Selection and Identification of Dense Granule Antigen GRA3 by Toxoplasma gondii Whole Genome Phage Display*

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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. Although normally opportunistic in healthy adults, it is a lethal pathogen in immunocompromised humans, particularly in AIDS patients. We present the application of a genomic phage display as a tool for the direct identification of antigens with potential value in diagnosis and/or as subunit vaccine components. Using a polycosmid cloning strategy, we constructed a large phagemid display library (>10⁶ 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 an enzyme-linked immunosorbent assay. These results demonstrate the accessibility of midsized eukaryotic genomes to display technology and the feasibility to screen these whole genome display libraries with antibodies for isolating novel antigenic determinants.

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 harbors 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). A primary infection during pregnancy can cause abortion or severe damage to the fetus. In animals, toxoplasmosis is recognized as a major cause of abortion and neonatal losses in sheep, goats, and pigs (3).

A 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 cultures. 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., the surface antigens SAG1 (P30) (6) and SAG2 (P22) (7), the dense granule antigens GRA2 (8) and GRA4 (9), and the rhoptry protein ROP2 (10).

Direct procedures by immunization with non-living vaccines are not as yet available for humans. Various attempts to develop an animal vaccine to both the asexual systemic stage and the sexual enteroprophylial 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, have 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 the 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 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 the panning of a genomic T. gondii display library against a monoclonal antibody directed toward an uncharacterized 30-kDa T. gondii antigenic protein.

EXPERIMENTAL PROCEDURES

Preparation of T. gondii Genomic DNA—The T. gondii strain Deelen (19) was maintained in permanent in vitro cultures by serial 3–4 day passages of parasites on monolayers of VERO cells in Opti-MEM medium (Invitrogen) supplemented with 4% fetal calf serum and 50 μg/ml gentamicin. 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 were 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 Biosciences) at 28,600 × g in a fixed angle 25° rotor. Batches of 10⁶ purified

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tachyzoite cells in 100 μl of PBS were lysed by the 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 heated 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 AarII and a BstEII site and a reverse primer bearing an AarII and a BstEII. The resulting PCR fragment was cloned into the AarII site of the phagemid vector pHEN1 (21). A number of clones containing the inserts in different orientations were sequenced and tested for transducibility after *in vitro* packaging of concatamers or so-called polycosmids in phage lambda particles. Therefore, the vector was linearized with BstEII, religated at a high vector concentration (500 ng/μl), and mixed with MaxPlax™ Packaging Extract (Epicentre Technologies, Madison, WI). Phage particles were titered by the transduction of *Escherichia coli* strain TG1 (*supE·hsdS2·thi·Dlac-proAB F*{*traD36 proAB’ lacIq galK2Δ(lacZAM15)*}). One positive clone was retained, and the phagemid/cosmid vector was termed the phosmid pHOS1. In this vector, multiple cloning sites (BglIII, SmalI and BstBI) for gene III fusion were created by the replacement/insertion of a synthetic Ncol/NotI cassette. Shotgun cloning of restriction fragments in the correct reading frame was made possible by the construction of a set of nine phagemid plasmids pHOS21–PHOS29, each with the multiple cloning sites in a different upstream/downstream gene III reading frame (Table 1).

**Genomic Library Construction**—*T. gondii* genomic DNA was cut with the four compatible restriction enzymes HinP1 (G ↓ CGG),MspI (C ↓ CGG),AciI (C ↓ CGG), and TsofI (T ↓ CGA). Equal proportions 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 (Applied Biosystems, Foster City, CA). Nine different size fractions ranging from 30 to 540 bp were sampled from 30 to 540 bp were selected and concentrated with Microcon YM-10 centrifugal devices (Millipore, 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 by digestion with BstEII (TT ↓ CGAA) followed by dephosphorylation. The vector and individual genomic DNA size fractions were ligated at a 1:2 molar ratio and a low total DNA concentration (~12.5 ng/μl). The heat-inactivated ligation mixtures were pooled, linearized with BstEII, and religated at a high concentration (~250 ng/μl). Five fractions of 1 μg of concentrated DNA were packaged in phage lambda and transduced into a single reaction into *E. coli* TG1 cells grown in LB supplemented with 0.2% maltose and 10 mM MgCl₂. *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 the collection of phage particles produced 3 h after co-infection. The number of independent clones was estimated at ≥ 10⁷ (see also “Results”). Representative portions of this library were stored at −70 °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 described previously (21). The library was biopanned against *Toxoplasma* antibodies essentially as described by Smith and Smith (32). Briefly, in the first selection round 10⁻¹² colony-forming unit phagemid particles (~1000 times the estimated library size) were allowed to react overnight at 4 °C with 1 μl biotinylated monoclonal antibodies preincubated with M13mp18 particles. Binding phagemid particles were captured for 10 min on 60-mm streptavidin-coated polystyrene Petri dishes blocked with 10% dialyzed fetal calf serum and 0.02% NaN₃ in Tris-buffered saline. After ten washing steps, binding phages were eluted with Tris-glycine, pH 2.2, neutralized, and used for the infection of TG1 cells. Recovered phage particles in general titered to 1.4 × 10⁸ transducing units in total from ~5 μg of ligated DNA. According to the size of the phosmid (5 kb), concatamers 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 (Fig. 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 1.1–1.4 × 10⁶. The total number of phagemid particles in the primary library had a titer of 4 × 10¹² 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 an amplified whole library using a set of 12 primers specific to sequences of the *T. gondii* P22 and P30 genes. All primer pairs located on a single, potentially cloned restriction 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). Because 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

**RESULTS**

**Library Construction**—*T. gondii* genomic DNA was semirandomly fragmented by combinatorial digestion with four restriction enzymes generating protruding 5'-CG ends compatible with the BstBI cloning site of the pHOS20 vector set. Size fractionation by HPEC of the pooled restriction fragments and A₂₆₀ monitoring of the eluted DNA showed a statistically expected asymmetrical size distribution with a peak around 100 bp and more than 90% of the 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 under “Experimental Procedures.” The packaged polycosmids were titered at 1.4 × 10⁸ transducing units in total from ~5 μg of ligated DNA. According to the size of the phosmid (5 kb), concatamers 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 (Fig. 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 1.1–1.4 × 10⁶. The total number of phagemid particles in the primary library had a titer of 4 × 10¹² 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.

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reading frame and consequently displayed as a peptide-g3p fusion (Table I). One sequence occurred 16 times out of 24 random clones and encoded a peptide of 42 amino acids (Table II). Sequence similarity search in the public sequence data bases showed that the peptide corresponds to a segment of the GRA3 protein of T. gondii (Fig. 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™ EST data base division (Release 041400; 10,741 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 AccI fragment, the smallest epitope encoding fragment potentially present in the genomic library.

**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 its corresponding genomic sequence. The whole genome phage display approach extends display procedures previously used for epitope mapping (e.g. Refs. 23 and 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 the 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. This genomic phage display for the purpose of tracing antigenic determinants of *T. gondii* is the first display application on a midsized 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 (50–200 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 three-dimensional structures (e.g. domains). Large fragments, on the other hand, may 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 one-eighteenth chance of insertion of coding sequences in the correct orientation and reading frames, a representative library of short genomic *T. gondii* fragments should contain >10^6 independent clones. We preferred to use enzymatic restriction as a DNA fragmentation method to maximize cloning efficiency. The combinatorial use of four different tetranucleotide-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 the inhibitory effects of methylation upon restriction fragmentation cannot be evaluated at present because of 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 50–500 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...
Toxoplasma Genomic Phage Display

Table II

| Frequency | Deduced amino acid sequence of displayed peptide | Comments |
|-----------|-----------------------------------------------|----------|
| 1         | DQFENHQALAEPTVGVEAGVSVPNEAGESYSSATSGQVEAT     | Identity with dense granule protein GRA3 (18) and with cluster Ctxoxqual2 533 of 33 Toxoplasma ESTs (17) |
| 2         | GVIDSAFCQRLRGPFCFLPS                          |          |
| 3         | RGF5WRETPIPWRRHGGNTDRGWDDG                   |          |
| 4         | RVEILFCRTY*ALPCDVGLUP                       | Probable plastic-binding peptide |
| 5         | LHPRSQALCADFSHPCVDIESY                      | Identity of 39 bases at 3'-end with cluster Ctxoxqual2 3434 of 2 ESTs (17). Possibly a chimeric clone |
| 6         | RPPCRDPRPELSQRLSDMAVSADS                     |          |

* Corresponding nucleotide sequences have GenBank™ EMBL database accession numbers AJ344353 to AJ344358; *, Amber stop codon, suppressed in E. coli strain TG1.

Fig. 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 no. Q27914). Discrepant amino acids are in boldface. 

Described for the efficient construction of phage libraries by lambda-mediated transduction of cos-engineered M13 phage vectors that are autonomously released from their concatamers (28). Our approach with phagemid vectors and helper phagemid excision allows us to increase the total number of vectors packaged from about 6–10 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%). A sequence similarity search of the encoded peptide reading frame correctly fused to gene III. One clone occurred in

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