Biochemical Characterization and Substrate Specificity of Autophagin-2 from the Parasite Trypanosoma cruzi*

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Background: Successful colonization of humans by the parasite Trypanosoma cruzi critically depends on intact parasite autophagy and autophagin-2.

Results: T. cruzi autophagin-2, in contrast to human autophagin-1, exhibits substantial preference toward the unnatural amino acid cyclohexylalanine in the P2 position.

Conclusion: Selective fluorogenic substrates, which are only recognized by T. cruzi autophagin-2, were synthesized.

Significance: These selective substrates have a potential for screening of T. cruzi autophagin-2 specific inhibitors.

The genome of the parasite Trypanosoma cruzi encodes two copies of autophagy-related cysteine proteases, Atg4.1 and Atg4.2. T. cruzi autophagin-2 (TcAtg4.2) carries the majority of proteolytic activity and is responsible for processing Atg8 proteins near the carboxyl terminus, exposing a conserved glycine. This enables progression of autophagy and differentiation of the parasite, which is required for successful colonization of humans. The mechanism of substrate hydrolysis by Atg4 was found to be highly conserved among the species as critical mutations in the TcAtg4.2, including mutation of the conserved Gly-244 residue in the hinge region enabling flexibility of the regulatory loop, and deletion of the regulatory loop, completely abolished processing capacity of the mutants. Using the positional scanning-substrate combinatorial library (PS-SCL) we determined that TcAtg4.2 tolerates a broad spectrum of amino acids in the P4 and P3 positions, similar to the human orthologue autophagin-1 (HsAtg4B). In contrast, both human and trypanosome Atg4 orthologues exhibited exclusive preference for aromatic amino acid residues in the P2 position, and for Gly in the P1 position, which is absolutely conserved in the natural Atg8 substrates. Using an extended P2 substrate library, which also included the unnatural amino acid cyclohexylalanine (Cha) derivative of Phe, we generated highly selective tetrapeptide substrates acetyl-Lys-Lys-Cha-Gly-AFC (Ac-KKChaG-AFC) and acetyl-Lys-Thr-Cha-Gly-AFC (Ac-KTChaG-AFC). Although these substrates were cleaved by cathepsins, making them unsuitable for analysis of complex cellular systems, they were recognized exclusively by TcAtg4.2, but not by HsAtg4B nor by the structurally related human proteases SENP1, SENP2, and UCH-L3.

Autophagy is a lysosome- and/or vacuole-dependent mechanism of degradation of intracellular constituents, highly conserved through evolution (1). It enables cells to adopt extracellular and intracellular stress such as nutrient and growth factor deprivation (2–4), hypoxia (5–7), removal of excessive and damaged cytosolic proteins (8), and organelles (9–12) and invading pathogens (13, 14). It also plays a crucial role during the development (15, 16), differentiation (17, 18), embryogenesis (19), and tissue remodeling in various organisms (20–22). Formation of autophagosomes and execution of autophagy critically depend on proteolytic processing of the Atg8 and its subsequent conjugation to the phosphatidylethanolamine in the expanding phagophore membrane. The protease responsible for the processing of proAtg8 homologues, the Atg4 cysteine protease, cleaves the peptide bond after the conserved glycine residue at the C terminus of the protein (23–25). The processed Atg8 is further transferred to Atg7 (E1 activating enzyme) and Atg3 (E2 conjugating enzyme) and finally conjugated to the phosphatidylethanolamine moiety in the phagophore membrane. After autophagosome formation, the Atg8-phosphatidylethanolamine complex is deconjugated by the same Atg4 protease, leading to the release of Atg8 from the outer phagosome membrane. The whole conjugating reaction is reminiscent of the ubiquitin-proteasome system, where deubiquitinating enzymes participate in the control of ubiquitin and ubiquitin-like modifiers. The core machinery of macroautophagy, which clears non-selectively engulfed cargo, is evolutionary highly conserved among all eukaryotes, from unicellu-
lar organisms, such as yeast, protozoan pathogens, and free living protists, to fungi, plants, and mammals including humans (26–28).

The flagellated protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease, a prevalent health problem in Central and Latin America, with an estimated 7–8 million people infected (29). The *T. cruzi* life cycle alternates between the insect vector and the mammalian host and involves four developmental stages, two replicative forms, and two infective forms. All four forms are needed for successful colonization of both hosts and for the survival of the parasite (30). The differentiation process is characterized by massive metabolic and morphological changes, which critically depend on an activated autophagic pathway. Using bioinformatic analysis, a functional Atg8 ubiquitin-like conjugating system has been identified in the parasite (Atg3, Atg4, Atg7, and Atg8), whereas the major components of the Atg12 conjugation system have not been identified as yet (26). *T. cruzi* genome encodes two Atg4 and two Atg8 homologues, TcAtg4.1 (Uniprot code A6XG54), which are proline rich (31). Moreover, TcAtg4.2 and TcAtg8.1 were already characterized as yeast Atg4 and Atg8 counterparts (2008-02-08 nomenclature). Moreover, TcAtg4.2 and TcAtg8.1 were previously (33–37).

Biochemical Characterization of TcAtg4.2

**Construction of Plasmids**—Preparation of the plasmids carrying the sequences encoding wild-type TcAtg4.2, TcAtg8.1, and TcAtg8.2 was described previously (31). Catalytically inactive mutant TcAtg4.2 C91S (Ser was selected as structurally most similar to Cys), mutant lacking the regulatory loop (amino acids 245–258) TcAtg4.2 A129H, F130S, and G131A mutants were generated using QuikChange™ (Stratagene) with oligonucleotides 5'-ACGTCATATGGCGCCAACAGAAATGGGCGCTTCTAATGGCTGTA-3' for TcAtg4.2 and 5'-AGTCATATGGCGCCAACAGAAATGGGCGCTTCTAATGGCTGTA-3' for TcAtg8.2. TcAtg8.2 mutants lacking the first 18 in TcAtg4.2 dN18 and 24 amino acid residues in TcAtg4.2 dN24 were generated by PCR using the forward primers 5’-AACATATGTTGGCTAGATCTGTG-3’ for TcAtg4.2 dN18 and 5’-AACATATGTTGGCTAGATCTGTG-3’ for TcAtg4.2 dN24 both carrying the Nd6l restriction site and the reverse primer 5’-AAGGATCTAATGCTGTA-3’ the included BamHI restriction site. The amplified PCR fragments were digested by Nd6l and BamHI (New England Biolabs) and cloned into the pET-28a(+) bacterial expression vector (Novagen). TcAtg8.2 S128G, A129H, F130S, and G131A mutants were generated using QuickChange™ (Stratagene) with oligonucleotides for TcAtg8.2 A129H, 5’-AACATATGTTGGCTAGATCTGTG-3’. The amplified PCR fragments were digested by NdeI and restriction site and the reverse primer 5’-AAGGATCTAATGCTGTA-3’ for TcAtg8.2 dF130S, 5’-CACG-TCATATGGCGCCAACAGAAATGGGCGCTTCTAATGGCTGTA-3’ for TcAtg8.2 dG131A, 5’-AGG-TCATATGGCGCCAACAGAAATGGGCGCTTCTAATGGCTGTA-3’ and 5’-CACG-TCATATGGCGCCAACAGAAATGGGCGCTTCTAATGGCTGTA-3’.

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**Expression and Purification of Recombinant Proteins**—All plasmids were introduced into the *Escherichia coli* BL21(DE3) strain. Transformants were stimulated to produce recombinant proteins by incubation with 0.5 mM isopropyl β-D-galactopyranoside at 27 °C for 4 h expressing TcAtg8 proteins and 25 °C for 6 h expressing TcAtg4.2 and TcAtg8.2. All proteins were expressed as fusion protein with a His, tag and were allowed to bind to HisTrap FF crude columns (GE Healthcare) and eluted with buffer containing 250 mM imidazole. DeSUMOylating and...
deubiquitinating enzymes were produced and purified as previously described (33, 34). Human capthensins were produced and purified as previously described (35–37). Purity and the relative molecular masses of the purified proteins were verified by SDS-PAGE (12.5% gel) followed by Coomassie Brilliant Blue staining.

Circular Dichroism (CD) Spectroscopy—Far-UV CD spectra of TcAtg4.2 were recorded in an Aviv 62 ADS CD spectropolarimeter (Aviv Biomedical, Lakewood Township, NJ) equipped with a thermoelectric sample holder for temperature control as described (38). Briefly, samples (0.1 mg/ml) were measured in a 0.1-cm cell and at a bandwidth of 1 nm, whereas the temperature was maintained at 25 °C throughout. Data were collected every nanometer and the mean residue ellipticity in deg cm2/°/mg was calculated using a mean weight of 109/residue (MRW).

In Vitro Cleavage of TcAtg8 Proteins—For cleavage assay of recombinant TcAtg8 constructs by recombinant TcAtg4.2, 1–4 μg of TcAtg8 proteins were incubated with 0–150 ng of TcAtg4.2 in 20 μl of reaction buffer, containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM DTT. To determine the ability of TcAtg4.2 to cleave wild-type and mutated TcAtg8 molecules with substituted amino acid residues in positions P4, P3, P2, and P1 the samples were incubated at 37 °C for the designated times. To determine the catalytic efficiency of the enzyme we used the equation: 

\[ k_{\text{cat}}/k_{\text{m}} = \frac{1}{t \times EC_{\text{cat}}}, \]

where \( EC_{\text{cat}} \) is the enzyme concentration at which 50% conversion of the substrate is obtained (39, 40). Reactions were stopped by addition of 6× SDS loading dye and incubation at 100 °C for 5 min.

Synthesis and Characterization of Single Tetrapeptide-AFC (7-Amino-4-trifluoromethyl-coumarin) Substrates—To synthesize Boc-Gly-AFC, 1 eq of Boc-Gly-COOH and 1 eq of AFC (7-amino-4-carbamoylmethylcoumarin) Positional Scanning-Synthetic Combinatorial Peptide Library (PS-SCL)—The PS-SCL was synthesized using semiautomatic FlexChem Peptide Synthesis System (model 202) as described earlier (33, 34, 40). All substrates from each sublibrary (P4, P3, and P2) were dissolved in DMSO at concentrations of 50 or 20 μM and stored at −20 °C until use.

Effect of pH and Salts on TcAtg4.2 Activity—The effect of different salts on TcAtg4.2 activity was determined by incubating 4 μM enzyme with 100 μM Ac-ETFG-AFC in the presence of 1 M salt in 25 mM Tris-HCl, pH 8.0, and 5 mM DTT. The optimal pH of TcAtg4.2 was determined by incubation of 4 μM enzyme and 100 μM Ac-ETFG-AFC in 50 mM reaction buffer, containing MES buffer (pH 6.0 and 6.5), HEPES buffer (pH 7.0 and pH 7.5), Tris-HCl (pH 8.0 and pH 8.5), and Bistris propane buffer (pH 9.0) in the presence of 500 mM K2HPO4 and 5 mM DTT. The enzyme was activated with DTT for 10 min at 37 °C before adding to the wells containing substrate. The reaction volume was 100 μl. Release of AFC fluorophore was monitored continuously with excitation at 400 nm and emission at 505 nm. The total assay time was 30 min, and only the liner portions of the progress curves was used to calculate velocity.

Assay of the PS-SCL—All reactions were assayed at 37 °C in 25 mM Tris-HCl, pH 8.0, containing 500 mM K2HPO4 and 5 mM DTT. The enzyme was activated with DTT for 10 min at 37 °C before being added to the wells containing substrates. Standard reaction conditions were reaction volume, 100 μl, final enzyme concentration, 4 μM, and final substrate concentration, 250 μM, which is similar to conditions used for DeSUMOylating and deubiquitinating enzymes, and HsAtg4B (250–500 μM) (33, 34, 40). Release of the AFC fluorophore was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm for 30 min. Only the linear portions of the progress curves were used for further analysis. In each sublibrary the substrate was solved in a minimal amount of N,N-dimethylformamide, followed by the addition of 1 eq of Ac-peptide-COOH and 1 eq of HBTU (O-benzotriazole-N,N,N′,N′′-tetramethyl-uronium-hexafluoro-phosphate). Finally, 3 eq of DIMEA (N,N-dissopro-pylethylamine) were added and the coupling reaction was carried out for 3 h. After the reaction was completed, as judged by HPLC analysis, the mixture was diluted in ethyl acetate and extracted with 5% citric acid (2x), 5% NaHCO3 (2x), and brine (2x). The organic fraction was dried over MgSO4 and the ethyl acetate was then removed under reduced pressure to obtain crude Ac-peptide-Gly-AFC. Next, all protecting groups were removed in 50% TFA in dichloromethane (with 2.5% of triisoproplysilane as scavenger) for 1 h. Finally, the crude product was purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using a semi-preparative Waters Spherisorb S100DS2 column. The molecular weight of each Ac-peptide-Gly-AFC substrate was determined by high-resolution mass spectrometry using a High Resolution Mass Spectrometer WATERS LCT premier XE with Electrospray Ionization (ESI) and Time of Flight (TOF) module.
Biochemical Characterization of TcAtg4.2

with the highest relative fluorescence unit value was set to 100%. The other results were adjusted accordingly to the 100% of the best substrate. The difference between individual values was in every case smaller than 10%. The \( k_{cat}/K_m \) of TcAtg4.2 was determined using nonlinear regression analysis of a series of progress curves using the equation: \( k_{cat}/K_m = \text{slope}/E \).

Assay of SENPs, DUBs, and Cysteine Cathepsins B, K, L, and S—The enzymatic activities of recombinant SENP1, SENP2, and UCH-L3 were previously confirmed with cleavage of the optimal fluorogenic synthetic tetrapeptidic substrates Ac-QTGG-ACC for SENPs and Ac-LRGG-AC for UCH-L3 (33, 34). All reactions were assayed at 37 °C in 25 mM Tris-HCl, pH 7.5, containing 500 mM Na\(_3\)C\(_3\)H\(_5\)(COO)\(_3\) and 5 mM DTT. The enzymes were activated for 10 min at 37 °C in the same DTT-containing buffer before they were added to the wells containing substrates. Standard reaction conditions were 100 µl reaction volume, 4–6 µM final enzyme concentration, and 100 µM final substrate concentration. The enzymatic activities of recombinant cathepsins were confirmed with cleavage of Cbz-FR-AMC, Cbz-RR-AMC, and Cbz-FVR-AMC. Standard reaction conditions for the cleavage of synthetic substrates were 100 µl reaction volume (100 mM BANA (0.1M phosphate buffer, 1.5 mM EDTA, pH 6.5), containing 0.1% PEG-6000 and 5 mM DTT), 10–50 mM final enzyme concentration, and 100 µM final substrate concentration.

To evaluate cleavage of recombinant Atg8 homologues TcAtg8.1, TcAtg8.2, and human GABARAPL2/GATE-16 with recombinant human cathepsins B, K, L, and S, substrates and enzymes were incubated at a 50:1 molar ratio in 20 µl of BANA buffer, pH 6.5, containing 0.1% PEG-6000 and 5 mM DTT for 1 h at 37 °C. Reactions were stopped by addition of 6× SDS loading dye and incubation at 100 °C for 5 min. The samples were then loaded on 16% Tricine SDS gels and separated (42).

Results

Kinetics of TcAtg8.1 and TcAtg8.2 Cleavage with TcAtg4.2—Recombinant TcAtg4.2 and human Atg4B autophagins, and Atg8 homologs TcAtg8.1 and TcAtg8.2 were expressed in E. coli BL21(DE3) host strain and purified using a Ni\(^{2+}\)-nitrioltriacetic acid column. Relative molecular masses and purity of proteins was verified by SDS-PAGE (12.5% gel) followed by Coomassie Brilliant Blue staining (Fig. 1A). To confirm that the recombinant TcAtg4.2 is catalytically active the enzyme was incubated with its natural substrates TcAtg8.1 and TcAtg8.2 (31). Processing of the substrates was monitored by SDS-PAGE and \( k_{cat}/K_m \) values of 3.7 × 10\(^5\) and 3.4 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) were determined for processing TcAtg8.1 and TcAtg8.2, respectively, using the same method as described previously for caspasases (39). In the control experiment, TcAtg8.1 and TcAtg8.2 proteins were incubated in the reaction buffer in the absence of TcAtg4.2 and no processing could be detected (Fig. 1B).

Alignment of amino acid sequences of multiple autophagin orthologues revealed a highly conserved amino acid sequence around Cys-74, which is the catalytic Cys in human autophagin-1 (Fig. 3D). To evaluate the importance of its equivalent in TcAtg4.2, Cys-91, the latter was mutated into a Ser. When TcAtg8.1 and TcAtg8.2 proteins were incubated with the TcAtg4.2 C91S mutant processing of proTcAtg8.1 or proTcAtg8.2 was abolished, suggesting that Cys-91 is indeed the active site Cys residue in TcAtg4.2. Prolonged incubation of TcAtg4.2 C91S with Atg8.1 (24 h) resulted in a very minor proteolysis, which was ~10,000 times lower than the enzymatic activity of the wild-type TcAtg4.2, similar to what was observed earlier for TcAtg4.1 (31) (Fig. 1B). This is in agreement with previous results on human Atg4B, where a very minor activity of C74S on Atg8 substrates was observed (43). In addition, in adenoviral protease replacement of the active site Cys to Ser also resulted in a protease with substantially diminished activity (44, 45).

Biochemical Characterization of TcAtg4.2—In addition to processing natural substrates, human autophagin-1 (HsAtg4B) was found to be able to process small synthetic fluorogenic tetrapeptide substrates (40). The cleavage of tetrapeptide fluorogenic substrates was not very efficient, but could be enhanced in the presence of an optimal kosmotropic salt. Based on the high homology between the human Atg4B and TcAtg4.2, four tetrapeptide substrates carrying AFC fluorophore, which were found to be cleaved by HsAtg4B, were therefore used for the initial biochemical analyses. Ac-GTFG-AFC and Ac-STFG-AFC substrates were derived from the HsAtg4B PS-SCL, whereas Ac-NTFG-AFC and Ac-ETFG-AFC were derived from human Atg4 substrates GATE-16 and LC3, respectively. The hydrolysis of Ac-ETF-G-AFC by TcAtg4.2 was substantially more efficient than of the other three substrates. However, all reactions exhibited very slow kinetics (Fig. 2A). The latter was therefore used for further biochemical characterization of TcAtg4.2. The enzyme was found to be active between pH 6.5 and 8.5 with the maximum activity at pH 8.0, whereas its activity dropped sharply at pH 9.0 (Fig. 2B). Because it is known that kosmotropic salts can significantly enhance proteolytic activities of various proteases (33, 34, 40) we applied this approach also to TcAtg4.2. The activity dramatically increased at a high concentration of various citrate, phosphate, and sulfate salts, with the highest activity observed in the presence of K\(_2\)HPO\(_4\) (Fig. 2C). A titration experiment showed that the proteolytic activity of TcAtg4.2 increased with the increasing salt concentration even above 1 M (Fig. 2D). However, at high salt concentrations (≥1 M) the enzyme started to precipitate. Circular dichroism measurements in the far-UV and deconvolution analyses made with the Dichroweb software (46, 47) showed unfavorable changes in the enzyme conformation when the K\(_2\)HPO\(_4\) concentration was increased from 0.5 to 1.0 M. A significant decrease in α-helix (from ~60 to 40%, respectively) and β-sheet (from 10 to 3%, respectively) content was thus observed at 1.0 M salt. Moreover, protein precipitation seen at 1.0 M K\(_2\)HPO\(_4\) also resulted in decreased ellipticity (Fig. 2E) and therefore 0.5 M K\(_2\)HPO\(_4\) was used in all further experiments. In addition, the cleavage efficacy of the enzyme was improved by elongation of the synthetic substrates, as demonstrated using tetra- (ETFG), hexa- (SQETF), and octapeptides (YASQETF) based on the sequence of the natural substrate LC3B, which were designed for HsAtg4B (40) (Fig. 2F). In a control experiment, active site Cys-91 was mutated into Ser, which has almost completely abolished substrate processing (Fig. 2G).
Structure-function Basis for TcAtg4.2 Substrate Hydrolysis—

During the evolution of eukaryotes ATG4 genes multiplied. There is only one Atg4 member in yeast possessing both, endopeptidase and isopeptidase activity (25). Two members were found in kinetoplastid parasites T. cruzi, Trypanosoma brucei, and Leishmania major (Atg4.1 and Atg4.2), where LmAtg4.1 possesses endopeptidase activity and LmAtg4.2 possesses isopeptidase activity (31, 48, 49), whereas four members have been found in mammals (autophagin-1/Atg4B, autophagin-2/Atg4A, autophagin-3/Atg4C, and autophagin-4/Atg4D). However, mammalian autophagins have different tissue distribution as well as catalytic properties. Among them, only Atg4A and Atg4B are catalytically active with Atg4B carrying the highest catalytic activity. In addition, Atg4B is the most important autophagin involved in basal macroautophagy in mammals (43, 50, 51).

To interpret substrate hydrolysis of TcAtg4.2 at the molecular level, we first analyzed the phylogenetic relationship between TcAtg4.2 and other Atg4 othologues, using a phylogenetic tree construction program MEGA6 based on distances of amino acid sequences from five species (human, yeast, T. cruzi, T. brucei, and L. major).

The dendrogram placed TcAtg4.2 near TbAtg4.2 and LmAtg4.1, the latter two carrying the majority of endopeptidase activity in these species, with sequence identity of 45 and 34.5%, respectively (Fig. 3A). Moreover, the TcAtg4.2 amino acid sequence exhibits 21.3 and 22.2% identity with the sequences of HsAtg4A and HsAtg4B, respectively, with several conserved regions that share higher local identity to both human autophagins. To explore whether the mechanism of action of TcAtg4.2 is indeed highly conserved as compared with HsAtg4B, the SWISS-MODEL structure homology-modeling server (52–55) and RaptorX web server (56) were used to model the three-dimensional structure of TcAtg4.2 as no crystal structure of autophagins from the trypanosomatids family of parasites is available. To construct the TcAtg4.2 three-dimensional model, the structures of the human homologues HsAtg4A (PDB code 2p82) and HsAtg4B (PDB code 2d1i) were
Biochemical Characterization of TcAtg4.2

FIGURE 2. Biochemical characterization of TcAtg4.2. All experimental details are described under "Experimental Procedures." A, TcAtg4.2 cleavage of Ac-ETF2-AFC, Ac-SFT2-AFC, Ac-NTF2-AFC, and Ac-GTF2-AFC. TcAtg4.2 cleaves Ac-ETF2-AFC 7.4, 6.3, and 2.2 more efficient than Ac-STF2-AFC, Ac-NTF2-AFC, and Ac-GTF2-AFC, respectively. The y axis represents relative fluorescence units/s. Only the linear portions of the progress curves were used to calculate velocity. B, pH dependence of the TcAtg4.2 activity was assayed in 50 mM buffers of various pH values in the presence of 0.5 M K2HPO4 and 5 mM DTT. The concentrations of enzyme and Ac-ETF2-AFC were 4 and 100 μM, respectively. C, to evaluate the effect of buffer composition on TcAtg4.2 activity the enzyme was incubated with non-Hoffmeister and Hoffmeister salts. The latter increase proteolytic activity of the enzyme. The non-Hoffmeister salts NaCl, KCl, and (NH4)2SO4 were used as a control to rule out the effects from alteration of ionic strength. D, effect of K2HPO4 concentration on proteolytic activity of TcAtg4.2. E, effect of K2HPO4 concentration on conformation of TcAtg4.2. Far-UV CD spectra of TcAtg4.2 in the absence or presence of different K2HPO4 concentrations shows unfavorable changes in the enzyme conformation when salt concentrations excess 0.5 M. F, the effect of substrate length on TcAtg4.2 activity. TcAtg4.2 activity was measured in the presence of synthetic tetra-, hexa-, and octapeptide substrates, and was found to increase with the increasing length of the substrate. G, Cys-91 to Ser mutation abolishes the activity of TcAtg4.2. The S.D. values in all experiments were calculated from at least three independent measurements.

used as templates (Fig. 3B). The SWISS-MODELs global and per-residue model quality was assessed using the QMEAN scoring function with integrated Z-score (57). The QMEAN value of 0.63 indicates a good quality of the predicted model. In addition, the quality evaluation parameters of RaptorXs p value (3.4 × 10−12), score (286), and uGDT/GDT (236/76) also indicate good quality of the predicted three-dimension model (Fig. 3C), suggesting that both constructed models are appropriate for further studies. Using this TcAtg4.2 model and sequence alignment (Fig. 3D) several TcAtg4.2 mutants were then prepared to study functional conservation of the structural elements involved in substrate hydrolysis (58, 59). The first is the regulatory loop region comprising residues 258–263 (HsAtg4B numbering), which shares very high sequence homology throughout different species. In human Atg4B the loop covers the active site Cys-74 residue and occupies the active site cleft in the free enzyme, thereby preventing substrate hydrolysis. The closed conformation of the loop in the enzyme-free form is maintained by limited interactions of the loop with the neighboring side chains, where the majority of van der Waals interactions are formed between the side chain of Trp-142, which is located in the loop between helices D and E, and Pro-260 in the regulatory loop. However, upon substrate binding Phe-119 and Gly-120 from the C-terminal tail of LC3B interact with Trp-142 of HsAtg4B and open the regulatory loop. In addition, neighboring interactions between Pro-260 (equivalent to Ser-246 in TcAtg4.2) in this loop and Trp-142 (equivalent to Trp-158 in TcAtg4.2) in the vicinity embrace the C-terminal tail of LC3B and position the scissile bond in proximity of the catalytic Cys residue, as seen in the crystal structure of Atg4B-LC3B complex (59), thereby enabling processing of the substrate. To address the role of the loop in TcAtg4.2, the corresponding residues (245–248) were deleted to generate the TcAtg4.2 dL mutant. Deletion of the regulatory loop resulted in a complete loss of processing capacity, indicating its critical role in substrate hydrolysis in particular in positioning the C terminus of Atg8 (Fig. 4A).

Next, the role of the highly conserved Pro-260 (Ser-246 in TcAtg4.2) in this loop was addressed. First, Ser-246 was mutated into Pro, which slightly enhanced the proteolytic activity of TcAtg4.2, in agreement with earlier data demonstrating that Pro-260 is not critical for the activity of HsAtg4B on its natural substrate LC3 (58). However, mutating Ser-246 into Asp resulted in a significant enhancement of the proteolytic activity of TcAtg4.2 (Fig. 4A). Because the flexibility of the regulatory loop in Atg4 is maintained by two conserved Gly residues (Gly-256 and Gly-257 in human Atg4B) at the N terminus of the regulatory loop, one of them (Gly-244 in TcAtg4.2/Gly-257 in HsAtg4B) was mutated into His, resulting in a complete loss of processing capacity (Fig. 4A). Having shown the importance of the regulatory loop and its flexibility, we next addressed the role of Trp-158, the TcAtg4.2 equivalent of HsAtg4B Trp-142. Upon mutation into Ala, only weak processing of TcAtg8.1 was observed, in agreement with previous
results on the HsAtg4B W142A mutant (58). Because in the crystal structure of the HsAtg4 complex with LC3 (59), Trp-142 was moved from its normal position, we next wanted to explore whether it would be possible to monitor this movement by changes of tryptophan fluorescence. Therefore, fluorescence spectra of the wild-type TcAtg4.2 and the W158A mutant were compared. Unfortunately, no difference was observed, suggesting that the fluorescence intensity of Trp-158 is not sufficient to allow its monitoring (data not shown).

The second important region in HsAtg4B is the N-terminal tail, which masks the exit of the catalytic site of the free enzyme (40, 59). We therefore prepared two TcAtg4.2 mutants, TcAtg4.2 dN18 and TcAtg4.2 dN24, lacking the first 18 or 24 amino acid residues, respectively. When compared with WT TcAtg4.2, the activity of the two mutants toward TcAtg8.1 was almost the same ($k_{cat}/K_m = 3.7 \times 10^3$, $3.1 \times 10^3$, and $2.8 \times 10^3$ M$^{-1}$ s$^{-1}$ for WT TcAtg4.2, TcAtg4.2 dN18, and TcAtg4.2 dN24, respectively; Fig. 4A). Similarly as for the natural sub-
strate, the activity of the mutants on the small synthetic substrate Ac-ETFG-AFC was only slightly affected. TcAtg4.2 dN18 exhibited somewhat diminished activity, whereas the activity of TcAtg4.2 dN24 was moderately increased (Fig. 4B).

As full-length Atg8 is required for efficient hydrolysis, we co-incubated TcAtg4.2 with full-length TcAtg8.1 and TcAtg8.2 and their C terminally truncated forms, lacking five amino acid residues upstream of the P1 glycine, TcAtg8.1 ΔC8 (residues 1–116) and TcAtg8.2 ΔC14 (residues 1–126) (Fig. 4C). Using a similar strategy, i.e. by co-incubating the deubiquitinating enzyme IsoT with its natural substrate ubiquitin, Drag et al. (33) observed a 20-fold enhancement of the cleavage of the synthetic tetrapeptide Ac-LRGG-AFC. However, the two truncated versions failed to significantly enhance hydrolysis of the Ac-ETFG-AFC substrate by TcAtg4.2, whereas both full-length natural substrates completely prevented cleavage of the synthetic substrate, suggesting a direct competition between both substrates for the binding into the active site cleft of the enzyme (Fig. 4D and E).

A PS-SCL Revealed High Preference for Cyclohexylalanine in the P2 Position—To identify the optimal amino acid sequence of a tetrapeptide substrate for TcAtg4.2 we designed a PS-SCL. In all sublibraries a glycine residue was fixed in the P1 position, due to the absolute requirement of ATG4 proteases for a glycine in this position (Fig. 5A) (31, 60). The P2 sublibrary was designed by fixing P1, P3, and P4 residues with Gly, Glu, and Ser, respectively. The P2 position sublibrary included 19 natural and three non-natural amino acids, nor-leucine (O), cyclohexylalanine (Cha), and homo-phenylalanine (hPhe). Because HsAtg4B shows high preference toward Phe in the P2 position, unnatural amino acids were used to possibly improve substrate specificity. P3 and P4 sublibraries included only natural L-amino acids. The PS-SCL contained two sublibraries for determination of the optimal amino acid residues in the P4 position.
The Ac-XTFG-ACC sublibrary with the last three amino acids proximal to the cleavage site identical to the natural substrate TcAtg8.1 and Ac-XAFG-ACC with the amino acid sequence corresponding to the sequence of the TcAtg8.2 homologue. All substrates in the PS-SCL were conjugated to the ACC fluorophore. Screening of the P2 sublibrary revealed an almost exclusive selectivity of TcAtg4.2 toward the unnatural amino acid residue Cha. In contrast, screening the P3 and P4 sublibraries revealed a much broader specificity in these two positions. In the P3 position the most preferred residue was arginine, followed by threonine, alanine, and lysine, whereas the enzyme exhibited preference for tryptophan, followed by lysine, in the P4 position (Fig. 5B). To further evaluate these findings several mutants of the natural TcAtg4.2 substrate, TcAtg8.2, were prepared including P2 F130S, P3 T129H, and P4 S128G mutants. All three included mutations to non-preferred residues based

**FIGURE 5.** **Substrate specificity of TcAtg4.2.** A, schematic representation of sublibraries used in the positional scanning. B, positional scanning of the tetrapeptide fluorogenic substrate library revealed high preference of TcAtg4.2 for cyclohexylalanine in the P2 position. In P3 and P4 positions broad spectrum of amino acids is tolerated. ACC fluorescence was monitored at excitation wavelength of 455 nm and emission wavelength of 460 nm. The y axis shows “natural” amino acids written in the standard one-letter code. “Unnatural” amino acids are indicated as follows: O, norleucine; hPhe, homo-phenylalanine. The y axis represents the average relative activity expressed as a percentage of that observed with the “best” amino acid. Standard deviation shown is <10%. Data represent the mean ± S.D. of at least three independent experiments. C, wild-type TcAtg8.2 and its mutants P1 G131A, P2 F130S, P3 T129H, and P4 S128G were incubated in the presence of different concentrations of TcAtg4.2. Replacement of P1 Gly and P2 Phe abolished the ability of TcAtg4.2 to process TcAtg8.2, whereas replacements of amino acids in P3 and P4 positions have only a minor effect on cleavage of the natural substrate TcAtg8.2. Cleavage was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.
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on PS-SCL results. In addition, the P1 G131A mutant, which is known to prevent the cleavage (31) was used as a positive control. In addition to Gly in the P1 position also Phe in the P2 position, as in HsAtg4B (59, 61), was found to be critical for cleavage of natural substrates, whereas substitutions in P4 and P3 positions had only a minor effect (Fig. 5C).

Searching for Optimal Synthetic Substrate for the TcAtg4.2—

Based on the TcAtg4.2 specificity obtained in PS-SCL and previous data for HsAtg4B (40) a new tetrapeptide library was synthesized consisting of 11 substrates. Seven of them carried Cha in the P2 position and were expected to be cleaved preferably by TcAtg4.2 rather than by the human orthologue HsAtg4B. The other four substrates carried Phe in the P2 position, which is preferred by HsAtg4B and were selected for comparison. Based on the screening results, Lys or Trp were fixed in the P4 position as the two preferred residues for TcATg4.2 in eight substrates, six in combination with Cha in the P2 position, and two with Phe in the P2. Among them, KLChaG and KLFAG contained a Leu in the P3 position, which was found unfavorable for TcATg4.2, but highly favorable in this position for HsAtg4B. The last Cha-containing substrate was GLChaG with the non-preferred amino acids Gly in the P4 position and Leu in the P3 position, which was expected to be only poorly cleaved by TcAtg4.2. Finally, the GLFG and GTFG substrates were included in the group as the preferred substrates of HsAtg4B (40).

As expected, TcAtg4.2 cleaved substrates containing Cha in the P2 position much more efficiently than those with a Phe. The best substrate for TcAtg4.2 was found to be Ac-KTChaG-ACC and Ac-KKChaG-ACC and were not processed by HsAtg4B. ACC release was monitored at excitation wavelength of 455 nm and emission wavelength of 460 nm. B, for improvement of catalysis the ACC fluorophore in tetrapeptide substrates was substituted with AFC. Tetrapeptide substrates indicated on the x axis were tested against TcAtg4.2 and HsAtg4B, desUMOylating enzymes SENP1 and SENP2, and deubiquitinating enzyme UCH-L3. The $k_{cat}/K_m$ was determined using nonlinear regression fitting of a series of progress curves using the equation: $k_{cat}/K_m = \text{slope} / E$. The y axis represents the average relative activity expressed as a percentage of that observed with the "best" amino acid. Standard deviation shown is <10%. C, Atg8 homologues, human GABARAPL2/GATE-16, and T. cruzi TcAtg8.1 and TcAtg8.2 were incubated with recombinant human cathepsins B, K, L, and S. All were found to process GABARAPL2/GATE-16 and all except cathepsin B were able to degrade TcAtg8.1 and TcAtg8.2. D, two best substrates for TcAtg4.2 Ac-KKChaG-AFC and Ac-KTChaG-AFC as well as Ac-SACHA-AFC and Ac-ATChaG-AFC, bearing amino acid sequences from the TcAtg8.1 and TcAtg8.2 with Cha in the P2 were tested on human cysteine cathepsins B, K, L, and S. Particularly cathepsin L was able to cleave all substrates very efficiently. The S.D. values in all experiments were calculated from at least three independent measurements.
of the ATG4 proteases through evolution and the potential of TcATG4.2 as a therapeutic target for Chagas disease (17, 26), there is a need to develop selective assays that would discriminate between Trypanosoma and human autophagins to avoid potential off target effects of TcATG4.2-targeting compounds. We took advantage of the PS-SCL, which had been successfully applied to define preferred specificity of a number of proteases, including caspases (64), SENP enzymes (34), DUB enzymes (33), and human autophagins (40). The expanded P2 sublibrary, which additionally contained several unnatural amino acids, enabled generation of selective substrates, with which we were able to distinguish between the activities of two closely related homologues human Atg4B and T. cruzi Atg4.2. The use of unnatural amino acids has been successfully applied to identify substantially improved substrates of several other proteases including human cathepsin C (65), neutrophil elastase (41), and caspases (66). Using this approach, we were able to identify Ac-KKChaG-AFC as an optimal substrate distinguishing TcATG4.2 from HsAtg4B. The substrate contains in the P2 position the unnatural Phe homologue Cha, which was found to be the major selectivity determinant, thereby confirming the validity of the approach to include unnatural amino acids. Because Cha contains a methyl group linked to the cyclohexyl ring, which in contrast to the planar phenyl ring occupies a chair or bath conformation, we suggest that the S2 binding site in TcATG4.2 has a different architecture than the human orthologue. In addition to Cha, which was found to be by far the preferred P2 residue for TcATG4.2, aromatic residues such as Phe and Tyr were also tolerated in this position. As found in human Atg4B and its natural substrate LC3 (40, 58) and also in TcAtg8.2, both P1 Gly and a P2 aromatic residue in the substrate polypeptide chain are critical for substrate recognition by TcATG4.2. In addition, the two enzymes exhibited some minor differences in P3 and P4 specificities as revealed on the basis of comparison of our results for TcATG4.2 with the previous results for HsAtg4B (40). Surprisingly, TcATG4.2 was found to cleave Ac-KLChaG-ACC with almost the same efficiency as Ac-KKChaG-ACC, and even Ac-GLChaG-ACC with about 3-fold lower efficiency. These results could be due to subsite cooperativity (27). Cha in the P2 position thereby seems to have an enormous effect on the adjacent positions, much stronger than Phe, which was fixed in this position in the original libraries (Fig. 6). Moreover, when this was combined with a Lys in the P4 position, the negative effect of a Leu in the P3 position was completely overcome. In addition, the finding that the replacement of the larger ACC fluorophore with the smaller AFC dramatically (≈50-fold) increased \( k_{cat}/K_m \) values for two best substrates, Ac-KTChaG-AFC and Ac-KKChaG-AFC, suggests that the S1’ subsite of TcATG4.2 is relatively small and tolerates smaller residues such as those found in the natural substrates TcAtg8.1 and TcAtg8.2, where Gly and Ser, respectively, are found in the P1’ position (31). Interestingly, Ac-KTChaG-AFC and Ac-KKChaG-AFC, which were the best substrates for TcATG4.2, were only very poor substrates for the functionally related human cytosolic proteins HsAtg4B, SENP1 and −2, and UCH-L3, but very good substrates for human cytosine cathepsins B and L. This might pose a serious problem in further design of selective Atg4 sub-
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strates that would work in cellular assays, as cathepsins, which are normally involved in the turnover of Atg8/LC3, may cleave them resulting in false positive results, but should not pose a problem for in vitro screening. Similarly, a cathepsin L-related enzyme cruzipain is present in T. cruzi (67, 68), which might additionally complicate in vivo screening in the parasite, because the enzyme, as TcAtg4.2, prefers Cha in the P2 position (69). Replacement of Cha with other unnatural Phe derivatives might therefore be an alternative to improve specificity of the substrates.

In addition to the residues intimately involved in substrate hydrolysis there are two additional domains critically important for substrate recognition and binding that require major conformational changes for efficient substrate hydrolysis as revealed by structural analysis of human Atg4B (58, 59). These are the regulatory loop, under which the catalytic Cys residue is buried and functions as an autoinhibitory peptide, and the N-terminal tail of the enzyme. Deletion of the regulatory loop or reduction of the loop flexibility as a consequence of mutation of one of the two conserved glycines preceding it resulted in a complete loss of processing capacity of the protein. In contrast, deletion of the N-terminal region had only a minor effect on substrate hydrolysis, suggesting that the N-terminal domain plays only a minor role in hydrolysis of both natural and small substrates. This supports the idea that the N-terminal region is more important for deconjugation activity of the enzyme, as suggested earlier (59). Atg4 contains a unique inserted regional exosite distant from the catalytic site that binds the ubiquitin-like domain of Atg8/LC3, termed the ubiquitin-binding domain. Binding to this site is hypothesized to induce a conformational change that activates the enzyme and enables entrance of the C terminus of the substrate into the active site cleft (58). The lack of this domain interaction explains the very inefficient hydrolysis of tetrapeptide substrates. However, addition of the ubiquitin-like domain in trans did not improve tetrapeptide substrate hydrolysis as shown by coinubcating the enzyme and tetrapeptide substrate with C-terminal truncation mutants of TcAtg4 lacking the C-terminal residues. This stands in contrast to the activation of the deubiquitinating enzyme IsoT by a similar approach (33), suggesting that substrate hydrolysis differs between these functionally related enzymes.

In summary, we have thoroughly characterized TcAtg4.2, the major autophagin of the T. cruzi parasite. The enzyme resembles its human orthologue Atg4B in many properties, in agreement with the very high conservation of the autophagy pathway through evolution. Although the two enzymes share very similar substrate specificity, using PS-SCL we were able to demonstrate that the Trypanosoma autophagin exhibited substantial preference toward the unnatural amino acid Cha in the P2 position, which was not accepted by the human orthologue, as well as for a smaller group in the P1’ position. This represents a solid basis for the development of T. cruzi Atg4.2-specific substrates, which should, however, be conjugated to moieties that could deliver the substrate specifically into the cytosol to avoid their nonspecific cleavage in the degradative organelles of living cells. If successful, the strategy might also allow tracking of autophagy in the living parasites.

Author Contributions—M. P., M. D., A. W., and A. B. synthesized synthetic fluorogenic substrates and libraries. R. V. performed MALDI-TOF analysis. D. C. designed pOPINF-Atg4B construct. D. C., J. R., G. S., V. T., and B. T. designed the experiments. J. R. and M. P. performed the experiments. J. R. and B. T. wrote the paper with editorial input from M. D. and G. S.

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