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A recombinant thioredoxin-glutathione reductase from *Fasciola hepatica* induces a protective response in rabbits

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ABSTRACT

Antioxidant systems are fundamental components of host–parasite interactions, and often play a key role in parasite survival. Here, we report the cloning, heterologous expression, and characterization of a thioredoxin glutathione reductase (TGR) from *Fasciola hepatica*. The deduced polypeptide sequence of the cloned open reading frame (ORF) confirmed the experimental N-terminus previously determined for a native *F. hepatica* TGR showing thioredoxin reductase (TR) activity. The sequence revealed the presence of a fusion between a glutaredoxin (Grx) and a TR domain, similar to that previously reported in *Schistosoma mansoni* and *Echinococcus granulosus*. The *F. hepatica* TGR sequence included an additional redox active center (ACUG; U being selenocysteine) located at the C-terminus. The addition of a recombinant selenocysteine insertion sequence (SECIS) element in the *Escherichia coli* expression vector, or the substitution of the native selenocysteine by a cysteine, indicated the relevance of this unusual amino acid residue for the activity of *F. hepatica* TGR. Rabbit vaccination with recombinant *F. hepatica* TGR reduced the worm burden by 96.7% following experimental infection, further supporting the relevance of TGR as a promising target for anti Fasciola treatments.

1. Introduction

A crucial survival mechanism for parasites inside their host is the evasion of the mammalian immune response. Among immune-effector mechanisms, reactive oxygen and nitrogen intermediates produced by inflammatory cells have been associated with toxic effects in metazoan parasites (McGonigle et al., 1998; Selkirk et al., 1998). Accordingly, parasite antioxidant proteins are considered to be first-line actors in host–parasite interactions that tilt the balance towards the parasite (Bogdan et al., 2000; Callahan et al., 1988). In this context, parasite reduced glutathione (GSH) and thioredoxin (Trx) systems are considered to be major thiol-dependent redox pathways involved in the control of cellular redox balance. Both systems act by transferring the reducing equivalents of NADPH to different substrates and substrate reductases, through reversible thiol oxidoreduction (Holmgren, 1989). The Trx system consists of the small protein Trx and thioredoxin reductase (TR). Trx is a powerful thiol-disulfide oxidoreductase, which reacts with numerous cellular proteins, providing electrons to enzymes such as thioredoxin peroxidase and ribonucleotide reductase (Nordberg and Arner, 2001). It also participates in the control of vital cellular processes, such as gene expression and signal transduction, through the redox regulation of kinases, phosphatases, and transcription factors (Arner and Holmgren, 2000). Trx is maintained in the reduced state by NADPH equivalents transferred by the flavoprotein TR, a member of the pyridine-nucleotide disulfide oxidoreductase family.

The other major thiol system, the GSH pathway, comprises glutathione reductase (GR), GSH, and glutaredoxin (Grx). In this system, GR transfers reducing equivalents from NADPH to oxidized GSH (GSSG). GSH in turns acts by recycling Grx and glutathione peroxidase to their reduced state. Grx, a thiol-disulfide oxidoreductase member of the Trx family, contains a glutathione binding site, and in turn transfers reducing equivalents to different substrates and substrate reductases.

Recently, a new member of the thioredoxin reductase (TR) family, named thioredoxin glutathione reductase (TGR), has been

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EST, expressed sequence tag; FAD, flavin adenine dinucleotide; GR, glutathione reductase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; HED, β-hydroxyethyl disulfide; IPTG, isopropyl β-D-thiogalactopyranoside; ORF, open reading frame; PBS, phosphate-buffered saline; SECIS, selenocysteine insertion sequence; TGR, thioredoxin glutathione reductase; TR, thioredoxin reductase; Trx, thioredoxin.

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described (Sun et al., 1999; 2001). This protein is an oxidoreductase showing TR, GR, and Grx activities, which gained this broad substrate specificity by a fusion of TR and Grx domains. Originally, it was described in mammals showing low levels of expression in various tissues and accumulation in the testicles after puberty. Additionally, it has been reported that TGR catalyzes the isomerization of protein disulfide bridges and internal proteins during the process of spermatid maturation (Su et al., 2005). TR is also present in *Drosophila melanogaster*, in which no GR has been detected. It has been postulated that, in this insect, the reduction of GSSG is carried out by the thioredoxin system (Kanzok et al., 2001). More recently, TGR has been identified in the platyhelminth parasites *Schistosoma mansoni*, *Echinococcus granulosus*, and *Taenia crassiceps* (Agorio et al., 2003; Alger and Williams, 2002; Rendon et al., 2004). In these organisms TGR appears to be the sole protein responsible for recycling Trx and also GSH. This hypothesis agrees with the results of inhibition studies of enzyme activity in parasite extracts, and the analysis of expressed sequence tags (ESTs). These findings suggest that platyhelminth parasites lack GR and TR, and that TGR provides the link between Trx and glutathione systems (Salinas et al., 2004).

The trematode *Fasciola hepatica* is the causative agent of fasciolosis, a zoonosis that affects mainly ruminants and causes important losses in agricultural-based economies. Using β-hydroxyethyl disulfide (HED) as a substrate, and bound GSH–Sepharose, we previously isolated and characterized the TR activity of a detergent-soluble extract of *F. hepatica* (Maggioli et al., 2004), which also showed Grx activity. These properties suggested that the purified protein might in fact be a TGR showing glutathione and thioredoxin specificities. Here, we report the cloning of a TGR from adult *F. hepatica* cDNA, its heterologous expression in *Escherichia coli*, and its use as a vaccine against metacercarial challenge in rabbits.

2. Materials and methods

2.1. Bacteria and plasmids

E. coli strains Y1088 and Y1090 were used for cloning and expression, using λgt11. The strains XL1-Blue and BL21 (DE3) were used in routine plasmid construction and expression experiments, respectively. The vectors pBluescript SK+(pSK+) (Stratagene) and pGEM-T (Promega) were used for cloning and sequencing purposes. The expression vectors used were pGEX-2T (Pharmacia Biotech) and pET28a(+) (Novagene). DNA manipulations, transformation, and *E. coli* cultures were performed according to standard protocols (Fritsch et al., 1989).

2.2. Purification of native TGR and antiserum production

The putative native TGR was purified to homogeneity from a detergent-soluble extract of adult *F. hepatica*, using a combination of ammonium sulfate fractionation, anion exchange, and affinity chromatography on 2′,5′-ADP-Sepharose as previously described (Maggioli et al., 2004). A rabbit immune serum was produced by subcutaneous immunization with the purified native enzyme. The protocol consisted of a priming immunization with 100 μg of native protein in Freund's complete adjuvant (Sigma), and two subsequent intramuscular immunizations on weeks 4 and 6 with 50 μg of the purified enzyme in Freund's incomplete adjuvant (Sigma). Serum was collected 10 d after the second booster, and stored at −20 °C prior to use.

2.3. Determination of the N-terminus sequence

Following SDS–PAGE, the putative native TGR band was electrotransferred onto a polyvinylidene difluoride (PVDF) membrane and stained with Coomassie Blue. The band was excised and subjected to automated amino acid sequence analysis at the proteomic service of Centro Nacional de Biotecnología-CSIC (Spain).

2.4. Cloning and sequencing of the TGR cDNA

We screened 7×10^3 independent recombinants of an unamplified cDNA expression library from adult *F. hepatica*, using the anti-TGR rabbit serum and standard procedures (Marin et al., 1992). After three rounds of screening, several positive clones were amplified, and the purified phage DNA was digested using restriction endonuclease *Not*I. The cDNA inserts were then subcloned into a *Not*I-digested, phosphatase-treated pSK + vector. The cDNAs were sequenced in both directions by the dideoxy chain-termination method (Sanger et al., 1977), using a Sequenase (U.S.B.) sequencing kit. We used T7 and T3 oligonucleotide primers (Promega), and also three specific primers, i.e., TRR5r, TRR5f, and TRR3r, for DNA sequencing (Table 1). The TGR coding cDNA was selected for further study, based on the observed sequence homology.

2.5. Cloning, expression, and purification of recombinant FhTGR into pGEX-2T

The full-length TGR cDNA from positive clones was amplified by PCR using forward primer 5TGRBgl (Table 1) and reverse primer 3TGREcoSecis. The latter encoded a minimal selenocysteine insertion sequence (SECIS) element for Sec incorporation, similar to the one present in *E. coli* formate dehydrogenase H (Li et al., 2000). Two other amplifications were made using forward primer 5TGRBgl and two alternative reverse primers: 3TGREcoCys, to produce a Sec/Cys mutation; and 3TGREcoStop, which contained the native *F. hepatica* TGA codon without an added SECIS element. The forward and reverse primers included *Bgl*II and *Eco*RI restriction sites, respectively, for cloning purposes. The PCR products were gel-purified and subcloned into the pGEM-T easy vector (Promega). The expression plasmids pGEX-*Fh*TGRsecys, pGEX-*Fh*TGRcys, and pGEX-*Fh*TGRstop were made by insertion of the corresponding *Bgl*II–*Eco*RI fragments from pGEM-T easy derivatives into the pGEX-2T vector (GE Healthcare), digested using *Bam*HI and *Eco*RI.

Overnight cultures from single colonies of *E. coli* (BL21), transformed with the plasmids pGEX-*Fh*TGRsecys, pGEX-*Fh*TGRcys, or pGEX-*Fh*TGRstop, were grown at 28 °C in 100 mL of Luria broth medium containing 100 μg/mL ampicillin. When the cultures reached an OD_{600} of 0.6–0.8, the recombinant gene expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 28 °C in the presence of 1 μM Na_2SeO_3 and a vitamin mixture (riboflavin, niacin, and pyridoxine, 20 μg/mL each). After induction, bacteria were harvested by centrifugation. The cell pellets were suspended in 10 mL of ice-cold PBS, and cell extracts were made by sonication. Cell debris and insoluble proteins were removed by centrifugation at 16,000*g* for 20 min at 4 °C. The soluble cell-free extracts were used for purification of GST-fusions by affinity chromatography by using a GSH–Sepharose 4B matrix (Amersham-Pharmacia), following the guidelines specified by the manufacturer. The *Fh*TGR-moieties were released from the gel-matrix by proteolysis using thrombin (Sigma–Aldrich). After purification, the recombinant proteins were kept at −20 °C prior to use.

2.6. Cloning, expression, and purification of recombinant FhTGR into pET28a

The expression plasmid pET-*Fh*TGR was made by insertion of the corresponding *Bgl*II–*Eco*RI fragment from pGEM-T easy derivative into the pET28a vector (Novagene) digested using *Bam*HI and *Eco*RI. The pET-*Fh*TGR was used to transform *E. coli* strain BL21 (DE3) in the presence of pSUABC, a plasmid that supports high-level expression