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Molecular Characterization of a Saposin-Like Protein Family Member Isolated from Bovine Lymphocytes

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ABSTRACT

Human and porcine T lymphocytes and natural killer (NK) cells produce antibacterial proteins that belong to the saposin-like family of proteins (SAPLIP). The objective of this study was to determine if a bovine homolog of SAPLIP exists in lymphocytes that exhibit antibacterial activity. Following stimulation with IL-2, bactericidal activity against *Staphylococcus aureus* was detected to some extent in most major subpopulations of T lymphocytes including $CD4^+$, $CD8^+$, $CD3^+$, and $WC1^+$ $\gamma\delta$ T lymphocytes. However, the majority of antibacterial activity was observed in the $CD2^+CD3^-$ lymphocytes, which are similar phenotypically to NK cells. A partial sequence of a bovine SAPLIP was generated using low specificity primers designed from regions of homology between other SAPLIP including porcine NK-lysin and human granulysin. Enhanced expression of the bovine lysin gene was detected in mRNA isolated from IL-2–stimulated $CD2^+CD3^-$ lymphocytes. The partial cDNA sequence was then used to make gene specific primers for a rapid amplification of cDNA ends (RACE) procedure that provided repeatable 5′ and 3′ cDNA ends. By examining overlapping regions from the RACE procedure, full-length sequence information was obtained for the bovine lysin homologue. Conceptual translation of the cDNA demonstrated conserved similarities to known SAPLIP members. Further characterization of the bovine lysin may facilitate its use in protecting dairy cattle against bacterial infections.
(**Key words:** saposin-like, lymphocyte, bovine)

Abbreviation key: CF = cell free, **GSP** = gene-specific primers, **HBSS** = Hank's balanced salt solution, **NK** = natural killer, **ORF** = open reading frame, **PBMC** = peripheral blood mononuclear cells, **QC RT-PCR** = quantitative competitive reverse transcription PCR, **RACE** = rapid amplification of cDNA ends, **SAPLIP** = saposin-like protein, **TIGR** = The Institute for Genomic Research.

INTRODUCTION

Lymphocytes are able to recognize antigens through specific membrane receptors that define the immunological characteristics of specificity, diversity, memory, and self/nonself recognition. Researchers have shown that there are at least 2 major lymphocyte subpopulations present in bovine tissues and secretions, T and B lymphocytes, that differ in function and protein products (Sordillo and Streicher, 2002). The T lymphocytes can be subdivided further into either $\alpha\beta$ T lymphocytes, which include $CD4^+$ (T-helper lymphocytes) and $CD8^+$ (T-cytotoxic or T-suppressor) lymphocytes, or $\gamma\delta$ T lymphocytes. Depending on stage of lactation and tissue location, the percentages of these cells can vary significantly in dairy cattle. For example, lower percentages of $\alpha\beta$ T lymphocytes during the postpartum period were associated closely with diminished mitogen-stimulated proliferation and spontaneous cytotoxic activity when compared with $\alpha\beta$ T lymphocyte populations obtained from midlactation cows. It also was shown that bovine $CD4^+$ lymphocytes primarily express a T-helper-2 phenotype (i.e., characteristic of humoral immunity) during the periparturient period and a T-helper-1 phenotype (i.e., characteristic of a cell-mediated immunity) at midlactation (Shafer-Weaver and Sordillo, 1996). Changes in the composition of blood and mammary gland mononuclear cell populations have been linked with heightened susceptibility to infectious diseases in dairy cattle including mastitis and Johne's disease (Kehrli et al., 1989). These findings indicate that the alterations in the overall phenotypic profiles of lymphocyte populations may affect the functional capacity of the immune system and possibly increase the cow's susceptibility to microbial challenge.

Whereas compositional changes in bovine lymphocyte populations with respect to lactation stage and disease susceptibility are well characterized, it is significant to note that the functional significance of spe-

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cific lymphocyte subpopulations is not defined fully. The majority of previous work has focused on the involvement of lymphocyte populations in orchestrating an effective, adaptive (i.e., humoral and cellular) immune response. Involvement of nonspecific soluble and cellular mechanisms in the defense against bacterial pathogens has primarily focused on that which was mediated by phagocytic cell populations including macrophages and neutrophils (Sordillo and Streicher, 2002). By comparison, considerably less is known about the nonspecific defensive role of bovine lymphocyte populations against the spectrum of bacterial pathogens capable of causing disease. However, research has shown that cytokine-stimulated porcine and human T lymphocytes and natural killer (**NK**) cells are capable of nonspecific killing of bacteria through the secretion of the proteins porcine NK-lysin and human granulysin, respectively (Andersson et al., 1995b, 1996a, 1999; Pena and Krensky, 1997). A parasitic protozoan of the human intestinal tract, *Entamoeba histolytica*, also kills bacteria through the generation of a pore-forming protein, amoebopore (Bruhn and Leippe, 1999). Despite the considerable evolutionary distance between single-celled parasitic organisms and mammalian lymphocytes, the bactericidal effector proteins porcine NK-lysin, human granulysin, and amoebopore all are members of same protein family, the saposin-like proteins (**SAPLIP**). The SAPLIP are small glycoproteins, often derived from larger precursor proteins in vivo, which carry out diverse functions through association with lipid membranes (Munford et al., 1995). The amino acid sequences of family members include highly conserved cysteine residues that form disulfide bonds and give SAPLIPs a stable structure, whereas their secondary protein structure consists mainly of α-helices joined by loops (Munford et al., 1995; Andreu et al., 1999). Previous research demonstrated that cytokine-stimulated bovine lymphocytes also possess antibacterial activity in vitro that is not major histocompatibility complex restricted (Sordillo et al., 1991; Shafer-Weaver and Sordillo, 1996). The objective of the current study was to determine if a bovine homologue (bovine lysin) to human granulysin or porcine NK lysin is expressed in lymphocyte subpopulations that possess antibacterial activity.

MATERIALS AND METHODS

Isolation of Mononuclear Cells

Peripheral blood mononuclear cells (**PBMC**) were isolated from 6 midlate lactating Holstein dairy cows. All experimental cows were free of mastitis and in their third or fourth lactation. Mononuclear cells were isolated and purified as previously described (Sordillo et al., 1991). Purified cells were washed 3 times in Hank's balanced salt solution (**HBSS**; Sigma Chemical Co., St. Louis, MO) and suspended in PBS containing 2% BSA (Sigma Chemical Co.). Mononuclear cell-enriched preparations contained more than 95% mononuclear cells as determined by Wright's Giemsa staining and were more than 95% viable as assessed by trypan-blue exclusion.

Cell Separation Using Magnetic Beads

To identify the effector phenotype responsible for bactericidal activity, lymphoid subpopulations were isolated from PBMC using the VarioMACS separation system (Miltenyi Biotech Inc., Sunnyvale, CA). The VarioMACS isolation yielded both enriched (+) and depleted (–) cultures of a given lymphoid subpopulation. Cell cultures for CD2 ($\alpha\beta$ T lymphocytes and NK cells, BAQ95A), CD4 (T-helper, CACT83B), CD8 (T-cytotoxic/suppressor, CACT80C), WC1 ($\gamma\delta$ T lymphocytes, B7A1), and B2 (B lymphocytes, BAQ44A) were obtained using the respective monoclonal antibody (VMRD, Pullman, WA) as described previously (Shafer-Weaver and Sordillo, 1996). Briefly, isolated lymphoid cells (1×10^7) were incubated with individual monoclonal antibodies (10 μL/1×10^7 cells) for 30 min at 4°C. The cells were then washed with PBS and incubated for an additional 30 min at 4°C with 10 μg of goat-antimouse Ig-coated magnetic beads (Miltenyi Biotech) per 10^7 target cells. The bead-cell complex was then extracted from noncomplexed cells using a magnetic field and the positive (enriched) and negative (depleted) fractions were collected. The purity of the subsets was >97% as determined by flow cytometric analysis. To obtain an NK-cell enriched population, $CD2^+CD3^-$ cells were isolated by first collecting a $CD3^-$ fraction and then selecting for $CD2^+$ cells. For use in the bactericidal assay, enriched (+) and depleted (–) cultures of individual lymphoid phenotypes were incubated for 48 h in the presence of recombinant human IL-2 or the absence of this cytokine as previously described (Sordillo et al., 1991; Shafer-Weaver and Sordillo, 1996).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described to characterize the phenotype of antibacterial effector cells and to ensure isolation procedures yielded lymphoid populations (Sordillo et al., 1991). All monoclonal antibodies (VMRD) were at a stock concentration of 1 mg/mL and diluted: CD2 ($\alpha\beta$ T lymphocytes and NK cells, BAQ95A at 1:100), CD4