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Chlamydial protease CT441 interacts with SRAP1 co-activator of estrogen receptor α and partially alleviates its co-activation activity

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ABSTRACT

Chlamydiae are obligate intracellular pathogens which secrete host-interactive proteins capable of directly modulating eukaryotic pathways. Using the PDZ domain of the protease CT441 of *Chlamydia trachomatis* as a bait in a yeast two-hybrid screen, we identified the SRAP1 co-activator of estrogen receptor α (ERα) as an interacting protein. SRAP1 is a unique modulator of steroid receptor activity, as it is able to mediate its co-regulatory effects both as a RNA and a protein. GST pull-down experiments confirmed the interaction of CT441 and SRAP1 *in vitro*. Furthermore, it was shown that the CT441-PDZ domain fused to a nuclear localization signal was able to bind and to target SRAP1 to the nucleus in mammalian cells. CT441 did not cleave SRAP1, but retained the protein in the cytoplasm and thereby partially alleviated its co-activation of ERα in a heterologous yeast system and in mammalian cells. Possible implications of chlamydial regulation of host metabolism by targeting ERα activity are discussed. Moreover, the property of CT441-PDZ domain to specifically sequester SRA1 protein but not SRA1 RNA may be used to distinguish between the cellular functions of the SRA1 RNA and protein. This has clinical relevance as it has been proposed that disturbance of the balance between SRAP1-coding and non-coding SRA1 RNAs in breast tumor tissues might be involved in breast tumorigenesis.

1. Introduction

Chlamydiae are obligate intracellular bacterial pathogens that infect a broad range of cell types, including those of the eye, lung and genital tract epithelia. The ability of these microorganisms to express and secrete host-interactive proteins capable of directly modulating relevant eukaryotic pathways represents an important adaptation strategy. Despite our current inability to genetically manipulate *Chlamydiaceae*, characterization of these effector proteins enables chlamydiologists to elucidate molecular mechanisms essential for chlamydial pathogenesis [1]. For secretion of effector proteins chlamydiae use type II and type III secretion systems [2,3]. In this context, proteases capable of interfering with cytoplasmic host proteins play an important role. The best characterized chlamydial protease is CPAF (chlamydial protease-like activity factor), a protein, found in nearly all chlamydiae, which is responsible for cleavage of host transcription factors and pro-apoptotic proteins [4,5]. A second chlamydial protease, i.e. CT441, which belongs to the group of bacterial tail-specific proteases (Tsp), is functionally less well characterized. By cleaving the p65 subunit of NF-κB, CT441 of *Chlamydia* (*C.*) *trachomatis* urogenital serovar LGV2 is capable of interfering with the NF-κB pathway of host antimicrobial and inflammatory responses [6,7]. An important structural feature of CT441 is a so-called PDZ domain. PDZ domains are modular protein–protein interaction domains that are specialized in binding to specific amino acid sequences located at the C-terminal of membrane proteins or Tsp substrate proteins. PDZ domains were originally identified as conserved sequence elements in **P**SD-95, the *Drosophila* tumor suppressor protein **D**lg and the mammalian tight junction protein **Z**O-1 [8].

Estrogen receptor α (ERα) is a ligand-activated nuclear receptor that plays an important role in normal development, organ and bone homeostasis and the neoplastic progression of some cancers. ERα encompasses a centrally located DNA binding domain and two activation functions; the one located in the amino-terminal domain is referred to as activation function-1 (AF-1), while a second located in the carboxy-terminal ligand binding domain is named AF-2. The molecular mechanisms of ERα-mediated transcription involve the binding of ligand estrogen to the receptor, receptor dimerization and subsequent localization and binding to DNA response elements in the promoter region of hormone-responsive genes [9]. Maximum stimulation of ERα-mediated gene expres-

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sion depends on co-activators, which associate with ligand-bound ERα and enhance transcription by interacting with basal transcription factors and/or altering the chromatin structure around hormone-responsive genes [10]. One of the most interesting ERα co-activators is the steroid receptor RNA activator 1 (SRA1), which was initially characterized as belonging to the growing family of functional non-coding RNAs [11]. However, recently, the detection of an endogenous SRA1 protein (SRAP1) in breast cancer cells was reported [12], which suggests that SRA1 is the first example of a new class of functional RNAs that are also able to encode a protein.

In an attempt to identify interacting proteins and, therefore, potential substrates of PDZ domain-containing protease CT441 of *C. trachomatis* we performed a yeast two-hybrid screen in a human HeLa cDNA library using the PDZ domain of CT441 as a bait. Our findings suggest that SRAP1 as a protein interacts with CT441 *in vitro* and *in vivo*.

2. Materials and methods

2.1. Mammalian expression constructs

Vector pRK5(HA)$_2$-CT441M (Fig. 1A, expressing the mature form of protease CT441) was kindly provided by Li (The Scripps Research Institute, La Jolla, USA). To construct pDsRED-C1-NLS-PDZ the PDZ domain of CT441 (aa251–335) (Fig. 1A) was amplified by PCR using primers: forward-5′-GCGAATTCTCCTCCAAAAAAGAAGAGAAAGGTAGCTATGTGTGGCATAGGAGTCGTG-3′, reverse-5′-GCGGTCGACGAGAATTTTTTCACGACGTAA-3′ and pRK5(HA)$_2$-CT441M as template. EcoRI-SalI-digested PCR fragment was transferred into pDsRed2-C1 (CLONTECH). To assemble pCEP4-HA-PDZ the PDZ domain was amplified by PCR using primers: forward-5′-GCGAAGCTTACCATGTACCCATACGATGTTCCAGATTACGCTATGTGTGGCATAGGAGTCGTG-3′, reverse-5′-GCGCTCGAGTTAGAGAATTTTTTCACGACGTAA-3′ and pRK5(HA)$_2$-CT441M as template. HindIII-XhoI-digested fragment was cloned into pCEP4 (Invitrogen). To obtain pEGFP-C1-SRAP1 full-length SRAP1 was amplified by PCR using primers: forward-5′-GCGAATTCCACCATGACGCGCTGCCCCGCTGGC-3′, reverse-5′-GCCGTCGACTTATGAAGCCTGCTGGAAGCC-3′ and full-length SRAP1 construct in p425TEF (see below) as template. EcoRI-SalI-digested PCR fragment was transferred into pEGFP-C1 (CLONTECH). HindIII-SalI-fragment of SRAP1 from p425TEF-SRAP1 (see below) was transferred into pCEP4 to obtain another SRAP1 mammalian expression construct. To receive a GAL4-AF-1 fusion construct the AF-1 domain of ERα was amplified by PCR using primers: forward-5′-GCGAATTCACCATGACCATGACCCTCCACACC-3′, reverse-5′-GCCGTCGACTCATTCCATAGCCATACTTCCCTT-3′ and ERα-construct pG/ER(G) (obtained from Picard, Geneve, Switzerland) as template. EcoRI-SalI-digested PCR fragment was cloned into pCMV-BD (Stratagene). Plasmid pFR-Luc was obtained from Stratagene.

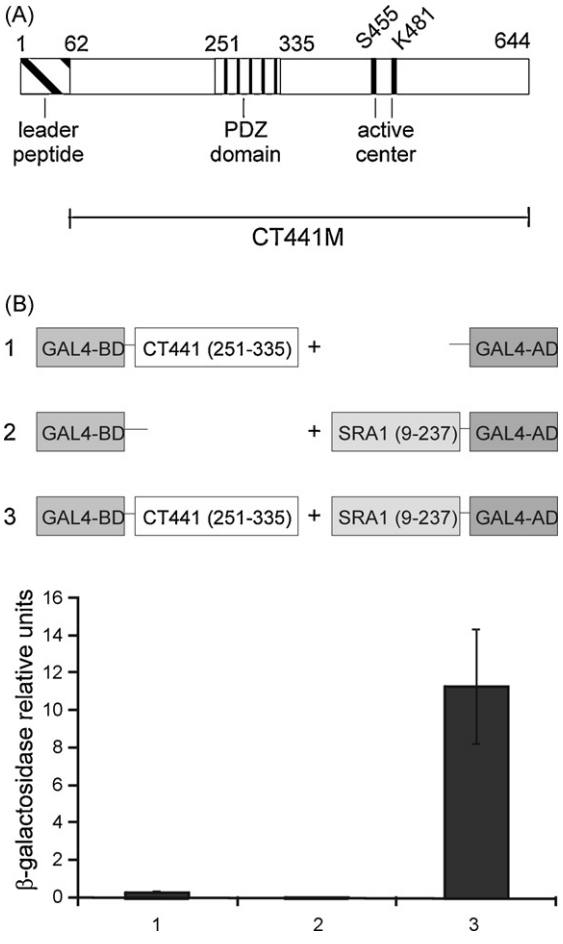

Fig. 1. The PDZ domain of CT441 interacts with SRAP1 in a yeast two-hybrid system. (A) Schematic structure of the protease CT441 of *C. trachomatis*. CT441M: mature form of protease CT441. (B) Specific interaction of the PDZ domain fused to GAL4 DNA binding domain (BD) with C-terminal part (aa9–237) of SRAP1 fused to GAL4-activation domain (AD) in yeast. Interaction was quantitatively assessed by β-galactosidase liquid culture activity assays. β-Galactosidase values represent the mean numbers and standard deviations of three independent experiments.

2.2. Cell culture and transfections

HEK293T cells were grown as monolayers in RPMI medium with L-glutamine (PAA) supplemented with 10% heat-inactivated fetal calf serum and maintained at 37 °C in an atmosphere of 5% CO_2. Cells were transfected with FuGENE® HD Transfection Reagent according to the manufacturer's instructions (Roche). For mammalian transactivation assay HEK293T cells were seeded in 60 mm culture dishes in phenol red-free RPMI medium with 10% charcoal-stripped FBS 24 h prior transfection. Following amounts of empty control or expression plasmids were added: 0.5 μg pFR-Luc, 3 μg pCMV-GAL4 DBD or pCMV-GAL4-DBD-ERα-AF1, 3 μg pCEP4-SRAP1 and/or pRK5(HA)$_2$-CT441M and pcDNA3.1 to fill up to equal total DNA ratios. After 24 h cells were washed twice with PBS and lysed 15 min at room temperature with passive lysis buffer (Promega). Lysats were centrifugated briefly and supernatants were assayed for luciferase activity using luciferase assay system (PJK-GmbH) according to the manufacturer's instructions and a Luminoscan luminometer (Labsystems). Relative luciferase units were normalized to total cellular protein, as determined by the Bradford assay. All the luciferase results presented are the average of at least three independent experiments performed in duplicate.

2.3. Immunofluorescence

HEK293T cells were seeded on cover slips and co-transfected with pEGFP-C1-SRAP1 and pDsRED-C1-NLS-PDZ or pCEP4-HA-PDZ. After 48 h cells were washed with PBS, fixed with 4% PFA and stained with 1 μg/ml DAPI. To detect HA-tagged PDZ domain cells were permeabilized with 0.5% NP-40/PBS, blocked with 5% FBS/0.1% NP-40 over night at 4 °C and incubated with monoclonal rat anti-HA antibody (Roche) and RRX-conjugated secondary antibody (Dianova) according to manufacturer's instructions. The subcellular localization of tagged proteins was monitored using a BX51 fluorescence microscope (Olympus).

2.4. Western blot

HEK293T cells were transfected with pRK5(HA)$_2$-CT441M or a control plasmid. After 24 h cells were harvested and lysed in

an aqueous solution containing 150 mM NaCl, 50 mM Tris/HCl (pH 8.0), 1% Triton-X-100 and a mixture of protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstantin and 1 mM PMSF). After incubation for 45 min on ice lysates were centrifuged briefly and equal amounts of supernatants were loaded on a 10% SDS-PAGE. Proteins were visualized with monoclonal rat anti-HA antibody (Roche), monoclonal mouse anti-NF-κB p65 antibody (Santa Cruz Biotechnology), polyclonal rabbit anti-SRA1 antibody (Lifespan) or monoclonal anti-β-actin antibody (Sigma) and HRP-coupled secondary antibody (Dianova) according to manufacturer's instructions. For detection ECL Plus Western Blotting Detection Reagent (GE Healthcare) was used as recommended.

2.5. Yeast two-hybrid screen

The PDZ domain of CT441 (aa251–335) was amplified by PCR using primers: forward-5′-GCGAATTCATGTGTGGCATAGGAGTCGTG-3′, reverse primer used for construction of pDsRED-C1-NLS-PDZ (see above) and pRK5(HA)$_2$-CT441M as template. The PCR fragment was digested with EcoRI-SalI and transferred into the GAL4-BD destination vector pGBKT7 (CLONTECH). *S. cerevisiae* Y190 (CLONTECH) expressing pGBKT7-CT441 (aa251–335) was mated for 6 h with *S. cerevisiae* Y187 expressing the HeLa cDNA MATCHMAKER 3 library in the activation domain (AD) vector pGADT7-Rec (CLONTECH). Interacting clones were selected by growth on vector-selective medium also lacking His but containing 5 mM 3-amino-1,2,4-triazole (3-AT). Emerging colonies were checked for activity of the second reporter gene lacZ by performing the colony-lift filter assay using X-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) as a substrate. HeLa cDNA-expressing plasmids were recovered from these strains, propagated in *Echerichia coli* (*E. coli*) and sequenced to identify coding sequences. For quantitative two-hybrid assays *S. cerevisiae* Y190 was transformed with corresponding pGBKT7- and pGADT7-constructs and β-galactosidase liquid culture assays, using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, were performed according to Breeden and Nasmyth [13]. The β-galactosidase values represent the mean numbers and standard deviations of three independent experiments.

2.6. Purification of GST fusion proteins, IVT, GST pull-down

To produce GST-tagged CT441 protein fragments, full-length maturated protease fragment (Fig. 1A, aa62–644) was amplified by PCR using following primers: forward-5′-GCGAATTCGCAGAGCCTCTTCGACGA-3′, reverse-5′-ATATGCGGCCGCTTATGATATAGATTTTAGAAG-3′ and pRK5(HA)$_2$-CT441M as template. EcoRI-NdeI-digested PCR fragment and PDZ domain as EcoRI-SalI-fragment from pGBKT7-CT441 (aa251–335) (see above) were transferred into expression vector pGEX-4T-1 encoding GST (GE Healthcare). To obtain GST-tagged SRAP1 protein fragment, full-length SRAP1 fragment was amplified by PCR using following primers: forward-5′-GCGAATTCATGACGCGCTGCCCCGCTGGCCAAGCGGAAGTGGAGATGGCGGAG-3′ and reverse primer used for construction of pEGFP-C1-SRAP1 (see above). EcoRI-SalI-digested PCR fragment was transferred into pGEX4T-1. Plasmids containing sequences encoding GST, GST-CT441, GST-PDZ and GST-SRAP1 were transformed into *E. coli* strain BL21-CodonPlus-RIL (Stratagene). An overnight culture with 13 ml LB medium containing 100 μg of ampicillin/ml was filled up to 20 ml. GST fusion derivates were induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) and incubation was continued for 2 h. Cells were collected by centrifugation, resuspended in 1.5 ml PBS containing 1% Triton-X-100, 1 mM PMSF, 10 mM DTT and then sonicated. Upon sonification the lysates were cleared by centrifugation and the supernatants were subjected to Glutathione Sepharose affinity chromatography for purification of the GST fusion proteins as described by the manufacturer (GE Healthcare). L-[^{35}S]-Methionine-labeled SRAP1 and CT441 proteins were synthesized *in vitro* by coupled T7 RNA polymerase-mediated transcription and translation (IVT) in a reticulocyte lysate system as described by the manufacturer (Promega). L-[^{35}S]-Methionine (specific activity: 1175 Ci/mmol) was obtained from Hartmann Analytic. For IVT a full-length SRAP1 fragment was amplified by PCR using primers: forward-5′-GCGAATTCATGACGCGCTGCCCCGCTGGCCAAGCGGAAGTGGAGATGGCGGAG-3′, reverse primer used for construction of pEGFP-C1-SRAP1 (see above) and SRAP1 yeast two-hybrid clone (see Fig. 1B) as template. Furthermore, for IVT a maturated CT441 fragment was amplified by PCR using forward primer used for construction of GST-CT441 (aa62–644) (see above) and reversed primer: 5′-GCGCTGCAGTTATGATATAGATTTTAGAAG-3′ and pRK5(HA)$_2$-CT441M as template. Both PCR fragments were cloned into pGBKT7 and used for IVT. Glutathione Sepharose beads (50 μl) liganded by either GST-tagged CT441 protein fragments or GST alone, were washed with HB-buffer (20 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4) and then resuspended in 50 μl HB-buffer containing 0.5% Igepal CA-630. To each sample, 10 μl L-[^{35}S]-Methionine-labeled SRAP1 full-length protein were added. After incubation at 4 °C by constantly rotating for 2 h the beads were washed with HB-buffer extensively. Proteins were eluted from the beads using SDS sample buffer. After separation by 10% SDS-PAGE, SRAP1 was revealed by exposure on KODAK X-OMAT AR film. For a reversed experiment fusion proteins for GST and IVT proteins were changed, using GST-SRAP1 and IVT of CT441.

2.7. Construction of yeast expression vectors

Full-length SRAP1 was amplified by PCR using primers: forward-5′-GCCAAGCTTATGACGCGCTGCCCCGCTGGC-3′ and reverse primer used for construction of pEGFP-C1-SRAP1 (see above). SRAP1 mutant protein containing a STOP codon after translational start was amplified by PCR using primers: 5′-GCCAAGCTTATGACGCGCTGACCCGCTGGCCAAGCGGAAGTGGAGATGGCGGAG-3′ and the reverse primer as above. HindIII-SalI-digested fragments were cloned into p425TEF (kindly provided by Funk, Medigene AG, Martinsried/Munich, Germany). The EcoRI-SalI-fragment from pGBKT7-CT441 (aa251–335) was transferred into expression vector p423TEF (M. Funk) to express PDZ domain of CT441 in yeast.

2.8. Estrogen receptor assay in yeast cells

The yeast strain YE1, containing ERα expression vector pG/ER(G) and estrogen responsive element – β-galactosidase-reporter construct pUCΔSS-ERE [14], was kindly provided by Munder (University of Applied Sciences, Jena). YE1 was transformed with the corresponding expression and control plasmids, cultivated over night at 30 °C, diluted to OD_{600} of 0.3 and dispersed 100 μl each on 96 well microtiter plates. 2 μl estradiol with 10-fold increasing concentrations (0.1 nM to 20 μM) were added and the plates were incubated for 1.5 h at 30 °C. The following β-galactosidase liquid culture assays, using ONPG as substrate, were performed as described above. All assays were conducted at least three times in triplicates of each sample.

3. Results

3.1. PDZ domain of CT441 interacts with SRAP1 in yeast two-hybrid system

To look for interacting proteins and, therefore, potential substrates of protease CT441 of *C. trachomatis* (Fig. 1A) we chose its

PDZ domain (aa251–335) as a bait in a yeast two-hybrid screen. Expression of the PDZ domain of CT441 from *C. trachomatis* fused to the GAL4 DNA binding domain (BD) in pGBKT7 in *S. cerevisiae* Y190 did not activate any of the reporter genes (*HIS3*, *lacZ*) (Fig. 1B). Therefore, this strain was mated with *S. cerevisiae* Y187 expressing the HeLa cDNA MATCHMAKER 3 library in the activation domain (AD) vector pGADT7-Rec. Interacting clones were selected by growth on vector-selective medium lacking His, and emerging colonies were checked for activity of the second reporter gene *lacZ*. One yeast clone contained in frame the coding sequence for the C-terminal part (aa9–237) of the steroid receptor RNA activator protein 1 (SRAP1) (Fig. 1B). To verify the validity of the interaction, *S. cerevisiae* Y190 expressing pGBKT7-PDZ (aa251–335) or only the GAL4-BD were co-transformed with the SRAP1-HeLa-library plasmid and the interaction was quantitatively assessed by β-galactosidase (β-gal) activity assays (Fig. 1B). Compared with the BD-PDZ and pGADT7 or pGBKT7 and AD-SRAP1 (aa9–237) vector controls, β-gal activity was significantly induced in *S. cerevisiae* Y190 expressing BD-PDZ (aa251–335) and AD-SRAP1 (aa9–237). These data indicate that the PDZ domain of CT441 specifically interacts with the C-terminal part of human SRAP1 in the yeast two-hybrid system.

3.2. Full-length CT441 and SRAP1 proteins directly interact in vitro

We next performed GST pull-down assays to investigate whether CT441 and SRAP1 directly interact *in vitro*. Glutathione Sepharose beads bound to GST-tagged full-length CT441 (aa62–644), GST-PDZ (aa251–335) or GST alone (Fig. 2A) were incubated with L-[^{35}S]-Methionine-labeled full-length SRAP1, processed as described in Section 2 and analyzed by autoradiography. As shown in Fig. 2B, full-length SRAP1 bound specifically to both the GST-full-length CT441 and the GST-PDZ matrix, while practically no binding was detected using GST as a control. In the reversed experiment full-length CT441 bound specifically to the GST-SRAP1 matrix (Fig. 2C and D), suggesting a direct physical interaction between CT441 of *C. trachomatis* and human SRAP1.

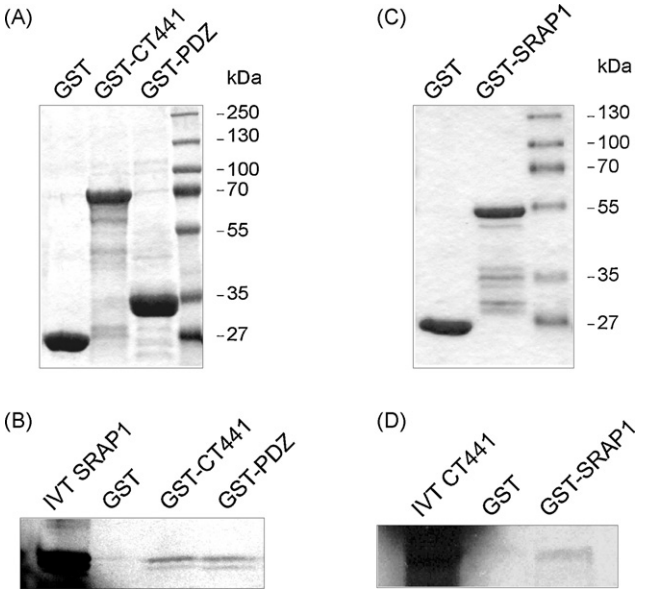

Fig. 2. Full-length CT441 and SRA1 proteins interact *in vitro*. (A) Purified GST, GST-CT441 and GST-PDZ or (C) GST and GST-SRAP1 stained with Coomassie. (B) Results of GST pull-down experiments performed with CT441 full-length and truncation clones fused to GST and L-[^{35}S]-Methionine-labeled full-length SRAP1 or (D) with SRAP1 fused to GST and L-[^{35}S]-Methionine-labeled full-length CT441. GST served as control. Input IVT: 10 μl of the *in vitro* translation product.

3.3. SRAP1 is directed to the nucleus of mammalian cells by the PDZ domain of CT441 fused to a nuclear localization signal but is not cleaved by CT441

Chimeric fusion proteins were constructed consisting of either SRAP1 fused C-terminal to EGFP or of the CT441-PDZ domain fused C-terminal to DsRed2 and additionally to a nuclear localization signal (NLS) [15] to target the PDZ domain to the cell nucleus. As a control a mammalian expression construct of the PDZ domain without a NLS was prepared. HEK239T cells were

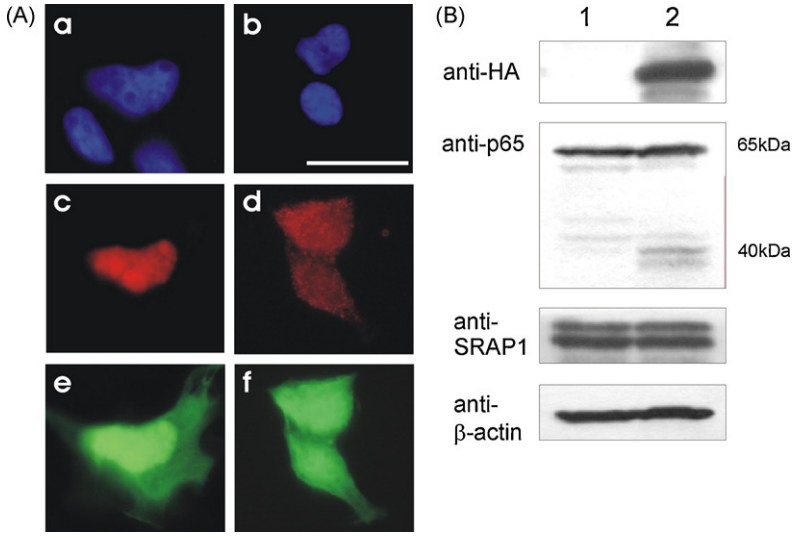

Fig. 3. SRAP1 is directed to the nucleus of mammalian cells by the PDZ domain of CT441 fused to a nuclear localization signal but is not cleaved by CT441. (A) PDZ domain of CT441 fused to a nuclear localization signal directs SRAP1 to the nucleus of mammalian cells. HEK293T cells were co-transfected with a full-length EGFP-SRAP1 construct and additionally with a DsRed2-PDZ(aa251-335) construct fused with a N-terminal nuclear localization sequence (NLS) (a, c, e) or with a HA-tagged PDZ construct without a NLS (b, d, f). Nuclei were stained with DAPI (a, b). In cells co-transfected with the HA-tagged PDZ construct both the PDZ domain (red) and EGFP-coupled SRAP1 (green) were distributed almost equally in cytoplasm and nucleus (d, f). In cells which were co-transfected with the NLS-containing PDZ construct the PDZ domain of CT441 localized in the nucleus (c) and initiated an enrichment of EGFP-SRAP1 in the nucleus, too (e). Scale bar = 20 μm. (B) CT441 does not cleave SRAP1. Cells were transfected with an empty control plasmid (lane 1) or a $(HA)_2$-tagged CT441M construct (lane 2). The cleavage of the p65 subunit of NF-κB is shown in the middle blot. No degradation of SRA1 protein and β-actin was detectable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

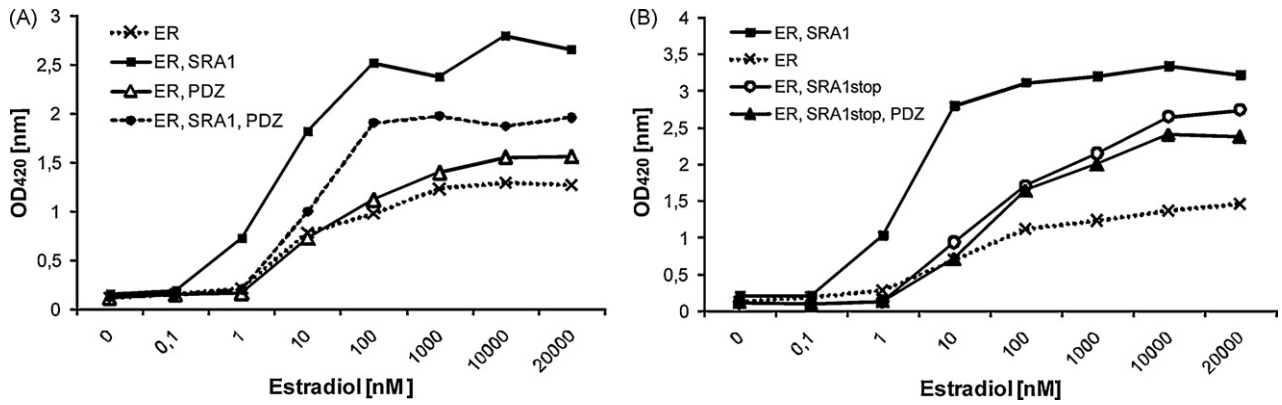

Fig. 4. PDZ domain of CT441 partially alleviates SRAP1 co-activtion of ERα in *S. cerevisiae*. For ER assay, the yeast strain YE1, containing the ERα expression vector pG/ER(G) and the estrogen responsive element – β-galactosidase-reporter construct pUCΔSS-ERE, was used. (A) Expression of the full-length SRAP1 in the yeast strain YE1 led to a significant stimulation of ERα with increasing estradiol concentrations, whereas expression of the PDZ domain of CT441 had no effect. Co-expression of SRAP1 and the PDZ domain showed a decreased stimulation of ERα, however, not as low as in the control strain YE1. (B) The YE1 strain expressing a SRAP1 mutant construct with a STOP codon after translational start showed an intermediate stimulation of ERα. Additionally, the co-expression with the PDZ domain of CT441 had no effect. Shown are the curves from one out of three independent experiments with triplicates from each sample.

transiently co-transfected with these constructs and after 48 h the subcellular localization of the fluorescent fusion proteins was observed using a fluorescence microscope (Fig. 3A). Expression of the DsRed2-NLS-PDZ domain in the nucleus (Fig. 3A, c) resulted in an enrichment of the co-expressed EGFP-SRAP1 in the nuclear compartment (Fig. 3A, e). This localization contrasted markedly with the diffusely distributed EGFP signal throughout the whole cell, which was observed in cells co-transfected with EGFP-SRAP1 and the PDZ domain without a NLS (Fig. 3A, f). These results show that CT441-PDZ domain is able to target SRAP1 to subcellular compartments in mammalian cells.

Subsequently, we wondered whether CT441 was able to cleave SRAP1 in the same manner as described for the p65 subunit of NF-κB [6,7]. For this purpose, HEK239T cells were transfected with pRK5$(HA)_2$-CT441M and examined by Western blotting, which detected $(HA)_2$-tagged protease, endogenous p65, SRAP1 and β-actin (Fig. 3B). While we were able to reproduce the p65 cleavage resulting in a p40 cleavage product (Fig. 3B, 2) [6,7], no cleavage of neither SRAP1 nor β-actin by CT441 could be obtained (Fig. 3B, 2).

3.4. PDZ domain of CT441 partially alleviates SRAP1 co-activation of ERα in S. cerevisiae

Since CT441 bound to SRAP1, but did not cleave it, we wondered whether SRAP1 was retained in the cytoplasm and thereby unable to co-activate ERα. To investigate this we chose a yeast system. Studying human ERα in *S. cerevisiae* has the advantage that yeast is a null host for nuclear receptors, albeit it was demonstrated that human ERα functions in yeast in a hormone-dependent manner [16]. At first, yeast strain YE1 containing an ERα expression vector and an estrogen responsive element – β-galactosidase-reporter construct was transformed with expression vectors encoding full-length human SRAP1 or PDZ domain of CT441. As a control, YE1 was transformed with the empty vectors. Fig. 4A shows that ERα is stimulated with increasing estradiol concentration in the control strain. Expression of SRAP1 led to a significantly stronger stimulation, whereas expression of the PDZ domain alone had no effect. Co-expression of PDZ domain of CT441 and SRAP1 decreased the stimulation of ERα significantly, but not completely (Fig. 4A). As already mentioned, SRAP1 stimulates human steroid receptors as much as its corresponding RNA does. Thus, we assumed that binding of PDZ domain to SRAP1 selectively blocks the stimulation of ERα by the protein. To show this, we generated a SRAP1 mutant construct containing a STOP codon after translational start which allowed the synthesis of a functional RNA, but not of the protein. Expression of this construct in YE1 led to an intermediate stimulation of ERα (Fig. 4B) and the co-expression of PDZ domain of CT441 had no effect on this stimulation. Summarizing this data, we were able to show that (i) SRA1 stimulates ERα in yeast as both, RNA and protein, and that (ii) the protein part of ERα stimulation was selectively inhibited by binding of the PDZ domain of CT441.

3.5. Full-length CT441 alleviates ligand-independent SRAP1 co-activation of ERα AF-1 domain in mammalian cells

In order to determine whether the full-length maturated protease CT441 affects SRAP1 co-activation of ERα also in mammalian cells we investigated the influence of CT441 on ligand-independent SRAP1 co-activation of ERα AF-1 domain [17]. For this purpose an expression vector encoding fusion of GAL4 DBD to ERα AF-1 domain was made. This construct was co-transfected into HEK239T cells

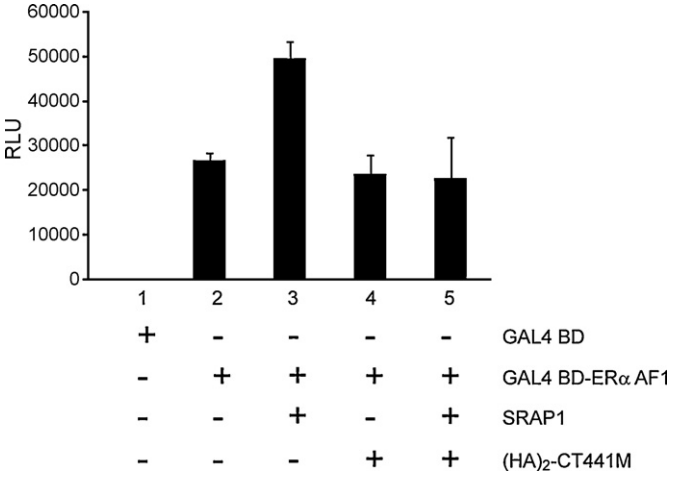

Fig. 5. Full-length CT441 alleviates ligand-independent SRAP1 co-activation of ERα AF-1 domain in mammalian cells. HEK293T cells were co-transfected with pFR-Luc and pCMV-GAL4 DBD (column 1) or pCMV-GAL4-DBD-ERα-AF1 (2) and pCEP4-SRAP1 (3) or $(HA)_2$-CT441M (4) or both (5). In comparison to GAL4 DBD alone (column 1) the AF-1 domain posses transcriptional activity (2). The overexpression of SRAP1 leads to a distinct stimulation (3), whereas overexpression of CT441M had no effect (4). When both proteins, SRAP1 and CT441M, were co-expressed no enhancement of the basic transcriptional activity of GAL4-AF-1 is observable (5). Luminescence values represent the average of at least three independent experiments performed in duplicate.

with a reporter gene containing five GAL4 DNA binding sites (pFR-Luc). In accordance with earlier findings [17] the AF-1 domain in the absence of exogenous co-activators has considerable transcriptional activity in comparison to GAL4 DBD alone (Fig. 5, columns 1 and 2). Overexpression of SRAP1 stimulated the ability of GAL4 DBD-AF-1 nearly twofold whereas overexpression of CT441M alone had almost no effect (Fig. 5, columns 3 and 4). However, when both SRAP1 and CT441M were overexpressed together no further stimulation of the basic transcriptional activity of GAL4-AF-1 was observed (Fig. 5, column 5). Altogether this results show that full-length CT441M was able to repress co-activation of ERα-AF-1 domain by SRAP1 and speak in favour of an interaction of SRAP1 and CT441M in mammalian cells.

4. Discussion

Being obligate intracellular parasites, chlamydiae must create and maintain a specialized intracellular niche, while simultaneously contending with potent host defenses. Recently, several examples of chlamydial effector proteins interfering with host metabolism were reported. This includes the description of potential functions of chlamydial TARP in invasion [18], Inc proteins in modulation of vesicular interactions [19] and chlamydial proteins in dysregulation of NF-κB signal transduction [20]. Among the latter is the tail-specific protease CT441 of *C. trachomatis* LGV2, which harbors a PDZ domain of protein–protein interactions and a Ser/Lys dyad catalytic unit. CT441 is able to cleave the p65 subunit of NF-κB and by this means to interfere with the NF-κB pathway of host antimicrobial and inflammatory responses [6,7]. However, the secretion of CT441 into the host cytoplasm remains to be shown experimentally [21]. In the present study, we found that CT441 specifically interacts with the human ERα co-activator SRAP1 in yeast and in mammalian cells, but failed to cleave it. The interaction between the PDZ domain of CT441 and SRAP1 led to a diminished co-activation of ERα in a yeast system. Moreover, CT441 was also able to repress co-activation of ERα-AF-1 domain by SRAP1 in mammalian cells. This suggests a new possibility of host control by *Chlamydiaceae*. Chlamydial attachment and infectivity *in vitro* as well as ascending disease and sequelae *in vivo* have been reported to be enhanced or modulated by estrogen [22]. Furthermore, products of many ERα target genes involved in signal transduction, cell proliferation and apoptosis may have an influence on chlamydial survival and development in the host [23]. Among estradiol-induced genes, *WNT11* (wingless-type MMTV integration site family member 11) was found to increase cell survival by significantly reducing apoptosis, a process tightly regulated by chlamydiae [23]. Another relevant ERα target gene encodes the pro-apoptotic protein Bcl2 (B-cell lymphoma) [24]. Downregulation of Wnt11 and/or Bcl2 caused by lower ERα activity may lead to increased apoptosis necessary for chlamydiae at the end of infection cycle [25]. Therefore, it seems plausible that chlamydiae regulate host metabolism by targeting ERα activity. For more detailed characterization, the development of a cellular system to identify the specific human target genes of chlamydial protease CT441 is required.

On the other hand, the specific interaction between PDZ domain and SRAP1 opens up interesting possibilities to use this domain as a biomedical tool. There have been several reports dealing with mutagenesis and selection of PDZ domains that bind new protein targets [26,27]. The goal of those experiments was the specific targeting and inactivation of oncogenes such as *c-myc* [26] or the targeting of intracellular proteins to different subcellular compartments [27]. The capability of the CT441-PDZ domain to specifically sequester SRA1 protein, but not SRA1 RNA, as shown in our ERα yeast expression system, may be used to distinguish between the cellular functions of the SRA1 RNA and protein. This may have clinical relevance as it was shown that SRAP1-coding and non-coding SRA1 RNAs co-exist in breast cancer cell lines [28]. The balance between these two genetically linked entities is controlled by alternative splicing and might be involved in breast tumorigenesis and tumor progression through regulating the expression of specific genes [28]. While the ERα-specific co-activation of SRA1 RNA had already been shown [17] we demonstrate for the first time that also SRA1 protein plays a role in ERα co-activation. While the complexity of the bi-faceted SRA1/SRAP1 system makes it difficult to address its function, the selective inhibition of SRA1 protein by the PDZ domain of chlamydial CT441 protease can be used to discriminate between the effects of SRA1 RNA or SRA1 protein on ERα target genes. Moreover, Leygue and co-workers recently showed that modulation of the balance coding/non-coding endogenous SRA1 RNAs altered T5 breast cancer cells growth. T5 cells treated with a modified anti-sense oligoribonucleotide, leading to an increased expression of non-coding SRA1 transcripts and a decrease in the level of SRA1 protein, showed a significantly lower viability. Authors propose that modifying splicing events might lead to establishing potential new breast cancer treatments [28]. We hypothesize that a similar effect on tumor cell growth may be achieved by titrating SRAP1 with the PDZ domain of *C. trachomatis*.

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