Deregulation of eIF4E: 4E-BP1 in Differentiated Human Papillomavirus-Containing Cells Leads to High Levels of Expression of the E7 Oncoprotein

Kwang-Jin Oh,1 Anna Kalinina,1 No-Hee Park,2 and Srilata Bagchi1*

Center for Molecular Biology of Oral Diseases, College of Dentistry (M/C 860), University of Illinois at Chicago, 801 South Paulina Street, Chicago, Illinois 60612,1 and School of Dentistry, University of California at Los Angeles, 53-038 CHS, 10833 Le Conte Ave., Los Angeles, California 900952

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Infections with high-risk human papillomaviruses (HPVs) are linked to more than 95% of cervical cancers. HPVs replicate exclusively in differentiated cells and the function of the HPV E7 oncoprotein is essential for viral replication. In this study, we investigated the mechanism that regulates E7 expression in differentiated cells. The level of E7 protein was strongly induced in HPV-containing Caski, HOK-16B, and BaP-T cells during growth in methylcellulose-containing medium, a condition that induces differentiation. Enhanced expression of E7 was observed between 4 and 8 h of culturing in methylcellulose and was maintained for up to 24 h. The increase was not due to altered stability of the E7 protein or an increase in the steady-state level of the E7 mRNA. Instead, the translation of the E7 mRNA was enhanced during differentiation. More than 70 to 80% of the E7 mRNA was found in the polysome fractions in the differentiated cells. Consistent with this observation, higher levels of the phosphorylated translational inhibitor 4E-BP1 were observed in differentiated HPV-containing cells but not in differentiated non-HPV tumor cells or primary keratinocytes. The mTOR kinase inhibitor rapamycin blocked phosphorylation of 4E-BP1 and significantly decreased the level of E7 protein in Caski cells, suggesting that phosphorylation of 4E-BP1 is linked to E7 expression. Prevailing models for the molecular mechanisms underlying E7 expression have focused largely on transcriptional regulation. The results presented in this study demonstrate a significant role of the cellular translation machinery to maintain a high level of E7 protein in differentiated cells.

The high-risk types of human papillomavirus are the main etiological factors for cervical cancers (reviewed in references 27, 30, and 45). Furthermore, epidemiological studies have shown that a significant percentage (30 to 40%) of oral, head, and neck cancers, as well as other anogenital cancer lesions, contain these high-risk human papillomaviruses (HPVs) (13). Cervical cancer alone accounts for almost 12% of all cancers in women (45). Therefore, elucidation of the mechanism that contributes to the induction of the HPV-associated cancers is of major importance. The HPV-infected proliferating epithelial cells, but the viral DNA replication and structural viral gene expression are restricted to only the differentiated layers of epidermis or mucosa (27, 30). All HPV-transformed cancer tissues express two HPV-encoded oncoproteins, E6 and E7. Both E6 and E7 possess immortalizing activity. Moreover, continued expression of the E6 and E7 genes is necessary for the maintenance of the transformed phenotype (30). Past studies showed that knocking down the expression of the E7 gene by RNA interference induced senescence in cervical cancer cells (18, 22). Therefore, the expression of E7 is directly linked to the growth and survival of the HPV-associated cancer cells.

The transforming activity of E7 is associated with its ability to interact with the retinoblastoma tumor suppressor protein Rb and its ability to induce proteolysis of Rb through the 26S proteasome (3, 5, 16, 31, 43). One of the major biochemical functions of Rb is to form repressor complexes with the E2F family transcription factors and to repress expression of the replication and cell division genes (reviewed in references 9 and 19). E7 converts the repressor form of E2F (Rb/E2F) to the activator form (E2F). The E7-mediated conversion of E2F to the activator form stimulates the expression of DNA replication enzyme genes, which allows E7 to reactivate cellular DNA replication in differentiated epithelial cells (reviewed in references 27 and 30).

The requirement for a differentiated layer has made it difficult to study the productive life cycle of HPVs. The proliferating keratinocytes are located in the basal layer in contact with the extracellular matrix glycoproteins in the basement membrane. Keratinocytes leave the basal layer to undergo a series of biochemical and phenotypic changes that constitute the differentiation program. Since the movement away from the basement membrane is associated with the initiation of the differentiation process, historically, suspension of keratinocytes has been used as a method of triggering differentiation (17). The HPV-containing epithelial cells express the early differentiation markers, involucrin, and different cytokeratins within 24 h of growth in methylcellulose-containing culture (12, 35). Many events of the HPV life cycles, including expression of differentiation-specific viral promoters, differentiation-dependent viral-genome amplifications, and viral DNA replication, could be efficiently achieved within 24 to 48 h of growth in methylcellulose-containing medium (6, 11, 12, 21, 35, 39, 40). However, the final steps of virus life cycles, including the
production of infectious viral particles, could not be achieved in the methylcellulose culture system (35). The organotypic raft culture system is the only in vitro system that allows late-stage differentiation for production of limited amounts of infectious viral particles from the HPV-infected cells. Therefore, differentiation in methylcellulose-containing (1.6%) semisolid medium has been extensively used for analysis of HPV promoters and enhancers, HPV DNA replication, gene expression, and late protein synthesis.

Previous studies in our laboratory and by other research groups showed that the HPV16 E7 protein has a short half-life and the level of E7 in tumor cells is regulated primarily through proteolysis by the ubiquitin proteasome (16, 34, 43). E7 plays a critical role in the replication of HPV16 in differentiated cells. However, how E7 expression is regulated during differentiation is currently unknown. In this study, we observed a sustained induction of E7 protein during differentiation in semisolid methylcellulose-containing medium. We show that the increased expression of E7 is due to enhanced translation of the E7 mRNA in differentiated cells.

MATERIALS AND METHODS

Cells and culture conditions. Caski cells, C33A cells, HaCaT cells, and SCC-25 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, Primary human oral keratinocytes, primary human foreskin keratinocytes, and HPV16-immortalized HOK-16B cells were grown in keratinocyte growth medium from Cambrex as previously described (32). Primary human oral keratinocytes, primary human foreskin keratinocytes, and HPV16-immortalized HOK-16B cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 4 g/ml hydrocortisone (24). Caski, HOK-16B, BaP-T, HaCaT, C33A, and SCC-25 cells and primary oral and foreskin keratinocytes were induced to differentiate by being cultured in medium containing 1.6% methylcellulose as previously described (35). For CaCl2-induced differentiation, primary human oral keratinocytes, primary human foreskin keratinocytes, Caski cells, and BaP-T cells were grown in medium containing 1.5 mM CaCl2.

Antibodies and chemicals. [35S]Methionine-cysteine was from ICN, mGTP-Sepharose, rapamycin, and MG132 were from EMD Bioscience Calbiochem. The following antisera were from Cell Signaling Technology: eIF4E, phospho-eIF4E (Ser209), 4E-BP1, and phospho-4E-BP1 (Ser65). The E7 antibody (ED17), p27 antibody (C-19), and pan-cytokeratin (C11) antibody were from Santa Cruz Biotech. Unless otherwise stated, all chemicals were from Sigma.

RNA protection assay. Total RNA was isolated from Caski, BaP-T, and HOK-16B cells with an RNeasy kit (QIAGEN, Valencia, Calif.). The entire HPV16 E7 open reading frame (ORF) was amplified in pGEM 4. For generation of the antisense E7 probe, pGEM-E7 plasmid was linearized with EcoRI and transcribed in vitro with SP6 polymerase and 32P-labeled UTP. For generation of the antisense Skp2 probe, a BamHI-KPN1-digested Skp2 cDNA fragment was subcloned into pcDNA3, linearized with XbaI, and transcribed in vitro with eukaryotic transcription system (Molecular Dynamics). HPVs replicate exclusively in differentiated epithelial cells (reviewed in references 27, 30, and 45). The E7 oncoprotein encoded by HPV plays a critical role in differentiation of keratinocytes, allowing replication of the viral DNA (8). However, the regulation of E7 expression during differentiation has not been studied. Previous studies show that culturing of HPV-containing keratinocytes in semisolid medium containing methylcellulose induces differentiation that supports HPV DNA amplification and expression of viral late genes (6, 11, 12, 21, 35, 39, 40). Therefore, to analyze E7 expression during differentiation, the HPV16–containing Caski cervical carcinoma cells were induced to differentiate by culturing them in medium containing 1.6% methylcellulose, and the cell lysates were analyzed for the E7 protein using an

RESULTS

Enhanced expression of E7 protein during growth in methylcellulose-containing medium. HPV16containing Caski cells were grown as a monolayer or in methylcellulose-containing medium for 14 h. The cells were then homogenized with 15 strokes of a Dounce homogenizer and the nuclei were removed by spinning at 1,000 g for 10 min. The supernatant was supplemented with 665 μg/ml of heparin and centrifuged at 12,000 × g for 5 min to remove mitochondria. A sucrose gradient (15 to 40%) was prepared in buffer A. Postmitochondrial extract (200 μl) was layered on the top of a 4-ml gradient and centrifuged in an SW60 Ti rotor at 40,000 rpm for 90 min. Nine fractions (450 μl) were collected and digested with proteinase K (80 μg) in 1% sodium dodecyl sulfate (SDS) for 15 min at 37°C. RNA was isolated from individual fractions by phenol-chloroform extraction. The RNA was analyzed by electrophoresis on a 1.2% formamide agarose gel and stained with ethidium bromide to visualize the distribution of subpolysomal and polysomal RNA. Fractions 1 to 4 contained mostly subpolysomal RNA, and fractions 5 to 9 contained predominantly polysomal RNA. RNA from each fraction was analyzed for E7 mRNA and Skp2 mRNA by RNase protection assay as described above. The percentages of the E7 mRNA bound to polynomials were quantified by phosphorimaging (Molecular Dynamics).

Pulse-chase analysis using [35S]methionine-cysteine. The Caski cells were grown as a monolayer or in methylcellulose-containing medium for 6 h, 12 h, or 18 h and were treated with 50 μg/ml cycloheximide for different time periods (between 1 and 4 h). The cells were lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors; 200 μg of cell extracts was separated by 12% SDS-PAGE and blotted to a nitrocellulose membrane, which was probed with E7 antibody.

Western blot analysis. Western blot analysis was performed using a previously described procedure (3, 43). Cell lysates (50 to 300 μg) were resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and probed with primary and horseradish peroxidase-conjugated secondary antibodies, and signals were detected by enhanced chemiluminescence (ECL; Amersham). Flow cytometry. For analysis of distribution, 1 × 106 cells were fixed in 70% ethanol in phosphate-buffered saline, treated with 100 μg/ml RNase, and stained with 20 μg/ml propidium iodide. For each sample, 10,000 cells were analyzed for DNA content using a Coulter EPICS 753 flow cytometer. The percentages of cells in G1, S, and G2/M were determined using the EASY2 computer system (Coulter Electronics).

mGTP affinity chromatography. eIF4E and 4E-BP1 were isolated by mGTP-Sepharose chromatography as previously described (41). Briefly, cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1.5 mM EDTA, 10% glycerol, 20 mM β-glycerophosphate, 50 mM NaF, 200 mM NaVO4, 2 mM PMSF, 1.5 μg aprotinin/ml, and 5 μg leupeptin/ml. Cell lysates (500 μg) were incubated with 20 μl of mGTP-Sepharose for 4 h at 4°C and washed extensively with lysis buffer followed by 1 mM GTP-containing buffer, and the beads were suspended in SDS sample buffer and boiled for 5 min. The bound proteins were separated by SDS-PAGE and analyzed by Western blot assay as described above.

Enhanced expression of E7 protein during growth in methylcellulose-containing medium. HPV16–containing Caski cervical carcinoma cells were induced to differentiate by culturing them in medium containing 1.6% methylcellulose, and the cell lysates were analyzed for the E7 protein using an
immunoblot assay. A significant increase (four- to fivefold) in E7 was observed in Caski cells during growth in methylcellulose (Fig. 1A). The increase in the E7 protein was noticed between 4 and 8 h, reached maximum level around 14 h, and remained elevated for up to 20 h of culturing in methylcellulose-containing medium (Fig. 1A). The increase of E7 was not restricted to the malignant Caski cells but was also observed in HPV16-immortalized HOK-16B oral epithelial cells and HPV16-transformed BaP-T cells. Both HOK-16B and BaP-T cells expressed low levels of E7 protein in actively dividing monolayer cultures that were barely detectable using Western blot assays (Fig. 1C and D). However, more than six- to eightfold increases in E7 protein were observed when these cells were allowed to grow in semisolid culture containing methylcellulose (Fig. 1C and D). As in the case of Caski cells, the increase in E7 protein in HOK-16B and BaP-T cells was rapid, reached its maximum level at around 14 h, and was maintained at a comparable level for up to 24 h.

Growth in methylcellulose induces differentiation of epithelial cells, and therefore we analyzed the expression of differentiation-associated genes. An induction of cytokeratins was observed after 20 h of differentiation in Caski cells (Fig. 1A). Exit from the cell cycle is a prerequisite for differentiation. A robust increase in the cyclin-dependent kinase inhibitor p27 expression began at 8 h and continued for the duration of the time course. In contrast, the downregulation of growth-promoting c-Myc protein occurred rapidly within 2 h of suspension (data not shown). Flow cytometry analysis of cell cycle distribution revealed that after 16 h of growth in methylcellulose-containing medium, more than 40% of the cells were arrested in G2 with 4 N DNA content (Fig. 1B). In HOK-16B cells and BaP-T cells, the induction of differentiation was similar to that in Caski cells; an induction of the differentiation marker protein involucrin was detected after 20 h of growth in methylcellulose-containing medium. Interestingly, the increase in the level of E7 was observed long before the induced expression of involucrin and keratins. This result suggests that the induction of E7 is not an effect of differentiation; rather, E7 is induced prior to the onset of differentiation.

To further confirm that the induced expression of E7 during growth in methylcellulose is linked to differentiation, the Caski and BaP-T cells were allowed to differentiate in medium containing 1.5 mM CaCl2. Up to two- to fourfold induction of E7 protein was observed in both Caski and BaP-T cells after CaCl2 treatment (Fig. 1E and F). To determine the level of differentiation during CaCl2 treatment, the expression levels of the differentiation marker involucrin in BaP-T cells and cytokeratins in Caski cells were analyzed. A marginal increase in involucrin was observed in BaP-T cells after 36 h of CaCl2 treatment (Fig. 1F). Similarly, only a marginal increase in cytokeratins was observed in Caski cells (data not shown). These results suggest that in HPV-containing cells, CaCl2 induces only limited differentiation in comparison to growth in methylcellulose-containing medium. However, the level of E7 protein was induced during both methods of differentiation.
Half-life of the E7 protein did not change significantly during differentiation in methylcellulose-containing medium. E7 protein has a short half-life (~1 to 2 h), and the steady-state level of E7 in tumor cells is maintained primarily through proteolysis by the 26S proteasome (16, 34, 43). To determine whether the increase in E7 in differentiating cells is due to increased stability, we analyzed the half-life of E7 using two different assays. First, we analyzed the half-life of E7 by a pulse-chase assay using [35S]methionine-cysteine. Caski cells grown in attached or methylcellulose cultures (14 h) were labeled with [35S]methionine-cysteine for 2 h and were chased for 1 to 6 h with unlabeled methionine. Interestingly, significantly more 35S-labeled E7 was obtained by pulse-labeling of differentiated Caski cells than by actively dividing cells. However, the half-life of E7 protein did not change during differentiation (Fig. 2A). The half-life of E7 was between 1 and 2 h both in actively dividing and in differentiating Caski cells (Fig. 2A). A modest 1.2- to 1.5-fold increase in the E7 mRNA was observed in differentiating Caski cells (Fig. 3A) but not in HOK-16B cells (Fig. 3B). Interestingly, the steady-state level of the E7 transcript did not change significantly in either cell type, even after 20 h of growth in methylcellulose (Fig. 3). In addition, the stability of the E7 mRNA did not change significantly during growth in methylcellulose-containing medium (data not shown).

Increased polysome association of the E7 mRNA during growth of Caski and BaP-T cells in methylcellulose-containing medium. To determine whether the induction of E7 was due to increased translation, we analyzed the association of the E7 mRNA with polysomes in both Caski and BaP-T cells during growth in methylcellulose-containing medium. Cytosolic extracts from cells grown in monolayer culture or in methylcellulose were fractionated by sucrose gradient (15 to 40%) to separate polysomes from monosomes, as described in Materials and Methods. The gradient fractions were assayed for the distribution of E7 mRNA by RNase protection assay. The results show a significant increase in polysome-bound E7 mRNA from 25 to 30% to more than 70%, indicating enhanced translation of the E7 mRNA.
in either cell type. The changes in the E7 mRNA distribution correlated with the induction of the E7 protein in both cell types. Taken together, these data show that the expression of E7 is regulated at the level of translation initiation during growth in methylcellulose-containing medium.

The translation inhibitor 4E-BP1 remains inactivated by phosphorylation during differentiation in HPV-containing Caski cells but not primary keratinocytes or non-HPV HaCaT cells. The association of mRNA with ribosomes is controlled largely by cellular translation regulators such as eIF4E and 4E-BP1 (reviewed in references 14, 15, and 26). Therefore, to gain insight into the molecular mechanism regulating translation of the E7 mRNA in differentiating cells, we sought to investigate whether the cellular translation initiators were deregulated during differentiation of the HPV-containing cells. The translation initiation factor eIF4E binds to the 5′ Cap structure (m7GpppN, where N is any nucleotide) found in the majority of eukaryotic mRNA. A major mechanism for control of eIF4E function is through its interaction with the translation inhibitory protein 4E-BP1. The inhibitory function of 4E-BP1 is

FIG. 3. The steady-state level of the E7 mRNA did not change significantly during growth in methylcellulose-containing medium. Total RNA was isolated from Caski cells or HOK-16B cells at the indicated times following growth in methylcellulose-containing medium. RNA (10 μg) was hybridized to 32P-labeled antisense-E7 and antisense-cyclophilin probes and processed by an RNase protection assay as described in Materials and Methods. The 32P-labeled E7 and cyclophilin probes are shown.

FIG. 4. Increased polysome association of the E7 mRNA during differentiation. Caski cells (left) and BaP-T cells (right) were grown in a monolayer culture (A) or in methylcellulose-containing medium for 14 h (B). Cytosolic extracts were fractionated on a sucrose gradient (15 to 40%) as described in Materials and Methods. RNA isolated from each fraction (numbers indicated at the top) was analyzed for the presence of the E7 mRNA and Skp2 mRNA by an RNase protection assay. Staining for rRNA by ethidium bromide in gradient fractions is shown for each sample. Fractions 1 to 5 represent subpolysomal RNA, and fractions 6 to 9 represent polysome-associated RNA.
regulated primarily by phosphorylation (2, 4, 10, 14, 15, 26, 33). The hypophosphorylated form of 4E-BP1 competes with another translation initiation protein, eIF4G, to bind eIF4E and blocks its function (2, 15, 33). In contrast, the hyperphosphorylated form of 4E-BP1 cannot bind to eIF4E and cannot repress translation. The function of eIF4E is also regulated by phosphorylation. We analyzed the phosphorylation status of both 4E-BP1 and eIF4E during the differentiation of Caski cells, using antibodies specific for phospho-eIF4E (Ser209) and phospho-4E-BP1 (Ser65). Increased phosphorylation of 4E-BP1 was observed in Caski cells within 4 h of growth in differentiating culture, and most remarkably, the phosphorylation of 4E-BP1 remained high throughout the differentiation phase, for up to 20 h (Fig. 5). More than 60% of 4E-BP1 was in a phosphorylated form in Caski cells after 14 h of growth in methylcellulose-containing medium (Fig. 5). As a control, we analyzed phosphorylation of 4E-BP1 during differentiation of normal human keratinocytes and non-HPV HaCaT skin keratinocytes. In both primary human keratinocytes and non-HPV HaCaT skin keratinocytes, 4E-BP1 was dephosphorylated at between 4 and 8 h of growth in methylcellulose-containing medium. In these cells, less than 5% of 4E-BP1 remained phosphorylated after 14 h of growth in methylcellulose-containing medium (Fig. 5). The higher level of dephosphorylated 4E-BP1 in differentiating cells suggests repression of Cap-dependent translation. A decrease in the steady-state level of 4E-BP1 was also observed in differentiating non-HPV cells. In contrast, the steady-state level or the phosphorylation status of eIF4E did not change during differentiation of keratinocytes. Incidentally, the kinetics of induction of phospho-4E-BP1 paralleled the induction of E7 in Caski cells. Taken together, these results suggest that the induced phosphorylation of 4E-BP1 is linked to increased Cap-dependent translation of E7 mRNA in differentiating Caski cells (Fig. 5).

Induced phosphorylation of 4E-BP1 was also observed in differentiating HPV-containing HOK-16B and BaP-T oral keratinocytes (Fig. 6). In comparison, in primary oral keratinocytes, a dramatic decrease in the phospho-4E-BP1 was observed during differentiation. The level of eIF4E or phospho-eIF4E, on the other hand, did not change significantly in differentiating normal or HPV-containing oral keratinocytes. We further analyzed the phosphorylation of 4E-BP1 in SCC-25, a non-HPV oral cancer cell line, and in C33A, a non-HPV cervical cancer cell line. Similar to primary keratinocytes, a significant decrease in phospho-4E-BP1 was observed during differentiation in methylcellulose-containing medium. The steady-state level of eIF4E was comparable in all the cell types studied (Fig. 6). In comparison to primary keratinocytes, a higher level of phospho-4E-BP1 was also observed in Caski cells during calcium-induced differentiation (data not shown).

To evaluate the relationship between the increased phosphorylation of 4E-BP1 and the initiation of translation, the interaction of 4E-BP1 with eIF4E was examined by chromatography of cell lysates on 7-methyl GTP-Sepharose (Cap affinity column). For this experiment, Caski and HaCaT cells were grown in monolayer cultures or in methylcellulose-containing medium for 14 h and the cell extracts were allowed to bind to Cap affinity resins. In Caski cells, there was no detectable change in the ratio of 4E-BP1 to eIF4E bound to the column during differentiation (Fig. 7A). However, there was a dramatic increase in the ratio of 4E-BP1 to eIF4E bound to the Cap affinity column when extracts from differentiating non-HPV cells were analyzed (Fig. 7A). The lack of increase in the ratio of 4E-BP1 to eIF4E upon differentiation of HPV-containing cells as opposed to that in non-HPV cells during differentiation is significant. Phosphorylation of 4E-BP1 disrupts its interaction with eIF4E and the Cap complex. The enhanced binding of 4E-BP1 with the 7-methyl GTP-Sepharose is consistent with the acute hypophosphorylation of 4E-BP1 in HaCaT cells during differentiation.

The FRAP/mTOR kinase pathway leads to phosphorylation of 4E-BP1 (2, 15, 33). To correlate the induced phosphorylation of 4E-BP1 with the expression of E7, we treated Caski cells with the inhibitor of mTOR kinase, rapamycin. Actively
dividing Caski cells in a monolayer culture or Caski cells growing in methylcellulose-containing medium were treated with rapamycin (100 ng/ml) for 12 h. As expected, during rapamycin treatment, a significant decrease of the phospho-4E-BP1 was readily observed in both the actively dividing and differentiating cells (Fig. 7B). Similarly, a significant decrease in E7 protein level was observed in rapamycin-treated Caski cells (Fig. 7B). Rapamycin treatment did not change the steady-state level of E7 mRNA (Fig. 7B). Taken together, these results suggest that the induced and sustained phosphorylation of 4E-BP1 allows enhanced translation of E7 mRNA in differentiated cells.

DISCUSSION

HPVs replicate in differentiated cells, and the viral oncoprotein E7 makes the host differentiated cells replication competent to support virus growth. In this study, we investigated the mechanism by which E7 is regulated during differentiation. Due to the lack of a simple cell culture system to grow the HPVs, studies on the HPV oncoproteins are restricted to HPV-containing cell lines. An important new observation of this study is the finding that a significantly higher level of E7 is expressed during differentiation of HPV-containing tumor cells. Studies on the mechanism of the E7 induction revealed that the increase was due to induced and sustained translation of the E7 transcript in the differentiated cells. Concomitantly, we observed significant changes in the phosphorylation pattern of a key translation regulatory protein, 4E-BP1, in HPV-containing tumor cells during differentiation. The enhanced phosphorylation of 4E-BP1 predicts increased Cap-dependent translation in differentiating HPV-expressing cells.

Mobilization of the translation machinery, instead of the transcription machinery, to induce E7 during differentiation of HPV-containing tumor cells is intriguing. Multiple recent studies have shown that the deregulation of Cap-dependent translation by overexpression or induced phosphorylation of key translational regulators plays a major role in transformation and tumor formation (reviewed in references 4, 26, 28, and 36). The translation factor eIF4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis (37). The activation of the translation complex has been shown to be essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells (1). The oncogene Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukemia cells to induce translation (25). Similarly, in this study we observed enhanced translation of the E7 mRNA in differentiated cells, which is linked to enhanced phosphorylation of 4E-BP1. Expression of high levels of E7 protein would support the ability of papillomaviruses to reinitiate the growth cycle in differentiated cells.

The translation of most eukaryotic mRNAs involves the Cap initiation complex eIF4F (15). The eIF4F complex involves three proteins: the Cap-binding protein eIF4E, the RNA helicase eIF4A, and the scaffold protein eIF4G (reviewed in references 14, 15, and 26). Among them, eIF4E is a limiting translation factor and a major target for translation regulation. 4E-BP1 regulates the initiation of Cap-dependent translation by association with the Cap complex through eIF4E. The binding of 4E-BP1 to the Cap complex depends on phosphorylation (2, 10, 15, 33). Hypophosphorylated 4E-BP1 competes with eIF4G to bind eIF4E to block formation of active translation

FIG. 6. Changes in the levels of 4E-BP1 and eIF4E during growth in methylcellulose-containing medium. Lysates of primary oral keratinocytes (200 µg), HOK-16B cells (150 µg), BaP-T cells (100 µg), Caski cells (50 µg), SCC-25 cells (100 µg), and C33A cells (50 µg) grown in monolayer cultures or in methylcellulose-containing medium were analyzed by a Western blot assay using 4E-BP1 and eIF4E antibodies.
Hyperphosphorylation of 4E-BP1 inhibits association of eIF4E to 4E-BP1 and results in stimulation of translation (2). We observed both induced and sustained phosphorylation of 4E-BP1 in differentiating HPV-containing cells. The induction of 4E-BP1 phosphorylation coincided with the induction of E7 protein in Caski cells, suggesting that the translation inhibitor 4E-BP1 plays a major role in the accumulation of E7. Treatment of Caski cells with the TOR kinase inhibitor rapamycin blocked both the phosphorylation of 4E-BP1 and the expression of E7 in Caski cells. The phosphorylation of 4E-BP1 was sustained for up to 20 h without significant loss during differentiation of multiple HPV-containing cells, including Caski, HOK-16B, and BaP-T. In contrast, the phosphorylation of 4E-BP1 was reduced in primary keratinocytes and non-HPV cancer cells during differentiation in methylcellulose-containing medium. Consistent with this observation, significantly higher levels of 4E-BP1 were found to be associated with the 7-methyl GTP affinity resins in differentiating HaCaT cells, suggesting repression of Cap-dependent translation.

The molecular mechanism of sustained phosphorylation of 4E-BP1 during differentiation of HPV-containing cells is not elucidated in this study. However, these results support the notion that the formation of the functional translation initiation complex is induced and maintained during differentiation of HPV-containing cells. A recent study showed that eIF4E, but not 4E-BP1, is strongly phosphorylated during the onset of TPO-induced differentiation of UT7-mpl cells (7). Increased levels of eIF4E were also observed in differentiating lung tumor cell lines (42). The level of eIF4E or phospho-eIF4E did not change significantly during differentiation of HPV-containing cells. It is possible that the modifications of translation regulators during differentiation are cell type specific.

Viruses often use host DNA replication machinery to replicate viral DNAs, cellular transcription machinery to transcribe viral mRNAs, and cellular translation machinery to ensure translation of the viral mRNA. Different viruses utilize different strategies to use the host translation machinery for enhanced synthesis of viral polypeptides (38). However, little is known about how the human papillomaviruses interact with the cellular translation machinery. In high-risk oncogenic HPVs, E7 is expressed from a bicistronic E6/E7 pre-mRNA (44). The most abundant mRNAs transcribed from oncogenic HPVs are derived from the p97 promoter. The HPVs contain a differentiation-specific enhancer sequence that induces expression of late genes in differentiated cells. The differentiation-dependent promoter is not responsible for expression of E6/E7 mRNA (20). The E7 ORF is the second ORF after the E6 ORF in the E6/E7 mRNA. No mRNA that encodes E7 as the first open reading frame has been identified. In Caski cells, the most abundant in vivo mRNA is E6’IE7 in which part of the E6 ORF is spliced out, suggesting that E7 is formed from this mRNA. Thus, the 5’ end of the E7 mRNA contains uAUGs and an sORF from E6 (29). It is possible that these uAUGs and the E6 ORF control translation of the E7 ORF.
The results described in this report show that Cap-dependent translation is enhanced in HPV-containing cells, in marked contrast to many viruses that impaired such translation. In herpes simplex virus type 1-infected cells, viral gene product ICP0 stimulates Cap-dependent translation to support viral DNA replication in quiescent differentiated cells (41). ICP0 stimulates phosphorylation of eIF4E and 4E-BP1 to enhance the assembly of active eIF4E complex, and this process is crucial for productive viral growth and efficient reactivation of latent infection (41). Since productive HPV infections in differentiated cells depend on the E7 function, the induction of E7 at the onset of differentiation might constitute a critical step in the HPV life cycle. The HPV proteins are translated exclusively in the differentiated epithelial cells, and the HPV late proteins are translated exclusively in the differentiated epithelial cells. Our studies show that Cap-dependent translation is enhanced in differentiating HPV-containing cells, in contrast to non-differentiated HPV cells. Parallel to the ICP0 protein of herpes simplex virus type 1, an HPV protein is likely involved in enhancing Cap-dependent translation in HPV-containing cells. A recent study showed that the HPV oncoprotein E6 associates with GADD34/PP1 to block PKR-initiated translation inhibition (23a). Future studies will be important in determining the identity of the HPV protein involved in enhancing Cap-dependent translation machinery in differentiating cells.

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