JOURNAL OF VIROLOGY, Aug. 1993, p. 4521–4532
0022-538X/93/084521-12$02.00/0

Vol. 67, No. 8

The E5 Oncoprotein of Human Papillomavirus Type 16 Transforms Fibroblasts and Effects the Downregulation of the Epidermal Growth Factor Receptor in Keratinocytes

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Received 11 February 1993/Accepted 21 April 1993

To determine the function of the E5 open reading frame (ORF) of the human papillomaviruses (HPVs), rodent fibroblast cell lines were transfected with the E5 ORF of HPV type 6 (HPV-6) and HPV-16 expressed from an exogenous promoter. Transfected fibroblasts were transformed to colony formation in soft agar, and the transformation frequency was increased by epidermal growth factor (EGF) but not by platelet-derived growth factor. In a transitory assay, the E5 ORFs from both HPV-6 and HPV-16 were mitogenic in primary human foreskin epithelial cells (keratinocytes) and acted synergistically with EGF. Investigation of keratinocytes expressing HPV-16 E5 showed that the number of endogenous EGF receptors (EGFRs) per cell was increased two- to fivefold. Immunofluorescence microscopy of HPV-16 E5-expressing keratinocytes indicated that there was an apparent delay in the internalization and degradation of EGFRs compared with controls. Kinetic studies with [^{125}I]EGF showed that the ligand underwent normal internalization and degradation in both HPV-16 E5-expressing and control keratinocytes, but in E5-expressing cells, a greater number of receptors recycled back to the cell surface within 1 to 6 h of ligand binding. Finally, ligand-stimulated phosphorylation of the EGFR on tyrosine, an indication of receptor kinase activity, was of greater magnitude in the HPV-16 E5-expressing keratinocytes than in control cells, although the basal level of receptor phosphorylation was similar.

Cervical carcinoma is among the most common cancer-related causes of death among women worldwide (35). Infection with human papillomavirus (HPV), a small DNA tumor virus, plays a role in the malignant transformation of cervical epithelium. Of the genital HPVs, HPV type 6 (HPV-6) and HPV-11 are the most common isolates and are associated primarily with benign lesions, such as condyloma acuminata, and a portion of the premalignanant cervical intraepithelial neoplasias (19). HPV-16 and HPV-18 are associated with 60 to 90% of cervical intraepithelial neoplasias and 90% of malignant disease (19), leading to a correlation between infection with these HPV types and progression to invasive cervical cancer.

The papillomaviruses show a strong conservation of genetic organization (9), and the genes of bovine papillomavirus type 1 (BPV-1) have analogous counterparts in HPV-16. However, the activities of these analogous genes are, in some cases, different. For example, the E5 open reading frame (ORF) of BPV-1 is responsible for the major transforming activity of the virus, producing foci in rodent cells, but cooperation with other viral ORFs, especially E6, is necessary for anchorage-independent growth and tumorigenesis (21). The major immortalization and transforming activities of HPV-16 reside in the E6 and E7 ORFs (8, 18), although the E5 ORF may have a complementary role (14, 15, 24). The E5 ORF of BPV-1 encodes a small, hydrophobic peptide (26) present predominantly in the membranous compartments in cells (4) and capable of forming dimers via disulfide bonds (3). HPV-16 E5 is structurally analogous to E5 of BPV-1, but it is nearly twice the size and there is little amino acid homology between them, although there is a high degree of correlation in the hydrophobicity profiles of the two proteins (2). The N-terminal two-thirds of each protein is hydrophobic, and the C-terminal third is hydrophilic, suggesting that E5 is cell membrane associated. HPV-16 E5 also has the potential for dimer formation (2). Therefore, while there are similarities between the E5 proteins of BPV-1 and HPV-16, there is no guarantee that they act via similar pathways.

Recently, BPV-1 E5 was shown to alter the downregulation of the epidermal growth factor receptor (EGFR), or c-*erbB*, and the colony-stimulating factor receptor, or c-*fms*, both receptor protein-tyrosine kinases (17). NIH 3T3 cells transfected with BPV-1 E5 and the human EGFR DNAs display anchorage-independent growth and hyperphosphorylation of the receptor in the absence of the ligand epidermal growth factor (EGF). Cotransfection of E5 and colony-stimulating factor receptor DNA also produced transformed cells in the absence of ligand. Transfection of cells with either receptor gene alone did not result in transformation in the absence of ligand. These studies also demonstrated that the half-life of the active EGFR at the cell surface is prolonged in the presence of ligand in the E5-cotransfected cells, indicating that the downregulation of the EGFR is inhibited. However, subsequent studies by another group (31) with similarly transfected fibroblasts demonstrated that the internalization of EGFR was normal in BPV-1 E5-expressing cells, although the interaction between ligand and receptor was stabilized. In either case, the persistence of

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active EGFR may be responsible for the increase in proliferation of these cells, since prolonged EGFR activation has a mitogenic effect on cells (5). This property is consistent with the hyperproliferative lesions produced in vivo by papillomaviruses. BPV-1 E5 has also been shown to interact with a complex containing the platelet-derived growth factor receptor (PDGFR) and cause hyperphosphorylation of the receptor in the absence of the ligand (23).

Having established the ability of the E5 ORF of BPV-1 to activate growth factor receptors, it is important to ask whether HPV-16 E5 may induce cell proliferation via the activation of growth factor receptors. Two recent studies (14, 24) demonstrated the ability of HPV-16 E5 to transform mouse fibroblasts to anchorage-independent growth but differed on the importance of growth factors in E5-mediated transformation. NIH 3T3 cells transfected with E5 required EGF for efficient anchorage-independent growth (24). 3T3-A31 cells expressing HPV-16 E5 grew in soft agar independently of growth factors, although EGF but not platelet-derived growth factor (PDGF) dramatically increased colony size (14). These cells also had a variable ability to form tumors in nude mice, possibly linked to the level of E5 expression (14). Another study (15) found that, unlike BPV-1 E5, HPV-16 E5 did not have focus-forming ability in mouse fibroblasts, nor did these cells form tumors in nude mice. However, a murine keratinocyte cell line expressing HPV-16 E5 was able to form tumors in the same assay (15), indicating a possible tissue tropism for tumorigenic activity. These diverse results clearly indicate the need for further study of the activity of HPV-16 E5.

The EGFR is present on all epithelial cells, including cervical mucosal cells (6), and is a transmembrane receptor protein with a ligand-activated tyrosine kinase activity (5, 27). The ligand EGF is a mitogen for keratinocytes and is essential for the growth of primary keratinocytes in vitro (25). Ligand binding to the extracellular domain of the receptor initiates receptor dimerization and stimulates the kinase activity of the intracellular domain of the receptor (5, 27), resulting in autophosphorylation of the receptor, which in turn initiates a cascade of events leading to the induction of cellular genes, such as c-*fos*, and the stimulation of DNA synthesis (7, 20). The downregulation of the active receptor begins with the rapid endocytosis of the ligand-receptor complex in clathrin-coated vesicles (5, 27). The endosomal compartments are acidified and subsequently fuse with lysosomes, resulting in the dissociation and degradation of the complex. Although some recycling of the EGFR to the cell surface has been observed (up to 10% in cultured epithelial cells [5]), reconstitution of EGFR on the surface of cells following downregulation primarily involves de novo synthesis (an 8-h process). This is in contrast, for example, to the receptors for transferrin and low-density lipoprotein, which lack an intrinsic tyrosine kinase activity and primarily recycle to the cell surface for reutilization (5, 27).

Members of the EGFR family are frequently involved in human cancer (1, 6), usually when there are perturbations in the control of receptor activation or down-regulation. For example, the EGFR gene is often amplified or overexpressed, or both, in squamous cell carcinomas (11, 34), and it has been demonstrated in vitro that internalization-defective mutant receptors that retain kinase activity (32) and exogenous overexpression of the receptor (28, 29) both result in ligand-dependent transformation. In addition, constitutive activation of the kinase domain, as in the truncated receptor oncogene v-*erbB*, can also lead to a transformed phenotype (5). Therefore, the inappropriate activation of the EGFR or the inhibition of downregulation of kinase activity can lead to transformation.

Here we examine the ability of the E5 ORF of the HPVs to induce mitogenesis and transform cells. We also determine the influence of growth factors and their receptors on this process, particularly in human foreskin keratinocytes, which are used as a model for the genital epithelium normally infected by HPV-16. Our results demonstrate that the HPV-16 E5 oncoprotein transforms rodent fibroblasts and stimulates mitogenesis in human epithelial cells and that both activities are enhanced by EGF stimulation. Furthermore, these activities may be attributed to the ability of HPV-16 E5 in human keratinocytes to increase the number of EGFRs at the cell surface, inhibit degradation of the receptor in endosomal vesicles, increase receptor kinase activity, and cause recycling of the receptor to the cell surface for restimulation by ligand.

MATERIALS AND METHODS

Plasmid construction. The entire HPV-16 genome (previously cloned in the laboratory [8a]) was cloned into plasmid pAT153 at the *Bam*HI site to create the vector pAT16. HPV early genes are expressed from the endogenous promoter (P97) in this construct. The E5 ORF was amplified from the HPV-16 genome by using primers adjacent to bp 3837 to 4110 in a polymerase chain reaction. The E5a ORF was amplified from the HPV-6b genome (a gift from H. zur Hausen) by using primers flanking bp 3875 to 4183. Each set of primers carried suitable restriction endonuclease sites to enable cloning into the expression vector pKV461 (a gift from S. Kingsman) downstream of the human cytomegalovirus (hCMV) immediate-early gene promoter and upstream of the simian virus 40 polyadenylation signal. The vectors are designated pKV16E5 and pKV6E5a, respectively.

Cell culture. J23T3 and F3T3 fibroblast cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum, and the C127 cell line was cultured in the presence of 10% fetal calf serum. SCC-13 cells, an immortalized human epithelial cell line (a gift from J. W. Rheinwald), were grown in E medium (33) plus 10% fetal calf serum. Primary human foreskin keratinocytes, including those transfected with various vectors, were cultured in either KGM (Clonetics Corp., San Diego, Calif.) or E medium supplemented with 10% fetal calf serum and 5 ng of human recombinant EGF (hrEGF; Austral Biologicals, San Ramon, Calif.) per ml in the presence of a mitomycin C-treated J23T3 feeder layer (10^6 cells per 90-mm dish). All sera tested negative for EGF-specific effects.

Transfection and selection. Fibroblast cell lines were transfected via lipofection. Briefly, a mixture of 1 μg of the selectable marker pSV2neo and 5 μg of either pKV461, pKV16E5, or pKV6E5a together with 30 μg of lipofectin reagent (Life Technologies, Gaithersburg, Md.) in a total volume of 100 μl was added dropwise to cells grown to 60 to 80% confluence in 60-mm dishes. After incubation overnight at 37°C, the medium was changed, and 48 h later, the cells were selected with 0.3 mg of G418 (Life Technologies, Gaithersburg, Md.) per ml for 14 days.

Primary human foreskin keratinocytes were electroporated with 10 μg of either pKV16E5 or pAT16 plus 1 μg of pSV2neo at 1,000 V and 25 μF. After 48 h, the cells were selected with 30 μg of G418 per ml for 14 days, and resistant colonies were cloned.

Soft-agar transformation. Cells were seeded at 8×10^3 to 1×10^4 per 60-mm dish in 3 ml of 0.35% agarose (Sigma

Chemical Company, St. Louis, Mo.), supported by a 5-ml layer of 0.5% ultrapure agarose (Bio-Rad, Richmond, Calif.). Growth medium (3 ml) with or without hrEGF (10 ng/ml; Austral Biologicals) or human recombinant PDGF AB (10 ng/ml; Austral Biologicals) was layered atop the agarose, and the medium was changed twice weekly. After 21 days, cells were stained overnight with *p*-iodonitrotetrazolium violet (1 g/liter) in phosphate-buffered saline (PBS) and photographed, and the agarose was dried on Whatman chromatography paper to score colonies. The number of colonies reported is the average from three experiments.

Mitogenesis assay. Cells were plated at 5×10^4 per 30-mm dish, serum starved for 48 h, and either stimulated with human EGF (10 ng/ml) or transfected by lipofection with a control plasmid or one of the E5 constructs described above, or both. After 24 h of stimulation, DNA synthesis was determined by measuring [^{3}H]thymidine uptake after a pulse of 2 h. The results reported are the averages of five experiments.

Immunofluorescence. The immunofluorescence experiments described in this article were carried out with concentrations of EGF (100 ng/ml, or 16.5 nM) necessary to give 99% saturation of the receptors during the 1-h incubation at 0°C, synchronizing receptor-ligand complex formation, internalization, and signal transduction.

Cells for the immunofluorescence experiments were passaged onto sterile 18-mm glass coverslips in six-well plates. When cells were 50 to 80% confluent, they were washed with PBS, and growth medium without EGF was added for an overnight incubation at 37°C to promote maximal surface expression of EGFRs. To synchronously saturate the EGFRs with EGF, the cells were chilled and incubated on ice with 1 ml of cold (0°C) growth medium plus hrEGF (100 ng/ml) for 1 h. After thorough washing with PBS to remove unbound EGF, 1 ml of warm (37°C) growth medium was added, and the cells were incubated at 37°C for up to 6 h. For surface immunostaining, the cells were fixed for 5 min with 3.7% formaldehyde in PBS and washed with PBS. For immunostaining of intracellular compartments, the cells were fixed and then permeabilized with 0.1% Triton X-100 in PBS for 5 min and then washed with PBS.

Nonspecific binding of antibodies to the fixed cells was prevented by incubation of the coverslips overnight at 4°C under bovine serum albumin (BSA) solution (2% BSA in PBS with 0.01% sodium azide as a preservative). Anti-EGFR monoclonal antibody R1 (a gift from M. Waterfield [30]), which recognizes an epitope on the extracellular domain of the receptor, was used at a concentration of 2 μg/ml in BSA solution, and 50 μl was added to each coverslip for 1 h at 37°C. Coverslips were rinsed and then washed twice with BSA solution to remove unbound primary antibody. Coverslips were incubated at 37°C for 30 min with 50 μl of goat anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) conjugate (Organon-Teknika Corporation, Durham, N.C.) at a concentration of 2 μg/ml. The secondary antibody was washed as above, and the coverslips were mounted on glass slides with 90% glycerol in PBS with 1 g of *p*-phenylenediamine per liter as an antiquenching agent. Control experiments included the binding of the anti-mouse IgG secondary antibody alone or a rabbit antiserum against the L1 capsid protein of HPV-16 diluted 1:100 and a goat anti-rabbit IgG-FITC conjugate (Organon-Teknika Corporation) as a secondary antibody. Both controls gave comparable staining.

Double labeling of cells was done by the protocol detailed above but with murine EGF (100 ng/ml) conjugated to FITC (EGF-FITC; Molecular Probes, Eugene, Ore.) instead of hrEGF during the 1-h incubation on ice. After incubation at 37°C, cells were fixed for surface or intracellular staining with R1 as above, but then the cells were incubated at 37°C for 30 min with 50 μl of goat anti-mouse IgG-tetramethylrhodamine isothiocyanate (TRITC) conjugate (Organon-Teknika Corporation) at a concentration of 2 μg/ml. These cells were examined on a Nikon fluorescence microscope with a video camera and image intensifier and analyzed with Image I software. Photographs were taken from the monitor image. The binding of EGF-FITC was demonstrated to be specific by competition with 100 ng of unlabeled human EGF per ml during the 1-h incubation on ice.

Receptor phosphorylation and antiphosphotyrosine immunoprecipitation. To determine the phosphorylation state of the EGFR, equal numbers of cells were seeded into five 60-mm dishes per cell type. When the cells reached 80 to 90% confluence, they were washed with PBS and incubated at 37°C overnight in medium not supplemented with EGF. One dish of cells of each type was washed and trypsinized, and the cells were counted on a hemacytometer. The other dishes of cells were washed with Tris-buffered saline (TBS; 137 mM NaCl, 20 mM Tris [pH 7.5]) and incubated for 2 h at 37°C in 1 ml of phosphate-free DMEM–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–1% BSA–0.5 mCi of $^{32}P_i$ (carrier-free, aqueous; Amersham, Arlington Heights, Ill.). The cells were chilled on ice, 100 ng of hrEGF was added to three of the four remaining dishes, and all dishes were incubated on ice for 1 h. The dishes were warmed to 37°C for 5, 60, or 180 min (the dish not treated with EGF was incubated for 180 min) and then rinsed twice with cold TBS. Cells were solubilized in 1 ml of solubilization buffer (50 mM Tris [pH 7.4], 1% Triton X-100, 1 mg of bacitracin per ml, 25 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride [a protease inhibitor], 50 mM NaF, 100 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium PP_i) and scraped into a 1.5-ml Eppendorf tube. The supernatant was cleared by centrifugation for 5 min in a microcentrifuge and transferred to a fresh tube. The anti-phosphotyrosine monoclonal antibody 4G10 (6.8 mg/ml; a gift from T. Roberts) was added (0.5 μl) to each tube, and the tubes were rotated overnight at 4°C. Phosphotyrosine-containing proteins were immunoprecipitated with 30 μl of a 50% solution of protein A-Sepharose CL4B (Sigma Chemical Company) and 2 h of rotation at 4°C. The protein A beads were washed sequentially with 1 ml of solubilization buffer, 1 ml of LiCl buffer (0.5 M LiCl, 50 mM Tris [pH 7.4], 0.1% Triton X-100), 1 ml of NaCl buffer (0.1 M NaCl, 50 mM Tris [pH 7.4], 0.1% Triton X-100), and 1 ml of Tris buffer (50 mM Tris [pH 7.4], 0.1% Triton X-100). Phosphoproteins were eluted from the beads with 100 μl of 50 mM Tris (pH 7.4)–0.1% Triton X-100–20 mM *p*-nitrophenylphosphate (Sigma Chemical Company)–0.1 mM sodium orthovanadate for 2 to 3 h on ice. The eluate was collected and stored at −70°C.

The phosphoproteins were electrophoresed on an 8% polyacrylamide–sodium dodecyl sulfate (SDS) gel. Each sample of phosphoprotein loaded was the fraction of the eluate equivalent to 10^6 cells (determined from the equivalent trypsinized plate) plus loading buffer (containing β-mercaptoethanol) and was boiled for 5 min. After electrophoresis the gel was fixed, soaked in 1 M sodium salicylate for 30 min, dried, and autoradiographed with Kodak X-AR film. Densitometry was performed with a Molecular Dynamics laser scanner and PDI Quantity One software. Careful control of sample loading and length of autoradiography ensured linearity of the densitometric readings.

TABLE 1. Number of colonies produced, as measured by anchorage-independent growth

Cells	DNA transfected[a]	No. of colonies/dish											
		No ligand				EGF (10 ng/ml)				PDGF (10 ng/ml)			
		Expt 1	Expt 2	Expt 3	Avg ± SD	Expt 1	Expt 2	Expt 3	Avg ± SD	Expt 1	Expt 2	Expt 3	Avg ± SD
J23T3	pKV461	1	0	0	0 ± 1	0	0	0	0 ± 0	0	0	0	0 ± 0
	pKV6E5a	16	22	23	20 ± 4	52	53	78	61 ± 15	26	41	24	30 ± 9
	pKV16E5	21	30	46	32 ± 13	83	97	123	104 ± 25	19	24	35	26 ± 8
F3T3	pKV461	0	0	0	0 ± 0	0	0	0	0 ± 0	0	0	0	0 ± 0
	pKV6E5a	30	22	22	25 ± 5	88	57	40	62 ± 24	44	28	22	31 ± 11
	pKV16E5	43	65	30	45 ± 16	113	153	100	122 ± 28	24	57	39	40 ± 17
C127	pKV461	0	0	0	0 ± 0	0	0	0	0 ± 0	0	0	0	0 ± 0
	pKV6E5a	37	26	10	24 ± 14	90	98	60	83 ± 20	10	28	15	18 ± 9
	pKV16E5	42	27	42	37 ± 9	68	122	105	98 ± 28	30	20	69	40 ± 26

[a] All transfected DNA included 1 μg of pSV2neo.

EGF-stimulated keratinocyte lysates for immunoblot analysis were prepared as described above, although without the P_i labeling. The cleared cell lysate was collected, and aliquots equivalent to 10^6 cells were electrophoresed on 8% polyacrylamide–SDS gels. After electrophoretic transfer of seperated proteins to nitrocellulose membranes, nonspecific binding sites were blocked by incubation in 5% nonfat dry milk in TTBS (TBS with 0.1% Tween-20). The position of the EGFR was determined by incubation with anti-EGFR monoclonal antibody R1 (2 μg/ml) in TTBS and goat anti-mouse IgG-alkaline phosphatase conjugate (1:3,000 in TTBS; Sigma Chemical Co.). A seperate blot of aliquots from the same lysates was incubated with anti-phosphotyrosine monoclonal antibody 4G10 (1.38 μg/ml in TTBS) and goat anti-mouse IgG-alkaline phosphatase conjugate. The blots were developed with alkaline phosphatase color reagents and buffer as per the manufacturer's (Bio-Rad) direction.

Kinetic studies with [^{125}I]EGF. The binding affinity of [^{125}I]EGF and the number of receptors per cell were measured as follows. Cells on 35-mm dishes were incubated in medium lacking EGF overnight and then incubated with serum-free culture medium supplemented with 0.25% BSA and human [^{125}I]EGF (1,350 Ci/mmol; Biomedical Technologies, Stoughton, Mass.) at the concentrations (0.05 to 0.25 nM) and temperatures (0 or 37°C) noted in the tables and figure legends. Parallel dishes received [^{125}I]EGF and a 100-fold excess of nonradioactive hrEGF to serve as nonspecific binding controls. Nonspecific binding, typically <5%, was subtracted from the total to give specific binding. After incubation at 37°C for the times noted, dishes were rinsed three times with 2 ml of 0.15 M NaCl, cells were scraped into 1.5 ml of water with a rubber policeman, and radioactivity was counted in a Beckman gamma counter.

Internalization was monitored by an acid-salt wash procedure as previously described (12). Following [^{125}I]EGF binding, the cells were washed three times with 0.15 M NaCl, and then 1 ml of ice-cold 0.2 N acetic acid–0.5 M NaCl, pH 2.5, was added for 15 s. The acid-salt wash was removed, the radioactivity in it was counted; the cells were scraped into 0.1 N NaOH, and the radioactivity in them was counted. [^{125}I]EGF removed by the acid-salt rinse was considered to be present on the cell surface, and [^{125}I]EGF resistant to the acid-salt wash was considered to be internalized.

In order to measure [^{125}I]EGF degradation, the total ^{125}I in a fraction was determined, 25 μg of BSA was added as a carrier, and trichloracetic acid (TCA) was then added to give a final concentration of 10%. Fractions were incubated in the cold for 30 min or more and centrifuged, and TCA-precipitable counts in the pellet were determined. Proteins were determined by the method of Lowry et al. (16) with BSA as a standard.

RESULTS

Transformation of rodent fibroblasts. To examine the possibility that transforming activity is encoded by the E5 ORFs of HPV-6 and HPV-16, these genes were cloned downstream of the immediate-early gene promoter of human cytomegalovirus (hCMV; pKV6E5a and pKV16E5) and stably introduced into mouse fibroblast cell lines. Several fibroblast cell lines were used so that a more comprehensive analysis of the growth properties, focus formation, and anchorage-independent growth of E5-expressing cells was performed. All cells transfected with E5 constructs were found to express E5 mRNA by Northern (RNA blot) hybridization (data not shown).

In tissue culture, E5-transfected cells grew at the same rate as parental cells and vector control cells but to a greater density, and the cells did not form foci in confluent monolayers (data not shown). Furthermore, neither EGF nor PDGF added to the culture medium was able to increase the growth rate of transfected cells.

E5-transfected cells demonstrated anchorage-independent growth in soft agar (Table 1). Cells transfected with either pKV6E5a or pKV16E5 grew in soft agar into large colonies for the three fibroblast lines tested. No significant colony formation was exhibited by vector-containing fibroblasts (pKV461) grown in medium or in medium supplemented with growth factors. Addition of EGF to the soft-agar medium increased the number of transformed colonies up to threefold in both HPV-6 E5a-expressing and HPV-16 E5-expressing fibroblasts, but PDGF had no significant effect. In addition, colonies grown in the presence of EGF tended to be larger than those grown in its absence.

Therefore, E5 from both a virus associated with benign lesions (HPV-6) and one associated with malignant disease (HPV-16) transformed fibroblast cell lines. Furthermore, this

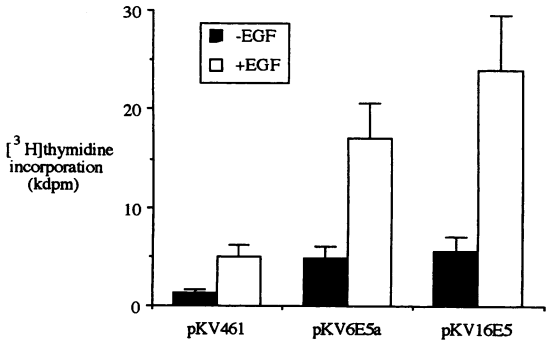

FIG. 1. Mitogenic effect of E5 and EGF on keratinocytes is synergistic. Human foreskin keratinocytes were serum-starved for 48 h and then either treated with 10 ng of EGF per ml, transfected with a control or an E5 expression vector, or both. After 24 h, a 2-h pulse of [^{3}H]thymidine was administered to measure DNA synthesis. The results are averages of five experiments.

activity was enhanced by the addition of EGF but not by PDGF.

Studies on epithelial cells. As the natural host of HPVs is the epithelial cell, further experiments involved the use of normal human keratinocytes. In a transitory-mitogenesis assay, the HPV-6 E5a (pKV6E5a) and the HPV-16 E5 (pKV16E5) constructs induced DNA synthesis, measured by [^{3}H]thymidine uptake in serum-starved primary keratinocytes (Fig. 1). EGF induced a similar mitogenic response in control cells. When EGF was added to the E5-transfected cells, the subsequent mitogenic response appeared to be the result of a synergistic interaction between EGF stimulation and the E5 ORF.

Further experiments also involved the use of normal human keratinocytes and keratinocytes transfected with HPV-16 E5 alone, expressed downstream of the immediate-early gene promoter of hCMV (pKV16E5), or keratinocytes transfected with the HPV-16 genome (pAT16). The keratinocyte transfectants used in the following experiments were used at less than the 23rd passage, as the lifespan of normal keratinocytes falls within this range. In this way, the behavior of normal keratinocytes could be compared with that of keratinocytes expressing E5 either from a heterologous promoter or in the context of the natural promoter in the HPV genome. In either case, the transfected cells expressed E5-specific RNA, as determined by Northern blot hybridization (data not shown), and maintained their dependence on EGF for growth.

Immunofluorescence studies. To investigate the effect of the HPV-16 E5 ORF on growth factor receptor activity, we chose first to examine the downregulation of the EGFR. Immunofluorescence with the anti-EGFR monoclonal antibody R1 allowed us to monitor the receptor from the cell surface through the endocytic pathway in cells stimulated by EGF. Figure 2 shows representative results of an immunofluorescence assay performed with normal keratinocytes and a keratinocyte line (pKV16E5.5) stably transfected with the pKV16E5 construct. Negative control staining with an unrelated primary antibody and appropriate secondary antibody gave no specific signal (Fig. 2A and B), nor did the secondary antibody alone (data not shown). Normal and E5-expressing keratinocytes exhibited abundant EGFRs at the cell surface after EGF starvation overnight (Fig. 2C and D). A similar surface staining pattern was observed with unpermeabilized cells (data not shown). After the addition of EGF for 1 h on ice and subsequent warming to 37°C, the normal keratinocytes followed the normal pathway of internalization and localization to perinuclear lysosomes within 60 min (Fig. 2E) and degradation within 3 h (Fig. 2G). Keratinocytes expressing HPV-16 E5 appeared to display two differences in downregulation of the EGFR. First, internalization of the EGFR at the plasma membrane after ligand binding appeared to be inhibited, since EGFR could be detected at the surface of the E5-expressing cells (Fig. 2J) long after it was internalized and degraded in normal epithelial cells (Fig. 2I). However, examination of these cells 10 min after EGF addition demonstrated that the majority of the EGFRs had been internalized soon after binding EGF (immunofluorescence data not shown; see next section), but the receptors apparently returned rapidly to the cell surface. Second, degradation of the receptor was delayed in E5-expressing cells, and receptors persisted in intracellular compartments for at least 3 h after the addition of EGF (compare Fig. 2G and H). In several experiments, the presence of EGFR in intracellular compartments was seen up to 6 h after the addition of EGF. The addition of 10 μM cycloheximide to inhibit protein synthesis from the time that EGF was added had no effect on the results described here (data not shown).

Table 2 shows a summary of the results from this assay with a variety of cell types. SCC-13 cells, an immortalized, EGF-dependent epithelial cell line not containing any HPV sequences, were included as a control for the effects of immortalization on this assay. Although immortal, SCC-13 cells exhibited the normal timing of EGFR downregulation seen with normal keratinocytes. Early-passage (<23rd passage) pAT16.2 and immortalized (>70th passage) K1/16 cell lines, both transfected with full-length HPV-16 DNA, had the same pattern of EGFR persistence, both at the cell surface and in intracellular compartments, as the cells expressing E5 alone. This assay demonstrates that in cells expressing HPV-16 E5, either from the homologous promoter in the viral genome or from a heterologous promoter, ligand-stimulated EGFRs persist at the cell surface and the degradation of internalized receptors is inhibited. Furthermore, as the SCC-13 cells demonstrate, these activities are the result not of immortalization but of E5 expression.

Receptor number, ligand-binding affinity, and kinetics of internalization in E5-expressing cells. To study the effect of the HPV-16 E5 ORF on EGFR kinetics, we performed several experiments with [^{125}I]EGF. The affinity of receptors for EGF was measured at both 0 and 37°C for normal keratinocytes and for keratinocytes expressing E5, either from the natural promoter in the HPV genome (pAT16.2) or from an exogenous promoter (pKV16E5.5). [^{125}I]EGF (0.05 nM) was displaced by identical concentrations of nonradioactive human EGF for all cells, yielding an apparent K_d of 0.7 nM. At equilibrium, nearly the same amount of [^{125}I]EGF was bound at 0°C, at which only cell surface receptors are occupied, and at 37°C, at which receptor internalization can take place, suggesting that the majority of EGFRs are on the cell surface in both normal and E5-expressing keratinocytes (Fig. 3).

Consistent differences in the total number of receptors were observed for normal keratinocytes and cells expressing E5 (Table 3). Cells expressing either the E5 gene (pKV16E5.5) or the entire HPV-16 genome (pAT16.2) bound from 2.5 to 4.3 times as much [^{125}I]EGF as normal keratinocytes.

It has been proposed that bovine E5 delays internalization of the ligand-occupied EGFR, thereby extending its activity

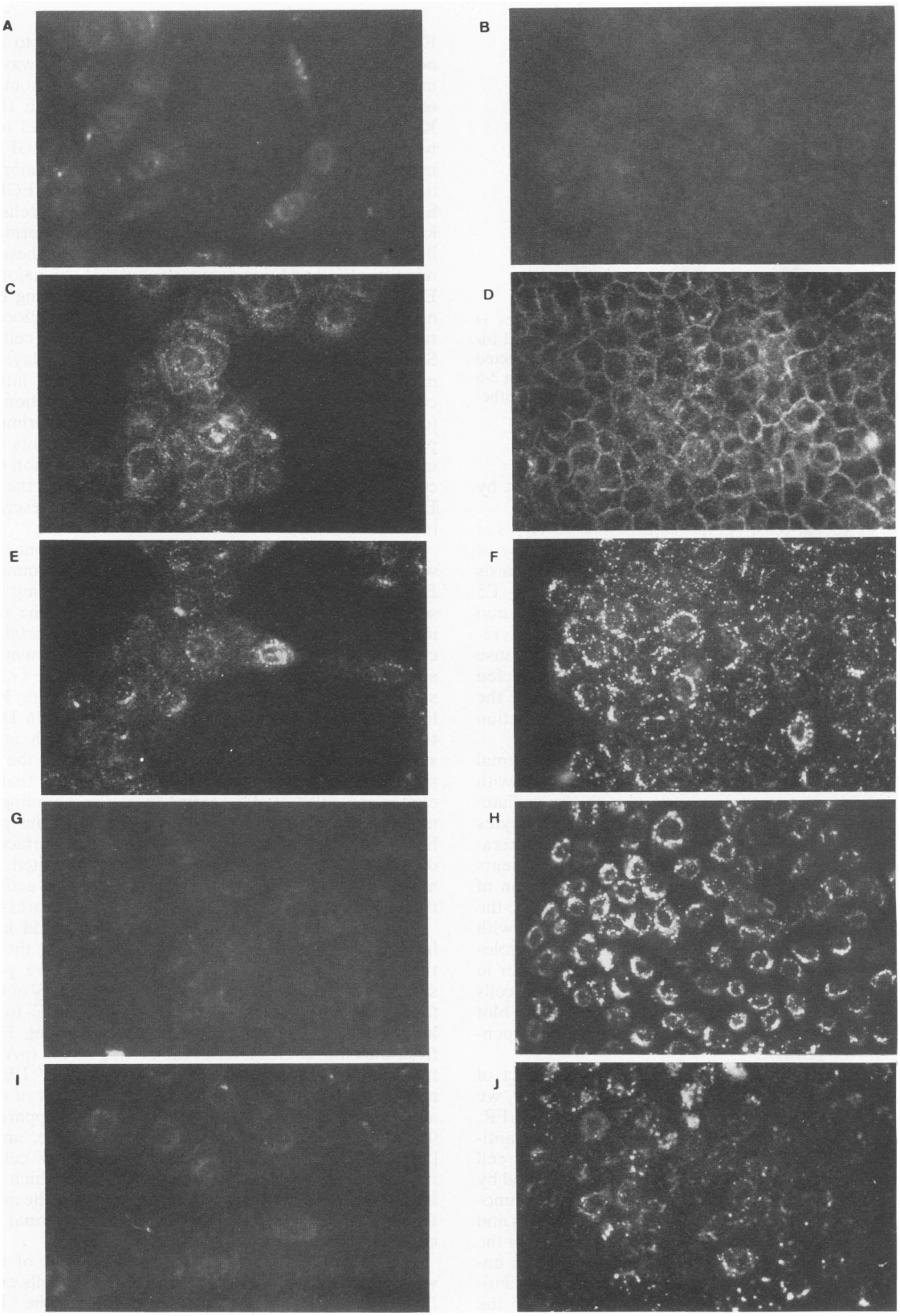

FIG. 2. EGFR immunofluorescence assay. Cells were saturated with EGF on ice, incubated at 37°C for various times, fixed, permeabilized, stained with anti-EGFR monoclonal antibody R1 and a goat anti-mouse IgG-FITC conjugate, and visualized by fluorescence microscopy. (A, C, E, G, and I) Normal keratinocytes; (B, D, F, H, and J) pKV16E5.5, a clonal isolate of keratinocytes stably transfected with pKV16E5. (A and B) Cells stained with a negative-control primary antibody and an appropriate secondary antibody (see Materials and Methods). Cells in all other panels were stained with R1 antibodies for (C and D) 0 min, (E and F) 60 min, or (G and H) 180 min; (I and J) surface staining of EGFR, represented by fixed but unpermeabilized cells, after 180 min.

TABLE 2. EGFR immunofluorescence

Cells[a]	EGFR detected[b] at time post-EGF stimulation:					
	0 min		60 min		180 min	
	Surface	Intracellular	Surface	Intracellular	Surface	Intracellular
Keratinocytes	+	−	−	+	−	−
SCC-13	+	−	−	+	−	−
K1/16	+	−	+	+	+	+
pAT16.2	+	−	+	+	+	+
pKV16E5.5	+	−	+	+	+	+

[a] Cell types: keratinocytes, human foreskin keratinocytes; SCC-13, squamous cell carcinoma cell line (no HPV DNA); K1/16 and pAT16.2, two clonal lines of keratinocytes immortalized by HPV-16 DNA; pKV16E5.5, clonal line of keratinocytes transfected with pKV16E5.

[b] +, detection of EGFR with monoclonal antibody R1 at different times after ligand stimulation; −, no detectable immunofluorescence.

TABLE 3. Effect of HPV-16 E5 on EGFR density

[^{125}I]EGF treatment			[^{125}I]EGF bound[a] (fmol/mg of cell protein)		
Concn (nM)	Time (h)	Temp (°C)	Keratinocytes	pKV16E5.5	pAT16.2
0.05	1	37	9.22 ± 0.65	28.10 ± 0.43	25.51 ± 0.43
0.05	1.5	37	14.60 ± 0.30	ND[b]	37.00 ± 2.40
0.10	2	0	19.92 ± 0.57	42.50 ± 1.19	50.70 ± 0.33
0.25	0.5	0	7.32 ± 0.26	31.54 ± 2.98	ND

[a] Binding of [^{125}I]EGF was measured in separate experiments with different preparations of normal keratinocytes, pKV16E5.5 (a clonal isolate expressing HPV-16 E5), or pAT16.2 (a clone of cells transfected with the HPV-16 genome). Values are the mean and range or standard error for two to three dishes.

[b] ND, not determined.

(17). To determine the effect of HPV-16 E5 on the rate and extent of internalization of the EGFRs, pKV16E5- and pAT16-containing cells and normal keratinocytes were incubated with [^{125}I]EGF at 4°C to occupy surface receptors with a near-saturating amount of radioligand. The cultures were then washed to remove unbound [^{125}I]EGF and incubated at 37°C, and intracellular and surface-bound [^{125}I]EGF levels were monitored over time. The rate of internalization was the same for normal keratinocytes and cells expressing E5 or the entire genome, with 90% of the radioligand internalized at 20 min (Fig. 4). To monitor the rate of [^{125}I]EGF degradation, cultures were incubated with [^{125}I]EGF at 37°C to allow binding and internalization and then washed, and the fraction of radioactivity that remained TCA precipitable was measured at intervals. The overall rate of degradation of receptor-bound [^{125}I]EGF to TCA-soluble fragments was the same with and without E5 (Fig. 5).

Recycling of EGFR in the presence of E5. To compare the behavior of receptor-bound ligand with that of the EGFR, we followed a protocol similar to the one used in the immunofluorescence studies. Cells were incubated at 0°C with [^{125}I]EGF (enough to saturate ~80% of receptors), washed, and then warmed to 37°C; surface and internalized [^{125}I]EGF were quantified at intervals. Since degraded [^{125}I]EGF rapidly leaves the cell, this procedure measures mostly intact peptide. As shown in Fig. 6, one difference between keratinocytes and pKV16E5 cells was that cells expressing E5 had more cell-associated [^{125}I]EGF at all times. In both the normal and E5-expressing cells, [^{125}I]EGF was present on the surface at time zero and internalized after 1 h. However, we observed very little [^{125}I]EGF associated with surface receptor in the E5-transfected cells at 3 h, when there was clear evidence of surface receptors in the microscopic studies.

This result suggested a dissociation between the receptor and the ligand in cells expressing E5. To pursue this, we carried out the following experiment to study the localization of receptor after EGF binding. We incubated cells with a saturating concentration of unlabeled EGF in the cold, washed them, and then incubated the cells at 37°C for various times before measuring the amount of [^{125}I]EGF able to bind in a 30-min binding reaction. This protocol was designed to determine how many receptors are cycling to the surface, are functional, and are free to bind [^{125}I]EGF. The results (Fig. 7) indicated that initially little [^{125}I]EGF could bind to either normal keratinocytes or pKV16E5 cells pre-

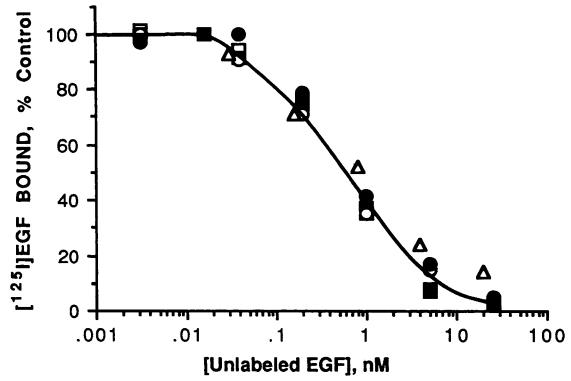

FIG. 3. Affinity of EGFRs. Cells on 35-mm dishes were incubated with 0.05 nM [^{125}I]EGF and the indicated concentrations of unlabeled human EGF for 90 min at 37°C (open symbols) or 0°C (solid symbols). [^{125}I]EGF bound is expressed as a percentage of control [^{125}I]EGF binding in the absence of unlabeled peptide. Control binding per dish was: normal keratinocytes, 13,100 cpm (37°C) and 10,581 cpm (0°C) (open and solid circles, respectively); second preparation of normal keratinocytes, 3,285 cpm (open triangles); and pKV16E5.5 cells, 64,100 cpm (37°C) and 54,700 cpm (0°C) (open and solid squares, respectively).

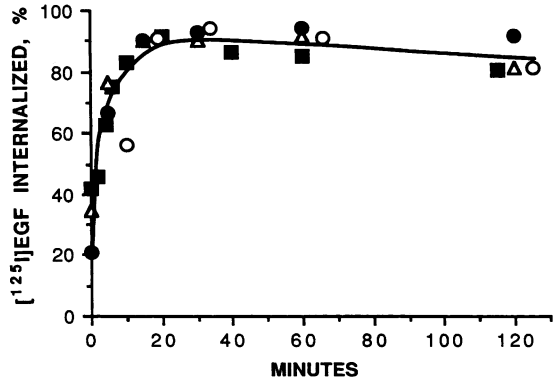

FIG. 4. Rate of [^{125}I]EGF internalization. Cells were incubated with 0.25 nM [^{125}I]EGF at 0°C for 3 h, washed, and then incubated at 37°C. At intervals, the fraction of specifically bound [^{125}I]EGF internalized was measured by the acid-salt wash procedure described in the text. The figure shows two preparations of normal keratinocytes (open and solid circles), pKV16E5.5 cells (solid squares), and pAT16.2 cells (open triangles).

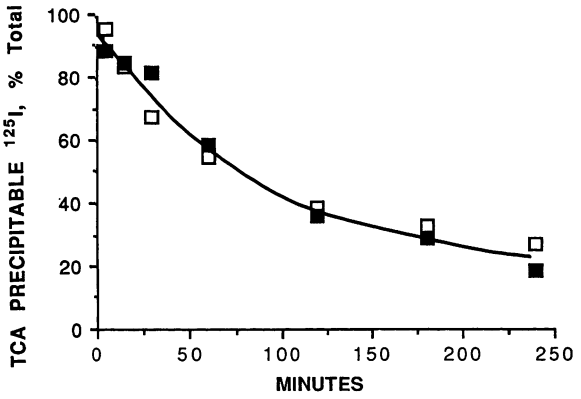

FIG. 5. Rate of degradation of receptor-bound [^{125}I]EGF. Cells were incubated at 0°C with 0.25 nM [^{125}I]EGF, washed as described in the legend to Fig. 4, and incubated at 37°C. At intervals, cells and medium were collected, and TCA-precipitable ^{125}I was measured to determine the extent of [^{125}I]EGF degradation for normal keratinocytes (open squares) and pKV16E5.5 cells (solid squares). The ordinate shows the percentage of ^{125}I (cells and medium) that was TCA precipitable. At time zero, ~95% of receptor-bound ^{125}I was TCA precipitable. At 240 min, ~60% of cell-associated ^{125}I was TCA precipitable, but only ~20% of ^{125}I in the medium was, indicating that once [^{125}I]EGF is degraded, the ^{125}I-labeled breakdown products diffuse from the cell rapidly.

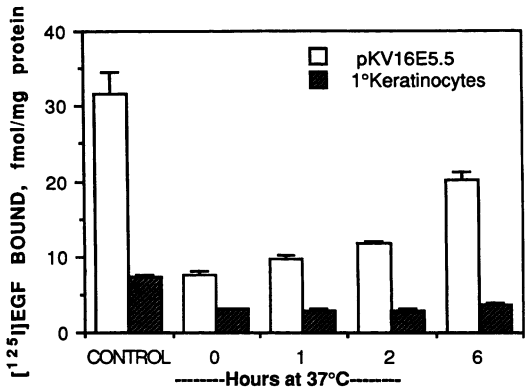

FIG. 7. Cycling of EGFRs. Normal keratinocytes (hatched bars) or pKV16E5.5 cells (open bars) were incubated with or without 100 ng of unlabeled EGF per ml for 1 h at 0°C. Cells were then washed and incubated at 37°C for 0, 1, 2, or 6 h, when 0.25 nM [^{125}I]EGF was added for a 30-min incubation to quantify the number of unoccupied, functional receptors. The control bars show cells not exposed to unlabeled EGF.

viously incubated with nonradioactive EGF, as expected, because the receptors were already occupied. After 2 to 6 h, there was a marked increase in the number of receptors available to bind [^{125}I]EGF on the pKV16E5 cells (40% recycled) but little increase in the normal keratinocytes (7% recycled). The EGFR was therefore more resistant to degradation in cells expressing E5, even though receptor-bound EGF seemed to be degraded at the same rate in normal keratinocytes and pKV16E5 cells (Fig. 5).

The use of radiolabeled EGF demonstrated the similarities and differences in EGFR kinetics between normal keratinocytes and E5-expressing cells. Both cell types displayed

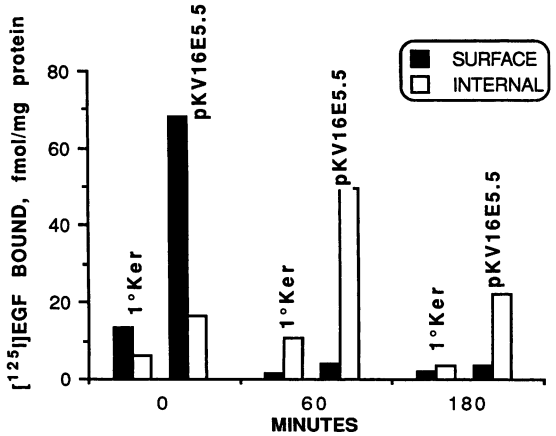

FIG. 6. Cycling of receptor-bound EGF. Either normal keratinocytes (1° Ker) or pKV16E5.5 cells were incubated with [^{125}I]EGF at 0°C, washed, and then incubated at 37°C as described in the legend to Fig. 4. At intervals, surface (solid bars) and internalized (open bars) [^{125}I]EGF were measured. Nonspecific binding has been subtracted from all values.

the same rate of receptor internalization, ligand affinity, and ligand degradation to TCA-soluble fragments. However, the E5-expressing cells possess a greater number of EGFRs than do normal keratinocytes, and after ligand stimulation, the receptors appear to be recycled to a greater degree in the E5-expressing cells than in controls.

Double-labeling experiments with cells stimulated with FITC-labeled EGF, fixed, and then stained for EGFRs with monoclonal antibody R1 and a rhodamine-labeled secondary antibody also demonstrated recycling of the EGFR in E5-expressing cells (Fig. 8). The EGFRs on the surface of the cells (Fig. 8A and B) internalized rapidly in both normal and E5-expressing keratinocytes (Fig. 8C and D), but then reappeared on the surface of the E5-expressing cells but not normal keratinocytes (Fig. 8E and F). Furthermore, EGF and the EGFR had different destinations after internalization in E5-expressing keratinocytes, since EGF-FITC was degraded in the perinuclear lysosomes (arrows, Fig. 8F), while a portion of the EGFRs reappeared at the cell surface (Fig. 8F). Permeabilized preparations allowed the observation of internalized EGFRs, and a portion were observed to be localized to the perinuclear compartments after stimulation (data not shown, but see Fig. 2E and F). In the normal keratinocyte, both EGF and the EGFR are degraded in lysosomes (Fig. 8E). Competition with unlabeled human EGF abolished the signal from EGF-FITC. It should be noted that EGF-FITC was soluble in the Triton X-100 solution, so the localization of EGF-FITC is solely from fixed, unpermeabilized cells. Finally, the addition of 10 μM cyclohexamide to inhibit protein synthesis during this experiment had no effect on the results described here.

Phosphorylation of the EGFR. To determine the biological activity of the EGFR in cells expressing HPV-16 E5, we examined the phosphorylation state of the EGFR as a measure of the tyrosine kinase activity of the receptor. Phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody from lysates of $^{32}P_i$-labeled cells starved of EGF or stimulated with a saturating concentration of ligand for various times. The immunoprecipitate from 10^6 cells was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiographed (Fig. 9). The tyrosine kinase activity and the phos-

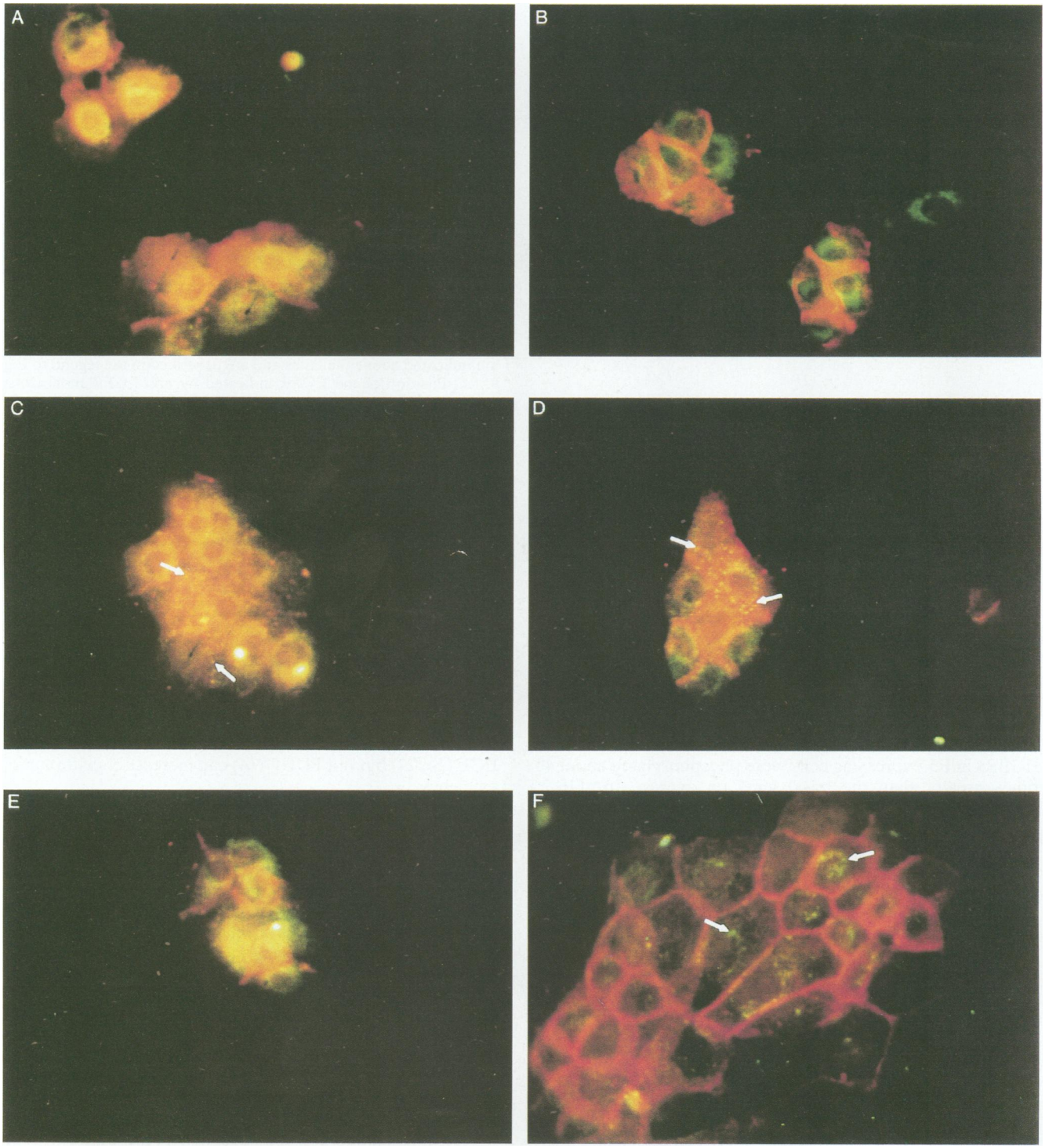

FIG. 8. Double labeling with EGF-FITC and monoclonal antibody R1 followed by anti-mouse IgG-TRITC. (A, C, E, and G) Normal keratinocytes; (B, D, F, and H) pooled clone of keratinocytes stably transfected with either pKV16E5 or pAT16.2. (A and B) 0 min, surface; (C and D) 10 min, surface; (E and F) 60 min, surface.

phorylation of the EGFR and of other proteins are clearly induced by EGF stimulation in all the cell types tested (compare the lanes marked 0 and 5 min). The location of the EGFR was determined by a similar experiment with the A431 vulval carcinoma cell line, which overexpresses the EGFR.

The magnitude of EGFR phosphorylation was determined by densitometric scanning. By this criterion, there was little difference in the constitutive or basal phosphorylation state of the EGFR in control and E5-expressing cells. Comparison among cell types in the EGF-stimulated lanes indicated a marked hyperphosphorylation of the EGFR in the E5-ex-

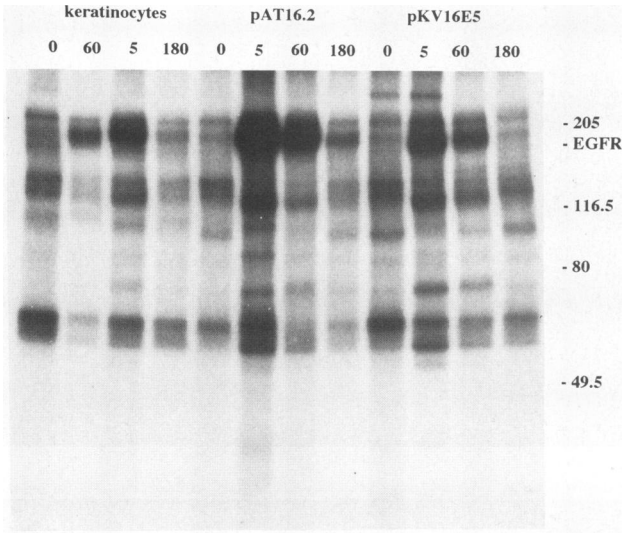

FIG. 9. Phosphorylation of the EGFR after stimulation with EGF. Keratinocytes were labeled with $^{32}P_i$, saturated with EGF on ice, and then warmed to 37°C for the times indicated above the lanes (in minutes), when a cell lysate was collected, and phosphoproteins were precipitated with an antiphosphotyrosine monoclonal antibody and separated by SDS-PAGE for autoradiography. The cell type is indicated above the lanes: keratinocytes are normal human foreskin keratinocytes, pAT16.2 are a clone of keratinocytes immortalized with the HPV-16 genome, and pKV16E5 are pooled keratinocytes stably transfected with pKV16E5. The positions of the EGFR and of size markers are shown (in kilodaltons).

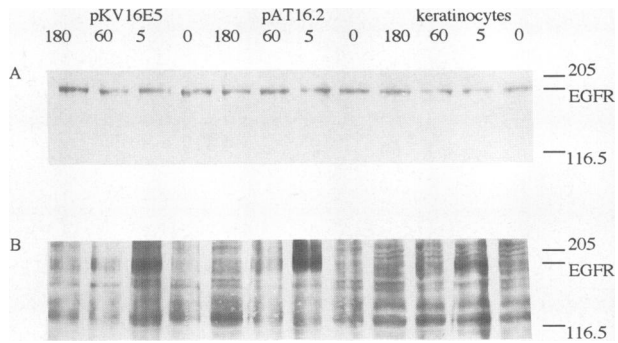

FIG. 10. Immunoblot analysis of EGF-stimulated cell lysates. Keratinocytes were saturated with EGF on ice and then warmed to 37°C for the times indicated above the lanes (in minutes), when a cell lysate was collected and separated by SDS-PAGE. The cell types are indicated above the lanes and are described in the legend to Fig. 9. (A) Position of the EGFR indicated by anti-EGFR monoclonal antibody R1 and goat anti-mouse IgG-alkaline phosphatase conjugate. (B) Same lysate reacted with an antiphosphotyrosine monoclonal antibody and goat anti-mouse IgG-alkaline phosphatase conjugate. The positions of the EGFR and of size markers are shown (in kilodaltons).

pressing cells when stimulated by ligand. At 5 min after EGF treatment, receptor phosphorylation increased 3.8-fold on average (range, 3.4 to 4.3) in normal keratinocytes, while the EGFRs in E5-expressing cells were phosphorylated about 40 times greater than the basal level on average (pAT16.2, >71.2-fold; pKV16E5 range, 9.5- to 19.5-fold). The difference in magnitude of receptor-kinase activity between control and E5-expressing cells at 5 min cannot be accounted for simply by the increase in EGFR number in E5-expressing cells. The phosphorylation of the EGFR in normal keratinocytes was transient, and label incorporation fell to basal levels or below by the 1-h time point. In contrast, the phosphorylation state of the EGFR persisted in the E5-expressing cells for up to 3 h, and at 1 h, the receptors retained on average seven times the phosphotyrosine present basally (pAT16.2 range, 3.0- to 19.6-fold; pKV16E5 range, 2.0- to 5.0-fold). This experiment was repeated five times with consistent results.

Immunoblots were performed (Fig. 10) to confirm that the observed increase in phosphorylation occurred on tyrosine residues in response to ligand-stimulated kinase activity. Keratinocytes with and without E5 were stimulated with EGF, and whole-cell lysates were prepared. Aliquots of the same lysates were electrophoresed, transferred to nitrocellulose, and stained with monoclonal antibodies to either the EGFR or phosphotyrosine. EGFR-specific staining was evident for all the time points examined for all the cell types tested (Fig. 10A). Staining for phosphotyrosine (Fig. 10B), however, was greater after ligand stimulation (5 min) and of greater magnitude in E5-expressing cells. The profile of proteins obtained by immunoblot with anti-phosphotyrosine antibody is similar to the profile of $^{32}P_i$-labeled proteins immunoprecipitated by antiphosphotyrosine antibody, an indication that there was an increase in tyrosine phosphorylation (13).

DISCUSSION

In our studies, HPV-6 E5 and HPV-16 E5 were able to transform a number of rodent fibroblast cell lines, and the transformation rate of fibroblasts by E5 was greater in the presence of EGF but not of PDGF. These results support the results of the previous studies on the ability of HPV-16 E5 alone to transform fibroblasts (14) and reinforce the fact that EGF (14, 24) but not PDGF (14) can increase transformation efficiency.

The mitogenic response of DNA synthesis occurs subsequent to the induction of early genes, such as c-*fos* (7). Fibroblasts transfected with HPV-16 E5 expressed increased amounts of c-*fos* mRNA after EGF stimulation compared with untransfected fibroblasts (14). In our work, we found that the E5 ORFs of HPV-6 and HPV-16 both stimulated mitogenesis in primary keratinocytes. Furthermore, synergistic stimulation of mitogenesis by EGF and the E5 ORFs was also demonstrated.

We have shown for the first time that HPV-16 E5 has several effects on the endogenous EGFR of human keratinocytes. First, there is a two- to fourfold increase in the number of functional receptors on E5-expressing cells, as measured by radioligand binding. The range of increase in receptor numbers may be the result of varying numbers of receptors present on the normal keratinocytes cultured from different human foreskins. Also, since the affinity of the receptor for ligand was unchanged in E5-expressing cells, the differences in the equilibrium binding of ligand must be due to the number of binding sites, i.e., the number of receptors per cell. Second, in contrast to the initial work on BPV-1 E5 (17) and in support of subsequent studies (31), we found that internalization of the receptor and ligand was normal in keratinocytes expressing HPV-16 E5. However, in our studies, the degradation of receptor but not ligand within endosomes was delayed in E5-expressing cells, and the receptor could be detected for up to 3 h after the addition of ligand, by which time the receptor is degraded in control

cells. Third, the immunofluorescence studies indicated that there is a significant amount of receptor on the surface of E5-expressing cells after the initial internalization, while the kinetic studies with [^{125}I]EGF showed that the internalization and degradation of the ligand were normal. One explanation for the separation of the receptor and ligand pathways after internalization would be that in the event of inhibition of degradation of the receptor, the receptors recycle back to the cell surface. Support for this explanation came from the results shown in Fig. 7. This experiment showed that 40% of the receptors in E5-expressing cells recycled, as opposed to 7% in the control cells. As these recycled receptors bind ligand, they are assumed to be restimulated for signal transduction. Further support for this explanation comes from the double-labeling experiments with EGF-FITC. In this experiment, EGF and the EGFR were observed to follow different routes after internalization in E5-expressing cells: EGF proceeded to the perinuclear lysosomes for eventual degradation, while a significant proportion of the EGFRs reappeared at the cell surface. The use of cycloheximide to inhibit translation of the EGFR had no effect on the immunofluorescence study results, indicating that the de novo synthesis of EGFR could not account for the reappearance of receptor at the cell surface.

The separation of the ligand and receptor indicates an alteration in the compartmentalization process in the E5-expressing cell following endocytosis of the ligand-receptor complex. This may occur if there is a delay in the acidification of the endosomal compartments of E5-expressing cells, since acidification is responsible for dissociation of the ligand-receptor complex in the endosome and is necessary for subsequent proteolysis. This delay in acidification may allow the partitioning of the receptor to a recycling pathway and may be due to the action of E5, since it has been shown that the BPV-1 E5 can bind to a subunit of the vacuolar proton-ATPase associated with ion transport pumps (10), including the pump responsible for endosome acidification. Recent work on BPV-1 E5 shows that the protein appears to decrease the rate of degradation of the EGFR by stabilizing intact EGF-EGFR complexes in fibroblasts transfected with human EGFR (31), perhaps also because of the effect of E5 on endosome acidification.

The activity of the EGFR can be assessed by its level of tyrosine phosphorylation after stimulation with ligand. Our data suggested that there was no difference in the constitutive level of phosphorylation between E5-expressing and control cells. This is a different situation than with BPV-1 E5, which causes phosphorylation of the EGFR (17) and PDGFR (23) on rodent fibroblasts in the absence of ligand. BPV-1 E5 interacts with a complex which contains the PDGFR (22), and, since BPV-1 E5 can dimerize, perhaps causes dimerization and autophosphorylation of the PDGFR. While HPV-16 E5 has the potential to dimerize (2), it is currently unknown whether it binds to the EGFR. Regardless, our data indicate that HPV-16 E5 does not cause autophosphorylation of the EGFR in the absence of ligand. Upon ligand stimulation, however, there was an increase in the level of phosphorylation of the receptor in E5-expressing cells that appeared greater than that predicted by the increased receptor number on these cells. This was true for cells containing the whole genome or E5 alone. Furthermore, this hyperphosphorylation persisted for at least an hour in the E5-expressing cells, while the control cells returned to their basal state. Finally, the immunoblots in Fig. 10 demonstrated that the hyperphosphorylation of EGFR in E5-expressing cells included tyrosine residues.

In our receptor phosphorylation studies, HPV-16 E5 did not constitutively activate the EGFR of keratinocytes. This is unlike BPV-1 E5, which activates growth factor receptors in the absence of exogenous ligand (17, 23). Additionally, HPV-6 E5 and HPV-16 E5 transformed fibroblasts to anchorage-independent growth in the absence of exogenous ligand. Both E5 ORFs were also mitogenic stimuli for keratinocytes in our transient-transfection assay. These facts demonstrate that HPV-16 E5 may act through several pathways, one involving the EGFR and another independent of it, possibly in a cell type-specific manner, or that E5 affects some point downstream of the EGFR in the receptor-mediated endocytosis/signal transduction pathway, such as the proton-ATPase pump. This second possibility would appear to conflict with the inability of PDGF to enhance transformation of rodent fibroblasts, since the EGFR and the PDGFR share a similar receptor-mediated endocytosis pathway. However, this could be explained by the fact that the PDGFR is not a major growth factor receptor on keratinocytes, and HPV-16 E5 may have some specificity for the primary growth factor receptors of the normal host cell type for HPVs. Clearly, there are several questions about the role of the HPV-16 E5 ORF in HPV infection and its interaction with cellular signal transduction pathways that warrant further study.

ACKNOWLEDGMENTS

We thank Eric J. Nelson, Stephanie Pretel, and Michael O'Banion for their technical help.

This work was supported by NIAID grants AI30798-03 awarded to D.J.M. and DK19974 awarded to P.M.H. and by Cancer Center Core Research Grant CA11098. S.W.S. is supported by NIH training grant T2GM07102D.

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