Transcriptional regulation of the human NaPi-IIb cotransporter by EGF in Caco-2 cells involves c-myb

Hua Xu, Michael Inouye, Eric R. Hines, James F. Collins, and Fayeiz K. Ghishan

Departments of Pediatrics and Physiology, Steele Memorial Children’s Research Center, University of Arizona Health Sciences Center, Tucson, Arizona 85724

Submitted 30 September 2002; accepted in final form 12 January 2003

Xu, Hua, Michael Inouye, Eric R. Hines, James F. Collins, and Fayeiz K. Ghishan. Transcriptional regulation of the human NaPi-IIb cotransporter by EGF in Caco-2 cells involves c-myb. Am J Physiol Cell Physiol 284: C1262–C1271, 2003. First published January 15, 2003; 10.1152/ajpcell.00456.2002.—The type IIb sodium-phosphate (NaPi-IIb) cotransporter mediates intestinal phosphate absorption. Previous work in our laboratory has shown that EGF inhibited NaPi-IIb cotransporter expression through transcriptional regulation. To understand this regulation, progressively shorter human NaPi-IIb promoter constructs were used to define the EGF response region, and gel mobility shift assays (GMSAs) were used to characterize DNA-protein interactions. Promoter analysis determined that the EGF response region was located between −784 and −729 base pair (bp) of the promoter. GMSAs and overexpression studies revealed an interaction between this promoter region and c-myb transcription factor. Inhibition of EGF receptor activation restored promoter function. Further studies suggested that MAPK, PKC, and/or PKA pathways are involved in this regulation. In conclusion, these studies suggest that EGF decreases human NaPi-IIb gene expression by modifying the c-myb protein such that it inhibits transcriptional activation. We further conclude that this downregulation of promoter function is mediated by EGF-activated PKC/PKA and MAPK pathways. This is the first study that demonstrates involvement of c-myb in the regulation of intestinal nutrient absorption.

type IIb sodium-phosphate cotransporter; epidermal growth factor

PHOSPHATE (Pᵢ) plays a major role in growth, development, bone formation, and cellular metabolism. Pᵢ (re)absorption occurs in the small intestine and the kidney, mainly through sodium-dependent pathways. Type II sodium-dependent Pᵢ (NaPi-II) cotransporters are the major proteins involved in these processes. The type IIb sodium-dependent Pᵢ (NaPi-IIb) cotransporter is responsible for intestinal Pᵢ absorption, whereas the type IIa sodium-dependent Pᵢ (NaPi-IIa) cotransporter is critical for renal Pᵢ reabsorption. Many physiological factors regulate Pᵢ homeostasis via modulating Pᵢ absorption in the kidney and intestine. Specifically in the small intestine, low-Pᵢ diet, as well as 1,25(OH)₂ vitamin D₃, stimulate Pᵢ absorption by elevating NaPi-IIb cotransporter activity (18, 44), whereas glucocorticoids reduce Pᵢ absorption by decreasing NaPi-IIb cotransporter function (4, 10, 39).

Epidermal growth factor (EGF), a growth hormone, also regulates renal and intestinal Pᵢ absorption. EGF has been shown to inhibit renal Pᵢ reabsorption by modulating NaPi-IIa mRNA expression (2, 3). Our previous study also showed that EGF reduced NaPi-IIb mRNA synthesis in rat intestine and in human intestinal epithelial (Caco-2) cells by a transcriptional mechanism (46). Furthermore, a putative EGF response element(s) was shown to be in the region of −1,103 to −380 base pairs (bp) of the human NaPi-IIb (hNaPi-IIb) gene promoter.

EGF response elements have been identified as a serum-response element and AP1 binding sequences from the c-fos gene (15) and as Sp1 binding sequences from the rat preprothyrotropin-releasing hormone (33) and the human gastrin genes (16, 17, 25). In the hNaPi-IIb gene promoter region (−1,103 to −380 bp), two sequences at position −792 to −786 bp (GG-GAAGG) and −479 to −474 bp (GGGGCGC) were found to have high homology with the EGF response elements identified as Sp1 binding sequences from the rat preprothyrotropin-releasing hormone gene (33). However, it is unclear whether these sequences are involved in EGF regulation of hNaPi-IIb cotransporter gene expression.

To determine whether these DNA sequences are involved in EGF regulation of hNaPi-IIb gene expression, we made a series of promoter constructs that contain different lengths of the 5’-flanking region of the hNaPi-IIb gene upstream of a luciferase reporter gene and transfected these plasmids into Caco-2 cells to test the promoter response to EGF. DNA gel mobility shift assay (GMSA) was then used to detect interactions between DNA and nuclear proteins. From these results, we have shown for the first time that EGF regulation of hNaPi-IIb gene expression involves modulation of c-myb binding affinity as mediated by EGF activated PKC/PKA and MAPK pathways.

MATERIALS AND METHODS

Cell culture. Human intestinal epithelial (Caco-2) cells were purchased from American Type Culture Collection...
(ATCC) and cultured according to ATCC guidelines. Cells were cultured at 37°C in a 95% air-5% CO₂ atmosphere and passaged every 72 h. In EGF treatment experiments, cells were incubated with 50 or 100 ng/ml EGF for 8 h before being harvested. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA). Cells between passages 40 and 46 were used in this study.

Assembly of reporter gene constructs. A series of progressively shorter hNaP₅-Iib promoter constructs in the pGL3/basic luciferase reporter vector (Promega, Madison, MI) were made by restriction enzyme digestion or PCR (46). Briefly, pGL3/–1,103 bp construct was made by subcloning a SacI–XmaI fragment of the hNaP₅-Iib promoter into the pGL3/Basic vector. For other deletion constructs, pGL3/–784 bp, pGL3/–729 bp, pGL3/–646 bp, pGL3/–624 bp, pGL3/–563 bp, and pGL3/–380 bp, different lengths of hNaP₅-Iib promoter were PCR amplified, utilizing the same reverse primer and different forward primers containing sequences for SacI. These PCR products were then ligated into SacI–XmaI digested pGL3/Basic plasmid and sequenced. All deletion constructs end on the 5’ end at +15 bp of the hNaP₅-Iib gene.

The mutation of the c-myb binding sequence was introduced by PCR-based site-directed mutagenesis (7). In this strategy, the desired promoter region was PCR amplified with primers containing mutated base pairs. The wild-type sequence in the promoter was then replaced by the PCR fragment containing the mutated bases using restriction enzyme digestion. All constructs were confirmed by sequencing on both strands.

Transient transfection, EGF treatment, and luciferase assays. Caco-2 cells were seeded in 24-well plates and maintained in a defined medium. When cells were 70% confluent, lipofectamine (Invitrogen, Carlsbad, CA)-mediated transfection was performed as previously described (46). Cells were treated with EGF (50 or 100 ng/ml; Australl Biological, San Ramon, CA) or vehicle for 8 h before harvest. Promoter reporter gene assays were performed using a dual luciferase assay kit according to the manufacturer’s instructions (Promega). For EGF-signaling pathway studies, various inhibitors or vehicles were added to the transfected cells 2 h before EGF was added. A mouse monoclonal antibody against the human EGF receptor (Ab-3) was purchased from Oncogene Research Products (Boston, MA). T-25 flasks were AG-1478 (called AG-1478 throughout) and PD-030594 were purchased from Sigma (St. Louis, MO). H7 was purchased from Calbiochem (San Diego, CA).

Preparation of nuclear extracts for GMSA. Nuclear extracts were prepared by a previously described method (38) from Caco-2 cells treated with EGF (100 ng/ml) or vehicle for 8 h. Synthetic double-stranded oligonucleotides were designed to cover the promoter region –799 to –727 bp. DNA oligonucleotides were end-labeled with [γ-32P]ATP, and 4 μg of nuclear extract were incubated with 1 ng of labeled probe in GMSA binding buffer containing 10 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, and 50 μg/ml poly(d(I-C)). After incubation at room temperature for 20–30 min, the mixture was electrophoresed on a 6% polyacrylamide gel in 0.25M Tris-boric acid-EDTA buffer. Gels were dried and exposed to X-ray film. For competition experiments, a 100-fold molar excess of unlabeled probe was added to the reaction mixture before the labeled probe was added. For supershift assays, 4 μg of a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 500–640 from the COOH terminus of human c-myb or nonspecific rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixtures.

Cellular protein preparation. Cells were rinsed twice with PBS buffer and incubated in cell lysis buffer containing 0.45 × PBS (pH 7.4), 0.5% Na-deoxycholate, 1% NP-40, 0.1% SDS, 2 mM PMSF, and protease inhibitor cocktail (Boehinger Mannheim, Indianapolis, IN) for 30 min at 4°C. Cells were scraped and passed through a 21-gauge needle several times. The cell lysates were then spun down at 10,000 g for 10 min at 4°C. The supernatant, containing cellular protein, was stored at –70°C.

Western blotting. Nuclear protein (20 μg) or cellular protein (40 μg) was subjected to SDS-PAGE (7.5%). The proteins were electrophoresed to a membrane and reacted with an affinity-purified rabbit c-myb polyclonal antibody raised against an immunogenic peptide corresponding to amino acid residues 2–16 of the human c-myb protein (Active Motif, Carlsbad, CA). Immunodetection was performed using the BM chemiluminescence Western blotting kit (Roche Molecular Biochemicals, Mannheim, Germany). Nuclear proteins from the human chronic myelogenous leukemia (K562) cells (Active Motif) were used as a positive control for c-myb immunoblotting.

mRNA purification and PCR amplification. mRNA was purified from Caco-2 cells using the Micro FastTrack mRNA purification kit (Invitrogen). A reverse transcription reaction was performed in the presence of oligo(dT)¹₅ primers and AMV reverse transcriptase. The primers used to amplify NaP₅-Iib and β-actin gene products were the same as described previously (46). The primers used to detect the c-myb gene product were designed to cover the human c-myb cDNA (accession no. NM005375) region from 439 to 701 bp. Semi-quantitative RT-PCR, as previously described (46), was used to determine the NaP₅-Iib and c-myb gene expression levels in the absence or presence of EGF.

Overexpression of human c-Myb in Caco-2 cells. Human c-myb cDNA was PCR amplified from Caco-2 cells between bp 110 to 2,037 bp. PCR products were cloned into the pCR2.1 vector (Invitrogen) and confirmed by sequencing. The PCR insert was then moved into the mammalian expression vector pTarget (Promega) by restriction enzyme digestion. Recombinant plasmids were transfected into Caco-2 cells by

![Fig. 1. Identification of the promoter region responsible for EGF down-regulation of human type IIb sodium-phosphate (hNaP₅-Iib) extran- porter gene expression by deletion analysis. A series of progressively shorter hNaP₅-Iib promoter constructs were transfected into Caco-2 cells, and EGF treatment was conducted before promoter assays were performed. Relative change is shown as the ratio of luciferase activity in EGF-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 9 independent experiments done on different days. *P < 0.01 for pGL3/–794 and pGL3/–1,103 vs. others. Basic indicates the promoterless pGL3/Basic vector.]
Lipofectamine (Invitrogen/GIBCO). Forty-eight hours after transfection, G418 was added (1 mg/ml) to the standard culture medium for 3 days, and the cells were lysed in 20 mM HEPES (pH 7.9), 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride on ice for 20 min. Cell extracts were then frozen, thawed, and centrifuged for 10 min at 13,000 g at 4°C. Supernatants were collected and stored at −80°C until use (40).

Statistical analysis. ANOVA post hoc tests (StatView 5.0.1; SAS Institute, Cary, NC) were used to compare values and p values of <0.05 were considered significant.

**RESULTS**

Identification of the EGF responsive region in the hNaPi-IIb promoter. A series of 5’ deletion constructs was made between −1,103 and −380 bp of the hNaPi-IIb promoter region. Caco-2 cells were transfected with these promoter constructs and treated with EGF (100 ng/ml) or vehicle for 8 h. Promoter reporter gene assays showed that deletion constructs pGL3/−563, pGL3/−624, pGL3/−646, and pGL3/−729 did not respond to EGF treatment, whereas the constructs pGL3/−784 and pGL3/−1,103 were suppressed by EGF (Fig. 1).

GMSA identification of DNA sequences involved in the EGF response of the hNaPi-IIb promoter. To identify the precise DNA sequences involved in the EGF response, four DNA oligos were designed to overlap the promoter region −799 to −727 bp. As shown in Fig. 2A, a low molecular weight DNA-protein interaction band was obtained from all the oligos used, whereas a higher molecular weight DNA-protein interaction band was observed with only the probe covering the promoter region −751 to −727 bp. Furthermore, the higher molecular weight-shifted band, but not the lower molecular weight-shifted band, was reduced by EGF administration. Therefore, the lower molecular weight-shifted band was considered nonspecific, and we felt that it was most likely not involved in EGF regulation of hNaPi-IIb cotransporter gene expression.

Mutation at promoter region −739 to −734 bp abolished nuclear protein binding. To further define the DNA-protein interaction at −751 to −727 bp, mutant DNA oligos (Fig. 2B) covering different parts of this region were used. Nuclear protein (4 μg) from Caco-2 cells was incubated with labeled probe 751/727 in the presence or absence of a 100-fold excess of mutant or wild-type DNA oligos. As shown in Fig. 2B, wild-type DNA oligos completely competed the binding of nuclear proteins in shifted complex a. Mutant oligos (m2 and m2a) containing the wild-type sequence (739 bp and 727 bp, respectively) were used. Nuclear protein (4 μg) from nontreated Caco-2 cells and was incubated with labeled probe 751/727 at 25°C for 30 min in the presence of 100-fold excess unlabeled competitors (oligo sequences shown above). The competitor oligos (m2, m2a, and m5) containing 5’-AAGTGG-3’ sequences (shown in boxes) competed for nuclear protein binding with the labeled probe. The competitor oligos (m1, m3, and m4) containing mutations within the 5’-AAGTGG-3’ sequence (mutations shown in bold) could not compete for nuclear protein binding. The image presented here is representative of 1 of 5 independent experiments that showed similar results. NE, nuclear extract; a indicates the specific DNA-protein complexes.
-AACTGG- 734 bp) also competed binding of nuclear proteins in shifted complex a. Other mutant oligos (m1, m3, and m4), which have mutations of the -AACTGG- sequence, had no effect on shifted complex a. The mutant oligo m5, which has upstream bps mutated, reduced the binding in complex a.

Functional confirmation of the DNA sequence involved in EGF regulation. To determine whether the DNA sequence (-AACTGG-) identified by GMSAs plays a functional role in EGF regulation of the hNaP_i-IIb gene expression, the wild-type sequence in the promoter construct pGL3/784 was replaced by mutant sequence 5'-TCTGTT- 3' in the pGL3/729 construct, and this construct was transfected into Caco-2 cells. Promoter assays were performed after EGF treatment. Relative change is shown as the ratio of luciferase activity in EGF-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 7 independent experiments done on different days. *P < 0.03 for pGL3/784 vs. others.

Identification of the protein interacting with the DNA sequence involved in EGF regulation by supershift assay. GMSA and functional studies suggested that the DNA sequence (bp 739 5'-AACTGG-3' bp 734) in the hNaP_i-IIb promoter is the potential cis-acting element involved in EGF regulation. A search for transcription factor binding motifs within this region suggested a potential consensus binding site (-AACT/GG-) for the c-myb transcription factor. To determine whether this DNA sequence could be bound by c-myb protein, supershift GMSAs were performed. As shown in Fig. 4A, the nuclear protein bound to the probe (751/727) caused a specific band shift, and this band could be partially

Fig. 3. Effect of the sequence mutation on the hNaP_i-IIb promoter response to EGF. The DNA region 5'-AACTGG-3', identified in Fig. 2, was replaced by the mutant sequence 5'-TCTGTT- 3' in the pGL3/784 construct, and this construct was transfected into Caco-2 cells. Promoter assays were performed after EGF treatment. Relative change is shown as the ratio of luciferase activity in EGF-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 7 independent experiments done on different days. *P < 0.03 for pGL3/784 vs. others.

Fig. 4. Identification of c-myb bound to the hNaP_i-IIb -751 to -727 bp promoter region. A: identification of c-myb protein by supershift assays. Nuclear protein (4 µg) from Caco-2 cells was incubated with 1 ng of labeled DNA oligos (751/727) in the presence or absence of 4 µg of c-myb supershifting antibodies (Ab) or rabbit IgGs (IgG) at 25°C for 30 min. c-Myb binding to the probes formed a shifted complex (a), which was supershifted by an anti-c-myb antibodies (s.a). Rabbit IgGs alone did not produce a supershift. In both panels, Probe indicates probe only, and NE indicates the addition of nuclear extract. B: overexpressed human c-myb protein also bound to DNA oligos (751/727) from the hNaP_i-IIb promoter. Cell lysates were prepared from c-myb cDNA (pT-myb)- or vector DNA (pT)-transfected Caco-2 cells. Cellular protein (12 µg) was incubated with 1 ng of labeled DNA oligos (751/727) at 25°C for 20 min. As a control, 4 µg of nuclear protein from Caco-2 cells were used in the reaction (indicated by NE). A shifted complex (a) is observed in c-myb-overexpressing cells, but not in vector DNA-transfected cells.
supershifted by an anti-c-myb antibody. As a control to demonstrate specificity of the c-myb antibody, we showed that the addition of rabbit IgG to the reaction did not produce a supershift. To further exemplify c-myb binding to the hNaPi-IIb promoter, human c-myb cDNA was overexpressed in Caco-2 cells, and the cytoplasmic extracts were used for GMSAs. As shown in Fig. 4B, the cell lysate from c-myb cDNA-transfected cells (pT-myb) generated an identical band shift to that seen with Caco-2 cell nuclear extracts. Conversely, cell lysate from vector DNA-transfected cells (pT) did not produce a band shift. These data, when considered together, indicate that the protein bound to this hNaPi-IIb gene promoter region is c-myb.

**Quantitation of endogenous c-myb gene expression in Caco-2 cells.** Our studies suggested that c-myb protein is possibly involved in regulation of hNaPi-IIb gene expression by EGF. To further confirm that Caco-2 cells endogenously express the c-myb gene, mRNA was purified from EGF- or vehicle-treated Caco-2 cells, and subjected to immunoblot analyses. Antibodies against the NH₂ terminus of the human c-myb protein were used to detect and quantitate c-myb protein expression levels. c-Myb protein was detected as an expected ~80-kDa molecular mass protein, as reported. As a positive control, 20 µg of nuclear extracts from human chronic myelogenous leukemia cells (K562) were loaded onto the gel. The image shows the result from 1 of 5 independent experiments. Bottom: optical density analysis of immunoblot data. Data are presented in arbitrary densitometric units. Results are means ± SE from 5 independent experiments.

**c-Myb involvement in hNaPi-IIb gene regulation is mediated through EGF receptor tyrosine kinase signaling transduction pathway.** It is known that EGF can exert its effects on gene expression through various pathways. To determine endogenous c-myb protein distribution and expression levels, total cellular proteins and nuclear proteins were prepared from Caco-2 cells treated with EGF or vehicle. As shown in Fig. 6, a protein at ~80 KDa was detected by the antibody against the NH₂ terminus of the human c-myb protein from both nuclear and cellular protein samples. EGF treatment did not alter c-myb protein expression levels or its cellular distribution in Caco-2 cells.
signal transduction pathways. To further elucidate the pathway involved in the regulation of hNaPi-IIb gene expression, various EGF receptor-signaling pathway blockers were used. Caco-2 cells were transfected with promoter construct pGL3/-784 and pretreated with various inhibitors for 2 h before EGF was added. As shown in Fig. 7, hNaPi-IIb gene promoter activity was reduced 32% by 50 and 100 ng/ml EGF in transfected Caco-2 cells. Administration of 50 nM monoclonal EGF receptor antibodies blocked the response of hNaPi-IIb promoter to EGF treatment (50 ng/ml; Fig. 7A). AG-1478, a specific inhibitor of EGF receptor tyrosine kinase activity, also abolished the EGF effect on the hNaPi-IIb promoter at concentrations of 1 and 5 μM (Fig. 7B). Furthermore, administration of inhibitors PD-098059 (25 μM) and H7 (10 μM) inhibited 30 and 60% of the promoter response, respectively. The combination of C-MYB INVOLVEMENT IN EGF REGULATION OF NaPi-IIb

![Graph A](image1.png)

**A**: Ab-3, an EGF receptor monoclonal antibody (Ab, 50 nM), abolished the promoter response to EGF (50 ng/ml) treatment. *P < 0.03 for EGF-treated vs. vehicle-treated and EGF plus Ab-3-treated cells. **B**: tyrphostin AG-1478 (1 and 5 μM) blocked the EGF (100 ng/ml) effect on hNaPi-IIb promoter. *P < 0.03 for EGF-treated vs. vehicle-treated and EGF plus AG-1478-treated cells. **C**: H7 (10 μM) and/or PD-098059 (PD, 25 μM) restored the activity of the hNaPi-IIb promoter in the presence of EGF (100 ng/ml). *P < 0.03 for EGF-treated cells vs. others; +P < 0.03 for PD-098059-treated cells vs. others. "AJP-Cell Physiol • VOL 284 • MAY 2003 • www.ajpcell.org"
Tyrphostin AG-1478 inhibited the response of hNaPi-IIb gene promoter to EGF by restoring the interaction between nuclear protein and DNA. Functional promoter analysis results showed that AG-1478 completely blocked the effect of EGF on hNaPi-IIb promoter activity. We therefore treated Caco-2 cells with 1 \( \mu \)M AG-1478 and purified nuclear protein for GMSA to determine whether the DNA-protein interaction was restored. As shown in Fig. 8, EGF treatment reduced the specific binding of nuclear protein to the probe 751/727. However, in the presence of 1 \( \mu \)M AG-1478, binding levels were restored. AG-1478 itself had no effect on this DNA-protein interaction.

DISCUSSION

\( P_i \) is an essential element of the body, and its homeostatic regulation is thus important. EGF, as a growth hormone, plays an important role in modulating intestinal \( P_i \) absorption in certain pathophysiological conditions, such as hyperphosphatemia induced by intestinal ischemia/injury (14, 20, 41). Previous work in our laboratory has shown that EGF reduced NaPi-IIb gene expression by inhibiting transcriptional activation in epithelial (Caco-2) cells. EGF affected NaPi-IIb gene expression by inhibiting transcriptional activation in Caco-2 cells. The DNA region involved in this regulation was hypothesized to be located between bp −1,103 and −380 of the hNaPi-IIb gene promoter (45). In the current studies, we further narrowed the EGF response region to bp −784 to −729 of the hNaPi-IIb promoter. This DNA region has no homology with known EGF response elements identified from the c-fos (15), the rat preprothryrotropin-releasing hormone (33), the rat prolactin (13), or the human gastrin genes (16, 17, 25). These findings suggest that a novel EGF response element may be present in the hNaPi-IIb promoter.

The EGF-responsive region was further refined to bp −751 to −727 by GMSA and functional analysis in Caco-2 cells. In GMSAs, the DNA-protein complex (called a) was decreased by EGF treatment. Competition studies with mutant oligos from the −751 to −727 region showed that the sequence at bp −739 to −734 (5′-AACCTGG-3′) was critical for this DNA-protein interaction. When this wild-type DNA sequence in the hNaPi-IIb promoter was replaced by a mutant DNA sequence (5′-TCTGTT-3′), this DNA-protein interaction and the functional response of the hNaPi-IIb promoter to EGF were abolished. These results strongly suggest that this region is involved in EGF regulation of hNaPi-IIb gene expression.

Transcription factor binding motif searches suggested that the DNA sequence (5′-AACCTGG-3′) identified in the hNaPi-IIb promoter could be recognized by the c-myb transcription factor. Additionally, supershift studies with an anti-c-myb antibody indicated that the c-myb transcription factor could bind to this EGF responsive sequence in the hNaPi-IIb promoter, suggesting a novel role for c-myb in EGF regulation. The fact that only a portion of the DNA-protein complex could be supershifted by c-myb antibody might be due to insufficient antibody affinity for c-myb protein or to low specific antibody concentration. Furthermore, we used cellular extracts from Caco-2 cells transfected with human c-myb cDNA in GMSAs and demonstrated that overexpressed human c-myb can bind to the hNaPi-IIb promoter oligo probe and produce an identical band shift to that seen with Caco-2 cell nuclear extracts. Overall, these data strongly suggest that the protein interacting at the EGF responsive site is c-myb.

C-Myb is a phosphorylated nuclear protein, which plays important roles in regulating cell growth, differentiation, and apoptosis (30). C-Myb protein has three distinct regions, which are individually responsible for DNA binding, protein-protein interactions, and negative regulatory functions (26). The COOH-terminal negative regulatory region of c-myb interacts with its DNA binding domain at the NH2-terminal region to influence trans interactions with transcriptional coactivators, cooperating proteins, or DNA (11, 29). Studies have shown that c-myb function is regulated at the posttranslation level (28). MAPK phosphorylates chicken c-myb protein at Ser-11/12, resulting in reduced DNA-binding activity (23, 24). MAPK also phosphorylates homologous serines in mouse (Ser-528) and human (Ser-532) c-myb proteins, which also interferes with c-myb activity (5, 6, 43). C-Myb is highly expressed in immature hemopoietic cells and in the human intestine (35, 42). It regulates the expression of many genes,
such as myb-induced myeloid protein-1 (mim-1) (12), T cell surface markers CD4 (27, 37), and CD34 (19) in immature hemopoietic cells. However, little is known about the target genes of c-myb in intestinal epithelial cells.

Our results demonstrated that mutation of the c-myb-binding site abolished the EGF response of the hNaPi-IIb promoter. Further data suggested that c-myb binding to the promoter under basal conditions has no effect on transcriptional activation because mutation of the putative binding site did not alter basal promoter activity. But, upon EGF stimulation, c-myb is likely modified in some way that then leads to an inhibitory effect on transcriptional activation and decreased binding affinity on the hNaPi-IIb promoter. In fact, our data (as discussed in detail later) show that inhibition of MAPK or PKC activity could partially restore the activity of the hNaPi-IIb promoter, which suggests that MAPK- and/or PKC-mediated phosphorylation of c-myb induced by EGF signaling may be this proposed modification of c-myb. Furthermore, we found that inhibition of EGF receptor tyrosine kinase activity and EGF-EGF receptor interaction also abolished the effect of EGF on hNaPi-IIb promoter activity, presumably by blocking this modification of c-myb, which leads to its inhibitory properties on promoter function. Additionally, the findings that EGF did not alter c-myb mRNA abundance, c-myb protein expression levels, or distribution between cytosol and nucleus further suggested that the c-myb protein was modified by EGF treatment in Caco-2 cells. This possible post-translational modification might involve changing protein–protein interaction(s) between c-myb and basal transcription factor(s) (28, 30), which together may mediate the EGF response of the hNaPi-IIb gene.

EGF receptor signaling transduction pathways have been extensively studied. EGF receptor activation by EGF binding initiates multiple cellular signaling pathways. Ab-3, a mouse monoclonal antibody against the human EGF receptor, inhibits EGF binding to the EGF receptor and thus inhibits EGF-dependent tyrosine protein kinase activity. In preliminary experiments, we determined that 50 ng/ml EGF led to a maximal decrease in hNaPi-IIb gene expression and that 50 nM Ab-3 (the maximal concentration provided by the manufacturer) completely abolished this downregulation. Furthermore, we used an inhibitor of EGF receptor tyrosine kinase activity (tyrophostin AG-1478), which has been previously used at concentrations between 1 and 10 μM (21, 34, 36). These experiments showed that the response of the hNaPi-IIb promoter to EGF treatment could be completely blocked by treating transfected cells with 1 or 5 μM tyrophostin AG-1478. Overall, these findings indicated that EGF downregulation of hNaPi-IIb gene expression involves EGF binding to its receptor, followed by EGF receptor tyrosine kinase activation.

Further experiments were performed to identify downstream pathways after EGF receptor activation by PD-098059, which is a highly specific inhibitor of MAPK activation and has been previously used at concentrations between 10 and 50 μM (1, 21, 34). We chose 25 μM for our experiments on the basis of these previous investigations. We also utilized H7, a potent inhibitor of both protein kinase C (PKC) and cAMP-dependent protein kinase (PKA). In previous PKC-signaling pathway studies, the H7 concentration used was between 10 and 50 μM (22, 31, 32). We used the minimal dose of 10 μM H7 for our experiments to avoid toxicity and nonspecific inhibition of other protein kinases. Our results with these inhibitors showed that administration of 10 μM H7 reduced 60% of the promoter response to EGF and that 25 μM PD-098059 reduced 30% of the promoter response to EGF. When used in combination, PD-098059 and H7 fully restored hNaPi-IIb promoter activity in the presence of EGF. These data suggest that both PKC and MAPK pathways are involved in EGF regulation of hNaPi-IIb gene expression, with less impact from the MAPK pathway. Because 10 μM H7 could also inhibit PKA activity, PKA pathway involvement is possible.

PKC activation is known to be one of the downstream signals for EGF-induced receptor tyrosine kinase activation (9). There are at least 12 PKC isoforms grouped into three subtypes identified to date, and each isoform has a unique, nonredundant role in signal transduction (8). As an intestinal epithelial cell model, Caco-2 cells express at least five different PKC isoforms encompassing the three subtypes (8). PKC activation by EGF has been shown to be related to protecting intestinal cells from oxidants/ethanol damage through PKC-β1 (9) and PKC-ζ (8) isoforms. Here, we observed that the downregulation of hNaPi-IIb gene promoter function by EGF could be partially restored by use of the PKC inhibitor H7, but this inhibitor is not isoform-specific, so it is currently unknown which PKC isoforms may be involved.

In summary, this study has identified the DNA sequence in the hNaPi-IIb gene promoter likely responsible for EGF downregulation of hNaPi-IIb gene expression. Supershift analysis and overexpression studies indicated that the transcription factor c-myb was responsible for binding to this sequence and that EGF treatment reduced binding affinity and concomitantly reduced promoter activity. Further work showed that EGF receptor tyrosine kinase-activated MAPK, PKC, and/or PKA pathways are involved in EGF modulation of hNaPi-IIb gene expression. These studies, for the first time, directly demonstrate c-myb regulation of a gene involved in intestinal nutrient absorption. Moreover, our data further exemplify a novel target gene (the NaPi-IIb cotransporter) of EGF receptor signaling in the mammalian intestine.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-33209.

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