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Functional Characterization of the CCL25 Promoter in Small Intestinal Epithelial Cells Suggests a Regulatory Role for Caudal-Related Homeobox (Cdx) Transcription Factors

Anna Ericsson,2* Knut Kotarsky,2,3* Marcus Svensson,*, Mikael Sigvardsson,† and William Agace*

The chemokine CCL25 is selectively and constitutively expressed in the small intestinal epithelium and plays an important role in mediating lymphocyte recruitment to this site. In this study, we demonstrate that CCL25 expression in murine small intestinal epithelial cells is independent of signaling through the lymphotoxin β receptor and is not enhanced by inflammatory stimuli, pathways involved in driving the expression of most other chemokines. We define a transcriptional start site in the CCL25 gene and a region −141 to −5 proximal of exon 1 that is required for minimal promoter activity in the small intestinal epithelial cell lines, MODE-K and mICc12. These cell lines expressed far less CCL25 mRNA than freshly isolated small intestinal epithelial cells indicating that they are missing important factors driving CCL25 expression. The CCL25 promoter contained putative binding sites for the intestinal epithelial-associated Caudal-related homeobox (Cdx) transcription factors Cdx-1 and Cdx-2, and small intestinal epithelial cells but not MODE-K and mICc12 cells expressed Cdx-1 and Cdx-2. EMSA analysis demonstrated that Cdx proteins were present in nuclear extracts from freshly isolated small intestinal epithelial cells but not in MODE-K or mICc12 cells, and bound to putative Cdx sites within the CCL25 promoter. Finally, cotransfection of MODE-K cells with Cdx transcription factors significantly increased CCL25 promoter activity as well as endogenous CCL25 mRNA levels. Together these results demonstrate a unique pattern of regulation for CCL25 and suggest a role for Cdx proteins in regulating CCL25 transcription. The Journal of Immunology, 2006, 176: 3642–3651.

Chemokines are a large family of low m.w. proteins primarily recognized for their role as leukocyte chemotactants and in regulating leukocyte trafficking. They function through seven transmembrane G protein-coupled receptors to induce directed cellular migration and enhanced integrin-mediated adhesion, which are processes critical for leukocyte extravasation (1). Chemokines can be divided into two groups, inflammatory and homeostatic chemokines, based on their regulation and function (2). Inflammatory chemokines control the recruitment of effector leukocytes, including cells from both the innate and adaptive immune response, to sites of infection or inflammation, and can be induced in a wide variety of cells upon exposure to host or pathogen-derived inflammatory stimuli (2). Homeostatic chemokines, by contrast, are constitutively expressed in primary and secondary lymphoid organs and in tertiary tissues, such as the skin and intestine, where they control lymphocyte migration during hemopoiesis, initiation of immune responses, and immune surveillance of healthy peripheral tissues (2). The expression of homeostatic chemokines in lymphoid organs and the intestine is largely dependent on lymphotoxin (LT)4 β receptor signaling (3–5). The division of chemokines into inflammatory and homeostatic chemokines is, however, not absolute because many homeostatic chemokines can be up-regulated in response to inflammatory stimuli (6, 7), and inflammatory chemokines can target noneffector leukocytes at sites of leukocyte development (8).

The chemokine CCL25 is selectively and constitutively expressed in the small intestine and thymus, primarily by resident epithelial cells (9–12). Its sole functional receptor, CCR9, is expressed on small intestinal lymphocytes, a subset of circulating gut tropic lymphocytes, and thymocytes (13, 14). Analysis of CCR9−/− mice, and in vivo studies using neutralizing anti-CCL25 Ab, or CCR9−/− TCR transgenic T cells have demonstrated a central role for CCL25/CCR9 in the generation of the small intestinal lymphocyte compartment (14–21). Despite the importance of CCL25/CCR9 in small intestinal immunity, the mechanisms underlying the selective and constitutive expression of CCL25 in the small intestine are not understood.

In the current study, we have examined expression and regulation of CCL25 in small intestinal epithelial cells. Our results demonstrate that CCL25 displays a unique pattern of regulation compared with other inflammatory or homeostatic chemokines and suggest a role for the Caudal-related homeobox transcription factors in enhancing CCL25 promoter activity in the small intestine.

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Materials and Methods

**Mice**

Germfree or conventional Swiss Webster mice were from Taconic Farms, and C57BL/6 mice were from the Microbiology, Immunology, and Gnotobiology animal facility and the Biomedical Centre animal facility (Lund University, Lund, Sweden). Small intestinal tissue from athymic mice with a truncated common cytokine receptor γ-chain (CγR−/−) was provided by Dr. H. Ishikawa (Keio University School of Medicine, Tokyo, Japan), tissue from TGFRI−/− mice was provided by Dr. N. Lycke (University of Gothenburg, Gothenburg, Sweden), and tissue from LTr−/− and LTB−/− mice was provided by Dr. D. Finke (University of Lausanne, Lausanne, Switzerland). All animal studies were approved by the local ethical committee.

**Epithelial cell isolation and cell line culture**

Epithelial cells were removed from the small intestine with EDTA. Briefly, the small intestine was rinsed with ice-cold PBS, inverted, and cut into 5-cm fragments. Intestinal fragments were then washed in PBS containing 30 mM EDTA for 30 min at 37°C on a rotating platform and EDTA was changed every 5 min. Murine small intestinal epithelial crypts were isolated and cultured as described (22). RT-PCR for cytokeratin 18 expression was performed to confirm the epithelial identity of cultured cells (data not shown). The murine small intestinal epithelial cell lines MODE-K and cloned into pBluescript and seven obtained clones selected by restriction endonucleases. The first PCR resulted in a product of 418 bp in length, which was cloned into pBluescript and eight obtained clones were sequenced.

**Construction of plasmids containing mouse CCL25 promoter fragments**

The different promoter regions were amplified by PCR using the following reverse primers: PE1 5′-GGGAGCTTCATCATCAGGCCTGTTGCTGTTGCTG-3′ for PF1 and PE2 5′-GGGAGCTTCAATGAGTCAGACTGTCGGC-3′ for PF2, and the following forward primers: 5′-GGGAGCTTCATCATCAGGCCTGTTGCTGTTGCTG-3′ for PF1 or 5′-GGGAGCTTCAATGAGTCAGACTGTCGGC-3′ for PF2 and 5′-GGGAGCTTCAATGAGTCAGACTGTCGGC-3′ for PF3 on mouse genomic DNA. PCR products were cut with SacI and XhoI, cloned into the pGL3-basic (Promega), and their identity verified by sequencing. Different parts of the promoter were deleted using restriction endonucleases. The HindIII digestion gave rise to the plasmids pGL3-PF4 and pGL3-PF5, whereas the PstI restriction resulted in the plasmid pGL3-PF6. The constructs pGL3-PF7-9 were constructed by ligating double-stranded oligonucleotides in the pGL3 vector.

**Immunohistochemistry**

Acetone-fixed small intestinal tissue sections (8-μm thick) were incubated with 1% H2O2 for 15 min to block endogenous peroxidase activity, followed by an avidin-biotin blocking kit (Vector Laboratories) to block endogenous alkaline phosphatase (Vector Laboratories, CA). Tissue sections were cut from Tissue-Tek OCT embedded tissue. The sections were placed on PEN membrane-coated slides (P.A.M. Microal, Rio de Janeiro, Brazil) and fixed in 70% ethanol for 30 s and acetone for 4 min. Fixed sections were stained with Harris hematoxylin for 10 s (Sigma-Aldrich), washed, overlaid with 10% DMSO, and placed on dry ice or kept at −80°C until use. All aqueous solutions were made from diethyl pyrocatecholic-treated water and supplemented with vanadyl-ribonucleoside complex RNase Inhibitor (Sigma-Aldrich). Laser capture was performed on a Zeiss microscope equipped with a microspatula knife system (P.A.M. Microal, Rio de Janeiro, Brazil). Total RNA was extracted from the catapulted samples using a StrataClone RNA Miniprep kit. For 5′ RACE total RNA was reverse transcribed onto magnetic beads (Dynal Biotech) using Superscript III (Invitrogen Life Technologies) according to manufacturer’s instructions. The single-stranded cDNA was tailed with dATP on the 3′ end using terminal transferase EC 2.7.7.31 (Roche). The second strand was synthesized with Pfx-polymerase (Invitrogen Life Technologies) and the following adaptor primer: 5′-GTCGACGAGGATGCTAGCTGAT-3′. Primers for cytokeratin 18 were sense 5′-AGATCGACATGTCGTTGTTGTAACCCT-3′ and antisense 5′-AGACTGTGGGAGCATGTTGTTGTAACCCT-3′, antisense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′ and sense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′, and antisense 5′-GAGTTTAAAACCCTCTTCTTCTTCT-3′ and sense 5′-GAGTTTAAAACCCTCTTCTTCTTCT-3′, and antisense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′ and sense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′, and antisense 5′-GAGTTTAAAACCCTCTTCTTCTTCT-3′ and sense 5′-GAGTTTAAAACCCTCTTCTTCTTCT-3′, and antisense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′ and sense 5′-GCGGATTTTACAGTGTTTCAGGTGCT-3′.
5 μl of Metafectene to 450,000 cells/well. For analysis of luciferase activity, cells were harvested 24 h posttransfection by adding passive lysis buffer and stored at −80°C until analysis. Luciferase assays were performed using the Dual Luciferase Assay kit (Promega) in a BMG LUMistar Galaxy instrument. For cotransfection experiments using Cdx-1, 0.5 μg of pGL3-3xPP1 or pGL3-3xP7 were cotransfected with 0.9 μg of pcDNA3Cdx-1 plasmid (provided by Dr. P. Soubeyran, Institut National de la Santé et de la Recherche Médicale, Marseille, France) (24) or empty pcDNA3 plasmid as control. For cotransfection experiments using Cdx-2, 0.5 μg of pGL3-3xP1 or pGL3-3xP7 were cotransfected with 0.5 μg of pTREgagaCdx-2 (provided by Dr. T. Uesaka, Hiroshima University, Hiroshima, Japan) (25) and 0.5 μg of pTet-ON (BD Biosciences). Cdx-2 expression was initiated by adding doxycycline (Sigma-Aldrich) to a final concentration of 10 μM. All transfections performed included 50 ng of the pTRE-Rl Renilla plasmid (Promega) for the normalization of transfection efficiencies. Expression of Cdx-1 and Cdx-2 mRNA was verified by RT-PCR. To assess the effect of Cdx-1 and Cdx-2 on endogenous CCL25 mRNA expression, miC12 cell lines were transfected with 1.5 μg of pcDNA3 Cdx-1, 1.5 μg of pcDNA3Cdx-2, or 0.75 μg of both, or 1.5 μg of pcDNA3-EGFP or of pcDNA3 as controls. Stable clones were selected in the presence of 250 μg/ml G-418 (Sigma-Aldrich) for 12 days. The percentage of pcDNA3-EGFP transfected cells expressing enhanced GFP after this time ranged from 65 to 85%. CCL25 and GAPDH mRNA copy numbers were determined as earlier described.

Database analysis of transcription factor binding sites and alignment of the mouse and human CCL25 promoters

Putative transcription factor binding sites in the CCL25 promoter were identified using Transcription Element Search System (TESS) (www.cbil.upenn.edu/tesse/) and MatInspector (www.genomatix.de/cgi-bin/matinpector/matinpector.pl). All reported hits were sorted by their matrix similarity (for TESS according to TRANSFAC and for MatInspector according to MatInspector matrices). All hits with a threshold over 0.8 were considered relevant. Factors predicted to interact with both human and murine sequences were checked for their predicted tissue distribution using Geneatlas (www.dsi.univ-paris5.fr/genatlas/), and transcription factors limited to cells of hemopoietic origin were excluded. The alignment of the human and mouse core promoter sequence was performed using ClustalX (26).

EMSA

The following dsDNA oligonucleotides were used: Cdx 5'-TCTGAGC TATATAAGATGAAAGCC-3' and mutant Cdx 5'-TCTGACTGGG GAGAATGAAAGCC-3'. The Cdx probe was 32P-labeled using T4 Polynucleotide kinase (Invitrogen Life Technologies) according to the manufacturer’s instructions. EMSA was performed in 15 μl of binding buffer (20 mM phosphate buffer (pH 6), 10 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 0.01% Nonidet P-40, 0.1 mM NaCl, 100 μg/ml BSA, 4% Ficoll) containing 5–10 μg of nuclear protein extract, 2 μg of poly(dI:dC), and 20,000–30,000 cpm of 32P-labeled DNA probe. Reactions were allowed to proceed for 30 min at room temperature. Complexes were separated on a 6% non-denaturing polyacrylamide gel, and the gels were dried and analyzed by autoradiography. For competition experiments, a 200-fold molar excess of unlabelled Cdx or mutant Cdx oligonucleotide was added before the addition of labeled probe. For Ab blocking experiments, 2 μg of rabbit polyclonal anti-mouse Cdx Ab (CeMines) or 2 μg of rabbit serum (DakoCyton) as control was added, and samples were incubated for 30 min at room temperature before the addition of probe.

Statistical analysis

Statistical analysis was performed using the Student’s unpaired t test with GraphPad InStat software.

Results

Epithelial cells are the major source of CCL25 mRNA in the murine small intestine

CCL25 mRNA was constitutively and selectively expressed in the murine small intestine and thymus (Fig. 1A) consistent with previous reports (9, 12, 14). In situ hybridization studies have demonstrated
that epithelial cells constitutively express CCL25 mRNA (10, 12), and immunohistochemical staining of small intestinal sections with anti-CCL25 Ab showed predominant staining in small intestinal epithelial cells (Fig. 1B) (20). To determine the contribution of epithelial cells to total CCL25 mRNA levels in the murine small intestine, epithelial cells were removed from small intestinal tissue with EDTA, and CCL25 mRNA expression assessed by quantitative real-time RT-PCR. Hematoxylin staining of small intestinal sections confirmed that villous and crypt epithelium were effectively removed by this procedure (Fig. 1C). Small intestinal epithelial cells expressed CCL25 mRNA, whereas CCL25 mRNA was barely detected in intestinal tissue devoid of epithelial cells (Fig. 1D). FACS sorted CD8+ intraepithelial lymphocytes (IEL) failed to express CCL25 mRNA (data not shown), excluding the possibility that any contaminating IEL in epithelial cell preparations are a significant source of CCL25 mRNA. CCL25 mRNA was expressed at high levels by both crypt and villous epithelium (Fig. 1, E and F). Previous immunohistochemical studies have suggested that lamina propria microvascular endothelial cells are a potential additional source of intestinal CCL25 (11, 20). However, CCL25 mRNA was not detected in laser capture microscopy samples taken from the lamina propria of EDTA-treated small intestinal tissue that contained microvascular endothelial cells (as assessed by a positive signal for MadCAM-1 mRNA) (Fig. 1G), indicating that CCL25 detected on these cells may derive from epithelial cells. Indeed, cellular presentation of exogenous-derived chemokines has been previously described in other systems (27–29).

Together, these results demonstrate that small intestinal epithelial cells are the major if not the sole source of the CCL25 mRNA in the murine small intestine.

CCL25 mRNA expression is not enhanced by inducers of homeostatic or inflammatory chemokines

Constitutive expression of homeostatic chemokines in secondary lymphoid organs and the small intestine is regulated by members of the LT/TNF family of cytokines (3–5). To determine whether these factors are required for high CCL25 mRNA in the small intestine, CCL25 mRNA expression was examined in the small intestine of LTα−/−, LTβ−/−, or TNFR1−/− mice. CXCL13 mRNA expression was reduced in the small intestine of LTα−/− and LTβ−/− mice (data not shown), consistent with a previous report (5). In contrast, CCL25 mRNA was expressed at similar levels in the small intestine of LTα−/−, LTβ−/−, and TNFR1−/− mice as in wild-type mice (Fig. 2A). To determine whether intestinal CCL25 mRNA was enhanced after exposure to inflammatory stimuli, mice were injected i.v. with LPS and intestinal CCL25 mRNA levels determined 3 h later. This procedure has been shown to induce expression of multiple chemokine mRNA species in the small intestine, including CCL20, a chemokine constitutively expressed by small intestinal epithelial cells (30, 31). As expected, injection of LPS i.v. enhanced levels of CCL20 mRNA in the murine small intestine (Fig. 2B); however, intestinal CCL25 mRNA levels remained unchanged (Fig. 2B). In a second set of experiments, we determined whether proinflammatory cytokines could enhance CCL25 mRNA expression in the small intestinal epithelial cell lines MODE-K, S1-H10, or mICcl2. TNF-α and IFN-γ were chosen because these cytokines enhance inflammatory chemokine expression in a wide range of intestinal epithelial lines including MODE-K cells (32–35). Addition of TNF-α (10–100 ng/ml) or IFN-γ (10–500 U/ml) alone to confluent MODE-K, S1-H10, or mICcl2 epithelial cell layers for 6 and 24 h failed to alter CCL25 mRNA levels (data not shown). Furthermore, whereas TNF-α and IFN-γ enhanced CXCL1 expression (Fig. 2C) as previously described (33), this cytokine combination failed to enhance CCL25 mRNA expression (Fig. 2C). Similarly these cytokines failed to induce CCL25 mRNA expression in human HT-29 and Caco-2 colonic epithelial lines, and FHs 74 Int cells (CCL-241; American Type Culture Collection), a morphologically epithelial-like cell line derived from the human small intestine (data not shown). Together, these results indicate that transcriptional regulation of CCL25 mRNA is unique compared with that of other homeostatic or inflammatory chemokines.

Small intestinal CCL25 expression is independent of the presence of intestinal microflora or IEL

The expression of CCL25 mRNA in the murine small intestine is increased between 2 and 3 wk of age (17), a time point correlating with increased numbers of IEL (17) and bacterial colonization of the intestine. Because the small intestinal epithelium is in intimate

![FIGURE 2. Intestinal CCL25 mRNA expression is independent of LT and TNFR signaling, inflammatory stimuli, and the presence of intestinal microflora or lymphocytes.](http://www.jimmunol.org/)

**A**. CCL25 mRNA levels in whole proximal small intestine of LTα−/− and LTβ−/− and TNFR1−/− mice as assessed by real-time RT-PCR. Results are the mean (±SEM) of three mice per group. **B**. LPS fails to enhance CCL25 mRNA expression in the murine small intestine. CCL20 and CCL25 mRNA levels were determined by semiquantitative and real-time RT-PCR, respectively, 3 h after administration of 200 μg of LPS (Escherichia coli, serotype O55:B5; Sigma-Aldrich) i.v. into C57BL/6 mice. For real-time RT-PCR results are the mean (±SEM) of three mice. For semiquantitative PCR, cDNA was serially diluted 1/10, and results are representative of three mice per group. **C**. TNF-α and IFN-γ fail to enhance CCL25 mRNA expression in MODE-K cells. MODE-K cells were stimulated with TNF-α (100 ng/ml; PeproTech) and IFN-γ (500 U/ml; PeproTech) for 24 h and CXCL1 and CCL25 mRNA expression determined by semiquantitative and real-time RT-PCR, respectively. For real-time RT-PCR results are the mean (±SEM) of triplicate wells from one representative experiment of three performed. For semiquantitative PCR, cDNA was serially diluted 1/10. Results are from one representative experiment of three performed. **D**, CCL25 mRNA expression in whole small intestine of athymic mice with a truncated common cytokine receptor γ-chain (CRγ−) and germfree mice as determined by real-time RT-PCR. Results are representative from three mice per group (germfree and conventional (convent.)) or from three intestinal pieces from different sites along the small intestine (athymic CRγ− mice).
contact with intestinal microflora and IEL, we determined whether the presence of these components was important in maintaining high constitutive CCL25 expression in the murine small intestine. CCL25 mRNA was expressed at similar levels in the small intestine of athymic mice with a truncated common cytokine receptor γ-chain (CRγ−/−nil/nul) (Fig. 2D), which contains a negligible TCR−/−IEL population (36). Furthermore, coinoculation of confluent MODE-K monolayers for 6 h with 50–500,000 freshly isolated syngeneic DBA/2 IEL failed to enhance epithelial CCL25 expression (data not shown). Finally, CCL25 mRNA was expressed at similar levels in the small intestine of germfree and conventional mice (Fig. 2D). Thus constitutive CCL25 mRNA expression in the murine small intestine is independent on interactions with mature IEL, signaling through the cytokine receptor γ-chain or the presence of intestinal bacteria.

Mapping of murine small intestinal epithelial CCL25 mRNA 5′ and 3′ ends and organization of the murine CCL25 gene

The findings suggesting that CCL25 expression is regulated in a manner different from other chemokines motivated us to search for regulatory elements involved in the transcriptional regulation of this gene. To identify the putative CCL25 transcriptional start site, 5′ RACE was performed on cDNA prepared from small intestinal epithelial cells (Fig. 3A). Seven clones obtained from 5′ RACE were sequenced, three of which showed identity to clone A, three to clone B, and one to clone C (Fig. 3A). These transcripts are a few base pairs longer at the 5′ end as compared with the presumed full-length CCL25 mRNA transcript previously described in mouse thymus (GenBank accession number NM_009138). Analysis of expressed sequence tags obtained in the mouse (GenBank) showed good agreement with these sequences (data not shown). Eight clones obtained from 3′ RACE were sequenced, four of which showed identity to clone 1 and four to clone 2 (Fig. 3A). Thus, murine CCL25 mRNA appears to have two alternative polyadenylation sites. Having identified mRNA ends of the murine CCL25 message, we determined the genomic organization of the murine and human CCL25 gene (Fig. 3B). Both murine and human CCL25 consists of six exons covering 10.5 and 9.9 kb of genomic DNA, respectively. Exon 1 of human CCL25 that was missing from the human CCL25 mRNA sequence (NM_005624) was identified from expressed sequence tags derived from the human small intestine (BX415301) (Fig. 3B).

Identification and characterization of the murine CCL25 promoter

To determine whether the region upstream of the predicted CCL25 transcriptional start site contained promoter activity, we generated a series of constructs comprising regions surrounding exon 1 (Fig. 4A) fused to a luciferase reporter gene. Next we attempted to identify a cell line that constitutively expressed high levels of CCL25 mRNA for use in transfection studies. Screening of a wide range of murine and human epithelial cell lines including MODE-K, mICc12, S1-H10, HT-29, FHs 74 Int, Caco-2, and T-84 cells failed to identify any cell line constitutively expressing CCL25 mRNA levels comparable to that of freshly isolated epithelial cells (Fig. 4B and data not shown). Furthermore freshly isolated epithelial cells showed a dramatic reduction in CCL25 mRNA expression after culture (Fig. 4B). We therefore determined whether any of the constructs displayed basal promoter activity in murine MODE-K and mICc12 cells. The murine fibroblast line BALB/c 3T3 was used as a control cell line because these cells failed to express full-length CCL25 mRNA as assessed by RT-PCR using a 5′ primer in exon 1 and 3′ primer in exon 2 of CCL25 (data not shown). Constructs PF1 to PF3 increased luciferase activity ~20 times compared with the control in MODE-K and mICc12 cells (Fig. 4C), but not in BALB/c 3T3 cells. Thus the area upstream of exon 1 contains the minimal CCL25 promoter, and intron 1 does not appear to contribute to promoter activity in these cell lines. Construct PF4 consistently induced greater luciferase expression compared with PF2, indicating that the region −583 to −311 contained elements that repressed promoter activity in these cell lines. In addition, PF6 failed to induce luciferase expression, demonstrating that a region between −45 and −167 is critical for promoter activity in these cells. Consistent with this finding PF7 covering region −141 to −5 had strong promoter activity, whereas PF8, covering region −99 to −5, and PF9, covering region −49 to −5, showed poor promoter activity (Fig. 4C). Constructs containing region −141 to −99 alone or region −141 to −99 fused to region −49 to −5 showed no promoter activity (data not shown).

Together these results demonstrate that the region upstream of exon 1 of the CCL25 gene contains the minimal murine CCL25 promoter including both activating and repressive elements (Fig. 4D).

The CCL25 promoter contains putative binding sites for Cdx transcription factors

To identify potential transcriptional factors that may contribute to the high CCL25 expression in normal small intestinal epithelial cells, we examined the CCL25 promoter for putative transcription factor binding sites. A consensus TATA box could be identified ~25 bp upstream of the transcriptional start site (Fig. 5). Additional binding sites were predicted for a number of transcription factors.
factors, including Cdx-1 and Cdx-2, Krueppel-like factor, GATA, MAZ, and TFII-I (Fig. 5 and data not shown). Binding sites for IRF-3 and classical NF-κB dimers, such as RelA:p50 implicated in the induction of inflammatory chemokines, or binding sites for nonclassical NF-κB dimers RelB:p52, implicated in driving homeostatic chemokine gene expression (7, 37–45), were not detected within a region covering -2000 to +25 bp of the CCL25 promoter, supporting the notion that these factors are not directly involved in the regulation of CCL25 expression.

Cdx interacts with the predicted binding site in the CCL25 promoter in vitro

The presence of putative Cdx-1 and Cdx-2 binding sites within the murine CCL25 promoter was of interest because Cdx-1 and Cdx-2 expression is restricted to the gut epithelium in adult mice (46), and these transcription factors have been implicated in regulating expression of intestinal specific genes (47–52). Importantly, although freshly isolated small intestinal epithelial cells expressed Cdx-1 and Cdx-2 mRNA (Fig. 6A), MODE-K and mICcl2 cells, and cultured primary small intestinal epithelial cells failed to express Cdx mRNA (Fig. 6). Thus expression of Cdx mRNA correlated with the cells ability to express high levels of CCL25 mRNA.

To determine whether small intestinal Cdx protein could interact with Cdx binding motifs within the CCL25 promoter, nuclear extracts were prepared from primary small intestinal epithelial cells, MODE-K and mICcl2 cells, and incubated with a 32P-labeled probe covering the Cdx-binding site predicted within the TATA box (Fig. 7A). A single complex was observed when labeled Cdx probe was incubated with nuclear extracts from primary small intestinal epithelial cells, and this complex was competed away with unlabeled probe, but not unlabeled mutant probe (Fig. 7A). The complex formation was also inhibited by the addition of anti-Cdx, but not control Ab, to the nuclear extracts before addition of labeled probe (Fig. 7A). In contrast, this complex was not observed using nuclear extracts from MODE-K cells (Fig. 7A), mICcl2 cells, or the thymocyte cell line 2017 (data not shown) (53). Thus, Cdx is present in freshly isolated epithelial cells, but not MODE-K or mICcl2 cells, and epithelial cell derived Cdx can bind to the putative Cdx binding motif within the TATA box of the CCL25 promoter.

Cdx-1 and Cdx-2 enhance activity of the CCL25 promoter in small intestinal epithelial cell lines

To determine whether Cdx could enhance the activity of the CCL25 promoter in epithelial cell lines, the PF1 CCL25 promoter construct or the PF7 CCL25 promoter construct (which contained a single Cdx binding site corresponding to that predicted within the TATA box) were cotransfected with expression plasmids encoding Cdx-1 or Cdx-2 into MODE-K cells. In initial experiments functionality of the Cdx expression plasmids in MODE-K cells was confirmed using a promoter construct containing a TATA box proceeded by three consensus Cdx binding sites or three mutant Cdx sites, as a negative control, upstream of the luciferase gene. Co-transfection with the Cdx expression plasmids leads to a 3-fold
increase in luciferase activity from the reporter construct containing 3 CdxA consensus sites but not from the mutant reporter (data not shown). MODE-K cells transfected with constructs encoding either Cdx-2 or Cdx-1 significantly enhanced CCL25 promoter activity compared with their relevant controls (Fig. 7, B and C). In contrast neither Cdx expression construct enhanced CCL25 promoter activity in BALB/c 3T3 cells (data not shown). Furthermore, stable ectopic expression of Cdx-2, or the combination of Cdx-1 and Cdx-2 in mICc12 cells, leads to an increase in endogenous CCL25 mRNA expression in these cells (Fig. 7D). Together these results suggest a role for Cdx transcription factors in enhancing CCL25 mRNA transcription in small intestinal epithelial cells; however, because transfection with Cdx failed to enhance CCL25 mRNA expression to levels observed in freshly isolated small intestinal epithelial cells, they also suggest that other factors in addition to Cdx are responsible for maintaining the high constitutive expression of CCL25 in these cells.

Discussion

Despite the importance of CCL25 in small intestinal immunity (14–20), the mechanisms underlying the tissue selective expression and regulation of this chemokine in the small intestine are unknown. In the present study we demonstrate that epithelial cells are the major source of CCL25 mRNA in the small intestine, and that constitutive CCL25 mRNA expression is independent of the presence of intestinal bacteria and lymphocytes. CCL25 expression was not regulated by the LTR and TNFR1 signaling pathways and was not enhanced by inflammatory mediators suggesting a unique pattern of regulation compared with homeostatic and inflammatory chemokines, respectively. The CCL25 promoter contained several putative binding sites for Cdx transcription factors and primary small intestinal epithelial cells that expressed high CCL25 mRNA levels expressed Cdx-1 and Cdx-2, whereas epithelial cell lines and cultured primary epithelial cells that expressed low levels of CCL25 mRNA failed to express Cdx-1 and Cdx-2. Finally EMSA and transfection studies suggested a role for Cdx transcription factors in contributing to the high CCL25 expression levels in small intestinal epithelial cells.

Chemokines have been broadly separated into homeostatic and inflammatory chemokines with partially distinct mechanisms of regulation. The LTα/β2 heterotrimer binds to the LTβR (3), signaling through which is required for maintaining constitutive expression of the homeostatic chemokines CXCL12, CCL21, CCL19, and CXCL13 in lymph nodes, spleen, and intestine (4, 5) as well as the epithelial-derived chemokine CCL20 (37). LTβR mediated expression of homeostatic chemokines functions through the alternative NF- κB pathway, involving translocation of RelB:p52 dimers into the nucleus (44). In this study we show that in contrast to homeostatic chemokine promoters (37, 43, 44, 54), putative binding sites for NF- κB and IRF-3, both of which are involved in the induction of inflammatory chemokines (40, 45, 54), were not present in the CCL25 promoter, and intestinal epithelial CCL25 mRNA levels remained unaltered in two inflammatory models known to induce epithelial expression of inflammatory chemokines (30–35). This inability of inflammatory mediators to induce CCL25 mRNA expression, is in apparent odds with the enhanced CCL25 expression reported in small intestinal crypts of Crohn’s disease patients in areas of lymphocytic infiltration; however, no quantitative data were presented in this study (55). Together, our results demonstrate that CCL25 is not regulated as other homeostatic or inflammatory chemokines.
We were unable to identify a cell line that could model freshly isolated small intestinal epithelial cells in their levels of CCL25 mRNA expression. Nevertheless, constructs encompassing the area immediately upstream of exon 1 of the CCL25 gene showed promoter activity in MODE-K and mICc12 cells. Constructs lacking region −311 to −511 showed enhanced promoter activity, indicating that this area contained potential suppressor elements. We were also able to identify important activating elements required for minimal promoter activity in these cells. A region between −45 to −167 appeared critical for minimal promoter activity, and constructs containing region −99 to −141 had significantly higher promoter activity than constructs lacking this sequence. Cdx-1 and Cdx-2 are clearly not involved in driving this activity because MODE-K and mICc12 cells failed to express these transcription factors and the region −99 to −141 contained no Cdx binding sites. However, two binding sites for the TFII family of general transcription factors, which make up part of the initiation complex involved in gene transcription (56), were located within this region and are thus likely to be critical for minimal promoter activity in these cells.

The far higher levels of CCL25 mRNA transcription in primary small intestinal epithelial cells compared with epithelial cell lines suggested that critical components required for driving CCL25 promoter activity were missing or not functional within the cell lines. Furthermore, because cultured primary small intestinal epithelial cells showed reduced CCL25 transcription, the intestinal environment appears important in maintaining the expression and/or activity of these components. Several results from the current study suggest that Cdx transcription factors are one such component. Firstly, analysis of the CCL25 promoter predicted several binding sites for Cdx proteins. Secondly, Cdx was expressed by primary small intestinal epithelial cells but not cultured primary epithelial cells or epithelial cell lines. Thirdly, Cdx present in nuclear extracts from freshly isolated small intestinal epithelial cells, which has previously been shown to interact with TATA boxes of several intestinal specific genes (49–52), bound to the Cdx site predicted in the CCL25 TATA box. Finally, transfection of Cdx expression plasmids caused a significant enhancement CCL25 promoter construct activity in MODE-K and mICc12 cells and enhanced CCL25 mRNA expression in mICc12 cells.

Nevertheless, because transfection of epithelial cell lines with Cdx expression plasmids failed to induce CCL25 mRNA expression to the levels observed in small intestinal epithelial cells, additional factors other than Cdx must be involved in maintaining high CCL25 transcription levels in these cells. Consistent with this, Cdx transcription factors are expressed by small intestinal and colonic epithelium (46), and colonic epithelial cells express ~50 times less CCL25 mRNA than small intestinal epithelial cells (Fig. 1A). The mechanisms driving selective gene expression in the

FIGURE 6. Small intestinal epithelial cell lines and cultured primary epithelial cells express reduced levels of Cdx. Cdx-1 and Cdx-2 expression in small intestinal epithelial cells (SIEC) (A) and in mICc12 and MODE-K cells (B), as determined by RT-PCR. Results are representative of one experiment from two performed for the cultured cells and one from three performed on the cell lines.

FIGURE 7. Cdx proteins bind to the CCL25 promoter and enhance promoter activity in small intestinal epithelial cell lines. A, EMSA is performed using nuclear protein extracts from small intestinal epithelial cells (SIEC) and MODE-K cells as indicated. Labeled probe and nuclear extract only (lanes 1 and 6), with excess of unlabeled probe (lanes 2 and 7), with excess of mutant probe (lanes 3 and 8), anti-Cdx Ab (lanes 4 and 9), and control Ab (lanes 5 and 10). Results are representative from one experiment of three performed. Cotransfection of Cdx-2 (pTREtightCdx-2) (B) or Cdx-1 (C) together with the pGL3b-PF1 or pGL3b-PF7 increased luciferase activity in MODE-K cells. Results are from one representative experiment of three performed. Bars indicate mean ± SEM. **, p < 0.01; ****, p < 0.0001. D, Transfection of Cdx-2, or Cdx-1 and Cdx-2 enhanced the endogenous CCL25 transcript level. Stable transfectants of mICc12 cells expressing Cdx-2, Cdx-1, or both were generated as described in Materials and Methods. The endogenous CCL25 mRNA level was determined by quantitative real-time PCR. Data are mean ± SEM (n = 3–4 wells). *, p < 0.05; **, p < 0.01.
small intestine vs colon are poorly understood. For the small intestinal epithelium, the network of transcription factors, including Cdx, responsible for maintaining the high and tissue selective expression of CCL25 in the murine small intestinal epithelium.

**Disclosures**

The authors have no financial conflict of interest.

**References**


