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Characterization of the human monothiol glutaredoxin 3 (PICOT) as iron–sulfur protein

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ABSTRACT

Mammalian glutaredoxin 3 (Grx3/PICOT) is an essential protein involved in the regulation of signal transduction, for instance during immune cell activation and development of cardiac hypertrophy, presumably in response to redox signals. This function requires the sensing of such stresses by a hitherto unknown mechanism. Here, we characterized Grx3/PICOT as iron–sulfur protein. The protein binds two bridging [2Fe–2S] clusters in a homodimeric complex with the active site cysteinyl residues of its two monothiol glutaredoxin domains and glutathione bound non-covalently to the Grx domains. Co-immunoprecipitation of 55-iron with Grx3/PICOT from Jurkat cells suggested the presence of these cofactors under physiological conditions. The $[2Fe–2S]^{2+}$ clusters were not redox active, instead they were lost upon treatment of the holo protein with ferricyanide or *S*-nitroso glutathione. This redox-induced dissociation of the Grx3/PICOT holo complex may be a mechanism of Grx3/PICOT activation in response to reactive oxygen and nitrogen species.

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1. Introduction

The first glutaredoxin (Grx) was identified as glutathione (GSH)-dependent reductase of the disulfide formed in ribonucleotide reductase during its catalytic cycle [1]. Today, two main groups of Grxs can be distinguished, defined by phylogeny, active site motif, and domain structure [2]. First, the 'classical' dithiol Grxs containing the active site consensus sequence Cys-Pro-Tyr-Cys and, second, the monothiol Grxs with a Cys-Gly-Phe-Ser active site consensus sequence. Although monothiol Grxs contain all functional residues and substrate binding motifs to catalyze the reduction of mixed disulfides between proteins and GSH, most of them lack this enzymatic activity entirely [3]. Monothiol Grxs can be further categorized into single domain monothiol Grxs that consist of only one Grx domain and multidomain monothiol Grxs that contain an N-terminal thioredoxin (Trx)-like domain and one to three C-terminal monothiol Grx domains, also named PICOT homology domains [4]. This type of Grx is restricted to eukaryotic cells. In humans this protein was named protein kinase C interacting cousin of thioredoxin (PICOT) [5], HUSSY-22 (human sequence similar to yeast, number 22) [6], and human thioredoxin-like 2 (TXNL2, early genomic annotation). Later, the HUGO gene nomenclature committee approved GLRX3 (glutaredoxin 3) as the official gene name.

In vertebrates, Grx3 consists of an N-terminal Trx domain that lacks a redox active motif and two monothiol Grx domains that both harbor the Cys-Gly-Phe-Ser active site. In mice, Grx3 is essential for embryonic development, $Grx3^{-/-}$ mice die between E12.5 and E14.5 with no obvious defects in organogenesis [7]. Human Grx3 was first identified in a two hybrid screen as a potential interaction partner of protein kinase C-θ (PKC-θ). Transient overexpression of Grx3 attenuated the activation of c-Jun N-terminal kinase and transcription factors AP-1 and Nf-κB in stimulated Jurkat T cells. This effect was dependent on both the Trx and Grx domains, while the Trx domain alone was sufficient for the interaction with PKC-θ [5]. In rat basophilic leukemia cells Grx3 was reported to mediate FcεRI signaling by acting as positive regulator of IL-4 and TNF-α expression as well as the NFAT pathway, and as negative regulator of JNK signaling [8]. It was suggested that these effects of Grx3 on cell activation signal transduction occur in response to stress signals by reactive oxygen species [9].

In addition to these effects on immune signaling, Grx3 expression was found to be up-regulated in hypertrophic adult rat hearts induced by transverse aortic constriction as well as in neonatal rat cardiomyocytes after exposure to endothelin-1 or phenylephrine. Mice overexpressing Grx3 in cardiomyocytes that were exposed to transverse aortic constriction displayed a significantly attenuated increase in heart to body weight compared to wild-type litter mates, a reduced induction of genes associated with cardiac hypertrophy, and an increase in contractility and improved ventricular

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function [10]. Corroboratively, reduced levels of Grx3 in heterozygous knockout mice exacerbated cardiac hypertrophy and enhanced calcineurin-NFAT signaling by pressure overload [7]. The direct interaction of Grx3, through its Grx domains, with the muscle LIM protein (MLP) that is associated with the Z-disk may explain the effect on calcineurin-NFAT signaling [11]. Noteworthy, the N-terminal Trx domain of Grx3 appeared to be dispensable for the inhibitory effect of the protein towards the development of cardiac hypertrophy.

Grx3 is an essential protein with suggested functions in a number of regulatory processes, i.e., in embryonic development, immune response, and cardiac physiology. All of these functions are thought to be mediated by the direct and specific interaction with other proteins, both through Grx3's Trx and Grx domains, and likely in response to redox signaling by reactive oxygen species. Given the fact that Grx3 lacks enzymatic activity, one of the most urging questions to fully comprehend the regulatory role of Grx3, from our point of view, was: how could the regulation of Grx3 be modulated by redox signals? Here, we proposed that human Grx3 can form a dimeric quaternary complex that involves bridging iron–sulfur clusters. The transition between such a holo complex and monomeric Grx3 could serve as the regulatory switch sought-after.

2. Materials and methods

2.1. General methods

Chemicals were purchased from Sigma–Aldrich (Munich, Germany) unless otherwise stated and were of analytical grate or better. Materials for molecular biology were purchased from Fermentas (St. Leon-Rot, Germany). DNA was purified from bacteria and agarose gels using the Nucleo spin kits from Macherey and Nagel (Düren, Germany). Disposable cell culture material was purchased from Sarstedt (Nümbrecht, Germany) and cell culture medium, FCS, trypsin, and penicillin/streptomycin were purchased from PAA (Cölbe, Germany). SDS–Page was run using the Novex Minicell and precasted NuPAGE gels (12% acrylamide, Bis–Tris), according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

2.2. Cell cultures

Jurkat cells were cultivated at 37 °C in a 90% humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated (45 min, 56 °C) FCS, 2 mM glutamine, and 100 U ml^{-1} penicillin/streptomycin. Radiolabeling of the cells was done with 1 mCi, 2 μM transferrin-bound ^{55}Fe (Perkin-Elmer, Boston, USA).

2.3. Molecular cloning and mutagenesis

Human Grx3 was cloned from pooled human cDNA (Biocat, Heidelberg, Germany) by PCR using the primer pair 5′-CACACAC ATATGGCGGCGGGGGCGGCTG (forward) and 5′-GGATCCTTAATTT TCTCCTCTCAGTATAGGCAGCAATTCACCATTTTCTTTCAGTTCCTTCAC AATATCCAATCCTCC (reverse). The PCR fragment was initially inserted into vector pJet (Fermentas) and subsequently cloned into the NdeI and BamHI sites of the vector pET15b (Merck, Darmstadt, Germany). Deletion mutants were generated from the pET15b-Grx3 plasmid by PCR constructs with the following primer pairs: Grx3 (1–120) 5′-CACACACATATGGCGGCGGGGGCGGCTG/5′-GGATC CTCAGGAGCCACTAGATGCATGTCGCTG (reverse); Grx3 (125–235) 5′-CATATGGCTAATGAACATCTTAAAGAAGATCTCAACC (forward) and 5′-GGATCCTCATAATTTGGGAGCTTTGGGACAAATTGTATC (reverse); Grx3 (235–335) 5′-CATATGTTAGAGGAAAGGCTCAAAGTG CTGAC (forward) and 5′-CACACAGGATCCTTAATTTTCTCCTCTCA GTATAGGCAGC (reverse).

Site directed mutagenesis was done by using two complementary oligonucleotides harboring the following mutations: Cys-159-Ser 5′-CTCCTCAAGAACCACGCAGTGGTTTCAGCAAGCAGATGGTGGA AATTCTTCACAAC (forward) and 5′-GTTTGTGAAGAATTTCCACCATC TGCTTGCTGAAACCACTGCGTGGTTCTTGAGGAG (reverse) and Cys-261-Ser 5′-CAAACAGGAAGCAAAATCTGGATTCAGCAAACAAATTCTG GAAATACTAAATAGTAC (forward) and 5′-GTACTATTTAGTATTTCC AGAATTTGTTTGCTGAATCCAGATTTTGCTTCCTGTTTG (reverse) and the pET15b-Grx3 plasmid as template. Mouse Grx3 was cloned into the NdeI/BamHI sites of pET15b following amplification of the cDNA using the following primer pair: 5′-CATATGGCGGCGGGGGCGGCCG (forward) and 5′-GGATCCCTAATTTTCTCCTTTCAGTATAGGCAGCAAT TCACCGTTGTCTTTCAGTTCCTTGACAATATCCAATCC (reverse). All plasmids were verified by DNA-sequencing (Seqlab, Göttingen, Germany).

2.4. Expression and purification of proteins

Grx3 and all its mutants were essentially expressed and purified as described for human Grx2 before [12]. In brief, 4.5 L of LB medium in a fermenter (Bioengineering, Wald, Switzerland) were inoculated with 100 ml *Escherichia coli* BL21(DE3)-codonplus containing one of the pET15b-Grx3 expression plasmids. At A_{600} = 0.6 expression was induced by application of 0.5 mM isopropyl-β-D-thiogalactosidase and the temperature was decreased to 22 °C. Pellets of bacterial cells were washed in lysis buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl) and lysed in lysis buffer containing 30 mM imidazole using a liquid homogenizer (Emulsifelx C3, Avestin, Mannheim, Germany). The proteins were purified from clarified extracts using Ni–NTA columns and an ÄKTA FPLC system (GE Healthcare, München, Germany) and eluted in the presence of 250 mM imidazole. When indicated buffers were exchanged using pre-packed Sephadex G-25 columns (PD-10 or Nap5, GE Healthcare). For the analysis of cluster content of the Grx3 domains, the proteins were purified under argon atmosphere. The apparent molecular mass of dimeric or monomeric Grx3 was determined by FPLC using a Superdex 75 precision column equilibrated with lysis buffer. Calibration was done using the supplier's molecular weight standards (GE Healthcare).

2.5. In vitro Fe/S cluster reconstitution

For the *in vitro* reconstitution of Fe/S clusters, 50–200 μM of the proteins were treated in an anaerobic glove box (Coy, Grass Lake, USA) as described in Ref. [13] including 2.5–5 M equiv of $FeCl_2$ and GSH. The amount of iron and acid-labile sulfide bound to Grx3 was determined as described in Refs. [14,15].

2.6. Spectroscopy

UV–visible spectra were recorded using a Specord S300 spectrophotometer (Analytic Jena, Germany). For the analysis of cluster content of the Grx3 domains, spectra were recorded during anaerobic purification using a flow cuvette (Hellma, Mühlheim, Germany) integrated into the flow path of the argon-purged FPLC system. Mössbauer data were recorded with a spectrometer of the alternating constant acceleration type. The minimum experimental line width was 0.24 mm s^{-1} (full width at half height). The sample temperature was maintained constant either in an Oxford instruments Variox or an Oxford instruments Mössbauer Spectromag cryostat. Isomer shifts are quoted relative to iron metal at 300 K. ^{57}Fe-labeled Grx3 was expressed and purified as described above by using ^{57}Fe-enriched Vogel-Bonner-Medium [16]. The stability of the cluster was analyzed after anaerobic purifica-

tion by incubation of 180 μM protein in normal atmosphere with 1 mM GSH, 1 mM GSNO, 1 mM H_2O_2 or 0.5 mM ferricyanide. The absorbance at 420 nm was followed in a 96-well plate reader (Tecan Infinite 200, Trading, Switzerland).

2.7. Co-immunoprecipitation of ^{55}Fe with Grx3

Prior to immunoprecipitation, IgGs were purified from polyclonal rabbit sera using Protein A coupled Sepharose as suggested by the supplier (GE Healthcare). Directly after elution with 0.1 mM glycine–HCl, pH 3, antibodies were neutralized by addition of 10% (v/v) of 1 M Tris–HCl, pH 9. Next, the purified IgGs were coupled to CnBr activated Sepharose (GE Healthcare). For immunoprecipitation, cells were lysed under anaerobic conditions and clarified protein extracts (4.5 mg of total protein) were adjusted to 1 ml with PBS and incubated with the Sepharose-coupled IgGs over night at 4 °C. The Sepharose was washed repeatedly with PBS until no ^{55}Fe radioactivity could be recorded in the supernatant following centrifugation. Finally, ^{55}Fe was quantified by scintillation counting of the resuspended Sepharose.

2.8. Western blot analysis

Proteins from SDS gels were transferred to PVDF membranes by wet transfer in a buffer containing 25 mM Tris–HCl, 150 mM glycine, pH 7.4, 20% methanol, and 0.02% SDS for 1 h at 30 V. The membrane was blocked in TBST (25 mM Tris–HCl, 150 mM NaCl, 2.7 mM KCl, 0,05% Tween 20) containing 5% fat free milk powder and 1% BSA for 1 h. Polyclonal rabbit anti-Grx3 serum was used at a dilution of 1:1000.

3. Results and discussion

3.1. Grx3 can form a dimeric Fe/S-bridged holo complex

We have cloned human Grx3 from cDNA and recombinantly expressed the protein in *E. coli*. As proposed, the purified protein showed a yellow-brownish color. When subjected to gel filtration chromatography, the primary eluate divided into two fractions with apparent molecular weights of 44.1 and 87.8 kDa, respectively (Fig. 1A, insert). These correspond to the monomer (theoretical molecular weight 39.6 kDa) and the homodimer. The dimeric, but not the monomeric, fraction displayed UV–vis absorption bands at 324, 412, 510, and 590 nm in addition to the band at 280 nm (Fig. 1A). Essentially identical features were observed for mouse Grx3 (data not shown). These features indicated the presence of a Fe/S cluster, similar to the ones described for the human dithiol Grx2 [12], the dithiol GrxC1 from poplar [17], and the monothiol Grx from *Synechocystis* [18]. Biochemical studies [13] and the crystal structure of human Grx2 [19] demonstrated a holo complex in which two monomers of the Grx complex a [2Fe–2S] cluster ligated by the N-terminal cysteinyl residues of the Cys-Ser-Tyr-Cys active site and the thiol groups of non-covalently bound GSH molecules. To clarify the nature of Grx3's Fe/S cluster, we have over-expressed the protein in *E. coli* in the presence of ^{57}Fe and analyzed the cell pellets by Mössbauer spectroscopy. The dominant subspectrum (40% relative contribution, not present in control cells, data not shown) of the zero-field spectrum shows a single symmetric quadrupole splitting of 0.622 mm s^{-1}, an isomer shift of 0.273 mm s^{-1}, and a Lorentzian line shape of 0.302 mm s^{-1}. These values are characteristic for ferric iron in tetrahedral sulfur geometry as seen in $[Fe^{III}–(S-Cys)4]$, $[2Fe–2S]^{2+}$, and $[3Fe–4S]^{+}$ clusters. Since holo Grx3 was not paramagnetic in EPR spectroscopy (data not shown), we could safely conclude the presence of a $[2Fe–2S]^{2+}$ cluster. Next, we have established an *in vitro* reconstitution assay for the holo complex. This reconstitution assay was dependent on the presence of reduced GSH in the assay (Fig. 1B), suggesting GSH as additional Fe/S ligand in the holo complex. Indeed, when the assembly of the holo complex was performed in the presence of radiolabeled GSH, significant amounts of GSH co-eluted with the holo complex when subjected to gelfiltration chromatography (Fig. 1B insert). Finally, we have examined the contribution of the cysteinyl residues of the two Cys-Gly-Phe-Ser active site by mutating these to seryl residues. The resulting C159S, C216S mutant did not contain the chromophore (data not shown), demonstrating the essential character of these residues for binding of the Fe/S cluster.

3.2. The Grx3 holo complex contains two Fe/S clusters bound to the two Grx domains

Unlike monothiol Grxs from bacteria and yeast, mammalian Grx3 contains two Grx domains [3]. This domain structure allows, in theory, different configurations of the holo complex. For in-

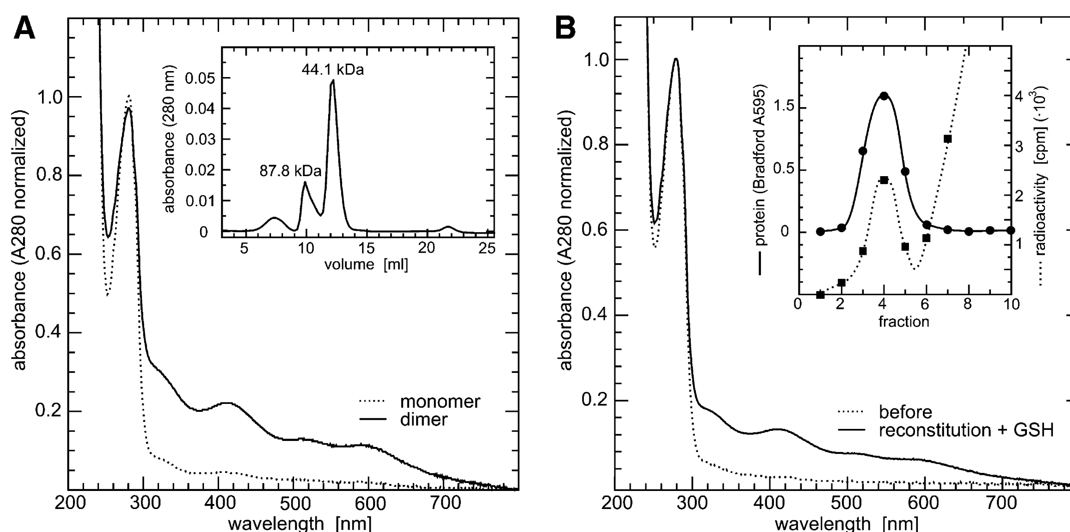

Fig. 1. Grx3 binds Fe/S cluster in a dimeric holo complex using GSH as ligand. (A) UV–vis absorption spectra (normalized to the A280) of Grx3 purified from *E. coli* following gel filtration chromatography (insert). Straight line, dimeric fraction; dotted line, monomeric fraction. (B) UV–vis spectra (normalized to the A280) of Grx3 before (dotted line) and following *in vitro* reconstitution of the Fe/S clusters in the presence of GSH (straight line). Insert: chromatogram showing the protein content (straight line) and the radioactivity (dotted line) of Grx3 following reconstitution in the presence of radiolabeled GSH and gelfiltration.

stance, binding of one or two Fe/S clusters or head-to-head versus head-to-tail configuration. Using colorimetric assays, we have determined 2.08 ± 0.29 Fe/monomer and 2.63 ± 0.17 S^{2-}/monomer of reconstituted Grx3, suggesting the presence of one [2Fe–2S] cluster per monomer. Next, we have cloned, expressed, and analyzed the spectra of the anaerobically purified individual domains (Trx, GrxA, and GrxB) and domain pairs (Trx–GrxA and GrxA–GrxB). Both individual Grx domains displayed spectral bands characteristic for the Fe/S cluster, while the Trx domain did not (Fig. 2A). The calculated sum of the absorbance coefficient-normalized spectra for the individual Grx domains was essentially identical to the spectrum of the GrxA–GrxB domain pair (data not shown), the sum of all individual domains was very similar to the spectrum of the wild-type holo protein (Fig. 2A). These results may be best explained by the presence of two [2Fe–2S] clusters in the wild-type holo complex bound to the two Grx domains. The presence of the chromophore in the Trx–GrxA domain pair (data not shown) as well as the geometric constraints derived from the structures of the holo complexes of human Grx2 and poplar GrxC1 [19,17] suggested a head-to-head rather than a head-to-tail configuration. This proposed structure is displayed in form of a speculative model in Fig. 2B. The active sites of the two consecutive Grx domains (GrxA and GrxB) face each other with axial symmetry complexing the two Fe/S clusters together with non-covalently bound GSH. The Trx domain at the N-terminus is connected to the Grx domains by a linker domain that contains both hydrophobic and hydrophilic amino acid side chains. These may allow for some conformational freedom and for interactions with the solvent. Thus, the Trx domain may adopt other positions relative to the Grx domains and may therefore allow different protein–protein interactions.

The multidomain structure of Grx3 is ideal for a variety of specific protein–protein interactions. The Trx-fold, common to both the Trx and Grx domains, allows specific substrate interactions with a variety of ligands in a binding cleft that includes the catalytic center (usually Cys-X-X-Cys) and a cis-proline loop [20]. The Trx domain of human Grx3 lacks the dithiol motif essential for redox activity. In fact, it has been recognized before that the Trx-fold, independent of any redox activity, can serve as platform for protein–protein interactions, for instance as processivity factor of bacteriophage T7 DNA polymerase [21] and activator of chloroplastic enzymes [22]. The specific interaction of the Trx domain with PKC isoforms and the interaction of the Grx domains with the MLP have been demonstrated in different model systems. So far, all activities described for Grx3 were dependent on the presence of the Grx domains, even if the Trx domain was sufficient for binding to the target protein [5]. The presence of the cluster may thus regulate the activity of Grx3. The sequestration of the Grx3 active site in the holo complex might regulate the protein–protein interactions of the Grx domains. It is also conceivable that the dimer itself mediates interactions between different proteins or protein complexes. It remains to be established which form (holo or apo) of Grx3 is active, for instance as inhibitor of PKC isoforms.

3.3. Stability of the holo complex

The suggested function of Grx3 in signal transduction in response to redox signals by reactive oxygen species implies an effect of such redox signals on the protein itself. Under aerobic conditions, the Grx3 holo complex gradually dissociated over a time of approximately 2 h (Fig. 3). In contrast to the human Grx2 holo complex [12], the Grx3 holo complex was only moderately stabilized by the presence of reduced GSH (Fig. 3). Likewise, hydrogen peroxide did not affect the decay of the Fe/S clusters. The (one electron) oxidant ferricyanide and *S*-nitroso glutathione (GSNO), an NO donor, greatly enhanced the dissociation of the holo complexes. Hence, the transition in the quaternary structure of Grx3 can be specifically induced by specific redox modifications induced by one-electron oxidation and reactive nitrogen species.

3.4. Binding of iron to Grx3 in vivo

The binding of iron to Grx3 under physiological conditions was investigated by propagation of Jurkat cells (growing in suspension) in the presence of transferrin-bound ^{55}Fe for 2 days. Following the lysis of the cells under anaerobic conditions, Grx3 was immunoprecipitated using Sepharose-linked polyclonal IgGs. Following several washing steps, iron bound to the antibody–antigen complexes was quantified by scintillation counting. The efficiency of Grx3 enrichment was tested in separate samples by Western

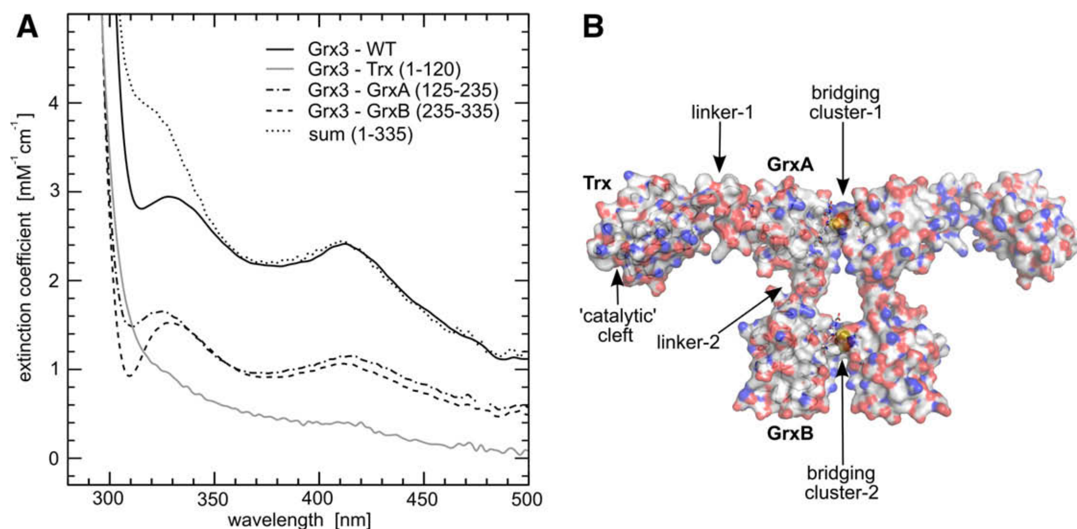

Fig. 2. Analysis of the cluster content of the individual domains suggests that Grx3 binds two [2Fe–2S] clusters at the dimer interface of the Grx domains. (A) UV–vis spectra of individual Grx3 domains and wild-type protein normalized to the theoretical absorption coefficients (A280). Straight black line: wild-type Grx3, straight gray line: the Trx domain, dotted-dashed line: the first Grx domain (GrxA), dashed line: the second Grx domain (GrxB). The dotted line depicts the sum of the spectra of the three individual domains. (B) Hypothetical model of the Grx3 holo complex. The structure of the Grx domains was modeled based on pdb entry 2wik (2nd Grx domain of mouse Grx3), the Trx domain represents entry 2diy (Trx domain of human Grx3). The linker-1 region is derived from pdb entry 3dxb (PUF60 fused to Trx). The Grx-cluster complex was modeled based on 2HT9 (human holo Grx2). Please note that the linker regions, especially the relative position of the Trx domains, are speculative.

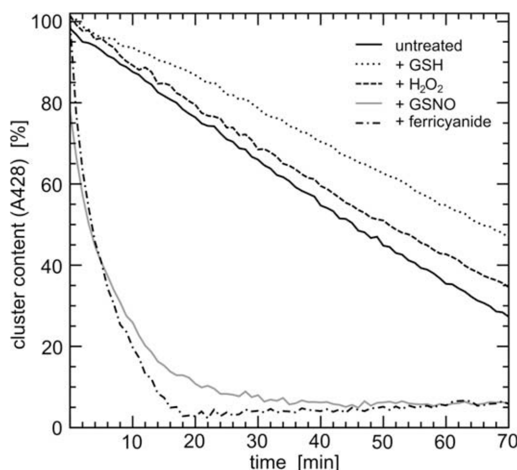

Fig. 3. Stability of the Grx3-bound Fe/S clusters under aerobic conditions. The stability of freshly purified Grx3 holo complex (180 μM) was followed by recording the decrease in A420 under normal atmosphere. The protein was treated with 1 mM GSH, 1 mM H_2O_2, 1 mM GSNO, 0.5 mM ferricyanide, or left untreated as indicated.

blotting before (data not shown). As depicted in Fig. 4, significantly more ^{55}Fe was co-precipitated with Grx3 IgGs compared to IgGs isolated from control sera. These results advocate for the presence of the holo complex *in vivo*, and support a regulatory function of the holo complex. Although an increasing number of Grxs was reported to complex Fe/S cofactors [17,18,23,24], this is only the second time that iron binding to a Grx has been demonstrated in a physiological context [12]. Unfortunately, the moderate signal to background ratio did not allow to quantify iron binding under different conditions using ^{55}Fe co-immunoprecipitation.

4. Conclusions

Here, we have characterized mammalian Grx3, an essential regulator of cell function, as iron–sulfur protein *in vitro* and *in vivo*. The two $[2Fe–2S]^{2+}$ clusters bound to the Grx domains of the protein cannot be reduced and are not redox active. Instead, they may likely serve as redox sensors for the suggested functions of Grx3 in signal transduction in response to redox signals by reactive oxygen and nitrogen species.

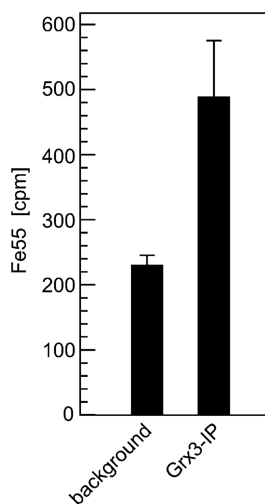

Fig. 4. Co-immunoprecipitation of iron with Grx3 from ^{55}Fe-labeled Jurkat cells. Cells were propagated in the presence of transferrin-bound ^{55}Fe for 2 days. Subsequently, Grx3 was isolated by immunoprecipitation. ^{55}Fe was quantified by scintillation counting and compared to control sera. The average of three experiments ± SEM is shown.

Acknowledgments

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