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Trypanosoma cruzi: Attenuation of virulence and protective immunogenicity after monoallelic disruption of the *cub* gene

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Abstract

Calmodulin–ubiquitin (*cub*) is a single-copy gene of *Trypanosoma cruzi*, which encodes a 208 aminoacid polypeptide of unknown function, containing putative calcium-binding domains. After targeted deletion, a clone (TulCub8) was derived where one of the two alleles was disrupted. This clone displayed a sharp and stable loss of virulence for mice. Parasitemias after inoculation of 10^6 trypomastigotes of the mutant, as compared to wild-type parasites were 68-fold lower ($p = 0.018$) in adult Swiss mice and 27-fold lower ($p = 0.002$) in newborn Balb/c mice. Epimastigote inocula of the mutant were strongly protective against infection by wild-type parasites. Virulence was not restored by serial passage in mice, showing that the attenuated phenotype is stable and gene-conversion from the intact *cub* allele does not occur at an appreciable rate. Retransfection of the missing cub allele restored virulence. Complementation experiments showed that the intact *cub* gene is necessary for full expression of virulence.
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Index Descriptors and Abbreviations: *Trypanosoma cruzi*; Calmodulin–ubiquitin; Virulence; Attenuation; Targeted deletion; DNA, deoxyribonucleic acid; Cub, calmodulin–ubiquitin-associated gene; TulCub8, *T. cruzi* clone with monoallelic deletion of *cub* gene; FBM, fresh blood mount; LIT, liver infusion-tryptose medium; WT, wild-type; PCR, polymerase-chain reaction; RL, retransfected line

1. Introduction

Chagas disease is a highly endemic parasitosis of Central and South America, caused by the kinetoplastid hemoflagellate *Trypanosoma cruzi*. With the complete sequencing of the *T. cruzi* genome (reviewed by El Sayed et al., 2005), the cumulative studies on the genetics of this parasite are being integrated into an orderly collection of genes and mutants. The organism is basically dipliod, both haplotypes displaying a high level of synteny. Protein sequences have an average difference of 2.2% between alleles and most divergence occurs at the intergenic regions. The number of genes is close to 12.000, with an annotated dataset of 60.4 Mb per haploid genome. A putative function has been assigned to 50.8% of the proteins, on the basis of comparison with previously characterized proteins or known functional domains. Fifty percent of the genome is composed of repetitive sequences and there is a growing list of single- and multiple-copy genes.

The creation of mutants with deleted or modified genes is at present a useful tool for analyzing the relationships between specific functions, proteins and genes. As long as growth in culture can be maintained, the normal *in vivo* invasive mechanisms of *T. cruzi* lineages can be profoundly altered by genetic manipulation, interfering or eliminating the capacity for *in vivo* infection of insect vectors and mammalian hosts. Virulence or infectivity is a complex function involving the highly evolved ability of *T. cruzi* to transform into infective stages, penetrating epithelia and cells and evading the complex immunologic reactions of the mammalian host. These mechanisms are related to the expression of certain genes, loosely referred-to as ''virulence genes or factors''. Remarkably, a variety of genetic manipulations, altering

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apparently unrelated genes, affect the virulence of this parasite. Studies on a null mutant of the *gp72* gene of *T. cruzi* (Cooper et al., 1993; Ribeiro de Jesus et al., 1993) showed major alterations in the expression of the GP72 surface glycoprotein, the invasion of mammalian cells *in vitro* and the colonization of insect vectors. Both protein synthesis and functions were restored by ectopic expression of gp72 by an episome-based shuttle vector (Nozaki and Cross, 1994). Further studies (Basombrío et al., 2002) demonstrated the inability of the mutant to sustain infection in mice. Norris (1998) demonstrated that the gene expression of a complement-regulatory protein of *T. cruzi* (CRP) in trypomastigotes is a virulence factor associated with the acquisition of resistance to complement by transfected epimastigotes. Caler et al. (1998) observed that the deletion of the oligopeptidase B gene produced a significant attenuation of virulence for mice and a 70% reduction in the ability to invade mammalian cells *in vitro*. Manning-Cela et al. (2001, 2002), working with a null mutant of the *lyt* gene demonstrated the involvement of this gene in the hemolytic, extracellular activity and the regulation of the intracellular life-cycle of *T. cruzi*. As shown with the *gp72* and *lyt* genes, elimination of a single allele often produces an intermediate degree of alteration of gene products (haploinsufficiency), as compared with complete elimination of both alleles. Metacyclogenesis and the infective ability of *T. cruzi*, was reduced, both *in vitro* and *in vivo*, by deleting one copy of *tc-52*, a thioredoxin and glutaredoxin-coding gene (Allaoui et al., 1999). The product of *tc-52* is an important factor regulating the immune response and the course of infection (Garzon et al., 2003).

Chung and Swindle (1990) identified two calmodulin and ubiquitin gene clusters in *T. cruzi*. These clusters are members of a highly conserved family of genes in kinetoplastids. This group of genes was assigned to allelic loci, referred-to as 2.65 and 2.8, after the kb length of BglII restriction fragments containing the gene. In the intergenic region between these two gene families, Ajioka and Swindle (1996) identified a single-copy gene, named calmodulin–ubiquitin related gene (*cub*). The 2.65/2.8 locus organization included, sequentially, a tandem of calmodulin genes, followed downstream by a single copy of the *cub* gene, by the ubiquitin-fusion gene and by the polyubiquitin locus (Fig. 1a). Cub's open reading frame is transcribed by trans-splicing and polyadenylation and is translated, encoding a 208 aminoacid polypeptide with calcium-binding domains (NCBI protein Databank, Accession No. AAA3017), homologous to the EFH5 protein of *T. brucei*. The specific function of *cub* is unknown. However, deletion experiments (Ajioka and Swindle, 1993, 1996) demonstrated that the *cub* gene is actively expressed and essential for parasite viability. Although either *cub* allele is individually dispensable, both can be deleted only when an additional copy is expressed from an alternative locus.

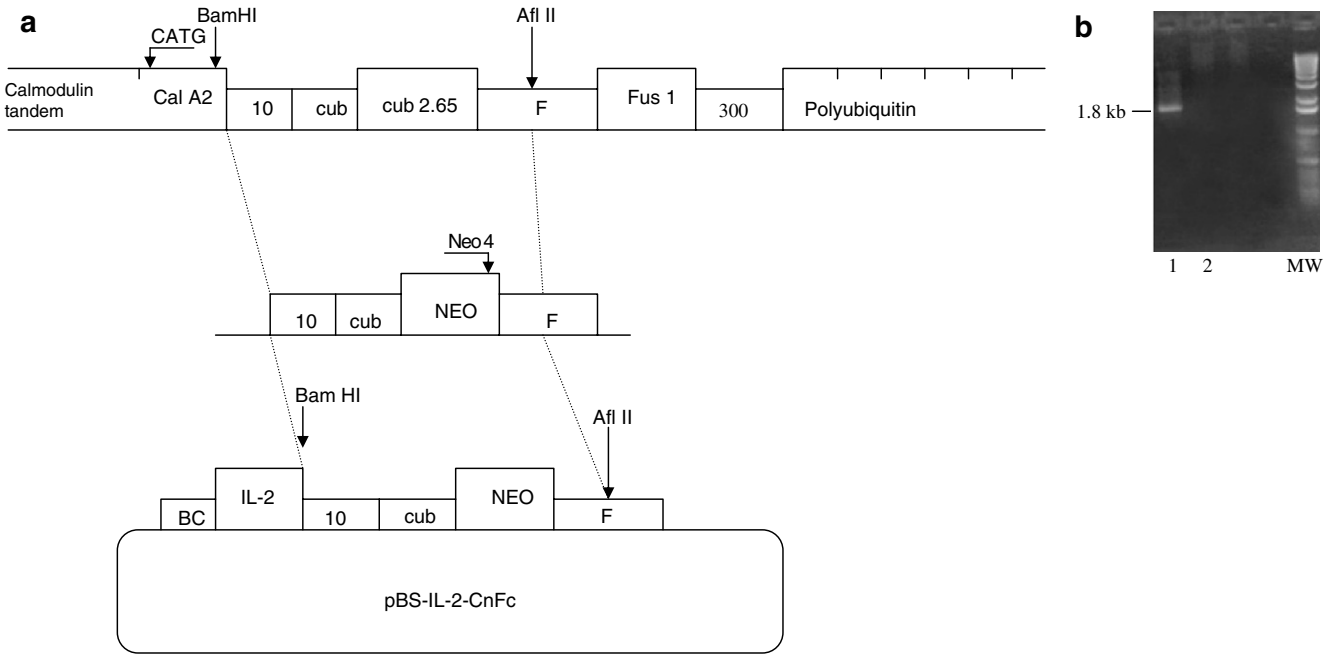

Fig. 1. (a) Diagram of relevant regions of the calmodulin–ubiquitin 2.65 locus and of plasmid pBS-IL2-CnFc, with the restriction sites selected for obtaining a 2.3 kb fragment, used for homologous recombination. Genes are shown as tall rectangles; intergenic regions are shown as flat rectangles. Restriction sites are indicated by straight arrows and primer annealing sites, by bent arrows. Explanation of *T. cruzi* genes is given in Section 1. Adapted from la Flamme et al. (1996) with permission from the authors. (b) PCR test of genomic integration of *neo* into the CUB locus of TulCub8 using primers CATG and NEO4. The hybrid segment is amplified in TulCub8 (lane 1) and not in Tulahuen WT (lane 2); MW, molecular weight marker.