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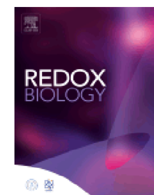

Degenerate cysteine patterns mediate two redox sensing mechanisms in the papillomavirus E7 oncoprotein

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ABSTRACT

Infection with oncogenic human papillomavirus induces deregulation of cellular redox homeostasis. Virus replication and papillomavirus-induced cell transformation require persistent expression of viral oncoproteins E7 and E6 that must retain their functionality in a persistent oxidative environment. Here, we dissected the molecular mechanisms by which E7 oncoprotein can sense and manage the potentially harmful oxidative environment of the papillomavirus-infected cell. The carboxy terminal domain of E7 protein from most of the 79 papillomavirus viral types of alpha genus, which encloses all the tumorigenic viral types, is a cysteine rich domain that contains two classes of cysteines: strictly conserved low reactive Zn^{+2} binding and degenerate reactive cysteine residues that can sense reactive oxygen species (ROS). Based on experimental data obtained from E7 proteins from the prototypical viral types 16, 18 and 11, we identified a couple of low pKa nucleophilic cysteines that can form a disulfide bridge upon the exposure to ROS and regulate the cytoplasm to nucleus transport. From sequence analysis and phylogenetic reconstruction of redox sensing states we propose that reactive cysteine acquisition through evolution leads to three separate E7s protein families that differ in the ROS sensing mechanism: non ROS-sensitive E7s; ROS-sensitive E7s using only a single or multiple reactive cysteine sensing mechanisms and ROS-sensitive E7s using a reactive-resolutive cysteine couple sensing mechanism.

1. Introduction

Reactive oxygen species (ROS) are a broad set of chemical oxidants, which include hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2$-), hydroxyl radicals ($\cdot OH$) and singlet oxygen ($^1O_2$), all involved in harmful and vital cellular processes [1,2]. The “dark side” of ROS involves the unwanted oxidation of proteins, lipid peroxidation, and DNA damage [3]. However, H_2O_2 is necessary for cell signaling and mediates vital processes such as hypoxic signal transduction, cell differentiation, proliferation, angiogenesis, apoptosis and immune response [4]. For cell signaling purposes, ROS are enzymatically produced in a defined time, on a defined cell compartment and upon specific stimuli by NAD(P)H oxidases or NADPH oxidases (NOXs) [5]. NOXs produce superoxide anion that is readily converted to H_2O_2 by superoxide dismutase (SOD). The homeostasis of redox balance that governs the pros and cons of ROS is based on an intertwined network of redox enzymes such as SOD, catalase, thioredoxin and peroxiredoxin [1,6] along with a plethora of redox-responsive proteins that includes transcription factors such as Nrf2 [7] or FOXO [8] and redox-sensitive kinase such as Src [9].

A direct link between ROS and cancer development has been recently established [10,11] where tumor cells show an altered ROS production resulting in the up-regulation of peroxide-dependent signaling pathways that regulate cell differentiation, growth and survival. A well-established case of this association is the effect of human papillomavirus (HPV) replication on infected and transformed

Abbreviations: CRD, cysteine richdomain; ROS, Reactive oxygen species; H_2O_2, hydrogen peroxide; NOXs, NADPH oxidases; SOD, superoxide dismutase; HPV, human papillomavirus; Rb, retinoblastoma protein; EK, enterokinase; ROIs, regions of interest; RT, retention time

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cells, where the overall redox balance is deregulated [12–15].

Papillomavirus are small DNA tumor viruses that are the causative agents of cervical cancer [16] and common warts [17]. Only a small fraction of the infected cells will eventually progress to cancer in a process that requires, at least, the persistent expression of E7 and E6 oncogenes [18] and an unknown triggering factor. In this scenario, it was suggested that the oxidative damage induced in HPV-infected cells could be the factor that eventually cooperates with viral oncoproteins to trigger cancer progression, in a very rare event.

Accumulated evidence supports the correlation between oxidative stress and HPV-induced transformation: i) ERp57 and GST involved in redox homeostasis were sharply elevated in dysplastic and neoplastic HPV-induced lesions [13]. ii) Oxidative damage reported by the increase of plasma malondialdehyde was demonstrated for patients with cervical intraepithelial neoplasia and cervical carcinoma [19], and an increase in the overall levels of protein oxidation was demonstrated in HPV-infected cells [13]. iii) The over-expression of NOX 1 accelerates neoplastic progression of human gingival mucosal keratinocytes immortalized by HPV16 E6/E7 [20,21]. iv) The solely expression of HPV16 E6 and E7 proteins induces a chronic oxidative stress response via NOX 2 [22].

We focused on the E7 oncoprotein, a 100 amino-acid protein with two well defined domains that differ in their conformational and functional properties [23]. The amino terminal domain (E7N, ca. 40 residues) is devoid of stable secondary and tertiary structure and shows properties resembling the intrinsically disordered domains. The E7N contains the highly conserved LXCXE motif, which is responsible for the high binding affinity of the retinoblastoma protein (Rb) [24]. The carboxy terminal domain (E7C, ca. 50 residues) is a well-folded domain with a globular hydrodynamic behavior. The E7C bears a tetracysteine Zn binding motif, unique among papillomavirus, and is composed of the CXXC-X_{29}-CXXC sequence. These four cysteines are highly conserved and Zn^{+2} coordination plays an essential structural role. The E7C is the oligomerization domain and is responsible for the dimeric structure observed in the published structures [24,25]. Still, a Kd of 1 μM indicates that the protein forms weak dimers along with the monomeric state and has been proved to be functional for transformation [26,27]. The four cysteines present in the E7C (Zn^{+2} binding motif) together with the cysteine in the LXCXE motif of E7N are considered as canonical cysteines. These five residues are highly conserved and are present in most of the E7 sequences regardless the genus, species or viral types. Many HPV types show an increased number of cysteines besides the canonical residues; however, these cysteines are much less conserved and we name them as non-canonical cysteines [28].

Along with the different conformational properties of the E7N and E7C, we previously showed that the cysteine residues in each domain, at least for HPV16, constitute different redox centers [29]. The cysteine residue located within the Rb binding motif (LXCXE) is sensitive to gluthationylation and this modification abolishes the binding of Rb. On the other hand, we have recently shown that the non-canonical cysteines, C59 and 68 in HPV16 E7, form a disulfide bond when exposed to an oxidative environment where the tetrahedral Zn^{+2} binding motif remains unaffected [29]. We further demonstrated that each domain can be oxidized independently and a mutant that lacks the LXCXE motif is able to form the observed disulfide bond.

The structures of the E7 proteins for the viral types HPV1a and HPV45 are dimeric and superimposable, and it is well accepted that the overall fold is a common feature of all E7s. It should be noted that residues 59 and 68 in the dimeric structure are 18.6 Å apart, too far to form a disulfide bridge. Therefore, an intermediary is more likely to occur [29].

In this work, we address the chemical mechanism by which a cysteine-rich oncoprotein senses and responds to the redox stimuli in an HPV-infected cell and how the protein can manage the potentially harmful oxidative environment of a tumor tissue. We address the differential conservation pattern and functional properties of canonical and non-canonical residues in the E7 proteins of alpha genus papillomavirus.

2. Methods

2.1. Protein expression and purification

Recombinant proteins were expressed in *E. coli* BL21(DE3)pLysS. Wild type E7 from HPV16 and cysteine-substituted mutants E7desLXCXE and HPV16E7(C59/68A) accumulate in inclusion bodies as fusions to a 19-aminoacid beta-galactosidase peptide with an enterokinase (EK) cleavage site as linker. The detailed purification protocol was previously described [30]. The truncated gene coding HPV16 E7Δ1–26 was cloned into a pMAL-c2 vector (New England Biolabs). The recombinant protein was purified as described for the full length HPV16 E7 rendering the E7Δ1–26 with two N-terminal aminoacids (glycine and serine) due to MBP excision with thrombin protease. GST-tagged wild type E7 from HPV11 and HPV18 were purified from soluble bacterial extracts in a glutathione-sepharose column. Untagged HPV11 E7 and HPV18 E7 were also obtained by thrombin cleavage.

A final purification step in a Superdex 75 column (GE Healthcare Bio-Sciences Corp, USA) with a reducing agent-free buffer was performed for all proteins. Purity was $> 95\%$ as confirmed by PAGE, with no more than 10% of oxidized species. Concentrated reductant-free protein stocks were maintained at -80C for 3 months with no oxidative damage.

2.2. Sequence alignment and naïve model for cysteine distribution

We retrieved all alpha genus papillomavirus types in the NCBI taxonomy database as October 1, 2015. At least 79 alpha-papillomavirus types had one ORF coding for E7 protein. Alignments for HPV E7 protein sequences were performed using the MUSCLE algorithm [31] and manually edited using the SeaView software. Naïve model for cysteine distribution were generated by a randomized assignment of cysteines at different positions in the sequence. To build the model this procedure was repeated 10000 times, the mean and standard deviation values for each combination of cysteines were calculated.

2.3. Ancestral states reconstruction and randomized trees

The tracing of the ancestral redox sensing state of each internal node was done using Mesquite and choosing reconstruction by parsimony [32]. The transformation types were defined as unordered, in which any state is allowed to transform to any other with one step of evolution. Each transition of states between nodes was counted. The redox state reconstructions from randomized trees were generated by reshuffling the same terminal taxa without altering the tree structure to obtain 100,000 trees. For each tree the ancestral redox state was reconstructed by parsimony using Mesquite and all transitions between node states were counted.

2.4. Quantitative thiol and Zn determination

Thiol content was quantified using DTNB; Zn content was determined using PAR-PMPS methods as described in [49].

2.5. Reactivity to H_2O_2 by RP-HPLC

Oxidation was triggered by the addition of 0.15 mM of H_2O_2 to a solution containing 3.5 μM of reduced protein in 50 mM potassium phosphate buffer pH 7.5, incubated at 37 °C. Samples were taken at the stated time; methionine was added to 2.5 mM final concentration to quench peroxide and immediately injected into a reverse phase HPLC

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