Activation and Inactivation Mechanisms of Na-HCO_3 Cotransporter NBC1

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Abstract: The electrogenic Na^+-HCO_3^- cotransporter NBC1, also known as NBCe1, is expressed in several tissues such as kidney, pancreas, eye, and brain. NBC1 has three variants, which may mediate different physiological functions. Inactivating mutations in NBC1 result in proximal renal tubular acidosis associated with ocular abnormalities. Functional analysis suggests that at least 50% reduction in NBC1 activity is required to induce severe acidemia invariably associated with stunted growth. Some mutations in the *NBC1* gene may also induce trafficking abnormalities. On the other hand, the variant-specific N-terminal regions seem to play an essential role in the full activation of NBC1. This review will focus on the recent progress in physiology and pathophysiology of NBC1.

Keywords: NBC1, *SLC4A4*, pRTA, IRBIT.

INTRODUCTION

Regulation of intracellular and extracellular pH is essential for many cellular functions. To accomplish this task, eukaryotic organisms have developed two types of bicarbonate transporter families, *SLC4* and *SLC26* transporters. The *SLC4A* gene family includes the Na^+-independent electroneutral Cl^-/HCO_3^- exchangers such as *SLC4A1*/AE1, *SLC4A2*/AE2, and *SLC4A3*/AE3 [1-3]. AE1 is expressed in erythrocytes and in renal collecting duct cells. Mutations of the human AE1 gene result in either erythrocyte abnormalities such as hereditary spherocytic anemia or autosomal dominant distal renal tubular acidosis (dRTA) [3,4]. The *SLC4* gene family also includes the electrogenic Na^+-HCO_3^- cotransporters *SLC4A4*/NBC1 (NBCe1) and *SLC4A5*/NBC4 (NBCe2) [1,2,5]. NBC1 is expressed in renal proximal tubules and in other tissues such as pancreas, eye and brain. Mutations of the human *NBC1* gene result in autosomal recessive proximal renal tubular acidosis (pRTA) associated with ocular abnormalities [4,6]. This review will focus on NBC1, highlighting disease-causing mechanisms by NBC1 mutations and variant-specific regulation.

NBC1 VARIANTS

The *NBC1* gene generates three spliced-variants as shown in Fig. (**1**): kNBC1 (NBCe1A) originally cloned from kidney, pNBC1 (NBCe1B) from pancreas, and bNBC1 (rb2NBC/NBCe1C) from brain [7-10].

As kNBC1 and pNBC1 variants are derived from alternate promoter usage in the *SLC4A4* gene, they differ in their structure only at the N-terminus, where the first 41 amino acids of kNBC1 replace the first 85 amino acids of pNBC1 [11]. bNBC1 is identical to pNBC1 except for the C-terminus, where the last 61 amino acids of bNBC1 replace the last 46 amino acids of pNBC1 [10]. These variants show distinct patterns of tissue expression [8,9]. kNBC1 is predominantly expressed in the basolateral membranes of renal proximal tubules, and mediates a majority of bicarbonate efflux from proximal tubules [12,13]. kNBC1 is also expressed in eye and brain [14-16]. pNBC1 is expressed in various tissues including pancreas, intestine, eye, and brain, and may mediate other biological processes such as bicarbonate secretion from pancreatic ducts [9,17-19]. bNBC1 seems to be exclusively expressed in brain [10].

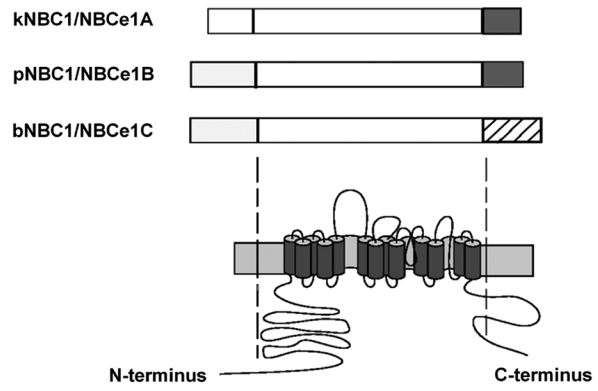

Fig. (1). Structures of NBC1 variants encoded by *SLC4A4*. While kNBC1 and pNBC1 differ only at the N-terminus, pNBC1 and bNBC1 differ only at the C-terminus.

The transport stoichiometry is one of the main factors, which determine the net transport direction of NBC1. In renal proximal tubules, for example, kNBC1 mediates HCO_3^- exit from cells with $1Na^+$ to $3HCO_3^-$ stoichiometry [20]. On the other hand, in other epithelial cells such as corneal endothelium or pancreatic duct cells pNBC1 seems to mediate HCO_3^- influx into cells with $1Na^+$ to $2HCO_3^-$ stoichiometry

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[1,21]. However, the difference in stoichiometry in these tissues may not be due to the expression of different NBC1 variants. On the contrary, recent studies suggest that the stoichiometry of NBC1 variants may not be fixed, but may vary according to the cell conditions or cell types [22-26]. Other studies suggest that either changes in cell Ca^{2+} concentrations [27] or phosphorylation by protein kinase A [28] may be responsible for the changes in stoichiometry.

NBC1 MUTATIONS CAUSE PRTA

Renal proximal tubules reabsorb more than 80% of the filtered bicarbonate. An impairment of this process results in pRTA, characterized by a decrease in renal bicarbonate threshold. pRTA usually occurs as one manifestation of generalized defects in proximal tubular functions (Fanconi syndrome). An isolated defect in proximal bicarbonate absorption (isolated pRTA) is rare, and mostly transient in infants or children. Extremely rare forms of hereditary isolated pRTA are divided into two types according to the clinical features; (I) stunted growth without other predominant clinical features and (II) stunted growth and ocular abnormalities such as band keratopathy, cataracts, and glaucoma [29-31].

We speculated that the mutational inactivation of NBC1 would be responsible for the type II hereditary isolated pRTA, because both renal proximal tubules and corneal endothelium had been shown to posses electrogenic Na^+-HCO_3^- cotransport and to express NBC1 mRNA [7,8,21,32,33]. Consistent with this speculation, we identified homozygous missense kNBC1 mutations (c.1034A>C corresponding to p.Arg298Ser and c.1678G>A corresponding to p.Arg510His) in two unrelated patients, who had severe pRTA, short stature, and ocular abnormalities. Functional analysis in ECV304 cells confirmed that both mutations reduced the NBC1 activity by approximately 50% [6]. These observations established that faulty *SCL4A4* gene is responsible to the development of the autosomal recessive pRTA associated with short stature and ocular abnormalities (Online Mendelian Inheritance in Man, OMIM 604278). Subsequent studies have revealed that NBC1 is expressed in several ocular tissues such as corneal endothelium, lens epithelium, ciliary epithelium, and trabecular meshwork cells, which can potentially explain the occurrence of ocular abnormalities associated with NBC1 mutations [14,15]. On the other hand, the stunted growth could most likely originate from the severe acidemia, since the intensive alkali therapy, if started early in the life, was able to significantly improve the body height and growth rate [34].

MECHANISMS OF NBC1 INACTIVATION

To date, ten homozygous mutations in the *kNBC1* gene have been reported, in which eight correspond to missense mutations p.Arg298Ser [6], p.Ser427Leu [35], p.Thr485Ser [36], p.Gly486Arg [37], p.Arg510His [6], p.Leu522Pro [38], p.Ala799Val [36], and p.Arg881Cys [36], one to a nonsense mutation p.Gln29X [39], and one to a frame shift mutation c.2311deA or p.Asn721ThrfsX29 [40]. Except for one patient with p.Leu522Pro mutation whose blood HCO_3^- concentration was not reported [38], all the patients presented the severe pRTA, defined as the reduction of blood HCO_3^- concentration to less than 13 mmol/L. Functional analysis of these mutants suggested that at least 50% reduction in the transport activity of kNBC1 would be necessary to induce the severe acidemia [6,36,37].

The nonsense mutation p.Glu29X is expected to abolish the kNBC1 function completely, while leaving the pNBC1 function intact [39]. On the other hand, the flame shift mutation p.Asn721ThrfsX29 is expected to abolish the functions of both kNBC1 and pNBC1. Consistent with this speculation, the p.Asn721ThrfsX29 mutant failed to induce any NBC1 activities in *Xenopus* oocytes [40]. It remains to be confirmed whether the lack of surface expression of p.Asn721ThrfsX29 in *Xenopus* oocytes [40] is due to the instability of mutant polypeptide.

Interestingly, several mutations such as p.Thr485Ser, p.Gly486Arg, p.Arg510His, and p.Leu522Pro accumulate in the third and fourth transmembrane domains as shown in Fig. (**2**), suggesting that these domains play an important role in the transport functions of NBC1.

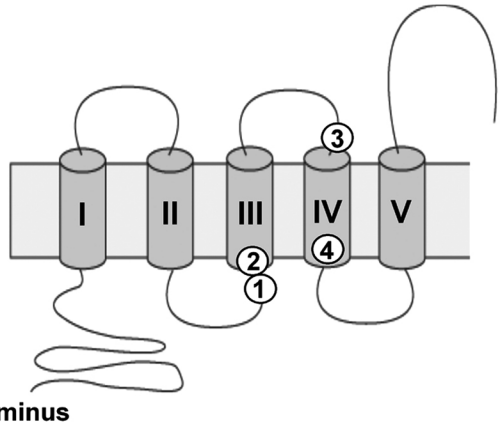

Fig. (2). Disease-causing mutations in the third and fourth transmembrane domains of NBC1. Circles indicate missense NBC1 mutations corresponding to p.Thr485Ser (1), p.Gly486Arg (2), p.Arg510His (3), and p.Leu522Pro (4).

However, recent studies revealed that these mutations inactivate the NBC1-mediated transport through different mechanisms. In the non-polarized ECV304 cells, p.Thr485Ser, p.Gly486Arg, and p.Arg510His showed an efficient surface expression, and their transport activities corresponded to approximately 50% of the wild-type kNBC1 activity [6,36,37]. However, p.Leu522Pro was exclusively retained in the cytoplasm of ECV304 cells, showing no transport activity [37]. In the polarized Madin-Darby canine kidney (MDCK) cells, p.Thr485Ser and p.Gly486Arg showed the predominant basolateral expression, which was indistinguishable from that of wild-type kNBC1. By contrast, p.Arg510His and p.Leu522Pro were predominantly retained in the cytoplasm of MDCK cells [37,41]. On the other hand, p.Thr485Ser, p.Gly486Arg, p.Arg510His, and p.Leu522Pro mutants had almost no electrogenic activity in *Xenopus* oocytes due to poor surface expression [36,37]. The reason why these NBC1 mutants showed different trafficking behaviors depending on the expression systems is unknown. However, the use of MDCK cells is thought to be the most reliable way to analyze the trafficking behaviors of NBC1 [37,41-43]. Moreover, the *Xenopus* oocyte expression system is not suitable for examination of trafficking abnor-

malities [4]. Therefore, p.Thr485Ser and p.Gly486Arg may induce the "pure" functional impairment without affecting the plasma membrane expression. While p.Arg510His seems to induce both functional impairment and abnormal trafficking, p.Leu522Pro seems to cause the clinical abnormalities predominantly through cytoplasmic retention.

Other mutations in the *NBC1* gene are also found to lead to abnormal trafficking in MDCK cells, but not in *Xenopus* oocytes. For example, NBC1 mutants such as p.Arg298Ser, p.Ser427Leu and p.Arg881Cys could be effectively expressed in the surface membrane of *Xenopus* oocytes [35,36,44]. In MDCK cells, however, p.Arg298Ser and p.Ser427Leu mutations led to mistargeted apical expression in addition to the normal basolateral expression, while p.Arg881Cys led to the predominant retention in the cytoplasm [37,41,43]. Interestingly, Li *et al.* demonstrated that NBC1 mutants lacking 23-67 amino acids from the C-terminus end also showed the aberrant apical expression in MDCK cells [42]. Taken together, the correct basolateral targeting of NBC1 seems to be dependent on multiple regions including both transmembrane domains and C-terminus. This situation is quite analogous to that of AE1. In case of AE1, mutations in either the C-terminus (p.Arg910X) or in the transmembrane domains (p.Gly609Arg) resulted in the aberrant apical expression, which might represent one of possible molecular mechanisms for dominant dRTA [45,46]. In contrast to autosomal dominant dRTA caused by mutations in the *AE1* gene, pRTA caused by mutations in the *NBC1* gene is of recessive inheritance. Moreover, all the NBC1 mutations with or without trafficking abnormalities induced the severe pRTA invariably associated with stunted growth. Therefore, the pathophysiological significance of NBC1 mistargeting in the systemic acid/base imbalance remains less clear compared to that of AE1 mistargeting. The reason why the NBC1 mutants such as p.Arg298Ser and p.Ser427Leu do not induce an autosomal dominant pRTA phenotype arising from the aberrant apical expression in renal proximal tubules is also unknown. It is possible, however, that the acidification capacity of renal distal tubules may be sufficient to compensate for the moderate reduction in bicarbonate absorption from proximal tubules.

VARIANT-SPECIFIC REGULATION OF NBC1

Although NBC1 variants have different physiological roles, the underlying molecular mechanisms for this difference remain speculative. In this context, we recently identified that an inositol 1,4,5-trisphosphate (IP_3) receptor binding protein termed IRBIT can specifically bind to and activate pNBC1 [47].

IRBIT was originally identified by its ability to bind to IP_3 receptors, and was later shown to regulate the affinity of IP_3 receptors to IP_3 [48,49]. IRBIT is ubiquitously expressed in adult mice tissues [48], and IRBIT mRNA is increased markedly during activation of blood and skin dendritic cells [50]. In search of an IRBIT binding protein, we identified that IRBIT can bind to pNBC1 but not to kNBC1. IRBIT binds to the pNBC1-specific N-terminus, and its binding seems to be dependent on the phosphorylation status of multiple serine residues of IRBIT. In *Xenopus* oocytes, the pNBC1 activity was found to be very low corresponding to less than 20% of the kNBC1 activity. Co-expression with IRBIT, however, markedly stimulated the pNBC1 current, which became comparable to the kNBC1 current over the entire voltage range. This marked stimulation of pNBC1 by IRBIT was not associated with changes in surface expression of NBC1. On the other hand, IRBIT did not change the kNBC1 current. Furthermore, the binding-deficient IRBIT mutants failed to activate the pNBC1 activity, indicating that the direct binding of IRBIT is indispensable for the full activation of pNBC1 [47].

In line with our findings, McAlear *et al*. reported that pNBC1 and bNBC1 had much lower activities than kNBC1 in *Xenopus* oocytes [51]. They also found that while the deletion of variant-specific N-terminal regions substantially enhanced the activities of pNBC1 and bNBC1 but rather reduced the kNBC1 activity, the further deletion of entire cytoplasmic N-terminal regions of NBC1 resulted in the complete functional loss [51]. These observations suggest that the transport activity of NBC1 is tightly controlled by subtle changes in interactions between the cytoplasmic N-terminal regions and the putative transmembrane core domain as shown in Fig. (**3**).

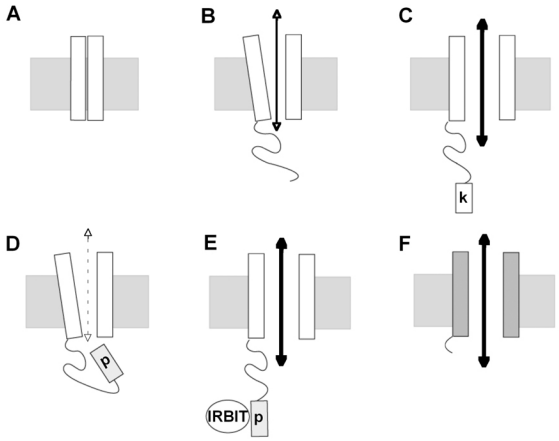

Fig. (3). Roles of N-terminal regions in the activation of SLC4 transporters. (**A**) The electrogenic cotransporter NBC1 has no activity in the absence of entire N-terminal regions. (**B**) NBC1 has the moderate activity in the presence of a substantial part of N-terminal regions. (**C**) NBC1 has the full activity in the presence of kNBC1-specific N-terminus (k). (**D**) NBC1 has only the low activity in the presence of pNBC1-specific N-terminus (p). (**E**) NBC1 regains the full activity after the binding of IRBIT to pNBC1-specific N-terminus. (**F**) The electroneutral anion exchangers AE2 and AE3 have the full activity in the absence of most of N-terminal regions.

According to this model, the electrogenic cotransporter NBC1 requires a substantial portion of cytoplasmic N-terminal regions to show the moderate activity. The presence of kNBC1-specific N-terminus may create the ideal conformation to allow ionic translocation through the core domain, resulting in the full transport activation (see Fig. (**3C**)). By sharp contrast, the presence of pNBC1-specific N-terminus may generate a different conformation that prevents ionic movement across the transmembrane domains resulting in the inactivation of the transporter (see Fig. (**3D**)), in a manner analogous to the ball-and chain model proposed for a voltage-dependent potassium channel [52]. However, the

binding of IRBIT to this pNBC1-specific N-terminus may somehow free its interaction with the core domain, again creating the ideal conformation required for the full transport activity (see Fig. (**3E**)). The cytoplasmic N-terminal regions of AE2 are also known to significantly modify the pH sensitivity of AE2-mediated anion exchange [3,53]. However, the structural requirement for the electroneutral anion exchange may be different from that for the electrogenic sodium/bicarbonate cotransport, because AE2 and AE3, unlike NBC1, can show the full transport activities even after the deletion of most of cytoplasmic N-terminal regions [54,55]. On the other hand, a recent study suggests that the electrogenicity of NBC1 requires interactions among most of the transmembrane domains [56].

At present the exact reason why pNBC1, but not kNBC1, requires IRBIT for the full activation remains unknown. However, the marked and specific effects of IRBIT on pNBC1 could be of great relevance to the different physiological roles of NBC1 variants. For example, pNBC1 is often expressed in exocrine tissues such as pancreas, where bicarbonate transport rates are known to change dramatically depending on cell conditions [57,58]. It is tempting to speculate that pNBC1 may require IRBIT in order to adapt to such a marked change in transport rates. By contrast, kNBC1 is predominantly expressed in renal proximal tubules, where the bicarbonate transport rates are rather constant at physiological conditions [5]. Therefore, kNBC1 may not have to change its transport rates so dramatically. Theoretically, the inactivation of pNBC1 function may induce pancreatic phenotypes. However, clinically overt pancreatitis or pancreatic insufficiencies were not reported in the pRTA patients with NBC1 mutations, and slight elevation in serum amylase or lipase concentrations were reported only in the three patients carrying homozygous NBC1 mutations p.Arg298Ser, p.Asn721ThrfsX29, and p.Arg881Cys [6,36,40]. The reason for lack of severe pancreatic phenotypes is not apparent, but pancreatic ducts may have other acid/base transporters, which can compensate for the inactivation of pNBC1 [57,58].

CONCLUDING REMARKS

Functional analysis suggested that at least 50% reduction of NBC1 would be necessary to induce severe pRTA associated with ocular abnormalities. Some mutations also seemed to induce trafficking defects. Most of the NBC1 mutations identified in patients with pRTA are found in the transmembrane domains. However, recent studies have revealed that the variant-specific N-terminal regions may also play an essential role in the regulation of NBC1 activity. Future studies are warranted to further clarify the transport mechanism and regulation of this important transporter.

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