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Human Papillomavirus Type 16 E5 Protein Affects Cell-Cell Communication in an Epithelial Cell Line

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The human papillomavirus type 16 (HPV16) E5 protein is considered to have weak oncogenic properties, and its function in infected human keratinocytes is unknown. HPV16 E5 protein has been found to localize to the Golgi apparatus and the plasma membrane. To analyze the effect of E5 on plasma membrane properties, cells from the human keratinocyte cell line HaCaT were transfected with the HPV16 E5 open reading frame under the control of an inducible promoter. The gap junction-mediated cell-cell communication of E5- and vector-transfected cells was analyzed by microinjection of Lucifer yellow to measure dye coupling of the cells. A strong impairment of dye transfer in E5-transfected cells but not in vector-transfected cells was observed, with more than 80% dye transfer inhibition 40 min after injection. This impairment correlated with dephosphorylation of connexin 43, the major gap junctional protein in HaCaT cells. Furthermore, the dye coupling inhibition was not the result of differentiation of the E5-expressing cells, since no overexpression of cytokeratin 1 or filaggrin, markers of HaCaT cell differentiation, could be observed. These results therefore strongly suggest a correlation between expression of the HPV16 E5 open reading frame, impairment of gap junction-mediated dye coupling, and dephosphorylation of connexin 43.

Human papillomaviruses (HPVs) have been associated with the development of epithelial neoplasias (30). Two different groups of HPVs have been defined according to their association with benign or malignant lesions: a high-risk group including types 16, 18, and 33, which are associated with cervical cancer, and a low-risk group including types 1, 6, and 11, which are found in warts and other benign epithelial tumors (31). Two proteins encoded by the high-risk viruses, the E6 and E7 proteins, have oncogenic properties in that they are able to immortalize primary keratinocytes and fibroblasts (9, 24). Although no direct evidence about the mechanisms by which these proteins contribute to cellular transformation has been presented, their interactions with p53 and the retinoblastoma gene product, respectively, suggest a direct effect on factors involved in cell cycle control (10, 25).

Whereas in case of the bovine papillomavirus type 1 (BPV1) the E5 protein seems to be the major transforming protein, HPV E5 proteins have only weak oncogenic properties (2, 17, 18, 23). It was suggested that HPV E5 plays a role in the first steps of cellular transformation. This hypothesis is supported by the fact that in cervical intraepithelial neoplasia (low-grade intraepithelial neoplasia) lesions large amounts of E5 mRNA as well as of E5 protein can be detected (16, 27).

In BPV1- and HPV type 16 (HPV16)-transfected cells, most of the synthesized E5 protein localizes to the Golgi apparatus but a small proportion can be found at the plasma membrane (5, 7). In yeast cells transfected with an HPV16 E5 open reading frame (ORF), most of the E5 protein is found within the vacuolar system and at the plasma membrane (our unpublished results). BPV1 E5 protein has been found to bind and enhance the effects of the receptors for platelet-derived growth factor and epidermal growth factor (EGF). In contrast, binding of the HPV16 E5 protein could not be observed (6, 8, 14, 22, 28). However, it seems that the presence of HPV16 E5 results in a reduced degradation of internalized EGF receptors, allowing their recycling to the plasma membrane, which may result in an enhancement of their biological effect (28). Simi-

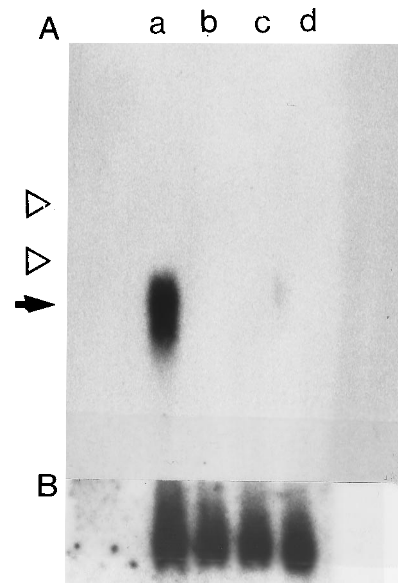

FIG. 1. RNA transcription in transfected HaCaT cells. Transfected cells were treated for 48 h with 1 μM dexamethasone, and RNA was extracted. Then 20 μg of each RNA was separated on agarose under denaturing conditions, blotted onto GeneScreen membranes, and hybridized to a radioactive DNA fragment comprising the entire E5 coding region. (A) Lanes: a and b, HaCaT-E5 with and without dexamethasone, respectively; c and d, HaCaT-pMSG with and without dexamethasone, respectively. Empty arrowheads show the positions of the 28S and 18S rRNAs. The black arrow shows the position of the 1.2-kb hybridizing band. Upon long exposition times a very faint band could also be identified in lane b. (B) After autoradiography, the membrane was stripped and rehybridized with a radioactive DNA fragment of the glyceraldehyde-3-phosphate dehydrogenase coding region to control for loading differences.

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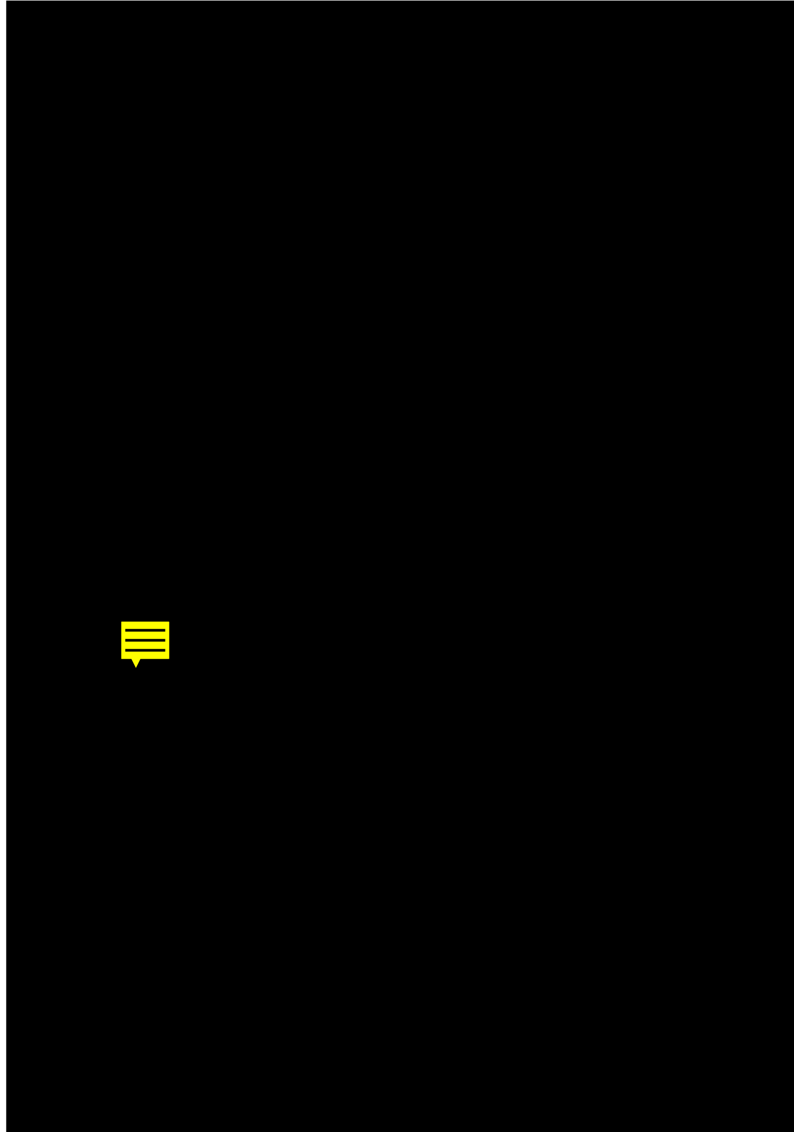

FIG. 2. Dye coupling in E5-transfected and control-transfected HaCaT cells. Cells were seeded on sterile glass coverslips and cultured in the presence of 1 μM dexamethasone for 48 h. At this time the cells reached about 80% confluency. The coverslips were taken out of the petri dishes, and the cells were microinjected with Lucifer yellow. E5-transfected (a, c, and e) and vector-transfected (b, d, and f) cells were photographed with a Leitz DMRBE microscope under fluorescent light at 2.5 (a and b), 10 (c and d), and 40 (e and f) min thereafter. Note that at 40 min a large amount of cells (scarcely visible in the picture) surrounding the strongly fluorescent cells were slightly fluorescent in control-transfected but not in E5-transfected cells.

larly to BPV1 E5, an association between the membrane-bound proton-ATPase and the HPV16 E5 protein has been reported previously (7). Interestingly, the proton ATPase has also been localized within the plasma membrane as being a part of the gap junctional complex (12).

Since the E5 protein is associated with the plasma membrane, we decided to investigate whether the E5 protein of HPV16 is able to modify the biological properties of the cellular membrane, which may be a first step in cellular transformation induced by HPVs.

The complete E5 ORF (nucleotides 3849 to 4100) was cloned immediately downstream of the mouse mammary tumor virus promoter into the expression vector pMSG (Pharmacia). The mouse mammary tumor virus promoter can be induced by dexamethasone, thus achieving in transfected cells a controlled expression of the E5 mRNA. The construct was transfected into HaCaT cells, and cells containing the construct were selected by growth in hypoxanthine-aminopterin-thymidine medium. As a control the vector alone was transfected. The HaCaT cell line is an immortalized epithelial keratinocyte cell line which differentiates in response to retinoic acid or tetradecanoyl phorbol acetate (1, 3). Cellular clones growing in hypoxanthine-aminopterin-thymidine were pooled and used for all further analyses.

To demonstrate that the selected cells transcribed the E5 ORF correctly, we cultured the hypoxanthine-aminopterin-thymidine-selected E5- and pMSG-transfected HaCaT cells (HaCaT-E5 and HaCaT-pMSG cells, respectively) in the presence of dexamethasone for 48 h. RNA was isolated, and Northern (RNA) blots were hybridized with a DNA fragment encompassing the E5 coding region. As shown in Fig. 1A, a hybridizing band could be observed in dexamethasone-treated

TABLE 1. Dye coupling of E5- and pMSG-transfected HaCaT cells

Cell line	Clone or condition[a]	Dye coupling (no. of fluorescent cells/microinjected cell ± SD)
HaCaT-pMSG	Poly	46 ± 10
HaCaT-E5	Poly	10 ± 7
HaCaT-pMSG	1	30 ± 9
HaCaT-E5	1	7 ± 2
	3	6 ± 2
	5	7 ± 5
HaCaT-E5*	−Dexamethasone	23 ± 5
	+Dexamethasone	1/2

[a] Pooled clones (poly, each containing more than 20 picked single clones) of HaCaT-E5 and HaCaT-pMSG and four independent clones (one pMSG-transfected and three individual E5-transfected clones) were cultured for 48 h in the presence of 1 μM dexamethasone. Individual cells (at least 25) were then microinjected with Lucifer yellow; after 40 min, they were photographed and the number of fluorescent cells was counted. Only strongly fluorescent cells were considered; therefore the coupling number in control cells is a minimal estimation (see text). Microinjections with pooled clones were repeated five times. HaCaT-E5* cells were E5-transfected cells cultured for 72 h in the absence (−) or presence (+) of hormone. Microinjections were repeated three times.

E5-transfected cells but not in untreated or control cells. After long exposure times, however, a faint hybridizing band could also be detected in the RNAs isolated from non-dexamethasone-treated HaCaT-E5 cells, indicating that the mouse mammary tumor virus promoter is not completely shut off in the absence of inducer. The length of the hybridizing RNA is approximately 1.2 kb, which corresponds well to the expected size of the E5-encoding mRNA. These results therefore strongly suggest that the E5 ORF is correctly transcribed in the transfected cells and that this transcription is largely dependent on the addition of dexamethasone to the culture medium.

As mentioned above, a small percentage of the HPV16 E5 proteins localizes to the plasma membrane. Therefore, we analyzed whether gap junctional communication was altered in E5-expressing cells. E5-expressing and control HaCaT cells were injected with Lucifer yellow, and the gap junction-mediated coupling was analyzed microscopically by monitoring diffusion of the fluorescent dye. A strong difference in the dye coupling between E5-expressing and control cells was observed. Whereas in control cells the dye rapidly diffused within 10 min after injection, only a few E5-expressing cells were labelled after this time (Fig. 2c and d). Forty minutes after injection, more than 50 cells were strongly labelled in pMSG-transfected cells but less than 15 were strongly labelled in E5-expressing cells (Fig. 2e and f; see Table 1). In addition, several hundred cells surrounding the strongly fluorescent group showed a slight fluorescence in the control but not in the E5-transfected cells. Table 1 gives a summary of the dye coupling experiments expressed as the number of fluorescent cells counted per microinjected cells. It should be noted that only the strongly fluorescent cells were counted; the results presented here are therefore a minimal estimation of dye transfer in the pMSG-transfected cells. To further substantiate these results, we cultured three E5-transfected clones which have been shown by Southern blot analysis to represent independent clones on the basis of the E5 integration pattern and one pMSG-transfected clone. The cells were treated with dexamethasone and microinjected with Lucifer yellow, and dye transfer was again measured as described before. As shown in Table 1, all three E5-expressing cell lines showed a restricted gap junctional communication in contrast to the pMSG-transfected clone. To show that impairment of dye coupling is dependent on HPV E5 transcription, we measured dye transfer in

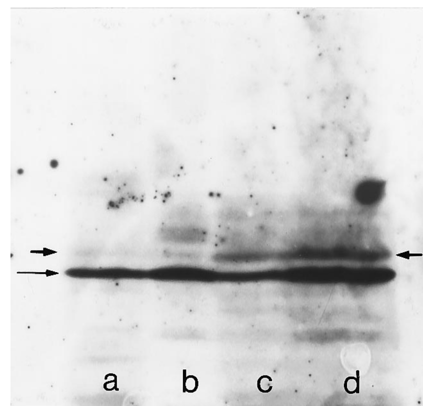

FIG. 3. Western blots of Cx43 in transfected cells. Transfected cells were cultured with dexamethasone (1 μM) and EGF (25 ng/ml) in Dulbecco modified Eagle medium without addition of serum. After 48 h the cells were washed twice with phosphate-buffered saline, and proteins were extracted with 2% SDS–0.5% β-mercaptoethanol–10 mM Tris, pH 7.5, at 100°C. Proteins were separated on a 10% polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Specific antibodies to Cx43 were purchased from Affinity, Nottingham, United Kingdom. Reacting bands were developed with the alkaline phosphatase-luminescence system from Tropix (Serva, Heidelberg, Germany). Lanes: a and b, HaCaT-E5 with dexamethasone and with dexamethasone and EGF, respectively; c and d, HaCaT-pMSG with dexamethasone and with dexamethasone and EGF, respectively. The thin arrow shows the position of the nonphosphorylated Cx43 major band. The thick arrows denote the position of the presumptive P1 phosphorylated Cx43 form.

HaCaT-E5 cells in the presence or absence of dexamethasone. Whereas HaCaT-E5 cells cultured in the presence of dexamethasone showed no or only minimal dye coupling (see HaCaT-E5* cells in Table 1), those cultured in the absence of hormone showed a strong coupling. Thus, these results strongly suggest that impairment of the gap junctional intercellular communication is associated with the expression of the E5 ORF. Interestingly, the degree of coupling of the untreated E5-expressing cells was smaller than that observed in the pMSG-transfected cells. This difference may be due to the low-level transcription of the E5 gene observed in HaCaT-E5 cells in the absence of dexamethasone, which may be sufficient to slightly restrict dye coupling.

Since connexins are known to be the major component of the gap junctions (29), the effect of E5 on connexin transcription was investigated. Cells were treated with dexamethasone, and RNA was extracted after 48 h. Northern hybridization experiments with specific probes for connexin 43 (Cx43), connexin 31.1, and connexin 27 did not reveal any differences in the steady-state levels of all three mRNAs in control versus E5-expressing cells, suggesting that E5 has no major influence on the transcriptional control of connexins (results not shown). Next we analyzed the status of the Cx43 protein, which is the major component of the HaCaT cell gap junctional system (13). Cells were again induced with dexamethasone, and protein extracts were prepared. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blots (immunoblots) were performed using Cx43-specific monoclonal antibodies. As shown in Fig. 3, no major differences in the total amounts of Cx43 in E5-expressing and control cells could be observed. However, a large difference in the phosphorylation degree of Cx43 in both types of cells was evident. Whereas about 30% of Cx43 was in the phosphorylated form in dexamethasone-treated control cells, only 5 to 10% was phosphorylated in E5-expressing cells. In the control cells, most of the phosphorylated Cx43 is found

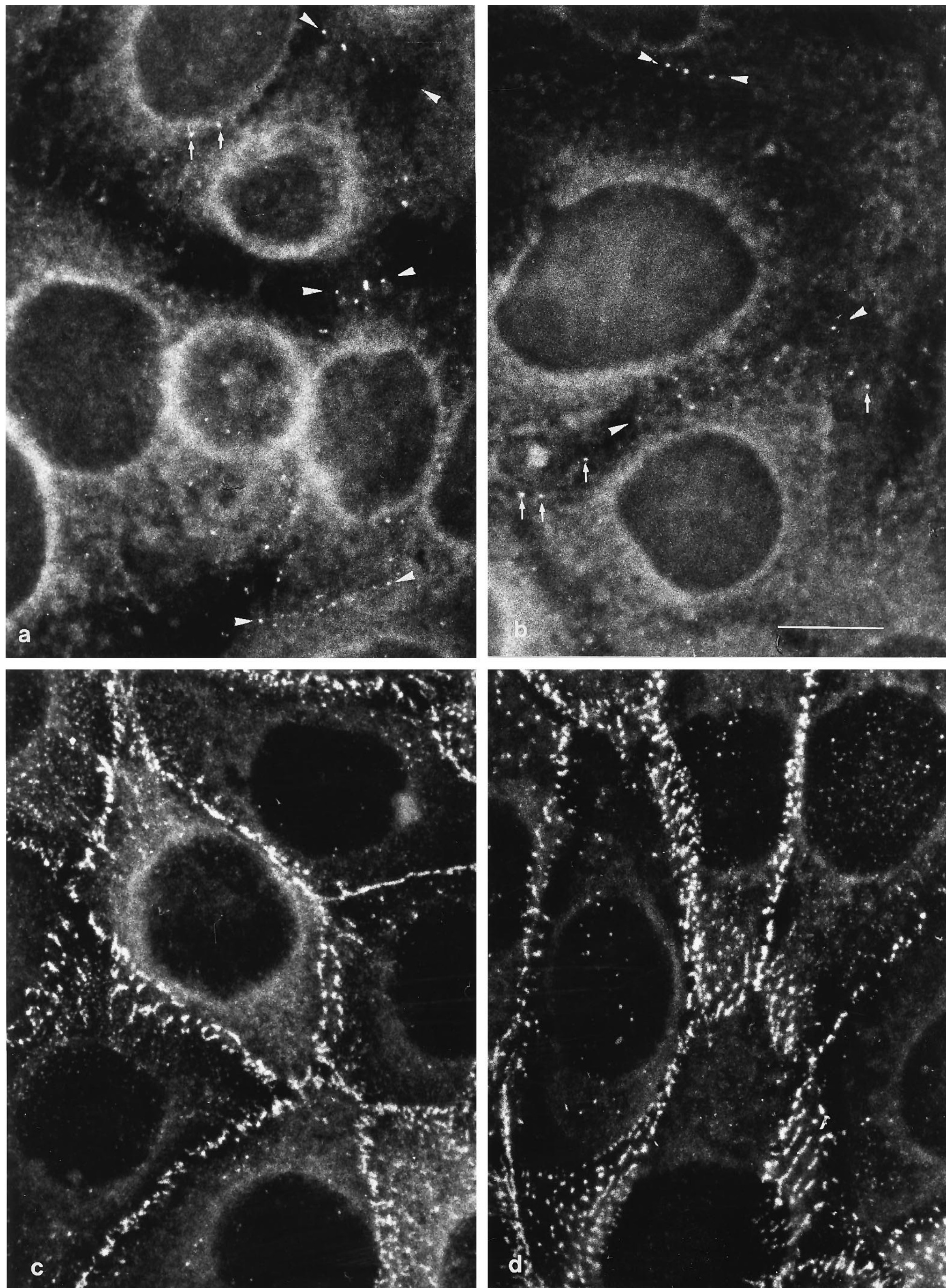

FIG. 4. Arrangement of gap junctions and desmosomes in cultures of HaCaT-pMSG (a and c) and HaCaT-E5 (b and d) cells. Cells were cultured for 48 h in the presence of 1 μM dexamethasone and fixed for 5 min in ice-cold methanol and subsequently for 10 s in ice-cold acetone. The primary and secondary antibodies were both applied for 45 min. Air-dried coverslips were mounted in Moviol and examined with a Zeiss Axiophot microscope. Immunofluorescence microscopy with antibodies against Cx43 (Affinity) (a and b) and desmoplakin (c and d) are shown. Gap junction plaques appear as single fluorescence spots (arrows) or are arranged in the form of continuous lines (arrowheads). Bar represents 10 μm.

in the P1 form (thick arrow in Fig. 3) (19), whereas only a slight P1 band is observed in E5-expressing cells. The phosphorylation degree of the connexins has been associated with dye-coupling activity (19, 21). In liver cells, the tumor promoters dieldrin and heptachlor epoxide, in contrast to tetradecanoyl phorbol acetate, produce a reduction in the P1 form of Cx43 together with a strong inhibition of dye coupling (19). A similar correlation between the phosphorylation status of Cx43 and dye coupling has been found in cultured cardiac myocytes (21). One may therefore speculate that E5 affects the gap junction-mediated coupling as measured by transfer of Lucifer yellow either directly or indirectly by influencing the phosphorylation status of connexins. The mechanism by which E5 affects connexin phosphorylation may be either decreasing the activity of

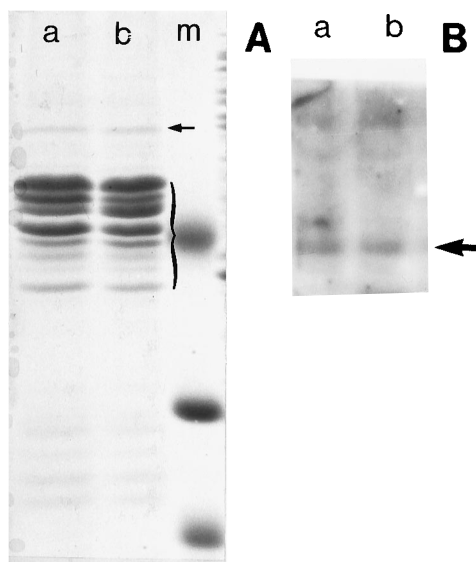

FIG. 5. Differentiation markers in E5- and pMSG-transfected cells. (A) Analysis of the cytokeratins in transfected cells. Cells were treated with dexamethasone for 48 h, and cytokeratins were extracted with 1.5 M KCl–10 mM Tris, pH 7.5. After the sediment was washed several times with phosphate-buffered saline, the proteins were placed in 1% SDS–0.5% β-mercaptoethanol and boiled for 5 min. Aliquots from E5-transfected (lane a) and pMSG-transfected (lane b) cells were separated on a 10% polyacrylamide gel. Proteins were stained with Coomassie blue and photographed. The arrow shows the position of cytokeratin 1; the other epithelial cytokeratins are enclosed in the brace. Note that small quantitative differences can be recognized for two of the cytokeratins between both types of cells. These differences are intrinsic to the HaCaT cells and not associated with differentiation events. Lane m, molecular weight markers. (B) Filaggrin contents of E5- and pMSG-transfected cells. Cells were treated with dexamethasone for 48 h, and a total cellular extract in 1% SDS–5% β-mercaptoethanol–protease inhibitors was prepared. Proteins were separated on a 10% polyacrylamide gel, blotted onto nitrocellulose, and incubated with a specific antibody to filaggrin (Paesel, Frankfurt, Germany). Lane a, pMSG-transfected cells; lane b, E5-transfected cells. The arrow shows the position of the filaggrin band.

certain kinases, increasing the activity of specific phosphatases, or both. In fact, it has been reported that EGF-stimulated phosphorylation of the EGF receptor was enhanced in E5-expressing cells (28). Whether such an enhancement is related to the specific connexin dephosphorylation observed in our experiments is unknown. Since HPV E5 is able to enhance the activity of the EGF receptor (28), we analyzed whether sustained culture in the presence of EGF would modify the phosphorylation status of Cx43 (Fig. 3, lanes b and d). We observed that EGF treatment slightly increased the total amount of Cx43 in both E5- and vector-transfected cells without affecting their relative phosphorylation degree (Fig. 3, compare lanes c and d).

To analyze whether E5-mediated dephosphorylation of Cx43 results in structural changes on the gap junctions, we performed immunofluorescence analysis using monoclonal antibodies to visualize the gap junction structures. As shown in Fig. 4, plaques of connexons are recognized as granulated structures, distributed more or less regularly on the cell membrane. Examination of the immunofluorescence micrographs revealed no differences in the structure and localization of the gap junctions between E5-expressing and control cells. Thus, the reduction in the gap junctional cell communication was not accompanied by a concomitant loss of gap junctional structures containing Cx43. Furthermore, immunofluorescence with desmoplakins I (Fig. 4c and d) and II (not shown) revealed a regular distribution and arrangement of desmosomal structures similar in both types of cells (26).

The dye coupling between keratinocytes has been found to be dependent on the differentiation status of the cell, and it seems well established that keratinocyte differentiation is accompanied by a marked reduction in dye transfer (4, 15). It could be argued that the differences in dye coupling between E5-expressing and -nonexpressing cells are the result of differentiation induced by the E5 protein. Consistently we determined the degree of differentiation of the transfected HaCaT cells by analyzing the cytokeratin expression pattern in the presence of dexamethasone. In HaCaT cells, differentiation is accompanied by a large increase in the amount of cytokeratin 1 together with changes in other markers like involucrin or filaggrin (3). We therefore prepared cytokeratins from E5- and control-transfected cells, separated them by SDS-PAGE, and determined the amount of cytokeratin 1. As shown in Fig. 5A (arrow), no differences in the expression of cytokeratin 1 could be observed between E5- and control-transfected cells, indicating that expression of E5 did not alter the differentiation status of the cells. Since it could be argued that E5 may induce an atypical differentiation program without modification of the cytokeratin expression pattern, we analyzed by Western blotting the amount of filaggrin, known to be another marker of HaCaT cell differentiation (3). As shown in Fig. 5B, no difference in the amount of filaggrin could be observed between E5- and pMSG-transfected cells. Thus, the differences in dye coupling between control and E5-transfected cells cannot be accounted for by differences in the degree of differentiation.

The results presented in this report demonstrate that E5 induces a significant reduction of gap junctional cellular communication in keratinocytes probably mediated by a dephosphorylation of Cx43. These results are in line with those of Faccini et al. (11), who recently reported that BPV4 E8, the protein analogous to HPV16 E5, is able to reduce dye coupling between fibroblasts and bind to the 16,000-Da subunit of the vacuolar proton ATPase, also called ductin and localized in part at the plasma membrane (12). Thus, it seems likely that both the E5 ORF of HPV16 and the corresponding E8 ORF of BPV4 are able to modify gap junction-mediated intercellular communication. Whether this modification is due to a direct or an indirect HPV E5 effect cannot be ascertained at the present. Reduced cell-cell communication has been observed in several tumor types and seems to be a normal finding in transformed cells (reference 20 and references therein). Thus, it seems reasonable to postulate that the effect of HPV16 E5 takes place early after infection and before the beginning of the malignization process. This effect may include the interruption of cell-cell communication, rendering infected cells insensitive to control signals arising from surrounding normal, i.e., non-HPV-infected, cells. Cells destabilized in this manner might be more susceptible to the action of the oncogenic proteins E6/E7 of the virus and to other external factors, which finally would lead to uncontrolled growth.

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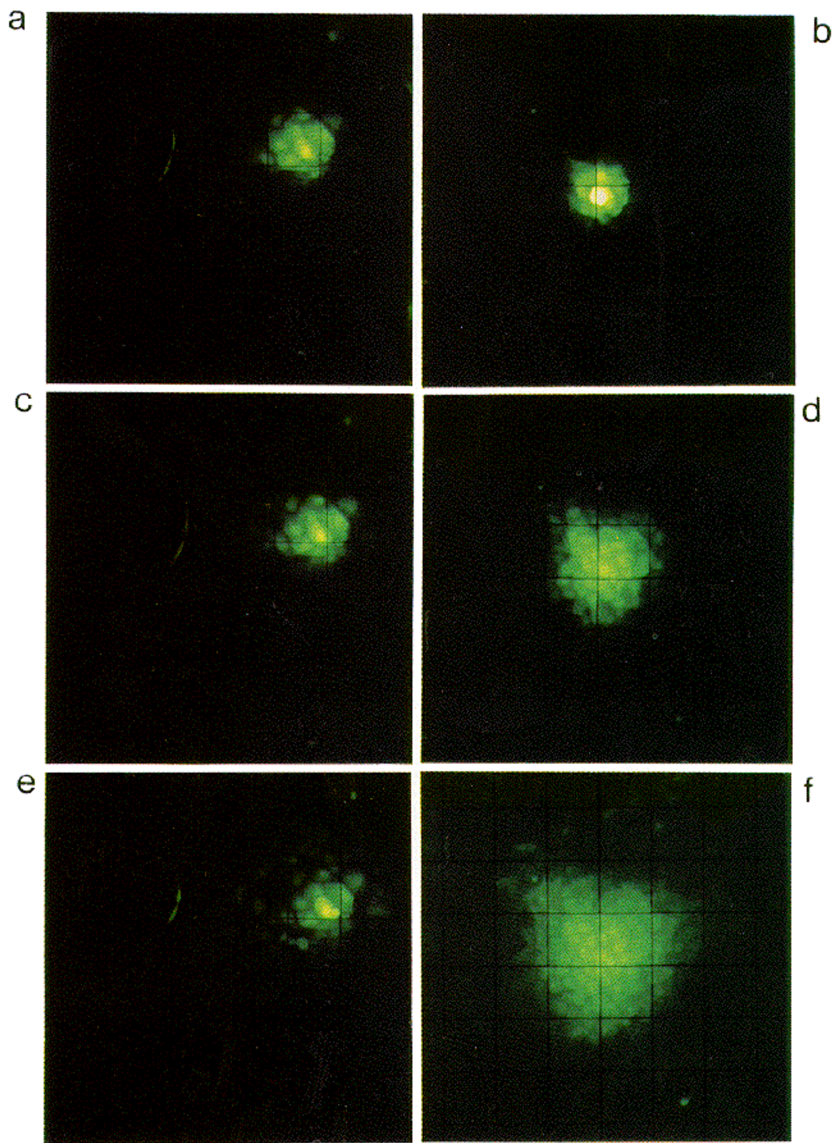
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