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Analysis of the Impact of Human Papillomavirus Type 5 E2 Serine 255 Phosphorylation on the Viral Genome Replication and E2 Protein Stability

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Analysis of the Impact of Human Papillomavirus Type 5 E2 Serine 255 Phosphorylation on the Viral Genome Replication and E2 Protein Stability

Abstract:

Human papillomaviruses (HPVs) are associated with the number of diseases from genital warts to cancer. While vaccination grants immunity to the wide range of HPV strains, it is important to develop effective treatment strategies to counter already established infections in order to prevent the egress of new strains of the virus. E2 is one of the most promising targets for development of therapeutic agents against the established HPV infections and, as many other proteins, it undergoes post-translational modifications changing the properties and the functions of this protein. This work is focused on investigating the effects of the phosphorylation at Serine 255 residue on the stability of HPV type 5 E2 protein and replication of the viral genome. The results of this work can benefit the projects focused on the treatment of HPV infection through the disruption of E2 functions and synthesis.

Keywords:

HPV5, stability, replication, E2 protein, PKA, phosphorylation

CERCS:

B230 Microbiology, bacteriology, virology, mycology

Inimese papilloomiviiruse tüüp 5 E2 seriin 255 fosforüülimise mõju analüüs viiruse genoomi replikatsioonile ja E2 valgu stabiilsus

Lühikokkuvõte:

Inimese papilloomiviirused (HPV) on seotud erinevate haigustega alates healoomulistest nahatüügastest kuni vähini. Kuigi vaktsineerimine annab immuunsuse paljude HPV tüvede vastu, on oluline välja töötada tõhusad ravistrateegiad juba olemasolevate nakkuste vastu võitlemiseks. E2 on HPV-nakkuse vastaste raviainete väljatöötamise kõige lootustandvaim sihtmärk. Nagu paljud teised valgud, läbib E2 translatsioonijärgsed modifikatsioonid, mis muudavad selle valgu omadusi ja funktsioone. Selle töö põhirõhk on uurida seriin 255 jäägi fosforüülimise mõju HPV5 E2 valgu stabiilsusele ja viiruse genoomi replikatsioonile. Selle töö tulemused võivad olla abiks projektidele, mis on suunatud HPV nakkuse ravi väljatöötamisele E2 funktsioonide ja sünteesi inhibeerimise kaudu.

Võtmesõnad:

HPV5, valgu stabiilsus, replikatsioon, valk E2, PKA, fosforüülimine

CERCS:

B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

DMSO - Dimethyl sulfoxide

E region - Early ORF encoding proteins for viral genome replication

E2^E8 - Repressor of viral replication

HPV - Human papillomavirus

HPV5 E2-Flag - vector containing HPV5 WT E2 sequence

HPV5-E1HA-Nluc-E2Flag - vector containing HPV5 WT genome

HR - High risk HPV types

IBMX - 3-isobutyl-1-methylxanthine

IMDM - Iscove's Modified Dulbecco's Medium

L region - Late ORF encoding proteins for virion assembly

LCR - Long control region

LR - Low risk HPV types

NLuc - NanoLuc luciferase

ORF - Open reading frame

PBS - Phosphate-buffered saline

PCR – Polymerase chain reaction

PKA - cAMP-dependent protein kinase A

PVs – Papillomaviruses

RPM - Rotations per minute

RT - Room temperature

ori - Origin of replication

WT – wild type

INTRODUCTION

Human papillomaviruses (HPVs) are double-stranded DNA (dsDNA) viruses infecting epithelial keratinocytes of mucosa or skin. HPV infections belong to the most common sexually transmitted diseases in both men and women worldwide. It is associated with the majority of cases in genital, mouth, tonsils, or throat cancer (Milner DA, 2015; Ljubojevic S *et al*, 2014; Anjum *et al*, 2020). Different HPV strains are divided into different genera (for instance, α, β, μ), and also into high and low risk groups depending on their oncogenic potential or ability to induce cancer (Rosa *et al*., 2013). The most common strategy to prevent HPV infection is vaccination (CDC, 2015), which grants immunity against some high-risk strains of HPVs. However, cervical and other types of cancers associated with some HPV subtypes are not covered by the vaccines (Guan P *et al*., 2015). In addition, the development of drugs against the already existing infections harboured by up to 20% of the population is currently a very pressing matter. Therefore, it is important to study the viral life cycle to develop possible strategies to counter the virus.

Inhibition of HPV replication is one of the most promising strategies in the treatment of HPV-related diseases. In order to interfere with the viral life cycle, it is vital to study the interactions between the virus and host cells. Protein kinases take part in the regulation of many important processes within the cell through the post-translational modification of proteins. E2 is one of HPV key replication proteins. It is also believed to be a substrate for many cellular kinases, which thereby may regulate the replication of the virus (Piirsoo *et al*, 2019; McBride, 2013).

Recent studies suggest that cAMP-dependent protein kinase A (PKA) phosphorylates cutaneous β HPV8 E2 protein at serine residue 253. This phosphorylation increases the half-life of the E2 protein, and promotes binding of E2 to chromatin through mitosis (Sekhar and McBride, 2012). This serine residue and PKA recognition motif (RRPSS) are highly conserved in many β HPVs and may be very important for regulation of their life cycle.

High risk β HPV5 infects cornified epithelium. Although it is the most common HPV type found on normal skin, it belongs to the types frequently associated with skin cancers (Mistry *et al*., 2017). The aim of this study was to generate HPV5 genomes and HPV5 E2 expression

constructs bearing the phosphomimetic and non-phosphorylatable mutations in the E2 serine residue 255 and to investigate the impact of these mutations on the E2 protein stability and viral genome replication efficiency. The chosen Serine 255 residue belongs to the highly conserved PKA recognition motif. To prove that the activity of E2 protein and the replication of the viral genome depend on the PKA-mediated phosphorylation of this specific residue, the generated mutants, where the serine residue 255 was replaced with either non-phosphorylatable alanine or with phosphomimetic glutamic acid, were analysed in the terms of the expression levels of the over-expressed E2 proteins and replication efficiency of the viral genomes in U2OS cells challenged either with the pharmacological activators of PKA or a PKA catalytic subunit.

The first part of the thesis provides an overview of the HPV genome and infection cycle with emphasis of the E2 protein functions, and describes the structure and roles of PKA. The experimental part describes the generation of the mutant constructs and the analysis of the E2 protein expression level and stability as well as HPV5 genome replication in the presence of the overexpressed catalytically active PKA subunit or elevated PKA activity caused by 3-isobutyl-1-methylxanthine (IBMX) or Forskolin.

This study was performed in the molecular virology research group, Institute of Technology, University of Tartu.

1 LITERATURE REVIEW

1.1 Human papillomaviruses and papillomaviruses

Papillomaviruses are small non-enveloped icosahedral viruses with double-stranded circular DNA. The general size of the viral genome is approximately 8 kb. It typically contains up to eight open-reading frames (ORFs) (IARC, 2007). Papillomaviruses belong to Papillomaviridae family that includes two subfamilies: Firstpapillomavirinae (includes 52 genera and more than one hundred species) and Secondpapillomavirinae (includes one genus and one unique specie - Alefpapillomavirus) (International Committee on Taxonomy of Viruses).

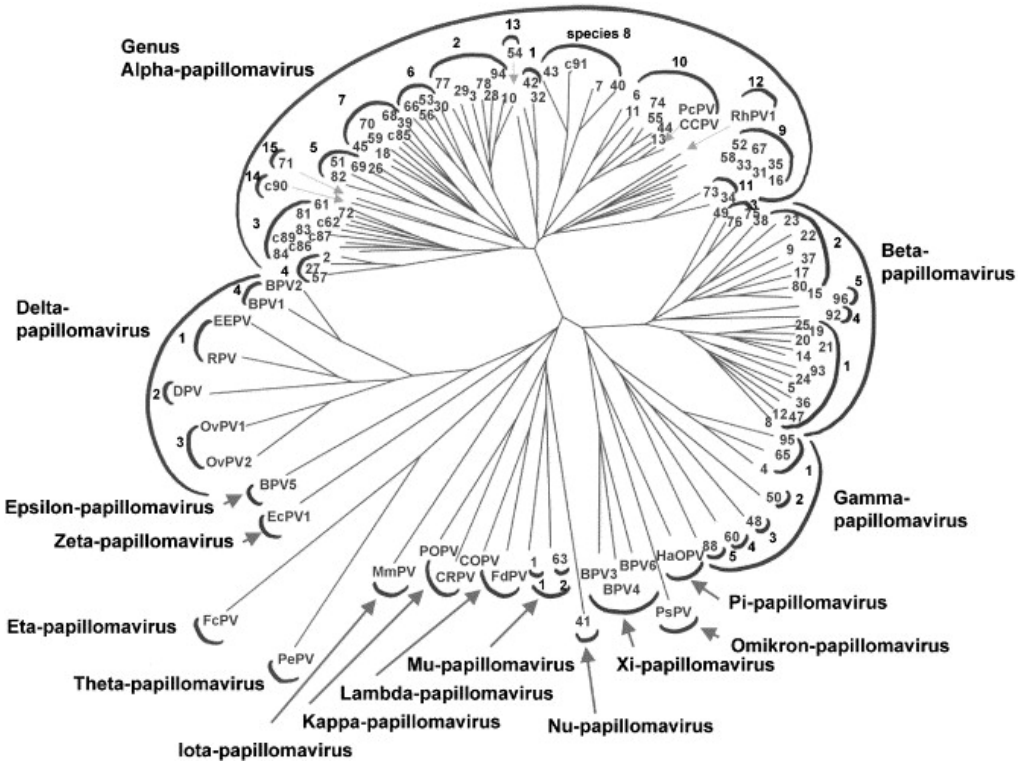

Figure 1. Phylogenetic tree containing the sequences of 118 papillomavirus types (de Villiers et al., 2004).

Papillomaviruses infect epithelial keratinocytes in a large variety of vertebrate species (mammalians, fish, bird). The infection can either persist without symptoms or cause neoplasms (Bernand *et al.*, 2010).

Human Papillomaviruses (HPVs) are a diverse group of Papillomaviruses consisting of more than 200 types phylogenetically divided into five major genera: alpha, beta, gamma, mu and nu HPVs (de Villiers *et al*., 2004). Alpha-HPVs are associated with the infections of oral and genital mucosal epithelia, while all other genera are believed to be tied to the infection of non-genital mucosa and skin (*Rosa et al.*, 2013).

Additionally, HPV types can be divided into two big categories depending on their ability to induce cancer. There are oncogenic or high-risk (HR) types (16, 18, 31, 33, 35, 39, 45, 51, 52, and 58) and non-oncogenic or low-risk (LR) types (6, 11, 40, 42, 43, 44, and 54). HR types are mainly are associated with cervical, vulvar, vaginal, and anal cancers, while LR types are associated with genital warts (Braaten and Laufer, 2008; Muñoz *et al*., 2003). In addition, HR types may serve as a reason for head and neck squamous cell carcinoma (SCC) (Leemans *et al*, 2011).

For example, HPV 16 and 18 together account for approximately 70% of cervical cancers. HPV 6 and 11 are the most common strains associated with genital warts and account for approximately 90% of these lesions (Clifford *et al*, 2003).

HPV DNA can be also found on healthy skin: DNA of beta-HPV can be detected in newborns after a few days of life (Antonsson *et al*., 2003). It only proves that not all types of this virus cause symptomatic infections.

1.2 HPV genome, functional ORFs and viral proteins

Different HPV types have similar genome organisation. The average length of an HPV genome is ~8000 bp (Doorbar, 2016). The open reading frames (ORFs) of the viral genome can be divided into three functional regions: the early (E) region encoding proteins necessary for viral replication; the late (L) region encoding the structural proteins required for virion assembly; and a non-coding part or long control region (LCR), which contains cis elements necessary for the replication and transcription of the viral DNA. E proteins are transcribed from early promoters, meanwhile L proteins are transcribed from late promoters which are inactive during the initial stages of the infection cycle (Fehrmann and Laimins, 2003).

The E1 and E2 proteins of HPV recognize and bind as a complex to the specific sequences on the origin of replication (ORI) (Mohr et al., 1990; Ustav and Stenlund, 1991; Chiang et al., 1992; Frattini and Laimins, 1994). The E2 protein is also the main regulator of viral gene transcription. It is believed that it might have a repressor activity on the viral early promoter that in turn may function as a part of a mechanism controlling a viral genome copy number (Thierry and Yaniv, 1987; Stubenrauch et al., 1998; Howley and Lowy, 2001). E4 is believed to be involved in the alteration of the cytoskeleton network. It is expressed as a fusion protein with five amino acids from the N-terminus of E1 protein (Doorbar et al., 1991). E5 may function during both early and late phases of HPV life cycle. The exact function of this

protein is still largely unknown, however, it seems that E5 encodes a protein with a weak transforming activity (Leptak et al., 1991). The E6 and E7 proteins target a number of negative regulators of the cell cycle. During the viral life cycle, E6 and E7 facilitate stable maintenance of viral episomes and stimulate differentiating cells to re-enter the S phase (Longworth and Laimins, 2004). The L1 and L2 proteins assemble in capsomers, which form icosahedral capsids around the viral genome during the generation of progeny virions (Fehrmann and Laimins, 2003).

The LCR is approximately 500 to 1000 bp long region located upstream of the coding region. It contains ORI, transcriptional enhancer and promoter elements, binding sites for cellular transcriptional factors and viral proteins E1 and E2 (McBride, 2008).

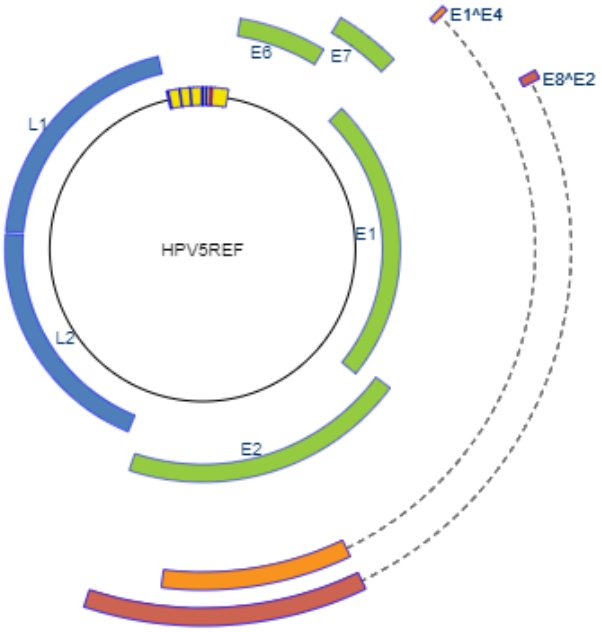

Figure 2. Schematic representation of HPV5 genome: ORFs for the E proteins are marked with green color, the late ORFs are marked with blue color, the LCR is marked with yellow. HPVs encode truncated forms of proteins from alternatively spliced mRNA (E1^E4 and E8^E2 - marked orange and pink, respectively). Binding sites for the viral proteins and host transcription factors are shown with red, blue and purple stripes in the LCR.

1.3 E2 viral protein

The E2 is around 50 kDa long regulatory protein (Rakjumar, 2016) However, the products of the corresponding ORF vary in size due to the expression from different promoters and

alternative RNA splicing. Such deviating products (E8^E2, E1^E2 and others) function as inhibitors of viral transcription and replication (McBride, 2013).

The E2 protein consists of two highly conserved domains: a carboxyl-terminal DNA binding and dimerization domain (CTD) that binds to palindromic 12 bp long sequences on the viral genome and an amino-terminal transactivation domain. Both of them play an important role in viral replication and transmission. They are separated by a highly flexible and variable hinge region (Sekhar and McBride, 2012).

The full-length E2 protein can influence the transcription of the viral genes both positively and negatively in a dose dependent manner (Steger and Corbach, 1997). Mostly the E2 proteins function by recruiting cellular factors to the viral genome thus leading to activation or repression of transcriptional processes. The E2 proteins bind specifically to sequence motifs in the viral genome and can activate or repress transcription, depending on the context of these binding sites and nature of the associated cellular factors (McBride, 2013).

The E2 proteins play an important role in the maintenance and transcription of the viral genome. E2 takes part in initiation of viral DNA replication by loading the E1 helicase onto ORI (Ilves et al., 1999; Sanders and Stenlund, 2000). After E1 is loaded, it converts to a double-hexameric helicase and E2 is displaced. E2 also displaces nucleosomes from the ORI to alleviate repression (Li and Botchan, 1994).

Some studies also suggest that E2 protein regulates pre-mRNA processing, namely alternative pre-mRNA splicing. According to the studies, E2 protein is capable of interacting with the SR proteins (conserved family of proteins involved in RNA splicing) that leads to association with the 5′ splice site of pre-mRNA intron. RS-rich hinge is required for E2 protein's function in promoting pre-mRNA splicing (Lai *et al*., 1999)

E2 may act as a transcriptional activator or repressor in healthy and infected cells. The mechanism of repression in HPV-positive cells has been already discovered: E2 inhibits one of the early viral promoters, thus down-regulating E6 and E7 expression. It leads to induction of growth arrest and senescence (Dowhanick *et al.*, 1995).

E2 also induces apoptosis even in the absence of other HPV proteins, however the mechanism is more controversial and involves both p53 dependent and independent pathways (Webster *et al.*, 2000), and also interaction with caspase 8 (Thierry and Demeret, 2008).

E2 protein is also responsible for the tethering of the viral genome to host chromosomes during mitosis ensuring the persistence of infection, retention, maintenance and partitioning

of the viral genome. Several studies suggest that phosphorylation is involved in regulation of this process (Sekhar and McBride, 2012; Ilves *et al*, 1999).

1.4 Truncated forms of E2 proteins from alternatively spliced mRNA

The shorter forms of E2 are produced from alternatively spliced mRNA. These truncated forms of E2 can act as repressors of viral genome replication. The mechanism of repression includes either competition for E2 binding sites (Lambert *et al.*, 1987, Lim *et al.*, 1998, Monini *et al.*, 1993) or recruitment of repressor complexes by the E8-derived peptide to viral DNA (Ammermann *et al.*, 2008, Fertey *et al.*, 2010, Powell *et al.*, 2010). There is also an alternative mechanism of repression through dimerization of full-length E2 with the shorter forms (Barsoum *et al.*, 1992). However, it has been stated that heterodimers with just one activation domain can still activate transcription (Kurg *et al.*, 2006).

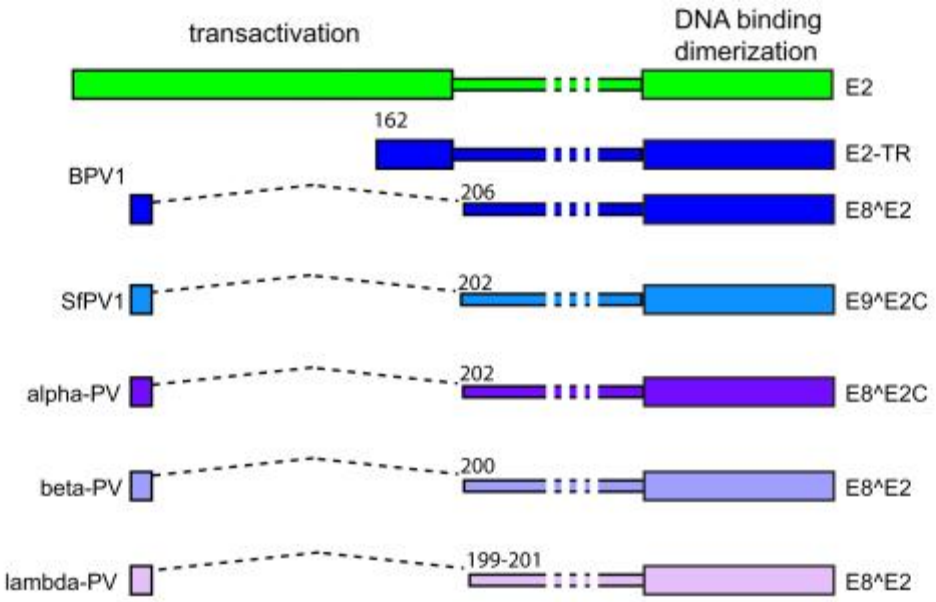

Figure 3. Examples of E2 repressor proteins. Two conserved regions of full-length protein are shown in green, the region between two domains is a highly variable region called hinge. Most PVs have the tendency to encode repressor E2^E8 and all of them encode homologous sequences and splice donor/acceptor sites. (Alison A. McBride, 2010).

1.5 HPV infection cycle

HPVs are strictly epitheliotropic. Therefore, stable and persistent infection can be established only in stratified epithelia of the skin, the anogenital tract and the oral cavity. Since these cells

are able to proliferate constantly, the viral life cycle is strongly connected to the differentiation of the infected epithelial cells. The infection begins with the viral particles invading the basal lamina through the micro wounds on the skin surface and mucosa (Schiller *et al.*, 2010). Some studies also suggest that active division of the cells and cell cycle progression are absolutely required for entry of the viral genome inside the nucleus. The main evidence supporting this statement would be the formation of lesions requiring the presence of mitotically active cells like the ones found in the healing wound (Pyeon *et al.*, 2009). The mechanism HPV uses to attach itself to the cell membrane is still largely unknown. Studies showed that the attachment occurs with the help of several surface proteins (Roden *et al*., 1994). Although now many studies have concluded that heparan sulfate proteoglycans (HSPGs) play the most crucial role in the attachment of viral particles to the surface (Giroglou *et al.*, 2001). In the basal layer, low HPV genome copy number and low expression of the HPV early genes is observed (Stoler & Broker, 1986).

In normal conditions, when basal cells divide, the daughter cell loses contract with the basal membrane and migrates to the suprabasal compartment. As a result, the cell leaves the cell cycle and enters the differentiation process. It does not happen in HPV-positive keratinocytes and as result of the disturbed cell cycle and constant re-entering to the S phase (Flores *et al.*, 1999). In the suprabasal compartment, the infected cell will start the amplification of the viral genome copies. In the upper layers, the late region ORFs are expressed to produce capsid proteins required for assembly of the viral progeny. At the end of the viral life cycle in the upper layers of the stratified epithelium, viral genomes are packed into capsids and assembled virions egress from the host cell (Peh *et al.*, 2002).

At this stage, late protein L2 plays a very important role in the number of processes happening within the cell. Encapsidation of the viral DNA into the new virions quantitatively depends on the expression of L2 (Holmgren *et al.*, 2005). It has been suggested that the L2 protein plays an important role in localization of the viral genome inside the nucleus of the infected cells (Day *et al.*, 2004).

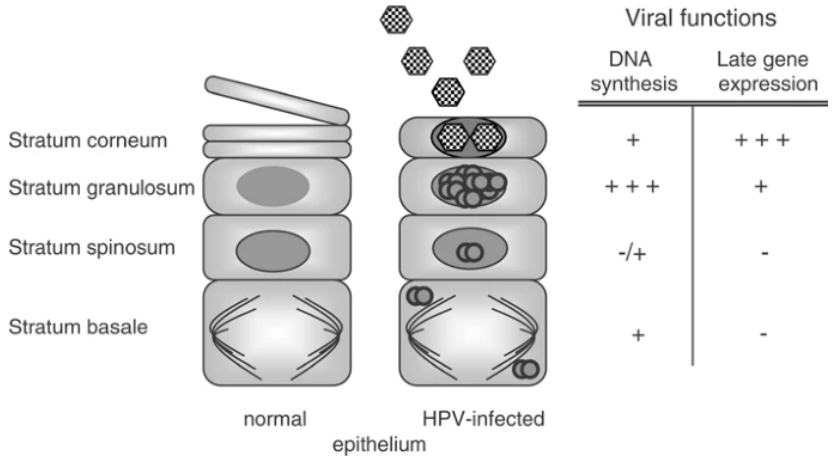

Figure 4. Abnormal epithelial differentiation induced by HPV infection (Fehrmann and Laimins, 2003).

1.6 Protein kinase A

Cyclic AMP (cAMP) dependent protein kinase A (PKA) is an important enzyme regulating a lot of processes in the cell. It's also the most studied and characterized member of the protein kinase superfamily. It was the first protein kinase that was successfully sequenced (Shoji *et al.*, 1983), and the first for which it was possible to obtain a crystal structure (Knighton *et al.*, 1991). As the name suggests, the activity of this enzyme depends on the concentration of cAMP in the cellular environment.

The inactive holoenzyme is a complex of two regulatory (R) and two catalytic (C) subunits.

The main function of the C subunit is the transfer of γ−phosphate from ATP to the target protein (Li et al., 2002). C subunit consists of three functional units: conserved core, small lobe and big lobe (Taylor and Radzio-Andzelm, 2010). Three isoforms of the C subunit were discovered - α, β, and γ. The alpha and beta subunits (Cα and Cβ, respectively) are expressed in all cells, the gamma subunit (Cγ) is found mostly in testes (Uhler et al., 1986; Showers et al., 1986; Beebe et al., 1990).

Two R subunits form a homodimer with disulfide bonds linking two molecules together (Bubis *et al.*, 1988). It contains cAMP binding sites located at the C-terminus and a pseudo-substrate inhibition site. In the absence of cAMP, the inhibitor binds to the active site cleft of the C subunit preventing access to the PKA substrates (Taylor and Radzio-Andzelm, 2010). Two major isoforms (I and II types) exist together with two subfamilies (alpha and beta). The members of the alpha subfamily (such as RIα or RIIα) are expressed in the biggest

part of mammalian cells, however, the expression of the beta subfamily members is more tissue-specific (Stratakis *et al.*, 2002).

There are two general recognition sequences for PKA C subunit: Arg-Arg-X-Ser/Thr-Hyd and Arg-X-X-Arg-X-X-Ser/Thr-Hyd where X is any amino acid and Hyd is a hydrophobic residue (Zetterqvist *et al.*, 1990). The list of substrates is continuously updated by NHLBI (Isobe *et al.*, 2017).

As it has been mentioned above, the cAMP-dependent protein kinase A plays a very important role in the number of cellular processes. As an example, glucose homeostasis and triglyceride storage require the activation of PKA. β-adrenergic stimulation by catecholamines leads to the increase in cAMP concentration. As a consequence, PKA is activated that gives lipases better access to the triacylglycerol droplet (Czech *et al.*, 2013).

1.7 Reasons to study

HPV infection is one of the most common sexually transmitted diseases in both men and women worldwide. It is associated with the majority cases of genital, mouth, tonsils, or throat cancer (Milner DA, 2015; Ljubojevic *et al.*, 2014; Anjum *et al.*, 2020). The most common strategy to prevent HPV infection include primary (vaccination) or secondary prevention programs (cervical screening) (Stern *et al.*, 2012). However, some HR HPV subtypes responsible for cervical cancer are not covered by vaccines (Guan *et al.*, 2012). To prevent the emergence of new strains of the virus, it would be important to develop therapeutic agents for already existing infections.

Several studies reported that the replication of HPV genome may be regulated via phosphorylation of certain viral proteins. Thus, suppression of the HPV infection may be achieved via disruption of the protein kinase – viral protein interplay by either inhibition of the specific protein kinases responsible for the positive regulation of the viral protein activities or activation of the negative regulators of the viral proteins (Piirsoo et al., 2019; Sekhar and McBride, 2012).

This study is aiming to investigate the consequences of the specific serine residue phosphorylation of HPV5 E2 protein in terms of the stability of this protein and the replication of the viral genome.

2 THE AIMS OF THE THESIS

- The generation of constructs encoding point mutations at Serine 255 residue in HPV5 genomes and E2 expression vectors.
- The evaluation of E2 stability in response to the pharmacological activators of PKA using Western Blot.
- The evaluation of the effects the introduced mutations have on the replication of HPV5 genome using co-transfection with the catalytic subunit alpha of PKA and Luciferase assay.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

- HPV5-E1HA-Nluc-E2Flag parental genome contains the fragments of bacterial DNA required for amplification of the plasmid in bacteria, HA tag encoding sequence in the E1 ORF, Flag tag encoding sequence in the E2 ORF and NanoLuciferase (Nluc) encoding sequence in between of E1 and E2 ORFs
- HPV5 E2-Flag WT expression vector contains HPV5 wild type (wt) E2 sequence cloned between HindIII and BamHI restriction sites in the pCMV-Flag-4 vector (Sigma-Aldrich)
- pEGFP-C1 vector encodes the green fluorescent protein (Clontech)
- PKA-Flag-pCMV vector contains a sequence encoding human PKA catalytic subunit alpha cloned into the pCMV-Flag-4 vector between HindIII and KpnI sites

The described plasmids are obtained from the Laboratory of Molecular Virology, Institute of Technology, University of Tartu

3.1.2 Generation of HPV5-E1HA-Nluc-E2Flag-A/E genomes / HPV5-E2-Flag-A/E expression vectors

3.1.2.1 The amplification of the HPV5-E1HA-Nluc-E2Flag parental genome

Even though the plasmid containing HPV 5 genome, protein tags and the sequence encoding NLuc has been provided, the initial concentration was not sufficient for further experiments. Therefore, the first aim was to amplify the plasmid through bacterial transformation and plasmid purification. Bacterial cells grow very quickly and produce a lot of copies of the genome that might be purified and used.

3.1.2.1.1 Heat-Shock Bacterial Transformation

Non-pathogenic *Escherichia coli* strain DH5α was chosen for the transformation, since these bacteria were engineered to maximize the transformation efficiency. Before usage, the bacterial cells were frozen at -70 ˚C. According to the Heat-Shock Transformation protocol, the cells were thawed on ice for 15 minutes, and 12 ng of the plasmid was added to the bacterial cells. After resuspension, the mixture was left on ice for 30 minutes, incubated at 37 ˚C for 4 minutes and then moved back on ice for one minute. Then, 1 ml of Lysogeny Broth

(LB, 10 g/l tryptone, 5 g/l yeast extract,10 g/l NaCl) was added to the tube, and the mixture was incubated at 37 ˚C for 30 minutes. Next, 100 µl of bacterial suspension was plated using spread plate method on LB agar containing 50 µg/ml kanamycin. The plates were incubated at 37 ˚C overnight. Next day, two bacterial colonies were transferred to 3 ml of LB containing 50 µg/ml kanamycin and incubated at 220 rpm and 37 ˚C overnight. The medium with the highest cell density was chosen and transferred to the flask containing 100 ml of LB supplemented with 50 µg/ml kanamycin. The cells were incubated at the same conditions overnight, transferred into a 50 ml Falcon tube, centrifuged at room temperature and 5000 RPM for 15 minutes in Eppendorf 5810 centrifuge and frozen at -20 ˚C for short-term storage.

3.1.2.1.2 Plasmid purification and DNA precipitation

Endotoxin-free DNA extraction and purification was made using NucleoBond® Xtra Midi EF kit (MACHEREY-NAGEL GmbH) according to the manufacturer's protocol. Bacterial cells were resuspended in 8 ml of RES-EF buffer. The vortex mixer Vortex-Genie® 2 was used to speed up the resuspension of the cell pellet in the buffer. Then, the cells were lysed with 8 ml of LYS-EF and incubated at RT for 4 minutes. Meanwhile, the column and filter were calibrated with 15 ml of EQU-EF buffer. In order to prevent contamination with bacterial DNA, the lysis was stopped with 8 ml of neutralisation buffer NEU-EF. Lysate was centrifuged at 5000 RPM for 2 minutes to separate the supernatant containing plasmid DNA and cell debris. After the separation, the supernatant was loaded on NucleoBond® Xtra Column Filter. The column was washed with 5 ml of FIL-EF buffer, and after the first wash the filter was discarded. To remove endotoxins, the second wash with 35 ml of ENDO-EF buffer was made. The final wash was made with 15 ml of WASH-EF buffer. Finally, 5 ml of ELU-EF buffer was added to the silica membrane to elute DNA.

Then, 3.5 ml of isopropanol was added to the eluted DNA. The sample was vortexed and centrifuged at 10 000 RPM, 4 ˚C for 30 minutes. The supernatant was removed with aspiration, and the pellet was washed with 2 ml of cold (previously stored at -20 ˚C) 75% ethanol and centrifuged under the same conditions for 5 minutes. The supernatant was removed, the sample was left to dry at room temperature for 5 minutes, and finally DNA was dissolved in 0.5 ml of water. The final concentration was 190 ng/µl. The concentration was measured using NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific) at 260 nm wavelength.

3.1.2.2 Introduction of the desired point mutations using PCR

3.1.2.2.1 PCR

In order to introduce the point mutations into the viral parental genome HPV5-E1HA-Nluc-E2Flag and HPV5-E2-Flag expression vector, PCR was used. To amplify the plasmids, the LongRange PCR Kit (QIAGEN) was used due to its low error rate ensured by high-fidelity enzymes and unique buffer system minimizing PCR optimization. To ensure successful introduction of point mutations, PCR mixtures were prepared in two replicates, and one replicate contained additionally the Q-reagent for amplification of GC-rich regions. The sequences of the used primers and PCR components are listed in Table 1 and Tables 2-3, respectively. The PCR was performed using an Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific).

Table 1. Primers used to introduce the point mutations into the HPV5 E2 serine 255 residue encoding sequence.

Primer	Sequence
E2 S255A FWD	GTACGGACGGAGGCCCGCCAGCAAGTCAAGGAGATC
E2 S255A RES	GATCTCCTTGACTTGCTGGCGGGCCTCCGTCCGTAC
E2 S255E FWD	GTACGGACGGAGGCCCGAAAGCAAGTCAAGGAGATC
E2 S255E RES	GATCTCCTTGACTTGCTTTCGGGCCTCCGTCCGTAC

Table 2. PCR mixtures without Q-reagent.

Reagent	Volume	Master Mix (x 8.5)
LongRange PCR Buffer	2.5 µl	21.25 µl
dNTP Mix	1.25 µl	10.625 µl
Primer 1	1.25 µl	
Primer 2	1.25 µl	
LongRange PCR Enzyme Mix	0.25 µl	2.125 µl
Template	1 µl	
RNase-Free Water	17.5 µl	148.75 µl

Table 3. PCR mixtures with Q-reagent.

Reagent	Volume	Master Mix (x 8.5)
LongRange PCR Buffer	2.5 µl	21.25 µl
dNTP Mix	1.25 µl	10.625 µl
Primer 1	1.25 µl	
Primer 2	1.25 µl	
LongRange PCR Enzyme Mix	0.25 µl	2.125 µl
Template	1 µl	
RNase-Free Water	12.5 µl	106.25 µl
Q-Reagent	5 µl	42.5 µl

The next PCR program was used: initial activation step 3 minutes at 93 °C; 35 cycles of denaturation (15 seconds at 93 °C), annealing (30 seconds at 55 °C), and extension (12 minutes at 68 °C).

3.1.2.2.2 Gel electrophoresis

A mixture of the restriction enzyme DpnI 0.5 µl (Thermo Fisher Scientific), 3 µl of 10x FastDigest Green Buffer (Thermo Fisher Scientific) and 1.5 µl of water was added to all PCR products. The samples were incubated at 37 ˚C for 1 h to digest the input DNA. The treated PCR products were analyzed using agarose gel electrophoresis in the presence of 1 µl of GeneRuler 1kb DNA ladder (Thermo Fisher Scientific). Then all samples were loaded on 0.8% agarose gel. The gel was run in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer solution under 220 mA for 40 minutes. After separation, the desired fragments were cut out of the gel under 260 nm UV and placed in 1.5 ml Eppendorf tubes.

3.1.2.2.3 Purification of DNA fragments from agarose gel

The DNA fragments were purified from the agarose gel using ZymoClean™ Gel DNA Recovery Kit (Zymo Research) according to the protocol provided by the manufacturer. Roughly 3 volumes of Agarose Dissolving Buffer (ADB) were added to each volume of agarose, and then the samples were incubated at 50 ˚C until the gel slice was completely dissolved. One additional volume of water was also added. Then melted agarose was transferred to Zymo-Spin™ Column in a Collection Tube and centrifuged at 5000 RPM and room temperature for 1 minute in Biofuge pico (Heraeus) centrifuge. The flow-through was removed by aspiration to prevent possible contamination. Following this step, 200 µl of Wash Buffer containing 70% ethanol was added to the membrane. The column was centrifuged for

another minute at the same conditions. The washing step was repeated. The column was transferred to a new Eppendorf tube, 40 µl of DNA Elution Buffer (10mM Tris-HCl, pH 8.5, 0.1mM EDTA, preheated to 65 ˚C) was added to the membrane, and DNA was eluted through centrifugation. The obtained DNA concentrations were measured using NanoDrop 1000 Spectrophotometer. The weights of the gel, the volumes of added ADB and water and the concentrations of eluted DNA are shown in Table 4.

Table 4. Used agarose weight, volumes of reagents added and the final concentrations of the eluted DNA. The samples used in further stages are shown in bold.

#	Name	Agarose weight (µg)	ADB volume (µl)	H2O volume (µl)	Final concentration (ng/µl)
1	**HPV5-E2-Flag-A**	**370**	**1100**	**370**	**77.2**
2	**HPV5-E2-Flag-A**	**380**	**1140**	**380**	**53.4**
3	**HPV5-E1HA-Nluc-E2Flag-A**	**400**	**1200**	**400**	**73.5**
4	**HPV5-E1HA-Nluc-E2Flag-A**	**250**	**750**	**250**	**38.4**
5	HPV5-E2-Flag-A	190	570	190	24.6
6	**HPV5-E2-Flag-A**	**260**	**780**	**260**	**72.6**
7	**HPV5-E1HA-Nluc-E2Flag-E**	**310**	**930**	**310**	**53.9**
8	**HPV5-E2-Flag-A**	**340**	**1020**	**340**	**73.3**
9	HPV5-E1HA-Nluc-E2 Flag-E	350	1050	350	11.6

The obtained purified DNA samples were transformed to the competent bacteria, as described above. The plasmid DNA was purified. The E2 encoding parts of the plasmids were sequenced in the Institute of Genomics Core Facility, University of Tartu. The correct clones were chosen for further manipulations.

3.1.2.2.4 Cloning into the original vector or genome to generate the HPV5-E2Flag-A/E expression constructs or HPV5-E1HA-Nluc-E2Flag-A/E genomes

Since the only E2 encoding parts of the whole plasmids were verified by DNA sequencing, it was necessary to subclone the sequences of interest to the original vector or genome to avoid possible PCR-generated mutations in the non-sequenced parts of the plasmids. The new expression constructs encoding the mutant E2 proteins and the pCMV-Flag-4 vector (Sigma-Aldrich) were restricted with 1.5 µl of HindIII, 1.5 µl of BamHI and 2 µl of 10x FastDigest buffer (Thermo Fisher Scientific) in 20 µL. Similarly, the generated mutant

genomes and the original HPV5-E1HA-Nluc-E2Flag genome were treated with 1.5 µl of restriction enzymes BstXI (Thermo Fisher Scientific) and NgoMIV (New England Biolabs) After incubation for 30 minutes at 37 ˚C, the samples were loaded on 0.8 % agarose gel. The gel was running for 50 minutes in 1x TAE buffer. The bands corresponding to the size of the sequence of interest and a plasmid backbone were cut out and purified from the gel using ZymoClean™ Gel DNA Recovery Kit (Zymo Research) as described above. The purified fragments were ligated together using 1 µl of T4 DNA Ligase in a respective buffer (Thermo Fisher Scientific) at 16 ˚C overnight. Next morning, the ligated DNAs was transformed to the competent bacterial cells using the heat-shock protocol. Bacteria positive for the E2 expression constructs were plated on LB agar containing 100 µg/ml of ampicillin, since the pCMV-Flag-4 vector has AmpR gene providing resistance to this drug. Bacteria containing the parental genomes were plated on the LB agar supplemented with 50 µg/ml of kanamycin. The chosen one colony from each plate was grown in 100 ml of LB supplemented with either 100 µg/ml of ampicillin or 50 µg/ml of kanamycin. The plasmids were purified with NucleoBond® Xtra Midi EF kit (MACHEREY-NAGEL GmbH) and the concentrations were measured. Concentrations of the HPV5-E2Flag-A and HPV5-E2Flag-E expression vectors were 550 ng/µl and 580 ng/µl, and concentrations of the HPV5-E1HA-Nluc-E2Flag-A and HPV5-E1HA-Nluc-E2Flag-E parental genomes were 330 ng/µl and 304 ng/µl, respectively.

3.1.2.2.5 Production of minicircles

The presence of bacterial DNA in the parental HPV genome is necessary to amplify the genome in bacteria. However, this approximately 3000 bp long DNA fragment negatively affects the replication of the genome. Therefore, it is required to remove the bacterial elements from the vector. The minicircle DNA technology was used to produce the HPV genomes without bacterial elements (Kay et al., 2010).

In order to produce minicircles DNA, *Escherichia coli* strain ZYCY10P3S2T was used. This strain contains specific recombinase ΦC31 and SceI endonuclease under the inducible L-arabinose PBAD promoter. The same protocol as before was used to transform HPV5-E1HA-Nluc-E2Flag, HPV5-E1HA-Nluc-E2Flag-A and HPV5-E1HA-Nluc-E2Flag-E parental genomes into the bacterial cells. Bacterial suspension was plated on plates containing 50 µg/ml kanamycin. The cells were incubated overnight at 37 ˚C. Two colonies from each plate were transferred into 3 ml of LB supplemented with 50 µg/ml kanamycin and incubated at 220 RPM and 37 ˚C overnight. The cells were transferred in 400 ml of Difco Terrific broth (Pancreatic digest of Casein 12g/L, Yeast extract 24g/L, Dipotassium Phosphate 9.4g/L,

Monopotassium Phosphate 2.2g/L) with 50 µg/ml kanamycin. All six flasks (two for HPV5-E1HA-Nluc-E2Flag, two for HPV5-E1HA-Nluc-E2Flag-A and two for HPV5-E1HA-Nluc-E2Flag-E) with cells and nutrient-rich medium were incubated at 220 RPM, 37 ˚C for 16 hours. After that bacteria were induced with 100 ml of LB supplemented with 0.4 M NaOH and 0.04% L-arabinose. The cells were incubated at the same conditions for an additional 8 hours. Then cells were collected using centrifugation with Sorvall LYNX 4000 (Thermo Fisher Scientific) at RT and 5000 RPM for 10 min. Finally, the genomes were purified using NucleoBond® Xtra Midi EF kit (MACHEREY-NAGEL GmbH) and subjected to the restriction analysis to verify the obtained genomes..

3.1.3 Electroporation of the U2OS cell line

3.1.3.1 U2OS cell line and incubation parameters

In this study, human osteosarcoma U2OS cell line was used since it is permissive for HPV genome replication. Cells were grown on 10 cm plates (Corning Inc.) in Iscove's Modified Dulbecco's Medium (IMDM, Corning Inc.) supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin (Sigma-Aldrich). Cells were incubated at 37 ˚C and 5% of CO_2.

3.1.3.2 Transfection of U2OS cells by electroporation

One day prior to the transfection, the cells grown on 10 cm plates with approximately 100% confluency were split to two 10 cm plates. Next day, the medium was aspirated and the cells were washed with 5 ml of Phosphate-Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.47 mM KH2PO4), detached using 1.2 ml of 0.25% Trypsin-EDTA solution incubated for 1 minute, transferred to 6 ml of fresh medium and then collected with centrifugation at 20 ˚C, 1000 RPM for 1 minute using Eppendorf Centrifuge 5810R (Thermo Fisher Scientific). The supernatant was aspirated, and the cell pellet was resuspended in the volume of fresh medium determined by the number of transfections - 250 µl per every transfection.

For the transfection of the expression vectors, 250 µl of cell suspension was added to every tube containing 2 µl of Salmon Sperm DNA (Thermo Fisher Scientific) as a carrier and either 900 ng of the E2 expression vectors HPV5-E2-Flag-WT, HPV5-E2-Flag-A or HPV5-E2-Flag-E or 900 ng of the GFP-encoding vector as a negative control. For the transfection of the genomes, 250 µl of cells was added to every tube containing 4 µl of carrier, 2000 ng of a wt or mutant genome and 240 ng of either an empty vector or a plasmid

encoding the catalytic subunit alpha of PKA. Mixtures were transferred into electroporation cuvettes. Electroporation cuvettes with a gap size of 4 mm were used. All electroporations were performed using a Gene Pulser XCell machine (Bio-Rad Instruments) at 220 V voltage and 975 µF capacity.

After electroporation, the cells transfected with expression vectors were resuspended in 3 ml of fresh medium and plated on a 12-well plate (Corning Inc.) using 1 ml of cell suspension per well. The cells were incubated for two days. The cells were treated with 0.5 mM IBMX, 20 µM Forskolin or DMSO as a control. The cells were induced for 6 hours, then medium was aspirated, the cells were washed with PBS and then frozen at -20 ˚C for further experiments.

The cells transfected with the genomes were plated on three separate 96-well plates (Corning Inc.) in triplicates. The cells were incubated for 48, 72 and 96 hours, the medium was aspirated, the cells were washed with PBS and frozen at -20 ˚C.

3.1.4 Western Blot

3.1.4.1 The preparation of the gel

For preparation of 8% a separating acrylamide gel, 4.6 ml of water, 2.7 ml of 30% acrylamide-bisacrylamide mix, 120 µl of 10% ammonia persulfate (APS), 12 µl of tetramethylethylenediamine (TEMED) and 2.5 ml of 4xSeparating buffer were mixed.

For preparation of a stacking gel, 2.1 ml of water, 0.5 ml of 30% acrylamide-bisacrylamide mix, 42 µl of 10% NAPS, 4.2 µl of TEMED and 750 µl of 4x Stacking buffer were used. The Mini-PROTEAN® Tetra Cell Casting Module (Bio-Rad) was assembled. The separating gel was loaded first, and then incubated for 20 minutes at RT. Ethanol was added on top to prevent the appearance of air bubbles. The ethanol was removed, and stacking gel and a 15-well comb were added on top. The gel polymerized for another 20 minutes, was removed from the cell casting module and placed in a Mini Trans-Blot® Cell (Bio-Rad) already filled with a running buffer (3.3 g Tris, 14.4 g glycine, 1% SDS, up to 1 L water). The comb was removed and the gel was ready to use.

3.1.4.2 SDS-PAGE

U2OS cells transfected with the expression constructs HPV5-E2-Flag-WT, HPV5-E2-Flag-A and HPV5-E2-Flag-E, or pEGFP-C1 vector and treated with IBMX, Forskolin or DMSO were

directly lysed with 100 µl of buffer containing 100 µl of reducing agent dithiothreitol mixed with 300 µl of 4x Laemmli sample buffer and 400 µl of RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 0.1 % Triton x100 buffer). The cell lysates were transferred to clean Eppendorf tubes and incubated at 100 ˚C for 10 minutes in order to denature DNA and proteins. Following denaturation, the samples were loaded on acrylamide gel together with PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific).

The gel was running at 20 mA per one gel for 1 hour and 30 minutes. After that, the gel was removed from the buffer tank and transferred into a gel holder cassette with two foam pads, four pieces of filter paper and PVDF membrane (Millipore). All components were soaked in the Protein Transfer buffer (3,3 g Tris, 14.2 g glycine, 7% ethanol) in advance and transfer of the proteins to the membrane was performed in the same buffer at 400 mA for 1 hour and 40 minutes. A cooling unit was added to the tank to prevent warming due to the higher voltage.

3.1.4.3 Membrane blocking, antibody incubation and X-ray film development

The membrane was blocked in PBS solution containing 0.1% Tween-20 (PBS-T) and 5% of nonfat dry milk for 30 minutes at RT. Following blocking, the membrane was incubated with ANTI-FLAG M2-HRP antibody diluted 1:3000 in PBS-T containing 2.5% of nonfat dry milk (Sigma-Aldrich) at 4 ˚C overnight. Next morning, the membrane was washed with 10 ml of PBS-T 3 times for 15 min, incubated with ECL Dura kit (Pierce) for 1 minute at room temperature and exposed to X-ray film. The film was developed and fixed using AGFA Developer and Rapid Fixer solutions.

3.1.5 Luciferase assay

The U2OS cells transfected with the wt or mutant genomes were lysed in 50 µl of 1x Passive Lysis Buffer (Promega) per one well of the 96-well plate and incubated for 15 minutes at -20 ˚C. After that, the lysates were unfrozen for 10 minutes at room temperature. Next, 10 µl of the lysate was transferred to two different 96-well plates for analysis of the Nluc and alkaline phosphatase (AP) activities. Nluc substrate furimazine was diluted with the Luciferase Assay Buffer (Promega) in 1:500 ratio and added to the lysates (20 µl/well).

To measure the AP activity, 20 µl of CSPD substrate (Applied Biosystems, Tropix) was added to the 10 µl of the lysate on the second plate. Chemiluminescence was measured using GloMax 96 Microplate Luminometer (Promega), and Nluc activity was normalized by AP activity.

3.2 RESULTS

3.2.1 Generation of HPV5-E1HA-Nluc-E2Flag-A/E / HPV5-E2-Flag-A/E constructs

In order to generate the required constructs, the point mutations at Serine residue 255 were introduced in vectors containing either wild type HPV5 genome or a sequence encoding the wt E2 protein of HPV5. The mutations were introduced using PCR mutagenesis. PCR products corresponding to the length of the template DNAs were obtained (Figure 5).

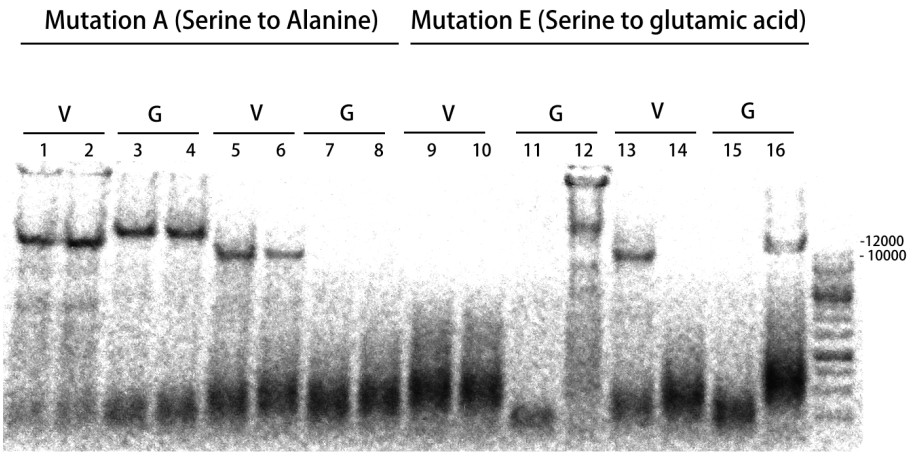

Figure 5. PCR products obtained using PCR-based mutagenesis. The plasmids HPV5-E1HA-Nluc-E2Flag ("G" on the image, approximately 12 kb) or HPV5-E2-Flag ("V" on the image, approximately 10 kb) were used as templates for 30 cycles of amplification using the primers containing the required mutations. The introduction of the point mutations was successful in samples 1,2,5,6 (HPV5-E2-Flag-A), 3,4 (HPV5-E1HA-Nluc-E2Flag-A), 12, 16 (HPV5-E1HA-Nluc-E2Flag-E) and 13 (HPV5-E2-Flag-E). 1kb GeneRuler ladder was used.

The obtained PCR products were purified from the agarose gel and transformed to bacteria. Plasmid DNA from several clones, either ampicillin-resistant (for HPV5-E2-FlagA/E expression vectors) or kanamycin-resistant (for HPV5-E1HA-Nluc-E2Flag-A/E genomes), was isolated.

3.2.1.1 Validation of HPV5-E2-Flag-A/E constructs

In order to validate that the HPV5-E2-Flag-A/E constructs were generated correctly, two methods were employed: DNA sequencing and restriction analysis. All samples were restricted using 0.5 µl of HindIII and 0.5 µl of BamHI together with 2 µl of FastDigest Green Buffer and 15 µl of water, and incubated for 30 minutes at 37 ˚C. All samples were loaded on 0.8 % agarose gel. The gel was running for 40 minutes in 1x TAE buffer solution under 220 mA then the resulting fragments were analyzed under UV light to see if they match to the expected lengths (Figure 6).

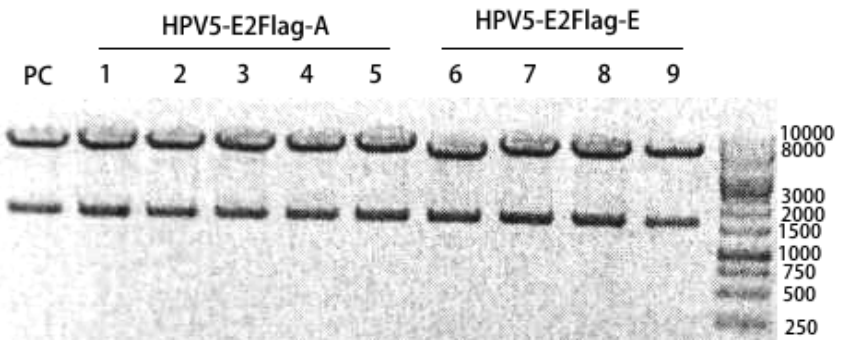

Figure 6. Restriction analysis of the obtained HPV5-E2Flag-A/E expression constructs using BamHI and HindIII restriction enzymes. Two 8 kb and 1.7 kb long fragments were corresponding to the expected ones. For the positive control, HPV5-E2Flag-WT was restricted with the same restriction enzymes. Samples from 1 to 5 are HPV5-E2Flag-A and samples from 6-8 are HPV5-E2Flag-E. 1kb GeneRuler ladder was used.

All tested HPV5-E2Flag-A constructs and HPV5-E2Flag-E clones 7, 8 and 9 were correct and used for further verification by DNA sequencing

3.2.1.2 Validation of HPV5-E1HA-Nluc-E2Flag-A/E constructs

To confirm that the HPV5-E1HA-Nluc-E2Flag-A/E constructs were generated correctly, restriction analysis was performed in two duplicates. The first one was performed using 1 µl of SacI restriction enzyme and 2 µl of FastDigest buffer, while the second one was performed with 1 µl of SacI, 1 µl of BstxI and 2 µl of FastDigest buffer. In the first case, we would expect to see the band equal in length to 12 kb (the length of the linearized parental plasmid HPV5-E1HA-Nluc-E2Flag) (Figure 7A). In the second case, we would expect to see two bands of 3 kb and 9 kb (Figure 7B). Restriction analysis showed that the length of fragments produced by all samples corresponded to the expected ones.

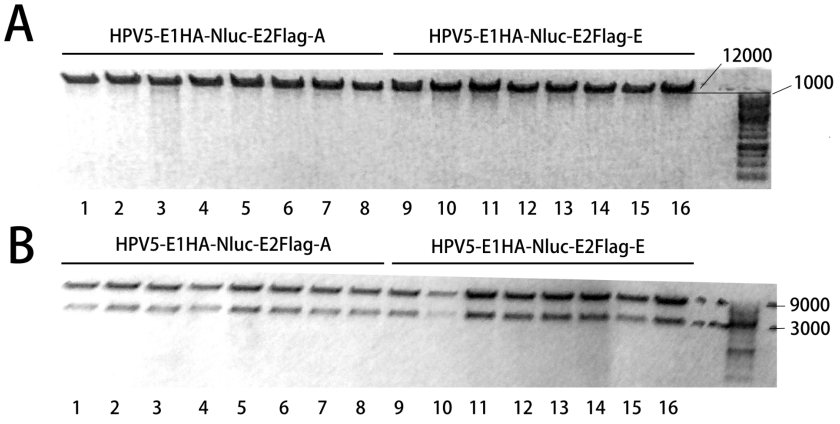

Figure 7. Restriction analysis of the obtained HPV5-E1HA-Nluc-E2Flag-A/E construct. A) Restriction with SacI linearizing the genome. The expected length of the fragment is 12 kb. B) Restriction with SacI and BstxI. The length of the expected fragments were 9 kb and 3 kb long. All bands match this expectation. 1kb GeneRuler ladder was used.

The E2 encoding sequence of the generated HPV5-E2-Flag-A/E or HPV5-E1HA-Nluc-E2Flag-A/E constructs were also verified by DNA sequencing. The correct DNAs were chosen and subcloned to the parental vectors in order to avoid possible PCR-generated mutations in the non-sequenced parts of the plasmids.

3.2.1.3 Validation of the generated HPV5-E1HA-Nluc-E2Flag-A/E minicircle genomes

To generate 8.5 kb long HPV5-E1HA-Nluc-E2Flag minicircle genomes, the obtained 12 kb long HPV5-E1HA-Nluc-E2Flag-A/E parental plasmids were subjected to the specific recombination in the *E. coli* strain ZYCY10P3S2T. Plasmid DNA was purified and the resulting minicircles were validated using restriction analysis to confirm that the recombination was successful and bacterial DNA was removed (Figure 8). In this case, the linearized length of HPV5-E1HA-Nluc-E2Flag genome should be around 8.5kb.

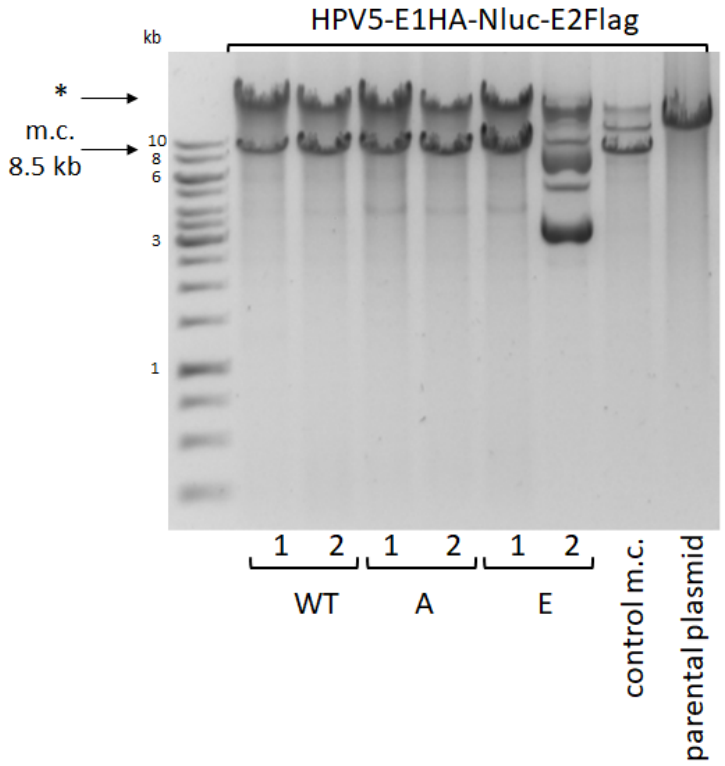

Figure 8. Restriction analysis of the obtained minicircle HPV5-E1HA-Nluc-E2Flag genomes. Two independent clones (1 and 2) of each genome were analysed. DNA was purified from bacteria and subjected to restriction analysis using BglI enzyme linearizing the HPV5-E1HA-Nluc-E2Flag genome. The required fragment corresponding to the full-length linearized HPV5-E1HA-Nluc-E2Flag genome is approximately 8.5 kb. The band indicated with an asterisk is another form of the HPV5-E1HA-Nluc-E2Flag minicircle genome produced by bacterial cells. WT - wild type, A - HPV5-E1HA-Nluc-E2Flag-A, E - HPV5-E1HA-Nluc-E2Flag-E, m.c. – minicircle, control m.c. – previously purified HPV5-E1HA-Nluc-E2Flag minicircle genome.

All purified DNAs except HPV5-E1HA-Nluc-E2Flag-E clone 2 were correct. The genomes HPV5-E1HA-Nluc-E2Flag WT clone 1, HPV5-E1HA-Nluc-E2Flag-A clone 1 and HPV5-E1HA-Nluc-E2Flag-E clone 1 were used for further experiments. Concentrations of the chosen minicircle genomes were 180 ng/µl for HPV5-E1HA-Nluc-E2Flag, 310 ng/µl for HPV5-E1HA-Nluc-E2Flag-A and 130 ng/µl for HPV5-E1HA-Nluc-E2Flag-E

3.2.2 Western Blot

It has been suggested that HPV8 E2 protein is regulated via PKA-mediated phosphorylation at serine residue 253. To analyse the expression levels and PKA-dependent regulation of the HPV5 wt and mutant E2, these proteins were over-expressed in U2OS cells. On the second day after transfection, the cells were treated with PKA activators IBMX or forskolin for 6 hours and lysed. Western Blot method and anti-Flag antibody were used to determine the effect of different PKA activators upon the stability of the wt or mutant E2 proteins (Figure 9).

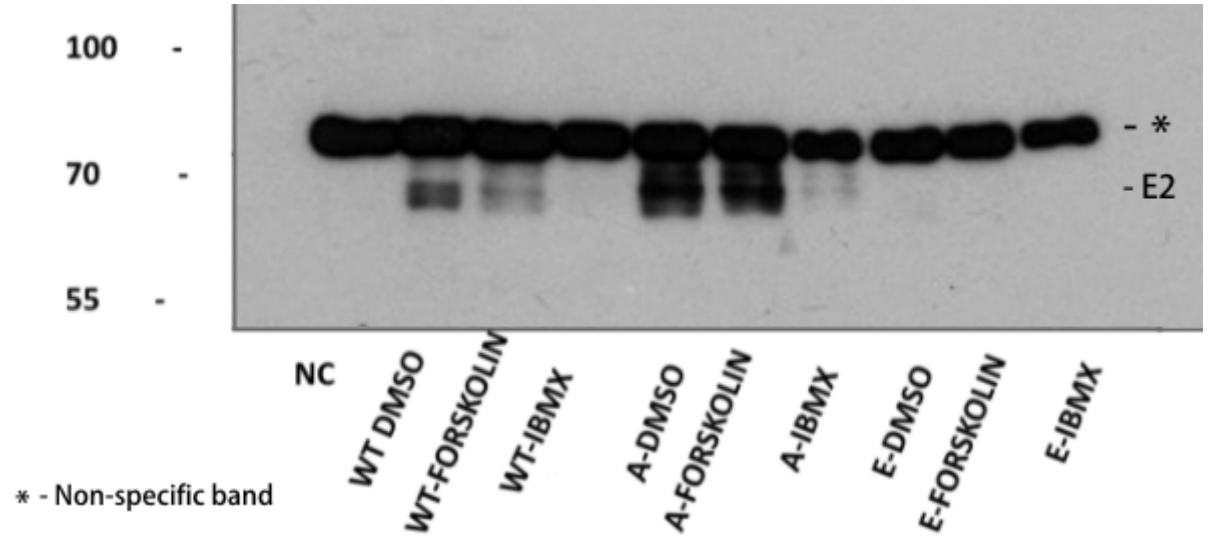

Figure 9. The results of the Western Blot experiment. U2OS cells were transfected with the E2-expression constructs (WT - HPV5-E2-Flag-WT, A - HPV5-E2-Flag-A and E - HPV5-E2-Flag-E), incubated for 2 days and treated with PKA activators or DMSO as a control. Immunoblotting analysis was performed using anti-Flag-HRP antibody. The expected molecular weight of the E2 protein is approximately 57 kDa. The signal corresponding to the Flag-tagged E2 was detected between 70 kDa and 55 kDa. Lysate of the cells with transfected GFP was used as a negative control (NC). Non-specific band (*) migrated at approximately 90 kDa.

The level of E2 protein containing phosphomimetic glutamic acid in position 255 was almost under the detection limit. In contrast, expression level of the E2 protein containing non-phosphorylatable alanine residue 255 was much higher than that of the wt E2. In the cells transfected with HPV5 wild type E2, treatment with forskolin led to reduction of the E2

protein level. However, the E2 protein containing non-phosphorylatable alanine residue 255 was not degraded extensively in response to the forskolin. The E2-corresponding signals in the IBMX-induced samples were weaker in both cases.

3.2.3 Luciferase Assay

The constructs encoding wt and mutated HPV5 genomes also include the NLuc encoding sequence. In this study, this bioluminescent enzyme is used as a reporter protein, since the level of Nluc activity correlates with copy number of the Nluc-containing genome and provides the linear dependence between the number of the copies of the viral genome and the strength of the chemiluminescence signal genome (Piirsoo 2019, Lototskaja 2021).

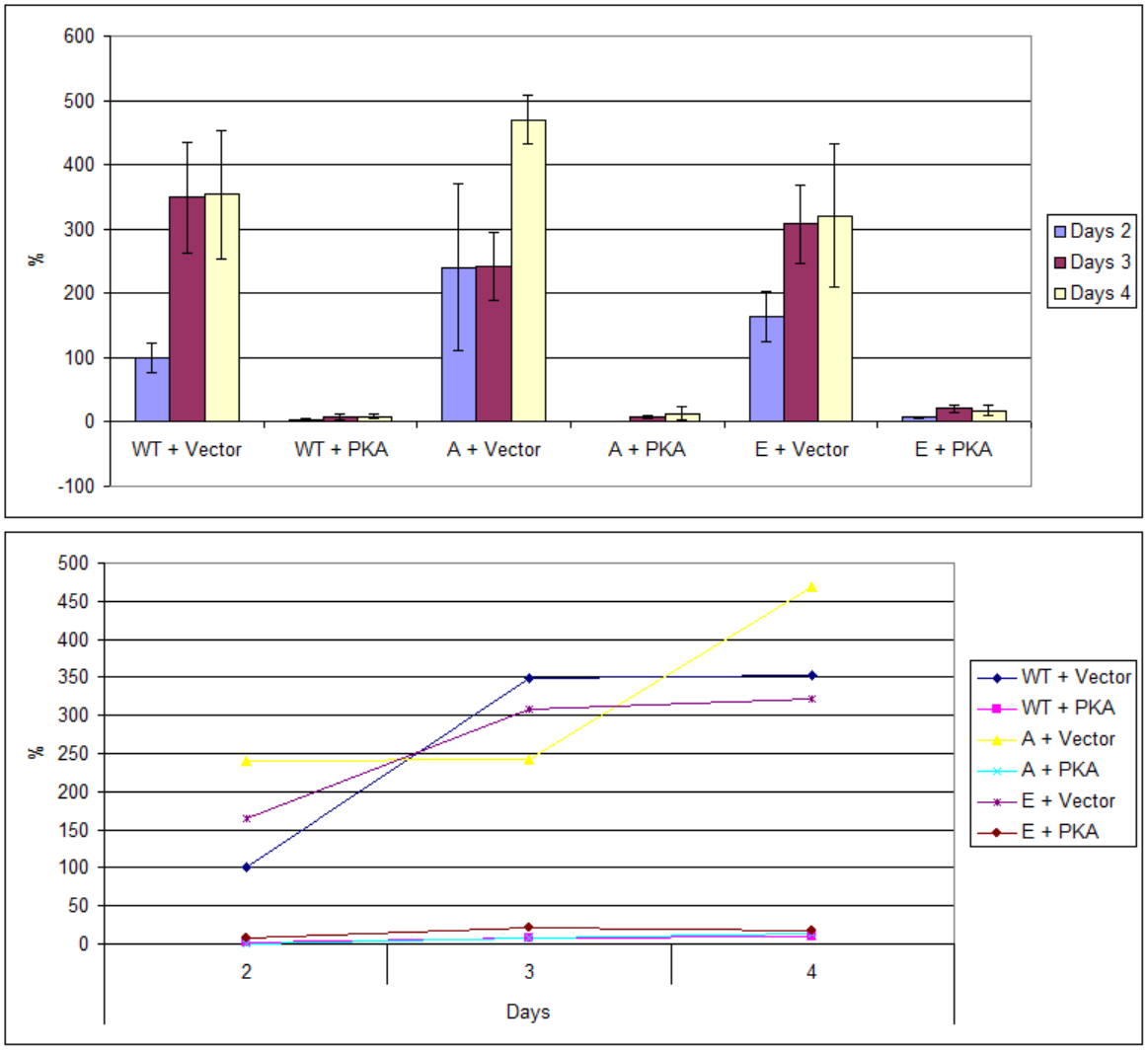

Figure 10. The results of the Luciferase assay. U2OS cells were transfected with the HPV5 genomes (WT - HPV5-E1HA-Nluc-E2Flag-WT, A - HPV5-E1HA-Nluc-E2Flag-A, E - HPV5-E1HA-Nluc-E2Flag-E) and either empty vector or vector encoding catalytic subunit alpha for the overexpression of PKA. Cells were incubated for 2, 3 and 4 days. Cells were lysed, the luciferase substrate was added and the bioluminescence was measured. The values were scaled to percentages for the better representativity. The first diagram shows the quantitative activity of the luciferase in different samples over the course of the experiment. The second diagram shows the dynamics.

3.3 DISCUSSION

It has been shown that HPV5 E2 protein is degraded in response to PKA activator IBMX (Lototskaja 2021). However, the residues subjected to the PKA-mediated phosphorylation remained unknown. It has been suggested that PKA may phosphorylate serine residue 253 in the E2 protein of the HPV8, which is highly similar to HPV5 (Sekhar and McBride, 2012). This serine residue and PKA consensus motif are conserved in the E2 proteins of HPV8 and HPV5. Therefore, we introduced point mutations to substitute this serine residue to phosphomimetic alanine and nonphosphorylatable glutamic acid residues and evaluated the effect of these mutations on the stability of E2 protein using Western Blot method. Our experiment has shown that the level of the E2 protein is affected by the status of this residue 255. In the cells transfected with HPV5-E2Flag-E construct, in which this specific serine residue was mutated to the phosphomimetic glutamic acid, the level of the E2 protein was under detection limit. The protein was not detected even in the cells not treated by the PKA activators. Since glutamic acid is a phosphomimetic residue, its structure highly resembles phosphorylated serine that makes the protein always “phosphorylated”. Therefore, we can suggest that phosphorylation at this specific residue leads to the rapid degradation of the E2 protein.

In contrast, the mutant A (HPV5-E2Flag-A, Serine 255 is mutated to alanine) demonstrated a stronger signal compared to the wt E2. Since alanine cannot be phosphorylated, degradation of E2 does not take place, which results in a stronger signal detected using immunoblotting. However, the levels of E2 proteins in the samples induced with IBMX are weaker in both cases, E2 wt and E2(S255A) proteins. The possible reason may be the increased level of cyclic guanosine monophosphate (cGMP) caused by IBMX, which non-specifically blocks all phosphodiesterases in the cells. Treatment with IBMX may lead not only to increase of cAMP and activation of PKA in cells, but also to increase of cGMP levels and the activation of cGMP-dependent protein kinase G (PKG) that can also contribute to the degradation of the E2 protein by phosphorylation at other recognition sites. That does not occur with Forskolin, because it increases only the level of cAMP by stimulation of adenylate cyclases.

According to our immunoblotting data, we would expect to obtain similar results in the Luciferase assay experiment, which was applied to estimate the copy number of the replicating wt and mutant genomes. However, we can see that the intensity of the bioluminescence signal representing the number of copies of the viral genomes are similar in all cases. The cells transfected with the HPV5-E1HA-Nluc-E2Flag-A construct show slightly

better performance compared to the mutant E and wild type HPV genomes, however, the difference is not significant enough. Replication and genome copy number of the mutant E were similar to the wt genome. The mutant A does not have the expected resistance to the overexpressed PKA catalytic subunit, which inhibits the replication of all used genomes in a similar manner. Taking together our data, we can suggest that there might be other recognition sites phosphorylated by PKA. We would also expect to see mutant E to have a very small number of copies of HPV5 genome due to the rapid degradation of E2 protein in the cells transfected with the corresponding construct. Even though the E mutant-produced NLuc activity is weaker than it is in the mutant A and wt samples, the observed difference does not match our expectations. We suggest that the reason behind that is the shorter form of E2 protein called E2^E8 repressor that acts as an inhibitor of viral genome replication. The rate of HPV replication is controlled by the proper balance between E2 and E2^E8. Different concentrations of full-length E2 protein and E2^E8 alternative transcript allow the virus to control the replication of its DNA at different stages of its life cycle. The E2^E8 protein has the same serine residue and hypothetically may be subjected to the PKA-mediated phosphorylation and regulation. However, it remains to be investigated. We suggest that not only the degradation of the activator occurs but also the degradation of the inhibitor may occur in response to the overexpressed PKA catalytic subunit. Similarly, if the HPV5-E1HA-Nluc-E2Flag-A expresses a more stable E2 protein, it may also express a more stable repressor E2^E8, which counteracts with full-length E2. Therefore, the copy numbers of all used genomes are similar. Finally, it is possible that regulation of the endogenous genome-derived and over-expressed at high levels E2 proteins are different. In this case, the results observed in the overexpression studies cannot be expanded to explain the behaviour of the endogenous proteins.

SUMMARY

E2 is one of the most promising targets for development of therapeutic agents against the established HPV infections. This regulatory protein plays a crucial role in the replication of the viral genome at different stages of the viral life cycle. As many other proteins, E2 undergoes post-translational modifications changing the properties and the functions of this protein. Therefore, in order to develop effective drugs against the virus, it is important to study the interplay between the host and the virus. The main focus of this work is to investigate the effects the phosphorylation at Serine 255 residue has on the stability of E2 protein and replication of the viral genome.

In order to accomplish this goal, we introduced point mutations in the constructs encoding either the whole HPV5 viral genome or E2 protein of HPV5. The mutation "A" is the replacement of Serine 255 with alanine, the amino acid that cannot be phosphorylated. This would give us an opportunity to observe the stability and the replication of the viral genome without phosphorylation at this specific residue. The mutation "E" is the replacement of Serine 255 with glutamic acid. This amino acid is a phosphomimetic one, and its structure highly resembles the phosphorylated serine. The point mutations were introduced using long-range PCR, and the obtained constructs were verified using DNA sequencing and restriction analysis. Then the constructs were transfected into U2OS cells to analyze either the replication of the wt and mutated viral genomes or the stability of the wt and mutated E2 proteins.

It was determined that phosphorylation at Serine 255 residue leads to the rapid degradation of E2 protein since the level of the E2(S255E) protein was under the detection limit. In contrast, the level of the E2(S255A) protein was higher than that of the wt E2. Two types of PKA activators were able to induce degradation of the wt E2 protein. However, their effects were controversial in the case of the E2(S255A) protein. The level of the E2(S255A) protein was reduced in the presence of IBMX, but remains similar to the control in response to forskolin. Further studies are needed to analyse the PKA-mediated effects on stability of HPV5 E2 protein.

It was expected to obtain in-line with immunoblotting assay results in the replication of the viral genome as well. However, we found that the introduced mutations did not provide substantial positive or negative effects on the replication of the HPV5 genome in U2OS cells. Besides, the introduced mutation A did not provide any resistance to the overexpressed PKA catalytic subunit alpha. We suggest that the reason might be the presence of other yet unknown PKA phosphorylation sites in the E2 protein. The mutation E did not have any effect on the replication of the viral genome as well. Possible reason might be degradation of the repressor of replication E8^E2 together with the degradation of the full-length E2 acting as the initiator of the replication of the viral genome, since both proteins contain the mutated serine residue 255 and therefore may be subjected to similar regulation.

The obtained results might be beneficial for further studies focused on the disruption of the E2 activity and for the development of new therapeutic strategies to prevent the HPV-related diseases.

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