FEBS Letters 389 (1996) 80–83 FEBS 17181

Minireview

Molecular genetic strategies in *Toxoplasma gondii*: close in on a successful invader

Dominique Soldati

Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany

Received 2 May 1996; revised version received 14 May 1996

Abstract ***Toxoplasma gondii*** **is an obligate intracellular parasite with an exceptional ability to invade, survive and replicate within nearly all nucleated cells. Upon differentation into an encysted form (bradyzoites), the parasites escape the host immune defenses and thus persist long enough in man and other hosts to ensure maintenance of transmission. This protozoan parasite has long been known to cause severe congenital infections in humans and animals but has recently gained additional notoriety as an opportunistic pathogen associated with AIDS. Development of a DNA transfection system for *T. gondii* has provided a new tool for exploring molecular aspects of important processes such as invasion and differentiation. Additional strategies associated with genetic transfomation have been devised and elaboration of even more desirable molecular tools is in progress.**

Key words: *Toxoplasma gondii*; Transfection, genetic system; Molecular parasitology; Invasion; Differentiation

1. Introduction

One of the most exciting recent breakthroughs in parasitology is the development of DNA transfection methodologies for protozoan parasites. In the early 1990s, the establishment of stable transformation and gene replacement for *Leishmania* species and *Trypanosoma brucei* has provided a tremendous boost to research on kinetoplastids [1–4]. *Toxoplasma gondii* was the first member of the phylum Apicomplexa to be transfected [5], and elaboration of appropriate methods for selection of stable transformants of this obligate intracellular parasite have followed [6–9]. As expected, overcoming the barrier of transfectability for one member of Apicomplexa led rapidly to the establishment of stable transformation and homologous recombination for others such as the important human pathogen *Plasmodium falciparum* [10] and the rodent malaria parasite *P. berghei* [11]. Two more medically important protozoan parasites, *Giardia lamblia* and *Entamoeba histolytica*, have been transiently and stably transfected [12,13].

Because of its simplicity and versatility for in vitro and in vivo studies, as well as the opportunity to combine classical and reverse genetics [14], *T. gondii* still ranks among the best experimental models to study intracellular parasitism. The newly developed methods have already had significant impacts on our understanding of the biology of *T. gondii* and afforded some tantalizing insights into the mechanisms governing invasion and differentiation Table 1.

*Corresponding author. Fax: (49) (6221) 545892.

2. Transfection

2.1. Transient transfection

The efficiency of transient expression varies between 30 and 50%. Plasmids carrying flanking sequences from a variety of *T. gondii* genes have been successfully used to drive expression of reporter genes upon introduction into parasites by electroporation. Chloramphenicol acetyltransferase (CAT) [5] and β-galactosidase [15] function as reporter enzymes in transfection studies, whereas attempts by several laboratories to use β-glucoronidase or the green fluorescent protein have been unsuccessful to date. Luciferase, although faithfully expressed in *T. gondii*, cannot be used because the quantification assay of this reporter is not reliable, presumably due to the massive production of potent nucleotide triphosphate hydrolases (NTPases) by the parasites [16,17].

2.2. Stable transformation

Most of the commonly used selectable marker genes for eukaryotic cells are not suitable for selection of stable transformants in *T. gondii* because of the inability of the parasites to replicate outside host cells. Only drugs selectively affecting the parasites and keeping the host cells intact can be considered. In spite of this restriction, various selection schemes have been developed. Chloramphenicol shows a potent but delayed parasiticidal effect, allowing the use of *E. coli* chloramphenicol acetyltransferase (*cat*) not only as a reporter enzyme but also as a tight selectable marker gene [6]. An artificially mutated dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene from *T. gondii* confers pyrimethamine resistance [8]. Extracellular parasites expressing the *ble* gene of *Streptoalloteichus* are fully protected against the DNA-breaking activity of bleomycin [9,18]. Two genes coding for non-essential nucleotide salvage pathway enzymes have been exploited as negative selectable markers. Loss of uracil phosphoribosyltransferase (*UPRT*) activity confers resistance to 5-fluorodeoxyuridine [19]. The hypoxanthine-xanthine-guanine phosphoribosyltransferase (*HXGPRT*) gene is particulary versatile as it works both in positive or negative selection strategies. The *HGPRT* gene of *T. gondii* converts 6-thioxanthine into an inhibitor of GMP syntase which kills the parasites, whereas mycophenolic acid efficiently kills parasites lacking the enzyme [20]. As an alternative to drug resistances, stable selection can be achieved by complementation of the naturally occurring tryptophan auxotrophy of *Toxoplasma* by the bacterial tryptophan synthase (*trpB*) gene [7]. Finally, expression of the components of the site-specific recombinase system *cre loxP* from bacteriophage P1 to *T. gondii* allows specific excision of DNA segment and thus an unlimited use of all the

PII S0014-5793(96)00572-8

above selectable marker genes (H. Erhardt and D. Soldati, unpublished results). In this procedure, the intervening DNA between two *loxP* sites positioned head-to-tail is excised along with one *loxP* site in the presence of cre. Alternatively, inversion of the intervening DNA occurs if the *loxP* sites are placed in head-to-head orientation. Upon transient expression of recombinase cre any target gene can be selectively and efficiently deleted or inverted in the parasites.

The precise frequency of genomic integration has been difficult to determine, but fluctuates between 10^{-3} and 10^{-5} depending on the type of selectable marker used and on the circular versus linear form of the plasmid vector. The use of restriction enzyme-mediated integration (REMI) increases up to 400-fold the frequency of transformation [21] and enables co-transfection of several unselected constructs together with a single selectable marker. The *dhfr-ts* based selection shows an exceptional frequency of chromosomal integration of over 5% [8].

3. Gene disruption

3.1. Gene replacement

Homologous gene targeting is an extremely valuable tool to selectively alter genes of interest and to examine the structure-function relationships of genes and their products. In contrast to kinetoplastids and yeast, where transfected plasmid DNA integrates into chromosomes exclusively by homologous recombination, stable integrative transformation in *T. gondii* can occur at random locations throughout the genome. However, providing sufficient contiguous homology (several kilobases) to the targeted locus significantly improves the rate of homologous recombination [22].

3.2. 'Hit-and-run'

Since gene replacement is rather inefficient in *T. gondii*, a counterselection strategy has been developed for producing targeted gene knock-outs or allelic replacement at any cloned locus. The system is based on a two-step 'hit-and-run' strategy using *HXGPRT* as a positive/negative selectable marker. A plasmid containing the HXGPRT gene and a large segment encompassing the gene of interest (but lacking essential coding sequences) is stably introduced at the endogenous locus of the target gene by single-site homologous recombination, under positive selection with mycophenolic acid and xanthine. Resolution of the pseudodiploids is selected with 6-thioxanthine which produces either wild-type (revertant) or allelic replacement (gene knock-out) with loss of *HXGPRT* (D. Roos, personal communication).

3.3. Random insertion mutagenesis

The extraodinarily high frequency of stable transformation obtained with *dhfr-ts*-based selection, coupled with the predominantly haploid nature of the rapidly replicating form of the parasite (tachyzoites) and its relatively small genome size, allows insertional mutagenesis to inactivate genes. Tagging the whole genome through random integration has been used to clone the non-essential UPRT and HXGPRT genes for whose inactivation can be specifically selected (see above) [19,20].

The haploidy of the tachyzoites, however, precludes the identification of essential genes for the parasites by insertional

Table 1
Strategies associated with genetic transformation of *Toxoplasma gondii*

Methodologies and tools		Applications	References
Transient transfection			
Reporter enzymes:	CAT		[5]
	LacZ		[15]
		Promoter analysis	[9,23]
		Gene expression	
Stable transformation			
Positive selectable markers:	*cat*		[6]
	dhfr-ts		[8]
	trypB		[7]
	ble		[9,18]
Negative selectable markers:	*UPRT*		[19]
	HXGPRT		[20]
		Gene knock-out	
		Gene expression	
		Mutational analysis	
		Functional complementation	
'Hit-and-run'		Gene knock-out	
Insertional mutagenesis		Cloning of non-essential, selectable genes	[19,20]
Restriction enzyme-mediated integration		Co-integration of multiple plasmids	[21]
Cre mediated site-specific recombination		Deletion/inversion of integrated sequences	
		Targeted chromosomal integration	
Missing tools:			
Episomal vector			
Shuttle cosmid vectors		Functional complementation	
Inducible promoter			
Controlled gene expression system		Conditional knock-out	
		Analysis of essential genes	
		Expression of toxic genes	

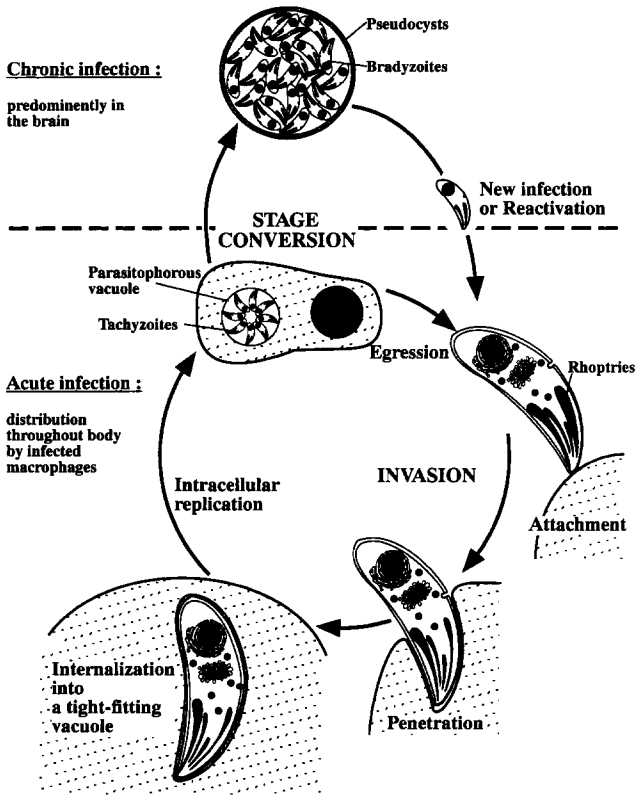

Fig. 1. Invasion and asexual life cycle of *Toxoplasma gondii* in intermediate hosts.

mutagenesis. A standard 'forward genetics' procedure is therefore required. Such a strategy is based on chemical mutagenesis of the entire organism to create an average of one hit per genome followed by the isolation of conditional temperature sensitive mutants (ts) affected in the process of interest. The high frequency of stable transformation should likely allow isolation of ts genes, by functional complementation of the specific mutation via transfection of DNA from wild-type parasites.

4. Applications and prospectives

4.1. Gene regulation and differentiation

Transient transfection has been utilized to outline and characterize DNA elements of the 5′ and 3′ of ORFs used by the parasite for gene expression. There are no typical eukaryotic promoter sequences such as TATA boxes conserved in 5′ flanking sequences of *T. gondii* genes but instead, the dissection of the upstream region of a developmentally regulated gene (*SAG1*) led to the identification of a promoter element that is essential for high-level expression of *SAG1* and specifies transcription initiation [9]. Deletion and mutational analysis performed on the 5′ flanking sequences of four distinct dense granule protein genes mapped an important *cis*-acting repeated element (A/TGAGACG) which is part of the *SAG1* promoter and is also present upstream of the tubulin (*TUB1*) gene [23]. Elucidation of the mechanisms governing stage-specific regulation of gene expression is an important step towards understanding developmental control in all organisms. Recently, several genes specifically expressed in bradyzoites have been cloned [24–27]. In addition, in vitro procedures mimicking the in vivo stage conversion have been established using a variety of stress inducers [28–30], allowing promoter activities to be examined during differentiation. This should contribute to the identification of the mechanisms triggering and controlling stage conversion and hopefully shed some light on a major clinical concern: reactivation of chronic toxoplasmosis infection in AIDS patients.

4.2. Invasion

T. gondii actively invades virtually any nucleated cells via a mechanism distinct from phagocytosis, resulting in the formation of a parasitophorous vacuole that is resistant to acidification and to fusion with the endocytic network [31–33] (Fig. 1). The basis of the unusual broad host range, and the molecular basis of resistance to host cell endocytic processing are unknown. The gliding and rotating motility of the parasites are the primary effectors of invasion, since nonmotile parasites do not get inside most host cells. In addition, active secretion by specialized secretory organelles (micronemes, rhoptries and dense granules) of the parasites participates in several phases of intracellular parasitism. Host cell recognition and cellular attachment, building of the parasitophorous vacuole and establishment of interaction machineries with the host cytoplasm are among the many functions for which proteins need to be assigned. The *ROP1* gene codes for a protein stored in the rhoptries and secreted at the time of invasion. ROP1 disappears shortly after invasion and was presumed to play a role in this process. However, *ROP1* knock-out mutants obtained by homologous recombination show no significant alteration in their growth and virulence in mice or in their ability to attach and invade host cells in culture [9].

Recently, a study combining approaches of forward and reverse genetics has demonstrated that invasion is critically dependent on the actin cytoskeleton of the parasite [34]. In the presence of cytochalasin D (CD) invasion is blocked even if the host cells are resistant to the drug which demonstrates the essential role of the parasite actin microfilaments. Mutant parasites resistant to CD, generated by chemical mutagenesis, show a point mutation in the single copy actin gene *ACT1*. The transfection of the mutant *act 1* allele into wild type parasites confers resistance to CD, and recovery of parasite gliding motility in the presence of the drug, conclusively demonstrating the crucial role of parasite actin microfilaments during invasion.

4.3. Genome sequencing

The general enthusiasm for genome sequencing projects has reached the field of parasitology as well. Many medically relevant protozoan parasite genomes are currently being sequenced. A pilot project in *T. gondii* has demonstrated the feasibility of generating expressed sequence tags (EST) from cDNAs derived from tachyzoites in order to uncover many new genes based on homology to existing genes in the databases [35]. A large EST effort is currently conducted by the Genome Center at Washington University (J. Ajioka, D. Sibley, B. Waterston and Merck Research laboratories). The aim of this project is to generate 10 000 partial sequences which should provide a wealth of information about genes expressed by the rapidly proliferating form of the parasite.

5. Missing tools

To benefit from the full power of 'reverse genetics', important molecular tools are still missing. A cosmid shuttle vector would greatly facilitate complementation of mutants and recovery of the gene responsible for functional rescue as demonstrated in *Leishmania* [36]. A pre-requisite for the creation of such vectors is the isolation of autonomously replicating sequences of *T. gondii*.

A controlled gene expression system is required to elucidate the function of essential genes in a haploid organism. An ectopically inducible promoter system renders possible the generation of conditional knock-out, the use of antisense RNA and ribozymes strategies (both alternative forms of gene knockout) and to express mutated forms of endogenous genes or toxic genes. Recently, a tight control of transcription based on the Tet repressor of *E. coli* has been successfully established in *Tr. brucei* [37].

The site-specific recombinase cre-*LoxP* shows 'cut and paste' activities. It remains to be determined whether targeted chromosomal integration ('paste') can be a faithfully accomplished in *T. gondii* with the assistance of the recombinase system. Targeting incoming DNA at a docking site would allow the analysis of the expression of numerous constructs integrated into the same chromosomal location, without differences caused by flanking sequences or position effects.

6. Conclusion

The methodologies available to investigate protozoan parasites have considerably expanded our knowledge of the basic cell and molecular biology aspects of these pathogens. A better understanding of the parasitic diseases and a considerable impact on the development of new drugs or efficient and safe vaccines are undoubtedly to be expected. In the case of *T. gondii*, elucidation of the mechanisms governing invasion and differentiation will potentially offer opportunities to interfere with acute and chronic infections, respectively.

Acknowledgements: Space constraints have dictated that many original references have been omitted, for which I apologize. I am grateful to colleagues who provided unpublished information and I would like to thank Dr. Christine Clayton Dr. Jean Francois Dubremetz and Dr. Thierry Soldati for their helpful comments on the manuscript.

References

[1] Laban, A., Tobin, J.F., Curotto de Lafaille, M.A. and Wirth, D.F. (1990) Nature 343, 572–574.
[2] Cruz, A. and Beverley, S.M. (1990) Nature 348, 171–173.
[3] Lee, M.G.-S. and Van der Ploeg, L.H.T. (1990) Science 250, 1583–1587.
[4] Ashbroek, A.L., Ouellette, M. and Borst, P. (1990) Nature 348, 174–175.
[5] Soldati, D. and Boothroyd, J.C. (1993) Science 260, 349–352.
[6] Kim, K., Soldati, D. and Boothroyd, J.C. (1993) Science 262, 911–914.
[7] Sibley, L.D., Messina, M. and Niesman, I.R. (1994) Proc. Natl. Acad. Sci. USA 91, 5508–5512.
[8] Donald, R.G.K. and Roos, D.S. (1993) Proc. Natl. Acad. Sci. USA 90, 11703–11707.
[9] Soldati, D., Kim, K., Kampmeier, J., Dubremetz, J.-F. and Boothroyd, J.C. (1995) Mol. Biochem. Parasitol. 74, 87–97.
[10] Wu, Y., Kirkman, L.A. and Wellems, T.E. (1996) Proc. Natl. Acad. Sci. USA 93, 1130–1134.
[11] Van Dijk, M.R., Janse, C.J. and Waters, A.P. (1996) Science 271, 662–665.
[12] Yee, J. and Nash, T.E. (1995) Proc. Natl. Acad. Sci. USA 92, 5615–5619.
[13] Vines, R.R., Purdy, J.E., Ragland, B.D., Samuelson, J., Mann, B.J. and Petri, W., Jr. (1995) Mol. Biochem. Parasitol. 71, 265–267.
[14] Sibley, L.D., Pefferkorn, E.R. and Boothroyd, J.C. (1993) Parasitol. Today 9, 392–395.
[15] Seeber, F. and Boothroyd, J.C. (1996) Gene, in press.
[16] Bermudes, D., Peck, K.R., Afifi, M.A., Beckers, C.J. and Joiner, K.A. (1994) J. Biol. Chem. 269, 29252–29260.
[17] Asai, T., Miura, S., Sibley, L.D., Okabayashi, H. and Takeuchi, T. (1995) J. Biol. Chem. 270, 11391–11397.
[18] Messina, M., Niesman, I., Mercier, C. and Sibley, L.D. (1995) Gene 165, 213–217.
[19] Donald, R.G. and Roos, D.S. (1995) Proc. Natl. Acad. Sci. USA 92, 5749–5753.
[20] Donald, R.G.K., Carter, D., Ullman, B. and Ross, D.S. (1996) J. Biol. Chem., in press.
[21] Black, M., Seeber, F., Soldati, D., Kim, K. and Boothroyd, J.C. (1995) Mol. Biochem. Parasitol. 74, 55–63.
[22] Donald, R.G. and Roos, D.S. (1994) Mol. Biochem. Parasitol. 63, 243–253.
[23] Mercier, C., Lefebvre-van Hende, S., Graber, G., Lecordier, L., Beauchamps, P., Capron, A. and Cesbron-Delauw, M.-F. (1996) Mol. Microbiol., in press.
[24] Parmley, S.F., Yang, S., Harth, G., Sibley, L.D., Sucharczuk, A. and Remington, J.S. (1994) Mol. Biochem. Parasitol. 66, 283–296.
[25] Yang, S. and Parmley, S.F. (1995) Mol. Biochem. Parasitol. 73, 291–294.
[26] Bohne, W., Gross, U., Ferguson, D.P. and Heesemann, J. (1995) Mol. Microbiol. 16, 1221–1230.
[27] Parmley, S.F., Weiss, L.M. and Yang, S. (1995) Mol. Biochem. Parasitol. 73, 253–257.
[28] Soete, M., Camus, D. and Dubremetz, J.F. (1994) Exp. Parasitol. 78, 361–370.
[29] Bohne, W., Heesemann, J. and Gross, U. (1994) Infect. Immun. 62, 1761–1767.
[30] Weiss, L.M., Laplace, D., Takvorian, P.M., Tanowitz, H.B., Cali, A. and Wittner, M. (1995) J. Eukaryot. Microbiol. 42, 150–157.
[31] Joiner, K.A., Fuhrman, S.A., Miettlnen, H.M., Kasper, L.H. and Mellman, I.(1990) Science 249, 641–646.
[32] Morisaki, J.H., Heuser, J.E. and Sibley, L.D. (1995) J. Cell Sci. 108, 2457–2464.
[33] Sibley, L.D. (1995) Trends Cell Biol. 5, 129–132.
[34] Dobrowolski, J.M. and Sibley, D.S. (1996) Cell 84, 933–939.
[35] Wan, K.-L., Blackwell, J.M. and Ajioka, W. (1996) Mol. Biochem. Parasitol. 75, 179–186.
[36] Ryan, K.A., Garraway, L.A., Descoteaux, A., Turco, S.J. and Beverley, S.M. (1993) Proc. Natl. Acad. Sci. USA 90, 8609–8613.
[37] Wirtz, E. and Clayton, C. (1995) Science 268, 1179–1183.