Two metallocarboxypeptidases from the protozoan *Trypanosoma cruzi* belong to the M32 family, found so far only in prokaryotes

Gabriela NIEMIROWICZ, Fabiola PARUSSINI, Fernán AGÜERO and Juan J. CAZZULO¹

Instituto de Investigaciones Biotecnológicas - Instituto Tecnológico de Chascomús (IIB-INTECH), Universidad Nacional de General San Martín-CONICET, Av. General Paz 5445, 1650 San Martín, Buenos Aires, Argentina

MCPs (metallocarboxypeptidases) of the M32 family of peptidases have been identified in a number of prokaryotic organisms, and only a few of them have been characterized biochemically. Members of this family are absent from eukaryotic genomes, with the remarkable exception of those of trypanosomatids. The genome of the CL Brener clone of *Trypanosoma cruzi*, the causative agent of Chagas' disease, encodes two such MCPs, with 64 % identity between them: *TcMCP-1* and *TcMCP-2*. Both genes, which are present in a single copy per haploid genome, were expressed in *Escherichia coli* as catalytically active polyHis-tagged recombinant enzymes. Despite their identity, the purified *TcMCPs* displayed marked biochemical differences. *TcMCP-1* acted optimally at pH 6.2 on FA {N-(3-[2-furyl]acryloyl)}-Ala-Lys with a K_m of 166 μ M. Activity against benzyloxycarbonyl-Ala-Xaa substrates revealed a P1' preference for basic C-terminal residues. In contrast, *TcMCP-2* preferred aromatic and aliphatic

residues at this position. The K_m value for FA-Phe-Phe at pH 7.6 was 24 μ M. Therefore the specificities of both MCPs are complementary. Western blot analysis revealed a different pattern of expression for both enzymes: whereas *TcMCP-1* is present in all life cycle stages of *T. cruzi*, *TcMCP-2* is mainly expressed in the stages that occur in the invertebrate host. Indirect immunofluorescence experiments suggest that both proteins are localized in the parasite cytosol. Members of this family have been identified in other trypanosomatids, which so far are the only group of eukaryotes encoding M32 MCPs. This fact makes these enzymes an attractive potential target for drug development against these organisms.

Key words: Chagas' disease, metallocarboxypeptidase, M32 family of peptidases, *Trypanosoma cruzi*.

INTRODUCTION

Trypanosoma cruzi, a flagellated protozoan parasite, is the causative agent of the American trypanosomiasis, Chagas' disease. The parasite has a complex life cycle, including two replicative forms: the epimastigote, present in the gut of the insect vector, and the amastigote, an intracellular form in the infected mammal. The two infective non-replicative forms are the metacyclic trypomastigote in the insect vector, and the bloodstream trypomastigote released from infected cells into the blood of the mammal. The infection is endemic in Central and South America where its prevalence is estimated at 16–18 million cases, with 120 million people at risk. No vaccines have been developed so far, and the low effectiveness of the chemotherapeutic agents available makes treatment of Chagas' disease very difficult [1]. There is therefore a pressing need for the identification of novel drug targets and virulence factors to improve the prevention and treatment of this disease. In this context, the study of peptidases in protozoan parasites in general, and in trypanosomatids in particular, has acquired considerable importance over the last decade, since some of them have been proposed to play central roles in diverse processes such as cell invasion, differentiation, cell cycle progression, catabolism of host proteins and evasion of the host immune response [2]. The possibility of developing selective inhibitors of key proteases of pathogenic parasites has been explored vigorously in recent years as a novel chemotherapeutic strategy.

T. cruzi has been shown to contain several proteolytic activities [3]. Among them, the best characterized are endopeptidases, such as the cysteine proteinase, cruzipain, which is the major proteolytic activity in the parasite. Other endopeptidases described more recently include a 30 kDa cathepsin B-like cysteine proteinase [4], two serine proteinases belonging to the prolyl oligopeptidase family [5,6], a GPI (glycosylphosphatidylinositol)-anchored membrane metalloproteinase of the leishmanolysin family [7] and the proteasome [8,9]. The study of exopeptidases in *T. cruzi*, on the other hand, has received little attention. Except for the detection of some activities (a dipeptidyl aminopeptidase and one or more aminopeptidases) in cell-free extracts [10], and the characterization of a lysosomal serine carboxypeptidase (*TcCBP*) [11], very little is known about these enzymes.

Carboxypeptidases are peptidases that cleave the C-terminal amino acid residue from peptides and proteins. On the basis of their catalytic mechanism, they are divided into two major classes, namely metallopeptidases and serine peptidases [12]. MCPs (metallocarboxypeptidases) are ubiquitous in Nature and typically have a single zinc ion bound to the active site [13]. Previous studies on *Thermus aquaticus*, an extremely thermophilic bacterium, led to the identification of a new zinc-dependent MCP [14]. The biochemical characterization of *Thermus aquaticus* carboxypeptidase (*TaqCP*) demonstrated that this enzyme was distinct from any other carboxypeptidase in both size and sequence [15]. *TaqCP* contained a HEXXH signature which is a common

Abbreviations used: ACE, angiotensin-converting enzyme; CPB, carboxypeptidase B; DAPI, 4',6'-diamidino-2-phenylindole; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; FA, N-(3-[2-furyl]acryloyl); GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; MCP, metallocarboxypeptidase; NTA, nitrilotriacetate; ORF, open reading frame; *PfuCP*, *Pyrococcus furiosus* carboxypeptidase; *TaqCP*, *Thermus aquaticus* carboxypeptidase; TBS, Tris-buffered saline; *TcCBP*, *Trypanosoma cruzi* serine carboxypeptidase; *TcMCP*, metallocarboxypeptidase from *Trypanosoma cruzi*; TOP, thimet oligopeptidase; ZAX, benzyloxycarbonyl-Ala-Xaa.

¹ To whom correspondence should be addressed (email jcazzulo@iib.unsam.edu.ar).

The nucleotide sequence data reported are available in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AJ704363 and AJ704364.

conserved sequence found so far in the active site of neutral metalloendopeptidases [15], but not described previously in a carboxypeptidase. These findings demonstrated that *TaqCP* was a novel type of MCP and the first member of a new and independent family (M32) named as the carboxypeptidase *Taq* family [16]. Another member of the M32 family that has been characterized is the archaeal *Pyrococcus furiosus* carboxypeptidase (*PfuCP*). As with *TaqCP*, *PfuCP* is a thermostable enzyme with a broad specificity towards a wide range of C-terminal substrates that include basic, aromatic, neutral and polar amino acids [17]. Important insights into the structural features of this family came from the structure of *PfuCP* to 2.2 Å (1 Å = 0.1 nm) resolution, which revealed that this enzyme has a similar fold to neurolysin, a zinc-dependent endopeptidase that cleaves neurotensin [18].

Sequence alignments have identified M32 family members in species from a very limited phylogenetic range that includes bacteria, archaea and trypanosomatids (including *T. cruzi*, *Trypanosoma brucei*, *Trypanosoma vivax* and *Leishmania major*), but no metazoa, suggesting that the genes have been acquired by horizontal gene transfer or retained for some special function that is no longer essential for higher organisms.

In the present paper, we report the cloning, sequencing, expression and biochemical characterization of two MCPs from the *T. cruzi* CL Brener clone belonging to the M32 family (*TcMCPs*). These are the first members of this family to be characterized in a eukaryotic organism. Our results add to our knowledge of the peptidases present in the parasite, and may offer a new target for the development of a rational chemotherapy against Chagas' disease and other diseases caused by trypanosomatids.

MATERIALS AND METHODS

Materials

Peptide substrates were purchased from Sigma–Aldrich and Bachem Bioscience. All other reagents were purchased from Sigma unless otherwise stated.

Parasite cultures

The different developmental forms of the parasite (epimastigote, metacyclic trypomastigote, cell-derived trypomastigote and amastigote) of the *T. cruzi* CL Brener cloned stock [19] were obtained as described previously [20].

DNA purification

DNA was obtained from epimastigotes of *T. cruzi* by using the Proteinase K/phenol/chloroform method [21].

Southern blot analysis

Genomic DNA (4 µg/reaction) was digested using restriction enzymes according to the manufacturer's instructions (New England Biolabs). DNA fragments were run on a 0.8 % agarose gel, transferred on to a nylon membrane (Hybond™-N+; Amersham Biosciences) [22] and UV cross-linked. PCR-amplified fragments of the complete *TcMCP-1* or *TcMCP-2* ORFs (open reading frames) were labelled with [γ -³²P]dCTP following standard procedures [21]. Filters were hybridized with the different probes for 12 h at 62 °C in 0.5 M sodium phosphate buffer, pH 7.4, 1 mM EDTA, 1 % BSA and 7 % SDS. The membranes were thoroughly washed at 62 °C with 20 mM sodium phosphate buffer, pH 7.4, and 0.1 % SDS, and subjected to autoradiography.

Molecular cloning of MCP genes from *T. cruzi* CL Brener clone

In order to clone both MCP genes from *T. cruzi* CL Brener clone, four synthetic oligonucleotide primers were designed: *TcMCP-1* ATG (5'-GGATCCATGAAGCCGTATAAAGAGCTG-3'); *TcMCP-1* STOP (5'-GAATTC TAATCCCCTATCGTCGCGAT-3'); *TcMCP-2* ATG (5'-GGATCCATGGAGGCATACAAGAA-GCTTG-3'); and *TcMCP-2* STOP (5'-GAATTCCTTAACCCAA-GTTGTCACGATAACG-3'). PCR amplification was carried out using genomic DNA. PCR conditions were as follows: initial denaturation (5 min at 94 °C), denaturation (1 min at 94 °C), annealing (45 s at 62 °C) and elongation (90 s at 72 °C); followed by a final extension step (10 min at 72 °C). The PCR products were isolated from a 1 % agarose gel, purified using the Qiaex II protocol (Qiagen) and cloned into pGEM-T Easy vector (Promega). Sequencing of the products was performed using an ABI 377 DNA sequencer (PerkinElmer).

Expression and purification of polyHis-tagged recombinant *TcMCPs*

TcMCP-1 and *TcMCP-2* genes were excised as BamHI/EcoRI fragments from the respective pGEM-T Easy plasmids, gel-purified and subcloned into the BamHI and EcoRI sites of the pTrcHis A expression vector. The resulting constructs presented a polyHis tag at the N-terminus and an enterokinase cleavage site.

pTrcHis A-*TcMCP* constructs were transformed into *Escherichia coli* XL1 Blue cells. N-terminally poly-His-tagged fusion proteins were expressed by induction of exponential phase cultures (200 ml; D_{600} = 0.6) with 1 mM IPTG (isopropyl β -D-thiogalactoside) for 7 h at 18 °C with vigorous (250 rev./min) shaking. Bacteria were harvested by centrifugation at 5000 g for 30 min at 4 °C, resuspended in 20 ml of 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.1 % Triton X-100, 1 mM PMSF and 1 mg/ml lysozyme, and centrifuged at 12000 g for 30 min at 4 °C to obtain the bacterial crude extract.

The recombinant enzymes were purified in one step using Ni- or Co-NTA (nitrilotriacetate) resin (ProBond) pre-equilibrated in 50 mM Tris/HCl buffer, pH 7.6, containing 150 mM NaCl and 20 mM imidazole. The bacterial crude extract was percolated twice through the column which was washed with 50 ml of the equilibration buffer. *TcMCPs* were eluted with 50 mM Tris/HCl buffer, pH 7.6, 150 mM NaCl and 300 mM imidazole. Samples were desalted using a PD-10 column (Amersham Biosciences) according to the manufacturer's specifications. Sample purity was evaluated on a Coomassie Blue-stained SDS/10 % polyacrylamide gel.

For an alternative purification procedure of *TcMCP-1*, the bacterial crude extract was prepared in the absence of NaCl and applied to a Q-Sepharose column (Sigma) equilibrated in 50 mM Tris/HCl buffer, pH 7.6. The column was washed with 50 ml of the same buffer containing 100 mM NaCl. Recombinant *TcMCP-1* was eluted with 50 mM Tris/HCl buffer, pH 7.6, and 200 mM NaCl. The eluate was concentrated using Centrprep YM-30 (Amicon) and applied to a Superdex 200 column (Amersham Biosciences) equilibrated with 50 mM Tris/HCl buffer, pH 7.6, and 250 mM NaCl. The active fractions were pooled, diluted 1/5 with 50 mM Tris/HCl buffer, pH 7.6, and loaded on a MonoQ HR 10/10 anion-exchange column equilibrated with the same buffer. Proteins were eluted with a 0–0.2 M linear NaCl gradient in 8 column vol. Fractions (0.5 ml) were collected at a rate of 1 ml · min⁻¹.

Expression and purification of *TcMCPs* fused to GST (glutathione S-transferase)

TcMCP-1 and *TcMCP-2* genes were also cloned into the BamHI and EcoRI sites of the pGex 2T expression vector (Amersham

Figure 1 Determination of *TcMCP* copy number

(A) Southern blot of *T. cruzi* genomic DNA digested with several low-frequency restriction enzymes and hybridized with the *TcMCP-1* gene labelled with [γ - 32]dCTP. The third band generated by *HaeII* is not evident because the *HaeII* site is located very close (24 bases) to the 3'-end of the coding sequence, yielding a small stretch of sequence with which the probe could not hybridize. (B) Blots probed with *TcMCP-2* gene labelled as in (A). The upper band generated by *HincII* and *KpnI* corresponds to the allele lacking the restriction site. Sizes are indicated in kb.

Biosciences) as described above. Both *TcMCP* constructs were transformed into *E. coli* BL21 Codon Plus (DE3) cells. GST-fusion proteins were expressed by induction of exponential phase cultures (200 ml; $D_{600} = 0.6$) with 1 mM IPTG for 12 h at 18 °C with vigorous (250 rev./min) shaking. Bacterial crude extracts were obtained as mentioned above.

Recombinant fusion proteins were purified using a glutathione-agarose resin (Sigma) equilibrated with 50 mM Tris/HCl, pH 7.6, and 150 mM NaCl. The column was washed with 5 column vol. of the equilibration buffer. Samples were eluted with 50 mM Tris/HCl, pH 7.6, and 150 mM NaCl containing 10 mM reduced glutathione.

Gel-filtration experiments

Gel filtration was conducted using a Superose 12HR 10/30 (Pharmacia Biotech) column, eluted with 50 mM Tris/HCl buffer, pH 7.6, containing 0.25 M NaCl. Molecular-mass markers were Vitamin B12 (1.3 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), BSA (66 kDa), and γ -globulin (158 kDa).

Enzyme assays

Routinely, recombinant *TcMCP-1* activity was assayed using FA {*N*-(3-[2-furyl]acryloyl)}-Ala-Lys (200 μ M) as substrate in 100 mM Mes, pH 6.2. The hydrolysis of FA-Phe-Phe (100 μ M) by recombinant *TcMCP-2* was measured in 50 mM Tris/HCl, pH 7.6. Initial steady-state velocity was determined by continuous assay for a range of substrate concentrations at 340 nm with a Beckman DU 650 spectrophotometer. One unit of activity was defined as the amount of enzyme that released 1 μ mol of the group being cleaved per min at 25 °C. Kinetic parameters for recombinant *TcMCP-1* were determined using FA-Phe-Phe and FA-Ala-Lys in 100 mM Mes, pH 6.2, in the presence or absence of 50 μ M CoCl_2 . Recombinant *TcMCP-2* activity was assayed with FA-Phe-Phe in 50 mM Tris/HCl, pH 7.6, also in the presence or absence of 50 μ M CoCl_2 . The substrate preference with respect to P1' position was determined using ZAX (benzyloxycarbonyl-Ala-Xaa) substrates. The *TcMCP* activities were assayed using

the ninhydrin method [17] with 1 mM ZAX as substrate at 30 °C in their optimal buffers. One unit of enzyme was defined as the amount of enzyme that produced a ninhydrin-positive substance corresponding to 1 μ mol of amino acid per min.

pH profile and thermostability

The pH profiles of the *TcMCPs* were determined by incubating the enzymes for 5 min at 25 °C before the addition of the substrate in the following buffers at 100 mM: Mes (pH 5–6.7), Mops (pH 6.5–7.9) and Hepes (pH 7–8.2). For *TcMCP-2*, 50 mM Tris/HCl, pH 7.6–9, was used in addition.

Thermostability was analysed by measuring residual *TcMCP* activities after pre-incubating both enzymes for 5 min at a given temperature in their optimal buffers.

Effects of metal ions

Metal-ion-dependence was investigated by assaying *TcMCP* activities after pre-incubation of bacterial crude extracts (5 μ l, 5 min) with the metal chlorides. Purified *TcMCPs* ($\approx$ 200 ng) were also tested to confirm the effects seen in crude extracts. *TcMCP-1* residual activity was assayed using 200 μ M FA-Ala-Lys in 100 mM Mes, pH 6.2, whereas *TcMCP-2* activity was analysed with 100 μ M FA-Phe-Phe in 50 mM Tris/HCl, pH 7.6.

Metal cations were also tested for their ability to reactivate metal-depleted *TcMCPs* (apo-*TcMCPs*). The recombinant *TcMCP*-GST fusion proteins were dialysed against 10 mM EDTA in 50 mM Tris/HCl, pH 7.6, for 16 h at 4 °C. EDTA was removed using a PD-10 column. Apo *TcMCPs* were incubated for 5 min with metal chlorides (0.01–10 mM) before activity was assayed.

Preparation of parasite extracts

T. cruzi CL Brener clone cells (1×10^9) were broken by three cycles of freezing at -20 °C and thawing. The parasite pellets were extracted with 200 μ l of 50 mM Tris/HCl, pH 7.6, containing 1 mM PMSF and 10 μ M E-64 [*trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane]. The preparation was centrifuged at

A

TcMCP-1	1	-----MKPKYKELERVFTKLYRYGHMLLLADWDSHTMPCCKGSDARGA	42
TcMCP-2	1	-----MEAYKKLETIFTKIYFRHLISMAFWDIQVLMRRGGAEARGA	42
<i>Vibrio cholerae</i>	1	MMKKRASHHLVICTLIDTLRFRLSSRAISMNAFNKLVKHSKKISNFNLSSIVGWDQAAMVPSGGAEARSQ	72
<i>Rickettsia rickettsii</i>	1	-----MNNNYTKLENEFATISHFNILSILYWDVAVNMPMGSGESRTN	43
<i>Bacillus subtilis</i>	1	-----MEIHTYEKEFFDLLKRIISHYSEAVALMHWDRTGAPKNGSEDRAE	48
<i>Pyrococcus furiosus</i>	1	-----MEEVFQNETIKQILAKYRRIWAIGHAQSVLGDLEVNMPKEGILERSV	48
<i>P. marinus</i>	1	-----MSEKEWNKLGAYLKETQILGSIQSTLYWDQNTGMKPKGASWRSE	44
TcMCP-1	43	AMAEQLHMHDTITAPKIRALIEEAESVSG-----DLEKLQRANLREMRAWELNLLPEEFVERKTV	105
TcMCP-2	43	ALAEQLHVHLLTDPKMRDLLEAEKNKE-----QLGTLQQANLREIRLEWERENLLPEEIKKKSE	105
<i>Vibrio cholerae</i>	73	AMAEQSVHIGLMTQPLADWFAEAESE-----TLNIEQQATLREMQRQWQATVLPALVEAQSL	133
<i>Rickettsia rickettsii</i>	134	AGSKCEHAWRSQKNDWTGFEKNWAEVVKLSQEEAQIRAEATGKTPYDAMLELYEPGTTTEQLNRVSDVK	205
<i>Bacillus subtilis</i>	44	EIVTLTSLVHSMKSPILKELLSKAKEESK-----NLDEWQNGNIREIERKVTDANCIDEQLQKKLVA	106
<i>Pyrococcus furiosus</i>	46	SIGQLSTDIFNIQTSDRMKELIDVLYERFD-----DLSEDTKKAVELAKKEYEENKKIPEAEYKEYVI	108
<i>P. marinus</i>	49	AQGEQSVLSHELLLHPEFVNLEKAGKLE-----NLNEYERGIVRVLDRIIRARAFPEFIREVSE	110
	45	QLTYIAKILHKNRSSEEFSDLIKAAQDEFRGGLDKSLNQEIRSKKRNLLELLIKEFNQKNLDAKLVEALAK	116
TcMCP-1	106	LTTKAHQVWKTCKREKNDFAFLPTLTKELIALFREEGKL RAGNSGKHPYEALVDIYEPGMTLQRLDEIFGNVR	177
TcMCP-2	106	LTTRAQQIWRKSREENDEFSQWLPMLKELVALFYETGVALAGNTGKHPYEALQLLYEPGMSLERIDEINEIK	177
<i>Vibrio cholerae</i>	134	AGSKCEHAWRSQKNDWTGFEKNWAEVVKLSQEEAQIRAEATGKTPYDAMLELYEPGTTTEQLNRVSDVK	205
<i>Rickettsia rickettsii</i>	107	ATTKAALVWREARKHNDYNLKSHLQKVLDTYKEVAKVRADAFNCELVNSLIDMFDPKSRKSSSEIKQVSVLK	178
<i>Bacillus subtilis</i>	109	LCSKAETAWEEAKGSDFSLSFYLEQLIEFNKRFITYWG--YQEHYPYDALDLFEPGVTVKVLQDLFAELK	178
<i>Pyrococcus furiosus</i>	111	TTSLATKAWEEAKAKDDFSKFEPLWDLKISLAKRAAEYLG--YEEEPYDALDLDEEGLRTRDVEKMEVLE	180
<i>P. marinus</i>	117	AKSKGYESWQEAKRKSDFEIFLPIFKNLIELRIEETKQIS--DKYTWETLAQPFEPDLTLEVLNKLIQPLK	186
TcMCP-1	178	SWLPELLEKEVQEKQKALGETVLEPKGPFVSKQALCRFFMDVWKFDFDG-GRLDVSAHPFCGNS-KEDVRI	247
TcMCP-2	178	SWLPALEIREVMEKRKTAGEEVLTPVGPFPLEAQKELSRVCMNVWKFDFDG-GRQDCSLHPPFCGGV-KEDVRI	247
<i>Vibrio cholerae</i>	206	TWLPSSLIDVVEIKQ--SSEFIPAKGHYPAESQKALGLDVMKLLQDFENH-GRLDVSAHPFCGGV-PEDVRI	273
<i>Rickettsia rickettsii</i>	179	KELPQLINKVLEKQ--KTEKELVVKSHKLAPEMOKRIKIMEIMQFDLTG-GRLDVSAHPFCGGT-PNDIRL	246
<i>Bacillus subtilis</i>	179	EAIPIPLVKQVTSAGNPKPT--SFITKAPFKEKQKELSLYFLQELGYDFDG-GRLDVSAHPFATTNLRGQVRI	247
<i>Pyrococcus furiosus</i>	181	KKLKPLLDKILIEGKVPREHLE-KEYEREMERVNWLWLQKFGPLGTRARLDVSAHPFTEFGIRDVRI	251
<i>P. marinus</i>	187	ETIPSLISDLNKSEK-----YHWDLPSPKQNLCSKLLDEFGRDEL-VVVAKSPHPPFISITLGPDIYRI	249
TcMCP-1	248	ITKYTEFETVTSLLGVITHETGLAKYEQNCGPKGFETQ--PVCMARSLGVHGGSLFAEMQIGRSGAFMEFLA	317
TcMCP-2	248	ITRYDESRETFETGLMAVITHETGLAKYEQNGGPKEMITQ--LIARYRSLGVHGGSLFAERMVARSKPIEFLLA	317
<i>Vibrio cholerae</i>	274	ITRYNESEFVQSLMGIVHETGHARYEQG-LPKHLATG--PAGEARSMQIHESQSLFFEMQVGRSPAFIAHLA	342
<i>Rickettsia rickettsii</i>	247	ITRYDKDNFISGLMGIHETGHALYEQN-LPEMYKAGT--PVGLAKGMAFHESQALFMEMQVGRSRETFEFLA	315
<i>Bacillus subtilis</i>	248	ITRYDEKDFRTAIFGTIHECGHAIYEQNID--EALSGTNLSDGASMGIHESQSLFYENFIGNKHFVTPYY	316
<i>Pyrococcus furiosus</i>	252	ITRYEGYDERRTILSTVHEFGHLYELQ-QDERFMFT--PIAGGVSLGIHESQSRFENIIGRSKEFVELIY	320
<i>P. marinus</i>	250	ITRVVEGEPLSSFLATAEHWGLSIEYEGPLPSQSHQWAWPLGOATSMGVHESQSLFENIRIVKSKSESKRFF	321
TcMCP-1	318	PRLVEYFGDQ--PAFTSSNMKRVIQRVSPGLIRIDADELCTPLHYMLRYEIERDLMDGNIIEAEVPRVWNEK	387
TcMCP-2	318	PKLKELFGDQ--PAFTPENMVRIRIQRVNPSFIRITSDDELCTYMHILRYEIERGLIEGKLREVDVPAVWNEK	387
<i>Vibrio cholerae</i>	343	ELAGKHFSAMNDPVRVENIQRLITRVQKDFIRVDADELTYPAHVILRFEIERDLMDGNIKHTDVPDLWQK	414
<i>Rickettsia rickettsii</i>	316	KLRLRDEFALKS-EEYSAASLYRKITRVTDFIRVDADELTYPMHVMRLFEIEEMLINSDLNDELPSCWDSK	386
<i>Bacillus subtilis</i>	317	KKIQEASPVQ-FKDISLDDFVRAINESKSPFIRVEADELTYPHLIIIRYEIEKAIFSNVSVEDLPSLWNQK	387
<i>Pyrococcus furiosus</i>	321	PVLKENLPFMS--NYTPEDVLYFNIRPDEIRTEADVVTYNFHIILRFKLERLMVSEEIKAKDLPPEMWNDE	390
<i>P. marinus</i>	322	KHFVSEG--CSLNHLELWKSINHLKAGLNVEADELSYGLHILIRTELEIDLIEGLEAKDVPSEWNKR	389
TcMCP-1	388	MKSYLGLLETGNDKEGCLQDQVHWSGMGFYFPTYSLGAMVAAQLMSCVRRELGEVVDCCIRKGLDKITLAK	459
TcMCP-2	388	MKSYLGLIETLGNDRGCLQDQVHWSGMGFYFPTYSLGALVAAQLMASIRRDGNDVDDCIRKGLGALILEK	459
<i>Vibrio cholerae</i>	415	MQAYLGLSTKGNQNGCMQDIHWTDGSGFYFPTYSLGAMVAAQLMAAMKKTVD--VDGAIRRGDLSPIFTW	483
<i>Rickettsia rickettsii</i>	387	MQEYLGVPVSFS-NGCLQDIHWSHGNFYFPTYSYALGYMYAAQLKQKMLEDLPE-EDFALLERGEFHPKQW	455
<i>Bacillus subtilis</i>	388	YQDYLGITP-QTDAEGILQDQVHWAGGDEGYFPTYSYALGYMYAAQLKQKMLEDLPE-EDFALLERGEFHPKQW	456
<i>Pyrococcus furiosus</i>	391	MERLLGIRPRKYS-EGILQDIHWAGSIFGYFPTYSITGLLSAQLYYHKKDIPD--FEKVAKAEFDPKAW	459
<i>P. marinus</i>	390	YEELGLIRP-SNDAEGCLQDQVHWSEGAIFYFPTYSLLGHLISAQISSQMEKEIG--LIDNLVEDGEYEKILW	458
TcMCP-1	460	QNEKIWOHGSSLTDELLRQATG-ETLNPEHYRRHLERRRDRRG-	503
TcMCP-2	460	QREKIWOHGSSYTTTEELLTKATG-EPLNPRFFREHLERRRDRNLG-	503
<i>Vibrio cholerae</i>	484	SENISWKGSLFSTDELVKQATG-ETLNPEHFKAHLSQRYLK----	524
<i>Rickettsia rickettsii</i>	456	LNKNFRNLGSLKNSADLLKSASGEKISPEVYIRYLEGKYL----	496
<i>Bacillus subtilis</i>	457	LTEKVIHIGKRKKPLDIKDATG-EELNVRYLIDVSNKYSNLVLL	501
<i>Pyrococcus furiosus</i>	460	LRKELHRWGSYIPPKELLKKAIG-EDMDAEYFVRWVKEKYL----	499
<i>P. marinus</i>	459	LKNNIHNYGRSVNSMKIVRVNVTG-EELTSKYEVNHLRSKIKDFC----	501

B

Figure 2 For legend see facing page

26 900 *g* for 30 min at 4 °C). The pellet was extracted again with the same buffer and centrifuged under identical conditions. The combined supernatants were centrifuged at 26 900 *g* for 30 min at 4 °C. The cell-free extract was retained and the pellet was discarded. Activity against FA-Ala-Lys substrate (200 μ M) was assayed in 100 mM Mes, pH 6.2. The hydrolysis of FA-Phe-Phe (100 μ M) was measured in 50 mM Tris/HCl, pH 7.6, containing 1 mM CoCl₂.

Antibodies

Polyclonal antibodies against *TcMCP-1* were obtained in rabbits using purified recombinant His-tagged *TcMCP-1* as immunogen. Additional antisera were also raised in rabbits by using the synthetic peptide DLMDGNIEAEEVPRC, corresponding to amino acid residues 371–384 of *TcMCP-1* and the synthetic peptide GLIEGKLRVEDVPAC, corresponding to the equivalent amino acid residues in *TcMCP-2*. The C-terminal cysteine residue was added in both cases for the covalent linking of the peptides to the carrier KLH (keyhole-limpet haemocyanin) (Pierce).

Western blot analysis

For Western blots, soluble *T. cruzi* extracts (100 μ g) were resolved on a SDS/10% polyacrylamide gel, transferred on to a nitrocellulose Hybond™ ECL® (enhanced chemiluminescence) membrane (Amersham Biosciences), blocked in 3% (w/v) non-fat dried milk powder, 2% glycine and 150 mM NaCl in 50 mM Tris/HCl, pH 7.6 [TBS (Tris-buffered saline)] and incubated with *TcMCP-1* antiserum (1:1000, 2 h) or anti-*TcMCP-2* peptide (1:500, 2 h) in blocking buffer. Blots were washed twice with TBS for 10 min, once with 0.05% Nonidet P-40 in TBS for 10 min and twice with TBS for 10 min. Horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma) (1:10 000, 1 h) in blocking buffer was used as secondary antibody. The membranes were washed again and developed using SuperSignal WestPico Chemiluminescent Substrate (Pierce).

Immunofluorescence experiments

For immunofluorescence experiments, parasites from different life cycle stages of the *T. cruzi* CL Brener clone were air-dried on poly(L-lysine)-coated glass coverslips, fixed with 4% (w/v) paraformaldehyde in PBS (20 min), incubated with 25 mM NH₄Cl (10 min), permeabilized with 2% BSA, 0.1% saponin, 2% normal goat serum in PBS (30 min) and incubated with anti-*TcMCP-1* rabbit serum (1:500) or anti-*TcMCP-2* peptide rabbit serum (1:500) with 2% BSA, 0.1% saponin in PBS (1 h). Coverslips were washed three times with PBS for 1 min. Alexa Fluor®

488-conjugated goat anti-rabbit IgG (H + L) (Molecular Probes) (1:1000) (1 h) was used as secondary antibody. Nucleus and kinetoplast were stained with DAPI (4',6-diamidino-2-phenylindole) (5 mg · ml⁻¹). Coverslips were washed again three times with PBS for 1 min and mounted using 15 μ l of Fluor Save (Calbiochem). Samples were examined with a Nikon Eclipse E6000 microscope using appropriate fluorescence filters and photographed with a digital Spot RT Slider camera.

RESULTS

The *T. cruzi* genome encodes two MCPs of the M32 family

Following previous work in which we characterized a serine carboxypeptidase [11], we obtained a partial genomic clone containing the sequence of what seemed like another (but different) carboxypeptidase. Screening of the then available *T. cruzi* genome data (unassembled genomic sequences) using this sequence as a query led us to assemble two DNA contigs encoding putative proteins with sequence similarity to thermostable carboxypeptidases of the M32 family. To avoid dealing with possible assembly artefacts we proceeded to (i) amplify, clone and sequence the genes, and (ii) check the accuracy of the assembly by Southern blot with selected restriction enzymes. Using specific primers (see the Materials and methods section), we amplified and sequenced both genes which we named *TcMCP-1* and *TcMCP-2* and deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AJ704363 and AJ704364. The *TcMCP* genes contained an ORF of 1512 bp and encoded polypeptides with predicted molecular masses of 57.7 kDa and 58.3 kDa. Not surprisingly, we found that our versions of these genes (also from *T. cruzi* CL Brener) have minor point differences when compared with those from the *T. cruzi* Genome Project (accession numbers Tc00.1047053504153.160 and Tc00.1047053504045.60). These differences are probably due to the merging of the two alleles into a single consensus sequence during genome assembly, since for all differing sites we were able to find support from individual (i.e. unassembled) GSS (Genome Sequence Survey) sequences.

In order to confirm the estimated copy number of *TcMCP* genes and check the accuracy of our assembly we performed Southern blot analysis. *T. cruzi* genomic DNA digested with restriction enzymes that do not cut within the *TcMCP-1* or *TcMCP-2* ORF (BamHI, BstXI, EcoRV, NcoI, NdeI and XhoI) yielded single bands when probed with the corresponding full-length *TcMCP-1* or *TcMCP-2* gene, whereas endonucleases with a single restriction site inside the ORFs (AvaI, PstI, AccI and SacII) rendered two bands (Figure 1). Some enzymes (HincII and KpnI) produced

Figure 2 Multiple sequence alignments of MCPs belonging to the M32 family

(A) Multiple alignment of *TcMCPs*. The complete *TcMCP-1* and *TcMCP-2* ORFs are 1512 bp long and predict polypeptides of 503 amino acid residues with calculated masses of 57.7 kDa and 58.3 kDa respectively. In the alignment, sequences were sorted by similarity to the *TcMCP-1* using the ClustalW program [45]. Similar residues according to the BLOSUM62 matrix are indicated by different tones of grey, according to the degree of similarity. The most highly conserved motifs of the enzyme (HPF, DXRX; HESQ, IRXXAD and GXQDXHW) are indicated with white letters. The metal-ion-binding motif (HEXXH) is marked with an asterisk (*) and the active-site glutamic acid residue is indicated by ●. GenBank® accession numbers with percentage identity with *TcMCP-1* and *TcMCP-2* respectively are as follows: *Vibrio cholerae* O1 biovar El Tor str. N16961, NP_231057, 46% and 45%; *Rickettsia rickettsii*, ZP_00153287, 36% and 36%; *Bacillus subtilis* subsp. *subtilis* str. 168, NP_390090, 33% and 34%; *P. furiosus*, 1KA4A, 31% and 33%; and *Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986, NP_892611, 31% and 27%. (B) Sequences were aligned using ClustalW. Starting from this initial alignment, a larger dataset (100 replicates) was obtained by bootstrap resampling using the SEQBOOT program with all the default options. Phylogenies from protein sequences were estimated using the PROTPARS parsimony analysis. The consensus tree was computed using the program CONSENSE (majority-rule consensus tree method). Bootstrap values are shown on the nodes. SEQBOOT, PROTPARS and CONSENSE are part of the Phylogeny Inference Package (Phylib version 3.6a2). MCP accession numbers other than those described in the legend to Figure 1(A) are: *Chromobacterium violaceum* A.T.C.C. 12472, NP_900962; *Yersinia pestis* biovar *Medievalis* str. 91001, NP_993430; *Shewanella* sp. MR-4, ZP_00881701; *Legionella pneumophila* str. Paris, YP_123472; *Mesorhizobium loti* MAFF303099, BAB50714; *Candidatus* 'Phytoplasma asteris', YP_456728; *Wolbachia* endosymbiont of *Drosophila melanogaster*, NP_966968 (pseudogene); *Enterococcus faecalis* V583, NP_814883; *Listeria monocytogenes* str. 4b F2365, YP_014507; *Deinococcus geothermalis* DSM 11300, ZP_00397346; *T. aquaticus*, P42663; *Sulfolobus* sp. EE-36, ZP_00954983; *Paracoccus denitrificans* PD1222, ZP_00631417; *Nanoarchaeum equitans* Kin4-M, NP_963336; *Sulfolobus solfataricus* P2, NP_344418; *T. brucei*, XP_828333; *L. major*, CAJ06700. 2e + 02 = 2 × 10².

three bands when probed with the *TcMCP-2* gene which correspond to the two *TcMCP-2* alleles. Thus *TcMCP* genes are present as a single copy per haploid genome.

Sequence and comparative genomics analysis

Both *TcMCP* proteins were subjected to domain analysis against Interpro [23] and Smart [24] databases which confirmed the presence in both *T. cruzi* enzymes of amino acid motifs related to M32 peptidases. Figure 2(A) shows the amino acid alignment of *TcMCP-1*, *TcMCP-2* and five prokaryotic members of the M32 family. The *TcMCPs* described here shared 64 % identity with each other and displayed the highest sequence identity (46–36 %) with those M32 proteins of the proteobacteria group. The sequence of *TcMCPs* contains the canonical HEXXH motif that includes the active-site glutamic acid residue at position 268 [18], flanked by two histidine residues (positions 267 and 271) that co-ordinate the catalytic metal ion (Figure 2A). *TcMCPs* also present the third metal ligand glutamic acid residue (position 296) of the HEGQ signature conserved in all M32 peptidases. Additional key residues, proposed to bind the C-terminal carboxylate group and promote release of the cleaved amino acid product, are conserved when comparing *TcMCPs* with the crystallized *PfuCP*, these residues include Arg³⁴⁸ and Asp⁴⁰⁷ from the IRXXAD and GXXQDXHW motifs respectively.

An extensive search of the publicly available genome sequences at the NCBI non-redundant (nr) database (<http://www.ncbi.nlm.nih.gov>) using the BLAST (basic local alignment search tool) allowed us to identify M32 MCP genes in multiple prokaryotic organisms. However, their phylogenetic distribution was quite peculiar, as trypanosomes and leishmanias were the only eukaryotic organisms in which members of this family were found (see Supplementary Table S1 at <http://www.BiochemJ.org/bj/401/bj4010399add.htm>). To analyse further the phylogenetic relationships of protozoan parasite MCPs, an unrooted tree using the full-length amino acid sequences of several M32 peptidases was constructed. As shown in Figure 2(B), MCPs from trypanosomatids seem to be closely related to the enzymes of the proteobacteria group.

Expression and purification of recombinant *TcMCPs*

On the basis of these particular findings, we decided to analyse the enzymatic properties of both *T. cruzi* enzymes. The full-length *TcMCP* genes were cloned into the bacterial expression vector pTrcHisA, and expressed in *E. coli* XL1 Blue cells as N-terminally polyHis-tagged recombinant enzymes. *TcMCP-2* was purified in one step using Co-NTA affinity chromatography with a yield of 1–2.5 mg/l of bacterial culture. As recombinant *TcMCP-1* was inhibited by Co²⁺, it had to be purified using a conventional purification scheme including three steps, namely gel filtration and two ion-exchange chromatography steps. In a standard preparation, 0.33 mg of homogeneous *TcMCP-1* was obtained from 21 mg of protein in the bacterial cell-free extract, with a 20.4-fold purification and 32 % recovery (Table 1). Recombinant *TcMCPs* were evaluated for purity on Coomassie Blue- or silver-stained SDS/10 % polyacrylamide gels. The apparent subunit molecular masses of both purified enzymes (approx. 58 kDa) were in good agreement with the calculated masses of the affinity-tagged products of the corresponding genes (approx. 62 kDa) (Figure 3). *TcMCP-1* and *TcMCP-2* eluted in a single peak from a Superose 12HR 10/30 column with an apparent molecular mass corresponding to 128 kDa and 132 kDa respectively, indicating that both enzymes associate into catalytically active homodimers.

Table 1 Purification of recombinant *TcMCP-1*

The enzyme was purified as described in the Materials and methods section. Activity was assayed with FA-Ala-Lys (200 μ M) as substrate in 100 mM Mes, pH 6.2.

	Total protein (mg)	Total activity (units)	Specific activity (units · mg ⁻¹)	Yield (%)	Purification (fold)
Cell-free extract	21.0	87.7	4.20	100	1
Q-Sepharose	5.04	76.2	15.1	87	3.60
Superdex 200	1.51	51.6	34.2	59	8.20
Mono Q	0.33	28.1	85.0	32	20.4

Figure 3 Expression and purification of recombinant *TcMCPs* from *E. coli*

(A) Samples obtained from the different steps of the *TcMCP-1* purification procedure were analysed by SDS/PAGE on a 10 % gel. Proteins were visualized by silver staining. Lane 1, crude extract; lane 2, Q-Sepharose eluate; lane 3, Centrprep; lane 4, Superdex G-200 pool; lane 5, MonoQ. (B) *TcMCP-2* was purified in one step using Co-NTA resin. Sample purity was evaluated on a Coomassie Blue-stained SDS/10 % polyacrylamide gel. Lane 1, soluble fraction derived from the disruption of induced *E. coli* cells; lane 2, fraction containing the purified recombinant *TcMCP-2* eluted with 300 mM imidazole. The positions of molecular-mass markers (kDa) are shown to the left of the gels in (A) and (B). (C) The molecular masses of active *TcMCPs* were determined by molecular-exclusion chromatography on a Superose 12HR 10/30 column: *TcMCP-1* (○), *TcMCP-2* (■), molecular-mass markers (▲) [thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.3 kDa)]. MW, molecular mass.

Purified recombinant *TcMCP* proteins were used in enzymatic assays with N-blocked furylacryloyl dipeptides commonly employed for screening carboxypeptidases. These experiments

Table 2 Effect of bivalent cations on recombinant *TcMCP* activities

Metal-ion-dependence was investigated by assaying the activity of recombinant *TcMCPs* (~200 μ g) after pre-incubation with the metal chlorides. Residual activity was measured as described in the Materials and methods section. *TcMCP*-1 activity was assayed with FA-Ala-Lys (200 μ M) as substrate in 100 mM Mes, pH 6.2. *TcMCP*-2 activity was determined in 50 mM Tris/HCl, pH 7.6, with FA-Phe-Phe as substrate. Basal activity (control) in the absence of added metal ions was 2.51 units/ml for *TcMCP*-1 and 5.9 units/ml for *TcMCP*-2.

	Concentration (mM)	<i>TcMCP</i> -1 relative activity (%)	<i>TcMCP</i> -2 relative activity (%)
Control	0.00	100 $\pm$ 4.0	100 $\pm$ 1.4
Co^{2+}	0.01	59.7 $\pm$ 1.3	108 $\pm$ 4.2
	0.10	48.6 $\pm$ 0.3	125 $\pm$ 7.0
	1.00	22.8 $\pm$ 4.6	168 $\pm$ 1.4
	1.00	8.98 $\pm$ 0.0	83.1 $\pm$ 1.4
Zn^{2+}	0.01	6.28 $\pm$ 0.2	27.5 $\pm$ 3.4
	0.10	0.35 $\pm$ 0.0	2.46 $\pm$ 0.1
	1.00	104 $\pm$ 0.0	95.8 $\pm$ 5.6
	1.00	76.0 $\pm$ 4.0	95.8 $\pm$ 2.8
Mn^{2+}	0.01	64.1 $\pm$ 0.7	95.8 $\pm$ 8.5
	0.10	44.6 $\pm$ 7.4	98.6 $\pm$ 2.8
	1.00	5.72 $\pm$ 0.3	90.1 $\pm$ 5.6
	1.00	1.81 $\pm$ 1.2	90.1 $\pm$ 2.8
Ca^{2+}	0.01	92.2 $\pm$ 4.0	100 $\pm$ 1.4
	0.10	84.4 $\pm$ 4.0	95.8 $\pm$ 5.6
	1.00	88.0 $\pm$ 8.0	95.8 $\pm$ 2.8
	1.00	75.1 $\pm$ 4.9	97.2 $\pm$ 1.4

Table 3 Kinetic constants for the hydrolysis of synthetic substrates by recombinant *TcMCPs*

Kinetic parameters of recombinant *TcMCPs* were determined from Lineweaver–Burk plots, as described in the Materials and methods section. N.D., not detected.

Substrate	Co^{2+} (50 μ M)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \text{mM}^{-1}$)
<i>TcMCP</i> -1				
FA-Ala-Lys	No	0.166	20	121
FA-Ala-Lys	Yes	0.169	5.7	34
FA-Phe-Phe	No	N.D.*	N.D.*	N.D.*
FA-Phe-Phe	Yes	0.073	0.7	9.6
<i>TcMCP</i> -2				
FA-Phe-Phe	No	0.024	7.6	315
FA-Phe-Phe	Yes	0.034	11	323

Figure 5 Differential expression of MCPs in *T. cruzi* life cycle stages

Western blots of soluble extracts (100 μ g) of epimastigotes (E), amastigotes (A), cell-derived trypomastigotes (T) and metacyclic trypomastigotes (M), probed with anti-*TcMCP*-1 (A) or with anti-*TcMCP*-2 peptide (B). Molecular-mass sizes are given in kDa. (C) Carboxypeptidase activity was assayed in parasite cell-free extracts prepared by the freezing and thawing method. Activity against FA-Ala-Lys (grey bars) was determined in 100 mM Mes buffer, pH 6.2. *TcMCP*-2 activity was measured in 50 mM Tris/HCl, pH 7.6, and 1 mM CoCl_2 with FA-Phe-Phe as substrate (white bars).

revealed that recombinant *TcMCP*-2 exhibits a significant hydrolytic activity against the carboxypeptidase A (subfamily M14A) substrate FA-Phe-Phe at pH 7.6–8, whereas recombinant *TcMCP*-1 optimally cleaved the CPB (carboxypeptidase B) (subfamily

Figure 4 P1' preference of recombinant *TcMCPs*

The enzyme activities were assayed by the ninhydrin method. (A) *TcMCP*-1 was measured at 30 °C in 100 mM Mes buffer, pH 6.2, containing 1.2 mM ZAX (X represents a different amino acid as indicated by the single-letter amino acid code) substrate. (B) *TcMCP*-2 activity on ZAX (1 mM) was measured in 50 mM Tris/HCl, pH 7.6.

Figure 6 For legend see facing page

M14B) substrate FA-Ala-Lys at pH 6.2–7.3. In both cases, carboxypeptidase activities declined under mildly acidic conditions (pH 5.5). Metal-ion chelators EDTA (10 mM) and *o*-phenanthroline (1 mM) were strong inhibitors of both proteins, consistent with the *TcMCPs* being metal-dependent enzymes. As expected, the serine peptidase inhibitor PMSF (2 mM) and the cysteine peptidase inhibitor E-64 (10 μ M) had no effect on *TcMCPs* activities (results not shown).

Effects of metal ions

Table 2 shows the effects of different metal ions on purified *TcMCPs*. Recombinant *TcMCP*-1 activity on FA-Ala-Lys was inhibited by metal ions such as Co^{2+} , Mn^{2+} and Ni^{2+} . Ca^{2+} and Mg^{2+} were also slightly inhibitory at higher concentrations. The pronounced inhibition by Ni^{2+} and Co^{2+} probably accounts for the poor specific activities that we obtained when recombinant *TcMCP*-1 was purified on Ni- or Co-NTA resin. In contrast, recombinant *TcMCP*-2 activity on FA-Phe-Phe was stimulated by Co^{2+} , whereas Mn^{2+} and Ni^{2+} had little effect. Zn^{2+} , the most common metalloprotease cofactor, inhibited both recombinant *TcMCPs* at micromolar excess. A similar phenomenon was reported for several zinc peptidases [25–27] and was attributed to a second Zn^{2+} ion that binds the active site [28,29].

To study the effects of bivalent cations on metal-depleted *TcMCPs* (apo-*TcMCPs*), the GST-fused recombinant enzymes were dialysed against the metal-ion chelator EDTA (10 mM) as described in the Materials and methods section, leading to the inactive apo-enzymes. Incubation with 0.1 mM Mn^{2+} or Co^{2+} reactivated apo-*TcMCP*-1 by up to 28 % and 20–24 % respectively. Zn^{2+} had less effect (5–6 % maximal reactivation at 10 μ M). Higher metal concentrations did not afford a higher recovery of activity and resulted in enzyme inhibition. Co^{2+} at 1 mM completely restored apo-*TcMCP*-2 activity on FA-Phe-Phe, whereas 1 mM MnCl_2 reactivated the enzyme up to 82–90 %. Zn^{2+} had no effect on apo-*TcMCP*-2. This behaviour was also reported for *PfuCP*, which was activated in the presence of Co^{2+} , but not in the presence of Zn^{2+} [17]. The poor activity recovery by apo-*TcMCP*-1 might be due to its lower stability, since *TcMCP*-1 control (dialysed against 50 mM Tris/HCl, pH 7.6) lost almost 50 % of its activity compared with the *TcMCP*-2 control. It has been suggested for several metalloproteins that removal of the bivalent cation induces conformational changes that result in enzyme inactivation [30,31].

Kinetic parameters and P1' preference

Kinetic parameters of recombinant *TcMCP*-1 activity in 100 mM Mes, pH 6.2, with FA-Ala-Lys as substrate were determined from a Lineweaver–Burk plot: K_m 166 μ M, k_{cat} 20 s^{-1} . When Co^{2+} was added to the reaction mixture, FA-Ala-Lys was hydrolysed at a lower rate, with a k_{cat} value 28 % of that seen in its absence. Interestingly, recombinant *TcMCP*-1 activity on FA-Phe-Phe was enhanced in the presence of Co^{2+} , although the substrate was much less efficiently hydrolysed: k_{cat}/K_m 9.6 $\text{s}^{-1} \cdot \text{mM}^{-1}$. In the absence of Co^{2+} , it was possible to detect activity on FA-Phe-Phe only when 10-fold more enzyme was used (up to 5 μ g). Recombinant *TcMCP*-2 purified by IMAC (immobilized metal-ion-affinity chromatography) Co^{2+} had a K_m value of 24 μ M for the hydrolysis of FA-Phe-Phe in 50 mM Tris/HCl, pH 7.6 (Table 3).

To characterize further the substrate preference with respect to the P1' position of the identified *TcMCPs*, several commercially available ZAX (X represents a different amino acid as indicated by the single-letter amino acid code) dipeptides were used. As shown in Figure 4, the recombinant *TcMCP*-1 acted best on substrates having a basic amino acid at P1' position. Activity was very low with hydrophobic C-terminal residues. In contrast, recombinant *TcMCP*-2 seems to have preference for aliphatic, even glycine, and aromatic amino acids, not acting on basic substrates (ZAR or ZAK). For both enzymes, no activity was seen against ZAH, ZAP, ZAD or ZAE.

We have also performed similar experiments with the full-length *TcMCP*-1 and *TcMCP*-2 produced as GST-fusion proteins. These experiments confirmed the above results for substrate specificity and sensitivity to metal cations obtained by using His-tagged *TcMCPs* (results not shown).

TcMCPs are not thermostable

As both M32 carboxypeptidases characterized previously were thermostable, many M32 sequences belonging to mesophilic organisms were subsequently annotated as putative thermostable carboxypeptidases, including the *TcMCP*-2 gene. To confirm or discard this possibility, we incubated recombinant *TcMCPs* at different temperatures. Both enzymes drastically decreased their activity in response to increasing temperatures. Residual activities measured under standard conditions after 5 min of incubation at 50 °C were 21.4 % and 27.5 % for *TcMCP*-1 and *TcMCP*-2 respectively, as expected from a mesophilic organism.

Expression of *TcMCPs* in *T. cruzi*

In order to analyse the expression of *TcMCPs* in all developmental stages of *T. cruzi*, we obtained antibodies against *TcMCP*-1 and *TcMCP*-2 (see the Materials and methods section). Cell-free extracts of epimastigotes, amastigotes, cell-derived trypomastigotes and metacyclic trypomastigotes were subjected to Western blot analysis. Antibodies raised against the recombinant *TcMCP*-1 recognized a single band with an apparent molecular mass of 59 kDa in all life cycle stages of the parasite (Figure 5A). On the other hand, polyclonal anti-*TcMCP*-2 peptide serum solely detected a 58 kDa band in soluble extracts prepared from the epimastigote and metacyclic forms (Figure 5B). In good agreement with the results presented above, carboxypeptidase activity against FA-Ala-Lys was detected in all developmental stages of *T. cruzi*, whereas the FA-Phe-Phe substrate was hydrolysed solely by the parasite insect stages (Figure 5C). These data suggest a differential pattern of expression of *TcMCP*-2 during the *T. cruzi* life cycle.

TcMCP subcellular localization

The subcellular localization of *TcMCPs* was studied by indirect immunofluorescence as described in the Materials and methods section. *T. cruzi* cells were fixed and permeabilized with saponin before antibody binding. Polyclonal antiserum raised against recombinant *TcMCP*-1 or *TcMCP*-2 peptide yielded a diffuse uniform fluorescence throughout the cytosol of the epimastigote and metacyclic forms of the parasite (Figure 6). Mammalian stages

Figure 6 *TcMCP* subcellular localization

(A) Indirect immunofluorescence using a *TcMCP*-1 rabbit antiserum on permeabilized *T. cruzi* cells (epimastigotes, metacyclic trypomastigotes, amastigotes and cell-derived trypomastigotes are indicated on the right-hand side) yielded a diffuse uniform fluorescence throughout the parasite cytosol. (B) Fluorescence microscopy analysis using a polyclonal antiserum raised against *TcMCP*-2 peptide. *T. cruzi* epimastigotes (e) and metacyclic trypomastigotes (m) are shown. The amastigote (a) and cell-derived trypomastigote (t) forms of the parasite are indicated in the lower panel.

solely revealed reactive material distributed throughout the cell bodies of the parasites when they were incubated with *TcMCP-1* antiserum. On the other hand, amastigotes probed with anti- (*TcMCP-2* peptide) antibody presented low labelling, whereas no signal could be detected in cell-derived trypomastigotes. These observations, together with those derived from analysis of the primary sequences (i.e. absence of a predicted signal peptide) suggest that both *TcMCPs* are cytosolic enzymes.

DISCUSSION

In the present paper, we report the cloning, genetic analysis and biochemical characterization of two novel MCPs of the M32 family from *T. cruzi* CL Brener clone. In contrast with cruzipain [32] and *TcCBP* [11], *TcMCP* genes are present in a single copy per haploid genome. Their encoded proteins displayed 20–60 % identity with other M32 peptidases, showing the higher similarity scores with those of the proteobacteria group. The *TcMCPs* described here lack the canonical HEXXE(X)_{123–132}H motif characteristic of the M14 family of carboxypeptidases (subfamilies M14A, M14B and M14C) and instead contain the HEXXH signature present in metalloendopeptidases, such as thermolysin (family M4) [18]. This work represents the first identification of a M32 peptidase from any eukaryotic organism.

To explore further the functional relevance of *TcMCP* proteins, we performed enzymatic analysis with both recombinant enzymes (*TcMCP-1* and *-2*) produced in *E. coli*. This analysis provided evidence that these eukaryotic proteins are catalytically active carboxypeptidases that exhibit sensitivity to general metalloproteinase inhibitors. Despite their identity (64 %), the *TcMCPs* showed marked differences. Whereas *TcMCP-2* could cleave aliphatic, neutral and aromatic residues at the P1' position, *TcMCP-1* exhibited a narrow substrate specificity, acting best on basic residues at a lower pH, resembling the subfamily M14B. This makes *TcMCP-1* different from those enzymes of the M32 family characterized to date: *TaqCP* and *PfuCP*, which exhibited broad substrate specificity for most peptides, except for those containing C-terminal acidic residues [14,17]. Intriguingly, *TcMCP-1* substrate specificity could also be modulated by metal cations. Thus the presence of Co²⁺ ions enhanced the activity on FA-Phe-Phe, whereas it inhibited the activity on FA-Ala-Lys. A change in substrate specificity, related to a change in the metal cofactor, has been reported for CPB from human plasma: with cobalt at the active site, CPB activity towards the ester substrate hippuryl-L-arginic acid was stimulated, whereas hydrolysis of the peptide substrate hippuryl-L-arginine was inhibited. The opposite trend was observed with cadmium at the active site [33]. Unfortunately, there are no data on the cytoplasmic levels of Co²⁺, Mn²⁺ and Ni²⁺ in *T. cruzi*, therefore we cannot speculate on the possibility that this substrate modulation is operating *in vivo*.

In solution, the recombinant *TcMCP-1* and *TcMCP-2* exist as homodimers of 128 kDa and 132 kDa respectively, as determined by gel-filtration chromatography. In good agreement with these results, the primary structure of *TcMCPs* show conserved hydrophobic amino acid residues (Ala²⁴, Pro³³, Gly³⁶, Ala⁴², Ala⁴³ and Leu⁴⁷) and residues involved in the formation of salt bridges (Lys¹³, Asp⁵³ and Asp²⁸, Arg²⁴⁶) that were supposed to be involved in *PfuCP* dimer stabilization, some of which are lost in the monomeric *TaqCP* [18]. There is no evidence of post-translational processing, since the native *TcMCPs* were detected by Western blot analysis at a molecular mass corresponding to the size deduced from the sequence data. A different pattern of expression of both *TcMCPs* was detected during the *T. cruzi* life cycle.

Whereas *TcMCP-1* is expressed at similar levels throughout the life cycle of the parasite, *TcMCP-2* is found mainly in the insect vector stages, suggesting that its expression is developmentally regulated.

Orthologue genes of *TcMCP-1* (displaying 59–72 % identity with the corresponding *T. cruzi* enzyme) have been annotated in the genomes of *T. brucei* and *L. major* (see Supplementary Table S1). Interestingly, all of these genes share the same genomic context (i.e. they are present in syntenic regions). In contrast, analysis of the *TcMCP-2* genomic locus suggest that this gene is species-specific, since it occurs at a non-syntenic chromosome region. Nevertheless, a phylogenetic analysis of parasite MCPs clustered *TcMCP-2* within *TcMCP-1*-related proteins, suggesting that it could have arisen by gene duplication (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/401/bj4010399add.htm>). Likewise, multiple M32 genes and pseudogenes were identified through searches against the *L. major* genome [34] and other *Leishmania* spp. genomes whose sequencing is underway [35]. These findings illustrate the complexity of the M32 family within the trypanosomatids which is not seen in prokaryotic organisms and might be the result of specific adaptations to distinct species-specific selection pressures and survival strategies of each organism.

An additional distinctive feature of the M32 family is its particular phylogenetic distribution. In fact, our *in silico* analysis revealed that these enzymes are widely distributed in bacterial and archaeal organisms, but are absent in most eukaryotic genomes fully sequenced so far. This phylogenetic distribution could be explained by considering different evolutionary scenarios. One possibility is that of horizontal gene transfer between an ancestral proteobacterium (the most likely donor based on the phylogenetic tree in Figure 2B) and an ancestral trypanosomatid. This hypothetical scenario explains the presence of M32 genes in trypanosomatids and their absence in other eukaryotic organisms. A similar suggestion has been made for the three last enzymes of haem biosynthesis (coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase and ferrochelatase) of *L. major* and the third enzyme of the aminoethylphosphonate pathway (aminoethylphosphonate transaminase), found in *L. major* and *T. cruzi* [34]. In another possible scenario, the M32 genes were lost in an ancestral eukaryote, sometime after the ancestor of present-day trypanosomatids had branched off the tree. This hypothesis can also explain the absence of M32 genes in eukaryotic organisms that evolved from this hypothetical ancestor lacking M32 genes. However, to explain the apparent absence of M32 genes in early branching eukaryotes (such as *Giardia lamblia*, 11.3× genome coverage; *Entamoeba histolytica*, 8× genome coverage; or *Trichomonas vaginalis*, 6× genome assembly), one would have to postulate additional gene loss events. In the light of these observations, the first scenario stands as the most parsimonious. However, given that there is limited coverage of complete genome sequences from the early branches of the eukaryotic tree (mostly because of the paucity of extant organisms), the second evolutionary scenario (gene loss in other early branches) cannot be discarded.

No biological function has yet been assigned to peptidases of the M32 family. Nevertheless, microarray experiments on the hyperthermophilic archaeon *P. furiosus* suggest that M32 peptidases may be involved in the utilization of peptides and proteins for nutrition in this organism, since the relative transcript levels of the ORF PF0456 (coding for an M32 peptidase) are up-regulated 2.6-fold when cells are grown in media containing peptides as the primary carbon source compared with those grown in maltose [36]. Additional experiments performed on *Bacillus subtilis* knockout for the *YpwA* gene have shown that the enzyme is

not essential for growth in glucose medium. Notably, this mutant showed improved growth in terms of rate and yield of biomass formation [37]. Additional clues about the possible roles of these enzymes can be derived from the analysis of the crystal structure of *Pfu*CP. This analysis revealed that the enzyme has a fold similar to those of ACE (angiotensin-converting enzyme) [38,39], ACE2 [40], neurolysin [18] and TOP (thimet oligopeptidase) [41], all of which are zinc metalloproteases with no detectable sequence similarity to M32 proteases. Whereas ACE is a crucial regulator of the renin–angiotensin system, converting the inactive decapeptide angiotensin I into the potent vasoconstrictor angiotensin II, many biologically active peptides could be hydrolysed by ACE2 [42]. TOP has been implicated in the metabolism of a number of small peptides in the central nervous system, and recent studies have also demonstrated that this enzyme is primarily responsible for degrading peptides released from proteasomes [43], thereby limiting the extent of antigen presentation by MHC class I molecules [44]. Thus we can speculate that a major role of the *Tc*MCPs might be the degradation of peptides, such as those produced by the proteasomes, in the cytosol. However, a possible role in the processing of proteins and small regulatory peptides cannot be excluded at present.

Previous studies on M32 proteases focused their attention on the possibility of using these enzymes in C-terminal ladder sequencing of proteins and peptides. Today, apart from the possible biotechnological applications, it is clear that M32 sequences are present in human pathogens such as *Vibrio*, *Rickettsia*, *Yersinia*, *Legionella*, *Leptospira*, *Listeria*, *Enterococcus*, *Bacillus* and also the Trypanosomatidae family. Thus the apparent absence of these enzymes in most eukaryotic organisms, including mammals, makes the M32 family a unique group of proteases, particularly suitable for exploration as possible drug targets against these organisms. Validation of *Tc*MCP-1 and *Tc*MCP-2 as targets will require further studies with deletion mutants and permeant inhibitors.

This work was performed with a grant from ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), SECYT (Secretaría de Ciencia y Técnica) (Argentina), PICT 01-15042. J.J.C. and F.A. are members of the research career, and G.N. is a research fellow of the Argentinean National Research Council [CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas)]. F.P. was a research fellow of the Universidad Nacional de General San Martín. We are indebted to Ms Ulla Engström and Dr Ulf Hellman from the Ludwig Institute for Cancer Research, Uppsala Branch, Sweden, for the synthesis of the peptides used to produce antibodies.

REFERENCES

- Barrett, M. P., Burchmore, R. J., Stich, A., Lazzari, J. O., Frasch, A. C., Cazzulo, J. J. and Krishna, S. (2003) The trypanosomiasis. *Lancet* **362**, 1469–1480.
- Klemba, M. and Goldberg, D. E. (2002) Biological roles of proteases in parasitic protozoa. *Annu. Rev. Biochem.* **71**, 275–305.
- Cazzulo, J. J. (2002) Proteinases of *Trypanosoma cruzi*: potential targets for the chemotherapy of Chagas disease. *Curr. Top. Med. Chem.* **2**, 1261–1271.
- García, M. P., Nobrega, O. T., Teixeira, A. R., Sousa, M. V. and Santana, J. M. (1998) Characterisation of a *Trypanosoma cruzi* acidic 30 kDa cysteine protease. *Mol. Biochem. Parasitol.* **91**, 263–272.
- Burleigh, B. A., Caler, E. V., Webster, P. and Andrews, N. W. (1997) A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{2+} signaling in mammalian cells. *J. Cell Biol.* **136**, 609–620.
- Bastos, I. M., Grellier, P., Martins, N. F., Cadavid-Restrepo, G., de Souza-Ault, M. R., Augustyns, K., Teixeira, A. R., Schrevel, J., Maigret, B., da Silveira, J. F. and Santana, J. M. (2005) Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells. *Biochem. J.* **388**, 29–38.
- Cuevas, I. C., Cazzulo, J. J. and Sanchez, D. O. (2003) gp63 homologues in *Trypanosoma cruzi*: surface antigens with metalloprotease activity and a possible role in host cell infection. *Infect. Immun.* **71**, 5739–5749.
- de Diego, J. L., Katz, J. M., Marshall, P., Gutierrez, B., Manning, J. E., Nussenzweig, V. and Gonzalez, J. (2001) The ubiquitin–proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. *Biochemistry* **40**, 1053–1062.
- Gonzalez, J., Ramalho-Pinto, F. J., Frevert, U., Ghiso, J., Tomlinson, S., Scharfstein, J., Corey, E. J. and Nussenzweig, V. (1996) Proteasome activity is required for the stage-specific transformation of a protozoan parasite. *J. Exp. Med.* **184**, 1909–1918.
- Healy, N., Greig, S., Enahoro, H., Roberts, H., Drake, L., Shaw, E. and Ashall, F. (1992) Detection of proteases in *Trypanosoma cruzi* epimastigotes using chromogenic and fluorogenic substrates. *Parasitology* **104**, 315–322.
- Parussini, F., Garcia, M., Mucci, J., Agüero, F., Sanchez, D., Hellman, U., Aslund, L. and Cazzulo, J. J. (2003) Characterization of a lysosomal serine carboxypeptidase from *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **131**, 11–23.
- Vendrell, J., Querol, E. and Aviles, F. X. (2000) Metalloproteases and their protein inhibitors: structure, function and biomedical properties. *Biochim. Biophys. Acta* **1477**, 284–298.
- Vendrell, J. and Aviles, F. X. (1999) Carboxypeptidases. In *Proteases: New Perspectives*. (Turk, V., ed.), pp. 13–34. Birkhäuser Verlag, Basel.
- Lee, S. H., Minagawa, E., Taguchi, H., Matsuzawa, H., Ohta, T., Kaminogawa, S. and Yamauchi, K. (1992) Purification and characterization of a thermostable carboxypeptidase (carboxypeptidase Tag) from *Thermus aquaticus* YT-1. *Biosci. Biotechnol. Biochem.* **56**, 1839–1844.
- Lee, S. H., Taguchi, H., Yoshimura, E., Minagawa, E., Kaminogawa, S., Ohta, T. and Matsuzawa, H. (1996) The active site of carboxypeptidase Tag possesses the active-site motif His-Glu-X-X-His of zinc-dependent endopeptidases and aminopeptidases. *Protein Eng.* **9**, 467–469.
- Rawlings, N. D., Morton, F. R. and Barrett, A. J. (2006) MEROPS: the protease database. *Nucleic Acids Res.* **34**, D270–D272.
- Cheng, T. C., Ramakrishnan, V. and Chan, S. I. (1999) Purification and characterization of a cobalt-activated carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Protein Sci.* **8**, 2474–2486.
- Arndt, J. W., Hao, B., Ramakrishnan, V., Cheng, T., Chan, S. I. and Chan, M. K. (2002) Crystal structure of a novel carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Structure* **10**, 215–224.
- Zingales, B., Pereira, M. E., Almeida, K. A., Umezawa, E. S., Nehme, N. S., Oliveira, R. P., Macedo, A. and Souto, R. P. (1997) Biological parameters and molecular markers of clone CL Brener the reference organism of the *Trypanosoma cruzi* genome project. *Mem. Inst. Oswaldo Cruz* **92**, 811–814.
- Franke de Cazzulo, B. M., Martinez, J., North, M. J., Coombs, G. H. and Cazzulo, J. J. (1994) Effects of proteinase inhibitors on the growth and differentiation of *Trypanosoma cruzi*. *FEMS Microbiol. Lett.* **124**, 81–86.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503–517.
- Quevillon, E., Silventoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R. and Lopez, R. (2005) InterProScan: protein domains identifier. *Nucleic Acids Res.* **33**, W116–W120.
- Letunic, I., Copley, R. R., Pils, B., Pinkert, S., Schultz, J. and Bork, P. (2006) SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res.* **34**, D257–D260.
- Mallya, S. K. and Van Wart, H. E. (1989) Mechanism of inhibition of human neutrophil collagenase by gold(I) chrysotherapeutic compounds: interaction at a heavy metal binding site. *J. Biol. Chem.* **264**, 1594–1601.
- Larsen, K. S. and Auld, D. S. (1989) Carboxypeptidase A: mechanism of zinc inhibition. *Biochemistry* **28**, 9620–9625.
- Kerr, M. A. and Kenny, A. J. (1974) The molecular weight and properties of a neutral metallo-endopeptidase from rabbit kidney brush border. *Biochem. J.* **137**, 489–495.
- Gomez-Ortiz, M., Gomis-Ruth, F. X., Huber, R. and Aviles, F. X. (1997) Inhibition of carboxypeptidase A by excess zinc: analysis of the structural determinants by X-ray crystallography. *FEBS Lett.* **400**, 336–340.
- Holland, D. R., Hausrath, A. C., Juers, D. and Matthews, B. W. (1995) Structural analysis of zinc substitutions in the active site of thermolysin. *Protein Sci.* **4**, 1955–1965.
- Kayestha, R., Sumati and Hajela, K. (1996) Studies on metal induced conformation changes in a peripheral blood lymphocyte lectin. *Biochim. Biophys. Acta* **1289**, 51–56.
- Kerovuo, J., Lappalainen, I. and Reinikainen, T. (2000) The metal dependence of *Bacillus subtilis* phytase. *Biochem. Biophys. Res. Commun.* **268**, 365–369.
- Campetella, O., Henriksson, J., Aslund, L., Frasch, A. C., Pettersson, U. and Cazzulo, J. J. (1992) The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic tandemly organized genes located on different chromosomes. *Mol. Biochem. Parasitol.* **50**, 225–234.

- 33 Tan, A. K. and Eaton, D. L. (1995) Activation and characterization of procarboxypeptidase B from human plasma. *Biochemistry* **34**, 5811–5816
- 34 Ivens, A. C., Peacock, C. S., Worthey, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M. A., Adlem, E., Aert, R. et al. (2005) The genome of the kinetoplastid parasite, *Leishmania major*. *Science* **309**, 436–442
- 35 Hertz-Fowler, C., Peacock, C. S., Wood, V., Aslett, M., Kerhornou, A., Mooney, P., Tivey, A., Berriman, M., Hall, N., Rutherford, K. et al. (2004) GeneDB: a resource for prokaryotic and eukaryotic organisms. *Nucleic Acids Res.* **32**, D339–D343
- 36 Schut, G. J., Brehm, S. D., Datta, S. and Adams, M. W. (2003) Whole-genome DNA microarray analysis of a hyperthermophile and an archaeon: *Pyrococcus furiosus* grown on carbohydrates or peptides. *J. Bacteriol.* **185**, 3935–3947
- 37 Fischer, E. and Sauer, U. (2005) Large-scale *in vivo* flux analysis shows rigidity and suboptimal performance of *Bacillus subtilis* metabolism. *Nat. Genet.* **37**, 636–640
- 38 Natesh, R., Schwager, S. L., Sturrock, E. D. and Acharya, K. R. (2003) Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. *Nature* **421**, 551–554
- 39 Brew, K. (2003) Structure of human ACE gives new insights into inhibitor binding and design. *Trends Pharmacol. Sci.* **24**, 391–394
- 40 Towler, P., Staker, B., Prasad, S. G., Menon, S., Tang, J., Parsons, T., Ryan, D., Fisher, M., Williams, D., Dales, N. A. et al. (2004) ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. *J. Biol. Chem.* **279**, 17996–18007
- 41 Ray, K., Hines, C. S., Coll-Rodriguez, J. and Rodgers, D. W. (2004) Crystal structure of human thimet oligopeptidase provides insight into substrate recognition, regulation, and localization. *J. Biol. Chem.* **279**, 20480–20489
- 42 Guy, J. L., Lambert, D. W., Warner, F. J., Hooper, N. M. and Turner, A. J. (2005) Membrane-associated zinc peptidase families: comparing ACE and ACE2. *Biochim. Biophys. Acta* **1751**, 2–8
- 43 Saric, T., Graef, C. I. and Goldberg, A. L. (2004) Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. *J. Biol. Chem.* **279**, 46723–46732
- 44 Kim, S. I., Pabon, A., Swanson, T. A. and Glucksman, M. J. (2003) Regulation of cell-surface major histocompatibility complex class I expression by the endopeptidase EC 3.4.24.15 (thimet oligopeptidase). *Biochem. J.* **375**, 111–120
- 45 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680

Received 26 June 2006/7 September 2006; accepted 28 September 2006

Published as BJ Immediate Publication 28 September 2006, doi:10.1042/BJ20060973