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Trichinella spiralis: genome database searches for the presence and immunolocalization of protein disulphide isomerase family members

C.P. Freitas[1], I. Clemente[2], T. Mendes[2] and C. Novo[2]*

[1]Centro Hospitalar de Lisboa Central, EPE – Serviço de Anatomia Patológica, Rua José António Serrano, 1150-199 Lisboa, Portugal: [2]Unidade de Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008, Portugal

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Abstract

The formation of nurse cells in host muscle cells during *Trichinella spiralis* infection is a key step in the infective mechanism. Collagen trimerization is set up via disulphide bond formation, catalysed by protein disulphide isomerase (PDI). In *T. spiralis*, some PDI family members have been identified but no localization is described and no antibodies specific for *T. spiralis* PDIs are available. In this work, computational approaches were used to search for non-described PDIs in the *T. spiralis* genome database and to check the cross-reactivity of commercial anti-human antibodies with *T. spiralis* orthologues. In addition to a previously described PDI (PDIA2), endoplasmic reticulum protein (ERp57/PDIA3), ERp72/PDIA4, and the molecular chaperones calreticulin (CRT), calnexin (CNX) and immunoglobulin-binding protein/glucose-regulated protein (BIP/GRP78), we identified orthologues of the human thioredoxin-related-transmembrane proteins (TMX1, TMX2 and TMX3) in the genome protein database, as well as ERp44 (PDIA10) and endoplasmic reticulum disulphide reductase (ERdj5/PDIA19). Immunocytochemical staining of paraffin sections of muscle infected by *T. spiralis* enabled us to localize some orthologues of the human PDIs (PDIA3 and TMX1) and the chaperone GRP78. A theoretical three-dimensional model for *T. spiralis* PDIA3 was constructed. The localization and characteristics of the predicted linear B-cell epitopes and amino acid sequence of the immunogens used for commercial production of anti-human PDIA3 antibodies validated the use of these antibodies for the immunolocalization of *T. spiralis* PDIA3 orthologues. These results suggest that further study of the role of the PDIs and chaperones during nurse cell formation is desirable.

Introduction

Trichinella spiralis is one of the most widespread nematodes and can infect humans and many other species of mammal. Trichinellosis remains an important food-borne parasitic zoonosis. The formation of nurse

*E-mail: cnovo@ihmt.unl.pt

cells in host muscle cells is a key step of the infective mechanism, as it provides a suitable long-term habitat for larvae (Polvere *et al.*, 1997; Capo *et al.*, 1998), comprising a constant and reliable system for the supply of nutrients and waste removal required for the worm to develop (Kang *et al.*, 2011). The *T. spiralis* surface includes a cuticle, an epicuticle and a surface coat, which are vital for physiological functioning (Maizels *et al.*, 1993). During their invasion and development, parasites produce numerous molecules as part of a survival strategy against adverse changes (Dzik, 2006). Among the antigens of *T. spiralis* infective larvae, excretory–secretory products (ESP) and surface antigens are the main target of the immune response, and play an important role in the invasion and development process of *Trichinella* larvae (Robinson *et al.*, 2007). *Trichinella* antigens are specific to different developmental stages (Parkhouse & Ortega-Pierres, 1984), evoking a protective stage-specific host immune response, owing to the uniqueness of both the cuticular antigens and the ESP antigens of each stage (Wang & Bell, 1992).

Briefly, the cuticle is a multilayered structure composed mainly of collagens. It is synthesized five times during development: during embryogenesis and during each of the four larval stages before each moult (Johnstone, 2000). Collagen biosynthesis is a complex multistep process, involving chaperones and numerous modifying, folding and processing enzymes. The first important cotranslational modification of procollagen is prolyl 4-hydroxylation, which allows its proper folding into a thermally stable form. The α-subunit of the prolyl 4-hydroxylase enzyme associates with the β-subunit of protein disulphide isomerase (PDI) to form active soluble enzyme complexes, which are resident in the endoplasmic reticulum. Collagen trimerization is set up via PDI-catalysed disulphide bond formation. In nematodes, the presence of PDI has been described in *Onchocerca volvulus* (Wilson *et al.*, 1994), *Ancylostoma caninum* (Epe *et al.*, 1998, 2007), *Ostertagia ostertagi* (Vercauteren *et al.*, 2003) and *Caenorhabditis elegans* (Winter & Page, 2000). The draft genome of *T. spiralis* has been published (Mitreva *et al.*, 2011), and different PDI family members are listed, such as PDI (PDIA2), ERp57 (PDIA3) and ERp72 (PDIA4). Endoplasmic reticulum molecular chaperones, such as calreticulin (CRT), calnexin (CNX) and the 78 kDa glucose-regulated protein precursor/immunoglobulin heavy chain-binding protein (GRP78/BIP) are also described. Both CRT and CNX are functionally coupled, establishing a calreticulin–calnexin cycle, which is physically associated with ERp57 (PDIA3). Protein disulphide isomerase is an essential catalyst of the endoplasmic reticulum in different biological systems, acting as a dithiol–disulphide oxido-reductase capable of reducing, oxidizing and isomerizing disulphide bonds, and also acting as a chaperone. It is the founding member of a family of about 20 related mammalian proteins that are mainly located and functioning in the endoplasmic reticulum. The members vary in length and domain arrangement, but share the common structural feature of having at least one domain with a thioredoxin-like structural fold. Most PDI family members (PDIs) contain both catalytic motifs and non-catalytic thioredoxin-like domains (Kozlov *et al.*, 2010). Although PDIs are endoplasmic-reticulum-resident proteins, they can be found at the plasma membrane, where they regulate the redox status of the cell surface.

No specific antibodies are available for the localization of *T. spiralis* PDIs. This fact prompted our group to search the *T. spiralis* databases of PDIs for the identity and similarity of amino acid peptide sequences used in the production of commercial antibodies for human-orthologous PDI family members. A search of the *T. spiralis* database (taxid 6334) revealed that some of these antibodies should cross-react with *T. spiralis* PDIs. This paper reports our immunocytochemical analysis of the localization of *T. spiralis* PDIs in muscle larvae. We demonstrate the presence of different members of the PDI family and chaperones, as well as their localization on the larvae.

Materials and methods

Experimental infection and sample preparation

Trichinella spiralis (Spanish strain) was maintained by serial passage infections in female rats (*Rattus rattus*) according to the method of Pozio *et al.* (1992). Tissue samples of the tongue, diaphragm and masseter muscle were collected from rats infected with *T. spiralis* for immunocytochemical analysis. Immediately after dissection, tissues were fixed by immersion in 10% formalin for 24 h, dehydrated by immersion in increasing concentrations of alcohol, incubated with xylene, infiltrated with paraffin (60°C) and embedded in paraffin blocks.

Trichinella spiralis *genome databases*

A string search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) for previously listed proteins was undertaken. A search of the *T. spiralis* genome database (taxid 6334) for orthologues to human PDI family members was made using the BLAST algorithm (Altschul *et al.*, 1997). The Scratch program (http://scratch.proteomics.ics.uci.edu/; Cheng *et al.*, 2005) was used for a linear B-cell epitope search of the amino acid sequences of the antigen used for commercial anti-PDI antibody production.

Immunocytochemistry

Paraffin sections, 3 μm thick, of routinely processed, formalin-fixed, paraffin-embedded material were cut and mounted on glass slides (SuperFrost Ultra Plus®, Thermo Scientific, Waltham, Massachusetts, USA), ensuring a firm electrostatic attraction. Immunostaining was performed using an automated immunostainer (Ventana BenchMark® ULTRA, Ventana Medical Systems Inc., Tucson, Arizona, USA). Briefly, sections were deparaffinized and rehydrated with EZ Prep (Ventana), and heat-induced epitope retrieval was accomplished using CC1 (Ventana). Primary antibody anti-PDIs (Sigma, St. Louis, Missouri, USA) were employed at optimized dilutions: polyclonal rabbit anti-human PDIA3 (HPA003230), 1:100; polyclonal rabbit anti-human GRP78 (G8918), 1:300; polyclonal rabbit anti-human TMX4 (HPA000399), 1:50. Reactivity was detected using the UltraView detection kit (Ventana). Slides were counterstained with haematoxylin and

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Table 1. An exploration of the *Trichinella. spiralis* genome database for human-orthologous PDI family members using accession codes from databases. Query cover: % protein in analysis (*T. spiralis*) matching the template protein (human); identity: % sequence alignment presenting identical amino acids in both query and template sequences; E-value: statistical significance of a pairwise alignment of sequences, reflecting the size of the database and the quality of alignment (score) used, the lower the E-value the higher the congruity between the query and retrieved sequences, where a value of 0 indicates a precise match.

Human		*Trichinella spiralis*				
PDI family member	Accession code (UniProtKB/ Swiss-Prot or GenBank)	PDI family member	Accession code (GenBank)	Query cover (%)	E-value	Identity (%)
PDIA4	P13667	PDIA4	EFV50993.1	90	0.0	46
PDIA3	P30101	PDIA3	EFV54589.1	95	1.00E-150	49
PDIA2	Q13087	PDI2	EFV59922.1	92	7.00E-119	39
TMX1	Q9H3N1	Thioredoxin domain-containing protein 1	EFV59702.1	68	1.00E-54	47
TMX2	Q9Y320	Thioredoxin domain-containing protein 14-like protein	*EFV56813.1	84	1.00E-40	29
TMX3	Q96JJ7	Conserved hypothetical protein	**EFV52404.1	69	5.00E-28	25
ERp44 (PDIA10)	Q9BS26	Thioredoxin domain-containing protein 4	EFV51905.1	87	1.00E-98	45
ERdj5 (PDIA19)	AAN73271.1	DnaJ protein subfamily C member 10	EFV52026.1	91	1.00E-107	32
Calnexin (CNX)	AAA21749.1	CNX	EFV58900.1	76	5.00E-168	53
Calreticulin (CRT)	BAD96780.1	Calreticulin precursor	EFV52676.1	83	1.00E-128	54
Calreticulin family protein	BAD96780.1	Calreticulin precursor	EFV60863.1	84	7.00E-165	64
GRP78/BIP	P11021.2	Heat-shock protein 70	AAK85149.2	92	0.0	83

Reverse search on Human genome database found *TMX2 and **TMX3 as the highest homologous proteins.

blue-dye solutions. Sections were dehydrated with a graded series of ethanol and cleared in xylene. A cover slip was placed over the sections and secured with mounting medium, to protect the tissue. Positive and negative controls were included with each assay.

The human appendix (from the hospital's histological archive) was used as a positive control. Negative controls were treated in the same way as samples and positive controls, except for the absence of the primary antibody. No stain was observed in negative control tissues (results not shown).

Comparative modelling and validation

A theoretical three-dimensional model for PDIA3 from *T. spiralis* was constructed by comparative modelling using the Swiss-Model server, version 3.7 (Arnold *et al.*, 2006; Kiefer *et al.*, 2009; Biasini *et al.*, 2014; http://swissmodel.expasy.org/), which is accessible via the ExPASy web server (http://expasy.org/). The model quality was checked using the QMEAN program (Benkert *et al.*, 2011). The Swiss-Pdb Viewer, version 4.0.4 (Guex & Peitsch, 1997) was used for model visualization.

Results and discussion

Trichinella spiralis *genome database*

The string search performed for *T. spiralis* PDI found a number of annotated proteins (table 1): PDI2, PDIA3 and PDIA4, and a DnaJ protein subfamily C member 10. In addition, the chaperones CRT, CNX and heat-shock protein 70 were listed in the database.

A search for human-orthologous accession codes on the *T. spiralis* genome database revealed other PDI family members and chaperones (table 1). Orthologues for the human thioredoxin-related-transmembrane protein (TMX) were found: the thioredoxin domain-containing protein 1, the thioredoxin domain-containing protein 14-like protein and the conserved hypothetical protein. In addition, human PDIA10 matched, with 45% identity, the sequence from *T. spiralis* thioredoxin domain-containing protein 4; both exhibited the same active site amino acid sequence (CRFS).

Immunolocalization of Trichinella spiralis *protein disulphide isomerase family members*

Analysis of the paraffin sections of rat muscle infected with *T. spiralis* larvae after staining with commercial antibodies showed positive results for CRT, CNX, GRP78, PDIA3, TMX1 and TMX4. These results suggest that, in a first approach, some commercial anti-human antibodies could be used to detect orthologous molecules in *T. spiralis.* To validate these results, the specificity of these antibodies was checked by searching the amino acid sequence of the peptides used for commercial production of antibodies on the *T. spiralis* genome database (taxid 6334). When large amino acid sequences were used as immunogens, the potential linear B-cell epitope propensity and respective amino acid sequence were also predicted using the Scratch prediction method (Cheng *et al.*, 2005) and a search for homologous amino acid sequences in *T. spiralis* genome (taxid 6334) was performed.

Immunocytochemical analysis of binding cross-reactivity between *Homo sapiens* and *T. spiralis* of the commercial antibodies gave a positive result (not shown), suggesting that both anti-human PDIA3 antibodies could be used for the orthologous proteins in *T. spiralis*. A search of the *T. spiralis* genome database for the whole sequences used as an immunogen, in addition to the predicted amino acid sequences for the linear B-cell epitope, identified a match with *T. spiralis* orthologue XP_003373217.1 (EFV54589.1).

With respect to CNX, CRT and TMX1, similar searches of the *T. spiralis* database did not provide any results, suggesting that the binding of antibodies does not confirm their potential use in immunolocalization.

However, a database search using the amino acid sequences of the immunogens used for production of commercial human antibodies to TMX4 and GRP78 gave a different result. The peptide corresponding to human GRP78 matched the entries in the *T. spiralis* database for heat-shock protein 70 (AAK85149.2) and heat-shock protein C (EFV60727.1). A reverse search of these two amino acid sequences on the human database (taxid 9606) found, with the highest amino acid sequence identities, the same three entries for AAK85149.2: the GRP78 precursor entries NP_005338.1 and AAA52614.1 (82% and 81% identity, respectively), and the BIP protein entry AAF13605.1 (82% identity), validating the results obtained by immunocytochemical analysis.

In the case of the antibody to human TMX4, the amino acid sequence used as an immunogen matched the entry in the *T. spiralis* database for thioredoxin domain-containing protein 1 (EFV59702.1), which corresponds to the TMX1 human orthologue. This result suggests that the anti-human TMX4 binds to the TMX1 orthologue in *T. spiralis*.

From the constructed three-dimensional model for *T. spiralis* ERp57 (PDIA3) (not shown), it is possible to

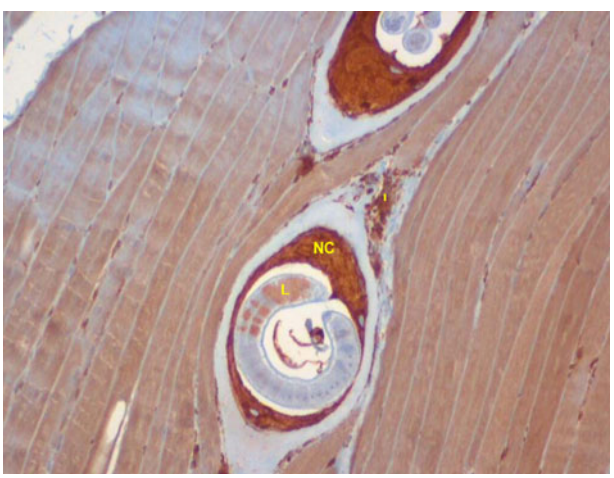

Fig. 1. Immunocytochemistry analysis. Rat muscle infected with *T. spiralis* larvae stained with commercial anti-human PDIA3 (ERp57) antibody. Magnification: ×100. NC, nurse cell cytoplasm; I, inflammatory infiltrate; L, larva.

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identify the expected four domains. It is also possible to see that the peptides predicted to be the linear B-cell epitopes for the commercial antibodies are located in domains b (127 GPSSKELKTADDFKK 141), b′ (256 DVDYERN 262; 323 KYVMKDEFS 331) and a′ (352 KSEPIPETNDNP 363; 448 PQSYTGGRTLDDFI 461) at the surface of the molecule, as expected, and accessible to the binding of commercial antibodies.

Paraffin sections stained using the antibodies whose predicted specificity suggested that they could be used for orthologous identification in *T. spiralis* larvae are shown in fig. 1 (for PDIA3; those for TMX1 and GRP78 are not shown). Analysing fig. 1, we can see intense immunostaining for PDIA3 in the developing nurse cell cytoplasm, moderate staining for PDIA3 in the surrounding inflammatory cell infiltrate and weak focal staining for PDIA3 in the larvae. The intense expression of PDIA3 in the nurse cell cytoplasm is suggestive of its role in the formation of the nurse cell. An intense immunostaining of TMX1 (cross-reacting with the anti-TMX4 antibody used) was observed in the nurse cell cytoplasm and in particular detail in the larvae. For GRP78, moderate immunostaining was noted in the developing nurse cell cytoplasm, while intense granular nuclear expression was observed in the larvae.

The immunocytochemical results suggest the localization of three important molecules belonging to the PDI family and chaperones, which were predicted by computational genome analysis. Unfortunately, the immunostaining results obtained with other anti-human antibodies did not agree with the computational cross-reaction prediction; these results are not shown. In particular, it is important to localize the CRT/CNX chaperone system, as it is intimately related with the activity of PDIA3. Moreover, the relationships between these molecules and induced endoplasmic reticulum stress (Yu *et al.*, 2014) and their role in the induced protective immunity from infection in the host (Fang *et al.*, 2014) suggests the importance of knowing the localization of these molecules during nurse cell formation.

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Conflict of interest

None.

Ethical standards

Work dealing with pathogens and drugs was performed under controlled conditions, and all safety measures were taken. If needed, the material used for laboratory practices was sterilized before being discarded appropriately. Good animal handling practices as well as good laboratory practices were used and all EU Directives were followed, namely: 2000/54/CE Exposure to biological agents, 88/320/CE Good laboratory practices, 90/679/CE, 405/98/PT and 1036/98/PT. Workers were protected against exposure to dangerous biological agents during work time. In no known way did this project contravene the social, ethical and environmental laws or principles accepted in Portugal and in the European Union. The person developing the project was certified by the national body for conducting experimental animal manipulations.

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