Channel-Forming Activity of the Perforin N-Terminus and a Putative α-Helical Region Homologous with Complement C9†

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ABSTRACT: Cytolytic lymphocytes are endowed with a pore-forming protein called perforin. Recently, a cytolytic domain was located in the first 34 residues of the perforin N-terminus. It has been proposed that the first 19 residues are composed of a 3-domain structure including a putative amphipathic β-sheet and that the 19 residues are sufficient for cytolytic activity. This model has now been tested by synthesizing peptides covering different portions of the N-terminus, and testing their ability to lyse lipid vesicles or increase the conductance of lipid bilayers or plasma membranes. It was found that the putative β-sheet is indispensable for lytic activity and that the first 19 residues of the N-terminus are required for optimal lytic activity but that shorter peptides, containing only 16 residues, can form pores in lipid bilayers and cell membranes. A putative amphipathic α-helix from the central portion of perforin, homologous to complement C9, is nonlytic to lipid vesicles, but it can form pores in lipid bilayers. Taken together, these results support the model that the perforin N-terminus is important in initial pore formation and that the putative α-helical domain may be involved in subsequent perforin polymerization into large pores.

Cytotoxic T lymphocytes (CTL)[1] and natural killer (NK) cells lyse cancerous and virally infected cells through a contact-dependent mechanism. According to a widely held view, recognition of the target cell triggers exocytosis of the killer cell, which results in vectorial release of a number of putative cytotoxins from the killer cell granules. Prominent among these toxins is a 70-kDa pore-forming protein called perforin or cytolysin (Tschopp & Nabholz, 1990; Podack et al., 1991; Young, 1989; Henkart, 1985). In its monomeric form, perforin is thought to bind to the target cell surface, where it inserts into the plasma membrane and polymerizes into nonspecific pores of up to 20 nm in diameter. The target cell thus swells and subsequently bursts due to the colloid osmotic pressure that ensues.

On the basis of electron microscopy and immunoblot analysis, it was known that the tubular lesions produced by perforin are ultrastructurally and immunologically related to the pores formed by the membrane attack complex (MAC) of complement (Young et al., 1986). Sequence analysis of the cDNA clones for perforin and the MAC components subsequently confirmed that there is significant homology between these 2 groups of pore-forming proteins, which is largely restricted to a region of about 270 amino acids (Lichtenheld et al., 1988; Ishikawa et al., 1989; Shinkai et al., 1988; Kwon et al., 1989; Lowrey et al., 1989); 40 amino acids from the N-terminus and 100 from the C-terminus of perforin are unique to perforin. The central one-third of the perforin protein is conserved in the complement components C6 through C9, and contains four candidate membrane-spanning domains that could potentially form amphipathic α-helices or β-sheets (Kwon et al., 1989). However, recent attempts to locate a lytic domain in this region have failed, as none of the synthetic peptides based on the amino acid sequences of the four candidate domains are able to lyse cells or lipid vesicles (Ojcius et al., 1991). Nevertheless, a search outside of the homologous region did yield lytic peptides comprising the first 34 amino acids of the N-terminus of human and murine perforin. These peptides were also shown to form pores in lipid bilayers, strongly suggesting that the N-terminus is involved in perforin insertion and pore formation (Ojcius et al., 1991). As these peptides lack hydrophobic segments long enough to span the membrane, they would have to assume an amphipathic configuration and to polymerize in order to form pores. It was therefore proposed that the lytic activity could be due to the first 19 amino acids, which can be broken down into 3 separate domains, consisting of amino acids 1–5 (domain A), 6–16 (domain B), and 17–19 (domain C). Figure 1 shows the proposed structure of the perforin N-terminus in the presence of a lipid bilayer. Both flanking regions of the N-terminus (domains A and C) are clearly hydrophobic, while the central region (domain B), drawn as a potential β-sheet, is predominantly hydrophilic, having several charged (mostly basic) residues.

This finding, however, did not lead us to the complete structure of the perforin pore. Although peptides have been used as models to study specific ion channels and pore-forming proteins, the complete structure of such channels/pores is usually formed by multiple domains organized in a complex form (Montal, 1990; Lear et al., 1988). In the case of perforin, part of the focus has also been concentrated on the putative amphipathic α-helix comprising amino acids 189–218 (α_1)

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[1] Abbreviations: CF, carboxyfluorescein; CTL, cytotoxic T lymphocyte(s); MAC, membrane attack complex; PC, phosphatidylcholine, SUV, small unilamellar vesicle(s).

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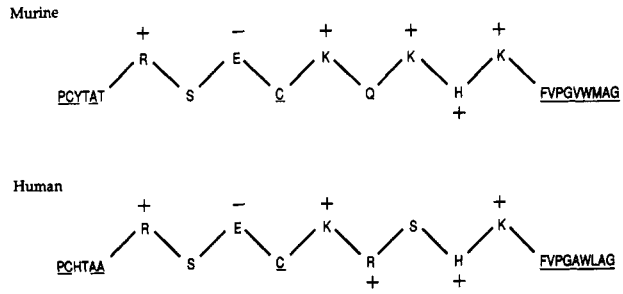

FIGURE 1: Model for the structure of the perforin N-terminus. Amino acid sequences (one-letter code) are given for the first 24 amino acids of the murine and human sequences. Strongly hydrophobic residues are underlined, and charged residues are indicated by a plus or minus sign.

(Lichtenheld et al., 1988; Ishikawa et al., 1989; Shinkai et al., 1988; Kwon et al., 1989; Lowrey et al., 1989). This is due not only to its potential amphipathic properties but also to the existence of a homologous domain in the complement proteins C6–C9 (Ishikawa et al., 1989; Kwon et al., 1989; Stanley & Luzio, 1988; Tschopp & Nabholz, 1990; Podack et al., 1991). Recently, published data have indeed suggested that, in the C9 molecule, the homologous domain contains an intramembrane region (Peitsch et al., 1990). It is thus necessary to design new experiments aimed at a better understanding of the pore structure of perforin and the mechanism whereby it causes damage to cell membranes.

We now describe the lytic activity and the pore-forming properties of various synthetic peptides covering different portions of the N-terminus. Moreover, in view of the possibility that other domains from the central portion of perforin and the homologous regions of complement could also be involved in protein polymerization, the putative membrane-spanning domain α_1 from perforin and the homologous regions of complement molecule C9 were investigated for their ability to form channels in a planar bilayer system.

Experimental Procedures

Cells and Peptide Synthesis. The murine cytolytic cell line CTLL-R8, the murine tumor cell lines P815 (mastocytoma) and EL-4 (T cell lymphoma), and the human tumor cell line K562 (erythromyeloid leukemia) were maintained as previously described (Jiang et al., 1990). Peptides were synthesized at the Sloan-Kettering Microchemistry Core Facility on an Applied Biosystems Model 431A automated peptide synthesizer using *N-tert*-butyloxycarbonyl amino acids by the Merrifield solid-phase method (Merrifield, 1963). Cysteines were not modified, but they were reduced with 2-mercaptoethanol before purification by HPLC. The peptides were purified by HPLC and then lyophilized. The purity was ascertained by amino acid analysis.

Preparation of Sonicated Vesicles and Measurement of Carboxyfluorescein (CF) Release. Small unilamellar vesicles (SUV) were made with phosphatidylcholine (PC, type IIS; Sigma, St. Louis, MO) by sonication in a water-bath sonicator for 30–60 min (Szoka & Papahadjopoulos, 1980) in the presence of 100 mM CF, 50 mM NaCl, and 10 mM Hepes, pH 7.4. Unencapsulated CF was removed by passing the SUV down a 5 × 200 mm Sephadex G-50 column with 150 mM NaCl/10 mM Hepes, pH 7.4. SUV were used at a final concentration of 0.01% (w/v) under constant stirring in a cuvette in an SLM Aminco 500C spectrofluorometer, and CF release was measured at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. SUV preparation and lysis were performed at room temperature (21–24 °C). For calibration of the fluorescence increase, 100% lysis was determined by adding 0.02% (final concentration) Triton X-100 into the cuvette.

Planar Bilayers and Whole Cell Experiments. Peptide-induced conductance increases in lipid bilayers were measured at room temperature in membranes formed by either the painting technique (Mueller et al., 1963) or the folding technique (Montal & Mueller, 1972). Asolecithin (type II, Sigma) was used at 15% in decane and 1% in hexane, respectively. In both cases, the cis compartment contained 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes, pH 7.4. The trans side was held at virtual ground and contained 5 mM NaCl, 150 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM Hepes, pH 7.4. Peptides were added to the cis side under constant stirring. Peptides were solubilized either in 1 mM EGTA (N-terminal peptides) or in 1 mM EGTA plus 6 M urea (α-peptides). In order to exclude pore formation due to contaminants, control experiments were always performed by adding buffer alone for at least 30 min before addition of peptides.

Whole cell voltage-clamp experiments were performed to room temperature using the standard patch-clamp technique (Hamill et al., 1981). Cells were kept in RPMI-1640 medium (Sigma) containing 0.1% bovine serum albumin (type V, Sigma) and 10 mM Hepes, pH 7.4, as described (Persechini et al., 1990). An EPC-7 amplifier (List Electronics, Darmstadt, Germany) was used to perform the voltage-clamp and current measurements. Patch pipets were heat-polished and filled with a solution containing 5 mM NaCl, 150 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM Hepes, pH 7.4. The final pipet resistance ranged from 5 to 10 MΩ. Peptides were added in a 1 mM EGTA solution either directly with a micropipet or by a hand-controlled microinjection system using another patch pipet filled with peptide solution and positioned at 5–100 μm from the cell surface.

Results

Lytic Activity of Various Peptides. A series of peptides spanning different regions of the human N-terminus were synthesized (Lichtenheld et al., 1988). The peptide originally described (Ojcius et al., 1991), Hu34, covers the first 34 amino acids of the N-terminus. Four new peptides, having at least 19 amino acids in length, are based on the human sequence, covering residues 16–38 (Hu38), residues 11–32 (Hu32), residues 5–24 (Hu24), and residues 1–19 (Hu19). The reasoning behind this choice was that only Hu19 would comprise the entire region hypothesized to be essential, containing domains A, B, and C (Figure 1). Hu24 would have more hydrophobic residues carboxy-terminal to domain C, but would completely lack domain A. Hu32 lacks domain A and half of domain B, while Hu38 was predicted to be nonlytic, as it contains neither domain A nor domain B. In addition, three other peptides were designed to estimate the minimal length requirement for lytic activity of the N-terminus: Hu17, Hu16, and Hu15 consisting of the first 17, 16, and 15 amino acids of the N-terminus, respectively.

The cytolytic potential of the several N-terminal peptides was compared to that of Hu34 for their ability to induce the leakage of CF from SUV (Table I and Figure 2). Hu19 was still able to lyse lipid vesicles, but the relative lytic potential decreased substantially as smaller peptides were tested (Table I). CF leakage can be detected shortly after peptide addition and reaches equilibrium in 5–10 min. These results show that although Hu34 has a higher lytic activity, amino acids 20–34 are not critical for membrane damage. Peptide Hu24 (residues 5–24) is also able to induce CF leakage from SUV, but pep-

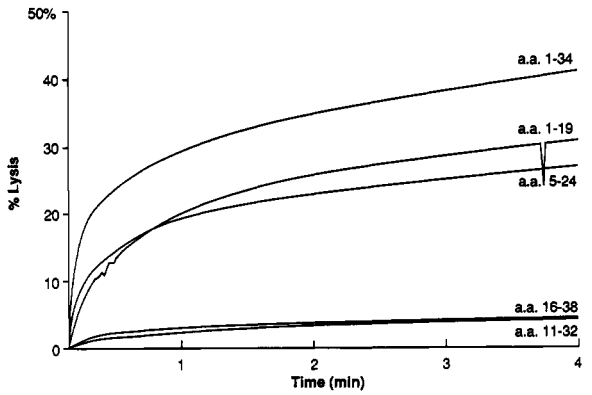

FIGURE 2: Lysis of CF-containing SUV induced by a 3 μM aliquot of the indicated peptides based on the sequence of the human N-terminus of perforin: Hu34 (residues 1–34), Hu19 (residues 1–19), Hu24 (residues 5–24), Hu38 (residues 16–38), and Hu32 (residues 11–32). Release of CF from the liposomes was measured as described under Experimental Procedures.

Table I: Relative Efficiency of CF Release from Liposomes Due to the Indicated Peptides

peptide	relative efficiency of lysis[a]	peptide	relative efficiency of lysis[a]
1–34 (Hu34)	10.0	104–132 (β_2)	0.0
1–19 (Hu19)	7.5	237–266 (β_1)	0.6
1–17 (Hu17)	3.2	402–422 (α_2)	0.4
1–16 (Hu16)	0.9	189–218 (α_1)	1.4
1–15 (Hu15)	0.4	516–534 (CP)	0.2
16–38 (Hu38)	0.2		
11–32 (Hu32)	0.2		

[a] CF release induced by a 3 μM aliquot of the indicated peptide was measured as described under Experimental Procedures. The relative efficiency of lysis was obtained from the fluorescence curves by measuring the percent lysis at 3 min, assigning an arbitrary value of 10 to the peptide with the highest lytic activity (Hu34) and normalizing the values of the other peptides with respect to HU34.

tides lacking either amino acids 1–10 or amino acids 1–15 (Hu32 and Hu38, respectively) have no activity in this system. In conclusion, the above results are consistent with the idea that domain B is important for N-terminal lytic activity.

Four regions from the central portion of perforin, bearing large homology with the MAC components, were also used: α_1 (HAYHRLISSYGTHFITAVDLGGRISVLTAL, residues 189–218) and α_2 (residues 402–422) would be amphipathic in the α-helical configuration, and β_1 (residues 237–266) and β_2 (residues 104–132) would be amphipathic in the β-sheet configuration. All of the above sequences have been previously reported in full (Ojcius et al., 1991). A C9 peptide displaying large homology to α_1 was also synthesized: α_{C9}, spanning residues 311–340 of the human C9 sequence (GEYFAFLETYGTHYSSSGSLGGLYELIYVL) (DiScipio et al., 1984). Finally, one peptide, CP, covering residues 516–534 of the perforin sequence (Ojcius et al., 1991), was chosen as a negative control. None of the four peptides showing homology with the MAC components (α_1, β_1, α_2, and β_2) nor the control peptide CP have significant lytic activity (Table I).

Pore Formation by N-Terminal Peptides. The ability to form ion pores in artificial bilayers and cell membranes has been associated with the cytolytic potential of perforin (Young, 1989; Young et al., 1986). Our recent experiments with the N-terminal peptides demonstrate that the first 34 amino acids of both the human and murine N-termini, Hu34 and Mu34, are able to form pores of 200–1500 pS in lipid bilayers (Ojcius et al., 1991). Here, we investigated first the action of Hu19

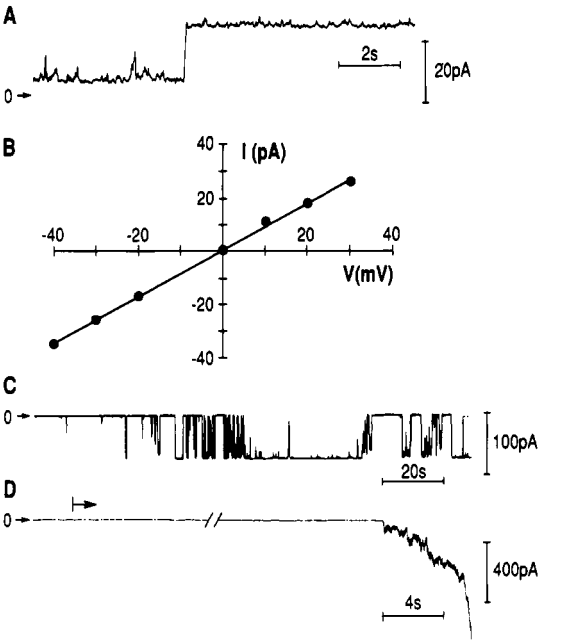

FIGURE 3: Conductance increases and pore formation due to Hu19. (A) Stable and steplike transition of approximately 1 nS at +20 mV. (B) I versus V curve of a stable pore similar to the one in (A); slope is 900 pS. (C) Large unitary channel activity of 1.9 nS in a membrane prepared by the folding technique; potential is −40 mV. (D) Conductance increase induced in an EL4 cell clamped at −30 mV. The peptide was continuously introduced at the cell surface using a microinjection pipet containing 0.5 mg/mL Hu19 and 1 mM EGTA (injection began at arrow; a 15-s interval was excised from the record).

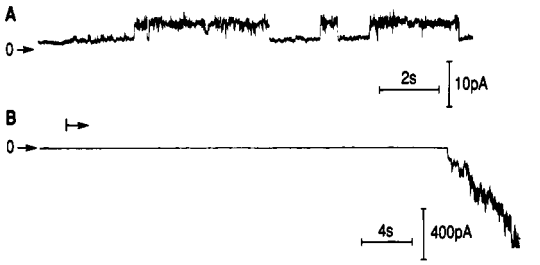

FIGURE 4: Conductance increases and pore formation due to Hu16. (A) Channels of 250 pS at +20 mV. (B) Conductance increase induced in an EL4 cell clamped at −30 mV. Peptide concentration and method of injection are the same as in Figure 3D.

on membranes (Figure 3). This peptide is able to form ion pores in lipid bilayers in a way that resembles the activity of the larger Hu34 peptide. Stable pores as well as unit-channel transitions with large conductances were observed (Figure 3A,C), but irregular and transient conductance changes were also frequently present. Conductance values ranged from 100 to 1900 pS, and the reversal potential was around 0 mV, indicating the absence of selectivity for K^+ over Na^+ (Figure 3B). When applied directly onto the surface of voltage-clamped cells, Hu19 is also able to induce irreversible increases in conductance (Figure 3D), a pattern also observed with Hu34 (not shown). These results show that Hu19 shares the main pore-forming properties of Hu34.

The relatively nonlytic peptide Hu16 (residues 1–16) was also tested in lipid bilayers and cell membranes. Single-channel conductance changes (Figure 4A) as well as stable steplike transitions (not shown) could be observed. Hu16 was also able to induce conductance increases in cells (Figure 4B). Conductance values ranged from 100 to 2000 pS, and the reversal potential was approximately 0 mV (not shown).

Pore Formation by Other Peptides of Perforin and Complement. In view of the already mentioned importance of

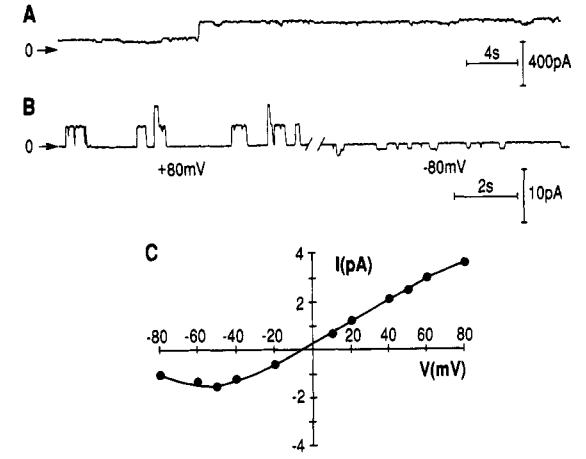

FIGURE 5: Pore-forming activity of the perforin peptide α_1. (A) Steplike transition of 60 pA (2 nS) induced by α_1 in a membrane that already had a stable pore of 1.3 nS. (B) Unitary channel activity of small conductance of α_1 at +80 mV and −80 mV. Note the strong rectification. (C) I versus V plot of the channel in (B). The conductance measured around 0 mV is 40 pS.

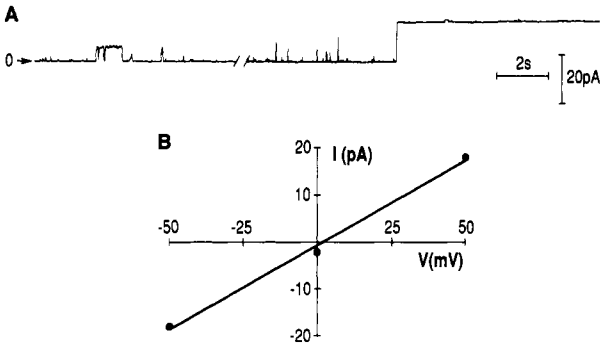

FIGURE 6: Pore-forming activity of the complement peptide α_{C9}. (A) Transitory and stable steplike transitions induced by α_{C9} at +50 mV; a 230-s interval was excised from the center of the record. (B) I versus V curve of the stable pore shown in (A); conductance is 360 pS.

perforin α_1, corresponding to amino acids 189–218, and its homologous region in C9, α_{C9}, we decided to use lipid bilayers in order to test directly the ability of these peptides to form ion pores (Figures 5 and 6). Although α_1 displayed negligible lytic activity in the liposome assay (Table I), it formed ion pores with variable size and kinetic properties in the planar bilayer system. Stable and steplike transitions were observed (Figure 5A) as well as irregular and transient conductance changes (not shown). Pore conductances ranged from 250 to 1500 pS, and the reversal potential was close to 0 mV, similar to the case for the N-terminal peptides. On rare occasions, a small and regular channel with unitary conductance of 40–60 pS was observed (Figure 5B). The $I \times V$ curve of this channel is linear between −40 and +80 mV, but a strong rectification was observed between −40 and −80 mV (Figure 5B,C).

The complement peptide α_{C9}, like α_1, was also found to be ineffective at lysing SUV (not shown). In the lipid bilayer system, however, α_{C9} forms ion pores, and the pattern obtained is essentially the same as seen for the other peptides tested, i.e., irregular conductance variations and steplike stable transitions, with conductance values ranging from 100 to 800 pS and a reversal potential around 0 mV (Figure 6A,B). Small and regular unitary transitions like the ones of Figure 5B for α_1 were not observed for α_{C9}.

Discussion

The lytic activity and pore-forming properties of several synthetic peptides from lymphocyte perforin and complement molecules were investigated using SUV, whole cells, and lipid bilayers. These experiments were performed in order to identify domains with the potential to form part of the intramembrane pores associated with the native proteins. We focused our attention on two regions of the perforin molecule: the N-terminus and a central domain called α_1 (residues 189–218). Evidence for the involvement of the N-terminal region came from our own previous data, which showed that the first 34 residues of the murine and human perforin N-termini have large lytic activity (Ojcius et al., 1991). On the other hand, the participation of the α_1 domain had been previously proposed by us and others on the basis of its putative amphipathic α-helix structure and its homology with complement molecules (Lichtenheld et al., 1988; Ishikawa et al., 1989; Shinkai et al., 1988; Kwon et al., 1989; Stanley & Luzio, 1988). The experiments with N-terminal peptides were aimed at uncovering the most important domains involved in pore formation and their possible secondary structure inside the membrane. The experiments with α_1 and α_{C9} were also performed in order to test experimentally the hypothesis that they can assume an amphipathic α-helical configuration, and thus that they can participate in the process of pore formation (Ojcius & Young, 1991).

The experiments described in Figures 2 and 3 clearly demonstrate that peptide Hu19 preserves the ability to lyse lipid vesicles and to make pores in lipid bilayers in a way similar to Hu34 (Ojcius et al., 1991). Peptide Hu24 is still able to lyse SUV, but peptides Hu32 and Hu38 are nonlytic, indicating that domain B, corresponding to residues 6–16, but not domain A is necessary for membrane disruption. These results are consistent with the previously proposed three-domain structure for Hu19 (Ojcius et al., 1991). The overall net charge of the N-terminal peptide (19 amino acids) is +5 for the human sequence and +4 for the murine sequence. In a β-sheet configuration, the murine central domain could be viewed as an imperfect amphiphile, since one side of the β-sheet is composed of strongly hydrophilic residues, while the other side contains residues that are either moderately hydrophobic (cysteine and histidine) or moderately polar (serine and glutamine) (Taylor & Kaiser, 1987). The sequence of the human domain B deviates even further from an idealized amphiphile, since a strongly polar residue, arginine, is found on the "nonpolar" side of the β-sheet. This deviation may not be critical, however, since interactions between proteins and membranes are not as specific as between protein receptors and protein ligands (Taylor & Kaiser, 1987). Furthermore, amphipathic β-sheets have a tendency to self-aggregate (Osterman & Kaiser, 1985), lending support to the model suggested here. Self-aggregation is also an important feature of perforin (Young, 1989), whose monomers must polymerize into large pores. An additional feature of β-sheets is that they can traverse the membrane as long as they are at least 10 amino acids long (Kleffel et al., 1985). We conclude that the domain consisting of the first 19 residues of perforin is cytolytic and pore-forming and we propose that the core of the pore may consist of a polymer of amphipathic β-strands formed by domain B.

Lipid bilayer experiments performed with α_1 and α_{C9} showed that these homologous peptides are also able to form ionic pores. The most probable secondary structure of these peptides compatible with a pore structure is an amphipathic α-helix. Our data are indeed in experimental agreement with the proposal that they form pores inside lipid bilayers by assuming an α-helical conformation (Lichtenheld et al., 1988; Ishikawa et al., 1989; Shinkai et al., 1988; Kwon et al., 1989; Stanley

& Luzio, 1988). The pore would consist of multiple units in such a way that the charged residues face the central aqueous region and the less polar residues face the acyl chains of the lipid bilayer. These results are also consistent with the possibility that the perforin N-terminus is involved in the initial insertion of the protein into the membrane and, together with α_1 and possibly other amphipathic domains, in subsequent polymerization of perforin into the large transmembrane pores. In agreement with this model, it has recently been shown that a C9 segment overlapping with α_{C9} traverses the membrane after pore formation, suggesting that α_1 may also span the membrane in the perforin pore (Peitsch et al., 1990). The fact that, in contrast to perforin, neither Hu19 nor Hu34 is hemolytic (data not shown) is a clear demonstration that the complete pore structure is still unsolved.

An important question raised by our experiments is the relationship between pore formation and lytic activity. Our data show that at least three peptides (Hu16, and α_1, and α_{C9}) are able to form pores in lipid bilayers even though they have little or no lytic potential directed to liposomes. Hu16 is a peptide that contains domains A and B but not domain C of the original Hu19 peptide. Consistent with the proposed role for domain B, this peptide is able to form ion pores in lipid bilayers and even increase the membrane conductance of whole cells (Figure 4). However, Hu16 is not as effective at lysing SUV. Because of the great variability observed in conductance values and patterns, we are not able to derive any mechanistic explanation for the distinct lytic activities displayed by the 16- and 19-mer peptides. Nevertheless, stable large-conductance pores like the ones observed with Hu34 and Hu19 (Figure 3A,C) were rarely observed with Hu16 under our conditions. Pores formed by Hu16 are also more unstable and difficult to observe at higher holding potentials. However, these observations could not completely explain the absence of lytic activity associated with the 16-mer peptide, since the α-peptides of perforin and C9 display large and stable pores but are similarly nonlytic to SUV. It thus seems clear that the ability to form pores in lipid bilayers and even in cell membranes is not a sufficient condition for a peptide to be lytic. Other factors such as ion selectivity, pore size and stability, and peptide concentrations have to be considered. The α-peptides are soluble only in high concentrations of urea, and it is reasonable to assume that they may self-aggregate in physiological buffers, decreasing their effective concentration. It may well be that, under special conditions, the insertion of some of these peptides into lipid membranes might be favored and lysis could be observed. A final word of caution should also be raised concerning the fact that we are studying isolated peptides that may have a different behavior when part of the native protein. Thus, although our data demonstrate that the peptides studied here can in fact form pores, further experiments are needed to establish whether they have the same behavior in the native protein. The analysis of pore-forming versus lytic properties of such peptides will also help us to understand the behavior of the pores formed by native perforin and other lytic molecules.

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