

Characterization of a probiotic-derived soluble protein which reveals a mechanism of preventive and treatment effects of probiotics on intestinal inflammatory diseases

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The beneficial effects of probiotics have been demonstrated in many diseases, such as inflammatory bowel disease. The known mechanisms for probiotic action include blocking pathogenic bacterial effects, enhancing the innate immunity and decreasing pathogen-induced inflammation, and promoting intestinal epithelial cell survival, barrier function, and protective responses. We purified and cloned a *Lactobacillus rhamnosus* GG (LGG)-derived soluble protein, p40. This protein ameliorated cytokine-induced apoptosis in intestinal epithelial cells through activation of the EGF receptor and its down-stream target, Akt. By using special hydrogel beads to protect p40 from degradation, we showed that p40 reduced intestinal epithelial apoptosis and preserved barrier function in the colon epithelium in an EGF receptor-dependent manner, thereby preventing and treating intestinal inflammation in mouse models of colitis. Further works regarding structural analysis of p40, regulation of EGF receptor activation and immunoregulatory effects by p40 are discussed. These results may provide insights into the clinical application of probiotics for intestinal inflammatory disorders.

engage in active cross talk with the host. Selective nonpathogenic living microorganisms, including some present as commensal microbiota, which have beneficial effects on host health and disease prevention and/or treatment were first described as probiotics by Lilly and Stillwell.¹ Most commonly used probiotics in humans and animals include *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*. A significant question regarding clinical use of probiotics is the mechanism underlying the wide range of actions. Three distinct cellular and molecular mechanisms have been demonstrated by both clinical and basic research, blocking pathogenic bacterial effects by production of antibacterial substances and competitive inhibition of pathogen and toxin adherence to the intestinal epithelium, regulating immune responses by upregulation of immune function may improve the ability to fight infections or inhibit tumor formation; downregulation may prevent the onset of allergy or intestinal inflammation, and modulating intestinal epithelial cell homeostasis, including restitution of damaged epithelial barrier, production of antibacterial substances and cell-protective proteins, and blockade of cytokine-induced intestinal epithelial cell apoptosis.²

Keywords: apoptosis, barrier, colitis, EGF receptor, inflammatory cytokine, intestinal epithelial cell, intestinal inflammation, *Lactobacillus rhamnosus* GG, probiotics

Submitted: 10/17/11

Revised: 12/06/11

Accepted: 01/03/11

<http://dx.doi.org/10.4161/gmic.19245>

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Addendum to: Yan F, Cao H, Cover TL, Washington MK, Shi Y, Liu L, et al. Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism. *J Clin Invest* 2011; 121:2242–53; PMID:21606592; <http://dx.doi.org/10.1172/JCI44031>

Introduction

A unique characteristic of the gastrointestinal tract is the continuous contact between the gastrointestinal epithelial cell monolayer and microbial flora, which

Probiotic-Derived Soluble Proteins

The current clinical applications of probiotics have raised two problems regarding the use of probiotic therapy. First is the difficulty of determining bioavailability of bacteria in the gastrointestinal tract. In

addition, use of live probiotic bacteria raises concerns about biosafety of probiotics. Probiotic administration has been reported to be associated with an increased risk of mortality in patients with severe acute pancreatitis.³ This condition may be related to bacteremia since several cases of bacteremia were found in immunocompromised patients.⁴ One approach to address these concerns of using probiotics may be through developing probiotic bacteria-derived proteins as innovative therapeutic reagents.

Analyses of current available probiotic genome sequences, including 8 *Lactobacillus* and 6 *Bifidobacterium* strains, have predicted a broad group of proteins, including secreted and cell surface proteins with potential regulatory effects on intestinal cells.⁵ For example, genomic analysis of LGG reveals that a mucus binding pilus on the surface of LGG is a key factor for adhesion of LGG to the host intestinal mucus.⁶ However, their *in vivo* effects on host health and disease prevention and/or treatment are not clearly demonstrated.

Thus, we have chosen LGG as a probiotic model to define the mechanisms of probiotic action. *Lactobacillus rhamnosus* GG (LGG) is a naturally occurring gram-positive bacterium originally isolated from the healthy human intestine.⁷ LGG is one of the best-studied probiotic bacteria in clinical trials for treating and/or preventing several disorders, including inflammatory bowel disease (IBD), diarrhea and atopic dermatitis.⁸ LGG treatment is more effective than standard treatment with mesalamine in maintaining remission and prolonging the relapse-free time in patients with ulcerative colitis.⁹

We have purified and cloned two LGG-derived soluble proteins, p40 and p75, and showed that LGG and LGG-derived soluble proteins prevent cytokine-induced epithelial damage and apoptosis^{10,11} and hydrogen peroxide disruption of epithelial barrier function.¹² Although both of these two proteins regulate intestinal epithelial homeostasis, we were focusing on studying p40 because p40 exerts more potent effects than p75. For example, p40 can stimulate two to 3-fold greater Akt activation and inhibition of apoptosis than p75, even at 5-fold lower molar concentration than p75. In addition, p40 may be a major

protein responsible for the cellular effects regulated by probiotic soluble proteins. *L. casei*-conditioned medium contains the same amount of p40, but very low amount of p75 compared with LGG-conditioned medium. However, *L. casei*-conditioned medium exerts the same effects of Akt activation and inhibition of apoptosis as LGG-conditioned medium. Other identified functions of LGG and soluble proteins include promoting cytoprotective protein production by intestinal epithelial cells¹³ and inhibiting lipopolysaccharide-induced tumor necrosis factor production in macrophages.¹⁴ These studies indicate that production of soluble proteins by probiotics may be a mechanism of probiotic action for regulating intestinal homeostasis and preventing and/or treating diseases.

We have recently performed studies to investigate the effects and mechanisms of p40 regulation of cellular responses in intestinal epithelial cells and p40s role in dextran sulfate sodium (DSS)-induced intestinal epithelial injury and acute colitis, oxazolone-induced Th2 cytokine-driven and TNBS-induced Th1 cytokine-driven chronic colitis in mice. We reported that p40 activated EGF receptor and its down-stream target, AKt, leading to amelioration of cytokine-induced apoptosis and disruption of epithelial barrier in

intestinal epithelial cells. Furthermore, specific delivery of p40 to the colon using a special hydrogel beads to protect p40 from degradation prevented and treated colon epithelial cell injury and inflammation in these colitis models in an EGF receptor-dependent manner (Fig. 1).¹⁵ Our results reveal a previously unrecognized mechanism of probiotic-derived soluble proteins in modulating intestinal inflammation.

Functional Domain(s) of p40

In order to gain further insights into the structure-functional relationship of p40, we performed sequence analysis of p40, which predicts that the N-terminal portion forms a coiled-coil structure and the C-terminal portion forms a β -sheet structure. Therefore, we expressed the N-terminal 1–180 aa and the C-terminal portion (181–412 aa) of p40 as recombinant peptides

p40 full-length and truncated peptides were used to detect their effects on EGF receptor activation and survival of intestinal epithelial cells. We found that the N-terminal 1–180 aa peptide is as potent as p40 full length for regulating signaling and cellular responses in intestinal epithelial cells. Thus, it may be possible to identify functional domain(s) of

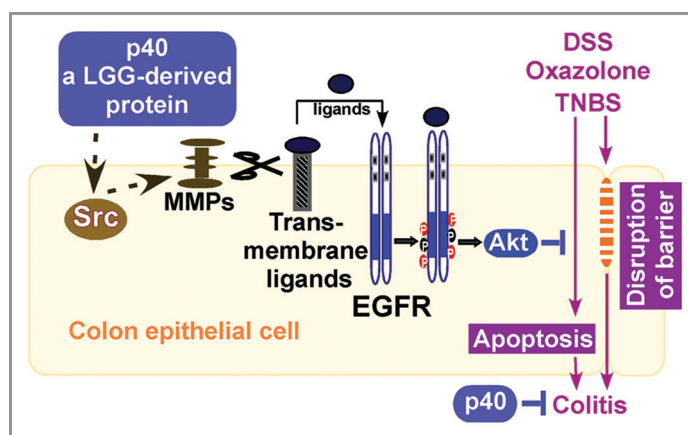


Figure 1. Regulation of colitis by p40. p40 stimulates Src activity, which leads to activation of MMPs. MMPs are proteolytic enzymes to induce release of EGFR ligands for transactivation of EGFR and its down-stream target, Akt, in the colon epithelial cells. Increased apoptosis and disruption of barrier function in the intestinal epithelial cells are two pathological factors involved in colitis induced by DSS, oxazolone, and TNBS. Activation of EGFR and Akt by p40 to prevent apoptosis and maintain intestinal integrity serves as a mechanism for p40s preventive and treatment effects on intestinal inflammation in these mouse models of colitis. EGFR, EGF receptor; MMPs, matrix metalloproteinases.

probiotic-derived proteins for novel therapeutic application in inflammatory intestinal disorders.

Upstream Regulators for EGF Receptor Activation by p40

One significant finding from this published paper that p40 transactivates EGF receptor in the intestinal epithelial cells leads to the question of what are the upstream regulators for EGF receptor activation by p40. Since several studies have demonstrated that the non-receptor tyrosine kinase, Src, activates EGF receptor directly or indirectly through activation of matrix metalloproteinases, which are proteolytic enzymes to induce release of EGF receptor ligands,¹⁶ we sought to determine the involvement of Src and metalloproteinase in p40 regulated signaling in intestinal epithelial cells. We found that p40 stimulated Src activation in intestinal epithelial cells and inhibition of Src or metalloproteinase kinase activity blocked p40 activation of EGF receptor. These data indicate that Src and metalloproteinase may serve as upstream signals to release ligands as the mechanism for p40 transactivation of EGF receptor in intestinal epithelial cells.

Immunoregulatory Effects of LGG p40

Modulation of the immune system is one of the most plausible mechanisms

underlying the beneficial effects of probiotics on human health. Probiotics regulate host innate and adaptive immune responses by modulating functions of dendritic cells, macrophages, and T and B lymphocytes.^{2,17} In addition, since the intestinal epithelium is integral to both discrimination of pathogens and commensal bacteria and is actively involved in immune responses in the intestinal tract, this monolayer is a target of probiotic immunoregulatory effects.¹⁸ Therefore, we detected whether p40 exerts immunoregulatory effects on colitis. To address this issue, we detected cytokine levels in the colon and intracellular cytokine protein levels in macrophages and lymphocytes. Our results suggest that p40 plays a role in regulation of innate immunity and Th1 immune response since p40 downregulated proinflammatory cytokines involved in innate immunity, including macrophage-produced TNF, IL-6, and KC TNF and Th1 response, such as TNF and IFN- γ (Supplementary data in ref. 15). It is important to note that it is likely that p40 associates with different aspects of immunity, such as regulation of innate immunity and Th1 immune response. p40 may have effects on macrophages, lymphocytes, intestinal epithelial cells to directly regulate immune responses during inflammation, such as proinflammatory cytokine production. LGG-conditioned cell culture medium decreases TNF production in macrophages, indicating that soluble molecules

derived from LGG exert this immunoregulatory role.¹⁴ The other possibility is that p40 may function on intestinal epithelial cells to decrease injury, which prevent production of these proinflammatory cytokines.

Conclusions

Our studies clearly demonstrated the preventive and treatment effects of p40 on intestinal inflammation. IBD is characterized by increased production of inflammatory cytokines, epithelial cell apoptosis, and immune cell infiltration, leading to disruption of the intestinal epithelial integrity. The first goal of medical therapy for IBD is to induce and maintain a clinical remission. Remission of these disorders requires both decreased apoptosis and restitution of the damaged epithelium. By using this colon-specific hydrogel delivery system, p40 may exert its protective effects on the intestinal epithelial cells and immunity. Therefore, p40 provides a novel therapeutic approach for preventing and/or treating IBD.

Acknowledgments

This work was supported by NIH grants DK065744, DK081134 and CCFA Senior Research Award (to F.Y.), DK56008 (to D.B.P.), P30DK058404 (Vanderbilt University Digestive Disease Research Center), and CA 68485 (Vanderbilt University Medical Center Imaging Core Research Laboratory).

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