Antigens Expressed in Feline Enteropelial-Stages Parasites of *Toxoplasma gondii*

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ABSTRACT. In an investigation aimed to identify *Toxoplasma gondii* antigens expressed in feline enteropelial-stages parasites, a cDNA library was constructed and fourteen positive clones were isolated by immunoscreening using sera from cats immunized with feline enteropelial-stages parasites. By DNA sequence homology analysis, these fourteen isolated clones were classified into four groups: hypoxanthine-guanine phosphoribosyl transferase (HGPRT) cDNA, heat shock protein 70 (HSP70) cDNA, 14–3–3 protein homologue cDNA, and cDNA encoding an unknown product. In an indirect immunofluorescence antibody test, sera from mice immunized with the recombinant protein encoded by the cDNA for HGPRT, HSP70, 14–3–3 protein, or the unknown product each showed a relatively high level of immunoreactivity with feline enteropelial-stages parasites.

KEY WORDS: cDNA, enteropelial-stages antigen, *Toxoplasma gondii*.

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*Toxoplasma gondii* (*T. gondii*), an intestinal coccidium of felids with a wide range of warm-blooded intermediate hosts, causes opportunistic infections in pregnant women and immunodeficient individuals. In feline intestinal epithelium, the parasite develops in a sexual stage form which is morphologically different from that in intermediate hosts, prior to gametogony, and these are known as enteropelial-stages parasites. Dubey and Frenkel [2] documented the morphological changes and characteristics of this parasite at each stage and Ferguson et al. [3] suggested that the majority of dense granule proteins (GRA proteins) of *T. gondii* show similar stage-specific expression. This is consistent with *T. gondii* having a different host parasite relationship in the feline enteropelial-stages.

Recently, we developed a technique for separation of enteropelial-stages parasites from the intestinal mucosa of acutely infected cats [7]. In an analysis of the antigens expressed by *T. gondii* feline enteropelial-stages parasites, we detected feline enteropelial-stages antigen(s) using antisera from cats immunized with parasites of these stages [12].

To further clarify the features of these stages antigens, we constructed a 3.265 × 10^6 pfu/ml-λ ZipLox® Not I-Sal I arms recombinant protein expression cDNA library (Superscript™ Lambda System for cDNA synthesis and Lambda cloning (GIBCO BRL, Grand Island, NY)) using 3.12 μg of mRNA from *T. gondii* Beverley strain feline enteropelial-stages parasites. SPF cats were immunized with 10^7 *T. gondii* feline enteropelial-stages parasites, previously frozen, thawed and ultrasonicated, together with an equal amount of Freund’s complete adjuvant and boosted on the 2nd week after the first immunization. Blood samples were collected in the 1st week after secondary immunization to obtain the sera. The library was screened using sera from these cats absorbed with *T. gondii* Beverley strain tachyzoites. Fourteen positive cDNA clones were isolated from the library. Both strands of the DNAs were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer; Foster City, CA) and a Perkin Elmer DNA sequencer 377A. These were compared with sequences in the DDBJ database using the DDBJ homology search system - FASTA version 3.0 - and the fourteen isolated clones were classified into four groups (Table 1).

Four positive cDNA clones, ES92, ES95, ES41 and ES51, were subcloned into the protein expression vector pGEMEX2™ (Promega, Tokyo, Japan) and the recombinant proteins were expressed in *E. coli*. To examine the antigenic specificity of the recombinant proteins, mice were inoculated with each recombinant protein and Freund’s complete adjuvant. After booster injection at 2 week intervals twice, sera were obtained from the mice on the 7th day after the final inoculation. Each serum sample was absorbed with *E. coli* cells which had been ultrasonicated to remove constituents contributing to non-specific reactions, and stored at – 80°C until use. Serum antibody reactivity against feline enteropelial-stages parasites, sporozoites and tachyzoites was examined by means of an indirect immunofluorescence test (IFAT) as described elsewhere [12].

The ES33, ES92, ES96 and ES109 sequences were found to be the same, except that the ES92 sequence was longer than the others (1,461 bp)(accession No. AB026835). The ES92 sequence contains an open reading frame (ORF) extending from bp 192–194 to a stop codon (TAA) at bp 1035–1037, which encodes a protein with a molecular mass of approximately 28 KDa. The results of homology analysis showed that ES92 has 98.6% homology at the nucleotide level and 100% homology at the amino acid level with *T.*
Table 1. Four groups of positive clones obtained from *Toxoplasma gondii* feline enteropelial-stages parasites cDNA library

<table>
<thead>
<tr>
<th>Clone</th>
<th>Restriction Enzyme Map</th>
<th>cDNA length</th>
<th>Homology</th>
<th>Expressed Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES33.92,96,109</td>
<td>1 791 1,402 1,461</td>
<td>1,461bp</td>
<td><em>Toxoplasma gondii</em> RH strain HGPRT</td>
<td>30 kDa</td>
</tr>
<tr>
<td>(*AB026835)</td>
<td>SalI Eco RI</td>
<td>bp192-1,037, 28 kDa</td>
<td>ATG: bp198-200</td>
<td></td>
</tr>
<tr>
<td>ES95</td>
<td>1 261 1,337 1,749 1,918</td>
<td>1,918bp</td>
<td><em>Toxoplasma gondii</em> RH strain Heat Shock Protein 70 (HSP70)</td>
<td>66kDa</td>
</tr>
<tr>
<td>(*AB031071)</td>
<td>NcoI Eco RV PstI</td>
<td>bp 3-1,898, 63 kDa.</td>
<td>ATG:bp198-200</td>
<td></td>
</tr>
<tr>
<td>ES14.41,66,99</td>
<td>1 180 374 1,501</td>
<td>1,501bp</td>
<td><em>Neospora caninii</em></td>
<td>31kDa</td>
</tr>
<tr>
<td>(*AB012775)</td>
<td>Hind III SstI</td>
<td>bp 1-930, 31 kDa</td>
<td>ATG:bp130-133</td>
<td></td>
</tr>
<tr>
<td>ES51.78,93,94,9101</td>
<td>1 272 426 1,256 1,794</td>
<td>1,794bp</td>
<td>unknown</td>
<td>60 kDa</td>
</tr>
<tr>
<td>(*AB031232)</td>
<td>PstI Hind III</td>
<td>bp 3-1,316, 44kDa</td>
<td>ATG:bp130-133</td>
<td></td>
</tr>
</tbody>
</table>

The clones that appear restriction enzyme maps were underlined.  
*GenBank, EMBL and DDBJ accession No.

*gondii* HGPRT [mRNA complete code comprising 1,369 bp; *T. gondii* RH strain HGPRT (hypoxanthine-guanine phosphoribosyl transferase); accession No. U100833]. The expected start codon (ATG) is at bp 198–200. The expressed protein was recognized as a 30 kDa antigen by immunoblotting. Sera from mice immunized with the recombinant protein showed immunoreactivity with sporozoites and small round-shaped feline enteropelial-stages parasites which we were unable to identify in terms of the morphological type or stage, in the IFAT. In analysis of the reactivity with tachyzoites, lower fluorescence intensity was observed than in the case of the feline enteropelial-stages parasites or sporozoites (Fig. 1 A, B, C, Table 2).

The ES95 gene sequence (1,918 bp) (accession No. AB031071) contains an ORF extending from the beginning of the insert to a stop codon (TAA) at bp 1,896–1,898, which encodes a protein with a molecular mass of approximately 63 kDa. In the homology analysis, ES95 showed 98.9% homology at the nucleotide level and 100% homology at the amino acid level with *T. gondii* heat shock protein 70 (HSP70) [mRNA complete code comprising 2,382 bp; *T. gondii* RH strain HSP70; accession No. AB026835]. This indicates that ES95 was lacking 248 bp at the 5' end of the HSP70 mRNA complete code. The expressed protein was recognized as a 63 kDa antigen by immunoblotting. Sera from mice immunized with the ES95 recombinant protein showed immunoreactivity with feline enteropelial-stages parasites, and the immunoreactive antigen was located over the whole body of merozoites and gametocytes and at the apical end of sporozoites, as observed in the IFAT. In this experiment, a signal was also observed in analysis of tachyzoites. It is reported that free tachyzoites in the brain of infected mice do not express HSP70 [14], however, it seems possible that, in the present study, this HSP70 signal may be induced by artificial stress imposed on the parasites in the course of preparation of the parasites used (Fig. 1 D, E, F, Table 2).

The four clones ES14, ES41, ES66 and ES99 were found to have the same sequence, except that the ES41 sequence was longer than the others (1,501 bp) (GenBank, EMBL and DDBJ accession No. AB012775). The results of homology analysis showed that ES41 has 87.2% homology at the nucleotide level and 98.5% homology at the amino acid level with the Neospora caninum 14–3–3 protein homologue (mRNA complete code comprising 1,757 bp; accession No. U31542) [3]. The expressed protein was recognized as a 31 kDa antigen by immunoblotting. Sera from mice immunized with the recombinant protein showed immunoreactivity with feline enteropelial-stages parasites (merozoites, gametocytes) and sporozoites (Fig. 1 G, H, Table 2) but did not with tachyzoites.

The five clones, ES51, ES78, ES93, ES94 and ES910, were found to have the same sequence and ES51 had a 1,794 bp ORF encoding a 45 kDa protein (accession No. AB031232). The ES51 nucleotide sequence and the deduced amino acid sequence of the ES51 product showed no similarity to any sequences deposited in the databases. The serum from a cat immunized with feline enteropelial-stages parasites reacted with a 60 kDa recombinant protein derived from ES51 (excluding the leader peptide). Sera from mice immunized with the ES51 recombinant protein showed specific immunoreactivity with feline enteropelial-stages parasites, and the antigen responsible for the signal was located over the whole body of gametocytes and merozoites, and at the apical end of sporozoites as observed in the IFAT (Fig. 1 I, J, Table 2) but did not with tachyzoites.

The 14 clones isolated in the present study were classified into 4 groups on the basis of the encoded proteins, which were recognized as antigens expressed in feline enteropelial-stages parasites. Three of the 4 groups showed high homology to HGPRT, HSP70 and 14–3–3 protein homologues which function in cell metabolism. Generally, HGPRT is recognized as an enzyme involved in porine metabolism. *T. gondii* is known to lack a *de novo* purine biosynthesis pathway, and it is totally dependent on a salvage
Fig. 1. Immunofluorescence patterns of each stage parasites of *T. gondii* treated with serum from mice immunized with ES92, ES95, ES41 and ES51 recombinant protein. (A, B, C: incubated with anti-ES92 recombinant protein mouse serum, D, E, F: incubated with anti-ES95 recombinant protein mouse serum, G, H: incubated with anti-ES41 recombinant protein mouse serum, I, J: incubated with anti-ES51 recombinant protein mouse serum, A, D, G, I : to *T. gondii* Beverly strain feline enteric-stages parasites (merozoites, gametocytes), B, E, H, J: to *T. gondii* Beverly strain sporozoites (oocysts), C, F: to *T. gondii* Beverly strain tachyzoites.
Table 2. Immuno reactivity of anti-recombinant protein mouse serum against each stage parasites of *Toxoplasma gondii* by IFAT

<table>
<thead>
<tr>
<th>Clone</th>
<th>Enteroepithelial-stages parasites</th>
<th>Sporozoite/Oocyst</th>
<th>Tachyzoite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Merozoite</td>
<td>Gametocyte</td>
<td>Small-round parasite</td>
</tr>
<tr>
<td>ES92</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>ES95</td>
<td>++</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>ES41</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>ES51</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

density were scored by four grade from - to ++.
(-: no reaction, ±: slight reaction, +: weak reaction, ++: strong reaction.)

* strong fluorescent were restricted one side of parasites.

pathway for its purine requirements [9]. In the process of transformation to gametogenesis, the *T. gondii* parasites multiply by schizontony. Therefore, it is considered likely that DNA synthesis in those stages may be activated and that purine metabolism in feline enteroepithelial-stage parasites may also be activated, showing a higher level of expression than that in other stages.

One clone was found to encode the HSP70 of *T. gondii*, a stress-induced protein, which may also play important roles in the course of differentiation of the parasites [14].

Interestingly, 4 clones showed extremely high homology to 14–3–3 protein (BBSP: bovine brain specific protein), a member of a protein family widely distributed in eukaryotes (animals, plants, yeast, etc.), which is thought to function as a signal transmitter playing a role in regulating signals related to cell proliferation, cell migration, and the cell cycle [4, 10]. The stage-specific expression of a 14–3–3 protein gene has been demonstrated in the case of *Schistosoma mansoni* [8], *Echinococcus multilocularis* [11] and *Plasmodium* (young trophozoite stage) [1]. From these findings, one possibility to be considered is that changes in the life cycle or the progress of transformation of *T. gondii* may be associated with some kind of cell-cycle regulation enzymes. Eukaryotic cell cycle control is concerned with the ordered progression of DNA replication and mitosis. Control of the life cycle in the case of most protozoa may also be closely associated with cell cycle control systems. In recent years, cyclin-dependent kinases (CDKs) have been recognized as the central components of cell cycle control in eukaryotes and CDK-related genes have been isolated from several species of protozoan parasites [5, 13, 15].

Further studies are necessary to examine the expression of each clone at mRNA level and protein level and to clarify the functions and biological roles of these protein homologues.

REFERENCES