NHA1 and NHA2 are essential for survival, but have distinct transport modalities

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The cation/proton antiporter (CPA) family includes the well-known NHE (SLC9A) family of Na+/H+ exchangers, and the more recently discovered and less well understood CPA2s (SLC9B), found widely in living organisms. In Drosophila – as in human – they are represented by two genes (Nha1 (Slc9b1) and Nha2 (Slc9b2)), which are enriched, and functionally significant, in renal tubules. However, the importance of their role in organismal survival has not been investigated in animals. Here, we show that single RNAi knockdowns of either Nha1 or Nha2 reduce survival, and in combination are lethal. Knockdown of either gene alone results in up-regulation of the other, suggesting functional complementation of the two genes. Under salt stress, knockdown of either gene decreases survival, demonstrating a key role for the CPA2 family in ion homeostasis. This is specific to Na+ stress – survival on K+ intoxication is not affected by NHA knockdown. Direct functional assay in Xenopus oocytes shows that Nha2 acts as an Na+/H+ exchanger. By contrast, Nha1 expressed in Xenopus oocytes shows strong Cl– conductance and acts as a H+–Cl– cotransporter. The activity of Nha1 is inhibited by chloride-binding competitors 4,4'-diiso-thiocyanato-2,2'-disulfonic acid stilbene (DIDS) and 4,4'-dibenzamido-2,2'-disulfenedisulphonate (DBDS). Salt stress induces a massive up-regulation of NHA gene expression, not in the major osmoregulatory tissues of the alimentary canal, but in the crop, cuticle and associated tissues. It is thus necessary to revise the classical view of the coordination of different tissues in the coordination of the response to osmoregulatory stress.

Drosophila | Drosophila | physiology | physiology | transport

Introduction

The NHA gene family

Ionic homeostasis is essential for life, and requires a significant fraction of an organism’s total energy budget. Primary ion-motive ATPases provide electrochemical ion gradients to drive an array of channels, cotransporters and antiporters. The cation/proton antiporter (CPA) family are ubiquitous, and best known for the NHE, or CPA1, branch exemplified by the classical Na+/H+ exchanger NHE1 (1, 2), which is a target for the potassium-sparing diuretic amiloride in treatment of hypertension and congestive heart failure. More recently discovered are the NHAS, a sub-branch of the CPA2 family, which is much less well understood (3, 4). Originally studied in bacteria, yeast and plants (5, 6), in humans there are two NHA genes in tandem. NHA1 (SLC9B1) is testis-specific, but NHA2 (SLC9B2) expression is ubiquitous (7, 8). In osteoclasts NHA2 colocalizes with the V-ATPase a3 subunit and the lysosomal marker LAMP2 (9), and resides in the plasma membrane of MDCK cells (7). In pancreas, NHA2 is necessary for insulin secretion, but localizes not to insulin containing vesicles, but to transferrin-positive endocytic vesicles (4, 10). NHA2 has also been linked to hypertension (8). NHA1 and NHA2 are essential for survival, but have distinct transport modalities

Son with their exhaustively studied NHE relatives, and a simple animal model is clearly needed.

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Drosophila melanogaster offers potent advantages as such a simple model system. As well as a sequenced genome and uniquely potent genetic tools, functional transport read-outs can be provided by the simple renal system (11, 12). As in humans and the malaria vector mosquito Anopheles gambiae (13, 14), the fruit fly Drosophila has two NHA genes, Nha1 and Nha2 (5). However, the NHA family is relatively divergent, and it is hard to assert orthology between human and insect NHAs (5); in terms of sequence identity, the insect NHA1s are slightly more similar to human NHA2 and vice versa. Both Drosophila NHAs are widely expressed, but at particularly high levels in epithelia, suggesting a role in organismal ion homeostasis (15, 16). As insect epithelia are energised by an apical plasma membrane H+ V-ATPase (17, 18), the NHAs have been hypothesised to act as the partner exchangers, predicted by Wieczorek (17, 19), that colocalize with V-ATPase and employ the proton electrochemical gradient to achieve transepithelial transport of sodium and potassium – similar to the position in mammalian MDCK cells (20). Consistent with this, over expression of GFP-tagged Nha1 or Nha2 labels the same apical membrane as the V-ATPase in the Malpighian (renal) tubule, a model epithelium in which transport and control mechanisms are conveniently studied (21, 22); and RNAi against one of the exchangers impacts on fluid secretion (15). Drosophila thus provides an ideal system in which to investigate the roles of NHAs in multicellular animals.

Significance

Cation/proton antiporters (CPAs) are essential to life; the sodium/proton exchanger (NHE) branch of the family has been exhaustively studied, and is an important drug target. However, much less is known about the recently discovered NHA branch, represented by two genes in both humans and flies. Here we show that NHA function is essential to life, and both NHAs protect against salt stress. However, their mechanisms are radically different, suggesting that function cannot be inferred from structural similarity: although NHA2 is a Na+/H+ exchanger as expected, NHA1 acts as an electroneutral H+–Cl– cotransporter. This is an important finding for future studies of these transporters.

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In this paper, we show that individual knockdowns of either Nha1 or Nha2 are deleterious, and that dual knockdowns are lethal. NHA activity is thus essential for survival. We further show that NHAs are essential for response to salt stress, specifically protecting against Na\(^+\) rather than K\(^+\), and that this is achieved by up-regulation of expression of both genes. However, the two NHAs have different transport properties; whereas Nha2 behaves as an electroneutral Na\(^+\)/\(\mathrm{H}^+\) exchanger, NHA1 is best modelled as a H\(^+\) - Cl\(^-\) cotransporter.

**Results and discussion**

NHAs are highly enriched in epithelia

The online atlas of gene expression, FlyAtlas.org, shows that both Nha1 and Nha2 are widely expressed, but particularly in the epithelia of the alimentary canal (15, 23, 24). To validate this pattern of gene expression, we performed quantitative real-time PCR, which demonstrated consistent enrichment levels at least as high as those seen in the Affymetrix-derived FlyAtlas (suppl. Fig. 1). In terms of investment in mRNA, NHAs must thus play major roles in epithelial function. Interestingly, a broader meta-analysis of the major transporting epithelia of *Drosophila* (salivary glands, midgut tubules and hindgut), showed that one of Nhe1, Nha1 or Nha2 was always very highly co-expressed with V-ATPase, implying that both branches of the CPA family can play epithelial roles in *Drosophila* (16).

A reverse genetic understanding of the roles of NHAs requires mutants of both loci. However, classical null alleles may be lethal, and cannot be targeted to specific tissues. In *Drosophila*, the GAL4/UAS binary system allows RNAi to be targeted to cell populations of choice. Accordingly, we both purchased available lines from the Vienna *Drosophila* Research Centre, and made our own using the pRISE vector, and screened them for knockdown efficiency using real-time PCR. We identified an insertional mutant of *Nha1*, and RNAi alleles of *Nha1* and *Nha2*, that each showed 60-80% knockdown when RNAi was expressed in whole flies with the actin-GAL4 driver (Fig. 1). Although these hypomorphic alleles are not true nulls, their viability allows physiological experimentation on NHA mutant animals.

NHAs show compensatory expression
A problem in reverse genetic analysis of any multigene family is that knockdown of any one member could be rescued either by functional redundancy with other family members, or by up-regulation of expression of other family members. To test whether such compensatory gene regulation occurred, we measured gene expression of the other gene in the Nha1 and Nha2 knockdowns (Fig. 1). Indeed, such compensatory regulation occurs, suggesting coordinated control of expression of the NHA gene family.

NHA s are essential for life

What is the impact of global RNAi-mediated NHA knockdown? This was assessed with both single and double knockdowns, using the Actin–GAL4 and stronger tubulin–GAL4 drivers, the latter in the presence of UAS–dicer2, a d-sRNA processing enzyme that increases the efficiency of RNAi in Drosophila. Additionally, we performed the experiments both at 22° and 26°C, as higher temperatures drive the GAL4/UAS system more strongly. The results (Fig. 2) show that NHA activity is essential for survival. When driven with the Actin–GAL4 driver, the Nha1 or Nha2 knockdowns individually show reduced viability (with Nha2 knockdown having a stronger effect); but both knockdowns combined are semi-lethal at 22°C (only a few flies escape to adulthood). At 26°C, the double knockdown demonstrates complete lethality. With the strong tubulin–GAL4 driver in the presence of UAS–dicer2, the double knockdown is completely lethal, even at 22°C (Fig. 2B).

NHA s are necessary for survival under salt (NaCl but not KCl) stress

Having established that NHA s are essential, it is important to identify the key physiological processes in which they are involved. An obvious candidate process – given their structural identity as alkali metal ion exchangers, and their enriched expression in epithelia - would be ionic homeostasis. Accordingly, we exposed the flies to salt stress. We and others have previously shown that diet supplemented with 4% w/v NaCl is lethal to wild-type Drosophila over several days (25). When wild-type flies were compared with either Nha1 or Nha2 knockdowns, or Nha1/Nha2 double knockdown escapers, it was obvious that NHA knockdowns reduced survival time on NaCl diet (Fig. 3). Therefore NHAs play a key role in acute defence against salt stress. Is this lethality a response to Na+, Cl− or both? When wild-type and knockdown flies were fed the (higher) levels of KC1 or NaCl required for lethality, NHA knockdown did not impact on survival, demonstrating that the pathology of salt stress is due mainly to the Na+ ion, and further that NHAs are not directly involved in K+ handling in the fly.

Nha1 and Nha2 have distinct ionic specificities

Given that all functionally characterized members of the CPA family are Na+/H+ exchangers (3, 5), that all members show structural similarity, and that we have shown compensatory over-expression in response to knockdown of either gene in Drosophila (Fig. 1), it would seem likely that both Nha1 and Nha2 proteins would act as canonical Na+/H+ exchangers. However, surprisingly this is not the case (Fig. 4; Table 1). When expressed in Xenopus oocytes, pH, of both Nha1 and Nha2 oocytes, responded to Na+/H+ activity (i.e., Nha) (Fig. 4A, 4B; Table 1). Intracellular Na+ activity (aNa) of Nha2 but not Nha1 oocyte responded to changes in both pH and [Na+]i (Fig. 4A, 4D; Table 1). Membrane potentials (Vm) of Nha1 and Nha2 oocytes were insensitive to Na+ (Fig. 4A, 4D). All these responses were not observed in water-injected control oocytes. Taken together, these results show that, in Drosophila, Nha1 and Nha2 have distinct ion specificities: Nha2 behaves as a classical electroneutral Na+/H+ (i.e., Nha) (Fig. 4G), whereas Nha1 acts as a novel class of H+ transporter with no Na+ activity.

Characterization of NhaNha1 as a H+-Cl- cotransporter or a Cl-/OH- exchanger

Analysis for intracellular Cl− activity (aCl) revealed the function of Nha1 as a Cl− transporter. aCl of Nha1 but not Nha2 oocytes increased in Cl−-free solution (Fig. 4B, 4C; Table 1). The pH increase of Nha1 oocytes was also observed in Cl-free solution (Fig. 4D). These results indicate that Drosophila Nha1 functions as a Na+-independent H+−Cl− cotransporter or a Cl-/OH- exchanger (Fig. 4F). In Cl−-free solution, Vm of Nha1 oocytes but not Nha2 oocytes were depolarized (Fig. 4B, 4C; Table 1). When Vm were clamped, membrane current of Nha1 oocytes did not significantly respond to changes in bath [Cl-]i whereas the pH, largely increased and decreased by removal and re-addition of bath Cl− (Fig. 5C). These results suggest that the H+-Cl− cotransport or Cl-/OH- exchange activity of Nha1 is electroneutral and the depolarization of Nha1 in Cl−-free solution is a secondary phenomenon possibly mediated by an endogenous membrane activity of Xenopus oocytes.

Footline Author

PNAS | Issue Date | Volume | Issue Number | 3
The Cl⁻ transport activity of Nha1 was not altered in the presence or absence of HCO₃⁻ (Fig. 5A). When similar analysis was performed on mouse Slc26a6, a well-known anion exchanger with both Cl⁻/ HCO₃⁻ and Cl⁻/OH⁻ exchange activities (26), Cl⁻ transport activity was significantly enhanced (Fig. 5A). These results indicate that Nha1 is not a Cl⁻/HCO₃⁻ exchanger. The Nha1 activity was sensitive to inhibitors of anion transport, DBDS and DIDS, but not to amiloride, an inhibitor of several Na⁺ exchangers and channels (Fig. 5B). The aNa⁺ of Nha1 oocytes were increased in Cl⁻-free solution (Fig. 5B, 5D; Table 1) but not in NaCl-free solution (Fig. 5E). It is not clear if this aNa⁺ change is mediated by an alternative mode of Nha1 activity (e.g., Na⁺/(H⁺, Cl⁻) exchange) which our experimental conditions cannot reveal or an endogenous membrane activity of Xenopus oocyte.

Salt-stress induces tissue-specific changes in NHA expression. If NHAs defend the organism against salt, then it is reasonable to expect that salt-loading would lead to increased expression of NHAs in key osmoregulatory tissues. Accordingly, NHA gene expression in salt-stressed flies by real-time PCR. The expression of both Nha1 and Nha2 increased in whole flies, in response to sodium, but not potassium exposure (Fig. 6 A-B); but surprisingly, the expression in the classical osmoregulatory tissues of the alimentary canal decreased (Fig. 6C-D), apart from a small rise in expression of Nha2 in midgut. By contrast, strong upregulation of both Nha1 and Nha2, albeit from very low resting levels (FlyAtlas.org), could be seen in the cuticle-dominated crop and abdominal carcass (Fig. 6). The cuticular epithelium has been known to play an active role in ion transport, forming the "moulting fluid" that helps to digest the old cuticle of moulting insects (27), so it is not merely a simple cuticle-secreting factory, and some ion transport competence must be inferred. It is also possible that one or more cell types within the cuticle are particularly sensitive to haemolymph Na⁺ levels, and protect themselves against Na⁺ intoxication with up-regulation of NHAs.

While these results show that NHAs are important players in organisinal epithelial transport, homeostasis, and survival, they also suggest that—in Drosophila at least— they can also be called on facultatively to defend individual tissues against failures in haemolymph homeostasis. This devolved model may provide extra robustness of the whole system. It will be interesting in future to identify exactly how tissues invoke a massive up-regulation of NHAs when under Na⁺ challenge, and whether it confers any adaptive advantage.

Conclusions

This work shows for the first time that Nha function is essential for survival, and that the Drosophila NHAs specifically protect against excess Na⁺. The applicability of these data to mammals will require the generation of not just Nha2 knockout mice (available now), but also Nha1 knockouts, and double knockouts. Meanwhile, the relative speed and low cost of Drosophila can provide a strong indicator of essentiality.

Nha1 the first member of the SLC9 family shown not to act as a Na⁺/H⁺ exchanger. This need not be surprising; there are examples of differing specificity in otherwise uniform families. For example, the Slc26 family can act as Cl⁻/HCO₃⁻ exchangers, Cl channels, or anion sensor (prestin) (28); ClC family of Cl⁻ channels includes Cl⁻/H⁺ exchangers (29); the Slc5 family show Na⁺/Cl⁻/HCO₃⁻ coupled transport of glucose, iodide, choline, or mono-carboxylates (30); and the Slc4 family of HCO₃⁻ transporters includes the borate transporter Slc4a11 (31, 32). While we cannot exclude that Nha1 is capable of handling Na⁺, we were unable to devise conditions under which this could be shown. The dominant transport modality appears to be an H⁺/Cl⁻ cotransport.

It is also significant that the close coupling of a V-ATPase to an exchanger to elicit net transmembrane efflux of Na⁺ or K⁺ (the Wieczorek model: (17)) is only partly explained by the Nhas. Nha1 does not function as an exchanger, and Nha2 is a Na⁺/H⁺, but not K⁺/H⁺, exchanger; and in mosquito, it may not colocalize with V-ATPase (33). So there may be no single partner K⁺/H⁺ exchanger to explain the net K⁺ transepithelial transport that characterizes most insect epithelia; indeed, transcriptomic studies show that different CPAs are co-expressed with V-ATPase in different insect epithelia (16). The search will need to be extended throughout
the CPA gene family, several of which show co-enrichment with V-ATPase in insect transporting epithelia (16). Significantly, although both Drosophila Nha1/Sle9b1 and Nha2/Sle9b2 clearly sit within the CPA2 family, the insect exchangers diverged relatively basally to the human exchangers (5), to the extent that it would be imprudent to assert direct homology between them. However, our results do imply that the functions of each member of the CPA2 family needs to be established experimentally, rather than inferred from the prototype.

Materials and methods

Drosophila methods

Drosophila were reared on standard diet (34) in vials, at 22°C with a 12:12 h photoperiod and at 45-55% relative humidity. Where required, they were anesthetized by brief exposure to carbon dioxide. Crosses were maintained at 22°C or at 26°C where noted, when it was desired to drive expression of the GAL4/UAS system as strongly as possible.

Generation of RNAi alleles

Commercially available UAS RNAi stocks were ordered from the Vienna Drosophila Research Centre (35). As only a minority of RNAI stocks display a phenotype when driven, we also generated our own RNAI stocks. Briefly, short regions throughout the gene were selected, and cloned in inverse short regions throughout the gene were selected, and cloned in inverse

Fig. 5. Functional characterization of Nha1 as an electroneutral H\(^{-}\)–Cl\(^{-}\) cotransporter or Cl\(^{-}\)/OH\(^{-}\) exchanger. (A) Intracellular Cl\(^{-}\) activity (aCl\(^{-}\)) of Xenopus oocytes expressing Drosophila Nha1 in response to changes in bath [Cl\(^{-}\)] and [HCO\(_3\)\(^{-}\)]. Oocytes expressing mouse Sle26a6, known anion exchanger with Cl\(^{-}\)/OH\(^{-}\) and Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange activity, is used as comparison. Representative traces of aCl\(^{-}\) (left) and quantitative comparison of changes in aCl\(^{-}\) (right) are shown (Student’s t test, N=3-5; significant changes are marked with an asterisk). Numbers beside the traces indicate the rate of changes in aCl\(^{-}\} (mM/min). (B) Inhibition of Nha1 activity by amiloride, DBDS and DIDS. Rates for pH\(^{+}\) increase in Cl\(^{-}\)-free bath solution were compared in the presence or absence of inhibitors (Student’s t test, N=3-4; significant changes are marked with an asterisk). (C) Electroneutral nature of H\(^{-}\)–Cl\(^{-}\) cotransport or Cl\(^{-}\)/OH\(^{-}\) exchange activity of Nha1. Representative traces of pH\(^{+}\) and membrane current (i) of voltage-clamped Nha1 and control oocytes are shown. (D) Na\(^{+}\)-independence of H\(^{-}\)–Cl\(^{-}\) cotransport or Cl\(^{-}\)/OH\(^{-}\) exchange activity of Nha1. Representative traces and quantitative comparison of changes in pH\(^{+}\) of Nha1 oocytes in Cl\(^{-}\)-free or NaCl-free solutions are shown (Student’s t test, N=11-14; P<0.31). (E) A representative trace and quantitative comparison of changes in intracellular Na\(^{+}\) activity (aNa\(^{+}\)) of Nha1 oocytes in Cl\(^{-}\)-free or NaCl-free solutions (Student’s t test, N=5; significant changes are marked with an asterisk).

Footline Author

PNAS | Issue Date | Volume | Issue Number | 5
Xenopus oocyte methods

The ORFs of the major transcripts of Nha1 (Nha1-RA) and Nha2 (Nha2-RA) were inserted into the pGEMHE Xeropus laevis expression vector. The plasmids were linearized with NotI, and cDNAs were transcribed in vitro using the T7 message machine kit (Ambion, Austin, TX). X. laevis oocytes were dissociated with collagenase and injected with 50 nl of water or a solution containing DNA at 0.5 μg/μl (25 ng/oocyte), as described previously (41). The oocytes were incubated at 16°C in OR3 medium, and studied 4-7 days after injection.

Intracellular pH (pHi), intracellular Na activity (aNa) or intracellular Cl activity (aCl) of oocytes were measured using H3, Na or Cl selective micro-electrodes prepared with a H3 ionophore I-mixture B ion-selective resin (Fluka Chemical, Ronkonkoma, NY), a Na ionophore cocktail A (Fluka), or a Cl ionophore cocktail A (Fluka) respectively, as described previously (41-43). The membrane potential (Vm) was measured as the difference between KCl microelectrode and an extracellular calomel electrode. The oocyte was held on a nylon mesh in a chamber and perfused with ND96 saline solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES (pH 7.5)). Vm and pH or aNa were constantly recorded at 0.4 Hz, and the bath solution was replaced with test solutions prepared as described follows. Na+/H+ exchangers were substituted using NaCl with choline chloride, Cl-free (OC) solution was prepared by substituting gluconate-salts for all Cl-salts. NaCl-free (0Na-0Cl) solution was prepared by substituting Na+ with choline. For CO3HCO3 equilibrated solutions, 33 mM NaCl was replaced with 33 mM NaHCO3 (33 mM sodium gluconate in non-CO3HCO3 solutions), and the HCO3- solutions were bubbled with 5% CO2 95% O2 during the experiments. Osmolarity and pH of these media were adjusted to ~200 mosM and 7.5, respectively. pH of high-pH ND96 (pH 8.5) solution was adjusted with NaOH solution.

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Statistical analyses were performed using Student’s t test or x2 test (two tailed). Significant differences in survival were assessed by testing Kaplan-Meier data with the logrank test. All testing used GraphPad Prism software. Throughout, the critical level is taken as P<0.05.