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Biological Relevance of Natural α-Toxin Fragments from *Staphylococcus aureus*

Young-Keun Kwak · Martin Högbom · Patricia Colque-Navarro · Roland Möllby · Beatrix Vécsey-Semjén

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Abstract Serine proteases represent an essential part of cellular homeostasis by generating biologically active peptides. In bacteria, proteolysis serves two different roles: a major housekeeping function and the destruction of foreign or target cell proteins, thereby promoting bacterial invasion. In the process, other virulence factors such as exotoxins become affected. In *Staphylococcus aureus* culture supernatant, the pore-forming α-toxin is cleaved by the coexpressed V8 protease and aureolysin. The oligomerizing and pore-forming abilities of five such spontaneously occurring N- and C-terminal α-toxin fragments were studied. ^{3}H-marked α-toxin fragments bound to rabbit erythrocyte membranes but only fragments with intact C termini, missing 8, 12 and 71 amino acids from their N-terminal, formed stable oligomers. All isolated fragments induced intoxication of mouse adrenocortical Y1 cells in vitro, though the nature of membrane damage for a fragment, degraded at its C terminus, remained obscure. Only one fragment, missing the first eight N-terminal amino acids, induced irreversible intoxication of Y1 cells in the same manner as the intact toxin. Four of the isolated fragments caused swelling, indicating altered channel formation. Fragments missing 12 and 71 amino acids from the N terminus occupied the same binding sites on Y1 cell membranes, though they inhibited membrane damage caused by intact toxin. In conclusion, N-terminal deletions up to 71 amino acids are tolerated, though the kinetics of channel formation and the channel's properties are altered. In contrast, digestion at the C terminus results in non-functional species.

Keywords α-Toxin fragment · V8 protease · Aureolysin · Limited proteolysis

Y.-K. Kwak (✉) · P. Colque-Navarro · R. Möllby
Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 171 77 Stockholm, Sweden
e-mail: Young-Keun.Kwak@ki.se

M. Högbom
Stockholm Center for Biomembrane Research, Department of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, 106 91 Stockholm, Sweden

B. Vécsey-Semjén
House of Science, Alba Nova University Center, Royal Institute of Technology, 106 91 Stockholm, Sweden

Introduction

The invasiveness of pathogenic bacteria from the primary infection site into the circulation and secondary infection sites in the host has long been associated with proteolysis. In staphylococcal infections, limited proteolysis of both host and bacterial proteins has been implicated, promoting bacterial spread (Arvidson 2000). *Staphylococcus aureus* is a multifactorial human pathogen (Mollby 1991) and one of the bacterial species neonates are first exposed to and colonized by (Mackie et al. 1999). In certain conditions, *S. aureus* can easily cause infections, from superficial skin acnes to severe postoperative wound infections and bacteremia. The bacteria's success largely depends on the production of an arsenal of virulence factors and the ability to adapt to changes in the environment and circumstances. Adaptation is based on quorum sensing. Bacterial colonization begins by the expression of attachment factors and advances by expression of factors involved in tissue degradation and invasion. In case of deep infections, high bacterial cell density leads to activation of the accessory gene regulator (agr) locus and expression of extracellular

toxins and enzymes that facilitate tissue degradation and ensure bacterial survival (Morfeldt et al. 1988). Furthermore, genes of exoproteins, including proteases and hemolysins, are upregulated during phagocytosis of *S. aureus* by the host cells (Voyich et al. 2005).

Successful host colonization leads to the simultaneous expression and activation of extracellular toxins and enzymes. In tissue degradation, the family of membrane-damaging toxins plays an essential role. Two subgroups in that family of toxins form oligomeric β-barrel pores in the plasma membrane: the single-component α-toxin and the bicomponent γ-toxins and leukocidines. That is, membrane-damaging toxins, so important for bacterial spread and survival, encounter coexpressed proteases at infectious focal points.

Though the pathology of *S. aureus* disease is highly variable, the production of α-toxin has always been considered as one of the most important factors and shown to play a major role in animal models (Bayer et al. 1997; Bhakdi and Tranumjensen 1991; Kernodle et al. 1997). In general, α-toxin is secreted by *S. aureus* in a fully active, monomeric form. The monomers bind to surface receptors (Hildebrand et al. 1991; Vijayvargia et al. 2004b) or to clustered phospholipid head groups in the cytoplasmic membrane of susceptible cells (Valeva et al. 2006), where they form oligomers. Oligomerization leads to membrane insertion and pore formation on the cell surfaces. The resulting heptameric transmembrane pores are comprised of three domains, the cap, rim and stem domains, each contributing one loop to form an antiparallel transmembrane barrel with an inner diameter of 1–2 nm (Gouaux et al. 1994; Jonas et al. 1994; Song et al. 1996; Walev et al. 1993). Insertion of α-toxin into the eukaryotic cell membrane and the formation of water-filled channels lead to disruption of the cellular chemiosmotic balance, release of intracellular contents and cell lysis. The primary target cells of α-toxin action are the circulatory cells. Different cell types react differently to α-toxin attack (Thelestam 1983), but the cellular factors and mechanisms responsible for sensitivity or resistance are largely unknown.

Recently, α-toxin-induced dephosphorylation of the EGF receptor in intact A431 cells was reported (Vijayvargia et al. 2004a). Pore formation has been shown to trigger secondary events such as endonuclease activation, increased platelet exocytosis and release of cytokines and inflammatory mediators (Bhakdi et al. 1996; Soderqvist and Hallberg 1994; Soderqvist et al. 1996).

Proteases such as trypsin, chymotrypsin and pronase attack α-toxin monomers primarily in the stem domain, which forms an exposed loop at about the middle of the monomer. The resulting N- and respective C-terminal halves of the toxin formed nonfunctional oligomers when cotranslated in vitro (Walker et al. 1993). In general, α-toxin fragments which are generated by in vitro mutagenesis show malfunction. When truncated at the N-terminus, α-toxin formed nonlytic oligomers, while the C-terminal truncation mutants remained as membrane-attached monomers (Walker et al. 1992a). The question of N-terminal truncated α-toxin molecules was readdressed by Jayasinghe et al. (2006), who, in contrast to previous publications, found that after truncation of up to 17 amino acids the ability of α-toxin to form functional pores was diminished but still substantial. This discrepancy was explained by a point mutation found in the first set of truncation mutants at position 217 (Jayasinghe et al. 2006).

In contrast, nicked α-toxin isolated from growth supernatant always retained some biological activity (Tomita et al. 1993). All of the aforementioned studies with α-toxin-truncated mutants were carried out on rabbit red blood cells (RRBCs) and, thus, did not give insight into the fragment's membrane-damaging ability and effect on eukaryotic cells. With this picture in mind, we set out to investigate the activity and significance of naturally occurring proteolytically degraded α-toxin molecules.

Materials and Methods

Growth of *S. aureus* Wood 46 and Preparation of Full-Length α-Toxin and Fragments from Culture Supernatants

S. aureus strain Wood 46 was cultured in Brain–Heart Infusion (BHI; GIBCO, Grand Island, NY) medium in a 5-liter bench-top fermentor. Cultures were inoculated with a 1:600 dilution of overnight culture, cultivated continuously to stationary phase (OD_{600} 2.0) and chilled quickly on ice. Bacteria were removed from culture media by centrifugation at 4°C. α-Toxin and fragments were purified from this clarified culture supernatant after $(NH_4)_2SO_4$ precipitation to 80% saturation. Precipitated proteins were collected by centrifugation and resolubilized in acetate buffer (10 mM sodium-acetate [pH 5.0] containing 20 mM NaCl). α-Toxin and fragments were separated on a cation exchanger column (Poros HS/M 4.6 mm D/100 mm L column; PerSeptive Biosystems, Cambridge, MA). Elution was carried out with a linear salt gradient (10 mM sodium-acetate [pH 5.0] containing 1 M NaCl) as previously described (Lind et al. 1987), including an isocratic step at 160 mM salt concentration for optimal separation. The purified toxin's activity on RRBCs was 60,000 hemolytic units (HUs)/mg protein. Hemolytic fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western-blotting. α-Toxin fragments were detected by monoclonal antibody Mab 12E (Blomqvist and Sjogren 1988). Fractions harboring α-toxin fragments were

concentrated (10,000 NMWL PLGC membrane units; Millipore, Bedford, MA) and after desalting (10 mM Na-acetate, 20 mM NaCl [pH 5.0]) separately reloaded to cation-exchange columns. Best separation was obtained on a Mono S 5/5 column (Pharmacia, Uppsala, Sweden) for fragments of 26 and 32 kDa, while fragments of 32.5 and 31 kDa were purified on a Poros HS/M column (PerSeptive Biosystems), using a linear salt gradient.

Separation of α-Toxin Fragments from a Nicked Molecule

Analysis of α-toxin fractions after isoelectric focusing (Thelestam et al. 1983) of *S. aureus* growth supernatant revealed a nicked molecule of 26- and 8-kDa fragments. The two fragments were separated by preparative electrophoresis in the model 491 Prep Cell (Bio-Rad, Richmond, CA). Samples of 3 ml (1 mg/ml in Laemmli buffer system, without β-mercaptoethanol) were loaded directly onto a 12.5% acrylamide gel column. Specific protein bands corresponding to the 8- and the 26-kDa bands were collected (Yamamoto and Munn 1986). α-Toxin fragments were concentrated and dialysed in TBS. The rest of SDS was adsorbed on Bio-Beads SM-2 (Bio-Rad) in several steps.

PAGE and N-Terminal Sequence Analysis

The sizes of the purified α-toxin fragments were estimated by comparison to standard molecular weight markers (Pharmacia) by 7.5–20% gradient SDS-PAGE. For N-terminal sequence determination, the protein bands were cut out from polyvinylidene fluoride (PVDF) membranes (Immobilon-Psq Transfer Membrane, Millipore) after 12% SDS-PAGE and stained with Coomassie brilliant blue as previously described (Matsudaira 1987). Sequence analysis was carried out on an Applied Biosystems (Foster City, CA) 477A Pulsed Liquid Phase sequencer with an on-line PTH 120A analyzer. The cycle programs were adapted to our reaction cartridges, and chemicals were purchased from the manufacturer. The initial yield was 47%, calculated from a sequenced standard protein β-lactoglobulin. Repetitive yield was 97–97.5%. As controls, the N-terminal sequence analysis of the 32.5- and 26-kDa fragments was repeated also from sulforhodamine B–stained membranes.

Limited Proteolysis of α-Toxin

α-Toxin was treated with two different staphylococcal exoenzymes after 10 min of preincubation at 37°C in different buffers (TBS, PBS and 145 mM NaCl buffered with 50 mM citric acid–disodium hydrogen phosphate), depending on the desired pH. α-Toxin, 1 mg, was cleaved either with 31.25 U of V8 protease (glutamyl endopeptidase; Sigma, St. Louis, MO) or with 440 U of aureolysin (Biocentrum, Krakow, Poland) in 1 ml final volume for different time intervals, ranging from 10 min to 6 h. Treatments with trypsin and pronase were included as controls. After incubation, samples were quickly cooled in ice, denatured in 4 × SDS sample buffer, heated to 96°C for 10 min and analyzed by electrophoresis in a 15% SDS-acrylamide gel. Bands were visualized by Coomassie brilliant blue staining.

Radiolabeling of α-Toxin and Fragments

Toxin and fragments were tritium-labeled with Bolton-Hunter reagent as previously described (Blomqvist et al. 1987). The labeled preparations' specific radioactivity ranged from 7 to 20 × 10^3 ct/min/mg protein. ^{3}H-α-toxin and fragments migrated in SDS-PAGE to the same position as the unlabeled samples.

Detection of Oligomers on RRBC Membranes

We mixed 0.5 ml of 10% RRBC suspension with 15 μg ^{3}H-α-toxin or ^{3}H-toxin fragment in an equal volume of TBS (pH 7.4) and incubated on ice for 15 min. Erythrocytes were pelleted by centrifugation and washed free of unbound radioactivity at 4°C. RRBCs with membrane-bound ^{3}H-α-toxin were resuspended in 1 ml TBS and incubated at 37°C for 45 min. For complete lysis, erythrocytes were treated with cold 5 mM phosphate buffer (pH 8) (Fairbanks et al. 1971). After centrifugation, the final membrane pellets were dissolved in 120 μl sample buffer (SB) and radioactivity of 10 μl was determined (Thelestam et al. 1991). For detection of ^{3}H-toxin-fragment oligomers, RRBCs were incubated for 14 h at 4°C and the membrane fractions were further incubated at 22°C for an extended period of 6 h. Oligomers formed by the intact toxin and respective fragment were separated from monomers by 7.5–20% gradient SDS-PAGE and detected by autoradiography.

Detection of Cytotoxicity and Irreversible Intoxication of Mouse Adrenocortical Y1 Cells

Mouse adrenal cortex tumor cells (FM Y1) were grown in Ham's medium (Flow Laboratories, Costa Mesa, CA) supplemented with 10% fetal bovine serum, 5 mM L-glutamine, penicillin and streptomycin (100 U/ml) in Costar (Cambridge, MA) six-well and 96-well plates. For determination of the α-toxin fragments' cytotoxicity, α-toxin or toxin fragment was diluted in two-step serial dilution in Ham's medium with a starting concentration of 20 μg/ml. FM Y1 cells were grown in 96-well plates to 80% confluence. Diluted toxin, 0.3 ml/well, was transferred to Y1 tumor cells and incubated at 37°C (Thelestam 1988). The rate of intoxication and cell death was

assessed by following morphological changes (detachment, rounding up) by light microscopy. Dead cells were counted as the number of rounded-up cells in one well and expressed as percentage of total number of cells/well determined after trypsinization and count in a Bürker chamber. Dead cells were counted more exactly after addition of trypan blue and expressed as a percentage of total cells in the Bürker chamber. Values are averages of duplicate samples in three independent experiments.

For determination of irreversible intoxication, FM Y1 cells were incubated with serially diluted toxin or fragment samples on ice. After 10 min, cells were washed toxin-free and further incubated in fresh medium at 37°C. Changes were observed at intervals as previously. As controls, untreated cells were used. All experiments were performed in duplicate and repeated at least thrice.

Assay of FM Y1 Cell Membrane Damage

Mouse adrenocortical FM Y1 cells were cultivated in HAM's F10 medium with 10% fetal calf serum (Flow Laboratories) in 24-well trays (Corning, Corning, NY) as described (Thelestam 1983). Near-confluent cultures of Y1 cells were treated with α-toxin (30 nM) or toxin fragments (32.5 kDa, 3 mM; 32 and 31 kDa, 30 nM; 26 kDa, 3 mM) diluted in TBS or acetate buffer (0.15 M Na-Cl, 10 mM Na-acetate [pH 5.0]) for 15 min on ice, rinsed three times before addition of toxin-free HAM's F10 medium and further incubated at 37°C (Blomqvist and Thelestam 1988). Nucleotide release was expressed as percentage of maximal release (Thelestam 1988). Maximal nucleotide release was measured after treatment with lysis buffer (40 mM Tris HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 1% SDS). Membrane damage was prevented by the neutralization of toxin by monoclonal antibodies: 1 ml α-toxin or fragment (3 μg/ml) was incubated with monoclonal antibody (Mab 12E) in TBS (1/200) for 30 min before transfer to FM Y1 cells (Blomqvist and Thelestam 1988).

Results

Purification and N-Terminal Sequence Analysis of α-Toxin Fragments

In the linear growth phase of cultivation of *S. aureus* Wood 46, α-toxin became degraded by simultaneously expressed staphylococcal proteases. The estimated concentration of the α-toxin monomer appeared to be constant, while the concentration of the degradation products increased progressively (data not shown). After 5 h of growth, approximately equal amounts of full-length toxin and α-toxin fragments ranging 32.5–8 kDa could be observed together with an increasing amount of degradation products, showing a continuous process. Isolated α-toxin fragments ranged in size from 32.5 to 8 kDa (Fig. 1a). However, although we were able to isolate an 8-kDa fragment from the bacterial culture supernatant, this fragment further degraded during storage.

N-terminal amino acid sequence analysis revealed that α-toxin was preferentially cleaved in the N-terminal latch (Fig. 1b). The 32-kDa fragment was determined to start at position 9-Y and the 31-kDa fragment at position 13-D. The 26-kDa fragment was the result of cleavage between amino acids E-71 and G-72.

Limited Proteolysis by Staphylococcal Exo-Enzymes

In order to confirm that the first appearance and relative concentration of fragments in the growth supernatant

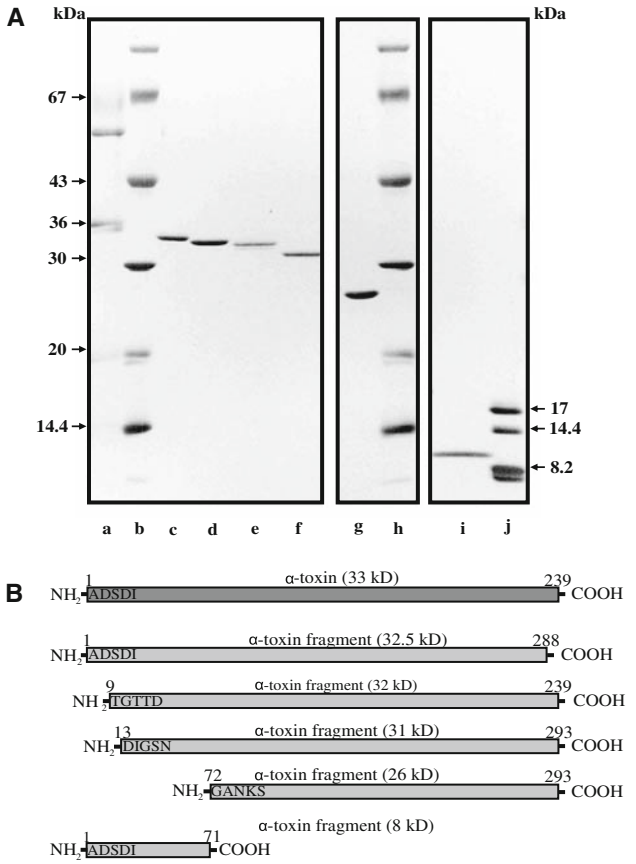

Fig. 1 **a** Size estimation of isolated α-toxin fragments on 7–20% gradient continuous SDS-PAGE. Samples of each purified fragment were mixed with SDS sample buffer and heated to 80°C for 5 min. *Lane a*, high–molecular weight marker (Pharmacia); *lanes b and h*, low–molecular weight marker (Pharmacia); *lane c*, full-length α-toxin; *lane d*, 32.5-kDa fragment; *lane e*, 32-kDa fragment; *lane f*, 31-kDa fragment; *lane g*, 26-kDa fragment; *lane i*, 8-kDa fragment; *lane j*, peptide marker proteins (Pharmacia). **b** Linearized size relationship of α-toxin and α-toxin fragments

indeed resulted from proteolysis by staphylococcal exo-enzymes, we analyzed the cleavage pattern of α-toxin after limited proteolysis with V8 enzyme and aureolysin, both secreted by *S. aureus*. Treatment with V8 protease and aureolysin produced several fragments (Fig. 2). As shown in Fig. 2, lane c, approximately eight or more fragments were produced by V8 treatment. The fragment of the estimated size of 26 kDa (VF26) ran to the same position as the naturally occurring 26-kDa fragment (Fig. 2, lane d). N-terminal sequence analyses of VF26 and the 26-kDa natural fragment were identical and confirmed a cleavage site between E-71 and G-72. The staphylococcal exo-enzyme aureolysin (Fig. 2, lane e) showed a similar cleavage pattern as the V8 protease but a lower efficiency of degradation. Trypsin treatment (Fig. 2, lane h), shown as control, gave a double band with molecular masses of 17 and 18 kDa. Weak bands below 20 kDa in the V8 enzyme and aureolysin cleavage pattern substantiate partial cleavage in the exposed central loop.

H^3-Labeled α-Toxin Fragments Bound to and Formed Homo-oligomers on RRBC Membranes

After toxin binding to RRBC membranes on ice, membranes were washed free of excess toxin or fragments and the temperature was elevated to 37°C to promote oligomerization and membrane insertion. Autoradiography of membrane-bound α-toxin and fragments revealed membrane-bound monomers but also high–molecular weight multimers. Full-length α-toxin formed toxin heptamers (Fig. 3a, lane 1). The 32.5-kDa fragment did not form multimers of any size, confirming the previous observation that C-terminal deletions led to inactive protein fragments (Jayasinghe et al. 2006; Walker et al. 1992b). A new band of about 60 kDa suggested dimers formed by the 32-kDa fragment (Fig. 3a, lane 5) as a possible intermediate for oligomer formation. Alternatively, as the 32-kDa fragment lost the first eight amino acids of its N-terminal latch, dissociation of the oligomers appeared reasonable. It was previously shown that oligomers were arrested in the pre-pore conformation on ice and could be dissociated by SDS at room temperature (Valeva et al. 1997). At low temperature the N terminus was still available to proteases and did not yet form the extensive contacts with its neighboring protomer as is the case in the final α-toxin pore. Surprisingly, the 26-kDa fragment formed SDS-stable oligomers of 10 and 12 subunits after 45 min incubation (Fig. 3a, lane 9). Previously, the same fragment was described as part of a nicked molecule with hemolytic properties (Tomita et al. 1993); thus, contamination by nicked molecules could have been the source of the observed oligomeric bands. Dissociation of the oligomers by heating the sample before gel electrophoresis did not reveal the presence of any minor band (Fig. 3a, lane 10), confirming that multimers were formed by the 26-kDa fragment and not by nicked molecules. However, we could not dismiss the presence of some such nicked α-toxin molecules in our sample, though in a concentration under our detection limit. To further address this question and obtain higher detection sensitivity, we chose Western blotting with polyclonal antibodies raised in rabbit (data not shown). Though bands of degradation products became visible in the α-toxin sample and weak but distinct bands appeared in a protein-free fraction, we could not detect any contamination in the 26-kDa fragment fraction (data not shown). In the next experiment, we extended both the binding and the oligomerization steps. After prolonged incubation (20 h), fragments with intact C termini formed oligomers (Fig. 3b, lanes 5, 7 and 9). After such a long incubation period, intact α-toxin formed not only heptamers but also large aggregates (Fig. 3b, lane 1). Also, we detected oligomers formed by varying numbers of subunits by the 32-kDa fragment, such as hexamers, heptamers and octamers (Fig. 3b, lane 5). We obtained only one oligomeric band by the 31-kDa fragment corresponding to a heptamer (Fig. 3b, lane 7), possibly due to low protein fragment concentration. The 26-kDa fragment formed decamers, with an indication of dodecamers (Fig. 3b, lane 9). The 8-kDa N-terminal part of α-toxin did not form oligomers, in line with our expectations, though it bound to RRBC membranes (Fig. 3b, lane 12). The two experiments show that purified α-toxin fragments were able to form homo-oligomers, though the kinetics of the oligomerization process seems to differ from that of the intact toxin. Although the radiograms did not yield quantitative data,

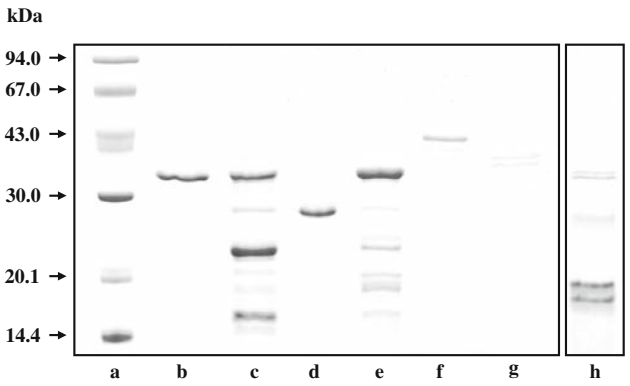

Fig. 2 Limited proteolysis of α-toxin. α-Toxin was treated with two different staphylococcal exo-enzymes: 1 mg of α-toxin cleaved either with 31.25 U/ml V8 protease or with 440 U/ml aureolysin for 2 h at 37°C. Treatment with trypsin was included as control. After incubation, samples were quickly cooled on ice, denatured in 4 × SDS sample buffer, heated to 96°C for 10 min and analyzed by electrophoresis in a 15% SDS-acrylamide gel. *Lanes a and h*, low–molecular weight marker (Pharmacia); *lane b*, α-toxin; *lane c*, V8 enzyme–treated α-toxin; *lane d*, 26-kDa fragment, *lane e*, aureolysin-treated α-toxin; *lane f*, V8 enzyme; *lane g*, aureolysin; *lane h*, trypsin-treated α-toxin

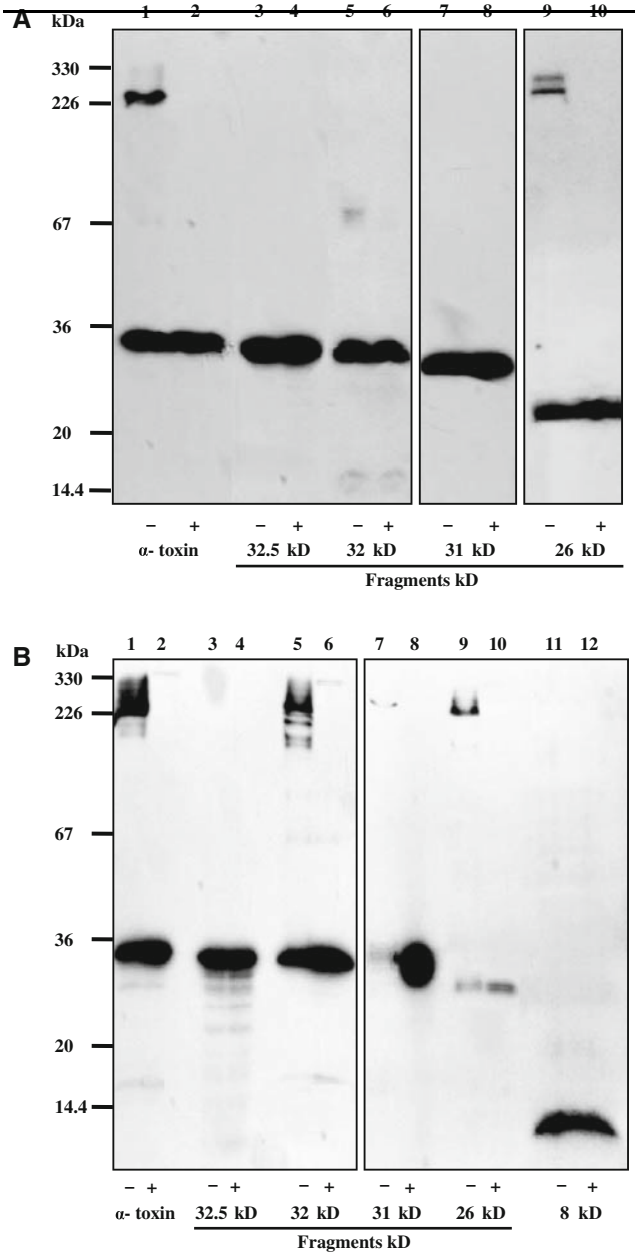

Fig. 3 Autoradiogram showing membrane binding and oligomerization of α-toxin and fragments on RRBC membranes. Ten percent of RRBCs in TBS (pH 7.4) were exposed to approximately 6 μg/ml final concentration of ^{3}H-α-toxin and respective fragments and incubated (**a**) on ice for 15 min (membrane-binding step), followed by washing and increase of temperature to 37°C (oligomerization step). Samples were further incubated in TBS for 45 min. After incubation, RRBCs were lysed in cold 5 mM phosphate buffer and the cell membranes washed and pelleted by centrifugation. The final membrane pellets were resuspended in sample buffer and the membrane proteins separated on continuous 7–20% SDS-PAGE, followed by autoradiography. Exposure time was 24 h. − *lanes*, two-thirds of the final sample was loaded without heating; + *lanes*, one-third of the sample was heated to 80°C for 5 min before loading. **b** The membrane binding step for α-toxin and fragments on RRBCs was extended to 14 h at 4°C, followed by washing and incubation in TBS for 6 h at room temperature (oligomerization step). Membrane proteins were separated and oligomers visualized as previously

they gave a good estimation of the membrane-bound and oligomerized fractions of α-toxin.

Intoxication of FM Y1 Cells

Intoxication was detected after continuous incubation with α-toxin and fragments in the growth medium at different time intervals. After the addition of intact α-toxin, changes in cell morphology were visible already after 20 min (data not shown). In contrast, only the 31- and 32-kDa fragments caused slight changes after 1 h incubation and at much higher concentrations (0.2 μg for α-toxin compared to 7 μg of fragments) (Fig. 4b). Not only was the toxic effect caused by the fragments strongly delayed but the morphological changes were also different. While the primary effect of both α-toxin and fragment led to the breaking up of cell–cell junctions of FM Y1 cells, treatment with α-toxin caused shrinking of cells (Fig. 4a [*b*]), as previously described (Thelestam 1983). Fragments of 31 kDa and 26 kDa caused swelling, rounding up and consequently detachment of cells from the plastic support (data not shown and Fig. 4a [*c*], respectively). After 6 h of incubation with 0.01 μg intact toxin/well, > 87% of FM Y1 cells were dead as determined by trypan blue count (Fig. 4b). Incubation for 24 h led to total cell death, at as low a concentration as 0.003 μg of toxin. A similar effect was obtained by 10 μg of 32-kDa fragment/well after 24 h of incubation. In the latter case, intoxication was delayed though (Fig. 4b). The 26-kDa fragment had much decreased toxicity as it needed 20 μg of fragment/well to cause visible intoxication (Fig. 4b) (19% dead cells, 24 h incubation). When the cells were reseeded and grown in toxin-free medium, the toxic effect was partially reversed. Irreversible intoxication of FM Y1 cells could be obtained only by intact α-toxin and the 32-kDa fragment.

To investigate whether the absence of intoxication was a consequence of the 31-kDa fragment's inability to bind to the cytoplasmic membrane or its abortive channel formation, FM Y1 cells were pretreated with 40 nM 31-kDa fragment for 15 min on ice, i.e., the same protein concentration as used for intact α-toxin. Thereafter, the cells were washed two times and reincubated with 40 nM of intact α-toxin. After 3 h incubation at 37°C, no changes were observed. The 31-kDa fragment inhibited cytoplasmic membrane damage by α-toxin.

Membrane Permeabilization and Nucleotide Release from FM Y1 Cells

Mouse adrenocortical (Y1) tumor cells were previously shown to be extremely sensitive and to become irreversibly

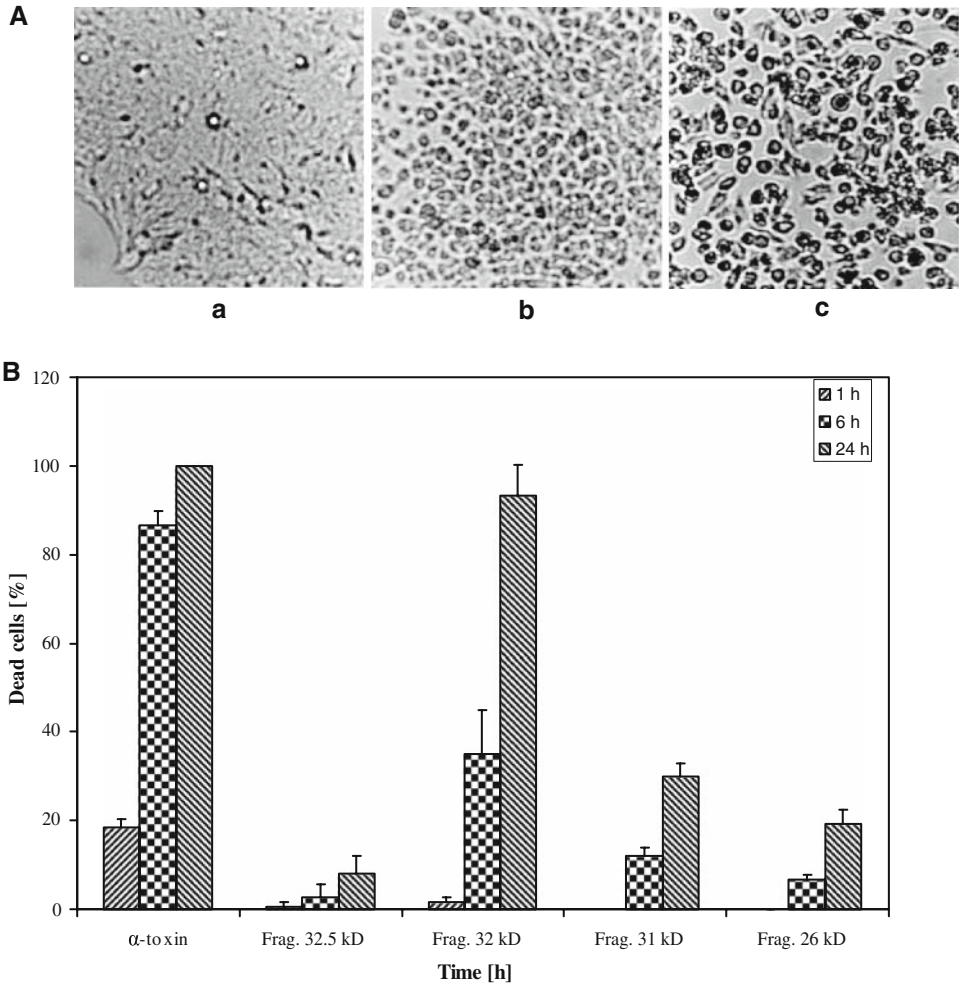

Fig. 4 Cytotoxic effect of α-toxin and fragments. **a** Irreversible intoxication of FM Y1 cells observed after treatment with full-length α-toxin (*a*) and 32-kDa fragment (*b*) on ice for 15 min, followed by washing and incubation in toxin-free medium. Irreversible intoxication was not observed after treatment with the 26-kDa fragment. After treatment with the 26-kDa fragment at 37°C, Y1 cells were rounded up and detached from the plastic (*c*). **b** Diagram showing intoxication of FM Y1 cells after continuous incubation for 1, 6 and 24 h with α-toxin or toxin fragments. Protein concentrations were 0.01 µg of α-toxin/well, 1 µg of 32-kDa fragment/well and 20 µg of 31- and 26-kDa fragments/well. Cytotoxicity was determined as the number of dead cells after trypsinization and trypan blue count. As an alternative, a field was marked out in the field of the light microscope and the number of rounded-up cells was determined and related to total cell counts. The number of dead cells was presented as a percentage of total cell count in a Bürker chamber

intoxicated by brief exposure to α-toxin. FM Y1 cells were prelabeled with the radioactive nucleotide probe (5-^{3}H) uridine. Nucleotide probes such as (5-^{3}H) uridine can easily pass through the lumen of the α-toxin pore and thereby provide a test for pore formation by the protein (Thelestam 1983). The pore formation of α-toxin fragments was measured as a linear function of the nucleotide release from prelabeled cells. Pore formation was in agreement with the fragments' membrane binding and oligomerizing capacity. Release of the radioactive nucleotide marker verified the C-terminal fragments' biological activity and the formation of transmembrane pores. FM Y1 cells showed dose-dependent membrane damage in response to the 32- and the 31-kDa fragments (data not shown), as previously observed for intact α-toxin (Thelestam 1983). Membrane damage had its maximum after 3 h of incubation at 37°C (48.5%), though the maximum, as a result of cell death, could be measured after 20 h (53.3%). The 32-kDa fragment–induced nucleotide release had the same kinetics as that of the intact toxin (Fig. 5) and caused nucleotide release in the same range (43.5%). In contrast, the nucleotide release induced by the 31- and 26-kDa fragments was very low (16.6% and 13.9% respectively) (Fig. 5). Furthermore, the nucleotide release induced by the 26-kDa fragment was independent of the applied concentrations (data not shown). These data suggest that the 26-kDa fragment binds to lipid bilayers but the oligomer-forming process and the following pore formation are much less efficient. The 31-kDa fragment effectively saturated specific binding sites when applied before α-toxin treatment (see above) but failed to form nucleotide-transmissible transmembrane channels with considerable efficiency. We could not obtain any nucleotide release by the 32.5-kDa fragment (0.36%). Thus, this fragment had no membrane-damaging effect on FM Y1 cells. There was complete inhibition of cell membrane damage when α-toxin or any of the fragments was mixed with rabbit polyclonal antibodies prior to incubation (Fig. 5).

Discussion

We investigated the effects of proteolysis of α-toxin by coexpressed proteases, such as aureolysin and V8 protease,

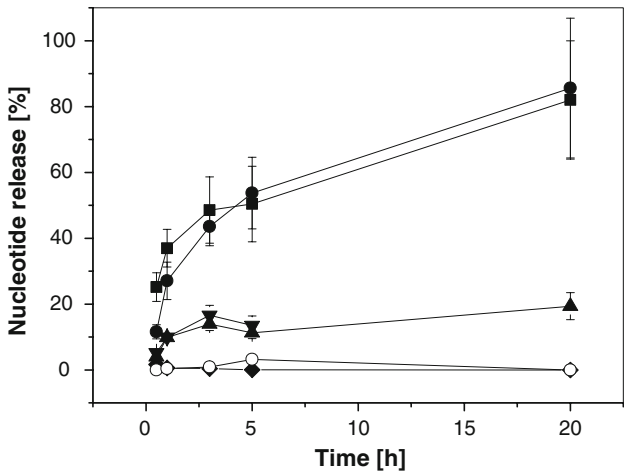

Fig. 5 Time course of membrane damage on sensitive mouse adrenocortical Y1 cells. Y1 cells were grown to near confluence and labeled with [^{3}H] uridine. Cells were transferred to ice and incubated with α-toxin (64 HU, 30 nM) and toxin fragments (32.5 kDa, 3 mM; 32 and 31 kDa, 30 nM; 26 kDa, 3 mM) for 15 min. After incubation, cells were washed free of toxin and further incubated in Ham's medium at 37°C. Plasma membrane damage was measured as a release of the radioactive cytoplasmic nucleotide marker. The measured nucleotide release was expressed as the percentage of maximal release after the addition of SDS-containing lysis buffer. Nucleotide release was measured by liquid scintillation from FM Y1 cells. Membrane damage was inhibited by preincubation of toxin and toxin fragments with (*open circle*), C-terminal-specific monoclonal antibodies E12; (*filled square*) α-toxin; (*filled circle*) 32-kDa fragment; (*filled inverted triangle*) 31-kDa fragment; (*filled triangle*) 26-kDa fragment; (*filled diamond*) 32.5-kDa fragment

during cultivation of *S. aureus* Wood 46. Cleavage took place in the monomer at exposed sites, such as the N-terminal latch after amino acids K8 and T12, in the connecting loop between β-strands 4 and 5 forming the outer β-sheets after E71 and at the C-terminal end, as we suppose is the case in vivo. Compared to our previous results with pronase (VecseySemjen et al. 1997), cleavage with V8 enzyme and aureolysin resulted mainly in fewer small fragment species. Fragments of 21 and 15 kDa were represented at the highest concentrations. Judged by α-toxin, primary sequence cleavage took place at position D92 or E94, possibly both, or alternatively at D208 and/or D212, where two aspartate residues are situated close to each other (Fig. 2). Weak bands of approximately 20 and 18 kDa suggest cleavage in the central glycine-rich loop, which represents the main cleavage site of the toxin monomer. This central loop has been shown to be sensitive to trypsin, pronase and proteinase K attacks and to V8 enzyme (Palmer et al. 1993; Tobkes et al. 1985). Although the aforementioned fragments were present after 1 h digestion with staphylococcal exo-enzymes at much higher concentrations than the purified fragments of 26, 31, 32 and 32.5 kDa, we were not able to separate any of them.

Staphylococcal enzymes such as aureolysin and V8 protease, staphylokinase, cysteine protease, serine protease, aminopeptidase, lipase and Xaa-Pro dipeptidase are virulence factors that promote invasion and spread to deeper tissues in the host (Burlak et al. 2007; Imamura et al. 2005; Karlsson et al. 2001; Massimi et al. 2002; McGavin et al. 1997). Protein degradation would counteract the promotion of bacterial spread if the coexpressed proteins and peptides would also become degraded and inactivated. Indeed, the N-terminus of α-toxin was thought to be essential in the formation of the transmembrane pores (Valeva et al. 2001). In truncation mutagenesis experiments loss of the N-terminal latch led to rapid inactivation as a result of premature oligomerization (Jayasinghe et al. 2006; Valeva et al. 1997; Walker et al. 1992a), while fragments with an inflicted nick or the coexpressed fragments in *Escherichia coli* retained their pore-forming ability (Palmer et al. 1993; Tomita et al. 1993; Walker et al. 1993). Previously, two of the separated fragments of 26 and 8 kDa in our study were reported as parts of a nicked molecule with slightly lower hemolytic activity than the intact toxin (Tomita et al. 1993). In neutral milieu, α-toxin retained its biological activity even after infliction of a nick, while in acidic milieu the compactness of the structure loosened and the smaller, N-terminal part went through further degradation, resulting in one 26-kDa fragment. This might be the case in phagocytosis of *S. aureus*. Indeed, α-toxin was shown to be essential for intracellular survival (Vann and Proctor 1988), and its production was triggered by phagocytosis in human neutrophils (Burlak et al. 2007). Numerous studies have reported individual *S. aureus* exoproteins that promote pathogenesis, including proteases (Imamura et al. 2005; Karlsson et al. 2001; McGavin et al. 1997) as well as leukotoxins and hemolysins (Kaneko and Kamio 2004).

Erythrocytes, but also nucleated cells, in the circulation have been known to be α-toxin-sensitive (Fussle et al. 1981; Jonas et al. 1994; Valeva et al. 1997). In contrast, most of the known, commercially available cell lines in culture show a surprising resistance against α-toxin action despite the fact that α-toxin was lethal in animal experiments. In this context, mouse FM Y1 cells represent an exception as they are known to be sensitive to very low toxin concentrations and cannot recover after α-toxin attack (Thelestam 1983). However, it is not known whether irreversible permeabilization of FM Y1 cells might be a result of mutations in specific binding sites or of deviant lipid composition (Pany et al. 2004; Valeva et al. 2006) or by other mechanisms such as the dysfunction of a cytosolic Ca^{2+}-related signaling pathway (Hocke et al. 2006). The isolated fragments induced irreversible intoxication and nucleotide release from prelabeled cells in a size-dependent manner, where the 32-kDa fragment was most effective. The loss of the first eight amino acids from the N-terminal latch had little influence on the 32-kDa fragment's membrane-binding and channel-forming abilities; thus, the fragment was

able to irreversibly intoxicate FM Y1 cells. Hence, the destabilized oligomers seem to be a consequence of their instability under denaturing conditions (SDS) rather than a malfunction of the oligomerization process itself (Valeva et al. 1997). Surprisingly, further degradation by only four more residues at the N terminus effectively abolished both irreversible intoxication and induction of nucleotide release from FM Y1 cells. Despite the fact that the 31-kDa fragment induced only negligible nucleotide release, it was found to bind to and saturate the same binding sites as α-toxin and, thus, prevented intoxication of FM Y1 cell. Consequently, the lack of irreversible intoxication by the 31-kDa fragment might be due to malfunction in channel formation or the instability and collapse of some part of the transmembrane channel. Indeed, oligomers of the 31-kDa fragment had further reduced SDS stability compared to the 32-kDa fragment, probably due to loss of the amino latch. Surprisingly, the 26-kDa fragment formed SDS-stable multimers of a varying number of subunits participating in the complexes. Oligomers of various sizes suggest variations in conformation and diameter of transmembrane pores, where only the larger ones would be permissible to nucleotides. Treatment of FM Y1 cells with the 26-kDa fragment resulted in rounded-up cells, detached from the surface of the culture dish (Fig. 4a [*c*]). The transmembrane channels were probably permissible only for smaller particles such as anions as the 26-kDa fragment caused only minor nucleotide release from FM Y1 cells. Indeed, in previous experiments Ca^{2+} influx activated signaling pathways and initiated cytoskeletal reorganization as a consequence of α-toxin treatment (Hocke et al. 2006; Menestrina et al. 1990; Suttorp et al. 1985).

The 32.5-kDa fragment, missing its C-terminal tail, bound to erythrocyte membranes; but neither formed oligomers or nucleotide-permissible transmembrane channels. This result is in accordance with previous studies where deletion of three, five or eight amino acids at the C-terminal end of α-toxin led to impaired oligomerization and pore formation (Walker et al. 1992a). In contrast to the N-terminus, the C-terminal end of α-toxin is more exposed in the oligomeric conformation and in the final transmembrane channel than in the monomer (Krishnasastry et al. 1994; Walker and Bayley 1995). In the absence of a C-terminus, the α-toxin monomer adopts a molten globule-like structure with exposed hydrophobic patches, leading to premature oligomerization and aggregation. Consequently, an intact C-terminus is necessary for stabilization of the native monomeric structure of α-toxin (Sangha et al. 1999).

The present results on oligomerization and pore-forming properties of the various proteolytic fragments of α-toxin are interesting from a structural point of view, both in relation to the transition between the monomeric and multimeric states as well as in the structural properties of the formed oligomers. The soluble α-toxin monomers are susceptible to protease cleavage in both the N-terminal latch as well as the glycine-rich region that forms the distal part of the membrane pore. Interestingly, these parts become protease-resistant after heptamerization of the full-length protein (Walker et al. 1992a). The crystal structure of the heptameric transmembrane pore (Song et al. 1996) shows that both the N- and C-terminal of the protein are completely ordered with low B factors and extensive interactions with the rest of the protein. The structure of the heptameric protein, monomeric structures of homologous proteins (Guillet et al. 2004; Pedelacq et al. 1999) and the proteolytic data on the monomeric and heptameric protein suggest structural rearrangements, mainly involving further ordering, in several regions upon oligomerization. The fact that the 32-kDa fragment forms multimers while the 31-kDa fragment's ability to do so is significantly impaired suggests that residues 9–12 are important for multimer formation (Valeva et al. 2001). These residues do interact with the adjacent monomer in the cap domain and are involved in the structural rearrangement in the heptamer. It seems most likely that this effect is mainly exerted in the early stages of multimerization rather than in destabilizing the formed multimer (Jayasinghe et al. 2006; Valeva et al. 2001). In this context, it is interesting to note that the 26-kDa fragment, lacking residues 1–71, is more prone to multimer formation than the 31-kDa fragment (lacking only residues 1–12). Residues 21–62 form three strands of a four-stranded antiparallel β-sheet that contributes the major interaction surface between the cap domains and includes several residues known to affect heptamer formation and activity (Walker and Bayley 1995). The increased potential for multimer formation upon removal of this large segment strongly suggests that the protomer interactions in the cap domain of the 26-kDa fragment multimers are fundamentally different from those in the full-length heptamers. It also seems most probable that this is accompanied by differences in the mechanism of multimer assembly and preference for various multimeric states.

The fact that severely truncated variants of α-toxin, unable to form the native protomer interactions of the full-length protein heptamer, still form pores and induce cell intoxication suggests an evolved biological role for these. Together, it seems possible that the function of the co-expressed proteases in part could be to modulate the properties of α-toxin by producing truncated variants with different pore-forming properties and permeabilities of the formed pores.

Although the clinical relevance of our finding is unclear, it is evident that the biological effects of bacterial protein toxins, such as α-toxin, in vivo may be due both to intact and to partially degraded molecules. Understanding the biological relevance of naturally occurring α-toxin

fragments and the possibility to create toxoid vaccines may be an important asset in the present fight against threatening methicillin-resistant *S. aureus* (MRSA) and recently observed vancomycin-resistant *S. aureus* (VRSA) strains.

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