Lactase-phlorizin hydrolase (LPH) participates in lactose digestion, which provides glucose and galactose for active transport in small intestinal absorptive cells. In particular, LPH is important for mammalian development during the suckling period, because human infants and animal pups consume large amounts of lactose from milk. Indeed, previous studies demonstrated that expression of the LPH gene is enhanced during the suckling period and its expression rapidly declines after weaning in rats (1, 2). In addition, lactose intolerance in human infants leads to developmental disorders (3). Nevertheless, regulation of the LPH gene expression during suckling and weaning periods as well as in adulthood is poorly understood.

Our recent study demonstrated that injection of triiodothyronine (T3) induced jejunal LPH gene expression during the suckling period, but its expression was reduced in the weaning period in rats (2). In addition, it was reported that serum concentrations of T3, tetraiodothyronine (thyroxine, T4), and glucocorticoid hormone were elevated during the suckling-weaning period in rodents (4). Previous studies demonstrated that both thyroid hormone and glucocorticoid hormone induced the expression of a matured intestinal gene, glucose transporter 5 (GLUT5), and that mRNA levels of GLUT5 were increased by T3 in rats during weaning (5) and in Caco-2 cells (6). In addition, other reports demonstrated that the mRNA levels of GLUT5 in Caco-2 cells were increased by dexamethasone (Dex), a glucocorticoid receptor (GR) agonist, through the GR pathway (7, 8). These results suggest that thyroid and glucocorticoid hormones synergistically enhance expression of the LPH gene in CDX-2/HNF-1α co-transfected IEC-6 cells.

Key Words lactase-phlorizin hydrolase, thyroid hormone, glucocorticoid hormone, CDX-2, HNF-1α

Thyroid and glucocorticoid hormones and several transcriptional factors such as caudal type homeobox (CDX)-2 and hepatocyte nuclear factor (HNF)-1α are important for the differentiation of small intestinal absorptive cells and the consequent expression of genes related to the digestion/absorption of carbohydrates. In this study, we investigated whether thyroid and glucocorticoid hormones enhanced the expression of lactase-phlorizin hydrolase (LPH) gene, an intestine-specific gene that encodes an enzyme for lactose digestion, in small intestinal stem-like IEC-6 cells co-transfected with CDX-2 and HNF-1α using a retrovirus system. Changes in expression of intestine-specific genes caused by treatment with thyroid and/or glucocorticoid hormones were monitored in empty vector-transfected cells and in CDX-2/HNF-1α co-transfected cells by qRT-PCR. Stable co-transfection with CDX-2 and HNF-1α evoked the expression of the LPH gene in IEC-6 cells. Furthermore, treatment with a thyroid hormone, triiodothyronine, and a glucocorticoid receptor agonist, dexamethasone, significantly enhanced expression of the LPH, CDX-2 and HNF-1α genes in CDX-2/HNF-1α co-transfected IEC-6 cells. These results suggest that thyroid and glucocorticoid hormones synergistically enhance expression of the LPH gene in CDX-2/HNF-1α co-transfected IEC-6 cells.
changes of genes related to nutritional digestion and absorption, using human colon cancer cell lines such as Caco-2 and HT-29 because these cell lines express several intestinal-specific genes including LPH, during cell differentiation (9, 10). However, the expression of intestinal genes in these cells is considerably low, and these cells respond inconsistently to nutrients and hormones. Therefore, we considered it important to establish an intestinal absorptive cell line, which expresses the LPH gene and retains responsiveness to hormones and nutrients. In this study, we used intestinal epithelial cell (IEC)-6 cells, a known un-differentiation model of cells of the small intestine. IEC-6 cells were derived from neonatal rat ileum, and have the characteristics of crypt-type intestinal cells, which do not exhibit differentiated morphology or gene expression (11). In addition, IEC-6 cells do not have differentiation phenotypes including disaccharidases such as LPH, and do not express transcriptional factors, such as caudal type homeobox (CDX)-2 and hepatocyte nuclear factor (HNF)-1 α, that are required for intestinal epithelial cell differentiation. To explore the underlying mechanism of differentiation phenotype gain, we used cells similar to un-differentiated intestinal cells compared with cells that express differentiation phenotypes constitutively. Therefore, we might reveal underlying mechanisms by which neonates gain matured intestinal phenotypes during suckling periods via transcriptional regulation and increased blood hormone levels such as thyroid and glucocorticoid hormones.

In this study, we co-transfected intestinal stem-like IEC-6 cells with expression vectors for CDX-2 and HNF-1α, two major transcriptional factors that play a pivotal role in intestinal differentiation and maturation by regulating the transcription of intestine-specific genes (12, 13). We then investigated whether co-expression of CDX-2 and HNF-1α in IEC-6 cells induced expression of the LPH gene. Furthermore, we explored whether thyroid and glucocorticoid hormones enhanced expression of the LPH gene in CDX-2/HNF-1α co-transfected IEC-6 cells.

**MATERIALS AND METHODS**

**Cell cultures and hormone treatments.** IEC-6 cells from the American Type Culture Collection (Rockville, MD) were seeded at a density of 1 × 10^7 cells/well in six-well plates (Iwaki, Tokyo, Japan) in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen, Carlsbad, CA), 20 mM HEPES (pH 7.4), 1×antibiotic–antimycotic mixed stock solution (Nakalai Tesque, Inc., Kyoto, Japan), and 2 mM l-glutamate (Invitrogen), at 37˚C in a humidified atmosphere of 5% CO₂. FBS was delipidated by treatment under charcoal for hormonal treatment. After re-seeding, the transfected cells were pre-cultured until they reached 100% confluence for 2 d, and were treated with 3,3′,5-triodo-l-thyronine sodium salt (T3) (Nakalai Tesque) and/or Dex (Sigma-Aldrich, St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO). Each hormone was treated at a final concentration of 1 µM for 1, 2 or 3 d in culture medium without antibiotics for selection.

**Retroviral constructs and infection.** The coding sequences for CDX-2 (NM_023963, from 110 until 1042) and HNF-1α (NM_012669, from 151 until 2037) were sub-cloned into the retroviral vector pMSCV-puro (Takarabio, Shiga, Japan) and pMSCV-neo (Takarabio), respectively. Each coding sequence was cut out from cDNA derived from rat total RNA, by restriction enzyme BamH I (Takarabio), and ligated into the restriction enzyme BgII site of the retroviral vector. HEK-293 cells, as host cells maintained in DMEM medium, were transfected by Polyfect (Qiagen, Tokyo, Japan) with the retroviral vectors and helper amphotropic DNA according to the manufacturer’s protocol. IEC-6 cells at 60% confluence were infected for 24 h by incubation with retrovirus supernatants containing empty vectors or the expression vectors with coding sequences of CDX-2 and HNF-1α in the presence of 4 µg/mL polybrene (Sigma-Aldrich). One day after infection, transfected IEC-6 cells were divided and selected in medium containing 5 µg/ml puromycin (Sigma-Aldrich) and 1.6 mg/mL neomycin (Nacalai Tesque) for 4 d. After selection by antibiotics, transfected cells were re-seeded at a density of 1 × 10^7 cells/well in new six-well plates. The cells were treated with hormones in new culture medium that excluded puromycin and neomycin.

**Quantitative RT-PCR analysis.** Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi (14), from transfected IEC-6 cells. Total RNA was subjected to reverse transcription using Superscript III reverse transcriptase (Invitrogen). To quantitatively estimate mRNA levels of the selected genes, PCR amplification was performed on a LightCycler® 480 instrument (Roche Diagnostics Japan, Tokyo, Japan), as previously described (15). The PCR primer sequences are listed in Table 1. The cycle threshold (CT) values of each gene detected by quantitative RT-PCR were converted into signal intensities by the delta–delta method (16), which calculates the difference of one CT value as twice the difference between the signal for each gene and the signal for a gene for normalization. The formula used was 2^[ΔCT of β actin–ΔCT of target gene].

**Immunoblotting.** To prepare crude nuclear extracts, pellets of IEC-6 cells scraped into PBS were homogenized by 10 strokes in buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, protease inhibitor tablet (Roche Molecular Biochemicals)/10 mL of buffer A). The cell homogenates were centrifuged in microcentrifuge tubes at 800 × g for 15 min at 4˚C. The precipitates were resuspended in buffer A, centrifuged, and collected. This step was performed twice to remove contaminating cytosolic proteins. The degree of cell lysis was monitored by light microscopy. Nuclear pellets were resuspended in RIPA (radio-immunoprecipitation assay) buffer [50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1.0% NP-40 substitute] containing a protease inhibitor tablet/10 mL of RIPA buffer, and incubated with rotation for 2 h at
4˚C. The nuclear debris was precipitated by centrifugation for 30 min at 8,900 × g, and the supernatants were collected as the nuclear fraction. The concentration of the nuclear fraction proteins were normalized using the Lowry method (17) and stored at 220˚C. Cell extracts (40 μg protein) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon paper (Millipore, Billerica, MA) at 80 V for 90 min in Tris/glycine/methanol transfer buffer. The membranes were blocked in 3% skimmed milk in PBS with 0.05% Tween 20, pH 7.4 (PBS-Tween) for 30 min at room temperature. Membranes were then incubated in 3% skimmed milk/PBS/Tween with the primary antibody at 4˚C for more than 7 h, using rabbit anti-CDX-2 (P A5-20891, Thermo Fisher Scientific Inc., Chicago, IL), rabbit anti-HNF-1α (sc-8986, Santa Cruz Biotechnology, Dallas, TX), or rabbit anti-general transcription factor IIB (TFIIB) (sc-225, Santa Cruz Biotechnology) antibodies. After washing in PBS/Tween, the membranes were incubated with biotin-conjugated anti-rabbit IgG (GE Healthcare, Tokyo, Japan) in 3% skimmed milk/PBS/Tween. After washing in PBS/Tween and incubation with horseradish peroxidase-conjugated streptavidin (Cell Signaling Technology, Danvers, MA), the signals were detected by chemiluminescence (ECL Plus; GE Healthcare) and scanned by a luminal image analyzer (Las-3000, Fujiﬁlm, Tokyo, Japan), according to the manufacturer’s instructions.

Statistical analysis. Data are presented as means ± SE. The Tukey-Kramer multiple range test based on one-way ANOVA was used to determine the significance of differences in relative mRNA abundance (STATVIEW, Abacus Concepts, Baltimore, MD). A p-value less than 0.05 was considered significant.

RESULTS

mRNA and protein levels of CDX-2 and HNF-1α in transfected IEC-6 cells

To explore mRNA and protein levels of CDX-2 and HNF-1α in empty vector-transfected and CDX-2/HNF-1α co-transfected IEC-6 cells, we performed quantitative real-time RT-PCR (qRT-PCR) and immunoblotting. Both CDX-2 and HNF-1α mRNA (Fig. 1A) and protein (Fig. 1B) were present in co-transfected cells, but not in empty vector (pMSCV-puro and pMSCV-neo) transfected cells.

Effects of T3 and Dex on LPH gene expression in CDX-2/HNF-1α co-transfected IEC-6 cells

We investigated whether gene expression of LPH and sucrase-isomaltase (SI), another enzyme responsible for starch and sucrose digestion, was induced by co-transfection with CDX-2 and HNF-1α. Furthermore, we analyzed whether expression of these genes in CDX-2/HNF-1α co-transfected IEC-6 cells was enhanced by thyroid and glucocorticoid hormones. Whereas IEC-6 cells transfected with empty vector did not express LPH mRNA even at 3 d after treatment with T3 and/or Dex, the CDX-2/HNF-1α co-transfected IEC-6 cells progres-

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sively expressed LPH mRNA over time after treatment with T3, Dex, or combined T3 and Dex. LPH mRNA levels in CDX-2/HNF-1α co-transfected IEC-6 cells were enhanced by treatment with T3, Dex or T3+Dex at 3 d after treatment (3.5-, 4.8- and 7.6-fold, respectively) compared with CDX-2/HNF-1α co-transfected IEC-6 cells treated with DMSO. Combined treatment of CDX-2/HNF-1α co-transfected IEC-6 cells with T3 and Dex for 3 d led to significantly greater LPH mRNA levels than treatment with T3 only (Fig. 2A). By contrast, SI gene expression was not found in either cell line, and was not induced by treatment with thyroid and/or glucocorticoid hormones (Fig. 2B).

**Effects of T3 and Dex on the expression of transcription factors in CDX-2/HNF-1α co-transfected IEC-6 cells**

We investigated whether expression of transcription factors, such as CDX-2, HNF-1α, GATA binding protein (GATA)-4, GATA-5, pancreatic and duodenal homeobox (PDX)-1, thyroid hormone receptor alpha (TRα)-1 and glucocorticoid hormone receptor (GR), which have vital roles in differentiation in the small intestine and the expression of absorptive-specific genes, was affected by treatment with T3 and/or Dex by quantitative RT-PCR. In empty vector-transfected IEC-6 cells, CDX-2 mRNA and HNF-1α mRNA were not detected even at 3 d after treatment with T3 and/or Dex. In CDX-2/HNF-1α co-transfected IEC-6 cells, mRNAs of CDX-2 and HNF-1α were detected at confluence before treatment with T3 or Dex, and they were significantly increased within 2 d after treatment with T3 or after treatment with Dex (Fig. 3A and B). Combined treatment of CDX-2/HNF-1α co-transfected IEC-6 cells with T3 and Dex for 1–3 d led to significantly greater CDX-2 and HNF-1α mRNA levels than individual treatment with either T3 or Dex (Fig. 3A and B). Other transcriptional factors such as GATA-4, GATA-5, PDX-1, TRα-1 and GR were expressed in both empty vector-transfected cells and in CDX-2/HNF-1α co-transfected IEC-6 cells. Expression of these transcription factors was unchanged by treatment with T3 and/or Dex in CDX-2/HNF-1α co-transfected cells (Fig. 3C–G).

**DISCUSSION**

This study demonstrated that changes in CDX-2 and HNF-1α expression in CDX-2/HNF-1α co-transfected IEC-6 cells was closely associated with induction of the
Induction of LPH Gene by CDX-2/HNF-1α

Induction of LPH Gene by CDX-2/HNF-1α

Previous studies demonstrated that CDX-2 and HNF-1α enhanced the promoter/enhancer activity region of the LPH gene (18, 19). Thus, CDX-2 and HNF-1α might regulate induction of the LPH gene in addition to intestinal absorptive cell differentiation. In this study, we employed IEC-6 cells, a stem-like cell line extracted from rat small intestine with crypt-type intestinal cell characteristics (11), and attempted to establish an intestinal cell line stably co-transfected with both CDX-2 and HNF-1α using a retrovirus system. Using the CDX-2/HNF-1α co-transfected IEC-6 cells, we examined whether LPH gene expression was induced by CDX-2 and/or HNF-1α at physiological expression levels.

We confirmed that CDX-2 and HNF-1α were not expressed in IEC-6 cells, consistent with a previous study (20). We demonstrated that CDX-2/HNF-1α co-transfected IEC-6 cells expressed LPH gene transcripts at 2–3 d after confluence. In our preliminary study, LPH mRNA was not detected in CDX-2 transfected IEC-6 cells or in HNF-1α transfected IEC-6 cells (data not shown). This study is the first to demonstrate the LPH gene is induced by co-expression of CDX-2 and HNF-1α in undifferentiated stem-like cells. Previous studies demonstrated that many genes related to digestion and absorption of nutrients, including LPH and SI, were induced in Caco-2 cells (9). Thus, it is likely that the CDX-2/HNF-1α co-transfected IEC-6 cells established in

LPH gene by T3 and/or Dex in IEC-6 cells.

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Fig. 3. Effects of T3 and Dex on gene expression of transcriptional factors in CDX-2/HNF-1α co-transfected IEC-6 cells. After antibiotics selection, transfected cells were seeded at a density of 1×10^7 cells/well, and were pre-cultured till 100% confluent for 2 d. Each hormone (T3 and/or Dex) was added at a final concentration of 1 μM for 1, 2 or 3 d. qRT-PCR analysis for mRNA expression of CDX-2 (A), HNF-1α (B), GATA-4 (C), GATA-5 (D), PDX-1 (E), TRα-1 (F) and GR (G). Relative mRNA levels of each gene normalized to the relative β-actin mRNA abundance are shown. Means±SE for three experiments are shown. Values not sharing a common superscript letter at each time point are significantly different by the Tukey-Kramer multiple range test at p<0.05.
this study have similar characteristics to Caco-2 cells in terms of expression of intestine-specific genes.

In addition, LPH gene expression was induced by both T3 and Dex in an additive manner in CDX-2/HNF-1α co-transfected IEC-6 cells, supporting the idea that thyroid and glucocorticoid hormones are important factors in LPH gene expression in intestinal cells. Indeed, previous studies showed that injection of T3 in suckling rats induced jejunal LPH gene expression (2), and that glucocorticoid receptor-null mice exhibited significantly reduced lactase activity (21). The mechanism underlying induction of LPH gene expression by thyroid and glucocorticoid hormones is unclear. Here we demonstrated that transcriptional factors CDX-2 and HNF-1α were induced by T3 and Dex in an additive manner as shown for LPH gene expression, consistent with a previous study, where CDX-2 gene expression and lactase activity in the small intestine were reduced in TRα-deficient mice (22). Thus, it is likely that induction of LPH gene expression by thyroid and/or glucocorticoid hormones could be caused by the endogenous induction of CDX-2 and HNF-1α by these hormones. Our previous study demonstrated that thyroid hormone induced expression of the LPH gene during suckling, while reducing expression of the LPH gene during weaning in rats (2). Thus, induction of LPH by thyroid and glucocorticoid hormones might be related to consumption of lactose from milk. The results of this study suggest that the CDX-2/HNF-1α co-transfected IEC-6 cells are useful for studies of hormonal and nutritional regulation of the LPH gene. However, it is unclear whether endogenous Cdx-2 and HNF-1α expression caused by hormonal treatment is directly regulated by TR and/or GR in CDX-2/HNF-1α co-transfected IEC-6 cells.

Further studies should determine whether CDX-2 and HNF-1α expression is directly regulated by TR or GR using promoter assays and Chromatin immunoprecipitation assays to detect binding levels of TR or GR to promoter regions of CDX-2 and HNF-1α genes, respectively. In addition, whether thyroid and glucocorticoid hormones enhance LPH gene expression in human intestinal stem-like cells should be examined.

It should be noted that an earlier study showed that a long-term culture (>50 d) of CDX-2-overtransfected IEC-6 cells induced SI gene expression (20). In this study, the SI gene was not expressed in CDX-2/HNF-1α co-transfected IEC-6 cells over a short period of time, i.e., 3 d after confluence. It is possible that the CDX-2/HNF-1α co-transfected IEC-6 cells are a model of ontogenically immature absorptive cells representing those during the suckling period in rats, where the SI gene is not expressed. In addition, confluence stimulation is important for intestinal cell differentiation. Caco-2 cells differentiate approximately 20 d after confluence (23), and develop polarization and differentiation functions such as digestion and absorption of nutrients, followed by cell cycle and cell growth arrest, known as contact inhibition. Interestingly, cell proliferation declined in CDX-2/HNF-1α co-transfected IEC-6 cells in comparison to CDX-2 or HNF-1α singly-transfected IEC-6 cells as detected by cytometric analysis and colony formation assay (unpublished data). These results suggest that expression of both transcriptional factors induces the differentiation of IEC-6 cells through a decrease in cell proliferation similar to differentiated Caco-2 cells by confluence stimulation. Further studies are required to investigate the mechanism of reduced cell proliferation, and to reveal the induction mechanism of intestinal differentiation phenotypes such as SI accompanied with confluence stimulation in CDX-2/HNF-1α co-transfected IEC-6 cells.

In conclusion, we demonstrated that expression of two transcriptional factors (CDX-2 and HNF-1α) at physiological expression levels in undifferentiated intestinal stem-like IEC-6 cells induced LPH gene expression, which is further enhanced by thyroid and glucocorticoid hormones. These findings support the idea that undifferentiated small intestinal absorptive cells gain tissue-specific functions for the absorption of nutrients through the expression of a set of essential transcriptional factors.

Acknowledgments

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