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Biochemistry

FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis

(antibacterial peptide/cathelin propart/amphipathic helix/cDNA cloning/solid-phase synthesis)

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ABSTRACT **PR-39, a proline/arginine-rich peptide antibiotic, has been purified from pig intestine and later shown to originate in the bone marrow. Intending to isolate a clone for a human counterpart to PR-39, we synthesized a PCR probe derived from the PR-39 gene. However, when this probe was used to screen a human bone marrow cDNA library, eight clones were obtained with information for another putative human peptide antibiotic, designated FALL-39 after the first four residues. FALL-39 is a 39-residue peptide lacking cysteine and tryptophan. All human peptide antibiotics previously isolated (or predicted) belong to the defensin family and contain three disulfide bridges. The clone for prepro-FALL-39 encodes a cathelin-like precursor protein with 170 amino acid residues. We have postulated a dibasic processing site for the mature FALL-39 and chemically synthesized the putative peptide. In basal medium E, synthetic FALL-39 was highly active against *Escherichia coli* and *Bacillus megaterium*. Residues 13–34 in FALL-39 can be predicted to form a perfect amphiphatic helix, and CD spectra showed that medium E induced 30% helix formation in FALL-39. RNA blot analyses disclosed that the gene for FALL-39 is expressed mainly in human bone marrow and testis.**

Animal peptide antibiotics were discovered about 15 yr ago, and in September 1993 at least some 50 different sequences were known (for reviews, see refs. 1 and 2). On a chemical basis, these peptides can be divided into five groups: (*i*) linear peptides lacking cysteine, often forming amphipathic helices; (*ii*) linear peptides with a high proportion of certain residues like proline and arginine; (*iii*) loop-forming peptides with one disulfide bond; (*iv*) peptides with two or more disulfide bonds, normally forming β-sheet structures; and (*v*) peptides derived from larger molecules with other known functions. The most studied peptides are the cecropins and the magainins in the first group and the defensin family in the fourth group; these three groups of peptides have antimicrobial spectra of almost the same broad type as classical antibiotics (1, 2).

On a functional basis, the animal peptide antibiotics can be divided into two groups: (*i*) those that, like the defensins, accumulate in the granule of phagocytes and (*ii*) those that are delivered into body fluids or epithelial layers. Peptides that have evolved to kill engulfed microbes inside phagocytic vacuoles can, in released form, be cytotoxic to the host, and this is the case for the defensins (3). On the other hand, peptides like the insect cecropins (4) and insect defensins (5), which are delivered into the circulatory system, are not harmful to the producing organism.

Animal peptide antibiotics differ from the "classical" antibiotics in several respects (2, 6). The animal peptides are all gene encoded, and they are made as prepro-proteins that are processed to the mature peptide by defined pathways. The actual processing steps have been studied for cecropins (7), for Bac5 and Bac7 (8), and for myeloid defensins (9), but in most other cases the processing is thus far only predicted or simply unknown. This biosynthesis is conceptually different from that for microbial peptide antibiotics like gramicidin or penicillin, which are made by a set of different enzymes that sequentially add different amino acid residues. Animal and microbial antibiotics also differ functionally; microbial antibiotics are often referred to as "secondary metabolites" (10), while the animal peptide antibiotics are considered important parts of the innate immunity of the producing organism (1).

Most animal peptide antibiotics have been purified from blood (hemolymph) or blood cells using the antimicrobial activity as an assay. cDNA and genomic clones were isolated later with the help of probes designed from the known amino acid sequences. However, Romeo, Zanetti, and colleagues (11) discovered that a number of antibacterial peptides from different mammals contained a conserved pro-region very similar to cathelin, a protein isolated from pig leukocytes and reported to be an inhibitor of cysteine containing proteases (12). This finding was used by the Trieste groups for 3′- and 5′-RACE (for rapid analysis of full-length cDNA) PCR experiments that gave the cDNA sequences corresponding to both previously known peptide antibiotics (13–15), as well as additional peptides (i.e., PMAP-36), which were synthesized and found to be antibacterial (16).

We report here a different PCR approach for cloning the precursor of another human peptide antibiotic that, unlike the human defensins, lacks cysteine. The gene was found to be expressed in bone marrow and in testis. The putative 39-residue peptide was named FALL-39 after the first four N-terminal residues and the total number of residues. The peptide was chemically synthesized and found to be antibacterial.¶

MATERIALS AND METHODS

cDNA Cloning. A liquid lysate of a human bone marrow λgt11 cDNA library (Clontech) was used to isolate template DNA by the Wizard DNA purification system (Promega). The following three primers, 5′-ACCATGGAGACCCAGAGGGC, 5′-CCTGTAGCTGAGGGCCTGGG, and 5′-TCCARYTC-CARCARNCKRTA (corresponding to underlined or dotted sequences in Fig. 1), were directed to the signal sequence and the pro-region of PR-39. These primers (at 0.4 μM) and template DNA (6 ng/μl) were used in a PCR experiment with the

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Abbreviation: LC, lethal concentration (lowest concentration that inhibits bacterial growth).

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z38026).

following thermal-cycle profile: 95°C for 3 min; 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and an extension step of 72°C for 7 min. Analyses of the reaction mixtures showed two bands with the expected sizes. The bands were purified and cloned into the pCR II vector by a TA cloning kit (Invitrogen). Positive clones were sequenced by the dideoxy nucleotide chain-termination method with a Sequenase kit (United States Biochemical). Sequencing confirmed that the two PCR bands were similar to the start of the open reading frame for prepro-PR-39. The larger band (183 bp) was radioactively labeled and used as a probe for screening a human bone marrow cDNA library (λgt11 from Clontech). About 150,000 plaque-forming units were screened using Hybond-N nylon membrane (Amersham), and positive plaques were purified to homogeneity. λDNA was prepared by a glycerol step gradient (17). The cDNA inserts were subcloned into pBluescript KS vector (Stratagene) and sequenced by the solid-phase sequencing method (18) on a Pharmacia A.L.F. (automated laser fluorescent) and an Applied Biosystems sequenator. The screening hybridization was done in 6× standard saline citrate (SSC)/5× Denhardt's solution/1% SDS/denatured herring sperm DNA at 100 μl/ml at 55°C overnight. Final washing was in 2× SSC/0.1% SDS at 55°C.

Nucleic Acid Analysis. Total RNA was isolated by an RNA separator kit (Clontech), and two filters preloaded with mRNA from different human tissues (Clontech) were used for hybridization. RNA was separated by electrophoresis in a denaturating formaldehyde gel, and the hybridizations occurred under high-stringency conditions (17).

Peptide Synthesis. Chemical peptide synthesis was done with an automatic peptide synthesizer (Applied Biosystems model 430A) using standard solid-phase procedure (for review, see ref. 19). Starting from *t*-butoxycarbonyl (t-Boc)-Ser(benzyl)-OCH_2-phenylacetamidomethyl (Pam) resin (0.67 mmol/g), t-Boc amino acid derivatives were used with reactive side chains protected as follows: serine and threonine with benzyl, lysine with 2-chlorobenzyloxycarbonyl, glutamate and aspartate with benzyl ester, arginine with 4-toluenesulfonyl. A standard program with preformed symmetric anhydrides and preformed 1-hydroxybenzotriazole esters was used for the synthesis. Double couplings were done for arginine, glutamine, and asparagine. The completed 39-residue peptide was cleaved from the resin with liquid hydrogen fluoride/anisole/methyl sulfide, 10:1:1, for 60 min at 0°C. The cleavage product was washed with ether to remove the scavengers and then extracted into 30% (vol/vol) acetic acid and lyophilized. The peptide was purified by HPLC on a Vydac C_{18} column, using a linear gradient of 80% acetonitrile (20–75% for 30 min) with 0.1% trifluoroacetic acid. The molecular mass was analyzed by a time-of-flight mass spectrometer (Biolon 20, Uppsala). The CD spectra were recorded with a J-710 spectropolarimeter (Jasco, Easton, MD) at the BioScience Center of Pharmacia (Stockholm).

Antibacterial Zone Assay. Thin plates (0.1 cm thick) were poured with LB broth/1% agarose and ≈4×10^5 cells of the two test bacteria. Small wells (diameter, 3 mm; volume, 3 μl) were punched in the plates and loaded with a dilution series of the peptide. After overnight incubation at 30°C the diameters of inhibition zones were recorded by using a magnification lens with an internal millimeter scale. Lethal concentration (LC) values, the lowest concentration that inhibits bacterial growth, were calculated as described (20). The experimental errors in the method come chiefly from determinations of the peptide concentration (the water content of dried peptides is often a source of error) and from the plate quality. It is important to use a level board and to make sure that the melted agarose is homogeneous when the plate is poured; if not, the thickness

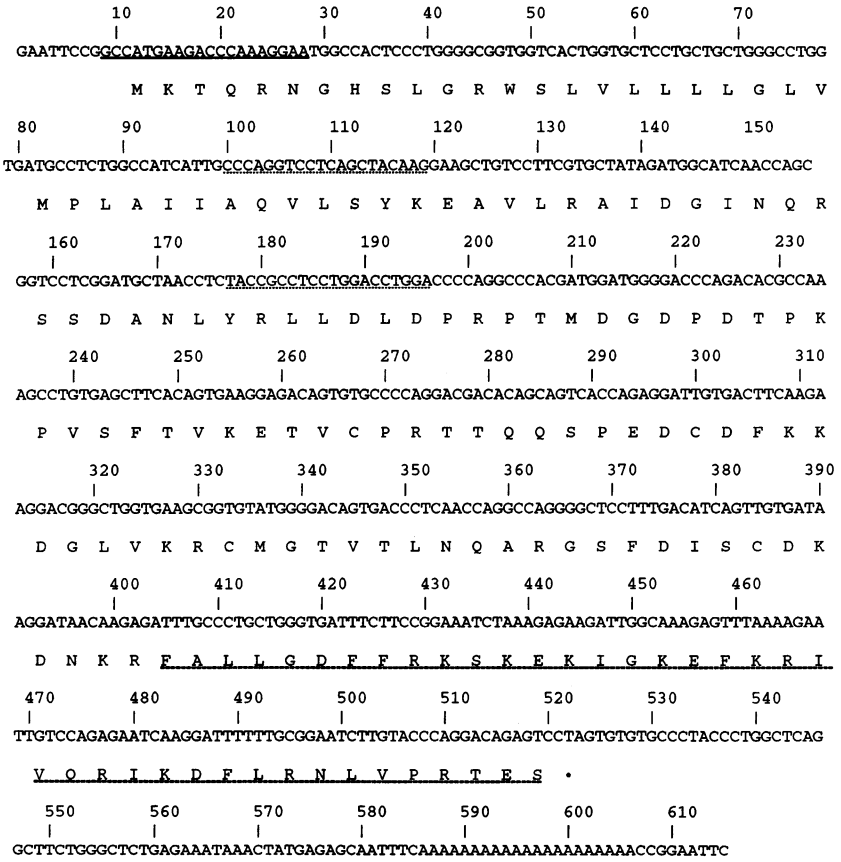

FIG. 1. cDNA sequence for prepro-FALL-39 with translation of the open reading frame. The putative peptide FALL-39 is indicated by dashed underlining. The regions toward which the three successful primers were directed are indicated as an unbroken line for the corresponding sequence and dotted lines for the two complementary sequences. Cathelin sequence starts with bases 101–115, translated to QVLSY-, etc. *, Stop signal.

will be uneven, and this may be the most common cause of poor reproducibility. For a highly active agent (LC < 0.5) with sharp zone boarders, the variation in LC values can be <20%. For peptides with moderate activity (LC = 10–15) the variation may be up to a factor of 2.

RESULTS

Isolation and Characterization of a cDNA Clone for FALL-39. To isolate the human counterpart to the porcine proline/arginine-rich peptide PR-39 (21), primers were initially designed by using both sequence information from the porcine gene for prepro-PR-39 (G.H.G., unpublished work) and conserved sequences of the prepro-regions in all published sequences for the cathelin family (13–16). A total of nine primers in 11 combinations were used for PCR, using DNA from a human bone marrow cDNA library as template. Analyses of these 11 reaction mixtures showed that only two combinations gave clear bands of the expected sizes. Cloning and sequencing showed both bands to be cathelin-like in structure. The larger band (183 bp) was then used as a probe to screen a human bone marrow library, and a number of positive clones were isolated. Partial sequences of the inserts indicated that all clones contained information for the prepro-form of the 39-residue peptide FALL-39.

Eight positive clones were fully sequenced and gave the same cDNA structure for FALL-39 as shown in Fig. 1. Comparison with the genomic DNA sequence for PR-39 shows that both the signal sequences and the pro-region are partly conserved. However, the C-terminal ends of the cathelin sequences differ in the last six residues: in prepro-FALL-39 the end is a typical dibasic cleavage site (aa KR, residues 130–131) instead of an elastase site (aa SV) in prepro-PR-39. The mature peptides are also totally different: there is no homology, and the PRP and PP motifs, typical of proline/arginine-rich peptides, are absent in FALL-39. A search in the GenBank data base for sequences similar to FALL-39 gave no significant relations to published peptides or proteins, and therefore we concluded that the putative peptide FALL-39 is distinctive.

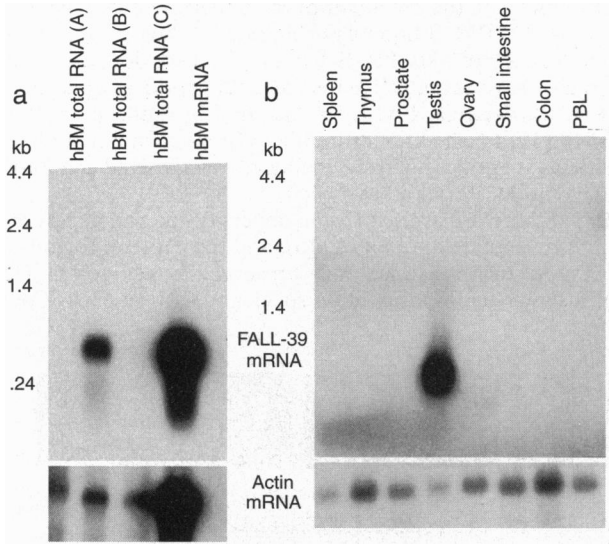

FIG. 2. Two Northern blot analyses. (*a*) Total RNA from human bone marrow (hBM) of a child with T-cell leukemia (lane A), a healthy child (lane B), a child with leukemia in remission (lane C), and mRNA from bone marrow of human adults (final lane) (Clontech). (*b*) Commercial preloaded filter with human mRNA from spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes (PBL). For both blots, an actin probe was used to show RNA amounts.

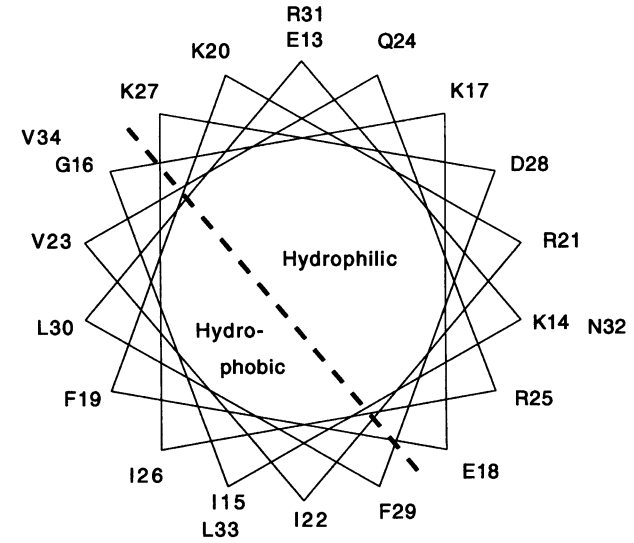

FIG. 3. An Edmundson wheel plot for residues E13–V34 in the sequence for FALL-39. Dashed line divides the helix into hydrophilic and hydrophobic parts. The helix starts at the top of the wheel. A plot of residues F7–V34 does not give a perfect amphipathic helix.

Three RNA blot analyses were done—one with a commercial sample of mRNA from human bone marrow and three samples of total RNA prepared from different human bone marrow samples. In addition, two commercial filters preloaded with mRNA from 16 different human tissues were used. The two filters that gave signals are shown in Fig. 2. Clear signals were seen only for bone marrow and testis from healthy individuals. Overexposure of the film in Fig. 2*b* showed a faint signal from peripheral blood leukocyte RNA. A filter preloaded with human mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas gave no signals.

Solid-Phase Synthesis of FALL-39 and Properties of the Peptide. FALL-39 was synthesized with residues 132–170 of the precursor structure. The synthetic peptide was analyzed in a time-of-flight mass spectrometer and found to have a M_r of 4707.9. The peak was slightly asymmetric, indicating some decomposition during the run. The mass value is therefore in reasonable agreement with the calculated value of 4711.6 for FALL-39.

An Edmundson wheel plot of the FALL-39 sequence showed that the central part of the molecule (residues 13–34) could form a perfect amphipathic helix (Fig. 3), a property

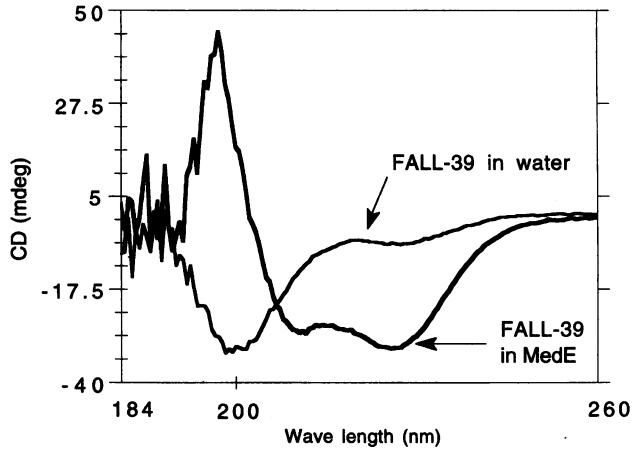

FIG. 4. CD spectra of synthetic FALL-39 in water (86 μM) and the same solution after adding 2% of 50 times concentrated medium E (MedE). Values below 190 nm are probably signals from the solvents. mdeg, Millidegrees.

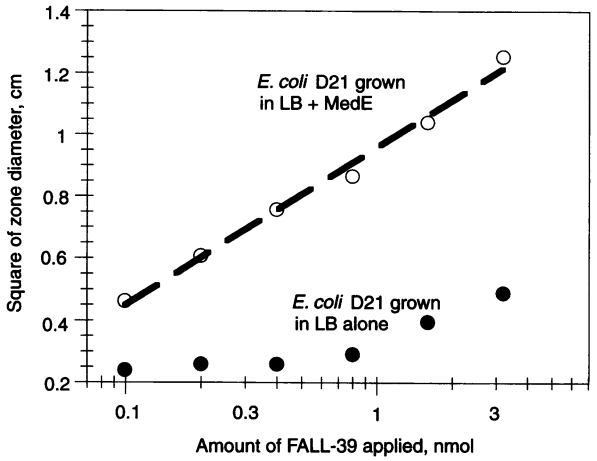

FIG. 5. Inhibition-zone assay for FALL-39 on *E. coli* D21 according to Hultmark *et al.* (20). Bacteria were grown in thin agarose plates with LB medium/basal medium E (MedE). The zone area should normally be a function of the logarithm of the amounts of peptide applied to each well (○). In the absence of medium E, there is a concentration dependance only for the three highest peptide amounts (●).

often found for antibacterial peptides like the cecropins and the magainins. The synthetic peptide was investigated for its helix content by CD spectroscopy. The spectra of an 86 μM solution of synthetic FALL-39 in water indicated a lack of structure (random coil), whereas addition of medium E (22) induced ≈30% of helix formation in the peptide (Fig. 4). About 50% of helix formation was induced in the presence of 30% (vol/vol) trifluoroethanol.

Preliminary experiments showed FALL-39 to be active against *Escherichia coli* and *Bacillus megaterium*, providing that the basal medium E (22) was added to the LB (Luria–Bertani broth, which contains 0.9% NaCl) plates. Fig. 5 shows the results of the inhibition-zone assay produced by FALL-39 on *E. coli* D21 in LB plates supplemented with basal medium E. The graph also shows the zones obtained with our standard assay conditions (LB alone). The LC value (20) obtained from the data in the upper "curve" was 0.7 μM, whereas a calculation based on the three highest amounts of peptide in LB medium gave an LC value of 8.9 μM. A similar salt dependence was also found for the action of FALL-39 on *B. megaterium* Bm11 (data not shown), giving LC values of 0.2 μM in LB/medium E. The LC values for the porcine peptides cecropin P1 and PR-39 on *E. coli* D21 were 0.4 and 0.3 μM, respectively (23). Flat and nonlinear concentration dependences, as seen for the standard assay conditions in Fig. 5, are difficult to interpret, and the LC value from the three highest amounts may be misleading. The wells in the bacterial plates with the highest amounts of FALL-39 sometimes showed a white halo edge, which could indicate that the peptide was bound to the agarose. FALL-39 rendered no detectable lysis of human erythrocytes. The presence of 10 μM of FALL-39 during 5 days of incubation did not affect incorporation of [^{3}H]thymidine in triplicate cultures of human peripheral blood lymphocytes stimulated with phytohemagglutinin. Thus, FALL-39 does not seem cytotoxic for the human cells analyzed so far.

DISCUSSION

Human phagocytes have long been known to produce antibacterial proteins like lysozyme and cathepsin G (24). However, four defensins are the only antibacterial peptides described that are produced as such in human blood cells (3). Three of these peptides were isolated by Lehrer's group (25) from human neutrophils, and the primary structures of these peptides were found to contain three disulfide bridges. The crystal structure of human defensin HNP-3 was determined, and the molecules were found to form dimeric β-sheets (26). A human gene has been found to express a defensin type of peptide (human defensin 5 or HD-5) in Paneth cells, epithelial cells of the small intestine (27). Human Paneth cells also express mRNA for another human defensin, HD-6 (28). However, the intestinal human defensins have not yet been isolated or synthesized, and their properties are unknown. Beside the reports cited here on the presence or the production of antibacterial peptides in human tissues, no others were found.

The cloning of the precursor and the synthesis of the postulated human antibacterial peptide FALL-39, to our knowledge the only human peptide antibiotic lacking cysteine, is here reported. The sequence of the clone for prepro-FALL-39 indicates that the molecule belongs to the family of cathelin-like precursors. Surprisingly, the cathelin family (with conserved prepro-sequences) was found to carry antibacterial peptides belonging to the first four groups of chemically quite different peptide antibiotics (11, 13–16). Fig. 6 gives the sequences for five prepro-proteins from four different mammals: the precursor of human FALL-39 (this report), prepro-PR-39 (15) and the cathelin protein from pig [only sequenced as protein (12)], the precursor of the dodecapeptide from cow (29), and CAP18, a lipopolysaccharide-binding protein from rabbit leucocytes (30). PR-39 from pig and the dodecapeptide from cow have actually been isolated as peptides (underlined in Fig. 6), whereas FALL-39 and an 18-residue peptide in CAP18 (31) (dashed underlining in Fig. 6) have not yet been isolated as peptides but were chemically synthesized and found to have antibacterial activity.

In pig there are at least five members of the cathelin family of prepro-peptide antibiotics (16), and the cathelin regions in these molecules are almost fully conserved to residue Thr-113. Fig. 6 shows that considerable species variations exist. Com-

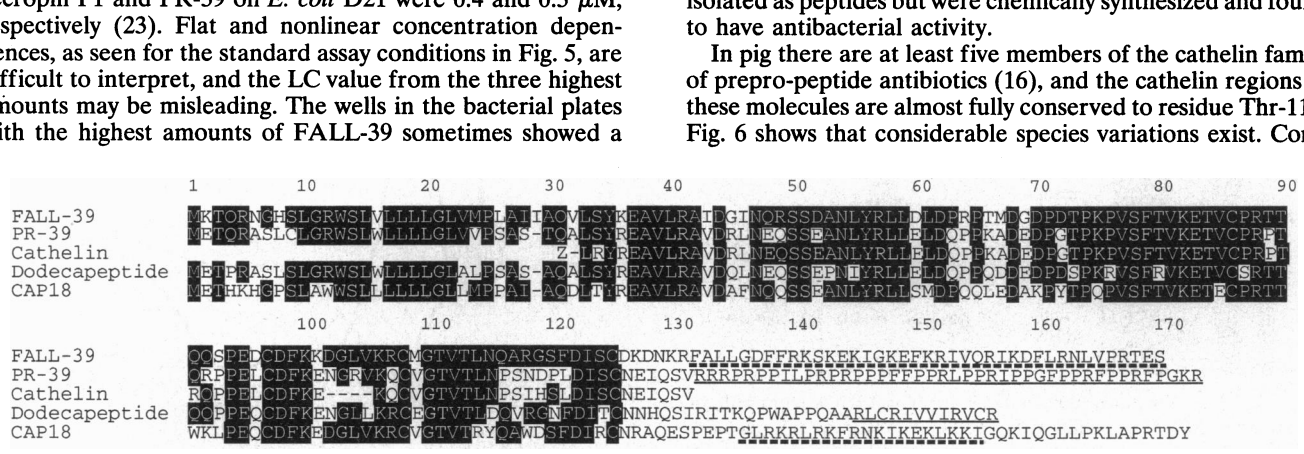

FIG. 6. Prepro-FALL-39 and three other cathelin-containing prepro-region sequences of peptide antibiotics from three other mammals shown together with the original sequence for the cathelin protein. The mature peptides PR-39 (pig) and dodecapeptide (cow) have been isolated as such (solid underlining), whereas FALL-39 (human) and the CAP18 (rabbit) peptide C18 have been postulated and synthesized (dashed underlining). The first residue in the cathelin protein (pig) is pyroglutamate (Z), whereas there is a glutamine residue (position 31) in all other known sequences. Residue numbers are placed so that the first character aligns with the indicated residue.

parison of the first 131 residues in the two top lines of Fig. 6 shows that the prepro-regions of FALL-39 (human) and PR-39 (pig) differ in 46 amino acid residues, whereas cow and rabbit (the two bottom lines) differ by 51 residues. Thus, these pro-regions differ to an extent that could make cross-specie isolations difficult. However, sequences for the PR-39 gene and the cDNA for FALL-39 did show as much as 76% identity.

The gene for FALL-39 was expressed mainly in bone marrow and in testis, organs that are not often infected. However, prevention of bacterial growth in semen is important, and an antibacterial protein (seminalplasmin) in bull semen has been found to contain a sequence for a peptide that is both antibacterial and hemolytic (32). The reproductive tract, but not the testis, of male *Drosophila* produces andropin, a 34-residue antibacterial peptide, first detected on the gene level, after which the putative product was synthesized (33). Interestingly, high andropin activity against *E. coli* D21 required 0.67 M phosphate buffer, a salt concentration too high to allow *Bacillus subtilis* growth.

The structural predictions for FALL-39 showed that residues 13–34 can form a perfect amphipathic helix (Fig. 3). Residue Gly-16 could be a potential helix breaker, but the fact that it is preceded by a large apolar residue (isoleucine) and followed by a lysine residue indicates that the long hydrophobic side chains in these two surrounding residues can interact and permit helix continuation (34). Outside the helix, the sequence for FALL-39 contains two sites for helix breaking, residues Gly-5–Asp-6 and Pro-35, the latter at the end of the helix. The two terminal parts could perhaps be partly, or fully, removed by further processing of FALL-39, hypothetical steps that can be settled only by the isolation of the mature peptide(s) from human testis (or possibly semen) and by more synthetic work. It is obvious that addition of medium E to a water solution of FALL-39 induces helix formation (Fig. 4) and at the same time the activity against *E. coli* is dramatically improved (Fig. 5). Thus, even though the processing of FALL-39 is yet unknown, we can conclude that helix formation is important for biological activity of FALL-39. Medium E is a basal salt medium developed especially for *E. coli* (22). It is of general interest that medium E (or the components thereof) can function as a physiological solvent, giving helix formation of a peptide. Normally 30% (vol/vol) trifluoroethanol or hexafluoropropanol is used to induce helix formation in peptides.

Resistance to classical antibiotics is an increasing clinical problem (35), especially since vancomycin resistance recently has been found in clinical isolates (36). Against this background, new human peptide antibiotics are of special interest. The LC value for FALL-39 on *E. coli* D21, 0.7 μM, is comparable to 0.9 μM found for tetracycline in the same assay (23). So far, no cytotoxic properties were found for FALL-39. However, the present data are not complete enough to allow judgment of the physiological importance of the peptide. On the other hand, human peptides like FALL-39 will gradually give better insight into the role of peptide antibiotics in human innate immunity and thereby facilitate an understanding of how bacterial infections are curbed. That FALL-39 is free of cysteine makes synthesis easier and inexpensive. If the peptide as such (or a fragment, such as the helix) can be demonstrated to exist and be active in human tissues, the peptide could potentially become a human drug.

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