Expression of a cDNA encoding a *Toxoplasma gondii* protein belonging to the heat-shock 90 family and analysis of its antigenicity

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Abstract

A cDNA clone (Tgzy85d11.r1) obtained from the *Toxoplasma* Expressed Sequence Tag project was chosen due to its homology with proteins of the heat shock 90 family. The cDNA encodes 137 amino acids of the C-terminal portion of the *Toxoplasma* Hsp90 protein (TgHsp90). Serum samples obtained from orally infected BALB/c and C57BL/6 mice showed reactivity against a recombinant TgHsp90 (rTgHsp90) after 8 weeks postinfection. Isotype analysis showed an anti-rTgHsp90 IgG2a/IgG3 response in infected BALB/c and anti-rTgHsp90 IgG1/IgG2a/IgG2b response in infected C57BL/6 mice. Serum samples from individuals chronically and putative acutely infected with *T. gondii* showed a similar anti-rTgHsp90 IgG response. Our work identifies TgHsp90 as a novel parasite antigen that seems to elicit a higher relation of anti-TgHsp90/anti-*T. gondii* IgGs during chronic infection in comparison with the acute stage.

Keywords: *Toxoplasma gondii*; Heat shock protein 90; cDNA; Humoral response

1. Introduction

Exposure of any living cell or organism to environmental stress results in the transcription of a highly conserved set of genes and the synthesis of the so-called 'heat shock proteins' (Hsps). Hsps are usually classified according to their apparent SDS–PAGE molecular mass. Parasite Hsps appear to play an important role in the parasite's adaptation to the mammalian host. In addition, heat shock protein families are among the major immunogens of several pathogens [1].

As observed in other heat shock protein families those of the Hsp90 family (sometimes named Hsp83) have been demonstrated to elicit a strong humoral response during candidiasis [2] and canine leishmaniasis [3], as well as in other parasitic infections. Bonnefoy et al. [4] showed that vaccination of monkeys with *Plasmodium falciparum* Hsp90 elicited a protective immune response. Recently, it was demonstrated that *Leishmania infantum* Hsp83 possesses remarkable immunostimulatory properties [5]. On the one hand, immunization with *L. infantum* Hsp83 fused to a reporter antigen elicits an enhanced humoral response against the reporter antigen. On the other, *L. infantum* Hsp83 recombinant protein stimulated spleen T- and B-cell proliferation from unprimed BALB/c mice.

Toxoplasma gondii is an obligate intracellular parasite that can infect mammals and birds. *T. gondii* invades and replicates in the intestinal epithelium from where infection is disseminated to other organs persisting as bradyzoite-containing cysts. Early in the infection a strong humoral and cellular immune response is elicited against *T. gondii*, which confers long-term protection [6].

In this study, we describe the cloning and expression of a tachyzoite *T. gondii* cDNA that encodes the C-terminal region of Hsp90 protein family. Serum samples from perorally *T. gondii* infected BALB/c and C57BL/6 mice, as well as from humans with *T. gondii* infection, were used to analyze the humoral response elicited by this protein.

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2. Materials and methods

2.1. Source of the cDNA clone, excision of insert and sequencing

Several cDNAs with high homology to Hsp90 proteins were identified in the expressed sequence tag (EST) database (www.cbil.upenn.edu./ParaDBs/Toxoplasma/index.html) [7]. The cDNA described here is the clone *Tgzy85d11.r1* obtained from *T. gondii* EST project (Genome System, USA). The Bluescript plasmid was excised from the lambda ZAP vector using the ExAssist kit (Stratagene). Plasmid DNA obtained from the positive clones was sequenced by Servicio de Secuenciación (INTA-Castelar, Argentina) using pBluescript primers (accession number: AF179480).

2.2. Host cells and parasite cultures

RH *T. gondii* strain tachyzoites were grown in PTP monolayers, a human foreskin fibroblast cell line established by Servicio de Cultivo de Tejidos (INEI, ANLIS Dr. Carlos G. Malbran), with Eagle's minimum essential media (Gibco) containing 1% fetal calf serum (Gibco). Parasites were purified from infected monolayers by filtration through 3-μm polycarbonate filters (Nuclepore). Cysts were obtained from brains of mice orally infected with Me49 *T. gondii* strain.

2.3. Subcloning of TgHsp90 insert into pQE32 vector, expression and purification of the recombinant protein

The *Tgzy85d11.r1* cDNA was digested with *Bam*HI and *Kpn*I restriction enzymes, purified from agarose gel by Qiaex II (Qiagen), cloned into the corresponding sites of the pQE32 plasmid (Qiagen), and sequenced. The plasmid pQE-*TgHsp90* produces a recombinant protein with a 6-His tag at the N-terminus (rTgHsp90). Expression and purification under denaturing conditions were performed as previously described [8].

2.4. Production of anti-recombinant TgHsp90 serum

A rabbit was immunized with 0.5 mg of affinity purified rTgHsp90 emulsified with Freund's complete adjuvant. Two subsequent boosters were given at 2-week intervals with 0.2 mg of rTgHsp90 emulsified in Freund's incomplete adjuvant. Serum was collected 1 week after the last booster.

2.5. Western-blot analysis

SDS–PAGE on 10% polyacrylamide gels was performed in the Mini-Protean system (Bio-Rad) using standard conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Mini-Protean-Blot system, Bio-Rad), and the membrane was blocked with 5% non-fat dried milk powder in PBS–0.5% Tween 20 (blocking solution). The nitrocellulose filters were probed with the first antiserum diluted in blocking solution. A peroxidase immunoconjugate (Jackson ImmunoResearch Laboratories) was used as the secondary antibody (1:5000), and specific binding was detected with H_2O_2 as substrate and diaminobenzidine as the chromogen.

2.6. Infections

Two-month-old female BALB/c ($n = 5$) or C57BL/6 ($n = 5$) inbred mice were orally infected with 20 cysts of Me49 *T. gondii* strain. Mice were bled at the following weeks: 1, 2, 4, 7 and 10. Infected C57BL/6 mice showed cysts in the brain at week 10, whereas no cysts were found in infected Balb/c mice.

2.7. Determination of IgG and isotypes in infected mice

Each well of a microtiter plate (Immuno Plate Maxisorp; Nunc) was coated overnight at room temperature with 100 μl of 5 μg ml^{-1} of recombinant proteins diluted in 0.05 M carbonate buffer (pH 9.6). Enzyme-linked immunosorbent assay (ELISA) was performed as described [9]. Immune complexes were developed with OPD (Sigma) as the chromogen and H_2O_2 as the substrate of a horseradish-peroxidase conjugated to anti-mouse IgG (Fc) antibody (Jackson) used as the second antibody. Absorbance at 450 nm (OD_{450}) was measured with an automatic ELISA reader (Dynatech MR4000). ELISA results were determined for each serum in duplicate. At least two independent ELISAs were performed for each serum. Isotype-specific analysis was performed by ELISA using the horseradish peroxidase conjugated anti-mouse IgG1 (1:2000), IgG2a (1:4000), IgG2b (1:2000) and IgG3 (1:1500) (Serotec).

2.8. Human serum samples

The Diagnostic Laboratory of our Institute provided sera. They were analyzed by anti-*T. gondii* IgG indirect-immunofluorescence (IgG-IIF), the double-sandwich IgM and IgA ELISA (Equipar, Italy), and grouped as either seronegative, chronic (IgG titer between 64 and 512) or putative acutely infected individuals (IgG titer between 16 284 and 130 272, and IgM^+/IgA^+). Serum samples were analyzed by ELISA as described above, using a horseradish peroxidase conjugated anti-human IgG (Fc) antibody (Jackson) diluted 1:4000.

3. Results and discussion

3.1. Sequence analysis and genomic organization of Tgzy85d11.r1 cDNA

The nucleotide sequence showed that *Tgzy85d11.r1* is a

413-bp cDNA encoding a 137 amino acid protein with a theoretical mass of 15.6 kDa. Alignment of the predicted amino acid sequence with *Eimeria bovis* [9], *Plasmodium falciparum* [10] and *L. infantum* Hsp90 [3] after a BLASTP analysis, revealed that *Tgzy85d11.r1* cDNA encodes for a both-side truncated version of a *T. gondii* Hsp90 protein (Fig. 1A). Using the nucleotide sequence of *Toxoplasma* EST clone *TgESTzy03c02.r1* it could be predicted that C-termini truncation was produced during the cloning by the presence of an internal *Xho*I site, losing the last 16 amino acids residues of the protein (Fig. 1B). Southern blotting using *Sal*I and *Eco*RI suggests that the *hsp90* is a single copy gene (data not shown).

3.2. Expression of the recombinant protein and identification of the natural protein

Toxoplasma C-terminal Hsp90 was expressed as a recombinant protein (rTgHsp90) in *Escherichia coli*. As it formed inclusion bodies, it was necessary to solubilize it with 8 M urea solution and was purified as described [9]. rTgHsp90 migrated with an apparent molecular mass of 20 kDa (Fig. 2A). Immunoblotting revealed that the rabbit polyclonal anti-rTgHsp90 serum recognized a strong band in the region of 90-kDa as well as others of lower molecular mass, which are likely to be degradation products of TgHsp90, in the whole *T. gondii* homogenate (lane 2, Fig. 2B). The serum showed cross reactivity with a putative Hsp90 of host cells (lane 1, Fig. 2B), which possess a higher molecular mass compared with TgHsp90. No bands were observed when preimmune serum was used (Fig. 2C).

3.3. Analysis of humoral response against rTgHsp90 in orally infected mice

The contribution of *Toxoplasma* Hsp90 to the humoral response was studied in orally infected BALB/c and C57BL/6 mice. Significative levels of anti-rTgHsp90 IgG antibodies were detected only after 8 weeks postinfection in both BALB/c and C57BL/6 infected mice, the latter showing a high standard deviation (Fig. 3A), inferring that rTgHsp90 could be a marker of chronic infection. These results are similar to those obtained by Mun et al. [11] with *T. gondii* cystic-stage Bag1/Hsp30 protein, which induced protective immunity in mice. By contrast, rRop2 [8] was shown to produce a significative humoral response early in the infection in both mouse strains (Fig. 3A).

Infected BALB/c mice elicited an anti-rTgHsp90 IgG2a and IgG3 response, whereas infected C57BL/6 mice produced an anti-TgHsp90 IgG1, IgG2a and IgG2b response (Fig. 3B). Since BALB/c mice are resistant and C57BL/6 are susceptible to avirulent *T. gondii* strains, differences observed in the isotype profile against rTgHsp90 could suggest a role for TgHsp90 in the immunopathogenesis during *T. gondii* infection.

3.4. Humoral response against TgHsp90 during human infection

Serum samples obtained from individuals with chronic and putative acute infection showed similar anti-TgHsp90 reactivities by IgG-ELISA (Fig. 4). Noteworthy, the absorbance value of the anti-rTgHsp90 response did not increase in concordance with the anti-*T. gondii* IgG-IIF titer

A

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Tg90    1 HEDKVEQVVVSNRITDSPCVLVTSEYGWSANMERIMKAQALRDNSMTTYMVSKKTMEINPTNPIMEELK
Eb90  288 LH...DK..L.............T.F.....................S.........V.GHH...V.I.
Pf90  599 ..-...K...GQ.............F.....................S..L...I....ARH..ISA..
Li83  550 LG....K.I..ECLST...I.....F....H..Q..RN.....S..AQ..M......L..RH..IK..R

Tg90   70 KKSNADKSDKTVKDLIWLLFDTALLTSGFSLDEPTQFAARIHRMIKLGLSIDEDDEELRAEEDLPPLE
Et90  355 N.AAV.............Y..........E......C.............---..D.EAKDD......
Pf90  667 Q.AD.................S......A.E...T.SK.............---...ENNDI......
Li83  619 RRVDA.EN..A....VF.....S......Q.ED..-Y.E..N........L..EE.VVA..ATVAETA
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B

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Tg90* 138*  evegaveetskmeevd
Pf90  732   .TVD.TD--.......
Li83  686   PA.VTAG-..S..Q..
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Fig. 1. (A) Alignment of the deduced TgHsp90 (Tg90) amino acid sequence with *E. bovis* (Eb90) [9], *P. falciparum* (Pf90) [10] and *L. infantum* (Li83) [3] Hsp90. BLASTP (www.ncbi.nlm.nih.gov/blast/) alignment between TgHsp90 and *E. bovis*, P. *falciparum* and *L. infantum* Hsp90/83 showed identities of 83, 79 and 65%, respectively. (B) To determine how many amino acids are necessary to complete the C-terminal region of *T. gondii* Hsp90, a C-terminal sequence was deduced from the nucleotide sequence of the clon TgESTzy03c02.r1 (Tg90*). Tg90* begins arbitrarily at position 138 (*), that corresponds to the next amino acid position after the last amino acid of TgHsp90 which has 137 residues. Since *E. bovis* Hsp90 sequence also is truncated at the C-termini region, TgHsp90* sequence was only aligned with *P. falciparum* and *L. infantum* Hsp90/83.

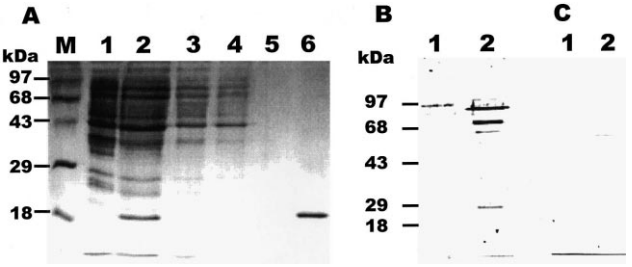

Fig. 2. (A) SDS–PAGE gel stained with Coomasie blue showing rTgHsp90 expression and purification. Recombinant *E. coli* protein extract in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–HCl) pH 8 (lane 2) was passed through a Ni^{2+}-nitrilotriacetic acid resin (lane 3). The resin was washed with lysis buffer at pH 6 (lanes 4 and 5) and rTgHsp90 was eluted with lysis solution at pH 4.2 (lane 6). Lane 1, non-induced recombinant *E. coli*. (B,C) Immunoblot analysis of fibroblast (lane 1) and whole RH tachyzoites (lane 2) extracts incubated with a rabbit anti-rTgHsp90 (B) or preimmune serum (C). M, molecular mass markers. Molecular masses are given on the left. Sera were used at 1:500 dilution.

between both groups, suggesting that a higher relation of anti-TgHsp90/anti-*T. gondii* IgGs is produced during chronic infection in comparison with the acute stage.

T. gondii Hsp70 could be associated with parasite differentiation, either from tachyzoite to bradyzoite or bradyzoite to tachyzoite [12,13]. Stage conversion associated with increased levels of Hsp90 protein levels within apicomplexa was described in *E. bovis* and *Plasmodiun yoelii* [9,14]. We are carrying out studies to investigate the role of TgHsp90 in the virulence and *T. gondii* stage-differentiation using the tools presented here. In addition, since *T. gondii* Hsp90 appears as a novel parasite antigen, its antigenic value and role in the immunopathogenic process will also be subject of future studies.

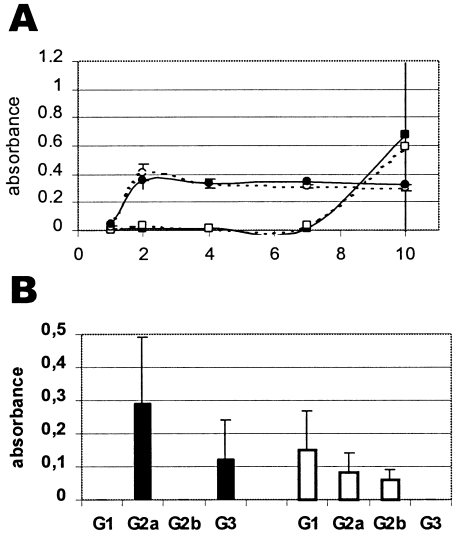

Fig. 3. (A) Anti-recombinant protein IgG profiles of serum samples obtained from infected BALB/c (filled squares and circles) and C57BL/6 (empty squares and circles) mice. Serum samples were obtained at weeks 1, 2, 4, 7 and 10 postinfection, diluted 1:100 and assayed by rTgHsp90- (squares) and rRop2-ELISA (circles). (B) Anti-rTgHsp90 IgG isotype profiles of serum samples obtained from infected BALB/c and C57BL/6 mice. Serum samples obtained at week 10 postinfection from infected BALB/c (black bars) and C57BL/6 (white bars) were analyzed by anti-TgHsp90 IgG1, IgG2a, IgG2b and IgG3. Sera were used at 1:100 dilution. Absorbance was measured at 450 nm and the mean value from each group was graphed with its standard deviation. Only those results showing significant absorbance values (higher than the mean of preimmune sera) were included in the graph. They were coincident with IgG absorbance values higher than 0.280. Sera were used at 1:100 dilution.

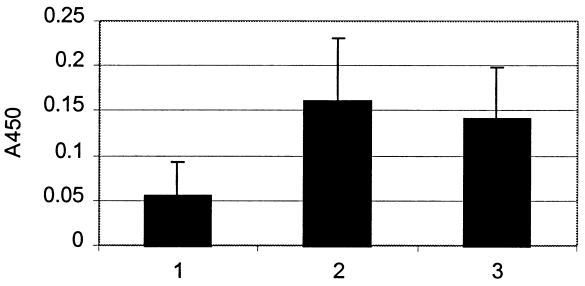

Fig. 4. Immunoreactivity of the rTgHsp90 with sera from 10 seronegative (lane 1), 10 *T. gondii* chronically infected (lane 2) and 10 potential acutely infected individuals (lane 3). Absorbance was measured at 450 nm and the mean value from each group was graphed with it standard deviation. Sera were used at 1:100 dilution.

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