BASIC—ALIMENTARY TRACT

The Solute Carrier Family 15A4 Regulates TLR9 and NOD1 Functions in the Innate Immune System and Promotes Colitis in Mice

SHIGEMI SASAWATARI,* TADASHI OKAMURA,‡ EIJI KASUMI,* KAORI TANAKA–FURUYAMA,* RIEKO YANOBU–TAKANASHI,‡ SENJI SHIRASAWA,§ NORIHIRO KATO,‖ and NORIKO TOYAMA–SORIMACHI*

*Department of Gastroenterology, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan; ‡Department of Infectious Disease, National Center for Global Health and Medicine, Tokyo, Japan; §Department of Cell Biology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan; ‖Department of Gene Diagnostics and Therapeutics, National Center for Global Health and Medicine, Tokyo, Japan

BACKGROUND & AIMS: Solute carrier family 15 (SLC15) A4 is a proton-coupled histidine and oligopeptide cotransporter expressed by the immune and nervous systems and associated with disorders such as inflammatory bowel diseases and systemic lupus erythematosus. High levels of *SLC15A4* transcripts were observed in human antigen-presenting cells, including dendritic cells, activated macrophages, and B cells. However, the roles of SLC15A4 in the immune regulation are not known. We investigated the function of SLC15A4 in the innate immune system. **METHODS:** We created SLC15A4-deficient (*SLC15A4*$^{-/-}$) mice and compared Toll-like receptor 9 and NOD1-dependent innate immune responses between *SLC15A4*$^{-/-}$ and control (*SLC15A4*$^{+/+}$) mice. **RESULTS:** SLC15A4 deficiency impaired CpG-induced production of interleukin-12, interleukin-15, and interleukin-18 by dendritic cells. Correspondingly, *SLC15A4*$^{-/-}$ mice developed a less severe form of Th1-dependent colitis than *SLC15A4*$^{+/+}$ mice. Increased lysosomal histidine, in the absence of SLC15A4, appears to negatively regulate Toll-like receptor 9 function by inhibiting the proteolytic activities of cathepsins B and L. *SLC15A4*$^{-/-}$ mice also had a severe defect in NOD1-dependent cytokine production, indicating that SLC15A4 functions as a transporter of the NOD1 ligand. **CONCLUSIONS: SLC15A4 promotes colitis through Toll-like receptor 9 and NOD1-dependent innate immune responses. Histidine homeostasis within intracellular compartments is important for eliciting effective innate immune responses.**

Keywords: Innate Immunity; Inflammatory Bowel Disease; Animal Model; Ulcerative Colitis.

Solute carrier family 15 (SLC15) is a subfamily of the proton-coupled oligopeptide transporter superfamily, which are all predicted to contain 12 transmembrane domains, with both the N- and C-termini facing the cytosol.[1,2] The proton-coupled oligopeptide transporter proteins vary in size from 450 to >700 amino acids, and their ancestral roots can be traced to peptide transporters of bacteria, fungi, and plants. However, only a few proton-coupled oligopeptide transporter family members have been characterized functionally.

The SLC15 family has 4 members, A1–A4, which transport short-chain peptides uphill by utilizing proton-motive force.[1] SLC15A1 and SLC15A2 possess around 70% sequence similarity and have very broad substrate specificity. They play a unique role in nutrient absorption, transporting all 400 different dipeptides and 8000 different tripeptides. In contrast, SLC15A3 and SLC15A4 have low sequence similarity with A1 and A2, and their substrate specificity is limited to the transport of histidine and certain oligopeptides. The in vivo roles of SLC15A4 are largely unknown. It is predominantly transcribed in the immune and nervous systems and transports free histidine and oligopeptides from inside the endosome to the cytosol.[3,4] Importantly, this transport activity is pH-dependent, with a higher uptake of histidine observed at pH 5.5 than at pH 7.0.[3] Therefore, it is conceivable that SLC15A4 functions in the course of endosomal acidification and lysosomal maturation. Recent studies suggest that SLC15A4 is associated with diabetes, inflammatory bowel disease, and systemic lupus erythematosus.[5–7] Moreover, tissue-specific transporters, including SLC15 family members, are of particular importance as physiological gatekeepers in pharmacological and toxicological drug-delivery systems. Therefore, we sought to elucidate the in vivo functions and physiological impact of SLC15A4.

SLC15A4 is proposed to be an endosomal transporter for NOD1 ligands because the knockdown of SLC15A4 in HEK293T cells results in a decrease in nuclear factor-κB activation by the NOD1 ligands, Tri-DAP and C12-iE-DAP.[7] NOD1 and NOD2 act as cytosolic pattern

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; DSS, dextran sodium sulfate; IL, interleukin; MDP, muramyl dipeptide; PBS, phosphate-buffered saline; SLC, solute carrier family; TLR, Toll-like receptor.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.01.041

recognition receptors for bacterial peptidoglycans and are essential for regulation of both innate and adaptive immunity.[8,9] NOD1 recognizes DAP, which is found primarily in Gram-negative bacteria, whereas NOD2 detects the peptidoglycan derivative muramyl dipeptide (MDP), a component of virtually all types of bacterial cell walls. NOD1 and NOD2 stimulation leads to the activation of nuclear factor-κB and mitogen-activated protein kinases and the induction of the innate immune responses; the importance of these molecules is underscored by their genetic association with inflammatory bowel disease.[10–13] Although NOD1 and NOD2 both detect peptidoglycans in the cytosol, how such microbial-derived peptidoglycans arrive in the host cytosol is unclear.

We found high transcript levels of SLC15A4 in human antigen-presenting cells, including dendritic cells (DCs), activated macrophages, and B cells (http://www.gnf.org/). In these cells, endosomes/lysosomes are important organelles for eliciting immune responses. Some Toll-like receptors (TLRs), such as TLR3, TLR7, and TLR9, are localized to endosomes and recognize molecular patterns unique to micro-organisms.[14,15] Among them, TLR9, which recognizes unmethylated DNA (CpG-ODN) and triggers the production of cytokines such as tumor necrosis factor-α, interleukin (IL)-6, and IL-12p70, is largely dependent on endolysosomal functions for its signal transduction.[16–18] In addition, endolysosomes are important for antigen processing.[19,20] Therefore, we reasoned that SLC15A4 may be involved in the endolysosome-dependent functions of antigen-presenting cells.

We demonstrated here that SLC15A4 positively regulates the TLR9-dependent production of Th1 cytokines such as IL-12 and IL-15. Concordantly, SLC15A4$^{-/-}$ mice exhibited a less severe form of Th1-dependent mouse colitis than control mice. We also showed, using the SLC15A4$^{-/-}$ mice, that SLC15A4 is responsible for NOD1-dependent cytokine production in vivo, probably by transporting an NOD1 ligand, tri-DAP, from the lysosomes to the cytosol. SLC15A4 does not appear to be important for the recognition of MDP, an NOD2 ligand, in vivo, because the MDP-dependent cytokine production in SLC15A4$^{-/-}$ mice was not affected. Our findings clearly indicate that SLC15A4 is important in the regulation of innate immune responses. Our data also suggest that the rigorous control of histidines in lysosomes may be important for efficient immune responses, probably because histidine has a unique property as an acid-base catalyst.

Materials and Methods

Mice

Mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Experiments were performed according to the Guidelines for Animal Use and Experimentation as set by the International Medical Center of Japan (Tokyo, Japan).

Generation of C57BL/6 Slc15a4-Deficient Mice

For the targeted deletion of *Slc15a4*, we designed a construct to introduce a neomycin-resistance cassette that resulted in the deletion of exons 2, 3, 4, and 5 of the *Slc15a4* coding sequence. The linearized vector with *Sal*I digestion was electroporated into embryonic stem cells; positive clones were selected by G418 on embryonic fibroblast feeder cells as described previously.[21] *Eco*RV digestion of the genomic DNA identified a 6.3-kb recombinant allele and 10.3-kb wild-type allele, when hybridized with an external probe. The targeted embryonic stem cells were microinjected into C57BL6/J blastocysts and the resulting male chimeras were mated with C57BL/6 mice. To generate *Slc15a4*$^{-/-}$ mice on the C57BL/6 background, we used marker-assisted selection protocols.[22] Male mice with the highest percentage of the C57BL/6 strain genotype were selected and used for breeding, and this process was repeated 3 times. The proportion of B6 homozygous loci in the N3 generation was 91.2% (93 of 102 markers). The N3 heterozygotes were then intercrossed to generate *Slc15a4*-deficient mice. From the N3 offspring, *Slc15a4*$^{-/-}$ and *Slc15a*$^{+/+}$ mice between 10 and 20 weeks of age were used in the experiments. In some experiments, *Slc15a4*$^{+/-}$ mice were also used. No differences between *Slc15a4*$^{+/-}$ and *Slc15a4*$^{+/+}$ mice were apparent in our experiments.

Cell Culture and Preparation of DCs

Cells were cultured in complete RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μM 2-mercaptoethanol, 1% (v/v) nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. The preparation of DCs in the presence of Flt3L was described previously.[18] The DCs were treated with CpG included in DOTAP liposomes (Roche Diagnostics, Indianapolis, IN), prepared following manufacturer's instructions.

Immunohistochemical Staining

Cells adhering to glass coverslips were fixed with 3.7% formalin in phosphate-buffered saline (PBS) at room temperature for 15 minutes, and then treated with 0.1% Triton X-100 in PBS for 20 minutes. After being washed with PBS containing 0.05% bovine serum album in, the cells were treated with 3% bovine serum album in in PBS to prevent nonspecific protein binding. Cells were then stained with the indicated antibodies or reagents, mounted, and analyzed by confocal (Zeiss, Stuttgart, Germany) or fluorescence (Olympus, Hamamatsu, Japan) microscopy.

Quantification of Cytokines

Proinflammatory cytokines were quantified using a BD cytometric bead array mouse inflammation kit (BD