

Effect of Human Papillomavirus Type 16 Oncogenes on MAP Kinase Activity

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The mitogen-activated protein (MAP) kinase signal transduction pathway is an intracellular signaling cascade which mediates cellular responses to growth and differentiation factors. The MAP kinase pathway can be activated by a wide range of stimuli dependent on the cell types, and this is normally a transient response. Oncogenes such as *ras*, *src*, *raf*, and *mos* have been proposed to transform cells in part by prolonging the activated stage of components within this signaling pathway. The human papillomavirus (HPV) oncogenes E6 and E7 play an essential role in the *in vitro* transformation of primary human keratinocytes and rodent cells. The HPV type 16 E5 gene has also been shown to have weak transforming activity and may enhance the epidermal growth factor (EGF)-mediated signal transduction to the nucleus. In the present study, we have investigated the effects of the oncogenic HPV type 16 E5, E6, and E7 genes on the induction of the MAP kinase signaling pathway. The E5 gene induced an increase in the MAP kinase activity both in the absence and in the presence of EGF. In comparison, the E6 and E7 oncoproteins do not alter the MAP kinase activity or prolong the MAP kinase activity induced with EGF. These findings suggest that E5 may function, at least in part, to enhance the cell response through the MAP kinase pathway. However, the transforming activity of E6 and E7 is not associated with alterations in the MAP kinase pathway. These findings are consistent with E5 enhancing the response to growth factor stimulation.

More than 60 human papillomavirus (HPV) genotypes have been identified, a subset of which, including HPV types 6 (HPV-6), -11, -16, and -18, is associated with genital infections (56). HPV-16 and -18 are commonly associated with cervical cancers, while HPV-6 and -11 are commonly associated with benign condylomas (55, 57). The association of high-risk HPV-16 and -18 with cancers is consistent with their ability to transform primary rodent cells (31, 36, 51) and human keratinocytes (17) in culture. In most of the advanced cervical neoplasias, the HPV DNA is integrated into the host chromosomal DNA, whereby only the E6 and E7 genes are retained and expressed (2, 45, 46, 48). These observations suggest that these viral oncogenes are involved in the progression and maintenance of transformation. Significant advances in our understanding of E6- and E7-associated cell transformation have come from the observations that these viral oncoproteins target cellular tumor suppressor proteins. For example, E7 binds to and inactivates the cellular tumor suppressor Rb (14) and related proteins such as p107 and p130 (13), whereas E6 targets the cellular tumor suppressor p53 (54) and promotes its degradation through the ubiquitin proteolysis pathway (43). These observations provide an understanding of the molecular basis on which E6 and E7 manipulate the cell towards the transformed phenotype.

In contrast to the E6 and E7 genes, the E5 gene is often deleted from the viral genome during viral DNA integration in the more advanced cervical neoplastic lesions (2, 46). However, in low-grade lesions prior to integration, among the most abundant mRNA transcripts are those which could potentially encode E5 and E4 (50). Therefore, if E5 does participate in the transformation process, this would be at an early stage when

the HPV DNA is episomal. Several reports have described HPV-16 E5 as a potential third oncogene which can transform established rodent cells to anchorage-independent growth (25, 26, 37, 53). The transforming activity of E5 may be due in part to its ability to inhibit downregulation of the epidermal growth factor (EGF) receptor (EGF-R). Evidence to support this view came from the observation that expressing HPV-16 E5 in human keratinocytes resulted in an increase in the number of EGF-Rs at the cell surface and that there was an inhibition of receptor degradation (53). Consistent with this observation, it was revealed that the inhibition of the downregulation of the EGF-R by E5 may be associated with the ability of E5 to bind a 16-kDa protein, a component of the vacuolar proton-ATPase pump complex (H⁺-ATPase) involved in receptor protein degradation (10). Finally, it has recently been demonstrated that HPV-16 E5 expression results in an impairment of acidification of endosomes (52). The acidification of endosomes is essential for their proteolysis function, including those involved in the degradation of the EGF-R (32, 44). The H⁺-ATPase pump is responsible for the acidification, and E5 interaction with the 16-kDa component may impair this process in HPV-infected human keratinocytes. Moreover, HPV-16 E5 has been demonstrated to cooperate with E7 to potentiate a mitogenic response which is enhanced in the presence of the EGF (5). The close association of E5 biological activities with growth factor receptors would suggest that E5 may contribute to the normal viral life cycle and the early stage of viral infection, by increasing cell responsiveness to growth factors such as EGF. One way to further investigate this possibility would be to examine signal transduction mediators such as mitogen-activated protein (MAP) kinase, which is downstream from the EGF-R, and this is the objective of the present study.

The EGF-R is present on all epithelial cells, including cervical mucosal cells (7), and is a transmembrane receptor protein with ligand-activated tyrosine kinase activity (6). Stimulation of the receptor with EGF activates a number of signal

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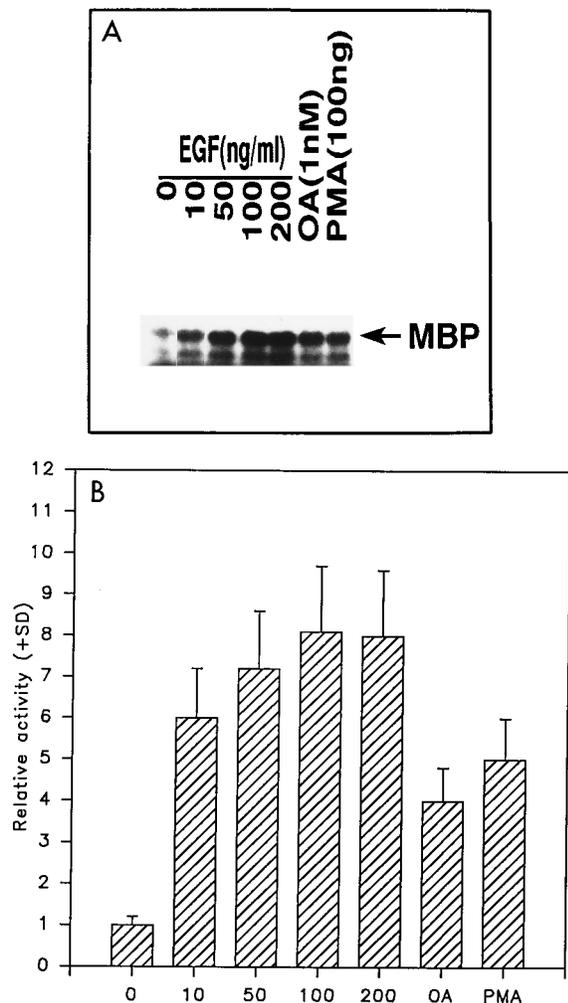


FIG. 1. Activation of MAP kinase in HT1080 cells. HT1080 cells were treated with 0, 10, 50, 100, or 200 ng of EGF per ml for 5 min or 1 nM OA or 100 ng of PMA per ml for 10 min. The kinase activities were assayed as described below. (A) MBP was used as the substrate for MAP kinase, and the phosphorylation of MBP was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by autoradiography. (B) A specific MAP kinase target peptide was used as the substrate for MAP kinase activity. Phosphorylation of this peptide was measured by liquid scintillation counting. The relative levels of radioactivity are shown as a bar graph and represent the mean \pm standard deviation of determinations from three experiments. Note that the data obtained by the two assay conditions were very similar. HT1080 human fibrosarcoma cells (40) were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For mitogen stimulation experiments, cells were starved with the same medium containing 0.1% fetal calf serum for 6 h and then treated with either EGF, PMA, or OA. The cell lysates were prepared as previously described (49). Briefly, cells were washed three times with ice-cold buffer containing 0.15 M sodium chloride and 25 mM sodium phosphate (pH 7.2) and lysed in 150 μ l of ice-cold homogenization buffer containing 20 mM Tris (pH 7.5), 50 mM *p*-glycerophosphate, 50 mM sodium fluoride, 2 mM dithiothreitol, 100 μ M sodium orthovanadate, 5 mM benzamide, 20 mM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, and 10 μ g of aprotinin per ml. Cell lysates were cleared by centrifugation at 100,000 \times g at 4°C for 30 min. The supernatants were assayed for protein by Bio-Rad protein assay reagent, and kinase activity was determined immediately, or the samples were frozen at -70° C. Two different MAP kinase assays were performed in order to have independent confirmation of the activities measured. The first MAP kinase assay was adapted from previously described methods (9, 49) with MBP as the substrate. Briefly, cell lysates (10 μ g) were assayed in a final volume of 25 μ l containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0), 10 mM manganese chloride, 1 mM dithiothreitol, 1 mM benzamide, 100 nM staurosporine, 50 μ M [γ - 32 P]ATP (1 μ Ci), and 0.4 mg of MBP per ml (Sigma, St. Louis, Mo.) at 30°C for 30 min. Assays were terminated with 2 \times SDS sample buffer and phosphorylated MBP as analyzed by SDS-polyacry-

lamide gel (15% acrylamide) electrophoresis and autoradiography. In the second assay, the phosphorylation of a MAP kinase target synthetic peptide (UBI, Lake Placid, N.Y.) which has the amino acid sequence APRTGGRR containing amino acids 95 to 98 (underlined) of bovine MBP (42) was measured as described previously (9). The phosphorylation reaction was similar to that for MBP phosphorylation. The reaction was stopped by spotting 20- μ l aliquots onto a 1.5-cm 2 piece of Whatman P81 phosphocellulose paper, then the papers were dried briefly at room temperature and washed seven times by shaking for 10 min in phosphoric acid (1% [wt/vol]), and radioactivity was then determined by scintillation counting.

transduction pathways including the MAP kinase pathway (4, 12, 29, 30). The MAP kinase pathway is associated with both cell proliferation and differentiation (1, 41). In response to growth factor receptor binding, a number of intermediates have been identified upstream of MAP kinase, including the activation of *ras* GTPase activity followed by the stimulation of c-Raf protein kinase, MAP kinase kinase, and MAP kinase (21, 29, 30). Activated MAP kinase is translocated into the nucleus upon activation, where it can potentially phosphorylate transcription factors, such as c-Jun, c-Myc, and c-Tal1 (8, 20, 28, 38, 39).

One of the hallmarks of this signaling pathway is its rapid transience (23, 33), and inappropriate activation or inhibition of downregulation of the pathway can contribute to cell transformation. Indeed, the components in the pathway, such as Ras and Raf, have been identified as the products of transforming oncogenes (15, 19). The current hypothesis is that oncogenes such as *ras*, *raf*, *src*, and *mos* transform cells by prolonging the activated stage of MAP kinase kinase and of components downstream in the signaling pathway (27). It has also been shown that the constitutive activation of the MAP kinase pathway by simian virus 40 small T could induce monkey kidney CV-1 cell proliferation (49), thus demonstrating for the first time that a DNA tumor virus protein could potentiate MAP kinase activity.

On the basis of these observations, we have examined whether any of the HPV-16 oncogene products could alter cellular MAP kinase activity. In this report, we show that the E5 gene was able to induce an increase in the MAP kinase activity. In comparison, the E6 and E7 genes did not stimulate or prolong MAP kinase activity.

Activation of MAP kinases in HT1080 cells. It has been shown that the MAP kinase signaling pathway is involved in cell growth, differentiation, and transformation. It was therefore of interest to investigate whether the biological activities of HPV oncogenes could influence this signaling pathway. Since there is no one cell type which can be readily transformed by each of the HPV-16 oncogenes individually, we chose to use a cell type which was already fully transformed to provide a more uniform background to compare MAP kinase in cells expressing the different HPV oncogenes. HT1080 human fibrosarcoma cells were transfected with HPV oncogenes, and then the MAP kinase activity of the resulting cells was measured. Before transfecting these cells, it was, however, necessary to verify whether it was possible to stimulate and measure MAP kinase activity in these cells. Therefore, MAP kinase activity was determined in these cells following stimulation with EGF, phorbol myristate acetate (PMA), and okadaic acid (OA). MAP kinase activity is enhanced by OA because OA inhibits 2A protein phosphatase, which in turn dephosphorylates MAP kinase (3, 18). PMA may stimulate serine/threonine phosphorylation of MAP kinase kinase (16).

As shown in Fig. 1, MAP kinase in HT1080 cells was stimulated with EGF in a dose-dependent manner and was maximally activated by eightfold with a concentration of 100 ng of

EGF per ml. These data are consistent with a previous study demonstrating that HT1080 cells contain normal levels of EGF-Rs (47). Treatments with PMA or OA increased MAP kinase activities by five- and threefold, respectively. EGF was used in subsequent assays because it induced the highest level of MAP kinase activity. As also shown in Fig. 1, two different MAP kinase assays were performed. In Fig. 1A, the phosphorylation of myelin basic protein (MBP) was determined by autoradiography. In Fig. 1B, the phosphorylation of a MAP kinase-specific peptide was determined by liquid scintillation counting. The data obtained by the two different MAP kinase assays were very similar, and therefore these two assays were used in subsequent analysis involving the HPV oncogene-expressing cells.

Expression of HPV-16 E5, mutant E5, E6, and E7 in HT1080 cells. The preceding data showed that the MAP kinase activity could be determined in HT1080 cells following EGF stimulation. This allowed us to use this cell line to examine the effects of expression of HPV oncogenes on EGF-activated MAP kinase signal transduction. HT1080 cells were cotransfected with HPV oncogene-expressing plasmid pJ4-16E5, pJ4-16M5, pJ4-16E6, or pJ4-16E7 or control plasmid pJ4 together with pWLN-Neo plasmid and selected in the medium containing G418. The resulting resistant colonies were pooled and expanded as polyclonal pools. The expression of the respective HPV oncogenes was verified by Northern (RNA) blot analysis. As shown in Fig. 2, the respective HPV oncogene transcripts were present in the resulting pooled cells. There was no visible change in the morphology or growth characteristics of the HPV oncogene-expressing cells. Given that these cells are already fully transformed (40), demonstrate a loss of contact growth inhibition in culture, form colonies in agar, and are tumorigenic in immunocompromised mice, it was perhaps not surprising that the expression of HPV oncogenes did not further alter the growth characteristics of these cells.

MAP kinase activities in HPV oncogene-expressing HT1080 cells. The major purpose of this study was to determine whether expression of HPV-16 oncogenes E5, E6, and E7 could affect the EGF-activated MAP kinase signaling pathway. Therefore, the MAP kinase activity in the absence or presence of EGF was determined in the cells expressing the individual HPV oncogenes. Cells were serum starved for 6 h, stimulated with EGF (100 ng/ml) for 5 min, and then lysed. Lysates containing 10 μ g of total protein were assayed for MAP kinase activities with MBP and MAP kinase target synthetic peptide as substrates. The results from both these assay conditions are presented in Fig. 3. These data demonstrate that expression of E6 or E7 in HT1080 cells did not affect the MAP kinase activity in these cells. In contrast, there was a modest yet reproducible increase in MAP kinase activity in the E5- and mutant E5-expressing cells as determined by both assay conditions. The mutant E5 used here was altered in order to potentially increase the translation efficiency of the corresponding E5 mRNA by introducing the Kozak consensus sequence around the initiation ATG of the E5 gene. This resulted in two amino acid changes following the initiation methionine. In vitro transcription-translation analysis of the mutant E5 compared with the wild-type E5 showed that the mutant E5 was at least five times more efficiently translated in vitro (data not shown). However, the wild-type E5- and mutant E5-expressing cells both demonstrated a similar increase in the MAP kinase activity, suggesting that this mutation did not increase translation efficiency in vivo. There was an approximately twofold increase in MAP kinase activity in the E5-expressing cells which was observed both in the presence and in the absence of EGF stimulation.

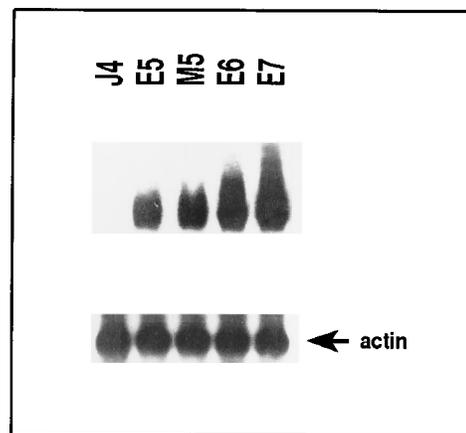


FIG. 2. Northern blot analysis of cell lines expressing individual HPV oncogenes. Ten micrograms of the total RNA from the individual cell lines stably transfected with control pJ4 vector and HPV oncogenes E5, M5 (mutant E5), E6, and E7 was probed with [α - 32 P]dCTP-labelled nick-translated DNA fragments from the open reading frames of each viral gene. The blot was re-probed with an actin probe to verify equal loading of RNA in each lane. The HPV oncogene-expressing plasmids were constructed by inserting the open reading frames into the pJ4 vector (51), which placed the HPV oncogenes under the transcriptional control of the Moloney murine leukemia virus long terminal repeat. The plasmids were designated pJ4-16E5, pJ4-16E6, pJ4-16E7, and pJ4-16M5. The plasmids pJ4-16E5 and pJ4-16M5 were generated as follows. A *Bam*HI-*Eco*RI restriction site-flanked E5 open reading frame (nucleotides 3849 to 4098) DNA fragment was synthesized by PCR and inserted into the pJ4 vector to generate pJ4-16E5. pJ4-16M5, a mutant in which the Kozak sequence was introduced to increase the potential translation level (24), was created by inserting a *Hind*III-*Eco*RI DNA fragment into pJ4 vector, which was synthesized by PCR with a pair of primers (5'-AGCAAGCTTAAATGGATCCGAATCTT and 5'-ATCGGAATTCCTATGTAAATAAAAA; Kozak consensus sequence is underlined). This generated a mutant E5 with the two amino acids aspartic acid and proline instead of threonine following the initiation methionine. HPV oncogene-expressing HT1080 cell lines were developed by using the standard calcium phosphate transfection procedure and selected with G418 (25). Briefly, the cells were cotransfected with 10 μ g of plasmid pJ4, pJ4-16E5, pJ4-16M5, pJ4-16E6, or pJ4-16E7 together with 1 μ g of pWLN-Neo plasmid and selected in medium containing G418 (150 μ g of G418 per ml). Two weeks after transfection, G418-resistant colonies were pooled and expanded. Northern blot analysis was performed to verify that transfected cells expressed the individual HPV oncogenes. Northern blot analysis was carried out as previously described (25). Briefly, total RNA was extracted with Trizol (Gibco-BRL). RNA samples (10 μ g) were denatured for 1 h at 50°C in the presence of 0.01 M NaH₂PO₄ and 1 M glyoxal and resolved in a 1.2% agarose gel. Following electrophoresis, RNA was transferred to Hybond-N membrane (Amersham, Oakville, Ontario, Canada), and pre-hybridized and hybridized at 42°C in 50% formamide with probes nick translated in the presence of [α - 32 P]dCTP (ICN, Toronto, Canada).

Effects of E5 expression on the duration of MAP kinase activity. The hallmark of the EGF-activated MAP kinase signaling pathway is its transience. Previous studies have shown that constitutive activation or prolongation of the activated stage of the MAP kinase pathway could lead to cell proliferation and transformation (11, 27, 49). Moreover, HPV-16 E5 has been shown to inhibit degradation of EGF-R and cause a greater number of EGF-Rs to be recycled back to the cell surface after 2 h of stimulation (53), and this may therefore lead to a prolongation of the MAP kinase pathway by E5.

On the basis of this information, we examined the influence of E5 expression on the duration of EGF-activated MAP kinase activity. As before, control cells and E5-expressing cells were serum starved for 6 h and then were left untreated or treated with EGF (100 ng/ml) for various times up to 3 h. Because the previous assays showed that the MBP phosphorylation data and the peptide phosphorylation data yielded comparable results, only the MBP was used as the MAP kinase substrate in this experiment. The results of two similar such

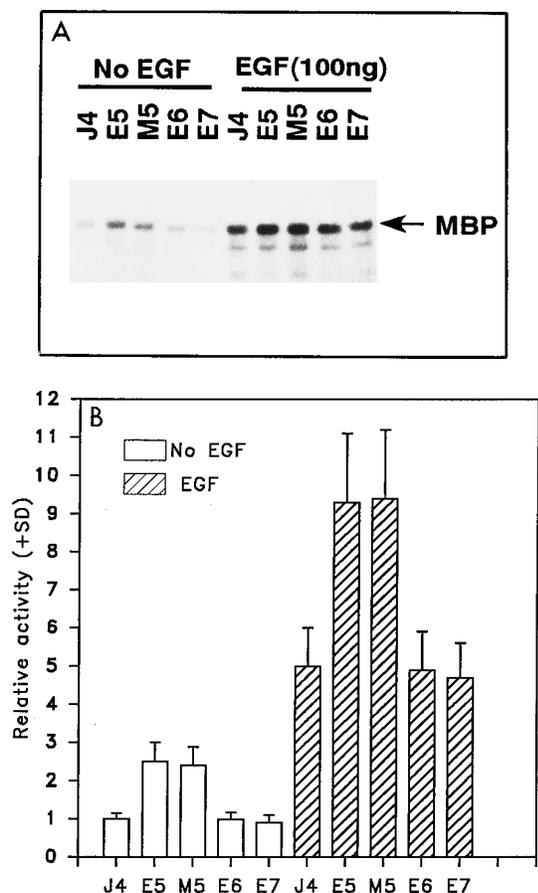


FIG. 3. MAP kinase activity in the HPV oncogene-expressing HT1080 cell lines. Cells expressing individual HPV oncogenes were grown for 24 h (80% confluence), serum starved for 6 h, and then treated with 100 ng of EGF per ml for 5 min or left untreated. (A) The MAP kinase activities were assayed by the MBP phosphorylation assay. (B) MAP kinase activity was determined by the peptide phosphorylation assay; the numbers represent relative MAP kinase activities and the mean \pm standard deviation of determinations from three experiments. J4 represents the cell line stably transfected with control pJ4 vector.

experiments are shown in Fig. 4, in which the upper and lower panels represents the MAP kinase activity determined for up to 2 and 3 h, respectively. These data show that the expression of E5 induced an increase in the MAP kinase activity by up to two- to threefold (as determined by densitometry of the X-ray films [data not shown]) compared with the control cells at each time point analyzed. These data also demonstrate that E5 could induce a sustained increase in MAP kinase activity for up to at least 3 h. Therefore, although E5 had a modest effect on MAP kinase activity, this effect was evident over an extended period. This is more significant than if the E5-mediated increase in MAP kinase activity was restricted only to the early 5-min interval.

MAP kinase activity in transiently transfected COS-1 cells.

Because of the modest increase in E5-mediated MAP kinase activity observed in the HT1080 cells, it was necessary to confirm this observation with an independent approach. Several previous reports have used COS-1 cells in transient transfection assays to characterize bovine papillomavirus E5 (10, 34) and HPV-16 E5 (22). We therefore have used this approach to determine whether a transiently transfected HPV-16 E5-expressing plasmid in COS-1 cells would result in a detectable increase in MAP kinase activity. As shown in Fig. 5, cells

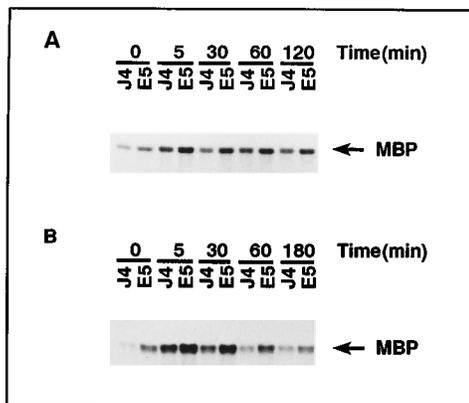


FIG. 4. Duration of EGF-induced MAP kinase activity in E5-expressing cells. Cells expressing E5 and control cells were assayed for MAP kinase activity following treatment with 100 ng of EGF per ml for various times. (A) Data are shown for a 2-h time course. (B) Data are shown for a 3-h time course. MAP kinase activity was determined as described for Fig. 1. Two such similar assays are shown here to demonstrate reproducibility in this assay. J4 represents the cell line stably transfected with control pJ4 vector. These data demonstrate that the increased level of MAP kinase activity in the E5-expressing cells was observed for at least 3 h following EGF stimulation.

transiently transfected with the E5-expressing plasmid demonstrated more MAP kinase activity than cells transfected with a control plasmid. As with the stably transfected HT1080 cells, the increase in MAP kinase activity in the E5-transfected cells was about twofold greater than in the control cells, and this was in both the presence and the absence of EGF stimulation. However, we estimated through the use of β -galactosidase activity staining in the presence of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) that about 20 to 30% of the cells were transfected in this manner. This argues that within the E5 plasmid-transfected cells, the MAP kinase as shown in Fig. 5 is an underestimate. These data are consistent with the experiments carried out in the HT1080 cells and provide further support for the argument that HPV-16 E5 can enhance cellular MAP kinase activity.

The stimulation of cell proliferation by growth factors is

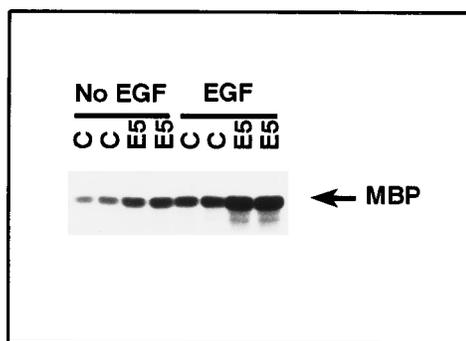


FIG. 5. MAP kinase activity in COS-1 cells transiently transfected with the E5 gene. COS cells transiently transfected with control or E5-expressing plasmid were assayed for MAP kinase activity 48 h after transfection. Cells were either untreated or treated with 100 ng of EGF per ml for 5 min as indicated. Each lane represents the MAP kinase activity determined from a separate dish of transfected cells. Duplicate dishes of transfected cells were used in this assay. Note that the E5-transfected cells contained more MAP kinase activity than the control cells. Cells were transfected with the E5-expressing plasmid pMT2-H16E5KC (22) by the calcium phosphate procedure as previously described (25). MAP kinase assays were performed as described for Fig. 1.

largely controlled by a series of specific kinases including MAP kinase. Subtle differences in the concentration, activity, or period of activity of these regulators could have profound influences on cell replication. Viruses such as HPV may influence growth factor responses in subtle but specific ways to influence host cell behavior to ensure virus replication and subsequent propagation. In the present study, we have demonstrated that expressions of HPV-16 E5 resulted in a modest enhancement of MAP kinase activity. In comparison, expression of E6 or E7 did not alter MAP kinase activity. The increase in MAP kinase activity in the E5-expressing cells was seen in both the presence and the absence of EGF and was observed for at least 3 h following EGF stimulation. This increase in MAP kinase activity may enhance DNA replication of infected cervical cells, thus ensuring replication of the viral genome in these cells.

Activation of the EGF-R results in the stimulation of a number of signal transduction pathways including the downstream activation of MAP kinase (4, 21, 29, 30). Therefore, the observation reported in this work that MAP kinase is more active in the E5-expressing cells is consistent with increased EGF-R activity in these cells. These data are in agreement with previous studies showing that expression of E5 inhibits degradation of EGF-R and causes EGF-R to be recycled back to cell surface (53) and that HPV-16 E5 expression could cooperate with EGF to induce anchorage-independent growth and proliferation of established and primary rodent cells (5, 25, 37).

It was also apparent that MAP kinase activity was increased in the E5-expressing cells in the absence of EGF stimulation. It is therefore possible that, like bovine papillomavirus E5 (34, 35), HPV-16 E5 can associate directly with growth factor receptors and that this results in an increase in growth factor receptor activity. This would be consistent with the recent observation that HPV-16 E5 is capable of complexing with a variety of growth factor receptors including the EGF-R, the platelet-derived growth factor receptor, the colony stimulating factor-1 receptor, and p185-neu (22). Furthermore, HPV-16 E5 has transforming activity in the absence of EGF (25, 26, 53), and this may be due in part to its association with and potential activation of growth factor receptors, thus resulting in the increased MAP kinase activity in the absence of growth factors. Another possibility is that E5 may inhibit the degradation of cellular growth factor receptors in addition to the EGF-R. These receptors may be able to respond to factors within the serum, and this may also contribute to increased MAP kinase activity in the absence of EGF.

The E5 gene is usually lost during viral DNA integration in the more advanced cancers, thus demonstrating that it is not required for disease progression to the later advanced stages. It is possible that the subtle activity which E5 has on the MAP kinase activity becomes redundant in the more advanced transformed cells because of the more dominant activity provided by the major transforming genes, E6 and E7. For example, loss of Rb regulatory activity due to E7 may override any requirement for increased MAP kinase activity. Likewise, E6-mediated p53 degradation could result in unchecked cyclin-dependent kinase activity because of reduced p21 activity, and this could also override a requirement for increased MAP kinase activity.

In summary, we have examined cellular MAP kinase activity in HPV-16 oncogene-expressing cells. Although E5 could enhance MAP kinase activity, E6 and E7 had no effect on this activity. These data suggest that one of the cellular targets of HPV-16 E5 may be the MAP kinase-associated signal transduction pathway.

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