2A, lane 5). The addition of insulin to the cells expressing E6 did not further increase the phosphorylation of S6 kinase and S6 (Fig. 2A, lane 6), thus suggesting the effect of E6 on the phosphorylation of S6 kinase and S6 was via insulin signaling pathway. More importantly, the effect of E6 on S6 kinase and S6 phosphorylation was overcome by coexpression of Tuberin (Fig. 2A, lanes 7 and 8), suggesting that E6 targets insulin signaling by eliminating Tuberin suppressor function.

A previous study (41) shows that high-risk E6 proteins degraded the membrane-associated guanylate kinase homologues MAGI-2 and MAGI-3. MAGI-2 and MAGI-3 were associated with PTEN (phosphatase and tensin homologue deleted on chromosome 10) and enhanced its ability to suppress Akt activation (42). Therefore, E6 may indirectly activate Akt. To confirm the phosphorylation of S6 kinase by expression of HPV16 E6 is a result of Tuberin elimination and not the indirect activation of Akt, the phosphorylation of Akt in these transfections was tested by Western blot using an anti-phospho-Akt antibody. In contrast to S6 kinase and S6, phosphorylation of Akt was not affected by expression of HPV16 E6 (Fig. 2A), indicating that the effect of E6 on phosphorylation of S6 kinase and S6 was mainly through abolishing TSC complex function.

To test whether the other E6s that do not bind to Tuberin cause the activation of S6 kinase independent of insulin stimulation, HEK293 cells were transfected with the E6 constructs. The S6 kinase phosphorylation was detected by Western blot. In contrast to HPV16 E6, the expression of 6E6 and 18E6 did not result in the phosphorylation of S6 kinase (Fig. 2B).
slightly reduced 4 h after treatment and dramatically reduced but did not vanish 6 h after treatment (Fig. 3C).

Previous studies revealed that HPV E6 proteins target and degrade intracellular partners through proteasomal degradation (43–46). To determine whether the Tuberin degradation is caused by this mechanism, HEK293 cells were transfected with Xpress-tagged Tuberin alone or with E6. 48 h after transfection, cells were treated with the proteasome inhibitor MG132 for 3 h prior to protein extraction. The expression of exogenous Tuberin was high in the cells without E6 in both the presence and absence of MG132. The presence of E6 greatly decreased the Tuberin level. However, the Tuberin expression was recovered in the presence of MG132. In the presence of MG132, degradation of Tuberin was inhibited (Fig. 3D).

**Fig. 3.** Degradation of Tuberin by expression of HPV16 E6. A, HEK293 cells were transfected by an increasing amount of Myc-tagged HPV16 E6. The degradations of the endogenous Tuberin and p53 upon increasing expression of HPV16 E6 were detected by Western blot. The anti-phospho-S6 antibody was used to determine the effects of E6 expression on S6 phosphorylation. B, Northern blot was performed in the transfected cells as in panel A. The lanes 1–8 correspond to the lanes in panel A. C, HEK293 cells were transfected with Xpress-tagged TSC2 plus or minus E6 as indicated. 48 h after transfection, 10 μg/ml cycloheximide was added for the indicated time. Cells then were harvested for Western blot. D, HEK293 cells were cotransfected by Xpress-Tuberin and either Myc-16 E6 (lanes 1 and 2) or vector (lanes 3 and 4). 40 μM MG132 was added (lanes 1 and 3) 3 h before cells were harvested. Tuberin was detected using anti-Xpress antibody. EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 4.** Identification of E6 binding domain in Tuberin. A, the Tuberin truncations used in binding assays. B, binding of HPV16 E6 to Tuberin truncations. HPV16 E6-GST fusion protein was prepared as in Fig. 1. HA-tagged Tuberin constructs were transfected into COS-7 cells. 48 h after transfection, cells were lysed. The binding assays were performed as described in Fig. 1. Tuberin was detected by anti-HA antibody. Lanes 1 and 5, transfected by pCMV-HA-TSC-C; lanes 2 and 6, pCMV-HA-T1; lanes 3 and 7, pCMV-HA-T2; and lanes 4 and 8, pCMV-HA-T3. Lanes 1–4, binding results; lanes 5–8, the inputs. C, the diagram of Tuberin mutants at the HPV16 E6 binding region. D, the Tuberin mutants were transfected into COS-7 cells. The binding ability of the mutants to HPV16 E6-GST fusions was tested as shown in Fig. 2, panel B. The anti-HA antibody was used to detect the exogenous Tuberin. The control lane was cells transfected with pCMV-HA-TSC2-C. Lane 1, pCMV-HA-TSC2-Mu1; lane 2, pCMV-HA-TSC2-Mu2; and lane 3, pCMV-HA-TSC2-Mu3.
domain, ELVG, is located between positions 1351 and 1354 of Tuberin. To confirm the binding domain for HPV16 E6, three truncated constructs were analyzed (Fig. 4A). The pCMV-HA-TSC2-C contains the cDNA fragment isolated from yeast two-hybrid screen. The pCMV-HA-T1, T2, and T3 represent the different proportion of the pCMV-HA-TSC2-C as shown in Fig. 4A. COS-7 cells were transfected with the pCMV-HA-TSC2-C construct and the three truncated constructs. Cell lysates were made for GST-16E6 pull-down experiments. Consistent with previous experiments, GST-16E6 bound to the pCMV-HA-TSC2-C (Fig. 4B, lane 1). The truncated construct, pCMV-HA-T1, also associated with the GST-16E6 (Fig. 4B, lane 2), whereas the pCMV-HA-T2 (Fig. 4B, lane 3) and T3 (Fig. 4B, lane 4) did not. These results indicate that the amino acids 1315–1560 of Tuberin containing both DILG and ELVG domains have the E6 binding activity. To determine the effect of DILG and ELVG domains on E6 binding, the binding domain mutations were generated (Fig. 4C). The pCMV-HA-TSC2-Mu1 containing the substitution for DILG and the pCMV-HA-TSC2-Mu2 containing the substitution for ELVG partially reduced the binding activity to HPV16 E6 (Fig. 4D, lanes 1 and 2 compared with lane C). However, the binding activity of pCMV-HA-TSC2-Mu3 was totally abolished in which both DILG and ELVG sites were replaced (Fig. 4D, lane 3). These results indicate that both DILG and ELVG domains are required for Tuberin to associate with HPV16 E6.

Identification of the Region in HPV16 E6 That Is Required for Interaction with Tuberin—To define the region of E6 responsible for binding to Tuberin, a series of HPV16 E6 deletions were generated as illustrated in Fig. 5A. GST-HPV16 E6 deletion fusions were used to test the binding capability with Tuberin. As shown in Fig. 5B, top panel, Tuberin binds to the HPV16 E6 deletion mutants 16E6-2-GST and 16E6-4-GST (lanes 2 and 4). In contrast, 16E6-1-GST, 16E6-3-GST, 16E6-5-GST, and 16E6-6-GST were unable to bind to Tuberin (lanes 1, 3, and 5). The amino acids 78–104 of 16E6 are overlapped in 16E6-2-GST and 16E6-4-GST and missed in the other constructs, indicating that the residues between 78 and 104 are the binding region for Tuberin. To further confirm the Tuberin binding region, a deletion mutant without the amino acids 78–104 was generated from the full-length HPV16 E6. The resulting GST fusion protein, 16E6-6-GST, failed to bind to Tuberin (lane 6), supporting that Tuberin binding region is located between amino acids 78 and 104 on HPV16 E6.

**DISCUSSION**

We have demonstrated here that HPV16 E6, which is present in >50% of cervical cancers, interacts with the tumor suppressor protein, Tuberin, and interferes with its suppressor function in insulin-induced cell growth and proliferation signaling pathway, leading to the activation of S6 kinase downstream of Akt activation. The exogenous expression of E6 resulted in the degradation of the endogenous Tuberin. Expression of E6 in serum-starved cells was sufficient to induce S6 kinase and S6 phosphorylation without the addition of stimuli, whereas insulin stimulated the phosphorylations of S6 kinase and S6, suggesting that E6 activated the signaling component downstream of insulin receptor tyrosine kinase. Conversely, the overexpression of Tuberin blocked the S6 kinase phosphorylation and activation induced by E6, indicating that E6 targeted and interfered with Tuberin function in the insulin signaling pathway. The possible model for interference of Tuberin by HPV16 E6 leading to S6 kinase activation is illustrated in Fig. 6.
activating protein activity toward RhoB. Overexpression of RhoB bypasses the nutrient induction to activate TOR signaling (31, 32, 51–53). The TSC2 mutations derived from TSC patients are defective in GTPase-activating protein activity, suggesting that failure in the control of RhoB plays an important role in the pathology of TSC. Abrogation of Tuberin function by HPV16 E6 may therefore mimic the naturally occurring TSC2 mutations and contribute to HPV16-induced proliferation and tumorigenesis.

Human papillomaviruses infect proliferating epithelial cells. The replication of high-risk types of HPV occurs in the differentiated cells, which contain little or none of the cellular replication components. To utilize the host replication machinery, the virus needs to modify the host cellular proteins and drive the differentiated cells to re-enter the cell cycle. HPV16 oncoproteins E6 and E7 have been found to independently modulate the ability of keratinocyte differentiation (54, 55). The potenti-ality of virus oncoproteins to re-program the cellular process is gained by interfering with the key cellular protein functions. Targeting and degradation of p53 by E6 oncoprotein represent the most critical functional event, leading to an increase in host genetic instability. In addition to p53, more than a dozen cellular proteins have been identified as E6-interacting proteins (9). Identification of Tuberin as the cellular partner of HPV16 E6 provides the direct evidence that E6 targets the major growth and proliferation signal transduction pathway. In addition, HPV E6 proteins also target the membrane-associated guanylate kinase homologues MAGI-2 and MAGI-3 for degradation (41). MAGI-2 and MAGI-3 have been reported to be associated with PTEN (42), the other negative regulator of the insulin-induced growth pathway, and enhance the ability of PTEN to suppress Akt activation. Therefore, the degradation of MAGI-2 and MAGI-3 by E6 may result in the abolishment of PTEN function. Taken together, these results highlight the importance of interference with the growth factor-mediated signaling pathway in E6-induced immortalization. Although the disruption of p53 by E6 shall demolish the fidelity of DNA replication, the abrogation of the cell cycle and proliferation pathway may lead to uncontrolled growth and immortalization. The diverse activities of HPV E6 may represent the different sides of the coin. How those activities of E6 are coordinated to achieve the full malignancy is currently unclear and needs to be elucidated.

In system used here, Tuberin bound to HPV16 E6 but not to HPV18 E6. The reason for this specificity might be due to the preferential binding motifs in different HPV E6 proteins. Several proteins are targeted by E6 through the PDZ motif. HPV16 E6 has a perfect PDZ binding motif, whereas HPV16 is suboptimal (56). Therefore, proteins with PDZ motif may bind HPV16 E6 more efficiently. In contrast, the other proteins, such as IRF-3 and Tuberin binding to E6 through ELLG motif identified as (E/D)L(L/V)G, may favor HPV16 E6 (14, 47, 57). HPV16 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the possibility that HPV16 E6 binds to Tuberin with very weak capability, which cannot be detected under current condition. Our results have shown that two E6 binding motifs in Tuberin are required to achieve the full binding activity. This is similar to the observation in tumor necrosis factor receptor 1 (57).

In contrast to its functional information, there is less clarity regarding the regions of E6 that are responsible for the association with the cellular partners. In this study, we have shown that residues 78–104 of HPV16 E6 are responsible for Tuberin binding. The corresponding region of HPV18 E6 was identified previously for the interaction with E6BP (58). However, unlike Tuberin, the associations of E6BP with E6 do not cause the degradation of E6BP. In addition, p53 and its binding partner E6AP bind to the separated regions of E6 that are as different as E6BP binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency compared with HPV16 E6. It cannot exclude the HPV18 E6 also binds to the ELLG domain but with much lower efficiency.
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