Establishment of Baculovirus Infected Insect Cell Line Expressing Porcine Salivary Lipocalin(SAL1) Protein

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ABSTRACT

Salivary lipocalin (SAL1) is a member of the lipocalin protein family that has a property to associate with many lipophilic molecules. The importance of SAL1 during pregnancy in pigs has been suggested by our previous study which has shown that SAL1 is expressed in the uterine endometrium in a cell type- and implantation stage-specific manner and secreted into the uterine lumen. However, function of SAL1 in the uterus during pregnancy in pigs is not known. To understand SAL1 function in the uterus during pregnancy, we generated recombinant porcine SAL1 protein in an insect cell line. Porcine SAL1 cDNA was cloned into a baculovirus expression vector using RT-PCR and total RNA from uterine endometrium on day 12 of pregnancy, and the expression vector was used to generate recombinant Bacmid containing the SAL1 gene. The recombinant Bacmid was then transfected Sf9 cell to produce recombinant baculovirus. By infecting Sf9 cell with recombinant baculovirus, we established a SAL1-expressing insect cell expression system. Immunoblot analysis confirmed SAL1 expression in the infected cells. Recombinant SAL1 produced by the Sf9 cell line will be useful for understanding physiological function of SAL1 during pregnancy in pigs.

(Key words : SAL1, Pig, Uterus, Endometrium, Implantation)

INTRODUCTION

Salivary lipocalin (SAL1) is a member of the lipocalin protein family that is composed of a large group of small extracellular proteins found in animals, plants, and bacteria. The lipocalin protein family has a common structural β-barrel feature, which allows these proteins to bind lipophilic molecules and primarily acts as a transporter in aqueous biological fluids (Flower, 1996). SAL1 was initially identified as sex pheromone-binding protein in submaxillary gland of boars (Marchese *et al*., 1998; Loebel *et al*., 2000). Later, we have shown that female pigs also express SAL1 in the uterine endometrium in a cell type- and pregnancy stage-specific manner, suggesting an important role in the establishment of pregnancy (Ka *et al*., 2008; Ka *et al*., 2009; Seo and Ka; unpublished data). Because SAL-1 is a member of the lipocalin family that binds to lipid molecules and acts as a transpoter, it has been speculated that SAL1 may be also a transporter of lipid molecules to the developing conceptus in pigs. However, function of SAL1 in the uterus has not yet been determined clearly. Thus, it is necessary to shed light on the ligand(s) for SAL1 to understand its physiological function in the uterine endometrium during pregnancy in pigs.

It is known that glycosylation has an effect on activity and functions of proteins. Uteroferrin (UF), iron-binding protein, possesses glycosylation chain, and its oligosaccaride moiety appears to mediate the uptake of UF by the fetal liver in pigs (Baumbach *et al*., 1984; Saunders *et al*., 1985; Roberts *et al*., 1986). In addition, glycodelin, which belongs to lipocalin family and has

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functions to inhibit human sperm-egg binding during fetilization and to immunosuppress during implantation appears to be mediated by its oligosaccharide moiety (Seppala *et al*., 2007). Given our previous finding shown that SAL1 protein may be N- glycosylated, SAL1 function may be dependent on its oligosaccharide moiety. Although a bacterial expression system is useful to produce recombinant protein and has been utilized to generate recombinant SAL1 protein (Loebel *et al*., 2000), bacterially-derived SAL1 protein is not glycosylated and may not be suitable to study its full biological functions. Recently, insect cells using a baculovirus expression system are widely applied for production of recombinant glycoprotein because its benefitial performing eukaryotic post-translation modifications (Altmann, 1999). Thus, in this study, an insect cell line expressing recombinant porcine SAL1 protein using baculovirus-insect cell system was established to understand physiological function of SAL1 in the porcine uterus during pregnancy.

MATERIALS AND METHODS

Porcine SAL1 cDNA Cloning

Total RNA was extracted from endometrial tissues using TRIzol reagent (Invitrogen Life Technolog, Carlsbad, CA) according to the manufacturer's instruction. The quantity of RNA was assessed spectrophotometrically, and the integrity of the RNA was examined by gel electrophoresis using 1% agarose.

Two micrograms of total RNA were reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNA. The cDNA templates were then diluted 1:4 with sterile water and used for cloning of SAL1. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) to amplify the open reading frame region of SAL1 (NM 213814.1). Polymerase chain reactions (PCRs) were carried out in 50 μl volumes containing 2 μl of cDNA, 4 μl of 25 mM dNTP mix, 5 μl of 10× PCR buffer, 1 μl of i-Pfu (Intron, Sungnam, Korea), 2 μl of right primer (5′-CACCATGAAGCTGCTGCTCTTGC-3′) and 2 μl of left primer (5′-TGGAGCCCACATGAGGAGG-3′) under the following conditions: 94℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 5 min, except for an pre-denaturation of 1 min and final extension for 5 min, 35 cycles of PCR were conducted. Approximately 600 bp fragments were excised and purified using NuceloGen Gel extraction kit (NucleoGen inc., Sihueng, Korea) after electrophoresis of PCR products on 0.7% agarose/EtBr gels. The purified fragments were inserted into pENTR/SD/ D-TOPO (Invitrogen). Cloned sequences were validated through DNA sequencing for the next experiments.

Construction of Expression Plasmid

Specific forward primer 5′-GAGAATTCATGAAGCTGCTGCTCTTGCTGTGTC-3′ for SAL1 gene amplification were designed to contain EcoRI restriction enzyme site. The reverse primer 5′-CTACTAGTCAGTGATGGTGATGGTGATGCTCAGCA-3′ was designed to contain Spe I restriction site for sub-cloning into insect expression vector and a 6His-tag. The SAL1-6His gene was amplified by PCR with iTaq DNA polymerase premixture (Intron). The PCR product of SAL1-6His gene was restricted with EcoRI and Spe I and separated by electrophoresis on a 1.2% agarose gel. The DNA in agarose gel isolated with gel extraction kit (Nucleogene, Seoul, Korea), and the SAL1-6His gene fragment was ligated with the pFastBac-Dual baculovirus specific vector (Invitrogen) with T4 ligase (Promega, Madison, WI) to complete SAL1-6His-pFastBac.

Formation of Bacmid DNA

Bacmid DNA was prepared from pFastBac vector by Bac-to-Bac system according to manufacturer's recommendations. Briefly, generated SAL1-6His-pFastBac was transformed into DH10Bac *E. coli* (Invitrogen). The SAL1-6His gene fragment in expression cassette, which was derived from pFastBac dual vector, was inserted into bacmid of *E. coli* (DH10Bac) by helper function in DH10Bac. Bacmid DNA was isolated from transformed DH10Bac *E. coli* with DNA Midi-prep kit (Qiagen, Hiden, Germany), and the concentration of bacmid was measured at 260 nm using spectrophotometer. The recombinant bacmid DNA was confirmed by PCR using the M13 forward and M13 reverse primers, which are specific primer of recombinant bacmid, and SAL1-6His primer.

Insect Cell Culture and Transfection

Sf9 (*Spodoptera frugiperda*) insect cells were suspension-cultured in T-75 flasks containing 25 ml of growth medium Sf-900 (Invitrogen), supplemented with antibiotics, penicillin and streptomycin (100 μg/ml), and incubated at 70 rpm shaking condition at 28℃. Sf9 cell was transfected with Cellfectin reagent (Invitrogen) following the manufacturer's instructions. Cells (9×10^5 cells/well) were seeded into a 6-well tissue culture plate (Nunc, International, Rochester, NY) in 2 ml of growth medium containing antibiotics and incubated at 27℃ for 1 h to attach the cells on the plate. One microgram of SAL1-6His bacmid DNA was solved in 200 μl of Grace's Medium (Invitrogen) containing 6 μl of Cellfectin reagent and incubated to accomplish DNA/lipid mixture for 30 min at room temperature. After 1 h for cell adherence, the cells were rinsed with the Grace's medium and then added in 1 ml of Grace's medium containing DNA/lipid complexes without antibiotics for 5 h at 27℃. After DNA transfec-

tion, the media were discarded and added to 2 ml of growth media containing antibiotics to the cells and incubated for 72 h at 27℃.

Virus Titration and Infection to Insect Cells

The P1 virus of cell supernatant was collected from transfected cells after 72 h culture by centrifugation, and measured titer of a P1 baculovirus. The concentration of baculovirus (SAL1-bacmid) was measured with BaculoELISA Titer Kit (Clontech, Mountain View, CA). Sf9 cells (1×10^5 cells/well) in Sf-900II growth medium were attached to a 96-well microplate for 15 min. Subsequently, diluted 1/2 of the recovered SAL1-6His baculovirus to 100 μl of medium were added into the 96-well plate. The Sf9 cell medium from each well was removed, and immediately replaced with 50 μl of the appropriate diluted virus. Cells with virus were incubated for 20 h at 27℃. At the same time, the capture antibody was prepared in 1× TBS buffer on 96-well plate, and incubated at 4℃ for overnight. After incubation of cells with SAL1-6His virus, the virus was transferred to virus capture antibody coated microplate. The concentration of SAL1 virus was measured compared with control virus and detected with TMB substrate. The virus concentration was represented as MOI (multiplicity of infection). According to MOI level, the SAL1-6His P1 virus infected insect cells for production of P2 virus. After collecting P2 virus, the virus concentration was measured again at the same way as described above.

Protein Expression of SAL1 Virus Infected Insect Cells

SAL1 protein concentration was measured with BCA protein assay kit (Pierce, Rockford, IL). Soluble protein (20 μg) from cell lysate and protein (90 μg) from cultured media were loaded into each lane and electrophoresed in 12% SDS-PAGE gels followed by electrotransfer to nitrocellulose membranes. Nonspecific binding was blocked with 3% (w/v) fat-free milk in PBST buffer (phosphate buffered saline with 0.1% tween-20) for overnight at 4℃. The blot was incubated with rabbit polyclonal anti-SAL1 antiserum (kindly provided by Dr. P. Pelosi) diluted in 2% milk/PBST. The blot was washed three times for 10 min in PBST at room temperature, incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Victoria, BC, Canada) for 1 h at room temperature, and rinsed

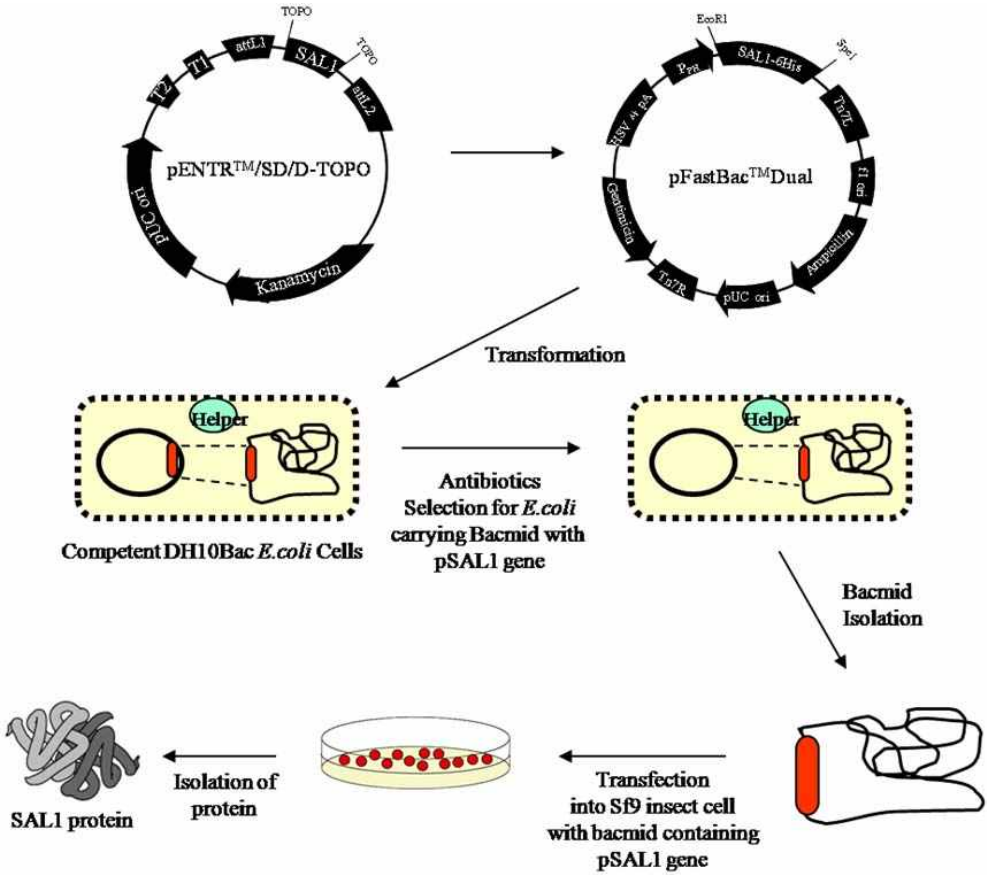

Fig. 1. Schematic overview of production of recombinant SAL1 protein using Bac-to-Bac Baculovirus expression system.

again for 30 min at room temperature with PBST. Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico, Pierce Chemical Co, Rockford, IL) according to the manufacturer's instruction.

RESULTS

Confirmation of the Expression SAL1-6His Gene in Bacmid

We developed Bac-to-Bac baculovirus expression system to generate recombinant baculoviruse expressing SAL1-6His protein in insect cells (Fig. 1). The porcine SAL1 cDNA was cloned into baculovirus vector pFastBac, and SAL1-6His-pFastBac vector was then transformed into DH10Bac competent cell for transposition of SAL1-6His gene fragment into bacmid. Successful insertion of SAL1-6His gene into bacmid was confirmed by PCR analyses using SAL1-6His primers and bacmid primers (Fig. 2B and 2C). After confirmation of recombinant bacmid DNA, the inserted DNA was verified by sequence analyses. In BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information), our product exhibits 100% sequence identity to SAL1 gene (NM_213814.1). To generate recombinant baculovirus, isolated recombinant bacmid DNA was used to transfect Sf9 cells and the recombinant virus was harvested 72 h posttransfection.

Expression of SAL1-6His Protein in Insect Cells

To analyze production of SAL1, the Sf9 cell was infected with recombinant baculovirus. At 48 h or 72 h postinfection, the media were collected and cell lysates were prepared. Both cell lysates and media collected 48 h and 72 h after infection were analyzed by immunoblotting to confirm SAL1-6His expression (Fig. 3). SAL1 protein was detected in infected cell lysate after 72 h, but not in the infected cell lysate after 48 h. SAL1 protein was not detectable in the media.

DISCUSSION

Implantation is a highly coordinated interaction between the apical plasma membranes of the conceptus trophectoderm and the uterine luminal epithelium to form placenta of a epitheliochorial type in pigs (Bowen and Burghardt, 2000). To succeed implantation, embryonic development is synchronized with the appropriate preparation of the uterus. During the period

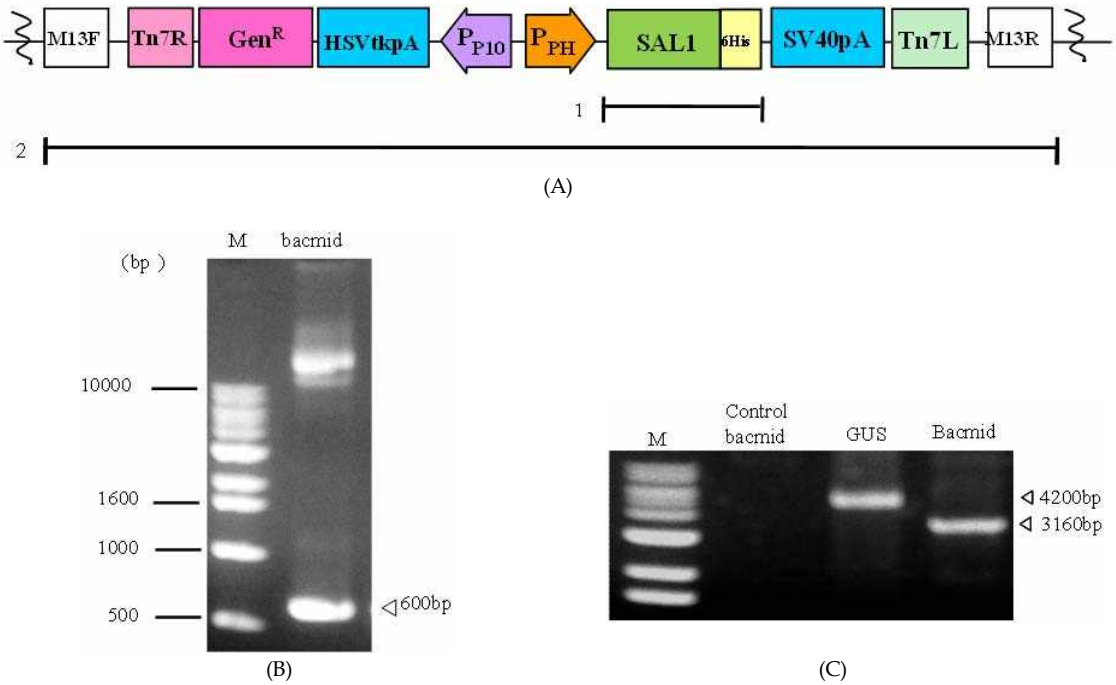

Fig. 2. Identification of recombinant bacmid with SAL1-6His gene. A. Construction of recombinant bacmid DNA by transposition of sequences from pFastBac which includes the Tn7R and Tn7L. B. The bacmid DNA was amplified with specific primer for SAL1-6His gene (600 bp). C. PCR analysis to confirm insertion of recombinant SAL1-6His gene in bacmid. The recombinant bacmid DNA was amplified with the M13 forward and M13 reverse primers. M, DNA size marker; Lane 1, control bacmid; Lane 2, GUS bacmid (4200 bp); Lane 3, SAL1-6His recombinant bacmid (3,160 bp).

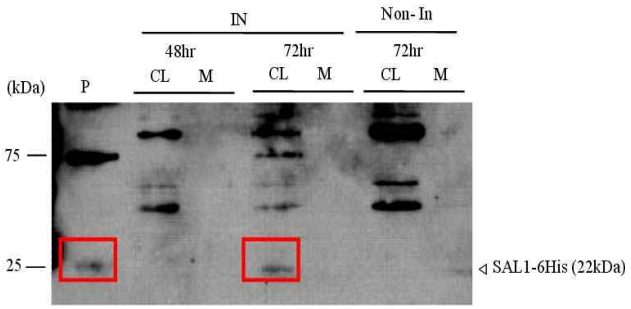

Fig. 3. Immunoblot analysis of SLA1-6His protein from insect isolated soluble protein. The soluble protein from insect cells was collected, and protein aliquots from either medium supernatant (M) or infected cell lysate (CL) were separated in SDS-PAGE gels, transferred to nitrocellulose membrane and blotted with anti-SAL1 antibodies. The SAL1 protein was expressed in culture cell lysate at 48 h and 72 h after virus infection. P, positive control; IN, virus infected; Non-In, non-infected; CL, cell lysate from insect cell culture; M, insect cell cultured media.

of pre-implantation the conceptus in pigs undergoes a dramatic morphological change and secretes estrogens, the signal for maternal recognition of pregnancy (Jaeger *el al*., 2001). The uterus also undergoes dynamic changes including alterations in expression of secretory products such as protease inhibitors, growth factors, cytokines, and adhesion molecules (Geisert and Yelich, 1997), which result in compositional change in uterine secretions to support development of the conceptus during the peri-implantation period. The uterine secretory proteins increasingly are released from the porcine endometrium during the peri-implantation period including UF, retinol binding protein (RBP) and folate binding protein (FBP), which trasnfer iron, retinol and folic acid to the develeoping conceptus, respectively. These proteins are considered to be necessary for normal conceptus development (Harney *et al*., 1993; Geisert *et al*., 1982; Vallet *et al*., 1998). In addition to these proteins, our previous studies have shown that SAL1 is secreted from the endometrium and composes uterine secretions during the peri-implantation period (Ka *et al*., 2008; Ka *et al*., 2009; Seo and Ka; unpublished data).

The lipocalin protein family has common structural feature, six- or eight-stranded continuously hydrogen-bonded β-barrel, in their tertiary structure (Flower, 1996). Due to β-barrel structure of this family, these proteins bind lipophilic molecules and act various physiological functions such as retinol transport, cryptic coloration, olfaction, pheromone transport, and the enzymatic synthesis of prostaglandins. SAL1 is a lipocalin protein with approximately 20 kDa in size. Since it was first identified in the boar submaxillary gland and not detectable in other tissues studied, and it binds to sex pheromones (Marchese *et al*., 1998; Loebel *et al*., 2000), SAL1 was considered as a boar-specific pheromone-binding protein. Later, our previous studies have shown that SAL1 is expressed in the uterine endometrium of female pigs in a cell type- and pregnancy stage-specific manner and secreted into uterine lumen, suggesting an important role in the establishment of pregnancy (Ka *et al*., 2008; Ka *et al*., 2009; Seo and Ka; unpublished data). It has been shown that SAL1 binds to the male sex pheromones 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol in the boar submaxillary gland (Loebel *et al*., 2000). These steroid pheromones have not been shown to be present in the uterine lumen during early pregnancy in pigs, while lipid molecules such as fatty acid, prostaglandins (Spencer *et al*., 2004), steroid hormones (Bazer *et al*., 1979; Heap *et al*., 1979), lysophosphatidic acid (Seo *et al*., 2008) are present. Thus, we speculated SAL1 has lipid molecule present uterine lumen during early pregnancy as its endogenous ligand and acts as lipid transporter. However, function of SAL1 in the uterus has not yet been determined. Thus, it is essential to identify ligand(s) for understainding the physiological function of SAL1 in the uterine endometrium during pregnancy in pigs. And for this reason, it is required to generate recombinant SAL1 protein for ligand binding experiment.

There are many protein expression systems using bacterial, yeast, insect and mammalian cells. So far, the recombinant SAL1 has been achieved in bacterial cells (Loebel *et al*., 2000). However, bacterially-derived protein is not glycosylated. Because SAL1 is N-glycosylated (unpublished data) and glycan structure affects the protein folding and binding activity (Kitts, 1996; Geisler *et al*., 2008), binding property of bacterially-derived recombinant SAL1 is likely different from that of physiological SAL1. Thus, to generate recombinant SAL1 protein we chose the insect cell system that has similar biological activities and glycan structures with mammalian derived protein (Hu, 2005; Kitts, 1996; Jarvis, 2003; Geisler *et al*., 2008). In this study, we used endometrial total RNA on day 12 of pregnancy to clone porcine SAL1 cDNA because endometrium on day 12 of pregnancy is known as a highly SAL1-expressing tissue (Seo and Ka; unpublished data). The SAL1 cDNA was cloned in pFastBac, baculovirus expression vector, to make recombinant Bacmid containing SAL1 gene. This recombinant Bacmid then transfected Sf9 cell to produce recombinant baculovirus. After infecting Sf9 cell with recombinant baculovirus, we obtained both cultured media and cell lysate to analyze SAL1 protein expression by immunoblotting. Immunoblot analysis using anti-SAL1 antiserum detected a band with the same size to uterine endometrial SAL1 in cell lysate, indicating that the recombinant SAL1 protein was successfully expressed in Sf9 cell infecting baculovirus containing SAL1-6His gene.

In summary, we developed a Sf9 cell line expre-

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porcine SAL1 protein using a baculovirus expression system. Although it is necessary to increase expression efficiency of recombinant SAL1, this recombinant protein will be very useful to determine the physiological function of SAL1 in the uterus during the implantation period in pigs.

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