Development 134, 147-155 (2007) doi:10.1242/dev.02710

A pump-independent function of the Na,K-ATPase is required for epithelial junction function and tracheal tube-size control

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The heterodimeric Na,K-ATPase has been implicated in vertebrate and invertebrate epithelial cell junctions, morphogenesis and oncogenesis, but the mechanisms involved are unclear. We previously showed that the *Drosophila* Na,K-ATPase is required for septate junction (SJ) formation and that of the three β-subunit loci, only Nrv2 isoforms support epithelial SJ barrier function and tracheal tube-size control. Here we show that Nrv1 is endogenously co-expressed with Nrv2 in the epidermis and tracheal system, but Nrv1 has a basolateral localization and appears to be excluded from the Nrv2-containing SJs. When the normally neuronal Nrv3 is expressed in epithelial cells, it does not associate with SJs. Thus, the β-subunit is a key determinant of Na,K-ATPase subcellular localization as well as function. However, localization of the Na,K-ATPase to SJs is not sufficient for junctional activity because although several Nrv2/Nrv3 chimeric β-subunits localize to SJs, only those containing the extracellular domain of Nrv2 have junctional activity. Junctional activity is also specific to different α-subunit isoforms, with only some isoforms from the major α-subunit locus being able to provide full barrier function and produce normal tracheal tubes. Importantly, mutations predicted to inactivate ATPα catalytic function do not compromise junctional activity, demonstrating that the *Drosophila* Na,K-ATPase has an ion-pump-independent role in junction formation and tracheal morphogenesis. These results define new functions for the intensively studied Na,K-ATPase. Strikingly, the rat α1 isoform has full junctional activity and can rescue *Atpα*-null mutants to viability, suggesting that the Na,K-ATPase has an evolutionarily conserved role in junction formation and function.

KEY WORDS: Na,K-ATPase, Epithelial junctions, *Drosophila*, Septate junctions, Trachea

INTRODUCTION

Cell junctions are multifunctional complexes that play many crucial roles in epithelial development by providing adhesion, diffusion barrier, polarity and signaling functions. These functions are generally evolutionarily conserved, but combinations of these functions in different junctions can be divergent. For example, although the adherens junctions (AJs) are very similar between vertebrates and *Drosophila*, the organization and function of junctions that create diffusion barriers show significant differences (reviewed by Knust and Bossinger, 2002; Tepass et al., 2001). In vertebrates, the barrier is provided by the claudin-based tight junctions, which are apical to the AJs and also contain apical polarity proteins such as Par-3, Crumbs and aPKC (Van Itallie and Anderson, 2006). In *Drosophila*, the barrier is provided by a claudin-containing junction termed the septate junction (SJ) (Behr et al., 2003; Wu et al., 2004). However, SJs are basolateral and contain basal polarity proteins such as Scribble (also known as Scribbled), Discs Large and Lethal Giant Larvae, and thus have similarity to both vertebrate tight junctions and basolateral regions (reviewed by Tepass et al., 2001; Wu and Beitel, 2004).

The functions and composition of SJs are only partially defined, but in addition to barrier function, SJs are required for proper regulation of tracheal tube size in *Drosophila* (Paul et al., 2003). The tracheal system is a ramifying network of epithelial tubes that serves as a combined pulmonary/vascular system to deliver oxygen to the body (reviewed by Affolter et al., 2003; Manning and Krasnow, 1993). After the tracheal system forms, the tubes are enlarged by a process called 'tube expansion' that is coincident with SJs assembly (Beitel and Krasnow, 2000). Without SJs, multicellular tubes such as the dorsal trunk (DT) become too long and can have diameter abnormalities, and some branches such as the ganglionic branches (GBs) lose staining of lumenal markers (Paul et al., 2003). These tracheal defects arise in SJ mutants because SJs mediate apical secretion of the lumenal matrix-associated protein Verm, which is required to control tracheal tube size (Luschnig et al., 2006; Wang et al., 2006). SJ-mediated secretion of Verm occurs via a specialized pathway, as SJs are not required for the secretion of other apical markers (Llimargas et al., 2004; Paul et al., 2003; Genova and Fehon, 2003; Hemphala et al., 2003). The nature of this specialized pathway is unclear, but it is an important and assayable cellular function of the SJ that is distinct from its paracellular barrier function.

In screens for tracheal tube-size or barrier junction mutants, we and others previously found that the Na,K-ATPase localizes to and is required for SJ formation (Genova and Fehon, 2003; Paul et al., 2003). This finding was unexpected because the Na,K-ATPase is expressed in essentially all animal cells and had not previously been reported to be part of a junctional complex. The Na,K-ATPase is a P-type ion transporter that is an α/β heterodimer (reviewed by Kaplan, 2002). The α-subunit is a large, ~1000 amino acid (aa) ten-transmembrane protein that contains the Na^+ and K^+ antiporter function coupled to ATPase activity. The β-subunit is a small, 330 aa single-transmembrane protein that is thought to chaperone the α-subunit from the ER to the plasma membrane and to modulate ion transport (reviewed by Geering, 2001).

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Accepted 23 October 2006

How could the ATPase function in SJ formation? One possibility is that Na,K-ATPase activity is required to keep intracellular Na^+ concentration low to allow junction formation, which has been demonstrated for MDCK cells (Rajasekaran et al., 2001a). Alternatively, there are multiple reports of ion-transport-independent roles for the Na,K-ATPase. For example, the human α3 Na,K-ATPase serves as a neural receptor for the agrin protein and, through mechanisms that are gradually being defined, the Na,K-ATPase appears to transduce a reactive oxygen-mediated signal initiated by ouabain (Hilgenberg et al., 2006; Xie and Askari, 2002). In this report, we show that the junctional activity of the *Drosophila* Na,K-ATPase is mediated by specific isoforms of the ATPα α-subunit and by the extracellular domain of the Nrv2 β-subunit, but is not affected by mutations predicted to block ion-pump activity. Furthermore, we show that expression of the rat α1-subunit in *Drosophila Atpα*-null mutants can completely restore junctional function and rescue the mutants to viability. This result suggests that the Na,K-ATPase could have an evolutionarily conserved role in cell junction formation and is consistent with evidence that the Na,K-ATPase can promote cell junction formation and cell polarity in vertebrate systems (see Discussion) (reviewed by Cereijido et al., 2004).

MATERIALS AND METHODS

Reagent construction

Nrv2/Nrv3 chimeras were generated by PCR and cloned into the pUAST vector (Brand and Perrimon, 1993). Breakpoints for the Nrv2/Nrv3 extracellular domain chimeras were as follows: Nrv2 Region A, W70-D131; Region B, F132-A186; Region C, P187-R260; Region D, G261-D322. Full-length *Atpα* clones were generated from RT-PCR amplicons utilizing whole adult RNA and the TOPO T/A Cloning System (Invitrogen). Clones were sequenced to identify inserts representing previously described cDNA isoforms without missense or nonsense mutations. The cloning primers contained *Eco*RI (5′) and *Xho*I (3′) linkers that were used to shuttle the insert into the pUAST transgenesis vector bearing the *w[mc+]* gene. The rat α1 clone with an N-terminal tag has been shown to be functional (Dada et al., 2003). Isoforms predicted to be catalytically inactive were generated by site-directed PCR mutagenesis with oligonucleotide primers producing the D355N (short isoform) or D394N (long isoform) mutation. The primers also introduced a silent GGC to GGT codon change 3′ to the mutation site, creating a unique *Age*I endonuclease site that was used to generate the *UASATPα [D→N]* constructs. All pUAST construct inserts were re-sequenced and standard P-element-mediated transgenesis techniques were used to generate transgenic flies.

The *da*-Gal4 on chromosome III (Wodarz et al., 1995) was crossed into the *ATPα[DTS2R3]* mutant (Palladino et al., 2003) background to generate a driver line for the α-subunit rescue experiments. *da*-Gal4 was mobilized to chromosome II and crossed into the *nrv2[23B]* (Genova and Fehon, 2003) background to generate a driver for the β-subunit rescues.

Polyclonal peptide antibodies were generated against intracellular epitopes of Nrv1 MSKNNGKGAKGEFEFPQPAKKQTFSE, Nrv2.1 MSKPVPMSPSFVDEDLHNLRKPKPF and Nrv3 MADKKIGEYYA-PPVKMGKWEGFKK. All peptides were synthesized by GenScript (Piscataway, NJ) and antibodies were generated by Harlan (Indianapolis, IN) in rabbits (Nrv2.1) or guinea pigs (Nrv1 and Nrv3). The pattern of anti-Nrv3 immunoreactivity precisely matched the expression of *nrv3* RNA determined by in situ hybridization.

Immunohistochemistry and microscopy

The lumenal antibody 2A12, embryo fixation, staining and staging procedures were as described in Samakovlis et al. (Samakovlis et al., 1996). Other antibodies used were anti-a5 (Lebovitz et al., 1989), anti-Coracle (Fehon et al., 1994), anti-GFP (Molecular Probes and Abcam) and anti-Verm (Luschnig et al., 2006). Anti-Nrv1, 2 and 3 sera required heat fixation (Peifer et al., 1994) and were used at 1:500 for anti-Nrv2.1 and 1:100 for anti-Nrv1 and anti-Nrv3. Confocal images were captured using a Leica TCS SP2. To assess protein levels, heterozygotes and homozygous mutants were imaged at the same settings on the same slide in the same session. Apparent variations in epithelial cell heights were artefacts resulting from optical sectioning of an irregular epithelial layer and/or differences between the angles at which optical sections intersected the epithelia. Post-processing image adjustments were applied equally to matched images. Embryos were at stage 16.

Assays of SJ function

SJ tube-size function was assayed by examining the tracheal DT, which is fairly straight in wild-type (WT) animals and tortuous in mutants, and GBs, which are contiguous in WT animals but have lumenal gaps in mutants. The GB gaps are a sensitive indicator of SJ function as they are more easily rescued than the DT phenotype. SJ barrier function was tested using the assay of Lamb et al. (Lamb et al., 1998) as modified by Paul et al. (Paul et al., 2003), which tests the ability of an epithelium to block paracellular diffusion of a 10 kDa fluorescent dye. SJ assembly was assessed by the localization of the canonical SJ marker Coracle to the normal position of SJs in the columnar epithelium of the salivary gland, rather than the trachea, because SJs occupy much of lateral cell surface in tracheal cells, precluding reliable assessment of changes in SJ component subcellular localization (Tepass and Hartenstein, 1994).

RESULTS

Drosophila Na,K-ATPase β-subunits have distinct subcellular localizations in epithelia

Previously, we demonstrated that of the three Na,K-ATPase β-subunits in the *Drosophila* genome, only Nrv2 isoforms have SJ and tube-size control functionality [compare Fig. 2D1-4 and 2E1-4 with C1-4 and Paul et al. (Paul et al., 2003)]. This finding was surprising because in vitro studies concluded that virtually any Na,K-ATPase β-subunit can associate with an α-subunit to generate a functional ion pump (reviewed by Blanco and Mercer, 1998; Schmalzing et al., 1991). To begin to understand the molecular basis of these Nrv2-specific activities, we created isoform-specific reagents to determine the endogenous subcellular localizations of these proteins (Figs 1, 2 and see Materials and methods). Although we were unable to generate antisera specific for Nrv2.2, an antibody specific for Nrv2.1 showed that Nrv2.1 is expressed in all ectodermally-derived tissues, consistent with the expression pattern of both Nrv2 isoforms determined using in situ hybridization (data not shown). Nrv2.1 is found at SJs (Fig. 1A1,B1,C1), where it co-localizes with the canonical pleated SJ marker Coracle (Fehon et al., 1994) (Fig. 2A4). SJs are basolateral to adherens junctions and occupy the apical third or fifth of columnar epithelia such as salivary glands (Fig. 1B1) and surface ectoderm (Fig. 1A1).

By contrast, Nrv3 is not expressed in epithelial tissues but was instead found in the central nervous system and the chordotonal organs in the peripheral nervous system (Fig. 1D1,D2; Fig. 2B2). When Nrv3 was ectopically expressed in epithelial cells such as the salivary glands using the Gal4/UAS system (Brand and Perrimon, 1993), it was not efficiently transported to the cell surface and either accumulated at low levels or appeared to become trapped intracellularly (Fig. 2E5). Although in many systems, Na,K-ATPase β-subunits must oligomerize with α-subunits in order to exit the ER (Jaunin et al., 1993), it seems unlikely that the general pool of α-subunit is limiting in this case because overexpression of Nrv2.1 or Nrv1 resulted in normal targeting (Fig. 2C5,D5).

Like Nrv2, Nrv1 was found to be expressed in ectodermal epithelia such as the epidermis (at relatively high levels) and the trachea, but was expressed at very low levels in salivary glands and was absent from the hindgut (Fig. 1A1-C4 and data not shown). In contrast to Nrv2, Nrv1 was also expressed in some endodermal epithelia including the Malpighian tubules and midgut, which lack pleated SJs (data not shown). Importantly, in all epithelial tissues, the large

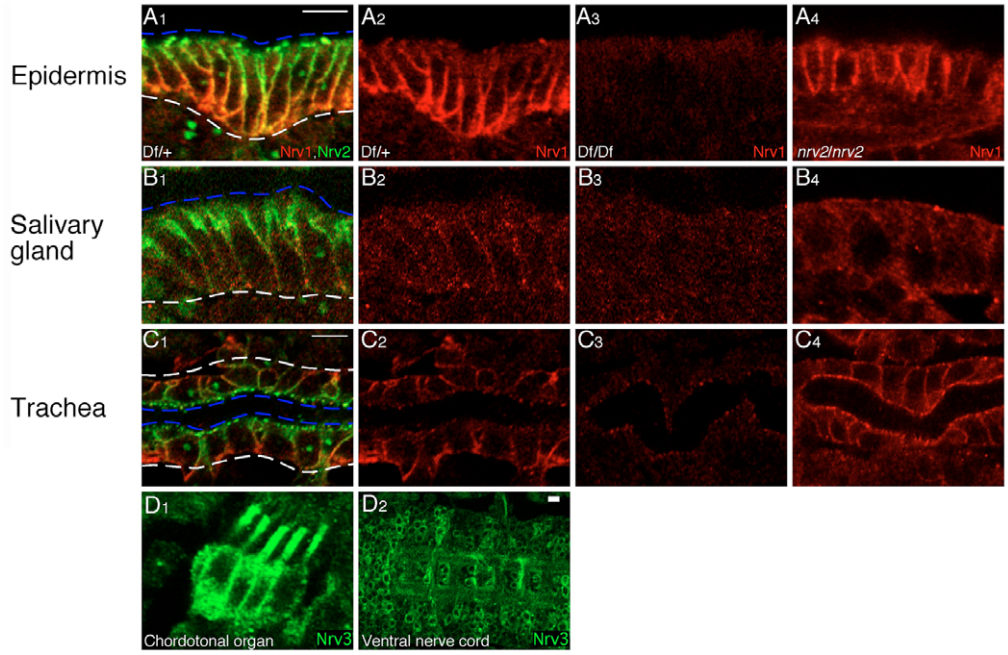

Fig. 1. *Drosophila* Na,K-ATPase β-subunits have distinct subcellular localizations. (**A-C**) Nrv1 (red) is expressed in many epithelial tissues, including the epidermis (A1,2), salivary gland (at low levels; B1,2), and trachea (C1,2). Nrv1 localizes to regions basal to and distinct from the SJ as marked by Nrv2 (green; A1,B1,C1). No Nrv1 protein is detectable in the trans-heterozygote of two small deficiencies [Df(ED)7007 and DF(ED)6569] that delete both *nrv1* and *nrv2* (A3,B3,C3). Levels of Nrv1 staining are unaffected by a *nrv2*-null mutation but the domain of Nrv1 localization is extended apically into the region normally occupied by SJs (A4,B4,C4). (**D**) Nrv3 is expressed in the chordotonal organ (D1) and the central nervous system (D2). In A1,B1,C1, the apical surface is denoted by dashed blue lines and the basal surface by dashed white lines. Scale bars: in A1, 5 μm for A1-B4,D1; in C1, 5 μm for C1-C4; in D2, 5 μm.

majority of Nrv1 localized to the basolateral surface and did not significantly co-localize with Nrv2 or Coracle at SJs (Fig. 1 and data not shown). Further, even when overexpressed in a WT background, Nrv1 was largely excluded from the SJs (Fig. 2D5).

Interestingly, in the *nrv2* mutant, both endogenous Nrv1 and *da*–Gal4-driven Nrv1 were found all along the lateral surface and were no longer restricted to the basolateral surface (Fig. 1A4,C4 and Fig. 2D4 versus D5). This is the first instance of a polarity defect in an SJ mutant and suggests that SJs may have a previously unrecognized role in polarity.

Since all three *Drosophila* β-subunits have different subcellular localizations when expressed in the same tissues, they are likely to have divergent functions and must contain different targeting signals. Furthermore, Nrv2 isoforms are the only isoforms that have an association with the SJ, suggesting that either targeting the Na,K-ATPase complex to SJs is important, or that Nrv2 isoforms might have a structural role in junctional assembly.

The extracellular domain of Nrv2 is required for septate junction function

We next investigated the ability of the different β-subunits to provide SJ function and to assemble SJ complexes. Expression of Nrv2.1 in a *nrv2*-null mutant completely rescued the tracheal and barrier function phenotypes and restored Coracle localization (Fig. 2 and see Materials and methods). Almost all Nrv2 protein localized to SJs. By contrast, expression of Nrv1 and Nrv3 did not rescue tracheal or SJ barrier defects (Fig. 2D1-D3,E1-E3) and did not restore localization of Coracle to SJs (Fig. 2D4,E4).

To define which regions of Nrv2 contained SJ function, we constructed a series of chimeras in which the intracellular (I), transmembrane (T) and extracellular (E) domains of Nrv2.1 were swapped with the corresponding domains of Nrv3. Because all β-subunits share the same basic structure we reasoned that the chimeric proteins should fold properly. We found that both chimeras containing the extracellular domain – Nrv3I/2TE and Nrv3IT/2E – had full tracheal and SJ barrier function activity and were phenotypically indistinguishable from WT (Fig. 2F1-F3 and data not shown). Both chimeras could also restore strong Coracle localization to the SJs of *nrv2* homozygotes. By contrast, although both chimeras with the Nrv3 extracellular domain appeared to be expressed at levels at least comparable to the UAS Nrv2 construct, these chimeras lacked detectable tracheal tube-size activity, SJ barrier function, or the ability to assemble SJ complexes as assayed by the ability to localize Coracle to SJs (Fig. 2G1-G4 and data not shown). Thus, the extracellular domain of the Nrv2 β-subunit has a specific activity required for tube-size control, SJ barrier function and SJ complex assembly.

Importantly, although the Nrv2IT/3E chimera did not have rescue activity, it did have the ability to associate with SJs when expressed in *nrv2*/+ heterozygotes (Fig. 2G5), which argues that simply bringing the Na,K-ATPase to the SJ is insufficient for organizing the SJ and that the extracellular domain of the β-subunit is involved in an extracellular complex required for SJ formation.

Multiple regions of the Nrv2 extracellular domain are required for junctional function

Having narrowed the essential functional region of Nrv2 to the extracellular domain, we constructed an additional series of chimeras between portions of the extracellular domains of Nrv2 and Nrv3 to further define the regions of Nrv2 containing the junctional and tube-size control activities. We divided the

extracellular domain into four sections of roughly equal length, using blocks of conserved regions as breakpoints for our chimera design given that a high resolution crystal structure of a Na,K-ATPase β-subunit is not available (see Materials and methods). Unexpectedly, when expressed in the *nrv2* mutant background, none of these extracellular domain chimeras rescued tracheal tube-size control or SJ barrier function (Fig. 3B1-G4), suggesting that the tube size and barrier junction function of the Nrv2 extracellular domain is a three-dimensional epitope formed from multiple regions of linear sequence or that full function of the extracellular domain requires several interactions.

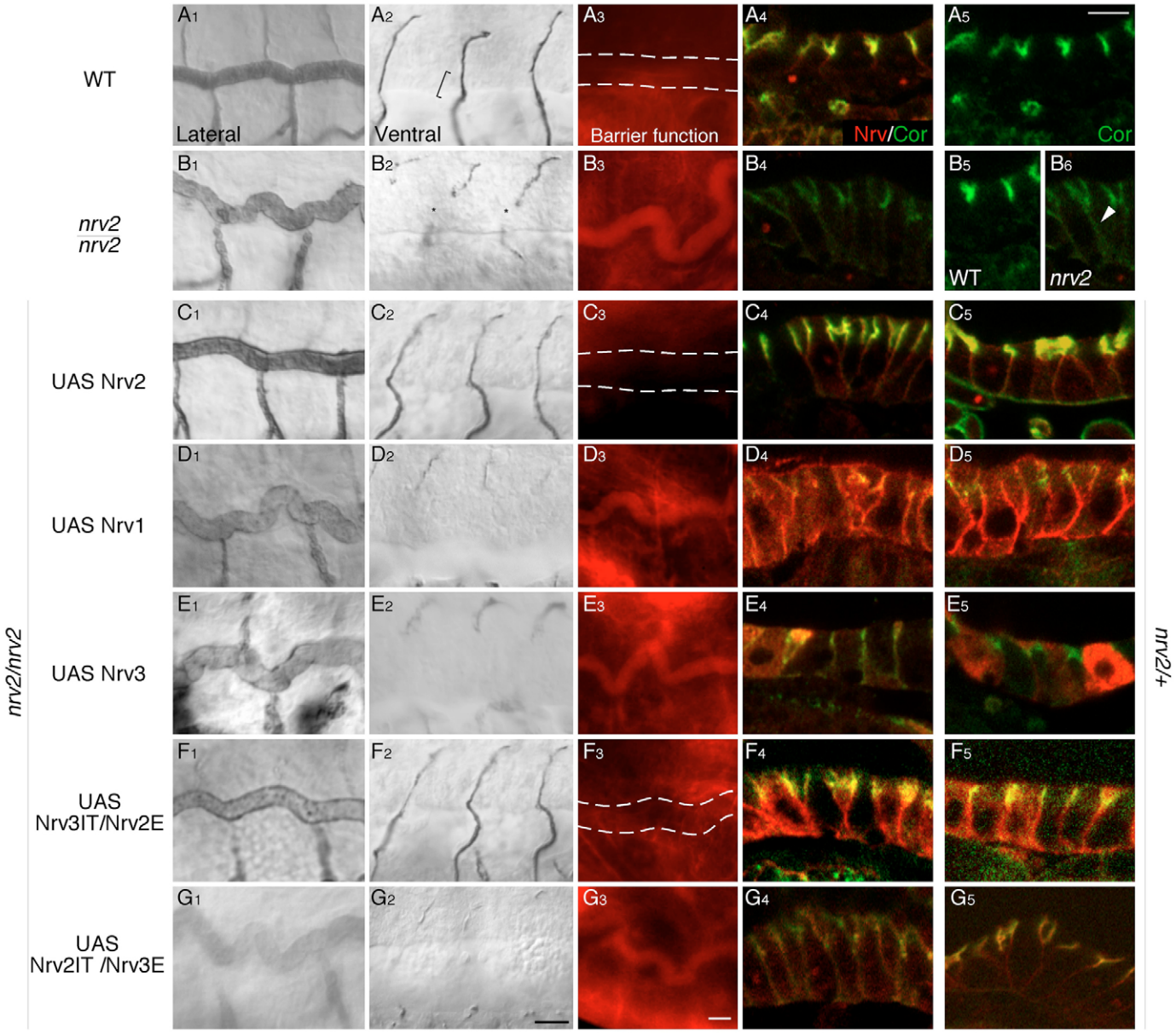

Fig. 2. The Nrv2 extracellular domain mediates SJ organization and tracheal tube morphogenesis. (**A**,**B**) When compared with wild-type (WT) animals (A1-5), *nrv2[23B]*-null mutants have tracheal defects including dorsal trunks (DTs) of increased length and irregular diameter (B1 versus A1) and lumenal staining gaps in ganglionic branches (GBs) (asterisks in B2 versus brackets in A2). *nrv2* mutants have also lost the paracellular barrier function of the SJs which allows a dye to penetrate the epithelium and accumulate in the lumen (red tracheal tube in B3 versus A3 where the trachea exclude the dye). Dashed lines delineate tracheal lumen. *nrv2* mutants also have disorganized SJs at the cellular level with reduced and partially mislocalized Coracle (B4 versus A4,A5). Lateral mislocalization of Coracle in the *nrv2* mutant (B6, arrowhead) is apparent in overexposed images that saturate signal levels for Coracle in WT animals (B5); B5 and B6 are higher gain images of A5 and B4, respectively. (**C-E**) The mutant phenotypes are rescued by expression of Nrv2 (C1-4) but not by expression of Nrv1 (D1-4) or Nrv3 (E1-4) using a *da*-Gal4 driver. Neither Nrv1 nor Nrv3 are incorporated into an established SJ in *nrv2* heterozygotes (D5,E5), whereas Nrv2 is incorporated (C5). (**F**,**G**) A chimera with the intracellular (I) and transmembrane (T) domains of Nrv3 and the extracellular (E) domain of Nrv2 rescued the *nrv2* defects (F1-4), whereas a chimera with the Nrv2 IT and Nrv3 E domains did not (G1-4). Both chimeras could be incorporated into established SJs (F5,G5). Coracle is green and Nrv protein is red (anti-Nrv2.1 in A4-C5 and G4,5; anti-Nrv1 in D4,5; anti-Nrv3 in E4-F5). All animals are stage 16. Scale bars: in G2, 10 μm for A-G images 1,2; in G3, 10 μm for A-G image 3; in A5, 5 μm for A-G images 4,5 and B6.

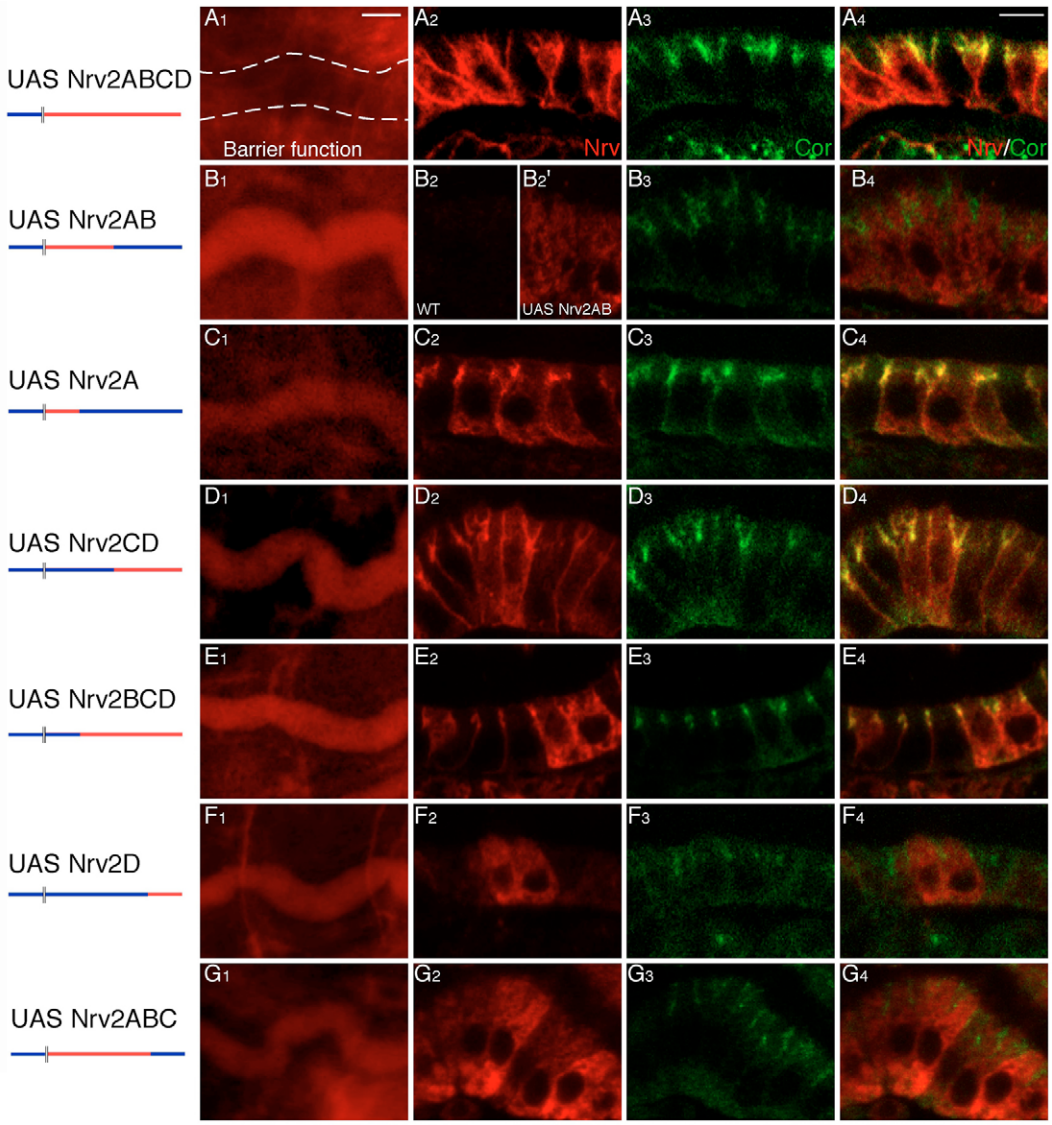

Fig. 3. Multiple regions of the Nrv2 extracellular domain are required for function. (**A**) A chimera containing the entire Nrv2 extracellular domain and the Nrv3IT domains (Nrv2ABCD) rescues tracheal tube size and paracellular barrier function (A1 and see Fig. 2F1), is targeted to the lateral membrane and SJ (A2), and restores proper Coracle localization (green) and levels (A3,4). (**B-G**) By contrast, chimeras missing any region fail to restore tracheal tube size (data not shown) and barrier function (B-G, image 1). All chimeras were expressed (B2′ and C-G image 2), with Nrv2A (C2), Nrv2CD (D2) and Nrv2BCD (E2) being targeted to the SJ and supporting SJ localization of Coracle at WT or near-WT levels (C3,D3,E3 and C4,D4,E4) indicating these chimeras nonetheless have some SJ-organizing activity. An anti-Nrv3 antibody recognizing the Nrv3I domain (red) was used to detect the chimeras because there is no expression of Nrv3 in *nrv2*/+ salivary glands [compare B2 (no chimera) and B2′ (UAS Nrv2AB)]. Scale bars: in A1, 10 μm for A-G image 1; in A4, 5 μm for A-G images 2-4 and B2′.

Consistent with the multiple-interaction hypothesis, there were differences in the subcellular localization of the chimeric proteins. For example, a chimera containing only the extracellular segment of Nrv2 adjacent to the transmembrane domain (UAS Nrv2A) was targeted to the SJ and restored Coracle levels at the SJ (Fig. 3C2), but the chimera containing the C-terminal two Nrv2 segments (Nrv2CD) also localized to the SJ (Fig. 3D2). It therefore appeared that there were targeting sequences within both Nrv2A and Nrv2CD. However, neither of these constructs had tube-size or junctional activity. These results independently demonstrate that targeting the Na,K-ATPase to the SJ is insufficient to provide these activities, and suggests that the extracellular domain mediates multiple interactions required for proper SJ assembly.

Although none of the extracellular domain constructs imparted full rescue activity, several of the constructs were able to restore apparently normal levels of Coracle localization to SJs, thus demonstrating that these chimeras could support some level of SJ complex assembly. Importantly, these constructs are the first mutant conditions that separate the localization of Coracle to SJs from the barrier and tube-size control activities. However, only those chimeras that rescued tracheal tube-size control were also able to rescue localization of the tracheal lumen matrix protein Verm (data not shown). Further analysis of these partial SJ complexes should provide an entry point into understanding which complexes are required to support the different SJ functions.

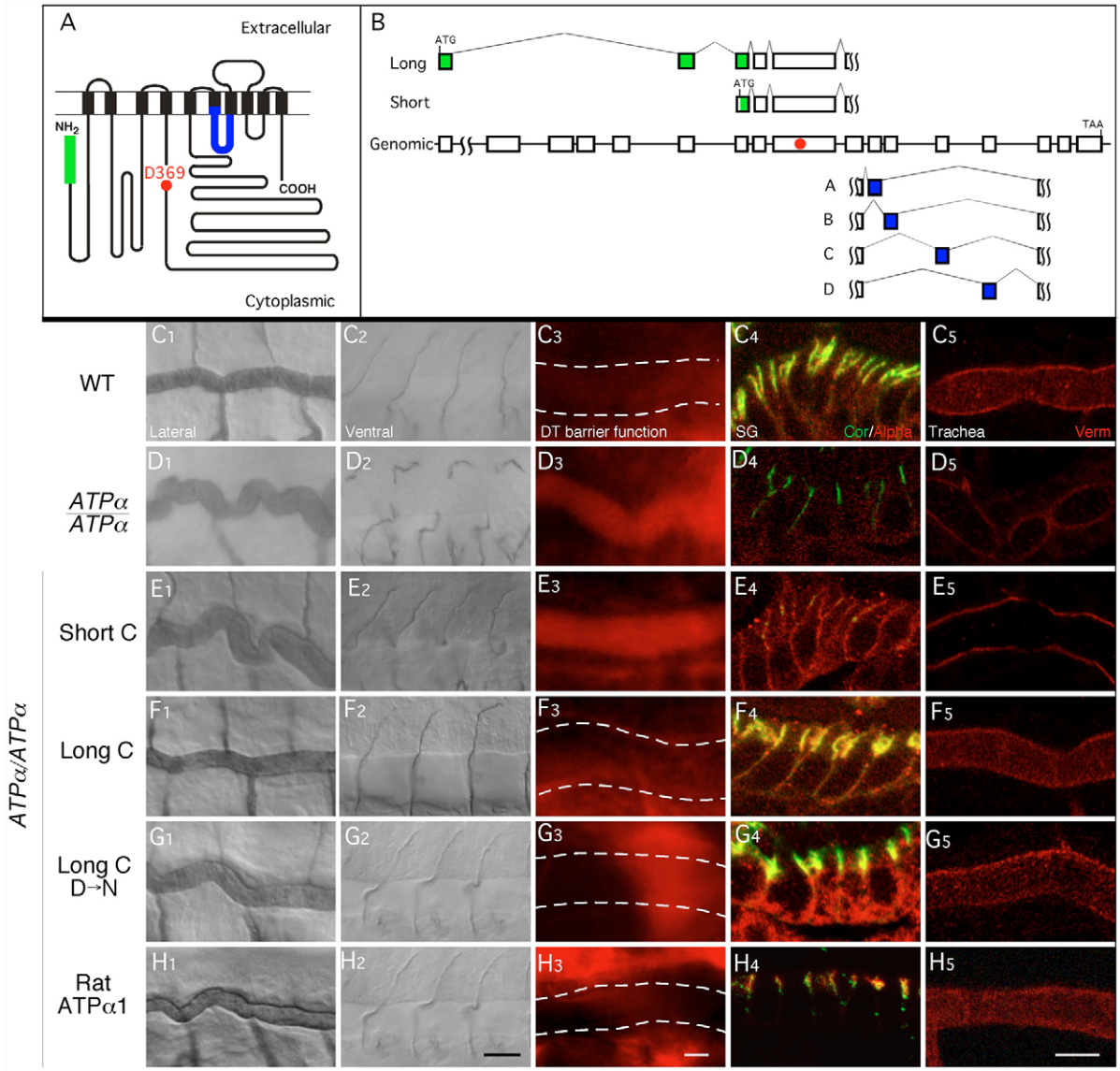

Fig. 4. Rat α1 and some *Drosophila* ATPα isoforms have SJ full activity. (**A**,**B**) The Na,K-ATPase α-subunit (A) is encoded by one major locus at 93A that generates numerous isoforms by alternative splicing (B). Splice forms differ in the N-terminal 39 aa (green) and in a mutually exclusive exon (blue). The catalytic residue D369 (D394 in the fly) is denoted by a red dot. (**C-H**) Compared with the WT (C1-5), *Atpα [DTS1R2]* mutants have long DTs with diameter defects (D1) and missing lumens in the GBs (D2). The mutant fails to exclude dye from the trachea (D3), has disorganized SJs (D4; Coracle, green; ATPα, red), and no longer accumulates Verm in the tracheal lumen [D5 and Wang et al. (Wang et al., 2006)]. DT and GB morphology, barrier junction, Verm accumulation defects and Coracle localization are fully rescued when Long C (F1,4,5), Long C D→N (G1,4,5), or rat α1 (H1,4,5) are expressed using a *da*-Gal4 driver. Expression of Short C gives only slight DT rescue (E1), no Coracle rescue (E4) and no Verm rescue (E5). Scale bars: in H2, 10 μm for C-H images 1,2; in H3,10 μm for C-H image 3; in H5, 5 μm for C-H images 4,5.

Only some α-subunit isoforms have full junctional activity

Given that only some of the Na,K-ATPase β-subunit isoforms had tube-size and barrier junction activities, we investigated whether all α-subunit isoforms would have junctional activities. In *Drosophila*, most Na,K-ATPase α-subunit isoforms are encoded by one primary locus, *Atpα*, that gives rise to numerous potential protein products by alternative splicing. These isoforms differ at the extreme N-terminus, where an additional 39 aa are found in the 'Long' isoforms, and in transmembrane domain six and the fourth intracellular domain, which together are encoded by a mutually exclusive sixth exon cassette (Fig. 4A,B) (Palladino et al., 2003). To date, the mutations in the *Atpα* locus that cause defects in tracheal tube-size control and SJ function (Paul et al., 2003; Genova and Fehon, 2003) affect constitutive exons and disrupt all known ATPα isoforms.

We asked which of the ATPα isoforms were capable of organizing the SJ and controlling tracheal tube size. To do this we generated UAS-expression constructs, each containing a cDNA encoding one ATPα isoform. When expressed in an *Atpα*-null mutant background under the control of the *da*-Gal4 ubiquitous driver, isoforms Long A, B and C were able to completely restore normal tracheal tube morphology, secretion of Verm and paracellular barrier function (Fig. 4F1-F3,F5 and Table 1). At the cellular level, the Long isoforms fully

Table 1. Transgenic rescue of *ATPα* mutant phenotypes by wild-type and mutant α-isoforms

Transgenic isoform*	DT†	GB†	Barrier function‡	Protein localization§	Adult lethality¶
Drosophila					
Short A	++	+++	+		No
Short B	++	++	++		No
Short C	+	++	+	BL	No
Short D	++	++	+++		No
Long A	+++	+++	+++		Yes
Long B	+++	+++	+++		No
Long C	+++	+++	+++	J/L	Yes
Short C (D→N)	+	++	+++	BL	No
Long B (D→N)	+++	+++	++		No
Long C (D→N)	+++	+++	+++	J/L	No
Rat					
ATPα1	+++	+++	+++	J/L	Yes

*Identical results were obtained with multiple independent insertion lines for isoforms Short C, Long A, B and C, Long C (D→N) and Rat ATPα1. For other isoforms, only a single insertion line was assayed.
†Degree of dorsal trunk (DT) and ganglionic branch (GB) rescue: +, mild rescue (e.g. Fig. 3E1,E2); ++, intermediate rescue; +++, perfect rescue (e.g. Fig. 3C1,C2,F1,F2,G1,G2).
‡Septate junction barrier function rescue measured by dye-exclusion assay (Lamb et al., 2001): +, 40-60% tracheal tubes impermeable to dye; ++, 60-80%; +++, 80-100%. *n*>7 for all lines.
§Localization of transgenic isoform: J, junctional; L, lateral; BL, basolateral. Localization of wild-type protein is junctional/lateral.
¶Lethality was considered to be rescued when *n*>50 adults were scored and rescued *ATPα* homozygotes were present.

supported junctional assembly, with both the Na,K-ATPase and Coracle localizing to SJs (Fig. 4F4). By contrast, none of the Short isoforms had full rescue ability despite being expressed at levels comparable to or greater than those isoforms capable of full rescue (e.g. Fig. 4, compare E4 with F4 and H4); instead, the Short isoforms had a range of activity for tracheal morphology and barrier function (Table 1). The Short C isoform had the least activity at the phenotypic level, showing little rescue of DT morphology, Verm secretion or barrier junction defects (Fig. 4E1-E3,E5). At the cellular level, Short C did not visibly support junctional assembly, as there was no concentration of Coracle at the SJ, and the Short C ATPase was uniformly distributed along the lateral membrane (Fig. 4E4). Importantly, the lateral distribution of the Short C isoform mimicked the lateral distribution of SJ components in the absence of SJs, indicating that the Short C isoform had little junctional organization activity. This result suggests that the Long N-terminus is required for the α-subunit to interact with cytoplasmic proteins and/or cytoplasmic portions of membrane proteins to support junctional assembly.

Ion-pump-independence of the Na,K-ATPase tube size and barrier junction activities

The key mechanistic question for the role of the Na,K-ATPase in SJ function is whether the ATPase simply acts as a pump or whether it has ion-transport-independent functions. We tested α-subunits predicted to be catalytically inactive for tube-size control and barrier junction activities. Across phylogeny, the Na,K-ATPase ion pump function is contained within the α-subunit and depends on an essential aspartic acid that interacts with the terminal phosphate group of ATP to allow hydrolysis of the high-energy bond (reviewed by Kaplan, 2002). Mutation of this invariant aspartic acid to asparagine (D369N in vertebrates) has been shown in several systems to completely abolish ATP hydrolysis and ion pumping (Ohtsubo et al., 1990; Kuntzweiler et al., 1995). Because regions of α-subunits containing catalytic function are nearly 100% identical between flies and vertebrates (for example, the 60 residues flanking asparatic acid 369 are 98% identical between fly ATPα and the vertebrate α-isoforms), it is highly likely that mutation of the essential aspartic acid in fly α-subunits completely eliminates ion pump function. Strikingly, we found that several ATPα isoforms containing the fly equivalent of the D369N mutation rescued tube-size and barrier junction functions as well as their WT counterparts (Fig. 4 and Table 1). For example, expression of Long C in the *Atpα* mutant background completely restored tracheal tube-size control to wild type levels (Fig. 4, compare C1 with F1). In the absence of pump function, Long C D394N still retained the ability to rescue tracheal morphology, Verm secretion and barrier function (Fig. 4G1-G3) at the phenotypic level, and SJ organization at the cellular level (Fig. 4G4). Therefore, *Drosophila* SJ formation requires an ion-pump-independent function of the Na,K-ATPase. These results define a new function for a protein complex that has been extensively studied for over 60 years.

Junctional activities are conserved between the *Drosophila* ATPα and vertebrate α1 Na,K-ATPase subunits

The vertebrate Na,K-ATPase has been implicated in cell-cell adhesion and polarity in a number of studies, although its exact role remains elusive. Given the high degree of conservation between α-subunits in eukaryotes (>70% identity between flies and vertebrates), and the fact that multiple combinations of α- and β-subunits of the different species can form functional complexes (Schmalzing et al., 1991), we investigated whether a vertebrate α-subunit could function in the fly context. We selected rat α1 because, of the four vertebrate α-subunit isoforms, α1 is the only one expressed in all epithelial tissues and therefore seemed the most likely to have a junctional function (Blanco, 2005). Strikingly, expression of this rat α1 with an N-terminal GFP tag in the *Drosophila Atpα* mutant rescued the tracheal mutant phenotypes to wild type (Fig. 4H1,H2), restored SJ barrier function (Fig. 4H3), rescued Coracle and Verm localization (Fig. 4H4,H5), and was able to rescue the lethality associated with null *Atpα* alleles. Thus, it appears that the functions of the Na,K-ATPase α-subunit in SJ formation are conserved between flies and vertebrates.

DISCUSSION

The Na,K-ATPase has been intensively studied as an ion-transporter over the last 60 years (reviewed by Kaplan, 2002), and although there is increasing evidence that it has roles beyond this, in most cases the details of these ion-transport-independent functions are

unclear. In particular, in vertebrate epithelia there are multiple reports that implicate a role for Na,K-ATPase in cell adhesion and/or polarity (Cereijido et al., 2004). For example, Shoshani et al. (Shoshani et al., 2005) provided evidence that the β1-subunit acts as a homophilic cell adhesion molecule, and Rajasekaran et al. (Rajasekaran et al., 2001b) showed that expression of the β1-subunit and E-cadherin, but not of either alone, could cause viral-transformed unpolarized MDCK cells to form adherens and tight junctions, and to polarize. However, to date the vertebrate Na,K-ATPase has not been shown to be a component of known cell adhesion or polarity complexes. In *Drosophila*, the Na,K-ATPase is part of the SJ which also contains the basolateral polarity proteins Scrib, Dlg and Lgl (Genova and Fehon, 2003; Paul et al., 2003). Here we show that the junctional activity of the Na,K-ATPase is mediated by the extracellular domain of the Nrv2 β-subunit and that the junctional activity does not require ion pumping by the α-subunit.

The extracellular domain of the β-subunit mediates junction formation

Using chimeric β-subunits composed of domains from the Nrv2 isoform that has junctional activity, and from the Nrv3 isoform that lacks junctional activity, we discovered that only chimeras containing the Nrv2 extracellular domain could properly target the chimera to the SJ and provide junctional activity. Although the extracellular domains of Na,K-ATPase β-subunits have previously been shown to mediate α-subunit ion-transport activity (Laughery et al., 2003; Noguchi et al., 1994) and cell-cell adhesion interactions (Contreras et al., 1999; Muller-Husmann et al., 1993), to our knowledge this is the first demonstration that the extracellular domain of the β-subunit organizes a junctional complex rather than simply acting as a cell adhesion molecule. Although the extracellular domain could simply serve to localize the Na,K-ATPase to the SJ, evidence that the Nrv2 extracellular domain has additional roles in junctional activity is provided by the observation that the Nrv2IT/3E chimera localized to the SJ but did not provide junctional activity. Thus, the Nrv2 extracellular domain is likely to interact with other extracellular SJ components to help organize SJ complexes. Whether these are cis interactions that organize the other transmembrane SJ components such as Neurexin, Neuroglian, or Lachesin (Genova and Fehon, 2003; Llimargas et al., 2004), or trans interactions that organize septa between cells, or both, remains to be determined.

Ion-pump-independent activity of the Na,K-ATPase in SJ function

One of the most surprising results from our studies is that the junctional and tube-size functions of the Na,K-ATPase apparently do not require ion pumping. This contrasts with the traditional view of the ATPase, as an ion pump required for ion homeostasis in many cellular functions and developmental events. For example, Rajasekaran et al. (Rajasekaran et al., 2001a) showed that the low intracellular Na^+ concentration maintained by the Na,K-ATPase is required for MDCK junction formation, and Lowery and Sive (Lowery and Sive, 2005) showed that ATPase-mediated ion transport was required for zebrafish neural tube inflation. Shu et al. (Shu et al., 2003) have shown that ion-transport by the α1β1 isoform is required for zebrafish heart morphogenesis. Hilgenberg et al. (Hilgenberg et al., 2006) recently showed that the neuronal α3 ATPase binds to and acts as a receptor for Agrin, but the signal is transduced via changes in ATPase ion transport activity. Thus, the apparent ion-pump-independent junctional activity of Nrv2 appears to be a novel activity for an Na,K-ATPase.

Although ion pumping by the α-subunit is not required for SJ formation, the α-subunit nonetheless appears to have an important role in organizing SJs. All isoforms with the Long N-terminus fully support junctional assembly, whereas isoforms with the Short N-terminus have only partial activity at the phenotypic level and do not significantly support junctional assembly at the cellular level. The alternatively spliced sixth exon also appears to contain some junctional activity because different sixth exon isoforms vary widely in their ability to provide tube-size and barrier function when the Long N-terminus is absent.

A model for the role of the ATPase in SJ assembly that is consistent with the combined results of the α- and β-subunit data is that the extracellular domain of the β-subunit interacts with multiple extracellular SJ components to assemble an extracellular complex, whereas the α-subunit interacts with cytosolic proteins or intracellular portions of transmembrane proteins to promote junction formation, paracellular barrier formation and tracheal tube-size control. An example of a protein that could interact with the α-subunit to organize junctions is the cytoskeletal protein Ankyrin, which has been shown to bind two distinct sites on the rat α-subunit (Jordan et al., 1995; Zhang et al., 1998), sites which are conserved in the *Drosophila* ATPα (data not shown).

An evolutionary conserved role for the Na,K-ATPase in cell junction and/or polarity formation?

The ability of the rat α1 isoform to rescue all junctional defects of *Drosophila Atpα*-null mutants is consistent with the 77% identity between the *Drosophila* ATPα and the rat α1 proteins, and supports the hypothesis that the Na,K-ATPase has a conserved role in cell junction formation. Why would the Na,K-ATPase have evolved and maintained a role in epithelial cell junction formation and/or polarity? Possibly, as metazoans first became multicellular, their epithelial cells would have needed to establish cell-cell junctions and asymmetrically distributed ion pumps (i.e. primitive cell polarity) to enable polarized ion transport. In the first epithelial cells, the asymmetric localization of the Na,K-ATPase may have been achieved by anchoring the pump to asymmetrically localized adhesion proteins. As cell junctional and polarity mechanisms evolved, the Na,K-ATPase could have transitioned from being associated with adhesion proteins only to serving an integral scaffolding role in a larger junctional complex. Although ATPase-mediated ion transport would still be required for ion homeostasis, the scaffolding function could be ion-transport-independent, consistent with our findings that Na,K-ATPase catalytic activity is required for *Drosophila* viability, but that ATPα-subunits predicted to be catalytically inactive fully support SJ formation.

We are grateful to I. T. Helenius, T. Krupinski and L. Swanson for comments on the manuscript; to B. Ganetzky for support during construct generation; to T. Le, Z. Liang, B. Kalish, R. Hurtado and C. Johnston for technical support; and to W. Russin of the Northwestern Biological Imaging Facility for assistance with confocal imaging. The GFP-rat α1 was a gift from J. I. Sznajder. Support for M.J.P. was provided by NIH R01 AG025046 and NIH R01 NS15390. G.J.B. was a recipient of an NSF Career Award IBN-0133411 and NIH R01 GM069540.

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