Identification and Biological Characterization of Mouse \( \beta \)-Defensin 14, the Orthologue of Human \( \beta \)-Defensin 3*

Johann Röhr†, De Yang‡, Joost J. Oppenheim§, and Thomas Hehlgans†,‡

From the †Institute of Immunology University of Regensburg, D-93042 Regensburg, Germany and the ‡Laboratory of Molecular Immunoregulation, Division of Basic Science, NCI, National Institutes of Health, Frederick, Maryland 21702-1201

\( \beta \)-Defensins are small antimicrobial polypeptides that are mainly expressed by epithelial cells and play an important role in the antimicrobial innate immune response. In addition to the direct microbicidal effects of these polypeptides, it became evident that certain members of the \( \beta \)-defensin super family have the capacity to promote local innate inflammatory and systemic adaptive immune responses by interacting with the CC-chemokine receptor CCR6. We have identified mouse \( \beta \)-defensin 14 (mBD14, Defb14) as an orthologue of human \( \beta \)-defensin 3 (hBD3 or DEFB103). Based on primary structural analysis, mBD14 demonstrates greater (68%) homology to its human orthologue, containing three conserved cystein linkages, characteristic for the \( \beta \)-defensin super family. mBD14 is expressed in a wide variety of tissues including spleen, colon, and tissues of the upper and lower respiratory tract. Interestingly, we also detected mBD14 expression in immature CD11c+ bone marrow-derived dendritic cells. The expression of mBD14 can be induced by Toll-like receptor agonists such as lipopolysaccharide and poly(I:C) and by pro-inflammatory stimuli e.g. tumor necrosis factor and interferon-\( \gamma \). Furthermore, expression of mBD14 seems to be regulated by activation of the intracellular pattern recognition receptor NOD2/CARD15 as revealed by reporter gene analysis. We prepared a recombinant mBD14-Ig fusion protein that retained potent antimicrobial activity against several Escherichia coli strains but not against various Gram-positive Staphylococcus aureus strains. hBD3 and also the newly identified mBD14 were chemotactic for cells expressing the mouse CC-chemokine receptor CCR6. In addition, both hBD3 and mBD14 were chemotactic for freshly isolated mouse resident peritoneal cells. Thus, mBD14, based on structural and functional similarities, appears to be an orthologue of hBD3.

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1 To whom correspondence should be addressed: Institute of Immunology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93042 Regensburg, Germany. Tel.: 49-941-944-5463; Fax: 49-941-944-5462; E-mail: thomas.hehlgans@klinik.uni-regensburg.de.
2 The abbreviations used are: mBD, mouse \( \beta \)-defensin; hBD, human \( \beta \)-defensin; TLR, Toll-like receptor; TNF, tumor necrosis factor; RT, reverse transcription; qPCR, quantitative PCR; DC, dendritic cells; BMDC, Bone marrow-derived dendritic cells; MHC, major histocompatibility complex; PC, peritoneal cells; RPC, resident peritoneal cells; CCR6, CC-chemokine receptor.
(Defb4). However, the name of the peptide remains hBD2. Human β-defensin genes were assigned a number greater than 100 if no orthologous gene has been identified so far. All mouse β-defensin genes were assigned sequential numbers based on their discovery and whether or not they have orthologues in the human genome (18). To identify and functionally characterize the human β-defensin 3 (hBD3, DEFBI03) orthologue in the mouse, we described the cloning of the mouse BD14 cDNA encoding the mouse Defb14 gene and analyzed its gene structure, expression pattern, regulation, antimicrobial, and chemotactic activity.

EXPERIMENTAL PROCEDURES

Cloning of the mBD14 cDNA—Total RNA from primary mouse tissue was reverse-transcribed using an RNeasy plus mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. For RT-PCR, 1 µg of total RNA was reverse-transcribed in a total volume of 20 µl using a RT system (Promega, Mannheim, Germany), according to the manufacturer’s instructions. The primers used for PCR amplification of the predicted entire open reading frame of mBD14 were: 5'-ATG AGG CTT CAT TAT CGT CTA TTT-3'; 5'-CTA CTT CTT CTT TCG GCA GCA TTT-3'. β-Actin: 5'-AAT CCT GTG GCA TCC ATG AAA C-3'; 5'-CGC AGC TCA GTA ACA GTC CG-3'. Samples were heated for 5 min at 95 °C, and the cycles were 95 °C for 45 s, annealing at 55 °C for 30 s, and 72 °C for 45 s for 35 cycles. PCR products were purified by gel electrophoresis, cloned using the pGemT easy vector system (Promega), and verified by sequencing.

Expression and Purification of mBD14 and hBD3 Fusion Proteins—Both fusion proteins were generated by insertion of the mBD14 and hBD3 cDNA encoding for the mature polypeptides into the Signal Ig plus vector (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Expression and purification of the fusion proteins were purified from the culture medium of the BMDC cultures was removed following the addition of fresh medium supplemented with granulocyte-macrophage colony-stimulating factor.

Stimulations were performed on day 8 of culture in triplicates using 24-well plates with 1–2 x 10^6 BMDC/ml with 100 µg/ml lipopolysaccharide from Salmonella enterica serotype abortus equi (Sigma-Aldrich, Steinheim, Germany). The CD11c^+ population was sorted from directly labeled BMDC fractions in a FACSria flow cytometer (BD Biosciences, Heidelberg, Germany). CD11c^+ , CD86^+ , and MHC class II^+ BMDC were identified by using a phycoerythrin-conjugated anti-mouse CD11c monoclonal antibody (clone N418; eBioscience, San Diego, CA), fluorescein isothiocyanate-conjugated anti-mouse CD86 (clone GL1; eBioscience), and allophycocyanin-conjugated anti-mouse MHC class II monoclonal antibody (Miltenyi Biotech, Bergisch Gladbach, Germany).

RNA Isolation and qRT-PCR—Total RNA from cultured BMDC was obtained by using an RNeasy plus mini kit (Qiagen), according to the manufacturer’s instructions. For qRT-PCR, the iQ Sybr Green mix (Bio-Rad, Munich, Germany) was used according to the manufacturer’s instructions. Sequences of primers used for qRT-PCR amplification were: mBD14, 5'-GTA TTC TCT CTC ATC TTC TCC TTC G-3', 5'-AGG TAT AGC ACA CCG GCC AC-3', and 18 s RNA, 5'-GTA ACC CTT TGA ACC CCA TT-3', 5'-CCA CTT AAA CAG GAC-3'. Samples were heated for 3 min at 95 °C, and 30 cycles were 95 °C for 10 s, annealing, and elongation for 10 s at 63.5 °C, and 50 cycles.

Target Cell Preparation and Chemotaxis Assay—HEK 293 cells expressing mCCR6 (mCCR6/293) are maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 400 µg/ml G418 and used in chemotaxis assay when they reach 60–70% confluence. Mouse resident peritoneal cells (RPCs) were obtained by lavage of the peritoneum of 8-week-old C57BL/6 mice with 5 ml of ice-cold phosphate-buffered saline containing heparin (20 units/ml) and EDTA (5 mM). Both mCCR6/293 and RPCs were suspended in chemotaxis medium (RPMI 1640 containing 1% bovine serum albumin) at 1 x 10^7/ml. The migration of both types of cells in response to chemoattractants (β-defensins or control chemotactic factors) was determined using the 48-well microchemotaxis chamber assay as described previously (20). In brief, che-
Mouse β-Defensin 14 (mBD14), the Orthologue of hBD3

a) exons 1
58 bp
652 bp
146 bp

b) tataataggtgataaagccacacatctttgtcaggtcggtgtggaaagcagtacact
ataaggggctctattctgtaattacgtcctttctctgttctttgtctgttacgtcctttcc
agcgcaccttcaccaaaaaaccctggaattttcttgagccgcttctgacgggg
agtatccttactgtccttgatggagaaagaacarattatctgtcaaggtctcga
mammal defensin super family are completely conserved in the mouse, and conserved residues among hBD3 and mBD14 are shown in gray.

moattractants diluted in chemotaxis medium at various concentrations were put into the lower wells of a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD), and cell suspension was added into the upper wells. The lower and upper compartments were separated by a 5-μm uncoated (for RPCs) or a 10-μm collagen-coated (for mCCR6/293 cells) polycarbonate filter membrane (Osmonics, Livermore, CA). After incubation at 37 °C for an indicated period of time (1.5 h for RPCs and 5 h for mCCR6/293 cells) in humidified air with 5% CO₂, the filters were removed, scraped, and stained, and the cells migrated across the filter were counted under a light microscope. The results (mean ± S.D. of triplicate wells) were presented as the number of cells per high power field.

Transfection and mBD14 Reporter Assay—A DNA fragment containing 1.7 kb of the regulatory region of mBD14 was amplified in a PCR, using the following primers: 5′-GGG GGG GAG CTC TTA GAA AGG CCT CTC AGA-3′; 5′-CCC CTC GAG AGT TGA CTG CTT CCC FFL AMP ACG-3′. This fragment was subcloned via ScaI and Xhol into the plg4L.11 firefly luciferase reporter vector (Promega). mBD14 promoter activation in response to NOD2 activation was done in 96well plates at 60–80% confluence and transfected with 50 ng of mBD14-luciferase reporter plasmid in combination with 10 ng of phRL-TK Renilla luciferase vector (Promega) to normalize transfection efficiency, 50 ng of NOD2 expression plasmid (pcDNA3.1) and 1 μg/ml muramyl dipeptide (Sigma) using DOTAP (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions. Cells were harvested 48 h after transfection, and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega), according to the manufacturer’s instructions.

Genomic Localization and Alignment—Genomic information of Defb14 refers to the Ensembl mouse data base. Alignment of the predicted amino acid sequence of mBD14 with other human β-defensins. Cysteine residues are shown in black boxes, and conserved residues among hBD3 and mBD14 are shown in gray.

RESULTS

Identification of Defb14 Gene—To identify the mouse orthologue of the human β-defensin 3 (hBD3) gene (DEFB103), we searched the Ensembl mouse data base using a combination of similarity searches against DNA and protein data bases. This resulted in the identification of the Defb14 gene within the β-defensin gene cluster located on mouse chromosome 8 at locations 19,194,334–19,195,187. The Defb14 gene comprises the conserved genomic structure consisting of two exons and one intron (Fig. 1a). The predicted transcript of 204 bp encodes for a 67-amino-acid polypeptide and results, according to protein prediction methods after posttranslational modifications including proteolytic cleavage of the signal sequence, in a mature β-defensin polypeptide of 45 amino acids (Fig. 1, b and c).

The exon-intron structure and the corresponding Defb14 transcript were verified by PCR using mouse genomic DNA and by RT-PCR on mouse lung cDNA using primers deduced from the putative exon sequences, respectively. A sequence comparison analysis confirmed the predicted open reading frame for the Defb14 gene (data not shown).

Defb14 demonstrates 72% homology to hBD3 on the cDNA level and 68% homology on the protein level, with significant lower homology to hBD1, hBD2, and hBD4. Alignment of the Defb14 cDNA demonstrates 21% homology to mBD1 and mBD2, about 39% homology to mBD3 and about 30% to mBD4, respectively. Furthermore, the characteristic cysteine residues of the β-defensin super family are completely conserved in the mature mBD14 polypeptide (Fig. 1d).

Tissue-specific Expression and Regulation of mBD14—RT-PCR analysis revealed that mBD14 is expressed in a variety of mouse tissues including lung, liver, and spleen but also in the thymus and tissue of the respiratory tract. The abundant expression in trachea, esophagus, and tongue seems to be conserved since earlier reports have demonstrated that the human orthologue (hBD3) is also expressed in these tissues (12). Amplified transcripts using primers spanning exon/intron boundaries were verified by sequencing (data not shown), and β-actin was amplified as an internal control (Fig. 2a). Interestingly, mBD14 is also expressed in BMDCs, which have been sorted for DC11c+ expression and for MHCII high/CD86 high (mature) and MHCII low/CD86 low for immature BMDCs. The expression level of mBD14 was higher in the mature BMDCs than in immature BMDCs. Furthermore, maturation of immature BMDCs induced by lipopolysaccharide results in an up-regulation of mBD14 expression (Fig. 2b). Furthermore,
stimulation of immature BMDCs with various TLR ligands (e.g. TLR4, TLR9, and TLR3) demonstrates an up-regulation of mBD14 mRNA expression as shown by quantitative RT-PCR (Fig. 2c). To our knowledge, this is the first report of a mouse β-defensin expressed and regulated in BMDCs.

Induction of hBD3 mRNA expression has been previously reported in primary epithelial cells in response to TNF or heat-inactivated bacteria (12). We observed up-regulation of mBD14 in the mouse lung epithelial cell line LA-4 in response to TLR ligands e.g. poly(I:C) and CpG. Additionally, proinflammatory cytokines such as TNF and interferon-γ also clearly up-regulated mBD14 expression on mRNA level in these cells (data not shown). These results are supported by the observation that several interferon regulatory factor binding sites are located within the proximal promoter region of the mBD14 gene. Thus, mBD14 is inducible by interferon and other proinflammatory stimuli.

A recent report demonstrated that in addition to the activation of membrane-bound TLRs, nucleotide-binding oligomerization domain protein 2 (NOD2/CARD15), a cytosolic protein involved in intracellular recognition of microbes by sensing peptidoglycan fragments, mediates β-defensin expression (21). To test whether mBD14 is also induced by NOD2, we used luciferase as a reporter gene fused to 1.7 kb of the mBD14 5′-flanking region. Activation of NOD2 in NOD2-overexpressing LA-4 cells through its ligand muramyl dipeptide clearly demonstrated that mBD14 is induced by activation of NOD2 (Fig. 3), indicating that NOD2 seems to serve as an intracellular pattern recognition receptor to enhance host defense by inducing mBD14 expression in epithelial cells.

To confirm the antimicrobial activity of mBD14, we expressed the putative mature mBD14 polypeptide as a fusion protein fused to the constant domain (Fc) of human IgG1, to obtain correct folding and increase solubility of mBD14, in the Drosophila S2 system (Fig. 4a). Stable expression of the mBD14-Ig fusion protein followed by purification and Western blot analysis using an anti-human IgG antibody resulted in detection of a single protein band with the expected apparent molecular mass of ~37 kDa (Fig. 4b). The recombinant expressed mBD14-Ig fusion protein exhibits antimicrobial activity against several strains of Gram-negative E. coli at a concentration as low as 1 μg/ml. E. coli was killed by recombinant mBD14 fusion protein at low and physiological salt concentrations. The antimicrobial effect of recombinant mBD14 is comparable with other recombinant β-defensins that have been reported earlier (12, 23). In contrast, the recombinant mBD14 fusion protein did not exhibit any antimicrobial activity against different Gram-positive strains of S. aureus that we tested so far (Fig. 4c).

hBD3 and mBD14 Are Chemotactic for CCR6-expressing HEK 293 Cells and Mouse resident Peritoneal Cells—Previous studies have revealed that hBD2 is chemotactic for CD45RO+ memory T cells and for immature, but not mature, dendritic cells. The chemotactic activity has been shown to be mediated by the human CCR6 (2). Furthermore, mBD2 and mBD3 are chemotactic for immature mouse DCs as well as HEK 293 cells transfected to express mouse CCR6 (24). In line with these experiments, we tested hBD3 and its mouse orthologue mBD14 for chemotactic activity of HEK 293 cells expressing mouse CCR6. As shown in Fig. 5a, recombinant mBD14-Ig and
hBD3-Ig fusion proteins induced the migration of mouse CCR6-expressing HEK 293 cells both in a dose-dependent fashion and in a typical bimodal manner. Human IgG as a control did not show any chemotactic activity for HEK 293 cells expressing mouse CCR6. Furthermore, we analyzed the chemotactic activity of recombinant hBD3-Ig and mBD14-Ig for mouse resident peritoneal cells (PCs) since both hBD3 and hBD4 have also been reported to chemoattract freshly isolated monocytes (24, 25). Both hBD3-Ig and mBD14-Ig fusion proteins were unexpectedly also chemotactic for freshly isolated mouse PCs (Fig. 5b). Since these cells do not express CCR6, hBD3 and mBD14 have to use at least one additional unidentified receptor mediating chemotaxis besides CCR6.

**DISCUSSION**

We report the identification of mBD14 as a novel member of the β-defensin super family. So far, the gene had only been predicted by genomic annotation based on its homology to the human orthologue hBD3. The exon-intron structure and the existence of a corresponding transcript were confirmed by RT-PCR on various mouse tissues and subsequent genomic PCR using primers deduced from the putative exon sequence. The genomic Defb14 sequence further revealed a TATA box within the proximal 5′-flanking sequence and the existence of several putative signal transducers and activators of transcription (STAT) binding sites and cAMP-responsive elements within the proximal promoter region. It is noteworthy that there are no putative NFκB binding sites within this region, which, in contrast, have been identified in several other β-defensin promoters.

The expression of mBD14 has been detected in many tissues and also in a variety of cell lines of epithelial origin. The mRNA transcript levels of mBD14 seem to be comparable with those of hBD3 with respect to those tissues that are known to express β-defensins including the respiratory and gastrointestinal tracts. The expression of mBD14 is induced and up-regulated in response to TLR ligands and pro-inflammatory stimuli e.g. TNF and interferon-γ in various cell lines of epithelial origin (data not shown).

Interestingly, mBD14 mRNA expression is also up-regulated by the activation of NOD2, a cytosolic protein involved in the recognition of microbial components, although no NFκB sites, which have been described to be essential for the induction of hBD2 by NOD2 (21), could be identified in the proximal promoter region of mBD14. Further detailed promoter analysis will be necessary to identify the corresponding transcription factors and the putative DNA-binding sites responsible for the NOD2-dependent induction of Defb14 gene transcription. Unexpectedly, mBD14 is also expressed and regulated on the transcriptional level in BMDCs, which, to our knowledge, is the first report of production of a mouse β-defensin expressed by the dendritic cell population. It is tempting to speculate that mBD14 expression is induced in DCs during an encounter with a bacterial challenge acting based on its antimicrobial activity and on the other hand contributing to the antimicrobial effector phase by supporting the adaptive immune response.

We were able to express mBD14 as a fusion protein, fused to the constant region of human IgG1 in Drosophila S2 cells. The expres-
since these cells do not express CCR6. Consequently, both of these β-defenseins use at least one additional yet unidentified receptor besides CCR6. The chemotactic activities of α- and β-defenseins predict that these β-defenseins may contribute to recruiting T cells, immature dendritic cells, and monocytes to the sites of infection and thereby initiate and support an adaptive immune response (22).

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