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**Title:** Structure-based insights into the repair of UV-damaged DNA  
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Cockayne Syndrome protein A (CSA) is one of the main players in mammalian Transcription-Coupled Nucleotide Excision Repair and mutations in this protein lead to the serious human disorder Cockayne Syndrome. CSA is thought to be the substrate adaptor protein of an E3-ubiquitin ligase complex, but its exact substrate is still a matter of debate. To get more insight into the function of CSA in Transcription-Coupled repair, we performed in vitro and in vivo experiments for determining its substrate. In this chapter we describe the results that show that CSA probably does not bind DNA, while the related protein DDB2 does, but that a more likely substrate is a negative stretch of a protein such as encountered in Cockayne Syndrome protein B.

E.M. Meulenbroek, M.G. Vrouwe, L.H.F. Mullenders, N.S. Pannu, *Insights into Transcription-Coupled Repair from the crystal structure of Cockayne Syndrome protein A.* (to be submitted)
4. SUBSTRATE-BINDING BY COCKAYNE SYNDROME PROTEIN A

4.1 Introduction

To repair DNA lesions blocking transcription, the DNA repair pathway of Transcription-Coupled Nucleotide Excision Repair (TC-NER) has evolved. In mammals, most of the proteins involved are shared with the Global Genome Nucleotide Excision Repair (GG-NER) pathway, but two factors are unique for TC-NER: Cockayne Syndrome protein A and B (CSA and CSB) (reviewed in a.o. Tornaletti, 2009). Their function in TC-NER is suggested to be as follows (reviewed in Fousteri & Mullenders, 2008). CSA is thought to interact dynamically with RNA polymerase II during transcription. Upon encountering a lesion past which RNA polymerase II cannot transcribe, it gets stalled on the lesion and this stabilizes its interaction with CSB. CSB recruits NER factors and chromatin remodelers to the lesion as well as CSA in an E3 ubiquitin ligase complex (constituted of CSA, DDB1, Cul4A and Rocl) inactive at the time of recruitment. The function of CSB might be to make space for the repair with its DNA-dependent ATPase activity. The CSA complex has been suggested to protect TC-NER factors from degradation in early stages of repair and/ or direct proteins for degradation at later stages of repair to prevent them from obstructing transcription (Groisman et al., 2006). The repair is eventually done by excision of the fragment containing the lesion by the NER factors. The biological importance of CSA and CSB is demonstrated by mutations in either CSA or CSB, that have been reported to cause the serious human recessive disorder Cockayne syndrome, which is characterized by a.o. dwarfism and premature aging (Nance & Berry, 1992).

The crystal structure of CSA has recently been solved (see Chapter 3). In the crystal structure, CSA was seen to form a complex with DDB1 that is similar to the DDB1-DDB2 complex, which is involved in initial detection of UV-lesions in GG-NER (Scrima et al., 2008). Like CSA, DDB2 also forms an E3-ubiquitin ligase complex with Cul4A, Rocl and DDB1. DDB2 binds to lesions in the DNA and there it promotes ubiquitination of the nearby XPC and itself, leading to the handover of the damage from DDB2 to XPC (Sugasawa et al., 2005).

In the structure of CSA it could be seen that the most likely substrate-binding site is positively charged and small, as is also observed in proteins that bind e.g. phosphorylated stretches of other proteins. This site was considerably smaller than the large positive groove that the DNA-binding protein DDB2 has. Therefore, it was suggested that CSA binds to a phosphorylated or otherwise negative stretch of another protein, but its substrate could not yet be identified from these results. In this chapter we describe experiments to get more insight into the substrate for CSA.

4.2 Material and methods

4.2.1 Protein overproduction and purification

Overproduction and purification of the CSA-DDB1 complex has been reported in Chapter 2 of this thesis. Briefly, CSA with a C-terminal 10x His-tag was co-
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expressed in Sf9 insect cells with N-terminally 6x-His tagged DDB1. The complex was co-purified to near homogeneity with Ni-column affinity chromatography, ion exchange and gel filtration.

4.2.2 Bandshift assay

The oligos containing CPD or 6-4PP were synthesized as described in Iwai, 2006, all the other oligos were bought at Eurogentec, Belgium. The top strands of the DNA substrates were 5’ radioactively labelled using polynucleotide kinase as reported previously (Verhoeven et al., 2002). Labelled DNA substrates were incubated for 10 min at 30 °C with up to 0.62 µM CSA-DDB1 in 20 mM Tris pH 6.5 and 100 mM NaCl in a reaction mix of 10 µl. Samples were then loaded on a 3.5 % native Tris-aceatate gel at pH 7.2, which was run at 4 °C in 1x TAE pH 7.2. The gel was dried and the result was visualized by irradiation of a photographic film.

4.2.3 Construction of CSA mutants

CSA mutants were created by PCR and cloned into the pDONR221 vector (Invitrogen) with a C-terminal 10x-His-tag as was CSAwt. The presence of the desired mutation was verified by sequencing analysis (Baseclear, the Netherlands). The gene with mutation and tag was then transferred to the pLenti4 vector (Invitrogen) via the Gateway LR recombination reaction. The resulting vector was transfected to 293FT cells for virus production and this virus was then used to infect CS3BE-sv cells. After selection with Zeocin, 250 cells of these mutant cell lines or of the positive control (VH10 cells) or the negative control (untransfected CS3BE-sv) were seeded on a 90 mm petridish and were irradiated with 0, 2, 4, 6, and 8 J/m². After two weeks, the colony-forming ability was assessed. All experiments were executed four to eight times.

4.2.4 CSA interaction studies

Around 30 * 10⁶ human cells were harvested by trypsination. After inactivation of the trypsin, centrifugation and washing with 1x PBS, the cells were frozen at -80 °C until use. The cell lines used were: VH10 cells or CS3BE-sv cells as negative controls (both gave similar results, hence only one negative control is shown in the remainder of the chapter), and CS3BE-sv cells into which the gene coding for CSA-His was transfected as described above.

Cells were resuspended in 1 ml lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 0.1% Triton-X-100, 5 mM β-mercaptoethanol and Complete mini EDTA-free protease inhibitor cocktail (Roche)) and lysed by sonification. Cells were spun down at 13,000 rpm in an Eppendorf table-top centrifuge for 10 min at 4 °C and the soluble fraction (lysate) was loaded on 200 µl Ni-NTA column material (Qiagen) equilibrated in Ni buffer A (20 mM Tris pH 8, 200 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). The small column was washed with 1.5 ml Ni buffer A and the CSA-His protein together with interacting proteins were eluted with
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300 µl Ni buffer B (20 mM Tris pH 8, 200 mM NaCl, 330 mM imidazol, 5 mM β-mercaptoethanol).

Samples of the lysate and the elution fractions were run on 10% SDS PAGE gels and blotted o/n at 90 mA per gel. The next morning, the blots were washed in 1xTBST and blocked with 5% protifar in TBST for 1 h. The first antibody (αCSA; W-16 from Santa Cruz biotechnology or αCSB; E-18 from Santa Cruz biotechnology) was added in 5% protifar in TBST and incubated for 2 h at a 1:200 dilution (αCSA), 1: 250 (αCSB) or 1:10,000 (αHis). After washing 3 times 15 min with 1x TBST, the secondary antibody (RoG and αG-M HRP) was added in 5% protifar in TBST and incubated for 1 h. After washing 3 times 15 min with 1x TBST, the blots were developed using a luminol - enhancer - hydrogen peroxide mixture.

4.3 Results

4.3.1 Verification of CSA’s substrate binding site

From the crystal structure of CSA, several residues have been identified as potential substrate-interacting residues (see Chapter 3): Glu103, Phe120, Lys122, Arg164, Lys247, Lys292, Lys293, Arg354 (Figure 4.1(a)). These residues were selected using the following three criteria: the side-chains should be probable candidates for interaction (e.g. charged or big hydrophobic side-chains), the side-chains should be interacting with or otherwise be very near substrate peptides in a superposition of CSA with co-crystal structures of WD40 proteins with peptides, and the side-chains should not be involved in any interaction that is likely to be important for the stability of the structure of the protein itself.

To verify the importance of these residues for the function of CSA, complementation studies were performed with these residues changed to alanine. As can be seen in Figure 4.1(b), cells with most of the mutants are only marginally more UV-sensitive than cells with wildtype CSA (VH10 and CSA wt), but one mutant shows a clear UV-sensitivity (Figure 4.1(c)). This is the only double mutant of the set of mutants (K292A + K293A), of which the mutations are located exactly in the centre of the proposed substrate-binding site in a positively charged loop that is unique to CSA (blue in Figure 4.1(a)). The phenotype of this mutant confirms that the top face of WD40 domain of CSA is important for CSA function and hence reinforces the idea that this face is a likely site for substrates to bind to CSA. However, it should be noted that the sensitivity is only partial, though one might have predicted a more severe sensitivity for a double mutant.

4.3.2 Determination of CSA’s binding capacity to DNA

Since the structurally related DDB1-DDB2 complex binds strongly and specifically to damaged DNA (Wittschieben et al., 2005), we decided to test whether DDB1-CSA also binds to DNA. To this end bandshift assays were performed in many different conditions. The only conditions in which binding of CSA to DNA was observed, was at pH 6.2 - 7.2 with a low amount of salt (0-100 mM) in gels run in
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Figure 4.1: Verification of CSA’s proposed binding site.
(a) Cartoon representation of the CSA structure with the residues potentially involved in substrate-binding indicated in magenta. The loop 290-296 is indicated in blue.
(b) Results of complementation assays with CSA’s substrate-binding mutants, showing that most mutants only have a marginal effect on UV-sensitivity, but that the double mutant K292A + K293A has a larger effect. The positive controls are in red (CSA-His construct) and blue (VH10; cell line with wt CSA) and the negative control is in green (CS3BEsv).
(c) Result of Figure 4.1(b) showing only the controls and the double mutant K292A + K293A to indicate more clearly the disturbed complementation of this mutant.
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Figure 4.2: DNA-binding by CSA
Representative bandshift assay with CSA showing that CSA only binds DNA at very high protein concentrations in a non-damage-specific way. Below the lanes is indicated what concentration of protein was used, whether the CSA-DDB1 complex (denoted: CSA) was used or only DDB1 (denoted: DDB1) and which DNA damage was used (no: no damage, 8-Ox: 8-oxoguanine, Fl: fluorescein). The bands were verified not to be in the slots of the gel.

TAE at pH 7.2. High concentrations of protein had to be used (0.5 µM) with DNA concentrations up to 1.8 nM to see a significant binding (Figure 4.2). Hardly any binding could be observed at lower protein concentrations, even not at 2x lower protein concentration (Figure 4.2 lanes 8-15). No discrimination between damaged and undamaged DNA was observed (Figure 4.2 lanes 2-4 show some representative examples; a wide range of lesions ranging from 8-oxoguanine to fluorescein to UV lesions as well as different DNA lengths were attempted; not all results shown). The bandshift was CSA-dependent as samples with only DDB1 did not show bandshift (Figure 4.2 lanes 5-7). Filter-binding assays (results not shown) confirmed the above results.

Binding of CSA to DNA could only be observed at high protein excess (250-fold or more molar excess) with no discrimination between different DNA substrates in the large range of conditions tested, while DDB1-DDB2 shows bandshifts at equimolar DNA-protein concentrations and an up to 80x higher affinity for damaged DNA (Wittschieben et al., 2005). Therefore, we are unsure of the biological relevance of the observed binding and should keep in mind that the binding we observed might be an artefact. The fact that we don’t see any shift in more biologically relevant ratios while many conditions were tested, shows that it is very unlikely that CSA indeed binds DNA \textit{in vivo}. 

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Figure 4.3: CSB as potential substrate for CSA
Western-blot with α-CSA (a) or α-CSB (b) antibodies on the elutions of small-scale Ni-affinity purifications with cell lines expressing wildtype CSA (VH10) or overexpressed CSA with a C-terminal His-tag (CSA-His). For the CSB Western-blot (b), also the lysates are shown to be able to assess the amount of CSB in the total soluble fraction. The arrows indicate the specific band for CSA or CSB and the orange star indicates the CSB-PiggyBac fusion protein, a splice-variant of CSB. Brightness and contrast of the left panel of (b) have been adapted by computer to improve the image quality and the right panel is taken from the same blot with a shorter exposure time of the film.

4.3.3 CSB is a possible substrate for CSA

The substrate for CSA in TC-NER is still uncertain, but one of the most likely options is CSB, since CSA has been reported to ubiquitinate CSB in vitro and a CSA-dependent degradation of CSB after UV-irradiation has been observed (Groisman et al., 2006). However, this matter has remained controversial due to contradicting results in literature, for example on the degradation of CSB after UV-irradiation and on the physical interaction between CSA and CSB in cells (see for a discussion Licht et al., 2003). To get more insight into the possibility of CSB as substrate for CSA, we performed pull-down analysis using the 10x His-tag on our CSA construct in complemented CS3BE-sv cells.

In Figure 4.3(a) it can be seen that small-scale Ni-column purification leads to a high enrichment of CSA in the samples from the cell-line with CSA-His overexpressed, as expected, showing that the experimental setup is correct. As can be seen in Figure 4.3(b), less CSB is present in the lysate of the cell line where CSA is overexpressed. This suggests that CSA is indeed involved in vivo ubiquitination and degradation of CSB. Moreover, when these samples are partially purified with Ni column affinity chromatography (using the His-tag of the overexpressed CSA), there is considerably more CSB in the Ni elution fraction of the sample with CSA-His overexpressed than in the negative control (Figure 4.3(b)), in which no tagged CSA is present. This shows that CSB co-purifies with CSA. This indeed suggests that there is a (direct or indirect) interaction between CSA and CSB. Similar experiments using CSA with a mutated top face are currently in progress.
4.4 Discussion

For understanding the function of CSA in TC-NER and how defects in CSA cause the disease phenotype, it is vital to know how CSA recognizes its substrate and what this substrate is. In this chapter site-directed mutagenesis results are reported that suggest that the top face of the WD40 domain of CSA is indeed the substrate-binding site, considering the effect a double mutation in the centre of the proposed site had. Single mutations in this site did not have a clear effect. It is possible that the affinity for the substrate is not strongly affected by mutation of only one residue and that the high expression of CSA in the WD40 top-face mutant cell lines might compensate for slight decreases in efficiency. A double mutation then perhaps has a too strong influence to be compensated for by a larger amount of CSA. Therefore, for the double mutation an effect is observed. Together with the fact that it is the most used substrate-binding site in WD40 proteins and that it is accessible in a model of CSA in the E3-ubiquitin ligase complex, the UV-sensitivity of the double mutant provides evidence pointing toward this being the substrate binding site.

Next, we investigated the identity of the substrate for CSA by looking at the two most likely options. The first option, CSA binds DNA like DDB2 does, is very unlikely based on our results, since binding to DNA could only be observed in one condition of salt and pH with a very large excess of protein (250 times or more) and no discrimination between different substrates was observed. This binding therefore most likely only represents aspecific binding of CSA to the DNA, e.g. to the negatively charged phosphate groups. Moreover, CSA was reported not to copurify with nucleosomes after UV-irradiation unlike DDB1-DDB2 (Groisman et al., 2003), which agrees with our results.

Our findings indicate that the second option, CSA binds CSB leading to its ubiquitination, is more likely, since less CSB was shown to be present in cell lines over-expressing CSA than in cell lines with no or normal amounts of CSA. Moreover, CSB could be pulled down using the His-tag on CSA. The results in literature on this matter contradict each other. A physical interaction between CSA and CSB was found with in vitro translated proteins and with the yeast-two hybrid system (Henning et al., 1995), but not in cells using gel filtration, co-immunoprecipitation or immunofluorescence (discussed in Licht et al., 2003). An explanation for this controversy can be that the amount of CSA in cells normally is so low that very sensitive methods and optimized experimental conditions are required to pick up a CSA-CSB interaction. Different methods have been used in literature and perhaps not all are sensitive enough to allow detection of weakly interacting, rare complexes. Our method, however, has the advantage in this regard that CSA-His is overexpressed, so it allows easier detection of CSA-interactions, though its disadvantage is that overexpression is less close to the normal condition of the cell. Moreover, our method does not prove that the observed interaction is direct or whether it is mediated via another protein, hence further research is needed to identify with certainty the substrate of Cockayne Syndrome protein A.