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Increased production of intestinal immunoglobulins in *Syntenin-1*-deficient mice

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ABSTRACT

Syntenin-1 is an intracellular PDZ protein that binds multiple proteins and regulates protein trafficking, cancer metastasis, exosome production, synaptic formation, and IL-5 signaling. However, the functions of Syntenin-1 have not yet been clearly characterized in detail, especially *in vivo*. In this study, we generated a *Syntenin-1* knock out (KO) mouse strain and analyzed the role(s) of Syntenin-1 in IL-5 signaling, because the direct interaction of Syntenin-1 with the cytoplasmic domain of the IL-5 receptor α subunit and the regulation of IL-5 signaling by Syntenin-1 have been reported. Unexpectedly, the number of IL-5-responding cells was normal and the levels of fecal immunoglobulins were rather higher in the *Syntenin-1* KO mice. We also found that IgA and IgM production of splenic B cells stimulated *in vitro* was increased in *Syntenin-1* KO mice. In addition, we showed that a distribution of intestinal microbial flora was influenced in *Syntenin-1* KO mice. Our data indicate that Syntenin-1 negatively regulates the intestinal immunoglobulin production and has a function to maintain the intestinal homeostasis *in vivo*. The analysis of *Syntenin-1* KO mice may provide novel information on not only mucosal immunity but also other functions of Syntenin-1 such as cancer metastasis and neural development.

Introduction

Syntenin-1 (Syndecan-binding protein, Sdcbp) was first identified as an intracellular scaffold protein interacting with the transmembrane heparan sulfate syndecans (Grootjans et al., 1997). It was also termed melanoma differentiation-associated gene-9 (*MDA*-9), which promoted cancer metastasis by regulating cell adhesion (Boukerche et al., 2005, 2007, 2008; Lin et al., 1998). Subsequently, Syntenin-1 has been reported to act as a multifunctional intracellular adapter protein and to regulate protein trafficking and recycling (Zimmermann et al., 2005), Notch signaling (Estrach et al., 2007), IL-5 signaling (Beekman et al., 2009; Geijsen et al., 2001), T cell chemotaxis (Sala-Valdes et al., 2012), HIV infection (Gordon-Alonso et al., 2012), exosome production (Baietti et al., 2012), and synaptic formation in CNS (Hirbec et al., 2005; Jannatipour et al., 2001; Ohno et al., 2004). Additionally, we previously found that extracellular Syntenin-1 in human colostrum could preferentially induce IgA production from cord blood naive B cells (Sira et al., 2009).

IL-5/IL-5R signaling, one of the pathways interacting with Syntenin-1, was reported to maintain mouse B-1 B cells and promote secretion of mucosal IgA (Moon et al., 2004). IL-5 also promotes eosinophil differentiation in humans and mice (Hiroi et al., 1999; Kopf et al., 1996; Moon et al., 2004; Yoshida et al., 1996). Structurally, IL-5R consists of two distinct subunits, an

Abbreviations: BAC, bacterial artificial chromosome; bp, base pair; CNS, central nervous system; ES, embryonic stem; IL-5Rα, interleukin-5 receptor α subunit; KO, knock out; L-LP, large intestinal lamina propria; LP, lamina propria; PC, peritoneal cavity; PDZ, PSD-95/Discs large/zO-1; PEC, peritoneal exudate cells; PP, Peyer's patch; rRNA, ribosomal RNA; sIgA, surface-IgA; S-LP, small intestinal lamina propria; WT, wild type.

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IL-5R-specific α subunit (IL-5Rα) and a common β subunit for the GM-CSF receptor family (Mita et al., 1989). Syntenin-1 was reported to associate with the cytoplasmic tail of IL-5Rα through the PDZ (PSD-95/Discs large/zO-1) domain and to interact directly with the transcription factor Sox4 (Beekman et al., 2009, 2012; Geijsen et al., 2001). Interestingly, Sox4 promotes B cell development, as determined by analysis of *Sox4* KO mice (Schilham et al., 1996; Sun et al., 2013).

Although these various functions of Syntenin-1 *in vitro* have been well reported including the relationship with IL-5R, little is known about the distribution and role of this protein *in vivo*. To clarify the physiological role(s) of Syntenin-1 *in vivo*, we generated a *Syntenin-1* KO mouse strain by gene targeting in this study. We found that *Syntenin-1* KO mice showed no obvious signs of diseases under specific pathogen-free conditions and the Syntenin-1 was widely expressed, particularly in immunologically related organs and CNS. In addition, we focused on the relationship between Syntenin-1 and IL-5 signaling in gut-associated tissues and found that Syntenin-1 was not essential for the maintenance of IL-5-responding cells, and rather negatively regulated immunoglobulin production in the intestine.

Materials and methods

Generation of Syntenin-1 KO mice

Animal care and experimental protocols were approved by the Animal Experiment Committee of the University of Toyama (Authorization No. A2012-MED-35) and were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animal of the University of Toyama.

A bacterial artificial chromosome (BAC) genomic clone (RP24-301N) originating from the DNA of C57BL/6 mice and containing *Syntenin-1* was obtained from BACPAC Resource Center CHORI (Oakland, CA). A counter-selection BAC modification kit (Gene Bridges, Dresden, Germany) and a MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen, Carlsbad, CA) were modified for the targeting vector construction. The nucleotide sequence of the mouse genome was obtained from the National Center for Biotechnology Information (NCBI Map Viewer, *Mus musculus* Build 37.1) and the initiation site of translation in *Syntenin-1* (the A of ATG) refers to position +1 and the proceeding residues are indicated by negative numbers in this report. The 5′ arm of ∼5 kbp (base pair) (Nos. −4769 to −334) and 3′ arm of ∼5 kbp (Nos. +322 to +4947) were subcloned into the pDONR P4-P1R and pDONER P2R-P3 vectors, respectively, using the counter-selection BAC modification kit. The 655-bp *Syntenin-1* (Nos. −333 to +321) gene fragment containing exon 2, part of intron 1, and part of intron 2 was amplified by PCR and subcloned between two loxP sequences of a modified pDONR 221 vector containing a pgk-Neo cassette flanked by two FRT sites. To construct the targeting vector, these three plasmids were directionally subcloned into pDEST R4-R3 containing the diphtheria toxin gene (MC1-DTA) by MultiSite Gateway LR recombination reaction. The targeting vector linearized with *Not*I was electroporated into the embryonic stem (ES) cell line RENKA derived from the C57BL/6N strain (Fukaya et al., 2006) as previously described (Miya et al., 2008). After the selection with G418, recombinant ES clone was identified by Southern blot analysis using the 5′ probe (Nos. −5412 to −4923) on *Spe*I-digested genomic DNA, the 3′ probe (+6740 to +7093) on *Apa*I-digested genomic DNA, and the Neo probe (Miya et al., 2008) on *Apa*I-digested genomic DNA. The obtained recombinant ES clone was transfected with the pCre-Pac plasmid (Taniguchi et al., 1998) and pCAGGS-FLP plasmid (Gene Bridges, Dresden, Germany) by electroporation to delete exon 2 and the pgk-neo cassette, respectively. The PCR amplified fragments were verified using the DNA sequencer ABI PRISM 3100 (Perkin-Elmer, Foster City, CA).

The obtained clone was injected into eight-cell stage embryos of the mouse strain ICR. The embryos were cultured to the blastocyst stage and transferred to the pseudopregnant ICR mouse uterus. The resulting male chimeric mice were crossed with female C57BL/6 mice to establish the mutant mouse line. The *Syntenin-1* KO mice were further genotyped by PCR using the following primers; 5′ forward, 5′-TGACCCTGGTTTAGCTGAGGA-3′; 5′ reverse, 5′-TCTGTTCCCACAGCTACCCAA-3′; and 3′ reverse, 5′-GCTCACAACCGTCTAACTCCAAC-3′ (Fig. 1A).

Western blotting

At the age of 6 weeks, wild type (WT) and *Syntenin-1* KO mice were deeply anesthetized with pentobarbital sodium (100 mg/kg body weight, intraperitoneal injection) and then perfused transcardially with ice-cold PBS. Tissues were quickly removed and homogenized in Mammalian Tissue Extraction Reagent (Pierce, Rockford, IL) with Protease Inhibitor (Nacalai, Kyoto, Japan). The homogenate was centrifuged at 14,500 rpm for 15 min to remove large debris. The protein concentration was determined using a BCA Protein Assay kit (Pierce) and the protein samples were diluted at 1:1 in a sample buffer (50 mM Tris–HCl, pH 8.2, 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue). After denaturation by heating at 95 °C for 5 min, 30 μg of proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Perkin-Elmer). After blocking with 5% skim milk in Tris buffered saline containing 0.1% Tween-20 for 1 h, the membranes were incubated with rabbit polyclonal anti-Syntenin-1 antibody (1:1000, Abcam, Cambridge, UK) or mouse monoclonal anti-β-actin antibody (1:10,000, Sigma–Aldrich, St. Louis, MO) overnight at 4 °C, then with HRP-conjugated goat anti-rabbit IgG (1:25,000, Bio-Rad, Richmond, CA) or goat anti-mouse IgG (1:25,000, Bio-Rad) for 1 h. Protein bands were detected using the ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK).

Antibodies and reagents for flow cytometry

Antibodies used for flow cytometry were anti-mouse B220 (RA3-6B2), CD3ε (145-2C11), CD5 (53–7.3), CD19 (1D3), CD23 (B3B4), and CD45 (30-F11) antibodies purchased from eBioscience (San Jose, CA) and anti-mouse CD21/35 (7G6), surface-IgA (sIgA) (C10-3), and Siglec-F (E50-2440) antibodies purchased from BD Biosciences (San Diego, CA). FcγRs were blocked with anti-mouse FcγR (2.4G2). Flow cytometry was performed using a FACSCanto II (BD Biosciences). Dead cells were gated out by 7-aminoactinomycin D staining (BD Biosciences). FlowJo (Tree Star, Ashland, OR) was used for analysis.

Preparation of lamina propria cells

To obtain lamina propria (LP) cells, the small and large intestines were harvested, and Peyer's patchs (PPs) and cecal patches were removed. The intestines were then opened longitudinally, washed twice with 40 ml of Ca^{2+}- and Mg^{2+}-free HBSS (Sigma–Aldrich) supplemented with 5% FCS, 1 mM DTT, and 5 mM EDTA and then incubated at 37 °C for 40 min with shaking at 150 rpm. Tissues were minced and incubated with RPMI 1640 (Invitrogen) supplemented with 5% FCS. To the small intestine tissues, 1 mg/ml collagenase type I