Unraveling the roles of CRK1 and CRK3 in cell cycle progression, metacyclogenesis, and viability of *Trypanosoma cruzi*.

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The cell cycle is a conserved and tightly regulated process in eukaryotes, occurring in an ordered manner and controlled by cyclins and CDKs. In *T. cruzi*, the etiological agent of Chagas disease, CRKs are involved in cell cycle control. However, it remains unclear how these kinases function, what their substrates are during the cell cycle, or whether they participate in the transition between the parasite's life forms. Therefore, the main objective of this study was to investigate the dynamics of the CRK1 and CRK3 kinases throughout the cell cycle, during metacyclogenesis, and in the viability of *T. cruzi*. To this end, CRISPR/Cas9 technology was used to generate cell lines expressing CRKs fused to mNeonGreen and Myc tags, in order to evaluate their expression and localization throughout the cell cycle. Additionally, Hemi-knockout and Knockout (KO) lines were generated using CRISPR/Cas9 to assess the impact of CRK1 and CRK3 depletion and deletion on the viability of epimastigote forms. Hydroxyurea (HU) synchronization was employed to analyze CRK protein expression during the cell cycle by Western blot (WB), using an anti-Myc antibody. Immunofluorescence (IF) assays were performed to investigate the localization of CRK1 and CRK3 in epimastigote cell cycle stages, also using the anti-Myc antibody. Metacyclogenesis was induced in TAU 3AAG medium to obtain metacyclic forms of the parasite, which were likewise subjected to WB and IF analyses using the same antibodies. The results demonstrated that both kinases are constitutively expressed throughout the cell cycle, with no significant variation in expression ($p < 0.05$). However, they display distinct localization patterns. While CRK1 exhibits a cytoplasmic distribution, lacks nuclear signal, and shows partial association with the mitochondria, CRK3 displays dynamic changes in localization: it translocates from the cytoplasm to the nucleus in G1, from the nucleus to the cytoplasm in S phase, remains predominantly cytoplasmic in G2/M with a perikinetoplast signal, and displays a perinuclear signal at the end of the cycle (cytokinesis). During metacyclogenesis, CRK1 expression is maintained, whereas CRK3 disappears at the end of the differentiation process. Likewise, CRK1 signal is detected in these non-replicative forms, with no detectable CRK3 signal. Partial deletion (Hemi-KO) of CRK1 and CRK3 in epimastigote forms did not impair cell viability, maintaining growth similar to controls. In contrast, complete deletion (KO) was lethal. CRK1-KO parasites lost viability within the first days after selection, whereas CRK3-KO parasites survived for up to two weeks, but without long-term culture maintenance. In conclusion, the data indicate that CRK1 and CRK3 are essential and functionally distinct kinases in *T. cruzi*. Although both are constitutively expressed, their differences in localization dynamics and behavior during metacyclogenesis suggest specific regulatory mechanisms and non-redundant roles in cell cycle progression and parasite viability.

Keywords: Regulation, kinases, cell viability, differentiation, trypanosomatid.