Stéphane Charpentier, Mohamed Amiche, Jan Mester, Véronique Vouille, Jean-Pierre Le Caer, Pierre Nicolas and Antoine Delfour

Structure, Synthesis, and Molecular Cloning of Dermaseptins B, a Family of Skin Peptide Antibiotics*

Stéphane Charpentier‡, Mohamed Amiche‡, Jan Mester§, Véronique Vouille‡, Jean-Pierre Le Caer¶, Pierre Nicolas‡, and Antoine Delfour ‡∥

From the ‡Laboratoire de Bioactivation des Peptides, Institut Jacques Monod, 2 Place Jussieu, 75251 Paris Cedex 05, France, §INSERM U 55, 184 rue du Faubourg Saint-Antoine 75571 Paris Cedex 12, France, and ¶Laboratoire de Neurobiologie et Diversité Cellulaire, CNRS URA 2054, ESPCI, 10 rue Vauquelin 75231 Paris Cedex 05, France

Analysis of antimicrobial activities that are present in the skin secretions of the South American frog *Phyllomedusa bicolor* revealed six polycationic (lysine-rich) and amphipathic α-helical peptides, 24–33 residues long, termed dermaseptins B1 to B6, respectively. Prepro-dermaseptins B all contain an almost identical signal peptide, which is followed by a conserved acidic propiece, a processing signal Lys-Arg, and a dermaseptin progenitor sequence. The 22-residue signal peptide plus the first 3 residues of the acidic propiece are encoded by conserved nucleotides encompassed by the first coding exon of the dermaseptin genes. The 25-residue amino-terminal region of prepro-dermaseptins B shares 50% identity with the corresponding region of precursors for D-amino acid containing opioid peptides or for antimicrobial peptides originating from the skin of distantly related frog species. The remarkable similarity found between prepro-proteins that encode end products with strikingly different sequences, conformations, biological activities and modes of action suggests that the corresponding genes have evolved through dissemination of a conserved "secretory cassette" exon.

Gene-encoded peptide antibiotics are an essential defense component of prokaryotic and eukaryotic organisms destined to circumvent invading pathogens and proliferation of commensals. Small-sized antimicrobial peptides, termed bacteriocins, have been produced by a number of Gram-positive and Gram-negative bacteria for million of years for containing the proliferation of microorganisms that are closely related or confined within the same ecological niche, thus helping the producing microbe to compete for limited resources (1–5). In vertebrates, polycationic antimicrobial peptides, 10–46 residues long, that are secreted by specialized epithelial cells offer a first line of defense against infectious agents by limiting bacterial invasion and/or colonization of the respiratory tract, the gastrointestinal and genital systems, and the skin (6, 7).

Gene-encoded peptides of the chemical defense so far isolated from vertebrates act completely unlike traditional antibiotic agents and may open novel perspectives in the conception of new therapeutic treatments of nosocomial infections and infections because of multidrug-resistant pathogens. Whereas many antibiotics or secondary metabolites disable microorganisms over a period of days by inhibiting critical enzymes, most of the antimicrobial peptides kill microorganisms rapidly by destroying or permeating the microbial membrane and impairing the ability to carry out anabolic processes (6, 7). In addition, these peptides are of small size and processed from prepro-proteins by dedicated pathways. Vertebrate antibiotics are thus unlikely to induce the acquisition of antibiotic resistance genes by bacterial pathogens.

In recent years, the secretions from amphibian skin have become a pivotal model for the discovery of new peptide antibiotics originating from the nonmyeloid cells of vertebrates. The frog dermatous glands synthesize and expel an extraordinarily rich variety of mammalian-like hormones and neuropeptides including thyrotropin-releasing hormone, angiotensin, bombesin, skin peptide tyrosine-tyrosine, and bradykinin as well as the astonishing D-amino acid containing heptapeptide opioids, dermorphin, and deltorphins (8). Glands also produce huge amounts of cytolytic and antimicrobial cationic peptides thought to be involved in the defense of the naked skin against microbial invasion and to aid in wound repair. To date, the microbicidal peptides of the frog skin are grouped into three broad families on the basis of their sequence/tridimensional structure characteristics (6, 7): (*a*) linear amphipathic helix forming peptides such as the magainins and related peptides from the African clawed frog *Xenopus laevis*, and the dermaseptins S from the South american arboreal frogs *Phyllomedusa sauvagei*. These peptides manifest broad spectrum antimicrobial activity against bacteria, yeast, protozoa, and fungi but have no or little harmful effect on human cells; (*b*) four different groups of related peptides isolated from various species of the Ranidae family, namely the brevinins-1 and -2, the gaegurins, the esculentins-1, and the esulentins-2. Different from the peptides mentioned above they all contain 2 cysteine residues at the COOH terminus that are linked in a disulfide bridge; (*c*) the temporins, isolated from *Rana temporari*, are the smallest antimicrobial peptides known so far being only 10–13 residues in length.

As a rule, a given amphibian species produces a unique repertoire of antimicrobial peptides that have overlapping structural features but often target specific microorganisms. It is thought that the simultaneous presence of closely related antimicrobial molecules acting in synergy provides frogs with a better shield against a wider range of harmful invaders, bacterial, fungal, and protozoan. The peculiar characteristic of amphibians belonging to different families, genera, and species to store distinct set(s) of antimicrobial peptides that differ with respect to their chain length, hydrophobicity, charge distribu-

tion, and spectrum of activity may be exploited for unearthing new antimicrobial agents targeting specific microorganisms for which the therapeutic armament is scarce. Also, the isolation of new members of old families is not of minor importance because they may exhibit remarkable differences in activity from their congeners. In addition, very little is known about these peptides at the gene level. In that regard, we report herein the isolation, characterization, and molecular cloning of cDNAs encoding precursors of several new members of the dermaseptin B family of antimicrobial peptides.

MATERIALS AND METHODS

Chemicals—9-Fluorenylmethoxycarbonyl (Fmoc)[1]-protected L-amino acids and polyethylen glycol polystyren-graph copolymer support (PEG-PS resin, substituted at 0.18 meq/g) were from Milligen (Bedford, MA). Chemicals for peptide synthesis (dimethylformamide, dichloromethane, diisopropylcarbodiimide, hydroxybenzotriazol, piperidin, trifluoracetic acid, and acetonitrile) were obtained from commercial sources and were of the highest purity available.

Breeding of the South American Frog Phyllomedusa bicolor—Frogs were placed in large wooden cages ($120 \times 90 \times 90$ cm; 12 animals/cage) covered on three sides by plastic mosquito net to provide good ventilation. Philodendron, pothos, and dracena were used as perches, and water bowls were made available for nocturnal baths. The frogs were fed crickets. Humidity was kept at 65% by a constantly operating humidifier. Temperature was maintained at 25 ± 1 °C.

Purification of Dermaseptins B from Frog Skin Extract—The skin and adherent tissues from one specimen of *P. bicolor* were minced with scissors and extracted for 1 min with a Braun press in 40 ml of 10% acetic acid at 4 °C. The extract was centrifuged twice for 30 min at $10,000 \times g$, and the supernatant was lyophilized. The dried extract was dissolved in 200 ml of 5% acetic acid and lyophilized (yield, 800 mg). The resulting powder was dissolved in 30 ml of 10% acetic acid and fractionated on a calibrated gel filtration column (Sephadex G-50 superfine, 2.4×100 cm). Fractions (9 ml) were collected at a flow rate of 10 ml/h, from which aliquots (250 μl) were lyophilized and assayed for mitogenic activity as described below. Antimitogenic fractions (fractions 32–36, see Fig. 1) were pooled and evaporated under vacuum (yield, 40 mg). This material was subjected to reversed-phase HPLC chromatography on a Waters RCM compact preparative cartridge Deltapak C18 (300 Å, 25×100 mm) loaded with 40 mg of extract in water containing 0.1% trifluoracetic acid. After an initial 1-min wash in 0.1% trifluoracetic acid/water, elution was achieved at a flow rate of 8 ml/min with a 0–60% linear gradient of acetonitrile containing 0.07% trifluoracetic acid (from 3 to 33 min). 10-ml fractions were collected, lyophilized, and resuspended in 1 ml of water from which an aliquot (60 μl) was assayed for cytotoxic activity (see below). Fractions P2, P4, P3, and P1 that eluted at 26.5, 29, 30.5, and 31.5 min, respectively, were further fractionated either on a Lichrosorb C18 analytical HPLC column (5 μm, 250×4.6 mm; flow rate, 0.75 ml/min) or a Deltapak C18 column (5 μm, 150×2 mm; flow rate, 0.75 ml/min) using a 20–60% linear gradient of acetonitrile containing 0.1% trifluoracetic acid.

Amino Acid Sequence Analysis—Sequence analyses were carried out as described (9) on a Milligen 6600 solid phase sequencer after covalent binding of the samples (500 pmol) to Sequelon arylamine membranes. Phenylthiohydantoin amino acids were detected with an on-line HPLC column (Waters MS HPLC; SequaTag C18 phenylthiohydantoin analysis column; 350×3.9 mm) developed with ammonium acetate (pH 4.8) and acetonitrile and calibrated with 15 pmol of phenylthiohydantoin amino acid standards. Data collection and analysis were performed with a Maxima-phenylthiohydantoin chromatography analysis software package (Dynamic Solution Corp., Division of Waters Chromatography, Milford MA). Alternatively, analyses were carried out on a gas phase automatic protein sequencer (Applied Biosystems, model 476 A) calibrated with 32.5 pmol of phenylthiohydantoin amino acid standards.

Electrospray Ionization Mass Spectrometry—Mass spectral analysis were performed as described (9) using a quadrupole coupled electrospray mass spectrometer (VG Platform). The mass scale was calibrated using myoglobin. The accuracy was ± 0.1 atomic mass unit. Samples (25 pmol) were dissolved in a water/acetonitrile (1:1, v/v) mixture con-

taining 0.2% formic acid and introduced via a capillary using a microliter syringe. An electrospray voltage of 5 kV was applied to the internal wall of the source at the origin of the liquid dispersion for an electrospray formation and ion extraction. Ions were detected and analyzed in the positive mode as a function of their *m/z* ratio.

Solid Phase Peptide Synthesis—Dermaseptins B3 and B4 were prepared by stepwise solid phase synthesis using Fmoc polyamide active ester chemistry on a Milligen 9050 pepsynthesizer (9). All Nα-Fmoc amino acids were from Milligen. PAL-PEG-PS resin was used for carboxamidated dermaseptin B4. Fmoc-SPC resin was used for dermaseptin B3. Side chain protections were *tert*-butyloxycarbonyl for lysine and tryptophan, *O-tert*-butyl ester for aspartic acid, *O-tert*-butyl ether for threonine; and trityl for asparagine and glutamine. Syntheses were carried out using a triple-coupling protocol; Nα-Fmoc-amino acids (4.4 molar excess) were coupled for 30–60 min with 0.23 M diisopropylcarbodiimide in a mixture of dimethylformamide and dichloromethane (60:40, v/v). Acylation was checked after each coupling step by the Kaiser test. Cleavage of the peptidyl resins and side chain deprotection were carried out at a concentration of 40 mg of peptidyl resin in 1 ml of a mixture composed of trifluoroacetic acid, phenol, thioanisole, water, and ethyl methyl sulfide (82.5:5:5:5:2.5, v/v/v/v/v) for 2 h at room temperature. After filtering to remove the resin and ether precipitation at 20 °C, the crude peptides were recovered by centrifugation at $5,000 \times g$ for 10 min, washed three times with cold ether, dried under nitrogen, dissolved in 20% acetic acid, and lyophilized. After lyophilization, the crude oxydized peptides were purified by preparative reversed-phase HPLC on a Waters RCM compact preparative cartridge Deltapak C18 (300 Å; 25×100 mm) eluted at a flow rate of 8 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Homogeneity of the synthetic peptides was assessed by solid phase sequence analysis, mass spectrum analysis, and analytical HPLC on a Lichrosorb C18 column (5 μm, 4.6×250 mm) eluted at a flow rate of 0.75 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid/water.

Mitogenic Assay—Benzo[α]pyrene-transformed Balb/c mouse 3T3 fibroblast (BP-A31) cells were maintained in minimum essential medium supplemented with 6% fetal calf serum. For studies of mitogenic effects, the cells were seeded in Limbro 24-well boxes (2×10^4 cells/well). After 24 h, the medium was replaced with 1 ml of minimum essential medium containing 0.05% fetal calf serum plus 2.5 μM FeSO$_4$, and the cells were allowed to enter quiescence during the next 3 days. Quiescent cells, aliquots of the fractions from G-50 column, and [^{3}H]thymidine (28 Ci/mmol, 4 nCi/ml) were incubated during 24 h. After 24 h the rate of [^{3}H]thymidine incorporation was monitored as described (10).

Cytotoxic Assay—Cytotoxic activity of fractions recovered from preparative HPLC column was measured on a human breast carcinoma cell line (MCF-7) originally isolated from a pleural effusion of a primary breast cancer patient (11). Nonquiescent MCF-7 cells were incubated with the fraction to be tested for 24 h and then incubated with [^{3}H]thymidine (4 and 28 Ci/mmol) for 30 min. The reaction was stopped with 1 M ascorbic acid, and the rate of [^{3}H]thymidine incorporation was monitored as described (10).

Antimicrobial Assays—Minimal inhibitory concentrations of synthetic dermaseptins were determined against *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (CIP A22 and ATCC 27853), and *Escherichia coli* (ATCC 25922 and 54127) in sterilized 96-well plates (Nunk F96 microtiter plates, Denmark) in a final volume of 100 μl composed of 50 μl of 10^6 bacteria/ml in Luria Bertani culture medium and 50 μl of synthetic peptide in serial 2-fold dilutions in water. Synthetic peptides were weighted in a microbalance and solubilized in water at the desired primary dilution. Concentrations were determined by measuring the optical density of primary dilutions at 280 nm. Positive and negative controls were, respectively, water or 0.4% formol/water added to the same suspension. Inhibition of growth was determined by measuring optical density at 540 nm with a Titertek Multiskan MCC after incubation time of 6 h at 37 °C.

Cloning Procedure—One adult specimen of *P. bicolor* was anaesthetized and immersed in liquid nitrogen and kept deep frozen until further processing. The skins were completely dissected on dry ice, and the tissues (approximately 7 g) were homogenized. Total RNAs were extracted as described by Chomczynski and Sacchi (12). Poly(A)$^+$ RNAs were purified over an affinity oligo(dT) cellulose spun column supplied by Amersham Pharmacia Biotech, and cDNA library was constructed from skin poly(A)$^+$ RNA as described (13) using standard procedure (14). cDNA template for reverse transcription PCR was synthesized from RNA poly(A)$^+$ by reverse transcription. We used Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and a 3′ antisense gene-specific primer (primer 2, 5′-GCTCTCCCACCGCAT-CAG-3′) corresponding to the 3′ nucleotide sequence of coding exon 2 in

---

[1] The abbreviations used are: Fmoc, 9-fluorenylmetoxycarbonyl; DRS, dermaseptin; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

FIG. 1. **Fractionation profile of the skin extract on a Sephadex G-50 column using 10% acetic acid as eluant.** The absorbance at 280 nm is represented as a *solid line*. Mitogenic activity (*hatched area*) was measured by following [³H]thymidine incorporation on quiescent serum-deprived BP-A31 cells. *Inset*, reversed-phase HPLC chromatogram of fractions 32–36 from the G-50 column on a Waters RCM compact preparative cartridge Deltapak C18. Elution was achieved with a 0–60% linear gradient of acetonitrile. *Solid line*, absorbance at 220 nm; *dashed line*, percentage of acetonitrile. Cytotoxic activity (*hatched area*) was measured on MCF 7 carcinoma cells as described under "Materials and Methods." The *arrows* point to the elution position of cytotoxic materials subjected to further purification.
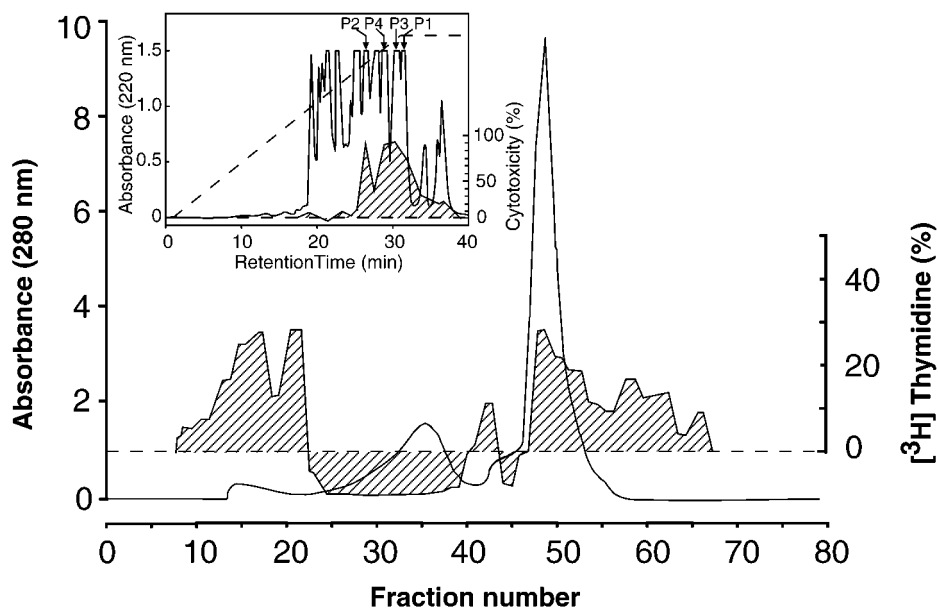
the dermaseptin *Drg2* gene (15). An aliquot of the cDNA was used for PCR. The reaction was performed using primer 2 and a 5′ sense gene-specific primer (primer 1, 5′-GGCTTCCCTGAAGAAATCTC-3′) corresponding to the 5′ nucleotide sequence of coding exon 1 in the dermaseptin *Drg2* gene under the following conditions: 94 °C for 180 s, followed by 30 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 180 s. At the end of the last cycle, the sample was further incubated at 72 °C for 5 min. The product were electrophoresed in 1.2% agarose gel, and the DNA fragments were excised from the gel and purified by Qiaquick gel extraction kit protocol (Qiagen). The PCR product was cloned in the pGEMT-Easy vector system (Promega). To determine the structure of the 3′ ends and 5′ ends of dermaseptins B3 and B4, we used the 3′ RACE and 5′ RACE strategy. Recombinant plasmids of the library were extracted from bacteria grown at 37 °C for 16 h in LB medium containing 100 µg/ml ampicillin and linearized by *Bam*HI or *Eco*RI. In the 3′ RACE, cDNA cleaved by *Eco*RI was used as template with an antisense universal primer (5′-GTAAAACGACGGCCAGTG-3′) and a 3′ sense gene-specific primer (primer 3, 5′-GAAGAAAAGAGAGC TCTGTGG-3′). In the 5′ RACE, cDNA linearized by *Bam*HI was used as template with a sense T7 primer (5′-AATACGACTCACTATAGGG-3′) and a 5′ antisense specific primer (primer 4, 5′-ACCTTTCAGCATATTTTTCCA-3′). The following thermal cycle profile was used for the RACE PCRs: 94 °C for 240 s, 25 cycles of 94 °C for 40 s, annealing at 56 °C for 30 s and 72 °C for 60 s, and a final extension step of 72 °C for 5 min. Race products were purified by Qiaquick Kit (Qiagen) and cloned in pGEMT-Easy vector (Promega). Nucleotide sequencing analysis was performed by dideoxy chain termination technique (16) in double stranded pGEMT-Easy vector. We used the fluorescence-labeled dye terminator method and an ABI 377 automatic sequencer.

<div align="center">RESULTS</div>

Isolation and Purification of Dermaseptins B—During the search for new growth factors in *P. bicolor* skin we have size-fractionated skin extracts on a calibrated Sephadex G-50 superfine column and tested aliquots of each fractions for the presence of mitogenic activities. As shown previously, restimulation of quiescent serum-deprived BP-A31cells with growth factors leads to a peak of [³H]thymidine incorporation during 24-h pulses (17). As shown in Fig. 1, several fractions of the skin extract were found to exert significant mitogenic activity. In sharp contrast, fractions eluting in the mid-volume of the column strongly inhibited [³H]thymidine incorporation by BP-A31 cells. Examination of the treated cells by light microscopy showed that cells began to swell after a very short period of time and to lose their intracellular content. As a first attempt to isolate this unexpected cytotoxic activity, fractions 32–36 from G-50 were pooled and fractionated on a reversed-phase HPLC preparative column. As depicted in Fig. 1, the initial

cytotoxic material was recovered under four wide UV-absorbing peaks eluting at Å 26.5, 29, 30.5, and 31.5 min, respectively. Each peak was collected and subsequently purified on a C18 Deltapak or Lichrosorb HPLC analytical columns. Six components were isolated, each emerging as a symetrical sharp peak accounting for >95% of the eluted material (Fig. 2). Inspection of the near UV spectra of each peak indicated the presence of the classical Trp signature.

Amino Acid Sequence and Mass Spectrometric Analysis—The primary structure of the purified peptide that was recovered under the 30.5 min eluting peak in Fig. 1 (Peptide P3) was successfully determined up to the 28th residue (Fig. 3) by automated Edman degradation of the peptide (150 pmol) using a solid phase sequencer after carboxylic covalent binding of the sample to a Sequelon arylamine membrane. Because the sequence analysis does not yield information on additional post-translational modifications of amino acid side chains, the purified peptide was also subjected to mass spectral analysis using electrospray ionization spectrometry. Two unequivocal pseudomolecular ions ($[M + {}^{3}H]^{3+}/3 = 927.7$ and $[M + {}^{4}H]^{4+}/4 = 695.9$) were observed that correspond to protonated species whose averaged molecular mass was 2780.4 (Fig. 2). This value corresponded to that expected theoretically for the experimentally determined amino acid sequence of 28 residues (Fig. 3).

Solid phase sequence analysis of the major peptide corresponding to the 29-min peak of Fig. 1 (Peptide P4) was interpretable until cycle 23. When subjected to electrospray mass spectrometry analysis, the peptide molecular mass was estimated to be 2997.15 ± 0.48 Da. The discrepancy of 587 Da between the measured and calculated molecular mass is because of the occurrence of a carboxamidated COOH-terminal residue because treatment of the peptide with 20% trifluoroacetic acid during 20 min at 110 °C gave then an interpretable sequence up to residue 28 (Fig. 3) whose theoretical molecular mass corresponded exactly to that experimentally determined by mass spectrometry. Using a similar procedure, the amino acid sequences of the 31- and 33-residue carboxamidated peptides, corresponding respectively to the 31.5-min (Peptide P1) and 26.5-min (Peptide P2) eluting peaks in Fig. 1, were determined (Fig. 3). Two other active peptides named P5 and P6 were also recovered during the final purification of peptide P4 and sequenced by a gas phase protein sequencer (Fig. 3). Whereas peptides P3, P4, P5, and P6 constitute previously
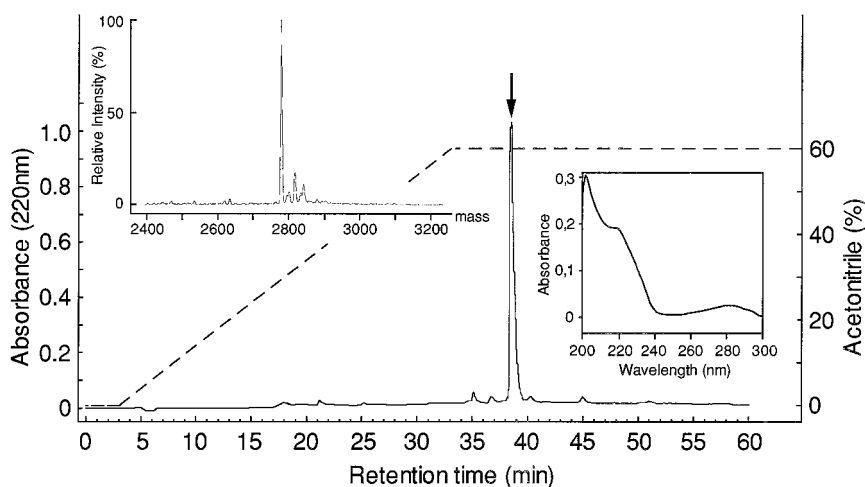
FIG. 2. **Example of the final purification step of the cytotoxic material that was recovered under the 30.5-min eluting peak in Fig. 1 (*inset*) (Peptide B3, *i.e.* dermaseptin B3).** The peak was loaded onto an analytical Deltapak C18 HPLC column. Elution was achieved with a 0–60% linear gradient of acetonitrile at a flow rate of 750 μl/min. The *arrow* points to the elution position of synthetic dermaseptin B3 under the same conditions. *Solid line*, absorbance at 220 nm; *dashed line*, percentage of acetonitrile. The *right-hand inset* represents the UV absorbance spectrum of the eluting peak at 38.19 min. The *left-hand inset* represents recalculated electrospray ionization mass spectrum of purified dermaseptin B3 showing an ion whose calculated average molecular mass was 2780.4 Da.

```
              1    5    10   15   20   25   30
DRS B2   GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV(CONH₂)
DRS S5   GLWSKIKTAG----KSVAKAAAKAAVKAVTNAV
DRS B5   GLWNKIKEA-------ASKAAGKAALGFVNEMV(CONH₂)
         ***.***          ***.***.  *  .  *
              1    5    10   15   20   25   30
DRS S1   ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
DRS S2   ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ
DRS B1   AMWKDVLKKIGTVALHAGKAALGAVADTISQ---(CONH₂)
         *.*  .******.************  *.****
              1    5    10   15   20   25   30
DRS S3   ALWKNMLKGIGKLAGKAALGAVKKLVGAES
DRS B3   ALWKNMLKGIGKLAGQAALGAVKTLVGA--
         ***************  *******  ****
              1    5    10   15   20   25
DRS B4   ALWKDILKNVGKAAGKAVLNTVTDMVNQ(CONH₂)
DRS S4   ALWMTLLKKVLKAAAKAALNAVLVGANA
DRS B6   ALWKDILKN----AGKAALNEINQLVNQ(CONH₂)
         ***  .**      *.** **  .    *
```

FIG. 3. **Maximized multiple sequence alignments of dermaseptins B from *P. bicolor* and dermaseptins S from *P. sauvagei*.** The numbering is indexed at the top of the longest of the dermaseptins. Alignments were performed by using CLUSTAL V Multiple Sequence Alignment software with fixed gap penalty. A Dayhoff PAM 250 matrix is used in peptide comparisons (40). The similarity scores for pairwise sequence alignments are calculated by the method of Wilbur and Lipman (41). Identical (*) and similar (·) residues among sequences are highlighted. Gaps (-) have been introduced to maximize sequence similarities.

unknown structures, peptide P1 exhibits strong sequence similarities with an antimicrobial peptide, designated dermaseptin B1, that have been recently isolated from *P. bicolor* skin (18). In particular, the sequence of dermaseptin B1 matches exactly that of the COOH-terminal core, residues 5–31, of peptide P1. Also, the missing NH₂-terminal tetrapeptide ALWK is present in the coding part of prepro-dermaseptin B1 (see below). These findings make it likely that the novel 31-residue peptide isolated herein represents the genuine native form of dermaseptin B1, whereas the shorter version arose through proteolysis *in vivo* or *in vitro*. In a similar vein, there is almost complete agreement between the amino acid sequences of the 33-residue peptide adenoregulin, also termed dermaseptin B2 (13), isolated by Daly *et al.* (19) from *Phyllomedusa* skin and that of peptide P2 of the present study. The only exception is the

presence of a carboxamidated valyl residue at the COOH terminus of B2 that was not detected in the former study.

The search for similarity in the complete amino acid sequences of dermaseptin B1 and B2 and that of the four novel peptides was performed using the Clustal V multiple alignment software (20). Pairwise and multiple alignments revealed a significant level of identity between the peptides (33–62% of amino acid positional identities). Typical conserved features are a tryptophan in position 3 and positive net charge because of the presence of lysine residues that punctuate an alternating hydrophilic and hydrophobic sequence. These properties, along with other characteristics (see below), suggest that the 6 peptides are homologous and are products of genes that are all members of the same family. Accordingly, the four novel peptides P3, P4, P5, and P6 were named dermaseptin B3, B4, B5, and B6, respectively.

Solid Phase Synthesis of Dermaseptins B3 and B4—Dermaseptins B3 and B4 were synthesized by the solid phase method to confirm their proposed structures and to demonstrate that cytotoxic/antimicrobial activities of the native peptides reflected intrinsic properties. Purification of the synthetic peptides was by reversed-phase HPLC. After purification, synthetic dermaseptins were shown to be indistinguishable from the natural products by the following chemical and physical criteria. HPLC analysis revealed that synthetic dermaseptins eluted exactly at the same positions as the natural corresponding products (Fig. 2); coinjection of the native and synthetic peptides resulted in only one symmetrical peak (not shown). The sequences of synthetic dermaseptin B3 and B4 could be determined up to Ala[28] and Thr[23], respectively, by automated Edman degradation after covalent binding of the peptides to a Sequelon arylamine membrane. Mass spectrometry of a sample of each synthetic peptide gave pseudo-molecular ions at *m/z* values corresponding to species whose molecular mass was identical to that obtained with the corresponding natural peptide. Because the synthetic products were found to be indistinguishable from their natural counterparts, they were used in the following to evaluate their antimicrobial spectra of activity.

Antimicrobial and Cytotoxic Activities—Synthetic dermaseptins B3 and B4 were tested for antibacterial and cytotoxic activities. As shown in Table I and Fig. 4, both peptides were highly active in inhibiting the growth of Gram-positive and Gram-negative bacteria with minimal inhibitory concentra-

TABLE I
Growth inhibition activity of DRS B3, DRS B4, and DRS S1

Organism	MIC[a]		
	DRS B3	DRS B4	DRS S1
	μM		
S. aureus (ATCC 25923)	1.3	3.0	60
P. aeruginosa (CIP A22)	2.3	4.6	43
P. aeruginosa (ATCC 27853)	5.0	11.6	50
E. (ATCC 25922)	2.6	5.0	46
E. (54127)	2.3	2.3	4.6
Cytotoxic activity[b]	12	15	ND[c]

[a] The minimal inhibitory concentration is defined as the dose at which 100% inhibition of growth was observed after 6 h of incubation at 37 °C in culture medium. These concentrations represent mean values ($\pm$30%) of two independent experiments performed in duplicates.

[b] 50% inhibition of [^{3}H]thymidine incorporation by MCF-7 carcinoma cells.
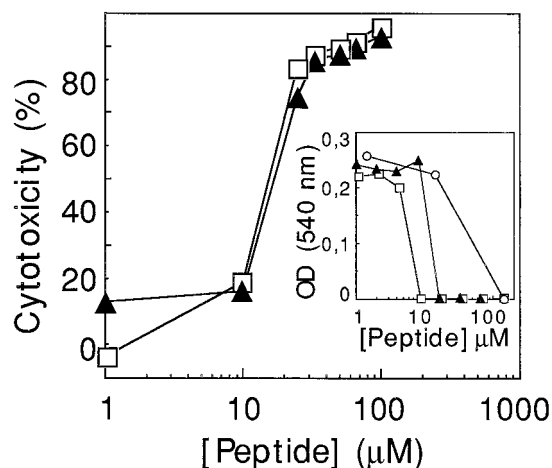
[c] ND, not determined.

FIG. 4. **Ability of synthetic dermaseptins B3 (□) and B4 (▲) to inhibit the incorporation of [₃H]thymidine by human transformed MCF-7 cells.** Each point represents the mean from two independent experiments performed in duplicate. Standard deviations were ≤10%. *Inset*, dose-dependent inhibition curves obtained for dermaseptins B3 (□), B4 (▲), and S1 (○) after 6 h of incubation with *P. aeruginosa* ATCC 27853. Each point represents the mean from two independent experiments performed in duplicate.

tions in the range 1–10 μM. The corresponding values for dermaseptin S1 from *P. sauvagei* (21) are also reported for comparison. The dose-response profiles obtained showed sharp curves in which 0–100% inhibition was generated within a 1–2-fold peptide dilution, suggesting that peptide aggregation occurs at a threshold concentration before membrane disruption (Fig. 4). No colony forming units were observed when plating bacteria after an overnight incubation with 1 μM dermaseptins B3 or B4, which indicated that the peptides are bactericidal. Cytotoxicity of dermaseptins B3 and B4 against mammalian cells was evaluated on a quantitative basis through their ability to inhibit the incorporation of [^{3}H]thymidine by human transformed MCF-7 cells. With this assay, no inhibition was detected at least up to a peptide concentration of 10 μM, a concentration corresponding respectively to the mean lethal dose against the least sensitive bacterial strain *P. aeruginosa* ATCC 27853 and 10-fold higher than that inhibiting the proliferation of the most sensitive strain *S. aureus*. Cytotoxicity, however, occurred at doses above 10 μM, with 50% inhibition at ~20 μM (Fig. 4).

Isolation and Identification of cDNA Clones Encoding Dermaseptins B—In cloning dermaseptins B cDNAs, we used polymerase chain reaction to amplify the mature prepro-sequence from skin poly(A)$^+$ mRNA using primers based on the NH$_2$-

and COOH-terminal coding sequences of dermaseptin gene *Drg2*. Because reverse transcription PCR experiments provided partial information on the target sequences, rapid amplification of cDNA ends was performed on linearized plasmids prepared from the cDNA library. Four different complete cDNAs were cloned and shown to encode the precursors of dermaseptins B1, B2, B3, and B4, respectively (Fig. 5). The deduced amino acid sequences of the precursor polypeptides all comprise that of a potential signal peptide for endoplasmic reticulum membrane translocation (residues 1–22) ending with a Cys residue. The peptide bond between Cys^{22} and Glu^{23} is the best point for cleavage of this signal sequence by a signal peptidase, as determined by the method of Von Heijne (22, 23). The putative signal sequence is immediately followed by an acidic proregion comprising 23 residues with a pair of basic residues (Lys^{22}-Arg^{23}) at its carboxyl terminus. Cleavage of the precursors at the carboxyl side of this cleavage signal for endoproteases and removal of the basic residues by carboxypeptidases would also liberate, in addition to mature dermaseptins, an acidic peptide containing 20–21 residues. This peptide is extremely rich in glutamic (43%, normal 6.2%) and, to a lesser extent, aspartic acid (30%, normal 5.3%).

The protein coding regions of prepro-dermaseptins B1, B2, B3, and B4 are strikingly similar, indicating that they were formed by gene duplication from a common ancestor (Fig. 5). The NH$_2$-terminal regions encompassing the signal peptides all contain 22 residues and are superimposable with only three exceptions (86% amino acid positional identity). The acidic prosegments contain 22–23 residues, exhibit 78% amino acid positional identity, and terminate with a pair of basic residues (Lys-Arg). Lastly, the 5′-untranslated regions of the corresponding cDNAs show about 53% sequence identity.

DISCUSSION

Analysis of the antimicrobial activities that are constitutively present in the skin secretions of *P. bicolor* revealed six related peptides, 24–33 residues long, termed dermaseptins B1 to B6, respectively. Although these peptides show diversity in length and sequence, they are all bona fide members of the dermaseptin B family characterized by a small size, a positive net charge because of the presence of lysine residues that punctuate an alternating hydrophobic and hydrophylic sequence, a conserved tryptophan residue in position 3, and the absence of Cys, Arg, and Tyr residues. Identities between different members of the family range from 33 to 62%. The proposal that dermaseptins from *P. bicolor* skin belong to a single family was further supported by comparing the nucleotide and amino acid sequences of the respective prepro-forms (Fig. 5). The four polypeptide precursors have a unique tripartite structure, that is, a signal peptide, an acidic proregion that terminates with two consecutive basic amino acids (Lys-Arg), and a progenitor sequence coding for a dermaseptin. Prepro-dermaseptins show a remarkable high conservation between the signal peptides (86% amino acid identity) and 78% identity between the acidic spacer sequences. The progenitor sequences are immediately followed by a Gly-Glu-Gln extension in prepro-dermaseptins B1, B2, and B4. Processing of these precursors should expose an extra Gly residue, which may serve as an amide donor for the COOH-terminal dermaseptin residue (24). The great overall similarities between the four precursors, both at the cDNA and polypeptide levels, make it likely that the corresponding genes belong to the same family and arose through duplications from a common ancestor.

Structure prediction using the Garnier-Osguthorpe Robson and Chou-Fasman methods indicated that all six dermaseptins have an overall propensity to assume an α-helical conformation encompassing residues 1–29, 1–33, 1–27, 1–18, 1–26, and 1–24

```
B1                              ccacgcgtccgattctgtcctccagtactcaacacattctgaattgtaagaacaaac   58
B2                                    tc*gg***t*********ac****c**g**   30
B3          c***********_____**********g**g***t*********ac****c**g**   51
B4                                cc*gggcagg******g*ac****c**g**   30

DRS B4    *    A   F   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   22
DRS B3    *    A   F   *   *   *   *   V   *   *   *   *   *   *   *   *   *   *   *   *   *   22
DRS B2    *    A   F   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   22
DRS B1    M    D   I   L   K   K   S   L   F   L   V   L   F   L   G   L   V   S   L   S   I   C   22
      B1  atg gat atc ctg aag aaa tct ctt ttc ctt gta tta ttc ctt gga ttg gtt tcc ctt tcc atc tgt  124
      B2  *** *c* t** **t *** *** *** *** *** *** *** c** *** *** *** *** **c **t *** **t *** **c   96
      B3  *** *c* t** *** *** *** *** g** *** *** *** *** *** *** *** *** **c **t **g **t *** ***  117
      B4  *** *c* t** *** *** *** *** a** *** *** *** c** *** *** *** c** **g **t *** **t *** ***   96

DRS B4    *   *   *   *   *   *   *   K   *   *   I   E   *   E   *   *   *   *   *   *   E   *   44
DRS B3    *   *   *   *   *   *   E   *   N   *   *   *   *   E   *   *   *   *   *   *   E   *   44
DRS B2    *   *   *   *   *   *   *   *   *   *   *   E   *   E   *   *   *   *   *   *   *   *   44
DRS B1    E   E   E   K   R   E   N   E   D   E   E   K   Q   -   D   D   E   Q   S   E   M   K   43
      B1  gaa gaa gag aaa aga gaa aat gaa gat gag gag aaa caa --- gat gac gag caa agt gaa atg aag  187
      B2  *** *** *** *** *** *** *** *** *** *** *** g** *** gaa *** *** *** *** *** *** *** ***  162
      B3  *** *** *** *** *** *** g*g *** a*c *** *** *** *** gag *** **t *** *** *** *** ga* ***  183
      B4  *** *** *** *** *** *** *** a** *** *** ata g** *** gaa *** **t *** *** *** *** gaa ***  162

DRS B4    *   A   L   W   K   D   I   L   K   N   V   G   K   A   A   G   K   A   V   L   N   T   66
DRS B3    *   A   L   W   K   N   M   L   K   G   I   G   K   L   A   G   Q   A   A   L   G   A   66
DRS B2    *   G   L   W   S   K   I   K   E   V   G   K   E   A   A   K   A   A   A   K   A   A   66
DRS B1    R   A   M   W   K   D   V   L   K   K   I   G   T   V   A   L   H   A   G   K   A   A   65
      B1  aga gct atg tgg aaa gat gtg tta aaa aaa ata gga aca gtg gcc tta cat gca gga aaa gcg gct  253
      B2  *** *gg c*t *** *gt a*a a*a aa* g** gt* gg* aa* ga* *ca **a aa* gc* *** *c* *** **t **a  228
      B3  *** *** c** *** *** a** a** c*g *** ggt **t **g *a* c*t **a ggg **a *** *ct tt* *gt **c  249
      B4  *** *** c** *** *** *** a*a *** *** **t g** *** *a* *ct **a gg* a*a **t *tt tt* aat a*a  228

DRS B4    V   T   D   M   V   N   Q   -   -   -   -   *   *   *                                   76
DRS B3    V   K   T   L   V   G   A   E                                                           74
DRS B2    G   K   A   A   L   G   A   V   S   E   A   V   *   *   *                               81
DRS B1    L   G   A   V   A   D   T   I   S   Q   -   -   G   E   Q                               78
      B1  tta ggt gca gtt gct gat aca ata agt caa --- --- gga gag caa TAA agtgaaaaaaatttaaaattgaa  318
      B2  gg* aaa **g *c* tta *g* g** g*t tc* g*g gcg gtg *** *** *** *** *tat**g****g*****ca**     299
      B3  ggt aag a*c c** *t* *gg g*c ga* --- --- --- --- --- --- --- *** ***t-------*a*****----   288
      B4  g*t ac* *at a*g *ta a** ca* --- --- --- --- --- **g *** *** *** *a*t**g****g****t-ca**   283

      B1  ttactctaaatagaacaattagcaataattgtgtcaaacctacattaaagcatactgaacc          (poly A)   379
      B2  **g****t**c***g*******t*****t****c*******tt*********a*t*****tgaa       (poly A)   363
      B3  *****t***ggg*t******tt*cc*****t*tc*c**at*ttt**t**atga*a**g*a*gtg      (poly A)   352
      B4  **g******gg**t*******t*********g********t**********gt*****tt          (poly A)   345
```

FIG. 5. **Nucleic and amino acid sequences of cDNAs encoding dermaseptin B1, B2, B3, and B4 from the skin of *P. bicolor*.** The predicted amino acid sequences of prepro-dermaseptins B are given in *capital letters* above the nucleotide sequences. Nucleotide and amino acid sequences of prepro-dermaseptins B2, B3, and B4 are indicated only where they differ from that of dermaseptin B1. *Asterisks* indicate nucleotides or amino acids identical to those of dermaseptin B1 cDNA. *Numbers* on the *right* indicate the positions of the nucleotides and amino acids. Nucleotides are numbered positive from the 5′ to 3′ ends of the cDNAs. Amino acids are numbered starting with position 1 in the open reading frames. The *dashed lines* indicate gaps in one nucleotide or amino acid sequence. A *solid line* is drawn under the amino acid sequence of the encoded dermaseptins B. The signal peptide and the polyadenylation signal are *doubly underlined*. The stop codon is in *capital letters*, and the basic doublet KR are in relief.

in DRS B1 to B6, respectively. When plotted on a Schiffer-Edmunson α-helical wheel, dermaseptins B reveal well behaved amphipathic helical structures, *i.e.* most of the basic and polar residues are aligned on a portion of the helical cylinder, whereas the lipophilic side chains occupy the remaining surface, the proportion of each surface varying from one peptide to another. Several lines of experimental evidence demonstrated that the ability to form well behaved α-helices at membrane interfaces is linked to antimicrobial/cytolytic properties of these peptides (6, 7). Although the activity spectrum of DRS B has not yet been analyzed in details, these peptides are certainly highly potent because they exhibit a nearly immediate lethal activity against Gram-positive and Gram-negative bacteria at micromolar doses.

In a previous work (25), we have isolated a whole family of dermaseptins from *P. sauvagei* (Fig. 3). It is interesting that some members of the dermaseptin B family resemble more closely their *P. sauvagei* counterparts rather than congeners originating from the same frog species. In that context, four subfamilies can be discerned on the basis of additional sequence characteristics. Although they differ widely in size (25–31 residues), DRS B2 and B5 constitute together with DRS S5 from *P. sauvagei* the first subfamily. By introducing a gap of four and eight residues into the sequence of DRS S5 and DRS B5, respectively, they can be readily hand-aligned with the longer type DRS B2. In this alignment, 15 positions are occupied by the same residues in all of the subfamily I dermaseptins, which amounts to about 45% identity. The overall impression that one gets from examining the alignment is that DRS S5 and B5 arose by deleting of the central portion, four to eight amino acid residues long, in DRS B2. Dermaseptins B1, S1, and S2 clearly form a separate subfamily of peptides of similar size (31–34 amino acids) where 23 positions are occupied by the same residues in all peptides (67% positional identity). DRS B3 and S3 are prototypical members of the third subfamily. They exhibit 26 identities in the 28 amino-terminal residues (86% positional identity). To this subfamily belongs DRS S4 because its 28-amino acid residue sequence is 53% identical to that of DRS B3. Dermaseptins B4 and B6 are allocated to a fourth subfamily and differ most from the others. Whereas these
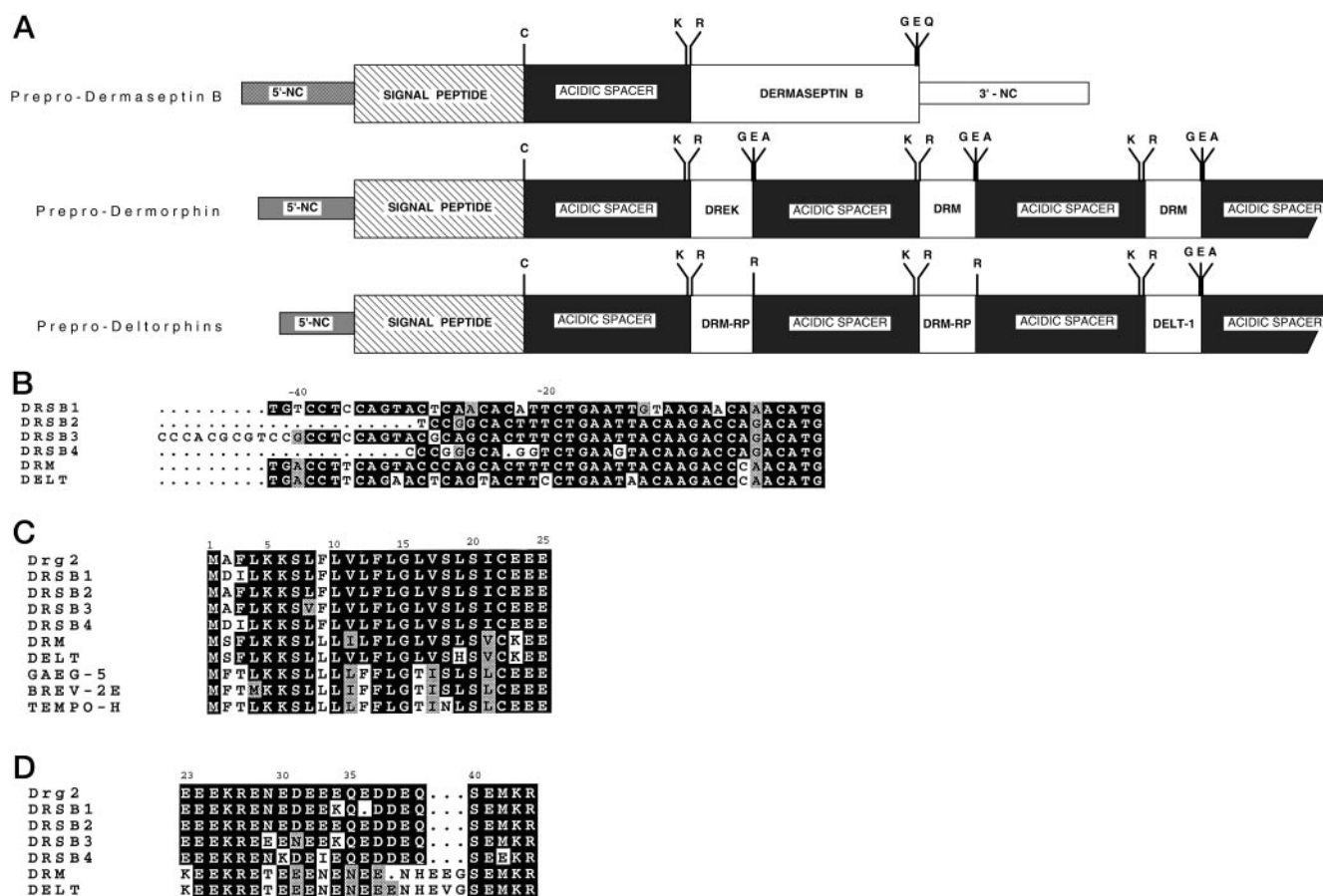
FIG. 6. *A*, block diagrams of prepro-dermaseptin B cDNAs, prepro-dermorphin (clone D-1/2) (27) and prepro-deltorphins (clone AD 2) (28) cDNAs. Regions of great similarity among all three cDNAs are labeled with the same boxed patterns as follows: *5′ NC*, 5′-noncoding region (*dark dotted box*); signal peptide (*hatched box*); acidic spacer peptide (*black box*); C, cysteine; *KR*, Lys-Arg dipeptide; *GEQ* and *GEA*, Gly-Glu-Gln and Gly-Glu-Ala tripeptides. *Open boxes* denote sequences with low or no homology among the four precursors: *3′ NC*, 3′-noncoding region; *DRM*, dermorphin; *DREK*, dermenkephalin; *DRM-RP*, dermorphin-related peptide; *DELT 1*, deltorphin I. Only partial sequences of the cDNAs encoding prepro-dermorphin and prepro-deltorphins are shown because the number of repeats varies and is clone-dependent. *B*, alignment of the 5′ DNA sequences of prepro-dermaseptins B1, B2, B3, and B4, prepro-dermorphin, and prepro-deltorphins. Identical nucleotides have *black backgrounds*. The nucleotides are numbered from the ATG. Gaps (·) have been introduced to maximize sequence similarities. *C*, alignments of the predicted amino acid sequences of the first coding exon in *Drg2* gene (15), the signal peptides from prepro-dermaseptins B1, B2, B3, and B4, prepro-dermorphin (27), prepro-deltorphins (28), prepro-gaegurin 5 (*GAEG-5*) (33), prepro-brevinin-2E (*BREV-2E*) (31), and prepro-temporin H (*TEMPO-H*) (34). The first three amino acids corresponding to the beginning of the acidic propiece are also shown. Identical (*black box*) and similar (*shaded box*) residues are shown. Gaps (·) have been introduced to maximize sequence similarities. *Numbers* above the prepro-dermaseptin B1 sequence indicate positions of residues corresponding only to the dermaseptin B precursors. *D*, alignments of the predicted amino acid sequences of the acidic propieces from gene *Drg2* exon 1, prepro-dermorphin, prepro-deltorphins, and the four prepro-dermaseptins B.

24–28-residue peptides have highly similar amino-terminal regions, their COOH termini are markedly different (64% identity). Despite the structural similarities between the DRS S and B subfamily members, they differ markedly in their ability to inhibit microbial proliferation (26). For instance, whereas DRS B3 and B4 are among the most effective peptides against the Gram-positive *S. aureus*, DRS S1 is 25–50-fold less efficient against this bacteria. These differences in potency are also observed against *P. aeruginosa* and *E. coli* ATCC 25922. In contrast, the three peptides inhibit the growth of *E. coli* 54127 with nearly the same efficiency. Hence, the biological significance of antimicrobial peptides with similar sequences in frog skin, such as the dermaseptins in *Phyllomedusa* sp., may be that they provide the frog with maximum protection against a wider range of potential invading microorganisms at a minimum metabolic cost.

The deduced amino acid sequences of prepro-dermaseptins B have striking similarities to plurifonctional precursor proteins prepro-dermorphin (27) and prepro-deltorphins (28) and to the precursors of brevinins, esculentins, gaegurins, and temporins (29–34), disulfide-containing antimicrobial peptides isolated from distantly related frogs of the Ranidae family (Fig. 6). Dermorphin, Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (35), dermenkephalin, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (36), also named dermorphin-gene associated peptide (37) or deltorphin A (38), and the deltorphins B and C, Tyr-D-Ala-Phe-Xaa-Val-Val-Gly-NH_2 (where Xaa is either Asp or Glu) (39) are opioid heptapeptide amides isolated from *Phyllomedusa* sp. skin that have been shown to be highly potent at and exquisitely selective for the μ and δ opioid receptors, respectively. These peptides are synthesized as part of two larger precursor proteins containing multiple copies of the heptapeptide bioactive sequences. As shown in Fig. 6*A*, prepro-dermaseptins B, prepro-dermorphin, and prepro-deltorphins are composed of three kinds of distinct domains arranged in a similar manner. The amino-terminal domain includes a signal peptide of 22 residues, followed by an acidic leader peptide domain containing 20–24 residues. The third domain consists of a progenitor sequence of variable length, coding either for a membranolytic or a neuroactive peptide, and is flanked by a paired basic residues at its amino end. The Gly residue at the carboxyl end of some of the progenitor sequences is involved in the formation of the COOH-terminal

peptide amide. The Glu residue that always flanks the carboxyl end of an antimicrobial or an opioid peptide sequence or that of the extra Gly residue may serve as cleavage site for an acidic carboxypeptidase during processing. Whereas prepro-dermaseptins each contain only a single copy of an acidic propeptide and an antimicrobial peptide assembled in tandem, prepro-dermorphin and prepro-deltorphins are made of five highly homologous repeats of this tandem module. A comparison of the cDNA and deduced amino acid sequences of the six prepro-proteins (Fig. 6) revealed that the signal peptides (72% identical at the amino acid level) and the acidic propeptides (43% identical at the amino acid level) are highly similar. This similarity also extends into the 5′-untranslated regions of the respective mRNAs. However, except for the presence of a conserved polyadenylation signal, the similarity is not quite as high in the 3′-untranslated regions of the mRNAs.

Most interesting, the signal peptide and part of the acidic proregion of the precursors of brevinins, esculentins, gaegurins, and temporins share ~50% amino acid identity with the corresponding regions of prepro-dermaseptins B, prepro-dermorphin, and prepro-deltorphins (Fig. 6). The remarkable similarity found between prepro-regions of precursors that encode end products with strikingly different origins, sequences, conformations, biological activities, and modes of action suggests that the corresponding genes have evolved with a conserved "secretory cassette". In that regard, we have recently characterized a gene, termed *Drg2*, involved in the production of a close variant of dermaseptin B2 (15). The gene has a two exon-coding structure interrupted by a small intron. The first coding exon contains codons for the first 25 amino acids of prepro-dermaseptin B2, comprising the conserved 22-residue signal peptide and the first three Glu residues of the acidic propiece. The second exon contains the sequence coding the remainder of the acidic propiece plus a Lys-Arg doublet and the dermaseptin progenitor sequence that is immediately followed by a stop codon. This finding reinforces the hypothesis that one exon encoding the signal peptide of several otherwise unrelated peptide precursors was present early in the evolution of amphibia. The duplication and recombinational events that promoted the association of such a homologous secretory exon with nucleotide sequences coding for a variety of end products in various amphibian species remain to be elucidated but are likely to have occurred at the very early stages of evolution.

Although the overall organization of eukaryotic signal peptides is evolutionarily conserved, no sequence similarity exists. The conservation of the signal peptide sequence within skin peptide precursors coding for a variety of end products in various amphibian species strongly suggests that this topogenic sequence may have an additional role that remains to be discovered.

REFERENCES

1. Jung, G. (1991) *Angew Chem. Int. Ed. Engl.* **30,** 1051–1068
2. James, R., Ladzunski, C., and Pattus, F. (eds) (1992) *Bacteriocins, Microcins and Lantibiotics*, Springer-Verlag, Berlin
3. Kolter, R., and Moreno, F. (1992) *Annu. Rev. Microbiol.* **46,** 141–163
4. Bierbaum, G., and Sahl, H. G. (1993) *Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis.* **278,** 1–22
5. Klaenhammer, T. R. (1993) *FEMS Microbiol. Rev.* **12,** 39–86
6. Boman, H. G. (1995) *Annu. Rev. Immunol.* **13,** 61–92
7. Nicolas, P., and Mor, A. (1995) *Annu. Rev. Microbiol.* **49,** 277–304
8. Lazarus, L. H., and Attila, M. (1993) *Prog. Neurobiol. (N. Y.)* **41,** 473–507
9. Fleury, Y., Abdel Dayem, M., Montagne, J. J., Chaboisseau, E., Le Caer, J. P., Nicolas, P., and Delfour, A. (1996) *J. Biol. Chem.* **271,** 14421–14429
10. Gray, H. E., Buchou, T., and Mester, J. (1987) *Exp. Cell Res.* **169,** 95–99
11. Zava, A. (1977) *Science* **196,** 663–664
12. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
13. Amiche, M., Ducancel, F., Boulain, J. C., Lajeunesse, C., Menez, A., and Nicolas, P. (1993) *Biochem. Biophys. Res. Commun.* **191,** 983–990
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Vouille, V., Amiche, M., and Nicolas, P. (1997) *FEBS Lett.* **414,** 27–32
16. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
17. Buchou, T., Charollais, R. H., Fagot, D., and Mester, J. (1989) *Exp. Cell Res.* **182,** 129–143
18. Mor, A., Amiche, M., and Nicolas, P. (1994) *Biochemistry* **33,** 6642–6650
19. Daly, W. J., Caceres, J., Moni, W. R., Gusovski, F., Moos, M., Seamon, B. K., Milton, K., and Myers, W. C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10960–10963
20. Higgins, D. G., and Sharp, P. M. (1989) *Comput. Appl. Biosci.* **5,** 151–153
21. Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., and Nicolas, P. (1991) *Biochemistry* **30,** 8824–8830
22. Von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21
23. Amiche, M., Ducancel, F., Mor, A., Boulain, J. C., Menez, A., and Nicolas, P. (1994) *J. Biol. Chem.* **269,** 17847–17852
24. Bradbury, F. A., and Smyth G. D. (1991) *Trends Biochem. Sci.* **16,** 112–115
25. Mor, A., and Nicolas, P. (1994) *Eur. J. Biochem.* **219,** 145–154
26. Mor, A., Hani, K., and Nicolas, P. (1995) *J. Biol. Chem.* **269,** 31635–31640
27. Richter, K., Egger, R., and Kreil, G. (1987) *Science* **238,** 200–202
28. Richter, K., Egger, R., Negri, L., Corsi, R., Severini, C., and Kreil, G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4836–4839
29. Morikawa, N., Hagiwara, K., and Nakajima, T. (1992) *Biochem. Biophys. Res. Commun.* **189,** 184–190
30. Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1993) *FEBS Lett.* **324,** 159–161
31. Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1994) *J. Biol. Chem.* **269,** 11956–11961
32. Park, J. M., Jung, J. E., and Lee, B. J. (1994) *Biochem. Biophys. Res. Commun.* **215,** 948–954
33. Park, J. M., Lee, J. Y., Moon, H. M., and Lee, B. J. (1995) *Biochim. Biophys. Acta* **1264,** 23–25
34. Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangone, M. L., and Barra, D. (1996) *Eur. J. Biochem.* **242,** 788–792
35. Montecucchi, P. C., De Castiglione, R., Piani, S., Gozzini, L., and Erspamer, V. (1981) *Int. J. Pept. Protein Res.* **17,** 275–283
36. Sagan, S., Amiche, M., Delfour, A., Mor, A., Camus, A., and Nicolas, P. (1989) *J. Biol. Chem.* **264,** 17100–17106
37. Lazarus, L. H., Wilson, W. E., de Castiglione, R., and Guglietta, A. (1989) *J. Biol. Chem.* **264,** 3047–3050
38. Kreil, G., Barra, D., Simmaco, M., Erspamer, V., Falconieri-Erspamer, G., Negri, L., Severini, C., Corsi, R., and Melchiorri, P. (1989) *Eur. J. Pharmacol.* **162,** 123–128
39. Erspamer, V., Melchiorri, P., Falconieri-Erspamer, G., Negri, L., Corsi, R., Severini, C., Barra, D., Simmaco, M., and Kreil, G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5188–5192
40. Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed) Vol. 5, Suppl. 3, p. 345, National Biomedical Research Foundation, Washington, D.C.
41. Wilbur, W. J., and Lipman, D. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 726–730