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Karyopherin β3: A new cellular target for the HPV-16 E5 oncoprotein

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ABSTRACT

Epidemiological and experimental studies have shown that high-risk human papillomaviruses (HPVs) are the causative agents of cervical cancer worldwide, and that HPV-16 is associated with more than half of these cases. In addition to the well-characterized E6 and E7 oncoproteins of HPV-16, recent evidence increasingly has implicated the HPV-16 E5 protein (16E5) as an important mediator of oncogenic transformation. Since 16E5 has no known intrinsic enzymatic activity, its effects on infected cells are most likely mediated by interactions with various cellular proteins and/or its documented association with lipid rafts. In the present study, we describe a new cellular target that binds to 16E5 in COS cells and in stable human ectocervical cell lines. This target is karyopherin β3, a member of the nuclear import receptor family with critical roles in the nuclear import of ribosomal proteins and in the secretory pathway.

High-risk human papillomaviruses (HPVs), the causative agents of cervical cancer, are considered a major health threat worldwide and represent one of the most common causes of cancer in women [1,2]. Out of 120 different HPV types that have been isolated, more than 40 infect the genital mucosa [3]. Some of these genital HPVs are low-risk (e.g. HPV-6 and -11) and are associated primarily with non-malignant lesions. However, there are 12–15 high-risk HPVs that have been linked to cervical cancer [1–4]. HPV-16 alone is responsible for approximately half of all cervical cancers [2,5,6].

Three HPV-16 genes possess activities that promote malignant transformation: E6, E7, and E5 (16E6, 16E7, and 16E5). 16E6 and 16E7 cooperate to induce cellular immortalization. The main role of 16E6 is to bind to the tumor suppressor protein p53 and facilitate its ubiquitin-mediated degradation. One of the primary functions of 16E7 is to inactivate the retinoblastoma tumor suppressor protein [4,7–9].

In contrast to 16E6 and 16E7, the biological functions of 16E5 are rather poorly understood [7]. 16E5 is a small (83 amino acids) hydrophobic protein that localizes to membranes of the endoplasmic reticulum (ER) [10,11]. It exhibits weak transforming activity *in vitro* [7,12], but can induce malignant tumors in transgenic mice [13]. 16E5 also is believed to play a role in immune evasion by HPV-16-infected cells [14]. Importantly, since 16E5 has no innate enzymatic activity, its ability to alter cellular function most likely relies on its association with numerous proteins and/or lipid rafts [14] of the host cell. Documented intracellular binding targets for 16E5 include the 16 kDa pore-forming subunit of the vacuolar H^+-ATPase [10,15], the heavy chain of HLA type I [16] and ErbB4, a member of the epidermal growth factor (EGF) receptor family [17]. In this study we identify a new cellular target for 16E5, karyopherin β3 (KNβ3). KNβ3 is a member of the nuclear import receptor superfamily [18–21], which includes more than 20 members in humans and 14 in yeast. These proteins regulate the nuclear import of various cargo, including ribosomal proteins, histones and viral proteins; however, they may also act as cytoplasmic chaperones and as microtubule motor adaptors [20]. KNβ3 localizes mainly to the cytoplasm, but also binds to some nucleoporins and can therefore be visualized in the nuclear envelope by immunostaining [18,22].

Materials and methods

Cells and viruses. Retroviruses encoding either HPV-16 E6 and E7 genes in the vector pBabePuro [23], or encoding codon-optimized 16E5 or a C-terminal deletion mutant of codon-optimized 16E5 lacking the last 10 amino acids (16E5 (-10)) in the vector pLXSN [24], were generated using the Phoenix cell system [25]. The cloning of codon-optimized 16E5 into the pJS55 expression vector has been described previously [11,26]. 16E5 (-10) was cloned into the BamHI restriction site of pJS55. Both 16E5 proteins were N-terminally tagged with the AU1 epitope, DTYRYI [27]. Primary human ectocervical cells (HECs) were derived from cervical tissue after hysterectomy for benign uterine disease as described [28], and were immortalized by infection with an HPV-16 E6/E7-encoding retrovirus and selection in the presence of puromycin (0.5 μg/ml). Stable 16E5-expressing cell lines were generated from immortalized HECs by infection with retroviruses encoding 16E5, 16E5

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(-10) or the empty pLXSN expression vector, and selection in the presence of geneticin G418 (100 μg/ml).

HEC lines were grown at 37 °C and 5% CO_2 in keratinocyte growth medium (KGM; Invitrogen, Carlsbad, CA), supplemented with gentamicin sulfate (10 μg/ml). To maintain constant levels of 16E5 and 16E5 (-10) expression, G418 was administered to cell cultures at alternating passages. COS cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen).

Transfection. Prior to transfection, COS cells were grown to 90% confluence in 10-cm or 15-cm tissue culture dishes, or on 22 × 22 mm sterile glass cover slips in six-well tissue culture plates, in antibiotic-free Opti-MEM (Invitrogen) containing 4% FBS. For each 10-cm dish, 15-cm dish, or cover slip, 32, 72, or 4 μg DNA (in pJS55) was mixed with 4, 9, or 0.5 ml serum-free Opti-MEM and 40, 90, or 5 μl Lipofectamine 2000 transfection reagent (Invitrogen) and added to the cells for 4 h.

Isolation and microsequencing of 16E5-associated proteins. Tissue culture dishes (15 cm) of COS cells were transfected with 16E5 or 16E5 (-10) in pJS55, as described above. For each DNA, eight dishes were placed on ice 24 h after transfection and washed with 25 ml/dish phosphate-buffered saline (PBS). Cells were scraped into 2.0 ml/dish modified RIPA buffer [29] and disrupted by vigorous shaking for 15 min at 4 °C. The protein concentration of lysates was determined using the Bio-Rad (Hercules, CA) DC protein assay with bovine IgG as the standard. Aliquots of lysates containing 20 mg protein were mixed with 400 μl of 50% (v/v) protein A–sepharose beads (ImmunoPure Plus; Pierce, Rockford, IL) and 40 μl of mouse anti-AU1 ascites (Covance, Princeton, NJ) and were rotated end-over-end for 90 min at 4 °C. Subsequently, the beads were washed 3 times with 5 ml RIPA buffer (5 min rotating end-over-end per wash) and 3 times with 5 ml PBS. Bound proteins were eluted in SDS lysis buffer [30] by incubating 15 min at 37 °C and 20 min at 110 °C. The SDS eluant was mixed with 0.1 volume of 20% (w/v) Triton X-100, 500 μl of 50% (v/v) protein A–sepharose beads and 400 μg of Pierce unconjugated rabbit anti-mouse IgG (H+L); and was rotated end-over-end overnight at 4 °C. About 75 μg of *E. coli* RNA was added to the unadsorbed fraction and proteins were precipitated with 10% trichloroacetic acid (TCA) for 1 h on ice. The precipitates were centrifuged for 5 min at 8000*g* at 4 °C and extracted with 800 μl of (95% acetone, 0.1 N HCl). Extracted TCA precipitates were solubilized in 50 μl of 2× Laemmli SDS–PAGE sample buffer (125 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol) for 20 min at 37 °C and 6 min at 110 °C. To maintain pH, 3 μl of 1 M Tris–HCl (pH 8.0) was added. Electrophoresis was performed in 1.5-mm polyacrylamide Tris–glycine mini-gels (Invitrogen). After electrophoresis, gels were washed 3 times with deionized H_2O, stained for 3 h with GelCode Blue Stain Reagent (Pierce) and rinsed with several changes of deionized H_2O over a period of 18 h. Bands of interest were excised for microsequencing by ProtTech, Inc (Eagleville, PA).

Metabolic labeling. Dishes (10 cm) of 80% confluent COS cells were washed with PBS (15 ml per dish) and incubated for 2 h in DMEM without cysteine and methionine (4 ml per dish; BioSource International, Carlsbad, CA). [^{35}S] methionine/[^{35}S] cysteine protein labeling mix (Perkin-Elmer "Easy Tag", Waltham, MA) was added (200 μCi per dish) for 3 h and the cells lysed in modified RIPA buffer.

Immunoprecipitation and immunoblotting. AU1 epitope-tagged 16E5 proteins were detected in RIPA lysates by immunoprecipitation and Western blotting [29], using 4 μg rabbit anti-AU1 polyclonal antibody for immunoprecipitation, and 1:5000-diluted mouse anti-AU1 ascites for immunoblotting (Covance). KNβ3 was detected in RIPA lysates using 4 μg Santa Cruz Biotechnology (Santa Cruz, CA) anti-KNβ3 rabbit polyclonal or mouse monoclonal antibodies for immunoprecipitation, and 1:200-diluted rabbit polyclonal or mouse monoclonal antibodies for immunoblotting, as described [30,31].

Confocal microscopy. HECs and COS cells were grown on 22 × 22 mm sterile glass cover slips in six-well tissue culture plates, fixed in 4% (w/v) paraformaldehyde and labeled as described [29], except that Alexa Fluor-conjugated donkey secondary antibodies (Molecular Probes, Carlsbad, CA) were used at a concentration of 5 μg/ml. The following primary antibodies were used: Covance anti-AU1 mouse monoclonal (1:7000 dilution of ascites) and Santa Cruz anti-KNβ3 rabbit polyclonal antibody (1:150 dilution). HECs were cultured in keratinocyte serum-free medium (KSFM; Invitrogen) for approximately 24 h prior to fixation to increase flatness. A Zeiss Axiovert confocal microscope was used for visualization and microphotography.

Results

To identify new potential targets for the 16E5 oncoprotein, COS cells were transfected with DNA encoding AU1 epitope-tagged wild-type (wt) 16E5 or a 16E5 mutant lacking the C-terminal 10 amino acids (16E5 (-10)) that is defective for altering endosomal pH [24] and enhancing ligand-dependent EGF receptor activation [12,15]. The cells were then labeled with [^{35}S] methionine and immunoprecipitated with an anti-AU1 antibody to isolate 16E5 and associated proteins. A unique 116 kDa polypeptide was found to be associated only with wt 16E5, and not with 16E5 (-10) (Fig. 1A, upper panel), even though both 16E5 proteins were expressed at similar levels (Fig. 1A, lower panel). To identify the 116 kDa polypeptide, 16E5 was immunoprecipitated from 20 mg of cell lysate, and the associated 116 kDa band excised from Coomassie blue-stained SDS–PAGE for microsequencing (Fig. 1B). As shown in Fig. 1C, 17 proteolytic fragments of the 116 kDa protein exactly match the amino acid sequence of human KNβ3.

To verify the binding interaction between 16E5 and KNβ3, COS cells were transfected with 16E5 or 16E5 (-10) DNA, and were immunoprecipitated with antibodies recognizing KNβ3. The immunoprecipitates were then analyzed on Western blots that were labeled for AU1 or KNβ3. The results clearly demonstrate an association between KNβ3 and wt 16E5, but not between KNβ3 and 16E5 (-10) (Fig. 2A, upper panel), even though equivalent amounts of KNβ3 are present in all immunoprecipitates (Fig. 2A, lower panel). A strong association of KNβ3 and 16E5 was also detected, as well as weak binding of KNβ3 to 16E5 (-10) when the AU1 antibody was used for immunoprecipitation and KNβ3 antibody was used for immunoblotting (Fig. 2B, upper panel). Both wt and mutant 16E5 proteins were present at similar levels in these immunoprecipitates (Fig. 2B, lower panel).

To verify the association of KNβ3 and 16E5 in a natural HPV host cell in the presence of other HPV-16 early genes, we performed similar immunoprecipitation/immunoblot experiments using 16E6/16E7-immortalized human ectocervical cell (HEC) lines that stably express wt and mutant 16E5 proteins [14]. A strong association between wt 16E5 and KNβ3, and slight association between 16E5 (-10) and KNβ3, was observed when the AU1 antibody was used for immunoprecipitation and KNβ3 antibody for immunoblotting (Fig. 2C, upper panel). Again, both 16E5 proteins were expressed at similar levels in the HECs (Fig. 2C, lower panel). However, we were unable to detect 16E5 in anti-KNβ3 immunoprecipitates of HECs, possibly due to the difference of 16E5 protein levels in HECs and in COS cells.

To further characterize the interactions of these proteins, we localized 16E5 and KNβ3 in COS cells and HECs using immunofluorescence confocal microscopy. KNβ3 shows a typical cytoplasmic and perinuclear staining pattern [18,22] in both cell types (Fig. 3A and B, left column), while 16E5 exhibits staining typical for ER-