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Polymorphic family of injected pseudokinases is paramount in Toxoplasma virulence

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Toxoplasma gondii, an obligate intracellular parasite of the phylum Apicomplexa, has the unusual ability to infect virtually any warm-blooded animal. It is an extraordinarily successful parasite, infecting an estimated 30% of humans worldwide. The outcome of Toxoplasma infection is highly dependent on allelic differences in the large number of effectors that the parasite secretes into the host cell. Here, we show that the largest determinant of the virulence difference between two of the most common strains of Toxoplasma is the ROPS locus. This is an unusual segment of the Toxoplasma genome consisting of a family of 4–10 tandem, highly divergent genes encoding pseudokinases that are injected directly into host cells. Given their hypothesized catalytic inactivity, it is striking that deletion of the ROPS cluster in a highly virulent strain caused a complete loss of virulence, showing that ROPS proteins are, in fact, indispensable for Toxoplasma to cause disease in mice. We find that copy number at this locus varies among the three major Toxoplasma lineages and that extensive polymorphism is clustered into hotspots within the ROPS pseudokinase domain. We propose that the ROPS locus represents an unusual evolutionary strategy for sampling of sequence space in which the gene encoding an important enzyme has been (i) catalytically inactivated, (ii) expanded in number, and (iii) subject to strong positive selection. Such a strategy likely contributes to Toxoplasma’s successful adaptation to a wide host range and has resulted in dramatic differences in virulence.

The evolutionary pressure on the molecules that form the interface between a pathogenic organism and its host has been likened to an arms race (1, 2) and has been proposed to give rise to highly polymorphic genes such as those that encode host MHC and viral capsid proteins. Whereas most pathogens have a single or restricted range of hosts with which they coevolve, the obligate intracellular parasite Toxoplasma gondii can infect virtually any cell of almost any warm-blooded animal, a remarkable feat from an evolutionary perspective.

Different strains of Toxoplasma cause significantly different disease in mice (3) and humans as well (4, 5). In North America and Europe, three strains have grown to dominate Toxoplasma’s genetic landscape, and they are characterized by distinct disease outcomes in mice: although a single Type I parasite is uniformly lethal to a mouse, Type II and III parasites have a lethal dose (LD50) ranging from 102 to 105 (3). Sexual crosses between members of these lineages (Type I × Type III and Type II × Type III) (6, 7) have shown that virulence in mice segregates among Type II and III lineages that led to the identification of ROP16 and ROP18 indicated that a third virulence locus (VIR1), not then identified, had the greatest impact on virulence in mice (9). Here, we identify that locus to be a cluster of tandemly duplicated polymorphic pseudokinases originally annotated as a single gene (ROP5). We further show that the ROPS cluster is required for Toxoplasma to cause lethal disease in mice, because targeted deletion of the entire locus attenuated an otherwise highly virulent strain. The evolutionary pressures and mechanisms that may have led to the emergence of ROP5 as the key virulence locus are discussed.

Results

Previously, in a QTL mapping study using F1 progeny from a cross (6) between a Type II and III strains of Toxoplasma, we identified a 0.98-Mbp region of chromosome XII (the VIR1 locus) that was responsible for over 50% of the inherited variation in virulence (logarithm of odds = 9.4) (9). In that study, the Type III allele was predicted to contain the more virulent version relative to Type II. To predict which gene encodes this major virulence determinant, we used several criteria, including predicted secretion of the gene product into the host cell, predicted function (e.g., those having predicted kinase or protease domains), and polymorphism between Type II and III strains. Based on these criteria, we determined that a strong candidate was ROP5, a member of the ROP2 superfamily. The ROP2 superfamily is made up of protein kinase-fold proteins (21) that originate in Toxoplasma’s apical secretory organelles (called rhoptries) and are injected into the host cytoplasm on invasion. Many rhoptry proteins, including ROP5, subsequently associate recognition by the host (15) and through direct action of the parasite effectors themselves (9, 10, 16). In the latter category, two rhoptry proteins (ROPs) with kinase folds, ROP16 (16) and ROP18 (9, 10), were identified as key virulence factors by QTL mapping. ROP18 seems to function by phosphorylating and inactivating host immunity-related GTPases (17, 18), and ROP16 is a tyrosine kinase that directly phosphorylates the host STAT family of proteins (19, 20).

The QTL analysis of a cross between members of the Types II and III lineages that led to the identification of ROP16 and ROP18 indicated that a third virulence locus (VIR1), not then identified, had the greatest impact on virulence in mice (9). Here, we identify that locus to be a cluster of tandemly duplicated polymorphic pseudokinases originally annotated as a single gene (ROP5). We further show that the ROPS cluster is required for Toxoplasma to cause lethal disease in mice, because targeted deletion of the entire locus attenuated an otherwise highly virulent strain. The evolutionary pressures and mechanisms that may have led to the emergence of ROP5 as the key virulence locus are discussed.
directly with the host cytosolic face of the parasitophorous vacuolar membrane (PVM) (22, 23). One member of the family, ROP18, is a catalytically active kinase that has been identified previously as a key determinant of virulence on chromosome VIIa (9, 10). ROP5, however, has substituted a basic residue for the catalytic HRD motif Asp (invariant in active kinases) (24) and thus, is considered a catalytically inactive pseudokinase (21). These features made ROP5 a particularly intriguing candidate for the VIR1 QTL. Although many pathogens disrupt host signaling with secreted enzymes (19, 20, 25) or through secreted nonenzymatic activators or inhibitors of host signaling (26, 27), we know of no reports of virulence factors in any pathogen that, like ROP5, possess an enzymatic fold but lack catalytic activity.

**ROP5 is a Crucial Virulence Locus.** As a preliminary test of whether ROP5 is indeed the locus responsible for the VIR1 QTL, we complemented the S22 strain of *Toxoplasma* (8, 28) with a cosmid containing a predicted high-virulence allele of the ROP5 locus (derived from the highly virulent Type I strain RH). S22 is a hypervirulent progeny of the Type II × Type III cross (8), carrying the low-virulence allele for ROP16 and ROP18 (9) and the low-virulence (Type II) allele at the VIR1 locus, and thus, it represents an ideal starting point for studies of virulence in this model. Based on the LD₅₀ in mice, the complemented strain showed an increase in virulence of >10⁶-fold compared with the parental strain (Fig. S1). This strongly supported the hypothesis that ROP5 was indeed the VIR1 locus; however, because there are two additional genes unrelated to ROP5 on the cosmid (TGGT1_042730 and TGGT1_042740), it was possible that one of these latter genes was responsible for the differences that we saw.

As discussed in detail below, preliminary indications were that the ROP5 locus consists of multiple tandem genes, and therefore, rather than deal with each of these in turn, we chose to knockout the entire locus as a way to quickly validate ROP5’s role in virulence. We chose to do our genetic manipulation in a Type I strain because Type I strains are generally more easily manipulated than Type II strains. In addition, the Type I strain is highly virulent (infection with one parasite kills most strains of mice 100% of the time) (29), making it particularly suitable for observing a potential reduction in virulence. Using a strain with increased efficiency of homologous recombination (parental; RHΔku80Δhxgprt) (30), we created parasites in which the entire ROP5 locus was replaced with an HXGPRT selection cassette (RHΔrop5) (Fig. S2). Lack of ROP5 protein expression was confirmed by Western blot (Fig. L4). RHΔrop5 parasites exhibited no detectable growth phenotype during repeated passage in vitro or any detectable differences in plaque size on a fibroblast monolayer (Fig. S3).

We found that, although mice injected intraperitoneally with 100 parasites of the parental strain all succumbed within 7 d (as expected), mice injected with as many as 10⁶ RHΔrop5 parasites uniformly survived (10⁶ was the highest dosage tested) (Fig. 1B). This dramatic result was not dependent on mouse strain, because both BALB/c and outbred CD-1 mice showed the same sensitivity to parental parasites and resistance to infection with RHΔrop5 (Fig. S4). It is important to note that 10⁶ parasites represents a dosage at least 10-fold greater than the LD₅₀ for any of the three major strains (8, 29). These results indicate that the ROP5 locus is indeed crucial for the RH strain to establish a lethal infection in mice and is likely the genetic basis for the VIR1 QTL.

**ROP5 is a Cluster of Paralogs.** To prove that the loss of virulence was caused by the absence of ROP5 rather than some other incidental effect of generating the knockout strain, we needed to complement the mutant with an ectopically expressed copy of ROP5. Surprisingly, when we attempted to clone ROP5 from either Type I genomic or cosmid DNA by PCR, we obtained several distinct sequences, none of which matched the sequence predicted in ToxoDBv6.1 (www.toxodb.org). A likely explanation for this is the presence of multiple, nearly identical genes at the ROP5 locus, and the algorithm used to assemble the genome had viewed these as one sequence and mistakenly compressed them all into a single chimeric sequence. Consistent with this hypothesis, we noted a high degree of oversampling of the ROP5 locus in genomic sequencing reads for each of the major strains (Fig. 2A), which provides an accurate estimate of copy number variation (31). To confirm the existence of tandem ROP5 copies experimentally, we digested genomic DNA from Types I, II, and III strains with Apal, which our preliminary sequencing indicated cut outside and not within the ROP5 locus in all three strains (Fig. 2B). The resulting DNA was separated by field inversion gel electrophoresis, transferred to a membrane, and probed with radiolabeled DNA derived from the region encoding the ROP5 arginine-rich amphipathic helix domain (RAH) (22). As predicted by the differential oversampling of the sequence reads, we observed bands of different sizes in each strain, ranging from ~19 kbp for Type III to ~46 kbp for Type II (Fig. 2C).

**Fig. 1.** ROP5 is required for virulence in mice. (A) Western blot of a parasite lysate probed with mouse anti-ROP5 polyclonal Ab and Sag1 loading control. (B) BALB/c mice (n = 10) were infected with either 100 parental RHΔku80 parasites or 10⁶ RHΔrop5 parasites and monitored for 30 d.

**Fig. 2.** The ROP5 locus is differentially expanded in different *Toxoplasma* strains. (A) Quantification of sequencing read oversampling of each of the strains (black, Type I/GT1; dark gray, Type II/ME49; light gray, Type III/VEG). (B) Diagram of ROP5 locus restriction sites. (C) Genomic DNA from each of the three dominant strains was digested with Apal and analyzed by Southern blotting using a probe from the conserved N-terminal-encoding region of ROP5. (D) Diagram of the most likely locus organization of the three isoforms (Fig. 3) found in each strain using diagnostic double digests (Fig. S5B). Copies whose order is ambiguous are indicated with braces.
results supported the case for a variable number of tandem copies of ROP5 in the genomes of the three dominant strains and confirmed their relative copy number in each strain. We also quantified the locus expansion by repeating our analysis on parasite genomic DNA digested with XhoI, which cuts within the repeat unit (Fig. S5A); these results confirmed the estimates from the other two methods. Collectively, these data established that each strain has a different locus structure: Type I has ∼6, Type II has ∼10, and Type III has ∼4 tandem copies of ROP5. We specifically amplified the distal genes in each cluster by PCR using primers upstream or downstream of the locus and cloned these copies A and C, respectively. Southern blots with diagnostic double digests (Fig. SSB) (based on the sequences discussed below) were used to determine the most likely organization of the remaining genes in the locus. The results showed that, between the A and C copies, each strain had a variable number of additional copies of the A or C isoforms and a third distinct isoform, dubbed B (Fig. 2D).

ROP5 Paralogs Are Highly Divergent. To better understand the evolution of the ROP5 paralogs and inform the decision of which to use in the complementation study, it was necessary to determine their individual sequences. To do this and avoid obtaining chimeric sequences from PCR, we cloned the ROP5 paralogs directly from genomic DNA. Genomic DNA from Type II (containing an avirulent cluster of ROP5 paralogs), Type I (containing a virulent cluster), or Type III (containing a virulent cluster) was digested with XhoI, an enzyme that cuts between the two domains of the protein (33), and the RAH secretory compartment within the parasite (33), and the RAH.

Fig. 3. The ROP5 isoforms are highly divergent in their pseudokinase domains. (A) A neighbor-joining tree of the coding sequences of the major isoforms of the virulent (Types I and III) and avirulent alleles (Type II) of ROP5. Bootstrap values are indicated at branch points. (B) The ratio of nonsynonymous to synonymous SNPs (Kd/Ks) of the various ROP5 isoforms within the three major domains of the protein is indicated in italicized numbers. The Kd/Ks for the N-terminal domains represents an average of all pair-wise comparisons (P = 0.03), although the value for the pseudokinase domain represents the highest significance pair-wise comparison (that of ROP5A and ROP5C, P = 0.002). Amino acid numbering is bold. Codons with nonsynonymous SNPs are marked with a black line in the graph to the right of the domain architecture. Amino acid sequences are shown for highly polymorphic regions; identity is noted with a dot. The HDR and DFG motifs required for kinase activity (and unconserved in ROP5) are indicated in the sequence alignments in the insets. The sequence near these motifs is boxed and gray and contains polymorphisms that are conserved between either A or B/C isoforms, regardless of strain.

of the pseudokinase domain, whereas very few nonsynonymous polymorphisms are found within either of the N-terminal domains (i.e., the pro and RAH domains). In fact, the pseudokinase domain seems to be under strong diversifying selection (Kd/Ks = 3.6), whereas the N-terminal domains may be under purifying selection (Kd/Ks = 0.2). The N-terminal prodomain has been shown to target homologous proteins to the correct secretory compartment within the parasite (33), and the RAH
domain drives ROP5’s association with the PVM when within the host cell (22). Overall, these data indicate that selection is acting differently on the different domains of these proteins. Importantly, although the former ROP5 active site represents a hot spot of divergence, none of the polymorphisms would be predicted to restore (or leave intact) a functional kinase activity. Thus, ROP5’s pseudokinase status seems to be conserved among the paralogs from all three lineages.

**Single ROP5 Paralog Restores Parasite Lethality.** It is possible that all paralogs encoded in a multicopy locus such as ROP5 must be expressed together for correct function in vivo. To determine what combinations of the ROP5 isoforms are sufficient to complement and rescue the RHΔrop5 virulence phenotype, we modified the RHΔrop5 strain to express either single isoforms or pair-wise combinations of isoforms from the high-virulence cluster in Type III. Because the RHΔkiu80 background of the parental strain severely reduces the efficiency of random integration (30), we chose to complement the RHΔrop5 strain by targeting the two genes for which there are viable negative selection strategies for *Toxoplasma*. Thus, the ROP5 isoforms were targeted either to the *Toxoplasma* uracil-phosphoribosyltransferase (*UPRT*) locus (34) or the HXGPRT cassette that we had originally inserted as part of the ROP5 knockout construct in RHΔrop5 (constructs described in Fig. S2). Expression and localization were verified by including a short sequence encoding a C-terminal HA or FLAG tag in the introduced copies (Fig. S7), which have been shown to allow normal trafficking of ROP5 protein to the rhoptries and PVM (22, 23).

Virulence of the complemented strains was assayed by mouse survival as above. Importantly, a single copy of ROP5AΔIIIi targeted to the *UPRT* locus, was able to partially rescue the virulence phenotype, because a dose of 10³ parasites could no longer be tolerated and was, in fact, now uniformly lethal (Fig. 4A). These results show that the loss of virulence in the RHΔrop5 is, in fact, because of loss of ROP5. They further reveal that the ROP5 locus is not a single functional unit; an individual isoform can significantly alter virulence in the absence of the other copies.

**Isoforms Differentially Affect Virulence.** Given the expansion of the ROP5 locus, we hypothesized that complementation with additional copies of ROP5 would lead to a more substantial recovery of the virulence phenotype. To test this, we first targeted an additional copy of ROP5AΔIII to the HXGPRT cassette. This resulted in a sharp increase in virulence, because a dose of 10³ parasites was now lethal; 80% of the mice infected at this dose now succumbed (Fig. 4B).

To determine if there are differences between ROP5 isoforms, these experiments were repeated using complementation with a combination of ROP5AΔIII and ROP5BΔIII. Remarkably, a strain expressing a single copy of both seemed to have its virulence largely, if not completely, rescued; infection of mice with 10³ parasites of such a strain was uniformly lethal by 9 d postinfection, a curve that is indistinguishable from the parental infection (Fig. 4C). Thus, expression of only two of three different isoforms from the virulent Type III locus is sufficient to rescue most, if not all, of the virulence phenotype. Taken together, these data show that different isoforms differ in the magnitude of their effect on virulence in this model. Although the extensive polymorphisms in the individual ROP5 isoforms play a large role in determining the virulence effects of the locus, given the ROP5 copy number variation among the strains, it is possible that expression level may also contribute to observed differences in virulence.

**ROP5 Acts Independently from ROP18 and ROP16.** Before this work, the effectors that had the greatest known roles in virulence were ROP16 and ROP18 (which had previously been shown to be the key genes at the VIR3 and VIR4 loci, respectively) (9, 10). Both the ROP16 and ROP18 loci consist of a single copy gene encoding a catalytically active kinase. Alleric differences in these genes mediate either a four- (ROP18) or one-log (ROP16) difference in virulence in mice (9, 10). To assess whether these two active kinase virulence factors might interact with ROP5, we analyzed the five significant previously identified QTLs for epistatic interactions. We found no significant interaction between ROP5 and ROP16, but we did find high statistical support (*P* = 6 × 10⁻⁵) for genetic interactions between ROP5 and ROP18. However, this interaction effect accounted for only 4% of the variation in virulence observed in the F1 progeny.

To further explore the relationship between the ROP5 and ROP18 loci, the same strategy used to create the RHΔrop5 mutant was used to generate an RHΔrop18 line, and this was tested for virulence in mice. If these two genes were members of the same pathway, we would expect the RHΔrop18 to phenocopy the RHΔrop5 strain. Surprisingly, deletion of ROP18 had a much less dramatic effect on virulence than the ROP5 deletion; one-half of mice infected with 100 parasites of RHΔrop18 survived ~5 d longer than those infected with WT, but infection with the RHΔrop18 still resulted in 100% mortality (Fig. S8). Taken together, these data suggest that ROP5 acts independently from both of the active kinases ROP16 and ROP18 and that any genetic interactions are likely indirect (i.e., they do not represent physical or biochemical interactions between the molecules). Further supporting this hypothesis are the data for the cosmid-complemented S22 strain (Fig. S1). As discussed above, S22 has the avirulent alleles for both ROP16 and ROP18. Moreover, the
avirulent allele of \textit{ROP18} contains an insertion disrupting its promoter, resulting in undetectable expression (9, 10). Thus, the fact that differences in the \textit{ROP5} allele can alter virulence by five logs in such a background shows that \textit{ROP5} acts independently from \textit{ROP18} in its direct effects on virulence.

In summary, despite lacking a catalytically active kinase domain, \textit{ROP5} seems to play a larger role than \textit{ROP18} in an in vivo infection, at least within the context of a Type I strain. This is consistent with the fact that only when the \textit{ROP5} locus is of the same (e.g., high) virulence type, as is the case for the Type I and III strains, does \textit{ROP18} become the predominant genetic determinant of virulence in F1 progeny (10). It is important to note that the Type III strain possesses the avirulent alleles of both \textit{ROP16} and \textit{ROP18} (9), which helps explain its low virulence. However, it is clear that many factors, both genetic and possibly, epigenetic, contribute to a complex phenotype such as virulence, and the identification of the major \textit{VIR} loci will assist in determining these other factors.

\textbf{Discussion}

We have identified a cluster of polymorphic secreted pseudokinases as being of paramount importance to virulence in \textit{Toxoplasma}. Recently, pseudokinases have emerged as key regulatory effectors in metazoan signal transduction (35), particularly in mammalian immune signaling (36, 37). Although it is difficult to determine the specific role of a catalytically inactive protein, other pseudokinases have been shown to act as molecular scaffolds (38–40) and thereby, coordinate signal transduction. The presence of hotspots of polymorphism within the \textit{ROP5} pseudokinase domains indicates evolutionary pressure to bind divergent partners. This strongly suggests that differences in virulence are mediated by differential interactions of \textit{ROP5} within the host cell. Because the \textit{ROP5} proteins are present on the cytosolic face of the PVM, they may interact with and thus, dysregulate host proteins that are the key to the immune response. Alternatively, they could partner with other parasite proteins that are injected into the host cells to affect host signaling. Intriguingly, over 30\% of \textit{Toxoplasma}’s ~160 protein kinases are predicted to be catalytically inactive pseudokinases (41), and over one-half of these are predicted to be secreted into the host cell. Thus, \textit{ROP5} may occupy a key, central role in a large and complex network of highly unusual effectors.

We have shown that, unique among other known virulence genes in \textit{Toxoplasma}, the ROP5 locus has been considerably expanded, such that there are multiple tandem copies of \textit{ROP5} in each of the predominant strains and each of these strains possesses a different copy number and different alleles. Remarkably, expression of just two of the isoforms (and only two of the six copies found in Type I) in the RH\textit{Δrop5} strain effectively rescued the parental virulence phenotype. Copy number variation is a major source of genetic diversity in metazoa (42, 43), and the \textit{ROP5} locus seems to represent a specialized case of this in a protozoan parasite. This expansion of the \textit{ROP5} locus may allow \textit{Toxoplasma} to sample a greater sequence space for these critical genes than it could otherwise, because there is likely to be minimal cost to a single copy of diverging \textit{ROP5}. Given ROPS’s importance in infection, this ability to acquire multiple flavors of a key effector might explain the extraordinary expansion of intermediate hosts that can be productively infected by \textit{Toxoplasma}. This represents a dark reflection of the diversification of rapidly evolving host proteins used to respond to varied infections, such as the MHC (44) and tripartite motif (TRIM) (45) families.

Diversification of effector molecules is a hallmark of the pathogenic lifestyle. Although viruses use error-prone polymerases and bacteria use both sexual recombination and transformation, \textit{Toxoplasma} has a clonal lifestyle; within a given clonal type, isolates from across Europe and North America are virtually identical (8, 46). This may, in part, be because of \textit{Toxoplasma}’s ability to forgo its sexual cycle by horizontal transmission between successive intermediate hosts (through carnivorous and scavenging) and/or self-mating of these haploid organisms. It is possible, then, that diversification of key virulence factors like \textit{ROP5} through duplication may be necessary to compensate for inefficient diversification through sexual recombination. This hypothesis finds direct support in the fact that, whereas the three \textit{ROP5} isoforms from Types I and III are nearly identical at the sequence level, these loci vary in copy number (four vs. six, respectively). Intriguingly, the virulent Type I/III version of the locus contains isoforms that are more divergent from each other than are the isoforms from the avirulent Type II allele. Because the Type II strain seems to be a P1 parent of the Type III strain and a P2 parent of the Type I strain (32), both Type I and III strains were sufficiently viable to rise to predominance in a genetic landscape presumably dominated by Type II. It is possible that the divergent isoforms present in the Type I/III virulent \textit{ROP5} alleles provide a selective advantage in certain host species compared with the Type II alleles. Because \textit{Toxoplasma} strains may simultaneously coevolve with not one but many host organisms, the divergent \textit{ROP5} isoforms may represent a Swiss army knife method of evolution; rather than existing as a single, precisely evolved tool for a single situation, the multiple \textit{ROP5} isoforms may be a collection that together equips \textit{Toxoplasma} to infect a variety of host species.

\textbf{Materials and Methods}
with the ROPS isoform of choice (34). Clonal parasites were grown from populations by limiting dilution.

**Mouse Survival Analysis.** Six- to eight-week-old female BALB/c (Jackson Labs) or CD-1 (Charles River) mice were infected by peritoneal injection with parasites diluted in PBS (pH 7.4) to the appropriate dose and monitored daily for survival. Infection of surviving mice was verified by testing serum for reactivity with Toxoplasma lysate.

**QTL Interaction Analysis.** Survival data for mice infected with Type II × Type III F1 progeny originally published in ref. 9 were reanalyzed for epistatic interactions using the rqtl module as implemented in R (51). For the F1 progeny originally published in ref. 9 were reanalyzed for epistatic QTL interaction analysis.

**Infection of surviving mice was verified by testing serum for reactivity with Toxoplasma lysate.**