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Interaction of Ddc1 and RPA with single-stranded/double-stranded DNA junctions in yeast whole cell extracts: Proteolytic degradation of the large subunit of replication protein A in *ddc1Δ* strains

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ABSTRACT

To characterize proteins that interact with single-stranded/double-stranded (ss/ds) DNA junctions in whole cell free extracts of *Saccharomyces cerevisiae*, we used [^{32}P]-labeled photoreactive partial DNA duplexes containing a 3′-ss/ds-junction (3′-junction) or a 5′-ss/ds-junction (5′-junction). Identification of labeled proteins was achieved by MALDI-TOF mass spectrometry peptide mass fingerprinting and genetic analysis. In *wild-type* extract, one of the components of the Ddc1-Rad17-Mec3 complex, Ddc1, was found to be preferentially photocrosslinked at a 3′-junction. On the other hand, RPAp70, the large subunit of the replication protein A (RPA), was the predominant crosslinking product at a 5′-junction. Interestingly, *ddc1Δ* extracts did not display photocrosslinking of RPAp70 at a 5′-junction. The results show that RPAp70 crosslinked to DNA with a 5′-junction is subject to limited proteolysis in *ddc1Δ* extracts, whereas it is stable in *WT*, *rad17Δ*, *mec3Δ* and *mec1Δ* extracts. The degradation of the RPAp70-DNA adduct in *ddc1Δ* extract is strongly reduced in the presence of the proteasome inhibitor MG 132. We also addressed the question of the stability of free RPA, using anti-RPA antibodies. The results show that RPAp70 is also subject to proteolysis without photocrosslinking to DNA upon incubation in *ddc1Δ* extract. The data point to a novel property of Ddc1, modulating the turnover of DNA binding proteins such as RPAp70 by the proteasome.

1. Introduction

DNA damage can arise as a consequence of normal cellular metabolism and the action of exogenous genotoxic agents [1]. To maintain the genome integrity, eukaryotic cells have evolved sophisticated mechanisms, one of which is DNA damage checkpoint control [2,3]. DNA damage checkpoint provides monitoring of genome integrity during cell cycle, delaying or arresting cycle progression after detecting of DNA lesions [2–4]. Impaired DNA damage checkpoint response increases DNA damage sensitivity in yeast and the risk of tumor development in humans [5,6]. The molecular mechanism of checkpoint control involves the action of three groups of proteins: sensors, signal transducers and effectors [2–4]. Sensor proteins act at the early steps of the checkpoint response that involves primary detection of DNA damage [2–4]. They recognize DNA repair/replication intermediates containing single-stranded DNA (ssDNA), double-stranded/single-stranded (ds/ss) DNA junctions, gaps or specific DNA–protein complexes such as ssDNA coated by replication protein A (RPA) [7,8]. Genetic and biochemical studies reveal that at least two complexes (Mec1-Ddc2) and (Ddc1-Rad17-Mec3), the (9-1-1) clamp in high eukaryotes, are involved in initial DNA damage recognition step in *Saccharomyces cerevisiae* [9,10]. These complexes independently localize at the sites of DNA damage [9,10]. Mec1-Ddc2 was shown to bind to ssDNA coated with RPA [11]. The (Ddc1-Rad17-Mec3) complex is structurally related to PCNA and its recruitment to DNA damage *in vivo* depends on the Rad24-RFC$_{2-5}$ clamp loader complex [12–15]. Biochemical studies have been carried out to determine DNA substrate specificity of the (9-1-1) complex *in vitro*

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[13–16]. It has been shown that the appropriate DNA substrates for loading of the (Ddc1-Rad17-Mec3) checkpoint clamp contain 3′- or 5′-ss/ds DNA junctions, that are generated during double-strand breaks repair, nucleotide excision repair (NER) or at stalled replication forks [3,17–21]. The efficiency and specificity of the (9-1-1) clamp loading can be modulated by RPA that directs 5′-loading [16,22,23]. Therefore, the identification of proteins acting at ss/dsDNA junctions is critical to understand DNA damage response (DDR) in eukaryotes [24].

The aim of this study was to characterize proteins interacting with partial DNA duplexes containing 3′-ss/dsDNA or 5′-ss/dsDNA junctions in whole cell free extracts of *S. cerevisiae* using the photoaffinity labeling approach. Photoreactive DNA probes were generated by inserting a photoreactive analog of dCTP (FABGd-CTP) at the margin of the ss/dsDNA junction. Photoactivation of such DNA probes results in photocrosslinking of proteins bound at ss/dsDNA junctions, which are identified by MALDI-TOF MS peptide mapping and genetic analysis [25]. Here, we found that the (9-1-1) checkpoint clamp component, Ddc1, was preferentially crosslinked to the 3′-junction DNA substrate. Interestingly, Ddc1 can interact with 3′-junction DNA in the absence of the two other checkpoint clamp partners, Rad17 or Mec3, under conditions when clamp cannot be formed. On the other hand, we found that RPAp70, the large subunit of RPA, is the predominant crosslinking product with the 5′-junction DNA substrate. Intriguingly, extracts deleted for Ddc1 did not display photocrosslinking of RPA with 5′-ss/dsDNA substrate. The data show that RPAp70 crosslinked to DNA is rapidly cleaved in *ddc1Δ* extracts yielding limit products, whereas it is stable in *WT*, *rad17Δ*, *mec3Δ* and *mec1Δ* extracts. Furthermore, the degradation of RPAp70-DNA adducts in *ddc1Δ* extract is reduced in the presence of the proteasome inhibitor MG 132. We also investigated the stability of RPA non-crosslinked to DNA using anti-RPA antibodies. The data show that RPAp70 is also subject to proteolysis without photocrosslinking to DNA upon incubation in *ddc1Δ* extract, whereas it is stable in *WT*, *rad17Δ* and *rad24Δ* extracts. These results led us to propose that Ddc1 is likely involved in cellular processes, independently of the (9-1-1) checkpoint clamp structure, regulating proteasome-dependent degradation of DNA-binding proteins, like RPA.

2. Materials and methods

2.1. Materials

Synthetic oligonucleotides were obtained from “Eurogentec S.A.” (Belgium) and Laboratory of Medical Chemistry (Institute of Chemical Biology and Fundamental Medicine (ICBFM), Novosibirsk, Russia). [γ-^{32}P]ATP, [α-^{32}P]dATP (5000 Ci/mmol or 3000 Ci/mmol) were from the Laboratory of Biotechnology (ICBFM, Novosibirsk, Russia) or “Perkin-Elmer”, respectively. Protein molecular mass markers were from “Fermentas” and “BioRad”. Protease inhibitors *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), pepstatine A, *o*-phenanthroline monohydrate and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and proteasome inhibitor MG 132 were from “Sigma”. A peptide calibration standard II was from “Bruker Daltonics” (Germany, Part No. 222570). Streptavidin Magne-Sphere Paramagnetic Particles, sequencing grade modified trypsin (porcine, part No. V5111), reagents for electrophoresis and basic components of buffers were from “Promega”. ECL detection system was from “Thermo Scientific”, hyperfilm-ECL was from “Amersham Biosciences”. Horseradish peroxidase-conjugated goat anti-rabbit IgG were from “Invitrogen”. The photoreactive dCTP analog, FABGdCTP (exo-N-[4-(4-azido-2,3,5,6,-tetrafluorobenzylidenehydrazinocarbonyl)-butylcarbamoyl]-2′-deoxycytidine-5′-triphosphate), was a kind gift from Dr. I.V. Safronov (ICBFM, Novosibirsk, Russia). The 5′-modified oligonucleotide with photoreactive exo-N-[4-(4-azido-2,3,5,6,-tetrafluorobenzylidenehydrazinocarbonyl)-butylcarbamoyl group was synthesized by Dr. Silnikov V.N. (ICBFM, Novosibirsk, Russia).

Table 1
Saccharomyces cerevisiae strains used in this study.

Strain	Genotype		Reference
FF18733/34	MATa/α	leu2-3,112, trp1-289, his7-2 ura3-52, lys1-1	[25]
AC91	FF18734	With rad17::KanMX6	[lc[a]]
BG49	FF18734	With ddc1::LEU2	[lc]
BG27	FF18734	With rad24::KanMX6	[lc]
BA055	FF18733	With mec3::LEU2	[lc]
AC53	FF18733	With mec1::KanMX6 sml1::URA3	[lc]

[a] lc: Our laboratory collection.

2.2. Proteins and cell extracts

T4 polynucleotide kinase was from “Fermentas” (Canada) and “Biosan” (Russia). Rat DNA polymerase β was overexpressed and purified as described [26]. Yeast RPA expression vector was a kind gift of M.S. Wold (University of Iowa, Iowa City, USA). Yeast RPA was overexpressed in *Escherichia coli* and purified as described [27]. Yeast Gst-Ddc1 was overexpressed in *E. coli* and purified as described [25]. Recombinant (Ddc1-Rad17-Mec3) complex was a kind gift of Peter M. Burgers (Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, USA). Rabbit polyclonal antibody against yeast RPA was a kind gift of X. Veaute (CEA, Fontenay aux Roses, France) and V. Gely (CNRS, Marseille, France). Rabbit polyclonal antibody against yeast Ogg1 was prepared as described [28]. Whole cell extracts from *WT*, *ddc1Δ*, *mec3Δ*, *rad17Δ* and *rad24Δ* yeast cells were prepared as described [25]. Protein concentration in extracts was 10–20 mg/ml determined by the Bradford assay.

2.3. Yeast strains and microbiological methods

S. cerevisiae strains used in this study are listed in Table 1. Deletion mutant strains were constructed in the FF18733/34 *wild-type* (*WT*) background. Deletions were obtained using PCR-based allele replacement techniques [29,30]. All disruptions were confirmed by PCR and genetic analysis. Cells were grown at 30 °C in standard media YPD (1% yeast extract, 1% bacto-peptone, and 2% glucose).

2.4. Preparation of photoreactive DNA substrates

The nucleotide sequence and structure of the two oligodeoxyribonucleotides probes, DNA*1 and DNA*2, used in this study are shown (Table 2). To generate DNA*1, the upper primer was 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (5000–6000 Ci/mmol), and the photoreactive dCTP analog (FABGd-CTP) was incorporated by DNA polymerase β (Polβ) into 3′-end of the primer (Table 2). Standard reaction mixtures (300 μl) contained 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 μM Polβ and 70 μM FABGdCTP, 0.5 μM 5′-[^{32}P]-labeled partial DNA duplex. The reaction mixtures were incubated for 15 min at 37 °C to allow elongation of the primer with FABGdCMP, and then the reaction was stopped by adding EDTA to final concentration 20 mM followed by precipitation by adding 1/10 volume of 3 M NaAc, pH 5.0 and 2.5 volume of 96% ethanol. The photoreactive DNA*1 was dissolved in TE buffer to a final concentration 2 μM.

To generate DNA*2, the upper primer carrying the photoreactive group (FABG) at the 5′-end was labeled by using [α-^{32}P]dATP and Polβ. Standard reaction mixtures (300 μl) containing 50 mM