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Nuclear import of high risk HPV16 E7 oncoprotein is mediated by its zinc-binding domain via hydrophobic interactions with Nup62

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ABSTRACT

We previously discovered that nuclear import of high risk HPV16 E7 is mediated by a cNLS located within the zinc-binding domain via a pathway that is independent of karyopherins/importins (Angeline et al., 2003; Knapp et al., 2009). In this study we continued our characterization of the cNLS and nuclear import pathway of HPV16 E7. We find that an intact zinc-binding domain is essential for the cNLS function in mediating nuclear import of HPV16 E7. Mutagenesis of cysteine residues to alanine in each of the two CysXXCys motifs involved in zinc-binding changes the nuclear localization of the EGFP-16E7 and 2xEGFP-16E7 mutants. We further discover that a patch of hydrophobic residues, $_{65}$**LRLCV**$_{69}$, within the zinc-binding domain of HPV16 E7 mediates its nuclear import via hydrophobic interactions with the FG domain of the central channel nucleoporin Nup62.

Introduction

Human Papillomaviruses (HPVs) are estimated to be the most common sexually transmitted infection in the United States. The largest group of HPVs is the alpha HPVs, and consists of both cutaneous viruses causing common warts, as well as the approximately 40 mucosal types known to infect the cervical epithelium. Mucosal HPVs are further divided into high and low risk groups, dependent upon the frequency with which they have been linked to the malignant progression of their resultant lesions (Doorbar, 2006; zur Hausen, 2000, 2009). Fifteen of the sexually transmitted genital HPVs can be classified as high risk, notably HPV16, HPV18, HPV31, HPV33 and HPV45, which may result in squamous intraepithelial lesions capable of progressing to invasive carcinomas (Doorbar, 2006; Munger et al., 2004; zur Hausen, 2000, 2009). Nearly 99% of cervical cancers and 20% of oropharynx cancers are positive for high risk HPV DNA, with HPV16 found in nearly 63% of the cervical cancers (Doorbar, 2006).

HPVs are dependent on the replication machinery of the host cells and consequently they have evolved the E7 oncoproteins to induce reentry into the S phase of the differentiated epithelial cells and establish the appropriate environment required to support viral DNA amplification. To accomplish this, the high risk HPV16/18 E7 oncoproteins bind and destabilize the retinoblastoma protein (pRB) and the RB-related pocket proteins, p130 and p107. E7 oncoproteins interact also with other components of the cell cycle machinery, such as cyclin A, cyclin E and the cyclin-dependent kinase inhibitors p27 and p21 (Jones and Munger,1996; Zwerschke and Jansen-Durr, 2000; McLaughlin-Drubin and Munger, 2009). In addition to these nuclear target proteins, HPV16 E7 binds to targets in the cytoplasm, such as the microtubule-associated N-end rule ubiquitin ligase p600 (Huh et al., 2005) and the nuclear mitotic apparatus protein (NuMA) (Nguyen and Munger, 2009).

Structurally, the E7 proteins consist of three biochemically distinct domains. Both NMR and X-ray crystal structures have been solved for the C terminal domain of E7, and the three-dimensional structure has been shown to organize into a tightly packed zinc-binding fold (McLaughlin-Drubin and Munger, 2009). The N terminus conserved regions (CR) CR1 (aa1–15) and CR2 (aa16–37) of HPV16 E7 possess significant sequence similarity to a portion of CR1 and the entirety of CR2 of adenovirus E1A and related sequences of SV40 large tumor antigen, as well as functional similarity, contributing to the transforming ability of HPV16 E7. The CR2 domain contains both the Leu–X–Cys–X–Glu (LxCxE) pRB binding motif as well as a consensus casein kinase II (CKII) phosphorylation site. The C-terminal CR3 domain (aa38–98) contains a zinc-binding domain with two Cys–X–X–Cys motifs separated by 29 amino acids (McLaughlin-Drubin and Munger, 2009).

High risk HPV16 E7 is predominantly nuclear in the CaSki cervical carcinoma cell line, when expressed transiently in HaCaT

Abbreviations: HPV, human papillomavirus; NLS, nuclear localization signal; NES, nuclear export signal; NPC, nuclear pore complex; EGFP, enhanced green fluorescent protein; GST, glutathione-S-transferase

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and U2OS cells, and in invasive cervical carcinoma (Guccione et al., 2002; Fiedler et al., 2004; Cid-Arregui et al., 2003). We have previously discovered that nuclear import of HPV16 E7 and HPV11 E7 is mediated by a Ran-dependent pathway that is independent of karyopherins/importins and it is mediated by their cNLS located within the unique zinc-binding domain (Angeline et al., 2003; Knapp et al., 2009; Piccioli et al., 2010). Both HPV16 E7 and HPV11 E7 proteins have also a leucine-rich nuclear export signal (NES) located within the zinc-binding domain that mediates their nuclear export via a CRM1 pathway (Knapp et al., 2009; McKee et al., 2013).

Nuclear import and export of different cargoes take place through nuclear pore complexes (NPCs) embedded at the nuclear envelope. There are some 30 nucleoporins (Nups) that assemble to form the NPC and they can be classified into: transmembrane Nups (Poms) that anchor the NPCs at the nuclear envelope, structural Nups, and FG-Nups containing phenylalanine–glycine (FG) repeats, and involved in nuclear import and export (Terry and Wente, 2009). Transport through the NPCs can be via passive diffusion for cargoes up to approximately 40 kDa, active transport mediated by karyopherins (importins and exportins) interacting with FG-Nups for cargoes over 40 kDa, and direct interaction of some cargoes with FG-Nups bypassing the requirement for karyopherins (Terry and Wente, 2009). The central channel for nucleocytoplasmic transport consists of three nucleoporins, Nup62, Nup58 and Nup54 and the structures of the Nup62–Nup54 and Nup54–Nup58 interacting domains has been recently determined revealing the architecture of the mammalian transport channel (Solmaz et al., 2011).

In this study we continue our characterization of the cNLS and nuclear import pathway of high risk HPV16 E7 oncoprotein. We determine that an intact zinc-binding domain within the CR3 domain is essential for the cNLS function in mediating nuclear import of HPV16 E7. Mutagenesis of Cys residues in each of the two CysXXCys motifs involved in zinc binding changes the nuclear localization of the resultant EGFP-16E7 and 2xEGFP-16E7 mutants. We further discover that a patch of hydrophobic residues, $_{65}$**LRLCV**$_{69}$, within the zinc-binding domain of HPV16 E7 mediates its nuclear import via direct hydrophobic interactions with the FG domain of the channel nucleoporin, Nup62.

Results

The zinc-binding domain of HPV16 E7 is essential for its nuclear localization

We have previously established the presence of a C-terminal NLS in the CR3 domain of HPV16 E7 containing the zinc-binding fold that consists of two copies of a Cys–X–X–Cys motif separated by 29 amino acids (Knapp et al., 2009). Moreover, we have determined that the zinc-binding domain of HPV11 E7 is essential for its nuclear import and localization (Piccioli et al., 2010). To examine the possibility that the intact zinc-binding domain of HPV16 E7 is essential for the nuclear import activity of the cNLS of HPV16 E7, we have now performed site-directed mutagenesis of cysteine residues in each of the two copies of Cys–X–X–Cys in the context of EGFP-16E7 and 2xEGFP-16E7 generating the CC58/59AA and C91A mutants. The CC58/59AA mutant is designated CC58AA for simplicity. HeLa cells were transiently transfected with the wild type EGFP-16E7, the EGFP-16E7$_{CC58AA}$ and EGFP-16E7$_{C91A}$ cysteine mutants and EGFP control, and the cellular localization of the expressed proteins was examined via confocal fluorescence microscopy. As previously reported, the wild type EGFP-16E7 had a nuclear localization in the majority of transfected cells with the rest of cells having a pancellular phenotype (Fig. 1); EGFP had a pancellular localization in all the transfected cells (Fig. 1). Both the

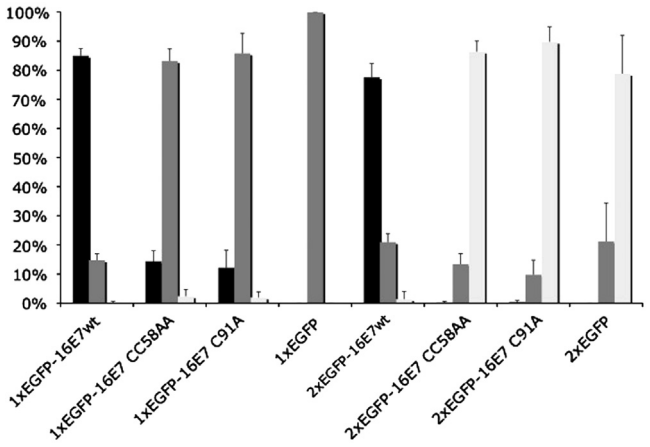

Fig. 1. Quantitative analysis of the effect of mutations of the zinc coordinating cysteine residues to alanine on the nuclear localization of EGFP-16E7 and 2xEGFP-16E7. Data from five experiments using EGFP-16E7, EGFP-16E7$_{CC58AA}$, EGFP-16E7$_{C91A}$, EGFP, 2xEGFP-16E7, 2xEGFP-16E7$_{CC58AA}$, 2xEGFP-16E7$_{C91A}$, and 2xEGFP plasmids were used for quantitative analysis and graphic representation of the localization distribution of transfected HeLa cells. Black bars represent cells exhibiting predominant nuclear localization; gray bars represent cells exhibiting pancellular localization; white bars represent cells exhibiting predominant cytoplasmic localization.

EGFP-16E7$_{CC58AA}$ and EGFP-16E7$_{C91A}$ cysteine mutants exhibited a pancellular localization, similar with that of EGFP, in the majority of transfected cells, 83.2% ± 4.1%, and 85.8% ± 6.9% respectively (Fig. 1). These data indicate that, while disruption of the zinc-binding domain in the context of EGFP-16E7 led to a change in the localization from mostly nuclear to pancellular, it did not completely prevent the EGFP-16E7$_{CC58AA}$ or EGFP-16E7$_{C91A}$ mutants from gaining some entry to the nucleus. As the cysteine mutations can affect the dimerization of 16E7, and in the absence of dimerization the EGFP-16E7$_{CC58AA}$ and EGFP-16E7$_{C91A}$ mutants could enter by passive diffusion (as the EGFP itself), we generated the same mutants in the context of 2xEGFP-16E7 (containing a 2xEGFP module) to exceed the limit for passive diffusion across the nuclear pore complex. HeLa cells were transiently transfected with the 2xEGFP-16E7$_{CC58AA}$ and 2xEGFP-16E7$_{C91A}$ mutants, along with the 2xEGFP-16E7 wild type and 2xEGFP control. As previously reported the 2xEGFP-16E7 wild type had a predominantly nuclear localization in the majority of cells (Figs. 1 and 2, panels B and C) and 2xEGFP had a cytoplasmic localization (Figs. 1 and 2, panels K and L). Significantly, the 2xEGFP-16E7$_{CC58AA}$ and 2xEGFP-16E7$_{C91A}$, exhibited predominantly cytoplasmic localization in the majority of transfected cells, 86.4% ± 3.6% and 89.8% ± 5.1%, respectively (Figs. 1 and 2, panels E, F, H and I). Together these data support the conclusion that the nuclear import activity of the cNLS of HPV16 E7 is dependent upon an intact zinc-binding domain. The loss of zinc coordination in the cysteine mutants can lead to structural changes in the zinc-binding domain that would make unavailable the critical residues of the cNLS.

We have previously showed that a cysteine residue at position 59 -conserved between the high risk and low risk HPVs, and not involved in coordinating the zinc ion – plays some role in the cNLS function of HPV11 E7 (Piccioli et al., 2010). To examine if this conserved cysteine residue performs a similar function in HPV16 E7, site directed mutagenesis was used to generate a pair of single amino acid substitutions of the N-terminal cysteines within the zinc-binding domain in the context of 2xEGFP-16E7. The 2xEGFP-16E7 wild type, 2xEGFP-16E7 single cysteine mutants and 2xEGFP, were transiently transfected into HeLa cells and their resultant subcellular localizations were examined via confocal fluorescence microscopy. Both 2xEGFP-16E7 wild type and 2xEGFP-16E7$_{C59A}$ exhibited predominantly nuclear localization (Fig. 3A, panels B and C; and panels H and I),