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Biochemical and physiological analyses of NADPH-dependent thioredoxin reductase isozymes in *Euglena gracilis*

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ABSTRACT

At least four peroxiredoxins that are coupled with the thioredoxin (Trx) system have been shown to play a key role in redox metabolism in the unicellular phytoflagellate *Euglena gracilis*. In order to clarify Trx-mediated redox regulation in this alga, we herein identified three NADPH-dependent thioredoxin reductases (NTRs) using a homologous search and characterized their enzymatic properties and physiological roles. Each *Euglena* NTR protein belonged to the small, large, and NTRC types, and were named EgNTR1, EgNTR2, and EgNTRC, respectively. EgNTR2 was phylogenetically different from the known NTRs in eukaryotic algae. EgNTR1 was predicted to be localized in mitochondria, EgNTR2 in the cytosol, and EgNTRC in plastids. The catalytic efficiency of EgNTR2 for NADPH was 30–46-fold higher than those of EgNTR1 and truncated form of EgNTRC, suggested that large type EgNTR2 reduced Trx more efficiently. The silencing of *EgNTR2* gene expression resulted in significant growth inhibition and cell hypertrophy in *Euglena* cells. These results suggest that EgNTRs function in each cellular compartment and are physiologically important, particularly in the cytosol.

1. Introduction

Thioredoxins (Trxs) are small proteins (~12 kDa) that act as redox regulators in numerous cellular processes including metabolism, the synthesis of DNA, photosynthesis, respiration, and protein folding and repair [1]. Since Trxs are able to reduce thiol-containing peroxidases, peroxiredoxins (Prxs), they also play crucial roles in the metabolism of reactive oxygen species (ROS) as well as cellular redox regulation and other metabolic functions including the regulation of gene expression and molecular chaperone [2,3]. The reducing power of Trxs is normally provided by NADPH in a reaction catalyzed by NADPH-dependent Trx reductase (NTR), which together form the so-called Trx system. NTR has been found in all types of organisms from bacteria to plants and animals. Two major classes of NTRs have been identified to date: (i) small type NTRs with a low molecular mass (~35 kDa), which are found in prokaryotes and some eukaryotes, and (ii) large type NTRs with a high molecular mass (~55 kDa) in some eukaryotes such as animals and protists [4]. Photosynthetic organisms such as plants and algae have the enzyme NTRC, which is a bimodular enzyme formed by an NTR and Trx module in the same polypeptide [5].

Among photosynthetic organisms, *Arabidopsis* NTRs have been characterized in detail. *Arabidopsis* NTRs are encoded by three genes and are distributed in three distinct cellular compartments: most NTRA are in the cytosol, most NTRB are in mitochondria, and NTRC exclusively exists in plastids [5,6]. The growth of an *Arabidopsis ntra ntrb* double knockout mutant was previously reported to be slower [7]. The growth of an *Arabidopsis ntrc* knockout mutant was also found to be inhibited and it also exhibited hypersensitivity to different abiotic stresses, such as oxidative, drought, and salt stresses [5]. Genetic and biochemical analyses using an *Arabidopsis ntrc* knockout mutant suggested that NTRC regulated the

Abbreviations: APX, ascorbate peroxidase; dsRNA, double-stranded RNA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EF1α, elongation factor 1α; FTR, ferredoxin-dependent thioredoxin reductase; GC–MS, gas chromatography–mass spectrometry; KD, knockdown; NTR, NADPH-dependent thioredoxin reductase; Prx, peroxiredoxin; RNAi, RNA interference; RNA-Seq, RNA sequencing; ROS, reactive oxygen species; TMHMM, transmembrane helices based on a hidden Marcov model; Trx, thioredoxin.

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biosynthesis of starch and chlorophyll [8,9]. These findings indicate that NTRs are key regulators in various biological processes in plants. In contrast, although NTR genes have been identified in a few algal species such as *Chlorella vulgaris* and *Emiliania huxleyi* [10,11], their physiological significance in algae has not yet been established.

Euglena gracilis is a motile unicellular flagellate that can grow by photosynthesis and possesses an unusual cell membrane complex, the pellicle complex, but not a cell wall [12]. Metabolism in this organism was previously exhibited to be unique. For example, *Euglena* accumulates a large amount of paramylon (β-1,3-glucan) under aerobic conditions [13]. Under anaerobic conditions, *Euglena* synthesizes medium-chain wax esters from paramylon by a unique metabolic process called wax ester fermentation [14]. We have been studying the molecular mechanisms underlying cellular redox regulation, including ROS metabolism, and their physiological roles in stress responses in *Euglena*. *Euglena* lacks catalase, but contains a single ascorbate peroxidase (APX), which is only localized in the cytosol. In contrast to APX in higher plants, the *Euglena* enzyme can reduce both H_2O_2 and alkyl hydroperoxides [15]. Furthermore, the activities of monodehydroascorbate, dehydroascorbate, and glutathione reductase have only been detected in the cytosol [16]. These findings indicate the physiological significance of an ascorbate-dependent antioxidant system in the cytosol in *Euglena*. We recently identified four genes encoding Prx proteins in *Euglena*. *Euglena* Prxs were predicted to be localized in the cytosol, chloroplasts, and mitochondria. All enzymes exhibited the reduction activities of H_2O_2 and alkyl hydroperoxides. Knockdown (KD) experiments suggested that cytosolic Prx isoforms were essential for the normal growth of *Euglena* cells [17]. These findings provided important evidence that the Trx-dependent antioxidant system is also a crucial regulator of cellular redox states in this alga. However, the roles of the Trx-dependent redox system(s) in ROS metabolism and other physiological processes remain largely unknown because Trx and NTR have not yet been identified in this organism.

We herein attempted to clarify Trx-dependent redox regulation in *Euglena* by searching for putative *NTR* genes. Three *NTR* genes were identified based on *Euglena* RNA-Seq data (Ishikawa et al., unpublished data), and named *EgNTR1*, *EgNTR2*, and *EgNTRC*. The enzymatic analysis of recombinant EgNTRs indicated that EgNTR2 had the highest catalytic efficiency, likely due to its molecular structure and catalytic mechanism of large type NTR. KD experiments suggested that EgNTR2 plays a key role in the homeostasis of cell growth and size in *Euglena* cells. To the best of our knowledge, this is the first study to have established the physiological importance of NTRs, especially in the cytosol, in eukaryotic microalga.

2. Materials and methods

2.1. Strain and culture

E. gracilis strain Z was grown in Koren–Hutner (KH) medium for heterotrophic growth [18] or Cramer–Myers (CM) medium for autotrophic growth [19] under continuous light conditions (50 μmol m^{-2} s^{-1}) at 26 °C with rotary shaking (120 rpm). Regarding the anaerobic treatment of the cells, the cultures were completely sealed and stood for 24 h without shaking after the replacement of air with nitrogen gas. Cell number and volume were determined using the electric field multi-channel cell counting system, CASY (Roche Diagnostics).

2.2. Construction of expression plasmids of recombinant Euglena NTRs

Total RNA was isolated from *Euglena* cells, as previously described [17]. First strand cDNA was synthesized using PrimeScript RT Master Mix (Takara) according to the manufacturer's instructions. The open reading frames of *Euglena* NTRs were amplified from first strand cDNAs using the following primer sets; EgNTR1-F (5′-GAGCTCATGTCCAAGCTGCTGC-3′), EgNTR1-R (5′-AAGCTTTCAGGGCTCGCCGT-3′), EgNTR2-F (5′-CATATGACGTCATACGACTATGACTACG-3′), EgNTR2-R (5′-AAGCTTTTAGCCGCACTTGC-3′), EgNTRCΔ-F (5′-GAGCTCCAATTGTTTTCGGG-3′), and EgNTRCΔ-R (5′-AAGCTTCTAGTACTCGATGAGCAGC-3′). The amplified DNA fragments were ligated into the pGEM-T easy vector (Promega) to confirm the absence of PCR errors. The resulting constructs were digested with Sac I and Hind III for EgNTR1 and EgNTRCΔ, and Nde I and Hind III for EgNTR2, and were ligated into the expression vector pCold II (Takara) to produce His-tagged proteins. The resulting plasmids were introduced into *Escherichia coli* strain BL21 Star cells (Agilent Technologies).

2.3. Expression and purification of recombinant proteins

E. coli strain BL21 Star cells transformed with each pCold II/EgNTR were grown in 3 mL of LB medium containing 50 μg mL^{-1} of ampicillin. After an overnight culture at 37 °C, the cultures were transferred to 600 mL of LB medium with the ampicillin and grown to an A_{600} of 0.5. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a concentration of 0.5 mM, and the cells were incubated for 20 h at 15 °C. All the buffers used for recombinant protein purification were prepared using metal ion-free water. The harvested cells were resuspended in 100 mM potassium phosphate buffer, pH 7.0, and were sonicated. His-tagged recombinant EgNTR proteins were purified using a TALON Metal Affinity resin (Clontech) according to the manufacturer's instructions. Protein contents were determined following the method of Bradford [20]. The purified enzymes were then desalted and concentrated using an ultrafiltration membrane (Amicon® Ultra-4, Millipore), and stored at −20 °C until later use.

2.4. Enzyme assay

NTR activity was determined by the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method described by Pascual et al. [21]. The reaction was performed in 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 5 mM DTNB, 150 μM NADPH, and purified enzymes at the concentrations indicated in the table legends. The reduction of DTNB was monitored by increases in absorbance at 412 nm.

2.5. RNA interference (RNAi) experiments

The KD of EgNTRs by RNAi was performed as described previously [17]. Approximately 500-bp partial cDNA templates of EgNTRs with the T7 RNA polymerase promoter (underlined in the primer sequences below) were amplified using the following primer sets EgNTR1/RNAi-F (TAATACGACTCACTATAGGGGCTTTTCCAAGGGCATC), EgNTR1/RNAi-R (TAATACGACTCACTATAGGGTCACCTTCACACCGGTC), EgNTR2/RNAi-F (TAATACGACTCACTATAGGGAAGAAGCTGATGCACTACGC), EgNTR2/RNAi-R (TAATACGACTCACTATAGGGTTCTCGGAGCACTGGC), EgNTRC/RNAi-F (TAATACGACTCACTATAGGGTACAACACAGCGGTGGAAG), and EgNTRC/RNAi-R (TAATACGACTCACTATAGGGCGACTCGTGGTACAGTTTC). Double-stranded RNA (dsRNA) was synthesized from PCR products as templates using the MEGAscript RNAi Kit (Life Technologies) according to the manufacturer's instructions. *Euglena* cells from seven-day-old cultures grown in CM medium were harvested and resuspended in CM medium. *Euglena* cells grown in KH medium for two days were used for heterotrophic conditions. Approximately 15 μg of dsRNA was introduced into