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Inhibition of Human Papillomavirus Type 16 E7 Phosphorylation by the S100 MRP-8/14 Protein Complex

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The human papillomavirus type 16 (HPV16) E7 is a major viral oncoprotein that is phosphorylated by casein kinase II (CKII). Two S100 family calcium-binding proteins, macrophage inhibitory-related factor protein 8 (MRP-8) and MRP-14, form a protein complex, MRP-8/14, that inactivates CKII. The MRP-8/14 protein complex may inhibit CKII-mediated E7 phosphorylation and therefore may alter its interaction with cellular ligands and reduce E7 oncogenic activity. We examined the inhibitory effect of the MRP-8/14 complex on CKII activity and HPV16 E7 phosphorylation. We have shown that CKII activity and HPV16 E7 phosphorylation were inhibited by uptake of exogenous MRP-8/14 and activation of endogenous MRP-8/14. MRP-8/14-mediated inhibition of E7 phosphorylation occurred at the G_1 phase of the cell cycle. Analysis of MRP expression in primary keratinocytes and in HPV16- and 18-transformed cervical and foreskin epithelial cell lines showed that expression of MRP-8, MRP-14, and the MRP-8/14 complex was detected only in primary untransformed keratinocytes and not in the HPV-infected immortalized epithelial cells. CKII activity in HPV-immortalized keratinocytes was approximately fourfold higher than in HPV-negative primary keratinocytes. Treatment of HPV-positive immortalized epithelial cells with exogenous MRP-8/14 resulted in E7 hypophosphorylation and complete inhibition of cell growth within 2 weeks, compared with HPV-negative primary and immortalized HPV-negative cervical epithelial cells, which showed 25 and 40% growth inhibition, respectively. Together these results suggests that the MRP-8/14 protein complex in HPV-infected epithelial cells may play an important role in regulation of CKII-mediated E7 phosphorylation and inhibition of its oncogenic activity.

Human papillomaviruses (HPV) are a large family of nonenveloped DNA viruses that infect epithelial cells and may cause condyloma, high-grade dysplasia, and invasive cancer. Certain high-risk genotypes of HPV, such as HPV type 16 (HPV16) and HPV18, are strongly involved in the pathogenesis of anogenital cancer in men and women (65, 66). HPV-induced malignant transformation is mediated by cooperative expression of the HPV E6 and E7 oncoproteins and their interaction with their host cellular target proteins (36, 37, 46). HPV E6 binds to tumor suppressor protein p53, and this interaction leads to inactivation of p53 and chromosomal instability (59). HPV E7 binds to pRb and to several other cellular proteins that are involved in cell cycle and transcriptional control mechanisms, and these interactions stimulate cell cycle progression (14).

The HPV16 E7 protein is a 98-amino-acid nuclear phosphoprotein (49) that induces DNA synthesis in quiescent cells, cooperates with the ras oncogene to transform primary rodent cells, and interacts with pRb to promote cell cycle progression (41, 57). The HPV16 E7 CR2 domain plays a critical role in its oncogenic function by interaction with the Rb family of proteins via its LxCxE motif (8). This domain contains two phosphorylation sites on its Ser 31 and Ser 32 positions that are phosphorylated by cellular casein kinase II (CKII) (3, 17). E7 is also phosphorylated at its C terminus at the serine 71 position by an unknown kinase (32). Furthermore, it has been shown that HPV E7 is differentially phosphorylated during the cell cycle at G_1 phase by CKII and at S phase by unidentified enzymes (32).

HPV18 E7 phosphorylation has been shown to be critical to promote S-phase entry (9). Consistent with these data, mutations of the serine 31/32 residues and a consensus sequence for HPV16 E7 phosphorylation by CKII (amino acids 30 to 37) resulted in defective E7 phosphorylation and impaired its transforming ability (3, 18). Replacement of the serine 31/32 residues with two negatively charged aspartic acid residues restored E7 transforming activity (3, 18). The level of E7 phosphorylation and transformation activity is lower in low-risk HPV types than in high-risk HPV types (3). These data suggest that E7 phosphorylation by CKII may play a critical role in enhancement of oncogenic functions of E7 and development of HPV-associated neoplasia. However, the molecular mechanisms of regulation of E7 phosphorylation and its role in E7 interaction with its intracellular ligands are not fully understood.

CKII is a pleiotropic, ubiquitous serine/threonine protein kinase composed of two α catalytic subunits (α and α′) and two β regulatory subunits which phosphorylate many key cellular and viral proteins (30, 42). CKII has been detected within the cytoplasm and nucleus and is associated with plasma membrane and intracellular organelles, including the Golgi and the endoplasmic reticulum (15, 44). CKII is a key component of the regulatory protein kinase network and is involved in reg-

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ulation of function of a broad spectrum of cellular proteins (30). Abnormally high levels of CKII expression have been detected in solid tumors and leukemia (11, 16, 29, 51, 58, 61), and its dysregulated expression in transgenic mice induces lymphoma (28, 48).

CKII activity is inhibited by mammalian cell proteins such as calprotectin (38). Calprotectin or MRP-8/14 is a protein complex of S100 family calcium-binding proteins macrophage inhibitory-related factor protein 8 (MRP-8; S100A8) and MRP-14 (S100A9). MRP-8 was previously known as L1 light chain, calgranulin A, and cystic fibrosis antigen, and MRP-14 was known as calgranulin B, p14, and L1 heavy chain (2, 4, 10, 13, 22, 60). MRP-8 and MRP-14, as well as their complex MRP-8/14, are expressed at high levels in neutrophils and monocytes (52, 54, 67). The MRP-8/14 complex has been shown to inhibit phosphorylation of casein by CKI and -II (38).

MRPs are expressed and secreted by monocyte/neutrophils in chronic and acute inflammatory diseases, such as rheumatoid arthritis and sarcoidosis (5, 20, 27). They may also be expressed at high levels in epithelium in inflammatory conditions such as psoriasis and eczema (12, 21, 39). MRP-8 and -14 are also found at high levels in the circulation (1).

Little is known about MRP expression in normal epithelium or in viral infections of the epithelium. We have shown that MRP-8 and MRP-14 expression is highly upregulated in HPV-associated dysplastic tissues compared with normal and cancerous tissues (unpublished data). Given the role of the MRP-8/14 complex in inhibiting CKII and given the role of CKII in phosphorylation of HPV E7, we hypothesized that the MRP-8/14 protein complex may regulate CKII kinase activity in HPV-infected cells and may play an important role in modulation of the oncogenic functions of E7. To test this hypothesis, we investigated MRP-8/14-mediated CKII inactivation, its effect on HPV16 E7 phosphorylation, and the antiproliferative activity of MRP-8/14 in HPV-infected and uninfected epithelial cells. Our data show that the MRP-8/14 complex inhibits CKII catalytic activity and leads to hypophosphorylation of the HPV16 E7 protein. Treatment of HPV-infected epithelial cell lines with exogenous MRP-8/14 protein complex inhibited E7 phosphorylation and cell growth, suggesting that MRP-8/14-mediated inhibition of E7 phosphorylation may play an important role in regulating oncogenic functions of E7.

MATERIALS AND METHODS

Cells and media. Primary normal human foreskin keratinocytes (NFK) were propagated from a neonatal foreskin as described previously (56). Primary normal human tongue keratinocytes (OCO) were propagated from a lateral tongue biopsy of a 35-year-old healthy volunteer. The protocol for use of primary OCO cells was approved by the Committee on Human Research Review Board of the University of California, San Francisco (UCSF CHR approval no. RS00908). Primary normal cervical keratinocytes (NCK) were purchased from Clontech (Palo Alto, Calif.). Primary NFK and OCO cells were grown in keratinocyte growth medium (Clontech). The HPV-negative human cervical carcinoma C33A and human oral squamous carcinoma HSC-3 cell lines were obtained from the American Type Culture Collection (Manassas, Va.) and Randall Kramer (University of California, San Francisco), respectively. We have shown that the NFK, OCO, and NCK cells were HPV negative using PCR (data not shown). The following HPV-positive cell lines were used: 16MT (NFK described above and immortalized with HPV16) (56), Caski (human cervical carcinoma cell line containing HPV16) (47), HOK-16 (oral carcinoma cell line immortalized with HPV16) (40), and HeLa (human cervical adenocarcinoma containing HPV18) and SiHa (human cervical squamous cell carcinoma containing HPV16) (47). All immortalized cell lines were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, N.Y.) with 10% fetal bovine serum (HyClone, Logan, Utah), 2.5 μg of streptomycin/ml, and 5 μg of penicillin (Gibco-BRL)/ml.

Cloning, expression, and purification of MRPs. To clone MRP-8 and MRP-14 genes, we purified mRNAs from white blood cells of a healthy volunteer and the mRNAs were reverse transcribed into cDNAs, which were used as templates for PCR to amplify these genes. The sequences of the primers used were as follows: for MRP-8, 5′-CGCGGATCCGTGGGCATCATGTTG-3′ and 5′-CCCAGTA ACTCAGCTACTCTTTG-3′; for MRP-14, 5′-CGCGGATCCAAGACGATGA CTTGCAAA-3′ and 5′-TCTTGGCCACTGTGGTCTTAGG-3′. The amplicons were TA cloned into the PCR II vector (Invitrogen, Carlsbad, Calif.). The clones obtained were sequenced, and their identity and orientation were confirmed. To generate MRP-8 and MRP-14 proteins, the genes were subcloned in frame into the pGEX-2T vector (Amersham Biosciences, Piscataway, N.J.). The pGEX-2T vector expresses glutathione *S*-transferase (GST) and the thrombin cleavage site in frame with the MRPs. To purify the fusion proteins, they were linked to a GST-Sepharose 4B column and eluted after cleavage with thrombin, and the purified MRPs were collected in the eluate.

Cloning HPV16 E6 and E7 genes into a mammalian vector. A PCR fragment (0.8 kb) containing the HPV16 E6 and E7 genes was amplified from a plasmid containing the entire HPV16 genome (ATCC 45113) using oligonucleotide primers (5′-CGGAAGCTTCCGAAATCGGTTGAA-3′ and 5′-CGGGAATTCGGT ACCTGCAGGATC-3′). The E6 and E7 genes are located tandemly and are translated using bicistronic mRNA. The PCR fragment was cloned into the pCR2.1-TOPO vector (Invitrogen). After confirming the orientation of the insert, the BamHI/XhoI fragment was cloned into a mammalian vector, pIRES-EGFP (Clontech). Expression of HPV16 E7 was confirmed in transiently transfected HSC-3 cells by green fluorescent protein (GFP) staining and Western blot assay using mouse monoclonal antibody (MAb) to HPV16 E7 (Zymed, San Francisco, Calif.).

Immunofluorescence assay. Expression of MRPs in HPV-positive and -negative epithelial cell lines and untransformed primary cells was examined in immunofluorescence assays. Cells were grown on chamber slides and fixed in methanol-acetone (1:1) for 20 min at −20°C. The cells were then incubated with the appropriate mouse MAbs to MRPs for 1 h at room temperature. MRP-8, MRP-14, and the MRP-8/14 complex were detected using mouse MAbs 8-5 C2, S 36.48, and 27 E 10, respectively (BMA Biomedicals AG, Augst, Switzerland). The cells were washed with phosphate-buffered saline and incubated with goat anti-mouse secondary antibodies conjugated with fluorescein isothiocyanate (Jackson Immunochemicals, West Grove, Pa.). Cell nuclei were stained with propidium iodide (Sigma, St. Louis, Mo.). The simultaneous expression of HPV16 E7, MRP-8/14, and CKII in HSC-3 cells transfected with HPV16 E7 was detected in a double immunofluorescence assay with antibodies to MRP-8/14 and CKII and by GFP staining for E7. For CKII detection, goat polyclonal antibody to the α′-subunit of CKII (Santa Cruz Biotechnology, Santa Cruz, Calif.) was used. To visualize MRP-8/14 and CKII signals, anti-mouse and anti-goat secondary antibodies conjugated with Cy5 or Texas Red were used, respectively (Jackson Immunochemicals). Cells were analyzed by laser-scanning confocal microscopy (MRC1024; Bio-Rad, Hercules, Calif.).

Northern blot assay. Total RNA was isolated from cell lines by using TRIzol reagent (Gibco BRL, Rockville, Md.). Equal quantities of total RNA from transformed and primary cell lines were loaded onto a formaldehyde-morpholinopropane sulfonic acid (MOPS) agarose gel and separated by electrophoresis. The RNA was blotted onto a Zetaprobe nylon membrane by using a semidry transfer cell, following the manufacturer's instructions (Bio-Rad). cDNA constructs of MRP-8 and MRP-14 in pBKCMV were used as MRP-8- and MRP-14-specific probes. A probe for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was generated using gene-specific primers (kindly provided by Mark Scott, University of California, San Francisco) in a SuperScript one-step reverse transcription-PCR system (Gibco BRL) with total RNA extracted from normal human keratinocytes. Northern blots were treated with prehybridization solution: 50% formamide, 0.12 M Na_2HPO4 (pH 7.2), 0.25 M NaCl, and 7% sodium dodecyl sulfate (SDS) for 30 min at 43°C. The γ-^{32}P-labeled probe was added (2×10^6 cpm/ml of hybridization solution) and incubated overnight at 43°C, blotted, and washed twice for 5 min each with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS. Blots were exposed to X-ray film.

Western blot assay. For Western blot analysis of endogenous MRP-8, MRP-14, and the MRP-8/14 complex, HSC-3 cells were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer containing nonionic detergents, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Tris (pH 8.0), and a cocktail of protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin A (10 μg/ml). Purified exogenous MRP-8 and MRP-14 proteins were denatured in sample buffer containing 2% SDS and 5%

β2-mercaptoethanol. To obtain the MRP-8/14 protein complex, MRP-8 and MRP-14 proteins in equal amounts were mixed in 0.5 mM $CaCl_2$ solution and incubated at room temperature for 30 min. MRP-8 and -14 proteins were separated using 16% denaturing reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the MRP-8/14 protein complex was subjected to nonreducing SDS-PAGE. The proteins were transferred to an Immobilon-P membrane, and nonspecific binding was blocked using phosphate-buffered saline (pH 7.2) containing 5% skim milk and 0.01% Tween 20. Specific bands representing MRPs were detected using MAbs to MRP-8, MRP-14, and the MRP-8/14 complex (BMA). The protein bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham Life Sciences).

CKII kinase assay. CKII kinase activity in HPV-positive and -negative epithelial cell lines and untransformed primary cells was examined using purified CKII obtained by immunoprecipitation. For immunoprecipitation of CKII, cells were extracted in RIPA buffer containing 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mg of aprotinin/ml, 1 mg of pepstatin/ml, 1 mg of leupeptin/ml, 1 mM Na_2V0_4, and 10 nM sodium pyrophosphate. CKII was then immunoprecipitated using rabbit polyclonal antibody α-subunit (70 to 89 amino acids) to CKII (EMD Bioscience Inc., San Diego, Calif.) and washed six times with RIPA buffer. The concentration of immunoprecipitated CKII was measured using a bovine serum albumin protein concentration kit (Pierce, Rockford, Ill.). Kinase activity of immunoprecipitated CKII was measured with a CKII assay kit (Upstate Biotechnology, Lake Placid, N.Y.). Ten micrograms of immunoprecipitated CKII was added to 50 μl of reaction mixture containing assay dilution buffer (20 mM MOPS [pH 7.2], 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol), substrate peptide (RRRDDDSDDD), inhibitor cocktail (2 μM protein kinase A inhibitor peptide), and 100 μM [γ^{32}-P]ATP (6,000 Ci/mmol; ICN Biomedicals, Irvine, Calif.). The reaction mixture was incubated for 10 min at 30°C, and the reaction was stopped by adding 20 μl of 40% trichloroacetic acid to each sample. Twenty-five microliters of the reaction mixture was transferred onto numbered P81 paper squares, and the papers were washed three times for 15 min with 0.75% phosphoric acid and once with acetone. The papers were dried and transferred into scintillation vials, and radioactivity was counted in liquid scintillation counter (LS1701; Beckman, Fullerton, Calif.). As a control for CKII inactivation, we used a specific inhibitor for CKII, apigenin (Sigma). Cells were treated with apigenin that was dissolved in dimethyl sulfoxide (DMSO; Fisher, Pittsburgh, Pa.).

HPV16 E7 phosphorylation assay. For in vitro HPV16 E7 phosphorylation assays, we used an E7-GST fusion protein. Phosphorylation of E7 was examined by measuring incorporation of 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; ICN Biomedicals). A 5-μg aliquot of E7-GST fusion protein was added to a phosphorylation reaction buffer (75 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.4), containing 500 U of purified CKII (New England Biolabs, Beverly, Mass.) reaction mixture and 0.1 mM ATP. As positive control for CKII kinase activity, we used 5 μg of partially dephosphorylated bovine casein (Sigma). A 100-ng aliquot each of MRP-8, MRP-14, and MRP-8/14 proteins in phosphorylation buffer was added independently into the phosphorylation reaction mixture. The reaction mixtures (50 μl) were then incubated for 30 min at 30°C, and the reaction was stopped by adding 2× loading sample buffer. E7 phosphorylation was examined by SDS-PAGE and autoradiography. HPV16 E7 phosphorylation in vitro also was examined using rabbit antiphosphoserine polyclonal antibody (Zymed), which detects serine-phosphorylated proteins. In this assay, in vitro phosphorylation of HPV16 E7 was performed as described above except using nonradioactive ATP as the source of phosphate molecules. The protein bands were visualized using the ECL Western blot assay. The film was imaged using a Molecular Dynamics personal densitometer (Amersham Biosciences, Little Chalfont, England), and the level of phosphorylation was quantitated by measuring the intensity of pixels (mean density) of protein bands using NIH image software.

HPV16 E7 phosphorylation in vivo was examined in HSC-3 cells transiently transfected with plasmid DNA containing HPV E6/E7 encoding sequences. Transfection of plasmid DNA was performed using the calcium-phosphate precipitation method (23). At 48 h posttransfection, 3×10^6 cells were extracted with RIPA buffer and E7 protein was immunoprecipitated with mouse MAb to HPV16 E7 (Zymed). Immunoprecipitated E7 was separated on a 16% gel by SDS-PAGE. Total and phosphorylated E7 were examined using mouse anti-E7 MAb and rabbit antiphosphoserine polyclonal antibodies, respectively (Zymed). The phosphorylated protein bands were detected using an ECL Western blot assay, and the level of phosphorylation was quantitated using NIH image software as described above.

To examine E7 phosphorylation during the cell cycle, HSC-3 cells were transfected with HPV16 E7 and 12 h later were growth arrested in medium containing 0.2% fetal bovine serum for the next 38 h. HSC-3 cells do not express MRP-8/14 without induction, and 10 ng of dexamethasone/ml was added at 24 h posttransfection to induce MRP-8/14 expression. At 38 h posttransfection, medium with 0.2% serum was replaced with fresh medium containing 15% serum, 10 ng of dexamethasone/ml, and 1 μCi of [^{3}H]thymidine/ml (1 μCi = 37 kBq) (Amersham Biosciences). At 2-h intervals, 10^6 cells were dissociated from the flask by using a cell scraper and extracted with RIPA buffer. E7 was immunoprecipitated, and its phosphorylation was analyzed using rabbit antiphosphoserine antibodies as described above. In the same experiment the cell growth, MRP-8/14 expression, CKII activity, and total E7 and phosphorylated E7 were analyzed. CKII activity and E7 phosphorylation were analyzed as described above. Cell growth was examined by measuring [^{3}H]thymidine incorporation into DNA-synthesizing cells. Incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a Beckman LS1701 liquid scintillation counter. For analysis of MRP-8/14 expression, a small drop of cells before extraction was placed on a glass slide (drop slide), fixed, and subjected to an immunofluorescence assay with mouse MAb to MRP-8/14.

E7 phosphorylation during the cell cycle in the presence of exogenous MRP-8/14 was examined as described above. Experiments were performed as above except that 3 μg of exogenous MRP-8/14/ml was added to the culture medium at 24 h posttransfection instead of dexamethasone.

To analyze HPV16 E7 phosphorylation in MRP-8/14-treated and untreated SiHa and Caski cells, E7 was immunoprecipitated using MAb to HPV16 E7 and E7 phosphorylation was examined using rabbit antiphosphoserine antibodies as described above. To examine dephosphorylation of E7, 100 μl of immunoprecipitated E7 from MRP-8/14-untreated cells was incubated with 400 U of λ protein phosphatase (New England Biolabs) for 30 min at 30°C.

Cell viability assay in the presence of exogenous MRP-8/14. Primary and immortalized cells were seeded in 24-well plates (Nunc; Fisher) at a density of 2×10^4 cells per well in 1 ml of appropriate medium for each cell line. After 24 h postseeding cells were treated with various concentrations of exogenous MRP-8/14 protein complex ranging from 0.1 to 10 μg/ml for 14 days. The culture medium was changed every other day with fresh medium containing MRP-8/14. To quantify the live and dead cells, cells treated with MRP-8/14 were trypsinized and stained every 2 days for 2 weeks with 2% trypan blue (1:1, vol/vol) for 5 min. Viable (unstained) and dead (stained) cells were counted from each well by using a hemocytometer (Neubauer chamber). Experiments were performed in triplicate and repeated three times.

RESULTS

Characterization of MRP-GST proteins. MRP-8 and MRP-14 proteins were produced in endotoxin-free host bacterial cells and subsequently purified and cleaved from GST protein. Western blot analysis of MRP-8 and MRP-14 proteins showed that their sizes were 10 and 14 kDa, respectively. These sizes of proteins corresponded well to their predicted authentic size. To examine the ability of MRP-8 and MRP-14 to form MRP-8/14 protein complexes, we examined a mixture of MRP-8 and MRP-14 in the presence of 0.5 M $CaCl_2$ in a Western blot assay with 27 E 10 MAbs that recognize only MRP-8/14 complexes. These experiments showed that MRP-8 and MRP-14 proteins formed a complex with each other in a 1:1 ratio (Fig. 1). MRP-8/14 complex formation was dependent on the presence of a high concentration of calcium, i.e., 0.25 mM and higher. Lower calcium concentrations of 0.05 mM and below were insufficient to form the MRP-8/14 protein complex.

Inhibition of CKII kinase activity and E7 phosphorylation by purified exogenous MRP-8/14. To determine whether exogenous MRP proteins inhibit CKII kinase activity in HPV-transformed cell lines, we examined CKII activity in MRP-8/14-treated HeLa and SiHa cells, which do not express either MRP-8 or MRP-14 proteins. The MRP-8/14 complex at various concentrations was added to the cells for 24 h at 37°C. In parallel experiments, the cells were treated with various concentrations of apigenin, a specific inhibitor for CKII (7, 55). CKII kinase activity was measured in an in vitro phosphoryla-

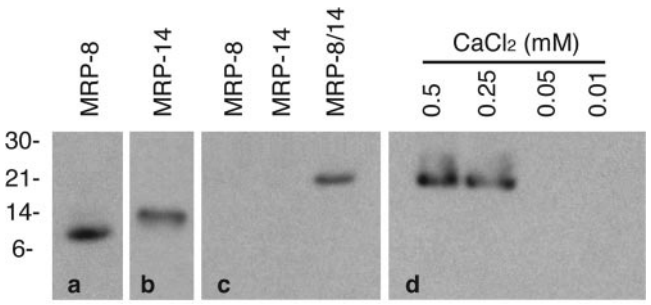

FIG. 1. Complex formation of MRP-8 and MRP-14. Equal concentrations, i.e., 1 μg each of MRP-8 (a) and MRP-14 (b), were mixed in the presence of 0.5 mM $CaCl_2$ (c) and incubated at room temperature for 30 min. To determine the dependence of MRP-8/14 complex formation on calcium ions, the MRP-8 and MRP-14 mixture was incubated with various concentrations of calcium (d). Proteins were resolved by SDS-PAGE, and MRP-8, MRP-14, and their complex formation were analyzed by Western blot assay using mouse MAbs 8-5 C2, S36.48, and 27 E10, which recognize MRP-8, MRP-14, and MRP-8/14, respectively. (a and b) Reducing; (c and d) nonreducing.

tion assay. These experiments showed that CKII activity was inhibited by MRP-8/14 in a concentration-dependent manner (Fig. 2A). The 50% inhibitory concentration of MRP-8/14 was about 2.5 μg/ml. HeLa cells were then incubated with 2.5 μg each of MRP-8, MRP-14, and MRP-8/14 per ml for 24 h, and CK activity was examined. Approximately 50% inhibition of CKII activity was observed only with the MRP-8/14 complex (Fig. 2B). MRP-8 alone did not inhibit CKII activity, but MRP-14 showed a weak inhibitory effect, i.e., approximately10% inhibition of CKII. HeLa cells treated with MRP-8, MRP-14, and MRP-8/14 were also examined for the kinase activity of protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaM kinase II). These experiments showed that neither MRP-8, MRP-14, nor the MRP-/14 complex inhibited PKC or CaM kinase II activity (data not shown), indicating that MRP-8/14 specifically inhibited CKII activity. Similar data were obtained in SiHa cells (data not shown). These data confirmed the earlier observations that the MRP-8/14 complex purified from neutrophils can inhibit CKII kinase activity.

To determine the role of MRPs in HPV16 E7 phosphorylation, we used the E7-GST fusion protein for an in vitro phosphorylation assay. MRP-8, MRP-14, and MRP-8/14 proteins were added to a phosphorylation reaction mixture containing purified CKII and [γ-^{32}P]ATP. Casein was used as a control substrate for CKII. E7-GST fusion protein and casein were extensively phosphorylated within 30 min by CKII (Fig. 3A). The MRP-8/14 complex reduced the CKII-mediated phosphorylation of E7 and casein by approximately 95 and 80%, respectively. MRP-8 alone did not inhibit E7 or casein phosphorylation. MRP-14 alone resulted in approximately 40% inhibition of E7 phosphorylation and casein phosphorylation by CKII (Fig. 3A). However, CKII inactivation in vivo in HeLa cells by MRP-14 alone was not significant (about 10%). It is possible that weaker inhibition of CKII catalytic activity by MRP-14 in the HeLa cells than was seen in the in vitro phosphorylation assay was the result of slow internalization of MRP-14. To address this possibility, we analyzed MRP internalization in HeLa cells. The results indicated that efficiency of internalization of MRP-8 and MRP-14 individually was significantly lower than that of the MRP-8/14 protein complex (data not shown).

CKII-mediated HPV16 E7 phosphorylation in the presence of various concentrations of MRP-8/14 proteins was examined in a Western blot assay using antiphosphoserine antibodies (Fig. 3B). These experiments showed that MRP-8/14-mediated inhibition of E7 phosphorylation was concentration dependent and that significant inhibition of E7 phosphorylation was detected when MRP-8/14 was added to the phosphorylation reaction mixture at 2 ng/μl or higher.

Inhibition of CKII activity and E7 phosphorylation by endogenous MRP-8/14 protein complex. To study the inhibitory role of endogenous MRP-8/14 protein complex on CKII activity and E7 phosphorylation, we established an epithelial cell model system using the HPV-negative human oral squamous carcinoma HSC-3 cell line. Immunofluorescence analysis of MRP protein expression in HSC-3 cells showed that these proteins were expressed in only a small proportion of the cells.

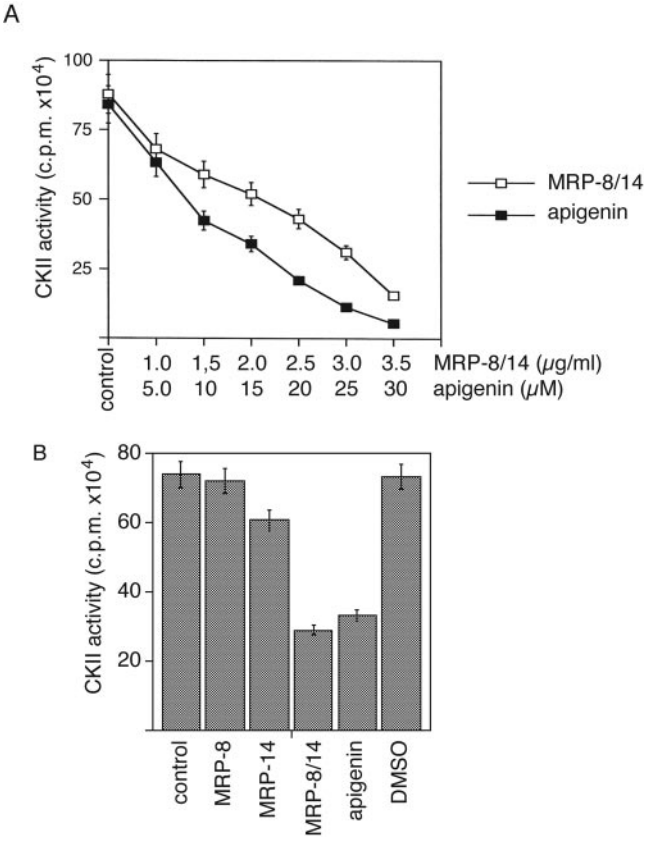

FIG. 2. Inhibition of CKII activity in HeLa cells by exogenous MRP-8/14. (A) HeLa cells were incubated with various concentrations of MRP-8/14 proteins for 24 h at 37°C and extracted in RIPA buffer. In parallel experiments cells were treated with an increasing concentration of apigenin, a CKII inhibitor, as a positive control. (B) HeLa cells were incubated with 2.5 μg of MRP-8, MRP-14, and MRP-8/14 proteins per ml and 10 μM apigenin for 24 h at 37°C and then extracted in RIPA buffer. CKII was immunoprecipitated, and kinase activity was measured in triplicate samples. Controls were untreated cells and DMSO-treated cells, since DMSO was used as an organic solvent for apigenin. Similar results were obtained in three independent experiments. The error bars indicate standard errors (n = 3).

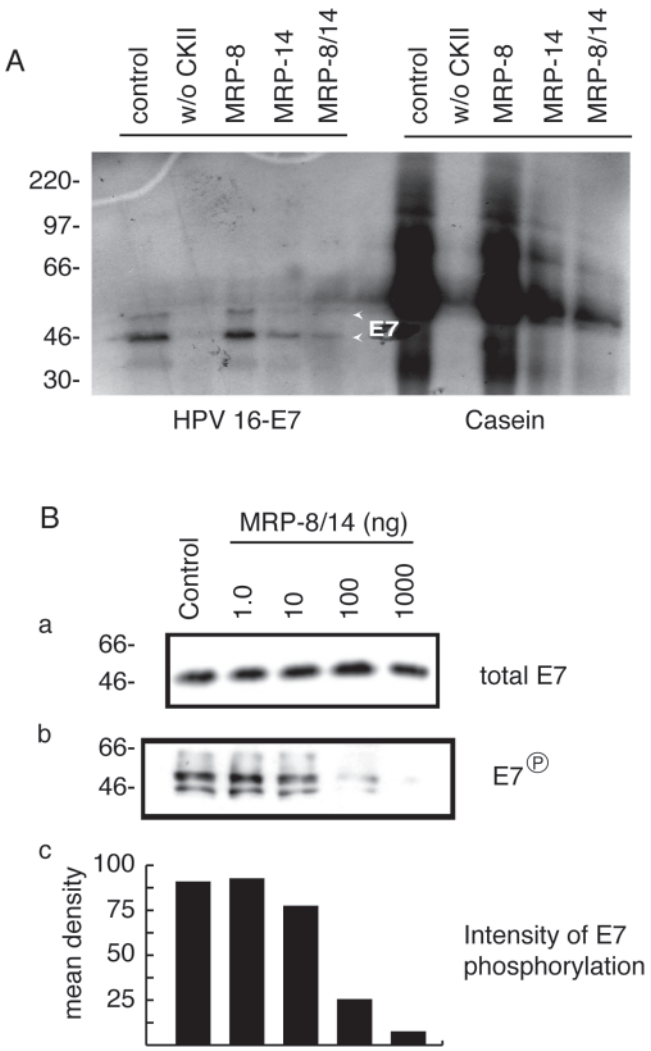

FIG. 3. Inhibition of HPV16 E7 phosphorylation by exogenous MRP-8/14. (A) HPV16 E7-GST phosphorylation was examined in an in vitro phosphorylation assay with CKII and [γ-^{32}P]ATP. Bovine casein was used as a positive control. One hundred nanograms each of MRP-8, MRP-14, and MRP-8/14 proteins was added into the reaction mixture for 30 min. The phosphorylated E7 and casein bands were visualized by exposing the gel to X-ray films. Control, E7 phosphorylation in absence of MRP proteins; w/o CKII, without CKII. (B) HPV16 GST-E7 phosphorylation was examined in an in vitro phosphorylation assay with nonradioactive ATP, CKII, and E7-GST in the presence of an increasing concentration of MRP-8/14 protein. (a) HPV16 E7-GST protein was detected by mouse MAb. (b) The phosphorylated E7 protein was detected by Western blot assay using rabbit polyclonal antiphosphoserine antibodies. (c) Intensity of E7 phosphorylation was measured by determining the mean density of pixels of phosphorylated E7 bands. Control, E7 phosphorylation in the absence of MRP-8/14.

MRP-8 was expressed in 10% of the cells, and MRP-14 and the MRP-8/14 complex were detected approximately in 1% of the cells. MRP expression was significantly increased when the HSC-3 cells were treated with the calcium ionophore A23187, phorbol ester, or dexamethasone. Time course analysis of MRP expression in an immunofluorescence assay after induction with 10 ng of dexamethasone/ml showed that MRP-8, MRP-14, and the MRP-8/14 complex were detectable in 20 to 30% of cells after 10 to 12 h of treatment of subconfluent cells. Expression of MRPs gradually increased and reached their highest levels within 4 days after induction, and this high-level expression was retained when cells reached 100% confluence at 5 days postseeding. The maximum number of cells expressing MRP-8 and MRP-14 individually was 60 to 80%, and the maximum number of cells expressing the MRP-8/14 complex was 50 to 60%. MRP-8 was found primarily in the cytoplasm, with small amounts in the nucleus. MRP-14 and the MRP-8/14 complex were found in the cytoplasm as well as the nucleus (Fig. 4A).

To detect MRPs in a Western blot assay, HSC-3 cells were treated with 10 ng of dexamethasone/ml for 4 days and then analyzed for MRP expression using appropriate MAbs. These experiments showed that approximately similar amounts of MRP-8, MRP-14, and the MRP-8/14 complex were detected in dexamethasone-treated HSC-3 cells (Fig. 4B). In contrast, in untreated HSC-3 cells only trace amounts of MRP-8 and no MRP-14 or MRP-8/14 complex were detected (data not shown). A Western blot time course analysis of MRP-8/14 induction by dexamethasone showed that after dexamethasone treatment MRP-8/14 expression gradually increased and reached the highest level within 4 days (Fig. 4C).

To evaluate the role of MRP-8/14 in CKII activity and E7 phosphorylation in vivo, HSC-3 cells were transiently transfected with HPV16 E7. To induce MRP-8/14 complex formation, the cells were treated with 10 ng of dexamethasone/ml, 10 ng of calcium ionophore/ml, or 10 μM apigenin. At 48 h posttransfection, MRP-8/14 and E7 expression (Fig. 5A and B, respectively), E7 phosphorylation (Fig. 5C), and CKII activity (Fig. 5E) were analyzed in the same samples. Together, these data showed that induction of endogenous MRP-8/14 was associated with inhibition of CKII activity and hypophosphorylation of E7 in vivo. Inhibition of E7 phosphorylation in MRP-8/14-induced cells was approximately 40 to 50%. Confocal immunofluorescence assays showed that MRP-8/14, CKII, and E7 proteins were colocalized in the nucleus, suggesting possible direct interactions between these proteins (Fig. 5F). Treatment of HPV16 E7-transfected but MRP-8/14-uninduced HSC-3 cells with exogenous MRP-8/14 also showed inhibition of CKII and phosphorylation of HPV16 E7 (data not shown).

Similar to the observation by Massimi et al. that E7 is differentially phosphorylated during the cell cycle at G_1 phase by CKII and at S phase by unidentified enzymes (32), we also observed the two highest peaks of E7 phosphorylation to be at G_1 and S phases in the HSC-3 cell line transfected with HPV16 E7 (data not shown). To study the role of MRP-8/14 in CKII-mediated E7 phosphorylation during the cell cycle, we examined E7 phosphorylation in HSC-3 cells in the presence of activated endogenous MRP-8/14. However, in our system MRP-8/14 expression was not significantly induced at the G_1 phase and therefore did not inhibit CKII-mediated phosphorylation of E7 at G_1 (Fig. 6A). During activation of the cell cycle, MRP-8/14 expression was gradually increased, and this correlated with gradually decreasing CKII activity. When cells entered into S phase, CKII activity declined substantially, but at this time point E7 phosphorylation reached a second peak, indicating that E7 phosphorylation at S phase was not mediated by CKII. In the next experiment we analyzed the role of exogenous MRP-8/14 in E7 phosphorylation during the cell

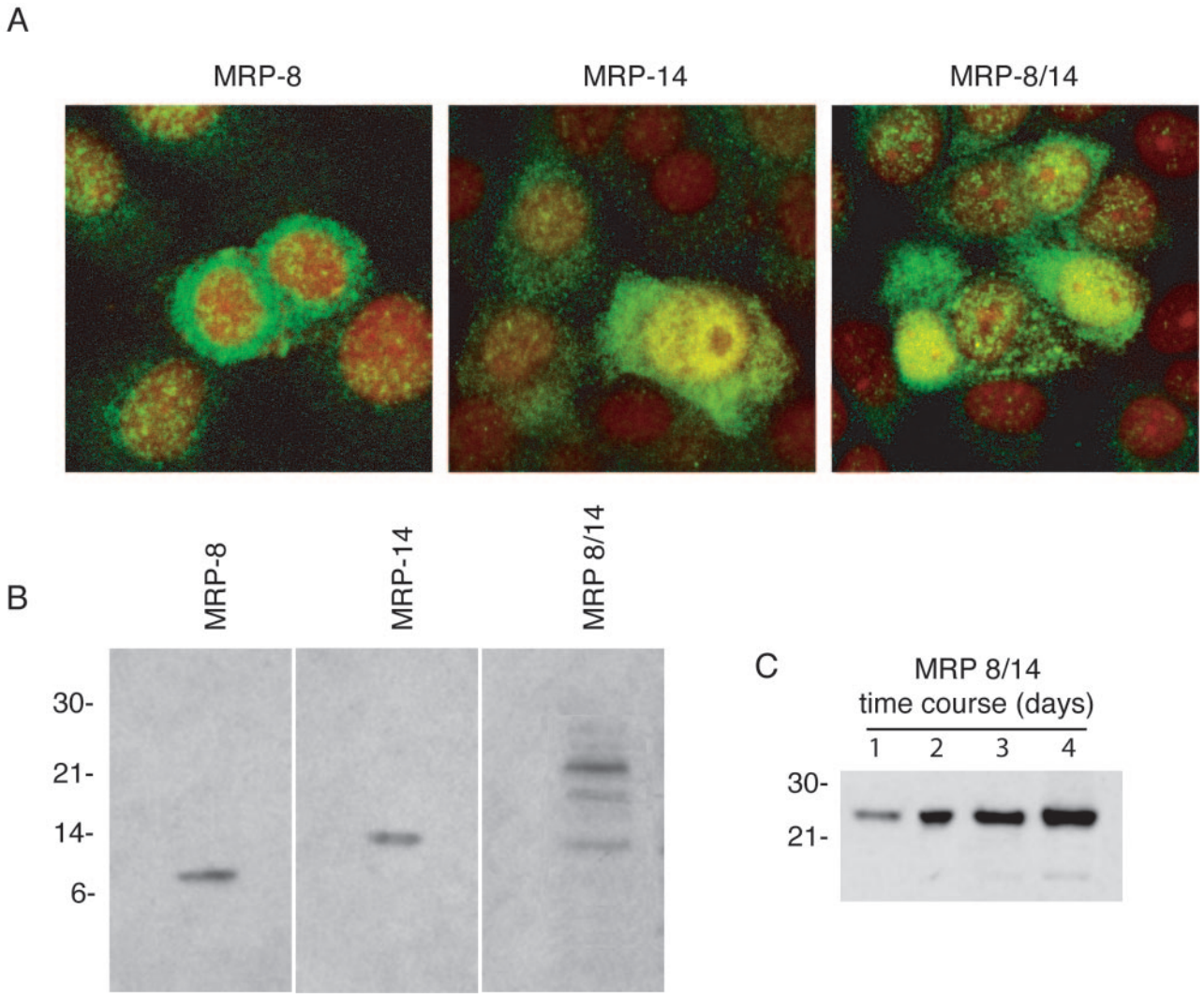

FIG. 4. Expression of MRPs in HSC-3 cells by confocal microscopy (A) and Western blot analysis (B and C). (A) Cells were grown on chamber slides and treated with dexamethasone for 4 days. For confocal microscopy analysis, cells were fixed and immunostained for MRPs with appropriate mouse MAbs (in green). The cell nuclei were stained with propidium iodide (PI; red). Yellow in the merged panels shows nuclear localization of MRPs. (B) For Western blot assays, approximately 10^6 HSC-3 cells were treated with 10 ng of dexamethasone/ml for 4 days and MRPs were detected using mouse MAbs. (a and b) Reducing; (c) nonreducing. (C) To analyze induction of MRP-8/14 expression in HSC-3 cells, approximately 3×10^6 cells were treated with 10 ng of dexamethasone/ml for 4 days. MRP-8/14 protein expression was examined in a Western blot assay under nonreducing conditions at 1, 2, 3, and 4 days postinduction.

cycle (Fig. 6B). This experiment showed that CKII activity was decreased by 45% in the G_1 phase, and this correlated well with a 90% inhibition of E7 phosphorylation. In contrast, inactivation of CKII at S phase did not affect the second peak of E7 phosphorylation. These data clearly demonstrate that exogenous MRP 8/14 protein complex specifically inhibits CKII-mediated E7 phosphorylation at the G_1 phase of the cell cycle and not at the S phase.

MRP expression in HPV-infected epithelial cell lines. Our data described above demonstrated that the MRP-8/14 protein complex inactivates CKII and inhibits phosphorylation of E7. Expression of MRP-8 and MRP-14 proteins in HPV-infected epithelial cells may therefore play an important role in E7 hypophosphorylation, with consequent reduction of its oncogenic potential. To examine the status of MRP-8 and MRP-14 proteins in HPV-infected cells, we analyzed MRP expression in HeLa, SiHa, and HOK-16 cell lines. Immunofluorescence (data not shown), Northern blotting (Fig. 7A), and Western blotting (Fig. 7B) assays showed that these cell lines did not express either MRP-8 or MRP-14. Treatment of these cells with dexamethasone or calcium ionophore did not induce MRP expression (data not shown). In contrast, HPV-negative primary oral (OCO), foreskin (NFK), and cervical (NCK) epithelial cells expressed both MRP-8 and -14 (Fig. 7A, B, D, and E). In parallel experiments, these cell lines were examined for CKII kinase activity. These data showed that MRP-8/14 expression in normal epithelial cells was associated with a low level of CKII activity, and their absence in HPV-infected immortalized epithelial cells was associated with a three- to five-fold-higher level of CKII activity than in primary cells (Fig. 7C and F).

We also compared MRP expression in HPV-negative (NFK)

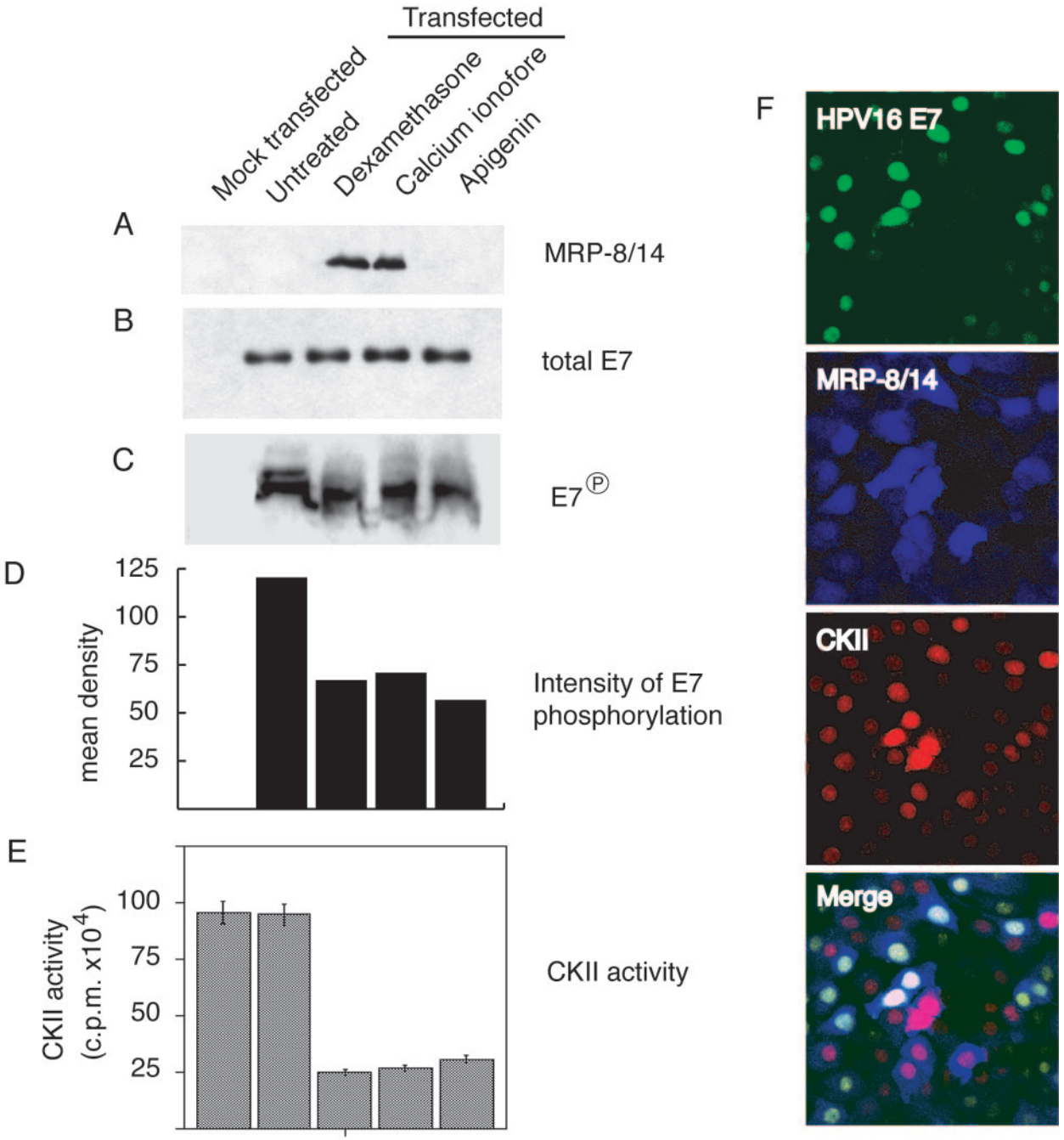

FIG. 5. Inhibition of HPV16 E7 phosphorylation in HSC-3 cells by endogenous MRP-8/14 protein complex. A total of 3×10^6 HSC-3 cells were transfected with HPV16 E7 and 8 h later were treated with 10 ng of dexamethasone/ml, 10 ng of calcium ionophore/ml, and 10 μM apigenin. (A and B) MRP-8/14 (A) and HPV16 E7 (B) were detected using a Western blot assay with appropriate mouse mAbs. (C) At 48 h posttransfection, HPV16 E7 was immunoprecipitated and E7 phosphorylation was examined in a Western blot assay with rabbit antiphosphoserine antibodies. (D) Intensity of E7 phosphorylation was measured by determining the mean density of pixels of phosphorylated E7 bands. (E) CKII activity was examined in an in vitro phosphorylation assay. The error bars indicate standard errors ($n = 3$). MRP-8/14 and E7 expression, E7 phosphorylation, and CKII activity were analyzed in the same samples. (F) Colocalization of MRP-8/14, E7, and CKII in HSC-3 cells. Cells were fixed and immunostained for CKII (red) and MRP-8/14 (blue). HPV16 E7 protein was detected by GFP (green). In the merge panel, white shows colocalization of MRP-8/14, CKII, and E7.

cells with the same cell line immortalized by HPV16 (16MT) cells of different passages to passage 100. Northern blotting (Fig. 7A), Western blotting (Fig. 7B), and immunofluorescence (Fig. 8A) analysis of the NFK cells showed expression of MRP-8 and MRP-14 and their MRP-8/14 complex, in contrast to the 16MT cells, which showed no expression of these proteins even at relatively early passages (passage 7 and up) (Fig. 8A). Analysis of CKII activity in parental NFK and immortal-

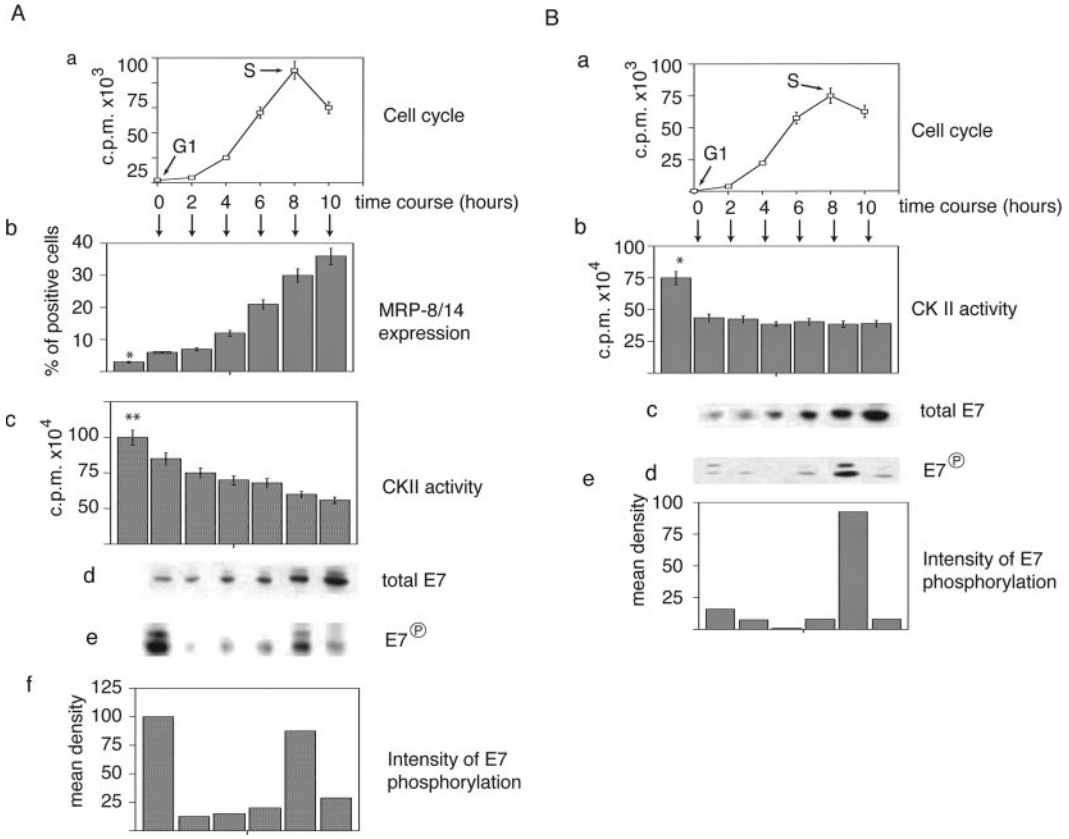

FIG. 6. MRP-8/14-mediated inhibition of HPV16 E7 during the cell cycle. (A) Inhibition of E7 phosphorylation by endogenous MRP-8/14 protein. HSC-3 cells were transfected with HPV16 E7, and 12 h later cells were growth arrested in 0.2% serum for the next 38 h. To induce MRP-8/14 expression at 24 h poststarvation, cells were treated with 10 ng of dexamethasone/ml. At 38 h posttransfection, cell growth was activated by adding 15% serum in the presence of 10 ng of dexamethasone/ml and 1 μCi of [^{3}H]thymidine/ml. At 2-h intervals after growth activation, the cell cycle (a), MRP-8/14 (b), CKII activity (c), total E7 expression (d), and E7 phosphorylation (e) were analyzed. *, MRP-8/14 expression in E7-transfected HSC-3 cells before MRP-8/14 induction; **, CKII activity in E7-transfected HSC-3 cells before MRP-8/14 induction. (B) Inhibition of E7 phosphorylation by exogenous MRP-8/14 protein. Experiments were performed as described above, but instead of induction of endogenous MRP-8/14 with dexamethasone, 3 μg of exogenous MRP-8/14/ml was added at 24 h posttransfection. At 2-h intervals after growth activation, the cell cycle (a), CKII activity (b), E7 expression (c), and E7 phosphorylation (d) were analyzed in the same samples. *, CKII activity in E7-transfected HSC-3 cells before adding MRP-8/14.

ized 16MT cells showed that CKII activity in 16MT cells was four- to fivefold higher than in the primary parental NFK cells (Fig. 8B).

To determine whether the loss of MRP-8/14 expression in HPV-infected cervical cancer cells was associated with HPV infection, we examined MRP-8/14 expression in the HPV-negative C33A cervical carcinoma cell line and normal primary cervical epithelial (NCK) cells. MRPs and their complex were expressed in NCK normal cervical epithelial cells, but not in the C33A cells (Fig. 7D and E). CKII kinase activity in C33A cells was higher than in the NCK cells, i.e., activation of CKII was correlated with loss of MRP-8/14 (Fig. 7F). Thus, absence of MRP expression in HPV-negative C33A cells indicated that loss of MRP expression was not specifically related to HPV infection.

Antiproliferative effect of MRPs on cancer cell lines. Inhibition of HPV16 E7 phosphorylation by the MRP-8/14 protein complex and its absence in HPV-associated tumor cell lines suggest that MRP-8/14 may play an important role in reduction of the oncogenic activity of E7. To study the antiproliferative effect of the MRP-8/14 complex, we examined the cell growth rate of the HPV-positive HeLa, SiHa, and Caski cell lines, as well as the HPV-negative C33A and NCK cervical epithelial cell lines, in the presence of exogenous MRP-8/14. As shown above, HeLa, SiHa, Caski, and C33A cell lines did not express MRP-8 and MRP-14 proteins and therefore did not form the MRP-8/14 complex. In contrast, NCK primary cervical epithelial cells did express MRP-8 and MRP-14 proteins and formed the MRP-8/14 complex. Cells were grown in the presence of purified MRP-8/14 at various concentrations ranging from 0.1 to 10 μg/ml for14 days. Concentrations higher than 3 μg of MRP-8/14 per ml were toxic for all cell lines, and cells began to die at 3 to 5 days after addition of MRP-8/14. At MRP-8/14 concentrations lower than 3 μg/ml, the HPV-negative and -positive cells remained morphologically healthy during the first 7 days. At the beginning of the second week the morphology of the HPV-positive cells at MRP-8/14 concentrations of 0.5 μg/ml and higher began to change. The cells detached from the substratum and began to die. At the end of the second week, almost 95% of these cells were dead (Fig. 9A). In contrast, HPV-negative NCK primary and C33A immortalized cervical epithelial cells were more resistant to the growth inhibitory

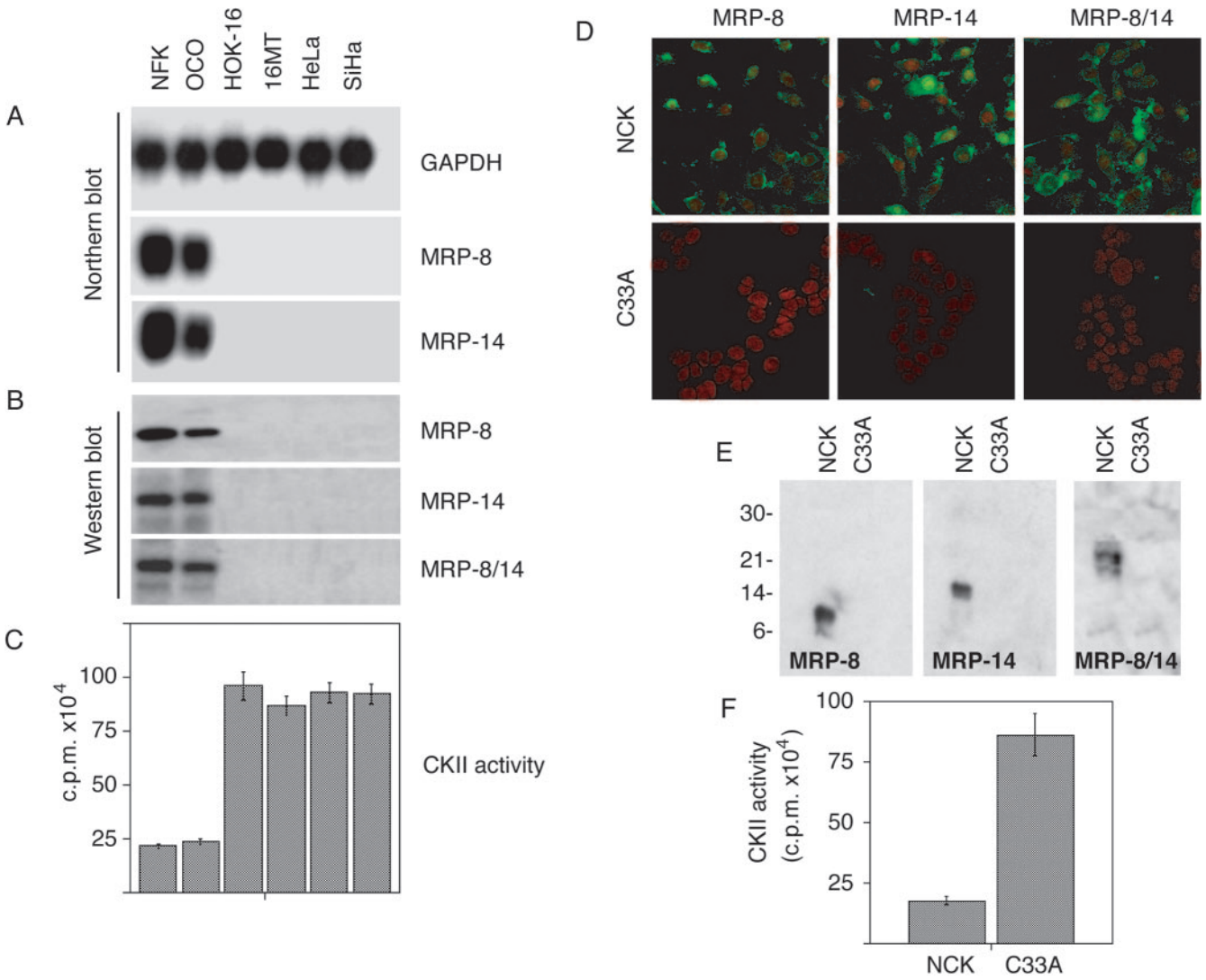

FIG. 7. Expression of MRPs and assessment of CKII activity in normal and tumor cell lines. MRP expression was examined in the HPV-negative primary NFK, OCO, and NCK and immortalized C33A cell lines, and in the following HPV-positive immortalized cell lines: 16MT (derived by HPV16-mediated transformation of NFK cells), HOK-16, HeLa, and SiHa cells. (A) For Northern blot assays, cellular mRNA was hybridized with probes to MRP-8 and MRP-14. A probe for GAPDH was used as a housekeeping gene control. (B and E) For Western blot assays, MRPs were separated under reducing (for MRP-8 and MRP-14) and nonreducing (for MRP-8/14 complex) conditions and immunoblotted with appropriate mouse MAbs to MRP-8, MRP-14, and MRP-8/14. (C and F) To measure CKII activity, CKII was immunoprecipitated and its kinase activity was measured in an in vitro phosphorylation assay. The error bars indicate standard errors ($n = 3$). (D) For the immunofluorescence assay, NCK and C33A cells grown on chamber slides were fixed and immunostained with MAbs to MRP-8, MRP-14, and MRP-8/14. The MRPs are shown in green, and the cell nuclei are red.

effect of MRP-8/14 than the HPV-positive cells. At a concentration of 1.5 to 2 μg of MRP-8/14 per ml, detachment and death of the HPV-negative cells was moderate, i.e., about 15 to 20% cells were dead. At a similar concentration of MRP-8/14, almost 90% of HPV-positive cells were dead. Substantial cell death, i.e., 25 to 40% of HPV-negative NCK and C33A cells, respectively, was seen only at concentrations of MRP-8/14 of 2.5 and 3 μg/ml (Fig. 9A). Continuous treatment of HPV-negative primary cervical NCK and tumor C33A cells with exogenous MRP-8/14 at concentrations of 1.5 μg/ml and above for 3 weeks completely inhibited their growth, indicating that high concentrations of exogenous MRP-8/14 were cytostatic, even for the HPV-negative cells.

To determine whether the antiproliferative effect of MRP-8/14 in HPV-infected cells was linked to E7 hypophosphorylation, we examined E7 phosphorylation in MRP-8/14-treated SiHa and Caski cell lines infected with HPV16 (Fig. 9B). Cells were treated with 1, 2, and 3 μg of MRP-8/14 per ml for 7 days, and E7 phosphorylation was examined. These experiments showed that MRP-8/14 inhibited the steady-state level of phosphorylation of HPV16 E7 in SiHa and Caski cells in a dose-dependent manner, similar to the dose-dependent antiproliferative effect (Fig. 9B). Treatment of E7 with λ protein phosphatase almost completely dephosphorylated it (Fig. 9C), showing high efficiency of phosphorylation of E7 in these cells.

Removal of MRP-8/14s from the culture medium of HPV-

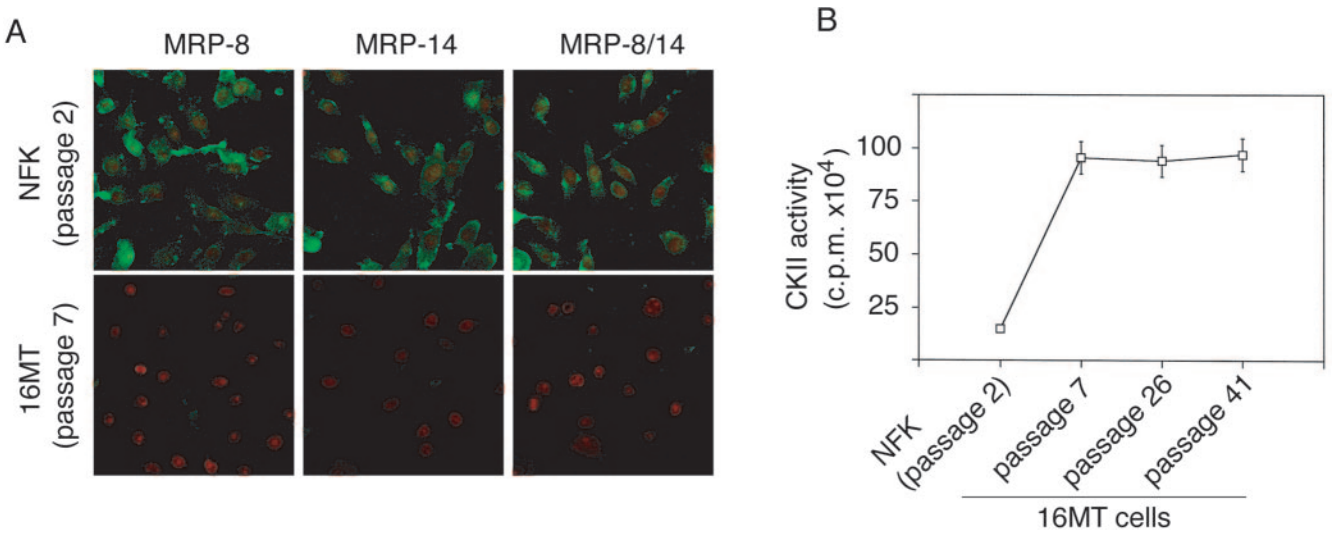

FIG. 8. Analysis of MRP expression and CKII activity in primary foreskin keratinocyte NFK cells and the HPV16-immortalized 16MT cell line. (A) For immunofluorescence assays, cells were fixed and immunostained with MAbs to MRP-8, MRP-14, and MRP-8/14. The MRPs are shown in green, and the cell nuclei are red. (B) CKII was immunoprecipitated, and its kinase activity was measured in an in vitro phosphorylation assay. The error bars indicate standard errors ($n = 3$).

positive and -negative cells after 7 to 10 days of MRP treatment resulted in the recovery of live cells. Cells in fresh medium without MRP-8/14 began to grow again, indicating that the cell growth inhibitory effect of MRP-8/14 was reversible in HPV-positive and -negative cell lines. However, recovery of cell growth in HPV-negative cells was faster than for HPV-positive cells. C33A cells took approximately 5 to 8 days to recover, while recovery of HeLa, SiHa, and Caski cells took approximately 2 to 3 weeks. These results indicate that MRP-8/14 may have a stronger antiproliferative effect in HPV-positive cells than in HPV-negative cells.

DISCUSSION

The MRP-8/14 complex has been shown to inhibit CKII (38). Since we have shown that the MRP-8/14 complex is expressed in normal epithelial cells that may be a target for HPV infection, we investigated the role of the MRP-8/14 protein complex in CKII inactivation and HPV16 E7 phosphorylation. The results presented here demonstrate that the MRP-8/14 protein complex inhibits HPV16 E7 phosphorylation in vivo and in vitro. MRP-8/14 inhibited CKII activity, which in turn was correlated with inhibition of E7 phosphorylation. MRP-14 alone also showed an inhibitory effect on CKII activity and E7 phosphorylation in vitro. However, MRP-14 did not show a significant inhibitory effect on CKII activity in vivo, possibly due to inefficient uptake. The exact mechanism by which MRP-14 or MRP-8/14 inactivates CKII is not yet clear. Murao et al. showed that MRP-8/14 inhibits only CKI/II but not other enzymes, including cyclic AMP-dependent protein kinase, PKC, v-abl tyrosine kinase, or insulin receptor protein kinase (38). Our data also confirmed that MRP-8/14 inhibited CKII but not PKC or CaM kinase II, indicating that MRP-8/14's inhibitory effect on CKII was highly specific.

Massimi and Banks (32) showed that CKII differentially phosphorylates E7 during the cell cycle; the highest level of E7 phosphorylation occurs during the G_1 phase at position Ser31/32 by CKII, and once cells enter S phase E7 phosphorylation decreases dramatically. However, E7 is phosphorylated again in S phase by an unknown kinase at position Ser71, and the functional significance of this phosphorylation is not understood. Analysis of E7 phosphorylation during the cell cycle in the presence of exogenous MRP-8/14 showed that MRP-8/14-mediated inhibition of E7 phosphorylation occurs at the G_1 phase of cell cycle. Activation of endogenous as well as uptake of exogenous MRP-8/14 did not inhibit E7 phosphorylation in S phase, which occurs through a protein kinase other than CKII. This may explain why we observed only 40 to 50% inhibition of the steady-state level of E7 phosphorylation in HSC-3 cells exposed to MRP-8/14. MRP-8/14-mediated hypophosphorylation of HPV16 E7 during transition from G_1 into S phase may play an important role in reducing E7's activation of cell cycle progression.

It is possible that MRP-8/14-mediated CKII inactivation and E7 hypophosphorylation in vivo may be modulated by other cellular proteins that interact with CKII and/or E7. For example, the S100A4 protein mst1 interacts with the regulatory β-subunit of CKII and inhibits CKII-mediated phosphorylation of nonmuscle myosin heavy chain (26). The $p21^{WAF1/CIP1}$ inhibitor of cyclin-dependent kinases also interacts with the β-subunit of CKII (19) and down-regulates the kinase activity of its α-subunit, and thereby it inhibits CKII-mediated phosphorylation of casein and p53 (19). Therefore, the potential intracellular interaction of MRP-8/14 with CKII may occur in the presence of other proteins, such as mst1 and $p21^{WAF1/CIP1}$, and hence its anti-E7 phosphorylation function may be regulated by those and perhaps by other proteins that interact with CKII and E7.

Absence of MRP expression was correlated with activation of kinase activity of CKII. In HPV-infected MRP-negative immortalized cell lines, CKII activity was four- to fivefold higher than in normal epithelial cells that expressed MRP-8 and

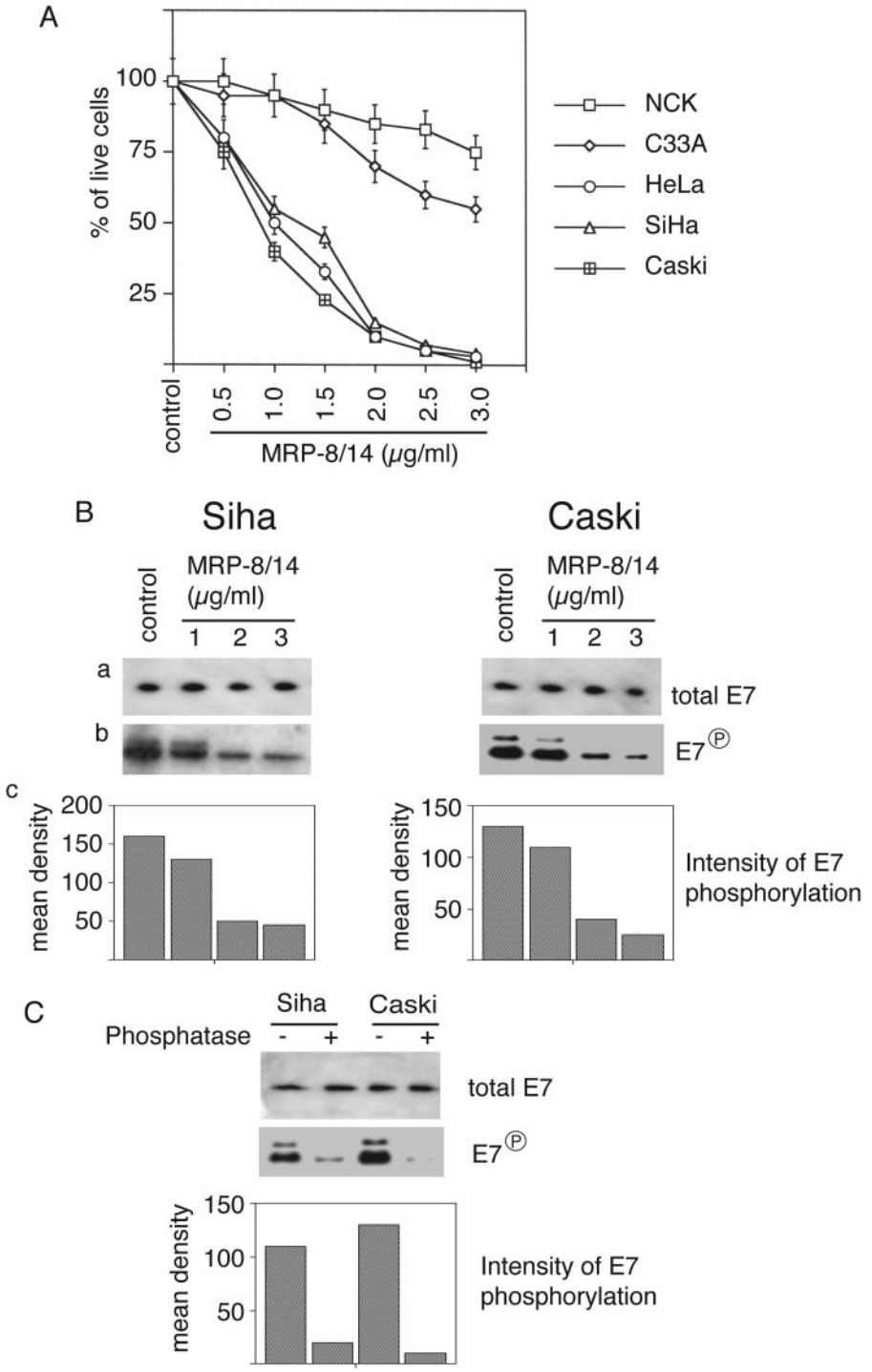

FIG. 9. Antiproliferative and anti-E7 phosphorylation activity of the MRP-8/14 protein complex. (A) Antigrowth activity of MRP-8/14 in HPV-positive and HPV-negative cervical cell lines. Cells were grown in the presence of the MRP-8/14 protein complex at various concentrations for 14 days. The number of live cells was quantified with trypan blue staining at day 14. Similar results were obtained in three independent experiments. The error bars indicate standard errors; $n = 3$. Control, live cells without treatment. (B) Inhibition of HPV-16 E7 phosphorylation by MRP-8/14 in HPV16-infected SiHa and Caski cell lines. Approximately 5×10^6 cells were grown in the presence of 1, 2, and 3 μg of exogenous MRP-8/14 per ml. (a) At 5 days posttreatment, cells were extracted and E7 was immunoprecipitated with mouse MAb and E7 was detected by Western blotting. (b) E7 phosphorylation in the same samples was examined using rabbit antiphosphoserine antibodies. (c) Intensity of E7 phosphorylation was measured by the mean density of pixels in the E7 phosphorylated bands. (C) Dephosphorylation of immunoprecipitated E7 by λ phosphatase treatment. Control (all panels), untreated cells.

MRP-14 proteins. Treatment of HPV-infected MRP-negative cells with exogenous MRP-8/14 complex led to inhibition of CKII activity in HPV16- and HPV18-infected cervical epithelial cells. Activation of endogenous MRP-8/14 also inhibited CKII activity and HPV16 E7 phosphorylation in HPV-negative HSC-3 oral squamous epithelial tumor cells. These data indicate that MRP-8/14 may play a role as a strong intracellular factor that may negatively regulate the activity of CKII.

It has been shown that treatment of various normal and transformed cell lines with 50 to 200 μg of MRP-8/14 per ml within 18 to 48 h leads to inhibition of DNA synthesis and cell growth (62–64). The minimum effective concentration to inhibit cell growth was approximately 50 μg/ml. We have shown here that prolonged uptake of a much lower concentration (1 to 10 μg/ml) of MRP-8/14 for 7 to 14 days by HPV-positive and HPV-negative tumor cells, as well as normal epithelial cells, caused detachment of cells from the substratum and inhibited their growth. CKII is a highly pleiotropic enzyme that phosphorylates more than 160 cellular proteins with a wide variety of functions, with consequent effects on gene expression, protein synthesis, cell cycling, and differentiation (15, 30). Therefore, the antiproliferative effect of MRP-8/14 during prolonged treatment may be due to its inactivation of CKII, which is required for cell proliferation and cell viability in both normal and cancer cells. However, MRP-8/14 more strongly inhibited growth of HPV-positive cells than HPV-negative cells, a difference that may have been due to their effect on E7. Our data therefore suggest that MRP-8/14-mediated CKII inactivation may lead to two groups of downstream antiproliferative effects. The first group of effects is more general, reflecting inhibition of phosphorylation of CKII substrates, i.e., housekeeping proteins that are required for cell proliferation and viability. This kind of antiproliferative effect occurred in both HPV-positive and HPV-negative cells. The second group of antiproliferative effects is more specific to HPV-positive cells, and it is possible this could be due to inhibition of HPV16 E7 phosphorylation.

Our data leave several questions open to future investigation. The mechanisms by which CKII inhibition leads to inhibition of cell growth are not fully understood, nor is the extent to which inhibition of E7 phosphorylation mediates the HPV-specific effects. While our data are consistent with a key role for E7 phosphorylation, the downstream effects of MRP-8/14-mediated inhibition of E7 phosphorylation are not clear. Storey et al. showed that substitution of one of the two serine residues of the E7 CKII site, Ser31 or Ser32, only slightly decreased its ability to cooperate with the EJ-ras oncogene to transform primary baby rat kidney cells (53). Barbosa et al. also showed that mutation of one of the two serines did not show any significant biological activity (3). However, simultaneous mutation of both serines impaired or significantly reduced its transforming activity (3, 18), suggesting that phosphorylation of both serines, Ser31 and Ser32, may be necessary for E7's full transforming activity.

The best-characterized E7 ligand is pRb, but the role of CKII-mediated E7 phosphorylation in its interaction with pRb and pRb-associated proteins is not understood. Using in vitro binding assays, it has previously been shown that mutation in the CKII phosphorylation site of E7 Ser 31/32 did not affect its binding to pRb (3, 18). However, it is not clear how well these in vitro binding assays reflect the more complex intracellular environment. Moreover, since inhibition of transformation occurred with mutation of both serines despite the lack of change in pRb binding (3, 18), it is also likely that the E7-pRb interaction is not the only mechanism for E7-mediated transformation or stimulation of cell cycling (6, 24, 45). Consistent with this, MRP-8/14-mediated CKII inactivation and E7 hypophosphorylation in vivo may affect other pRb indirect and/or pRb-independent cell cycle regulatory pathways.

E7 phosphorylation of high-risk HPV types may be involved in E7-mediated degradation of pRB (25). Phosphorylation of HPV16 E7 has been shown to increase the binding affinity of E7 to the basic subunit of the TFIID complex, the TATA box-binding protein (TBP), and TBP-associated factor 110 (TAF-110) protein (31, 34, 35, 36). E7 binding to F-actin requires HPV16 E7 phosphorylation (43), and HPV16 E7 phosphorylation may play a role in E7 interaction with p53 (33). A CKII phosphorylation-defective HPV16 E7 mutant severely impaired its ability to induce tetrasomy in primary foreskin keratinocytes, suggesting that E7 phosphorylation may be involved in induction of genetic instability (50). Taken together, these data indicate that CKII-mediated E7 phosphorylation may be critical for its oncogenic functions and suggest that MRP-8/14-mediated inhibition of CKII may affect HPV-induced cell cycling through multiple mechanisms.

In summary, we have found that the S100 calcium-binding protein complex MRP-8/14 inhibits CKII-mediated HPV16 E7 phosphorylation in vivo and in vitro. We showed MRP expression in normal primary epithelial cells in culture, with loss of their expression in several HPV-positive cervical cancer cell lines. The mechanisms of loss of MRP-8 and -14 protein expression in tumor cells are not well understood but may occur during the multistep cell transformation process. Consistent with this, loss of MRP expression was observed in 16MT cells during the process of HPV-induced immortalization. MRP-8/14 protein expression was detected only in parental cells, and once the primary keratinocytes were immortalized with HPV16 MRP-8/14 expression was lost and CKII activity was elevated. However, absence of MRP expression in HPV-negative C33A cervical carcinoma cells indicated that inactivation of MRP expression was not specifically due to HPV infection. Activation of CKII has been reported in tumor cells of different origins (11, 16, 29, 51, 58, 61), and mechanisms of its activation, particularly the role of MRP-8/14 in its regulation, are not well understood. It is possible that loss of MRP expression and elevation of CKII activity in high-risk HPV-infected precancerous lesions may lead to higher levels of phosphorylation of E7, increasing E7 oncogenic activity and possibly progression of HPV-associated neoplasia.

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