c/ebp\(\delta\) Null Mouse as a Model for the Double Knock-out of slc5a8 and slc5a12 in Kidney* 

Received for publication, July 18, 2006; and in revised form, July 25, 2006. Published, JBC Papers in Press, July 26, 2006, DOI 10.1074/jbc.C600189200

Muthusamy Thangaraju1, Sudha Ananth1, Pamela M. Martin1, Penny Roos1, Sylvia B. Smith1, Esta Sternek1, Puttur D. Prasad1, and Vadivel Ganapathy2,3

From the Departments of 1Biochemistry and Molecular Biology, and 2Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia 30912-2100 and the 3Laboratory of Protein Dynamics and Signaling, Center for Cancer Research, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702-1201

slc5a8 and slc5a12 represent the high affinity and low affinity Na\(^+\)/lactate co-transporters, respectively, in the kidney. Here we show that these transporters are expressed in the apical membrane of the proximal tubular cells in mouse kidney, indicating that these transporters are likely to mediate the first step in the renal reabsorption of lactate. Interestingly, the renal expression of both transporters is almost completely ablated in mice homozygous for the deletion of the transcription factor c/ebp\(\delta\). This effect is tissue-specific since the expression of the transporters is not affected in non-renal tissues. The functional role of C/EBP\(\delta\) in the expression of SLCA5A8 and SLCA5A12 is demonstrable in HEK293 cells in reporter assays using genespecific promoters. The ablation of the transporters in the kidney is accompanied by a marked increase in urinary excretion of lactate as well as a decrease in blood levels of lactate in c/ebp\(\delta\)\(^{-/-}\) mice. These data provide evidence for an obligatory role for slc5a8 and slc5a12 in the renal absorption of lactate. In addition, we show that urinary excretion of urate is significantly elevated in c/ebp\(\delta\)\(^{-/-}\) mice even though the expression of URAT1, the transporter responsible for the apical membrane uptake of urate in renal proximal tubule, is not altered. These data provide in vivo evidence for the functional coupling between lactate reabsorption and urate reabsorption in the kidney. Thus, the fortuitous double knock-out of slc5a8 and slc5a12 in kidney in c/ebp\(\delta\)\(^{-/-}\) mice reveals the physiologic role of these transporters in the renal handling of lactate and urate.

Lactate is the product of glucose metabolism under anaerobic conditions and is also the sole end product of glucose metabolism in erythrocytes. Normal levels of lactate in blood are significant (~1.5 mm). Lactate is an important precursor for gluconeogenesis in the liver and kidney and also plays a vital role in the maintenance of brain function (1, 2). This metabolite is reabsorbed in the kidney with great efficiency (>98% filtered load) (3). This process involves two different Na\(^+\)-coupled transport systems in the apical membrane and a Na\(^{+}\)-independent transport system in the basolateral membrane of the proximal tubular cells (4). The basolateral membrane transport system is mediated by MCT1, a monocarboxylate transporter. The molecular identities of the apical membrane transport systems remain unknown. Recently, we isolated two transporter clones from a mouse kidney cDNA library which function as Na\(^{+}\)-coupled transporters for lactate, SMCT1 (slc5a8) as the high affinity transporter (5–7) and SMCT2 (slc5a12) as the low affinity transporter (8). C/EBP\(\delta\) is a member of the C/EBP family (9). c/ebp\(\delta\)\(^{-/-}\) mice are viable with no impairment in embryonic/postnatal development but exhibit subtle defects in adipocyte differentiation (10), specific types of learning and memory (11), mammary gland involution (12, 13), and chromosomal stability (14). The only known renal phenotype reported in these mice is the altered myofibroblast transdifferentiation in an experimental model of glomerulonephritis (15).

SLC5A8 was originally identified as a putative tumor suppressor in colon (16). Subsequently, the expression of SLC5A8 has been shown to be down-regulated not only in colon cancer but also in cancers of various other tissues (reviewed in Refs. 17 and 18). However, the mechanisms responsible for the regulation of the expression of SLC5A8 are not well understood. A search for transcription factor binding sites in SLC5A8 promoter revealed the presence of consensus sequences for the binding of C/EBP\(\delta\). Since SLC5A8 is expressed most abundantly in the kidney, we investigated the potential role of this transcription factor in the renal expression and function of slc5a8 using c/ebp\(\delta\) null mice. These studies led to the surprising finding that not only slc5a8 but also its close functional relative slc5a12 are down-regulated in the kidneys of c/ebp\(\delta\) null mice. The fortuitous double knock-out of slc5a8 and slc5a12 in the kidneys of these mice helped us determine the physiologic role of these two transporters in the renal absorption of lactate.

EXPERIMENTAL PROCEDURES

Animals—Adult male wild type, c/ebp\(\delta\)\(^{+/-}\), and c/ebp\(\delta\)\(^{-/-}\) mice were obtained from the National Cancer Institute. 

Collection of Blood and Urine Samples—Mice were anesthetized with isoflurane and urine was collected from bladder and blood was collected from cardiac puncture. Urine was centrifuged, and clear supernatant was frozen immediately for later use in lactate and uric acid measurements. Blood collected in glass tubes was allowed to clot and serum was separated by centrifugation. Serum was also frozen immediately for later use in lactate and uric acid measurements. Urine and serum samples from each mouse were treated individually for measurements of lactate and uric acid (wild type, n = 7; heterozygous, n = 5; homozygous, n = 8).

Measurements of Lactate and Uric Acid—Lactate levels in urine and serum samples were measured using a commercially available kit (Lactate Assay Kit; Biomedical Research Service Center, University at Buffalo, Buffalo, NY). Uric acid levels in urine and serum samples were also measured using a commer-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. E-mail: vganapat@mail.mcg.edu.
cially available kit (uric acid assay kit; BioAssay Systems, Hayward, CA).

RT2-PCR—RNA was isolated from whole kidney, colon, jejunum, ileum, and brain collected from wild type, c/ebpΔ+/−, and c/ebpΔ−/− mice and used for RT-PCR to determine the steady-state levels of mRNAs for various transporters. PCR primers were designed based on the sequence information available in GenBank for mouse cDNAs for these transporters.

**Immunofluorescence Localization**—Antibodies were raised in rabbits against synthetic peptides specific for mouse slc5a8 and slc5a12. The sequences of the antigenic peptides were ELNFDTHS5KNGTRL for slc5a8 (the last 16 amino acids of the C-terminal tail) and CGVQHDPREQYLD for slc5a12 (amino acids 559–573). The antibodies were affinity-purified against immobilized antigenic peptides before use in immunofluorescence studies. The specificity of these antibodies was confirmed by immunoreactivity with cloned mouse slc5a8 and mouse slc5a12, which were expressed heterologously in HRPE cells (a human retinal pigment epithelial cell line). Anti-c/ebpΔ antibody, raised in rabbits, and the antibody specific for 4F2hc (the heavy chain of the 4F2 antigen, also known as CD98), raised in goat, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). This allowed us to perform double labeling studies with slc5a8 and 4F2hc or with slc5a12 and 4F2hc using appropriate fluorophore-conjugated secondary antibodies (slc5a8 or slc5a12, red fluorophore; 4F2hc, green fluorophore). 4F2hc is a marker for basolateral membrane of renal tubular epithelial cells (19). Tissue sections were prepared from kidneys collected from wild type and c/ebpΔ−/− mice and used for immunolocalization by methods described in our earlier publications (20, 21).

**Generation of SLC5A8- and SLC5A12-specific Promoter-Reporter Constructs**—The cDNAs for human C/EBPα, C/EBPβ, and C/EBPδ were cloned into the pcDNA3.1 vector. The human SLC5A8 promoter-EFGR and SLC5A8 promoter-luciferase constructs were generated by first subcloning the 2.4-kb SLC5A8 promoter (obtained by PCR using human genomic DNA as the template) into the TOPO-cloning vector and then using the HindIII/EcoRI-digested insert to clone into pUIIR3-EFGR and pUBT-luc vectors. The primers used for PCR were: 5′-CTCGACTAAGAAATGCCAGAAGACAACAG-3′ (sense) and 5′-AAGCTTGGCCGCACGGTCGCTGACC-3′ (antisense). Similarly, human SLC5A12 promoter-luciferase and SLC5A12 promoter-EFGR constructs were generated by first subcloning the 2.0-kb SLC5A12 promoter (obtained by PCR using human genomic DNA as the template) into pGEM-T Easy vector and then cloning the HindIII/PstI-digested promoter insert into pUBT-luc vector and the HindIII/XhoI-digested promoter insert into pUIIR3-EFGR vector. The primers used for PCR were: 5′-AACATGACTATCTTAACTG-3′ (sense) and 5′-AGTTCCTTCCAACGGGTGTC-3′ (antisense). For the transactivation assays, HEK293 cells were seeded (2 × 10³ cells) in 35-mm tissue-culture dishes and allowed to grow in Dulbecco’s modified Eagle’s medium containing 10% FBS for 24 h. The effector and reporter plasmids were transfected using FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science). The enhanced green fluorescent protein (EGFP) expression was monitored by epifluorescence after 36 h post-transfection under the fluorescence microscope. With the luciferase reporter, cells were collected after 36 h transfection, and the lysates were used for measurement of luciferase activity. The activity was normalized for protein levels and compared with vector-transfected cells.

**RESULTS AND DISCUSSION**

We first compared the renal expression of slc5a8 and slc5a12 between c/ebpΔ+/− and c/ebpΔ−/− mice by RT-PCR (Fig. 1A). Kidneys from wild type mice (n = 7) expressed slc5a8 mRNA and slc5a12 mRNA, whereas these mRNAs were almost undetectable in kidneys from c/ebpΔ−/− mice (n = 8). In heterozygous mice (c/ebpΔ+/−) (n = 5), the mRNAs were detectable, but the levels were markedly reduced compared with those in wild type mice, indicating a dose-response relationship between c/ebpΔ levels and slc5a8 and slc5a12 mRNA levels in kidney. In contrast, the levels of mRNAs for many other transporters (MCT1, a H+-coupled monocarboxylate transporter; URAT1, an anion exchanger responsible for the first step in the renal absorption of uric acid; OAT1, an anion exchanger; NaDC3, a Na+-coupled transporter for dicarboxylates) were comparable between wild type mice and knock-out mice (n = 4 for each genotype) (Fig. 1B). Interestingly, there were no changes in the expression of slc5a8 and slc5a12 in non-renal tissues such as the brain and intestinal tract in c/ebpΔ−/− mice compared with wild type mice (n = 2 for each genotype) (Fig. 1C). The changes in mRNA levels for the two transporters in kidney paralleled with the expression of corresponding proteins (Fig. 1D). In kidneys from wild type mice, slc5a8 and slc5a12 (red fluorescence) were found to localize to the apical membrane of proximal tubules as evident from the lack of co-localization with 4F2hc (green fluorescence), a marker for basolateral membrane in renal epithelial cells. The expression of slc5a12 in the kidney was more abundant, and detectable in more proximal tubule sections than slc5a8. In c/ebpΔ−/− mice, the expression of both transporters was markedly reduced. We also examined the expression of c/ebpΔ in these kidney sections, confirming the expression of the transcription factor protein in wild type mouse kidneys and its absence in c/ebpΔ−/− mouse kidneys. Taken collectively, these studies show that c/ebpΔ controls the expression of slc5a8 and slc5a12 in the kidney.

The in vivo findings that c/ebpΔ controls the renal expression of slc5a8 and slc5a12 were confirmed by two different reporter assays (luciferase and EGFP) using promoter constructs of human SLC5A8 and SLC5A12 (Fig. 2, A–D). In the human embryonic kidney cell line HEK293, expression of C/EBPΔ induced the reporter activity with both gene-specific promoters (n = 3; p < 0.05). This effect was specific for C/EBPΔ as the other two members of the family, C/EBPα and C/EBPβ, had no effect in these reporter assays. The expression of these transcription factor proteins in cells transfected with respective clones have been confirmed by immunodetection with appropriate antibodies (data not shown).

The apical membrane localization of slc5a8 and slc5a12 suggests that these two transporters are likely to mediate the first step in the active reabsorption of lactate. The lack of expression
of these two transporters in \( c/ebp\delta^{--} \) mouse kidneys suggests that lactate reabsorption is likely to be severely impaired in these mice. To test this hypothesis, we determined lactate levels in urine and serum samples collected from \( c/ebp\delta^{+/+} \), \( c/ebp\delta^{++/-} \), and \( c/ebp\delta^{+-/-} \) mice. Urinary lactate levels were low in wild type mice (48 ± 9 nmol/ml), but the levels increased 4-fold in heterozygous mice \( (p < 0.05) \) and 29-fold in homozygous mice \( (p < 0.05) \) (Fig. 3A). This was accompanied by a parallel decrease in the serum levels of lactate in homozygous mice \( (p < 0.05) \) (Fig. 3B). The level decreased 78% in heterozygous mice \( (p < 0.05) \) and 90% in homozygous mice \( (p < 0.05) \) (Fig. 3B). There is strong evidence for a functional coupling between lactate reabsorption and urate reabsorption in the kidney in vivo (22). This suggests that impairment in renal lactate reabsorption would lead to defective reabsorption of urate. This was indeed the case in \( c/ebp\delta^{--} \) mice. Urinary excretion of urate increased 2.5-fold in heterozygous mice \( (p < 0.05) \) and 6-fold in homozygous mice \( (p < 0.05) \) (Fig. 3C). This was accompanied by a significant decrease in the serum levels of urate in homozygous mice \( (p < 0.05) \) (Fig. 3D). The serum levels of urate were not significantly different between wild type and heterozygous mice \( (p > 0.05) \). These data confirm the functional coupling between renal reabsorption of lactate via slc5a8/ slc5a12 and renal reabsorption of urate via URAT1. slc5a8 and slc5a12 mediate active influx of lactate into proximal tubular cells and intracellular lactate in turn serves as the exchangeable anion for URAT1 (an anion exchanger), thus facilitating the reabsorption of urate (Fig. 3E). The lack of expression of slc5a8 and slc5a12 in \( c/ebp\delta^{--} \) mouse kidneys therefore leads to defective reabsorption of urate despite the normal expression of URAT1.

It has to be noted that there may be additional isoforms of monocarboxylate transporters in the basolateral membrane of renal tubular cells, which work along with MCT1 in the renal handling of lactate. Similarly, the molecular identity of the transporter responsible for the transfer of uric acid across the basolateral membrane has not been established. We do not know if the expression of any of these transporters is affected in \( c/ebp\delta^{--} \) null mouse kidneys. The first step in the renal absorption of lactate and urate occurs at the apical membrane of the renal tubular cells and the present studies focused on the transporters, which play a critical role at this step.

These studies establish slc5a8 and slc5a12 as the Na\(^+\)-coupled transporters responsible for the first step in the renal reabsorption of lactate, namely the uptake across the apical membrane of the proximal tubular cells. Though the relative importance of each of these transporters in the renal absorption of lactate cannot be assessed from the present studies, the two transporters together are obligatory for this important renal function. In addition, these studies demonstrate that C/EBPδ is a critical regulator of the expression of these two transporters in the kidney. The control of their expression by \( c/ebp\delta \) seems to be kidney-specific as there is no detectable change in non-renal tissues such as the intestinal tract and brain. These studies also provide unequivocal evidence for the coupling between lactate reabsorption and urate reabsorption in the kidney in vivo. Lactate is an important gluconeogenic substrate and the principal component of the Cori cycle, which links glycolysis in skeletal muscle and gluconeogenesis in the liver. Lactate is also the preferred energy substrate for neurons in the brain and retina (2).
Therefore, the conservation of lactate by the kidney via reabsorption from the glomerular filtrate is critical for the maintenance of energy homeostasis not only in the skeletal muscle but also in the central nervous system. c/ebpδ−/− mice as a model for the kidney-specific double knock-out of slc5a8 and slc5a12 would be useful in future studies to investigate the biological role of lactate under physiological and pathological conditions.

REFERENCES

1. Brooks, G. A. (2002) Biochem. Soc. Trans. 30, 258–264
2. Pellerin, L. (2005) Mol. Neurobiol. 32, 59–72
3. Ullrich, K. J. & Rumrich, G. (1993) Clin. Investig. 71, 843–848
4. Wright, S. H. & Dantzler, W. H. (2004) Physiol. Rev. 84, 987–1049
5. Gopal, E., Fei, Y. J., Sugawara, M., Miyauchi, S., Zhuang, L., Martin, P. M., Smith, S. B., Prasad, P. D. & Ganapathy, V. (2004) J. Biol. Chem. 279, 44522–44532
6. Miyauchi, S., Gopal, E., Fei, Y. J. & Ganapathy, V. (2004) J. Biol. Chem. 279, 13293–13296
7. Gopal, E., Fei, Y. J., Miyauchi, S., Zhuang, L., Prasad, P. D. & Ganapathy, V. (2005) Biochem. J. 388, 309–316
8. Srinivas, S. R., Gopal, E., Zhuang, L., Itagaki, S., Martin, P. M., Fei, Y. J., Ganapathy, V. & Prasad, P. D. (2005) Biochem. J. 392, 655–664
9. Johnson, P. F. (2005) J. Cell Sci. 118, 2545–2555
10. Tanaka, T., Yoshida, N., Kishimoto, T. & Akira, S. (1997) EMBO J. 16, 7432–7443
11. Sterneck, E., Paylor, R., Jackson-Lewis, V., Libbey, M., Przedborski, S., Tessarollo, L., Crawley, J. N. & Johnson, P. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10908–10913

FIGURE 2. C/EBPδ-specific induction of SLC5A8- and SLC5A12-specific promoters in reporter assays. C/EBPδ-specific induction of SLC5A8- and SLC5A12-specific promoter activities using luciferase (A and B) or EGFP (C and D) as the reporter. Human SLC5A8- and SLC5A12-specific promoters were generated by PCR using human genomic DNA as the template and then subcloned into pU8T-luc and pU1IR3-EGFP vectors. For the transactivation assays, HEK293 cells were seeded (2 × 10^5 cells) in 35-mm tissue culture dishes and allowed to grow in DMEM medium containing 10% fetal bovine serum for 24 h. The reporter plasmids were co-transfected with pcDNA3.1, C/EBPα cDNA, C/EBPβ cDNA, or C/EBPδ cDNA using FuGENE 6 according to manufacturer’s instructions (Roche Applied Science). At 36 h post-transfection, luciferase activity was measured in cell lysates (the activity was normalized with protein concentration), and EGFP expression was monitored by epifluorescence.

FIGURE 3. Urinary excretion and serum levels of lactate and uric acid in wild type (c/ebpδ+/+), heterozygous (c/ebpδ+/−), and homozygous (c/ebpδ−/−) mice. A and B, urinary excretion and serum levels of lactate in wild type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Urine and blood were collected from seven wild type male mice, five heterozygous male mice, and eight homozygous male mice, and lactate levels were measured using a commercially available kit. Values for urinary excretion of lactate are expressed as nmol/ml (means ± S.E.) and values for serum levels of lactate are expressed as mM (means ± S.E.). C and D, urinary excretion and serum levels of uric acid in wild type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Urine acid levels in urine and blood samples were measured using a commercially available kit. Values for urinary excretion and serum levels of uric acid are expressed as mg/ml (means ± S.E.). *, significantly different from values in wild type mice (p = 0.008). The values were not significantly different between wild type and homozygous mice (p = 0.075). E, a model for functional coupling between slc5a8/slc5a12 and URAT1 in kidney.
12. Gigliotti, A. P., Johnson, P. F., Sterneck, E. & DeWille, J. W. (2003) *Exp. Biol. Med. (Maywood)* **228**, 278–285
13. Thangaraju, M., Rudelius, M., Bierie, B., Raffeld, M., Sharan, S., Hennighausen, L., Huang, A. M. & Sterneck, E. (2005) *Development (Camh)* **132**, 4675–4685
14. Huang, A. M., Montagna, C., Sharan, S., Ni, Y., Ried, T. & Sterneck, E. (2004) *Oncogene* **23**, 1549–1557
15. Takeji, M., Kawada, N., Moriyama, T., Nagatoya, K., Oseto, S., Akira, S., Hori, M., Imai, E. & Miwa, T. (2004) *J. Am. Soc. Nephrol.* **15**, 2383–2390
16. Li, H., Myeroff, L., Smiraglia, D., Romero, M. F., Pretlow, T. P., Kasturi, L., Lutterbaugh, J., Rerko, R. M., Casey, G., Issa, J. P., Willis, J. Wilson, J. K., Plass, C. & Markowitz, S. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8412–8417
17. Ganapathy, V., Gopal, E., Meyauchi, S. & Prasad, P. D. (2005) *Biochem. Soc. Trans.* **33**, 237–240
18. Gupta, N., Martin, P. M., Prasad, P. D. & Ganapathy, V. (2006) *Life Sci.* **78**, 2419–2425
19. Palacin, M. & Kanai, Y. (2004) *Pfluegers Arch.* **447**, 490–494
20. Dun, Y., Mysona, B., Van Ells, T., Amarnath, L., Ola, M., Ganapathy, V. & Smith, S. B. (2006) *Cell Tissue Res.* **324**, 189–202
21. Martin, P. M., Gopal, E., Ananth, S., Zhuang, L., Itagaki, S., Prasad, B. M., Smith, S. B., Prasad, P. D. & Ganapathy, V. (2006) *J. Neurochem.* **98**, 279–288
22. Hediger, M. A., Johnson, R. J., Miyazaki, H. & Endou, H. (2005) *Physiology (Bethesda)* **20**, 125–133