(a) Title

Selection and identification of dense granule antigen GRA3 by *Toxoplasma gondii* whole genome phage display

Authors

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(b) Running title

Toxoplasma genomic phage display
(c) Summary

Toxoplasma gondii is a ubiquitous unicellular eukaryotic parasite with a complex intracellular life cycle, capable of invading and chronically infecting a wide variety of vertebrate host species including man. While normally opportunistic in healthy adults, it is a lethal pathogen in immunocompromised humans, particularly in AIDS-patients. We present the application of genomic phage display as a tool for the direct identification of antigens with potential value in diagnosis and/or as subunit vaccine component. Using a polycosmid cloning strategy, we constructed a large phagemid display library (> 10⁹ independent clones) of mixed short genomic restriction fragments (≤ 500 bp) of T. gondii genomic DNA (80 Mbp genome size) fused to gene III of the filamentous phage M13. Biopanning of the library with monoclonal Toxoplasma antibodies resulted in the isolation and identification of an epitope of GRA3, an antigen located in the dense granules of T. gondii tachyzoites. The reactivity of the phage displaying the GRA3 epitope with the monoclonal antibody was confirmed by ELISA. These results demonstrate the accessibility of mid-sized eukaryotic genomes to display technology and the feasability to screen these whole genome display libraries with antibodies for isolating novel antigenic determinants.
(d) Introduction

*Toxoplasma gondii* is an obligate intracellular protozoan parasite with a complex life cycle [1]. Humans usually acquire infection by ingesting either infectious oocysts through contact with cats or cat faeces, or by eating meat that contains tissue cysts. In healthy adults, infection normally results in a benign self-limiting disease. However, once infected, the host harbours the parasite for life. In a chronically infected individual developing immunodeficiency on response to drug treatment or disease, e.g. AIDS, the infection can reactivate and cause severe disease and mortality [2]. Primary infection during pregnancy can cause abortion or severe damage to the foetus. In animals, toxoplasmosis is recognized as a major cause of abortion and neonatal losses in sheep, goats and pigs [3].

Diagnosis of toxoplasmosis is usually based on serological assays, although in recent years, molecular biology techniques such as PCR have been applied for the detection of *T. gondii* DNA in clinical samples [4]. Most commercial serological assays detect antibodies by means of natural tachyzoite antigens from infected mice or cell culture systems. However, the use of whole tachyzoite antigens is rather expensive and sometimes results in false-positive reactions [5]. The use of recombinant antigens may overcome these drawbacks. Until now, only a limited number of recombinant antigens have been studied, e.g., surface antigens SAG1 (P30) [6], SAG2 (P22) [7], dense granule antigens GRA2 [8] and GRA4 [9], and rhoptry protein ROP2 [10].

Direct procedures by immunization with non-living vaccines are not yet available for humans to date. Various attempts to develop an animal vaccine to both the asexual systemic stage and the sexual entero-epithelial stage of the *Toxoplasma* life cycle have been reported over the last thirty years. Immunizations with whole killed [11] or irradiated [12] parasites had limited success. Effective vaccines have been produced using live attenuated parasites that induce immunity but do not persist as tissue cysts [13]. Vaccination with pure native P30 antigen [14] or with selected
fractions of parasite lysates [15] has been partially successful. Attempts with recombinant antigens, however, failed so far [16].

Further immunological characterization of the *Toxoplasma* parasite deserves undiminished attention. The search for new antigens by cDNA screening as well as EST sequencing [17] is hampered by the complex life cycle and the limited availability of certain parasite developmental stages. In this paper, we present the application of phage display of peptides encoded by fragmented genomic DNA as a useful tool to directly select and identify antigenic determinants from the genome. To facilitate the construction of essentially large genomic libraries, a novel display vector was constructed so as to allow highly efficient cloning. The feasibility of this approach was confirmed by the isolation and identification of an epitope from the dense granule antigen GRA3 [18] by panning of a genomic *T. gondii* display library against a monoclonal antibody directed towards an uncharacterized 30 kDa *T. gondii* antigenic protein.
(e) Experimental Procedures

**Preparation of *T. gondii* genomic DNA.** *T. gondii* strain Deelen [19] was maintained in permanent *in vitro* cultures by serial 3 to 4 day passages of parasites on monolayers of VERO cells in OptiMEM medium (Life Technologies, Gaithersbury, MD) supplemented with 4% fetal calf serum and 50 µg/ml gentamycin. After lysis of the monolayer, parasites were harvested by centrifugation of the supernatant. Pellets were resuspended in 1 ml of PBS buffer (phosphate, 0.01 M; NaCl, 0.14 M; pH 7.2) and the remaining unlysed host cells ruptured by repeated forced passage through a 27 gauge needle. The suspension was further purified by continuous density gradient centrifugation on 36% of Percoll® (Amersham Pharmacia Biotech, Uppsala, Sweden) at 28,600xg in a fixed-angle, 25° rotor. Batches of 10^9 purified tachyzoite cells in 100 µl PBS were lysed by addition of 1 ml of Qiagen lysis buffer G2 (Qiagen GmbH, Hilden, Germany) supplemented with 0.5 mg/ml proteinase K and 0.3 mg/ml RNase A, and heating at 50°C for 45 min. Genomic DNA was prepared from the lysate with Qiagen genomic-tip 100/G according to the supplier’s instructions.

**Display vector construction.** To create a phagemid display vector with cosmid properties, the phage lambda *cos* site from position -201 to +186 [20] was amplified by PCR with a forward primer extended at the 5′ end with an *Aat*II and *Bst*XI site, and a reverse primer bearing an *Aat*II and *Bst*EII. The resulting PCR fragment was cloned into the *Aat*II site of phagemid vector pHEN1 [21]. A number of clones containing the insert in different orientation were sequence-verified and tested for transducibility after *in vitro* packaging of concatemers, or so-called polycosmids, in phage lambda particles. Therefore, the vector was linearized with *Bst*EII, religated at high vector concentration (500 ng/µl) and mixed with MaxPlax™ Packaging Extract (Epicentre technologies, Madison, Wisconsin). Phage particles were titered by transduction of *E.*
coli strain TG1 (\textit{supE} hsd\Delta 5 thi \Delta (lac-proAB) F\{traD36 proAB+ lacI\^q lacZ\Delta M15\}). One positive clone was retained and the phagemid/cosmid vector was termed 'phosmid' pHOS1. In this vector multiple cloning sites (\textit{BglII}, \textit{SmaI} and \textit{BstBI}) for gene III fusion were created by replacement / insertion of a synthetic \textit{NcoI}/\textit{NotI} cassette. Shotgun cloning of restriction fragments in the correct reading frame was made possible by the construction of a set of nine phosmids (pHOS21 – pHOS29) each with the multiple cloning sites in a different upstream/downstream gene III reading frame (Table 1).

**Genomic library construction.** \textit{T. gondii} genomic DNA was cut with the four compatible restriction enzymes \textit{HinP1} (G/C\textit{GC}), \textit{MspI} (C/\textit{CGG}), \textit{AciI} (C/\textit{CGC}) and \textit{TaqI} (T/\textit{CGA}). Equal portions of genomic DNA were subjected to separate digestions or to consecutive double, triple or quadruple digestions in all 15 possible combinations. The restriction fragments were pooled and fractionated according to size by preparative electrophoresis through a 10 cm 2.5% agarose column on a model 230A HPEC System (PE Biosystems, Foster City, CA). Nine different size fractions ranging from 30 to 540 bp were selected and concentrated with Microcon YM-10 centrifugal devices (Millipore Corp., Bedford, MA) prior to ligation into the display vector. This display vector was prepared by mixing equimolar amounts of the nine plasmids from the pHOS20 series and digestion with \textit{BstBI} (TT/CGAA) followed by dephosphorylation. Vector and individual genomic DNA size fractions were ligated at a 1:2 molar ratio and low total DNA concentration (~12.5 ng/\mu l). The heat-inactivated ligation mixtures were pooled, linearized with \textit{BstEII} and religated at high concentration (~250 ng/\mu l). Five fractions of 1 \mu g of concatenated DNA were packaged in phage lambda particles and transduced in a single reaction to \textit{E. coli} TG1 cells grown in LB supplemented with 0.2% maltose and 10 mM MgCl\textsubscript{2}. \textit{In vivo} excision of the vector monomers was realized by co-infection with excess VCSM13 helper phage (Stratagene, La Jolla, CA). The primary library was obtained by collection of phage particles produced 3
hours after co-infection. The number of independent clones was estimated at $\geq 10^9$ (see also Results section). Representative portions of this library were stored at $-70^\circ\text{C}$ and amplified prior to biopanning.

**Biopanning.** *Toxoplasma* specific monoclonal antibodies were obtained from a mouse immunized with sonicated tachyzoites. Phage amplification, expression and display of cloned fragments were performed as previously described [21]. The library was biopanned against *Toxoplasma* antibodies essentially as described by Smith and Scott [22]. Briefly, in the first selection round, $10^{12}$ cfu phagemid particles (~1000 times the estimated library size) were allowed to react overnight at 4$^\circ\text{C}$ with 1 µM biotinylated monoclonal antibodies preincubated with M13mp18 particles. Binding phagemid particles were captured for 10 minutes on 60-mm streptavidin-coated polystyrene Petri dishes blocked with 10% dialyzed fetal calf serum and 0.02% NaN$_3$ in TBS. After ten washing steps, binding phages were eluted with Tris-glycine pH 2.2, neutralized and used for infection of TG1 cells. Recovered phage particles in general titered to $\sim 10^{7-8}$ cfu. In further rounds, 1-5x$10^{11}$ amplified phagemid particles were biopanned with monoclonal antibodies at 100 pM.

Selected clones were characterized by automated fluorescent DNA sequencing on an ABI 373 Sequencer using BigDye Terminator™ chemistry (PE Biosystems). Binding specificity was confirmed by phage ELISA. Microtiter plates were coated overnight with 500 ng/well specific monoclonal antibody in PBS and blocked for 2 hours with 200 µl/well blocking buffer (PBS supplemented with 5% BSA and 0.02% NaN$_3$). After three washes with PBS + 0.5% Tween 20, about 2x$10^{10}$ phages in 100 µl blocking solution were allowed to react for 3-4 hours. Bound phages were revealed by a 1 hour incubation with secondary horse radish peroxidase-conjugated
anti-M13 antibodies (Pharmacia Biotech) and 30 min staining with ABTS. The difference between $A_{405}$ and $A_{495}$ was taken as the ELISA signal.

(f) Results

Library construction. *T. gondii* genomic DNA was semi-randomly fragmented by combinatorial digestion with four restriction enzymes generating protruding 5'-CG ends compatible with the *Bst*BI cloning site of the pHOS vector set. Size fractionation by HPEC of the pooled restriction fragments and OD$_{260}$ monitoring of the eluted DNA showed a statistically expected, asymmetrical size distribution with a peak around 100 bp, and more than 90% of total DNA contained within fragments shorter than 500 bp. The 30 to 540 bp fragments were cloned by phage lambda-mediated polycosmid transduction as described in experimental procedures. The packaged polycosmids were titered at 1.4x10$^8$ transducing units in total from ~5 µg of ligated DNA. According to the size of the phosmid (5kb), concatemers of 8-10 vector units are expected to be packaged into lambda particles and transduced into *E. coli* cells. We could indeed show the presence of up to 8-10 vectors by PCR analysis of the cloned inserts in overnight colonies from control transduced cells not co-infected with M13 helper phage (Figure 1). Co-infection resulted as expected in *in vivo* excision and packaging of the phagemid monomers into M13 particles (PCR analysis results not shown). Hence, the total number of independent phagemid clones produced after *in vivo* excision was estimated at 1.1-1.4x10$^9$. The total number of phagemid particles in the primary library had a titer of 4x10$^{11}$ colony forming units. PCR analysis of the whole library and of individual clones confirmed the targeted size distribution of cloned fragments and showed that less than 5% of the clones were without insert.

Clonal representation of the library was evaluated by PCR on plasmid DNA prepared from amplified whole library using a set of 12 primers specific of sequences of the *T. gondii* P22 and
Toxoplasma genomic phage display

P30 genes. All primer pairs located on a single, potentially cloned, restriction fragment produced PCR products of the expected size. Primer pairs not located on potentially cloned restriction fragments yielded PCR products from genomic control DNA, but not from library DNA (data not shown). Since the library was analyzed after supplementary reinfection with M13 helper phages and phage rescue, we presume that the possible loss of clones from the library due to the supplementary amplification step had no significant influence on clonal representation.

**Biopanning.** The library was biopanned against monoclonal antibody 2F2, directed against a 30 kDa tachyzoite antigen, as determined by western blotting, and originally presumed to correspond to the major surface antigen P30. DNA sequence analysis of 24 individual clones randomly picked from the library obtained after three selection rounds showed that all selected DNA fragments were inserted in the correct gene III reading frame and consequently displayed as a peptide-g3p fusion (Table 1). One sequence occurred 16 times out of 24 random clones and encoded a peptide of 42 amino acids. Sequence similarity search in the public sequence databases showed that the peptide corresponds to a segment of the GRA3 protein of *T. gondii* (Figure 2). The sequence of the cloned DNA fragment is identical to the published cDNA sequence [18] with the exception of a T>G, resulting in a Gly instead of a Cys in position 80 of GRA3. In addition, 28 highly significant matches with tachyzoite and bradyzoite cDNA clones were found in the GenBank dbEST division (Release 041400; 10741 entries for *Toxoplasma*). ESTs overlapping with GRA3 residue 80 all encode for Gly at this position in strains RH and ME49, in agreement with our sequence. The specificity of monoclonal antibody 2F2 for the GRA3 epitope was confirmed by phage ELISA. Restriction analysis of the GRA3 cDNA showed that the panned fragment corresponded to an *Aci*I fragment, the smallest epitope encoding fragment potentially present in the genomic library.
(g) Discussion
We have developed an approach to screen for novel antigenic determinants, based on the display of genomic DNA fragment-encoded peptides on filamentous phage. The procedure allows the enrichment of relevant peptides from the library by affinity selection with specific antibodies as well as the identification of their corresponding genomic sequence. The whole genome phage display approach extends display procedures previously used for epitope mapping (e.g. [23] [24]). In the latter cases, a single gene, cDNA or whole plasmid containing the cloned target gene is fragmented with DNaseI, and the resultant clone library is biopanned against monoclonal antibodies. Jacobsson and Frykberg [25] were first to use genomic display libraries prepared from sonicated genomic DNA of Staphylococcus aureus to screen for ligand-binding bacterial receptors. Recently, Lin and Lis [26] applied a randomly sheared yeast genomic library expressing protein fragments on the surface of lambda phages for the identification of proteins that interact with the heat shock factor repression region. Genomic phage display for the purpose of tracing antigenic determinants of T. gondii is the first display application on a mid-sized eukaryotic genome, expanding the display selection range by an order of magnitude, and dealing with the occurrence of introns.

Statistical analysis of the size distribution of the exons in known T. gondii genes containing introns indicated that the exons are often rather short (50 to 200 bp). These data indicate the need to include fragments shorter than 100 bp in the library. In contrast, antibodies recognizing conformational epitopes require the incorporation of larger fragments encoding peptides capable of adopting native-like 3D structures (e.g. domains). Large fragments, on the other hand, risk to include the gene stop codon and thus be unsuitable for display in N-terminal g3p fusion based systems. Hence, a fragment size range from 50 to 500 bp was assumed as most appropriate.

Taking into account a genome size of 80 Mbp [27] and a 1/18 chance of insertion of coding
sequences in the correct orientation and reading frames, a representative library of short genomic
*T. gondii* fragments should contain ≥10^8 independent clones. We preferred to use enzymatic
restriction as DNA fragmentation method to maximize cloning efficiency. The combinatorial use
of four different tetra-nucleotide recognizing restriction enzymes all generating 5'-CG ends
allowed for the generation of short overlapping DNA fragments and the presence of a given
amino acid sequence in peptides of different lengths in the display library. This will theoretically
increase the chance that an epitope is expressed and recognized by antibodies. A drawback of the
use of restriction cleavage for fragmentation as opposed to mechanical shearing is the fixed and
reduced resolution of the fragment library. Possible effects of non-random distribution of CG
dinucleotide sequences and inhibitory effects of methylation upon restriction fragmentation
cannot be evaluated at present due to insufficient data on the *Toxoplasma* genome. Experimental
analysis of the restriction fragment distribution, however, showed that by far most of the
fragments fell within the preferred size range of 50 to 500 bp.

To accomplish the desired library size, we developed a highly efficient cloning strategy building
on the observation that empty vectors and cosmids/phosmids with small inserts can tandemly
ligate into so-called polycosmids of appropriate length, packagable into phage lambda particles.
As such, phosmid vectors can be transduced very efficiently and subsequently rescued
successfully in the form of M13 packaged monomers by M13 helper phage infection. The
method is fast, simple and easily applicable on a large scale, facilitating the construction of
extremely large libraries. A related system was described for the efficient construction of phage
libraries by lambda mediated transduction of *cos*-engineered M13 phage vectors that are
autonomously released from their concatemers [28]. Our approach with phagemid vectors and
helper phage mediated excision allows to increase the total number of vectors packaged from
about 6 to 10 units per phage head. SurfZAP™ [29] and lambdaZLG6 [30] are other examples of
phage display systems taking advantage of the cloning efficiency of phage lambda. In these systems, however, only a single phagemid is subjected to in vivo excision.

The proof of principle was delivered by biopanning of the constructed T. gondii genomic display library against monoclonal antibodies obtained by immunization of mice with sonicated tachyzoites. After three selection rounds, almost exclusive enrichment was observed of clones containing an open reading frame correctly fused to gene III. One clone occurred in excess (67%). Sequence similarity search of the encoded peptide in public protein databases analysis revealed identity with a segment of the T. gondii GRA3 protein. GRA3 is a 220 amino acid protein with a mature molecular weight of 30 kDa [18] and belongs to a group of antigenic proteins located in the dense granules of tachyzoites. Hence, the identified 30 kDa antigen was not the abundantly expressed p30 surface antigen (SAG1) initially presumed to react with the monoclonal antibody in western blots of total tachyzoite proteins, but a different highly expressed [17] tachyzoite protein. GRA proteins are secreted into the tachyzoite parasitophorous vacuole after invasion of the host cell and are also found in the parasitophorous vacuole membrane and in the bradyzoite cyst wall. Dense granule antigens are recognized by an important fraction of the specific G, M and A immunoglobulins in the serum of patients suffering from acute toxoplasmosis [31].

The isolation of the GRA3 gene fragment by biopanning confirmed the feasibility of the use of phage display to screen randomized genomic expression libraries with antibodies for novel antigen genes. Since only protein fragments are displayed, the approach is expected to be most appropriate for biopanning against antibodies recognizing linear epitopes, although discovery of non-linear epitopes may not be impossible. Whether the genomic display approach is also amenable to biopanning with polyclonal antibodies remains to be investigated.
(h) References


(j) Figures

**Figure 1**: PCR analysis of *Toxoplasma* genomic fragment cloning by polycosmid transduction in the absence of excision helper phage. Clones were subjected to standard PCR with vector primers and reaction products analysed by 2% agarose electrophoresis. Amplified vector sequence corresponds to 197±2 bp. Multiple bands reflect the presence of up to 10 vectors with different inserts per cell line.

**Figure 2**: Sequence alignment of the deduced amino acids of the monoclonal antibody 2F2-binding peptide obtained by genomic biopanning with the *Toxoplasma* GRA3 protein (Swiss-Prot accession # Q27914). Discrepant amino acids are in bold face.
(k) Tables

**Table 1: pHOS vector set for gene III fusion of restriction fragments**

<table>
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<tr>
<th>Vector</th>
<th>Multiple cloning sites and encoded amino acid sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>g3p frame</th>
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<td>CCATGGCTGCAACACACACCCCAGGATCTTTGGAAGGCGGCCGCAGAA</td>
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<tr>
<td></td>
<td>... M A A N T P G R S F E A A A E +</td>
<td>...</td>
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<tr>
<td>pHOS22</td>
<td>CCATGGCTGCAACACACCCAGGAGATCTTTGGAAGGCGGCCGCAGAGAA</td>
<td>...</td>
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<tr>
<td></td>
<td>... M A A N T P G R S F E M G R R -</td>
<td>...</td>
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<tr>
<td>pHOS23</td>
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<td></td>
<td>... M A A N T P G R S F E V R P Q -</td>
<td>...</td>
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<td>pHOS24</td>
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<td></td>
<td>... M A A N T Y P G D L S K C G R -</td>
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</table>

<sup>a</sup> Sequences from NcoI to NotI in pHEN1 [21] derivative pHOS1 were replaced by oligonucleotide cassettes. Frame adapting bases are in bold face. Restriction sites are underlined. -1/+1: PelB signal sequence cleavage site
Table 2: Clones isolated from the *Toxoplasma* genome phage display library after three biopanning rounds against monoclonal antibody 2F2

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<th>#</th>
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<td>1</td>
<td>16</td>
<td>DQPENHQALAEPTGVGEAGVSPVNEGYESYSSATSQGVAET</td>
<td>Identity with dense granule protein GRA3 [18] and with cluster Ctoxqual2_533 of 33 <em>Toxoplasma</em> ESTs [17]</td>
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<tr>
<td>2</td>
<td>3</td>
<td>GVTDTSAFCQRLGPFCLPS</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>RGPSWWRTPWWRHHGGNTDRWGWGWA</td>
<td>Probable plastic binding peptide</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>RVPLPRCY*ALPCDVGGLVP</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>LHPQSRQALCDFSHPCDVSDIEY</td>
<td>Identity of 39 bases at 3' end with cluster Ctoxqual2_3434 of 2 ESTs [17]. Possibly chimeric clone</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>RPFCRDRPELSQRLSDMAVSDS</td>
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<sup>a</sup> Corresponding nucleotide sequences have EMBL database Accesion numbers AJ344353 to AJ344358; <sup>*</sup>: Amber stop codon, suppressed in *E. coli* strain TG1
(l) Figures

Figure 1

see accompanying TIF-file (figure1.tif)
### Figure 2

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