Antisense Oligonucleotides Targeting Parasite Inositol 1,4,5-Trisphosphate Receptor Inhibits Mammalian Host Cell Invasion by *Trypanosoma cruzi*

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Chagas disease is caused by an intracellular parasitic protist, *Trypanosoma cruzi*. As there are no highly effective drugs against this agent that also demonstrate low toxicity, there is an urgent need for development of new drugs to treat Chagas disease. We have previously demonstrated that the parasite inositol 1,4,5-trisphosphate receptor (TcIP3R) is crucial for invasion of the mammalian host cell by *T. cruzi*. Here, we report that TcIP3R is a short-lived protein and that its expression is significantly suppressed in trypomastigotes. Treatment of trypomastigotes, an infective stage of *T. cruzi*, with antisense oligonucleotides specific to TcIP3R decreased TcIP3R protein levels and impaired trypomastigote invasion of host cells. Due to the resulting instability and very low expression level of TcIP3R in trypomastigotes indicates that TcIP3R is a promising target for antisense therapy in Chagas disease.

The parasitic protist, *Trypanosoma cruzi*, is an etiologic agent of Chagas disease1. Human infection is initiated by invasion of infective, metacyclic trypomastigotes in the urine of blood-sucking reduviid bugs2. After invasion, the parasite transforms into amastigotes inside the host cells and begins to proliferate. Parasite proliferation is often accompanied by destruction of the vital tissues, such as heart muscle. Chemotherapy of Chagas disease relies exclusively on two drugs, benznidazole and nifurtimox, but their effects are limited and often evoke severe side effects. Therefore, development of new therapeutic measures are urgently needed.

Ca\(^{2+}\) serves as an second messenger of cellular signaling and its concentration is strictly maintained in the cytosol at 0.1 \(\mu\)M order3. Transient increase of intracellular Ca\(^{2+}\) concentration plays a crucial role for its functions and is mediated in response to both external and internal stimuli. D-myo-inositol 1,4,5-trisphosphate (IP3) is a second messenger generated by phosphoinositide phospholipase C (PI-PLC) upon external stimuli via cell-surface receptors4 and provokes activation of its receptor, IP3R. IP3R is a Ca\(^{2+}\) channel located on the endoplasmic reticulum (ER) and is activated by the binding of IP3, which initiates Ca\(^{2+}\) release from ER as a Ca\(^{2+}\) pool5.

Recently, we reported that a homologue of IP3R in *T. cruzi* (TcIP3R) is an essential protein and participates in the growth and transformation of the parasite and its ability to infect the host cell. Furthermore, we demonstrated that TcIP3R is a determinant of the virulence of the parasite *in vivo*. Combined with the fact that the primary structure of TcIP3R has low similarity to that of human IP3Rs, TcIP3R is a promising drug target for Chagas disease.

Antisense oligonucleotides are a new generation of therapeutic agents that work by silencing the genes responsible for the diseases. One example is fomivirsen (marketed as Vitravene®), which has been approved by the US Food and Drug Administration (FDA) in 1998 for the treatment of cytomegalovirus retinitis. In parasitic infections, use of antisense oligonucleotides to inhibit specific mRNA synthesis and translation may represent a good chemothapeutic strategy. There are several reports showing that treatment of *T. cruzi* *in vitro* with
antisense oligonucleotides decreased expression levels of the target proteins. Thus, an antisense strategy against pivotal proteins of *T. cruzi* holds promise for a new treatment for Chagas disease.

In the present study, we examined whether TcIP3R is a potential target for antisense oligonucleotide treatment against *T. cruzi* by phenotypic analysis of trypomastigotes in an *in vitro* culture system. We show considerably reduced levels of parasite invasion of host cells, implying that antisense oligonucleotide chemotherapy against TcIP3R may be a viable approach to treatment in Chagas disease.

**Results**

**TcIP3R is a short-lived protein in epimastigotes.** Since antisense oligonucleotides specifically interfere with both mRNA stability and its translation into protein, short-lived proteins are desirable targets to ensure effective, functional knock-down by antisense oligonucleotides. In order to establish whether TcIP3R is suitable as a target for antisense strategy, we treated *T. cruzi* epimastigotes with cycloheximide (CHX), an authentic inhibitor of protein synthesis, for 0.5–10 h, and monitored degradation of TcIP3R by western blot analysis (Fig. 1A). Expression levels of TcIP3R declined after CHX treatment, whereas it was difficult to estimate its half-life, due exclusively to its low levels of expression.

We have recently established *T. cruzi* that overexpress recombinant TcIP3R fused to enhanced green fluorescent protein (EGFP) at its N-terminal (EGFP-TcIP3R), which is physiologically functional in the parasite. We examined the inhibitory effect of CHX treatment on expression of EGFP-TcIP3R to ascertain whether TcIP3R domain-specific protein degradation occurs. Western blots showed that the protein signals of EGFP-TcIP3R decreased rapidly and became undetectable by 8 h after CHX treatment, whereas the band for EGFP remained almost intact (Fig. 1B). These results clearly indicated that the degradation of EGFP-TcIP3R is specific to the TcIP3R domain. The half-life of EGFP-TcIP3R was estimated to be about 3 h, while the half-life of mammalian IP3Rs in unstimulated cultured cells is 10–12 h, suggesting that TcIP3R is more unstable than mammalian IP3Rs. We concluded that TcIP3R is a short-lived protein at least in epimastigotes, and possibly other forms of *T. cruzi*.

**Protein level of TcIP3R is very low in trypomastigotes.** We have recently shown that transcription of TcIP3R mRNA occurs throughout the parasite life cycle, but that its transcription level was much lower in trypomastigotes than in epimastigotes. In the present study, the protein levels of TcIP3R were examined by western blotting using an anti-TcIP3R monoclonal antibody, and were compared between epimastigotes and trypomastigotes. TcIP3R was detected in epimastigotes, but was undetectable in trypomastigotes, while the levels of β-tubulin, a control protein, were consistent between the 2 parasite forms (Fig. 1C). These results indicated that the protein level of TcIP3R is very low in trypomastigotes.

Because the native TcIP3R protein is undetectable in trypomastigotes, we tested whether EGFP-TcIP3R was detectable in trypomastigotes of the transgenic *T. cruzi*. We could detect EGFP-TcIP3R by western blots using an EGFP-specific antibody, confirming that these EGFP-TcIP3R-expressing *T. cruzi* were suitable for further analysis (Fig. 2). Notably, expression levels of EGFP-TcIP3R in trypomastigotes (Fig. 2A, untreated) was very low and was reduced to less than 10% of that in epimastigotes (Fig. 2B, epimastigotes), consistent with the results found in the wild-type parasite.

**Expression of TcIP3R is blocked by the antisense oligonucleotide treatment.** It has been reported that antisense oligonucleotides can be incorporated into non-dividing, infective trypomastigotes by co-incubation in medium, without specific treatment, leading to an effective knock-down of target protein expression. Given this, and the fact that trypomastigotes are the exclusive invasive form in non-phagocytic cells, we selected trypomastigotes as the target stage of the parasite for antisense treatment.

Because of the difficulty to determine the stability of native TcIP3R, we addressed whether treatment with antisense oligonucleotides inhibits expression of EGFP-TcIP3R in trypomastigotes (Fig. 2). Expression levels of EGFP-TcIP3R in trypomastigotes treated with the antisense oligonucleotide (Antisense 5995) was significantly reduced to 54% (vs. untreated; p = 0.003) and 41% (p = 0.0006) after 4 h and 8 h treatment, respectively (Fig. 2B). Although the stability of TcIP3R in trypomastigotes is unclear, reduction of the protein levels of EGFP-TcIP3R is only attributable to the degradation of premade proteins under the conditions that protein synthesis is suppressed by antisense oligonucleotide treatment, as well as by CHX treatment (see also Fig. 1). This is also supported by the fact that the reduction of EGFP-TcIP3R levels is time-dependent. Therefore, it is likely that EGFP-TcIP3R protein, and possibly native TcIP3R, are a short-lived protein in trypomastigotes.

Treatment with the complementary sense oligonucleotide (Sense 5995S) also showed 54% reduction of EGFP-TcIP3R expression after 4 h treatment (vs. untreated; p = 0.017), whereas the effect was rather limited. This was probably due to association of the sense oligonucleotides with the target mRNA.
Infectivity of trypanosomatids is decreased by treatment with antisense oligonucleotides targeted against TcIP3R mRNA. To establish whether treatment with oligonucleotides led to impairment of trypanosomatid infectivity, we compared the inhibitory effects between Antisense 5995 and Sense 5995S in trypanosomatids treated with Antisense 5995 or Sense 5995S. Intensity of the bands was measured densitometrically using a freeware, ImageJ version 1.47. Bars indicate the mean ± S.E. (n = 3). Statistical differences (*p < 0.05, **p < 0.01) are given as a comparison between untreated (0) and treated (2, 4, and 8 hours) groups.

oligonucleotide with the antisense DNA strand, which may interfere with transcription and lead to the inhibition of transcription.

**Discussion**

Chemotherapy of Chagas disease currently relies essentially on 2 old compounds, benznidazole and nifurtimox, both of which elicit harmful side effects. Therefore, development of novel drugs for treatment of this disease is crucial. New therapeutic measures against infectious diseases include antisense oligonucleotides, which aim to knock down an essential component in the responsible pathogens.

We further tested the inhibitory effects of additional 3 antisense oligonucleotides (Antisense 5531, 1777, and 8646) on invasion of HeLa cells by trypomastigotes. Treatment of trypomastigotes with each of these antisense oligonucleotides resulted in impaired invasion of HeLa cells by the parasite (Fig. 3B). These results indicated that suppression of expression of TcIP3R is dependent on the antisense oligonucleotide sequence, and can result in impaired trypanosomatid infectivity.
In the present study, EGFP-TcIP3R was expressed using pTREX expression vector via the ribosomal RNA promoter that facilitates constitutive, high-level transcription. Therefore, it is likely that TcIP3R-specific protein degradation is more active in trypanomastigotes than in epimastigotes, while we cannot exclude the possibility that the transcription level of EGFP-TcIP3R, as well as its protein level, in trypanomastigotes is very low.

Reduction of infectivity of trypanomastigotes after treatment with TcIP3R-specific antisense oligonucleotides strongly suggested that the suppression of transcription of this gene led to reduced levels of TcIP3R protein, which occurred in conjunction with rapid turnover at the protein level in trypanomastigotes. Thus, expression levels of TcIP3R is tightly regulated in trypanomastigotes at both transcription and protein levels.

Low expression levels of TcIP3R in trypanomastigotes, as well as its rapid turnover, are advantageous for further development of antisense therapy against TcIP3R. Firstly, trypanomastigotes are the only invasive stage of T. cruzi, and are responsible for the virulence of the parasite. Therefore, efficient targeting of the relatively small copy number of TcIP3R transcripts by introduction of antisense oligonucleotides followed by suppression of infectivity of trypanomastigotes should be possible. Secondly, trypanomastigotes represent the non-dividing stage, so that the intracellular concentration of antisense oligonucleotides can be stabilized. It is worth noting that when antisense nucleotides are incorporated in the dividing stage of the parasites (e.g. epimastigotes or amastigotes), the concentration of the oligonucleotide in the parasites may become diluted as the parasite replicates. Thirdly, antisense oligonucleotides can be incorporated into trypanomastigotes without artificial treatment, which facilitates the strategy.

In terms of Chagas disease, the antisense approach is suitable particularly in the acute phase, in which the blood-circulating trypanomastigotes predominate, whereas they are often undetectable in other phases, such as the indeterminate and chronic stages of infection. In addition, it is important to know whether this therapeutic approach is effective not only in trypanomastigotes, but also in amastigotes. We are now planning to investigate this using an in vitro infection system and an experimental animal model. In conjunction with the fact that TcIP3R shares far less similarity with mammalian IP3R isoforms, TcIP3R holds great promise as a target for antisense treatment with reduced side-effects.

It has been reported that PI-PLC of T. cruzi (TcPI-PLC) is essential for the parasite, and TcPI-PLC has been shown to be related to trypanomastigote-to-amastigote differentiation by experiments using antisense oligonucleotides. Since PI-PLC synthesizes IP3, followed by suppression of infectivity of trypomastigotes with this knock-down showed only a 40% reduction in infectivity of the host cell. In the present study, antisense treatment showed comparable levels of reduction in infectivity. Therefore, it is possible that in vivo antisense treatment can be effective for preventing development of the disease.

Knock-down efficacy may be improved by using antisense oligonucleotides with 2′-O-Me-2′-C-ethylene-brided nucleic acid species (ENAs). ENAs have higher binding affinity for the complementary RNA strand and are more resistant to nucleases than are phosphorothioate nucleic acids. Therefore, ENA antisense oligonucleotides are more favorable from a therapeutic viewpoint. Further analysis using ENA-based antisense oligonucleotides and an experimental animal model is necessary to optimize the conditions for this therapeutic strategy against Chagas disease.

Methods

Parasite and host cells. Epimastigotes of the T. cruzi Tulahuen strain were cultured as described. Metacyclic development was induced as previously described. Mammalian stages of the parasites were maintained in in vitro culture using 3T3 Swiss albino cells (Health Science Research Bank, Tokyo, Japan) and tissue culture-grown trypanomastigotes were collected from the culture supernatants by centrifugation, essentially as previously described. For in vitro experimental infection, 3T3 Swiss albino cells and human-derived HeLa cells were used.

Antibodies and reagents. The anti-TcIP3R monoclonal antibody was prepared as described previously. The anti-EGFP and anti-tubulin antibodies were purchased from Molecular Probes, Inc. (Eugene, OR) and Thermo Fisher Scientific, Inc. (Rockford, IL), respectively. Cycloheximide was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Quick-CBB PLUS (Wako) was used for CBB staining. Western blotting was performed as described.

Oligonucleotides. The following phosphorothioate oligonucleotides were designed and purchased from Integrated DNA Technologies, Inc. (Diego, CA). Anti-sense oligonucleotides can be stabilized by addition of antisense oligonucleotides to the complementary sequence of TcIP3R gene, 5′- TTCCCAAGCTCTACCATCCCGCCTCTCCCTCTCCACCATCCC-3′, 5′- GCCCCTCTCCCTCCGTGCTGT-3′, 5′-TCTCCTCCCTCCGTCGT-3′, and 5′-TCTCCTCCCTCCGACCATCCTCGGACACGGACT-3′, respectively. Sense Oligonucleotide, 5995S (5′-ACAGCAGGGAAAGGAGGAC-3′), is complementary to Antisense 5995.

Statistical analysis. Statistical analysis between the groups was performed using one-way ANOVA and Fisher’s PLSD post hoc test.
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Author contributions
M.H., T.N. and K.M. designed the study. M.H., H.H. and T.N. did the experiments. M.H. and T.N. wrote the manuscript. M.H., M.E., J.M. and K.M. interpreted the data. All authors reviewed the manuscript.

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