Antimicrobial and Pore-forming Peptides of Free-living and Potentially Highly Pathogenic Naegleria fowleri Are Released from the Same Precursor Molecule*

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The pore-forming polypeptides of Naegleria fowleri, naegleriapores A and B, are processed from separate multipeptide precursor structures. According to their transcripts, each precursor molecule appears to contain additional naegleriapore-like polypeptides, all of which share a structural motif of six invariant cysteine residues within their amino acid sequence. To identify the putative pronaegleriapore-derived peptides at the protein level, amoebic extracts were screened for small cysteine-rich polypeptides by fluorescently labeling their cysteine residues. Three novel naegleriapore isoforms derived from the precursor molecule of naegleriapore B were identified. Two of the isoforms were purified to homogeneity and tested for their biological activity. The pore-forming activity of the novel peptides was remarkably lower than that of the originally isolated naegleriapores, but both peptides killed bacteria by permeabilizing their cytoplasmic membranes. Collectively, these results indicate that naegleriapores isoforms with antibacterial and pore-forming activity are proteolytically released from the same precursor protein, presumably to generate a phylogenetically ancient complementary antimicrobial arsenal.

Naegleria fowleri is a free-living amoebal flagellate of soil and freshwater habitats throughout the world. Although the trophozoites are able to fulfill their life cycle without the intervention of a parasitic stage, they can invade the human brain via the nasal mucosa and olfactory nerve and cause primary amoe bic meningoencephalitis (1, 2). This disease is characterized by massive host tissue destruction indicating that the cytolytic activity of the amoebae is a major factor for their pathogenicity.

In vitro, intact amoebae (1), as well as cell-free extracts thereof (3), lyse efficiently a variety of target cells. The amoebic cytolytic capacity has been attributed mainly to pore-forming molecules that have been identified more than a decade ago (4). Recently, we characterized at the molecular level naegleriapores A and B, proteins that potently display pore-forming activity that kills prokaryotic as well as eukaryotic target cells (5). According to the respective transcripts, both naegleriapores were processed from larger precursor structures, each of which contains several naegleriapore isoforms that share the structural motif of six invariant cysteine residues within their highly diverse amino acid sequences. The precursor molecule of naegleriapore A contains three isoforms, and that of naegleriapore B contains five isoforms (5). For convenience, the putative isoforms within the precursor molecules were designated according to their location on the precursor of naegleriapore A as A1–A3 and on that of naegleriapore B as B1–B5. Isoforms A2 and B1 represent proteins that have been isolated recently by us according to their pore-forming activity (5).

As the existence, fate, and function of additional naegleriapores contained in the precursor molecules are unclear, we initiated a survey pursuant to their identification in crude extracts of N. fowleri. To detect naegleriapore-like peptides without prominent pore-forming activity at the protein level, we screened for small cysteine-rich proteins by fluorescently labeling their cysteine residues. Using this approach, we identified three novel isoforms, which are apparently proteolytically released from the precursor molecule of naegleriapore B. Two of the isoforms could be purified to homogeneity and in sufficient quantity to allow for testing of their pore-forming capacity and antibacterial activity.

EXPERIMENTAL PROCEDURES

Amoebae—A highly virulent strain of N. fowleri (ATCC 30894), LE-Emp, was used in this investigation. Samples of brain tissue from infected mice containing amoebae were cultured at 37 °C in Oxoid Emp, was used in this investigation. Samples of brain tissue from infected mice containing amoebae were cultured at 37 °C in Oxoid medium supplemented with serum and hemin to obtain the amoebae. The amoebae were cultured axenically for 4 days prior to use (1).

Protein Purification—Freshly harvested and washed amoebae were extracted with volumes of 10% acetic acid overnight at 4 °C. The extract was centrifuged at 150,000 × g at 4 °C for 1 h, and the resulting supernatant was passed through a C18-12 ml (2 g) Sep-Pak cartridge (Waters). The cartridge was washed with 0.1% trifluoroacetic acid, and the adsorbed material was eluted stepwise with 30–100% acetonitrile, 0.0% trifluoroacetic acid (10 ml for each 10% step). Each fraction was analyzed for cysteine-rich small proteins in the range of 5–20 kDa. Naegleriapore isoforms were eluted with 60–80% acetonitrile, 0.1% trifluoroacetic acid, lyophilized and subsequently resuspended in 10% acetonitrile, 0.1% trifluoroacetic acid, subjected to reversed phase HPLC® using a C3 PRP-3 column (Hamilton) connected to a 130A separation system (Applied Biosystems) and equilibrated with 0.1% trifluoroacetic acid. The column was washed with 0.1% trifluoroacetic acid (3 min), and peptides were eluted with a linear gradient of 0–40%
acetonitrile, 0.1% trifluoroacetic acid for 45 min at a flow rate of 0.2 mL/min. Naegleriapores B1, B4, B2, and B5 were eluted with 52, 56, 62, and 65% acetonitrile, respectively, from the column.

Detection of Cysteine-rich Proteins—Proteins of each fraction obtained during the purification process were tested for cysteine-rich peptides using the fluorescent dye monobromobimane (MBBr, Sigma) (6). The proteins were lyophilized briefly, resuspended in 200 mM Tris, 1% SDS, 3 mM EDTA, 3 mM dithiothreitol, pH 8.0, followed by denaturation at 70 °C for 20 min. After the samples were cooled to room temperature, MBBr was added to a final concentration of 6 mM, and the samples were incubated at room temperature for 2 min in the dark. The reaction was stopped with 10 mM cysteine, and the proteins were subjected to Tricine-SDS-PAGE under reducing conditions. Excessive MBBr was washed out of the gel with 50% methanol, and cysteine-rich peptides were detected under UV light as bright fluorescent bands.

Protein Analysis and N-terminal Sequencing—Tricine-SDS-PAGE was performed according to Schägger and von Jagow (7) using 13% separation gels. For N-terminal sequencing, peptides purified by reversed phase HPLC were analyzed using a gas phase protein sequencer (model 437A, Applied Biosystems). The method applied did not allow for the unequivocal detection of cysteine residues. The concentration of purified naegleriapores was determined by measuring absorbance at 214 nm. The extinction coefficients were calculated using the respective sequence information (8).

Pore-forming Activity Assay—The pore-forming activity of the samples was determined by measuring fluorometrically the dissipation of a valinomycin-induced membrane potential in liposomes (9). Fluorescence was measured by a fluorescence spectrophotometer (model LS 50B, PerkinElmer Life Sciences) using excitation and emission wavelengths of 620 and 670 nm, respectively. Pore-forming activity was measured as the initial change in fluorescence intensity over time after adding the sample. One unit of activity was defined as a fluorescence increase to 5% of the prevemoinycin-induced intensity in 1 min at 25 °C.

Antibacterial Assay—The bacterial strains used were Bacillus subtilis (strain 60015) (10) and Pseudomonas aeruginosa (ATCC 10145). The bacteria were grown in Luria-Bertani (LB) medium and subsequently inoculated in LB medium for growth to mid-logarithmic phase. After centrifugation, the bacteria were washed twice with, and resuspended in, 20 mM MES, pH 5.5, containing 25 mM NaCl. A 96-well microtiter plate (Greiner, Frickenhausen, Germany) was precoated with 0.1% bovine serum albumin for 15 min prior to use in the assay. Peptides in 0.01% trifluoroacetic acid were 2-fold serial diluted in 20 mM MES, pH 5.5, 25 mM NaCl. 1 × 10^6 bacteria (25 μl) were incubated with the diluted peptides (25 μl) and 2 μl of the fluorescent dye Sytox green (50 μl); in 20 mM MES, pH 5.5, 25 mM NaCl) (Molecular Probes) at 37 °C for 1 h. Compromised bacterial cytoplasmic membranes allowed the dye to cross this membrane and to intercalate with the DNA. When excited at 495 nm, the binding of the dye to DNA results in an increase of emitted fluorescence at 538 nm; this was measured in a fluoroscan II microtiter plate reader (Labsystems, Helsinki, Finland). The antibacterial activity of the peptides was expressed as percent of permeabilized bacteria. For maximum permeabilization of the bacteria (100% value), cells were incubated with 70% ethanol for 5 min. Cecropins A and B were purchased from Sigma as synthetic peptides and used as positive controls.

RESULTS

Identification and Purification of Small Cysteine-rich Proteins—To identify small cysteine-rich proteins in extracts of N. fowleri, the cysteine residues of the proteins in each fraction obtained during the purification process were labeled with the fluorescent dye MBBr. The bright fluorescence compared with the amount of protein loaded onto a polyacrylamide gel suggested the presence of numerous cysteine residues within their amino acid sequence. Several fluorescent proteins in the range of 5–25 kDa were eluted stepwise from a hydrophobic solid phase matrix. These proteins were purified further using reversed phase HPLC and subsequently were subjected to N-terminal sequencing. Altogether, 11 proteins were analyzed, and 4 of 11 were identified as naegleriapores B1, B2, B4, and B5 by their N-terminal sequence. All naegleriapores were found in the 60–80% acetonitrile fraction eluted from the hydrophobic matrix column. The elution profile of the final purification step is shown (Fig. 1A). From the HPLC elution profile, the relative amount of naegleriapore isoforms B2, B4, and B5 in the amoebic extracts was estimated to be 0.6, 0.3, and 0.8, respectively, when compared with B1. The peptides possessed apparent molecular masses of 13, 7, 10, and 10 kDa, respectively, as judged by Tricine-SDS-PAGE (Fig. 1B). This result indicates that virtually all peptides originating from the precursor molecule of naegleriapore B are proteolytically released (Fig. 2) and are present as mature peptides in crude extracts of N. fowleri.

Biological Activity—Two of the three new isoforms, naegleriapores B2 and B5, were purified to homogeneity and tested for their pore-forming activity. The specific pore-forming activities of naegleriapores B2 and B5 were 0.2 ± 0.05 units pmol⁻¹ (n = 4) and 0.4 ± 0.05 units pmol⁻¹ (n = 4), respectively, which are 10–20-fold lower than that of the major isoform naegleriapore B1 (4.1 ± 1.0 units pmol⁻¹, n = 3) measured in parallel.

The primary task of naegleriapores is thought to be the rapid
killing of engulfed bacteria. Therefore, we tested the antibacterial activities of naegleriapores B1, B2, and B5 against B. subtilis and P. aeruginosa in comparison with cecropins A and B (Fig. 3). Naegleriapores B1 and B2 exerted potent antibacterial activity against Gram-positive B. subtilis, which was comparable with that of the well known cecropins. However, they were less potent against Gram-negative P. aeruginosa.

The permeabilization of bacterial membranes was also detectable for isoform B5, but the activity displayed was lower.

**DISCUSSION**

The pore-forming peptides naegleriapores A2 and B1 of N. fowleri are encoded in larger multipeptide precursor structures. As no other entities displaying pore-forming activities have been detected after separation of amoebic extracts, it remained unclear whether all of the peptides were released from the precursor molecules. When we began a survey to identify the other naegleriapore-like peptides at the protein level, we used an alternative approach, which entailed screening for small cysteine-rich peptides. Three additional naegleriapore B isoforms were identified by N-terminal sequencing. With the exception of the pore-forming naegleriapore A (i.e. isoform A2), we were not able to identify additional isoforms arising from the second precursor structure. The unexpected finding that several mature peptides derived from a single precursor molecule were not present in equimolar amounts in crude extracts may be due to different stabilities of these peptides against degradation processes mediated by proteases. Naegleriapore B1 is a glycoprotein containing two glycan moieties (5). It is likely that naegleriapores B2 and B5 are glycoproteins as well because (i) both peptides contain a putative N-glycosylation motif within the N-terminal region of their respective amino acid sequences, and (ii) they display a migration behavior upon SDS-PAGE similar to that of naegleriapore A (5), which possesses one glycan moiety and hence migrates at a higher molecular mass than calculated from its primary structure. Naegleriapores B3 and B4 do not possess an N-glycosylation motif within their amino acid sequences. The aforementioned N-terminal N-glycosylation motif of naegleriapores is situated at the same position as in other SAPLIPs, e.g. saposins A, B, and D, and in surfactant protein B (see Fig. 2). Deglycosylation of naegleriapores A2 and B1 and saposin B does not have a negative effect on their respective activities (5, 11). However, for the latter, it was found that the absence of the sugar moiety led to aberrant folding, rendering the protein metabolically unstable (11). We suggest that the degree of glycosylation of naegleriapores may reflect their stability against degradation by proteases and may account for the variant amounts of the different isoforms in crude amoebic extracts. Interestingly, the missing naegleriapore A isoforms 1 and 3 do not possess a glycosylation motif at the assigned position.

The organization of several SAPLIP elements within one precursor molecule is not a unique feature of naegleriapores, as saposins A–D and the surfactant-associated protein B also are proteolytically processed from a multipeptide molecule (12–15). The processing of multiple antibacterial peptides from one large precursor molecule may constitute an efficient mode for the simultaneous synthesis of different effector molecules, thereby resulting in amplification of the antibacterial response and an expansion of the target cell spectrum. This strategy is found with other antibacterial peptides as well, e.g. magainins from frog skin and the apidaecins from honey bees (16–18). It may reflect the continuous “arms race” between the high mutational rate of microorganisms and the protective armament.
Fig. 3. Antibacterial activity of naegleriapores B1, B2, and B5 and cecropins A and B against *B. subtilis* and *P. aeruginosa*. Naegleriapore B1 (black squares), B2 (white squares), B5 (gray squares), cecropin A (black triangles), and cecropin B (white triangles) were incubated with *B. subtilis* (upper panel) and *P. aeruginosa* (lower panel) in various concentrations at 37 °C for 1 h. The amount of bacteria with compromised membranes was determined fluorometrically using the dye Sytox green. Antibacterial activity of the peptides was expressed as percent permeabilized bacteria.

of potential host organisms, in this case the amoebae. Notably, the primary translation products of members of the SAPLIP family with antibacterial and cytolytic activity from other species known so far, e.g. amoebapores, disparpores, NK-lysin and granulysin (10, 19–21), contain a single SAPLIP element. Assaying the activity spectrum of naegleriapores against a broad variety of target cells was not possible because of the minute amounts of proteins that can be purified from the natural source. The novel naegleriapore B isoforms exert poor pore-forming activity (not due to their different glycosylation states, as already mentioned above). All naegleriapore isoforms tested exhibited antibacterial activity against Gram-positive and Gram-negative bacteria. Collectively, our results confirm our postulate that the naegleriapores that display high pore-forming activity, i.e. the isoforms A2 and B1, constitute the unrivaled cytolytic factors of *Naegleria* and that they represent a component of the archaic antimicrobial system of a free-living bacteria-hunting amoeba, which may, incidentally, also be instrumental in the fatal parasitic disease.

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