Published in final edited form as:

Crit Rev Biochem Mol Biol. 2012 ; 47(4): 349–359. doi:10.3109/10409238.2012.678285.

Organization of the ENaC-regulatory machinery

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Abstract

The control of fluid and electrolyte homeostasis in vertebrates requires the integration of a diverse set of signaling inputs, which control epithelial Na⁺ transport, the principal ionic component of extracellular fluid. The key site of regulation is a segment of the kidney tubules, frequently termed the aldosterone-sensitive distal nephron, wherein the epithelial Na⁺ channel (or ENaC) mediates apical ion entry. Na⁺ transport in this segment is strongly regulated by the salt-retaining hormone, aldosterone, which acts through the mineralocorticoid receptor (MR) to influence the expression of a selected set of target genes, most notably the serine-threonine kinase SGK1, which phosphorylates and inhibits the E3 ubiquitin ligase Nedd4-2. It has long been known that ENaC activity is tightly regulated in vertebrate epithelia. Recent evidence suggests that SGK1 and Nedd4-2, along with other ENaC-regulatory proteins, physically associate with each other and with ENaC in a multi-protein complex. The various components of the complex are regulated by diverse signaling networks, including steroid receptor-, PI3-kinase-, mTOR-, and Raf-MEK-ERK-dependent pathways. In this review, we focus on the organization of the targets of these pathways by multi-domain scaffold proteins and lipid platforms into a unified complex, thereby providing a molecular basis for signal integration in the control of ENaC.

Keywords

Aldosterone signaling; ENaC-regulatory complex; scaffold protein; PDZ domains; lipid rafts; mTOR; PI3K; GILZ1; SGK1; Nedd4-2

Hormonal regulation of sodium (Na⁺) transport

Regulating blood pressure is intimately linked to the kidney's ability to maintain normal Na⁺ balance and circulatory volume. The kidneys of virtually all vertebrates use a strategy of nonselective filtration of water and solutes in the glomerulus coupled to their selective reabsorption by the renal tubules. This provides for tremendous capacity to adapt to wide variations in environmental conditions, particularly changes in water and ion availability.

Even subtle disruptions in kidney function play a role in most (if not all) cases of high blood pressure, a major risk factor for atherosclerotic vascular disease that can lead to malignant hypertension, stroke or even death.

The mineralocorticoid aldosterone is the principal regulator of Na^+ homeostasis, and is arguably the single most important physiological regulator of extracellular fluid volume and blood pressure in mammals. The epithelial Na^+ channel (ENaC), mediates apical entry of Na^+ in the aldosterone-sensitive distal nephron (aldosterone-sensitive distal nephron: last third of the distal convoluted tubule, connecting segment, and cortical and medullary collecting ducts, and is referred to here for brevity as “collecting duct” (CD)), and constitutes the rate-limiting step for Na^+ reabsorption by the kidney (Thomas & Itani, 2004). Aldosterone is an important physiologic regulator of ENaC activity and along with its principal receptor, the mineralocorticoid receptor (MR), has been implicated in a variety of disease states in humans (Pearce *et al.*, 2003). Aldosterone also has pronounced effects on K^+ , Cl^- , and H^+ transport. The reabsorption of Na^+ and Cl^- in particular establish osmotic gradients, which provide the driving force for water reabsorption, thus sustaining extracellular fluid volume. The critical roles of aldosterone and MR in mammalian homeostasis are well-established. Indeed, survival on a low NaCl or high K^+ diet is not possible in the absence of aldosterone or MR (Berger *et al.*, 1998, Bleich *et al.*, 1999), while hormone overproduction or constitutive receptor activity cause severe hypertension and electrolyte disturbances (Lifton *et al.*, 2001, Soundararajan *et al.*, 2010a).

Aldosterone acts primarily through a transcriptional mechanism via the MR, a hormone-activated transcription factor, which upon binding hormone undergoes a conformational change, translocates to the nucleus and regulates (stimulates or represses) a subset of target genes (Pearce *et al.*, 2003). The identification and characterization of aldosterone-regulated genes involved in the control of ion transport thus forms a crucial part of aldosterone research. In tight epithelia (most notably CD and colon), MR targets include transporter or channel subunits such as αENaC and the α -subunit of the basolateral Na , K -ATPase, and regulatory proteins such as SGK1 (serum-and-glucocorticoid-induced-kinase-1) (McCormick *et al.*, 2005) and GILZ1 (glucocorticoid-induced leucine zipper protein 1) (Muller *et al.*, 2003), and possibly K-WNK (the kidney isoform of “with-no-lysine” kinase 1) (Naray-Fejes-Toth *et al.*, 2004) and K-Ras (Stockand *et al.*, 1999). Much of the early work on MR target genes focused on the transporters themselves (most notably, Na , K -ATPase, ENaC, or proton pump subunits), and enzymes of intermediary metabolism (most notably citrate synthase) (Verrey, 1999). Recently, it has become clear that another group of targets—likely the most important category for the early effects of aldosterone and other hormones—is comprised of signaling proteins that modulate channel/transporter trafficking and/or activity.

ENaC regulation: a complex multicomponent system involving pleiotropic signaling pathways

ENaC is an apically located Na^+ -selective ion channel comprised of three structurally related subunits, termed α , β , and γ . Each subunit has two membrane spanning domains separated by a large extracellular region, and short N- and C-terminal cytoplasmic tails

(Canessa *et al.*, 1995, Canessa *et al.*, 1994). In principle, regulation can impact on the inherent activity of the channel (for example, by altering open probability (P_o), single channel conductance or cycling rates), or its plasma membrane abundance (by altering synthesis, delivery, removal or degradation) and has been extensively reviewed (Snyder, 2002, Rossier, 2002, Bhalla & Hallows, 2008, Sheng *et al.*, 2008, Kleyman *et al.*, 2009, Eaton *et al.*, 2001). P_o is markedly influenced by proteolytic processing and by interactions of channel cytoplasmic domains with specific membrane acidic phospholipids. Aldosterone profoundly stimulates ENaC activity, and also its expression. Transcriptional regulation and post-translational modifications of ENaC subunits constitute important regulatory mechanisms (Zhang *et al.*, 2007, Bhalla & Hallows, 2008, Sheng *et al.*, 2008, Kleyman *et al.*, 2009). Various studies have suggested crosstalk and convergence between aldosterone and these other stimulatory and inhibitory pathways (reviewed in (Bhalla *et al.*, 2006) and (Humphreys, 2004, Soundararajan *et al.*, 2010b)). These multiple modes of regulation of expression, protein-protein interactions, and processing of ENaC are integrated to result in net membrane incorporation and activity of the channel.

The downstream events of aldosterone action include complex alterations in intracellular signaling pathways that share common components with the insulin-signaling machinery. Aldosterone-mediated Na^+ transport includes the function of the mammalian target of rapamycin complex-2 (mTORC2)-dependent serine-threonine kinase SGK1, whose activity is governed by phosphatidylinositol 3'-kinase (PI3K) and the glucose-regulatory hormone, insulin (Pearce *et al.*, 2003). Insulin binding to its transmembrane tyrosine kinase receptor triggers two major kinase cascades: the PI3K- and the mitogen-activated protein kinase (MAPK) pathways, which mediate the various functions of insulin. Integration of multiple downstream signals determines the net effect of these hormones on Na^+ transport.

The Raf-MEK1/2-ERK1/2 kinase cascade and ENaC

Growth factors like the epidermal growth factor (EGF), act through receptor tyrosine kinases (RTKs) (EGF receptor), and inhibit Na^+ current in renal CD cells, by activating the MAPK pathway (Grossmann *et al.*, 2004). There are at least five distinct MAPK-signaling modules in mammalian cells, which impact on a wide array of cellular activities (Schaeffer & Weber, 1999). The major module implicated in ENaC regulation proceeds through Raf, MEK1/2, and extracellular-signal-regulated kinase (ERK)1/2. The general mechanisms leading to ERK1/2 activation have been well-characterized (Kolch, 2000, Baccarini, 2005). Importantly, several interconnected inputs are recognized, including RTKs, such as the EGF receptor, integrins acting through focal adhesion kinase and protein kinase C. RTK activation through autophosphorylation results in recruitment of adaptor proteins GRB2 and SOS; SOS is a guanine nucleotide exchange factor, which triggers the replacement of GDP by GTP in the small G-protein, Ras. GTP-Ras recruits the MAPK kinase, Raf, to the plasma membrane (and possibly endosomal membranes) where it is phosphorylated by multiple activating phosphorylations, which trigger recruitment and phosphorylation of MEK1/2, and ultimately ERK1 and ERK2 (also known as p44 and p42, respectively, and commonly clubbed together as ERK1/2 or simply ERK). Over 160 substrates have been described for these ERKs including transcription factors, protein kinases and phosphatases, cytoskeletal

elements, and regulators of apoptosis, which mediate the various effects of this complex signaling cascade (Yoon & Seger, 2006).

In the mammalian kidney, ERK is expressed in all nephron segments, and plays an important role in multiple physiological (O'Brien *et al.*, 2004) and pathological processes (Nagao *et al.*, 2003). In the distal nephron, most evidence supports an inhibitory effect on ENaC, either tonic or in response to EGF (Shen & Cotton, 2003, Falin *et al.*, 2005, Shi *et al.*, 2002, Soundararajan *et al.*, 2005), although one report suggested that short term (<1 h) blockade of ERK with pharmacologic inhibitors may diminish Na⁺ current in CD cells (Michlig *et al.*, 2004). The mechanistic basis of ERK inhibition of ENaC has also been addressed (Shi *et al.*, 2002, Booth & Stockand, 2003). In one report, robust *in vitro* evidence supported the hypothesis that ERK directly phosphorylates threonines in the cytoplasmic tail of β - and γ -ENaC subunits, leading to enhanced interaction of the channel with the E3 ubiquitin ligase Nedd4 (neural precursor cell expressed, developmentally downregulated 4). Mutant channels with Thr-Ala substitution at the ERK substrate site had enhanced activity when expressed in *Xenopus* oocytes, consistent with an earlier report (Schild *et al.*, 1996). In another report, protein kinase C-induced degradation of β - and γ -ENaC subunits was shown to require MEK-ERK (Booth & Stockand, 2003). Potential physiologic roles for this inhibition include: (i) control of ENaC activity in states of hypotonic osmotic shock; (ii) desensitization of the aldosterone response (Husted *et al.*, 2000); (iii) mediating Ca²⁺-induced inhibition of ENaC; (iv) establishing basal levels of transport, which are then upregulated through inhibition of ERK. These physiologic roles are not mutually exclusive, however, the baseline activation of ERK in CD (Masaki *et al.*, 2004) and cultured CD cells (Soundararajan *et al.*, 2005) are notable.

In kidney CD as well as in mpkCCD_{c14} cells, a highly differentiated mouse CD cell line, aldosterone coordinately induces the expression of a small chaperone protein called GILZ1 (Robert-Nicoud *et al.*, 2001, Soundararajan *et al.*, 2007). Studies in multiple model systems have shown that GILZ1 stimulates ENaC cell surface expression and activity, at least in part by inhibiting ERK (Soundararajan *et al.*, 2005, Soundararajan *et al.*, 2009, Soundararajan *et al.*, 2010b, Soundararajan *et al.*, 2007). GILZ1 also protects SGK1 (a well-described ENaC-stimulator) from rapid endoplasmic reticulum-associated degradation (ERAD) by controlling its protein-protein interactions and availability in specific subcellular compartments, thereby indirectly facilitating ENaC stimulation (Soundararajan *et al.*, 2010b).

One role that has been suggested for ERK-dependent inhibition of ENaC is that it serves as a brake on the effects of activating hormones such as aldosterone. However, in contrast to other nephron segments, ERK appears to have basal activity in distal nephron cells (Andreucci *et al.*, 2003, Fujita *et al.*, 2004, Michlig *et al.*, 2004, Masaki *et al.*, 2004), consistent with tonic inhibition of Na⁺ transport by the ERK pathway. It appears that tonic ENaC inhibition by ERK is implicated in setting baseline Na⁺ transport, and that aldosterone stimulates Na⁺ transport in part by increasing GILZ1 expression, which inhibits ERK signaling, and hence acts in concert with SGK1 to enhance ENaC-mediated Na⁺ current. In this regard, it should be noted that the well-described ENaC-stimulatory effects of Ras are mediated through PI3K, and not MEK-ERK (Staruschenko *et al.*, 2004b) (more below).

The PI3K pathway

PI3K is a lipid kinase that is activated by insulin/growth factor RTKs and G-protein-coupled receptors (Figure 1). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce phosphatidylinositol 3,4,5-trisphosphate (PIP₃). In turn, PIP₃ recruits to the plasma membrane several pleckstrin homology (PH) domain-containing proteins, including the phosphoinositide-dependent kinase-1 (PDK1), which then phosphorylate specific downstream targets.

It is well-established that the PI3K pathway is stimulatory to ENaC. PI3K signaling is believed to stimulate ENaC activity through at least two distinct mechanisms, although these may not be mutually exclusive. The first of these proposed mechanisms involves activation and recruitment of SGK1 to ENaC. Aldosterone stimulates SGK1 expression, and insulin activates it in an mTORC2/PI3K-dependent manner (Bhargava *et al.*, 2004, Wang *et al.*, 2001, Lu *et al.*, 2010) (more on mTORC below). SGK1 stimulation of ENaC involves negative regulation of the E3 ubiquitin ligase, Nedd4-2 (neural precursor cell expressed, developmentally downregulated 4-2). Nedd4-2 interacts with the C-terminal tails of ENaC subunits, decreases surface expression of the channel via channel ubiquitinylation, and hence inhibits Na⁺ currents (Staub *et al.*, 1996). SGK1 physically interacts with Nedd4-2, phosphorylates and inhibits it (Debonneville *et al.*, 2001, Flores *et al.*, 2005, Snyder *et al.*, 2002) within an ENaC-regulatory complex (ERC) (Soundararajan *et al.*, 2009), and hence indirectly enhances cell surface expression of ENaC (Alvarez de la Rosa & Canessa, 2003, Alvarez de la Rosa *et al.*, 1999). Increased residency time of the channel at the apical membrane results in increased Na⁺ reabsorption. It is now known that in renal cells, SGK1 phosphorylation inhibits Nedd4-2 by inducing an inhibitory interaction with 14-3-3 proteins (reviewed in (Bhalla *et al.*, 2006)). SGK1 also phosphorylates and inhibits Raf (Soundararajan *et al.*, 2009), thereby further contributing to ENaC activation by “disinhibiting” ENaC-inhibitory pathways. Besides impeding retrieval of ENaC from the apical membrane, there is also recent evidence demonstrating that cell surface associated-SGK1 can directly regulate ENaC P_o (Thomas *et al.*, 2011).

The second mechanism of PI3K stimulation of ENaC involves the phosphoinositide products generated by this kinase in renal epithelial cells. PIP₂ and PIP₃ are both physiologically important second messengers. Emerging evidence suggests that these phosphoinositide products bind ENaC directly and enhance ENaC P_o within the apical membrane (Tong *et al.*, 2004, Staruschenko *et al.*, 2004b, Pochynyuk *et al.*, 2005, Staruschenko *et al.*, 2007). Small G proteins such as K-RasA (within the Ras family) and RhoA and Rac-1 (within the Rho family) have been shown to augment ENaC activity by increasing PI(4,5)P₂ and PI(3,4,5)P₃ levels that influence both ENaC P_o as well as its plasma membrane levels (Staruschenko *et al.*, 2004b, Staruschenko *et al.*, 2005, Staruschenko *et al.*, 2004a). In contrast to aldosterone and insulin, ATP, most likely via PLC linked to a G_q-coupled receptor, decreases PI(4,5)P₂ levels in epithelial cells, and hence decreases ENaC activity (Ma *et al.*, 2002, Kunzelmann *et al.*, 2005).

mTOR complexes and the regulation of ENaC

mTOR is a highly conserved serine-threonine kinase, which integrates multiple inputs including nutrient abundance and hormonal signals to orchestrate a variety of cellular processes including growth, proliferation, and survival (Huang & Manning, 2009, Inoki & Guan, 2006, Laplante & Sabatini, 2009a, Laplante & Sabatini, 2009b, Wullschlegel *et al.*, 2006). This pathway has been intensely investigated for understanding fundamental aspects of cellular physiology, immune cell function, as well as development of therapeutic targets for a broad range of disease states. mTOR is organized into two distinct complexes, mTORC1 and mTORC2, which have distinct targets, and control distinct cellular processes (reviewed in (Su & Jacinto, 2011)). mTORC1 consists of mTOR, raptor, PRAS40, mLST8, and Deptor, whereas mTORC2 contains mTOR, rictor, mSIN1, mLST8, and Deptor. mTORC1 is central to the control of cell growth through its well-characterized effects on protein synthesis and cell cycle progression, which are due in part to hydrophobic motif (HM) phosphorylation of the AGC kinase, p70-S6K, and the oncogene, 4E-BP1 (Fingar *et al.*, 2004, Hay & Sonenberg, 2004). Elucidation of mechanisms of action of mTORC1 has been greatly aided by availability of rapamycin, a small molecule that acutely inhibits mTORC1 (Brown *et al.*, 1994, Kunz *et al.*, 1993). Rapamycin has also been widely used clinically as an immune suppressant and chemotherapeutic agent (Yocum, 1996). By contrast, mTORC2 molecular targets and physiologic functions have been less well-characterized, in part due to lack of availability of specific inhibitors, and unambiguous assignment of physiological functions to this complex has been difficult. A breakthrough in understanding the mechanisms of action of mTORC2 emerged from its identification as the kinase complex responsible for phosphorylation of a serine residue at position 473 (S473) within the HM of mammalian Akt, a key survival kinase (Sarbasov *et al.*, 2005). The HM motif of Akt is homologous to that found in other AGC family kinases including SGK1, raising the possibility that mTORC2 may mediate phosphorylation of the SGK1 HM as well.

Using recently developed chemical inhibitors of mTOR (Feldman *et al.*, 2009) in conjunction with shRNA-mediated knockdown of specific components of mTORC1 or mTORC2, it has been demonstrated that SGK1 HM phosphorylation and SGK1-stimulated Na⁺ transport in kidney epithelial cells are both dependent on mTORC2 activity (Lu *et al.*, 2010). Furthermore, in co-immunoprecipitation experiments, SGK1 was shown to interact selectively with the mTOR-rictor complex (mTORC2), and not with mTORC1 (Lu *et al.*, 2010). Together, these findings strongly support the idea that mTORC2 is the essential SGK1 HM kinase, and that it is essential for the physiologic control of ENaC-mediated Na⁺ transport. A search to identify the specific component(s) within mTORC2 that mediated interaction with SGK1, pointed to mSIN1 (mammalian stress-activated protein kinase-interacting protein) (Lu *et al.*, 2011). A recent study used random mutagenesis to select an mSIN1 mutant that assembles appropriately into mTORC2 but fails to bind SGK1 (Lu *et al.*, 2011). The regulatory characteristics of wild-type and mutant mSIN1 are fully consistent with a role for mSIN1 in mediating mTORC2 regulation of SGK1. In addition to its phosphorylation at the HM by mTORC2, SGK1 is also phosphorylated in a region termed the activation loop by the PI3K-dependent kinase, PDK1 (Figure 1). Both these events are required for full kinase activity (Park *et al.*, 1999, Webster *et al.*, 1993). Activated SGK1 then regulates Na⁺ transport by inhibiting inhibitors of ENaC (Park *et al.*, 1999,

Soundararajan *et al.*, 2009, Soundararajan *et al.*, 2007, Soundararajan *et al.*, 2005, Webster *et al.*, 1993), most notably, the ubiquitin ligase Nedd4-2 (Kamynina *et al.*, 2001a, Kamynina & Staub, 2002, Kamynina *et al.*, 2001b, Snyder *et al.*, 2002, Soundararajan *et al.*, 2009).

Several features of ENaC regulation have contributed to an emerging paradigm in hormone-regulated ion transport: (i) the earliest, and possibly most important actions of mineralocorticoids and other hormones are to alter levels and/or activities of signaling proteins that are themselves ion transporter regulators; (ii) pleiotropic signaling pathways with a wide range of potential targets and activities are “pressed into service” in the context of ion transport regulation, with a remarkable degree of specificity. In the case of ENaC-mediated Na⁺ transport, SGK1 acts within the PI3K/mTORC2-dependent signaling pathway, and GILZ1 acts within the Raf-MEK-ERK pathway; (iii) ENaC is subject to tonic inhibition, and regulatory factors such as SGK1 and GILZ1 are disinhibitory (Bhalla *et al.*, 2006); (iv) hormone regulation impacts substantially on ENaC trafficking, although possibly in conjunction with effects on P_o.

The mTOR-, the PI3K- as well as the MAPK-signaling pathways are all highly pleiotropic—they each have multiple regulatory inputs and several possible cellular outcomes including transformation, proliferation, differentiation, apoptosis, and ion transport. It is interesting then to consider how the cell utilizes the same existing machinery to selectively achieve highly specific effects, for instance Na⁺ reabsorption, without inappropriately activating other possible events. In other words, how is signaling specificity achieved within the cell? In the context of ENaC activity, the answer to this question lies in understanding how ENaC-regulatory signals are organized.

Organization of ENaC-regulatory signals

The control of fluid and electrolyte homeostasis in vertebrates requires the integration of a diverse set of signaling inputs, which must be coordinated to control epithelial Na⁺ transport and thus extracellular fluid volume. It is therefore conceivable that these various regulatory signals are harnessed and channeled appropriately by one or more scaffold protein/s.

In biology, scaffold proteins are critical regulators of many key signaling pathways. They are known to interact with multiple members of a signaling pathway, tethering them into complexes (Peterson-Kaufman *et al.*, 2010). In a recent review (Shaw & Filbert, 2009), Shaw and Filbert define a scaffold protein as a molecule that binds to at least two other regulatory proteins thereby allowing assembly of the signaling complex. Inherent in this definition, is the ability of the scaffold protein to regulate signal transduction and localize signaling complexes (and hence the signaling response) at specific locations of the cell, such as plasma membrane, the cytoplasm, the Golgi, the nucleus, the mitochondria or the endosomes (Kholodenko, 2009).

Interestingly, one of the best studied scaffold proteins in biology regulates signaling via the ERK pathway. The kinase-suppressor-of-Ras (or KSR) was originally described as a positive regulator of the Ras-MAPK signaling cascade in *Drosophila* and *Caenorhabditis elegans* (Therrien *et al.*, 1995, Kornfeld *et al.*, 1995, Sundaram & Han, 1995). It contains five conserved domains: a unique domain-of-unknown-function at the N-terminus, a proline-

rich domain, a cysteine-rich domain, a serine/threonine-rich domain, and a pseudokinase domain at the C-terminus. KSR binds many proteins, including the three main components of the ERK-signaling pathway (Raf, MEK, and ERK). Binding of ERK to KSR depends on the serine/threonine-rich domain of KSR, whereas binding to MEK and Raf occurs through the pseudokinase domain (Claperon & Therrien, 2007). KSR also binds 14-3-3 proteins through two phosphorylation sites at Ser 297 and Ser 392, and this is thought to localize KSR to the cytoplasm (Cacace *et al.*, 1999, Shaw & Filbert, 2009). Following cell stimulation, protein phosphatase 2A (PP2A) dephosphorylates these Ser residues, which releases KSR from 14-3-3 proteins in the cytoplasm. This then allows KSR to bind to the cell membrane where it can interact with activated Ras (Muller *et al.*, 2001) and bring it together with the other members of the complex (Raf, MEK and ERK), thus facilitating the MAPK1/2 signaling cascade. It has been suggested that another scaffold protein, the connector-enhancer-of-KSR-1 (CNKSR1 or CNK1), facilitates the interaction of KSR with Raf (Therrien *et al.*, 1998, Douziech *et al.*, 2006). There are two isoforms of KSR expressed in *Caenorhabditis elegans* and mammals—KSR1 and -2 (Ohmachi *et al.*, 2002, Channavajhala *et al.*, 2003). Deletion of both isoforms in *Caenorhabditis elegans* drastically impairs ERK activation downstream of many receptors (Ohmachi *et al.*, 2002). In mammals, KSR1 is highly expressed in the spleen, thymus, and brain. Peripheral T cells isolated from KSR1-deficient mice exhibited defective proliferation and markedly attenuated cytokine response, confirming that KSR1 is required for efficient ERK signaling (Nguyen *et al.*, 2002). The scaffolding function of KSR thus serves to consolidate the signaling inputs, bring key members of the complex together, and promote efficient signal transduction through the ERK cascade.

It has long been known that ENaC activity is tightly regulated in vertebrate epithelia, and recent evidence suggests that several of the regulatory proteins physically associate with one another and with ENaC in a multiprotein complex, the ERC (Soundararajan *et al.*, 2009, Soundararajan *et al.*, 2012). Organization of diverse regulatory inputs into a unified complex such as the ERC, provides for a novel mechanism for selecting out ENaC-specific signals, and transmitting them appropriately. Through this mechanism, ion transport can be selectively stimulated while having little or no effect on context-inappropriate targets. Harnessing and channeling of signals through such multi-protein complexes would permit the cell to perform coordinate regulation of several simultaneous, possibly divergent, signals at multiple levels, with a remarkable degree of specificity. While there is evidence for the existence of an endogenous ERC (Soundararajan *et al.*, 2009), the integrating component of this complex is still unknown.

It is, however, known that GILZ1 acts as an adaptor protein, that interacts with—and modulates the activity of—multiple components of this ENaC-regulatory machinery (Soundararajan *et al.*, 2005, Soundararajan *et al.*, 2010b, Soundararajan *et al.*, 2009). In the absence of aldosterone, GILZ1 and SGK1 levels are low and the ERC is inhibitory. In this situation, Nedd4-2 and Raf-1 are associated with, and inhibit ENaC. In the presence of aldosterone, GILZ1 and SGK1 levels rise, and they are recruited into the complex, where together they inhibit Raf-1 and Nedd4-2 activity, and therefore stimulate ENaC. Although GILZ1 plays a key role in recruiting SGK1 into this complex (Soundararajan *et al.*, 2010b),

it is difficult to conceive this small adaptor protein as the predominant scaffolding platform that physically organizes the association of a multitude of regulatory factors to selectively govern ENaC surface expression and activity.

Among other proteins reported to directly interact with ENaC and modulate its activity are syntaxins, which are plasma membrane localized t-SNAREs (target-SNAP (Soluble N-ethylmaleimide-sensitive factor Attachment Protein) REceptor proteins) hypothesized to mediate ENaC vesicle trafficking to the membrane (Qi *et al.*, 1999). Plasma membrane-resident SNAREs have been shown to be present in distinct microdomains or clusters, the integrity of which is essential for the exocytotic competence of the cell. Substantial evidence suggests that membrane ENaC levels can be regulated by controlling its exocytic insertion or endocytic retrieval. The SNARE protein syntaxin 1A (S-1A) was initially demonstrated to inhibit ENaC activity when coexpressed with the channel in *Xenopus* oocytes. This inhibition was associated with decreased cell surface ENaC levels, and could be prevented by munc18, an inhibitor of S-1A (Qi *et al.*, 1999). Brefeldin-A experiments showed that S-1A does not influence the retrieval or degradation of ENaC, but rather inhibits its plasma membrane insertion (Peters *et al.*, 2001). Further, in contrast to subunit truncation, a β -ENaC PY mutation did not relieve S-1A inhibition of Na^+ transport confirming that S-1A does not perturb Nedd4 interactions that lead to ENaC endocytosis/degradation (Condliffe *et al.*, 2003). Instead, S-1A was found to interact with ENaC C-terminal tails, and shown to influence surface ENaC levels via direct protein–protein interactions at the C-termini (Condliffe *et al.*, 2003). Studies done in HT-29 cells, a physiologically relevant cell line that expresses both endogenous ENaC and S-1A, and also responds to aldosterone stimulation, suggested that ENaC interacts with multiple domains of S-1A, which differentially regulate channel function (Saxena *et al.*, 2007). It appears that S-1A possesses distinct inhibitory and stimulatory domains that interact with ENaC subunits and regulate its overall activity under physiological conditions, in association with another SNARE protein, SNAP-23 (Saxena *et al.*, 2006). SNAP-23 is believed to interact with the N-terminus of α -ENaC, within a quaternary complex that includes S-1A, and modulate channel activity. S-1A-mediated regulation of ENaC involves multiple mechanisms including the PKA-, PLC-, PI3K-, and ERK1/2-signaling pathways, again, suggesting the assembly of ENaC-regulatory signals at or near the plasma membrane (Saxena *et al.*, 2007).

Such an extensive assembly allowing for complex regulation of ENaC by multiple elements, would require the presence of distinct/ordered microdomains within the cell membrane. Not surprisingly, ENaC has been shown to exist in discrete transitory lipid domains or “lipid rafts” (Hill *et al.*, 2007). The concept of lipid rafts originated from studies on epithelial cell polarity more than 20 years ago (Simons & van Meer, 1988, Simons & Ikonen, 1997, Brown & London, 1998), and its central postulate is the existence of dynamic assemblies of cholesterol and saturated sphingolipids in the exoplasmic/outer leaflet of the plasma membrane lipid bilayer. The membrane surrounding lipid rafts is more fluid and rich in phospholipids with unsaturated fatty acyl chains and cholesterol. In other words, lipid rafts form thicker or more closely packed distinct ordered phases (microdomains) in the bilayer. One of the most important properties of lipid rafts is that they can include or exclude proteins to variable extents. In fact, lipid rafts are thought to serve as dynamic assembly

platforms for proteins on their way to the apical membrane and allow clustering of key proteins in close proximity for functional interactions and signal transduction (Simons & Toomre, 2000). Transmembrane proteins are known to have raft affinity, and in fact may also play a role in stabilizing the coupling between the exoplasmic and cytoplasmic leaflets of the cell membrane (Simons & Toomre, 2000).

Interestingly, while lipid rafts are most abundant at the plasma membrane, they can also be found in the biosynthetic and endocytic pathways. Cholesterol is synthesized in the endoplasmic reticulum whereas sphingolipid synthesis and head-group modification are largely completed in the Golgi, suggesting that cholesterol-sphingolipid rafts proceeding to the plasma membrane first assemble in the Golgi (Vesicles going back to the endoplasmic reticulum contain little sphingomyelin and cholesterol) (van Meer, 1989, Brugger *et al.*, 2000). The inclusion of proteins into rafts is important for polarized delivery to the cell surface. One subset of lipid rafts is found in cell surface invaginations called “caveolae”. Caveolae are formed from lipid rafts by polymerization of caveolins that are palmitoylated integral membrane proteins that tightly bind cholesterol. Interestingly, ENaC was found to segregate into lipid rafts and colocalize with vesicular trafficking proteins such as S-1A and SNAP-23, as well as with caveolin-1, a marker protein for lipid rafts, using two independent approaches in mpkCCD_{c14} cells, a cell line that expresses endogenous ENaC and responds to aldosterone stimulation with increased Na⁺ reabsorption (Hill *et al.*, 2007). Further, cholesterol depletion of the plasma membrane prevented activation of ENaC by cytosolic SGK1, implicating the importance of an intact lipid environment and/or of lipid rafts as a signaling platform for ENaC activation by SGK1 (Krueger *et al.*, 2009).

Another interesting mode of channel regulation involves essential polyunsaturated fatty acids and their interaction with lipid bilayers within channel-containing complexes. Eicosapentanoic acid, for instance, activates ENaC in a cAMP-dependent manner involving stimulation of cAMP-dependent protein kinase A (PKA). This stimulation of ENaC also occurs via SGK1 in membrane-bound compartments containing an A-kinase-anchoring-protein (AKAP), activated PKA, and a phosphodiesterase (Mies *et al.*, 2007).

Prostasin, a channel-activating protease is a GPI-anchored protein and the physical association of ENaC with lipid rafts may therefore be important for its activation (Verghese *et al.*, 2006). The coordinated downregulation of ENaC by caveolin-1 and Nedd4-2 also suggests colocalization of these proteins in lipid rafts (Lee *et al.*, 2009). Indeed, both Nedd4 and Raf-1 have been reported to be present in detergent-resistant membrane fractions (Plant *et al.*, 2000, Rizzo *et al.*, 2001). Not surprisingly, ENaC itself is Cys-palmitoylated in the β -subunit, and this modification has been shown to modulate channel gating by facilitating interactions between cytoplasmic domains and the plasma membrane (Mueller *et al.*, 2010). Furthermore, phosphoinositide products such as PI(4,5)P₂ and PI(3,4,5) P₃ bind ENaC directly and enhance ENaC P_o within the apical membrane (Tong *et al.*, 2004, Staruschenko *et al.*, 2004b, Pochynyuk *et al.*, 2005, Staruschenko *et al.*, 2007). These observations suggest that ENaC is indeed present in lipid rafts, and that raft association is important for regulating its activity.

Although lipid rafts provide an attractive candidate platform for organizing the ERC, there are some key observations that this model fails to explain. As described above, the small GTPase Ras is central to many signaling processes, including those that regulate ENaC. Interestingly, the two Ras isoforms, K-Ras and H-Ras, are almost identical in sequence but have distinct signaling properties (Roy *et al.*, 1999). While K-Ras has a poly-basic region required for plasma membrane localization, H-Ras is palmitoylated and therefore, more likely to partition into lipid rafts. K-Ras does not appear to operate in rafts. Aldosterone-stimulated ENaC activity, however, does include K-Ras activity (Staruschenko *et al.*, 2004b), suggesting that the lipid raft model cannot fully explain the functional clustering of the ENaC-regulatory machinery. Further, one study reported that although membrane cholesterol depletion decreases Na⁺ transport and ENaC P_o, it has no detectable effect on ENaC cell surface expression (Balut *et al.*, 2006). Also, ENaC stimulation by either forskolin or aldosterone did not alter the lipid raft distribution of ENaC in mpkCCD_{c14} cells (Hill *et al.*, 2007).

Therefore, while lipid rafts may still play a role, it is perhaps a combination of the surrounding lipid environment and key direct protein–protein interactions (including the function of multi-domain scaffold proteins) that is needed to efficiently harness and organize the wide spectrum of ENaC-regulatory components at the cell surface. None of the previously well-characterized ENaC-regulatory proteins has the structural characteristics of a scaffold, and there is no functional evidence that any of them serves as a molecular scaffold to nucleate the other regulatory elements.

An interesting candidate to consider in this context is the recently identified MR target gene CNKSR3 (connector enhancer of kinase suppressor of Ras-3), commonly referred to as CNK3. CNK3 is the third isoform of the mammalian CNK protein family, whose members possess a modular structure and contain common protein–protein interaction domains: an N-terminal sterile α -motif (SAM), followed by a conserved region in CNK (CRIC) and a classic PSD-95–DLG-1–ZO-1 (PDZ) domain (Douziech *et al.*, 2003, Therrien *et al.*, 1998, Soundararajan *et al.*, 2012). CNK3, like SGK1 and GILZ1, is rapidly induced by physiological concentrations of aldosterone, and its promoter harbors two functional MR binding loci in close vicinity to the transcription start site, suggesting a direct mode of regulation (Ziera *et al.*, 2009).

CNK3—a potential scaffold for ENaC and its regulators

Evidence that CNK3 might be involved in regulating Na⁺ reabsorption came from expression analyses along different nephron segments microdissected from mice kidneys that revealed that CNK3 is highly expressed in the CD, the prime target segment of aldosterone-regulated Na⁺ retention in the kidney (Ziera *et al.*, 2009). Indeed, CNK3 expression correlates with, and is critically required for, ENaC-mediated Na⁺ transport in renal epithelial cells (Ziera *et al.*, 2009). The first study addressing the mechanistic aspects of CNK3 function revealed that CNK3 expression significantly interferes with the activation of the Raf-1/MEK1/2/ERK1/2 signaling cascade (Ziera *et al.*, 2009), a mechanism that is reminiscent of how GILZ1 indirectly stimulates ENaC activity (Soundararajan *et al.*, 2005).

Unlike the small adaptor GILZ1, CNK3 possesses a number of interaction motifs that are suggestive of a scaffold. SAM domains are known to exert protein–protein interactions by homo- or heterodimerization (Green *et al.*, 2003). Interestingly, a related member of the family, CNK1, binds to the angiotensin II type 2 (AT₂) receptor via its SAM domain although the receptor itself lacks one (Fritz & Radziwill, 2005). The CNK-specific CRIC domain has also been shown to interact with the AT₂ receptor (Fritz & Radziwill, 2005). The CNK3 PDZ domain is of special interest. PDZ domains are intermediate sized (80–100 amino acids) regions that bind to specific sequences at the C-termini of transmembrane proteins, and are frequently found in molecular scaffolds (Brone & Eggermont, 2005). Several PDZ domain-containing proteins play an important role in the transport, localization, and assembly of multicomponent signaling complexes (Harris & Lim, 2001). Since PDZ domains can also interact with each other, some PDZ domains can hetero-oligomerize in a head-to-tail fashion. PDZ domain-containing scaffolding proteins can therefore function not only to bind an array of target proteins, but also to crosslink one another into complex assemblies, factors which make PDZ domain-containing scaffolds such as CNK3 intriguing candidates for the assembly of regulatory components into a large supramolecular ENaC-regulatory complex.

Based on the observations that CNK3 has been shown to be an aldosterone-regulated gene product, which stimulates ENaC, and has multiple protein–protein interaction motifs, it is tempting to speculate that it can serve as a scaffold to assist in the assembly of ENaC-regulatory factors into a stimulatory complex (the ERC), thus providing tight control of Na⁺ reabsorption via ENaC. Both SGK1 and GILZ1 harbor consensus class I PDZ-binding-motifs (or PBMs) at their C-termini (Soundararajan *et al.*, 2012), consistent with the idea that they interact with CNK3 through its PDZ domain. However, although it is known that CNK3 positively regulates ENaC activity (Ziera *et al.*, 2009), it remains to be seen if it can directly interact with and modulate the channel. It is also not known if CNK3 enhances ENaC surface expression or P_o. A similar scaffolding mechanism has been suggested for CNK1 as a positive regulator of insulin signaling: CNK1 helps to localize cytohesins at the cell surface to generate a PIP₂-rich microenvironment, which is critical for PI3K/Akt signal transduction (Lim *et al.*, 2010).

Thus, many aspects of a scaffolding model involving CNK3 or other potential scaffolds remain to be elucidated: does CNK3 play a role in anchoring ENaC to the underlying cytoskeleton? Does the ERC vary in various subcellular compartments, and with respect to time in a scaffold-dependent fashion? Finally, does CNK3 (or other potential scaffolds such as AKAP or NHERFs (Wang, 1999, Ali *et al.*, 1998, Weinman *et al.*, 2001)) interact with ENaC within lipid rafts and if so, what is the nature of this interaction and what are the factors modulating this? The molecular identities of other players that facilitate this process, and the specific subcellular compartments wherein these interactions occur, are key areas for future research in ENaC regulation—and indeed in epithelial ion transport in general.

Acknowledgments

Declaration of interest: This work is supported by National Institutes of Health grants K01-DK078679 (to RS), R01-DK56695 (to DP), and R01-DK85101 (to DP).

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Figure 1.

Hypothetical model for SGK1 activation and regulation of ENaC by the ENaC-regulatory complex (or ERC). ENaC regulation occurs within a unique multi-protein complex called the ERC. The various components of the complex are regulated by diverse signaling networks, including steroid receptor-, PI3K-, mTOR- and Raf-MEK-ERK-dependent pathways. Aldosterone coordinately induces the expression of SGK1, GILZ1 as well as the scaffold protein CNK3, among many others. Highlights 1 through 5 depict sequential steps in the selective recruitment and activation of SGK1 (not meant to depict spatial/subcellular localization). GILZ1 protects SGK1 from rapid endoplasmic reticulum-associated degradation and redirects it away from the endoplasmic reticulum and into the ERC, that possibly includes CNK3. CNK3 provides a platform for organizing various components of the ENaC-regulatory machinery by virtue of its ability to interact with multiple proteins including mSin1. mTOR couples with Rictor and mSin1 to form the functional TORC2 complex, which phosphorylates and activates SGK1 at S422. Subsequent phosphorylation of SGK1 at T256 by PDK1 is required for its full activation. It is possible that activation of SGK1 occurs at the ERC, and that mTORC2 is recruited to the ERC via mSin1. It is also possible that SGK1 is brought into the ERC after it has been activated by mTORC2 and PDK1 elsewhere. ENaC, epithelial Na⁺ channel; ERAD, endoplasmic reticulum-associated

degradation; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase-1; SGK1, serum-and-glucocorticoid-induced-kinase-1.