Thrombocidins, microbicidal proteins of human blood platelets

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Structural Requirements Of Recombinant Thrombocidins For Microbicidal Activity

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ABSTRACT

Thrombocidin (TC)-1 and TC-2, microbicidal proteins of human blood platelets, are derivatives of NAP-2 and CTAP-III respectively, missing the two C-terminal amino acids of these CXC-chemokines. TC-1, TC-2, a TC-1 variant C-terminally truncated by 5 residues, and an N-terminally His-tagged TC-1 variant (rH-TC) were produced recombinantly in E. coli, and their microbicidal activities were determined. Recombinant TCs had activity patterns against bacteria and fungi identical to those of natural thrombocidins. Bactericidal activity of TC-2, but not of TC-1, was dependent on protein folding. The C-terminally truncated TC-1 retained bactericidal activity. Furthermore, rH-TC had enhanced microbicidal activity, and evidence is given that this is caused by the N-terminally fused histidines.
INTRODUCTION

Antimicrobial proteins are components of the innate immune system of a wide variety of organisms (5,6), and in humans are present most abundantly in neutrophils and epithelial tissue (16,19,32). For over two decades, human and rabbit blood platelets also have been recognized to contain antibacterial proteins (13,34). These proteins are believed to be involved in the clearance of bacteria in blood borne infections (9,10,12,36). Recently we have shown that human platelet granules contain at least 10 antimicrobial proteins, which we named thrombocidins (TCs) (22). TC-1 and TC-2 were fully characterized and appeared to be structurally different from the known classes of antimicrobial proteins. They are derivatives of the CXC-chemokines neutrophil activating peptide-2 (NAP-2) and connective tissue activating peptide-III (CTAP-III), respectively. CTAP-III is a platelet-specific peptide with fibroblast mitogenic activity (33) and is thought to be involved in wound healing. NAP-2 is an N-terminal cleavage product of CTAP-III, and is a mediator in the inflammatory response by its activity as a potent attractant and activator of neutrophils (2). TC-1 and TC-2 differ from NAP-2 and CTAP-III by a C-terminal truncation of two amino acids (22). This truncation appeared to be imperative for microbicidal activity, since NAP-2 and CTAP-III had no microbicidal activity (22).

The present study was designed to produce TC-1 and TC-2 recombinantly, and to investigate the influence of folding as well as C- and N-terminal modification on microbicidal activity.

MATERIALS AND METHODS

Construction of expression vectors containing TC-1, TC-2, CTAP-III and H-TC coding DNA.

Since the chemokines NAP-2 (15,30), CTAP-III (14,30) and IL-8 (25), which are all closely related to TCs, as well as several antibacterial proteins (20,26,29) have been produced in biologically active form in bacterial systems, we chose for an E. coli expression system to produce TCs recombinantly.

DNA coding for TC-1, TC-2, and CTAP-III was obtained in a 2-step PCR protocol. Primers were designed based on the cDNA sequence of platelet basic protein (PBP), which is the precursor protein of TCs, NAP-2 and CTAP-III (35). In the first PCR step a product coding for PBP was amplified from 2 ng of a human bone marrow cDNA library (Clontech, Palo Alto, CA, USA) using Pfu polymerase (Stratagene, La Jolla, CA, USA) and oligonucleotides PBPf and PBPr as forward and reverse primers, respectively (Table 1). The PCR protocol consisted of 5 min melting at 95°C followed by 30 cycles of 1 min 95°C, 2 min 55°C and 2 min 72°C. The resulting PBP amplicon (400 bp, not shown) served as a template in a set of subsequent PCR reactions generating products coding for TC-1, TC-2 and CTAP-III (Table 1). All primers were from Perkin Elmer-Applied Biosystems, Warrington, UK. The forward and reverse primers contained NdeI and BamHI restriction sites (underlined in Table 1), respectively, to allow cloning of the PCR products. Conditions for these PCR reactions were the same as for the PBP PCR. NdeI / BamHI
Table 1. Sequences of forward (f) and reverse (r) primers used in PCR reactions. Restriction sites used for cloning are underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Amplification of sequence encoding</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBGF</td>
<td>TATAGGATCCATGAGCCTGACGACTTGTGATACGCAGACC</td>
<td>PBP</td>
</tr>
<tr>
<td>PBP r</td>
<td>TATAGGATTCCTCAATCATAGGATACCACCTGCCTGCAATT</td>
<td>PBP/rH-TC</td>
</tr>
<tr>
<td>TC1f</td>
<td>GTGTAACATATGGCTGAAACTCCGCTGCATGTGTG</td>
<td>TC-1</td>
</tr>
<tr>
<td>TC1r</td>
<td>TATAGGATCCATAGGATACCACCTGCCTGCAATT</td>
<td>TC-1/TC-2/NAP-2</td>
</tr>
<tr>
<td>TC2f</td>
<td>GTGTAACATATGAAACTGGGAAAGGCAAAGAG</td>
<td>TC-2</td>
</tr>
<tr>
<td>NAP2vf</td>
<td>GTGTAACATATGGTGATCTCCGCTGCAATT</td>
<td>rH-TC</td>
</tr>
</tbody>
</table>

double-digested PCR products were purified from agarose gel (Qiagen), ligated to pET9a expression vectors (Novagen) digested with the same enzymes, and transformed to E. coli BL21DE3(LysS) cells (Novagen) by heat shock. Individual colonies on selective LB agar plates were checked for the presence of an insert by colony PCR using a direct primer recognizing the T7 promoter sequence of the pET vector (TAATACGACTCACTATAGGG) and the reverse primer specific for the respective constructs (Table 1). Both strands of three positive clones of each construct were sequenced. Bacteria containing the correct constructs were stored in glycerol broth at -70°C until further use.

In addition, we investigated the microbicidal potency of a TC-1 derivative, rH-TC, which we originally designed as part of a study to evaluate neutrophil chemotactic activity of thrombocidins and CXC-chemokines. In rH-TC the ELR motif needed for neutrophil chemotaxis is incomplete, and therefore expected to be non-functional. A PCR product encoding rH-TC was generated as described for the TC and CTAP-III coding fragments using primers NAP2vf and PBPr (Table 1). The PCR product was ligated to a Ndel I / BamHI digested pET16b expression vector (Novagen) to provide rH-TC with an N-terminal His-tag. Sequencing, transformation and storage were as described above.

Expression of recombinant proteins

E. coli BL21DE3(LysS) cells containing pET9a and pET16b-derived constructs were cultured in LB medium with chloramphenicol (50 μg/ml) + kanamycin (50 μg/ml) or chloramphenicol (50 μg/ml) + ampicillin (50 μg/ml), respectively. When cultures had reached an optical density (A_{620}) of 0.3, isopropyl-β-D-thiogalactoside (IPTG, Boehringer, Mannheim, Germany) was added to a final concentration of 0.5 mM (pET9a) or 1 mM (pET16b) to induce expression of the cloned genes. After 5 h of incubation at 37°C, cells were harvested by centrifugation (5 min, 5000 x g), pellets were resuspended in lysis buffer (20 mM Tris pH 7.2, 6 M urea, 1/40 of the culture volume) and kept at -20°C o/n. Bacteria were lysed by sonication (5 min, 0°C), cell debris was removed by centrifugation (20 min, 5000 x g), and the supernatant was dialyzed extensively against 10 mM sodium phosphate buffer, pH 7.0.
Purification of thrombocidins and chemokines

Recombinant TC-1 (rTC-1), rTC-2 and rCTAP-III were purified in a two-step procedure as described previously for the native thrombocidins (22), using CM-Sepharose cation exchange chromatography and continuous acid urea polyacrylamide gel electrophoresis (CAU-PAGE). rH-TC was purified using a Ni-NTA column (Novagen) followed by CAU-PAGE. After each purification step, activity of antibacterial proteins was analysed by AU-PAGE and gel overlay assays (see below). Natural NAP-2 and CTAP-III were purified as described (7,15), and recombinant NAP-2 and NAP(1-63) were produced as described in (14).

Characterization of recombinant proteins

N-terminal sequencing of recombinant proteins was performed by automated Edman degradation (Applied Biosystems Protein Sequencer, model 476A). Molecular masses were determined by electrospray ionization mass spectrometry performed on a hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK).

Biochemical analyses

Acid urea PAGE (21) and Tricine-SDS-PAGE (31) were performed using 15 x 10 x 0.7 cm gels. Silverstaining was done as described by Blum (4).

Influence of folding of natural and recombinant thrombocidins and rCTAP-III on microbicidal activity was evaluated by reduction of disulfide bridges in these proteins. Human neutrophil defensin (HNP1-3, a kind gift of Dr. P.S. Hiemstra, Leiden, The Netherlands) was included as a folding-dependent antibacterial peptide. Protein (ca. 1 mg/ml) was dissolved in water and boiled for 5 min to test heat-stability, or in 100 mM Tris-HCl pH 8.5 containing 6 M guanidine hydrochloride and 2 mM EDTA followed by incubation at 50°C for 30 min prior to reduction. Dithiothreitol (DTT, Sigma, St. Louis, MO, USA) was added (1 mg/ml final concentration) and incubation was allowed to proceed for 4 h under nitrogen at 50°C. Sulhydryl groups were alkylated by the addition of iodoacetamide (Sigma; 4 mg/ml final concentration) to prevent refolding. Alkylated protein was purified by HPLC using a Hypersil PEP C18 RP column (150 x 4.6 mm; Alltech, Deerfield, USA), lyophilized and dissolved in 0.01% acetic acid. Efficiency of alkylation was confirmed by mass spectrometry. Protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL, USA).

Peptide synthesis

Peptides His(7), His(10) and His(13), consisting of 7, 10 and 13 histidines, respectively, and the His-tag peptide (MGHHHHHHHHHHHHSSGHIEGRH) were synthesized on an Abimed 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany) at 10 umol scale (17,18). Peptides were isolated and purified by repeated ether precipitations, dissolved in 10% acetic acid and lyophilized. Purity of peptides was analyzed by HPLC, and peptide identity was confirmed by MALDI mass spectrometry.
Antibacterial activity testing

Antimicrobial activity was tested using *Bacillus subtilis* ATCC6633, *Escherichia coli* ML35p (23), *Staphylococcus aureus* 42D, and clinical isolates of *Cryptococcus neoformans*, *Candida albicans* and *Candida glabrata*. Fractions collected during preparative chromatography and electrophoresis as well as purified proteins were tested in overlay assays (24) using the *Bacillus* strain as the test organism. Minimal bactericidal concentrations (MBCs) and minimal fungicidal concentrations (MFCs) defined as the concentration of protein killing >99.9% of the inoculum in 2 h, were determined using microdilution assays as described previously (22). Inocula of 0.5-1 × 10^4 cfu in 50 μl of 10 mM phosphate buffer pH 7.0 + 0.06% (w/v) tryptic soy broth (TSB) were incubated with protein.

RESULTS

Production and characterization of recombinant TC-1, rTC-2, rCTAP-III, and H-TC

Recombinant TC-1 (rTC-1), rTC-2, rCTAP-III, and rH-TC (Fig. 1) were produced in *E. coli* BL21DE3(LysS). rTCs and rCTAP-III were purified by CM-Sepharose chromatography and CAU-PAGE, and rH-TC by Ni-NTA chromatography and CAU-PAGE. Antibacterial activity of recombinant proteins was assessed after each purification step using overlay assays with *E. coli* ML35p. Like natural CTAP-III (22) rCTAP-III had no antibacterial activity. Despite this, rCTAP-III could easily be recognized in acid urea gels after silver staining since this protein migrates faster than all *E. coli* proteins. The final yield of purified recombinant proteins was 3 to 5 mg per liter of culture.

Partial N-terminal sequencing revealed that the recombinant proteins had the expected sequences in all cases, but that proteins carrying an additional N-terminal methionine were also present. These additional products varied in abundance from 10% (rTC-1) to 90% (rTC-2). The molecular weight of rTC-1, determined by electrospray mass spectrometry, was 7436.05 Da. This is in accordance with the theoretical molecular weight of oxidized TC-1 (two cysteine bridges, 7436.88 Da). Mass spectrometric analysis of rTC-2 revealed products with molecular weights of 9231.19 and 9099.8 Da, matching the theoretical weights of oxidized TC-2 with and without N-terminal methionine (9232.88 and 9101.5 Da), respectively. The molecular weight of rCTAP-III was 9418.28 Da, and is in accordance with the expected mass of oxidized CTAP-III carrying an additional methionine (9418.90 Da). A minor peak of 9286.9 Da observed in the mass spectrum represented CTAP-III without N-terminal methionine. Partial sequencing of rH-TC confirmed the presence of the His-tag and the first three residues beyond the histidines.
Structural requirements for activity of thrombocidins

CTAP-III
NAP-2
TC-1
TC-2
rH-TC

NLAKGKEESLDSDLYAELRCMIKTTSGIHHPKNIQSLEVI
AELRCMIKTTSGIHHPKNIQSLEVI
AELRCMIKTTSGIHHPKNIQSLEVI
AELRCMIKTTSGIHHPKNIQSLEVI
MGHHHHHHHHHHSSGHIERGRHYLRCMIKTTSGIHHPKNIQSLEVI

Figure 1. Sequences of connective tissue activating peptide-III (CTAP-III), neutrophil activating peptide-2 (NAP-2) and thrombocidin-1 (TC) and -2 and the His-tagged rH-TC.

The migration of recombinant and natural TC-1, TC-2 and CTAP-III were compared in SDS-tricine and AU gels (Fig. 2). The recombinant and natural proteins, either in reduced form (SDS-tricine gel) or in non-reduced form (AU gel), migrated identically. rH-TC migrated faster than NAP-2 in AU gels due to the presence of positively charged histidines. As migration of a protein in AU gels is strongly dependent on its conformation, charge and oxidative state of the proteins (exemplified below), the identical migration of recombinant and natural proteins implied that these proteins were folded in an identical way.

Fig. 2. Analysis of natural and recombinant TCs, CTAP-III and recombinant H-TC on silverstained tricine SDS-PA (panel A) and acid urea PA gels (panel B).
Table 2. Microbicidal activity of natural and recombinant thrombocidins and CTAP-III, and of rH-TC.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MBC or MFC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nTC-1</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>0.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>3.4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>6.8</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>1.9</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>&gt;30</td>
</tr>
<tr>
<td>C. albicans</td>
<td>ND</td>
</tr>
</tbody>
</table>

MBC and MFC values were assessed in microdilution tests as described in Materials and methods. All experiments were carried out twice in duplicate.

Microbicidal activity of rTC and rCTAP-III

Bactericidal and fungicidal activities of recombinant and natural proteins were determined by the microdilution method, and were expressed as MBC or MFC (Table 2). Both rTC-1 and rTC-2 were bactericidal for the three bacterial species tested, and were fungicidal for Cryptococcus neoformans, but not for Candida species. This activity pattern is identical to that of nTCs, but the MBCs of rTCs were 2-10 fold higher than those of the natural thrombocidins. rCTAP-III was not microbicidal for any of the tested organisms (Table 2).

Antibacterial activity of thrombocidins in reduced state

To study the relation between protein folding and antibacterial activity, native and recombinant TC-1, TC-2 and CTAP-III were boiled in the absence of DTT, reduced by DTT treatment at 100°C, or reduced and alkylated, and subsequently analyzed on acid urea gels. Reduced natural as well as recombinant TC-1, TC-2 or CTAP-III had decreased migration velocity in AU gels as compared to the folded proteins (Fig 3, top panels). Migration of alkylated proteins and proteins which had only been reduced was identical, which was as expected since alkylation does not change the charge of reduced proteins and increases molecular weight only marginally. Thus, change in migration properties easily allowed discrimination between folded and unfolded TCs. In overlay assays of AU gels on B. subtilis-containing agar layers, antibacterial activity of nTC-1 appeared not to be neutralized by boiling nor by reduction or alkylation (Fig. 3, bottom panel). Identical results were obtained with rTC-1. Activity of both nTC-2 and rTC-2 was retained after boiling, but, in contrast to TC-1, was strongly decreased after reduction or reduction/alkylation. Recombinant CTAP-III (Fig. 3) and nCTAP-III (not shown) were inactive in all cases. Reduced HNP1-3, included as a control for folding-dependent antibacterial activity, indeed had no antibacterial activity (Fig. 3).
Figure 3. Influence of linearization of thrombocidins, CTAP-III and HNP on antibacterial activity. Proteins were left untreated (U), were boiled (B), reduced in DTT (R) or were reduced and alkylated (A). Proteins were analyzed in acid urea gels, which were silver stained (top panels) or used in overlay assays using *B. subtilis* as the test organism (bottom panels).

The microbicidal activity of alkylated rTCs was analyzed in more detail by determination of their MBCs for *B. subtilis*. The MBC of reduced and alkylated rTC-1 was 4 μM, and thus was not changed compared to untreated rTC-1 (Table 2). Alkylated rTC-2 was inactive (MBC > 50 μM). Collectively, these results indicate that folding is of little importance for bactericidal activity of TC-1, whereas TC-2 appears to be critically dependent on folding for full bactericidal activity.

Table 2. Microbicidal activity of natural and recombinant thrombocidins and CTAP-III, and of rH-TC.

<table>
<thead>
<tr>
<th>Organism</th>
<th>nTC-1</th>
<th>rTC-1</th>
<th>nTC-2</th>
<th>rTC-2</th>
<th>nCTAP-III</th>
<th>rCTAP-III</th>
<th>rH-TC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>0.4</td>
<td>4</td>
<td>0.7</td>
<td>6</td>
<td>&gt;30</td>
<td>50</td>
<td>0.4</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.4</td>
<td>15</td>
<td>2.7</td>
<td>15</td>
<td>&gt;30</td>
<td>&gt;40</td>
<td>0.9</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>6.8</td>
<td>30</td>
<td>11</td>
<td>30</td>
<td>&gt;30</td>
<td>&gt;40</td>
<td>1.9</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>1.9</td>
<td>7.5</td>
<td>30</td>
<td>30-60</td>
<td>&gt;30</td>
<td>&gt;60</td>
<td>0.4</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>&gt;30</td>
<td>&gt;60</td>
<td>&gt;30</td>
<td>&gt;60</td>
<td>ND</td>
<td>&gt;60</td>
<td>&gt;15</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>ND</td>
<td>&gt;60</td>
<td>ND</td>
<td>&gt;60</td>
<td>ND</td>
<td>&gt;60</td>
<td>&gt;15</td>
</tr>
</tbody>
</table>

MBC and MFC values were assessed in microdilution tests as described in Materials and methods. All experiments were carried out twice in duplicate.
Impact of C-terminal truncation of rTC-1 on antibacterial activity.

Although CTAP-III and TC-2 only differ by two C-terminal amino acids (Fig. 1), they strongly differ in antibacterial activity ((22) and Fig. 2). Similarly, we have shown that NAP-2 is inactive against *B. subtilis*, while TC-1 is highly active (22). To assess whether specifically the two C-terminal residues should be removed, or whether activity would be retained after further truncation we tested bactericidal activity of and rNAP-2 truncated by 7 amino acids (rNAP(1-63), which is TC-1 truncated by 5 residues) against *B. subtilis*, using full length recombinant NAP-2 (rNAP(1-70)) as a control. Like natural NAP-2, rNAP(1-70) was inactive (MBC > 30 μM). However, rNAP(1-63) was bactericidal at 7.5 μM, and thus almost equally potent as rTC-1 (Table 2).

Microbicidal activity of rH-TC

Bactericidal and fungicidal activity of rH-TC, the variant of TC-1 with an N-terminal His-tag, was tested for a number of organisms. This protein was highly active against all species except *Candida* (Table 2), with MBCs and an MFC in the low- to sub-micromolar range, being up to 8-fold more active than the natural TCs.

Since NAP-2 (22) and rNAP-2 (above), which have the same carboxyl-terminus as rH-TC, had no microbicidal activity, the potent microbicidal activity of rH-TC most likely was due to the N-terminal His-tag. To investigate this possibility a peptide was synthesized comprising the entire N-terminal sequence which is fused to the NAP-sequence in rH-TC ('His-tag peptide', MGHHHHHHHHHHSSGHIEGRH). In addition, three peptides consisting exclusively of histidines (13, 10 and 7 residues) were synthesized. The His-tag peptide was microbicidal for *B. subtilis*, *S. aureus* and *C. neoformans* (Table 3), indicating that the His-tag of rH-TC indeed was involved in the microbicidal activity of this protein. Neither the His-tag peptide nor the oligo-histidine peptides had significant activity against *E. coli*. His(13) was highly active against *B. subtilis*, *S. aureus* and *C. neoformans*, with MBCs/MFC almost equal to those of rH-TC (Tables 2 and 3). Activity of His(10) was moderate, and His(7) had no detectable microbicidal activity (Table 3). Clearly, the microbicidal activity of oligo-histidines depended on peptide length.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MBC or MFC (μM) of peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>His-tag</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>7.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>&gt;120</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>60</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>60</td>
</tr>
</tbody>
</table>

MBC and MFC values were assessed in microdilution tests as described in Materials and methods. All experiments were carried out twice in duplicate.
We have recently found that thrombocidins 1 and 2 are closely related to the CXC-chemokines NAP-2 and CTAP-III, respectively (22). The aim of the present study was to produce TCs recombinantly in an *E. coli* expression system to study the influence of length and folding of these proteins on their microbicidal activity.

The expression system and the two-step purification protocol yielded recombinant proteins of correct sequence. Furthermore, mass spectrometrical analyses and examination of migration characteristics of untreated, reduced, and alkylated recombinant proteins in tricine SDS acrylamide and acid urea acrylamide gels, indicated that these proteins were produced in folded form. Thus, recombinant and natural TCs were indistinguishable in all structural and electrophoretic experiments performed.

Recombinant TCs were less active than natural TCs, but exhibited an identical activity spectrum (Table 1). *B. subtilis* was the most susceptible organism, followed by *E. coli* and *S. aureus*. Additionally, rTCs were fungicidal for *C. neoformans*, while *Candida* species were not killed. Natural and recombinant NAP-2 and CTAP-III were inactive against all organisms tested (Table 2), illustrating the importance of C-terminal truncation of recombinant TCs for microbicidal activity, as has been shown for the natural proteins before (22).

At this point no obvious explanation can be given for the difference in antimicrobial activity between natural and recombinant proteins. Some antibacterial proteins, like cecropins, have an amidated C-terminus, increasing the bactericidal activity compared to the non-amidated forms (1,8). It is unknown whether natural TCs are amidated but it is unlikely that rTCs and nTCs differ by an amide group. Amidation would have resulted in a decrease in their net positive charge and thus in an altered migration in an AU gel compared to the non-amidated protein. This was clearly not the case (Fig. 2). A second possible explanation would be misfolding of rTCs. NAP-2 and CTAP-III have 4 cysteines which are 1-3 and 2-4 interlinked (2) and this most likely is also the case in the natural TCs. Since the first two cysteines are separated by just one amino acid, only a 1-4 and 2-3 interlinkage would be a possible alternative for disulfide bridge formation. A detailed study, involving protease fragmentation of rTCs and analysis of generated fragments, would be required to resolve the folding of rTCs. Furthermore, folding of IL-8, NAP-2 and CTAP-III, either chemically synthesized or produced recombinantly in bacteria, has repeatedly been shown to proceed correctly (25,30).

Based on crystallization and NMR studies, the C-terminal part of NAP-2 (37) and CTAP-III (27,30) is considered to form an α-helix. This structure may well be involved in the bactericidal activity of TCs. The helix is to a large extent amphipathic, a feature promoting peptide-membrane interactions required for the activity of many helical antimicrobial peptides (19). From structural studies of NAP-2 it appeared that the ultimate two C-terminal residues form a flexible element that does not take part in the α-helix (37). It is conceivable that these residues hinder lipid-helix interactions and subsequent insertion into microbial membranes, while this hindrance is relieved by the removal of these two C-terminal residues. Moreover, the penultimate Asp85 in NAP-2 is an acidic residue possibly impeding initial electrostatic interaction with negatively charged constituents of the target.
membrane. The increased activity due to removal of Ala84 and Asp85, as in TC-1, or even of five more residues (of which 2 are acidic) as in rNAP(1-63) would then increase microbicidal activity both by proper exposition of the amphipathic α-helix and by increasing the net positive charge of the proteins. Apparently, the length of the helical domain in NAP(1-63) (11 residues) is still sufficient for cidal activity. This is in agreement with the fact that a 13-residue peptide derived from the helical domain of CXC-chemokine platelet factor-4 (PF-4) (11), and numerous (synthetic) α-helical peptides consisting of 8 to 15 amino acids (3) have antimicrobial activity.

Our experiments showed that antimicrobial activity of TCs was not fully dependent on folding. Unfolded or reduced and alkylated nTC-1 or rTC-1 had no decreased activity against *B. subtilis* compared to the folded forms. This suggests that activity of TC-1 does not depend on specific tertiary structures imposed by disulfide bridge formation, but predominantly on primary or secondary structure characteristics. A crucial role of the helical C-terminal domain, as discussed above, would fit in such a hypothesis. However, this helix is not likely to be the only determinant of antimicrobial activity since, in contrast to TC-1, reduction of nTC-2 and rTC-2 abolished activity. Since the only difference between TC-1 and TC-2 is an N-terminal extension in the latter, it is conceivable that this extension prevents the activity of TC-2 in unfolded form, possibly by interfering with the active region in the C-terminal helix.

rH-TC had the highest microbicidal activity of all recombinant TCs. Although this protein is not truncated C-terminally it was even more active than natural TCs (Table 1). The enhanced activity of rH-TC supposedly is caused by the positively charged histidines in the His-tag, as was indicated by the activity of the oligo-histidines. Histidines could be as important for antimicrobial activity of rH-TC as they are for histatins, a family of antimicrobial peptides rich in these amino acids (28). Apparently, the decrease in microbicidal activity which might have been expected due to the presence of the C-terminal Ala and Asp is compensated by the N-terminal His-tag. Addition of a His-tag might enhance activity of other microbicidal proteins, and should be considered when producing such proteins recombinantly.

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REFERENCES


