Na$^+$–K$^+$–2Cl$^-$ cotransporter type 2 trafficking and activity: The role of interacting proteins

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The central role of Na$^+$–K$^+$–2Cl$^-$ cotransporter type 2 (NKCC2) in vectorial transepithelial salt reabsorption in thick ascending limb cells from Henle's loop in the kidney is evidenced by the effects of loop diuretics, the pharmacological inhibitors of NKCC2, that are amongst the most powerful antihypertensive drugs available to date. Moreover, genetic mutations of the NKCC2 encoding gene resulting in impaired apical targeting and function of NKCC2 transporter give rise to a pathological phenotype known as type I Bartter syndrome, characterised by a severe volume depletion, hypokalaemia and metabolic alkalosis with high prenatal mortality. On the contrary, excessive NKCC2 activity has been linked with inherited hypertension in humans and in rodent models. Interestingly, in animal models of hypertension, NKCC2 upregulation is achieved by post-translational mechanisms underlining the need to analyse the molecular mechanisms involved in the regulation of NKCC2 trafficking and activity to gain insights in the pathogenesis of hypertension.

Introduction

Na$^+$–K$^+$–2Cl$^-$ cotransporter type 2 (NKCC2) is a member of the superfamily of electroneutral cation-coupled cotransporters [solute carriers family 12A (*SLC12A*)] encompassing two Na$^+$–K$^+$–2Cl$^-$ cotransporters (NKCC1 and NKCC2), the Na$^+$–Cl$^-$ cotransporter (NCC) and at least four K$^+$–Cl$^-$ cotransporters (KCC1–KCC4). Genes encoding these transmembrane proteins, sharing common predicted membrane topology of 12 transmembrane domains with both N-terminus and C-terminus located intracellularly, are highly homologous.

NKCC2 in the kidney is exclusively expressed in the apical membrane of the thick ascending limb (TAL) and macula densa cells, and in interplay with basolaterally located chloride channel complex (ClC-Kb/Barttin) and with Na$^+$–K$^+$–ATPase, transports NaCl from the lumen back to the blood compartment. Another key component in this system is the apical inwardly rectifying ATP-sensitive K$^+$ channel, designated as ROMK (renal outer medullary potassium channel), which ensures adequate presence of luminal K$^+$ critical for continuous Na–K–Cl uptake by NKCC2 and generates lumen-positive electrical gradient driving paracellular Ca^{2+} and Mg^{2+} reabsorption (for review, see Greger, 1985) (Figure 1).

Differential splicing of fourth exon of NKCC2 results in three variants termed isoform F, isoform A and isoform B (Payne and Forbush, 1994), which vary in amino acid sequence in a region of the protein involved in the predicted second transmembrane domain and part of the subsequent first intracellular loop. These isoforms differ in their transcript levels, localisation and ion affinity properties (for review, see Castrop and Schnermann, 2008).

Despite the importance of NKCC2 in NaCl homeostasis, little is known about the membrane trafficking pathways controlling its apical membrane levels

Figure 1 | Schematic representation of ion transporters operating in the cells lining the TAL of Henle's loop
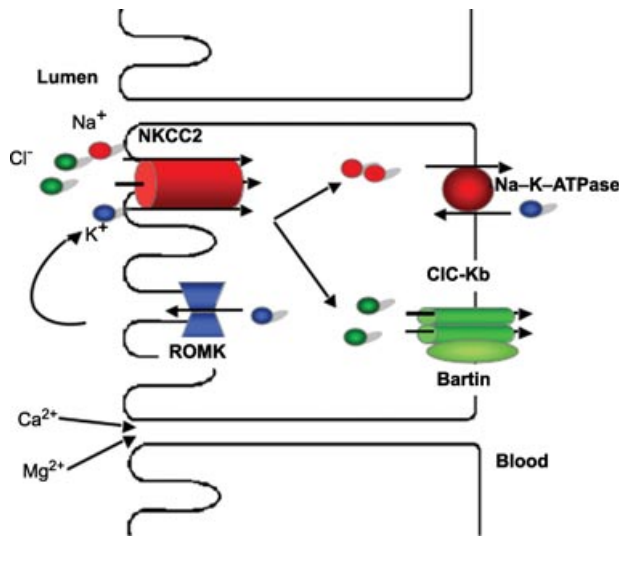
or the physiological relevance of such mechanisms in TAL.

Several advances towards understanding how NKCC2 is regulated by hormones, phosphorylation, glycosylation, Soluble NSF attachment protein receptors (SNAREs), lipid rafts and cytoskeleton have been done. The present review, after an overview on the role of NKCC2 in the regulation of blood pressure and in the pathogenesis of hypertension, however, will focus on particular elements of the NKCC2 regulatory machinery such as the NKCC2-interacting proteins.

An old adage says: 'Show me your friends, and I'll know who you are'. In the same way, finding interacting partners for NKCC2 can help to clarify its physiological function and give new insight on the molecular basis of hypertension and Bartter syndrome, providing new therapeutic tools to detect, prevent and treat these diseases.

NKCC2 in the regulation of blood pressure

The most compelling evidence for the role of kidney in the pathogenesis of hypertension comes from cross-transplantation studies between hypertensive strains of rats such as Dahl salt-sensitive (DS) rats (Dahl et al., 1974), Milan hypertensive rats (Bianchi et al., 1974) and Prague hypertensive rats (Heller et al., 1993) and their corresponding normotensive

strains. In these animal models, it has been demonstrated that arterial hypertension can be transferred with a renal graft from hypertensive strains to normotensive histocompatible recipients. Furthermore, renal grafts from the respective normotensive control strains lowered arterial pressure in these three genetically hypertensive rat strains. Thus, it has been consistently shown that renal mechanisms play a major role in the maintenance of genetic forms of hypertension.

NKCC2 is responsible for 25% of active sodium reabsorption in kidneys. It is, therefore, an important factor in the regulation of circulating fluid volume and long-term blood pressure control. The physiological importance of NKCC2 cotransporter in the regulation of blood pressure has been well established with the use of loop diuretics bumetanide and furosemide that act as functional blockers of the cotransporter and are amongst the most powerful antihypertensive drug available to date (Shankar and Brater, 2003). Moreover, mutations of NKCC2 have been linked to the Bartter syndrome type I, a severe human genetic disease characterised by hypokalaemic alkalosis, hypercalciuria, polyuria and salt wasting (Simon et al., 1996). Hypotension, that can be associated with this rare autosomic recessive disease, represents a lower extreme in the spectrum of salt reabsorption and blood pressure. Of note, the analysis of more subtle phenotypes associated with the heterozygous state of this disease showed that nine rare independent mutations in the gene encoding NKCC2 are associated with clinically reduced blood pressure and protection from hypertension (Ji et al., 2008). Interestingly, two independent studies showed that these mutations result in NKCC2 proteins with impaired transport activity caused by defects in protein processing, trafficking, turnover, regulation and ion affinity (Acuna et al., 2011; Monette et al., 2011).

Opposite to genetic inactivation of NKCC2, enhanced activity of NKCC2 has been linked to salt-sensitive hypertension and hypertensive disorders in humans. For instance, in African Americans, the incidence of salt-sensitive hypertension is higher than in whites (Aviv et al., 2004). This race group produces more concentrated urine, retains more Na^+, Ca^{2+} and Mg^{2+} and has higher TAL NaCl reabsorption based on furosemide-induced diuresis and water-loading studies, as reviewed by Jung et al. (2011).

NKCC2 in animal models of hypertension and Bartter syndrome

Interestingly, NKCC2 was found to be upregulated in several genetic animal models of hypertension. In 2004, Sonalker et al. showed that NKCC2 expression was fourfold higher in spontaneous hypertensive rats (SHR) as compared with the normotensive strain Wistar–Kyoto rat. This increase was not related to increased levels of NKCC2 mRNA, suggesting that NKCC2 overexpression was due to a greater translational efficiency or enhanced protein stability in SHR rats. The same group later showed that progression from prehypertensive to hypertensive state (5–8 wks) in SHR was accompanied by a twofold to fourfold increase in surface-to-intracellular ratio of NKCC2 as compared with the control rats (Sonalker et al., 2007). This indicates that NKCC2 trafficking may be affected in such a way in SHR rats that enhanced membrane NKCC2 levels correlates with the development of hypertension.

Another animal model of salt-sensitive hypertension is represented by DS rats, in which the kidney possesses a genetic alteration that determines an inability to excrete sodium (Dahl and Heine, 1975). Alvarez-Guerra and Garay (2002) found that the NKCC2 activity was higher in DS rats than in Dahl salt-resistant strain. Moreover, salt-sensitive rats exhibited higher bumetanide-induced diuresis and natriuresis, consistent with enhanced NKCC2 activity (Alvarez-Guerra and Garay, 2002). Interestingly, renal NKCC2 expression decreased in DS rats, thus suggesting that a post-transcriptional mechanism was again involved in the increased activity of NKCC2 in these rats. Of note, Ortiz and co-workers (2011) recently showed that high salt (HS) intake decreased surface NKCC2 expression in TALs from salt-resistant strain without affecting both total and phosphorylated NKCC2 (p-NKCC2) expression. In contrast, in salt-sensitive rats, HS intake significantly increased surface NKCC2 expression, again without affecting either total or p-NKCC2 expression levels, thus suggesting that HS intake affects the intracellular trafficking of NKCC2 towards the apical membrane in these rats. The enhanced NKCC2 surface expression in salt-sensitive rats might contribute to enhanced NaCl reabsorption and consequent hypertension during HS intake (Haque et al., 2011).

Another model of genetic hypertension is represented by the Milan hypertensive strain (MHS) that develops hypertension (Bianchi et al., 1975) because of increased activity and expression of Na^+–K^+–ATPase (Ferrandi et al., 1990; Melzi et al., 1991), associated with a point mutation of adducin (Tripodi et al., 1996), a cytoskeletal protein involved in actin polymerisation and cell signal transduction (Hughes and Bennett, 1995). This mutation affects the endocytosis of the sodium pump that may be an important contributing factor for the increased ability of the renal tubule cells to reabsorb sodium (Efendiev et al., 2004; Torielli et al., 2008).

It has been demonstrated that the increase in Na^+ entry during the induction phase of hypertension in young MHS rats is paralleled by an upregulation of NKCC2 at the level of TAL, suggesting a key role of this cotransporter in the genesis of hypertension in this rat model (Capasso et al., 2005). In contrast, the expression of NKCC2 in the TAL of adult Milan rats with established hypertension remained unchanged as compared with the age-matched controls (Capasso et al., 2008). However, Carmosino et al. recently demonstrated that NKCC2 activity is significantly upregulated in the TAL of adult MHS rats and that the mechanism, mediated by NKCC2 phosphorylation and consequent cotransporter activation (Carmosino et al., 2011), does not rely on the increases in NKCC2 mRNA and protein levels, in agreement with previously reported data (Capasso et al., 2008). Moreover, acute furosemide administration induced a greater natriuresis and diuresis response in MHS than in normotensive strain, confirming that NKCC2 activity is actually upregulated in MHS rats. Of note, chronic administration of furosemide *in vivo* revealed that the increase in the blood pressure in MHS rats was prevented convincingly, demonstrating that NKCC2 is implicated in the pathogenesis of salt-sensitive hypertension in MHS rats (Carmosino et al., 2011).

A mouse model of the Bartter syndrome type I has been generated by disrupting the *NKCC2* gene. However, mice homozygous for this loss-of-function mutation of NKCC2 do not survive (Takahashi et al., 2000).

Indeed, to study the phenotype resulting from a loss-of-function mutation of NKCC2, mice lacking one copy of the *NKCC2* gene (NKCC2+/–) have been generated and studied (Takahashi et al., 2002).

Interestingly, the disruption of one copy of the *NKCC2* gene had no direct effect on the physiologic

phenotype of the heterozygous mice in terms of blood pressure, plasma renin concentration and water and electrolyte handling by the kidney.

Of note, the amount of NKCC2 protein expressed in the kidney of NKCC2+/–mice was not distinguishable from that of wild-type (WT) mice, despite the 50% level of NKCC2 mRNA, and the amount and cellular distribution of NKCC2 protein in NKCC2+/–mice was likewise indistinguishable from the WT (Takahashi et al., 2002). These observations can be explained only by an increase in effective half-life and membrane stability of NKCC2 protein in NKCC2+/–mice.

It is worthy to underline that in all animal models described so far, NKCC2 appears to be upregulated by post-translational mechanisms such as increased membrane expression stability and phosphorylation, suggesting the importance of studies on the factors regulating the activity and trafficking of NKCC2 to gain insight into the regulation of NKCC2 in physiopathological conditions.

NKCC2 intracellular trafficking

Immunolocalisation experiments revealed that NKCC2 is expressed not only at the cell surface, but also in a population of subapical vesicles, raising the hypothesis that intracellular trafficking may regulate NKCC2 membrane expression (Nielsen et al., 1998). In agreement with this hypothesis, vasopressin (AVP) induces shuttling of these vesicles to the cell membrane, leading to an increase in apical NKCC2 protein expression and activity (Gimenez and Forbush, 2003). Likewise, recent reports showed that cAMP increases surface expression of NKCC2 in rat TALs (Ortiz, 2006). However, Caceres et al. (2009) showed that NKCC2 undergoes constitutive exocytosis in TALs, suggesting that trafficking of NKCC2 is a continuous dynamic process rather than a triggered event. These data suggest that steady-state surface levels are maintained by endocytosis and perhaps recycling of internalised NKCC2 in the absence of stimuli. Moreover, Ares and Ortiz (2011) studied the endocytosis and recycling of NKCC2 in the absence of stimulation and found that NKCC2 undergoes constitutive retrieval from the plasma membrane at a rate that closely matches the reported exocytic insertion. In addition, a fraction of internalised NKCC2 (30–40% in 30 min) recycles back to the surface (Ares

and Ortiz, 2011). In chase experiments, the surface NKCC2 pool showed a relatively slow rate of degradation with 25% of the surface NKCC2 pool degraded over 60 min in TALs (Ares and Ortiz, 2011). These data again indicate that dynamic trafficking of NKCC2 occurs under baseline conditions in the absence of stimuli.

Interestingly, the same authors found that cholesterol depletion completely blocked NKCC2 endocytosis, enhanced surface NKCC2 and net Cl⁻ transport by TALs, indicating that constitutive trafficking of NKCC2 is essential for reabsorptive function of TAL (Ares and Ortiz, 2011).

NKCC2-interacting proteins

Both constitutive and stimulated intracellular trafficking mainly involves protein–protein interactions, making the study of macromolecular complexing an emerging field of research.

Currently, little is known about NKCC2-interacting proteins and regulation in renal cells, mainly because of the difficulty in stably expressing the cotransporter protein in epithelial cells. It has been found that the presence of the N-terminus of NKCC2 in any construct appears to prevent good functional expression in human embryonic kidney (HEK) cells as well as stable expression in epithelial cells (Isenring et al., 1998). Indeed, the study of NKCC2-interacting proteins can be performed either in epithelial cells stably expressing NKCC2 chimeric constructs containing the N-terminal tail of close homologous NKCC1 cotransporter or alternatively in cells transiently transfected with full-length NKCC2.

Ste20-related praline–alanine-rich kinase–oxidative stress response kinase interacts with and phosphorylates NKCC2

Ste20-related praline–alanine-rich kinase (SPAK) and oxidative stress response kinase (OSR1) were the first 'putative' NKCC2-interacting proteins identified by using yeast two-hybrid screening by Piechotta et al. (2002). They are two serine–threonine kinases which have been linked to several key cellular processes, including cell differentiation, cell transformation and proliferation, cytoskeleton rearrangement and, most recently, regulation of ion transporters.

Only recently, Richardson et al. (2011) finally established by co-immunoprecipitation experiments in NKCC2-expressing HEK cells that the SPAK and OSR1 kinases interact with an Arg-Phe-Gln-Val (RFQV) motif on NKCC2 N-terminal tail. Interestingly, constitutively active mutant of SPAK–OSR1 directly phosphorylates the regulatory sites Thr95, Thr100, Thr105 and Ser91 in the N-terminal tail of NKCC2, significantly increasing NKCC2 activity. Of note, there was a significant amount of NKCC2 localised at the plasma membrane under basic conditions, and mutation of all identified phosphorylation sites on NKCC2 did not alter the membrane localisation of NKCC2 in HEK cells. These data suggest that interaction of NKCC2 with SPAK and consequent NKCC2 phosphorylation stimulates NKCC2 activity independently of trafficking into and out of the apical membrane, but by a mechanism regulating protein expression, degradation and membrane stability. Moreover, a knocked-in mouse expressing mutant inactive SPAK (T243A) showed not only a decrease in NKCC2 phosphorylation, but also in NKCC2 expression, corroborating this hypothesis (Rafiqi et al., 2011).

Nevertheless, it is important to note that it has been reported that AVP induces NKCC2 phosphorylation at the same residues as that of SPAK and, consequently, its trafficking to the apical membrane of the TAL *in vivo*, suggesting that under certain stimulation conditions, NKCC2 traffics to the plasma membrane in response to phosphorylation (Gimenez and Forbush, 2003). It still remains unclear whether SPAK activity is necessary for AVP-induced trafficking of NKCC2.

To address these issues, it would be important to study, in both steady state and under AVP stimulation, membrane localisation of endogenous NKCC2 in animal knockout (KO) for either SPAK or OSR1, so far reported to show impaired NKCC2 phosphorylation (Yang et al., 2011; Lin et al., 2011).

Ponce-Coria et al. (2008) added a new tassel to the puzzle of NKCC2–SPAK functional interaction. The intracellular chloride depletion in *Xenopus laevis* oocytes, achieved by low-chloride hypotonic stress, activates NKCC2 by promoting phosphorylation of the three regulatory threonines in the amino terminus. Interestingly, they found that the chloride-sensitive activation of NKCC2 requires interaction not only of SPAK, but also another serine–threonine kinase–with-no-lysine kinase (WNK3, related to WNK1 and WNK4; genes mutated in a Mendelian form of hypertension). WNK3 is positioned upstream of SPAK and appears to be the chloride-sensitive kinase, which interacts with and phosphorylates SPAK–OSR1 in response to low Cl$^-$ concentration, enabling SPAK–OSR1 to physically associate with, phosphorylate and activate NKCC2.

Recently, mice KO for kidney-specific WNK1 isoform (KS-WNK1) were generated and shown to exhibit higher blood pressure and higher NKCC2 expression without a change in phosphorylation-to-total ratio as compared with the WT mice. Conversely, transgenic mice overexpressing KS-WNK1 showed reduced blood pressure, lower NKCC2 expression (60% decrease) and unchanged phosphorylation-to-total NKCC2 ratio. Thus, it appears that KS-WNK1 is involved in the regulation of NKCC2 expression, stability and/or turnover (Liu et al., 2011).

Interestingly, SPAK–OSR1 kinases also associate and phosphorylate the secretory NKCC isoform NKCC1 and the structural related transporters KCC1–KCC4 and NCC, suggesting that interaction with these kinases is a common feature of the superfamily of SLC12 cotransporters (Ponce-Coria et al., 2008).

From the data presented above, it is evident that SPAK interacts and phosphorylates NKCC2, increasing its activity. However, whether phosphorylation by SPAK stimulates NKCC2 membrane expression and which are the physiological mechanisms that stimulate SPAK or OSR1 in the TAL are still open questions.

Interestingly, Carmosino et al. (2011) found that in Milan hypertensive rats, the increase in NKCC2 phosphorylation is paralleled by an increase in SPAK activation in the kidney's outer medulla, suggesting that the pathogenesis of hypertension in these animals might rely on a disregulation of the NKCC2-associated SPAK kinase rather than of NKCC2 itself.

Of note, a recent work by McCormick et al. demonstrated that WNK signalling via SPAK–OSR1 to NKCC2 is neither unidirectional nor always stimulatory because of the existence of either stimulatory or inhibitory SPAK isoforms in the kidney (McCormick et al., 2011). The authors suggested the fascinating hypothesis that extracellular fluid volume depletion shifts SPAK isoform abundance to favour NaCl

retention along TAL, shedding light on a possible physiological mechanism involved in WNK–SPAK–OSR1–NKCC2 complex modulation in the kidney.

NKCC2 forms homodimers

It has been shown that a number of membrane transport proteins are oligomeric. The close NKCC2 homologue, the secretory cotransporter NKCC1, exists as a homodimer in the plasma membrane and this dimer is sufficiently stable that it remains intact after membrane solubilisation in mild detergents (Moore-Hoon and Turner, 2000). Subsequent studies provided evidence that NCCs (de Jong et al., 2003) and KCCs (Casula et al., 2001; Simard et al., 2007) also occur as dimers, suggesting that this may be a common feature of the SLC12 family.

Starremans et al. (2003) showed that human NKCC2 (hNKCC2) when expressed in the plasma membrane of oocytes, forms at least homodimers. Moreover, sucrose gradient centrifugation experiments revealed that hNKCC2 sedimented predominantly as an approximately 320-kDa complex, suggesting at least a dimeric configuration (Starremans et al., 2003).

To investigate the NKCC2 dimeric function more closely, the authors analysed the activity of two hNKCC2 concatemers. The bumetanide-sensitive ^{22}Na$^+$ uptake of the WT–WT concatemer was comparable to that of complexes formed upon injecting of monomeric hNKCC2 constructs. Moreover, the introduction of the Bartter mutation G319R in one of the subunits of the concatemer resulted in a dimer normally processed and expressed at the plasma membrane, but the associated bumetanide-sensitive ^{22}Na$^+$ uptake was half that of the WT–WT concatemer (Starremans et al., 2003). This suggested that both hNKCC2 subunits in a dimer can function as separate transporters. Moreover, these results are in line with the fact that Bartter syndrome is a recessive disorder and thus no pathogenic phenotype would be present in a heterozygous situation. In this condition it is possible either that enough transport capacity remains in WT-mutated dimers or WT–WT dimers are preferentially formed.

Recent studies demonstrated that the C-terminal tails of both NKCC1 and NKCC2 are involved in homodimerisation (Parvin and Turner, 2011). In their studies, Parvin and Turner (2011) showed that NKCC1 molecules lacking their C-termini failed to dimerise and that replacing the C-terminus of NKCC1 with that of NKCC2 produced a fully functional chimeric protein that formed homodimers with another chimeric NKCC cotransporter but did not dimerise with NKCC1.

Different isoforms of NKCC2 are expressed differentially along the distal tubule of the kidney, with the A isoform present in both the medullary and cortical segments of TAL, whereas the F and B isoforms are expressed predominantly in the medullary region and the macula densa, respectively (Payne and Forbush, 1994; Igarashi et al., 1995). Interestingly, differences in ion affinity for the A, B and F NKCC2 isoforms have been shown (Gimenez et al., 2002). Thus, it is possible that where coexpressed, different combinations of NKCC2 isoforms within the dimer can produce NKCC2 transporters with a variety of functional properties.

It would be worth to investigate whether there is any mechanism that regulates the formation of NKCC2 dimers and their physiological role. Dimerisation and oligomerisation can confer several different structural and functional advantages to NKCC2, including improved membrane stability, control over the accessibility and specificity of ion-binding sites and facilitate a rise in the local concentration of Na$^+$, Cl$^-$ and K$^+$.

Aldolase B is involved in regulating the membrane expression of NKCC2

To identify potential interacting partners of NKCC2, Laghmani's group screened a kidney cDNA expression library by the yeast two-hybrid assay using NKCC2 C-terminus as bait (Benziane et al., 2007). Amongst the positive identified clones, several matched the sequence of aldolase B, a glycolytic enzyme that not only has specialised functions in fructose metabolism and gluconeogenesis, but also exerts other functions in the cell as it is an actin-binding protein that exhibits a dynamic interaction with the cytoskeleton (Kusakabe et al., 1997).

The interaction of NKCC2 with aldolase B was confirmed in NKCC2 transiently transfected opossum kidney (OK) cells by co-immunoprecipitation experiments and dual immunolabelling fluorescence microscopy. In addition, aldolase B overexpression promotes NKCC2 retention within these cells, thus decreasing its abundance at the cell surface and its cotransport activity (Benziane et al., 2007).

Aldolase B is involved in the exocytosis of H^+–ATPase (Lu et al., 2007) and the glucose transporter GLUT4 (Kao et al., 1999). Thus, the authors suggested in the hypothesis that this glycolytic enzyme is most likely involved in regulating the constitutive membrane insertion of NKCC2.

Although novel, the physiological relevance of this finding is questionable because aldolase B–NKCC2 interaction has not been investigated in the native tissue and the intracellular trafficking of NKCC2 *in vitro* has been analysed in single nonpolarised renal cells.

Moreover, these authors found that the presence of aldolase substrate, fructose 1,6-bisphosphate in the medium prevents aldolase binding to NKCC2 and aldolase coexpression had no further effect on the cell surface level of NKCC2 (Benziane et al., 2007). These data suggest that changes in the relative concentration of intracellular fructose 1,6-biphosphate in TALs may regulate the interaction of NKCC2 with aldolase B; however, this has not been tested in *vivo*.

Secretory carrier membrane protein 2 retains NKCC2 in a recycling compartment

The same yeast two-hybrid screen described above leads to the identification of another protein interacting with the C-terminus of NKCC2: the vesicle scaffolding protein secretory carrier membrane protein 2 (SCAMP2) (Zaarour et al., 2011). SCAMP2 is a member of the tetraspan family of proteins, which has been shown to be relevant to a broad cross-section of renal transport systems. It has been found that aquaporin-2 (AQP2) water channel (Kamsteeg et al., 2007), Na^+–K^+–ATPase (Pagel et al., 2003) and H^+–K^+–ATPase (Duffield et al., 2003) interact with the members of the tetraspan family of membrane proteins. Furthermore, these interactions appear to play important roles in determining the distribution, stability and regulatory properties of these transport proteins. The interaction between NKCC2 and SCAMP2 has been confirmed in opossum kidney (OK) cells transiently transfected with NKCC2 (Zaarour et al., 2011). Functional studies demonstrated that coexpression of SCAMP2 specifically decreases NKCC2 transport activity by promoting its intracellular retention and therefore reducing its abundance at the cell surface. Endocytosis–exocytosis assays demonstrated that SCAMP2-induced decrease in surface NKCC2 is due to decreased membrane insertion.

A fraction of SCAMP2 colocalises with NKCC2 in Rab11-positive intracellular structures. These data suggest that SCAMP2 retains NKCC2 in a recycling compartment and inhibits the membrane insertion of this recycling fraction. However, the authors did not check whether SCAMP2 and NKCC2 interact in the native tissue, raising the question whether this interaction and its functional role is only a feature of a cellular model exogenously expressing NKCC2.

It is of interest that SCAMP2 also interacts with the COOH-terminus of Na^+–H^+–exchanger-5 (NHE5) (Diering et al., 2009), NHE7 (Lin et al., 2005) as well as with serotonin and dopamine transporters (Muller et al., 2006; Fjorback et al., 2011), and it regulates the surface expression of all of them, suggesting that the interaction with SCAMP2 is rather a common feature of trafficking transporters in both epithelial and neuronal systems.

Myelin and lymphocyte-associated protein–vesicle integral protein of 17 kDa stabilises NKCC2 at the plasma membrane

Carmosino et al. (2011) found that in the rat's kidney, the NKCC2 cotransporter colocalises and interacts with myelin and lymphocyte-associated protein (MAL)–vesicle integral protein of 17 kDa (VIP17), another member of the tetraspan family of proteins. Interestingly, they found that MAL–VIP17 interacts with fully glycosylated and phoshorylated form of NKCC2, suggesting that this interaction occurs specifically at the plasma membrane of TAL cells. To study the physiological role of this interaction, the authors used Lilly Laboratories cell porcine kidney (LLC-PK1) cells cotransfected with MAL–VIP17 and a chimeric NKCC2 construct in which the apical sorting determinants of NKCC2, identified in its C-terminus, have been exchanged into the NKCC1 backbone. The chimera (c-NKCC2) is selectively expressed at the apical domain in epithelial cells and it is functionally active, thus rendering it a useful tool for studying the regulation of NKCC2 in an epithelial cell system (Carmosino et al., 2008). They demonstrated that the interaction with MAL–VIP17 involves the C-terminal tail of NKCC2 and that it is not involved in the apical sorting of NKCC2, but rather it stabilises c-NKCC2 at the apical membrane of LLC-PK1 by inhibiting the steady-state rate of endocytosis of the cotrasnporter (Carmosino et al., 2011).

This result is in agreement with the previous observation made in the same cellular model in which the interaction with MAL–VIP17 slows the internalisation of AQP2, resulting in an increase in the population of the water channel that is present in the apical membrane in the absence of hormonal stimulation (Kamsteeg et al., 2007). Moreover, Carmosino et al. (2011), using the R5 antibody that is specifically directed against p-NKCCs, showed that the stabilisation of c-NKCC2 at the apical membrane of MAL–VIP17 expressing LLC-PK1 coincides with an increase in the extent of cotransporter phosphorylation, underlining the possibility that this interaction has important implication not only in the trafficking but also in NKCC2's function.

It is unclear whether the trafficking mechanism and phosphorylation affected by MAL–VIP17 is the same for the full-length NKCC2 expressed in TAL cells as it is for the NKCC2 chimeric construct in the LLC-PK1 model system.

However, the authors showed that both glycosylation and phosphorylation patterns of NKCC2 were significantly increased in MAL–VIP17-overexpressing transgenic mice as compared with their controls. MAL–VIP17-overexpresing mice develop dramatically amplified renal epithelial cell apical membranes as well as cysts in the tubular system of the kidney, consistent with an important role of MAL–VIP17 in the genesis and development of apical plasma membranes in the kidney (Frank et al., 2000). It has been demonstrated that the glycosylation of NKCC2 is necessary for the correct surface expression of the cotransporter and for the stabilisation of its functional conformation (Paredes et al., 2006). Moreover, p-NKCC2 seems exclusively expressed at the apical membrane of TAL cells (Gimenez and Forbush, 2003). Indeed, the increase in the extent of NKCC2 glycosylation and phosphorylation observed in MAL–VIP17-overexpressing mice might be actually consistent with an increase in the NKCC2 membrane stability, confirming the data obtained in the cellular model. The measure of renal functional parameters in transgenic mice, such as natriuresis and diuresis, would clarify whether the increase in NKCC2 glycosylation and phosphorylation upon MAL–VIP17 overexpression coincides with an increase in the NKCC2 activity. However, the short survival of MAL–VIP17 transgenic mice makes these experiments unfeasible. Of course, further studies are needed to identify the molecular mechanism by which MAL–VIP17 inhibits NKCC2 internalisation.

Interestingly, the predicted membrane-spanning helices of MAL–VIP17 constitute the MAL and related proteins for vesicle trafficking and membrane link (MARVEL) domain, which is found in approximately 20 open reading frames in the human genome (Sanchez-Pulido et al., 2002). The MARVEL domain is also found in the tight-junction-localised protein occludin and in the synaptic-membrane-localised proteins synaptophysin and synaptogyrin. Thus, a common feature of proteins containing the MARVEL domain is their localisation to specialised region within surface membranes. Indeed, it is possible that MAL–VIP17 recruits the NKCC2 cotransporter into a MAL–VIP17-enriched specialised membrane domain in which additional partner proteins involved in NKCC2 regulation are included.

Tamm–Horsfall protein facilitates NKCC2 activation

NKCC2 colocalises with Tamm–Horsfall protein (THP) and both are unique proteins of TAL (Bachmann et al., 2005; Nielsen et al., 1998). The role of THP has remained obscure for many years, but recent studies have permitted a clearer distinction between its intracellular and extracellular functions. The former are related with the urinary concentrating mechanism and potentially interfere with transcellular electrolyte transport (Bachmann et al., 2005; Nielsen et al., 1998), whereas the latter have been associated with anti-inflammatory roles of the glycoprotein in the urinary tract and renal interstitium (Saemann et al., 2005).

In a recent elegant work, Mutig et al. (2011) proposed that THP interacts with NKCC2 and that this interaction facilitates the NKCC2 activation.

By immunogold labelling they revealed a disproportionate accumulation of NKCC2 in the subapical vesicle-enriched compartment of the TAL epithelium in THP KO mice as compared with WT strain with no difference in the membrane expression of NKCC2 in both strains of mice. This may reflect an altered NKCC2 turnover rather than an impaired trafficking of the cotransporter, possibly via impaired degradation of NKCC2 in the absence of THP.

Moreover, the lack of THP coincided with decreased steady-state levels of phospho-NKCC2 in

these mice, likely reflecting diminished activity of the cotransporter under this condition (Mutig et al., 2011). The influence of this interaction on NKCC2 activity has been corroborated in both *in vivo* and *in vitro* experiments. Cultured TAL cells with low endogenous THP levels and low steady-state phosphorylation of NKCC2 displayed a sharp increase in NKCC2 phosphorylation level along with a significant increase in NKCC2 activity upon the ectopic expression of THP (Mutig et al., 2011).

In addition, vasopressin (AVP) stimulation resulted in enhanced NKCC2 phosphorylation in WT mice, whereas in THP KO mice, NKCC2 phosphorylation was significantly blunted upon AVP administration, suggesting a reduced activation of the transporter *in vivo* in the absence of THP. In addition, acute furosemide administration resulted in a greater natriuretic response in THP KO than in WT mice, further supporting the hypothesis of a permissive role of THP for NKCC2-mediated salt reabsorption in TAL *in vivo* (Mutig et al., 2011).

These data clearly demonstrated that NKCC2 and THP functionally interact, but the proof of whether THP is an NKCC2-interacting protein is still lacking. THP is an abundantly synthesised protein of TAL cells specifically expressed in the membrane lipid rafts as well as NKCC2, thus indicating that the two proteins share lipid raft localisation.

The same authors demonstrated that activation of NKCC2 is dependent on its presence in lipid rafts (Welker et al., 2008). Thus, it is possible that THP could affect the function of rafts to provide scaffolding platforms that may promote interactions of relevant kinases with NKCC2.

Interestingly, mutations of THP are associated with disease such as familial juvenile hyperuricemic nephropathy and autosomal dominant medullary cystic kidney disease type 2, characterised by impaired urinary concentrating ability. Therefore, the NKCC2–THP interaction provide convincing support for the role of THP in the functioning of the renal concentrating mechanism and it may further explain urinary concentration defects in patients with THP mutations.

Conclusions and perspectives

In animal models of hypertension, NKCC2 appears to be regulated by post-translational mechanisms such as increased membrane expression/stability and phos-

Figure 2 | Working model for the NKCC2-interacting proteins identified so far
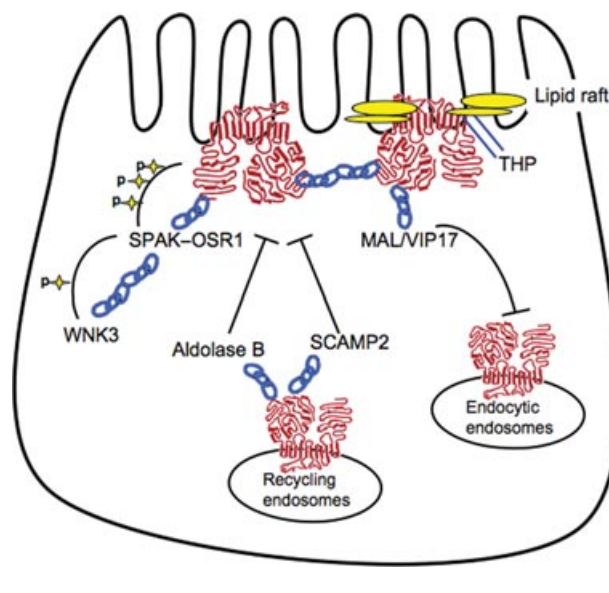
phorylation rather than NKCC2 expression level, thus underlining the clinical relevance to analyse the molecular mechanisms involved in the regulation of NKCC2 trafficking.

It is becoming increasingly clear that ion transport proteins do not exist as isolated units in the membranes of living cells. Instead, they appear to participate in an extremely wide array of interactions that help to regulate their localisation, intracellular trafficking, life span and susceptibility to control transduction machinery by signals. Of note, it has been demonstrated that NKCC2 is expressed at the plasma membrane as functional homodimer and that the interaction with MAL–VIP17 through the C-terminal tail stabilises the cotransporter in this compartment, increasing its susceptibility to be phosphorylated. Moreover, SPAK and OSR1 directly bind the N-terminal tail of NKCC2, dramatically increasing the phosphorylation and the activity of the cotransporter. Two NKCC2-interacting proteins appear to be involved in the intracellular trafficking of this cotransporter, aldolase B and SCAMP2, both of them interact with the C-terminal tail of NKCC2 and when overexpressed, inhibit the insertion of NKCC2 in the apical membrane. THP has been shown to be a crucial regulator of NKCC2 activity *in vivo*, most likely introducing NKCC2 in lipid rafts (Figure 2).

NKCC2 expression is restricted to the TAL and macula densa cells. Most likely interacting proteins specifically expressed in these nephron cell types are required for correct apical trafficking, maturation, functional expression and regulation of NKCC2.

Unfortunately, almost all NKCC2-interacting proteins found so far have been identified and studied in heterologous systems using nonepithelial cells, transient transfections or chimeric constructs and thus their role in controlling NKCC2-dependent TAL function remains elusive. Indeed, it is an essential and challenging task to unravel NKCC2–protein interactions in their actual *in vivo* context.

Several advances have been made in the biochemical approaches coupled to mass spectrometry to study the protein–protein interactions in the physiological environment and in native conditions. It would be worth to apply these techniques to isolated TAL preparations from different animal models to identify NKCC2 containing multiprotein complexes in different physiopathological conditions.

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Conflict of interest statement

The authors have declared no conflict of interest.

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