Type II Toxoplasma gondii KU80 Knockout Strains Enable Functional Analysis of Genes Required for Cyst Development and Latent Infection

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Type II Toxoplasma gondii KU80 knockouts (Δku80) deficient in nonhomologous end joining were developed to delete the dominant pathway mediating random integration of targeting episomes. Gene targeting frequency in the type II Δku80 Δhxgprt strain measured at the orotate (OPRT) and the uracil (UPRT) phosphoribosyltransferase loci was highly efficient. To assess the potential of the type II Δku80 Δhxgprt strain to examine gene function affecting cyst biology and latent stages of infection, we targeted the deletion of four parasite antigen genes (GRA4, GRA6, ROPT, and gdh57) that encode characterized CD8+ T cell epitopes that elicit corresponding antigen-specific CD8+ T cell populations associated with control of infection. Cyst development in these type II mutant strains was not found to be strictly dependent on antigen-specific CD8+ T cell host responses. In contrast, a significant biological role was revealed for the dense granule proteins GRA4 and GRA6 in cyst development since brain tissue cyst burdens were drastically reduced specifically in mutant strains with GRA4 and/or GRA6 deleted. Complementation of the Δgra4 and Δgra6 mutant strains using a functional allele of the deleted GRA coding region placed under the control of the endogenous UPRT locus was found to significantly restore brain cyst burdens. These results reveal that GRA proteins play a functional role in establishing cyst burdens and latent infection. Collectively, our results suggest that a type II Δku80 Δhxgprt genetic background enables a higher-throughput functional analysis of the parasite genome to reveal fundamental aspects of parasite biology controlling virulence, pathogenesis, and transmission.

Toxoplasma gondii is an extremely widespread obligate intracellular protozoan pathogen of virtually all warm-blooded animals. Infection is initiated following oral ingestion of transmissible parasite stages included within cysts contained in infected meat or within oocysts released in the environment by cat feces. Primary T. gondii infection rarely causes significant infection and causing a potentially lethal toxoplasmic encephalitis (12). T. gondii has rapidly developed as an outstanding model organism for obligate intracellular eukaryotic pathogens (40, 53). Within the single genus and species T. gondii, three major strain types were defined by their virulence phenotype in mice (58). Currently, type I strains of T. gondii are excellent in vitro models, but this acutely virulent strain type does not readily develop tissue cysts or latent infection in laboratory mice. In contrast, type II strains of T. gondii easily establish latent infections in mice that are characterized by the presence of tissue cysts, the key obligate developmental and latent stage required for the remarkable high transmission potential of this parasite (52). Oral transmission appears to have driven the recent clonal expansion of strains that predominate in North America and Europe (62). Type II strains represent the most prevalent strain type chronically infecting North American and European populations (33). After oral ingestion of a tissue cyst in a naïve host, the encysted bradyzoite reactivates and converts back to the rapidly dividing tachyzoite form that causes disseminated acute infection, followed later by cyst development and the establishment of a latent infection in the host (19).
While induction of tachyzoite to bradyzoite conversion in vitro can be triggered by various stress responses (temperature, high pH, chemical stress, nutrient stress, and cytokines) (67), little is currently known regarding fundamental parasite biology underlying acute and latent phases of infection in vivo.

Host-parasite interaction in acute and latent phases of type II T. gondii infection is dynamic. During acute infection T. gondii selectively invades host cell types primarily of the dendritic, macrophage, and neutrophil lineages (4, 11, 31). By invading host cell types that are critical to mounting effective innate responses to infection, the parasite actively seeks to manipulate the host through several mechanisms (14). Despite or because of this strategy, the host rapidly mounts highly effective CD8+ T cell responses associated with strong interferon gamma (IFN-γ) responses (26, 58). These innate and adaptive immune responses are required to achieve control of acute infection and may also trigger tachyzoite to bradyzoite conversion and development of the latent transmissible tissue cyst (29, 63, 67).

Several landmark studies have recently identified four parasite antigen genes (GRA4, GRA6, ROP7, and tgd057) that possess CD8+ T cell epitopes that elicit corresponding antigen-specific CD8+ T cells associated with the immune control of T. gondii infection (5, 27, 42, 68). The ROP7 protein of unknown function belongs to a family of rhoptry proteins localized to the parasite rhoptry organelles. These Apicomplexa-specific secretory organelles discharge their contents into the host cytosol during invasion of the host cell (7). The parasite dense granule (GRA) proteins GRA4 and GRA6 are members of the Toxoplasma prominent dense granule protein family (46, 49). Dense granule proteins are highly expressed proteins. The dense granule secretory organelles discharge their contents into the parasitophorous vacuole (PV) space following host cell invasion and the initial formation of the parasitophorous vacuole membrane (PVM) (9). A newly proposed member of the dense granule protein family called GRA15 localized to the dense granules, the PV space, the space outside of the PVM, and was also associated with evacuoles, suggesting that GRA15 is also a rhoptry-like protein in potentially being secreted during invasion of the host cell (54). GRA15 plays a significant role in host cell NF-kB nuclear translocation and NF-kB-mediated transcription and regulates the induction of IL-12 secretion in infected mouse macrophages (54). Various potential biological roles for GRA proteins have been previously proposed and significant studies have been performed on GRA protein secretion, traffic, and interaction biology (10, 49). At the tachyzoite stage, most of the GRA proteins associate with the PV membranes, meaning either the PVM and its digitations or the intravacuolar network of membranous tubules (49). At the bradyzoite stage, most of the GRA proteins were localized at the cyst wall or in the cyst matrix (64).

Type I mutants deleted in GRA2 or GRA6 showed a phenotype of reduced acute virulence during infection in mice (48; C. Mercier, unpublished data). However, to date, the only GRA proteins successfully disrupted in the type II background are GRA3 (13) and GRA15 (54). Disruption of type II GRA3 resulted in a phenotype of reduced acute virulence (13). Disruption of type II GRA15 increased parasite burdens in infected mice without influencing virulence (54). These previous studies did not address a functional role for GRA proteins in cyst development during infection. While GRA proteins appear to be central to the host-parasite relationship, it is currently unknown whether GRA proteins are required to establish latent infection.

Tachyzoite-to-bradyzoite conversion, cyst development, and latent infection are critical to parasite survival and transmission. Nine distinct targeted gene deletions (1, 6, 13, 32, 38, 54, 55, 57, 65, 69) have been successfully developed in type II strains since genetic transformation of T. gondii was reported nearly 20 years ago (18, 39, 61). In contrast, recently reported type I KU80 knockout strains that exhibit highly efficient gene replacement frequencies has significantly accelerated the development of targeted gene deletions (2, 3, 15, 20, 23, 26, 34, 37, 44). Increased efficiency of double-crossover homologous recombination at targeted loci in KU80 knockouts is due to the functional loss of the nonhomologous end-joining DNA repair pathway that mediates a major mechanism underlying frequent random insertion of linear episomes in T. gondii (26). We report here the development of KU80 knockouts (Δku80) in type II T. gondii. Eight genetic loci were targeted for the development of mutant strains with targeted single-gene deletions or multiple-gene deletions to functionally examine candidate genes required for cyst development and latent infection. In particular, we examined the hypothesis that a host response to parasite antigen genes that elicit antigen-specific CD8+ T cell responses during host infection may be essential to cyst development. Our results support a critical role for dense granule proteins GRA4 and GRA6 in fundamental biology at the host-parasite interface that is essential for cyst development and transmission of T. gondii.

**MATERIALS AND METHODS**

**Primers.** All oligonucleotide primers used in the development of plasmids for targeting gene deletions are listed in Table S1 in the supplemental material. All oligonucleotide primers used in validation of mutants with gene deletions are listed in Table S2 in the supplemental material.

**Plasmid constructs.** All plasmids were developed using the yeast shuttle vector pRS416 and yeast recombination cloning which fused three distinct genetic elements (an ~1-kb 5′ target flank, an ~2-kb hypoxanthine-xanthine-guanine phosphoribosyltransferase [HXGPRT] selectable marker cassette, and an ~1-kb 3′ target flank, all 5′ to 3′ and in this order) with pRS416 using 31- to 33-bp homologous crossovers at recombination junctions (23). DNA elements for yeast recombination were amplified from type II Prugniaud (Pru) genomic DNA or type I RH genomic DNA as indicated. Targeting plasmids constructed in pRS416 were verified by using restriction enzyme digests, and target DNA flanks were sequenced to verify DNA sequence homology.

Plasmid pΔTPR1 was constructed to delete nucleotides 2741952 to 2737843 in the Pru KU80 locus, defined as TME49_112510 on chrXI of the ToxoDB database version 6.0 (www.toxodb.org). This deletion strategy deletes 1,228 bp of the KU80 5′ upstream putative gene regulatory region and the first three exons of the predicted four protein coding exons of the KU80 gene. The HXGPRT minigene cassette was fused between a 2,997-bp 5′ genomic targeting flank and a 3,304-bp 3′ genomic targeting flank amplified from type II Prugniaud (Pru) genomic DNA.

Plasmid pΔTPR1C was constructed by NotI and PmlI digestion of pΔTPR1 at the 3′ end of the 3′ targeting flank, followed by ligation with the cytosine deaminase (CD) selectable marker contained on a NotI/Pmel restriction fragment isolated from plasmid pMDC44.4 (26).

Plasmid pΔTPR2C was constructed by digesting pΔTPR1 with KspI, followed by self-ligation to delete the HXGPRT minicassette fragment from pΔTPR1.

Plasmid pΔUPR was constructed to delete nucleotides 2709350 to 2713372 of the uracil phosphoribosyltransferase (UPRT) chromosomal locus on chrXI annotated as TME49_112480. The HXGPRT minigene cassette was fused between a 1,131-bp 5′ UPRT genomic targeting flank and a 1,119-bp 3′ UPRT genomic targeting flank amplified from Pru genomic DNA.

Plasmid pΔUPP was constructed to delete nucleotides 1491873 to 1488688 of...
the uridine phosphorylase (UP) chromosomal locus (23) on chrXI annotated as TGME49_110860. The HXGPRT minigene cassette was fused between a 1,141-bp 5' UP genomic targeting flank and a 955-bp 3' UP genomic targeting flank amplified from Pru genomic DNA.

Plasmid pαOPT was constructed to delete nucleotides 2733578 to 2735556 of the orotate phosphoribosyltransferase (OPRT) locus defined as TGGT1_010360 on chrVIIb. The HXGPRT minigene cassette was fused between a 1,050-bp 5' OPRT genomic targeting flank and a 1,118-bp 3' OPRT genomic targeting flank amplified from RH genomic DNA.

Plasmid pαOPP was constructed to delete nucleotides 2719166 to 2721145 of the OPRT locus defined as TGME49_059660 on chrVIIb. The HXGPRT minigene cassette was fused between a 1,050-bp 5' OPRT genomic targeting flank and a 1,118-bp 3' OPRT genomic targeting flank amplified from Pru genomic DNA.

Plasmid pαOPPC was constructed by digesting pαOPP with SacII, followed by self-ligation to delete the HXGPRT minicassette.

Plasmid pαGRA4P was constructed to delete nucleotides 7216123 to 7215219 of the GRA4 locus on chrX annotated as TGME49_110780. The HXGPRT minigene cassette was fused between a 1,124-bp 5' GRA4 genomic targeting flank and a 989-bp 3' GRA4 genomic targeting flank amplified from Pru genomic DNA.

Plasmid pαGRA4PC was constructed by digesting pαGRA4 with SpeI, followed by self-ligation to delete the HXGPRT minicassette.

Plasmid pαROP4P was constructed to delete nucleotides 1414364 to 1404626 of the ROP4/7 locus on chrIa annotated as TGME49_095110. This gene locus is incomplete in the genome database. Analysis of the Tnxodb database identified common flanking and unique DNA surrounding the ROP4/7 locus and suggested the gene structure for the ROP4/7 locus consists of a 5' ROP4 gene, followed by a complete ROP7a allele and ROP7b allele (with identical coding DNA but minor polymorphisms in their UTRs) and a 3' truncated and incomplete ROP7c allele (L. M. Rommelein and D. J. Beik, unpublished data).

Consequently, we targeted the deletion of the entire locus (~14,297 bp) and all ROP4 and ROP7 alleles by using target DNA flanks derived from unique DNA sequences that reside just 5' or just 3' of the ROP4/7 locus. The HXGPRT minigene cassette was fused between a 1,179-bp 5' ROP4/7 genomic targeting flank and a 989-bp 3' ROP4/7 genomic targeting flank amplified from Pru genomic DNA.

Plasmid pαTGDO57P was constructed to delete nucleotides 6486828 to 6487525 of the tgd057 locus on chrXI annotated as TGME49_015980. The HXGPRT minigene cassette was fused between an 800-bp 5' tgd057 genomic targeting flank and a 1,259-bp 3' tgd057 genomic targeting flank amplified from Pru genomic DNA.

Plasmid pαGRA4X was constructed to insert the coding region of GRA4 into the UPRT locus under the control of the UPRT 5' transcriptional region (5' UTR). Targeted insertion of the pGRA4X cassette deleted nucleotides 2709350 to 2713372 of the coding region of the UPRT chromosomal locus on chrXI annotated as TGME49_112480. The GRA4 coding region (plus 12 bp 5' of the ATG) and 635 bp of the GRA4 3' UTR (nucleotides 1579415 to 1581100 on ChrX) was amplified from Pru genomic DNA and inserted between the 5' UPRT target flank and the HXGPRT marker of plasmid pαUPPR.

Plasmid pαGRA6X was constructed to insert the coding region of GRA6 into the UPRT locus under the control of the UPRT 5' transcriptional region (5' UTR). Targeted insertion of the pGRA6X cassette deleted nucleotides 2709350 to 2713372 of the coding region of the UPRT chromosomal locus on chrXI annotated as TGME49_112480. The GRA6 coding region (plus 15 bp 5' of the ATG) and 542 bp of the GRA6 3' UTR (nucleotides 7214586 to 7215188 on ChrX) was amplified from Pru genomic DNA and inserted between the 5' UPRT target flank and the HXGPRT marker of plasmid pαUPPR.

**Culture conditions and strains.** All parasite strains were continuously maintained in vitro by serial passage in Eagle modified essential medium supplemented with 1% fetal bovine serum in diploid human foreskin fibroblasts (HFF) at 36°C (26). Pyrimidine auxotrophs were supplemented with uracil (250 μM). The parental Pru strain (Δhxgprt) was previously made transgenic for green fluorescent protein (GFP) under the control of the LDH2 bradyzoite stage-specific promoter and was designated BSG-4 (60). The T. gondii strains used and developed in the present study are shown in Table 1. We previously reported the RHαku80 strain (26).
Negative for the PCR 1 product. Genotypes with an intact knockout is positive for the PCR 2, PCR 3, and PCR 4 products and no template (lane 10). DNA size ladder (lane 11). A targeted 4 clones from lanes 5, 9, and 10 (lanes 6 to 8), parental Pru (lane 9), and 10 (lanes 1 to 3), parental Pru (lane 4), no template (lane 5); PCR KU80 (lane 18), no template (lane 19), DNA size ladder (lane 20). Clones in (Top agarose gel panel) PCR 1 and PCR 2 products: 17 randomly locus (not to scale) and the expected PCR product sizes are shown.

FIG. 1. Construction of the type II Δku80 Δhxgprt genetic background. (A) Strategy for disrupting the KU80 gene in the parental Pru strain via integration of theHXGPRT marker. Approximate locations of PCR products using primer pairs to verify the genotype at the KU80 locus (not to scale) and the expected PCR product sizes are shown. (Top agarose gel panel) PCR 1 and PCR 2 products: 17 randomly selected clones isolated after selection (lanes 1 to 17), parental Pru (lane 18), no template (lane 19), DNA size ladder (lane 20). Clones in lanes 5, 9, and 10 appear negative for PCR 1 showing deletion of the KU80 gene. (Bottom agarose gel panel) PCR 3: clones from lanes 5, 9, and 10 (lanes 1 to 3), parental Pru (lane 4), no template (lane 5); PCR 4: clones from lanes 5, 9, and 10 (lanes 6 to 8), parental Pru (lane 9), no template (lane 10). DNA size ladder (lane 11). A targeted KU80 knockout is positive for the PCR 2, PCR 3, and PCR 4 products and negative for the PCR 1 product. Genotypes with an intact KU80 locus are positive for PCR 1 and PCR 2 and negative for PCR 3 and PCR 4. (B) Excision of theHXGPRT marker from the KU80 locus in the parental Pru strain. The strategy for excision ofHXGPRT is depicted with negative selection in 6TX after transfection with plasmid pΔTPRC2. Approximate locations of PCR products using primer pairs to verify genotype are depicted (not to scale). The expected PCR product sizes are shown for a positive result. (Agarose gel panel) PCR 1 and PCR 5 products: 11 clones isolated after 6TX selection (lanes 1 to 11), parental Pru (lane 12), parental PruΔku80::HXGPRT (lane 13), DNA size ladder (lane 14). The PruΔku80::hxgprt strain is positive for PCR 5 and negative for PCR 1 (lanes 1 to 11), the parental Pru strain is positive for PCR 1 and negative for PCR 5 (lane 12), and the parental PruΔku80::HXGPRT strain is negative for PCR 1 (496-bp product) and PCR 5 (lane 13).
Transferase (OPRT) strategy for genotype verification of deletion of nucleotide polymorphisms in the 3'H11032 to scale). The expected PCR product sizes are shown for a positive Pru double-crossover homologous recombination in type II strain VOL. 10, 2011 ANALYSIS OF CYST DEVELOPMENT AND LATENT INFECTION 1197

FIG. 2. Targeted gene replacement at the orotate phosphoribosyltransferase (OPRT) locus. (A) Strategy for disruption of OPRT by double-crossover homologous recombination in type II strain Pru ku80::hxgprt using plasmid pΔOPP and in type I strain RH ku80::hxgprt using plasmid pΔOPT. Compared to plasmid pΔOPP, plasmid pΔOPT contains seven single-nucleotide polymorphisms (indicated by *+) in the 5' targeting DNA flank and three nucleotide polymorphisms in the 3' targeting DNA flank. The PCR strategy for genotype verification of deletion of OPRT is depicted (not to scale). The expected PCR product sizes are shown for a positive

sured as a relative unit of diameter. Average cyst size was determined at a magnification of ×150 by measuring bradyzoite diameter units (BDU). BDU were determined for each randomly selected cyst by counting the number of GFP+ bradyzoites residing in a direct line from one edge of the cyst wall to the opposite edge of the cyst wall at the maximum point of the cyst diameter. BDU were determined for 25 randomly selected cysts from each mouse brain tissue sample. At least five mice were used to determine BDU for each parasite strain tested. Cyst data samples were subjected to a Student t test and are represented as means ± the SEM. Differences in cyst burdens between groups of infected mice were significant if the P value was <0.05. Cysts were also evaluated using confocal microscopy. Images were acquired using a Zeiss LSM 510 Meta laser scanning confocal microscope equipped with a 20× Plan Apo NA 0.75 objective lens (Carl Zeiss Microimaging, Thornwood, NY). Confocal settings were set to 1 Airy unit (1.8-μm optical section). A zoom setting of six generated a pixel size of 0.15 by 0.15 μm. LSM 510 software version 3.2 was used.

RESULTS

Generation of T. gondii strains PruΔku80::HXGPRT and PruΔku80::hxgprt. Alignments of the predicted type I (26) and type II (TGME49_112510, 583.m05492 [www.toxodb.org] version 4.0) KU80 loci revealed frequent strain specific nucleotide polymorphisms. Plasmid pΔTPR1 was developed to functionally delete the type II KU80 gene. Multiple independent transfections of various type II strains, selections, and screens of more than ~800 MPA resistant clones failed to identify a targeted KU80 knockout in any type II strain.

We constructed plasmid pΔTPFC1 with the cysteine deaminase (CD) gene inserted 3' of the 3' KU80 target DNA flank (Fig. 1A). Inclusion of the CD gene provided a more robust selection strategy for double crossovers at the KU80 locus by using negative selection to eliminate transformants that still retained expression of CD by growth of the transfected parasite population in 5-fluorocytosine (Fig. 1A). After continued attempts using the CD negative selection strategy, we detected a low frequency of KU80 knockout clones (PruΔku80::HXGPRT) that were positive for PCR 2 and were negative for PCR 1 that probes for the presence of the targeted deletion in the KU80 gene (Fig. 1A, lanes 5, 9, and 10, top gel panel). Three PCR 1-negative clones were then validated as

result. (Top agarose gel panel) PCR 1 and PCR 2 products: 6 clones isolated after targeting type I OPRT deletion (lanes 1 to 6), 6 clones isolated after targeting type II OPRT deletion (lanes 7 to 12), parental type I RHΔku80::hxgprt (lane 13), parental type II PruΔku80::hxgprt (lane 14), no template (lane 15) DNA size ladder (lane 16). All six type I clones and all six type II clones appear to be OPRT knockouts (PCR 1 negative and PCR 2 positive). Type I and type II clones were evaluated in PCR 3 and PCR 4 to verify integration of HXGPRT at the deleted OPRT locus. (Bottom agarose gel panel) PCR 3 products: type I clones (lanes 1 to 4), type II clones (lanes 5 to 8); PCR 4 products: type I clones (lanes 9 to 12), type II clones (lanes 13 to 16); PCR 3 and PCR 4 on parental type I RHΔku80::hxgprt (lane 17), PCR 3 and PCR 4 on parental type II PruΔku80::hxgprt (lane 18), PCR 3 and PCR 4 using no template (lane 19), DNA size ladder (lane 20). (B) PFU assays were performed at various times after transfection of pΔOPT in the type I RHΔku80::hxgprt (top) or after transfection of plasmid pΔOPP into the type II PruΔku80::hxgprt (bottom) to determine the gene replacement frequency at the OPRT locus based on the fraction of parasites that exhibited resistance to MPA and grew with uracil supplementation compared to the fraction of parasites that were resistant to MPA without uracil supplementation (Table 2). The PFU assays shown were sampled 25 days posttransfection with or without uracil. All PFU assays contained MPA selection medium.
targeted KU80 knockouts using PCR 3 and PCR 4 to demonstrate precise 5' and 3' integration of HXGPRT at the deleted KU80 locus (Fig. 1A, bottom gel panel). To delete the HXGPRT marker from the PruΔku80::HXGPRT strain, two clones were transfected with plasmid pATPRC2, which is identical to plasmid pATPR1 but lacks the HXGPRT gene (Fig. 1B). 6TX-resistant parasite clones were obtained, and these clones uniformly exhibited the genotype Δku80 Δhxgprt based on PCR analysis using PCR 1 and PCR 5 that was designed to span the deleted region of KU80 and to demonstrate the targeted deletion of the 2-kb HXGPRT marker (Fig. 1B).

Influence of DNA sequence homology on gene replacement frequency at the OPRT locus. Disruption of de novo pyrimidine synthesis induces uracil auxotrophy (23, 24). Here, the OPRT locus was targeted for deletion to induce uracil auxotrophy as a phenotype. Type II OPRT deletion was targeted using plasmid pΔOPT and type I OPRT deletion was targeted using plasmid pΔOPR (Fig. 2A). OPRT knockouts were obtained in type I and type II backgrounds based on the identification of clones that were positive for PCR 2 and negative for PCR 1 that probes for the presence of the targeted deletion in the OPRT gene (Fig. 2A, top gel panel). Using PCR 3 and PCR 4, four of these clones from each strain type were then shown to be targeted OPRT deletions based on precise 5' and 3' integration of the HXGPRT marker at the OPRT locus (Fig. 2A, bottom gel panel).

The efficiency of gene targeting at the OPRT locus in the I versus the type II Δku80 Δhxgprt strain was measured using target DNA flanks with a strain-specific DNA homology of 100% (Fig. 2A) (Table 2). PFU assays performed on the MPA-selected population of parasites at 18 and again at 25 days posttransfection of the targeting plasmids revealed that targeted disruption of OPRT created the uracil auxotroph phenotype at a high frequency in both the type I and the type II Δku80 Δhxgprt strains (Fig. 2B). The gene targeting frequency at the OPRT locus was found to be slightly higher in the type II strain PruΔku80 Δhxgprt (99.8% ± 0.1%) compared to the type I strain RHΔku80 Δhxgprt (92.9% ± 1.5%) (Table 2).

 Naturally occurring nucleotide polymorphisms present within the 5' and 3' noncoding DNA between type I and type II strains enabled a test of homology requirements necessary for efficient gene targeting at the OPRT locus in T. gondii (Fig. 2A). These nucleotide polymorphisms divided the 5' target flank into segments of 94, 162, 53, 127, 39, 240, 88, and 247 bp of homology and the 3' target flank into segments of 530, 22, 359, and 208 bp of homology (sequence alignments between type I and type II OPRT are not shown). Type I targeting plasmid pΔOPT with 7 nucleotide mismatches in the 5' target DNA flank and 3 mismatches in the 3' target DNA flank relative to type II was inefficient in targeting gene deletion at the type II OPRT locus (Table 2).

Targeted disruption of the key enzymes of the pyrimidine salvage pathway. Disruption of UPRT was used to measure gene replacement efficiency in strain PruΔku80 Δhxgprt (26). The frequency of gene replacement at the UPRT locus was determined at different time points after transfection of plasmid pΔUPRP by plating equal numbers of parasites either in MPA or in MPA plus FUDR selection. The nonreverting PruΔku80 Δhxgprt::HXGPRT genotype was also confirmed in several MPA resistant clones (data not shown). The frequency of gene replacement at the UPRT locus in strain PruΔku80 Δhxgprt was found to be only 3.3% ± 0.4% at 10 days posttransfection, but this frequency steadily increased to 98.2% ± 1.1% by day 32 posttransfection (Table 3). In contrast, the gene replacement efficiency in the parental Pru strain was <0.10% at 32 days postinfection (Table 3).

It is currently unknown whether the two critical enzymes of the pyrimidine salvage pathway (23) (UPRT and uridine phosphorylase [UP]) are required for cyst development and latent T. gondii infection. To complete a genetic dissection of the major pyrimidine salvage activities in the type II T. gondii and to assess the potential role of the salvage pathway in cyst development, we deleted the UP gene using plasmid pΔUPP (data not shown) (Table 1). We found that 23 of 24 randomly selected MPA-resistant clones (96%) had the UP gene deleted.

Efficient targeted excision of the HXGPRT selectable marker from PruΔku80 mutant strains. HXGPRT was targeted for deletion from the OPRT locus in strain PruΔku80 Δoprt::HXGPRT using plasmid pΔOPPC and negative selection in 6TX. Strain PruΔku80 Δoprt was easily isolated and validated by PCR analysis (data not shown) (Table 1). PruΔku80 Δhxgprt stably maintains the ability to develop cysts and chronic infection in mice. We observed no significant difference (P = 0.20) between the in vitro intracellular replication rates of strain PruΔku80 Δhxgprt (14.2 ± 0.50 parasites/vacuole) compared to the parental Pru strain (15.0 ± 0.15 parasites/vacuole) (Table 4). The type II strain PruΔku80 Δhxgprt stably maintained the ability to develop brain tissue cyst burdens in mice after more than 16 months of continuous in vitro culture. Brain tissue cysts obtained from mice infected with the strain PruΔku80 Δhxgprt revealed a cyst wall structure (Fig. 3A), as well as the expected bradyzoite stage-specific expres-
sion of GFP (Fig. 3B and C) (60). In contrast, GFP expression was not detected in strain Pru\textsubscript{ku80/H9004 hxgprt} during in vitro culture of the tachyzoite stages (data not shown). In addition, brain cyst burdens in C57BL/6 mice at 3 weeks postinfection with strain Pru\textsubscript{ku80/H9004 hxgprt} (942 ± 184) were essentially identical (\( P = 0.70 \)) to the brain cyst burdens observed after infection with the parental Pru strain (1,065 ± 262) (Fig. 3D). Furthermore, cyst sizes in strain Pru\textsubscript{ku80/H9004 hxgprt} (13.0 ± 0.46 BDU) were essentially identical (\( P = 0.63 \)) to the cyst sizes measured in the parental Pru strain (13.3 ± 0.47 BDU) (Fig.

### TABLE 4. Intracellular replication rate of selected strains used in this study

| Strain (reference) | Mean no. of parasites per vacuole\(a\) ± SEM | \( P \) compared to the control strain\(b\) | Control strain genotype |
|--------------------|---------------------------------------------|---------------------------------------------|------------------------|
| Pru\textsubscript{ku80/gra4::HXGPRT} | 14.1 ± 0.48 | 0.74 | NS | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 13.9 ± 0.29 | 1 | NS | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 13.2 ± 0.14 | 0.09 | NS | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 14.2 ± 0.13 | 0.33 | NS | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 15.2 ± 0.31 | 0.02 | S | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 14.9 ± 0.20 | 0.03 | S | \( \Delta ku80::HXGPRT \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 13.9 ± 0.31 | 0.20 | NS | \( \Delta hxgprt \) (parental) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 15.0 ± 0.17 | 0.70 | NS | \( \Delta ku80 \Delta hxgprt \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 14.2 ± 0.57 | 0.51 | NS | \( \Delta ku80 \Delta hxgprt \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 14.5 ± 0.36 | 0.36 | NS | \( \Delta ku80 \Delta hxgprt \) |
| Pru\textsubscript{ku80/gra6::HXGPRT} | 13.8 ± 0.23 | 0.36 | NS | \( \Delta ku80 \Delta hxgprt \) |

\( a \) Parasites per vacuole were scored after 45 h of intracellular replication.

\( b \) To determine the \( P \) value, strains were matched for the presence or absence of \textit{HXGPRT}. S, significant; NS, not significant; ND, not determined.

**FIG. 3.** The Pru\textsubscript{ku80/Δhxgprt} strain elicits GFP\textsuperscript{+} cysts and chronic and/or latent infection in genetically susceptible C57BL/6 mice. (A to E) C57BL/6 mice were infected by intraperitoneal inoculation of 200 of the tachyzoites of Pru\textsubscript{ku80/Δhxgprt} strain or the parental Pru strain. (A, B, and C) An example of a cyst observed at 5 weeks postinfection of C57BL/6 mice with strain Pru\textsubscript{ku80/Δhxgprt} (942 ± 184) were essentially identical (\( P = 0.70 \)) to the brain cyst burdens observed after infection with the parental Pru strain (1,065 ± 262) (Fig. 3D). Furthermore, cyst sizes in strain Pru\textsubscript{ku80/Δhxgprt} (13.0 ± 0.46 BDU) were essentially identical (\( P = 0.63 \)) to the cyst sizes measured in the parental Pru strain (13.3 ± 0.47 BDU) (Fig.
Finally, peroral infection of the genetically susceptible C57BL/6 background with 10 brain cysts of strain PruΔku80Δhxgprt caused an acute infection with morbidity, and infected mice recovered and then developed the expected chronic infection that eventually led to mortality (Fig. 3F). Deletion of the Ku80 gene in strain PruΔku80Δhxgprt did not lead to any significant defects in regard to the intracellular growth rate, the development of brain cyst burdens, the cyst size, or the establishment of a chronic or latent infection.

**Targeted deletions at the GRA4, GRA6, ROP4/7, and tgd057 loci.** To assess the interplay of host response and cyst development, targeted deletions were developed at the four gene loci known to encode CD8$^\text{+}$ T cell epitopes that elicit corresponding antigen-specific CD8$^\text{+}$ T cell populations during T. gondii infection. Targeting plasmids pGRA4P, pGRA6P, pROP4/7P, and pTGD057P were used to develop mutant strains with deletions of the GRA4, GRA6, ROP4/7, or tgd057 loci. Plasmids pGRA4PC and pGRA6PC were then used to target the removal of HXGPRT from the disrupted GRA4 and GRA6 loci in 6TX-negative selections. Finally, the GRA4 and GRA6 strains with HXGPRT deleted were used to develop several mutant strains with targeted deletion of two genetic loci (Δku80 Δgra6::HXGPRT, Δku80 Δgra4 Δtgd057::HXGPRT, and Δku80 Δgra6 Δtgd057::HXGPRT) (Table 1).

**Determination of the replication rate of mutant strains in vitro.** None of the mutant strains examined exhibited any significant decrease in their intracellular growth rate in a 45 h growth assay (parasites per vacuole) compared to the control strain matched for the presence or absence of HXGPRT (Table 4). Two of the mutant strains, Δku80 Δtgd057::HXGPRT ($P = 0.020$) and Δku80 Δrop4/7::HXGPRT ($P = 0.032$), were found to exhibit a significant increase in the number of parasites per vacuole in the 45-h in vitro growth assay.

**Measurement of brain tissue cyst burdens in mice infected with mutant strains.** Cyst development was examined in the genetically susceptible C57BL/6 murine background that exhibits higher cyst burdens, reactivation, and chronic infection compared to the genetically resistant BALB/c murine background (30, 45, 56). Cyst burdens measured at 3 weeks postinfection of C57BL/6 mice were 462 ± 111 for the PruΔku80::HXGPRT strain and 942 ± 184 for the PruΔku80Δhxgprt strain (Fig. 4A; $P = 0.0499$). Nonetheless, all of the knockouts reported in the present study were developed in the PruΔku80Δhxgprt parent strain that is normal in regard to intracellular replication rate, cyst development, and cyst burdens (Fig. 3). The mutant strains developed in the

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**FIG. 4.** Brain cyst burdens measured at 3 weeks postinfection. C57BL/6 or CBA mice were infected by intraperitoneal inoculation of 200 tachyzoites of mutant or parental strains. (A, B, and C) Brain cyst burdens in C57BL/6 mice. (A) Cyst burdens were measured at 3 weeks postinfection of C57BL/6 mice infected with the strain genotypes Δku80::HXGPRT (6 mice), Δku80 Δhxgprt (6 mice), Δku80 Δuprt::HXGPRT (5 mice), Δku80 Δtgd057::HXGPRT (6 mice), and Δku80 Δrop4/7::HXGPRT (6 mice). (B) Cyst burdens were measured at 3 weeks postinfection of C57BL/6 mice infected with the strain genotypes Δku80 Δhxgprt (6 mice), Δku80 Δgra4::HXGPRT (6 mice), Δku80 Δgra6::HXGPRT (6 mice), Δku80 Δgra4 Δhxgprt (4 mice), and Δku80 Δgra6 Δhxgprt (4 mice). (C) Cyst burdens were measured at 3 weeks postinfection of C57BL/6 mice infected with the strain genotypes Δku80 Δhxgprt (6 mice), Δku80 Δgra4 Δtgd057::HXGPRT (6 mice), Δku80 Δgra6 Δtgd057::HXGPRT (6 mice), Δku80 Δgra4 Δgra6::HXGPRT (6 mice), and Δku80 Δgra4 Δgra6 Δhxgprt (4 mice). (D) Brain cyst burdens in CBA mice. Cyst burdens were measured at 3 weeks postinfection of CBA mice infected with strain genotypes Δku80 Δhxgprt (6 mice), Δku80 Δgra4 Δtgd057::HXGPRT (6 mice), Δku80 Δgra6 Δgra4::HXGPRT (6 mice), and Δku80 Δgra4 Δgra6 Δhxgprt (4 mice).
Prūku80Δhxgprt parent share the identical disrupted KU80 locus and differ only in regard to the presence of HXGPRT that is inserted at the gene locus targeted for deletion. Consequently, several gene knockouts developed in the Prūku80Δhxgprt strain using HXGPRT selection were subsequently deleted of HXGPRT to allow more optimal assessment of phenotypes in comparison to the parental Prūku80Δhxgprt strain.

Several mutant strains, including Prūku80Δup::hxgprt (data not shown), Prūku80Δsup::hxgprt (P = 0.75), Prūku80Δrop4/7::hxgprt (P = 0.33), and Prūku80Δg6d057::hxgprt (P = 0.11), exhibited no significant difference in cyst burdens compared to the parental Prūku80Δhxgprt strain at 3 weeks postinfection (Fig. 4A). In contrast, at 3 weeks postinfection of C57BL/6 mice, a 91% reduction (P = 0.0010) in cyst burden was observed in the Δgra6 mutant with HXGPRT (Δku80 Δgra6::HXGPRT) and a 91% reduction (P = 0.0010) in cyst burden was observed in the Δgra4 mutant with HXGPRT (Δku80 Δgra4::HXGPRT) (Fig. 4B). Similarly, a 93% reduction (P = 0.0051) in cyst burden was observed in the Δgra6 mutant with HXGPRT deleted (Δku80 Δgra6 Δhxgprt) and an 89% reduction (P = 0.0066) in cyst burden was observed in the Δgra4 mutant with HXGPRT deleted (Δku80 Δgra4 Δhxgprt) (Fig. 4B). Double mutants involving the GRA4 and/or the GRA6 loci were also examined for cyst development in C57BL/6 mice. Although the Prūku80Δgra4Δg6d057::HXGPRT (91% reduction; P = 0.0009) and Prūku80 Δgra6Δg6d057::HXGPRT (86%; P = 0.0032) mutant strains revealed similar reductions in cyst burden (Fig. 4C) compared to the Δgra4 and Δgra6 single mutants with or without the HXGPRT marker (Fig. 4B), the double-mutant strain Prūku80Δgra6Δgra4::HXGPRT revealed a markedly more severe defect in cyst burdens at 3 weeks postinfection (99% reduction; P = 0.0005) (Fig. 4C). This more significant defect in cyst burden in the Δku80 Δgra6 Δgra4::hxgprt double mutant was then verified by construction of the Δku80 Δgra6 Δgra4 Δhxgprt strain that exhibited a 99.8% reduction (P = 0.0037) in cyst burden (Fig. 4C). Collectively, these results demonstrate a significant defect in the development of brain cyst burdens in mutant strains with GRA4 and/or GRA6 deleted in C57BL/6 mice.

We also measured cyst burdens in the CBA murine (H-2Kb) background because the four currently characterized CD8+ T cell epitopes encoded by the GRA4, GRA6, ROP7, and g6d057 genes are not recognized by the H-2Kb-restricted CBA background. In the CBA murine background a 77% reduction (P = 0.018) in cyst burden was observed in the Δgra6 mutant and an 87% reduction (P = 0.011) in cyst burden was observed in the Δgra4 mutant at 3 weeks postinfection (Fig. 4D). In contrast, no significant difference in cyst burden was observed in the Δg6d057 mutant (P = 0.0099).

In addition, the cyst burdens for many of the mutant strains were also measured at 5 weeks postinfection of C57BL/6 mice. The Prūku80::HXGPRT (P = 0.28), Prūku80Δsup::HXGPRT (P = 0.25), and Prūku80Δg6d057::HXGPRT (P = 0.90) strains exhibited no significant difference in cyst burdens compared to the Prūku80Δhxgprt strain at 5 weeks postinfection (Fig. 5A). In contrast, the Prūku80Δrop4/7::HXGPRT mutant exhibited a 70% reduction in cyst burden at 5 weeks postinfection (P = 0.0009) (Fig. 5A). Significant reductions in cyst burdens compared to the parental Δku80 Δhxgprt strain were also observed in Cyst data samples were subjected to a Student's t test and are represented as means ± the SEM. Cyst burdens determined in mutant strains were compared to cyst burdens in the parental Δku80 Δhxgprt strain to establish significance (P < 0.05).
PCR 6 to verify precise integration of the GRA6 coding region at the deleted uptr locus. PCR 4 measures correct 5' integration, PCR 5 measures correct 3' integration, and PCR 6 measures the presence of the GRA4 coding region. (Agarose gel panel) PCR 4, PCR 5, and PCR 6 products: DNA size ladder (lane 1), PCR 1 from parental PruΔku80Δgra4Δhxgprt (lane 11), PCR 2 from parental PruΔku80Δgra4Δhxgprt (lane 12), PCR 3 from parental PruΔku80Δgra6Δhxgprt (lane 13), PCR 4 from parental PruΔku80Δgra6Δhxgprt (lane 11), PCR 5 from parental PruΔku80Δgra6Δhxgprt (lane 12), PCR 6 from parental PruΔku80Δgra6Δhxgprt (lane 13), PCR 6 from PruΔku80Δhxgprt (lane 14).

Two independent cloned isolates of each complemented strain were examined for cyst burden in C57BL/6 mice at 3 weeks postinfection. GRA4 complemented clones exhibited a 402 to 426% increase in cyst burdens compared to the Δgra4 mutant strain also lacking HXGPRT (GRA4, P = 0.0010 and P = 0.024) (Fig. 7). GRA6 complemented clones exhibited a 291 to 352% increase in cyst burdens compared to the Δgra6 mutant strain also lacking HXGPRT (GRA6, P = 0.032 and P = 0.031) (Fig. 7).

FIG. 6. Genetic strategy for complementation of the Δgra4 and Δgra6 deletion mutants. The coding region of the deleted GRA gene and the associated GRA 3' UTR was placed under regulatory control of the endogenous UPRT locus 5'UTR. (A) Plasmid pGRA4X was transfected into the PruΔku80Δgra4Δhxgprt strain, and parasites were selected in MPA and then selected in FUDR to select for the Δuprt phenotype. Clones isolated from this selection were examined using PCR 1, PCR 2, and PCR 3 to verify precise integration of the GRA4 coding region at the deleted uptr locus. PCR 1 measures correct 5' integration, PCR 2 measures correct 3' integration, and PCR 3 measures the presence of the GRA4 coding region. (Agarose gel panel) PCR 1, PCR 2, and PCR 3 products: DNA size ladder (lane 1), PCR 1 from 3 complemented clones (lanes 2 to 4), PCR 2 from 3 complemented clones (lanes 5 to 7), PCR 3 from 3 complemented clones (lanes 8 to 10), PCR 1 from parental PruΔku80Δgra4Δhxgprt (lane 11), PCR 2 from parental PruΔku80Δgra4Δhxgprt (lane 12), PCR 3 from parental PruΔku80Δgra6Δhxgprt (lane 14). (B) Plasmid pGRA6X was transfected into the PruΔku80Δgra6Δhxgprt strain, and parasites were selected in MPA and then selected in FUDR to select for the Δuprt phenotype. Clones isolated from this selection were examined using PCR 4, PCR 5, and each of the Δgra4 and Δgra6 mutant strains examined at 5 weeks postinfection (PruΔku80Δgra4::HXGPRT [P = 0.0020], PruΔku80Δgra6::HXGPRT [P = 0.0029], PruΔku80Δgra4Δhxgprt [P = 0.011], PruΔku80Δgra6Δhxgprt [P = 0.0087], PruΔku80 Δgra4Δgra6Δhxgprt::HXGPRT [P = 0.0015], PruΔku80Δgra4Δgra6Δhxgprt::HXGPRT [P = 0.0010], PruΔku80Δgra4Δgra6::HXGPRT [P = 0.0007], PruΔku80Δgra4Δgra6Δhxgprt [P = 0.0010] (Fig. 5B and C).

Complementation of the Δgra4 and Δgra6 mutants. To more clearly establish that the defect in cyst burdens observed in the Δgra4 and Δgra6 mutant strains was due to the deletion of GRA protein function, we complemented the Δgra4 and Δgra6 mutants by reinserting a functional allele of the deleted GRA gene. Rather than simply recreating the parental strain with an intact GRA locus, we complemented the Δgra4 and Δgra6 mutants by targeting the insertion of the GRA coding region and the HXGPRT marker into the UPRT locus, deleting the coding region of the GRA6 coding region at the UPRT locus (Fig. 6A and B). This strategy was based on our observation of normal cyst burdens in the Δku80 Δhxgprt mutant (Fig. 4A). In addition, by design we deleted the extremely strong GRA4 and GRA6 transcriptional promoters and their 5' transcribed, untranslated region (5' GRA UTRs), and via homologous recombination placed the GRA protein coding region(s) under the control of the endogenous UPRT locus 5'UTR which ranks at the ~78% for expression level percentile (mi
croarray data [www.toxodb.org]) (Fig. 6). In contrast, the relative expression level percentile ranking is ~94.2% for the endogenous GRA4 locus and ~99.2% for the endogenous GRA6 locus. Parasites transfected with the pGRA4X and pGRA6X complementation cassettes were selected in MPA for 10 days then the population was selected in FUDR. Complemented clones were examined using a PCR strategy (Fig. 6) and correctly targeted clones with the genotypes Δku80 Δgra4 Δuprt::gra4 coding region + 3'UTR HXGPRT and Δku80 Δgra6 Δuprt::gra6 coding region + 3'UTR HXGPRT were validated (Fig. 6).
FIG. 7. Cyst burdens are significantly increased in the GRA-complemented strains. C57BL/6 mice were infected by intraperitoneal inoculation of 200 tachyzoites of mutant parental or complemented strains. Cyst burdens were measured at 3 weeks postinfection of C57BL/6 mice infected with the strains PruΔku80Δgra4::HXGPRT (clone 1, 4 mice, and clone 2, 4 mice), PruΔku80Δgra4::HXGPRT (clone 1, 4 mice, and clone 2, 4 mice), PruΔku80Δgra6::HXGPRT (6 mice), PruΔku80Δgra6::HXGPRT (clone 1, 4 mice, and clone 2, 4 mice). Cyst data samples were subjected to a Student t test and are represented as means ± the SEM. Cyst burdens determined in complemented strains were compared to cyst burdens in the parental Δgra strain to establish significance (P < 0.05).

DISCUSSION

Type II isolates of *T. gondii* represent a prevalent strain type infecting humans and causing life-long infections characterized by the presence of the bradyzoite stage encysted tissue cyst. Previously, targeted genetic dissection of type II *T. gondii* strains was limited by inefficient homologous recombination pathways relative to highly efficient pathways for random integration of targeting episomes. By eliminating the dominant pathway of nonhomologous recombination via knockout and functional disruption of the *KU80* gene (26), we now report a new genetic model that enables efficient gene replacements and the reliable development of targeted gene deletions in type II *T. gondii*.

The PruΔku80Δhxgprt strain exhibited efficient gene targeting at the *HXGPRT* locus, at the *OPRT* locus, and at all other targeted loci. Efficient gene targeting was demonstrated to be dependent on perfect DNA sequence homology. We successfully targeted seven genetic loci for deletion (*UPRT*, *UP*, *OPRT*, *GRA4*, *GRA6*, *ROP47*, and *tdg057*), we retargeted the deletion of *HXGPRT* inserted at three disrupted loci (*OPRT*, *GRA4*, *GRA6*), we constructed three mutant strains with two targeted gene deletions (Δgra4 Δgra6, Δgra4 Δgd057, and Δgra6 Δgd057), and we functionally complemented the *GRA4* and *GRA6* knockouts. These results show that the type II Δku80 Δhxgprt genetic background is amenable for rapid and reliable development of targeted gene deletions, and other more complex genetic manipulations involving sequential targeting, removal of *HXGPRT* at the deleted locus, and retargeting using *HXGPRT*. The ability of the PruΔku80Δhxgprt strain to develop cyst burdens and latent infection was maintained after long-term in vitro culture or multiple genetic manipulations. In addition, the PruΔku80Δhxgprt strain retained a normal intracellular replication rate compared to the parental Pru strain, and this strain also retains normal acute virulence in mice (data not shown). The type I *KU80* knockout strain RHΔku80Δhxgprt retains a normal intracellular replication rate, acute virulence in mice (26), and this strain also exhibits an identical profile of gene expression during the cell cycle as parental RH (3). Collectively, these results support the PruΔku80Δhxgprt genetic background as an improved genetic model for dissecting type II *T. gondii* biology.

Type II mutants deleted in pyrimidine salvage activities for *UPRT* and *UP* were not defective in cyst development, demonstrating that pyrimidine salvage through the *UPRT* or *UP* enzyme activities is not required for cyst development or the establishment of latent infection. These results suggest that *de novo* pyrimidine synthesis is functional and essential during bradyzoite stage conversion, cyst development, and latent infection.

We examined the hypothesis that host CD8\(^+\) T cell responses directed against specific parasite antigens containing CD8\(^+\) T cell epitopes (GRA4, GRA6, ROP7, and tdg057) (5, 27, 42, 68) may influence cyst development. Our analysis was hindered by the low cyst burdens (<60 cysts per brain) that we observed in the H-2L\(^d\)-restricted BALB/c background. The H-2L\(^d\)-restricted BALB/c background is genetically resistant to infection and establishes a latent infection with reduced cyst burdens compared to genetically susceptible murine backgrounds such as C57BL/6, which exhibit higher cyst burdens, reactivation, and chronic infection (30, 45, 56). Recent observations suggest that infection of genetically susceptible C57BL/6 mice with the Pru strain produces the highest brain tissue cyst burden at 3 weeks postinfection (56), then cysts rupture (reactivate) and are lost at a higher rate than any potential rate of cyst reformation during chronic infection. Immune control of type II *T. gondii* infection has been proposed to be dependent on type II strain-specific H-2L\(^d\)-restricted cytotoxic T cells (35). This hypothesis is supported by the identification of the immunodominant and protective decapeptide HF10 epitope within the type II GRA6 protein that is presented in the context of the H-2L\(^d\) major histocompatibility complex I molecule (5).

The previously identified GRA4, GRA6, and ROP7 CD8\(^+\) T cell epitopes are H-2L\(^d\)-restricted (5, 27), whereas the tdg057 CD8\(^+\) T cell epitope is H-2K\(^\beta\) restricted. Consequently, in C57BL/6 mice a CD8\(^+\) T cell response is mounted against the known tdg057 epitope (68), but not against the known GRA4, GRA6, or ROP7 epitopes. At 3 weeks postinfection of C57BL/6 mice, we observed no significant differences in cyst burdens in either the Δrop4/7 or the Δgd057 mutants compared to the Δku80 Δhxgprt parent. The absence of CD8\(^+\) T cell response to the tdg057 epitope, as well as the loss of tdg057 protein function in the Δgd057 mutant, did not significantly affect cyst burdens. Similarly, the absence of CD8\(^+\) T cell response to ROP7 in both the parental strain and the Δrop4/7 mutant, along with the loss of ROP4/7 protein functions, did not significantly affect cyst burdens at 3 weeks postinfection. In
contrast, while the CD8⁺ T cell response to the GRA4 and GRA6 epitopes is absent in the parental strain in the C57BL/6 murine background, the deletion of the GRA4 (Δgra4) and GRA6 (Δgra6) genes markedly reduced cyst burdens independently of any potential CD8⁺ T cell response to the known epitopes within these GRA proteins. However, we cannot conclusively rule out the possibility that the GRA4 and/or GRA6 genes encode as-yet-uncategorized H-2Kb-restricted epitopes. We also observed a significant defect in cyst burdens in the Δgra4 and Δgra6 mutants in the CBA murine background (H-2Kb-restricted). These results suggest that the GRA4 and GRA6 proteins directly mediate functions necessary for the development of normal cyst burdens, rather than cyst development being strictly dependent on the host response to these proteins.

Previous studies have also reported defects in cyst development following gene disruption in type II strains. Disruption of the SRS9 gene or the SAG2CDXY gene locus revealed a phenotype of normal cyst burdens at 4 weeks postinfection, and then reduced cyst burdens were observed later during latent infection (41, 55). Disruption of a pseudouridyl synthase homologue (PUS1) revealed a phenotype of slightly smaller cyst size and slightly increased cyst burdens (1). Targeted disruption of the heat shock protein BAG1 gene (6, 69) resulted in a minor defect in cyst development. A significant defect in cyst burden was observed at 5 weeks postinfection in the Δrop4/7 mutant. Interestingly, the Δrop4/7 mutant and the Δgd057 mutant exhibited an increase in their in vitro growth rate. The recently reported type II GRA15 knockout was also reported to exhibit an increased growth rate in vitro (54).

Cross-linking studies suggest that a multimeric protein complex of GRA2, GRA4, and GRA6 exists within the nanotubular network of membranes within the parasitophorous vacuole space (43). However, a recent study of protein interactions using a more comprehensive panel of HA-FLAG-tagged GRA proteins did not reveal a direct interaction of GRA2, GRA4, and GRA6 in the membrane fraction (8). Disruption of GRA6 in type I tachyzoites revealed an altered intravacuolar network of membranes characterized by small vesicles instead of elongated nanotubules (47). The N terminus of GRA6 was also recently shown to be a critical domain in interacting with negatively charged lipids and is necessary for association of the protein with the vacular membranous network of nanotubules (28).

Type II mutants with GRA4 or GRA6 deleted exhibited drastically reduced cyst burdens during T. gondii infection. The defect in cyst development in these strains was independent of the presence or absence of HXGPRT. Thus, these GRA proteins appear to be involved in some aspect of cyst development that significantly reduces the likelihood of cyst formation rather than completely abrogating the parasites biological ability to convert to the bradyzoite stage and create a tissue cyst. Complementation of the Δgra4 and Δgra6 mutants using a functional allele of the corresponding GRA protein significantly restored cyst burdens. However, cyst burdens were not completely restored to the expected levels when GRA4 and GRA6 expression was placed under regulatory control of the UPRT locus. The relative strengths of the GRA4 and GRA6 promoters (GRA4, ~94.2%; GRA6, ~99.2%; [www.toxodb.org]) are significantly higher than that of the UPRT locus (~78%) used in the complementation study. These observations suggest that an extremely high level of expression of GRA4 and GRA6 may be necessary for the development of normal cyst burdens.

A more severe defect in cyst burden in the Δku80Δgra6Δgra4::HXGPRT and the Δku80 Δgra6 Δgra4 Δhxgprt mutants compared to mutants with single deletions of GRA4 or GRA6 suggests that GRA4 and GRA6 most likely play independent functional roles required for cyst development during infection. Ultrastructural localization is also consistent with the likelihood of independent roles for GRA4 and GRA6 in cyst development since, while GRA4 is abundant in the tachyzoite stage, PV it is not detectable in the bradyzoite stage wall, whereas GRA6 is easily detected in both stages (21). Since the cyst wall structure is thought to arise directly from modifications to the PVM during tachyzoite to bradyzoite conversion (59, 67), the function of GRA4 in cyst development is likely to be exerted prior to or during formation of the cyst wall.

Our study did not specifically address the mechanisms by which GRA4 or GRA6 influence cyst development. With the exception of uracil auxotrophy of the Δhxt mutants, no significant decreases were observed in the in vitro replication rate of any of the mutants isolated in the present study. The defect in cyst burden in the Δgra4 and Δgra6 mutants may be due to a more successful innate or adaptive host response that suppresses parasite burden or dissemination necessary for development of brain tissue cysts. Alternatively, GRA4 and GRA6 may play a direct role in parasite biology associated with the probability of successful tachyzoite to bradyzoite conversion or cyst development. Additional studies are therefore necessary to assess acute virulence, parasite tissue burdens, dissemination patterns, and host response during infection to further gauge potential mechanisms underlying the defect in cyst development in the Δgra4 and Δgra6 mutants.

The GRA protein family is extremely interesting because they are a remarkable family of highly expressed and compartmentalized proteins restricted thus far to the cyst-forming parasites Toxoplasma and Neospora (49). No identifiable ortholog can be discerned even in closely related Conoidiasida such as Cryptosporidium or Eimeria or Acanthoecida such as Plasmodium, which do not form tissue cysts (10). Our results suggest that in addition to GRA4 and GRA6 other members of the prominent GRA protein family may also play critical roles in biology underlying successful cyst development and latent infection.

The type II Δku80 Δhxgprt strain developed in the present study provides a reliable genetic model for targeted genetic dissection of parasite biology occurring during T. gondii infection. The ability to now control both parasite genes and host cell genes can reveal the complex interplay of parasite biology and host response during T. gondii infection. These genetic approaches will accelerate the development of improved strategies for vaccines, immunological interventions, and other therapeutics. Targeted genetic approaches using a type II Δku80 Δhxgprt genetic background has the immediate potential to functionally reveal parasite genes playing critical biological roles that, when intercepted during in vivo infection, block parasite development, pathogenesis, or transmission.
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