**Lactobacillus acidophilus** stimulates intestinal P-glycoprotein expression via a c-Fos/c-Jun-dependent mechanism in intestinal epithelial cells

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**MATERIALS AND METHODS**

**PROBIOTICS ARE VIABLE nonpathogenic microorganisms that have been shown to have beneficial effects on human health (8) beyond their intrinsic nutritional value.** Probiotics have been used to treat a variety of gastrointestinal disorders (11, 46), such as inflammatory bowel disease (IBD) (3, 19, 40), irritable bowel syndrome, and IBD susceptibility has been reported in other experimental models of IBD, including dextran sulfate sodium (DSS)-induced colitis (DSS-colitis) (24), IL-10 knockout (6), and T cell receptor-α knockout (36) mice, where Pgp expression/activity is significantly decreased.

Thus agents that alleviate Pgp inhibition in intestinal inflammation may prove to be effective against gut inflammatory disorders such as IBD. Our previous studies showed that LA gavage demonstrated an increase in Pgp expression in the ileum and colon and attenuated decreased Pgp expression in the colon of DSS-colitis mice (48), suggesting that the effects of LA CS on intestinal Pgp may have clinical significance. These studies also demonstrated that LA CS-induced upregulation of Pgp in IECs occurred via a transcriptional mechanism. However, the molecular mechanisms involved in the transcriptional modulation of Pgp by LA CS in IECs are not known.

Therefore, the present study was undertaken to elucidate the cis element(s) and transcription factors involved in the modulation of intestinal Pgp gene expression by LA CS. Our results showed the involvement of c-Fos and, partly, c-Jun in the stimulation of intestinal Pgp gene expression by LA CS. These findings define novel mechanisms of transcriptional regulation of Pgp by LA CS at the promoter level that may contribute to the beneficial effects of LA CS in intestinal inflammatory disorders.

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nal rabbit IgG antibody, and consensus and mutant oligonucleotides for activating protein 1 (AP1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); total and phosphorylated Erk1/2 MAPK antibodies from Cell Signaling Technology (Boston, MA); all restriction endonucleases and other modifying enzymes from New England Biolabs (Beverly, MA); luciferase assay system from Promega (Madison, WI); and β-galactosidase assay kit from BD Biosciences Clontech (Palo Alto, CA). All other chemicals were of at least reagent grade and were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**Bacterial culture.** LA (strain 4357, American Type Culture Collection) was grown overnight, and CS was obtained as described previously (48). For our studies, LA CS was diluted in a ratio of 1:10 in cell culture medium supplemented with 1% FBS.

**Cell culture and treatment.** Caco-2 cells were grown routinely in 75-cm² plastic flasks in minimum essential medium (pH 7.2) supplemented with high-glucose 20% FBS, 20 mM HEPES, 100 IU/mL penicillin, and 100 μg/mL streptomycin in 5% CO₂-95% O₂ at 37°C (48, 49). Cells at passages 25–45 were used for these studies. For promoter studies, Caco-2 cells were transiently transfected with full-length or 5'-deletion Pgp promoter constructs or cotransfected with Pgp promoter construct (p-1073/+703) along with c-Fos and/or c-Jun expression vectors (13, 48, 49) by electroporation utilizing Amaxa technology and plated at a density of 1 × 10⁵ cells/cm² on 12-well collagen-coated plates (plastic supports). At 24 h posttransfection, cells were treated from the apical side with LA CS diluted in a ratio of 1:10 in cell culture medium supplemented with 1% FBS for 24 h. In experiments involving Erk1/2 MAPK phosphorylation, gel shift [electrophoretic mobility shift assay (EMSA)], and chromatin immunoprecipitation (ChIP) assays, Caco-2 cells plated at a density of 4 × 10⁴ cells/cm² on six-well plastic supports for 21 days were treated from the apical side with LA CS diluted in a ratio of 1:10 in cell culture medium supplemented with 1% FBS for 1, 3, or 24 h.

**Reporter plasmid construction.** Plasmids used for functional analysis of Pgp promoter activity were generated using pGL2 basic vector (Promega) that contains a promoterless luciferase reporter gene. With the use of the Pgp promoter construct (p-1073/+703, 1,776-bp fragment) as template, five 5'-deletion constructs (p-772/+703, p-472/+703, p-172/+703, p+128/+703, and p+428/+703) were generated by PCR amplification. Five different forward primers containing anti-c-Fos or anti-c-Jun or phosphorylated Erk1/2 (Thr202 and Tyr204 of Erk1; Thr185 and Tyr187 of Erk2) or total Erk1/2 antibody. Bands were visualized with enhanced chemiluminescence detection reagents.

**Transient transfection and luciferase assays.** Caco-2 cells were transfected with full-length or 5'-deletion Pgp promoter constructs and p-cytomegalovirus (CMV) β-galactosidase expression vector (BD Biosciences Clontech) by electroporation using the Amaxa Nucleofector system as described previously (48, 49). Briefly, ~1 × 10⁶ cells were harvested and then electroporated in 100 μL of Nucleofector Solution T (supplied by Amaxa) with one of the Pgp promoter-luciferase constructs (30 μg) and 2.0 μg of pCMVβ. Cells were transferred to full medium and plated on 24-well collagen-coated plastic supports. Transfected Caco-2 cells were treated from the apical side with LA CS diluted in a ratio of 1:10 in cell culture medium supplemented with 1% FBS for 24 h. At 48 h posttransfection, cells were washed with PBS and lysed using a passive lysis buffer (Promega). Pgp promoter activity was expressed in terms of relative luciferase activity normalized to β-galactosidase activity as described previously (48, 49). In a separate set of experiments, Caco-2 cells were transiently cotransfected with 10 μg of Pgp promoter construct (p-1073/+703) or 20 μg of c-Fos or c-Jun expression vector or a combination of c-Fos and c-Jun vectors along with 2.0 μg of pCMVβ per 24-well plate as described previously (13). Control cells without c-Fos or c-Jun expression vector were transfected with equal amounts of the empty vector pcDNA3.1. The c-Fos and c-Jun vectors were a generous gift from Dr. Nancy Colburn (National Cancer Institute, Frederick, MD) (13). At 24 or 48 h postcotransfection, Pgp promoter activity was assessed as described above.

**Real-time PCR.** Total RNA was prepared from Caco-2 cells transfected with empty vector or c-Fos and c-Jun expression vectors alone or in combination utilizing the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA formation and PCR amplification were carried out with SYBR Green one-step real-time PCR master mix and the MX3000p machine (Stratagene). Human MDR1 and GAPDH (internal control) were amplified with gene-specific primers as described previously (14, 48, 49). Relative levels of human MDR1 mRNA are expressed as percentage of control normalized to GAPDH.

**Nuclear extracts and EMSA.** Nuclear extracts were prepared from control or LA CS-treated Caco-2 cells grown on glass supports using a commercially available kit (Thermo Scientific, Rockford, IL). The sequences of the potential AP1 binding sites utilized for EMSA are as follows: ATTCAATCCGGGCGGGA (-119/-98 bp) for the proximal binding site, GAGCCAGGTTCTGAGGGAGG (-99/-78 bp) for the AP1b (distal) binding site, and CAGAGGCTAACCCGAGATCTG (-175/-156 bp) for the AP1c binding site. Duplex oligonucleotides of potential AP1 binding sites of Pgp promoter (custom-synthesized from IDT, San Diego, CA) were end-labeled by a nonradioactive digoxigenin (DIG)-labeling method, and DNA/protein binding reactions by EMSA were performed utilizing a commercially available kit (Roche Diagnostics, Mannheim, Germany) as previously described (13).

**ChIP.** ChIP assays were performed utilizing the commercially available CHIP One-Day Kit essentially according to the manufacturer’s instructions (Qiagen) as previously described (14). Briefly, untreated or LA CS-treated confluent Caco-2 cells (grown in 6-well plastic supports) were cross-linked with 1% formaldehyde and chromatin was cross-linked and then immunoprecipitated with 5 μg of c-Fos or c-Jun antibody (overnight) at room temperature (Santa Cruz Biotechnology). Normal rabbit IgG (Santa Cruz Biotechnology) was used as a control. After reverse cross-linking and DNA extraction, immunoprecipitated DNA was used as the template for real-time quantitative PCR utilizing the primers (AP1) flanking the potential AP1 binding sites 5'-AGAACATTTCCGCGGTGAAGAAGTCGAGGCA-3' (forward) and 5'-TCAAGAAGAGGGTAGTCGAGGCACTGTTCCGTA-3' (reverse) and primers (non-AP1) that do not contain the AP1 binding sites and are ~0.8 kb away from this site 5'-CTCAGCCTTTCTGCCTGGCCCTGCAGG-3' (forward) and 5'-TTGGAAGCAATGCTCAATGCAATGGG-3' (reverse). At the end
CS induced Erk1/2 MAPK phosphorylation as early as 1 h that persisted until 24 h. Treatment of Caco-2 cells with LA CS for 24 h increased the protein expression of c-Fos (2-fold) and c-Jun (1.89-fold) in Caco-2 cells (Fig. 2B). Since c-Fos and c-Jun are downstream effectors of the Erk1/2 MAPK pathway, these results suggest a role for c-Fos or c-Jun in mediating the stimulatory effects of LA CS on Pgp promoter activity in Caco-2 cells.

Overexpression of c-Fos and/or c-Jun increases Pgp promoter activity and mRNA expression in Caco-2 cells. To examine whether c-Fos or c-Jun directly influences Pgp promoter activity, Caco-2 cells were cotransfected with the Pgp promoter along with mammalian expression vectors for c-Fos or c-Jun hemagglutinin (HA)-tagged protein. We previously showed increased expression of c-Fos- and c-Jun-HA-tagged fusion protein in cells transfected with the expression vectors for these proteins compared with transfection with empty pcDNA3.1 vector alone (13). Overexpression of c-Fos or c-Jun increased Pgp promoter (p-1073/+703) activity by nearly twofold. However, when c-Fos and c-Jun were simultaneously overexpressed, the increase in Pgp promoter activity was further enhanced by ~14-fold (Fig. 3A). These data suggest that the presence of one of these transcription factors potentiates the effect of the other, resulting in a remarkable stimulation of Pgp promoter activity. Furthermore, our data indicate that c-Fos and c-Jun are involved in LA CS-mediated stimulation of Pgp promoter activity. We next wanted to examine whether overexpression of c-Fos and c-Jun increases endogenous Pgp mRNA levels in Caco-2 cells. Our data showed that cotransfection of c-Fos and c-Jun in Caco-2 cells significantly increased Pgp mRNA levels by nearly threefold (Fig. 3B). These results further suggest that c-Fos and c-Jun play an important role in stimulation of Pgp gene expression in Caco-2 cells in response to LA CS.

RESULTS
Identification of the LA CS-responsive region in the Pgp promoter. LA increases Pgp promoter activity in Caco-2 cells (48). To determine which region of the Pgp promoter is responsible for LA CS-mediated stimulation of Pgp promoter activity, a series of 5'-truncated Pgp reporter constructs were generated in pGL2 basic vector containing progressive deletions from the 5' end of the full-length Pgp promoter construct p-1073/+703. Figure 1 depicts promoter activity of the full-length (p-1073/+703) and other 5'-deletion constructs in response to LA CS (1:10 dilution, 24 h). Incubation with LA CS resulted in a ~2.5-fold increase in the activity of three constructs of Pgp promoter (p-1073/+703, p-772/+703, p-472/+703, and p-172/+703). However, deletion from -172 to +428 abrogated the stimulatory effects of LA CS. These results suggested that the putative LA CS-responsive element(s) is located in the -172/+428-bp region.

LA CS induces Erk1/2 MAPK activation and c-Fos or c-Jun protein expression in Caco-2 cells. Sequence analysis of the region spanning the -172/+428-bp region revealed potential binding sites for AP1/c-Fos/c-Jun. Previous reports show that c-Fos and c-Jun transcription factors are activated by the Erk1/2 MAPK pathway (7, 18, 26, 37, 53, 57). Our previous studies showed that stimulation of Pgp function by LA CS was mediated via an Erk1/2 MAPK pathway (48). We then examined Erk1/2 MAPK phosphorylation in Caco-2 cells in response to LA CS at 1, 3, and 24 h. As shown in Fig. 2A, LA CS induced Erk1/2 MAPK phosphorylation as early as 1 h that persisted until 24 h. Treatment of Caco-2 cells with LA CS for 24 h increased the protein expression of c-Fos (2-fold) and c-Jun (1.89-fold) in Caco-2 cells (Fig. 2B). Since c-Fos and c-Jun are downstream effectors of the Erk1/2 MAPK pathway, these results suggest a role for c-Fos or c-Jun in mediating the stimulatory effects of LA CS on Pgp promoter activity in Caco-2 cells.

Overexpression of c-Fos and/or c-Jun increases Pgp promoter activity and mRNA expression in Caco-2 cells. To examine whether c-Fos or c-Jun directly influences Pgp promoter activity, Caco-2 cells were cotransfected with the Pgp promoter along with mammalian expression vectors for c-Fos or c-Jun hemagglutinin (HA)-tagged protein. We previously showed increased expression of c-Fos- and c-Jun-HA-tagged fusion protein in cells transfected with the expression vectors for these proteins compared with transfection with empty pcDNA3.1 vector alone (13). Overexpression of c-Fos or c-Jun increased Pgp promoter (p-1073/+703) activity by nearly twofold. However, when c-Fos and c-Jun were simultaneously overexpressed, the increase in Pgp promoter activity was further enhanced by ~14-fold (Fig. 3A). These data suggest that the presence of one of these transcription factors potentiates the effect of the other, resulting in a remarkable stimulation of Pgp promoter activity. Furthermore, our data indicate that c-Fos and c-Jun are involved in LA CS-mediated stimulation of Pgp promoter activity. We next wanted to examine whether overexpression of c-Fos and c-Jun increases endogenous Pgp mRNA levels in Caco-2 cells. Our data showed that cotransfection of c-Fos and c-Jun in Caco-2 cells significantly increased Pgp mRNA levels by nearly threefold (Fig. 3B). These results further suggest that c-Fos and c-Jun play an important role in stimulation of Pgp gene expression in Caco-2 cells in response to LA CS.

![Fig. 1. Functional analysis of various deletion constructs in response to Lactobacillus acidophilus (LA) culture supernatant (CS). Caco-2 cells were transiently transfected with different 5’-deletion constructs of P-glycoprotein (Pgp) promoter along with P-cytomegalovirus (CMV) β-galactosidase (pCMVβ) vector. At 24 h posttransfection, cells were treated with a 1:10 dilution of LA CS for 24 h in medium containing 1% FBS. Cells were harvested at 48 h posttransfection, and promoter activity was measured by luciferase (Luc) assay. Data were normalized to β-galactosidase activity to correct for transfection efficiency. Values are means ± SE of 4 separate experiments and are expressed as percentage of control [i.e., transfected cells treated with LA CS vs. untreated cells (control)]. *P < 0.05 vs. respective control.](http://ajpgi.physiology.org/ by 10.22033.4 on October 14, 2017)
LA CS induces binding of potential AP1a or AP1b, but not AP1c, cis elements of Pgp promoter to Caco-2 nuclear proteins. We next examined the ability of the three potential AP1 cis elements, namely, AP1a (proximal, 98 bp), AP1b (distal, 78 bp), and AP1c (196 bp), to bind Caco-2 nuclear proteins. EMSA was performed utilizing the three potential AP1a, AP1b, or AP1c cis elements as DIG end-labeled probes. As shown in Fig. 4, binding of labeled potential AP1a or AP1b sites to Caco-2 nuclear proteins was significantly increased in the presence of LA CS (lanes 3 and 7) compared with control (lanes 2 and 6). The DNA-protein complex was competed out in the presence of an excess of unlabeled probe (lanes 4 and 8), indicating the binding specificity of the complexes. However, binding of the labeled potential AP1c site to Caco-2 nuclear proteins was not increased in the presence of LA CS (lane 11) compared with control (lane 10). These results suggest that potential AP1a and AP1b, but not AP1c, sites of the Pgp promoter are crucial in DNA-protein interactions in Caco-2 cells in response to LA CS.

c-Fos strongly binds to potential AP1a cis element of Pgp promoter compared with c-Jun in response to LA CS. Since the above-described studies indicate that c-Fos and c-Jun together transactivate Pgp promoter and that the AP1 transcription complex is a family of dimeric transcription factors composed of c-Fos and c-Jun proteins (40), we next investigated the ability of c-Fos or c-Jun to bind the potential AP1a or AP1b site in response to LA CS. As shown in Fig. 5A, incubation of the DIG-labeled proximal AP1a cis element with the nuclear extracts from untreated (control) or LA CS-treated cells resulted in a band representing binding of the probe to c-Fos or c-Jun. Binding of the labeled potential AP1a site to Caco-2 proteins (DNA-protein complexes) was significantly increased in the presence of LA CS (lane 3) compared with control (lane 2). Competition experiments were performed to examine the binding specificity of the complexes. The DNA-protein complex...
In Fig. 5B, competition experiments with an excess of cold unlabeled AP1a (lane 2) or consensus AP1 (lane 3) or unlabeled AP1b oligonucleotide (lane 4) show results similar to those in Fig. 5A. Competition experiments with mutant oligonucleotides showed that the DNA-protein complex was not eliminated in the presence of an excess of consensus mutant AP1 oligonucleotide (lane 5) or unlabeled cold mutant AP1a (M3) oligonucleotide (lane 8). However, unlabeled cold mutant AP1a (M1 and M2) eliminated formation of the DNA-protein complex (lanes 6 and 7), indicating that mutations within the potential AP1a site (M3), but not away from the site (M1) or near the site (M2), were important in binding of AP1a to Caco-2 nuclear proteins. Similar findings were observed with the distal AP1b cis element of the Pgp promoter (data not shown). These results further indicate the role of c-Fos and c-Jun in LA CS-mediated stimulation of Pgp promoter activity.

LA CS induces association of c-Fos/c-Jun with Pgp promoter in vivo. The interactions of c-Fos or c-Jun transcription factor with the Pgp promoter in vivo were further confirmed by ChIP assays. Untreated and LA CS-treated Caco-2 cells were cross-linked using formaldehyde, and sheared chromatin was isolated and subjected to immunoprecipitation using anti-c-Fos or anti-c-Jun or normal rabbit IgG antibody. Immunoprecipitated DNA was purified and subjected to real-time PCR using AP1 primers flanking the AP1 binding sites [p−172/+1 bp (primer 1)] and non-AP1 primers [−0.8 kb away from the AP1 binding sites (primer 2)]. Figure 6A shows PCR products of the expected size amplified by primers flanking the AP1 binding sites, with normal rabbit IgG or c-Fos- or c-Jun-immunoprecipitated DNA used as template. Enrichment with c-Fos or c-Jun antibody, but not with IgG (negative control), was observed. Also, enrichment with c-Fos or c-Jun antibody was increased in the presence of LA CS compared with control. Our results show that LA CS increased the association of c-Fos with the Pgp promoter region (containing AP1 binding sites) by nearly fourfold compared with untreated control (Fig. 6B), while only a modest (~1.5-fold) increase in the association of c-Jun with Pgp promoter was observed. These findings further confirm the prominent role of c-Fos and, to a lesser extent, c-Jun in mediating the stimulatory effects of LA CS on Pgp promoter activity.

DISCUSSION

Reduced effectiveness of the intestinal epithelial barrier contributes to chronic inflammation and diarrhea in IBD (12, 29, 42). Pgp/MDR1 acts as a biological barrier in the protection of IECs by pumping xenobiotics and bacterial toxins into the intestinal lumen. Several studies have linked the dysregulation of Pgp function and expression to the pathogenesis of gut inflammatory disorders including IBD (2, 4, 27, 32). Thus, Pgp/MDR1 is slowly emerging as an important target for treatment of intestinal inflammation and, therefore, could be a novel target for the management of IBD. Probiotics are considered a promising alternative therapy for IBD (11, 46). Previous studies have shown that treatment with probiotics alleviated inflammation in patients with IBD (3, 19, 20, 34, 40). Probiotics in most of these studies were used as formulations containing a mixture of Lactobacillus sp., Bifidobacteria sp., and Streptococcus thermophilus. Thus, while it is possible that a mixture of strains of probiotics may beneficially impact...
the overall profile of intestinal bacteria, different probiotic strains may not provide the same efficacies. Also, they may show no beneficial effect or may be detrimental because of the promotion of latent pathogenicity in specific intestinal regions. However, there are very limited reports on the beneficial role of an individual probiotic strain in the treatment of IBD; therefore, it is important to thoroughly understand the mechanisms underlying regulation of intestinal Pgp function and expression by a given probiotic species.

Our previous studies showed that administration of the well-known anti-inflammatory probiotic LA to mice significantly attenuated decreased Pgp expression in the colon of DSS-colitis mice compared with control mice (48). Also, our studies demonstrated an increase in Pgp expression in the ileum and colon of LA-treated mice (48). These studies, for the first time, show a strong correlation between attenuation of intestinal inflammation by LA and stimulation of Pgp, which is involved in the protection of intestinal epithelial barrier integrity. Moreover, utilizing intestinal Caco-2 cells as an in vitro cell culture model, we found that LA CS significantly increased Pgp function (48). This increase in function was consistent with an increase in Pgp mRNA and protein levels and occurred via a transcriptional mechanism, as LA CS increased Pgp promoter activity in Caco-2 cells (48). However, the molecular mechanisms underlying stimulation of Pgp gene expression by LA CS in the intestine are not known. In the present study we provide novel data showing the involvement of c-Fos and, partly, c-Jun in stimulation of Pgp gene expression by LA CS in the intestine.

We previously showed involvement of the Erk1/2 MAPK pathway in mediating the stimulatory effects of LA CS on Pgp function in IECs (48). Moreover, previous studies have shown that Erk1/2 MAPK plays an important role in activation of the AP1 transcription complex comprising c-Fos and c-Jun family members (7, 18, 26, 37, 53, 57). Our current studies demonstrate that LA CS induced an increase in Erk1/2 MAPK phosphorylation in Caco-2 cells within 1 h that persisted for 24 h. Parallel to an increase in Erk1/2 MAPK phosphorylation at 24 h, LA CS also induced protein expression levels of c-Fos and c-Jun in Caco-2 cells. The AP1 transcription factor complex is composed of hetero- and homodimers of the Jun and Fos families of transcription factors, which bind to a specific DNA consensus sequence [TGA(C/G)TCA] (1, 43). Progressive deletions from the 5′-flanking region of the Pgp promoter showed that the LA CS-responsive region is located in the −222/−172-bp region, as deletion from this region, which harbors the potential AP1a and AP1b sites, abrogated the stimulatory effects of LA CS on Pgp promoter activity. Furthermore, EMSA studies using DIG-labeled oligonucleotide probes containing the AP1 potential binding sites AP1a (proximal, −56/−196 bp), AP1b (distal, −99/−78 bp, lanes 6 and 7), or AP1c (+175/+196 bp, lanes 10 and 11). Protein-DNA complexes competed in the presence of an excess of cold unlabeled oligonucleotide probe (lanes 4, 8, and 12) showing specificity of binding. Representative gels of 3 separate experiments with similar results are shown.
Jun/Jun homodimers, resulting in increased AP1 activity (22). This was further evident from EMSA studies showing a strong binding of c-Fos to potential AP1a and AP1b cis elements of Pgp promoter compared with c-Jun in response to LA CS. Moreover, competition experiments showed that mutations in the consensus AP1 oligonucleotide failed to eliminate the DNA-protein complex, further suggesting the involvement of AP1. These findings were substantiated in vivo by ChIP assays coupled with real-time PCR analysis in control and LA CS-treated Caco-2 cells. We showed that the association of c-Fos

Fig. 5. c-Fos or c-Jun binds to potential AP1 cis elements of Pgp promoter. A: electrophoretic mobility shift assay was performed using a double-stranded oligonucleotide as DIG-labeled proximal AP1a probe (−119/−98 bp) and nuclear extracts from untreated (control) Caco-2 cells or Caco-2 cells treated with LA CS (1:10 dilution, 24 h). Lane 1 depicts only probe. DNA-protein binding in control (lane 2) was significantly increased (lane 3) in response to LA CS. Competition experiments were performed in the presence of unlabeled cold AP1a oligonucleotide (lanes 4 and 5) and consensus AP1 oligonucleotide (lanes 6 and 7). DNA-protein complex was completely blocked in the presence of c-Fos antibody (2 μg; lanes 8 and 9) or partially blocked in the presence of c-Jun antibody (2 μg; lanes 10 and 11). B: DNA-protein complex was eliminated in the presence of an excess of cold unlabeled AP1a (lane 2) or consensus AP1 (lane 3) oligonucleotide or cold unlabeled AP1b oligonucleotide (lane 4), but not in the presence of consensus mutant AP1 oligonucleotide (lane 5) or unlabeled cold mutant AP1a oligonucleotide (M3; lane 8). Unlabeled cold mutant AP1a oligonucleotides M1 and M2 (lanes 6 and 7) eliminated the DNA-protein complex. + and −, Presence and absence of reaction components in the reaction mixture. Gels are representative of 3 separate experiments with similar results.
Fig. 6. LA CS induces binding of c-Fos/c-Jun to endogenous Pgp promoter. Cross-linked chromatin was isolated from untreated (control) and LA CS-treated Caco-2 cells subsequent to formaldehyde treatment. Chromatin immunoprecipitation assays were performed with c-Fos or c-Jun antibody. Coimmunoprecipitated DNA as template and primers were used to amplify the Pgp promoter region (see MATERIALS AND METHODS). A: amplified PCR products of expected size resolved on 1% agarose gel. B: quantification of binding of API cis elements of Pgp promoter with c-Fos or c-Jun immunoprecipitates (IP) in the presence and absence of LA CS utilizing primers spanning the API binding sites (primer 1, −172/+1 bp) or −0.8 kb away from the API1 binding sites (primer 2, −973/+801 bp). Results represent 3 separate experiments. *P < 0.05 vs. control.

or c-Jun with the fragment of the Pgp promoter that contains the AP1α and AP1β cis elements (Fig. 6B) was significantly increased in the presence of LA CS compared with control. However, in parallel to the EMSA results, the increase in the association of c-Fos with the Pgp promoter region flanking the API binding sites was much higher (~4-fold) than the association of c-Jun (~1.5-fold) in response to LA CS. Overall, these data indicate that, compared with c-Jun, c-Fos plays a major role in stimulation of Pgp promoter activity in IECs in response to LA CS. Furthermore, mutagenesis studies are needed to confirm the role of the proximal API1α and/or distal API1β cis element in stimulation of Pgp promoter activity by LA CS.

Moreover, the beneficial effects of probiotics have been attributed to the soluble bioactive factors released into the CS that are capable of eliciting responses in epithelial cells (33, 55). These metabolites are also capable of traversing the intestinal barrier and have been shown to contribute to intestinal homeostasis and barrier function. Two isolated and purified proteins secreted by Lactobacillus rhamnosus GG (LGG) have been shown to promote intestinal homeostasis and cell growth by inducing Akt activation and inhibiting cytokine-induced epithelial cell apoptosis (55). Other low-molecular-weight peptides secreted from LGG induce expression of heat shock proteins in a p38/JNK MAPK-dependent manner (52). Madsen et al. (31) showed that epithelial barrier function and resistance to Salmonella invasion are enhanced by exposure to soluble factor secreted by the bacteria found in the VSL#3 probiotic formulation. Ewaschuk et al. (10) showed that CS of Bifidobacterium infantis resulted in an increase in trans-epithelial resistance and expression levels of the tight junction proteins zonula occludens-1 and occludin in colonic T84 cells. Furthermore, the bioactive factors secreted in the CS were found to be effective in reducing colonic permeability and attenuation in an IL-10 knockout model of colitis (10). Our studies demonstrated that low-molecular-weight (3- to 10-kDa) peptides secreted from LA increased Cl−/HCO3− exchange activity in Caco-2 cells concomitant with an increase in the apical membrane levels of DRA (SLC26A3) protein (5). However, the molecular identity of the bioactive factors secreted by LA is not known.

Although Petrof et al. (44, 45) showed that the CS of VSL#3 or Lactobacillus plantarum exerts its anti-inflammatory effects in IECs via attenuation of activity of the proinflammatory nuclear transcription factor NFκB through proteasome inhibition, our studies, for the first time, provide evidence that c-Fos and, partly, c-Jun contribute to the anti-inflammatory effects of LA CS in IECs, leading to increased Pgp gene expression. Further studies are needed to identify the bioactive factors secreted by LA and to test whether the functional efficacy of the identified bioactive factors in inducing Pgp gene expression in Caco-2 cells via activation of Erk1/2 MAPK, c-Fos, and c-Jun is similar to that of LA CS.

In summary, our findings reveal that treatment of IECs with LA CS results in induction of c-Fos and c-Jun protein expression levels via Erk1/2 MAPK activation. The c-Fos/c-Jun heterodimer then binds to the API cis element of the Pgp promoter, leading to stimulation of Pgp promoter activity. Our findings provide novel mechanistic insights into the effects of LA on Pgp gene expression in IECs.
REFERENCES


AUTHOR CONTRIBUTIONS


