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Short communication

ORPHAN NUCLEAR HORMONE RECEPTOR NR4A1 INTERACTS WITH HPV16 E2 REGULATORY PROTEIN

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Abstract: The human NR4A1 orphan receptor is a member of the TR3 steroid receptor superfamily, which binds DNA at the NBRE and NurRE responsive elements. The TR3 receptors are involved in the regulation of differentiation, proliferation and apoptosis. We report that NR4A1 interacts with human papillomavirus type 16 (HPV16) E2 protein – a key papillomavirus regulatory factor. This interaction might be involved in the transcription regulation of the HPV16 genes and the regulation of infected cell homeostasis.

Key words: NR4A1, Nur77, E2, HPV16, Two-hybrid system, Nuclear localization, Colocalization studies

INTRODUCTION

Orphan receptors are transcription factors, but little is known about the nature and function of their ligands. They belong to a large family of endocrine hormone receptors (including steroid, retinoid, and thyroid) that reside in the cell cytoplasm or nucleus and target specific DNA sequences after acquiring their

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Abbreviations used: HPV – human papillomavirus; EGFP – enhanced green fluorescent protein; HA – hemagglutinin.

ligands. The members of this receptor family share a number of structural features. Their DNA-binding domain, located in the central portion of their aminoacid chain, is highly conserved and recognizes specific DNA sequences, termed hormone response elements. The carboxy-terminal part of the receptors, which interacts directly with the hormone (the ligand-binding domain), functions as a ligand-dependent transcriptional activation domain. The NH_2-terminal part varies in structure and contains the ligand-independent transactivation domain [1, 2]. The orphan nuclear hormone receptors are believed to be able to interact with DNA in the absence of any ligand. The NR4A1 protein (Nur77, TR3, NGFI-B) is involved in the regulation of differentiation, proliferation and apoptosis [3, 4]. This orphan receptor can bind DNA as a monomer to an estrogen receptor half-site element (NBRE 5'-AAAGGTCA-3') or as a homodimer to a palindromic DNA-binding motif (NurRE 5'-TGATATTTN$_6$AAATGCCA-3') [5]. In this report, we show that NR4A1 is able to interact with the E2 protein of human papillomavirus type 16 (HPV16). HPVs are small DNA tumor viruses that can cause benign proliferative lesions and cancer [6]. Over 100 HPV types have been identified, and HPV16 is the predominant cervical cancer-associated type [7]. E2 is a regulatory protein able to either activate or repress the transcription of viral oncoproteins from the long control region (LCR) of the HPV genome [8, 9]. This 42-kDa protein is composed of an NH_2-terminal transcriptional activation domain, a hinge region and a COOH-terminal DNA-binding and dimerization domain. The viral regulatory protein binds to multiple copies of an ACCN$_6$GGT motif; these occur in the LCR of all HPVs [10]. The LCR regulatory sequence also contains multiple binding sites for cellular transcription factors, with three steroid hormone response elements among them [11]. The interaction between the E2 and NR4A1 proteins reveals a novel potential relationship in the complex regulatory mechanism of the HPV replication cycle.

MATERIAL AND METHODS

Plasmid vectors

The human papillomavirus type 16 coding sequence of the E2 regulatory protein was cloned at full-length into a yeast two-hybrid system vector yielding a fusion protein with a Gal4-DNA-binding domain (pDBLeu-E2) and into a mammalian vector yielding a fusion protein with a green fluorescent protein (pEGFP-E2). Truncated NR4A1 cDNA (a 1.9-kbp coding protein lacking 148 N-terminal aminoacids) was obtained from a human epithelial cell library in a yeast two-hybrid system vector yielding a fusion protein with a Gal4-activation domain (pPC86-NR4A1), and was cloned into a mammalian vector yielding a fusion protein with a red fluorescent protein (pDsRed-NR4A1).

***In vitro* cell culture**

African green monkey kidney fibroblast Cos-7 and cervical cancer-derived epithelial C33-A cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37ºC in 5% CO_2.

The introduction of plasmid DNA into the cells

Two-hybrid system vectors were introduced into chemically competent yeast cells via LiAc/PEG transformation according to the manufacturer's instructions (ProQuest Two-Hybrid System, Invitrogen). Mammalian fluorescent protein expression vectors were transfected into Cos-7 cells by lipofection using a Tfx-20 Reagent (Promega) as recommended by the reagent manufacturer.

Two-hybrid analysis

Two-hybrid analysis was performed on yeast *S. cerevisie* strain MaV203 cells grown on complex YAPD or minimal synthetic SC medium. Where necessary, the SC medium lacked leucine (-Leu), tryptophane (-Trp), histidine (-His), or uracil (-Ura) and/or was supplemented with 10 mM 3-aminotriazole (+3AT) and 0.2% 5-fluorouracil (+5FOA). A ß-Gal assay was performed in Z buffer supplemented with 2-mercaptoethanol and X-Gal as described in the manufacturer's instructions (ProQuest Two-Hybrid System, Invitrogen).

***In vitro* transcription-translation and co-immunoprecipitation**

A co-immunoprecipitation study was performed using *in vitro* synthesized Myc epitope-tagged HPV16 E2 protein and HA epitope-tagged NR4A1 protein. The proteins labeled with ^{35}S-Met/Cys (ICN) were obtained using a TnT T7 Coupled Reticulocyte Lysate System (Promega) on PCR products used as templates. To amplify the coding sequences of the proteins and incorporate the T7 polymerase promoter and the Myc- and HA- epitope-tag sequences, the Advantage 2 PCR Enzyme System (BD Biosciences) was used with starters designed to anneal to plasmid vector sequences as described in the Matchmaker Co-IP Kit (Clontech) instruction manual. The co-immunoprecipitation study was performed using a Matchmaker Co-IP Kit (Clontech).

Colocalisation studies

The Cos-7 and C33-A cells were grown on microscope chamber cover glass and cotransfected with pEGFP-E2 and pDsRed-NR4A1 vectors. Forty-four hours after transfection, the Cos-7 cells were fixed in 4% paraformaldehyde in phosphate buffered saline for 20 minutes at room temperature, washed three times with PBS and immersed in mounting medium containing DAPI. The C33-A cells were observed with no fixation. Fluorescence microscopy was carried out using an Axiovert200 inverted epifluorescent microscope with GFP, dsRed and DAPI filter sets and a 20x air objective (Zeiss). An LSM 510 laser-scanning microscope (Zeiss) with an LD Plan-Neofluar 63x/0.75 objective were used for confocal imaging experiments. EGFP- or dsRed-tagged proteins were respectively excited with 488 nm (Argon laser) or 543 nm (HeNe laser). The

emission filters were a 500-530 nm band pass for EGFP and a 565-615 nm band pass for dsRed.

RESULTS AND DISCUSSION

The yeast two-hybrid analysis indicated an interaction between orphan nuclear receptor NR4A1 and human papillomavirus type 16 E2 regulatory protein. *S. cerevisiae* MaV203 cells transfromed with pDBLeu-E2 and pPC86-NR4A1 plasmid vectors grew on plates with the SC-Leu-Trp-His+3AT medium. The growth of the transformed MaV203 cells on a minimal synthetic medium lacking histidine was possible due to the two-hybrid-dependent activation of a chromosomally-integrated *HIS3* reporter gene. This activation was conditional on the reconstitution of functional Gal4 transcription factor [12] through the interaction of the NR4A1 with the E2. The transformant phenotype analysis (inability to grow on SC-Leu-Trp-His-Ura medium, growth on SC-Leu-Trp-His+5FOA medium and weak blue color in ß-Gal assay) indicated interaction between NR4A1 and E2 (Fig. 1). The strength of the interaction between these two proteins in the yeast cells is similar in strength to the interaction between the human retinoblastoma (Rb) and the human E2F1 proteins [13, 14].

A co-immunoprecipitation study using Myc epitope-tagged HPV16 E2 and HA epitope-tagged NR4A1 did not confirm the interaction between these two proteins (data not shown). Therefore, we did further study to analyze the possible interaction. In this experiment, we showed colocalization of the expressed EGFP-E2 and dsRed-NR4A1 fluorescent fusion proteins in the cell nucleus (Fig. 2). The epifluorescence and confocal microscopy study confirmed the interaction between the two proteins. Both *in vivo* tests are conducted in the complex environment of the living cell, which makes them artifact-impervious, and they indicated interactions between the E2 and NR4A1 proteins. An *in vitro* experiment of co-immunoprecipitation failed to demonstrate the interaction, probably due to a lack of post-transcriptional modifications of one or both of the proteins [15, 16]. Both E2 [17] and NR4A1 [18] are phosphoproteins, the action of which is dependent on the modification [19].

The orphan nuclear receptors emerge to play a significant role in the regulation of viral activity (SV40 [20], MMTV [21], HTLV-1 [22], HIV-1 [23]), and notably, in the case of HPV16 [24]. Further studies on the interaction of these two proteins would give a new insight into the transcription regulation of the HPV16 genome [25].

An interesting fact is that the NR4A1 orphan receptor is apparently a common target for viral proteins: E2 (this study) and the EBNA2 of EBV [26], which suggests a universal mode of interaction between viruses and infected cells.

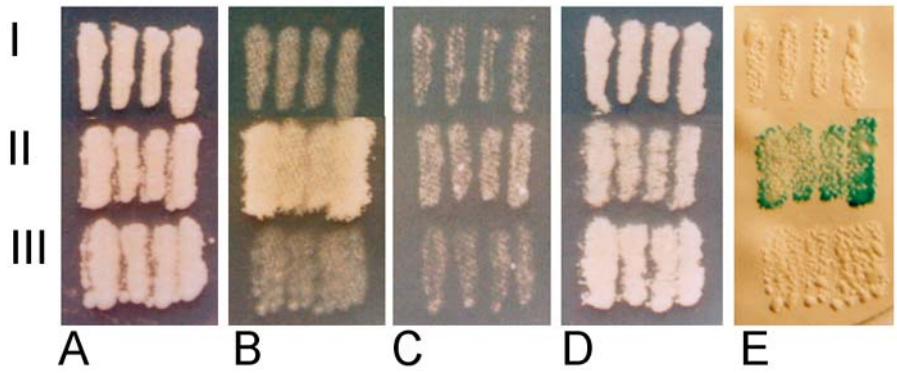

Fig. 1. A phenotype analysis of growth on selective media of the transformed *S. cerevisiae* MaV203 cells and ß-Gal assay. The yeast cells were transformed with (I) pDBLeu-E2 and pPC86, (II) pDBLeu-E2 and pPC86-NR4A1, or (III) pDBLeu and pPC86-NR4A1. Four colonies of each transformation were seeded on SC-Leu-Trp (A), then replica plated on selective media: SC-Leu-Trp-His+3AT (B), SC-Leu-Trp-His-Ura (C), SC-Leu-Trp-His+5FOA (D) and YAPD (for the ß-Gal assay) (E).

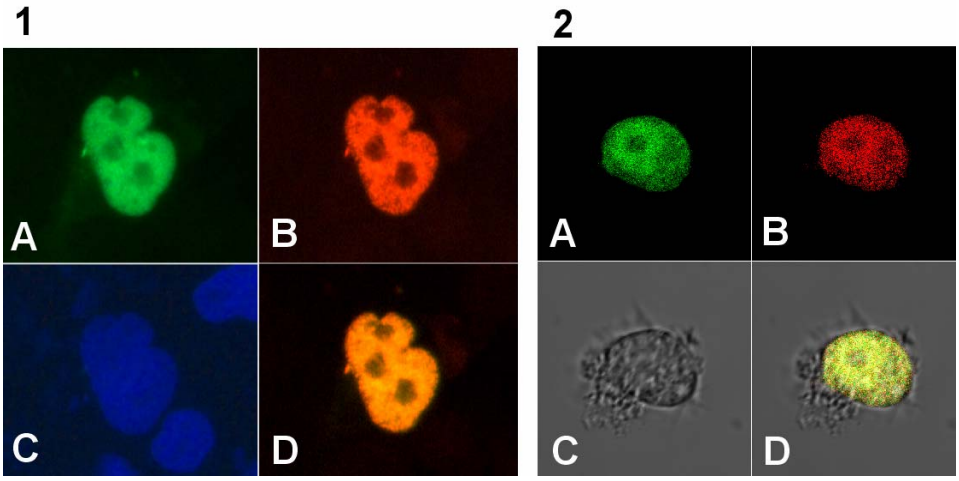

Fig. 2. Panel 1. Fluorescence microscopy of Cos-7 cells cotransfected with EGFP-E2- and dsRed-NR4A1-tagged proteins. (A) The localization of EGFP-E2 within the nucleus. (B) The localization of dsRed-NR4A1 within the nucleus. (C) DNA in the nucleus stained with DAPI. (D) Merged images show the superimposition of the EGFP and dsRed signals, which look yellow in the case of colocalization (D). Panel 2. Confocal microscopy of C33-A cells cotransfected with EGFP-E2- and dsRed-NR4A1-tagged proteins. (A) Localization of EGFP-E2 within the nucleus. (B) Localization of dsRed-NR4A1 within the nucleus. (C) Picture in transmitted light allowing to see the cell morfology. (D) Merged images show superimposition of EGFP and dsRed signals, which look yellow in the case of colocalization.

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