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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.

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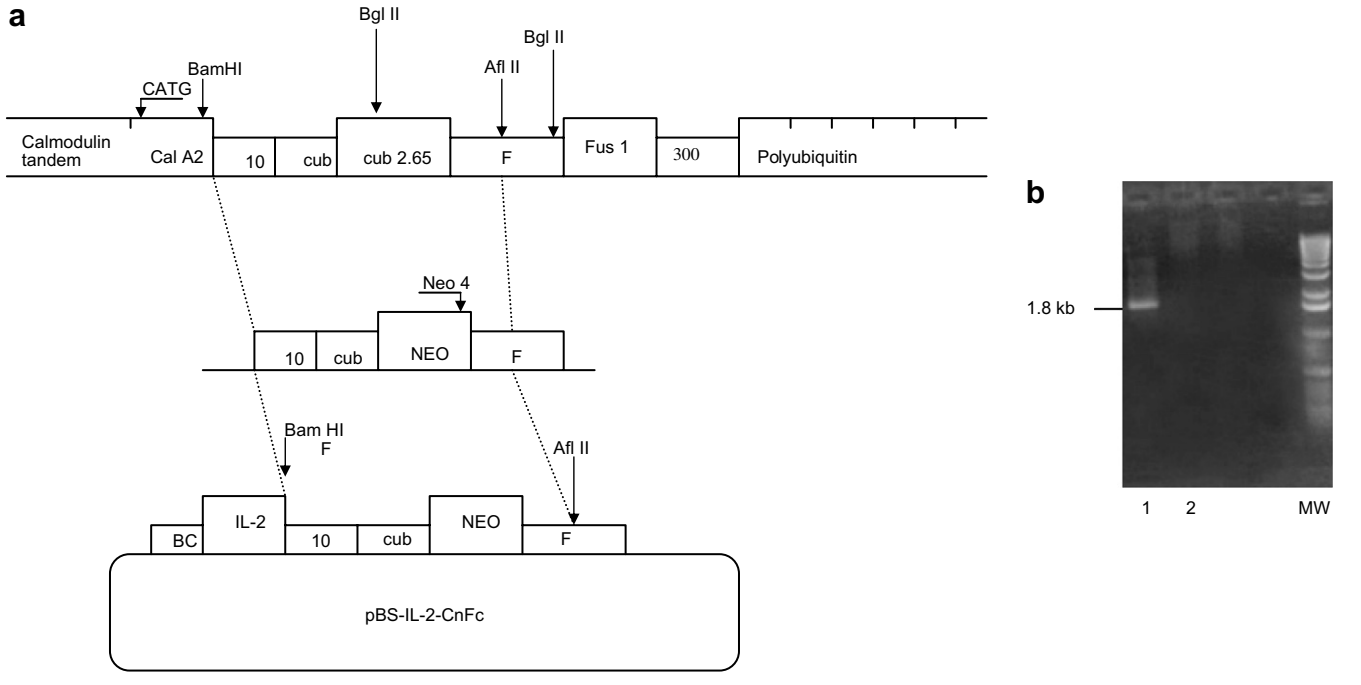

Fig. 1. (a) Diagram of relevant regions of the calmodulin–ubiquitin 2.65 locus and of plasmid pBS-IL2-CnFc, with the restriction sites selected for obtaining a 2.3 kb fragment, used for homologous recombination. Genes are shown as tall rectangles; intergenic regions are shown as flat rectangles. Restriction 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.

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Calmodulin, through its association with calcium, plays a critical role in many cellular processes. Ubiquitin is a multifunctional protein, which can target proteins for degradation (Chung and Swindle, 1990). The association of *cub* with these two genes and the fact that at least one copy is indispensable for parasite survival, point to the broad role that this gene may play in essential cellular functions. Moreover, being a single-copy gene, it represents a convenient target for genetic analysis.

The original purpose of this work was to explore the potential of targeted mutants of *T. cruzi* as experimental live vaccines for studies on protective immunity and clonal interactions in pathogenicity. In particular, we attempted to exploit the advances in the molecular identification of monoallelic deletions of *cub* to analyze alterations in infectivity occurring in the highly virulent Tul strain. Since disruption of one allele produced a highly attenuated transfectant, we tested the stability of the virulence/attenuation phenotype after culture and mouse passage, and the *in vivo* protective capacity of an attenuated, single-allele disrupted clone.

2. Materials and methods

2.1. Mice

Inbred Balb/c and outbred Swiss mice, bred in our colony following adopted institutional procedures of care and maintenance (Grossblatt, 1996) were used.

2.2. Parasites

The Tul strain, a Type IIe *T. cruzi* lineage derived from the Tulahuen strain was used. This strain is highly virulent and has been propagated for over 25 years in our laboratory. All parasites used in this work belong to a previously cloned population. Standard procedures (Basombrío et al., 1982) were used for mouse passage, measurement of parasitemia levels in fresh blood mounts (FBM) and hemoculture. Parasites were serially cultured in glass hemolysis tubes at 29 °C, containing 2 ml of liver infusion-tryptose medium supplemented with 20 μg hemin, 100 IU of penicillin and 100 μg streptomycin per ml (LIT). The monoallelic *cub* deletion mutant clone described here is named TulCub8, and after retransfection with a complementing cub copy, it is named RL.

2.3. Virulence tests

Culture epimastigotes were transformed into infective trypomastigotes by adding 0.25% triatome intestinal homogenate to the culture for 10 days and eliminating the remaining epimastigotes by complement-mediated lysis (Isola et al., 1986). Equal numbers of wild-type or genetically altered trypomastigotes were inoculated by intraperitoneal route (ip) into mice and the level of parasitemia was measured in FBM and compared between groups.

2.4. Plasmids

The pBS-IL2-CnFc plasmid (la Flamme et al., 1996) was digested with BamH1 and AflII enzymes in two linear fragments of 2.3 and 6.0 kb, respectively. A neo:cub 2.3 kb fragment, containing the *neo* gene flanked by the *cub* intergenic regions was gel-purified (GenecleanTM protocol) and used in electroporation to replace one copy of the *cub* gene by the *neo* gene. *Cub*'s coding sequence and the immediate flanking untranslated regions (utrs) was amplified by PCR using primers Cub1 (5′-CTGTTTGCCTGTTCACT-3′) and Cub2 (5′-CCTAACAACGTTCATATTT-3′) and 1 μg of *T. cruzi*, Tul strain genomic DNA as template. The 1.3 kb amplification product of *cub* was cloned into the pGem-T plasmid vector (Promega). The resulting construct, pGem-T:1.3 was sequenced, amplified and used for transfection of *cub* into *T. cruzi* after linearization with Not1 enzyme.

2.5. Electroporation, selection and cloning of trypanosomes

Ten micrograms of the linear 2.3 kb neo:cub fragment was used for electroporation to replace one allele of *cub* in WT epimastigotes (la Flamme et al., 1996), under the following conditions: BTX electroporator, 330 V, 400 μF capacitance, 25 Ω in a 0.4 ml cuvette. After keeping the parasites at 4 °C for 10 min, they were seeded into LIT medium. After 48 h, 250 μg/ml of antibiotic G-418 (geneticin) was added and replaced at 2-week intervals. The surviving parasites were cloned by limiting dilution in absence of G-418 in multiwell plastic plates (Falcon). Viable cultures were expanded and tested for resistance to 500 μg/ml G-418. For the reconstitution assay, 100 μg of the linearized p-GemT:1.3 cub plasmid was electroporated into TulCub8 under the above described conditions. After the electroporation, the RL parasites were transformed into trypomastigotes and inoculated into mice.

2.6. PCR

A standard PCR protocol (Sambrook and Russel, 2001) was followed.

2.7. Southern blot

The blotting, hybridization and washing conditions used in Southern analysis were as described previously for the nylon membrane protocol (Sambrook and Russel, 2001). The 625 bp cub coding sequence probe was obtained by Pml1 digestion of pGem-T1.3 and radiolabeled with 32P by random priming. Alternatively, a digoxigenin-labeled probe (DIG DNA labeling and detection Kit, Roche) was used.

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2.8. Statistical analysis

The significance of differences between parasitemia levels was evaluated with the Mann–Whitney test for single-day measurements and with the Wilcoxon test for time-course plots.

3. Results

3.1. Similarity of cub sequences between CL and Tul T. cruzi strains

Using primers complementary to sequences in the flanking regions of *cub*, a fragments containing the 625 bp *cub* gene were amplified from Tul strain. The size of this fragments was 1.3 kb and was sequenced for comparison to the already known cub gene of the CL strain (2.65 and 2.8 alleles). The similarity of the *cub* coding region was shown to be 95.5% between both alleles of the CL strain. The Tulahuen sequence had 94.7 and 97.7 similarity with the 2.65 and the 2.8 CL *cub* alleles, respectively.

3.2. Transfection, selection and characterization of the cub monoallelic deletion in T. cruzi

The purified 2.3 kb fragment from the pBS-IL2-CnFc plasmid was electroporated into Tulahuen-strain epimastigotes. After placing the transfected parasites in 250 μg/ml G-418, sham (no DNA)-electroporated parasites died, but those transfected with the 2.3 kb neo:cub fragment recovered and multiplied. To test whether this fragment was inserted in genomic DNA or was propagated as episome, a PCR was run using forward primer CATG (5′-ATGGCTGATCAACTGTCCAA-3′) homologous to the first 20 nucleotides of the codifying region of CalA2 gene, and reverse primer NEO4 (5′-TGTTGTGCCCAGT CATAGCC-3′), homologous to the NEO-codifying region (Fig. 1a). If genomic insertion occurred, a 1.8 kb product was expected from PCR, and it was indeed found (Fig. 1b). Furthermore, the deletion of the 2.65 cub allele was shown by Southern blot (see below and Fig. 4a).

3.3. Virulence tests

T. cruzi strains Tulahuen WT and TulCub8 were transformed to infective stages and either 10^3 or 10^6 trypomastigotes were inoculated ip into either adult Swiss 3-month-old female or highly susceptible, Balb/c, 2- to 7-day-old mice. Table 1 summarizes the peak parasitemia levels, occurring between days 12 and 18 in all groups. A clear reduction was found in the TulCub8 group, reaching clearly lower maximum levels of parasitemia than WT in all experiments. Parasitemia levels after inoculation of adult mice with low numbers (10^3) of TulCub8 trypomastigotes were reduced to zero ($p = 0.0057$) and 14-fold ($p = 0.035$) in the adult and newborn mice, respectively. The corresponding reductions for the high dose (10^6) were 68-fold ($p = 0.018$) and 27-fold ($p < 0.002$), respectively. Infections with WT, but not with TulCub8 were lethal in newborn mice.

Table 1
Compared infectivity of complement-resistant trypomastigotes from Tulahuen wild-type and cub-deleted clone TulCub8 after inoculation into mice

Inoculum and mice	Peak parasitemia (*T. cruzi*/100 fields) ± standard deviation		p^5
	WT	TulCub8	
10^3 T in ASM	16.5 ± 1.7 ($n = 5$)	0.0 ± 0.0 ($n = 5$)	0.0057
10^6 T in ASM	27.3 ± 2.6 ($n = 6$)	0.4 ± 0.1 ($n = 7$)	0.018
10^3 T in NBBM	82.7 ± 18.9 ($n = 7$)	6.0 ± 0.6 ($n = 5$)	0.025
10^6 T in NBBM	6512 ± 1478 ($n = 6$)	237 ± 126 ($n = 12$)	0.002

T, complement-resistant trypomastigotes. ASM, adult Swiss mice. NBBM, Newborn BALB mice; *p* values, calculated with Mann–Whitney "U" test. Peak parasitemia was observed between days 12 and 18 post inoculation. ASM survived infections. Most infections in NBBM were lethal.

3.4. Restoration of virulence after retransfection of the missing allele

A vector to retransfect the cub gene back into TulCub8 was constructed by ligation of the 1.3 kb fragment, containing the cub gene and flanking utrs of Tulahuen *T. cruzi*, into pGem-T. This plasmid (named pGem-T1.3 cub) did not contain a drug-selectable marker. Selection of transfectant parasites was not based on antibiotic pressure, but instead on preferential survival of virulent progeny in the living animal. The plasmid was linearized and 100 μg were electroporated into 10^9 TulCub8 epimastigotes. The retransfected cultures (RL) were subsequently transformed, passaged in mice and characterized by Southern blot. Selection of virulent progeny was obtained by serial, *in vivo* passages in BALB mice. Four such passages were serially inoculated (Fig. 2a–d). At each passage, trypanosomes were recovered by hemoculture, transformed and inoculated simultaneously and in uniform dose (10^5 trypomastigotes ip) into the next group of animals. After inoculation, the level of parasitemia was measured in FBM twice a week, to allow parallel comparison between TulCub8 and the retransfected line (RL). In the first passage (Fig. 2a) the levels of parasitemia of RL approximately tripled those of TulCub8 ($p < 0.002$). In passages B, C and D (Fig. 2) the peaks of parasitemia became higher in RL, whereas in TulCub8 they remained almost undetectable by FBM ($p < 0.0001$), even though the parasites could still be recovered by hemoculture. Although the "restored" lines were clearly more virulent than the mutant, they were never as virulent as WT. Typical average peak parasitemias (*T. cruzi*/100 fields) were: 100 for WT, 0–1 for the mutant and 7–35 for the "restored" lines. Thus, *cub* retransfection apparently restored virulence and this effect remained stable after serial *in vivo* passage of mammalian blood stages. TulCub8 never regained a virulent phenotype without retransfection to complement the missing *cub* allele, even

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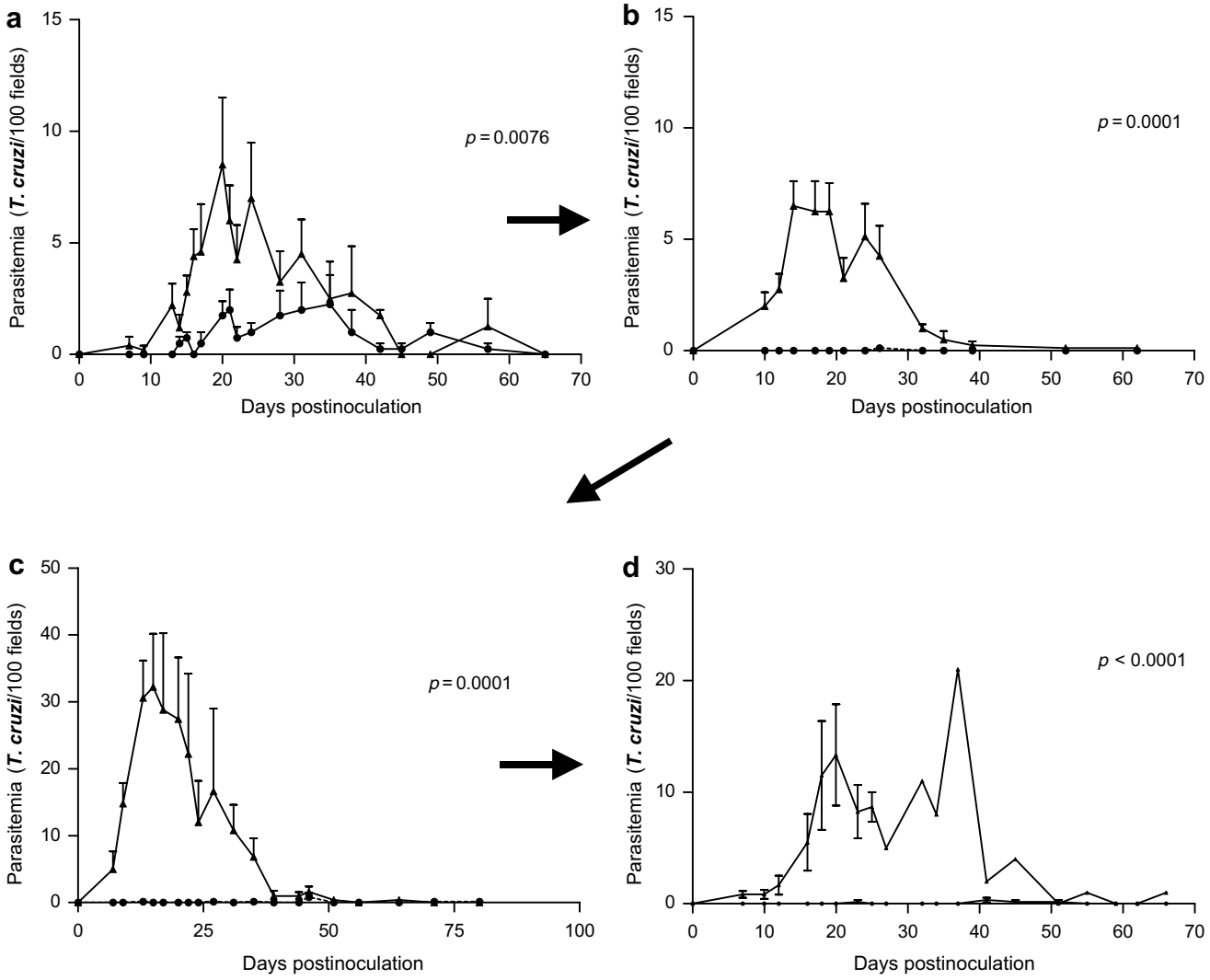

Fig. 2. Recovery of virulence by retransfection of *cub* gene and stability of virulent and attenuated phenotype in Tulahuen strain *T. cruzi* (·····•·····: TulCub8; —▲—: retransfected (RL) strain). (a) Levels of parasitemia after inoculation, into newborn BALB mice, of 10^4 *T. cruzi* trypomastigotes, previously transfected and transformed in culture. (b–d) Further serial mouse passages, where levels of parasitemia were measured after inoculation of parasites recovered by hemoculture from the previous passage. All inocula, in (b), (c) and (d) were standardized at 10^5 trypomastigotes ip. In (d), group RL, a single mouse survived after day 25. Note the stability, after serial passage, of the attenuated phenotype in TulCub8, and of the virulent phenotype in the *cub*-retransfected line.

after multiple mouse passages. A confirmatory experiment was done where the fourth, low virulence passage of TulCub8 (Fig. 2d) was recovered by hemoculture, retransfected with plasmid pGem-T1.3 cub and reinoculated into mice (Fig. 3). Again, there was an increase, 8-fold this time, of the level of parasitemia after retransfection.

3.5. Southern blot analysis of cub-deleted and retransfected *T. cruzi* lines

To demonstrate the deletion of the 2.65 *cub* allele in the different *T. cruzi* lines, a Southern blot analysis was carried out using the *cub* and *neo* probes for hybridization. Genomic DNA's from wild-type strains Tul, CL, TulCub8 and RL were digested with enzyme BglII (Ajioka and Swindle, 1993). The wild-type parasites displayed both 2.65 and 2.8 bands but TulCub8 appeared to lack a 2.65 kb band

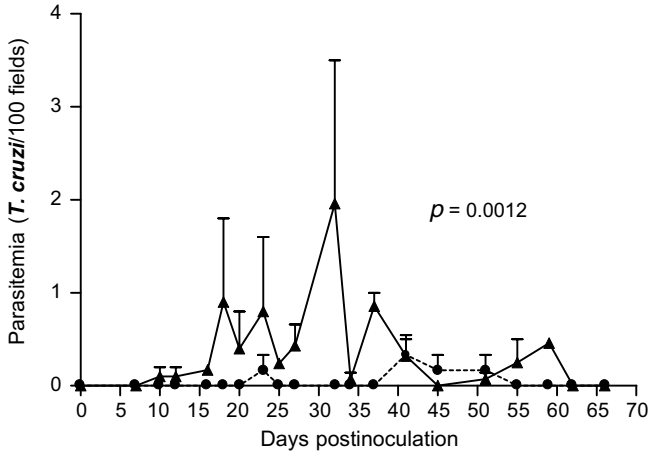

Fig. 3. Low levels of parasitemia of a fourth mouse passage of TulCub8 (·····•·····: $n = 6$), and increased infectivity ($p < 0.019$) after retransfection with *cub* gene (—▲—: $n = 12$).

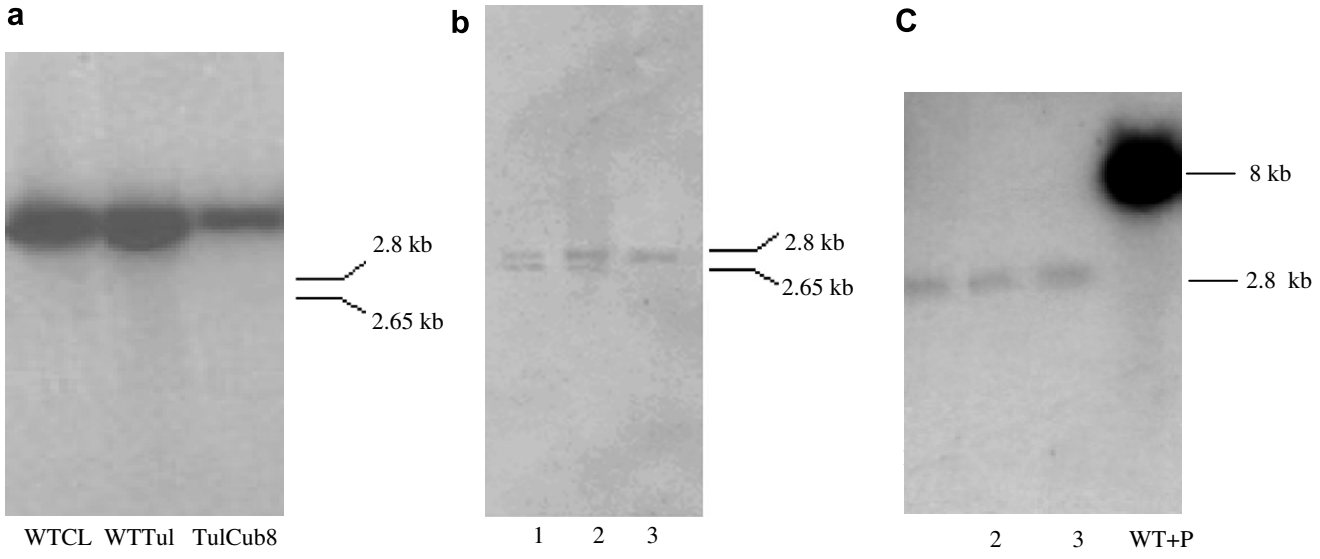

Fig. 4. Southern blot analysis of transfected *T. cruzi* lines. Parasite DNA was digested with BglII and hybridized with either CUB (a and b) or NEO (c) probes. (a) Hybridization with CUB probe. WTCL and WTTul are wild-type *T. cruzi* strains; TulCub8 is the transfected clone. Note thick band in WT, indicating the presence of both 2.8 and 2.65 alleles. In TulCub8 a single 2.8 band indicates the deletion of one *cub* allele. (b) Mouse-passaged, retransfected lines (RL). Digested DNA was hybridized with a digoxigenin-labeled *cub* probe. Lanes 1 and 2: two retransfected lines, RL1 and RL2, respectively; lane 3: TulCub8. (c) Hybridization with NEO probe. Lanes 1, 2 and 3 are three different TulCub *T. cruzi* populations selected in G-418 after transfection with the 2.3 kb neo:cub fragment. WT + P is a transfection control run in which parasites were transfected with the whole plasmid pBS-IL2-CnFc, which replicated episomally, generating a thick signal. Note genomic integration and apparent lack of episomal replication after retransfecting a linear vector as opposed to the complete plasmid. The size of the hybridization band in the transfected lines indicates the replacement of *cub* by *neo*.

(Fig. 4a). When DNA of the retransfected lines (RL1 and RL2) was hybridized with the *cub* probe, the mentioned 2.65 signal was again detectable (Fig. 4b). When a *neo* probe was hybridized to BglII digests of transfected lines and wtTul, the hybridization signal was found only in the 2.8 position of the transfected TulCub populations (Fig. 4c). The hybridization signal was placed in a 2.8 kb position because the *neo* gene is 159 bp longer than the replaced 2.6 *cub* allele. This indicated that the neo gene was inserted in the calmodulin–ubiquitin locus, replacing the 2.6 allelic copy of the *cub* gene.

3.6. Protective immunization with attenuated clone TulCub

Since the TulCub8 mutant displayed much lower infective capacity than the WT, we next explored whether it would retain, in spite of being attenuated, the protective ability against virulent challenge. Swiss, 40-day-old female mice were inoculated subcutaneously on days 0 and 7 with 10^3 TulCub8 epimastigotes. On day 38 these animals, together with naive, sham-preinoculated controls, were challenged ip with 10^6 WT Tulahuen strain blood trypomastigotes. Parasitemia was measured in FBM twice a week in all animals. Mice preinoculated with TulCub8 epimastigotes were strongly protected against WT challenge, peak parasitemias being 0.19 ± 0.1 in vaccinated versus 85.1 ± 16.3 in controls ($p < 0.0004$). Wild-type TUL cultures were also as "protective" as TulCub8 against secondary challenge but, in this case, resistance was achieved at the expense of a highly virulent, primary infection.

4. Discussion

The results here presented show that a monoallelic disruption of the *cub* gene markedly attenuates the infectivity of a highly virulent *T. cruzi* strain. Previous studies (Chung and Swindle, 1990; Ajioka and Swindle, 1993, 1996) established the molecular framework of the calmodulin–ubiquitin locus of *T. cruzi* and developed the vectors for monoallelic replacement and characterization of mutants. This gene was shown to be a valuable tool for genetic analysis, since it is represented by a single, diploid copy and is essential for parasite viability. *In vitro* propagation of mutants is dependent on the conservation of at least one *cub* copy, after replacement. Even the elimination of both native alleles has been possible, provided that the *cub* coding sequence was placed at a different genomic site. Although in those studies (Ajioka and Swindle, 1996) the monoallelic disruption of cub did not affect the *in vitro* growth of the parasites, we here show that this gene participates in the phenotypic expression of virulence. Both copies of the gene are necessary for high parasitemia and serial *in vivo* blood passage. The monoallelic mutant can only be propagated in mice by means of highly sensitive, hemoculture recovery. Although our work was restricted to *in vivo* virulence assays and Southern blot characterization, transcription analysis through Northern blot was done by Ajioka and Swindle (1996), demonstrating that the transcription product of the gene replacement site in the mutant is different from cub transcripts of wild-type parasites.

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Transfection and selection in *T. cruzi* is tricky. Strains are typically comprised of individual clones having different pathogenic potential, so serial passage and selection can bring out a particular clone. To minimize these confounding alternatives, we cloned the original, WT strain and the TulCub8 mutant before characterization. Moreover, *in vivo* inoculations/passages were controlled by standardizing the stage and dosage of inocula and by keeping parallel, comparative mouse passages of mutant and WT as reference, in transfected lines.

Four patterns of parasitemia were found after infecting mice with *T. cruzi*, genetically modified at the cub locus. (1) TulCub8 displayed drastically reduced parasitemia levels, as compared with the WT. (2) Serial passages of TulCub8 did not increase virulence. (3) TulCub8 retransfected with cub displayed a pattern of low, sporadic parasitemia. (4) Serial passages of retransfected TulCub8 progressively raised more virulent sublines, not reaching the level of the WT.

The difference in infectivity between TulCub8 and WT was detected across a wide range of infective doses and levels of host susceptibility. Infections with WT *T. cruzi* varied from massive parasitemia and acute death (10^6 trypomastigotes in newborn Balb/c mice) to the presence of barely detectable parasites in blood (10^3 trypomastigotes in Swiss mice). In each of the 4 host/dose combinations tested, TulCub8 trypomastigotes were less infective, by one or more orders of magnitude, down to the absence of detectable infection, with inocula of 10^3 TulCub8 trypomastigotes in adult Swiss mice.

Recovery of virulence after retransfection of the 1.3 *cub* plasmid appeared to be a slow process. This may have been due to stepwise selection of virulent variants from mixed populations after retransfection and optimal *cub* gene expression *in vivo*. The shift from low to high virulence did not happen spontaneously, even after multiple mouse passages, but appeared to be primarily driven by the presence of both *cub* copies. Moreover, variants of low virulence in TulCub8 or of high virulence in RL were favored by antibiotic pressure and virulence-driven selection, respectively. *In vitro* growth under geneticin pressure first produced a parasite population, and subsequently a clone (TulCub8) bearing the monoallelic deletion. The first inoculation of trypomastigotes from these cultured parasites into mice, revealed a sharp difference in infectivity between the clone and the wild-type *T. cruzi* strain. The reverse process, e.g., recovery of virulence by retransfection of additional *cub* copies, was only driven by the intrinsic ability for *in vivo* infection in the retransfected parasites. No antibiotic-selectable markers were used in this process. During the electroporation and transformation procedures, many parasites were lost and the virulent progeny was slowly recovered from infected mice, requiring serial mouse passage before regaining high virulence. That this recovery required additional *cub* copies and was not solely the result of serial mouse passage, was evidenced by the stable attenuation of the non-transfected TulCub8 clone which, in spite of parallel mouse passage and transformation, retained the attenuated phenotype.

Upon inoculation and serial passage in mice, the retransfected line displayed fluctuations contrasting with the rather smooth, bell-shaped pattern of parasitemia induced by laboratory strains stabilized by long-term passage. The process of *in vivo* selection after a recent transfection event may in part account for the observed fluctuations. After the first inoculation into mice of TulCub8 retransfected with cub (Figs. 2a and Fig. 3), parasites became sporadically visible in blood, following a pattern of low, delayed parasitemia. The difference between the patterns observed in two trials may reflect a higher proportion of retransfected parasites in the first (Fig. 2a) than in the second trial (Fig. 3). Between passages B and C, selection of virulent variants was apparent, C displaying a 5-fold higher parasitemia level. Moreover, in passage D, most infected animals died. An apparent double peak of parasitemia was generated by a single survivor, who displayed a delayed peak.

Reproducible reductions of the infective function of *T. cruzi* have been obtained in previous studies after targeted deletion of apparently unrelated, single-copy genes (Caler et al., 1998; Manning-Cela et al., 2001; Ribeiro de Jesus et al., 1993; Basombrío et al., 2002; Garzon et al., 2003). One of these mutants (Garzon et al., 2003) is comparable to TulCub8 in the fact that a single allele of a diploid gene (Tc-52) was also disrupted, causing impairment of virulence in spite of the presence of a single-allelic copy. The term "virulence factor" has been applied to gene products where a functional component of infectivity, such as cell invasion or immune evasion, has been proven. Proteins such as gp72 (Basombrío et al., 2002), Tc-52 (Garzon et al., 2003), complement-regulatory protein (Norris et al., 1998) or oligopeptidase B (Caler et al., 1998) of *T. cruzi* have been implicated in infectivity roles. No such functions have so far been proven for the 208 aa polypeptide encoded by *cub*. Therefore, the sharp abrogation of virulence here found after monoallelic disruption may well respond to regulatory effects on the calmodulin–ubiquitin locus, or on housekeeping genes required for invasion of the living mammal.

Measurement of parasitemia levels may allow an approximate comparison of the degree of attenuation obtained after different genetic manipulations of *T. cruzi*. In a *gp72*-null, biallelic mutant (Basombrío et al., 2002) the loss of long-term infectivity was apparently complete, as shown in athymic mice or immunocompetent animals tested for long periods after inoculation. A biallelic, single-gene knockout (L16) lacking both alleles of the *Lyt-1* (porin) gene also had a marked reduction of virulence, comparable to that observed in TulCub8 (unpublished results). Compared to these changes, studies on the Tc-52 −/+ mutant displayed a reduction of virulence of rather low magnitude (<4-fold in parasitemia levels), but showed that the animals infected with the mutant failed to induce the downregulation of T-cell proliferation and IL2 production which occurred in the wild-type infections.

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Non-lethal infections with virulent *T. cruzi* induce a strong and long-lasting protection against subsequent virulent challenge (Basombrio et al., 1992). Knowing that TulCub8 is attenuated and has a specific genetic alteration, we developed an interesting model of protective immunization. We injected mice with 10^6 epimastigotes (non-infective forms for mammalian hosts) of TulCub8, followed by challenge with infective, WT *T. cruzi*. A strong protective effect against virulent infection was observed, mirroring previous work (Basombrío et al., 1982, 1993), which demonstrated the vaccine effect of epimastigotes of the TCC, live-attenuated *T. cruzi* strain. The use of live epimastigotes from *T. cruzi* mutants obtained by genetic engineering opens a new perspective for the analysis of pathogenicity through the interaction of attenuated and virulent *T. cruzi* clones. This interaction may be highly relevant for natural parasite spread. The genetic basis for attenuation, which was unknown in spontaneously attenuated strains such as TCC (Basombrío et al., 1982) or Cl 14 (Lima et al., 1990), may now be characterized genetically and biochemically in targeted mutants. Moreover, dual or multiple deletions can be added to *T. cruzi* progenies, as a safety mechanism against reversion to the virulent phenotype.

5. Uncited reference

Wong et al. (1992).

Acknowledgments

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