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Structure-Activity Determinants in Paneth Cell α-Defensins

LOSS-OF-FUNCTION IN MOUSE CRYPTDIN-4 BY CHARGE-REVERSAL AT ARGININE RESIDUE POSITIONS*

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Paneth cells secrete microbicidal enteric α-defensins into the small intestinal lumen, and cryptdin-4 (Crp4) is the most bactericidal of the mouse α-defensin peptides *in vitro*. Here, site-directed Arg to Asp mutations in Crp4 have been shown to attenuate or eliminate microbicidal activity against all of the bacterial species tested regardless of the Arg residue position. R31D/R32D charge-reversal mutagenesis at the C terminus and mutations at R16D/R18D, R16D/R24D, and R18D/R24D in the Crp4 polypeptide chain eliminated *in vitro* bactericidal activity, blocked peptide-membrane interactions, as well as Crp4-mediated membrane vesicle disruption. Lys for Arg charge-neutral substitutions in (R16K/R18K)-Crp4 did not alter the bactericidal activity relative to Crp4, showing that bactericidal activity appears not to require the guanidinium side chain of Arg at those two positions. Partial restoration of (R31D/R32D)-Crp4 bactericidal activity occurred when an electropositive Arg for Gly substitution was introduced at the peptide N terminus and the (G1R/R31D/R32D)-Crp4 peptide exhibited intermediate membrane binding capability. Also, the loss of peptide bactericidal activity in (G1D/R31D/R32D)-Crp4 and (R16D/R24D)-Crp4 mutants corresponded with diminished phospholipid vesicle disruptive activity. Fluorophore leakage from anionic phospholipid vesicles induced by the charge-reversal variants was negligible relative to Crp4 and lower than that induced by pro-Crp4, the inactive Crp4 precursor. Thus, Arg residues function as determinants of Crp4 bactericidal activity by facilitating or enabling target cell membrane disruption. The role of the Arg residues, however, was surprisingly independent of their position in the polypeptide chain.

Paneth cells at the base of the crypts of Lieberkühn in the small intestine secrete apically oriented granules as components of innate immunity. The secretory granules are discharged in response to cholinergic stimulation or exposure to bacteria or their antigens (1–3), and they contain several antimicrobial peptides and proteins (4) including lysozyme (5–7), secretory phospholipase A_2 (8), angiogenin-4 (9), and α-defensins (10–12).

The α-defensins are cationic, amphipathic 3–4-kDa peptides with a defining tridisulfide arrangement and β-sheet polypeptide backbone that have a broad spectrum of antimicrobial activities (13–15). For example, mouse α-defensins, termed cryptdins (Crps),[1] account for ~70% of the bactericidal peptide activity in secretions elicited from Paneth cells (3) and Crp4 is the most potent of the known mouse α-defensin peptides (16, 17). Also, Crps became implicated as components of mouse innate enteric immunity *in vivo* when mice that lacked matrix metalloproteinase-7, the pro-Crp-activating enzyme, were shown to have impaired host defense against oral infections (18, 19). Remarkably, mice transgenic for human Paneth cell α-defensin HD5 expressed the minigene specifically in Paneth cells and they were immune to oral infection by high doses of *Salmonella enterica serovar Typhimurium* (*S. typhimurium*) (20). Accordingly, an understanding of structure-activity relationships in α-defensins will improve the understanding of mucosal innate immune mechanisms.

To investigate the role of α-defensin primary structure in innate immunity, amino acid substitutions that alter charge were introduced into the Crp4 peptide and tested for effects on microbicidal activity and on Crp4-membrane interactions. Regardless of the site of mutagenesis, charge-reversal substitutions at Arg positions altered Crp4 bactericidal activity profoundly and the loss of activity correlated directly with quantitative effects on peptide binding to phospholipid vesicles and with peptide-induced vesicular permeabilization.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Crp4 Peptide Variants—Recombinant Crp4 peptides were expressed in *Escherichia coli* as N-terminal His_6-tagged fusion proteins from the EcoRI and SalI sites of the pET28a expression vector (Novagen, Inc., Madison, WI) as described previously (21, 22). The Crp4-coding cDNA sequences were amplified using forward primer [ER1-Met-C4-F], 5′-GCGCGAATTCATCGAGGGAAGGATGGGTTTGTTATGCTATGT-3′, paired with reverse primer [pMALCrp4-R], 5′-ATATATGTCGACTCAGCGACAGCAGAGCGTGTACAATAAATG-3′.

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[1] The abbreviations used are: Crp, cryptdin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; %CR, percent colorimetric response; PIPES, 1,4-piperazinediethanesulfonic acid; CFU, colony forming units; PDA, polydiacetylene; LUV, large unilamellar phospholipid vesicles; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid.

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N-terminal Variants—To introduce substitutions at the N terminus, the common reverse primer, [pMALCrp4-R], was paired with the following forward primers: (G1D)-Crp4, [ER1-Met-Gly1AspC4-F], 5′-GCGCGAATTCATCGAGGGAAGGATGGACTTGTTATGCTATTGT-3′; (G1V)-Crp4, [ER1-Met-Gly1ValC4-F], 5′-GCGCGAATTCATCGAGGGAAGGATGGTTTTGTTATGCTATTGT-3′; and (G1R)-Crp4, [ER1-Met-Gly1ArgC4-F], 5′-GCGCGAATTCATCGAGGGAAGGATGCGCTTGTTATGCTATTGT-3′, as described previously (23, 24).

Mutagenesis at Arg Residue Positions—Mutations were introduced into Crp4 molecules by PCR as described previously (21). In the first reaction, a mutant forward primer, *e.g.* Crp4-R16D-F, containing the mutant codon flanked by three natural codons was paired with reverse primer, [pMALCrp4-R], the normal reverse primer at the 3′-end of the desired sequence. In the second reaction, the mutant reverse primer, Crp4-R16D-R, the reverse complement of the mutant forward primer, was paired with the normal forward primer, [ER1-Met-C4-F], at the 5′ end of Crp4. After amplification at 95 °C for 5 min followed by successive cycles at 60 °C for 1 min, 72 °C for 1 min, and 94 °C for 1 min for 40 cycles, samples of purified products from reactions 1 and 2 were combined as templates in PCR in the third reaction using the Crp4 external primers ER1-Met-C4-F and SLpMALCrp4R as amplimers. All of the mutated Crp4 constructs were verified by DNA sequencing, subcloned into pET28a plasmid DNA (Novagen, Inc.), and transformed into *E. coli* BL21(DE3)-CodonPlus-RIL cells (Stratagene) for recombinant expression. The *underlined* codons in forward primers denote Met codons introduced upstream of each peptide N terminus to provide a CNBr cleavage site (21, 22, 24).

The following C-terminal variants of Crp4 were prepared as noted above with the following mutant reverse primers: (R31D/R32D)-Crp4, [3′PDD-Crp4R], 5′-ATATATGTCGACTGTTCAGTCGTCGGGGCAGCAGTACAA-3′; (R31G/R32G)-Crp4, [3′PGG-Crp4R], 5′-ATATATGTCGACTGTTCACCCCCCGGGGCAGCAGTACAA-3′; (R31V/R32V)-Crp4, [3′PVV-Crp4R], 5′-ATATATGTCGACTGTTCAAACAACGGGGCAGCAGTACAA-3′; (ΔR31/R32)-Crp4, [3′PXX-Crp4R], 5′-ATATATGTCGACTGTTCAGGGGCAGCAGTACAA-3′. Similarly, Crp4 variants with C-terminal extensions resembling that of the Crp4(B6b) peptide (21) were prepared using the following primers: (Arg-33/Arg-34)-Crp4, [3′-C4-PRRRR], 5′-GCGCGTCGACTCAGCGGCGGCGGCGTGGGCAGCAGTACAAAAATCG-3′; (Δ-Pro-30/Arg-33/Arg-34)-Crp4, [3′-C4-RRRR], 5′-GCGCGTCGACTCAGCGGCGGCGGCGGCAGCAGTACAAAAATCG-3′.

The following primers were used to prepare (R16D/R18D)-Crp4 as noted above: [Crp4-R16D/R18D-F], 5′-AGAGGAGAAGACGTTGACGGGACT-3′, and [Crp4-R16D/R18D-R], 5′-AGTCCCGTCAACGTCTTCTCCTCT-3′. Primers [Crp4-R16K/R18K-F], 5′-AGAGGAGAAAAAGTTAAAGGGACT-3′, and [Crp4-R16K/R18K-R], 5′-AGTCCCTTTAACTTTTTCTCCTCT-3′ were used to prepare (R16D/R18D)-Crp4 and (R16K/R18K)-Crp4, respectively, by the same strategy noted above. To prepare (R16D/R24D)-Crp4 and (R18D/R24D)-Crp4 variants, (R24D)-Crp4 was prepared first using [Crp4-R24D-F], 5′-ACTTGTGGAATAGACTTTTTGTA-3′, and [Crp4-R24D-R], 5′-TACAAAAAGTCTATTCCACAAGT-3′ as mutagenizing primers as above. Subsequently, the (R24D)-Crp4 amplification product was used as template to prepare (R16D/R24D)-Crp4 and (R18D/R24D)-Crp4 double mutants with the following respective primer sets: [Crp4-R16D-F], 5′-GGAGAAGACGTTCGTGGGACT-3′; [Crp4-R16D-R], 5′-AGTCCCACGAACGTC TTCTCC-3′; [Crp4-R18D-F], 5′-GGAGAACGAGTTGACGGGACT-3′; and [Crp4-R18D-R], 5′-AGTCCCGTCAACTCGTTCTCC-3′.

Purification of Recombinant Crp4 Proteins—Recombinant proteins were expressed at 37 °C in Terrific Broth medium by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 37 °C, lysed by sonication in 6 M guanidine-HCl in 100 mM Tris-Cl (pH 8.1), and clarified by centrifugation (21, 22, 24). His-tagged Crp4 fusion peptides were purified using nickel-nitrilotriacetic acid (Qiagen) resin affinity chromatography from bacterial cells lysed in 6 M guanidine-HCl, 20 mM Tris-HCl (pH 8.1) as described previously (21). After CNBr cleavage, Crp4 peptides were purified by C18 reverse-phase high performance liquid chromatography and quantitated by bicinchoninic acid (Pierce) and molecular masses of purified peptides were determined using matrix-assisted laser desorption ionization mode mass spectrometry (Voyager-DE MALDI-TOF, PE-Biosystems, Foster City, CA) in the Mass Spectroscopy Facility, Department of Chemistry, University of California (Irvine, CA).

Bactericidal Peptide Assays—Recombinant peptides were tested for microbicidal activity against *E. coli* ML35, *S. typhimurium* (*PhoP*−), *Vibrio cholera*, *Staphylococcus aureus* 710a, and *Listeria monocytogenes* 104035 (25). Bacteria growing exponentially in trypticase soy broth at 37 °C were deposited by centrifugation at 1700 × *g* for 10 min, washed in 10 mM PIPES (pH 7.4), and resuspended in 10 mM PIPES (pH 7.4) supplemented with 0.01 volume of trypticase soy broth (21, 22). Bacteria (~5×10^6 CFU/ml) were incubated with test peptides in 50 μl for 1 h in a shaking incubator at 37 °C, and then 20-μl samples of incubation mixtures were diluted 1:100 with 10 mM PIPES (pH 7.4) and 50 μl of the diluted samples were plated on trypticase soy agar plates using an Autoplate 4000 (Spiral Biotech Inc., Bethesda, MD). Surviving bacteria were counted as colony forming units per milliliter after incubation at 37 °C for 12–18 h.

Peptide Interactions with Phospholipid/Polydiacetylene (PDA) Mixed Vesicles—Crp4 and three mutant peptides with varied Arg charge reversals were investigated for their relative membrane perturbation activities. Colorimetric phospholipid/PDA vesicles were prepared using dimyristoylphosphatidylcholine (Sigma) as described previously (22). Vesicles consisting of two parts phospholipid to three parts PDA were prepared by dissolving the phospholipids and 10,12-tricosadiynoic acid monomer (GFS Chemicals, Powell, OH) together in chloroform/ethanol (1:1), drying *in vacuo* to constant weight, suspending in H_2O, probe-sonicating for 3 min at 70 °C, and incubating overnight. PDA was polymerized by irradiation at 254 nm for 10–20 s, producing suspensions with an intense blue appearance. Peptides (0.2–20 μM) were added to 60 μl of vesicle solutions (0.5 mM total lipid) in 25 mM Tris-HCl (pH 8) and diluted to 1 ml, and spectra were acquired at 28 °C between 400 and 700 nm on a Jasco V-550 spectrophotometer (Jasco Corp., Tokyo, Japan) using a 1-cm optical path cell. Blue-to-red color transitions within the vesicle solutions, defined as the percent colorimetric response (%CR), were calculated as described previously (22, 26).

Fluorescence-based Vesicle Leakage Assays of Peptide-Membrane Interactions—Crp4, (R31D/R32D)-Crp4, (G1D/R31D/R32D)-Crp4, (R16D/R24D)-Crp4, and pro-Crp4 were tested for their relative abilities to induce leakage from large unilamellar phospholipid vesicles (LUV) of defined composition. LUV of palmitoyl-oleoyl-phosphatidylglycerol (Avanti Polar Lipids, Birmingham, AL) were loaded with a fluorophore/quencher system (27, 28). Aqueous lipid solutions consisting of 17 mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS, Molecular Probes, Eugene, OR), 60.5 mM DPX (*p*-xylene-bis-pyridinium bromide, Molecular Probes), 10 mM HEPES, 31 mM NaCl, and 19.5 mM NaOH (260 mOSM/liter, pH 7.4) were vortexed, frozen, and thawed for five cycles and then extruded through 100-nm pore size polycarbonate filters. Vesicles were separated from unencapsulated ANTS/DPX by gel-permeation chromatography with 130 mM NaCl, 10 mM HEPES, and 4.5 mM NaOH (260 mOSM/liter, pH 7.4) as column eluant. Vesicular suspensions diluted with eluant buffer to ~74 μM of total lipid were incubated with peptides at ambient temperature. Time-dependent fluorescence produced by ANTS release was monitored at 520 nm (excitation at 353 nm) as described previously (24, 28). The kinetics of vesicular leakage was a function of peptide concentration, and equilibrium was attained ≤4 h. Thus, 4-h values were expressed relative to fluorescence obtained by vesicular solubilization with Triton X-100.

RESULTS

Bactericidal Activities of Recombinant Crp4 Variants—Recombinant peptides (Fig. 1*A*) were prepared using the pET-28 vector system (21). All of the peptides were purified to homogeneity by reverse-phase high performance liquid chromatography after chemical cleavage with CNBr, migrating as single entities and as expected relative to native Crp4 and pro-Crp4 molecules (21, 24). Purity was verified by analytical reverse-phase high performance liquid chromatography (data not shown) and acid-urea-PAGE analyses (Fig. 1*B*). The molecular masses of individual recombinant peptides were determined by MALDI-TOF mass spectrometry, and they matched the respective theoretical values. Thus, the purified recombinant peptides were homogeneous (Fig. 1*B*) and their biochemical features were consistent with the modifications introduced to the natural Crp4 molecule (29).

As a first step toward investigating structure-activity relationships in Crp4, the *in vitro* microbicidal activities of Crp4, pro-Crp4, and selected N-terminal Crp4 variants were measured against a panel of bacterial test species (data not shown) (24). The overall bactericidal activities of the N-terminal variants differed only slightly over a range of 0.6–10 μg/ml of peptide as observed previously (24). In contrast to the bactericidal activities of these mature Crp4 peptide variants, pro-Crp4 lacks microbicidal activity because matrix metalloproteinase-

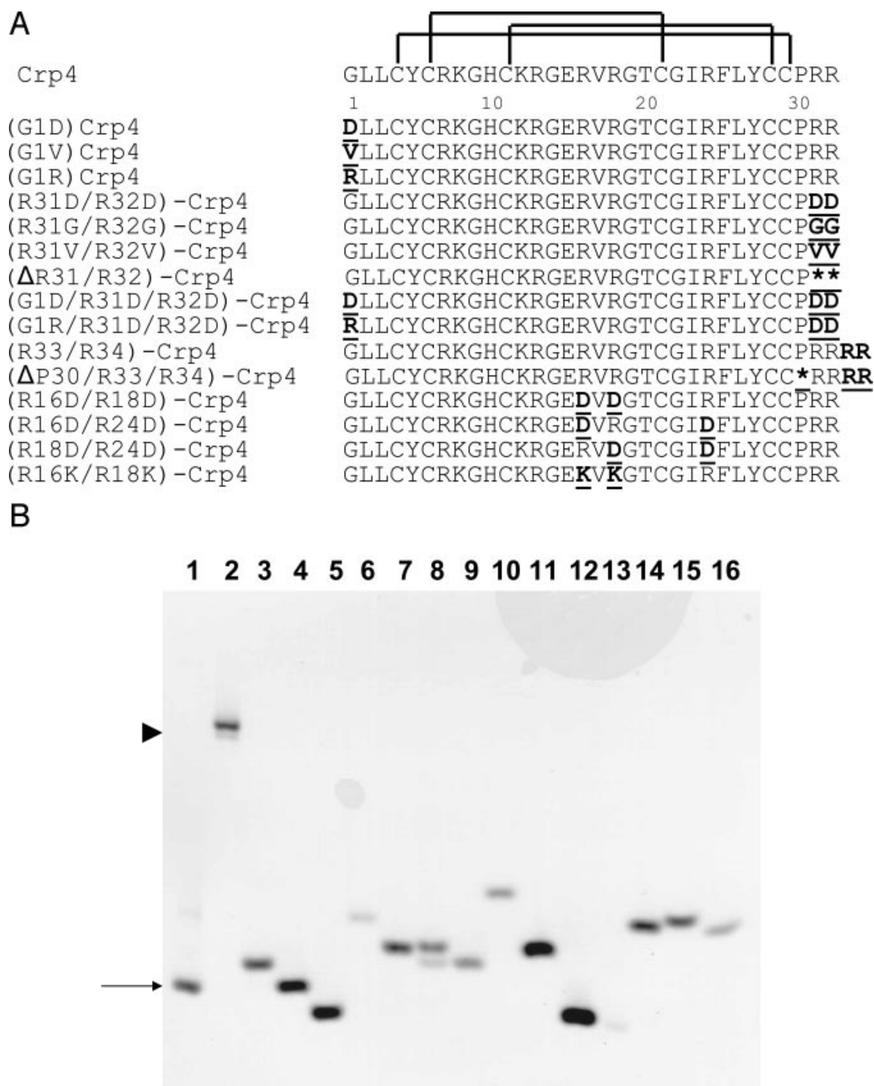

FIG. 1. **Recombinant Crp4 and Crp4 variants prepared by site-directed mutagenesis.** *Panel A*, the primary structures of the recombinant Crp4 peptides prepared and investigated in these studies are aligned with the exception of pro-Crp4, which is not shown. *Numerals* below the Crp4 sequence refer to residue positions at the beginning of N-terminal sequences detected in the digests numbered with the native Crp4 N-terminal Gly as residue position 1. Altered residues in mutant Crp4 peptides are shown in *underlined boldface* with *asterisks* representing deleted residues. The conserved α-defensin intramolecular disulfide bond arrangement is identified by the connecting bars above the Crp4 sequence. *Panel B*, 2-μg samples of purified recombinant Crp4, Crp4 variants, and pro-Crp4 were resolved by acid-urea-PAGE and stained with Coomassie Blue. *Lane 1*, pro-Crp4 (*arrowhead*); *lane 2*, Crp4 (*lower arrow*); *lane 3*, (G1D)-Crp4; *lane 4*, (G1V)-Crp4; *lane 5*, (G1R)-Crp4; *lane 6*, (R31D/R32D)-Crp4; *lane 7*, (R31G/R32G)-Crp4; *lane 8*, (R31V/R32V)-Crp4; *lane 9*, (ΔArg-31/Arg-32)-Crp4; *lane 10*, (G1D/R31D/R32D)-Crp4; *lane 11*, (G1R/R31D/R32D)-Crp4; *lane 12*, (Arg-33/Arg-34)-Crp4; *lane 13*, (ΔPro-30/Arg-33/Arg-34)-Crp4; *lane 14*, (R16D/R18D)-Crp4; *lane 15*, (R16D/R24D)-Crp4; and *lane 16*, (R18D/R24D)-Crp4.

7-mediated proteolysis is required for Crp4 activation (18, 19, 21). Although the (G1D)-Crp4 peptide was consistently less active at ≤5 μg/ml of peptide (data not shown), modifications at the Crp4 N terminus had very modest effects on bactericidal activity (24).

Modifications at the Crp4 C terminus—Because the Cys-1 to Cys-6-disulfide bond common to α-defensins places the Crp4 N and C termini in proximity (13), we prepared a series of Crp4 peptide variants with charge-modified C termini including (R31D/R32D)-Crp4, (R31G/R32G)-Crp4, (R31V/R32V)-Crp4, and (ΔR31/R32)-Crp4 (Fig. 1). Bactericidal activity assays of these variants showed that deleting the two C-terminal Arg residues or converting them to Gly or Val altered peptide activity variably depending on the bacterial target species (Fig. 2, compare *B* with *D*). In contrast, however, the R31D/R32D charge-reversal mutation eliminated bactericidal activity, even against the *S. typhimurium PhoP−* strain (data not shown), which is very sensitive to membrane-active cationic peptides (Fig. 2) (30, 31). As expected, wild-type *S. typhimurium* and the *PhoP*-constitutive (*PhoP*^{c}) CS022 strain were less sensitive to all of the peptides (data not shown).

Additional (R31D/R32D)-Crp4 variants with modified N termini were prepared and analyzed including an Arg_1 for Gly_1 substitution ((G1R/R31D/R32D)-Crp4) and an Asp_1 for Gly_1 variant ((G1D/R31D/R32D)-Crp4). Under the conditions of the assays, no bactericidal activity was detected with the (G1D/R31D/R32D)-Crp4 peptide but the (G1R/R31D/R32D)-Crp4 activity improved with the added electropositive side chain at the N terminus (Fig. 3). These findings showed that modifications of combined N- and C-terminal charge can modulate Crp4 bactericidal activity.

To investigate the effect of added C-terminal Arg residues on Crp4 bactericidal activity, additional modifications were introduced at the Crp4 C terminus. C57/BL6 mice express a Crp4 variant (Crp4(B6b)) that has four Arg residues at its C terminus (21), but because Crp4 and Crp4(B6b) differ at several other positions, the specific role of C-terminal charge in these peptides cannot be compared directly. Accordingly, the natural Crp4 C terminus was extended by two Arg residues to place the Crp4-(B6b) C terminus in the context of the Crp4 primary structure. The peptide, (Arg-33/Arg-34)-Crp4, had greater *in vitro* bactericidal peptide activity than Crp4 against *E. coli* and wild-type and *PhoP*^{c} *S. typhimurium* strains (Fig. 4), suggesting that C-terminal charge could be a determinant of bactericidal activity. However, the Crp4(B6b) molecule lacks the Pro-30 residue present in Crp4 between Cys-29 and Arg-31. Therefore, the effect of the Pro-30 residue was tested by assaying the bactericidal activity of (ΔPro-30/Arg-33/Arg-34)-Crp4, a variant of (Arg-33/Arg-34)-Crp4 from which Pro-30 was deleted (Fig. 1). (ΔPro-30/Arg-33/Arg-34)-Crp4 was less active than Crp4 and (Arg-33/Arg-34)-Crp4 against *E. coli* and *S. typhimurium PhoP*^{c} (Fig. 4), and the results in Fig. 4 also are representative of the relative bactericidal activities of these peptides against *V. cholera*, *S. aureus*, and *L. monocytogenes*

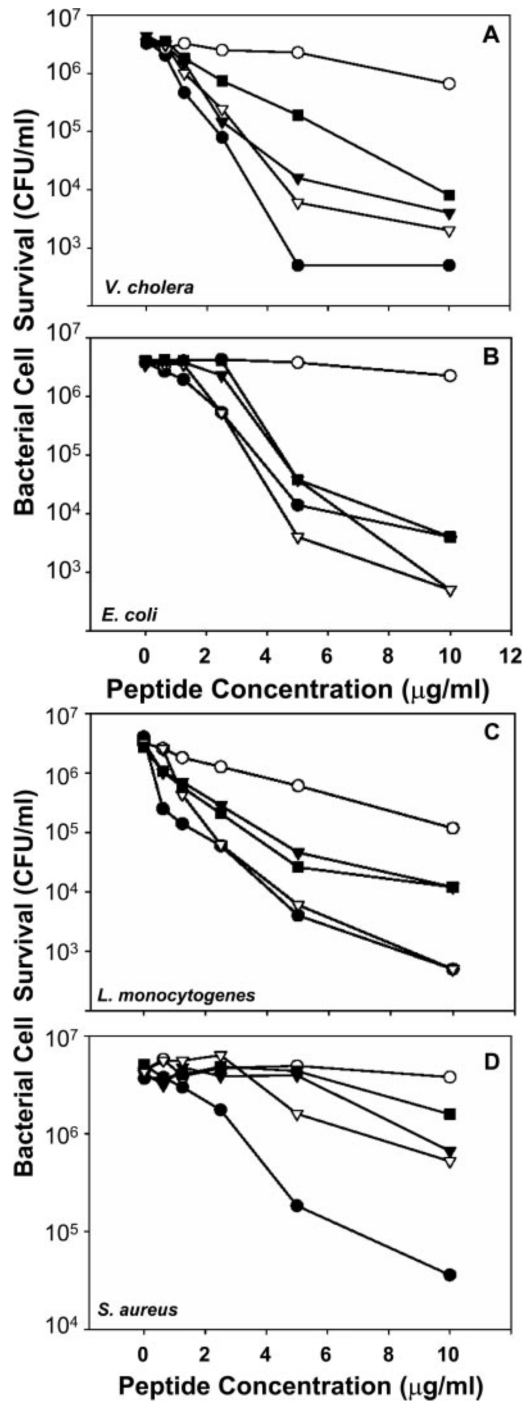

FIG. 2. **Bactericidal activity of recombinant Crp4 and C-terminally modified Crp4 variants.** Exponentially growing *V. cholera* (*A*), *E. coli* ML 35 (*B*), *L. monocytogenes* (*C*), and *S. aureus* (*D*) were exposed to the peptide concentrations shown: 50 μl of 10 mM PIPES (pH 7.4), 1% trypticase soy broth for 1 h at 37 °C (see "Experimental Procedures"). Following exposure, bacteria were plated on semi-solid media and incubated for 16 h at 37 °C. Surviving bacteria were quantitated as CFU/ml for each peptide concentration. Colony counts below 1×10^3 CFU/ml indicate that no colonies were detected. Symbols: Crp4 (-●-); (R31D/R32D)-Crp4 (-○-); (R31G/R32G)-Crp4 (-▼-); (ΔArg-31/Arg-32)-Crp4 (-▽-); and (R31V/R32V)-Crp4 (-■-). Against all of the species tested, the (R31D/R32D)-Crp4 charge-reversal mutation attenuated bactericidal activity to the greatest extent.

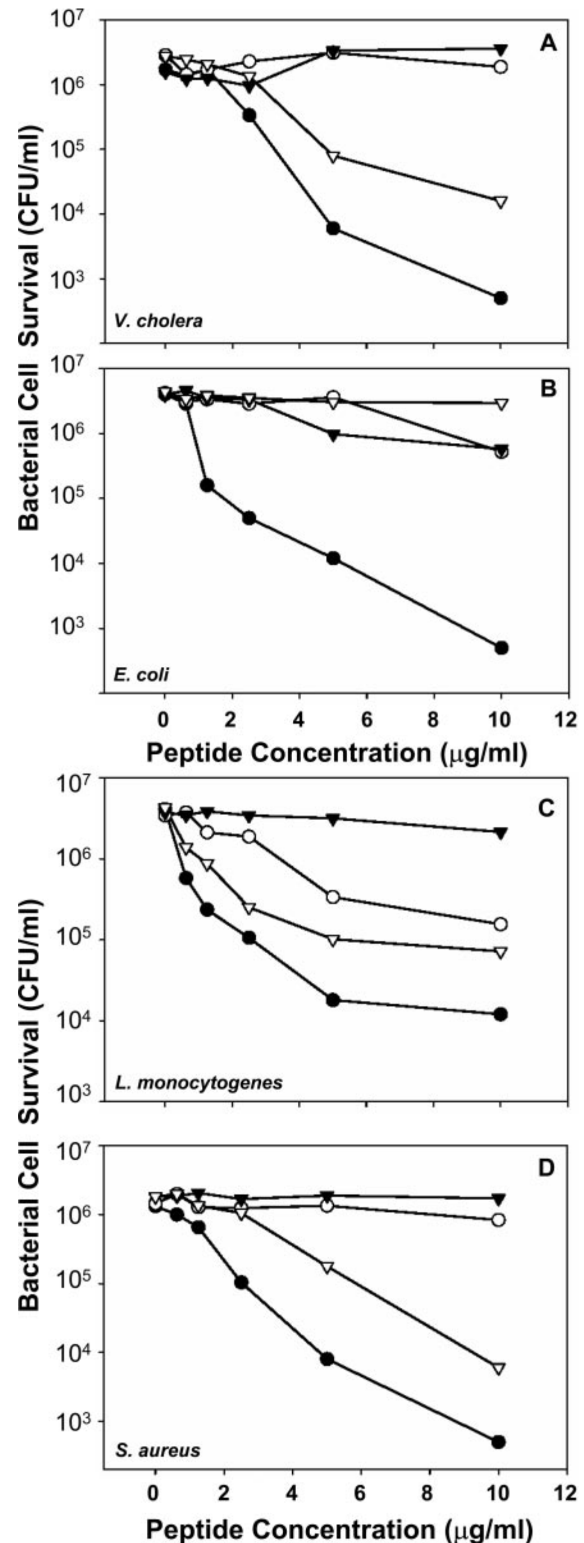

FIG. 3. **Introduction of N-terminal charge alters bactericidal activity of C-terminal Crp4 mutants.** Exponentially growing *V. cholera* (*A*), *E. coli* ML 35 (*B*), *L. monocytogenes* (*C*), and *S. aureus* (*D*) were exposed to the peptides, and surviving bacterial cells were quantitated as CFU/ml (Fig. 2). Symbols: Crp4 (-●-); (R31D/R32D)-Crp4 (-○-); (G1D/R31D/R32D)-Crp4 (-▼-); and (G1R/R31D/R32D)-Crp4 (-▽-). (G1R/R31D/R32D)-Crp4 has bactericidal activity intermediate between that of Crp4 and (R31D/R32D)-Crp4, and the addition of the (G1D) mutation to (R31D/R32D)-Crp4 abrogates activity.

(data not shown). Thus, addition of Arg residues at the C terminus *per se* is not sufficient to improve peptide activity in the absence of proline at residue position 30. Because the R31D/R32D C-terminal charge reversal eliminated Crp4 bactericidal activity, the effects of charge alterations at other residue positions in the Crp4 polypeptide chain were studied to test whether the effects were specific to C-terminal modification.

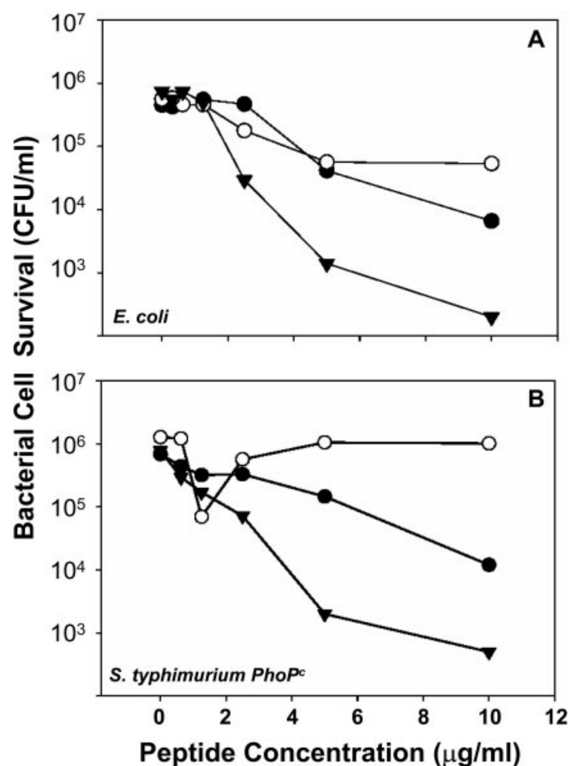

FIG. 4. **C-terminal cationicity is insufficient to augment Crp4 bactericidal activity.** Exponentially growing *E. coli* ML 35 (*A*) and *S. typhimurium* (*PhoP*^{c}) (*B*) were exposed to the peptides, and surviving bacterial cells were quantitated as CFU/ml (Fig. 2). Symbols: Crp4 (-●-); (Arg-33/Arg-34)-Crp4 (-▼-); and (ΔPro-30/Arg-33/Arg-34)-Crp4 (-○-). C-terminal addition of Arg residues does not augment bactericidal activity in the absence of Pro-30.

Charge Reversal at Arg Positions Result in Loss-of-function—Crp4 mutants with double Asp for Arg substitutions were introduced at three additional Arg pairs, purified, and assayed for bactericidal peptide activity (see "Experimental Procedures"). As was observed for (R31D/R32D)-Crp4, the R16D/R18D, R16D/R24D, and R18D/R24D variants of Crp4 all lacked bactericidal activity under the conditions of these *in vitro* assays (Fig. 5). Thus, the loss of activity by charge reversal is not a specific effect at the C terminus, because all of the peptides with double charge-reversal mutations were inactive. Perhaps, Asp for Arg substitutions eliminate bactericidal activity by reducing the overall electropositivity of the Crp4 peptide. Nevertheless, it is unlikely that Arg residues at specific positions are required for Crp4 bactericidal activity because all of the mutations introduced resulted in equivalent loss-of-function independent of the position of the replacements.

To determine whether a specific requirement exists for the guanidinium group of the Arg side chain in Crp4 or whether charge-neutral substitutions at Arg positions would result in peptides with equivalent bactericidal activity, we prepared (R16K/R18K)-Crp4 (Fig. 1*A*) and evaluated its antimicrobial activity. Because all of the Crp4 charge-reversal mutants were attenuated similarly including (R16D/R18D)-Crp4, the (R16K/R18K)-Crp4 internal charge-neutral mutant was taken to be representative of such peptide alterations. As stated earlier, the Lys for Arg substituted peptide was purified to homogeneity as in Fig. 1*B* (data not shown) prior to comparing its bactericidal peptide activity relative to Crp4. The bactericidal peptide activities of Crp4 and (R16K/R18K)-Crp4 were not distinguishable against *E. coli* or *L. monocytogenes* (Fig. 6) or against other bacterial species (data not shown). Thus, with regard to bactericidal peptide activity, Lys could replace Arg in Crp4 without effect.

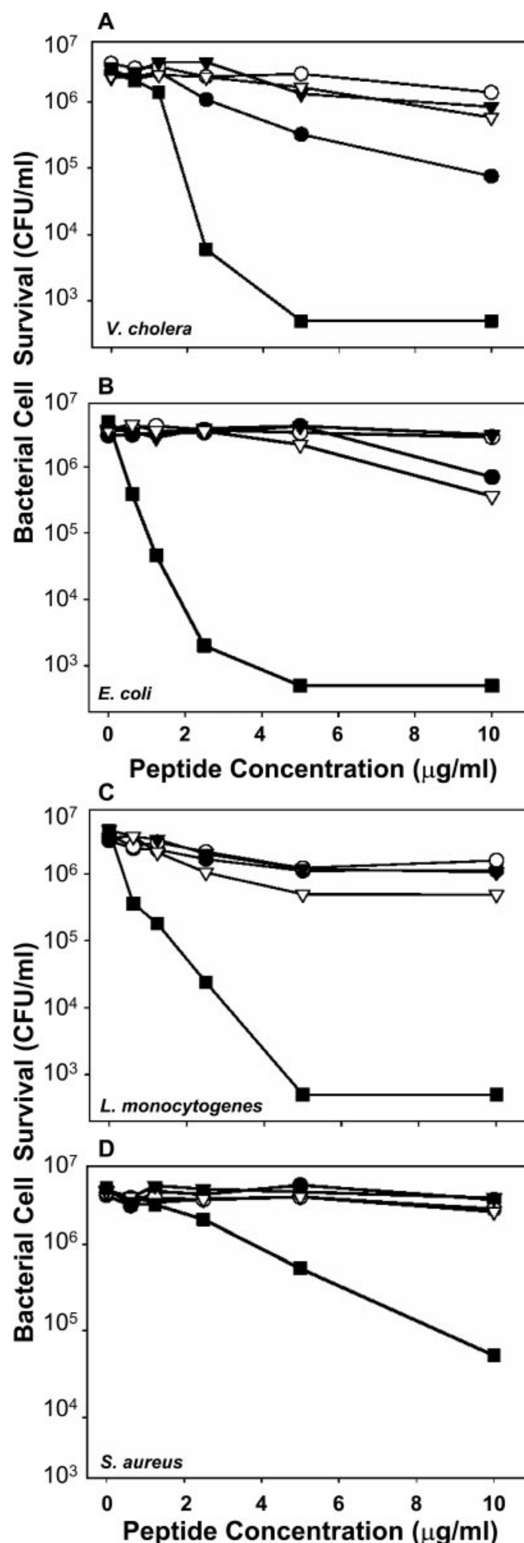

FIG. 5. **Equivalent loss-of-function by charge reversal at multiple Arg residue positions in Crp4.** Exponentially growing *V. cholera* (*A*), *E. coli* ML 35 (*B*), *L. monocytogenes* (*C*), and *S. aureus* (*D*) were exposed to the peptides, and surviving bacterial cells were quantitated as CFU/ml (Fig. 2). Symbols: Crp4 (-■-); (R16D/R18D)-Crp4 (-●-); (R16D/R24D)-Crp4 (-○-); (R18D/R24D)-Crp4 (-▼-); and (R31D/R32D)-Crp4 (-▽-). All pairs of Asp for Arg charge reversals eliminate bactericidal activity of the parent Crp4 peptide.

Charge-reversal Mutations Block Crp4-Membrane Interactions—To investigate the mechanisms by which charge-reversal mutagenesis of Crp4 eliminates peptide activity, we compared interactions of Crp4 and charge-reversal Crp4 mutants

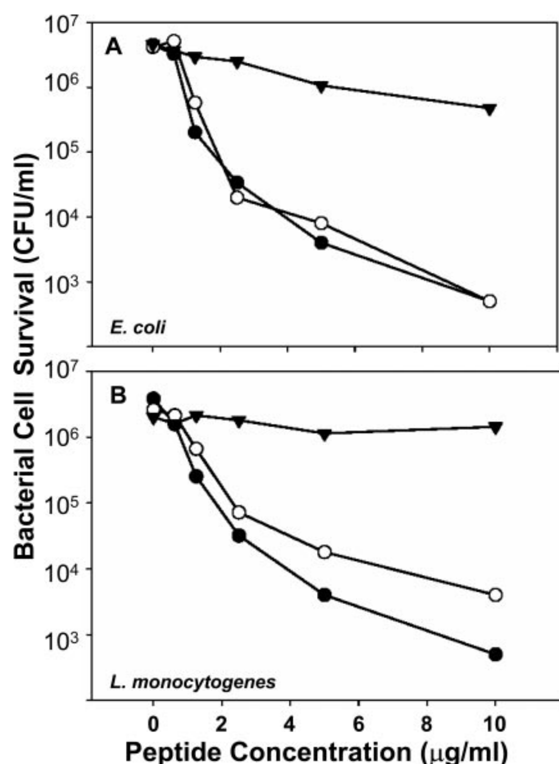

FIG. 6. **Bactericidal activity of Crp4 and Crp4 with charge-neutral substitutions at two internal Arg residue positions.** Exponentially growing *E. coli* ML 35 (*A*) and *L. monocytogenes* (*B*) were exposed to the peptides, and surviving bacterial cells were quantitated as CFU/ml (Fig. 2). Symbols: Crp4 (-●-); (R16K/R18K)-Crp4 (-○-); and (R16D/R18D)-Crp4 (-▼-). Lys for Arg charge-neutral substitutions do not alter the bactericidal activity of the parent Crp4 peptide. Findings are representative of bactericidal assay results against other species of bacteria that are not shown.

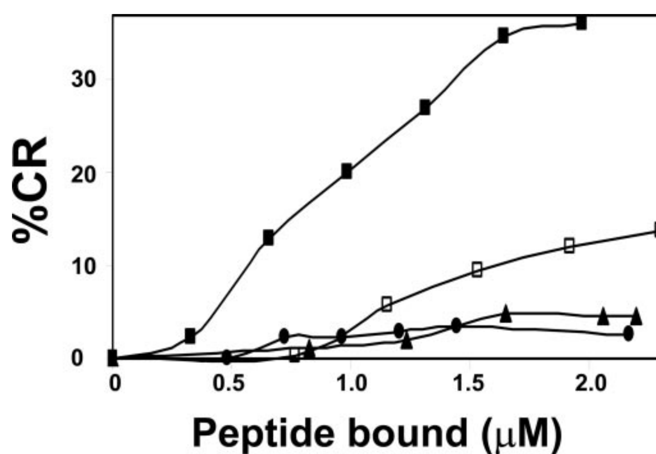

FIG. 7. **Crp4 Arg charge-reversal variants are defective in lipid/PDA vesicle interactions.** Dimyristoylphosphatidylcholine/PDA vesicles (2:3 mol ratio) were exposed to Crp4 (-■-), (G1R/R31D/R32D)-Crp4 (-□-), (R16D/R24D)-Crp4 (-●-), or (R16D/R18D)-Crp4 (-▼-) as described under "Experimental Procedures." The relative increase in %CR is proportional to the extent of bilayer disruption and depth of peptide insertion into the membrane. Charge-reversal mutants fail to interact with the mixed vesicles, and (G1R/R31D/R32D)-Crp4 shows an intermediate binding that is consistent with its intermediate bactericidal activity.

with membranes using colorimetric binding assays based upon lipid/PDA-mixed vesicles (22) and leakage assays from large unilamellar vesicles (24, 28).

Crp4 loss-of-function by Arg to Asp mutagenesis correlated with the inability of mutant peptides to interact with membranes at the vesicular lipid/water interface and to perturb the hydrophobic membrane core. In the dimyristoylphosphatidylcholine/PDA-mixed vesicle system, higher %CR values corresponded to interfacial lipid binding because peptides that localized at the lipid bilayer surface induced greater perturbation in the head-group region of the lipid/polymer assembly than peptides that penetrate deeper into the hydrophobic core (32, 33). Crp4 induced blue-red chromatic transitions, reaching a plateau of ~35%CR upon interaction with the phospholipid/PDA vesicles. The %CR value was dependent on peptide concentration (Fig. 7), and previous work has determined that the colorimetric response indicates an interface localization of Crp4 (22, 32). In contrast and without exception, only base-line %CR values were induced by inactive Crp4 mutants with Asp for Arg substitutions (Fig. 7). Consistent with its intermediate bactericidal activity (Fig. 4), (G1R/R31D/R32D)-Crp4 induced somewhat higher intermediate %CR values between those of Crp4 and the Crp4 variants (Fig. 7). Thus, the loss of bactericidal activity by Arg charge reversals was consistent with defective Crp4 interaction with the vesicular lipid/water interface and an inability to perturb the membrane core of the leaflet.

The membrane-disruptive activities of Crp4 and inactive Crp4 variants were analyzed by comparing induced leakage of the low molecular weight fluorophore, ANTS, from LUV (see "Experimental Procedures"). Crp4 induces leakage from LUV via a "graded leakage" mechanism (24, 28), and pro-Crp4, consistent with its lack of bactericidal activity (21, 22), induces markedly less leakage than Crp4. For both peptides, leakage is dependent on LUV phospholipid composition (Fig. 8) (28). When exposed to (R31D/R32D)-Crp4, (G1D/R31D/R32D)-Crp4, and (R16D/R24D)-Crp4, ANTS-loaded LUV prepared with the anionic lipid palmitoyl-oleoyl-phosphatidylglycerol exhibited leakage that was ≤5% of that induced by Crp4, much less than leakage induced by pro-Crp4 (Fig. 8). Thus, regardless of the position of Arg charge reversals in the polypeptide chain, the loss-of-function was the same and corresponded directly to a loss of peptide binding to membranes and an inability to disrupt phospholipid vesicles *in vitro*.

DISCUSSION

In this report, site-directed Arg to Asp mutations in Crp4, a mouse Paneth cell α-defensin, were shown to attenuate or eliminate microbicidal activity against all of the bacterial target cells examined. Whether charge reversals occurred at the C terminus, at R16D/R18D, R16D/R24D, or at R18D/R24D, mutagenesis eliminated Crp4 *in vitro* bactericidal activity, blocked peptide-membrane interactions, and eliminated Crp4-mediated membrane vesicle disruption. Because Lys for Arg charge-neutral substitutions in the (R16K/R18K)-Crp4 peptide did not modify Crp4 bactericidal peptide activity, it appears that it is the charge rather than a specific requirement for Arg *per se* that determines bactericidal activity in Crp4. Fluorophore leakage from anionic phospholipid vesicles induced by the charge-reversal variants was negligible relative to Crp4 and lower than that induced by pro-Crp4, the inactive Crp4 precursor. Thus, Arg residues function as determinants of bactericidal activity in native Crp4 by facilitating or enabling target cell membrane disruption but the role of the Arg residues appears to be independent of their position in the polypeptide chain.

The findings reported here support the conclusion that Arg residues in the Crp4 polypeptide chain facilitate peptide-membrane binding and interactions that lead to or are coincident with disruption of phospholipid bilayers (Figs. 7 and 8). Despite the insights provided by α-defensin crystal and solution structures (14, 34–36), structure-activity relationships in the α-defensin peptide family remain obscure. The possibility that Asp for Arg-substituted Crp4 molecules expressed in *E. coli* may

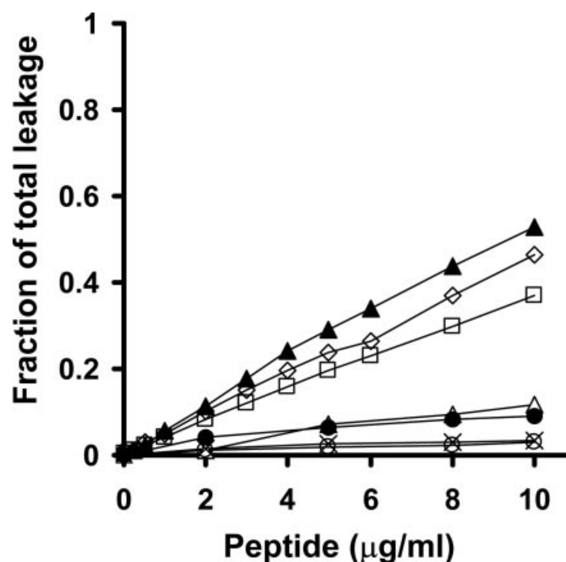

FIG. 8. **Crp4 variant induced leakage from LUV correlates with bactericidal activity**. Vesicles composed of 100% palmitoyl-oleoyl-phosphatidylglycerol were prepared as described under "Experimental Procedures." After exposure of vesicles to differing concentrations of Crp4 (-▲-), (G1R)-Crp4 (-◇-), (des-Gly)-Crp4 (-□-), pro-Crp4 (-△-), (R31D/R32D)-Crp4 (-●-), (G1D/R31D/R32D)-Crp4 (-○-), or (R16D/R24D)-Crp4 (-×-), increased fluorescence due to release of ANTS fluorophore from quenched conditions of the vesicle interior was measured at 520 nm (excitation at 353 nm, see "Experimental Procedures"). Fractional leakage is reported as calculated by the ratio of fluorescence increase relative to maximum fluorescence increase upon destroying the vesicles with the surfactant Triton X-100. The low extent of leakage observed for the Crp4 charge-reversal mutants is consistent with their bactericidal loss-of-function.

not be folded properly or form a correct disulfide array is a potential concern. However, preliminary one- and two-dimensional TOCSY NMR determinations indicate that the mutant peptides have proper disulfide connectivities.[2] Also, when natural α-defensins have been available as standards, recombinant mouse α-defensins and their precursors that we have prepared co-elute from reverse-phase high performance liquid chromatography. The recombinant peptides also co-migrate in acid-urea gels with the native molecules, a sensitive system for resolving misfolded defensins. It is generally accepted that peptide amphipathicity confers an ability on antimicrobial peptides to interact with and disrupt microbial cell membranes (37–43), leading to dissipation of cellular electrochemical gradients and microbial cell death (44). Because early events in bacterial cell killing by human neutrophil α-defensins depend on electrostatic interactions, our results are consistent with the Arg residues in Crp4 facilitating such interactions. Also, implicating the particular Arg residues noted does not exclude the possibility that additional polar or hydrophobic amino acids may be equally important for Crp4 bactericidal activity. The evidence shows that Crp4 bactericidal activity does not require that arginines occupy specific positions in the peptide primary structure, because all of the charge reversals caused equivalent loss-of-function independent of individual replacements.

The bactericidal activity of mouse Paneth cell Crps is dependent on the activation of pro-Crps from inactive 8.4-kDa proforms by matrix metalloproteinase-7-mediated proteolysis (18, 19, 21). For example, full-length pro-Crp4 (pro-$Crp4_{20-92}$) lacks bactericidal activity and that lack of activity corresponds with a diminished ability to bind to model membranes (22) and to permeabilize LUV (Fig. 7) (28). Interestingly, the two pro-Crp4 processing-intermediates, pro-$Crp4_{44-92}$ and pro-$Crp4_{54-92}$ have the same bactericidal activity as the mature Crp4 peptide.[3] That finding implicates amino acids in the N-terminal 24 residues of the pro-$Crp4_{20-92}$ molecule as inhibitory to Crp4 peptide activity in the unprocessed pro-Crp4 molecule. Because nine of the residues in the N-terminal moiety of the pro-region are Asp or Glu, it is reasonable to hypothesize that those acidic side chains in the pro-region interact with the Arg residues blocking their interactions with membranes. As has been proposed for human neutrophil α-defensin precursors (45, 46), we speculate that the formation of favorable yet hypothetical Asp-Arg or Glu-Arg salt bridges (47) could disable the ability of the Crp4 moiety of the pro-$Crp4_{20-92}$ molecule to bind and disrupt target cell membranes by neutralizing their cationicity at the peptide surface.

The α-defensins from human and rabbit neutrophils achieve bacterial cell killing by distinctive membrane disruptive mechanisms. Neutrophil α-defensins permeabilize the outer and inner membranes of *E. coli* sequentially, inducing the formation of ion channels in lipid bilayers (48), and both of these peptide-elicited effects are influenced by membrane energetics (49). The crystal structure of human neutrophil peptide 2 is a noncovalent dimer (14), and human neutrophil peptide 2 dimers form stable 20-Å multimeric pores in LUV after insertion into model membranes (40). In contrast, rabbit neutrophil peptide 1 is a monomer in solution (35) and neutrophil peptide 1 permeabilizes the membrane by creating large short-lived defects in model phospholipid bilayers (50). Although the structure of Crp4 is not yet reported, the peptide, which has the greatest *in vitro* bactericidal activity among the mouse α-defensins, exhibits strong interfacial binding to model membranes (22) and induces graded fluorophore leakage from LUV (24, 28). Thus, Crp4 resembles the rabbit neutrophil peptide 1-type α-defensin mechanism of bactericidal action and site-directed Arg to Asp mutagenesis targets Crp4 residues that facilitate or enable that membrane-disruptive mechanism.

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