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Identification and expression of a novel marsupial cathelicidin from the tammar wallaby (*Macropus eugenii*)

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Abstract

Cathelicidins are important components of the innate immune system and have been identified in skin and epithelia of a range of mammals. In this study molecular techniques, including RACE-PCR, were used to identify the full cDNA sequence of a cathelicidin gene, MaeuCath8, from the Australian marsupial, the tammar wallaby, *Macropus eugenii*. This cathelicidin was not homologous to other such genes previously isolated from a tammar wallaby mammary gland EST library, however, it did contain 4 conserved cysteine residues which characterise the pre-propeptide and had 80% identity with a previously isolated bandicoot cathelicidin. Reverse transcriptase-PCR established the expression profile of MaeuCath8 in a range of tissues, including spleen, thymus, gastrointestinal tract, skin and liver, of the tammar wallaby from birth to adulthood. Expression of MaeuCath8 was observed in spleen and gastrointestinal tract of newborn animals and was observed in most tissues by 7 days post-partum. The results indicate that pouch young could synthesize their own antimicrobial peptides from an early age suggesting that this ability most likely plays a role in protecting the pouch young from infection prior to the development of immunocompetence.

Keywords

Cathelicidin; Antimicrobial peptide; Marsupials; RACE-PCR; m-RNA expression

1. Introduction

Antimicrobial peptides are gene-encoded proteins with broad-spectrum antimicrobial activity. They form part of the innate immune system and have been identified in a large variety of plants, insects and animals. Online databases now contain peptide sequences of over 800 of these antimicrobial peptides (e.g. <http://aps.unmc.edu/AP/main.html>). As well as forming part of the innate immune system, they also stimulate an adaptive immune response in vertebrates (Shinnar et al., 2003). More recently antimicrobial peptides have been shown to have potential

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as alternatives to antibiotics as micro-organisms continue to develop antibiotic resistance. The advantages of using antimicrobial peptides include their broad target range, minimal side effects and low development of resistance by target organisms (Hancock and Lehrer, 1998).

Cathelicidins are a group of antimicrobial peptides found predominantly in mammals and are distinguished by a gene structure composed of 4 exons and 3 introns. Exons 1–3 encode a well conserved pre-propeptide while exon 4 encodes the functional, but variable, antimicrobial peptide (Zanetti et al., 1995; Zanetti, 2005). The name, cathelicidin, is based on a similarity to cathelin, a serine protease inhibitor first identified from pig leukocytes (Ritonja et al., 1989). Most species produce several related cathelicidins, while humans, rats and mice have only one (Zanetti, 2005). The only human cathelicidin, hCAP18/LL37, was first isolated from bone marrow (Agerberth et al., 1995). In all cathelicidins the functional peptide is activated through the proteolytic processing of the pre-propeptide. The signal sequence is removed first by a signal peptidase (Shinnar et al., 2003) with the resulting product stored in the granules of neutrophils. The active antimicrobial peptide is released from the cathelin region by a serine protease in response to a microbial invasion coinciding with the need for the antimicrobial activity at the sight of infection (Tomasinsig and Zanetti, 2005). In hydrophobic solutions most active cathelicidin peptides form an α -helical structure that is thought to form membrane pores in target organisms, thus disrupting metabolic activity (Oren et al., 1999; Turner et al., 1998; Brogden, 2005).

Although predominately stored in the granules of neutrophils, cathelicidin expression has been demonstrated in a range of tissues in the pig (Wu et al., 1999), mouse (Gallo et al., 1997) and rat (Termen et al., 2003). In species producing multiple cathelicidins, these expression patterns appear to differ depending on the type of cathelicidin synthesized (Zanetti, 2005).

Marsupials have become a recent focus of research due to an increased interest in the opportunities afforded by their unique reproductive strategies. They have a short gestation period, ranging from 11 days in the striped-faced dunnart, *Sminthopsis macroura*, to 35 days in the koala, *Phascolarctos cinereus* (Tyndale-Biscoe and Renfree, 1987), after which the pouch young undergoes the majority of its physical and immunological development in a non-sterile environment of an external maternal pouch. During this time the young marsupial is apparently dependant on maternal immune defences and behaviour for protection. Studies of the development of immune tissues of marsupial pouch young suggests immunocompetence develops close to midway through pouch residency, for example, at approximately 90 days post-partum in the tammar wallaby, *Macropus eugenii* (reviewed by Old and Deane, 2000). It has been hypothesised that marsupials have developed specific strategies to fight infection prior to the development of immunocompetence and antimicrobial peptides may form an important component of this system. Multiple cathelicidin genes have been previously identified in 3 marsupials using different methods. These include a single gene in the Australian northern brown bandicoot, *Isodon macrourus*, using expressed sequence tags (ESTs) (Baker et al., 2007); 7 genes in the tammar wallaby, *M. eugenii*, from a mammary gland cDNA library screening protocol (Daly et al., 2008) and 12 genes from the American grey-short tailed opossum, *Monodelphis domestica*, using *in silico* sequence database screening (Belov et al., 2007).

The current study reports the identification of a novel eighth cathelicidin gene, MaeuCath8, from the tammar wallaby, *M. eugenii*. By applying the technique of reverse transcriptase (RT)-PCR and RACE-PCR, the complete cDNA sequence of MaeuCath8 was obtained and used to determine its expression profile in a range of tissues of the marsupial from birth to adulthood.

2. Materials and methods

2.1. Collection of tissue

Tissue samples were collected opportunistically from a breeding colony of tammar wallabies (*M. eugenii*) maintained at the Macquarie University Fauna Park, NSW, Australia. Sampled tissues included blood, skin, liver, lung, kidney, thymus, spleen, bone marrow and gastrointestinal tract (GIT). Samples were collected as either part of routine management of the captive population or surplus from other approved research protocols. Age of the pouch young was estimated based on head length measurements (Poole et al., 1991). Tissues were dissected and frozen immediately at -80°C until required.

2.2. Preparation of cDNA

Total RNA was extracted from each tissue using TRIreagent (Molecular Research Center Inc., OH, USA) according to the manufacturer's instructions. Synthesis of cDNA was performed using the Superscript First-strand Synthesis System for RT-PCR (Invitrogen Corporation, CA, USA).

2.3. Initial RT-PCR for isolation of tammar cathelicidins

Initial primers were designed to amplify the conserved region of the cathelicidin gene based on a partial sequence of a cathelicidin gene identified in the northern brown bandicoot, *I. macrourus* (GenBank accession number: EE744556) (Baker et al., 2007). The upstream forward primer, 5'-AATGCTTTCCGACTTCTC-3' and the downstream reverse primer, 5'-ACATTCTTTCACCGAGCCC-3', produced a 168 bp product. A 1 μl aliquot of test cDNA was added to a 25 μl master mix consisting of 5 pM of each primer (Sigma-Genosys, NSW, Australia) and 0.25 mM of each dNTP, 1 mM MgCl_2 and 1.25 U of GoTaq DNA Polymerase (Promega Corporation, WI, USA) in 1 $\times$ Green GoTaq Flexi Buffer (Promega Corporation, WI, USA). Amplification was undertaken using a hot-lid thermal cycler with the PCR conditions set at 94°C for 7 min followed by 35 cycles of amplification at 94°C for 2 min, 55°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were subsequently isolated by electrophoresis on a 2% (w/v) agarose gel and the excised bands purified using a Wizard gel clean up kit (Promega Corporation, WI, USA).

2.4. Cloning and sequencing of cDNA products

Amplified cDNA was cloned into TOP10 competent *E. coli* cells using a TA cloning kit (Invitrogen Corporation, CA, USA) according to the manufacturer's instructions. Plasmid DNA was extracted and purified using the QIAprep Spin Miniprep kit (Qiagen, NSW, Australia). Both strands of the partial cathelicidin gene encoding the conserved region of the preprotein were sequenced using a DNA capillary sequencer (Applied Biosystems, CA, USA) using the M13 primers available in the TA cloning kit.

2.5. Rapid amplification of cDNA 5' and 3' ends (RACE)-PCR

RACE-PCR was employed to extend the partial cathelicidin cDNA gene sequence obtained above. The same primer site, 5'-GCCCAACGACTGAGGAGCTCCTGCTG-3', was used for amplification in both the 5' and 3' direction. The method made use of the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., CA, USA) protocol and reagents from the Advantage 2 PCR Enzyme System (Clontech Laboratories Inc., CA, USA) using a cDNA template synthesized from adult tammar wallaby spleen.

2.6. Expression of the full length cathelicidin in adult and pouch young tissues

The extended cDNA sequence obtained from RACE-PCR was used to design primers to amplify the complete cathelicidin gene, MaeuCath8. The upstream forward primer was 5'-

ATGGAGCACCTCAGGAAGGT-3', while the downstream reverse primer was 5'-GTGTGAGAT-TAAGGGGGTGG-3'. These primers were used to determine the level of expression of MaeuCath8 in various tissues covering a range of ages from birth to adulthood. The same PCR conditions and amplification protocol as described above were used in each instance. Cloning and sequencing confirmed that the PCR products from various tissues originated from the targeted MaeuCath8 gene.

2.7. Sequence analyses

Analyses of the cathelicidin gene, MaeuCath8, and its translated protein made use of the tools available through BioManager on the Australia National Genomic Information Service (ANGIS) web site (www.angis.org.au). Multiple sequence alignments were constructed using the CLUSTAL W program (Thompson et al., 1994), while phylogenetic trees were generated from the translation start site to the final cysteine residue of the cathelicidin pre-propeptide using the Neighbour-joining method and the Kimura model of DNA distance (Felsenstein, 1989). The trees were bootstrapped 1000 times. The most likely site for signal sequence cleavage was calculated by the Signal P program using both neural networks and hidden Markov models trained on eukaryotes. The cathelin domain was determined by pfam analysis and confirmed on the NCBI site, while Garnier secondary structure prediction (Garnier et al., 1996) made use of the NPS@ web server (Network Protein Sequence Analysis) (Combet et al., 2000).

The MaeuCath8 gene sequence has been assigned the accession number EU 883635 by NCBI.

3. Results

3.1. Identification and analysis of the cathelicidin MaeuCath8

Use of primers to the conserved region of the cathelicidin gene of the northern brown bandicoot (Baker et al., 2007) resulted in the PCR amplification of a 168 bp fragment. Cloning and sequencing of the amplified region confirmed that it had 88% identity to the cathelicidin gene of the northern brown bandicoot. Subsequent RACE-PCR produced a 279 bp product in the 5' direction and 981 bp product in the 3' direction resulting in a 1234 bp product covering the full 507 bp length of the expressed tammar wallaby cathelicidin gene which was named MaeuCath8 consistent with previous nomenclature (Fig. 1). While recent genome sequence projects have led to the putative identification of multiple complete and incomplete cathelicidin genes in the tammar wallaby (Daly et al., 2008), none of these gene sequences were homologous to the MaeuCath8 identified in this study.

The translated MaeuCath8 encoded a 168 amino acid protein of 18.8 kDa with a calculated *pI* of 9.7. Consistent with all other cathelicidins identified to date, the protein contained 4 cysteine residues in the conserved region of the putative pre-propeptide (Ramanathan et al., 2002) (Fig. 2), and possessed 80% identity to the bandicoot cathelicidin.

The α -helical structure of most active cathelicidin peptides is thought to be responsible for the formation of pores in the membranes of target organisms, thus disrupting metabolic activity (Oren et al., 1999; Turner et al., 1998). Garnier secondary structure prediction, however, suggested that the putative active MaeuCath8 peptide is not an α -helical peptide, unlike those of MaeuCath1, MaeuCath3 and MaeuCath7 (Fig. 3) and several of the putative opossum antimicrobial peptides (data not shown). Interestingly, the predicted antimicrobial peptide from MaeuCath8 (*M_r* 5094; Figs. 1 and 3) is a very basic protein (*pI* 11.14) implying that it would be readily attracted and adhere to the negative surface charge of bacteria.

3.2. Phylogenetic relation between MaeuCath8 and other cathelicidins

A phylogenetic tree was constructed from the amino acid sequences of the conserved regions of the proteins from the first amino acid to the fourth conserved cysteine residue (see Fig. 2). The phylogenetic tree showed that MaeuCath8 did not cluster with the other *M. eugenii* cathelicidins which formed a separate branch on the tree, nor with the majority of *M. domestica* cathelicidins, but was closely related to a cathelicidin from the bandicoot, *I. macrourus*, on a branch of the phylogenetic tree that also included the *M. domestica* cathelicidins, MdoCATH1, MdoCATH3 and MdoCATH9 (Fig. 4). The closest related non-marsupial sequence was from the chicken (Lynn et al., 2004), whilst the nearest eutherian cathelicidin was the canine protein, K9CATH, isolated from bone marrow (Sang et al., 2007) (Fig. 4).

3.3. Expression of MaeuCath8 in tammar wallaby tissues

Expression of MaeuCath8 was observed in at least one stage of development in all tissue types tested (upper bands, Fig. 5). For each positive result G3PDH expression was also detected (lower bands, Fig. 5). Where duplicate samples were tested only one band is shown. Expression was detected in blood, gastrointestinal tract (GIT) and spleen less than 24 h after birth. Between the ages of 7 and 144 days MaeuCath8 cathelicidin expression was observed in all tissues types. Expression was most difficult to detect in blood, skin, GIT and lung.

4. Discussion

Using RACE-PCR a new marsupial cathelicidin, Maeu-Cath8, has been isolated and sequenced and its expression documented in the developing and adult tissues of the tammar wallaby. The approach used in this study contrasts with the protocols used to isolate the other tammar wallaby cathelicidin genes, MaeuCath1-7, which were detected during sequencing of contigs from a mammary cDNA library of *M. eugenii* (Daly et al., 2008). Phylogenetic analysis of the propeptide domain of MaeuCath8 showed that it had little sequence identity to MaeuCath1-7, and, like most cathelicidins, the putative active antimicrobial region was unique. This identification of multiple cathelicidins puts marsupials in a class with other mammals such as horses, cows, pigs and goats, all of which have several related cathelicidins (Tomasinsig and Zanetti, 2005).

The exact cleavage site of tammar wallaby cathelicidins leading to the production of an antimicrobial peptide, as well as the processing enzyme responsible for this cleavage, remains unknown. However, based on evidence from equivalent eutherian mammalian proteins which are generally processed by the serine proteinase, elastase, a marsupial elastase (-like) enzyme would cleave the propeptide on the carboxyl side of a small hydrophobic amino acid such as glycine, alanine or valine, with valine being preferred (Fig. 2; Daly et al., 2008). On this basis, the most likely site of cleavage of MaeuCath8 would be between the valine and threonine residues after the fourth conserved cysteine residue at the site, CDPV/TPEL (Fig. 1). This would result in a positively charged 46 amino acid peptide with little α -helical structure (Fig. 3). In horses, cows, pigs and goats at least one of the antimicrobial peptides possesses an α -helical structure (Tomasinsig and Zanetti, 2005). Similarly for the tammar wallaby the putative antimicrobial peptides, MaeuCath1, MaeuCath3 and MaeuCath7 are all predicted to possess α -helical structure (Fig. 3) as do the putative antimicrobial peptides MdoCATH1, MdoCATH2 and MdoCATH12, and to a lesser degree, MdoCATH7 and MdoCATH9, from the opossum, *M. domestica* (data not shown).

Development of immune competence to protect self requires maturation of the lymphoid organs in the tammar wallaby as in any other mammal. In the case of the tammar wallaby, this remains incomplete until midway through development in the maternal pouch (Old and Deane, 2000).

A cathelicidin, such as MaeuCath8, would be anticipated to play a crucial role in the innate immune protection at early stages of development, particularly at surfaces prone to microbial colonisation—the skin and GIT, and cathelicidin expression in skin is commonly observed in young mammals (Marchini et al., 2002). MaeuCath8 could be detected at various stages of development in a range of tissues including the spleen and thymus from day 1 and day 7, respectively, but was not detected in skin or consistently in the GIT during this early period (Fig. 5). Interestingly, the skin samples from the adult wallaby where MaeuCath8 was detected were obtained from the maternal pouch, suggesting that secretion of MaeuCath8 by epithelial cells within the pouch may itself provide protection to young (Fig. 5). It is of note that expression in both adult and pouch young tissues was observed in the absence of specific stimulation. This is consistent with observations of expression in other mammals and regulation of expression is an area that warrants further study (Zanetti, 2005).

Expression of MaeuCath8 was observed in the GIT at an early age, though this appeared to decline in juvenile and adult animals as the GIT becomes colonized with an increasingly more complex microflora (Chhour et al., 2008, Fig. 5). These observations suggest that MaeuCath8 may contribute to protecting the pouch young from external bacteria during the early stages of colonization of the GIT during a period when the immune system and tolerance are developing and the intestinal microbiome required by the juvenile and adult herbivore is not yet fully established but is instead predicated by a milk diet. Similarly MaeuCath8 expression was observed in the lung tissue at an early age (>7 days) as the nasopharynx remains open to the external environment, the lungs, like skin and GIT would be exposed to potential pathogens (Tyndale-Biscoe, 2005; Frappell and MacFarlane, 2006).

MaeuCath8 was also expressed in the kidney and bone marrow of pouch young and this expression continued to be observed in older pouch young tissues. Unfortunately no bone marrow samples were available from juveniles or adult animals to confirm MaeuCath8 expression in mature animals. The human cathelicidin, hCAP18/LL37, has been shown to protect the urinary tract from infection in humans (Chromek et al., 2006; Saeemann et al., 2007) and it is likely that antimicrobial peptides such as that derived from MaeuCath8 may play a similar role in controlling infection in the tammar wallaby. The greater levels of expression in the liver and blood of the pouch young compared with juvenile and adult animals paralleled expression observed in the GIT. Although such expression could not be attributed to potential exposure to micro-organisms it could reflect the most likely presence of this cathelicidin in neutrophil granules, as occurs in other mammals (Zanetti, 2005).

Previous studies have investigated the role that maternal strategies play in the protection of the tammar wallaby pouch young in the non-sterile pouch environment. The current study of the cathelicidin gene, MaeuCath8, and its expression in the tammar wallaby indicate that pouch young synthesize an antimicrobial peptide in a range of tissues from an early age and that the production of this cathelicidin may contribute to the survival of the young wallaby prior to the full development of its immune system.

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1 agaggaaggaacATGGAGCACCTCAGGAAGGTCCTGCTGTTGGCCAGTGTGGCCATCCTCATCTCCACAC 70
-M--E--H--L--R--K--V--L--L--L--A--S--V--A--I--L--I--S--T-- 19

71 AAGCTTTACCGCTGTCCAGCCTGAGCTATCAGCAGACCCCTTCCACAGCCATCCATTTCTACAATGAGGT 140
20 Q--R--L--P--L--S--S--L--S--Y--Q--Q--T--L--S--T--A--I--H--F--Y--N--E--V 43
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141 ACACAGAGGAGAAAATGCCTTCCGTCTTCTCCAGATCTACTCTCCTTCACCCAATCAGGGTCCCATGAG 210
44 -H--R--G--E--N--A--F--R--L--L--Q--I--Y--S--P--S--P--N--Q--G--P--H--E-- 65

211 CAAACTCTGAAGCGTCTGAACTTTAOCCTGAAGGAACTGTGTCCTCAACGACTGAGGAGCTCTGCTG 280
66 -Q--T--L--K--R--L--N--F--T--L--K--E--T--V--C--P--T--T--E--E--L--L--L-- 88
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281 ATCAATGTGACTTCAAACTGATGGGCTGCTGAAAGAATGTCAAGGCTCAGTATCCAATGAACAGGGCAT 350
89 D--Q--C--D--F--K--T--D--G--L--L--K--E--C--Q--G--S--V--S--N--E--Q--G--I 112
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351 TGCTGCCATCATCCTCACCTGTGACCCAGTGACCCAGAGCTCTTTCGCTCCAGAAGAAGCCCACTTCCC 420
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421 GGACGAAAGAAAGGTTCAAAGAGACACAACTGGGTCTACTCTGTGATTGCCCTTGAAAGCCCGGTG 490
136 -G--R--K--K--G--S--K--R--H--K--P--G--S--Y--S--V--I--A--L--G--K--P--G-- 158
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491 TAAAGAAATCCCATATATGGAGGCTCTAGGctttaaaccaacataaccagagcttgctttctccctta 560
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561 tattttactctttttcaatttccaccaccccttaactctcacacatacacacacaaacccacacacct 630
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1051 cttccttgagctcttggggaattgcactttggatgaaattgaagagcttctctcctggggtgatctgaaa 1120
1121 tatctcacgctccatggggaactgttgattctatgtatcttctggcgtagctaacctatgtaacttgc 1190
1191 ctaatttctggttgctatgtgctcagataaatggatttactcgtaaaaaaaaaa... 1234

```

Fig. 1.

Tammar wallaby cathelicidin gene cDNA and deduced amino acid sequence. 5' and 3' untranslated regions are in lowercase letters. Amino acid sequence is bold. Primer sites are indicated: original gene primers (dashed arrows), expression primers (dark arrows) and RACE primer site (boxed). Most likely signal peptide cleavage site is indicated by a star. Most likely active peptide cleavage site is indicated by a cross. The proposed conserved cathelin domain is shaded.

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1                                100
Pig PR39      ---METQRASLCLGRWS--LWLLLLLALVVPAS--AQALSREAVLRAVDRLNEQSSEANLYRLLELDQPP--KAEDPGTKPKVSVFTVKETVQSPPTTRQPP
Water Buffalo ---MQTRASLSLGRWS--LWLLLLLGLVVPAS--AQDLSREAVLRAVDQLNERSSSEANLYRLLELDPL--KDDADLGTRKPVSVFTVKETVQSPRTTQPPA
Cow Bac4      ---METQRASLSLGRWS--LWLLLLLGLVLPAS--AQALSREAVLRAVDQLNERSSSEANLYRLLELDPPP--DVEDLGARKPVNFRVKETVQSPRSLQPP
Cow CATH1     ---METQRASLSLGRWS--LWLLLLLGLVLPAS--AQALSREAVLRAVDQLNEQSSEANLYRLLELDQPP--QDDEDPSPKRVSVFRVKETVQSPRTTQPP
Sheep SNAP-29 ---METQRASLSLGRWS--LWLLLLLGLVLPAS--AQALSREAVLRAVDQLNEQSSEANLYRLLELDPPPQDDENSHIPKRVSVFRVKETVQSPRTTQPPA
Cow CATH7     ---METQRASLSLGRWS--LWLLLLLGLVVPAS--AQDLSREAVLRAVDQLNERSSSEANLYRLLELDPPPQDVEHPGARKPVSVFTVKETVQSPRTTQPP
Horse CATH1   ---METQRNTRSLGRWS--PLLLLLLGLVIPAT--TQALSREAVLRAVDGLNQRSSSEANLYRLLELDPL--KGDKDSOTPKPVSPFMVKETVQSPKINKQTP
Dog K9CATH    ---METQRDPSLGRWS--LWLLLLLGLVIPAT--SRALSREAVLRAVNGFNQRSSSEANLYRLLELDQNSQP--KGDGDPNIPKPVSVFTVKETVQSPRTTQPP
Human LL37    ---METQRDGHSLGRWS--LWLLLLLGLVMPALAIQVLSYREAVLRAIDGQNRSSSEANLYRLLELDLDP--TMDGDPOTPKPVSVFTVKETVQSPRTTQPP
Mouse CRAMP   ---MQPQRDVPFSLMWRSLSLLLLLLGLGFSQTP-----SYRDAVLRAVDQFNQSSLDTNLYRLLELDLDP--KGDGDPOTPKPVSVFRVKETVQSGAERQLP
MaeuCath1     ---MQVLLLVLLGLLS-----LMTPLGYAQDQ-----PYQDVLRNFQIEYNTRKSESESLFRLSVNLNPS--QESNDPTAFQLLFTIRETVQSKSEHRNP
MaeuCath2     MEGLTHQVLLLVLLGLLS-----LMTALGYAQDQ-----PYQDVLRNFQIEYNTRKSESESLFRLSVNLNPS--QESNDPTAFQLLFTIRETVQSKSEHRNP
MaeuCath5     MEQSTMQVLLLVLLGLLS-----LMTSLACADQ-----PYQDVLRNFQIEYNTRKSESESLFRLSVNLNPP--EE-----
MaeuCath6     MEQSTMQVLLLVLLGLLR-----LMTSLACADQ-----PYQDVLRNFQIEYNTRKSESESLFRLSVNLNPP--EE-----
MaeuCath4     ---MQVLLLVLLGLLS-----LMTSLACADQ-----PYQDVLRNFQIEYNTRKSESESLFRLSVNLNPP--EESNDPAV--PLKFTIRETVQSKTEHRNA
MaeuCath3     ---MQVPLLVLLGLMS-----LMTPLAYAQDQ-----RYQDVLRNFQIEYNTRKSGSESLFRLSVNLNPP--EESNDPAVPRLLFTIRETVQSKTEHRNA
Bandicoot     ---MEHLKLLILLASVA---LILPTQALPLSS---LNYQKALSMHIFYNQAHKGENAFRLQLTYLPSNQPGQEQTLKHLSTFTLETVMQPMMEELL
MaeuCath8     ---MEHLKLLILLASVA---LILSTQALPLSS---LSYQQLSTAIHFYNEVHRGENAFRLQLIYSPSNQPGHEQTLKHLSTFTLETVMQPTTEELL
Chicken       -----MLSCWV--LLLALLGGACALPAP---LGYSQLAQAVDSYNQRPEVQNAPRLLSADPEP--GPNVQLSSLHNLNFTIMETRQARSGAQL

101                                201
Pig PR39      ELQDFKENGVRVQGVGTVTNLNFSIHSLSISNEIQSVRRR-----PRPPYLPFRPPPPFPPLPFPPIPGFPFPFPFPFGR--
Water Buffalo EQCDFKFKGRVKGVGVTTLDFSDQFDLNCNALQSVG-----LWILLR---WLFRRG-----
Cow Bac4      EQCDFKENGVLVKQGVGTTLNLSYRSDPFDLNCNELQSVRR-----LHPQHQRFPFRPRWPKPLSLPLPRPGPRPWPKPL-----
Cow CATH1     EQCDFKENGVLVKQGVGTTLNLSYRSDPFDLNCNELQSVRR-----LHPQHQRFPFRPRWPKPLSLPLPRPGPRPWPKPL-----
Sheep SNAP-29 EQCDFKENGVLVKQGVGTVTTLNLSYRSDPFDLNCNELQSVRR-----LHPQHQRFPFRPRWPKPLSLPLPRPGPRPWPKPL-----
Cow CATH7     EQCDFKENGVLVKQGVGTVTTRYWIRGDFDITCNISAGLFRLRD-----SIRRGQKILEKARRIGERIKIDIFRG-----
Horse CATH1   EQCDFKENGVLVKQGVGTVTILGPVKDFDVSQGEFQVRKR-----FGRLAKSLR--MRILLPRKILLAS-----
Dog K9CATH    EQCDFKENGVLVKQGVGTVILDEDTGYFDLNCDSILQVKKIDRLKE---LITGGQRIGERIRRIQGRKIDFFNQLPREES-----
Human LL37    EQCDFKDKGLVRKEMGTTLNKAQSGFDISSEKDNRRFALLG---D---FFKSEKIKGEKFRIVQRIKDFLRNLVFPES-----
Mouse CRAMP   EQCAFKEGQVVRQGMCAVTTNPAADSFDISNEPQAQPFPRFKISRLAGLLKGGKIGEKLRKIQGRKKNFFQKLVQEQEJ-----
MaeuCath1     EECDFKKNGLVVEEIGCTVDLSSPSVDISCDGP-----EKVRGFGKLRKRLKFKNSIKK--RLKNFMVVPVPIPLPG
MaeuCath2     EECDFKKNNG-----VRVSWAG-----EGF-----
MaeuCath5     ---LAVKQIGTIDLSDPNPSVDISCDRP-----AKVKGRLWESLKRKATKLGDDIRN--TLRNFRIKFPVFRG
MaeuCath6     DECDFKKNGLVKQIGTIDLSDPNPSVDISCDRP-----AKVKGRLWESLKRKATKLGDDIRN--TLRNFRIKFPVFRG
MaeuCath7     DECDFKKNGLVKQIGTIDLSDPNPCVDISCDRP-----AKVKGRLWESLKRKAPKLGDDIRN--TLRNFRIKFPVFRG
MaeuCath4     DECDFKKNGVVSVTREGV-----REKKRFGFLNFIITLLRRVGEKVKTRFIAAIEKLIDTFLP-
MaeuCath3     DECDFKSGLVKEIGMVLDSSNPSDSISCDGVPGGDLNTE-----REKKRFGFLNFIITLLRRVGEKVKTRFIAAIEKLIDTFLP-
Bandicoot     DECDFKSGLVKEIGMVLDSSNPSDSISCDGVPGGDLNTE-----REKKRFGFLNFIITLLRRVGEKVKTRFIAAIEKLIDTFLP-
MaeuCath8     DECDFKTDGLLKEIGGSVNEQGIAAIILTDVPTPELFRS-----RPSPLPGRKKGSKRHKPGSYVIALGKPGVKKSPYMEAL--
Chicken       DSECFKEDGLVKDGAAPVVVLQGGRAVLDTVCVDSMADP-----VRVWVPLVIRTVIAGNYLYRAIKK-----

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Fig. 2.

Alignment of amino acid sequences of 20 cathelicidin sequences. Alignment conducted using CLUSTALW from BioManager (ANGIS). Cysteine residues conserved across all species are highlighted in dark grey. Identified active antimicrobial portions of the cathelicidin protein are highlighted in light grey including the predicted sequence of MaeuCath8.

Fig. 3. Secondary structure prediction. Widest lines represent predicted regions of α -helical structure. Sequences used were: Human (*Homo sapiens*) hCAP18/LL37 CR457083, pig (*Sus scrofa*) PR-39 X87236 and tammar wallaby (*Macropus eugenii*) MaeuCath1 EF624481, MaeuCath3 EF624483, MaeuCath7 EF624487 and MaeuCath8. The region of the known antimicrobial peptide (AMP) for hCAP18/LL37 is shown.

Fig. 4.

Phylogenetic tree of cathelicidin sequences based on amino acid sequence alignment. Sequences were trimmed to include only the non-variable region of the gene from the start of translation to the final cysteine residue. Bootstrap values are based on 1000 trees. Sequences used were: bandicoot (*Isodon macrourus*) EE744556; cow (*Bos taurus*) CATHL1 Y09472, CATHL3 Y09741, CATHL4 X67340, BMAP-28/CATHL5 X97609, BMAP-27/CATHL6 X97608, CATHL7 Y12729; chicken (*Gallus gallus*) AY534900; dog (*Canis lupus familiaris*) K9CATH AY392089; gorilla (*Gorilla gorilla*) CAMP DQ471359; horse (*Equus caballus*) eCATH-1 AJ224927, eCATH-2 AJ224928, eCATH-3 AJ224928; human (*Homo sapiens*) LL37 CR457083; mouse (*Mus musculus*) CRAMP AF035680; pig (*Sus scrofa*) PR-39 X87236; rat (*Rattus norvegicus*) rCRAMP AF484553; sheep (*Ovis aries*) SMAP-29 L46854; Tammar wallaby (*Macropus eugenii*) MaeuCath1 EF624481, MaeuCath2 EF624482, MaeuCath3 EF624483, MaeuCath4 EF624484, MaeuCath5 EF624485, MaeuCath6 EF624486, MaeuCath7 EF624487, MaeuCath8; water buffalo (*Bubalus bubalis*) AJ812216.

Fig. 5.

Expression of cathelicidin in tissues from RT-PCR using gene specific primers. PCR products were run on a 2% (w/v) agarose gel. This figure shows gel bands corresponding to the product of the correct size. For each age the cathelicidin gene expression is on the top (507 bp) with G3PDH underneath (220 bp). G3PDH amplification was included as a positive control.

Samples were run in duplicate where possible with only one positive result shown. 'N' indicates a negative result and 'X' indicates there was no tissue available for testing. Marker band for cathelicidin is 517 bp and G3PDH is 222 bp (not shown). In all cases the no template control was negative (not shown).