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Transcriptional regulation of PCFT by KLF4, HNF4α, CDX2 and C/EBPα: Implication in its site-specific expression in the small intestine

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ABSTRACT

Proton-coupled folate transporter (PCFT), which is responsible for the intestinal uptake of folates and analogs, is expressed only in the proximal region in the small intestine. The present study was to examine its transcriptional regulation, which may be involved in such a unique expression profile and potentially in its alteration, using dual-luciferase reporter assays in human embryonic kidney (HEK) 293 cells. The luciferase activity derived from the reporter construct containing the 5′-flanking sequence of −1695/+96 of the human PCFT gene was enhanced most extensively by the introduction of Krüppel-like factor 4 (KLF4). The KLF4-induced luciferase activity was further enhanced by hepatocyte nuclear factor 4α (HNF4α) synergistically. To the contrary, caudal-type homeobox transcription factor 2 (CDX2) and CCAAT/enhancer-binding protein α (C/EBPα) extensively suppressed the luciferase activity induced by KLF4 alone and also that induced by KLF4 and HNF4α. Western blot analysis using the rat small intestine indicated uniform expression of KLF4 along the intestinal tract, proximal-oriented expression of HNF4α, distal-oriented expression of CDX2 and C/EBPα. These results suggest that the activity of PCFT promoter is basically induced by KLF4 and the gradiented expression profile of PCFT may be at least in part accounted for by those of HNF4α, CDX2 and C/EBPα.

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1. Introduction

Proton-coupled folate transporter (PCFT) has been recently identified as the transporter responsible for the intestinal uptake of folates and analogs that include antifolate drugs such as methotrexate [1]. Its characteristics, which feature specific affinity for reduced folates as well as folate and optimum operation driven by H^+ at acidic pH, are in agreement with those of the folate transport system that had long been hypothesized to be present in the small intestine [1–5]. Furthermore, the initial study on PCFT by Qiu et al. indicated that a loss of its function due to a homozygous mutation in its gene is responsible for hereditary folate malabsorption, underscoring its pivotal role in folate absorption [1]. It is also notable that PCFT is expressed abundantly in the upper and middle parts of the small intestine, but not at all in the lower part [3,4], and this unique expression profile is in excellent agreement with that of the activity of folate uptake, as we demonstrated in a study using rats [4]. Elucidating the mechanism regulating the unique expression profile of PCFT in the small intestine should be of interest and help understanding its potential alteration, which could have an impact on the absorption of antifolate drugs as well as folates.

Studies on PCFT have been expanding rapidly since its identification. They include those on its transcriptional regulation, which have identified Yin Yang 1 (YY1), activator protein 1 (AP1), AP2, nuclear respiratory factor 1 (NRF1) and vitamin D receptor (VDR) as transcription factors potentially involved in that [6–8]. However, all those studies are for transcriptional regulation in the cell lines of HeLa and Caco-2, and those identified transcription factors, which are present ubiquitously in various organs, are unlikely to be able to account for the specifically high expression of PCFT in the small intestine and its unique expression profile in that organ. We, therefore, attempted in the present study to identify transcription factors that could account for such a specific and unique expression profile of PCFT, assuming the involvement of those which are more specifically or abundantly expressed in the small intestine, and to elucidate the transcriptional regulation mechanism. We also examined the status of DNA methylation, because it has been reported to be an important epigenetic factor that determines the expression of PCFT in various cell lines [9,10].

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibody for Krüppel-like factor 4 (KLF4), rabbit polyclonal antibody for CCAAT/enhancer-binding protein α (C/

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EBPα) and rabbit polyclonal antibody for caudal-type homeobox transcription factor 2 (CDX2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal antibody for hepatocyte nuclear factor 4α (HNF4α) was from Abnova (Taipei, ROC), mouse monoclonal anti-β-actin was from Sigma–Aldrich (St. Louis, MO, USA) and goat anti-rabbit IgG (Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All other reagents were of analytical grade and commercially obtained.

2.2. Animals

Male Wistar rats, weighing about 300 g, were used without fasting in experiments to assess the expression of transcription factors and the status of DNA methylation in the small intestine. All the experiments were conducted with the approval of the Animal Ethics Committee of Nagoya City University Graduate School of Pharmaceutical Sciences.

2.3. Cell cultures

Human embryonic kidney (HEK) 293 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.4. Cloning of the 5′-flanking region of the PCFT gene and preparation of deletion reporter constructs

The region spanning positions −1695 to +96 (relative to the transcription start site), which encompasses the 5′-flanking region of the human PCFT gene was cloned from the genomic DNA of HEK293 cells and incorporated into pGL4.10(luc2) vector containing the firefly luciferase reporter gene (Promega, Madison, WI, USA), as detailed in Supplementary data. Deletion constructs (−1036/+96, −526/+96, −243/+96, −185/+96, −94/+96, −24/+96) were prepared from −1695/+96 construct, as also detailed in Supplementary data.

2.5. Cloning of transcription factors

HNF1α, HNF4α, HNF4γ, GATA binding protein 4 (GATA4), CDX2, C/EBPα, KLF4, KLF5 and KLF13 were cloned, as detailed in Supplementary data.

2.6. Luciferase assay

HEK293 cells were seeded on 96-well plates coated with poly-L-lysine (4.0×10^4 cells/100 μl/well), transfected with one of the reporter plasmids and one or a few plasmids carrying the cDNA of a transcription factor by lipofection using HilyMax (Dojindo Laboratories, Kumamoto, Japan) as a transfection reagent, according to the manufacturer's instructions, and cultured for 48 h for transient expression before luciferase assays. The cells were transfected with 200 ng/well of total plasmids of a reporter construct and transcription factors at a constant ratio in each set of experiments. The total amount of plasmids was kept constant by the addition of an appropriate amount of the empty pCI-neo vector when fewer transcription factors were introduced into the cells. The cells were also cotransfected with 20 ng/well of pGL4.74 (hRluc/TK) vector (Promega) for the expression of *Renilla* luciferase as an internal control of transfection efficiency.

Firefly and *Renilla* luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's protocol, and an ARVO MX plate reader (PerkinElmer Life Sciences, Boston, MA, USA) for the detection of luminescence. The firefly luciferase activity was normalized to the *Renilla* luciferase activity.

Because HEK293 cells have little PCFT expressed [2], we assumed that they are transcriptionally inactive for PCFT in practice and can be used for the investigation of its transcriptional regulation, using exogenously introduced PCFT promoter and transcription factors. To assess the effect of transcription factors on the promoter activity, the firefly luciferase activity, which was normalized to the *Renilla* luciferase activity, was evaluated in terms of the relative one further normalized to the basal activity in the absence of exogenously introduced transcription factors.

2.7. Western blot analysis

The 5 or 10 cm sections of duodenum, jejunum, midgut and ileum were isolated from male Wistar rats and the expression profiles of KLF4, HNF4α, CDX2, and C/EBPα proteins were examined by Western blotting, as detailed in Supplementary data.

2.8. ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed to examine the binding of KLF4 to the region between −209 and −18, which was suggested to include a promoter element for KLF4 binding, of the human PCFT promoter, as detailed in Supplementary data.

2.9. DNA methylation analysis

Genomic DNA (10 μg) was extracted from the jejunum and ileum of the male Wistar rats and DNA methylation was assessed by a bisulfite DNA sequencing method, as detailed in Supplementary data.

2.10. Data analysis

Experimental data are presented as the means ± SE, and statistical analysis was performed using two-tailed, unpaired Student's *t*-test or, when multiple comparisons were needed, analysis of variance (ANOVA) followed by Dunnett's test, with $p < 0.05$ considered significant.

3. Results

3.1. Activation of PCFT promoter by KLF4

As the first step to identify transcription factors involved in the transcriptional regulation of the expression of human PCFT in the small intestine, luciferase reporter assays were performed with the reporter construct containing −1695/+96 segment of the 5′-flanking region of the human PCFT gene in HEK293 cells, assessing changes in luciferase activity induced by cotransfection of plasmids for the expression of several transcription factors, which are known to be specifically present in the small intestine. Those tested were HNF1α, HNF4α, HNF4γ, GATA4, CDX2, C/EBPα, KLF4, KLF5 and KLF13. As shown in Fig. 1A, luciferase activity was enhanced extensively by a factor of about 16 by KLF4, compared with the basal activity in the absence of any exogenously introduced transcription factor (no factor). HNF4α also enhanced the luciferase activity, though only modestly by a factor of about 2. All the other transcription factors were, however, found not to alter the luciferase activity. These results suggest that KLF4 could be the principal activator of the PCFT promoter.

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