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Protein disulfide isomerase of *Toxoplasma gondii* is targeted by mucosal IgA antibodies in humans

Bob Meek[a,*], Jaap Willem Back[b], Vincent N.A. Klaren[a], Dave Speijer[c], Ron Peek[a]

[a] *Department of Molecular Immunology, the Netherlands Ophthalmic Research Institute, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands*
[b] *Swammerdam Institute for Life Sciences, Mass Spectrometry Group, University of Amsterdam, Amsterdam, The Netherlands*
[c] *Department of Biochemistry, University of Amsterdam, Amsterdam, The Netherlands*

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Abstract Mass spectrometric analysis identified a 49 kDa antigen from *Toxoplasma gondii* as protein disulfide isomerase (PDI). This antigen is generally recognized by IgA in tears of healthy humans. We determined the complete open reading frame and expressed PDI recombinantly. Recombinant PDI was recognized by IgA in human tears known to contain antibodies specific for the 49 kDa antigen. High expression level and similarity to other protozoan PDIs suggest that *T. gondii* PDI might be a suitable target for recently described anti-protozoan drugs. PDI-specific antibodies clearly constitute part of the mucosal antibody repertoire possibly involved in defence against parasites.

Key words: Human; Parasitic protozoan; Mucosa; Natural antibody; IgA; Protein disulfide isomerase; Mass spectrometry; *Toxoplasma*

1. Introduction

Toxoplasma gondii is a protozoan parasite that has the remarkable ability to infect a wide variety of mammalian species and birds [1]. In humans, infection can cause serious complications like systemic, ocular and congenital toxoplasmosis [1,2].

To prevent toxoplasmosis, several immunological mechanisms are available of which the mucosal system forms the first line of defence. In this context, our recent finding that 80% of individuals have anti-*T. gondii* IgA antibodies in their tears is of interest [3]. The presence of these IgAs was independent of systemic immunity against *T. gondii* and anti-49 kDa IgA antibodies in human tears could be pre-absorbed by incubation with intact, live *T. gondii* tachyzoites, indicating that the antigen is present on the exterior of the parasite [3]. Tears are continuously secreted by the lacrimal gland, an effector organ of the common mucosal immune system, and contain high concentrations of IgA antibodies [4]. Besides constituting the continuous immunologic barrier of the ocular surface and conjunctiva against pathogens during the day, tears are continuously drained by the nasolacrimal ducts, implying that tear IgA antibodies protect epithelial layers of these ducts, the upper respiratory tract, throat and esophagus. These anti-toxoplasma antibodies might be part of the continuous mucosal defence against protozoan parasites, either as a natural adaptation to co-existence with protozoan parasites or due to regular mucosal contact with parasitic antigens. Interestingly, a 49 kDa antigen was prominently recognized by IgA antibodies in tears [3]. Identification of this antigen will give indications whether these antibodies are actively involved in defence against *T. gondii*, and whether targeting of the antigen offers new opportunities in prevention of toxoplasmosis.

2. Materials and methods

2.1. Volunteers and sample collection

Tear fluid was collected from eyes of four healthy adults (V1–V4), using glass capillaries [4] (Assistent, Karl Hecht, Sondheim, Germany). Tears were stored at −20°C until use. This study was conducted in accordance with the Declaration of Helsinki.

2.2. Parasites

T. gondii tachyzoites of the RH strain were propagated in vitro in RK13 cells in RPMI medium with 3% fetal calf serum (Invitrogen). Alternatively, RH strain tachyzoites were propagated in vivo in Swiss mice. Parasites were filter purified using filters with a pore size of 3 μm supplemented with a pre-filter (Millipore). Following two washes with phosphate-buffered saline (PBS, pH 7.4), parasites were resuspended in PBS containing protease inhibitors (Complete EDTA free, Roche) and kept at −20°C until use. Following freeze/thawing, parasite suspensions were sonicated on ice (8×15 s, 30 kHz microprobe, Soniprep 150, MSE, Loughborough, UK), and insoluble material was removed by centrifugation (16 000×*g*, 20 min, 4°C). The water-soluble extract of *T. gondii* parasites (lysate) was frozen in small aliquots.

2.3. Ammonium sulfate precipitation

Ammonium sulfate (AS) was added stepwise within 15 min to 10 ml of *Toxoplasma* lysate to saturation percentages of 26, 38, 51, 64, 78, and 100%. At each of the percentages, the suspension was gently stirred for another 20 min. Following centrifugation for 20 min at 10 000×*g* and 4°C, supernatants were used for the next precipitation step, while the pellet was dissolved in TE buffer (10 mM Tris pH 8, 1 mM EDTA) containing protease inhibitors. Ten μl of each fraction was loaded on a gel, size-fractionated, and subsequently blotted.

2.4. SDS–PAGE, Western blotting and detection of peroxidase-conjugated antibodies

Procedures were performed as described earlier [3,5]. β-Mercaptoethanol (β-ME) was added to a final concentration of 5% prior to electrophoresis. Following electrophoresis, protein preparations were transferred to polyvinylidene difluoride membranes (Millipore). Bound antibodies were visualized by chemiluminescence and exposed to X-ray film.

2.5. Mass spectrometry

The 49 kDa antigen, one of the few antigens in the pellet of the 100% AS fraction, was cut from the gel after staining and destaining with bio-safe Coomassie stain (Bio-Rad) according to the manufac-

*Corresponding author. Fax: (31)-20-5666121.
E-mail address: b.meek@ioi.knaw.nl (B. Meek).

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turer's instructions. For MALDI analysis, protein-containing gel slices were *S*-alkylated with iodoacetamide, digested with trypsin (Roche Molecular Biochemicals, sequencing grade), and extracted according to Shevchenko et al. [6]. Only peptides eluted with 20 mM NH_4HCO_3 were used in the analysis. After drying in a vacuum centrifuge, peptides were dissolved in 10 μl of a solution containing 1% formic acid and 60% acetonitrile. Eluted peptides were mixed 1:1 (v/v) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie BV) in 50% ethanol/50% acetonitrile. Prior to dissolving, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted on target and allowed to dry at room temperature. Reflectron matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) spectra were acquired on a Micromass Tof Spec HT (Wythenshawe, UK). The resulting peptide spectra were used to search ABCC Non-Redundant Protein Database release 20000301 (Advanced Biomedical Computing Center, Frederick, MD, USA) (http://www-fbsc.ncifcrf.gov/). For further MS-MS analysis, a few microliters of several peptide solutions were concentrated on a C18 ZipTip (Millipore), eluted in 5 μl 60% acetonitrile/1% HCOOH and analyzed on a Micromass Q-TOF mass spectrometer. Low-energy collision-induced dissociation (CID) experiments were performed using argon as a collision gas. Homology searches with fragmented peptides were performed using the BLASTx program with default settings and dbEST database (at http://www.ncbi.nlm.nih.gov:80/blast/Blast.cgi and http://www.ncbi.nlm.nih.gov/dbEST/).

2.6. Primers

The following primers were based on *T. gondii* EST dbEST ID# 1374495: PDI 1, 5′-CGGCTTCGTCTTGCTTCTC-3′ (antisense (as)); PDI 3, 5′-AACGTGCCAAGAAACTCCG-3′ (sense (s)); PDI 5, 5′-CGGGACAGGCTCAGACTTC-3′ (as); PDI 6, 5′TAGCAGCGTCCTTCAGGGA-3′ (as). Primers used to amplify full-length cDNA and genomic sequence: PDI 23, 5′-GAGACATATGGAGGAGGAAGCTGTGACT-3′ (s); PDI 24 5′-AGACTCGAGTTACAGTTCTTCACCCTT-3′ (as). Other primers: PDI 7, 5′-GAGACCCGGGTTTTTTTTTTTTTTTV-3′ (with V = A, C or G); PDI 11, 5′-TAAGGTCGATGCCACCAG-3′ (s); PDI 22, 5′-GAGACCATGGGTCAAGGTTGTCGTCGGA-3′ (s).

2.7. Cloning of PCR products, RT, and RACE

PCR products were either ligated into the pGEM-T Easy cloning vector as described by the manufacturer (Promega), or digested and ligated into expression vector pRP261, a derivative of vector pGEX-3X (Amersham Biosciences).

Rapid amplification of unknown cDNA ends (RACE) was performed as described [7], with minor modifications. RNA was extracted from *T. gondii* tachyzoites using RNAzol reagent (Campro Scientific, Hilversum, The Netherlands). cDNA was synthesized using 1 μg RNA and Superscript II reverse transcriptase (RT) (Invitrogen), RNA was subsequently removed by RNase H treatment (Promega, Leiden, The Netherlands). For 5′ RACE, primer PDI 1 was used for first strand synthesis, followed by dA tailing by terminal deoxynucleotidyltransferase (Invitrogen). Second strand cDNA was synthesized using primer PDI 7 (s). Semi-nested PCR reactions were carried out with primers PDI 7 (s) and PDI 6. Primers PDI 3 and PDI 7 (as) were used for 3′ RACE. Four independent clones from both the 5′ and 3′ RACE procedure were sequenced, using pGEM-T Easy vector-specific primers (SP6 and -40) and PDI 7, PDI 11, PDI 1 and PDI 6.

2.8. Sequencing and analysis of nucleotide and protein sequences

All sequencing was performed on an Applied Biosystems Prism 310 dye terminator fluorescent-based genetic analyzer (PE Applied Biosystems, Warrington, UK). Homology searches were done using the BLASTx program with default settings. Multiple sequence alignments were performed using the CLUSTAL W program with default settings (at http://www.ebi.ac.uk/clustalw/). Splice sites in genomic sequences were predicted using the Fgenesm program (version FGENES-M 1.5.0, http://genomic.sanger.ac.uk/gf/gf.shtml). Signal P program was used for analysis of potential signal peptide cleavage sites (version 1.1, at http://www.cbs.dtu.dk/services/SignalP/).

2.9. Northern blotting, Southern blotting and autoradiography

These were performed as described earlier [8], with minor modifications.

Northern blots containing 2 and 10 μg size-separated *T. gondii* RNA were incubated with [α-^{32}P]dCTP-labeled probes specific for SAG1 (∼1 kb fragment) and the protein disulfide isomerase (PDI) EST (∼300 bp fragment).

2.10. Construction of the PDI expression vectors

To release the fragment encoding the 5′ part of PDI, the 5′RACE-pGEM-T Easy construct was digested with *Bst*XI (Invitrogen), followed by deletion of the 3′ overhang using Klenow enzyme (Roche) and digestion with *Bsu*36 (New England Biolabs). The 3′RACE-pGEM-T Easy construct was digested with *Nco*I, its 5′ overhang filled by Klenow, followed by digestion with *Bsu*36. The fragment encoding 5′ PDI was ligated into this digested vector, resulting in a full-length PDI-pGEM-T Easy construct. Upon digestion of this latter construct with *Nco*I and *Spe*I, the resulting fragment with the PDI open reading frame was transferred to pRP261. The 3′ RACE-pGEM-T Easy construct was digested with *Mae*II (New England Biolabs) and Klenow (Roche) filled. Following purification, the 3′ RACE product was released by digestion with *Spe*I. This fragment was ligated into pRP261, previously digested with *Sma*I and *Spe*I, and will be referred to as 3′ rPDI.

2.11. Expression, purification, and detection of recombinant proteins coupled to GST

All proteins were expressed in *Escherichia coli* strain BL21 according to standard procedures [8]. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) at 30°C. Glutathione *S*-transferase (GST), with or without fusion protein, was purified using glutathione Sepharose 4B beads according to the manufacturer's instructions (Amersham Biosciences). For protein gels, beads were directly solubilized in sample buffer. The amount of each recombinant protein used for immunoblots was normalized based on intensity of Coomassie staining on gel and staining with anti-GST antibody on immunoblot. For the detection of GST and GST-coupled proteins, antibodies were removed by incubation of blots in erase buffer (62.5 mM Tris pH 6.8, 100 mM β-ME, 2% w/v SDS) for 1 h at 70°C. Subsequently, blots were blocked for 1 h and incubated with anti-GST-peroxidase (diluted 10 000×, Sigma).

2.12. PDI specific antibodies

A rabbit previously selected for being non-responsive on a *T. gondii* lysate immunoblot was immunized once s.c. and i.m. with 500 μg of the purified PDI–GST fusion protein, using Specol (ID-DLO, Lelystad, The Netherlands) as adjuvant. Plasma was stored at −20°C until use.

3. Results and discussion

3.1. Fractional precipitation and mass spectrometry

The 49 kDa antigen was partially purified from an extract of *T. gondii* by AS precipitation. Western blot analysis of the AS fractions showed that the 49 kDa antigen precipitated most efficiently at 100% saturation with only few other *T. gondii* proteins, as these had been precipitated at 78% saturation (see Fig. 1A). To exclude the possibility that the major band at 49 kDa in this fraction consisted of multiple proteins, a gel fragment containing this band was loaded onto a protein gel with a different acrylamide/bisacrylamide ratio. Coomassie staining revealed a single band that was sliced out and used for mass spectrometric analysis.

Upon digestion with trypsin, resulting peptides were analyzed by MALDI-TOF. Because the peptide mass fingerprint did not identify the 49 kDa protein, selected peptides were analyzed by collision-induced dissociation on an ESI-Q-TOF. Some of the peptides matched dbEST entry ID# 1374495 (Fig. 1B,E). This EST of 300 bp encodes part of PDI of *T. gondii*, a protein not yet described for this parasite.

PDIs belong to the superfamily of thioredoxins (Trx), a group of oxidoreductases characterized by one or more thio-

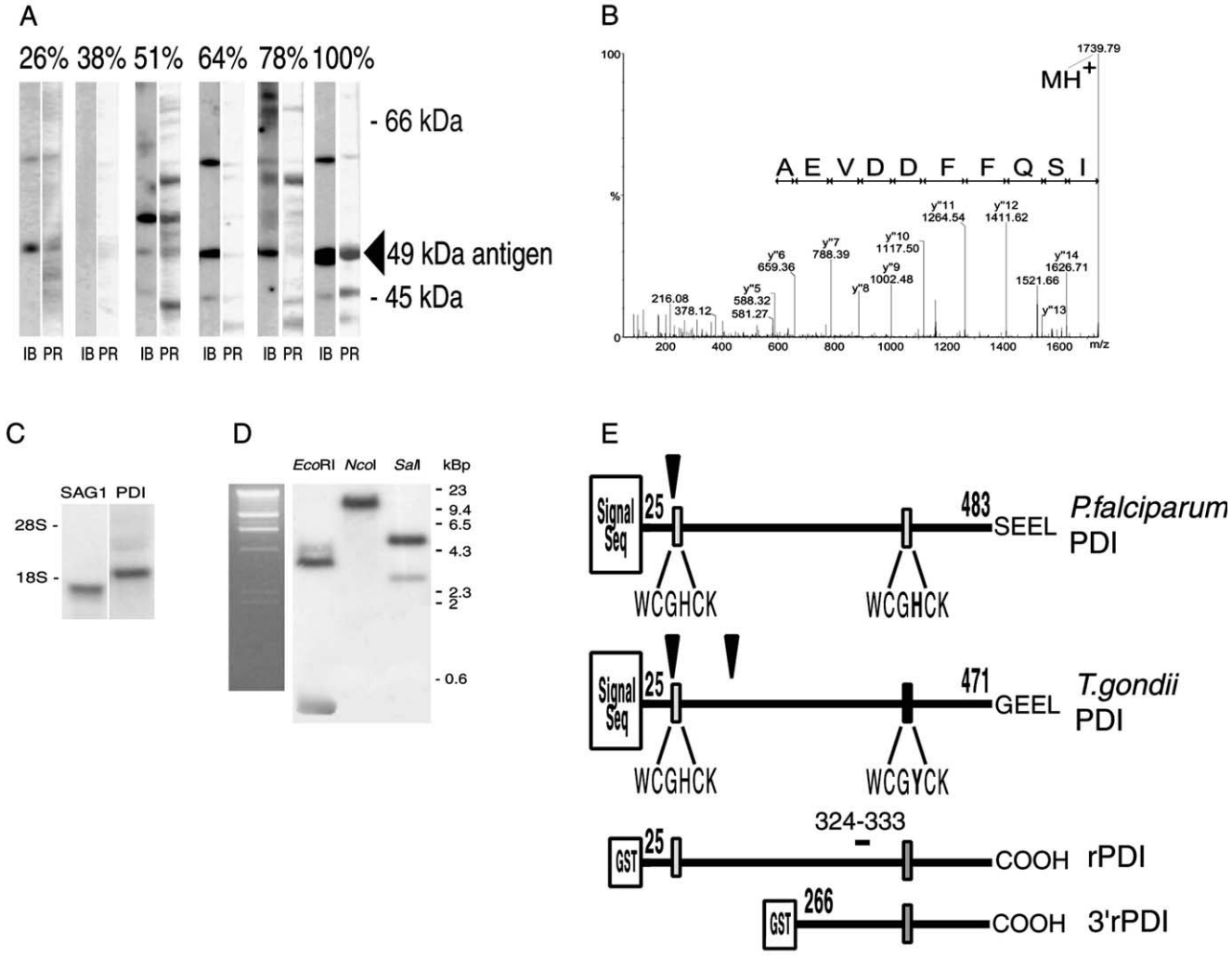

Fig. 1. Identification and characterization of the 49 kDa antigen of *T. gondii*. A: AS was added to saturation percentages indicated above the lanes and precipitated proteins were analyzed. Ponceau red staining patterns on blots (PR) are shown, as well as IgA staining patterns of strips incubated with tear sample V1 (IB). Tear samples of volunteer V1 were used for this experiment, because they predominantly contained IgA antibodies for the 49 kDa antigen. B: A triply protonated tryptic peptide of *m/z* 580.6 was fragmented from which a deconvoluted MS-MS spectrum with a partial sequence tag, ISQFFDDVEA (569.3), was constructed. This tag was used to screen databases and matched with dbEST entry ID# 1374495, an EST of 300 bp identified as part of the message encoding *T. gondii* PDI. *X*-axis: *m/z*, *y*-axis: relative intensity. C: Northern blots containing size-separated *T. gondii* RNA were incubated with labelled probes specific for SAG1 and the PDI EST. The ribosomal RNA bands of *T. gondii* run at ~3400 (28S) and ~1850 (18S) nt, while SAG1 mRNA is about 1500 nt [18]. Shown are the lanes representing 10 μg (SAG1) and 2 μg RNA (PDI). Blots were exposed to film for 5 h. PDI mRNA is estimated to be about 2000 nt in length. D: *T. gondii* DNA was digested with the restriction enzymes indicated above each lane. Southern blots were probed with a labelled PDI cDNA fragment of 1350 bp. *Eco*RI digestion resulted in three fragments. Since PDI cDNA has two *Eco*RI sites, this pattern indicates a single copy gene for PDI. E: Positions of introns in genes encoding PDI of *Plasmodium falciparum* and *T. gondii* are indicated by arrowheads. The position in the Trx domain of the first intron in the coding sequence is identical to the single intron of *P. falciparum*. *T. gondii* is unique in having a second intron at nt position 815. Light gray boxes represent Trx-like domains with CGHC motif, while a Trx-like domain with CGYC motif at *T. gondii* aa positions 380–383 is indicated by a dark gray box. Recombinant proteins were expressed as GST fusion proteins. The position of the peptide identified by mass spectrometry is given.

redoxin-like boxes with a CXXC motif [9]. One of the main functions of oxidoreductase is facilitating de novo formation of disulfide bridges between cysteines (oxidation), and/or rearranging existing disulfide bridges (isomerization). Besides acting as oxidoreductase, PDI also assists in protein folding, functions as a chaperone, and forms a subunit of prolyl-4-hydroxylase and triacylglycerol transfer proteins [9].

All major surface antigens of *T. gondii*, SAG1 and related proteins, have high numbers of intramolecular disulfide bonds [10] which might explain the high expression level of PDI observed in *T. gondii* (see below).

3.2. Characterization of the cDNA encoding T. gondii PDI

The full-length cDNA sequence of PDI was obtained by 5′ and 3′ RACE using primers based on dbEST entry ID #1374495. This resulted in cDNA products of approximately 1100 bp (5′ RACE) and 600 bp (3′ RACE). Northern blot analysis of total *T. gondii* RNA with a PDI cDNA fragment showed a single transcript of about 2 kb (Fig. 1C), abundantly present (compare with signal of the SAG1 message) and roughly matching the combined length of the 5′ and 3′ RACE products.

Sequence analysis showed that *Toxoplasma* PDI contained

the two Trx-like domains with active site sequence motif CXXC characteristic for PDIs (indicated in Fig. 1E; accession number AJ306291). Twenty-eight tryptic peptide peaks present in the MALDI spectrum, including all predominant peaks, matched the sequence obtained, yielding a sequence coverage of 60%, confirming the 49 kDa protein as PDI.

T. gondii PDI differs from all other known eukaryotic PDI sequences in an amino acid substitution of the consensus histidine (H) by a tyrosine (Y) in the C-terminal Trx-like domain. This H→Y substitution was confirmed by sequencing DNA from two different sources of *T. gondii* RH strains (data not shown). In addition to Trx-like domains other functional elements were predicted (see Fig. 1E), including a hydrophobic N-terminal signal sequence of 25 amino acids and a C-terminal ER retention signal (GEEL).

The replacement of H by Y in one of the *Toxoplasma* CXXC boxes is unique for eukaryotic PDIs described so far. It is not likely to affect the oxidoreductase function of PDI. Besides being a neutral substitution, it is not unique to Trx-like proteins in general, as one of the oxidoreductases of *E. coli*, DsbC, also has Y in its CXXC box. However, compared with DsbA, an oxidoreductase containing a 'classic' box, the function of DsbC is shifted towards isomerization [11], so this might hold true for PDI of *Toxoplasma*.

Based on amino acid similarities, PDI of *Toxoplasma* most closely resembles PDI of other members of the family of protozoan parasites like *Plasmodium falciparum* [12] and, somewhat less, *Cryptosporidium parvum* [13]. For alignments of *T. gondii* PDI with closely related PDIs and associated features, see supplemental figure (http://www.elsevier.com/PII/S0014579302029113).

3.3. Characterization of the genomic sequence encoding *T. gondii* PDI

Analysis of a genomic fragment containing the complete coding sequence of *T. gondii* PDI (accession number AJ312317) showed the presence of two introns at bp positions 303 and 815 (Fig. 1E). The position in the Trx domain of the first intron in the coding sequence is identical to the single intron of *P. falciparum* [12], the second intron is unique for *T. gondii*. The genomic sequence encoding PDI of *C. parvum* does not contain introns (see accession number U48261). Southern blotting demonstrated a major fragment of approximately 3 kb with *Eco*RI-digested chromosomal *T. gondii* DNA (Fig. 1D) indicating a single locus for PDI in the *T. gondii* genome.

3.4. Recognition of recombinant PDI by IgA in human tears

Full-length *T. gondii* PDI and its C-terminal part were expressed as GST fusion protein (Fig. 1E, rPDI and 3′rPDI, respectively) in *E. coli*, purified and blotted. Both recombinant PDI proteins were recognized by IgA antibodies in tears of individuals known to have anti-49 kDa antibodies (Fig. 2A). This recognition was not due to the GST part of the recombinant protein as none of the volunteers had IgA antibodies recognizing GST protein alone (see Fig. 2A, second panel). Furthermore, rabbits challenged with recombinant PDI generate IgG antibodies reacting with the 49 kDa antigen, now identified as PDI, on a *T. gondii* lysate blot (Fig. 2B).

As mentioned, PDI acts in the endoplasmic reticulum (ER). However, the binding of PDI-specific IgA antibodies to the exterior of parasites clearly demonstrated the presence of PDI

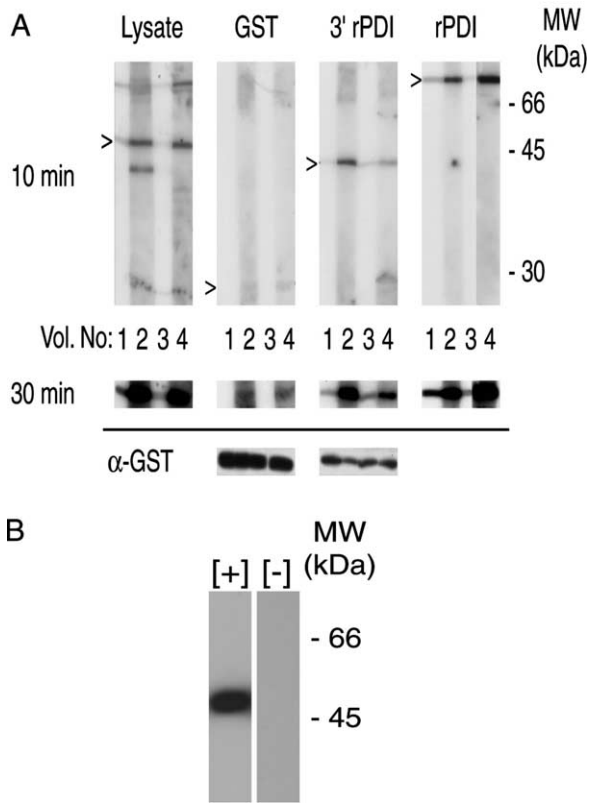

Fig. 2. Recognition of recombinant and native PDI by IgA in tears and immune serum. A: IgA antibodies in diluted tear samples of volunteers were incubated with blot strips containing either soluble *T. gondii* lysate proteins (Lysate), purified GST (GST, ~28 kDa) protein, GST coupled to 3′ part of PDI (3′ rPDI, ~45 kDa) and full-length PDI (rPDI, ~77 kDa). Positions of native PDI (~49 kDa) on the Lysate strips, and recombinant proteins are marked (>). IgA in tears recognized rPDI and 3′rPDI. Two exposures are shown (10 and 30 min). The anti-GST staining patterns were obtained after GST and 3′rPDI containing immunoblot strips were 'erased'. Vol. No. indicates volunteer number 1 to 4. Tear samples were diluted 200×. B: The antibody specifically generated against recombinant PDI stained a 49 kDa antigen on lysate blot. [+] indicates post-immune serum following immunization with rPDI and [−] pre-immune serum. Rabbit serum was diluted 20 000×.

on the surface [3]. There are examples of an extracellular location of PDI, despite the presence of a carboxy-terminal ER retention signal, e.g. on the surface of lymphocytes, involved in adhesion of human immunodeficiency virus [14], and on the surface of platelets, mediating adhesion to integrin [15]. It is possible that *T. gondii* PDI exits the ER bound to its targets (e.g. SAG1 and related proteins, TgAma-1 [16,17]) functioning as a chaperone for these membrane proteins. Together with proteins known to be involved in host cell adhesion like SAG3 and TgAma-1 [16,17], PDI may also participate in adhesion to host cells during the initial phase of infection. Although PDIs of protozoan parasites resemble their human equivalents, PDI of *P. falciparum* is selectively targeted by compounds inhibiting growth of *Plasmodium* [12]. These compounds are screened for anti-malarial capacity, and may also be of use in treatment of toxoplasmosis, especially in the light of a possible adhesion function for PDI.

With respect to its function, PDI can be regarded as a conserved protein. This suggests that these anti-*T. gondii* PDI antibodies constitute part of the natural antibody mem-

ory repertoire of humans, which might have evolved to cope with co-existing protozoan parasites. To confirm that these anti-PDI antibodies are natural antibodies, experiments are performed to characterize the regions on PDI they recognize. Other mucosal sites will be analyzed to determine whether these antibodies are unique for the lacrimal gland or are a general characteristic of the common mucosal immune system.

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