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A novel mouse beta defensin, *Defb2*, which is upregulated in the airways by lipopolysaccharide

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Abstract Studies have shown that beta defensins are present in the human airways and may be relevant to the pathogenesis of cystic fibrosis lung disease. Here we report the identification of a novel mouse gene, *Defb2*, which shows sequence similarity to previously described mouse and human airway beta defensins. *Defb2* does not appear to be expressed in the airways of untreated mice but it is upregulated in response to lipopolysaccharide. The induced expression of this gene by an inflammatory stimulus strongly suggests that this defensin contributes to host defence at the mucosal surface of the airways.

Key words: Antimicrobial peptide; Defensin; (Mouse)

1. Introduction

Defensins are a large family of antimicrobial cationic peptides of which two groups are present in vertebrates (alpha and beta) distinguishable by the spacing and connectivity of the conserved cysteine residues within the mature peptide.

Recent studies have shown beta defensins to be present in the airway epithelia of several species including humans, rodents and cattle where they contribute to the host defence system by the eradication of pathogens at the mucosal surface. There have been two beta defensins identified in humans to date, *hBD-1* and *hBD-2* [1–3]. HBD-1 synthetic peptides have been shown to have a broad spectrum bactericidal activity which is markedly reduced in elevated salt concentrations similar to those reported for cystic fibrosis (CF) airway surface fluid (ASF) [1,4,5]. HBD-2 has also been shown to have a salt-sensitive antimicrobial activity [6] and similar to the bovine beta defensin, TAP, but unlike *hBD-1*, can be transcriptionally induced by bacterial products [3]. It was therefore proposed that the reduced ability of these beta defensins to kill bacteria in the CF environment was of relevance to the pathogenesis of CF lung disease and gave some form of explanation for the early onset of bacterial colonisation in the CF lung [1]. We recently reported the identification and characterisation of a mouse beta defensin, *Defb1*, whose protein sequence had 57% similarity to HBD-1, was expressed in the airway epithelia and whose synthetic peptide was shown to have salt-sensitive antimicrobial activity [4]. Here we report the identification of a second mouse beta defensin, *Defb2*, from a BAC containing *Defb1*. We show that its expression, unlike *hBD-1* or *Defb1* but in common with *hBD-2*, is induced in the airways by lipopolysaccharide (LPS). This supports the hypothesis that beta defensins contribute to a dynamic host defence at the mucosal surfaces.

2. Materials and methods

2.1. Isolation of mouse defensin-related gene

Defb1 is located on BACs I8 and I20 (Research Genetics Inc.) and these were screened for the presence of related defensin genes by Southern hybridisation using the *Defb1* cDNA sequence as the probe at a reduced stringency hybridisation temperature of 55°C. A 2 kb fragment of BAC DNA was subcloned which only hybridised to the *Defb1* probe at the reduced stringency temperature, indicating it was not *Defb1*.

2.2. DNA sequencing

Purified plasmid DNA was sequenced from both strands using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

2.3. RACE

Total RNA was prepared from C57BL/6N adult mouse kidney tissue using RNAzol B (Biogenesis) as described by the manufacturer. First strand cDNA synthesis was performed using SuperscriptII (Gibco BRL) and primer R1 (CATTTCATGTACTTGCAACAG). Products were size selected by microdialysis on a 0.025 μM filter against TE for 4 h and then poly-A tailed. Second strand cDNA synthesis was accomplished using primer R2 (CCTGCTCGAATTCAAGCTTCTTTTTTTTTTTTTTTT) and the products microdialysed on a 0.1 μM filter against TE for 4 h. PCR amplification of the resultant cDNAs was performed using primers R3 (CCAGGACGCCTGACAGAAGG) and R4 (CCTGCTCGAATTCAAGCTTC) and the following conditions: 30 cycles of 94°C for 1.5 min, 60°C for 1.5 min and 72°C for 3 min. Products were then microdialysed on a 0.1 μm filter against TE for 4 h and a nested PCR performed using primer R4 and R5 (CCCTCCATTGGTGTGGCAGTG) with cycling conditions as above. PCR products were analysed on a 1% agarose gel by electrophoresis and cloned using Original TA Cloning Kit (Invitrogen). Cloned products were analysed by hybridisation with an exon-specific oligo (GGATACGAAGCAGAAC) and an intron-specific oligo (CGAGACAATTGCTTGG). Clones positive for the exon-specific oligo and not the intron-specific oligo were sequenced as above.

2.4. Long-range PCR

Long-range PCR was performed on 50 ng BAC I8 or 1 μg genomic DNA using the Extend Long Template PCR System (Boehringer Mannheim) and primers dr1 (GCCATGAGGACTCTCTGCTC) and dr2 (TGTCACTTGACTTCCATGTGC) and the following conditions: denaturation 92°C for 2 min; 30 cycles 92°C for 10 s, 65°C for 30 s and 68°C for 15 min; final extension 68°C for 7 min. The amplified products were analysed on a 0.7% agarose gel by electrophoresis. The gels were then blotted and probed with an internal oligo (R5).

2.5. Tissue expression of Defb2

Total RNA was isolated from a variety of tissues from C57BL/6N mice using RNAzol B as described by the manufacturer. The samples were DNase-treated then cDNA synthesis was accomplished using a first strand cDNA synthesis kit (Boehringer Mannheim). The resultant cDNAs were used as a template in a PCR reaction for *Defb2* with primers dr1 and dr2 and the following conditions: 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The amplified products

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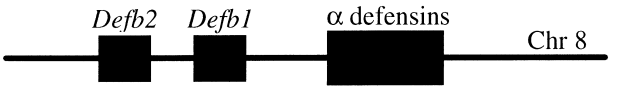

Fig. 1. Diagrammatic representation of mouse chromosome 8 indicating the relative positions of *Defb2, Defb1* and the alpha (α) defensin gene cluster.

(250 bp) were then analysed on a 2% agarose gel by electrophoresis. Amplification of *Hprt* was carried out in parallel as previously described [4]. Reactions were verified for RNA amplification by including controls without reverse transcriptase.

2.6. LPS administration and semi-quantitative RT-PCR

40 μg of LPS (*Escherichia coli* O55:B5, Sigma) was administered to mice by intratracheal delivery and the mice were killed at various timepoints after instillation. Total RNA was isolated from the lungs and trachea and RT-PCR for *Defb2* was performed as described above.

2.7. Quantification of tumour necrosis factor-α (TNF-α)

C57BL/6N mice were treated with 40 μg of LPS as described above. The mice were killed 1, 2, 4, 24 or 48 h after treatment and their lungs lavaged with 3×1 ml sterile PBS. Undiluted lavage fluid and a 1:10 dilution were assayed in duplicate for murine TNF-α using a commercially available ELISA kit (Genzyme).

2.8. Culture of primary mouse epithelial cells

Tracheal epithelial cells were isolated from eight male C57BL/6N mice, seeded onto four collagen-coated, cell culture inserts (Costar Transwell-Clear 3470, 0.4 μm pore size, 0.5 mm diameter) and grown with an air-liquid interface to confluent, polarised, ciliated monolayer as described (Davidson et al., in preparation).

2.9. LPS administration to primary mouse epithelial cells

Primary cultures were used 12 days after seeding, adding 80 μg of LPS (*E. coli* O55:B5, Sigma) in 25 μl of PBS to the apical surface of the primary culture or 25 μl of PBS alone on the controls. The cells were incubated at 37°C for 4 h then washed three times with PBS before isolation of RNA.

A.

Defb2

```
ctctctggagtctgagtgccctttctaccagcc atg aggactctctgctctctgctgctgatatgctgcctccttttctcatatacc
ctctctgcactctggaccctggctgccaccact atg aaaaactcattactttctcctggtgatgatatgttttcttttctcccagatg
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Defb1 ↓

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actccagctgttggaagtttaaaaagtattggatacgaagcagaacttgaccactgccacaccaatggagggtactgtgtcagagcc
gagccaggtgttggcattctcacaagtcttggacgaagaacagatcaatacaaatgccttcaacatggaggattctgtctccgctcc

atttgtcctccttctgccaggcgtcctgggagctgtttcccagagaagaaccccctgttgcaagtacatgaaa tga ttagaaggaagc
agctgcccatctaataccaaactacagggaacctgtaaaccagataagcccaactgttgtaagagc tga cagtagtttgaagaatgg

acatggaagtcaagtgacaga.tgtgtaattgatgtttca ataaa
acataaaggacgagcgatggattgtaaaattagtgttttta ataaa
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B.

Defb2

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1 MRTLCSLLLICCLLFSYTTPAVGSLKSIGYEAELDHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKYMK 71
1 MKTHYFLLVMICFLFSQMEPGVGILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS   69
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Defb1

C.

	Defb1	Defb2	HBD1	HBD2
Defb1	-	50.0	57.4	42.4
Defb2	50.0	-	41.2	35.6

Fig. 2. A: Comparison of *Defb2* and *Defb1* nucleotide sequence. Start codon, stop codon and poly-A signal are highlighted in bold. ↓ indicates the boundary between exon 1 and exon 2. B: Comparison of the predicted amino acid sequences of Defb2 and Defb1. Vertical line, direct match; two dots, close similarity; one dot, some similarity. C: Peptide similarities between the mouse beta defensins Defb1 and Defb2 and the human beta defensins HBD1 and HBD2.

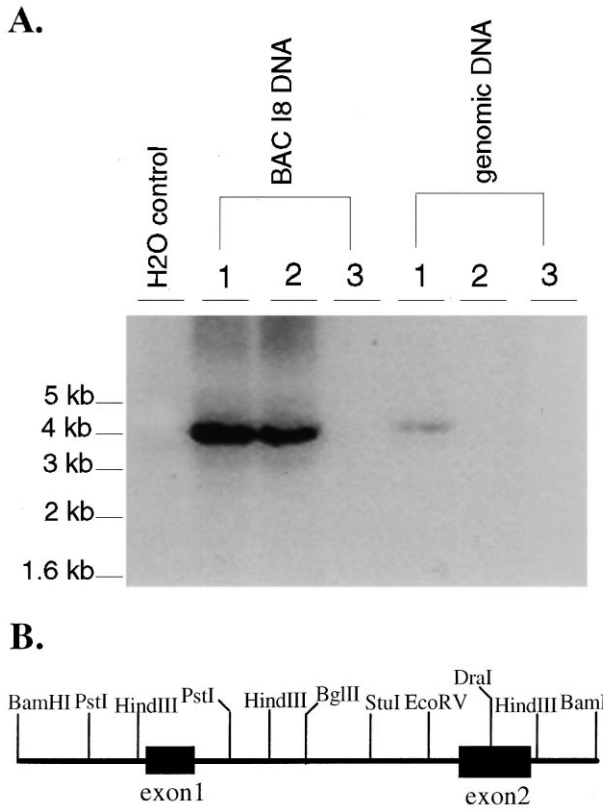

Fig. 3. A: PCR across the *Defb2* intron using BAC I8 or mouse total genomic DNA. 1, 2 and 3 refer to the buffers used in the PCR reactions containing 17.5 mM $MgCl_2$, 22.5 mM $MgCl_2$ or 22.5 mM $MgCl_2$ and detergents, respectively. *Defb2* PCR products were verified by hybridisation to a *Defb2* internal probe. B: Restriction map of *Defb2*.

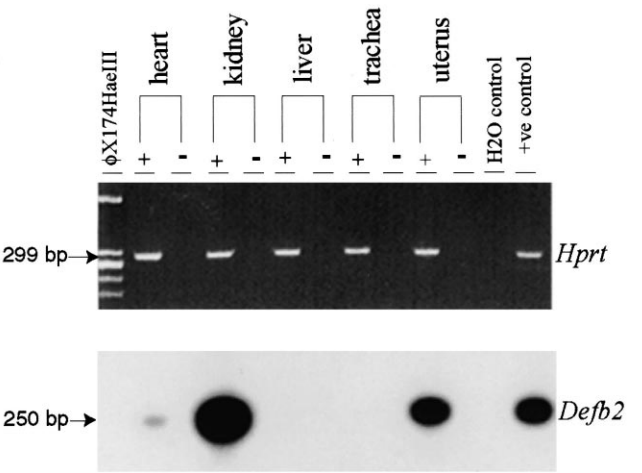

Fig. 4. RT-PCR of *Hprt* and *Defb2* from various mouse tissue RNAs. A plus (+) and minus (−) reverse transcriptase reaction is shown for each sample. *Defb2* PCR products were verified by hybridisation to a *Defb2* internal probe.

3. Results

3.1. Identification of a novel mouse sequence related to Defb1

Defb1 had previously been located on BACs I8 and I20 [4] and following low-stringency hybridisation, with *Defb1* cDNA as a probe, several novel bands were observed for BAC I8. No novel bands were observed for BAC I20. Since BAC I20 has been found to contain murine alpha defensin sequences [4] it indicated that these novel bands came from sequences more distal to the alpha defensin gene cluster than *Defb1* (see Fig. 1). A 2 kb hybridising fragment from BAC I8 was cloned and sequenced and a region of the sequence was shown to have homology to exon 2 of *Defb1*. An exon/intron predictor program indicated an intron/exon boundary site for the new sequence in the same position as that of *Defb1*. Therefore the 2 kb cloned region from BAC I8 was shown to contain a region showing homology to exon 2 of *Defb1* and also 1.5 kb of an intronic region 5′ to the putative exon 2. The 5′ end of the cDNA was successfully cloned by RACE and this novel gene was named *Defb2* (EMBL accession number AJ011800). Its nucleotide sequence was shown to be 60% identical to *Defb1* and the peptide translation of the entire coding sequence was found to have 50% similarity to *Defb1* (see Fig. 2A,B). Fig. 2C shows the similarity of this predicted peptide to Defb1 and the human beta defensins HBD1 and HBD2. Long-range PCR, shown in Fig. 3A, confirmed the presence of an intron of 4 kb between exons 1 and 2 and Fig. 3B is a restriction map of *Defb2* which was found to extend over approximately 5 kb of genomic DNA.

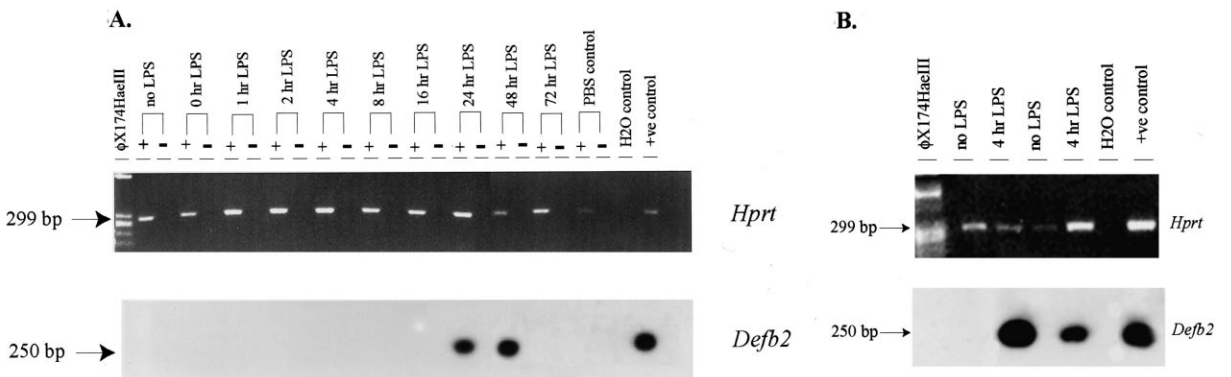

Fig. 5. A: RT-PCR of *Hprt* and *Defb2* from tracheal RNA following LPS instillation. The hours shown represent the time after LPS instillation when the mice were killed with a plus (+) and minus (−) reverse transcriptase reaction shown for each sample. B: RT-PCR of *Hprt* and *Defb2* from mouse primary epithelial cells in the absence of LPS and 4 h following LPS instillation. *Defb2* PCR products were verified by hybridisation to a *Defb2* internal probe.

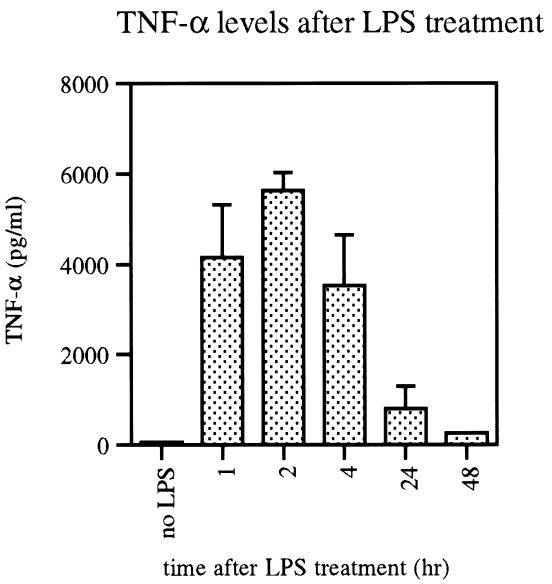

Fig. 6. Levels of TNF-α retrieved from mouse airways following LPS instillation. Each timepoint represents the mean TNF-α value of five mice. Standard deviation bars have been applied to all the mean values.

3.2. Expression of Defb2

Fig. 4 shows expression of *Defb2* by RT-PCR was detected in kidney and uterus and at much lower levels in the heart. RT-PCR failed to detect any *Defb2* expression in any other tissue tested. We examined the airways in response to LPS administration and found *Defb2* expression to be induced in the trachea (see Fig. 5A).

Expression of *Defb2* was not found in primary cultures of mouse tracheal epithelium under normal conditions but its expression was found to be induced to a detectable level 4 h after LPS administration to the cells as shown in Fig. 5B.

3.3. TNF-α expression following LPS treatment

Fig. 6 shows TNF-α levels to be increased in the mouse airways in response to LPS treatment. The maximum level of TNF-α was found to be approximately 100-fold higher than normal levels and was detected 2 h after LPS administration.

4. Discussion

The amino acid sequence, chromosomal localisation and expression data of *Defb2* indicate it to be a member of the murine beta defensin family. We have previously demonstrated the close physical proximity of the alpha defensin gene cluster to *Defb1* on mouse chromosome 8 [4] and here we have identified a second mouse beta defensin within the same BAC indicating that the two mouse beta defensins are also in close proximity.

It is likely that gene duplication of a common ancestral defensin gene occurred before man/mouse species divergence to create the alpha and beta defensin genes [7]. The differences between the mouse and human alpha defensins indicates that this branch of the defensin family expanded after man/mouse species divergence. The nucleotide sequence identity of 60% between *Defb1* and *Defb2* suggests that these two genes diverged from each other a long time ago, possibly before mammalian radiation. However, further studies on the evolution of these genes are required before any predictions can be made on when beta defensin gene expansion occurred in these two species. *Defb2* does appear to be homologous to the two human beta defensins found to date but due to the dissimilarity in nucleotide sequence to these human genes it is unlikely that *Defb2* is the orthologue of *hBD-1* or *hBD-2*. However, it is possible that an, as yet undiscovered, human beta defensin exists which is more similar to *Defb2* and work is currently under way to explore this possibility. It is hoped that the identification of additional members of the defensin family in mouse and man, if they exist, will help to clarify the evolution of these peptides. To date, this is only the second murine beta defensin identified that is expressed in the airways. The fact that its expression, unlike *Defb1*, was not found to be constitutive in the lung but that it is induced following an inflammatory stimulus suggests that this defensin may be an important component of the host response to pathogens. Expression was detectable from primary mouse epithelial cells 4 h after LPS administration whereas *Defb2* expression was not detected from tracheal tissue until 24 h after LPS treatment. The difference in the kinetics of the *Defb2* response may simply be a reflection of the different experimental conditions employed or it may indicate that non-epithelial cells found in the trachea may have a role in the regulation of *Defb2* expression.

Functional studies have not been performed on this novel defensin but, due to its predicted structural similarities to the other defensins with conserved cysteine domains capable of forming disulphide bridges, it is anticipated that it will be antimicrobial. The mechanisms which lead to the upregulation of *Defb2* in response to LPS are unknown. Studies on the bovine airway beta defensin TAP have revealed the presence of the nuclear transcription factors NF-κB and NF-IL6 binding sites in the 5′ untranslated region (UTR) of the gene [8] offering a possible explanation as to inducible gene expression via interactions with these *cis*-acting sequences. It will be of interest to investigate whether there are any similar regulatory control elements present in the 5′ UTR or the promoter region of *Defb2*. It has also been shown that TAP expression can be induced by the addition of the pro-inflammatory cytokine TNF-α and we have demonstrated in the mouse that treatment with LPS also leads to an induction of TNF-α expression in the airways. TNF-α mediates its downstream signals via NF-κB and therefore the actions of raised levels of TNF-α may cause the upregulation of *Defb2*. TAP expression has also been shown to involve the actions of the LPS binding protein CD14 [9]. CD14 is known to be released from mouse bronchial epithelial cells [10] and therefore it is also possible that it is also involved in the induction of *Defb2*.

The RT-PCR analysis performed was at best semi-quantitative but indicated that *Defb2* is expressed at a low level. It is possible that the Defb2 peptide may only be required at low levels to have a potent antimicrobial activity or that it has synergistic effects with other beta defensins in the mouse airways or with other antimicrobial substances such as lysozyme.

In conclusion, we have identified a novel mouse beta defensin whose expression is upregulated in the airways in response to an inflammatory stimulus. Studies are currently ongoing to elucidate the antimicrobial actions of this peptide, whether its activity is salt-sensitive and the effects of different pathogens on the inducibility of this gene in the airways. It will also be

interesting to examine whether this defensin has any synergistic effects with other mouse beta defensins or antimicrobial substances.

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