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Covalent and Non-Covalent Associations Mediate MED28 Homo-Oligomerization

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Abstract

The Mediator is a multi-protein complex that plays a key role in modulating gene expression. Our previous studies suggested that the MED10a, MED28, MED32 complex subunits could be subject to redox regulation. In this study we tested the capacity of different thioredoxins (TRXs) from poplar (TRX-H3 and TRX-H5) and Arabidopsis thaliana (TPR repeat-containing thioredoxin, TDX) as well as glutaredoxins (GRXs) from poplar (GRX-C3 and GRX-C4) to reduce MED28 oligomers *in vitro* and found that these proteins were less efficient than the the previously tested poplar TRX-H1 and Arabidopsis GRX-C1. Concerning the susceptibility of MED28 to oxidation, both hydrogen peroxide (H2O2) and glutathione disulfide (GSSG) are efficiently mediating the formation of intermolecular disulfides. In fact, MED28 forms homo- oligomers *in vivo* as assessed by yeast two-hybrid experiments but also *in vitro* in solution as shown by size-exclusion chromatography, the latter also demonstrated the formation of non-covalent homo-oligomers. These findings suggest that both the redox-dependent and - independent MED28 oligomerization could regulate its biological activities, could it be linked or not to the Mediator. In particular, it would be important to assess MED28 oligomerization state during senescence considering the previously observed phenotype of med28 plants.

Keywords: Mediator; Redox; Disulfide bond; Thioredoxins; Oligomerization; Oxidative stress

Introduction

Mediator is a protein complex formed by multiple subunits which are arranged in four modules named head, middle, tail and separate kinase module [1-3] that functions as a transcriptional coactivator in all eukaryotes. In addition to its central role as scaffold within the preinitiation complex (PIC) to regulate RNA polymerase II activity, Mediator also relays regulatory information from promoter-bound transcription factors (TFs) to the RNA polymerase II for the proper expression of specific target genes [4,5] in response to developmental or environmental cues [6].

Mediator complex exists in all eukaryotes. It was first discovered in yeast, but has later been identified in other eukaryotes like human and plants [7-9]. Mediator complex was isolated from plants for the first time in 2007 from Arabidopsis cell suspension culture [10]. Since then, genomic analyses allowed identifying genes coding for subunits of Mediator complex in other plant species such as rice [11]. In plants, individual mediator subunits have been implicated in development and stress responses [12-15]. Attention has recently been given to a possible redox regulation of the Mediator complex. The link between Mediator and redox regulation has been primarily proposed from reports on the involvement of Mediator complex subunits in plant immunity and root hair differentiation [16,17]. MED25, the most studied Mediator subunit, plays important roles in flowering time and light signaling [12,18], organ growth [19], jasmonic acid and (JA) and abscisic acid (ABA) signaling [19,20], abiotic stress [13], and root hair development [16]. In these processes, reactive oxygen species (ROS) have an important role and therefore MED25 might be regulated by or relay the changes in redox homeostasis [16]. Another mediator subunit, MED8, was found to interact with the YY1 transcription factor which associates with promoter regions of two GRX genes *GRX-S13*, *GRX-C9*, and one TRX gene, *TRX-H5*. Moreover, impaired expression of *GRX-S13, GRX- C9* and *TRX-H5* in yy1 and med18 mutants suggested a synergistic role for GRXs and TRXs in plant immunity [21].

We have recently shown that the MED10a, MED28, and MED32 Mediator subunits are sensitive to oxidation forming covalent oligomers upon hydrogen peroxide (H2O2) treatments that could be reduced by TRX- and GRX-dependent systems [22]. These subunits associate with the redox-responsive transcription factor GeBPL and block its ability to bind the CryR2-P DNA fragment [22]. Moreover, med32 and med28 mutants display phenotypes (altered root development and senescence) associated with redox changes [23]. The aim of the present study was to evaluate the effects of disulfide bond formation and homo-oligomerization on the quaternary structure of MED28 Mediator complex subunit.

Materials and Methods

MED28 protein expression and purification

The expression of recombinant MED28 was performed at 37°C in LB medium supplemented by ampicillin (50 µg/ml) using E. coli BL21 (DE3) or Rosetta strains transformed with pET-AtMed28 plasmid [22]. When the cell culture reached an OD600 nm of 0.7, AtMed28

expression was induced by 0.1 mM IPTG and cells further grown for 4 h. Cells were harvested by centrifugation, resuspended in a 30 mM Tris-HCl at pH 8.0 and NaCl 200 mM buffer with or without 10 mM DTT and stored overnight at -20°C. The cell lysis was completed by sonication and cell extract centrifuged at 12,000 g for 40 min to remove cellular debris and aggregated proteins. Recombinant Med28 was purified from the resulting supernatant as described previously [22] in presence or in absence of 1 mM DTT. The protein was expressed mainly in the soluble fraction, purified with quite high yield and purity which was assessed by SDS-PAGE. The protein concentration was determined either with the Bio-Rad Protein Assay (Biorad) or using the theoretical molar extinction coefficient at 280 nm of 6085 M-1 cm-1.

TRX- and GRX-dependent reduction of MED28

The reduction of MED28 protein was performed with either NADPH/NADPH-TRX reductase (NTR)/TRX or the NADPH/glutathione reductase (GR)/reduced glutathione (GSH)/GRX systems. For the NADPH/NTR/TRX system, the reactions contained 100 mM Tris-HCl pH 7.5, 0.5 mM NADPH, 2 µM AtNTRB and 70 µM PtTRX-H3, PtTRX-H5 or AtTDX. For the NADPH/GR/GSH/GRX system, the reactions contained 100 mM Tris-HCl pH 7.5, 0.5 mM NADPH, 0.5 unit baker's yeast GR (Sigma), 10 mM GSH and 70 µM PtGRX-C3 or PtGRX-C4. In all cases, the proteins were incubated for 20 min at room temperature before adding non-reducing SDS sample buffer to the protein mixture followed by separation on 10% non-reducing SDS-PAGE.

Treatment of reduced proteins by oxidizing agents

200 µL MED28 protein (~33 µM) was reduced with 50 mM DTT for about 1 h at room temperature. To eliminate DTT, the protein was dialyzed against 100 mM Tris-HCl pH 7.5 using centricons (10 KDa cut-off; Millipore) and the protein was diluted to the initial 200 µL volume (~33 µM). For oxidation, 20 µM pre-reduced MED28 was incubated with 1 mM oxidizing agent (H2O2, oxidized DTT, GSSG and GSNO) for 30 min at room temperature. After incubation, the protein samples were loaded on 10% SDS-PAGE and the signals were detected by Coomassie staining.

Yeast two-hybrid assays

The yeast two-hybrid interactions were performed with the DUAL hybrid system (Biotech) according to the manufacturer's instructions. Full-length coding sequence of MED28 was cloned in yeast two-hybrid bait (pLexA-N) and prey (pGAD-HA) vectors, respectively. Bait and prey constructs/vectors were transformed into the yeast reporter strain NMY51 and the transformants were selected on selective media lacking tryptophan and leucine (SD/-Trp-Leu) or tryptophan, leucine, histidine and adenine (SD/-Trp-Leu-His-Ade). *LacZ* activation was detected with β-galactosidase overlay assay in which an overlay buffer containing 0.5 M potassium phosphate, pH 7.0, 6% dimethylformamide, 0.1% SDS, 50 mL/100 mL β- mercaptoethanol, 5 mg/mL low melting agarose and 0.05% X-Gal was used.

Size-exclusion chromatography

Size-exclusion chromatography was performed with an Äkta purifier chromatography system (GE healthcare) on HiLoad 16/60 Superdex 200 prep grade column, equilibrated with 30 mM Tris-HCl pH 8.0, 200 mM NaCl with or without 1 mM DTT, and operated with a flow rate of 0.8 ml/min at 4°C. The approximate molecular masses of the different species were deduced by comparison with the elution profile of known protein standards ranging from 12.4 to 669 kDa (Sigma) separated under the same conditions.

Determination of free thiols

Free thiol content of the protein was estimated using Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, DTNB) at 412 nm as described previously with some modifications. The "as purified" protein was diluted to 20 µM in 495 µL of TE (30 mM Tris-HCl pH 8.0, 1 mM EDTA) buffer or of TE buffer containing 1% SDS or 6M urea. Alternatively, MED28 protein was reduced with 1 mM DTT in TE in the presence of 6M urea. The protein was precipitated for 15 min on ice by adding 10% TCA before a centrifugation step at 13,000 rpm for 15 min. The pellet was washed 2 times with 1% TCA and finally resuspended in 495 µL TE-6M urea. DTNB was added at 100 µM final concentration and the reaction proceeded in the dark for 45 min before the formation of TNB was monitored at 412 nm.

Results and Discussion

The oxidation of cellular thiols, i.e., the formation of protein disulfides, protein S- glutathionylation and protein S-nitrosylation, by ROS and reactive nitrogen species (RNS) represents an important signaling mechanism for fast responses to environmental changes [24]. The MED28 protein produced as recombinant protein was previously shown to oligomerize in the absence of oxidation treatment or upon H2O2 treatment [22]. The predominant form observed under non-reducing SDS-PAGE is a dimer while higher oligomer forms were present in smaller amounts [22]. The only visible forms remaining upon reduction and separation using non-reducing SDS-PAGE were monomers. A complete reduction was observed with high concentrations of the chemical reductant, DTT, whereas this was only partial with the two tested disulfide reductases, GRX-C1 and TRX-H1. In the following work, we sought to identify more efficient, possibly physiological, reductants, to delineate which agents could control MED28 oxidation and to determine the effects on the oligomerization state of the protein in solution.

Reduction of MED28 by TRX- and GRX-dependent reducing systems

TRXs and GRXs usually have different target proteins and employ different reducing mechanisms [25,26]. Moreover, within these large families, several isoforms also have peculiar features, in terms of reduction mechanisms, subcellular localization or simply domain architecture. Having shown before that PtTRX-H1 and AtGRX-C1 were able to reduce MED28 with a similar efficiency (81% and 82% respectively; [22]), we sought to test the capacity of additional TRXs and GRXs to serve as electron donors for MED28. Using the same physiological NADPH/NTR/TRX coupled system PtTRX-H3 and PtTRX-H5 were able to reduce MED28 oligomers, although to a lower extent compared to PtTRX-H1 (41% and 51% respectively; Figures 1A and 1C). The nucleo-cytosolic AtTDX was inefficient (Figures 1A and 1C) [27]. The absence of activity is likely not due to a problem of TDX reduction, since it is able to reduce the disulfide bond found in DTNB in the presence of a NADPH/NTR recycling system [28]. TRX-H proteins are mainly localized in the cytoplasm, but were also identified in other compartments [25]. The differences between TRXs H i.e., TRX-H1, H3 and H5 are more surprising because they belong to same

subgroup within the TRX H family [29]. In fact, TRX-H1 was generally shown to be very efficient in reducing several target proteins [30]. This may be due to its particular WCPPC active site signature compared to the regular WCGPC sequence found in TRX-H3 and H5. These differences in the active site signature are also found in the GRX family. We have tested two other glutaredoxins, GRX-C3 and GRX-C4 which possess the YCPYC active site and compared them to AtGRXC1, the active site of which is YCGYC. These three isoforms belong to class I glutaredoxins but GRX-C3 and GRX-C4 form a different subgroup with slightly different properties [31]. Using the NADPH/GR/GSH/GRX coupled assay, we observed that GRX-C3 and GRX-C4 demonstrated very weak efficiency to reduce MED28 (4% and 16% respectively; Figures 1B and 1C), in contrast to AtGRX-C1 which revealed 82% reduction. This might appear surprising because GRX-C3 and C4 were found to be more efficient than GRX-C1 when their activity was tested with conventional substrates [31]. Nevertheless, other subtle differences in the structures or charge surfaces for instance may make GRX-C1 a better catalyst for MED28 reduction. Also, it is interesting to note that GRX-C1 isoforms are localized in the cytoplasm and possibly in the nucleus e.g. in the same subcellular compartment as MED28 [32] whereas GRX-C3 and GRX-C4 are predicted to be secreted proteins which might also confer them specific properties.

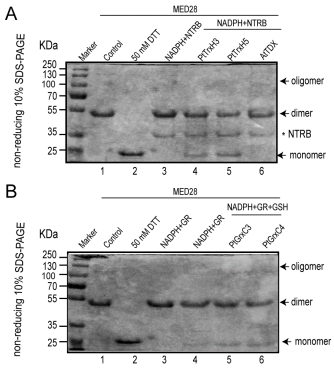

Figure 1: Efficiency of TRXs and GRXs in the reduction of MED28. The MED28 protein was incubated in the presence of the TRX (NADPH/AtNTRB/PtTRXH3 or PtTRXH5) or GRX (NADPH/GR/GSH/AtGRXC3 or AtGRXC4) regeneration systems. Controls missing key components (TRX, GSH, GRX) of each system are included. Protein separation was performed on 10% SDS-PAGE and detected with Coomassie staining. Positions of monomers, dimers, and oligomers are indicated by arrows. Molecular weight of a protein marker is shown. Results are representative of atleast three separate experiments.

Oxidation induced disulfide linkage of MED28

MED28 was previously shown to migrate predominantly as an apparent dimer under non- reducing conditions while some oligomers were present in small amount (Figure 2A, left panel;). This prompted us to analyze the sensitivity of MED28 to oxidizing treatments and the resulting changes in its redox state. For this purpose, the first step was to confirm that recombinant MED28 was indeed fully reduced with 50 mM DTT (left panel in Figure 2A). Then DTT was removed by extensive dialysis and the reduced protein was incubated for 30 min at room temperature with 1 mM of the selected oxidizing agents. Even in the absence of oxidizing treatment, a small amount of dimer is visible (Figure 2B, lane 2) revealing that the protein is quite sensitive to oxidation since a simple air-exposure during dialysis or SDS- PAGE migration was sufficient to promote oxidation. The longer we wait before the treatment, the more is the protein oxidized? A 1 mM H2O2 treatment promoted an oxidation quite similar to the one observed without reduction with a majority of dimers and some higher order oligomers (Figure 2B, lane 3). It is interesting to note that the presence of GSH interfere with the oxidation pattern, H2O2 alone promoting the formation of 2 bands in the dimeric region whereas the presence of GSH led to the formation of a single band in this region (Figure 2B, lane 3). This suggests that the doublet might represent different oxidation forms including the formation of overoxidized forms (sulfinic or sulfonic acids) which can be prevented if GSH reacts with sulfenic acids to form glutathione adduct. Both oxidized dithiothreitol (DTTox), nitrosoglutathione (GSNO) were poorly efficient in promoting dimer/oligomer formation (Figure 2B, lanes 5 and 7). DTTox is a relatively weak oxidizing agent and it is generally unable of rapidly oxidizing target proteins to a fully oxidized state. On the other hand, GSSG was able to promote the formation of substantial amounts of dimers but not oligomers (Figure 2B, lane 6), thus possibly generating a mixture of intermolecular disulfides and glutathione adducts. In the light of this result, the one obtained with GSNO is puzzling because it is often more efficient than GSSG for glutathionylation reactions and it is also able to catalyze nitrosylation depending on the substrates. Hence, it may either be inefficient in oxidizing MED28 or on the contrary too much efficient forming stable glutathione- or nitroso-adducts on all cysteinyl residues, which prevents disulfide bond formation.

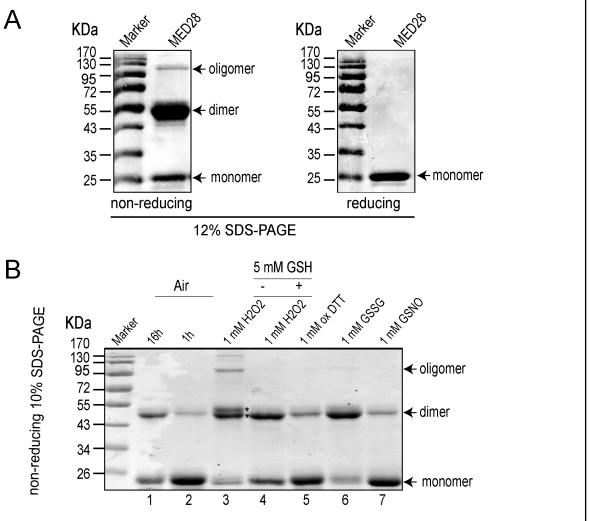

Figure 2: Oxidation induces disulfide bond formation in MED28. (A) Non-reducing and reducing 12% SDS-PAGE gel of recombinant MED28 protein. (B) Oxidation of pre-reduced MED28. The treatments and concentrations used are indicated at the top of each lane. After incubation, sample proteins were separated by 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Monomer, dimer, and oligomer positions are indicated by arrows. Molecular weight of a protein marker is shown. Results are representative of at least three separate experiments.

MED28 forms homo-oligomers through covalent and non-covalent interactions

The capacity of MED28 to interact with itself and thus to form at least dimers was examined by performing binary yeast two-hybrid experiments. The activation of the reporter genes *HIS3* and *LacZ* is possible only when the transcriptional regulator is functional due to the interaction of both chimeric constructs, indicates that MED28 is able to form homo-oligomers. While it shows the capacity of MED28 to oligomerize in an *in vivo* context, the yeast two-hybrid system is not suitable to determine whether the interaction is covalent or non-covalent (in fact, disulfide linkages are unlikely to occur in the reducing environment of the yeast nucleus) and to delineate a potential stabilizing influence of the covalent interactions for protein quaternary structure (Figure 3). Hence, the oligomerization state of the protein was further explored in solution using size exclusion chromatography (SEC) by comparing proteins whose purification steps were performed under reducing (1 mM DTT) or non-reducing conditions. In these experiments, MED28 was eluted in a single major fraction (Figure 4) with no substantial difference between reducing and non-reducing conditions. From the calibration with known molecular weight standards, the estimated molecular mass is around 140 KDa that would approximately correspond to a MED28 hexamer/heptamer considering that the theoretical molecular mass of the monomer is 21.2 KDa. The fact that MED28 forms such non-reducible oligomers was somewhat unexpected considering the results shown above upon reduction and separation under denaturing SDS PAGE and previous data demonstrating that substitution of both cysteines (Cys44 and Cys55) totally abolished the capacity to form both oligomers and dimers, although variants with a single mutation have variable behaviors [22].

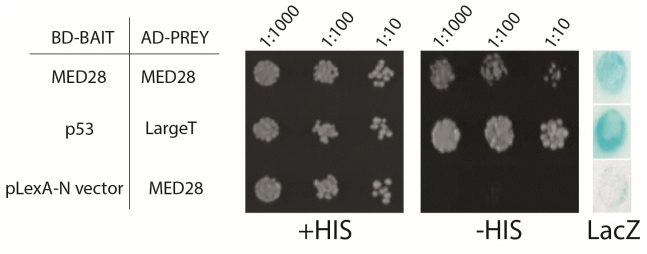

Figure 3: Homo-dimerization of MED28 visualized by yeast-two-hybrid experiments NMY51 yeast cells have been co-transformed with a couple of vectors expressing different combinations of baits and preys as indicated and grown for 5-7 days at 30°C on SD-Trp/-Leu (+HIS) and SD-Trp/-Leu/-His/-Ade (-HIS) media. The combinations between an empty vector and the one expressing AD-MED28 and between the vectors expressing p53 and Large T represent the negative and the positive controls, respectively. MED28 homo-dimerizes in the yeast two-hybrid system as indicated by the activation of *HIS3* reporter gene and by the *LacZ* activation. *LacZ* activation can be visualized using the ß-Galactosidase overlay assay by the development of a blue color. A growth on a -His medium indicates the activation of the *HIS3* reporter gene which is an indirect evidence for MED28 homo-dimerization.

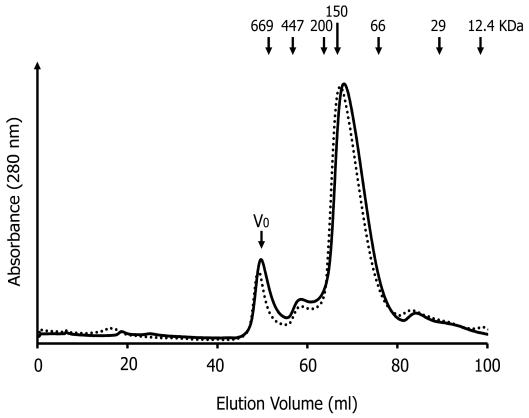

Figure 4. Quaternary structure of MED28 in solution. Molecular mass of AtMED28 was estimated by exclusion size chromatography under reducing (+DTT; dotted line) or non-reducing (-DTT; continuous line) conditions. Standard molecular weight markers including bovine thyroglobulin (669 KDa), horse spleen apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine carbonic anhydrase (29 kDa) as well as horse cytochrome c (12.4 kDa) were used as references to determine the apparent molecular weight of MED28. Void volume (V0) was determined by using dextran blue (2000 kDa).

Hence it is likely that the intermolecular disulfide bond(s) between MED28 monomers is (are) somehow partially buried in the hydrophobic core of the protein and cannot be efficiently reduced by DTT unless the protein is denatured. Accordingly, using thiol titration experiments performed with DTNB, the expected number of thiol groups (2.3 ± 0.15 moles of SH/moles proteins) can only be obtained when the protein was reduced in the presence of urea. Performing a similar experiment using a protein purified without DTT and denatured in TE, TE-1% SDS or TE-6M urea gives only a ratio of 1.25 ± 0.2 moles of SH/moles proteins instead of the expected number of 2.

Altogether, these results establish that both covalent and non-covalent interactions have a role in the oligomerization of MED28 but that the isolated protein mostly oligomerizes through non-covalent (ionic or hydrophobic) interactions. Concerning the covalent linkages, both Cys residues may not be equally exposed and accessible for disulfide bond formation, clearly establishing a role of non-covalent interactions in oligomerization of MED28. It could be tempting to hypothesize that changes in MED28 quaternary structure are important for its function, modulating for instance Mediator composition or the binding of TFs towards their DNA targets as shown already for GeBPL, whose interaction with MED28 was found to reduce its DNA binding activity [22]. On the other hand, it is important to keep in mind that these *in vitro* analyses performed with single recombinant proteins can sometimes be very different from the *in vivo* situation. Indeed, MED28 is supposed to be part of a large protein complex and it is possible that its oligomerization and redox state is very different in this context. It would be interesting In the future to investigate the MED28 redox state in planta under various

developmental and physiological conditions in order to assess whether these changes could indeed have an impact on its biological activity.

Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgment

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