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Short communication

Characterization and expression profile of complete functional domain of granulysin/NK-lysin homologue (buffalo-lysin) gene of water buffalo (*Bubalus bubalis*)

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ABSTRACT

Granulysin (GNLY)/NK-lysin (NKL) is an effector antimicrobial cationic peptide expressed in the cytotoxic and natural killer lymphocytes. We report here cDNA sequence (405 bp) encoding the complete functional domain of buffalo-lysin (bu-lysin), and its expression profile in the various tissues. The nucleotide sequence of bu-lysin exhibited >85% identity with the bovine lysin. Comparison of the deduced amino acid sequence of bu-lysin with those of GNLY/NKL of different species revealed the conservation of six cysteine (Cys) residues and five alpha helices. Unlike the homologues in other species, bu-lysin composed of 11 positively charged Lys residues as in equine. The expression of bu-lysin mRNA in the *in vitro* cultured lymphocytes was inducible and increased markedly ($p < 0.05$) in a dose dependant manner when incubated with Concanavalin A (ConA). The expression of bu-lysin mRNA in the different tissues was variable: comparatively higher in the spleen and lymph node, moderate in the uterine endometrium and low in the liver and kidney. These results indicate the existence and active expression of GNLY/NKL homologue in water buffalo having a significant influence in immune response.

1. Introduction

Antibacterial peptides play an important role in host defense against microbial pathogens (Boman, 1995). The natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), together with neutrophils and macrophages, are the primary effector cells of the immune system. These cells, on stimulation, release cytolytic granules, which contain a wide range of proteins, including those of perforin and the granzyme family, capable of causing membrane lysis and eventual cell apoptosis (Gamen et al., 1998). Jongstra et al. (1987) identified a novel gene from human T-cell lymphocyte, later renamed as granulysin (GNLY) due to its location in cytolytic granules of NK and CTL cells (Krensky, 2000). The gene encodes a peptide having broad lytic abilities against bacteria, fungi, protozoa, and parasites (Ernst et al., 2000; Gansert et al., 2003; Stenger et al., 1998). Subsequently, porcine homologue of human GNLY, referred to as NK-lysin (NKL), having similar structure and antimicrobial properties (Andreu et al., 1999; Jacobs et al., 2003) was identified from the intestinal tissue (Andersson et al., 1995a, 1996). The native as well as several synthetic GNLY/NKL peptide fragments displayed potent antimicrobial activity against several gram-negative and gram-positive bacteria (Andersson et al., 1995b; Andreu et al., 1999).

Antimicrobial peptides, although diverse in size, sequence, structure and spectrum, play an important role in immune responses of the host (Hancock and Diamond, 2000). Peptide mediated innate defense is an evolutionary

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old mechanism to combat microbial pathogen; therefore, it is quite likely that different species should evolve similar molecules to combat infection. During last decades several antimicrobial peptides have been isolated from a wide range of animal species. Further, the threat of the emergence of antibiotic-resistant bacteria and the non-availability of newer classes of antibiotics has prompted the need to identify novel antimicrobial peptides for the development of alternative therapeutics for both human and animal. Since the discovery of human GNLY (Jongstra et al., 1987), its homologues genes encoding GNLY/NKL peptides have been identified in different species including bovine (Endsley et al., 2004), equine (Davis et al., 2005), porcine (Andersson et al., 1995a), chicken (Hong et al., 2006) and catfish (Wang et al., 2006).

Water Buffalo (*Bubalus bubalis*) being a major contributor of milk production serves as one of the most important livestock species in India as well as in Southeast Asian countries (FAOSTAT, 2003). Further, the buffalo is a sturdy animal, well adopted to the tropics, and often anecdotally referred to as being resistant to many infectious diseases. However, only a few antimicrobial peptides of bubaline origin including β-defensins (Bera et al., 2007; Das et al., 2005), cathelicidin (Das et al., 2006), and cathelicidin-7 like antibiotic peptide (Das et al., 2008) have been characterized. Consequently, the immune mechanism behind the widely believed disease resistance is not known due to limited studies about bubaline immune effector molecules (Mingala et al., 2007). In this study, we report the cloning, characterization and the expression profile of the cDNA sequences encoding the entire mature peptide of GNLY/NKL homologue in buffalo, the bubaline-lysin (bu-Lys).

2. Materials and methods

2.1. Isolation of peripheral blood mononuclear cells (PBMC)

Venous blood was collected aseptically from healthy adult buffaloes. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Histopaque (Sigma, USA) following manufacturer's instructions. Briefly, 8 ml of PBS-diluted (1:1) blood samples were layered over 4 ml of Histopaque in 15 ml tube and centrifuged at $400 \times g$ for 30 min. PBMC at interphase were collected and washed three times with sterile PBS.

2.2. cDNA synthesis

The total RNA was isolated from PBMC using RNAgents system (Promega, USA) according to the manufacturer's instructions. The isolated RNA sample was treated with DNase using DNA-free™ DNase Treatment & Removal Reagents (Ambion, USA). The concentrations and purities of RNA preparations were determined spectrophotometrically at OD_{260} and OD_{280}. The total RNA was reverse-transcribed using Reverse Transcription (RT) System (Promega, USA) following the manufacturer's instructions. Briefly the cDNA was synthesized from approximately 2 μg total RNA using oligo-dT primers and avian myloblastosis virus reverse transcriptase in a final volume of 20 μl. The resultant first strand of cDNA was stored at −20 °C until use.

2.3. PCR amplification

A part of bu-lysin gene corresponding to the mature peptide was amplified using a pair of bovine-lysin primers (Forward: 5′CTGCTGCTCCAAGGAGAAGA3′ and Reverse: 5′GCAGTGGAGGGAGTTTGGT3′) as described by Endsley et al. (2004). PCR amplification was carried out in a total volume of 25 μl that contained 5 pmol of each primer, 5 μl of cDNA, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM dNTPs and one unit of *Taq* DNA polymerase (Fermentas, USA). Amplification was carried out in a Thermal Cycler (Eppendorf, Germany) for 35 cycles in the following conditions: 94 °C for 2 min and 35 cycles of 30 s at 94 °C, 60 s at 60 °C, and 30 s at 72 °C and a final extension of 10 min at 72 °C. The amplification products were resolved by agarose gel (1%) electrophoresis and visualized using ethidium bromide (EtBr) staining under UV light.

2.4. Cloning and sequencing

The amplicon was purified using Gel Cleanup kit (Eppendorf, Germany) and cloned into pDrive vector (Qiagen, USA) following the manufacturer's instructions. Positive recombinant clones were identified using blue and white screening. Further, the presence of the insert was confirmed by restriction digestion and plasmid PCR. The positive clones were sequenced using an ABI PRISM automatic sequencer (version 2.0) using standard cycle conditions by Sanger's dideoxy chain termination method with the standard T7 and SP6 sequencing primers. The sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST). The nucleotides as well as the deduced amino acid sequences were aligned with those of available species in the GenBank database using the Clustal method of MegAlign Programme of Lasergene Software (DNASTAR, USA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). Phylogenetic tree was constructed using Neighborhood Joining method.

2.5. Expression analysis of bu-lysin mRNA

The inducibility as well as the expression of bu-lysin in different tissues was studied using semi-quantitative RT PCR (Sundaresan et al., 2005).

2.5.1. Inducibility in ConA stimulated PBMC

Following the adjustment of concentration of PBMC to 5×10^6 cells per ml, the PBMC were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (Sigma) and supplemented with 100 U Penicillin G and 100 μg Streptomycin per ml in 24-well tissue-culture plates in triplicates. PBMC were induced with ConA at final concentrations of 0, 5 and 10 μg per 5×10^6 cells. After 24 h of ConA induction, total RNA was isolated and was subsequently reverse transcribed as described above. Approximately 5 μl of 1:5 diluted first strand cDNA was