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# Hydralysins, a New Category of β-Pore-forming Toxins in Cnidaria*[S]

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**Cnidaria are venomous animals that produce diverse protein and polypeptide toxins, stored and delivered into the prey through the stinging cells, the nematocytes. These include pore-forming cytolytic toxins such as well studied actinoporins. In this work, we have shown that the non-nematocystic paralytic toxins, hydralysins, from the green hydra *Chlorohydra viridissima* comprise a highly diverse group of β-pore-forming proteins, distinct from other cnidarian toxins but similar in activity and structure to bacterial and fungal toxins. Functional characterization of hydralysins reveals that as soluble monomers they are rich in β-structure, as revealed by far UV circular dichroism and computational analysis. Hydralysins bind erythrocyte membranes and form discrete pores with an internal diameter of ~1.2 nm. The cytolytic effect of hydralysin is cell type-selective, suggesting a specific receptor that is not a phospholipid or carbohydrate. Multiple sequence alignment reveals that hydralysins share a set of conserved sequence motifs with known pore-forming toxins such as aerolysin, ε-toxin, α-toxin, and LSL and that these sequence motifs are found in and around the pore-forming domains of the toxins. The importance of these sequence motifs is revealed by the cloning, expression, and mutagenesis of three hydralysin isoforms that strongly differ in their hemolytic and paralytic activities. The correlation between the paralytic and cytolytic activities of hydralysin suggests that both are a consequence of receptor-mediated pore formation. Hydralysins and their homologues exemplify the wide distribution of β-pore formers in biology and provide a useful model for the study of their molecular mode of action.**

Cnidarians (corals, sea anemones, jellyfish, and hydrae) are an evolutionarily ancient group of sessile, soft-bodied animals that rely heavily on offensive and defensive allomones for capture of prey and protection from predators. As such, they have developed a highly complex and versatile array of protein and polypeptide toxins (neurotoxins, cytolysins, and phospholipases) aimed to paralyze the prey and prepare it for digestion (1). These toxins are generally thought to be stored and delivered into the prey through the highly developed stinging cells, nematocysts (2).

Cytolytic toxins, which permeate target cell membranes causing lysis of affected cells, form an important part of the cnidarian allomonal inventory (3). Such toxins can cause toxic systemic effects, such as paralysis and pain, and are probably involved in the life threatening stings of the Portuguese Man-O-War (*Physalia physalis*) and of box jellyfish (4–6). Several types of cnidarian cytolytic toxins have been isolated and characterized, with the most common and most studied being the actinoporin family of pore-forming toxins (PFTs).[1] This family, highly abundant in sea anemones but found in all cnidarian classes (Hydrozoa, Chyphozoa, and Anthozoa) (1), has been used extensively as a model to study the interactions between proteins and biological or model membranes (7–10).

We have recently described a novel paralytic (presumably neurotoxic) protein, hydralysin (Hln), produced by the green hydra *Chlorohydra viridissima* (11). Hln is unique in that, unlike other cnidarian toxins, it is not found in the stinging cells (nematocytes) involved in prey capture. Hln was shown to exhibit a cell type-selective cytolytic activity, but no proteolytic or phospholipase activity. The possibility that Hln acts as a pore former was suggested by the existence of a low level of sequence similarity with aerolysin and several other bacterial PFTs (11).

With this background, in the present study we have shown that hydralysins comprise a novel family of highly diverse cnidarian PFTs and have functionally characterized this family. Although Hlns differ from the previously characterized groups of cnidarian cytolytic toxins, they reveal structural and functional similarities with known bacterial and fungal toxins. Using sequence alignment and site-directed mutagenesis of Hln isoforms, we characterized a specific pore-forming sequence motif shared by a diverse group of proteins from animals, plants, and bacteria.

## MATERIALS AND METHODS

*Test Animals*—Green hydrae (*C. viridissima*) were bred in glass trays in “M” medium as described (12) at 20 °C and fed newly hatched larvae of *Artemia salina*. For toxicity assays we employed laboratory-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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[1] The abbreviations used are: PFT, pore-forming toxin; Hln, hydralysin; rHln, recombinant Hln; AT, α-toxin from *C. septicum*; εT, ε-toxin from *Clostridium perfringens*; PEG, polyethylene glycol; PBS, phosphate-buffered saline; MS, mass spectrometry; MS-MS, tandem MS; HEK, human embryonic kidney; $PD_{50}$, dose that causes paralysis to 50% of the test animals.

bred blowfly larvae (*Sarcophaga faculata*) (13).

*Mass Spectrometry*—Mass spectrometry was performed at the Qtof Laboratory Interdepartmental Equipment Unit, The Hebrew University Medical School. Hln samples were separated by electrophoresis on 12% SDS-PAGE gels as described below and the Coomassie-stained bands excised. Reduction, alkylation, and trypsinization steps were carried out in the gel (14). After extraction of the peptides from the gel with 60% $CH_3CN$ 1% CHOOH and evaporation to dryness, the samples with the peptide mixtures were redissolved in 1% CHOOH and solid phase-extracted with a C18 resin-filled tip. Nanospray setup (15) was employed as a mode of injection of the samples (in 50% $CH_3CN$ 1% CHOOH) into the QtofII MS system (Micromass, England) for both mass and MS/MS measurements. Data analysis, including amino acid sequence assignments, was performed using the Biolynx package (Micromass).

*Cloning and Sequence Analysis of Hln Isoforms*—Hln-2 and Hln-3 were cloned directly into the expression vector pHIS-parallel-2 (a kind gift from Dr. P. Sheffield, University of Virginia) using the primers 5′-ACT TCT CAC ATA TGG GTA AAG AGC TTC TA A CCT TTA GTG-3′ and 5′-CAA GAA AGG AAT TCT TAC AAA GGC TCA CTT GG T CCG A-3′, which include the restriction sites for the enzymes NdeI and EcoRI, respectively (underlined). The plasmids were sequenced at the Genome Center, Hebrew University. Sequences were aligned using CLUSTALW 1.82 at EBI (16), and the phylogenetic tree was drawn using Multalin version 5.4.1 at prodes.toulouse.inra.fr/multalin/multalin.html (17). Secondary structure prediction from protein sequences of Hlns was performed using all the algorithms available in the ExPASy web server (except AGADIR, which is dedicated to peptides) (www.expasy.org/tools). To search for proteins homologous to Hln, we used PSI-BLAST (18), although other algorithms (BLAST, FASTA, Mpsearch, and scanPS) also detected several of the homologous proteins. The search was performed during February, 2005. The sequences were downloaded from the NCBI data base and aligned using MUSCLE (19), edited using CINEMA (20) to better match the PSI-BLAST output, and finally colored using CLUSTAL X (21) with the default color settings. The accession numbers of the proteins presented in Fig. 5*A* are as follows: Hln-2 (AY655142), Proaerolysin (gi 1827609), ε-toxin (gi 52696091), LSLa (gi 32261216), α-toxin (gi 27531076), Lysenin (gi 2274812), Phi ctx (gi 17313218), Flammutoxin (gi 30961841), Cry 14–4 (gi 56089905), Mosquitocidal (gi 1302632), Mtx2 (gi 1378030), 34-kDa cry (gi 282459), Parasporin (gi 48290384), Crystal protein (gi 32469226), Yolk protein (gi 4235368), Archaeal protein (gi 21227968), Spherulin (gi 84166), Chicken protein (gi 50784562), ep-37 (gi 2339973), Nematode protein (gi 21913095).

*Reverse Transcription PCR of Different Isoforms*—Total RNA was extracted from ~100 mg of hydrae using the method of Chomczynski and Sacci (22) and reverse-transcribed to cDNA using the SMART cDNA kit (Clontech). PCR was performed using the isoform-specific upper primers 5′-CAG AGG TGA CTC GTC AAT CGA (Hln-1), 5′-TCG AGG TGA CTC GCC GAT TAC (Hln-2), and 5′-CCG ATT ACT TTG ACG TTT GG-3′ (Hln-3) and the lower primer 5′-TTA CAA AGG CTC ACT TGG TCC GA-3′. The PCR program used was 2 min at 94 °C, followed by 32 rounds of 94 °C for 30 s, 60 °C (Hln-1 and Hln-2) or 65 °C (Hln-3) for 45 s and 72 °C for 1 min and ending with a 7-min extension at 72 °C, in a Crocodile II Thermal cycler (Appligene). The enzymes used were Biotools *Taq* (Biotools) for Hln-1 and Hln-2 and Bio-x-act *Taq* with the high specificity buffer (Bioline) for Hln-3. PCR products were separated on a 1.5% agarose gel containing ethidium bromide.

*Mutagenesis*—The double mutant rHln-$2^{(Q28E,V140L)}$ (in this report rHln-1 stands for the recombinantly expressed protein, whereas Hln-1 stands for the native one) was created by inserting the central portion (amino acids 42–147) of a clone similar to Hln-2, obtained during the initial characterization of Hln-1, into the pDEST14 expression vector containing Hln-1 (11), using the restriction sites for BstEII and BamHI. The single and triple mutants rHln-$2^{(G125E)}$ and rHln-$2^{(Q28E,V140L,G125E)}$ were created from rHln-2 and the double mutant, respectively, using the QuikChange site-directed mutagenesis kit (Stratagene). PCR was performed using the primer 5′-GTC AAG TGA GTT TGA AGT TGA AGG AGC TTT CAA AAT GGG-3′ and its antisense, according to the instructions provided by the manufacturer with the addition of a primer extension stage according to Ref. 23. The introduction of the planned mutations was verified by sequencing the expression plasmids.

*Expression of Recombinant Toxins*—Hln isoforms and mutants were expressed in BL-21(DE3)-SI bacteria (Invitrogen) and purified at the Protein Purification Facility, Wolfson Center for Applied Structural Biology, Hebrew University, by anion exchange chromatography followed by gel filtration. The first separation was carried out on a Q-Sepharose FF 15 × 1.5-cm column (Amersham Biosciences), equilibrated with 20 mM Tris-HCl, pH 8.8, and eluted by a gradient of molarity of NaCl (0.5 M) in the same buffer in an fast protein liquid chromatography system (Amersham Biosciences). The active fraction was concentrated by ultrafiltration (Amicon) and charged on a Sephacryl S-100 960 2.6-cm or a Superdex 75 prep 96 1.6-cm gel filtration column (Amersham Biosciences), equilibrated, and eluted in PBS. The single and triple mutants rHln-$2^{(G125E)}$ and rHln-$2^{(Q28E,V140L,G125E)}$ and native Hln (from hydrae sonicated in a Vibra-Cell sonicator (Sonics) for 2 s) were purified in essentially the same manner using Hi-Trap Q 5-ml or Hi-Trap Q HP 1-ml anion exchange columns followed by a Superdex 75 HR 10/30 gel filtration column (Amersham Biosciences) on an high pressure liquid chromatography system (LKB or Jasco). Purity was assessed by SDS-PAGE followed by staining with Coomassie.

*Far UV-CD Spectroscopy*—Circular dichroism spectra were recorded in the wavelength range 195–250 nm in a 0.1-cm path length cuvette on a Jasco J-810 spectropolarimeter at room temperature. Data were collected every 0.2 nm at 50 nm $min^{-1}$. At least four scans were performed for each spectrum, and the baseline was subtracted. The CD spectra are presented as mean residue ellipticity weight, *i.e.* [$\theta$] (degree × $cm^2$ × $dmol^{-1}$). The contents of secondary structure were estimated by data fitting using the SELCON3, CDSSTR, and CONTINLL programs in the CDPro software package (24).

*Activity Assays*—Toxicity was tested by injection to blowfly larvae as described previously (11, 13) and the paralytic dose ($PD_{50}$) determined from sampling and calculation according to Reed and Muench (25). Paralysis was defined as any locomotory disturbance that prevents the animal from moving and changing its location freely 1 min after injection. Hemolysis was assayed essentially according to Ref. 26, using human type A+ erythrocytes. One hemolytic unit was defined as the amount of toxin causing 50% hemolysis in a standard assay. Cytolytic activity was assayed using the Cyto-Tox One membrane integrity assay kit (Promega). HEK293, JAR, and HeLa cells (generously provided by Prof. Joseph Orly, Hebrew University) were maintained as described previously (27). Cells were used 1 day after seeding to 24-well plates at ~90% confluence. Hln in 300 μl of a 1:1 mixture of the cell-growth medium and PBS was added to the cells, which were incubated at 37 °C. After 30 min, 300 μl of assay buffer were added and the samples processed according to the manufacturers' instructions. Fluorescence was measured using a PerkinElmer LS50B luminescence spectrometer fitted with a 96-well plate reader (excitation 560 nm slit 10, emission 590 nm slit 5). Cytotoxicity assays using Trypan blue staining were performed as described previously (11).

*Binding of Hln to Erythrocyte Membranes*—To monitor Hln binding to membranes, human type A+ erythrocytes were washed in PBS as described, lysed with 5 mM phosphate buffer for 5 min at 37 °C, and centrifuged for 5 min at 14,000 rpm in order to recover membranes. The lysis with 5 mM phosphate buffer was repeated until membrane pellet was colorless. Following incubation of membranes (from 20 μl of erythrocyte suspension) with the rHln isoforms (200 nM) for 15 min at 37 °C, the membranes were pelleted by centrifugation for 5 min at 14,000 rpm, washed once with PBS, and subjected to Western blotting using anti-Hln antibodies (11). Immunocytochemistry was performed using a Cy-2-conjugated secondary antibody (Jackson) and visualized using confocal microscopy (Bio-Rad 1024 scanhead connected to a Zeiss Axiovert 135 M microscope at the Confocal Microscopy Unit, Life Science Institute, Hebrew University).

*Osmotic Protection Assays*—The functional size of the Hln pores was determined by performing hemolysis assays as described above in the presence of 30 mM of polyethylene glycols (PEGs; Fluka) of various molecular weights in PBS (28, 29).

## RESULTS

*Hydralysins Are a Diverse Family of Toxins in Hydrae*—We have recently described the cloning, expression, and functional characterization of a novel paralytic and cytolytic protein called hydralysin (11). An intriguing result obtained was that the recombinant and natively isolated Hlns migrate differently on SDS-PAGE gels, raising the possibility that the native toxin undergoes proteolytic cleavage as part of its maturation, similar to other known cytolytic toxins (30–32). To see whether, indeed, a fragment of the native protein is proteolytically removed, we subjected both recombinant and native Hln to tandem mass spectrometry (MS-MS). Careful study of the mass spectra (Fig. 1*A*) and manual analysis of all data collected allowed for coverage of over 75% of the proteins, revealing that

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Hln-1 MGKELLTFSDLTWLDSSPDTVRSAFTREYGTTPDGIALNSEGYFGYHSPPITEQYGRPCY 60
Hln-2 MGKELLTFSDLTWLDSSPDTVRSAFTRQYGTTPDGIALNSEGYFGYHSPPITEQYGRPCY 60
Hln-3 MGKELLTFSDLRWLDSSPDTVRSAFTREYGTTPDGIALNSEGYFGYHSPPITEQYGRPCY 60

Hln-1 KQTGETKITLQELGPSTDAVLGNSTAVNRGDSSIDLTVSVEGKWSDSTSWSTSTETGVKM 120
Hln-2 KQTGETKITLQDLAPPTDAILGNSIARNRGDSPITLTVSVEGKWSDSTSWSTSTETGVKM 120
Hln-3 KQTGETKITSQDLSAPTDDVLGSSAAINRGDSPITLT----------------------- 97

Hln-1 SSEFEVEGAFKMGGEFSLTLSVGKSGSSSVEKTSTSSVQVTVPPRSKVVVSMVGIMKKEK 180
Hln-2 SSEFGVEGAFKMGGEFSLTVSVGKSGSSSVEKTSTSSVQVTVPPRSKVVVSMVGIMKKEK 180
Hln-3 ---FGVEGVFQMGGEFSLTVSVRKSGSSSVEKTSTSSVQVTVPPRSKVVVSMVGIMKKEK 154

Hln-1 VFFEVPVTVDGSFGANFPSTVQGHYFWFMDARSCLNKTSGVIKGTIDHANVFDVSVEVGP 240
Hln-2 VFFEVPVTVDGSFGANFPSTVQGHYFWFMDARSCLNKTSGVIKGTIDHANVFDVSVEVGP 240
Hln-3 VFFEVPVTVDGSFGANFSSTVQGHYFWFMDARSCLNKTSGVIKGTIDHANVFDVSVEVGP 214

Hln-1 SEPL 244
Hln-2 SEPL 244
Hln-3 SEPL 218
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FIG. 1. **Hydralysin belongs to a diverse family of proteins.** *A*, mass spectra of recombinant Hln-1 (*upper panel*) and the native hydralysin (Hln-2) (*lower panel*). Note the prominent peptide ($m/z$ = 760) found only in the recombinant protein, and the peptide $m/z$ = 1040 found only in the native protein (marked by *); these correspond to amino acids 75–89, which contain most of the differences between the proteins. *B*, phylogenetic tree of the protein sequences of 16 cDNA clones of Hln. Sequences also detected using MS-MS are *underlined*. The sequences of the specific clones expressed and characterized as Hln-1, 2, and 3 are *circled*. *Hm*, *H. magnipappilata*, *Hv*, *H. vulgaris*. The sequences of the different clones are found in supplemental Fig. 1. *C*, sequence comparison of Hln-1, 2, and 3. Gaps in the sequence are marked by –. Identical amino acids are marked by *. Similar amino acids are marked by . or :. Amino acid substitutions between Hln-1 and 2 are marked *above* the alignment with a − (negative in rHln-1 to uncharged in rHln-2), + (uncharged in rHln-1 to positive in rHln-2) or by *0* (no change). *D*, hydrae express Hln-1, 2, and 3. Reverse transcription PCR was performed using primers that differentiate between Hln-1, 2, and 3, revealing that hydrae express all three isoforms. *MWM*, molecular mass marker, the strong band corresponds to 500 bp. *1*, *2*, and *3*, plasmids containing the clones of rHln-1, 2, and 3. *H*, total hydra cDNA−, negative control (no DNA). No band was observed when the reverse transcriptase enzyme was omitted (not shown). *E*, hydrae contain several Hln proteins differing by their electrophoretic mobility. Western blotting was performed using pure recombinant Hln-1 and 2 and native Hln isoforms that differ in their elution time from the anion exchange column. *H*, water extract of hydrae. *MWM*, molecular mass marker with sizes in kDa. *n-E*, fraction of hydra crude extract eluting early from the anion exchange column (~0.15 M NaCl). *n-L*, fraction eluting later (~0.2 M NaCl) from the same column. Each lane contains concentrated fractions from several rounds of chromatography. *rHln-1* and *2*, purified recombinant isoforms.

rather than being the result of proteolytic cleavage the native Hln in fact represents a second isoform, differing from the recombinantly expressed protein in eleven amino acid residues. We named this isoform Hln-2, the previously described one being Hln-1.

To verify the occurrence of more than one Hln isoform, we sequenced 14 different plasmids containing the cloned cDNA of Hln, several of them partial clones obtained during the initial cloning of the toxin (11) and others that were cloned directly into an expression vector. Thirteen distinct sequences were obtained, which can be clustered into three main groups as shown in Fig. 1*B*. A comparison of the sequences of Hln-1, Hln-2, and Hln-3, as representatives of these groups, is presented in Fig. 1*C*, and the full alignment can be found in supplemental Fig. 1. The Hln-1 and Hln-2 groups differ mainly in a region found between amino acid residues 72–95, with eight amino acid substitutions, whereas Hln-3, in addition to several amino acid substitutions, differs from the two other groups in that amino acids 98–123 are missing.

The diversity of different Hln cDNA sequences found raised

the suspicion that these may have been caused by mutations inserted during the cloning process, rather than representing true isoforms. Several controls were performed to eliminate this possibility. First, PCR was performed using the same polymerase enzyme and PCR program on a specific plasmid and the PCR products subcloned and sequenced. In five such cases, no differences were observed between the PCR product and the original template, eliminating the possibility that the differences between the cDNA isoforms were due to mutations introduced by the DNA polymerase. Second, reverse transcription PCR was performed on hydra cDNA using PCR primers that differentiate between Hln-1, 2, and 3. As can be seen in Fig. 1*D*, all three isoforms are expressed by the hydrae. Finally, we were able to separate the native Hln into two fractions, with apparent molecular masses (as measured by SDS-PAGE electrophoresis) of 27 and 31 kDa, using anion exchange chromatography (Fig. 1*E*). MS-MS analysis of the two native fractions revealed that the 27-kDa band is composed mainly of a protein identical in sequence to Hln-2, with a minor contaminant containing the amino acid substitution I80V corresponding to one of the sequences obtained from the cDNA clones (clone f6 in supplemental Fig. 1. The 31-kDa band contained an Hln isoform not represented in the cloned cDNA sequences but also belonging to the Hln-2 family (data not shown). Taken together, these data (MS-MS, sequencing of clones, reverse transcription PCR, and protein chromatography) show that Hlns are, in fact, a highly diverse family of toxins expressed by the green hydra.

Two other species of hydra, *Hydra vulgaris* and *Hydra magnipappilata*, also express proteins belonging to the Hln family. We cloned three such cDNAs from *H. vulgaris* cDNA using the same primers as those used for the green hydra. The cDNA sequences of the Hln orthologues from *H. vulgaris* (Hv-Hln) were very similar, with two possible amino acid substitutions (Asn or Ser at position 105 and Thr or Ala at position 211). Another sequence was obtained from *H. magnipappilata* as part of the hydra Expressed Sequence Tag project (ace_4352.y, created by joining the partial sequences found in the GenBank™ as gi 47545809, gi 47547418, gi 47541336, and gi 47541099) (1). Hln orthologues are therefore found not only in the green hydra but also in other hydra species.

*Hln Isoforms Differ in Their Biological Activities*—Many venom systems are known to be composed of polypeptides that exhibit some degree of sequence microheterogeneity that provide the organism with a set of functionally diverse, although structurally similar, proteins (33). This is especially well exemplified by the Cry family of PFTs from *Bacillus thuringienesis*, in which a wide array of similar proteins is combinatorially expressed by the bacteria in order to kill a specific type of host insect (34). The diversity of the cloned Hln sequences, the fact that almost all of the differences between the isoforms are found in the N terminus half of the protein, and the similarity of Hlns to bacterial PFTs, including several Cry toxins (see below), suggested that this sequence heterogeneity may provide diversity in the biological functions of the proteins. To test this, we recombinantly expressed Hln-1, 2, and 3 as representatives of the three subfamilies of Hln found in the green hydra and compared their activities. Hln-2 was recombinantly expressed and purified essentially in the same manner as recombinant Hln-1 (rHln-1) (11). The first purification step, anion exchange chromatography on Q-Sepharose FF, revealed significant differences between the isoforms in the NaCl concentration in which the proteins eluted, indicating variations in pI values (Table I). Conversely, gel filtration chromatography revealed that both isoforms are monomeric with apparent molecular masses of ~27 kDa. The final purity of the protein was confirmed by SDS-PAGE (Fig. 2*A*), revealing also that the recombinant protein migrates as a 27-kDa protein, similar to the natively isolated Hln (11).

TABLE I
*Properties of Hln isoforms and mutants*

For $PD_{50}$ determination in each experiment 19–25 animals were injected with 5–6 different concentrations of Hlns and the $PD_{50}$ calculated according to Ref. 25. ND, not determined.

| | Anion exchange elution | $PD_{50}$ |
|---|---|---|
| | *mM NaCl* | *ng/100 mg body weight* |
| rHln-1 | 155 | 55.7 ± 15.2 ($n$ = 2) |
| rHln-2 | 128 | 10.3 ± 0.5 ($n$ = 2) |
| rHln-2$^{(G125E)}$ | ND | 24.6 ± 11.6 ($n$ = 3) |
| rHln-2$^{(Q28E,V140L)}$ | 160 | 10.0 ± 1.2 ($n$ = 2) |
| rHln-2$^{(Q28E,V140L,G125E)}$ | 200 | 20.8 ± 15.4 ($n$ = 4) |

Recombinant Hln-1 was previously shown to have both paralytic and cell type-specific cytotoxic activities (11). When injected into blowfly larvae, rHln-2 revealed the same symptomatology as rHln-1, *i.e.* a fast contractile paralysis followed by body flaccidity. However, rHln-2 was approximately five times as toxic as rHln-1 (Table I). Both isoforms revealed cell type-specific cytotoxicity, with a strong activity against HeLa cells, a weaker activity against JAR cells, and no activity at all against HEK293 cells (Fig. 2*B*). Comparable results were obtained using the trypan blue method (not shown). In all cells affected, rHln-2 had a stronger effect, permeating cell membranes at lower concentrations. Finally, to corroborate the membrane-damaging effect of hydralysins we characterized their hemolytic activities on human type A+ erythrocytes. As can be observed in Fig. 3, both isoforms exhibit hemolytic activity, rHln-2 being approximately 20 times as potent as rHln-1.

The third isoform, rHln-3, in which amino acids 98–123 are missing, was also expressed using the same bacterial system. Western blotting revealed the protein in the soluble fraction of the lysate at a concentration of ~70 ng/μl (data not shown). However, injection of the bacterial lysate containing up to 300 ng of protein (>6 rHln-1 $PD_{50}$ doses) to blowfly larvae did not cause any paralytic effect. In addition, the lysate (containing rHln-3 at a final concentration of ~500 nM) did not cause any hemolytic effect. We therefore conclude that rHln-2 reveals a similar biological activity to, but is more potent than, rHln-1, whereas rHln-3 is an inactive isoform in the tests and concentrations used.

*Hln-1 and Hln-2 are β-Pore-forming Toxins*—The membrane-damaging effect of Hlns to the cell lines presented above, together with the similarity in the primary structure to bacterial pore-forming toxins (11), raised the possibility that the toxins act through a molecular mechanism of membrane pore formation. Pore formation by toxins is a process that takes place in several stages (35). In the first stage, the toxin is found in its water-soluble form. As described above, gel filtration chromatography revealed that both rHln-1 and 2 are water soluble as monomers. Far UV-CD spectroscopy (Fig. 4*A*) of rHln-1 and 2 revealed that they have essentially the same secondary structure in solution, which is rich in β-structure (18% α-helix and 36% β-structure). This result agrees with those obtained from the sequence (40% β-structure) using an extensive array of computerized prediction methods (see "Materials and Methods") and is in accordance with the amino acid similarity to aerolysin and other bacterial PFTs rich in β-structure (11, 36).

Upon encountering a target membrane, the water-soluble PFTs bind to it and oligomerize. Membrane binding is often mediated by a specific receptor, which can be either a lipid

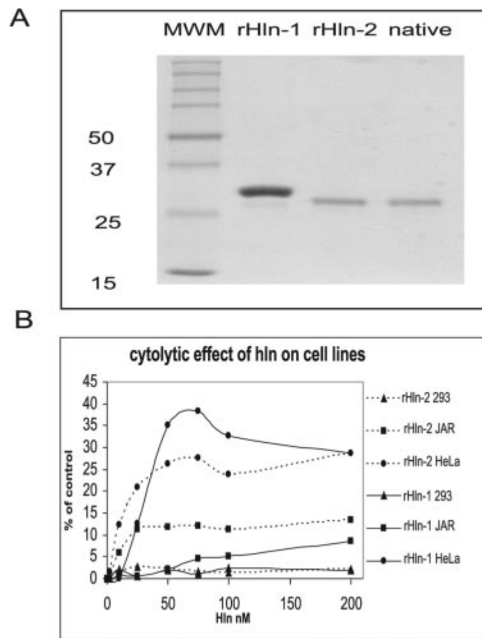


FIG. 2. **Hln-1 and 2 differ in their cytolytic activity.** *A*, Coomassie-stained SDS-PAGE gel of native and recombinant Hln isoforms, showing that they differ by their electrophoretic mobility. *MWM*, molecular mass marker with sizes in kDa; *rHln-1* and *2*, purified recombinant isoforms; *native*, natively isolated Hln, identified by MS-MS as being comprised mainly of Hln2. *B*, rHln-1 and 2 differentially permeate cell membranes in a cell type-specific manner. Three human cell lines were incubated with various concentrations of recombinant Hlns (*rHlns*), and the plasma membrane permeability was assayed by measuring the release of lactate dehydrogenase from the cells. Note that although the specificity of the two isoforms is similar, rHln-2 is more potent, affecting cells at lower concentrations. Results are presented as percent of positive control using 0.18% Triton X-100.

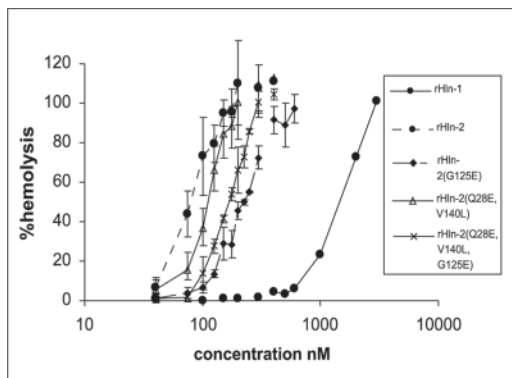


FIG. 3. **Hemolytic effects of recombinant Hln isoforms and mutants.** Hemolysis caused by rHln-1, rHln-2, and the mutants rHln-$2^{(G125E)}$, rHln-$2^{(Q28E,V140L)}$, and rHln-$2^{(Q28E,V140L,G125E)}$ was assayed by measuring the absorbance at $A_{540}$ of the supernatant after incubation of 4% v/v human type A+ erythrocytes with the toxins at 37 °C for 1 h. Results are presented as percent of the hemolysis caused by the addition of double-distilled water and are averages of two to five experiments. *Error bars* represent S.D. when three or more experiments were performed.

membrane component, such as cholesterol as in cholesterol-dependent-cytolysins (37), sphingomyelin in pore-forming toxins from sea anemones (8, 38), or a membrane protein or carbohydrate (39). rHln-1 and rHln-2, as well as the non-active isoform rHln-3, bind to erythrocyte membranes, as revealed by Western blot experiments (Fig. 4*B*). In some Western blots of Hln bound to erythrocyte membranes, high molecular mass bands were observed (Fig. 4*B*, *arrows*), possibly corresponding to weakly SDS-resistant oligomers (40). No significant difference could be seen between the binding of rHln-1 and rHln-2. These results reveal that the region absent in Hln-3 and the non-conserved residues between the isoforms are both not essential for membrane binding. The hemolytic activity of both active isoforms was unaffected by preincubation of the proteins with cholesterol, sphingomyelin, or phosphatidylcholine (up to 10 mM total lipid concentration), suggesting that these membrane components are not the primary receptors responsible for the Hln membrane binding. Similarly, preincubation of Hln with galactose, mannose, and lactose at concentrations of up to 100 mM did not affect the hemolytic activity of the protein, suggesting that these carbohydrates do not constitute direct receptors for the toxin. When rHln-2 bound to erythrocyte membranes was visualized by immunofluorescence (Fig. 4, *C* and *D*), a non-homogenous (punctuate) pattern was observed, suggesting that Hlns bind definite environments on the membrane. In this aspect, Hlns may be similar to aerolysin and other PFTs that have been shown to bind receptors located on specific lipid rafts (41–43).

Finally, after membrane binding and oligomerization, PFTs undergo conformational changes that cause a part of the toxin to enter into the membrane, lining a transmembrane pore. The pores allow leakage of small molecules such as ions and metabolites, followed by an influx of water causing the eventual bursting of the cell. This type of hemolysis, termed colloid-osmotic lysis, can be inhibited by the addition of large molecular mass nonelectrolytes to the extracellular solution: if the hydrated diameter of the nonelectrolyte protectant is such that it cannot enter through the pores it will counterbalance the osmolarity of the intracellular hemoglobin, thus inhibiting hemolysis. The hemolytic activity of both rHln-1 and rHln-2 is notably reduced by PEGs of average molecular mass of 1500 Da and above (Fig. 4*E* and data not shown). When the osmotically protected erythrocytes were transferred to PBS, hemolysis occurred almost instantly, indicating that PEGs did not inhibit the binding of the toxin to the membrane. The critical dependence of the hemolytic inhibition on the PEG molecular size permits us to discard a membrane permeabilization based on a detergent-like mechanism and also to estimate the size of the putative membrane pore (28), in this case ~1.2 nm. This is in good agreement with the sizes of pores created by aerolysin (44, 45), α-hemolysin from *Staphylococcus aureus* (45, 46), and α-toxin from *Clostridium septicum* (AT) (31).

*A Sequence Signature Common to Diverse βPFTs*—The characterization of Hln as a βPFT, and the low but significant level of sequence similarity between Hln and known pore-forming toxins from bacteria (11), prompted us to see whether we can use these similarities to describe sequence motifs common to these distant toxins. This is important because, although three-dimensional structural similarities have been noted (47, 48), these have been difficult to translate into sequence motifs that would allow extrapolation of structural data to novel sequences (49, 50). To do this, we searched the GenBank™ data base and aligned the hits found as previously described under "Materials and Methods." As can be seen in Fig. 5*A*, many of the proteins detected by PSI-BLAST as having sequence similarity to Hln are in fact known pore-forming or cytolytic toxins, including three (aerolysin, LSL, and ϵ-toxin) whose three-dimensional structures have been determined (47, 48, 51). All of these proteins share in part of their sequences a similar arrangement of amino acids that can be described as follows. Located within the region of significant similarity lies a motif showing an alternating pattern of hydrophobic residues (Fig. 5*A*) that is flanked by two motifs rich in hydroxylated (serine or threonine) amino acids. The second (C terminus) flanking Ser/Thr-rich region is followed by another region rich in hydrophobic residues, with a conserved diad of proline residues in the center.

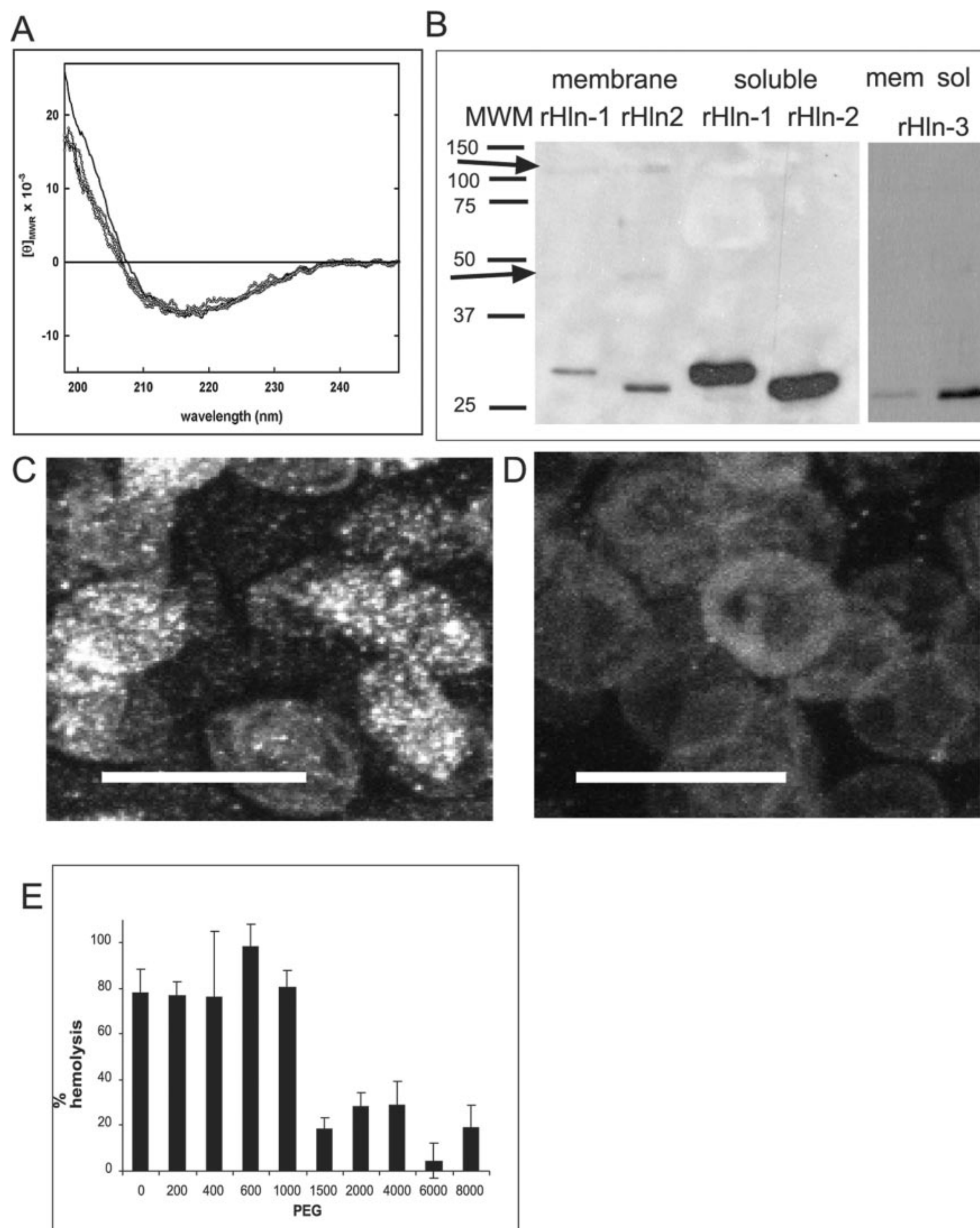


FIG. 4. **Hydralysins are βPFTs.** *A*, Hlns are rich in β-structure. The far UV-CD spectra of rHln-1, 2, and the mutants rHln-2$^{(Q28E,V140L)}$ and rHln-2$^{(Q28E,V140L,G125E)}$ are presented as mean residue ellipticity weight, *i.e.* [θ] (degree × cm2 × dmol$^{-1}$). The *continuous line* represents rHln-1, the other *overlapping lines* represent rHln-2, rHln-2$^{(Q28E,V140L)}$, and rHln-2$^{(Q28E,V140L,G125E)}$. *B*, rHln-1, 2, and 3 bind to erythrocyte membranes and undergo oligomerization, forming SDS-insoluble oligomers. A Western blot is shown of erythrocyte membranes incubated with 200 nM of rHln-1 or rHln-2 and of the toxin left in the supernatant after the incubation. Note that at this concentration rHln-1 reveals no hemolytic activity, whereas rHln-2 causes 100% hemolysis (see Fig. 3). *MWM*, molecular mass marker with sizes in kDa. *Arrows* indicate oligomers of ~46 and 120 kDa. Note the lack of high molecular mass oligomers in the supernatant despite higher concentrations of the monomeric toxins. The *right panel* represents a similar experiment in which a bacterial supernatant containing rHln-3 was incubated with the erythrocyte membranes. *C* and *D*, immunofluorescent detection of Hln-2 bound to erythrocyte membranes. Erythrocytes were incubated with either two hemolytic units of rHln-2 (*C*) or control PBS (*D*), washed extensively, and the location of Hln on the membrane was visualized immunocytochemically using a Cy-2-conjugated secondary antibody. *Bright dots* represent Hln, which binds the erythrocyte membrane in a non-homogenous (punctate) manner. *Bar* represents 20 μm. *E*, osmotic protection of erythrocytes from toxin-induced hemolysis by PEG. Two hemolytic units of rHln-2 in PBS were incubated with human erythrocytes in the presence of 30 mM PEGs of various molecular masses. Results are presented as percent of double-distilled water-induced hemolysis and represent averages of three experiments. *Error bars* represent S.D.

Interestingly, this conserved region lies in the heart of the pore-forming domain of several toxins (aerolysin, LSL, and ϵ-toxin) whose structure has been determined (47, 48, 51): the central motif with the alternating hydrophobic residues corresponds to the experimentally determined transmembrane domain of AT (52) and to the flexible loops of three toxins whose structures have been determined (Fig. 5*B*) (47, 48, 53), while the motifs flanking the conserved transmembrane domain form

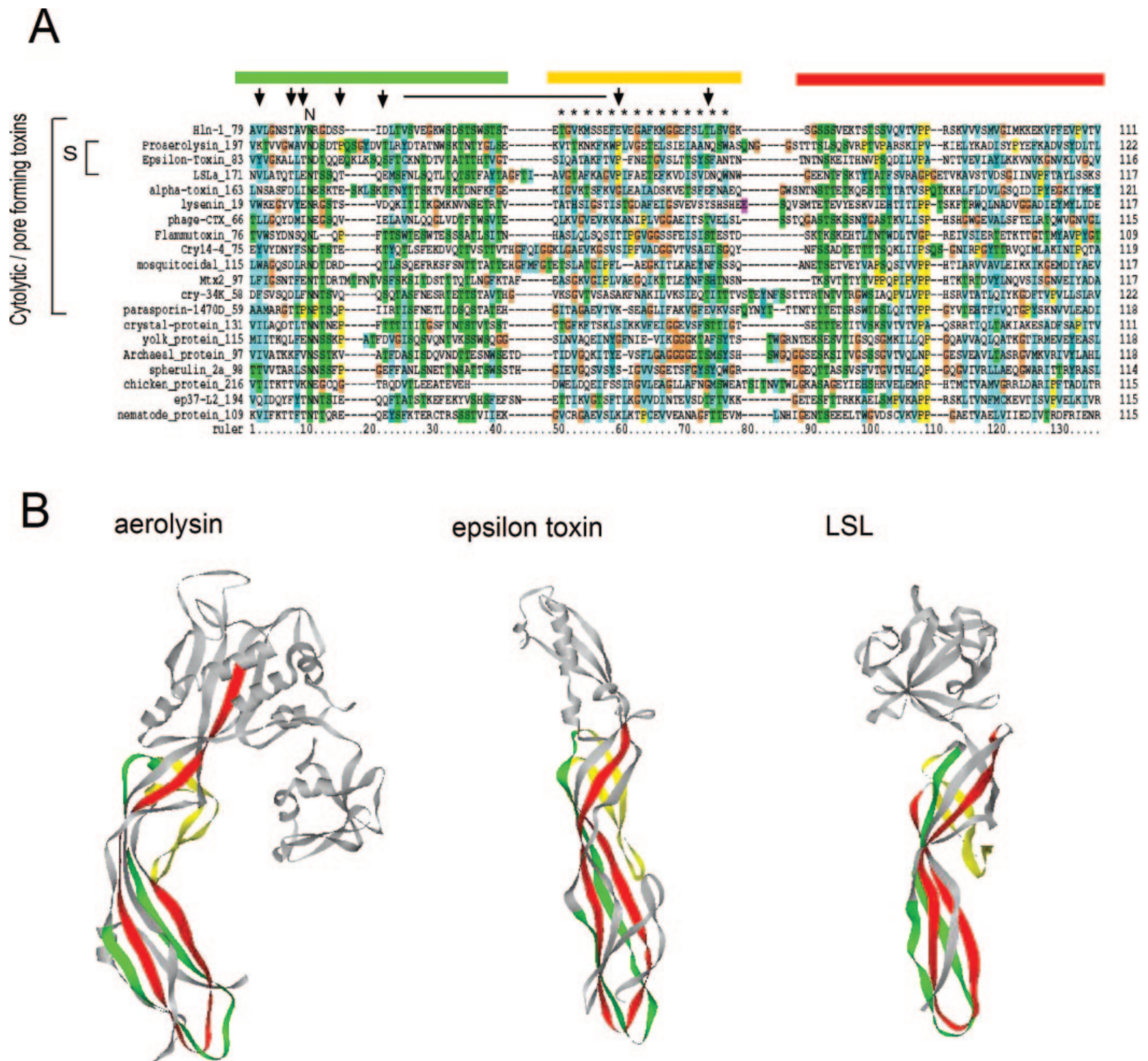


FIG. 5. **Hydralysins reveal a sequence similarity with βPFTs in pharmacologically active regions.** *A*, multiple sequence alignment of proteins detected by PSI-BLAST as being homologous to Hln, with the conserved sequence motifs marked above the sequence in *yellow* (transmembrane pore), *green* (upper flanking motif), and *red* (lower flanking motif). Conserved residues (based on the default Clustal X parameters) are colored according to their type (*blue*, hydrophobic; *green*, hydroxylated; *yellow*, proline). The residues predicted by Melton *et al.* (52) to line the transmembrane pore facing into the membrane are marked by *. Residues differing between Hln-1 and 2 are marked by *arrows*, and the region deleted in Hln-3 is marked by a *horizontal black line*. The asparagine residue conserved in all of the proteins is marked by *N*. The sequences of the toxins whose three-dimensional structure has been solved are marked by an *S*. For clarity only the sequence of Hln-2 has been shown. The accession numbers of the sequences are found under "Materials and Methods." *B*, structures of three βPFTs, with the sequence motifs described above highlighted in *yellow* (flexible transmembrane loop), *green*, and *red* (β-sandwich).

the β-sandwich of the pore-forming domain of the above mentioned proteins (Fig. 5*B*). Finally, the proline diad found in the middle of the second hydrophobic motif lies in one of the flexible loops of the β-sandwich of the pore-forming domain.

*Mutations in the Putative Transmembrane and Oligomerization Domains Are Responsible for the Difference between the Hln Isoforms*—Only a small number of amino acid substitutions are responsible for a significant change in activity between the hydralysin isoforms rHln-1 and 2, and several of these residues are found in the sequence motifs described above. Therefore, these two isoforms form an ideal model system for analyzing structure-function relationships of βPFTs; thus, we produced point mutants that change specific residues in Hln-2 to those found in Hln-1. First, we concentrated on residue 125, as it lies in the putative transmembrane domain of Hln. Unlike residue 140 (which also lies in the same region), the nonconservative change from glycine in Hln-2 to glutamate in Hln-1 is predicted to cause the insertion of a large, negatively charged group instead of a small, nonpolar one into the lumen of the pore, thus possibly changing the membrane-permeating activity of the protein. As expected, the paralytic and hemolytic activities of the mutant rHln-2$^{(G125E)}$ were much reduced compared with rHln-2 (Fig. 3, Table I), although not to the levels exhibited by rHln-1. This result indicates that the amino acid residue at position 125 is partly responsible for the differences between the isoforms. Conversely, the double mu-

tant rHln-2$^{(Q28E,V140L)}$, in which the amino acids at positions 28 and 140 of Hln-2 were changed to those found in Hln-1, revealed essentially the same paralytic and hemolytic activities as rHln-2, indicating that these residues do not contribute to the functional differences between the isoforms. Finally, the triple mutant rHln-2$^{(Q28E,G125E,V140L)}$, in which the central cluster between residues 72–95 (in which most of the differences between Hln-1 and Hln-2 are found, Fig. 1*C*) is the same as rHln-2, whereas the rest of the protein (including residues 28, 125, and 140) is the same as rHln-1, revealed essentially the same hemolytic and paralytic activities as the single mutant rHln-2$^{(G125E)}$, which are lowered compared with rHln-2 although not to the level of rHln-1. This suggests that the hydrophobic cluster between amino acids 72–95 (in which most of the differences between Hln-1 and Hln-2 are found, Fig. 1*C*) is also important for the pore-forming activity of the protein. It is noteworthy that the double and triple mutants revealed the same far UV-CD spectrum as the native rHln-2, indicating that they are correctly folded (Fig. 4*A*); thus, the differences in activity are presumably because of changes in the local environment around specific amino acids rather than to a general perturbation of the protein structure. Taken together, these results reveal that the local environment around residue 125, which is found in the predicted transmembrane domain, as well as the region between amino acids 72–95, part of which is homologous to the β-sandwich at the lower lobe of aerolysin, LSL, and εT, are important for the difference in activities between the Hln isoforms. The correlation between the paralytic and hemolytic activities suggests that both are caused by the same mechanism, pore formation.

## DISCUSSION

*Hydralysins, Novel βPFTs in Cnidaria*—Hydralysin was first described by us as a toxin from hydra that possesses both animal group-selective paralytic-neurotoxic and cell type-specific cytolytic activities but that does not originate from a delivery system such as the stinging cells (11). One way to explain such an assembly of pharmacological activities was to assume that Hln acts through a mechanism of receptor-mediated pore formation. Our interest in characterizing the biochemical-pharmacological activity of Hln is aimed at providing a working hypothesis as to the biological role of such a protein in the context of the hydra.

In this work, we have shown that Hlns can be defined as βPFTs based both on their sequences and on their structural and functional properties; they are water-soluble monomers, rich in β-structure, and upon encountering biological membranes they bind them, oligomerize, and form transmembrane pores (36, 51, 52, 54). Their cell type-specificity hints that membrane binding is mediated by a specific receptor, and the fact that lipid membrane components and carbohydrates do not inhibit the cytolytic effect of Hlns suggests that the receptor is a specific membrane protein, possibly located in specific membrane regions.

The biochemical characterization presented here reveals the uniqueness of Hlns as cnidarian pore-forming toxins, differentiating them from the well known actinoporin family (3, 8, 10) as well as from another possible non-cnidocystic toxin, coelenterolysin, which is known to require sphingomyelin as its receptor (38, 55). The wide diversity of Hln sequences expressed by the hydrae is accompanied by large differences in activity between these isoforms, including one subfamily (Hln-3) that does not seem to have the expected toxic-hemolytic activities. This suggests that in addition to their biochemical and morphological uniqueness, Hlns may also fulfill novel biological roles in cnidaria.

*A Sequence Signature for βPFTs*—While setting them apart from other cnidarian PFTs, the functional characterization of Hlns as βPFTs and their similarity to known bacterial and fungal PFTs such as aerolysin, AT, εT, and LSL broadens the scope of this family of proteins. Using a bioinformatic approach (data base searches and multiple alignment using several different algorithms), we suggest that Hlns share a conserved sequence signature with a wide array of diverse pore-forming toxins, including aerolysin, AT, εT, and LSL. This sequence signature corresponds to the "functional heart" of these PFTs, the pore-forming domain. The suggested sequence signature explains many of the observed differences between the Hln isoforms, such as the differences in hemolytic and paralytic activities between rHln-1 and 2, the inactivity of rHln-3, in which the deleted region spans part of the transmembrane domain, and the effect of the single mutation in residue 125 that is expected to face into the lumen of the transmembrane pore (Figs. 1*C*, 3, and 5). In addition, the region in which sequence similarity is observed is restricted to the pore-forming domain, suggesting that the receptor binding domain is a functionally and structurally autonomic module as suggested previously (48, 56). This would explain the similarity in the binding of all Hln isoforms (including the inactive isoform rHln-3) to erythrocyte membranes, as most of the differences between these isoforms are found in the suggested pore-forming region, whereas the rest of the proteins (presumably including the receptor binding domain) are similar. In addition, this would explain why the other βPFTs that reveal similarities in their mode of action (such as the size of the transmembrane pore) bind to different target cells. For example, aerolysin requires both the polypeptide moiety and the glycan core of glycosylphosphatidylinositol-anchored proteins (39), LSL specifically binds carbohydrates (29, 48), and lysenin (another PFT that reveals similarity to Hlns, see Fig. 5*A*) specifically binds sphingomyelin (57, 58).

The usefulness of the sequence signature described in Fig. 5*A* is that, in addition to detecting the similarity between pharmacologically and evolutionarily distinct proteins, it suggests the importance of specific conserved residues to the activity of the protein family. These include, apart from the suggested transmembrane domain, an asparagine residue conserved in all of the proteins, as well as the conserved tip of the second β-sandwich that contains proline residues in a region of hydrophobic and positively charged ones. Site-directed mutagenesis experiments performed on one or several of the proteins described in Fig. 5*A* could shed light on the functional roles of these residues.

Another point worth mentioning is that this sequence signature is shared by other proteins (also shown in Fig. 5) that have not previously been shown to act as PFTs. This can be experimentally validated using functional assays. We suggest that these proteins, which are found in diverse organisms, expressed in diverse tissues, and probably fulfill diverse biological roles (59, 60), may also be pore-forming proteins.

*What Is the Role of Hln in the Biology of Hydra?*—It appears that the sessile yet predatory cnidarians have achieved a high degree of specialization in the production and employment of pore-forming proteins (3). In several cases (including *H. magnipapillata*), it has been shown that the same organism produces more than one type of PFT, which presumably fulfil different biological roles (1). When dealing with the eco-chemical aspects of these toxins, two major differences between Hlns and the other cnidarian PFTs should be noted. First, members of all of the other cnidarian PFT families have been shown to originate from nematocysts (1), whereas Hln is clearly derived from non-nematocystic body tissue (11). Secondly, actinoporins, the most abundant cnidarian PFTs, recognize their target

membrane by binding sphingomyelin, which is replaced in anemone membranes by its phosphono analogue (38). This is in contrast to Hlns, which do not recognize sphingomyelin but rather a different, probably protein, receptor.

The fact that Hln can form pores in target membranes in a receptor-mediated manner led us previously to suggest that Hln can act as a defensive or offensive allomone despite the lack of a delivery system (11). However, a protein that creates pores in a specific manner can also perform a myriad of other biological roles in which the effect of the protein is endogenously directed toward the hydra and not prey or enemy organisms (61–63). Therefore, two major alternative hypotheses can explain the abundance of a soluble pore-forming toxin such as Hln in non-nematocystic hydra tissue. One, as described previously (11), is that Hlns fulfill an allomonal role, either deterring predators or prolonging the paralysis of prey in the gastrovascular cavity. In this case, the putative Hln receptor would be found on the target organism cells and the diversity of Hln toxins could reflect an evolutionary trend toward enhancing the specificity and activity of these toxins, as seen in other venomous organisms and pathogenic bacteria (34, 64). Alternatively, Hln could fulfil an endogenous role in the biology of the hydra, as shown for other pore-forming proteins (61–63). The putative receptor would then be found on the hydra cells themselves and allow the Hln pores to be formed in a specific controlled manner without causing general lysis of the hydra cells. In this case, the diversity of Hln isoforms could reflect a need to fine-tune the activity of these proteins, including the production of a non-active isoform (Hln-3) that can antagonize the effect of the active ones. Intense study is currently being performed to elucidate the biological role of this protein family.

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