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Purification and primary structure of murine cryptdin-1, a Paneth cell defensin

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We have purified and determined the amino acid sequence of cryptdin-1, a murine Paneth cell defensin. The peptide corresponds to a previously characterized mRNA that accumulates to high abundance during postnatal ontogeny of the small bowel. Acid-extracted intestinal protein was fractionated by cation-exchange chromatography and fractions were assayed for antimicrobial activity. One peak of anti-*Salmonella* activity contained a putative defensin, based on its predicted electrophoretic migration in acid-urea PAGE. The peptide was purified to homogeneity by RP-HPLC and sequenced. These studies demonstrate defensin expression in non-myeloid tissue. The N-terminal extension of cryptdin-1 is a unique structural feature of this novel epithelial defensin.

Antimicrobial peptide; *Salmonella*; Cation-exchange chromatography; High performance liquid chromatography (HPLC); Peptide sequencing

1. INTRODUCTION

Many low molecular weight mRNAs accumulate during postnatal ontogeny of the mouse small bowel [1], including an mRNA predicted to code for a Paneth cell defensin [2]. The putative peptide was termed 'cryptdin' (*crypt defensin*) because of its expression in intestinal crypts and its defensin-like structure. Using the cryptdin cDNA clone, asb4/134, the developmental regulation and high abundance of cryptdin mRNA were confirmed in jejunum and ileum, and the mRNA was localized to Paneth cells by in situ hybridization [1,2]. Furthermore, the mRNA was shown to be present at low levels in 5–10% of crypts in 10-day-old mice, at higher levels in 70–80% of crypts by 16 days of age, and at the adult level in every crypt in 20-day-old mice [2], consistent with crypt differentiation and coincident with the appearance of Paneth cells [3]. Cryptdin mRNA is the first defensin-coding sequence found in epithelial cells.

All previously characterized defensins have been isolated from phagocytic leukocytes, i.e. neutrophils and macrophages [4,5]. Defensins have broad spectrum antimicrobial activities and are characterized by their size (approx. 4 kDa), cationicity, and six conserved cysteine residues arranged in an invariant disulfide bonding pattern: 1–6, 2–4, 3–5 [6]. The presence of the defensin motif in the predicted cryptdin gene product [2], its DNA sequence homology with the defensins [7–9], the conserved linkage homology of the cryptdin gene locus with the human defensin gene(s) on 8p23 [10,11], and the existence of defensin resistance genes in the enteric pathogen *Salmonella typhimurium* [12–14] support the hypothesis that cryptdin is an enteric antimicrobial peptide that may function to restrict microbial colonization of the intestinal mucosa.

To ascertain whether a defensin with the sequence predicted by cryptdin cDNA existed in small bowel we sought to purify the cryptdin peptide and to determine its primary structure.

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2. MATERIALS AND METHODS

2.1. *Mice and preparation of intestinal extracts*

Outbred Swiss mice [(Crl:CD-1)(ICR)BR], 45-day-old males (30–35 g) were purchased from Charles River Breeding Laboratories, Inc. (North Wilmington, MA). Mice were housed under 12-h cycles of light and darkness and had free access to food and water.

Jejunum and ileum were removed intact from mice killed by cervical dislocation. Intestinal lumens were rinsed with 35 ml PBS, and the intestines of individual mice were disrupted in 35 ml of ice-cold 10% acetic acid using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were stirred continuously for 18 h at 4°C, clarified by centrifugation at 27,000 rpm in an SW28.1 rotor for 30 min at 4°C, dialyzed against 10% acetic acid, lyophilized, and stored at −85°C.

2.2. *CM–Sepharose chromatography of antibacterial intestinal proteins*

Lyophilized intestinal extract from 12 mice was resuspended in 100 mM ammonium formate (pH 6.2), applied to a 300 ml column of CM–Sepharose equilibrated with the same ammonium formate buffer, and washed with 200 ml of ammonium formate buffer. Cationic peptides were fractionated with a 2 l 0.10–1.0 M ammonium acetate (pH 5.2) gradient followed by a 300 ml 2.0 M ammonium acetate wash [15]. Gradient fractions were lyophilized, dissolved in 1.5 ml 0.01% acetic

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acid, and tested for antibacterial activity against wild-type *S. typhimurium* (ATCC10428) and an isogenic *phoP* mutant of *S. typhimurium* (strain CS015 *phoP102*::Tn*10*d-Cam [16]) using a well diffusion assay [15]. Wells were created in agarose containing 0.3% tryptone after seeding with 1×10^7 log-phase cells into which 2 μl samples of reconstituted column fractions were pipetted, and plates were incubated at 37°C for 14–18 h. Antibacterial activity was apparent as clear zones around wells containing antibacterial peptides, and the zone sizes were concentration-dependent. There was no evidence of an ionic strength effect from the column buffer.

Column fractions containing antimicrobial activity were also analyzed in acid–urea gels. Samples were lyophilized, dissolved in 30 μl of 5% acetic acid containing 3.0 M urea, and electrophoresed on 12.5% acid–urea acrylamide gels [17].

2.3. *Reverse-phase HPLC*

Cryptdin-1 was purified to homogeneity from active CM–Sepharose column fractions by reverse-phase HPLC on a 0.46 × 25 cm Vydac C-18 column [6]. A cryptdin-1-containing fraction was purified by sequential RP-HPLC, first with 0.1% trifluoroacetic acid (TFA) and then with 0.13% heptafluorobutyric acid (HFBA). Columns were developed using gradients of acetonitrile (1% per min).

Cryptdin-1 was tentatively identified by comparing its migration with authentic rabbit neutrophil defensin NP-1 [18] and with a synthetic defensin in acid–urea gels. The latter peptide, termed cryptdin-C which corresponds to residues 62–94 in the deduced preprocryptdin polypeptide sequence [7], was synthesized by solid phase chemistry using *N*-butoxycarbonyl protection [19]. After cleavage/deprotection of synthetic cryptdin-C the reduced peptide was diluted with 30% acetic acid and folded by exhaustive dialysis, first against 0.1 M sodium phosphate (pH 8.2), 20 mM guanidine-HCl, 100 mM NaCl, then against 5% acetic acid. The peptide was purified by RP-HPLC on a 1 × 25 cm Vydac C-18 column and used subsequently as a reference peptide in acid–urea PAGE.

2.4. *Biochemical analysis*

Amino acid analyses were performed on 6 N HCl hydrolysates (150°C, 2 h) of unmodified or performic acid-oxidized peptide. Hydrolysates were derivatized with phenylisothiocyanate, and the resulting phenylthiocarbamyl amino acids were quantitated as described previously [17].

Sequence determinations were performed twice by automated Edman degradation on an ABI model 477 system (Applied Biosystems Inc., Foster City, CA) with on-line PTH amino acid analysis. Peptide samples were reduced with dithiothreitol and pyridylethylated with 4-vinylpyridine for sequencing [20]. The carboxyl terminal sequence was confirmed by amino acid analysis of a purified tryptic peptide derived from the C-terminus.

3. RESULTS AND DISCUSSION

3.1. *Purification of mouse cryptdin-1*

Cryptdin-1 was purified from acid extracts of intact mouse jejunum and ileum by a combination of CM–Sepharose chromatography and sequential C-18 reverse-phase HPLC. Identification of antibacterial peptides in column fractions was facilitated by bioassay using a defensin-sensitive *phoP* mutant of *S. typhimurium* in combination with analysis on acid–urea PAGE.

Samples of small bowel were homogenized in 10% acetic acid, and high-speed supernatants of acid-soluble protein were eluted with a 2 l linear gradient of 0.1–1.0 M ammonium acetate from a 300 ml CM–Sepharose column (data not shown, see [15]). A plate diffusion

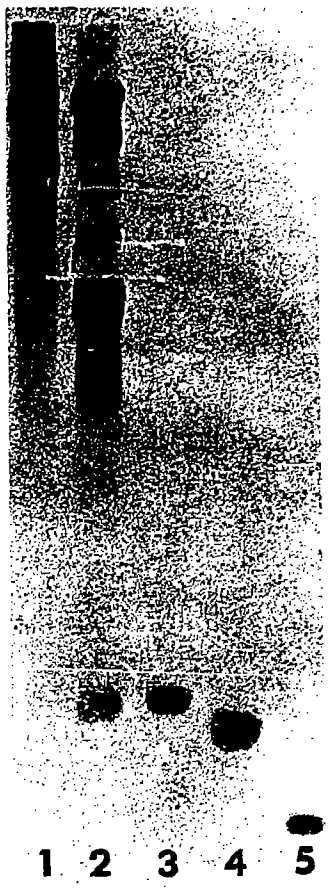

Fig. 1. Purification of mouse cryptdin-1. A 12.5% acrylamide acid–urea gel was loaded with (lane 1) 50 μg crude intestinal acid extract, (lane 2) 60 μg of CM–Sepharose fractions 124–140, (lane 3) 1 μg purified cryptdin-1, (lane 4) 1.5 μg synthetic cryptdin C, and (lane 5) 1 μg rabbit defensin NP-1. The gel was stained with Coomassie blue.

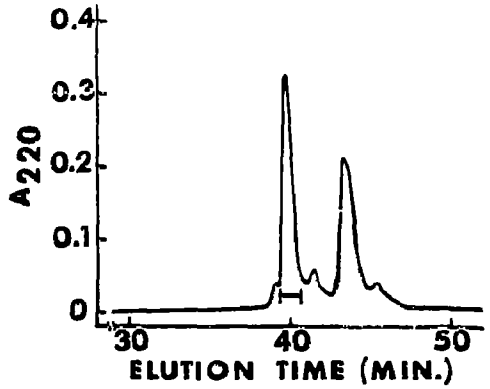

Fig. 2. HPLC purification of mouse cryptdin-1. Cryptdin-1 was purified to homogeneity in this final RP-HPLC step. 75 μg of a cryptdin-containing protein fraction was purified on a C-18 column equilibrated with 0.13% HFBA (see section 2) and eluted with an acetonitrile gradient. The peak fraction indicated in the bracket was collected and lyophilized.

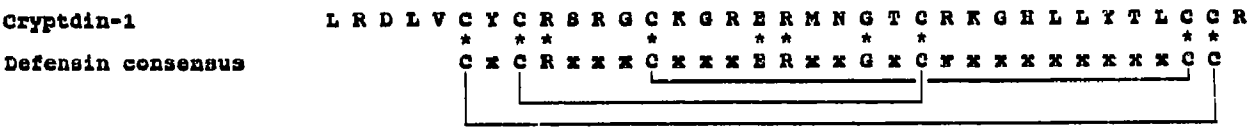

Fig. 3. The primary structure of cryptdin-1. The primary structure of purified cryptdin-1 is shown in single letter amino acid notation and aligned with the defensin consensus. Asterisks denote residues that are invariant in 13 of 16 myeloid defensins; 'x' characters in the defensin consensus represent residues that vary between defensins. The defensin disulfide bonding pattern is depicted by solid lines connecting cysteines.

assay [15] of the column fractions was performed using wild-type and attenuated strains of *S. typhimurium*. The latter strain, mutated in the *phoP* locus, had previously been shown to be sensitive to defensins [12–14]. Column fractions exhibiting greater antibacterial activity against the *phoP* mutant than the wild-type *Salmonella* were selected for further purification by HPLC.

A broad peak of antimicrobial activity, consisting of fractions 90–135, eluted over the range of 0.55–0.8 M ammonium acetate and contained three subpeaks of apparent antimicrobial activity. Fractions 124–140 within this active peak contained peptides characteristic of defensins: apparent molecular weights of 4 kDa on SDS-PAGE, rapid migration in acid–urea PAGE (>0.6 × R_F of the methyl green tracking dye), and presence of an abundant peptide with an R_F very similar to that of synthetic cryptdin-C in acid–urea PAGE (Fig. 1). Treatment of the sample with performic acid caused the apparent cryptdin peptide to disappear from acid–urea gels, demonstrating that it contained numerous half-cystines (not shown).

The cryptdin-1 peptide was purified from CM-Sepharose column fractions 124–140 by RP-HPLC by successive chromatographic separations using water–acetonitrile elution with 0.1% TFA and 0.13% HFBA as ion pairing agents (Fig. 2). The amino acid composition of the 4 kDa peptide was completely consistent with the composition of cryptdin-C, except that cryptdin-1 contained three additional amino acids: leucine, arginine and aspartic acid.

3.2. *The primary structure of mouse cryptdin*

A sample of the purified peptide was reduced, *S*-pyridylethylated, and subjected to two gas-phase sequencing determinations (Fig. 3). The carboxyl terminal sequence was confirmed by amino acid analysis of the tryptic peptide consisting of residues 26–35. These determinations confirmed the amino acid analysis and the sequence predicted by the cryptdin cDNA sequence [2]. However, the data show that the N-terminus of cryptdin-1 is extended in relation to other defensins. Although known leukocyte-derived defensins contain a maximum of two residues prior to the first cysteine, the first cysteine in cryptdin-1 is preceded by five amino acids. Cryptdin-C, which migrates slightly faster than cryptdin-1 in acid–urea PAGE, was synthesized with the expectation that the N-terminus of cryptdin-1 would be structurally similar to that of the myeloid defensins. Although the significance of the pentapeptidyl N-terminal sequence is not known, the relative R_F's of cryptdin-1 and synthetic cryptdin-C are consistent with the difference in their primary structures.

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