Brief Definitive Report

The Exocytosis-regulatory Protein Synaptotagmin VII Mediates Cell Invasion by Trypanosoma cruzi

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Abstract

The intracellular protozoan parasite Trypanosoma cruzi causes Chagas’ disease, which affects millions of people in Latin America. T. cruzi enters a large number of cell types by an unusual mechanism that involves Ca$^{2+}$-triggered fusion of lysosomes with the plasma membrane. Here we show that synaptotagmin VII (Syt VII), a ubiquitously expressed synaptotagmin isoform that regulates exocytosis of lysosomes, is localized on the membranes of intracellular vacuoles containing T. cruzi. Antibodies against the C$_2$A domain of Syt VII or recombinant peptides including this domain inhibit cell entry by T. cruzi, but not by Toxoplasma gondii or Salmonella typhimurium. The C$_2$A domains of other ubiquitously expressed synaptotagmin isoforms have no effect on T. cruzi invasion, and mutation of critical residues on Syt VII C$_2$A abolish its inhibitory activity. These findings indicate that T. cruzi exploits the Syt VII–dependent, Ca$^{2+}$-regulated lysosomal exocytic pathway for invading host cells.

Key words: trypanosome • parasite • intracellular • secretion • calcium

Introduction

Human infection with Trypanosoma cruzi is prevalent in extensive areas of South and Central America, where more than 15 million people are estimated to be chronically infected. The acute phase of the infection is often fatal in children, and survivors frequently develop serious conditions such as cardiomyopathy and megacolon, which can lead to premature death. A large number of wild animals serve as reservoirs for T. cruzi, and this broad host range correlates well with the parasite’s ability to invade and replicate inside a large variety of mammalian cell types.

Previous studies revealed that infection of fibroblasts and epithelial cells by trypomastigotes, the infective forms of T. cruzi, occurs by an unusual mechanism, distinct from phagocytosis. Parasite entry is independent of host cell actin polymerization and requires mobilization of host cell lysosomes to the invasion site (1, 2). Time-lapse imaging of the invasion process revealed a directional movement and clustering of lysosomes at the site of trypomastigote attachment, followed by progressive fusion of lysosomes with the plasma membrane as the parasites entered the cell (3). Recently and partially internalized trypomastigotes are found in acidic intracellular vacuoles containing lysosomal markers, strongly suggesting that the parasite-containing compartment is formed through lysosomal fusion (2). A trypomastigote-triggered signaling cascade resulting in a localized elevation of the host cell intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) is also required for cell invasion and for the efficient establishment of infections in mice (4–7). Thus, the T. cruzi cell entry process has several features that resemble regulated exocytosis: it involves elevation in [Ca$^{2+}$], and mobilization of lysosomes to the plasma membrane, followed by fusion.

The similarities between the T. cruzi invasion process and regulated exocytosis led us to investigate a possible role for synaptotagmin VII (Syt VII), a ubiquitously expressed member of the synaptotagmin family previously implicated in the regulation of lysosomal exocytosis (8). Synaptotagmins are transmembrane proteins with a short NH$_2$-terminal ectodomain, a single transmembrane region, and two highly conserved, independently folding Ca$^{2+}$-binding C$_2$ domains (C$_2$A and C$_2$B) homologous to the C$_2$ regulatory region of protein kinase C. Genetic studies in mice, Drosophila, and Caenorhabditis elegans revealed that synaptotagmin I (Syt I), which is present on the membranes of synaptic vesicles in neurons, is essential for normal Ca$^{2+}$-dependent neurotransmitter release (9, 10). The C$_2$A domains of Syt I and of several additional isoforms interact with the
t-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) syntaxin and with acidic phospholipids in a Ca\(^{2+}\)-dependent manner, albeit with slightly different properties (11). A Ca\(^{2+}\)-dependent interaction was also detected between the Ca\(^{2+}\) domain of Syt I and SNAP-25 (12), another core component of the conserved SNARE membrane fusion “machine” (13). Although several of the 12 rat and mouse synaptotagmin isoforms described to date are primarily found in brain, some are also expressed in other tissues (11, 14). Syt VII, in particular, is expressed at significant levels on most mouse tissues (15). Consistent with this ubiquitous pattern of expression, recent work from our laboratory showed that Syt VII is localized on the membrane of lysosomes in NRK cells (8).

Recently proposed models for the role of Syt I in synaptic vesicle exocytosis suggest that Ca\(^{2+}\)-triggered interactions involving the Ca\(^{2+}\) domains alter the physical relationship between the SNARE complex and the lipid bilayers, facilitating fusion (12, 16). In particular, several lines of evidence indicate that the Ca\(^{2+}\) domain is central for the function of synaptotagmins in the regulation of Ca\(^{2+}\)-triggered exocytosis (12, 17–20). Antibodies generated against the Syt I Ca\(^{2+}\) domain and recombinant peptides containing the Syt I Ca\(^{2+}\) domain are powerful inhibitors of Ca\(^{2+}\)-triggered exocytosis when introduced into neuronal cells (18–20). Similarly, the Ca\(^{2+}\) domain of Syt VII or antibodies to this domain efficiently block Ca\(^{2+}\)-triggered exocytosis of lysosomes in permeabilized NRK cells (8). Importantly, inhibition of lysosomal exocytosis in NRK cells is only observed in the presence of the Syt VII Ca\(^{2+}\) domain, and not the Ca\(^{2+}\) domain of the exclusively neuronal isoform Syt I (8). These observations indicate that Syt VII functions as a specific regulator of lysosomal exocytosis, which is triggered at low micromolar [Ca\(^{2+}\)]\(_i\), in several cell types (21–23). In this report, we show that modulation of Syt VII function inhibits host cell invasion by *T. cruzi*, strongly suggesting that this parasite utilizes the Ca\(^{2+}\)-regulated lysosomal exocytic pathway for establishing intracellular infections.

**Materials and Methods**

*Antibodies, Western Blot, and Immunofluorescence.* Polyclonal antibodies against the Syt VII NH\(_2\)-terminal ectodomain were generated by immunization of a rabbit with the synthetic peptide MYRDPEAASPAGAC and purified as described previously (8). Rabbit polyclonal antibodies against *Esherichia coli*–expressed Syt VII Ca\(^{2+}\) domain were also generated and affinity purified as previously described (8). For Western blots, extracts of NRK (rat), Hela, HEK-293 (human), 3T3 (mouse), and CHO (hamster) cells were prepared in 150 mM NaCl, 50 mM Tris, pH 8.6, and 1% NP-40 containing protease inhibitors. 20 μg of cell extract was loaded on each lane for SDS-PAGE, transferred to immobilon filters, and probed with purified anti–Syt VII IgG followed by ECL (New England Nuclear) detection (9). Immunofluorescence assays were performed on HEK-293 and 3T3 cells fixed in 100% methanol at 20ºC for 10 min and rehydrated in PBS for 30 min, followed by anti–Syt VII rabbit IgG (8) and a mouse mAb to human Lamp-1 (H4A3) or a rat mAb to mouse Lamp-1 (1D4B; Developmental Studies Hybridoma Bank, Iowa City, IA). CHO cells were fixed in 2% paraformaldehyde, incubated for 15 min in 50 mM NH\(_4\)Cl, permeabilized with 0.1% Triton X-100 for 30 min, and incubated with rabbit anti–Syt VII and a mouse mAb to hamster Lamp-1 (UH1), followed by the appropriate secondary antibodies (goat anti-rabbit, -mouse, or –rat conjugated to Alexa 488 or 546; Molecular Probes). Images were acquired in a Zeiss Axiovert 135 microscope through a 100× objective using a Hamamatsu Orca II cooled CCD camera, controlled by Meta morph software (Universal Imaging).

**Reverse Transcription PCR, Site-directed Mutagenesis, and Transfections.** cDNA was synthesized from 10 μg of total RNA previously treated with RNase-free Dnase I (GIBCO BRL). Forward and reverse primers used for reverse transcription (RT)-PCR amplification of the synaptotagmin Ca\(^{2+}\) domains were designed based on nonconserved regions encoded by two separate exons of the individual rat DNA sequences and were as follows: 5’-GAGAGAAGCTTGGGAAGGCTC-3’ (forward) and 5’-TTTCTCAGCGGCTCTGGAGAT-3’ (reverse) for Syt I Ca\(^{2+}\), 5’-GAAGGCGGAGAACCTTG-3’ (forward) and 5’-ATTCTCTGATGCGCTGTCGTAAG-3’ (reverse) for Syt II Ca\(^{2+}\), 5’-CTTTGCGAGCCAGGCACTCCC-3’ (forward) and 5’-AGATGTAGCGCTAGGGGCTGG-3’ (reverse) for Syt III Ca\(^{2+}\), 5’-CAAGAGAAGCCTGGCCACTCC-3’ (forward) and 5’-GCTTATTACATTTTCCGCTGAC-3’ (reverse) for Syt IV Ca\(^{2+}\), 5’-GCAAGACGTGTCGGAGAC-3’ (forward) and 5’-AGATGAGGCTCTGGGAGAC-3’ (reverse) for Syt VI Ca\(^{2+}\), 5’-AGCGGAGAGAACC-3’ (forward) and 5’-ATGGAGGCTGGAGAGGCAAG-3’ (reverse) for Syt VII Ca\(^{2+}\), 5’-TGCTGCTGGAGGGGACCAACAG-3’ (forward) and 5’-ACCAGCTCTCCAGGAGCACAGTG-3’ (reverse) for Syt VIII Ca\(^{2+}\), and 5’-CAGTTATTGGGACAAGCAGCA-CAG-3’ (forward) and 5’-TGAACCTCATAAGCACCAGCCAC-3’ (reverse) for Syt IX Ca\(^{2+}\).

The amplified products were first cloned into blunt-ended pBluescript plasmid and then into the EcoRI site of the pEGFP-N2 vector (Clontech). PCR-based site-directed mutagenesis reactions (Stratagene) were used to generate point mutations in the Syt VII Ca\(^{2+}\) domain, using the Ca\(^{2+}\)-pEGFP fusion plasmid as template. For mutations of aspartate residues to asparagines (D166N/D172N/D225N/D227N), two rounds of PCR mutagenesis were done using the following primers: 5’-AGGCGCCAGGAGCTTCGCGCGCAAGAAACTGATGGGAC-3’ (forward) and 5’-CTTGCCCAGTGCCCAGTCTCATC-3’ (reverse) for Syt VI Ca\(^{2+}\), 5’-AGCGGAGAGAACC-3’ (forward) and 5’-ATGGGAGGCTGGAGAGGCAAG-3’ (reverse) for Syt VII Ca\(^{2+}\), 5’-TGCTGCTGGAGGGGACCAACAG-3’ (forward) and 5’-ACCAGCTCTCCAGGAGCAGATCTGTTG-3’ (reverse) for Syt VIII Ca\(^{2+}\), and 5’-CAGTTATTGGGACAAGCAGCA-CAG-3’ (forward) and 5’-TGAACCTCATAAGCACCAGCCAC-3’ (reverse) for Syt IX Ca\(^{2+}\).
Quantitation of GFP Expression Levels. The average GFP fluorescence intensity associated with transfected cells was measured on 8-bit data mode images acquired through a 100× objective with a Hamamatsu Orca II cooled CCD camera, controlled by Metamorph imaging software (Universal Imaging). The focal plane and outline of each cell was initially established by phase-contrast observations. Fluorescent images were acquired for 50 ms without autoscale, under conditions that ensured linear dynamic range detection of E-GFP emission at 507 nm. Individual cells on the images were outlined, and average fluorescence intensity values for each outlined region were obtained using the Metamorph “region statistics” function. Background fluorescence intensity values were acquired in an identical area of the same microscopic field containing no transfected cells and subtracted from each value obtained for transfected cells. For each transfection group (GFPv, Syt I C2A–GFP, Syt VII C2A–GFP, Syt VIII C2A–GFP, Syt IX C2A–GFP, and mutated Syt VII C2A–GFP [mSyt VII C2A]), measurements were made in 40 randomly chosen individual cells. Cells expressing very high or very low levels of GFP, previously determined by eye examination, were excluded from the analysis.

Glass Bead Antibody Loading. Confluent monolayers of NRK cells were plated on 12-mm round coverslips 48 h before the experiment. Coverslips were rinsed in PBS2+ (containing Ca2+ and Mg2+) on a heated stage at 37°C and covered with 50 μl of PBS2+ containing 5 mg/ml Texas Red–dextran and 20 μg/ml affinity-purified rabbit anti–Syt VII C2A or preimmune rabbit IgG. The cells were then sprinkled with 0.05 g (~200 beads) of acid-washed glass beads (425–600 μm; Sigma-Aldrich) from a culture tube held 1–3 cm above the coverslip (24). The coverslips were gently rocked four times to let the beads roll over the cells, rinsed in PBS2+, and exposed to T. cruzi trypomastigotes.

Microinjection. Microinjection of NRK and CHO cells was performed on a Zeiss Axiovert 135 microscope equipped with a heated stage, using an Eppendorf micromanipulator 5170 and microinjector 5242. Histidine (his)-tagged constructs of Syt VII and Syt I C2A were expressed in E. coli, purified (8), and concentrated to 1 mg/ml in microinjection buffer (27 mM K2HPO4, 8 mM Na2HPO4, and 26 mM KH2PO4, pH 7.2) containing 1 mg/ml 10,000 MW Texas Red–dextran to visualize injected cells. Approximately 500 cells were microinjected in each experiment,
followed by incubation at 37°C in a 5% CO2 atmosphere for 1 h before exposure to parasites.

**Cell Invasion Assays.** *T. cruzi* invasion assays of NRK and CHO cells were performed by exposing cells plated 48 h before (10^4 cells/cm^2) to 5 × 10^7 trypomastigotes per milliliter in DMEM 2% FBS for 1 h at 37°C. After PBS washes and 2% paraformaldehyde fixation, the number of intracellular and extracellular trypomastigotes associated with cells was determined by immunofluorescence, as previously described (2). For *T. gondii* invasion assays, 5 × 10^7 parasites per milliliter were added to CHO cells plated as above and incubated at 37°C for 1 h. After PBS washes and fixation in 100% methanol for 1 min, intracellular parasites were quantitated by immunofluorescence using the mAb T62H11 specific for the dense granule protein GRA3, which is incorporated into the parasitophorous vacuole membrane during invasion (25). Invasion and immunofluorescence quantitation of intracellular *S. typhimurium* strain SL1344 after a 30-min infection of CHO cells was done as described (26). In all experiments, cells expressing very high or very low levels of GFP, as determined by fluorescence microscopy examination, were excluded from analysis. In all assays, a minimum of 200 transfected cells was analyzed for each point, and results were normalized to parasites per 100 cells.

**Results and Discussion**

As mentioned above, *T. cruzi* invades a large variety of vertebrate cells, through a mechanism involving early interaction with host lysosomes. Previous studies showed that the synaptotagmin isoform Syt VII is localized on the membrane of lysosomes of NRK cells, where it regulates Ca^{2+}-triggered exocytosis (8). Thus, to investigate a possible role for Syt VII on *T. cruzi* invasion, it was first necessary to verify if Syt VII was also present on the membranes of lysosomes of additional cell types. A protein with the expected migration in SDS–PAGE for Syt VII (~65 kDa) was detected on extracts of different cell types (human, rat, mouse, and hamster) with antibodies against the unique NH₂-terminal domain of Syt VII (Fig. 1 A). In immunofluorescence assays, Syt VII was found to colocalize with the lysosomal marker Lamp-1 in human HEK293, mouse 3T3, hamster CHO (Fig. 1 B), human HeLa, and mouse L cells (not shown). Taken together with the previous detailed characterization of Syt VII expression in NRK cells (8), these results indicate that Syt VII is present on the membranes of lysosomes of a large variety of mammalian cell types. Immunofluorescence of *T. cruzi*-infected CHO cells with specific anti–Syt VII antibodies also demonstrated that this isoform is incorporated into the membranes of recently formed intracellular vacuoles containing trypomastigotes (Fig. 1 C). Very similar images (not shown) were obtained in NRK cells transfected with an expression plasmid encoding full-length Syt VII fused to GFP, previously shown to be targeted to lysosomes (8).

To investigate whether modulation of Syt VII function had an effect on *T. cruzi* invasion, affinity-purified antibodies against the Syt VII C2A domain were introduced into the cytosol of CHO cells by glass bead loading. In this procedure, wounds created on the plasma membrane by contact with the glass beads are rapidly resealed at 37°C in the presence of Ca^{2+}, trapping extracellularly added molecules inside the cells (24). Previous studies in NRK cells showed that these anti–Syt VII C2A antibodies, when added to streptolysin O–permeabilized cells, are effective inhibitors of the exocytosis of lysosomes triggered by 1 μM Ca^{2+} (8).

As shown in Fig. 2, a marked inhibition was observed on *T. cruzi* invasion of CHO cells loaded with anti–Syt VII C2A antibodies. Whereas trypomastigotes entered normally unloaded cells or cells loaded with preimmune rabbit IgG, a reduction in the number of intracellular parasites of ~50% was observed in cells preloaded with anti–Syt VII C2A antibodies (Fig. 2, A–C).

Earlier studies showed that soluble recombinant constructs containing the C2A domain of Syt VII also inhibit Ca^{2+}-dependent exocytosis of lysosomes in permeabilized parasites by streptolysin O–permeabilized cells, are effective inhibitors of the exocytosis of lysosomes triggered by 1 μM Ca^{2+} (8).
cells in a dose-dependent manner. Equivalent constructs containing the C2A domain of Syt I, the exclusively neuronal isoform, have no effect (8). To verify if a similar effect was observed with *T. cruzi* invasion, CHO cells were transiently transfected with vectors encoding GFP-tagged constructs of Syt I C2A and Syt VII C2A or GFP alone. The GFP expression levels 24 h after transfection were determined by image analysis as described in Materials and Methods and found to be within a similar range (Table I). When the number of intracellular trypomastigotes was evaluated, no statistically significant difference was found between the number of intracellular parasites in untransfected and in GFPv- and Syt I C2A–GFP-transfected cells ($P = 0.7906$, one-way analysis of variance test). In contrast, an extremely significant inhibition of *T. cruzi* entry was observed in Syt VII C2A–GFP-transfected cells ($P = 0.0001$, unpaired Student’s *t* test; Fig. 3 A). Taken together with the inhibitory effect of anti–Syt VII C2A antibodies (Fig. 2 C), these results strongly suggest that the C2A domain of Syt VII interferes specifically with the *T. cruzi* cell invasion process, similar to what is observed with Ca2+-triggered exocytosis of lysosomes.

We thus proceeded to verify whether invasion by intracellular pathogens different from *T. cruzi*, which enter host cells by mechanisms not involving Ca2+-dependent lysosome recruitment and fusion, was affected by the Syt VII C2A domain. CHO cells were transfected with GFPv, Syt I C2A–GFP, and Syt VII C2A–GFP and infected with the protozoan parasite *T. gondii* or the enteric bacterial pathogen *S. typhimurium*. Toxoplasma enters mammalian cells by a motility-based mechanism, generating a plasma membrane–derived parasitophorous vacuole that does not fuse with lysosomes (27). *Salmonella* triggers extensive actin polymerization and membrane ruffling in host cells, being internalized in macropinocytic vacuoles (26). No significant difference in *T. gondii* invasion levels was detected among cells expressing the three different constructs ($P = 0.8484$; Fig. 3 B). In the *S. typhimurium* invasion assays, a marginally significant ($P = 0.0526$) enhancement in the number of intracellular bacteria (reproducible in several experiments) was observed in cells transfected with Syt VII C2A–GFP (Fig. 3 C). These findings thus indicate that the mechanism by which Syt VII C2A reduces host cell suscep-

### Table I. Expression Levels of GFP and Synaptotagmin C2A–GFP Constructs in Transiently Transfected CHO Cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Average intensity</th>
<th>SD</th>
<th>Intensity range</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFPv</td>
<td>30.7</td>
<td>15.8</td>
<td>14.9–46.5</td>
</tr>
<tr>
<td>Syt I C2A</td>
<td>38.5</td>
<td>17.3</td>
<td>21.2–55.8</td>
</tr>
<tr>
<td>Syt VII C2A</td>
<td>26.2</td>
<td>13.3</td>
<td>12.9–39.5</td>
</tr>
<tr>
<td>Syt VIII C2A</td>
<td>28.1</td>
<td>16.3</td>
<td>11.8–44.4</td>
</tr>
<tr>
<td>Syt IX C2A</td>
<td>20.0</td>
<td>10.3</td>
<td>9.7–30.3</td>
</tr>
<tr>
<td>mSyt VII C2A</td>
<td>29.9</td>
<td>18.2</td>
<td>11.7–48.1</td>
</tr>
</tbody>
</table>

Average fluorescence intensity values correspond to the means of measurements made on images of 40 individual transfected cells, as detailed in Materials and Methods. Background values (subtracted from each fluorescence intensity value) were 22.4 ± 0.5 for cells expressing synaptotagmin C2A–GFP constructs and 25.9 ± 1.1 for cells expressing the pEGFP-N2 vector alone (GFPv).

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** Expression of the Syt VII C2A domain inhibits cell invasion by *T. cruzi* but not by *T. gondii* or *S. typhimurium*. (A) *T. cruzi* invasion of CHO cells untransfected (gray bars; unt) or transfected (white bars) with GFPv, Syt I C2A–GFP, and Syt VII C2A–GFP. (B) *T. gondii* invasion of CHO cells transfected with GFPv, Syt I C2A–GFP, and Syt VII C2A–GFP. (C) *S. typhimurium* invasion of CHO cells transfected with GFPv, Syt I C2A–GFP, and Syt VII C2A–GFP. The data is expressed as the mean ± SD of triplicate infections. The color images below panels A, B, and C illustrate the immunofluorescence detection assays used to distinguish intracellular and extracellular pathogens: GFP is shown in green, antibodies against *T. cruzi*, *Toxoplasma*, or *Salmonella* in red, and DAPI DNA stain in blue. Arrows point to extracellular organisms and arrowheads to intracellular ones.
somes with the plasma membrane (8). Transfected with Syt VII C 2A constructs (Fig. 3 A), could be achieved by microinjection. Earlier studies demonstrated those reached in transiently transfected cells were probably not expressed in intact cells, we investigated whether the residual invasion observed in Syt VII C2A–transfected cells had the hallmarks of lysosome-mediated entry, extensively characterized previously in our laboratory (2). All results indicated that this is the case (not shown). First, the intracellular trypomastigotes found in Syt VII C2A–transfected cells after short infection periods were inside intracellular compartments containing the lysosomal markers Syt VII and Lamp-1. Second, when the actin cytoskeleton of host cells was disrupted by pretreatment with cytochalasin D, invasion levels of cells expressing GFP, Syt I–GFP, or Syt VII–GFP were equally increased in ~50–60%. Such enhanced susceptibility to T. cruzi infection after cytochalasin D treatment was previously described in several cell types and functionally correlated with facilitated lysosome recruitment and fusion (2, 7). This finding clearly rules out a phagocytic, actin-based alternative invasion mechanism. Third, the intracellular parasites escaped normally from the acidified lysosome-derived vacuoles and replicated in the cytosol (28), regardless of the presence of expressed GFP, Syt I C2A–GFP, or Syt VII C2A–GFP in the cells.

In spite of extensive similarity, characteristic sequence differences exist between the C2A domains of Syt I and Syt VII (11, 17). Our present and previous (8) results also revealed important functional differences between these domains. The inhibitory effect of Syt VII C2A and not of Syt I C2A on both T. cruzi invasion and lysosome exocytosis may be related to the previously reported distinct properties exhibited by recombinant forms of these isoforms on protein binding assays. Recombinant Syt I was reported to require Ca2+ concentrations in the 200 μM range for binding to syntaxin in vitro, whereas Syt VII–syntaxin interactions were detected at Ca2+ concentrations below 10 μM (11). Interestingly, exocytosis of lysosomes, which is specifically inhibited by the C2A domain of Syt VII, is also triggered at low micromolar [Ca2+]i (8, 21). A similar differential requirement for [Ca2+]i was also reported recently for the interaction of distinct synaptotagmin isoforms with SNAP-25 (12). In addition, Ca2+-dependent oligomerization has been recently proposed to play a central role in the regulation of exocytosis by synaptotagmins (29–31).

To further test the specificity of the inhibitory effect of Syt VII C2A, we investigated whether the C2A domains of additional synaptotagmin isoforms expressed in intact cells also influenced T. cruzi entry. Specific primers were designed to amplify the C2A domains of Syt I, II, III, VI, VII, VIII, and IX from NRK and CHO cell mRNA by RT–PCR. In addition to Syt VII, only the C2A domains of Syt VIII and Syt IX were specifically detected. As expected, the C2A domain of the synaptic vesicle–specific isoform Syt I, which is very abundantly expressed in the brain (11), was not amplified from NRK or CHO cell mRNA. Syt VIII was previously detected in several different tissues and defined as a ubiquitously expressed isoform (11, 14). Specific amino acid substitutions that abolish

Figure 4. The inhibitory effect on T. cruzi invasion is specific for Syt VII and is abolished by mutation of critical C2A domain residues. The number of intracellular trypomastigotes was quantitated after infection of: (A) NRK cells transfected with GFPv (pEGFP-N2 vector alone), Syt I C2A–GFP, or Syt VII C2A–GFP; (B) NRK cells microinjected with his-tagged Syt I C2A or Syt VII C2A; (C) CHO cells transfected with GFPv or GFP–fused C2A domains of the synaptotagmin isoforms Syt I, VII, IX, or VIII; (D) CHO cells transfected with GFPv, Syt VII C2A–GFP, or mSyt VII C2A. The data is expressed as the mean ± SD of triplicate infections.
Ca²⁺ binding were identified in the C₂A domain of Syt VIII, consistent with the lack of Ca²⁺-dependent binding of this domain to either phospholipids or syntaxin (11, 17, 32). Very little is known about the Syt IX isoform (also referred to as Syt V [reference 14]), except that it appears to be expressed in nonneuronal cells, consistent with our findings (33). When T. cruzi invasion assays were performed in CHO cells transiently transfected with C₂A–GFP fusion proteins (33), when expressed in nonneuronal cells, consistent with our findings (33). When T. cruzi invasion assays were performed in CHO cells transiently transfected with C₂A–GFP fusion constructs of Syt I, VII, VIII, and IX, only the Syt VII C₂A domain had a significant inhibitory effect on parasite entry (extremely significant: P < 0.0001; Fig. 4 C). The expression levels of all constructs, as determined from the GFP cytosolic fluorescence intensity, were within a similar range (Table I).

The results described above strongly suggested that the C₂A domain of Syt VII has unique properties responsible for both the modulation of T. cruzi entry and Ca²⁺-dependent exocytosis of lysosomes (8). Although the structure of the Syt VII C₂A domain has not yet been solved, the structure of the Syt I C₂A domain is known to consist of an independently folded, eight-stranded β sandwich formed by two four-stranded antiparallel β sheets (34). Structural and biochemical studies showed that Ca²⁺ ions bind at the top of the β sandwich, through five clustered aspartic acid residues, conserved in several Syt isoforms. Interaction with Ca²⁺ does not lead to conformational changes on the C₂A domain of Syt I but instead causes a marked change in the molecule’s electrostatic potential, increasing its affinity for effector molecules such as syntaxin and phospholipids (10). It has also been suggested that the unique electrostatic properties of synaptotagmins may allow a positively charged, polybasic region present at the center of both the C₂A and C₂B domains to interact with the negatively charged surface at the core of the SNARE complex, facilitating membrane fusion (35). Indeed, recent studies demonstrated that the polybasic KKKK motif at residues 189–192 of the C₂A domain of Syt I plays a central role in the regulation of Ca²⁺-triggered exocytosis in PC12 cells. Replacement of these residues by alanines did not interfere with proper folding of the C₂A domain, as indicated by normal interaction with syntaxin and phospholipids in vitro, but reduced its inhibitory activity in dense core granule secretion assays (20). We found that recombinant Syt VII C₂A constructs containing mutations either in the corresponding polybasic motif (K183,184A/H185A/K186A and D166,172,225,227N) or in the putative Ca²⁺-binding aspartic acid residues (D166,172,225,227N) were still inhibitory in β-hexosaminidase secretion assays with streptolysin O-permeabilized NRK cells (unpublished data), reinforcing the conclusion that these amino acid replacements do not appear to interfere with proper folding of the Syt VII C₂A domain. The lack of reduced inhibitory activity after these mutations was not surprising, considering the variation in sequence between the polybasic motif of Syt I and Syt VII C₂A (11), and particularly in view of recent findings indicating that functional Ca²⁺-binding sites remain in Syt I after mutation of Ca²⁺-coordinating aspartic acid residues (36). Interestingly, however, when both sets of mutations

References


