

In Vitro* Selection of RNA Aptamers That Bind to Cell Adhesion Receptors of *Trypanosoma cruzi* and Inhibit Cell Invasion

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***Trypanosoma cruzi* causing Chagas' disease needs to invade host cells to complete its life cycle. Macromolecules on host cell surfaces such as laminin, thrombospondin, heparan sulfate, and fibronectin are believed to be important in mediating parasite-host cell adhesions and in the invasion process of the host cell by the parasite. The SELEX technique (systematic evolution of ligands by exponential enrichment) was used to evolve nuclease-resistant RNA ligands (aptamer = to fit) that bind with affinities of 40–400 nM to parasite receptors for the host cell matrix molecules laminin, fibronectin, thrombospondin, and heparan sulfate. After eight consecutive rounds of *in vitro* selection four classes of RNA aptamers based on structural similarities were isolated and sequenced. All members of each class shared a common sequence motif and competed with the respective host cell matrix molecule that was used for displacement during the selection procedure. RNA pools following seven and eight selection rounds as well as individual aptamers sharing consensus motifs were active in inhibiting invasion of LLC-MK₂ monkey kidney cells by *T. cruzi* *in vitro*.**

Trypanosoma cruzi, the protozoan causing Chagas' disease, must invade host cells to complete its biological cycle. The parasite exists in three developmental forms. Epimastigotes extracellularly multiply in the gut of insect vectors. The mammalian stage of the parasite life cycle includes amastigotes, an intracellular form that replicates, an epimastigote-like form and trypomastigotes, released to the extracellular milieu to invade new cells or biting insects, thus propagating the infection (1, 2).

To contact and invade a mammalian host cell trypomastigotes must cross a network of extracellular matrix proteins on the surface of cells of the blood vessel walls and target organs. Evidence for receptor-ligand interactions in the early events of cell infection by trypanosomes was collected by several laboratories (3, 4). Host cell matrix molecules such as fibronectin (5), laminin (6, 7), heparan sulfate (8, 9), and proteins of the cy-

toskeleton exposed at the cell surface (4) bind to the parasite surface and affect host cell invasion.

Recognizing the importance of host cell-parasite interactions, our laboratory has investigated a 85-kDa glycoprotein, termed Tc85-11 (7) and belonging to a large family of 85,000 molecular mass glycoproteins, that is expressed only on cell surfaces of the infective trypomastigote stage and binds to laminin and cytokeratin-18 on host cells (4, 7). Tc85-11 has an extensive sequence homology with the gp-85 glycoprotein family that is related to the sialidase-trans-sialidase supergene family (10–11) and possesses at least two sequence motifs that have been proved to bind to different proteins of the cell matrix (4). Based on collected evidence that strongly supports a crucial function of receptor-ligand interaction between host cell and parasite enabling *T. cruzi* to invade cells, an *in vitro* selection technique was herein employed for the isolation of nuclease-resistant RNA aptamers that compete with host cell matrix molecules for their binding sites on *T. cruzi* cell surfaces (*cf.* Ref. 12).

The SELEX¹ method (systematic evolution of ligands by exponential enrichment) (13, 14) is an oligonucleotide-based combinatorial library selection procedure that has been used extensively to isolate high affinity ligands that bind with picomolar and low nanomolar affinities to a wide variety of proteins and cell surface epitopes, *i.e.* CD4 and selectins (15–17), as well as to complex targets such as red blood cell membranes (18), the membrane-bound nicotinic acetylcholine receptor (19–20), whole virus particles (21), and live African trypanosomes (22). The incorporation of modified nucleotides into the RNA transcripts, resulting in resistance against nuclease attack, has considerably increased the use of aptamers as probes to inhibit protein functions in *in vitro* and *in vivo* assays (*cf.* Ref. 23).

Nuclease-resistant RNA and DNA aptamers selected to block cell adhesion events involved in disease have gained importance in the last years. The biological activity of such aptamers have been shown in *in vitro* assays where RNA antagonists inhibited selectin-dependent adhesion (17, 24). Wang *et al.* (25) selected RNA aptamers that bind to infectious human cytomegalovirus and inhibit viral infection *in vitro*, showing the feasibility of the SELEX technique for the evolution of novel compounds that protect cells against infection by pathogens. Furthermore, combinatorially synthesized nuclease-resistant RNA and DNA ligands are promising candidates for use in pharmaceutical and therapeutic applications (*cf.* Refs. 26 and 27).

The SELEX technique was used to evolve nuclease-resistant RNA aptamers that block *in vitro* receptor-ligand interactions between *T. cruzi* trypomastigotes and epithelial monkey kid-

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¹ The abbreviations used are: SELEX, systematic evolution of ligands by exponential enrichment; PBS, phosphate-buffered saline, pH 7.4.

ney LLC-MK₂ host cells and partially inhibit cell invasion by these parasites.

EXPERIMENTAL PROCEDURES

Materials

[³²P]ATP was purchased from Pharmacia Biosciences. Two'-fluoropyrimidines (2'-F-dCTP and 2'-F-dUTP) were from TriLink BioTechnologies (San Diego, CA). Heparan sulfate was purified from bovine mucosa and kindly provided by Professor Helena B. Nader (Universidade Federal de São Paulo, São Paulo, Brazil).

Culture of Trypomastigotes and Epimastigotes of *T. cruzi* (Strain Y)

Trypomastigotes from *T. cruzi* were obtained by infection of confluent monkey kidney epithelial cells (LLC-MK₂) as described previously (28). LLC-MK₂ cells infected with trypanosomes were grown in Dulbecco's modified Eagle's medium containing 2% of fetal bovine serum at 37 °C and 5% CO₂. The trypomastigotes released from the infected cells after 5–6 days are washed three times in Dulbecco's modified Eagle's medium, purified by differential centrifugation through lymphoprep (Nycomed, Oslo, Norway) and resuspended in phosphate-buffered saline (PBS) containing 20 mM glucose at a density of 5 × 10⁶ parasites per microliter.

SELEX for Isolation of Aptamers That Displace Matrix Molecules from Cell Surfaces of *T. cruzi* Trypomastigotes

Laminin (human placenta) and fibronectin (human plasma) were purchased from Invitrogen and thrombospondin (human platelets) from Sigma. The following oligonucleotide, consisting of 108 nucleotides with a 40-nucleotide randomized region flanked on both sides by constant sequences, was synthesized at a 200 nM scale by Biomol, Berlin, Germany: 5'-ACCGAGTCCAGAAGCTTGTAGTACT(N₄₀)GCCTAGATGGAGTTGAATTCTCCCTATAGTGAGTCGTATTAC-3' (19). The product of the oligonucleotide synthesis was purified on a denaturing PAGE. To create the initial DNA pool for transcription of partial randomized RNA molecules, 10.8 nmol of purified DNA were amplified in a total volume of 12 ml using 11 rounds of error-prone PCR in the presence of 0.5 mM MnCl₂ and 7 mM MgCl₂. The primers used for amplification were 5'-GTAATACGACTCACTATAGGGAGAATTCAACTGCCATCTA-3' and 5'-ACCGAGTCCAGAAGCTTGTAGT-3'. The PCR product was gel-purified and ethanol-precipitated. For *in vitro* selection, 20 μg of the synthetic DNA pool were transcribed using 1,000 units of T7 RNA polymerase in the presence of 50 μCi of [³²P]ATP and 1 mM 2'-OH-(ATP and GTP) and 3 mM 2'-F-(dCTP and dUTP), the latter to provide stability of the transcript against RNase activity (29). The RNA pool was diluted to a final concentration of 50 μM in the reaction mixture containing 20 mM glucose and heat denatured and renatured to allow for proper secondary structure formation. *T. cruzi* trypomastigotes, purified by differential centrifugation, were resuspended in 200 μl at an approximate concentration of 500 nM possible binding sites for host cell matrix molecules and incubated for 60 min with 100 μl of the RNA pool. *T. cruzi* trypomastigotes and RNA molecules bound to their surfaces were pelleted by centrifugation for 45 s at 13,000 × *g*, washed once with 500 μl of binding buffer, and incubated for 20 min in 50 μl of a solution containing 1 μM each laminin and fibronectin and 5 μM heparan sulfate and 100 nM thrombospondin. The trypomastigotes were pelleted by centrifugation, and the supernatant containing displaced RNA molecules was collected. The collected eluate was phenol-chloroform- and chloroform-extracted using 1:1 supernatant:solvent volume ratios and ethanol-precipitated (80%) in the presence of 10 μg of yeast tRNA.

In round seven of SELEX a preselection procedure was employed to remove RNA molecules from the SELEX pool that also bind to non-infective epimastigote surfaces. The RNA pool (3 nmol) that had been obtained by collection and amplification from SELEX round seven was incubated for 60 min with 3 × 10⁸ epimastigotes in a total reaction volume of 300 μl. The supernatant containing 1.95 nmol of unbound RNA was collected after centrifugation, phenol-chloroform-extracted, ethanol-precipitated, and used for selection of cell matrix molecule-displaceable RNA molecules bound to trypomastigotes as described.

Following each round of selection, the ethanol-precipitated RNA was reverse-transcribed using avian myeloblastoma reverse transcriptase and amplified. The obtained DNA pools were transcribed for the next round of selection using an input of 10 μg of DNA, 1 mM of 2'-OH' purines and 3 mM of 2'-F' pyrimidines, and 500 units of T7 RNA polymerase (Invitrogen). Following transcription, the RNA was phenol-chloroform- and chloroform-extracted, ethanol-precipitated, and resuspended in diethylprocarbonate water.

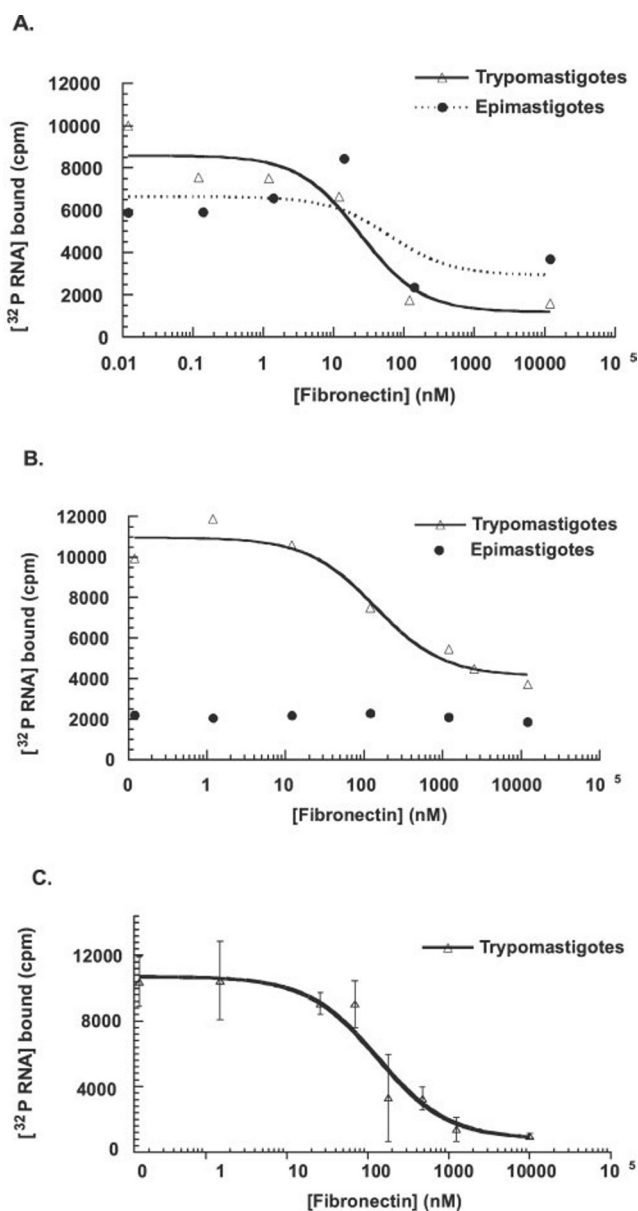


FIG. 1. Displacement of SELEX pool-5 RNA (A), SELEX pool-7 RNA (B), and SELEX pool-8 RNA (C) using fibronectin as a competitor. For IC₅₀ experiments samples of an identical preparation with a concentration of ³²P-labeled SELEX pool-5 RNA (10 nM) (A) or ³²P-labeled SELEX pool-7 RNA (10 nM) (B) were incubated with either 5 × 10⁷ trypomastigotes or 1 × 10⁷ epimastigotes as described under "Experimental Procedures." C shows an IC₅₀ determination of SELEX pool-8 that was then used for cloning and isolation of individual aptamers. The concentration of fibronectin to displace 50% of the RNA from the binding site, the IC₅₀ values, were calculated by fitting the data to the equation $Y = B_{\min} + (B_{\max} - B_{\min}) / (1 + [\text{fibronectin}] / \text{IC}_{50})$. The estimated IC₅₀ values for the displacement of SELEX pool-5 RNA bound to trypomastigotes were 23 ± 17 nM and to epimastigotes 67 ± 52 nM. An IC₅₀ of 119 ± 49 nM was calculated for the binding of SELEX pool-7 RNA to trypomastigotes. The binding of SELEX pool-7 RNA to epimastigotes was too low to allow IC₅₀ estimations. SELEX pool-8 RNA bound with an IC₅₀ of 142 ± 52 nM to trypomastigotes.

Individual Aptamer Characterization

After SELEX cycle 8, the cDNAs of the pools of aptamers that were eluted using laminin, fibronectin, heparan sulfate, or thrombospondin as a displacement agent were cut with *EcoRI* and *HindIII* at the constant regions and ligated into pGEM-3Z plasmids (Promega) (19). The recombinant vectors were transformed into *Escherichia coli* strains JM109 or DH5α and plated on selective medium. Colonies were picked, and inserts were determined by automatic sequencing using the ABI PRISM big dye terminator kit (PerkinElmer Life Sciences). The se-

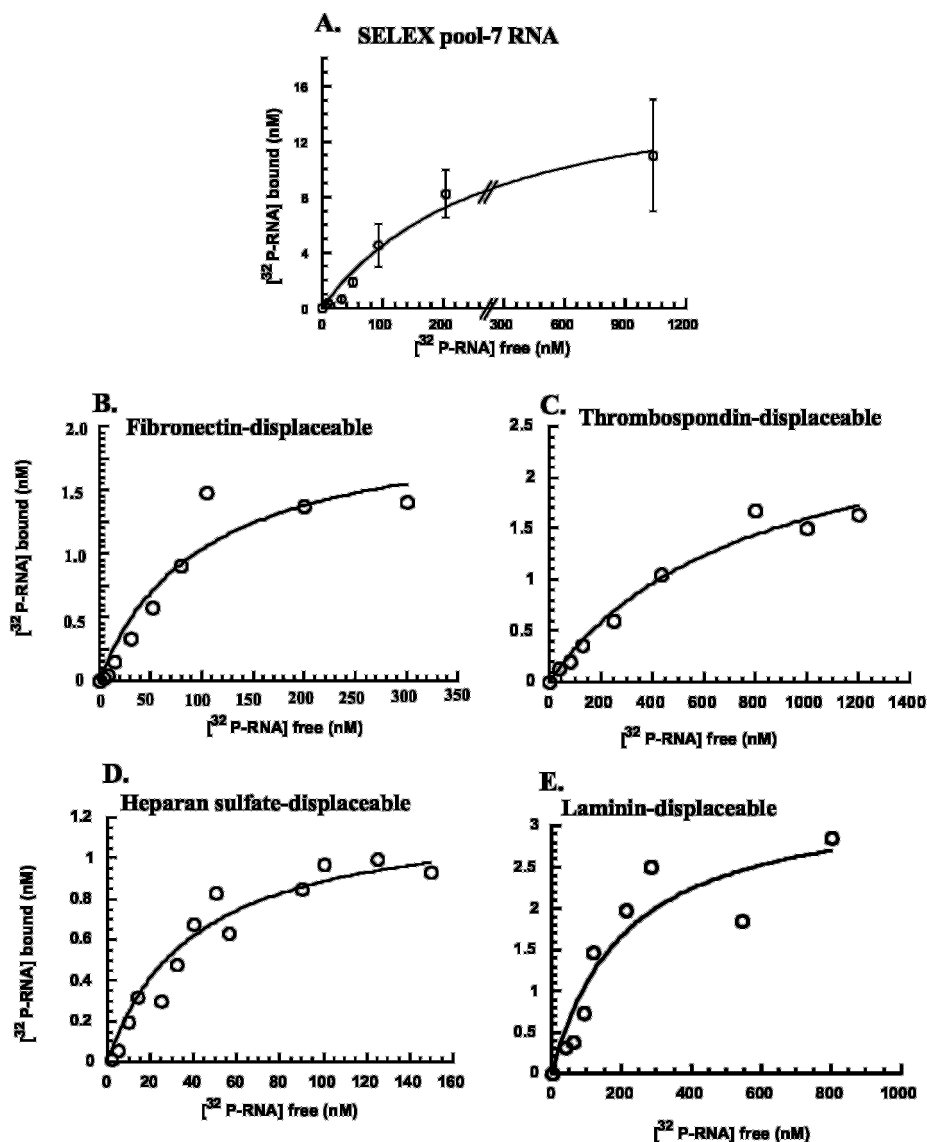


FIG. 2. Binding affinities of SELEX pool-7 RNA and aptamers containing the common consensus motifs of classes I–IV. Constant amounts of trypanosomes (5×10^7) in A and 2×10^7 in B–D were incubated with increasing concentrations of 32 P-labeled RNA in a reaction volume of 60 μ l. Unspecific binding was determined in the presence of one hundred-fold excess of unlabeled RNA. A K_d of 172 ± 54 nM was estimated by non-linear curve fitting for the binding of SELEX pool-7 RNA (A) and 142 ± 94 nM (B), 400 ± 239 nM (C), 40 ± 14 nM (D), and 209 ± 99 nM (E) for, respectively, fibronectin-, thrombospondin-, heparan sulfate- and laminin-displaceable consensus aptamers.

quences of the previously random inserts were aligned and searched for sequence similarities. RNA structures were predicted by using the MFOLD program (30) at bioinfo.math.rpi.edu/~mfold/RNA/form1.cgi.

Binding Analysis

IC₅₀ Determinations—The IC₅₀ value is the concentration at which a competitor displaces 50% of a radioactive ligand from its receptor. One microgram of DNA template of SELEX RNA pool-5, -7, or -8 or individual aptamers containing consensus motifs in 20 μ l of transcription buffer was transcribed in the presence of 0.5 mM GTP, 1.5 mM 2-F' pyrimidines, and 50 μ Ci of [α - 32 P]ATP and purified on a Spin Column 30 (Sigma). 32 P-Labeled RNA (2×10^5 cpm) diluted with unlabeled RNA to a final concentration of 20 nM was incubated for 60 min at room temperature with 5×10^7 trypomastigotes (130 μ g of protein) or with 1×10^7 epimastigotes (92 μ g of protein) in the presence of 0.1 μ g/ μ l t-RNA and increasing concentrations of fibronectin from 0 to 10 μ M in 45 μ l of selection buffer. The reaction mixtures were separated by centrifugation (45 s, $12,000 \times g$). The pellets containing parasites and RNA bound to their surfaces were washed once with 1 ml of selection buffer and scintillation counted.

Saturation Analyses—For saturation binding analysis, constant amounts (2×10^7 or 5×10^7 trypomastigotes) were incubated with increasing concentrations of 32 P-labeled SELEX pool-7 or individual cloned RNAs falling into structural classes in the presence of 0.1 μ g/ μ l yeast t-RNA in 60 μ l of selection buffer for 60 min at room temperature. Non-specific binding was determined in the presence of 8 μ M unlabeled RNA. The K_d was estimated by fitting the data to the equation $y = (x + B_{max}) / (x + K_d)$, where B_{max} is the concentration of binding sites for the RNA molecules.

Inhibition of Cell Invasion

Prior to infection, *T. cruzi* trypomastigotes were preincubated for 15 min with the aptamer solution containing SELEX RNA pool-0, -7, or -8 or individual pooled RNAs based on their consensus sequences in PBS, 20 mM glucose. LLC-MK₂ cells attached to eight-well dishes were infected in a 100:1 parasites/cell ratio with trypomastigotes in the absence or presence of SELEX RNA pools for 2 h at 37 $^{\circ}$ C. The incubation was terminated by removing the medium containing free trypomastigotes and washing twice with PBS. The non-adherent parasites were removed by 5-min incubation with lymphoprep (Nycomed), followed by two washes with PBS. The cells were incubated with Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum for 48 h at 37 $^{\circ}$ C, fixed with 100% methanol, and stained with chromomycin A (Molecular Probes), as described previously (4). All experiments were done in duplicates, and 12 photos of each replicate were made, enabling the counting of infected and non-infected cells in samples containing 100 cells each. The data were compared for statistical significance by the unpaired Student's *t* test. The viability of cells and trypomastigotes in the presence of aptamer solution was confirmed by staining with trypan blue and microscopic analysis.

RESULTS

Isolation of RNA Aptamers That Bind to Host Cell Matrix Molecule Receptors on *T. cruzi*—The SELEX technique has been used to evolve nuclease-resistant RNA ligands (aptamers) that bind with high affinity and specificity to receptors of host cell matrix molecules on *T. cruzi*.

A SELEX protocol was established to enrich RNA molecules that bind specifically to the receptors of laminin, thrombospondin, heparan sulfate, and fibronectin on infective trypanostigote stages of *T. cruzi*. After five SELEX rounds, RNA molecules that bind with high affinity to trypanostigotes were observed. They also bound to some extent to non-infective epimastigote forms as shown by IC₅₀ studies using fibronectin as a displacement agent (Fig. 1A). The RNA pool was therefore purified by using a preselection procedure where binders to epimastigotes were discarded, followed by selection of RNA molecules that bound only to trypanostigotes and were displaceable by the above-mentioned cell matrix molecules.

Following this preselection, increasing concentrations of fibronectin only displaced ³²P-labeled SELEX pool-7 RNA from trypanostigotes, but not from epimastigotes, indicating that the selected RNAs bind specifically to cell surfaces of infective

forms of *T. cruzi* (Fig. 1B). The fraction of RNA molecules that was adsorbed by epimastigotes was analyzed by sequencing of individual RNA molecules, and none of the consensus motifs of fibronectin, thrombospondin, laminin, and heparan-sulfate-displaceable RNA aptamers were similar to those that bound to trypanostigotes (data not shown). Saturation analyses revealed that the pool of RNA molecules after seven rounds of *in vitro* selection bound with a binding affinity (K_d) of 172 nM to trypanostigotes (Fig. 2A). Since further selection rounds did not result in any improvement of the binding affinity to *T. cruzi* cell surfaces, as shown by IC₅₀ studies using fibronectin as a displacement agent (Fig. 1C), the post-8 RNA pool that was selected using fibronectin, laminin, thrombospondin, and heparan sulfate as displacement agents was split and eluted with each individual cell matrix molecule.

Binding Affinities and Structures of Selected Aptamers—Aptamers from the four pools obtained after elution with each individual cell matrix molecule were cloned, isolated, and sequenced. Four classes of aptamers were obtained based on structural similarities (classes I–IV). The consensus region found in aptamers that were obtained by displacement with the same cell matrix molecule was specific and was not observed in aptamers that were isolated using another cell matrix molecule (Table I). Twenty-four individual fibronectin-displaceable sequences were cloned and sequenced. Five aptamer sequences contained a consensus motif. Fibronectin clone 4 was represented eight times in the sequenced pool. In 20 individual thrombospondin-displaceable, 28 heparan sulfate-displaceable, and 24 laminin-displaceable RNA aptamers, 8, 13, and 8 sequences were found that contained a respective consensus region (Table I). Based on sequence similarities, the respective individual clones that are displaceable by each matrix molecule employed were pooled and tested for binding affinity and biological activity.

The MFold program (30) was used to predict the secondary structure of individual RNA aptamers from classes I–IV. Interestingly, the structure prediction program integrated the consensus sequence of aptamers from all four classes within stem-loop structures (Fig. 3), suggesting that truncated aptamers containing only this stem-loop region may bind to their targets and be biologically active.

Binding Specificity and Affinity of Classes I–IV to Their Receptors on Trypanostigote Surfaces—RNA aptamers of each

TABLE I
Representative structural classes of individual aptamers

Conserved regions within individual classes of aptamers that are displaceable by the same host-cell matrix molecule are underlined. The consensus regions were: CGGACCAC(A/U)GAUGCCA (12/24 clones) for fibronectin-displaceable aptamers, AAAGC(G/C)XGGXCC (8/20 clones) for thrombospondin-displaceable aptamers, UUGGGC (13/28 clones) for heparan sulfate-displaceable aptamers, and GAUCCCC (8/24 clones) for laminin-displaceable aptamers.

Clone	Structure
Class I – Fibronectin-displaceable aptamers	
01	5' <u>GACCCUUC</u> CCGACUUG <u>ACGGCUGGAG</u> AGUCCAUUU 3'
03	5' <u>GGACCCUUC</u> CCGACUUG <u>ACGGCAGG</u> AGUCCCAUUG 3'
04	5' <u>GACCCUUC</u> CCGACGCA <u>CGGACG</u> AGUCCGCAACCCACC 3'
09	5' <u>ACAUGAUA</u> UCAUAC <u>CAACCAAC</u> AGUCCGACAUUGCA 3'
10	5' <u>CGGACCAC</u> UUGGCCAUCAUCC <u>CCUCCUAC</u> CCCAAGCA 3'
Class II – Thrombospondin-displaceable aptamers	
03	5' <u>CAAAACG</u> UGSGGSAUAAUU 3'
05	5' <u>GGGGAGC</u> UCGCA <u>CCCAUA</u> CAUCC <u>CGACUG</u> AGGUC 3'
08	5' <u>CAUAJUC</u> AAAGCA <u>AGCCAG</u> CCUAGCAACCCGGA 3'
10	5' <u>UUCUCUC</u> CGCA <u>AAAGC</u> GGGCGCAGCUGCAGAGCC 3'
18	3' <u>CUUGUCG</u> UACCCGACGCAU <u>GCAGCA</u> AGCGUCCAUUG 5'
19	5' <u>AAAGC</u> GGGAGCUCGCA <u>CGGUG</u> CAGG 3'
Class III – Heparan sulfate-displaceable aptamers	
03	5' <u>AUCAUCA</u> AAAC <u>GUUCG</u> CAUCGCAUUC <u>CCCCG</u> CCU 3'
06	3' <u>AUCGCUA</u> UCGCGCGGUGC <u>GGUUGCG</u> UUGCAAGCGCA 5'
09	5' <u>AAUACUC</u> UGGUCGCA <u>CGCCUUC</u> CUUUGGCA 3'
21	5' <u>CUACUCA</u> AGCCG <u>UUGG</u> CCUACCAAGAAACAGG 3'
24	5' <u>UACCCUA</u> AGUAGUCC <u>CAACCU</u> UUGCAGCUGUUGGGCC 3'
26	3' <u>CACCCGU</u> ACAGCCCGUAAU <u>UUGG</u> CCCAACCAACCCAGU 5'
27	5' <u>AACCCG</u> CCGCCCAGAUAA <u>AGG</u> UUGGCAUUC <u>CCG</u> UG 3'
Class IV – Laminin-displaceable aptamers	
01	5' <u>GGCCCAU</u> ACUGUACAU <u>AGAU</u> UCC <u>CGAGU</u> UCC 3'
05	3' <u>CGAGAU</u> UCCGUUCCAU <u>UCGAC</u> AGGCAUGAUU <u>CCCC</u> 5'
27	5' <u>GCCGCA</u> AGCAAGCA <u>AGCC</u> CCU <u>CAUC</u> UAGCCCUAGGG 3'
28	3' <u>UCUCU</u> AGCCAGUUCU <u>AGUG</u> CAAGCAGC <u>UCCU</u> UUAU 5'
38	3' <u>UCAUCU</u> UUGCCGUGU <u>UUAU</u> UUGCUGUCC <u>UUGA</u> 5'

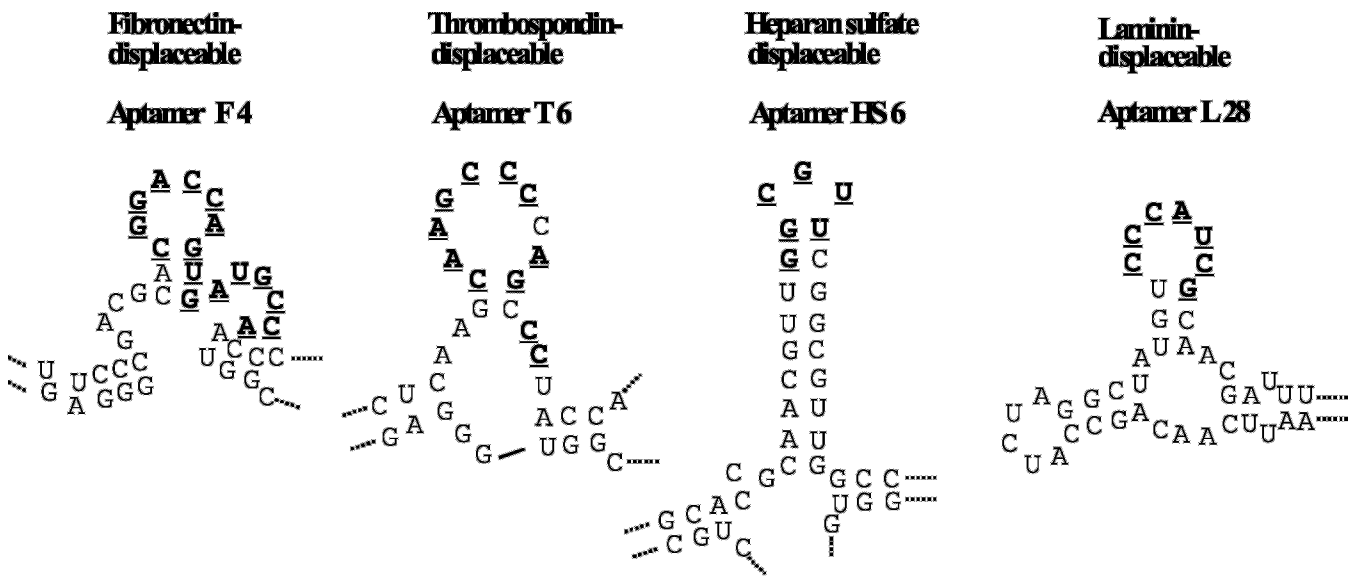


FIG. 3. Prediction of secondary structures of aptamers containing consensus motifs. Secondary structures of individual aptamers from each class were predicted using the MFold program (30). Conserved sequences within the previous random regions are in bold and underlined. The segments shown are those containing the conserved regions forming stem-loop structures.

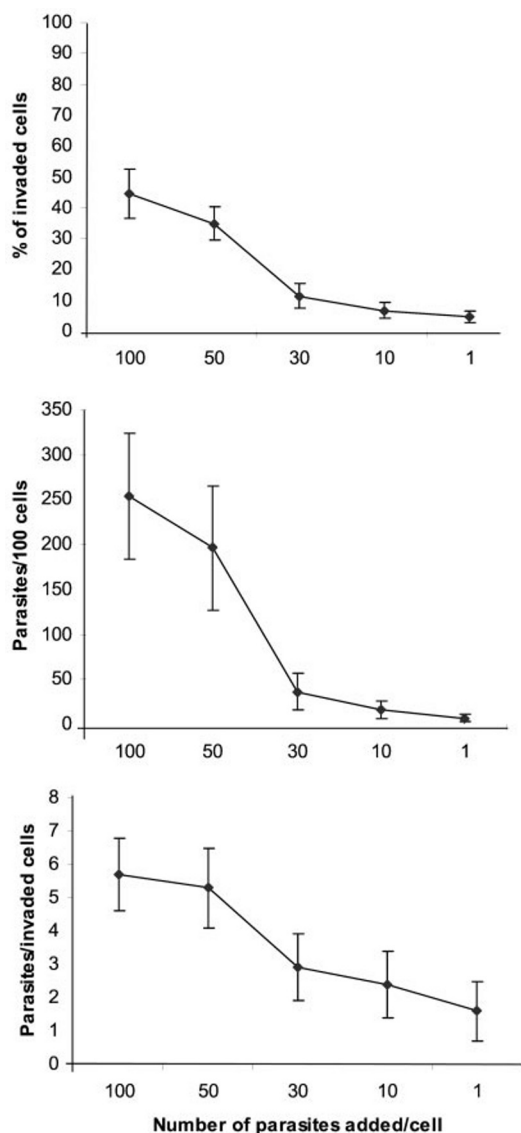


FIG. 4. Invasion of LLC-MK₂ cells by *T. cruzi* trypomastigotes. LLC-MK₂ cells were incubated for 2 h with increasing ratios of parasites to cells. The cells were fixed and stained with chromomycin A and analyzed by fluorescence microscopy.

individual class containing a consensus motif in the previous random region were pooled and tested for their binding affinity toward infective trypomastigotes. Saturation analyses of the binding of consensus aptamers from classes I–IV (see Table I and Fig. 3) to the corresponding matrix receptors on the parasite surface gave the following binding affinities: 124 nM (fibronectin, Fig. 2B), 400 nM (thrombospondin, Fig. 2C), 40 nM (heparan sulfate, Fig. 2D), and 209 nM (laminin, Fig. 2E). The selected aptamers recognized specifically their targets on trypomastigote surfaces and were not toxic to parasites nor reduced parasite motility. Neither the RNA mixture of SELEX-8 nor individual aptamers falling into structural classes bound specifically to cell membranes of LLC-MK₂ cells and HeLa human fibroblasts (not shown).

Do the Selected Aptamers Inhibit Cell Invasion by *T. cruzi*?—Invasion of LLC-MK₂ cells was tested *in vitro* in the presence of increasing amounts of trypomastigotes to determine the optimal ratio of parasites over host cells (Fig. 4). A 100:1 ratio of trypomastigotes over cells and an incubation time of 2 h in the presence and absence of RNA aptamers was a condition that gave a high enough infection to differentiate between infection

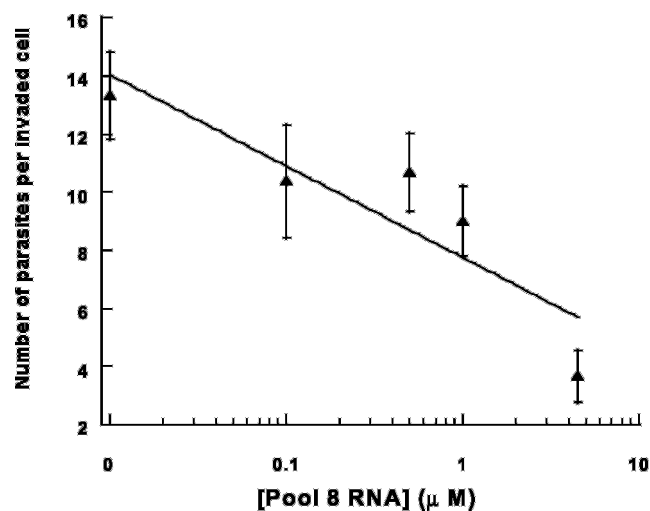


FIG. 5. Dose dependence of SELEX pool 8 RNA activity *in vitro*. LLC-MK₂ cells were incubated for 2 h with trypomastigotes (ratio of 1:100) in the presence of increasing amounts of SELEX pool-8 RNA. The cells were fixed and stained with chromomycin A and analyzed by fluorescence microscopy.

rates with confidence. The effect of SELEX round 8 RNA pool was dose-dependent as shown in Fig. 5. RNA pools from SELEX rounds 0, 7, and 8 as well as individual aptamers from classes I–IV that were respectively displaceable by each matrix component were tested in this assay. A representative invasion experiment is shown in Fig. 6. The pools following SELEX rounds 7 and 8 as well as fibronectin-, thrombospondin-, heparan sulfate-, and laminin-displaceable aptamers (classes that were pooled based on consensus sequences) inhibited at a concentration of 1 μM the invasion of LLC-MK₂ cells by 50–70% (Fig. 6; Table II), with thrombospondin-displaceable aptamers being the less effective. Interestingly, the presence of these selected aptamers, which were non-toxic to the cells, also decreased the absolute number of parasites per cell (Fig. 6; Table II). A mixture of structural classes such as laminin- and fibronectin-displaceable consensus aptamers did not increase inhibition of cell invasion by *T. cruzi*. In addition, cell invasion could not be totally aborted by the mixture of RNA aptamers following eight cycles of *in vitro* selection. These results suggest that the interaction of trypanosomes with their host cells also depends on further receptor-ligand interactions that are not affected by the selected aptamers. Nevertheless, the biological activity of the selected RNA aptamers in inhibiting cell invasion by *T. cruzi* provides direct evidence that receptor-ligand interactions between the host cell matrix molecules fibronectin, laminin, heparan sulfate, and thrombospondin with their receptors on cell surfaces of trypanosomes are events that concur for the successful invasion of the host cell by the parasite.

DISCUSSION

The present work demonstrates the importance of the interaction between host cell matrix molecules such as fibronectin, laminin, thrombospondin, and heparan sulfate with their respective host cell receptors on infective trypomastigote forms of *T. cruzi*. Thrombospondin is a family of secreted proteins widely distributed in the extracellular matrices of several tissues. Whereas fibronectin (5), laminin (6, 7) and heparan sulfate (8, 9) are known ligands for *T. cruzi* in the mammalian host, the involvement of thrombospondin in *T. cruzi* invasion is subjected to investigation. Invasion of Plasmodia is mediated by a thrombospondin-related adhesive protein (TRAP) (31), and thrombospondin is involved somehow with tumor progression (32, 33). Notwithstanding, present evidence favors fibronectin

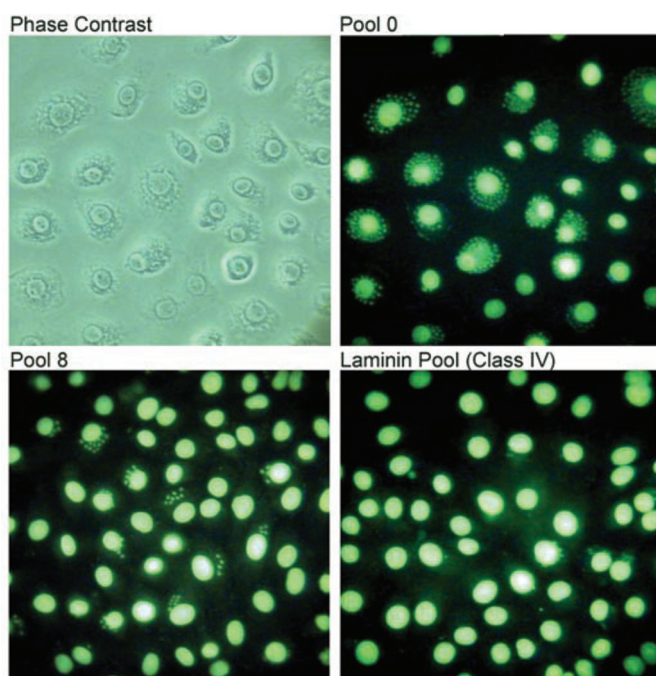


FIG. 6. Selected aptamers inhibit *in vitro* invasion of LLC-MK₂ cells. For testing the biological activity of the selected aptamers, LLC-MK₂ cells were infected with 100-fold excess of trypomastigotes over cells in the presence or absence of 1 μ M concentration of the respective aptamer solution for 2 h. The cells were fixed and stained with chromomycin A and analyzed by fluorescence microscopy.

TABLE II
Selected aptamers inhibit the *in vitro* invasion of LLC-MK₂ cells by *T. cruzi*

The percentage of infected cells from 2,000 cells as well as the number of parasites per 100 cells and the number of parasites per invaded cells were determined, and results were compared with the control for statistical significance using the unpaired Student's *t* test. Results are the average of triplicate determinations \pm S.D. In all three columns SELEX 0 RNA and SELEX 7 RNA do not differ from each other within a significance level of $p < 0.05$. All other conditions were $p < 0.005$, with the exception of the earmarked results of the third column. Infection rates in the control experiments made in the absence of RNA aptamers were normalized to 100%. For each condition the concentration of aptamer added was 1 μ M. Infection and staining were performed as described in the legend of Fig. 6.

Condition	Invaded cells	Number of parasites per 100 cells	Number of parasites per invaded cells
	%		
Control	100 \pm 25	669 \pm 261	12 \pm 3
SELEX 0 RNA	83 \pm 28	520 \pm 155	11 \pm 3
SELEX 7 RNA	61 \pm 18	338 \pm 104	10 \pm 3 ^a
SELEX 8 RNA	32 \pm 17	155 \pm 87	8 \pm 2
Consensus aptamers			
Fibronectin	41 \pm 19	225 \pm 112	10 \pm 4 ^a
Heparan sulfate	43 \pm 18	219 \pm 91	10 \pm 3 ^b
Thrombospondin	45 \pm 25	231 \pm 157	9 \pm 3
Laminin	19 \pm 6	41 \pm 23	4 \pm 2
Laminin plus fibronectin	21 \pm 10	33 \pm 18	3 \pm 1

^a $p < 0.1$.

^b $p < 0.05$.

tin, laminin, and heparan sulfate as more important molecules in host cell invasion by *T. cruzi* than thrombospondin.

It is known that trypomastigotes shed molecules bound to their cell surface (34), which may explain quantitative differences between binding affinity and biological effect *in vitro*. The failure of the selected RNA aptamers, even in higher concentrations, to inhibit completely the invasion of the host cell by the parasite suggests that *T. cruzi* may use several different mechanisms to invade host cells. This is expected due

to the large repertoire of molecules described in the literature as involved in host cell invasion by the parasite (3–11). In addition to the interaction of the parasite membrane with cell matrix components, parasite proteases (35–36), and host cell surface proteins such as bradykinin B2 (36), TGF- β (37), or mannose (38) receptors have been shown to participate in host cell infection, some of them preparing the host cell for invasion. As *T. cruzi* invades nearly every cell type except erythrocytes, it may express a variety of receptors and ligands to ensure its interaction with different pattern of molecules expressed on the surface of the respective cell type.

Thus, the SELEX method opens new avenues to block pathogen-host cell interactions. Considering that the 2'-hydroxyl group of the ribose is substituted by fluorine, the RNA aptamers are less amenable to RNase attack and, therefore, are ideal for *in vivo* experiments. Thus, stable oligonucleotides that may inhibit parasite-host cell interaction could be used to develop administrable drugs that would hopefully halt the progress of disease.

Combinatorial library approaches have been successfully employed to block ligand-receptor interactions that are necessary for completion of the life cycle of other parasites such as *Plasmodium*. Selection *in vitro* of peptide ligands from a combinatorial phage display library that block adhesion and invasion of insect midgut cells by *Plasmodium* was described previously (39). Combinatorial libraries may be advantageous for drug development when compared with the rational drug design approach, as the selection of aptamers, for instance, do not require a full understanding of infection by pathogens.

The development of agents that block adhesion events between parasites and host cells have not only become important regarding protection of cells against invasion by pathogens but also to prevent metastatic cancer cells from adhering to endothelial cells of blood vessels and invading the respective tissues. Interestingly the same host cell matrix molecules involved in cell invasion by *T. cruzi* as shown in this study are used as adhesion ligands by tumor cells (40, 41).

Recently, the *in vivo* use of aptamers is providing more consistent results by improving aptamer pharmacokinetics. Keeping intact aptamers in the blood from hours to days has been accomplished by conjugation with high molecular weight carriers, such as polyethyleneglycol, or by embedding into liposomes (42). As an example, one aptamer to vascular endothelial growth factor (VEGF), patented as NX 1838 is currently been used in clinical trials as a potential therapeutic agent for age-related macular degeneration (27). Several aptamers that inhibit adhesion events have been shown to be effective in animal models such as those directed against VEGF (43), P-selectin (17) and PDGF (44–45). Thus, it should be feasible to find active aptamers against *in vivo* infection by *T. cruzi* that may open new perspectives to future approaches in the treatment of Chagas' disease.

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