Amoebapores and NK-lysin, members of a class of structurally distinct antimicrobial and cytolytic peptides from protozoa and mammals: a comparative functional analysis

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Amoebapores, the pore-forming polypeptides of the protozoan parasite *Entamoeba histolytica*, and NK-lysin, an effector molecule of porcine NK (natural killer) and cytotoxic T cells, belong to the same protein family, the saposin-like proteins. As both types of protein are implicated in the killing of microbes *in vivo*, it appears that phylogenetically diverse organisms such as amoebae and mammals use similar effector molecules to fulfil a comparable task. However, structural features have led to the assumption that the proteins display their activities according to different modes of action. To address this question, we analysed the antibacterial, cytotoxic and pore-forming activities of these proteins in parallel and in comprehensive detail. Interestingly, the comparison of activities revealed significant differences. Whereas NK-lysin, recombinantly expressed, is efficient at a broad range of

pH values, the amoebapores exhibited a pronounced pH dependence of all their activities, with markedly decreased activity at pH values above 6. Moreover, increasing salinity affects amoebapores more drastically than NK-lysin. All of the proteins compared were found to be potently active against Gram-positive bacteria, but only NK-lysin was equally efficient against Gram-negative bacteria. However, the amoebapores displayed five times higher pore-forming activity than NK-lysin, which is in accordance with the more hydrophobic character of the amoebapores compared with the essentially cationic NK-lysin.

Key words: amoebapore, antimicrobial, cytolytic, NK-lysin, pore-forming peptide, protozoan parasite.

INTRODUCTION

Antimicrobial peptides are widespread in Nature, and are implicated in the internal defence of all species of plants and animals [1–3]. To date, more than 750 different antimicrobial peptides are known, which consist typically of 10–40 amino acid residues (see <http://www.bbcm.univ.trieste.it/~tossi/pag1.html>). Traditionally, these peptides are divided into different classes according to their structural features. The first class comprises mainly linear, helical and amphipathic peptides without disulphide bonds. Well known representatives are the cecropins of the silk moth *Hyalophora cecropia* [4]. Linear peptides with an over-representation of a single amino acid, such as histatin 5, a peptide from human saliva [5], belong to the second class. The third class comprises peptides with loop structures connected by one disulphide bridge, e.g. the brevinins from frog skin [6]. Peptides with two or more disulphide bonds are combined in the fourth class. These peptides usually possess a defined β -sheet structure, as found in the well characterized mammalian defensins [7,8].

Recently, with the discovery of porcine NK-lysin and human granulysin, an additional class of antimicrobial polypeptides has been recognized in NK (natural killer) and T cells [9,10]. Compared with the classical antimicrobial peptides, NK-lysin and granulysin are extraordinary, in that they can be considered as small proteins rather than peptides, and, in accordance with their size of 78 and 74 residues respectively, they possess a globular three-dimensional structure. Interestingly, the archetype of this

novel class of defensive weapons exists in amoebae: the pore-forming proteins of *Entamoeba histolytica*, termed amoebapores [11].

From an evolutionary point of view, successful molecules – i.e. those best suited for their function – are conserved between a wide range of species. Nevertheless, the fact that the highly specialized and complex immune system of mammals and a primitive phagocyte such as an amoeba use similar molecules to fulfil a similar (namely antimicrobial) function appears remarkable. The amoebapores may be viewed as the phylogenetically most ancient embodiment of membrane-disruptive polypeptides that are used as tools of innate immunity in higher eukaryotes. The amoebapores as well as NK-lysin and granulysin possess membrane-perturbing and antibacterial activity, which in mammals permits defence against invading microbes [9,12,13] and which the amoeba may use to kill phagocytosed bacteria [14]. Moreover, in addition to displaying similar biological activities, the amoebapores and NK-lysin and granulysin belong to the same protein family, the SAPLIPs (saposin-like proteins) [15].

The amoebapores exist in three isoforms, all with a length of 77 residues. They were identified on the basis of their pore-forming activity, molecularly cloned and characterized [16–19]. As *Entamoeba histolytica* is the causative agent of human amoebiasis, the amoebapores have been studied primarily as a putative pathogenicity factor in view of their cytolytic activity *in vitro* [20,21]. By using antisense RNA technology, their impact on the pathogenicity of the amoeba has been confirmed *in vitro* and in a hamster model *in vivo* [22].

Abbreviations used: BCECF AM, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; LB medium, Luria–Bertani medium; MALDI-TOF, matrix-assisted laser-desorption/ionization–time-of-flight; MIC, minimal inhibitory concentration; NK cells, natural killer cells; rNK-lysin, recombinant NK-lysin; SAPLIP, saposin-like protein; TFA, trifluoroacetic acid.

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With NK-lysin, the first structure of a member of the SAPLIP family was solved in 1997, revealing the common fold of five helices connected by three disulphide bonds [23]. The only exception is the human homologue granulysin, which is post-translationally processed at the C-terminus, thereby losing one cysteine residue, and its structure is therefore stabilized by two disulphide bonds only [10]. Granulysin has been reported to act in concert with perforin against intracellular pathogens [12]. Both of the mammalian proteins exhibit cytotoxic activity against human cells [9,10].

In spite of their similar functions, the amoebapores, NK-lysin and granulysin show only limited sequence identity. Beyond the SAPLIP-specific cysteine array, which builds the backbone for these membrane-interactive proteins [15], there is no shared motif that may account for their specific function. A detailed analysis of the known structure of NK-lysin and models derived from it for the amoebapores and granulysin revealed significant structural differences in spite of their common fold [24]. Both mammalian proteins show extended electropositive regions on their surface. Strikingly, the overall neutral amoebapores possess a loosely packed core which may facilitate conformational changes. Based on these structural features, different modes of action have been postulated [24,25]. The present study is directed to see how these structural differences are reflected in their activities. We performed a detailed comparison of the activities of the amoebapores with those of recombinantly expressed NK-lysin (rNK-lysin), as a representative of the mammalian effector molecules, under identical conditions.

EXPERIMENTAL

Cloning and recombinant expression of NK-lysin

For cloning of NK-lysin (GenBank accession no. X85431), porcine peripheral blood mononuclear cells were isolated by using a Ficoll gradient and subsequently cultivated in RPMI 1640 medium supplemented with 5 % (v/v) fetal bovine serum at a cell concentration of 3×10^6 /ml. For stimulation, cells were treated with interleukin-2 (20 units/ml; Chiron, Ratingen, Germany) after 48 h, and again after 7 days using half the interleukin-2 concentration. After 17 days, total RNA was purified by the use of TRIZOL (Invitrogen) according to the supplier's protocol. The RNA was reverse-transcribed by Superscript II (Invitrogen) and the resulting cDNA was taken as a template for PCR with two oligonucleotides specific for the sequence coding for the mature protein containing the additional restriction sites for *NdeI* and *HindIII* in the sense and antisense primers respectively (sense, 5' GATATACATATGGGCTCATCTGTGAGTCTTG; antisense, 5' GCTTGCAAGCTTTCAGATGAGACCTGTCTTCT). The fragment was cloned into the *NdeI* and *HindIII* sites of PET28a (Novagen) and the sequence was verified by DNA sequencing.

Expression was performed using transformed *Escherichia coli* BL21(DE3) in a 2-litre fed-batch fermentation starting with LB (Luria-Bertani) medium supplemented with 2 % (w/v) glucose, 40 mM potassium phosphate, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 35 μ g/ml kanamycin. Upon reaching a D_{600} of 2.5, the pH was kept at 7.0 using ammonium hydroxide; for every 1 g of ammonia, 10 g of glucose was added. At a D_{600} of 20, expression was induced with 2 mM isopropyl β -D-thiogalactopyranoside for 2 h. The cells were harvested, resuspended to a cell suspension of 30 % (w/v) in 20 mM Tris/150 mM NaCl, pH 7.4, and mechanically disrupted by grinding with glass beads at 4 °C for 10 min. The insoluble material was sedimented at 10 000 g for 20 min and washed twice with 20 mM Tris/150 mM NaCl, pH 7.4. The inclusion bodies within the pellet were solubilized in 6 M guanidine

hydrochloride/50 mM Tris, pH 7.8, at 20 °C for 30 min under constant shaking.

For purification, Ni^{2+} -ion affinity chromatography was performed on a nitrilotriacetic acid–Sepharose matrix under denaturing conditions according to the supplier's instructions (Qiagen). Briefly, the supernatant of a 10 000 g centrifugation step was applied on a Ni^{2+} -loaded 5 ml column equilibrated with 6 M guanidine hydrochloride/50 mM Tris, pH 7.8. The column was washed with 40 mM imidazole and bound proteins were eluted with 500 mM imidazole in the same buffer. For refolding, the eluate was dialysed in two steps against 3 M and 1 M guanidine hydrochloride in 20 mM Tris, pH 7.8. Subsequently, 10 mM GSSG, 1 mM GSH and 50 mM KCl were added and the sample was incubated at 4 °C for 16 h. Afterwards, the protein was dialysed first against 20 mM acetic acid, pH 3.0, and secondly against PBS. For cleavage, thrombin protease (20 units; Amersham Pharmacia) was added and the sample was incubated at 4 °C for 16 h. The cleaved rNK-lysin possesses the additional amino acid residues Gly-Ser-Met-His at the N-terminus. For further purification, cation exchange chromatography was performed on a 1 ml Resource S column (Amersham, Pharmacia) in 50 mM sodium acetate, pH 4.5, using a gradient from 0 to 1 M NaCl. rNK-lysin was eluted at 550 mM NaCl and its homogeneity was verified by Tricine-SDS/PAGE [26]. For buffer exchange, the protein was applied to a 0.5 g C_{18} Sep-Pak cartridge (Waters) equilibrated with 50 mM sodium acetate, pH 4.5, and eluted with 80 % (v/v) acetonitrile. The eluate was lyophilized and redissolved in 0.01 % TFA (trifluoroacetic acid) at a concentration of 1 mg/ml to serve as a stock solution for all tests performed. The protein concentration was determined photometrically using a molar absorption coefficient of $254\,811\text{ mol}^{-1} \cdot \text{cm}^{-1}$ at 214 nm [27].

Structural characterization of rNK-lysin

The identity and homogeneity of purified rNK-lysin were validated by N-terminal sequencing and mass determination. Mass determination was performed using a Bruker RELEX MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS instrument, as described previously [28].

For analysis of free thiol groups, 1 nmol of rNK-lysin was incubated with 15 μ mol of 5,5'-dithionitrobenzoic acid in PBS or PBS including 6 M guanidine hydrochloride. Absorbance was measured at 412 nm, and the molar concentration of thiols present was calculated using a molar absorption coefficient of $13\,700\text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in the presence of guanidine hydrochloride and of $14\,150\text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ in its absence [29].

CD was performed using a Jasco J-720 spectrometer and quartz cells of 0.1 cm path length. Spectra from 190 to 250 nm wavelength were measured for a solution of 20 μ M rNK-lysin in 50 mM sodium phosphate, pH 6.0. Protein secondary structure content was analysed using the program CONTIN [30].

Purification of the amoebapores

The amoebapores were purified according to a procedure described previously [18,31]. Briefly, frozen trophozoites of the amoebic strain HM-1:IMSS were extracted twice in 10 vol. of 1 M HCl, 5 % (v/v) formic acid, 1 % (v/v) TFA and 1 % (w/v) NaCl at 4 °C overnight. After centrifugation at 150 000 g for 1 h, the supernatants were applied on a C_{18} 12 cc (2 g) Sep-Pak cartridge (Waters), the column was washed with 0.1 % (v/v) TFA/35 % (v/v) acetonitrile, and bound material was eluted with 0.1 % TFA/80 % acetonitrile. The eluate was lyophilized, redissolved in 0.1 % TFA and subjected to reverse-phase

HPLC using an Aquapore Butyl 300 column (2.1 mm $\times$ 200 mm; Brownlee Labs). The polypeptides were eluted using a non-linear gradient of increasing concentrations of acetonitrile in 0.1 % TFA. The eluted fractions were tested for pore-forming activity as described below. Active fractions were rechromatographed using the same column and a linear gradient of 0–70 % acetonitrile in 0.1 % TFA. The homogeneity of purified amoebapores A, B and C, which were eluted in this order between 55 % and 70 % acetonitrile, has been verified by N-terminal sequencing and MS [18], and was routinely confirmed by analytical reverse-phase HPLC and Tricine-SDS/PAGE. The pure proteins were lyophilized repeatedly, twice redissolved in 10 mM HCl and finally dissolved in 0.01 % TFA. For the preparation of a mixture of amoebapores A, B and C, all amoebapore fractions eluted from the first HPLC column were collected into a single tube instead of separating them. This mixture should consist of the natural amounts of amoebapores A, B and C found in the amoebic granules [18]. The protein concentration was determined photometrically at 214 nm using the molar absorption coefficients calculated from the sequence information [27].

Antibacterial activity

The MIC (minimal inhibitory concentration) against bacterial growth was determined by a microdilution susceptibility test. *Bacillus subtilis* (strain 60015) [19] and *E. coli* K12 (A.T.C.C. 23716) were grown in LB medium (1 % tryptone, 0.5 % NaCl, 0.5 % yeast extract, pH 7.0) overnight and subsequently inoculated in the same medium to reach the mid-exponential phase. A bacterial suspension (25 μ l; 1×10^2 bacteria) was added to 2-fold serial dilutions of peptides in 10 mM Mes, pH 5.2 (25 μ l), and incubated for 16 h at 37 °C. The MIC was defined as the lowest concentration of peptide at which the bacteria did not exhibit visible growth.

Lesions of bacterial membranes were determined by monitoring the fluorescence of the DNA-binding dye SYTOX Green (Molecular Probes). *Bacillus subtilis* (strain 60015 [19]) and *E. coli* F515 (Research Center Borstel, Germany) were grown in LB medium overnight and subsequently inoculated in the same medium to reach the mid-exponential phase. Bacteria were washed twice with 10 mM Mes/25 mM NaCl, pH 5.5, or 10 mM Hepes/25 mM NaCl, pH 7.4, and were resuspended in the same buffer containing 2 μ M SYTOX Green. Microtitre plates were precoated with 0.1 % BSA (Sigma-Aldrich) at 20 °C for 10 min. The bacterial suspension (50 μ l; 1×10^5 *B. subtilis* or 1×10^6 *E. coli* F515) was added to 2-fold serial dilutions of the proteins in 20 mM Mes/25 mM NaCl, pH 5.5 (50 μ l), or 10 mM Hepes/25 mM NaCl, pH 7.4 (50 μ l), respectively, and incubated at 37 °C for 2 h. The buffer used for the protein dilutions was supplemented with the amount of salts needed to reach the final concentration indicated (i.e. 137.5, 287.5 or 587.5 mM NaCl, or 4 mM CaCl_2 or MgCl_2). The fluorescence was determined in a microtitre plate fluorescence spectrophotometer (Fluoroskan II; Merlin) using an excitation wavelength of 495 nm and an emission wavelength of 538 nm. The fluorescence intensity difference between bacteria treated with and without 1 μ M melittin under the same conditions was taken as the value for 100 % permeabilization of bacteria. When data for melittin are included, 100 % permeabilized bacteria was achieved by treatment with 70 % (v/v) ethanol at 20 °C for 10 min.

The kinetics of the permeabilization of the inner and outer bacterial membranes were determined by monitoring the activity of the intracellular galactosidase and the periplasmic β -lactamase in permease-deficient *E. coli* ML-35p in parallel [32]. *E. coli* ML-35p were grown in LB medium supplemented with

100 μ g/ml ampicillin overnight, washed twice with 20 mM Mes/25 mM NaCl, pH 5.5, and resuspended in the same buffer. To start the reaction, a solution containing the proteins and the substrate dyes CCF2/FA (Aurora Biosciences) or 6,8-difluoro-4-methylumbelliferyl β -D-galactopyranoside (Molecular Probes) respectively in buffer (75 μ l) were added to 25 μ l of the bacterial suspension with 1×10^7 cells in black flat-bottomed 96-well plates (Costar, Corning). The final concentrations were 1 μ M for the proteins and 10 μ M for the dyes. The time-dependent increase in fluorescence was measured in a microtitre plate fluorescence spectrophotometer (Fluoroskan II; Merlin) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for both reactions. The fluorescence difference between bacteria treated with 1 μ M polymyxin B, 0.04 % Triton X-100 and 80 μ M EDTA in buffer and bacteria incubated with buffer alone under the same conditions was taken as the value for 100 % permeabilization of the respective membrane.

Cytotoxic and cytolytic activities

The influence of the proteins on the viability of human cells was determined by monitoring the increase in the fluorescence of alamarBlue (BioSource) caused by metabolically active cells only. Jurkat cells were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10 % (v/v) fetal bovine serum (PAA Laboratories) at 37 °C and 5 % CO_2 , washed twice in 20 mM Mes, pH 5.5, 150 mM NaCl and resuspended in the same buffer. Then 5×10^5 cells (40 μ l) were added to 2-fold serial dilutions of the proteins in the same buffer (50 μ l) in a precoated microtitre plate [0.1 % BSA (Sigma-Aldrich) at 20 °C for 10 min] and incubated at 37 °C and 5 % CO_2 . After 30 min, 10 μ l of AlamarBlue (final concentration 10 %) was added and the cells were incubated for another 30 min. The increase in fluorescence was measured in Fluoroskan II microtitre plate fluorescence spectrophotometer (Merlin) using an excitation wavelength of 538 nm and an emission wavelength of 590 nm. The fluorescence difference between Jurkat cells treated with 0.1 % Triton X-100 and cells treated with buffer only under the same conditions was taken as the value for 100 % cytotoxic activity.

Permeabilization of the cell membrane was monitored by the release of the fluorescent dye BCECF AM [2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester] (Calbiochem) from dye-loaded Jurkat cells [21]. Jurkat cells were sedimented at 400 g and resuspended in 20 mM Mes (pH 5.5)/150 mM NaCl or PBS, pH 7.4, resuspended in the same buffer containing 10 μ M BCECF, labelled at 37 °C for 30 min, washed twice and resuspended in the same buffer without dye. For activity determination at different pH values, the buffers employed were 20 mM Mes/150 mM NaCl or 20 mM phosphate/150 mM NaCl, according to their buffering capacity. Cells (1×10^5) were incubated with various amounts of the proteins in the respective buffer (final volume 200 μ l) for 1 h. For the measurement of the pH-dependence of the amoebapores, a 5 μ M mixture of amoebapores A, B and C (approx. 35:10:1) was used [18]. After sedimentation at 400 g, the fluorescence of the supernatant was determined in a Fluoroskan II microtitre plate fluorescence spectrophotometer using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The fluorescence difference between supernatants of cells incubated with 0.1 % Triton X-100 and with buffer only was taken as 100 % lysis.

Pore-forming activity

Pore-forming activity was determined by monitoring the dissipation of a valinomycin-induced diffusion potential in

liposomes, as described previously [16]. Briefly, liposomes prepared from azolectin (40 mg/ml) in 50 mM K₂SO₄, 0.5 mM EDTA and 50 mM Tris maleate, pH 5.2, were diluted 1:4000 in a similar buffer containing Na⁺ instead of K⁺ and adjusted to pH 5.2 or 7.4. Addition of 1 nM valinomycin induced a diffusion potential that was monitored by the fluorescence quenching of 3,3'-diethylthiodicarbocyanine iodide dye (1 µM; Kodak) using a fluorescence spectrophotometer (LS50B; PerkinElmer) with excitation and emission wavelengths of 620 nm and 670 nm respectively. Pore-forming activity was measured as the initial change in fluorescence over time after the addition of sample. One unit is defined as a fluorescence increase to 5 % of the pre-valinomycin level in 1 min at 25 °C.

The lipid specificity of pore formation was measured by the release of entrapped calcein from liposomes comprising phosphatidylcholine (from fresh egg yolk; Sigma; 10 mg/ml) or 3:1 (w/w) compositions of phosphatidylcholine and phosphatidylglycerol (from egg yolk; Fluka), phosphatidylethanolamine (from bovine brain; Fluka) or cardiolipin (Sigma) prepared in a similar way in 50 mM Na₂SO₄, 0.5 mM EDTA, 50 mM Tris/maleate and 30 mM calcein (Sigma), pH 5.2. Untrapped calcein was removed by gel filtration using Sephacryl S-300 HR (Amersham). The increase in fluorescence due to liposome lysis upon addition of the proteins (10 nM amoebapore A or 50 nM rNK-lysin) to a 1:1000 dilution of liposomes in the same buffer was monitored fluorimetrically at excitation and emission wavelengths of 484 and 503 nm respectively for several minutes. The difference in fluorescence between before addition of the proteins and after achieving total lysis by addition of Triton X-100 (0.1 %) was taken as representing 100 % lysis. Lysis (%/min) was computed from the linear part of the graph.

RESULTS

Source of proteins

An evident prerequisite for a detailed comparison of the activities of these extraordinary polypeptides is the availability of isolated material in sufficient quantity. Whereas the amoebapores were purified to homogeneity from their natural source, i.e. trophozoites of *Entamoeba histolytica* HM-1:IMSS, NK-lysin was recombinantly expressed in *E. coli* in a fed batch fermentation. The resulting inclusion bodies were solubilized and purified by metal ion affinity chromatography, taking advantage of an N-terminal His-tag fusion. The denatured protein was refolded and cleaved to eliminate the His tag. Subsequent cation exchange chromatography yielded the homogeneous protein. The sequence was validated by MALDI-TOF analysis and N-terminal protein sequencing (results not shown). The structural integrity was verified by CD analysis, which revealed the spectrum of a helical protein devoid of β -structures. The content of α -helices was calculated to be 49 %, which is in good agreement with the known three-dimensional structure containing 52 % α -helix [23]. Moreover, no free thiol groups could be detected, which clearly indicates the presence of three disulphide bonds. It is concluded that the recombinantly expressed NK-lysin is properly folded and hence represents NK-lysin.

Antibacterial activity

The major function *in vivo* of all of the proteins studied here is considered to be based on their antibacterial activity. Therefore the MIC towards bacterial growth was determined, and revealed exceptionally low values of 1.3, 0.16, 0.3 and 0.16 µM for amoebapores A, B and C and rNK-lysin respectively against

Table 1 Concentration-dependent antibacterial activity of the amoebapores and NK-lysin

Membrane permeabilization was determined fluorimetrically using the dye SYTOX Green. (A) Influence of target bacterium and pH. Antibacterial activity is expressed as LD₅₀, which is the concentration at which 50 % of the bacteria have permeabilized membranes. The data points represent the means of triplicate experimental values. (B) Influence of NaCl concentration and the addition of bivalent cations on lysis of *B. subtilis* at pH 5.5. The bacteriolytic activity in the presence of 25 mM NaCl without addition of bivalent cations according to the standard assay is normalized to 100 %. The calculation of the residual activity (%) is based on the LD₂₀ values (the concentration at which 20 % of bacteria have permeabilized membranes), because LD₅₀ values were not reached upon addition of higher concentrations of NaCl. The data points are determined in duplicate. AP, amoebapore; NK-I, NK-lysin.

(A)

Species	pH	LD ₅₀ (nM)			
		AP-A	AP-B	AP-C	NK-I
<i>B. subtilis</i>	5.5	2.5	0.3	5.2	22
	7.4	> 10 000*	> 10 000*	> 10 000*	10
<i>E. coli</i>	5.5	3300	> 10 000†	> 10 000	47.5

(B)

Addition	Concentration (mM)	Relative bacteriolytic activity (%)		
		AP-A	NK-I	Melittin
NaCl	75	5.2	58.8	166
	150	5.4	16.3	n.d.
	300	5.4	3.8	100
Ca ²⁺	2	0.8	3.9	30.2
Mg ²⁺	2	0.7	4.8	51.0

* A concentration of 10 µM AP-A, AP-B and AP-C induced 32 %, 23 % and 13 % lysis respectively.

† A concentration of 10 µM AP-B induced 5 % lysis.

Gram-positive *B. subtilis*. Against Gram-negative *E. coli* K12, only rNK-lysin showed a similar high activity (MIC 0.16 µM), whereas the amoebapores inhibited growth only at concentrations of 10 µM and above, indicating different target specificities.

It is known that most, but not all, antibacterial peptides display their activity directly on the membrane, e.g. the porcine peptide PR-39 was shown to inhibit DNA synthesis before cell lysis occurred [33]. To verify that the antibacterial activity observed here was due to membrane permeabilization, we monitored the integrity of the bacterial membrane by measuring the fluorescence of SYTOX Green in polypeptide-treated bacteria samples. This dye displays fluorescence after intercalating into the DNA, a process that suggests that the cell membranes are compromised. In this assay, the amoebapores and rNK-lysin were highly active against Gram-positive bacteria in the nanomolar range (Table 1A). An increase in ionic strength due to the addition of NaCl resulted in a decrease in activity for amoebapore A as well as for rNK-lysin, but the effect was more pronounced for the former protein, which even at a concentration of 1 µM did not achieve 50 % lysis of the bacteria in the presence of 150 mM NaCl. Even in the presence of only 75 mM NaCl, the activity of amoebapore A decreased by 20-fold (Table 1B). By contrast, the activity of melittin, a well-described membranolytic peptide from bee venom, is virtually unaffected by salinity. Interestingly, addition of a low concentration of the bivalent cations Mg²⁺ or Ca²⁺ also substantially decreased the activity of all tested polypeptides against *B. subtilis*. For the amoebapores, but not for rNK-lysin, the potent antimicrobial activity is pH-dependent, and was shown to decrease with increasing pH.

Figure 1 Kinetics of the permeabilization of the outer and inner membranes of *E. coli* ML-35p by amoebapores and NK-lysin

The activities of periplasmic β -lactamase (■) and the cytosolic galactosidase (□) were monitored during incubation with amoebapore A, B or C or rNK-lysin (1 μ M each). Alamethicin and melittin served as model peptides. The data points represent the means of triplicate experimental values.

Against the Gram-negative representative *E. coli*, only rNK-lysin showed a strong activity comparable with that against Gram-positive bacteria; the activity of amoebapore A was about 10-fold less pronounced, and that of amoebapores B and C was virtually undetectable up to a concentration of 10 μ M. To analyse the mechanism of lysis of the Gram-negative bacteria in more detail, we performed an assay in which the permeabilization of the outer and inner bacterial membranes can be distinguished. This method allowed us to record the time scale of these two processes by observing the activities of two enzymes localized in different cellular compartments in permease-deficient *E. coli* ML-35p: the plasmid-encoded periplasmic lactamase and the cytosolic galactosidase. For comparison, the well known membranolytic peptides alamethicin and melittin were analysed in parallel, taking advantage of the comprehensive characterization of their different modes of action (reviewed in [34,35]). The kinetics of membrane permeabilization monitored here by enzymic amplification of the signal clearly show that all of the polypeptides studied are active against Gram-negative bacteria (Figure 1). For the amoebapores, the kinetics resemble those of alamethicin. In contrast, rNK-lysin perforates the inner membrane much earlier, and therefore its mode of action appears to be intermediate between those of alamethicin and melittin.

Activity against eukaryotic cells

According to their structural features and biological functions, membranolytic peptides possess different selectivities towards the different lipid compositions of natural membranes. Nonetheless, the pronounced harmful effects of the SAPLIP family members amoebapores and NK-lysin reported previously are quite peculiar for antimicrobial peptides. Therefore the cytotoxic activity of these polypeptides was also compared here; it was monitored by the increase in fluorescence of alamarBlue, which is reduced to the fluorescent form by metabolically active cells only (Figure 2). Again, the well characterized activity of alamethicin served

as a comparison. The amoebapores and rNK-lysin displayed cytotoxic activity in the same micromolar range, comparable with that of alamethicin. Whereas the dose-response curve of the major amoebapore isoform A was indistinguishable from that of rNK-lysin, amoebapore C exhibited even more pronounced cytotoxic activity. To verify that this toxic effect is indeed caused by membrane disruption, the release of fluorescence of BCECF-loaded Jurkat cells was examined (Figure 2). The results clearly demonstrate that the death of the cells induced by the various proteins in each case is due to lysis, and emphasize the extraordinary cytotoxic activity of amoebapore C, which even slightly exceeds the potency of the standard lytic peptide melittin.

Notably, in all assays performed and shown here for BCECF release in detail (Figure 2B, inset), the amoebapores show a strong pH dependence, with virtually no activity detectable at pH values above 6. rNK-lysin, in contrast, appears to act pH-independently (Figure 2C).

Pore-forming activity towards liposomes

Natural cell membranes are complex entities consisting of a variety of components which may interact with the antimicrobial polypeptides, thereby influencing their activity. The most minimalistic system with which to analyse the pore-forming capability of peptides and proteins is the use of artificial vesicles consisting of lipids only. Here, the pore-forming potential of the amoebapores and rNK-lysin was assayed in parallel by the dissipation of an induced diffusion potential in liposomes, which can be measured by the increase in fluorescence of a potential sensitive dye. Interestingly, the amoebapores display a more than 5-fold higher activity than the mammalian protein at a pH of 5.2 (Figure 3, inset). The liposomes employed here consisted of azolectin, a complex lipid mixture. However, most known antimicrobial peptides possess a specificity for negatively charged lipids. We tested these characteristics for amoebapore A and rNK-lysin by monitoring the release of calcein from preloaded

Figure 2 Concentration and pH dependence of the cytotoxic and cytolytic activities of the amoebapores and rNK-lysin

(A) The concentration dependence of the cytotoxic activity of amoebapore A (AP-A), amoebapore B (AP-B), amoebapore C (AP-C), rNK-lysin (NK-I) and alamethicin against Jurkat cells was determined fluorimetrically using the dye AlamarBlue at pH 5.5. (B) The concentration-dependent cytolytic activity of amoebapores A (■), B (●) and C (◆) and melittin (×) was measured by the release of the fluorescent dye BCECF from Jurkat cells at pH 5.5. The pH dependence of the activity of a 5 μM mixture of amoebapores is shown in the inset. (C) The concentration-dependent cytolytic activity of rNK-lysin (□, △) and melittin (×, +) is shown at pH 5.5 (△, +) and pH 7.4 (□, ×).

liposomes of defined lipid compositions. Both proteins also acted preferentially on negatively charged lipids; moreover, rNK-lysin showed a remarkably strong preference for phosphatidylglycerol (Figure 3).

DISCUSSION

Obviously, mammals and the unicellular amoeba *Entamoeba histolytica* are phylogenetically extremely diverse organisms, but, as shown here for the antimicrobial SAPLIPs, they may use similar proteins to fulfil comparable functions. In general, if proteins

Figure 3 Lipid specificity of the pore-forming activity of amoebapore A and rNK-lysin measured by calcein release from preloaded liposomes of defined lipid composition

PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin. For comparison, the activity toward liposomes of azolectin is given a value of 1 for both proteins. The data represent median and range of three measurements. Inset: pore-forming activity of the amoebapores (APs) and rNK-lysin (NK-I) measured by the dissipation of a valinomycin-induced membrane potential in liposomes at two different pH values. Means and S.D.s of ten independent measurements are shown.

are conserved during evolution, their functional mechanism is conserved, i.e. the amino acid residues that are responsible for and involved in the specific interactions leading to the function of the proteins are similar. The common functional feature of all members of the SAPLIP family to which the amoebapores and NK-lysin belong is their general ability to interact with lipids. This activity is provided by their structural characteristics, comprising a bundle of five mostly amphipathic helices connected by three conserved disulphide bonds. This extremely stable structural motif appears to be well suited for lipid binding, as each known SAPLIP – without any further sequence identity – has this capability. Within the family members, this common property gives rise to many different functions: they act as cofactors in glycosphingolipid metabolism, form a lipid-binding domain of several enzymes, or act as antimicrobial defence molecules (for a review, see [15]). The special biological function of the SAPLIPs described here, namely their antibacterial and cytolytic activity, does not manifest itself in conserved amino acid residues. Nevertheless, the proteins exhibit comparable activities in the same concentration range against mammalian cells and bacteria, causing membrane permeabilization. Only a closer look reveals slight differences, which may indicate different mechanisms of membrane interaction.

The most prominent difference in the activities of the proteins is the pH dependence of the amoebapores, with a sharp decrease in all activities when the pH is raised above 6, whereas rNK-lysin is apparently pH-independent. The dependence of amoebapores' activity on acidic pH may be based on the existence of a sole histidine residue. Beside the cysteine residues, this residue is one of only 24 that are conserved between all the amoebapore isoforms. The importance of this residue is supported by the facts that the pore-forming activity of amoebapore A is reversibly abolished by chemical modification of the histidine residue, and that the protein is no longer able to oligomerize in membranes after this treatment [19]. The pK value of a histidine side chain is (dependent on its local environment) approx. 6.2, and this residue is therefore a good candidate for conferring pH dependence on the protein. Hence the oligomerization of amoebapore A on membranes appears to depend on intermolecular electrostatic interactions, which may explain in part the dramatic decrease in its

antibacterial activity with increasing ionic strength. NK-lysin possesses no histidine residue, and the corresponding position is occupied by a lysine residue, which is positively charged up to pH 10.5.

In spite of the vast number of well known and comprehensively characterized antibacterial peptides from various sources, the molecular explanation for their target specificity or, conversely, the reason for the different susceptibilities of different bacterial species to these agents, is not well understood. The surface of bacteria is negatively charged, due to the lipopolysaccharide layer in Gram-negative bacteria and to teichoic acid bound to peptidoglycan in Gram-positive bacteria. Therefore, besides the common electrostatic interaction with the surface molecules, more subtle differences in affinity toward different lipid compositions may play a major role. All of the polypeptides studied here display extraordinary activity against Gram-positive bacteria, but apparently rNK-lysin is much better suited for the killing of Gram-negative bacteria. The cytoplasmic membranes of both groups of bacterial species contain large amounts of the negatively charged phospholipids phosphatidylglycerol and cardiolipin, which corresponds particularly well to the lipid selectivity of rNK-lysin. Monitoring the kinetics of the permeabilization of bacterial membranes gives insight into mechanistic features [32]. rNK-lysin is well adapted to permeabilize the outer membrane, as shown by the relatively steep increase in β -lactamase activity localized in the periplasmic space and the instantaneous measurement of cytoplasmic galactosidase activity. This assay has the advantage of catalytic amplification of the signal and therefore allowed the antimicrobial activity of the amoebapores against Gram-negative bacteria to be detected. However, the amoebic proteins act with different kinetics towards the different membranes compared with rNK-lysin. Following a lag phase, the inner membrane is perforated at a lower rate by the amoebapores. As NK-lysin possesses highly electropositive regions on its surface that are postulated to be responsible for the membrane interaction [25], it is reasonable to assume a higher affinity for the lipopolysaccharide molecules. Moreover, the lipopolysaccharide layer is stabilized by the bivalent cations Ca^{2+} and Mg^{2+} , which may be displaced by cationic antibacterial molecules such as NK-lysin, leading to membrane destabilization. In contrast, the amoebapores do not present any pronounced electropositive surface patch [24]. However, since not only the outer membrane of Gram-negative bacteria, but also other membranes, are known to be influenced by these bivalent cations [36], the decreasing effect of Ca^{2+} and Mg^{2+} on antibacterial activity against the representative Gram-positive bacterium *B. subtilis* observed here may be due to a change in the characteristics of the membrane rather than in those of the polypeptides tested.

For antibacterial peptides, several models of pore formation have been proposed. The first model developed to account for the observed characteristics of the action of alamethicin on membranes describes the formation of defined water-filled pores shaped like a barrel, with the staves built by oligomerization of a defined number of peptides [37]. Apparently, however, currently only alamethicin conforms to this model. The activity of most other peptides can be explained by the combined models of Shai, Matsuzaki and Huang, according to which the peptides cover the membrane like a carpet [38–40]. Once a threshold concentration is reached, the peptides insert into the membrane, causing strain within the bilayer [38], which may result in the formation of transient, torus-like pores lined by the peptides and phospholipid headgroups [39,40]. This mode of action is well accepted for the magainins from frog skin and also proposed as the most likely mechanism for other amphipathic and charged peptides [41,42]. Recently, neutron scattering experiments provided strong

indications that the amphipathic, cationic peptide melittin from bee venom also forms toroidal pores [35]. Inside the pore, the phospholipids shield the positive charges of the peptides, the repelling force of which would otherwise prohibit pore formation. Therefore it has been suggested that only essentially neutral peptides such as alamethicin are able to form barrel-stave-like pores. The initial binding of the antibacterial molecules studied here to membrane phospholipids is caused by electrostatic interactions, as is suggested by the electropositive surface of NK-lysin [25] and by the lipid affinity shown here for amoebapore A as well as for rNK-lysin. Moreover, the antibacterial activity decreases with increasing NaCl concentration, indicating the influence of electrostatic interactions. However, only the overall neutral amoebapores allow insertion into the membrane without the necessity of shielding positive charges. This behaviour is reflected in the higher pore-forming activity of the amoebapores using liposomes corresponding to a membrane-permeabilizing effect at a very low protein/lipid ratio characteristic of proteins acting according to the barrel-stave model.

To this end, the biological activities support the hypothesis that has arisen based on structural features, that – in spite of essentially similar biological activities – the amoebapores and NK-lysin act according to different modes of action. The amoebapores appear to form stable pores with channel-like characteristics [24,43], whereas the membrane-active behaviour of NK-lysin is caused mainly by electrostatic interactions described as molecular electroporation [25] which resemble vaguely the carpet model as far as the mode of action of such a large and structured polypeptide can be described by such a model.

Remarkably, however, rNK-lysin is as highly cytotoxic against human cells as the amoebapores. The composition of the outer surface of the membrane of mammalian cells consists predominantly of zwitterionic lipids and cholesterol. Therefore the membrane-lytic activities of interacting peptides are mostly driven by hydrophobic interactions. Notably, even the overall neutral amoebapores, which display an extended unpolar surface region [24], do not permeabilize liposomes consisting only of phosphatidylcholine and phosphatidylethanolamine, but require negatively charged lipids for their binding. NK-lysin does not even present any surface patch suitable for hydrophobic interactions. This discrepancy indicates that, on the complex cellular membrane, more interwoven interactions take place than can be mimicked by phospholipid vesicles.

The antimicrobial activity of members of the SAPLIP family has been maintained during evolution from bacteria-hunting amoebae to mammalian T and NK cells. It is tempting to suggest that amoebapores are the progenitors of NK-lysin, and maybe also of all SAPLIPs, but one has to admit that such a statement would require the identification and functional characterization of additional members of that protein family from various invertebrate species.

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