Molecular & Biochemical Parasitology 198 (2014) 71–74

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Short technical report

Conditional removal of selectable markers in *Trypanosoma cruzi* using a site-specific recombination tool: Proof of concept

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ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 12 January 2015
Accepted 14 January 2015
Available online 22 January 2015

Keywords:
DiCRE
loxP
Selectable markers removal
Conditional knockout
Chagas disease

ABSTRACT

The scarcity of molecular tools for genetic manipulation is a critical obstacle for functional genomics studies on *Trypanosoma cruzi*. The current study adapted an inducible site-specific recombination system based on Dimerizable CRE recombinase (DiCRE). Two vectors for stable transfection were created, a first one to express inactive portions of DiCRE recombinase, and a second plasmid containing the *lox*P sites to test DiCRE activity. After integrating both constructs into the *T. cruzi* genome, it was shown that DiCRE recombinase can be efficiently used to manipulate its genome by allowing the removal of selectable markers thus generating homogeneous populations. The DiCRE recombinase success allows conditional knockout and the removal of selectable markers without prior parasite modification, which also facilitate the transferring of DiCRE recombinase to different *T. cruzi* strains.

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease, affecting from 7 to 8 million people worldwide [1]. Functional genomics approaches are very useful for understanding key mechanisms such as host parasite interaction, drug resistance and parasite differentiation, and they can allow the development of alternative therapies to Chagas' disease. However, the list of approaches to perturb gene expression in *T.cruzi* is reduced, since many proteins are encoded by multicopy genes, and the RNAi system is not functional [2,3]. Furthermore, there are only few options of resistance genes to promote genomic modifications in *T. cruzi*. The establishment of molecular tools that allow the inducible excision of large DNA segments to generate knockouts or to remove selectable markers is a remarkable necessity for improving molecular biology research on *T. cruzi*.

The CRE recombinase system is a versatile tool in which the recombinase catalyzes the recombination between two *lox*P sites, thus promoting the excision or inversion of the *lox*P flanked DNA [4–6]. Despite its feasibility in several organisms, the CRE recombinase expression shows some degree of toxicity and it reinforces the necessity of a tight control of its expression. With respect to *Trypanosoma brucei*, Barrett et al. (2004) expressed CRE recombinase by using a GPEET promoter controlled by Tet repressor. However, CRE activity and toxicity were detected even in the absence of tetracycline [6]. In order to improve the CRE expression regulation, several modifications were introduced by Scahill and cols. [4]. Although there were some concerns about the regulated expression, the CRE recombinase system has been successfully used to manipulate gene expression or to remove selectable markers in *T. brucei* [4,6,7]. These scenarios prompt us to test the regulatable CRE recombinase to achieve genomic manipulation in *T.cruzi*. Nonetheless, the T7RNA polymerase/TetR inducible expression system has shown a high leakage rate, as it was detected by different approaches [8,9]. For this reason, we considered the dimerizable CRE recombinase (DiCRE) system as an alternative regulatory method. The DiCRE system was developed by splitting CRE recombinase into two inactive portions, where the CRE activity can be restored by rapamycin addition [10]. DiCRE has been successfully used in mice, *Toxoplasma gondii*, and *Plasmodium falciparum* [11–13].

Aiming to adapt the DiCRE system to *T.cruzi*, two plasmids were built in pROCK vectors [14], which use gGAPDH untranslated sequences for mRNA processing. The first plasmid, named pODiCRE-Hygro (Fig. 1A), contains the coding regions for DiCRE_59.F2, DiCRE_60.F2 [10], and the Hygromycin B resistance gene. The second plasmid, named pROCK-FEKO-PUR-Neo, works as an indicator construct for CRE recombination. This plasmid

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http://dx.doi.org/10.1016/j.molbiopara.2015.01.001
0166-6851/

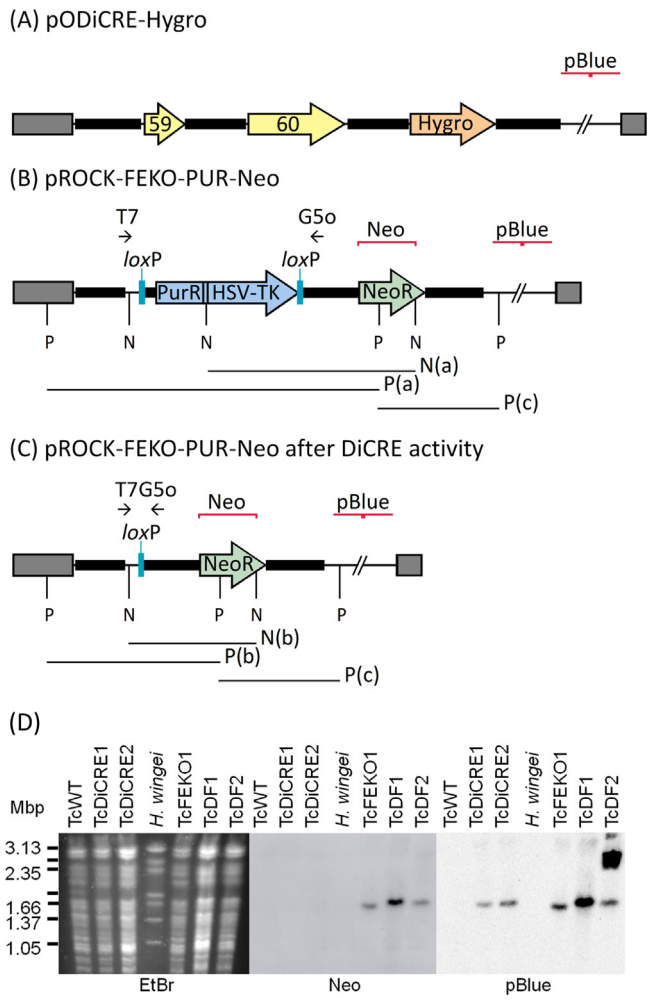

Fig. 1. Construction of DiCRE recombinase system in *T. cruzi*. (A) Schematic representation of plasmid pODICRE-Hygro linearized with *Not*I. Grey boxes represent β-tubulin fragments for plasmid integration. Intergenic regions of GAPDH (narrow black boxes) regulate gene (arrows) expression. Both portions of DiCRE genes are represented by yellow arrows with the number 59 indicating DiCRE_59.F2, and 60 indicating DiCRE_60.F2 [13]. Hygromycin resistance gene is represented by orange arrow. The red bar indicates the region used as probe pBlue, which is a 692 bp fragment of the vector backbone. (B) Schematic representation of tester plasmid pROCK-FEKO-PUR-Neo linearized with *Not*I. The blue arrow represents the gene fusion of puromycin resistance gene and the HSV-TK gene, which is flanked by two *lox*P sites, showed as blue bars. Red bars indicate pBlue probe, also represented at pODiCRE-Hygro, and Neo probe, which consists in a fragment from nucleotide 1 to 541 of the Neomycin resistance gene. In parasites containing both pODiCRE-Hygro and pROCK-FEKO-PUR-Neo integrated plasmids, DiCRE becomes active after rapamycin addition and it promotes the excision of the floxed DNA leaving a single remaining *lox*P. The vector after recombination is represented in (C). This excision can be detected by Southern blot performed by using genomic DNA digested with *Nco*I and *Pvu*II and revealed with ^{32}P-dCTP labeled Neo probe. The fragment sizes detected with Neo probe are depicted by black bars named N(a) and N(b), for *Nco*I digestion, before and after recombination, respectively. For *Pvu*II digestion, bars are named P(a) and P(b), before and after recombination. The segment size of P(c) remains unshaken (internal control). Small arrows above vectors scheme B and C represent T7 and GAPDH5'out (G5o) primer positions flanking *lox*P sites. (D) Corroborating the integration of pODiCRE-Hygro and/or pROCK-FEKO-PUR-Neo constructs at tubulin locus of CL Brener genome. Chromosomal bands were separated by Pulsed Field Gel Electrophoresis (PFGE) and its blots were hybridized with Neo or pBlue ^{32}P-dCTP labeled probe, as previously described [17]. The obtained clones were TcDiCRE1 (HygroR), TcDiCRE2 (HygroR), TcFEKO1 (NeoR), TcDF1 (double resistant) and TcDF2 (double resistant). TcWT refers to wild type parasites. *H. wingei* chromosomal bands were used as weight marker.

was generated by transferring a DNA fragment containing two loxP sites flanking a sequence encoding Thymidine kinase (TK) fused to puromycin N-acetyl-transferase (PurR) from pyrFEKO-PUR [4] to a pROCK vector containing the Neomycin (G418) resistance gene (NeoR) (Fig. 1B). After *Not*I linearization, combinations of plasmids were integrated into the β-tubulin locus of *T.cruzi* CL Brener genome, thus generating Hygromycin resistant parasites (named TcDiCRE–parasites carrying pODiCRE-Hygro), G418 resistant parasites (TcFEKO–harbors pROCK-FEKO-PUR-Neo), and double resistant cultures (named TcDF–parasites carrying both constructs). All transfections were carried as described previously [14]. The drug resistant populations were cloned by serial dilution and subjected to PFGE followed by Southern blot hybridization using NeoR or part of the plasmid backbone as probe in order to ensure that all plasmids were integrated at tubulin locus. As shown in Fig. 1D, both constructs integrated at the same chromosomal band (1.66 Mbp), which is compatible with previous data on pROCK vectors [14,15].

Recombination tests were performed by induction with rapamycin and followed by Southern blot analysis. Initially, epimastigotes clones in exponential growth were incubated in rapamycin ranging from 10 nM up to 1 μM for different time periods (12–144 h). The Southern blot analyses were done by using genomic DNA from induced and non-induced clones digested with *Pvu*II, which cleaves the tester construct as indicated in the restriction maps (Fig. 1B and C). The blots were probed with ^{32}P-labeled NeoR gene and it allowed the detection of site specific recombinations by RFLP, as indicated by the bars named P(a), P(b) and P(c) (Fig. 1B and C). After such tests, the clones presented both recombined and non-recombined profiles, i.e., heterogeneous profile (Fig. 2A). Aiming to increase recombination rates, we tested whether fetal bovine serum (FBS) components were able to interfere in rapamycin induction, however, the presence or absence did not change the profile, as shown at the last panel of Fig. 2A. The increasing rapamycin concentration, induction time and the FBS removal led to some slight enrichment of the recombined population, but the high rate of non-recombined parasites hampers its use on dynamic approaches such as RITE (Recombination-Induced Tag Exchange) [16].

In order to obtain a homogeneous *T. cruzi* population after the activation of DiCRE, we planned to take advantage of the presence of the *Herpes simplex* virus thymidine kinase (*HSVTK*) gene in the fragment to be deleted. This gene confers sensitivity to the ganciclovir and it was assumed that only parasites that underwent successful recombination would grow in the presence of the drug [4]. Unfortunately, it seemed that *T. cruzi* carrying *HSVTK* gene was not sensitive to ganciclovir (data not shown). It corroborated previous findings which showed that *T. cruzi* epimastigotes are not responsive for the pro-drug [17]. These findings made the use of the herein described strategy impossible, however the development of defined media may allow its use in *T. cruzi*.

By facing the challenge of selecting homogeneous population of *lox*P recombined parasites, we decided to induce recombination during parasite cloning step. For that, *T. cruzi* clones containing both plasmids, named TcDF1 and TcDF2, were serially diluted in the presence of rapamycin (1 μM) and grown until the turbidities were compatible to 1×10^7 parasites per mL (~30 days). As depicted in Fig. 1B and 1C, the recombination removed from the genome both the puromycin resistance and the *HSVTK* genes, thus switching the recombined parasites from puromycin resistant to sensitive. This allowed us performing a simple and quick screening by exposing recovered parasites to puromycin antibiotic. All clones were diluted 20 times and split into two wells, one without and the other with puromycin (2 μg/mL). After 7–10 days of incubation in humid chamber, the growth inhibition and morphological modifications were followed by optical microscopy using wild type CL Brener and