Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica*

(amoebiasis/amphipathic α-helices/melittin/membrane-active peptides)

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ABSTRACT   The pore-forming peptide amoebapore is considered part of the cytolytic armament of pathogenic *Entamoeba histolytica*. Amoebapore is composed of 77 amino acid residues arranged in four α-helical domains. For structure–function analysis, synthetic peptides were constructed corresponding to these four domains: H1 (residues 1–22), H2 (25–39), H3 (40–64), and H4 (67–77). The peptides H1 and H3, representing two highly amphipathic α-helical regions of amoebapore, possessed pore-forming activity. Peptide H3 displayed cytolytic and antibacterial functions similar to those of natural amoebapore. The most potent antibacterial activity and the broadest activity spectrum were expressed by H1-Mel, a hybrid molecule composed of the N-terminal α-helix of amoebapore and the C-terminal hexapeptide of melittin from the venom of *Apis mellifera*.

Infection with pathogenic *Entamoeba histolytica* is the cause of invasive amoebiasis in man which may lead to severe host tissue damage, colitis, and extraintestinal abscesses (1). The protozoan parasite is characterized by its extraordinary killing efficacy, which is potentially directed against almost all eukaryotic cells, including the effector cells of the host's immune system (2). In analogy to cytotoxic lymphocytes and perforin (3), amoebae kill target cells in a contact-dependent reaction which is thought to involve the secretion of membrane-disrupting material into the intercellular space between effector and target cell (4, 5).

The primary candidate for the molecular entity conferring cytolytic activity on *E. histolytica* is the pore-forming peptide amoebapore (4, 6). This peptide is capable of inserting well-defined ion channels into artificial phospholipid membranes (6–8), most likely by oligomerization according to the barrel-stave model (9); it also depolarizes eukaryotic cells *in vitro* (8, 10).

Amoebapore has been isolated from pathogenic *E. histolytica* (11) and its primary structure has been elucidated by sequencing the purified peptide and the corresponding cDNA (12). It is composed of 77 amino acid residues including 6 cysteine residues and has a calculated molecular mass of 8244 Da. Circular dichroism spectroscopy revealed an all-α-helical conformation. Three disulfide bonds provide the molecule with a relatively rigid structure. Computer-aided secondary-structure prediction yielded a molecule with four α-helices. Helix 1, starting at the N terminus, and helix 3, beginning approximately in the middle of the molecule, are highly amphipathic (12).

Amphipathic α-helices are the preferred structural elements of membrane-penetrating polypeptides (13). Helices 1 and 3 are long enough to span a lipid bilayer membrane and fulfill the criteria for putative transmembrane helices (12). In

a hydrophobic-moment plot (14) these helical segments of amoebapore clustered with small peptides of known membranolytic activity of various origin and thus may be classified as membrane-seeking domains (15). Both helices show some sequence similarity to melittin of bee venom (6), which is an amphipathic α-helical peptide of 26 residues and represents the prototype of membrane-damaging peptides (16). It is hypothesized here that the capacity of amoebapore for membrane perturbation resides within these two amphipathic helices. To test this hypothesis we synthesized peptides representing all four helical domains, including modifications thereof, and compared their pore-forming, antibacterial, and cytolytic activities with those of naturally occurring amoebapore.

MATERIALS AND METHODS

Peptide Synthesis, Purification, and Analysis. All peptides were synthesized by the solid-phase technique (17) using an automatic peptide synthesizer (model 431A; Applied Biosystems) and the small-scale "fastmoc" synthesis protocol of the manufacturer. Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids in the L configuration (Bachem) were used with side-chain-protecting groups. Peptides were synthesized and the final products were removed from the resin and deprotected (18, 19). Peptides were dissolved in 0.1% trifluoroacetic acid and dialyzed exhaustively against 0.9% NaCl in tubing with a molecular weight cut-off of 1000 (Spectra/Por 6; Spectrum Industries, Los Angeles) at 4°C. Homogeneity was determined by analytical reversed-phase HPLC. The primary structure of each peptide was confirmed by protein sequencing on a gas-phase protein sequencer (model 437A; Applied Biosystems). Peptide concentration was determined from A_{214}. The extinction coefficients were calculated (20) from the respective sequence information. Tricine–SDS/PAGE (21) and subsequent immunoblotting were carried out as described (11).

Other Peptides. Amoebapore was purified from trophozoite extracts of pathogenic *E. histolytica* HM-1:IMSS as described (11). Melittin from the venom of *Apis mellifera* (in sequencing grade) and gramicidin S from *Bacillus brevis* were purchased from Sigma.

Bacteria, Cytoplasts, and Eukaryotic Cells. The bacterial strains used were *Bacillus megaterium* (ATCC 14581), *Bacillus subtilis* (strain 60015), *Escherichia coli* K-12 (ATCC 23716), and *Micrococcus luteus* (strain 004) from the Botanical Institute, University of Hamburg, and clinical isolates of *Enterobacter cloacae* and *Staphylococcus aureus* obtained at our institute. Bacteria were grown in Luria–Bertani (LB) medium and subsequently inoculated in LB medium for

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Abbreviation: BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein.
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growth to midlogarithmic phase. *B. megaterium* and *B. subtilis* were dispersed on LB/agar plates containing 1% glucose, grown overnight, and subsequently inoculated in Penassay broth (antibiotic medium 3, Difco). Protoplasts of *B. megaterium* were prepared (22) by treating logarithmic-phase cells with lysozyme (Boehringer Mannheim). Spheroplasts of *Escherichia coli* K-12 were prepared (23) using lysozyme in combination with EDTA. Both cytoplasts were in 0.5 M sucrose/0.02 M maleate/0.02 M $MgCl_2$/2× Penassay broth, pH 5.2, to give an OD_{500} of 0.8. The monocyte-like tumor cell line U-937 and Jurkat T cells were maintained at a density of 1–6 × 10^5 per ml in suspension cultures with RPMI 1640 medium (GIBCO) supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin sulfate (0.1 mg/ml), and Amphotericin B (0.25 μg/ml).

Assays for Biological Activity. *Pore-forming activity.* Determination of pore-forming activity by monitoring the dissipation of a valinomycin-induced diffusion potential in liposomes and absorption of activity with phospholipid vesicles have been described (11).

Antibacterial activity. Bacteria in midlogarithmic phase were suspended in 1% tryptone/0.5% low-melting-point agarose to give 10^5 colony-forming units per ml. For *M. luteus* the medium contained additionally 1% NaCl and 0.5% yeast extract. Aliquots (80 μl) of bacterial suspension were added to peptides in 0.9% NaCl/0.1% trifluoroacetic acid (20 μl) in flat bottom 96-well microtiter plates and incubated at 28°C (24). OD_{595} of the microcultures was measured with a plate reader after 30 min and after 24–48 hr, when the control without peptides had reached a value of ≈0.2. The final values were corrected by subtraction of the initial values. Percent growth inhibition is defined as 100 × [1 − (corrected OD of the test microculture/corrected OD of the control microculture)].

Lysis of bacterial cytoplasts. Peptide samples in 0.9% NaCl/0.1% trifluoroacetic acid (150 μl) were added to the bacterial cytoplast suspension (850 μl) in a plastic cuvette and gently mixed, and OD_{500} was monitored during 30 min at 20°C. Lysis of cytoplasts was measured as a decrease of OD. Percent lysis was defined as 100 × (experimental lysis − spontaneous lysis)/(maximal lysis − spontaneous lysis). For maximal lysis the assay mixture was sonicated (80 W) three times for 15 sec in an ice bath.

Hemolytic activity. Human erythrocytes (20 μl; 5 × 10^8 per ml) in Mes-buffered saline [20 mM 2-(N-morpholino)ethanesulfonic acid/140 mM NaCl, pH 5.5] were incubated with 80 μl of peptide in the same buffer for 30 min at 37°C. Lysis was determined by measuring the concentration of released hemoglobin at 412 nm.

Cytotoxic activity. (*i*) Dye-exclusion assay. U-937 cells (2 × 10^5) in Mes-buffered saline were incubated at 37°C with peptides in the same buffer. After 1 hr, cells were examined microscopically in a hemocytometer chamber. Viability was indicated by exclusion of trypan blue (0.1%). The cytotoxic activity is expressed as percentage of stained cells minus the control (without peptide). (*ii*) Dye-release assay. Peptides in Mes-buffered saline were added to 2 × 10^5 Jurkat cells loaded with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF AM; Calbiochem). After 2 hr at 37°C, cytolysis was estimated by measuring the release of fluorescent dye (25).

RESULTS

Construction of the Synthetic Peptides. Based on the secondary-structure prediction for amoebapore, peptides H1, H2, H3, and H4 were synthesized which represent the four helical segments of the molecule (Fig. 1). Amino acid residues adjacent to the C terminus of the respective helix were

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         ┌──────── helix 1 ────────┐  ┌── helix 2 ─┐
         1                      21 25              36
Amoebapore   GEILCNLCTGLINTLENLLTTKGADKVKDYISSLCNKAS

         GFIATLCTKVLDFGIDKLIQLIEDKVDANAICAKIHAC
         40                          63 67         77
         └──────── helix 3 ──────────┘  └ helix 4 ┘

             1        10        20
H1       GEILCNLCTGLINTLENLLTTK

H1+6     GEILCNLCTGLINTLENLLTTKGADKVK

H1(Ala)+6    GEILANLATGLINTLENLLTTKGADKVK

H1-Mel   GEILCNLCTGLINTLENLLTTKRKRQQ*

H2       DKVKDYISSLCNKAS

H3       GFIATLCTKVLDFGIDKLIQLIEDK

H4       ANAICAKIHAC
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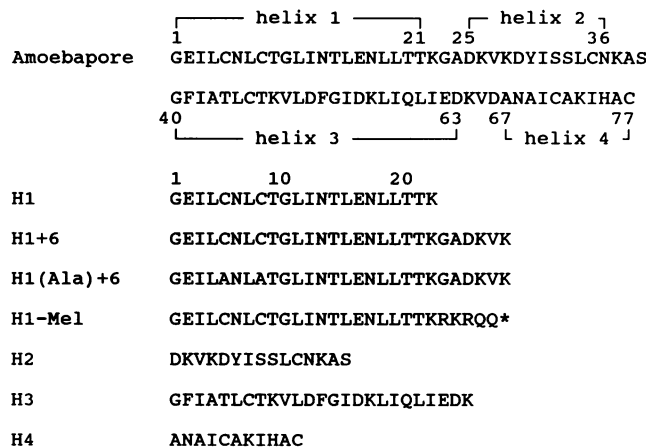
FIG. 1. Peptide designations and amino acid sequences of the synthetic peptides derived from amoebapore. The one-letter code is used. The four α-helical segments of amoebapore as obtained by computer-aided secondary-structure prediction are indicated. In H1(Ala)+6 the two cysteine residues of H1+6 have been replaced by alanine. H1-Mel constitutes a hybrid of the N-terminal α-helix of amoebapore and the C-terminal hexapeptide of melittin from the bee *Apis mellifera*. The C-terminal glutamine is amidated (★) as in melittin.

included when thereby an additional lysine residue was introduced (H1, H2, H3). Since positively charged residues are considered crucial for the activity of lytic peptides, two peptides were constructed in which amino acid stretches with a positive net charge were attached to the C terminus of H1: in H1+6 the additional residues were the six residues following the H1 sequence in the natural amoeba peptide, whereas the synthesis of H1-Mel gave a construct of the N-terminal helix of amoebapore and the C-terminal hexapeptide (Lys-Arg-Lys-Arg-Gln-Gln-NH_2) of melittin from *Apis mellifera*. In another peptide, the two cysteine residues in H1+6 were replaced by alanine residues [H1(Ala)+6]. The synthesized peptides were homogeneous and of correct primary structure as evidenced by analytical HPLC and protein sequencing. Upon dialysis of the peptides against saline the stock solution of H3 became very viscous at concentrations > 1 mg/ml, probably due to reversible self-aggregation. A similar observation was observed with H1 and H1+6 at concentrations > 2 mg/ml. Aggregation was reversed by acidification with trifluoroacetic acid (0.1%). For cytotoxicity assays, solubilization was achieved by dilution only.

SDS/PAGE and Recognition of the Peptides by Anti-Amoebapore. Upon SDS/PAGE, the peptides appeared homogeneous (Fig. 2A) with the exception of H1+6 and H3, where higher molecular mass entities were also visible. In a Western blot analysis anti-amoebapore primarly recognized H3 (Fig. 2B), and only faintly so the H1 derivatives. A putative dimer of H3 was considerably more reactive than the respective monomer.

Pore-Forming Properties. In the liposome depolarization assay, pore-forming activity was detected particularly for the H3 peptide and to a much lesser extent for H1. Peptides H2 and H4 were inactive (Table 1). The rather low activity of H1 was remarkably exceeded by that of H1+6 and even more by that of H1-Mel. Exchange of cysteine for alanine residues [H1(Ala)+6] did not increase but instead lowered the activity of H1+6. To determine whether the synthetic pore-forming peptides showed the same phospholipid preference as amoebapore, the peptides were preincubated with vesicles of various pure phospholipids and the samples were subsequently assayed for residual pore-forming activity (Fig. 3). The three active peptides exclusively adsorbed to negatively
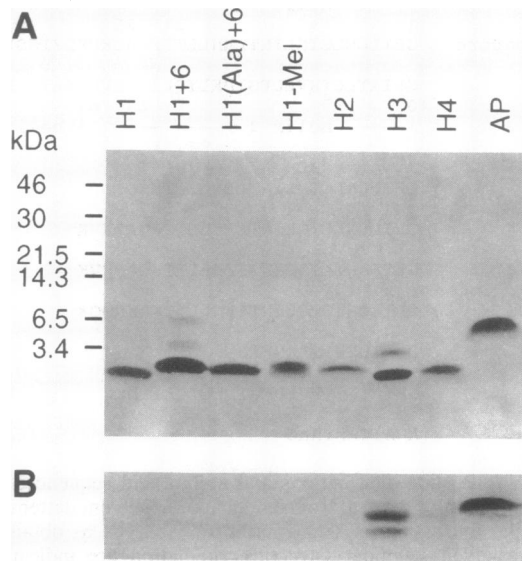
FIG. 2. SDS/PAGE of synthetic peptides and Western blot analysis with anti-amoebapore. (*A*) Peptide samples (0.1 μg) were subjected to tricine–SDS/13% PAGE under nonreducing conditions and silver stained. The peptides are designated as shown in Fig. 1. (*B*) The corresponding immunoblot of the peptide samples (0.3 μg) was developed with an antiserum to amoebapore. As a control, purified amoebapore (AP) was used (0.1 μg); the immediately appearing color reaction with AP in the immunoblot had to be stopped separately.

charged vesicles, as was previously found with amoebapore (11).

Cytotoxic and Hemolytic Activity. Although amoebapore at ≥2 μM induced membrane blebbing of U-937 cells (data not shown), much higher concentrations of the peptide were needed to effect cell death. As documented by trypan blue uptake, 20% of the target cells were killed at a concentration of 25 μM (≈200 μg/ml) (Fig. 4*A*). A release assay using BCECF-prelabeled Jurkat cells was more sensitive to monitor the cytolytic activity of amoebapore; 14 μM amoebapore (≈115 μg/ml) was sufficient to kill 50% of the cells (Fig. 4*B*). Hemolytic activity of amoebapore was found to be negligible at concentrations up to 10 μM (Fig. 4*C*). H3 was the only structural element of the amoeba peptide that was active in all three assays. H1-Mel was more active than amoebapore, but melittin was clearly the most active peptide tested.

Table 1. Pore-forming activity of amoebapore (AP) and related peptides

Peptide	M_r	Activity,* units/pmol	$n^\dagger$	Relative activity
AP	8244	1.32 ± 0.45	9	100
H1	2377	0.03 ± 0.005	6	2
H2	1671	<0.001	4	<0.1
H3	2795	0.23 ± 0.06	8	17
H3‡	2795	0.52 ± 0.01	10	39
H4	1114	<0.001	4	<0.1
H1+6	2970	0.21 ± 0.04	8	16
H1(Ala)+6	2912	0.07 ± 0.012	6	5
H1-Mel	2587	0.53 ± 0.14	10	40
Melittin	2844	0.17 ± 0.04	8	13

*Estimated by measuring liposome depolarization induced by various amounts of peptide (mean ± SD); a value of <0.001 indicates that no activity was found with up to 1 nmol of peptide. Peptide samples were dialyzed against 0.9% NaCl and acidified with trifluoroacetic acid (0.1%).
†No. of experiments.
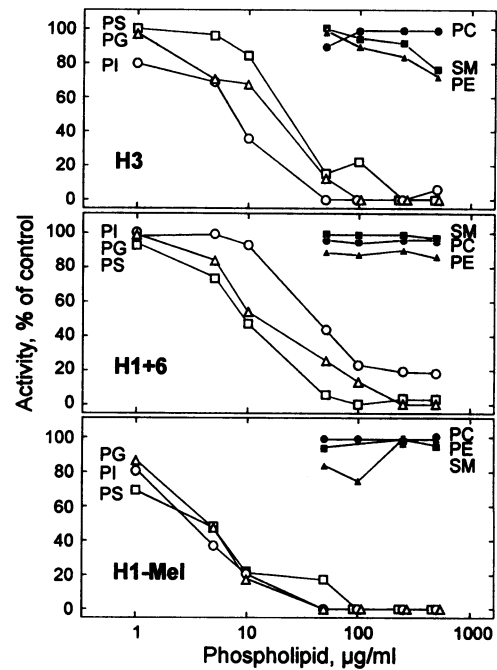‡Undialyzed peptide in 0.1% trifluoroacetic acid (0.1% trifluoroacetic acid alone had no effect).

FIG. 3. Absorption of pore-forming activity from peptide samples by phospholipids. Synthetic peptides H3, H1+6, and H1-Mel (20, 15, and 5 μM, respectively) were incubated for 3 hr at 4°C with vesicles composed of phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SM), or phosphatidylethanolamine (PE). As controls, peptides were incubated without phospholipids. After incubation the remaining pore-forming activity was determined by measuring the dissipation of liposome membrane potential. Open symbols indicate negatively charged phospholipids; filled symbols represent the absence of a net charge.

Antibacterial Activity. Peptides were tested for growth-inhibiting activity in cultures of four Gram-positive and two Gram-negative isolates (Table 2). For comparison, the antibacterial effect of melittin and gramicidin S was determined. The natural amoebapore displayed antibacterial activity against all four Gram-positive isolates, being most potent against *M. luteus*. The concentrations tested (up to 15 μM, ≈120 μg/ml) were not sufficient to affect the growth of the Gram-negative bacteria *Escherichia coli* and *Enterobacter cloacae*, which, however, were found to be less susceptible to the action of all membranolytic peptides tested than the other bacterial strains. H3 exhibited the highest antibacterial activity of the structural elements of amoebapore. The hybrid molecule H1-Mel was remarkably effective against all strains, exceeding the antimicrobial efficacy of amoebapore and almost reaching the potency of the antibiotic peptide gramicidin S and of melittin.

Lysis of Bacterial Cytoplasts. To investigate whether the cytoplasmic membrane of bacteria constitutes a target for the peptides, bacterial cytoplasts were prepared. Both protoplasts of *B. megaterium* and spheroplasts of *Escherichia coli* were lysed by the peptides with pore-forming activity (Fig. 5), indicating that these peptides can act on bacterial cytoplasmic membranes irrespective of their origin. Spheroplasts of *Escherichia coli* appeared to be slightly less susceptible to the peptides than protoplasts of *B. megaterium*.

DISCUSSION

The present study shows that (*i*) amoebapore is cytolytic toward metabolically active eukaryotic cells and also has antibacterial activity; (*ii*) of the four synthetic peptides representing the helical structural elements of amoebapore,
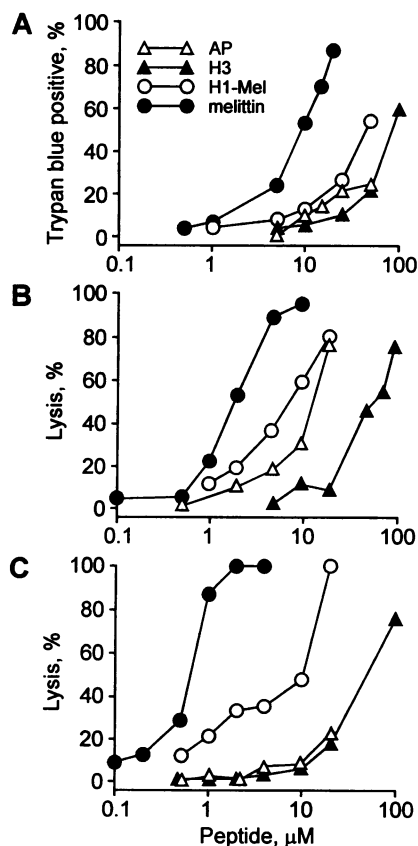
Fig. 4. Cytolysis induced by membrane-active peptides. Cell injury after exposure to various concentrations of peptides was measured by uptake of trypan blue by U-937 cells (A), release of the fluorescent dye BCECF from prelabeled Jurkat cells (B), and release of hemoglobin from human erythrocytes (C), respectively. Experiments were done in duplicate. AP, amoebapore.

H3 exhibits the most pronounced activity in all biological assays employed; and (*iii*) the marginal biological activity of H1 can be greatly enhanced by attachment of a stretch of residues with a positive net charge.

Amphipathic α-helices are the preferred structural entities of pore-forming peptides and proteins. In simple model systems for small lytic peptides, α-helices are visualized to form an ion channel by aggregation to a parallel bundle

Table 2. Antibacterial activity of membrane-active peptides

	Inhibitory concentration* of peptides†				
Bacteria	AP	H3	H1-Mel	Melittin	Gramicidin S
M. luteus	1.9	46	0.7	0.3	0.7
B. megaterium	13	36	0.5	0.3	0.7
B. subtilis	27	80	1.5	0.3	0.8
S. aureus	>15‡	83	3.3	0.3	2.2
Escherichia coli	>15	>100	6.2	0.9	3.3
Enterobacter cloacae	>15	>100	21	0.6	3.5

*Expressed as the concentration of peptides (μM) needed for a growth inhibition of 50% as calculated from dose-response curves; > denotes no inhibition detected at the concentration indicated. Experiments were done in duplicate.
†The synthetic peptides H1, H2, H4, H1+6, and H1(Ala)+6 did not show antibacterial activity against the above-mentioned bacterial strains at the maximal concentration tested (100 μM) with the exception of the activity of H1+6 and H1(Ala)+6 against *M. luteus*, where a growth inhibition of 50% was found at 60 μM and 75 μM, respectively.
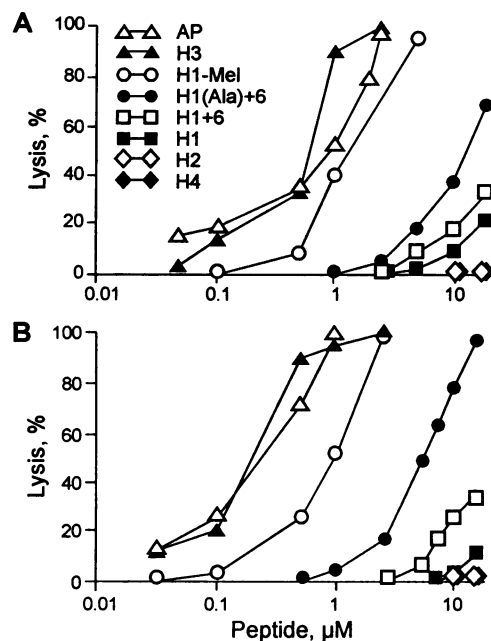‡At 15 μM, a growth inhibition of 10% was found.

Fig. 5. Lytic activity of amoebapore (AP) and synthetic peptides using bacterial cytoplasts as targets. Peptide samples in various concentrations were mixed with spheroplasts of *Escherichia coli* (A) or protoplasts of *B. megaterium* (B). Lysis of cytoplasts was estimated by monitoring decrease of OD_{500} for 30 min at 20°C. A sonicated cytoplast suspension represented 100% lysis. Experiments were done in duplicate.

traversing the membrane (26, 27). To span a lipid bilayer an amphipathic α-helix has to be at least 20 residues long and arranged so that the hydrophobic residues interact with the hydrocarbon chains of the phospholipids whereas the hydrophilic residues line the inside of the channel. Shorter amphipathic helices may perturb the integrity of a membrane by interacting with phospholipids in the plane of the lipid bilayer (27).

Previous studies on synthetic analogues of amphipathic α-helical peptides indicated that amino acid residues within a helix may be replaced without substantial loss of activity as long as the segregation of polar and apolar residues on opposite faces of the helix is preserved (28, 29). However, positively charged residues are considered critical for the lytic function of peptides (30). For example, melittin without the positively charged C-terminal hexapeptide is completely inactive toward erythrocytes (28). We showed that the rather low activity of H1 can be remarkably enhanced by the addition to the C terminus of the six residues that follow the H1 sequence in the natural peptide. Also, by conjugating the positively charged hexapeptide of melittin to helix 1 a hybrid molecule was obtained that was virtually as potent as the antibiotic peptide gramicidin S in antibacterial assays and more efficient than the parent molecule amoebapore. As suggested by the absorption studies, insertion into negatively charged phospholipid vesicles is probably influenced by the added positively charged lysine residues.

Among the four domains of amoebapore helix 3 and helix 1 appear to be instrumental in the disruption of membranes. In the intact amoebapore molecule the helices are embedded within a rigid tertiary structure constrained by three disulfide bonds which do not allow a major conformational rearrangement. This implies that the helices are already in an orientation appropriate for membrane perturbation while the molecule is in solution. Reduction of the disulfide bonds results in complete loss of activity, but activity is fully restored by reformation of the disulfide bonds (12). The precise function of helical elements acting in concert in the natural molecule

cannot be deduced from these studies. Their importance for the function of amoebapore, however, is emphasized by analogous structural features of other pore-forming molecules (31–33).

The extension of the target-cell spectrum for amoebapore to bacteria and bacterial cytoplasts is relevant to its biological role. The ability of amoebapore to damage bacterial cytoplasmic membranes, together with its localization inside amoebic vesicles (12), suggests that the actual function of this peptide is the killing of ingested bacteria, thereby preventing microbial growth inside the digestive vacuoles. Such a function has been attributed to the bactenecins (34) and defensins (35), found in mammalian neutrophils. On a molar basis amoebapore exhibits a similar antibacterial potency, at least against the Gram-positive strains tested. A variety of natural antimicrobial peptides appear to function as membrane-active agents, and some of them have been found to exert their activity through channel formation in bacterial membranes—e.g., the defensins, the cecropins from immune insect hemolymph, and the magainins from frog tissues (for review, see refs. 36 and 37). Probably to provide a broad target spectrum, none of these peptides interacts with a specific membrane receptor and hence relatively high doses are needed to affect the growth of bacteria.

The finding that the pore-forming activity of amoebapore and of the synthetic peptides virtually parallels their bacterial growth-inhibiting potencies suggests that also the antibacterial activity is due to membrane perforation. The different susceptibilities of Gram-positive and Gram-negative bacteria are most likely due to the different composition of their external walls. The nonsusceptibility of intact *Escherichia coli* was abrogated when spheroplasts were produced. Their cytoplasmic membrane is susceptible to the membrane-disrupting activity of amoebapore and the synthetic peptides derived from it. We therefore assume that the antibacterial activity of amoebapore constitutes a bacteriolytic rather than a bacteriostatic effect.

Defensins and magainins are also cytotoxic to eukaryotic cells by permeabilizing their membrane (38–40). Since cytolysis of host cells is a pronounced feature of pathogenic *E. histolytica*, amoebapore may be, at least in part, responsible for this activity. However, the killing of metabolically active eukaryotic cells such as Jurkat and U-937 cells *in vitro* needs relatively high concentrations of isolated amoebapore, probably to overcome ion pumps and effective repair mechanisms. Upon amoeba–target cell conjugation, the narrow space of the contact zone may allow amoebapore to achieve concentrations sufficient to destroy host cell membranes. With regard to the low hemolytic activity of amoebapore, it is likely that the lack of negatively charged headgroups on the outer leaflet of the membrane of erythrocytes retards the interaction of the peptide with the cell surface.

It is unclear whether the susceptibility of the various target cells to the isolated peptide *in vitro* reflects *in vivo* conditions, since ingested bacteria and attacked host cells are exposed not only to amoebapore but also to an array of aggressive components, such as other membrane-active substances and hydrolytic enzymes, which all may act synergistically.

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