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Diversity of function and mechanism in a family of organic anion transporters

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Abstract

Originally identified as transporters for inorganic phosphate, solute carrier 17 (SLC17) family proteins subserve diverse physiological roles. The vesicular glutamate transporters (VGLUTs) package the principal excitatory neurotransmitter glutamate into synaptic vesicles (SVs). In contrast, the closely related sialic acid transporter sialin mediates the flux of sialic acid in the opposite direction, from lysosomes to the cytoplasm. The two proteins couple in different ways to the H^+ electrochemical gradient driving force, and high-resolution structures of the *Escherichia coli* homolog D-galactonate transporter (DgoT) and more recently rat VGLUT2 now begin to suggest the mechanisms involved as well as the basis for substrate specificity.

Introduction

The solute carrier 17 (SLC17) family comprises organic anion transporters with diverse physiological roles. In mammals, there are nine members (SLC17A1–9) that group into four subfamilies (Figure 1), all of which transport organic anions. Despite high sequence similarity, the driving force and transport mechanism vary widely. The founding members of this family, SLC17A1–4 localize to the plasma membrane and were originally reported to mediate inorganic phosphate (Pi) uptake driven by cotransport of Na^+ [1]. However, subsequent studies showed that they also exhibit an associated Cl^- conductance and transport organic anions including penicillin, probenecid and uric acid [2–4]. These transporters may thus contribute to the excretion of metabolites and drugs. The SLC17A5 (sialin) confers the export of sialic acid (derived from protein degradation) out of lysosomes driven by the outwardly directed lysosomal pH gradient (ΔpH) [5–7]. Sialin thus functions as a H^+/sialic acid symporter. In contrast to SLC17A1–4, sialin lacks an associated Cl^- conductance. The three vesicular glutamate transporters (VGLUTs, SLC17A6–8) use membrane potential ($\Delta\Psi$) to drive uptake of glutamate into synaptic vesicles and require allosteric activation by both H^+ and Cl^- [8,9]. They do not apparently couple the flux of glutamate to H^+ exchange, distinguishing them from all other vesicular neurotransmitter transporters [10,11]. The VGLUTs also exhibit an associated Cl^- conductance that appears

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to compete with glutamate transport [12,13]. The most recently identified mammalian member of SLC17, the vesicular nucleotide transporter (VNUT, or SLC17A9) concentrates purine nucleotides such as ATP into the synaptic vesicles of neurons and lysosomes of glia cells, enabling their release by exocytosis [14–17]. Like the VGLUTs, VNUT is driven by $\Delta\Psi$ and requires allosteric activation by cytoplasmic Cl^- [15,18]. Similar to the mammalian proteins, six SLC17 family members from *Arabidopsis thaliana* were initially identified as Na^+ - or H^+-coupled phosphate transporters [19]. Recent studies suggest that they also function as organic anion transporters: localized to the chloroplast, they use membrane potential to drive ascorbate uptake and depend on Cl^- [20]. Although apparently diverse in function, the SLC17 family thus exhibits a limited set of distinct properties that confer the transport of organic anions in organisms from mammals to plants. Recent structures of two SLC17 proteins, one a H^+ symporter from bacteria and the other a membrane potential-dependent VGLUT from mammals [21,22] now provide a basis for understanding the mechanisms responsible.

Diversity of function

SLC17 transporters have thus been proposed to perform many apparently disparate activities, including Na^+/Pi cotransport, H^+/organic anion cotransport, $\Delta\Psi$-dependent organic anion flux and a Cl^- conductance. How can closely related proteins subserve such different functions? The identification of organic anions as substrates for the SLC17 family suggested that their original role in Pi transport might reflect a contaminating activity [2,23]. However, subsequent work using purified, reconstituted VGLUT2 (the main subcortical isoform) demonstrated both Pi uptake across the plasma membrane and glutamate transport into secretory vesicles [24]. The two activities appear to be independent, but extracellular glutamate inhibits Pi uptake across the plasma membrane and cytoplasmic Pi may inhibit glutamate flux into synaptic vesicles [25,26]. Despite the inhibition of Pi uptake, external glutamate was not accumulated by VGLUT at the plasma membrane [27], but the reported inhibition of vesicular glutamate uptake by Pi was competitive [26].

More recently, the main cortical isoform VGLUT1 has been shown to confer Pi uptake by neurons, presumably when they reside at the cell surface between synaptic vesicle exo- and endocytosis [27]. However, it has not been possible to test the role of Pi transport by the VGLUTs in excitatory transmission because mutations that affect Pi flux but spare glutamate transport have not been identified.

It is also remarkable that closely related SLC17 proteins can mediate either $\Delta\Psi$-driven uptake by secretory vesicles or H^+ cotransport out of lysosomes. These modes of coupling to two different components (ΔpH and $\Delta\Psi$) of a H^+ electrochemical gradient ($\Delta\mu_H^+$) confer transport in opposite directions across membranes of the secretory pathway but the mechanism that accounts for this difference remains unclear. Although not apparently coupled to ΔpH [10,11,28], the VGLUTs are nonetheless allosterically activated by low lumenal pH [29], raising the possibility that this regulatory mechanism evolved from H^+ coupling. It has also been suggested that sialin mediates vesicular uptake of glutamate and aspartate as well as H^+-coupled sialic acid efflux from lysosomes, thus exhibiting two

entirely independent activities [30]. However, loss of the VGLUTs eliminates glutamatergic neurotransmission, with no residual release due to sialin [31].

The relationship between the flux of organic anion and the Cl^- conductance associated with many SLC17 family members also remains unknown. In the case of the VGLUTs, both glutamate transport and Cl^- currents show allosteric activation by lumenal H^+ and Cl^-, suggesting they are both subject to the same regulatory mechanisms [29]. However, the Cl^- conductance does not depend on glutamate, showing that it is not coupled to glutamate transport [12,29]. Further, the Cl^- currents do not saturate even at very high Cl^-, suggestive of a channel, whereas glutamate currents saturate in the low millimolar range consistent with the alternating access mechanism of a transporter [12]. Consistent with this, the Cl^- currents (10–30 pA/pF in endosomes) are larger than the glutamate currents (2–17 pA/pF) but only modestly. Taken together, this suggests that permeation of the two ions shares regulatory mechanisms but the modes of permeation differ for glutamate and Cl^-. It has been suggested that lumenal Cl^- may exchange for cytoplasmic glutamate [13], but this seems likely to reflect $\Delta\Psi$ generated through the VGLUT-associated Cl^- conductance [29]. Indeed, this suggests a role for the Cl^- conductance. As synaptic vesicles fill with glutamate, it becomes necessary to remove the Cl^- trapped by endocytosis for charge and osmotic balance, and this has been inferred from the indirect effects of external Cl^- on vesicle acidification [32]. However, the role of the VGLUT conductance in the effects of lumenal Cl^- and the consequences of this conductance for glutamate release has not been demonstrated.

In addition to the Cl^- conductance, a subset of SLC17 proteins requires allosteric activation by Cl^-. A biphasic dependence on Cl^- was originally observed for glutamate uptake by synaptic vesicles [8,9,33]. Activation by 2–20 mM cytoplasmic Cl^- appears allosteric [9], although this mechanism is difficult to disentangle from effects of Cl^- on ΔpH and the driving force $\Delta\Psi$. Chloride also acts allosterically from the lumen of the synaptic vesicle [12,29]. A highly conserved arginine (R184 in VGLUT2) in transmembrane (TM) 4 accounts for this form of allosteric regulation: neutralization of R184 to mimic the interaction with Cl^- eliminates the allosteric requirement for lumenal Cl^- to activate the Cl^- conductance [12]. This mutation may also eliminate the requirement for allosteric regulation of glutamate transport by Cl^-.

In summary, Cl^- interacts in multiple ways with the VGLUTs. Both cytoplasmic and lumenal Cl^- allosterically activate the VGLUTs. Chloride also competes for permeation with glutamate, accounting for the inhibition of glutamate uptake by high concentrations of cytosolic Cl^- [12,34]. In addition, efflux of Cl^- through the associated conductance can balance the charge and osmotic strength as glutamate enters. Although the two anions compete for permeation, the mechanisms appear to differ. The Cl^- currents do not depend on glutamate [12], arguing against stoichiometric exchange and raising questions about the coordination of flux between the two ions.

Diversity of mechanism

Substrate recognition—To understand the molecular basis for the disparate functions of SLC17 family members, we determined high-resolution structures of first a bacterial relative and more recently a mammalian VGLUT. The D-galactonate transporter (DgoT) from

Escherichia coli shares ~20% sequence identity with other members of the SLC17 family. Using x-ray crystallography, structures of wildtype DgoT in inward-open conformation and a mutant (E133Q) in outward-open partially occluded conformation were determined [21]. The structures reveal an MFS fold with 12 TM helices divided into 2 six-helix domains. The substrate D-galactonate is resolved at the center of the outward-open partially occluded E133Q structure, consistent with rocking of the N- and C-domains back and forth around the substrate to allow alternating access from either side of the membrane (Figure 2a). More recently, the structure of rat VGLUT2 in complex with an Fab was determined by single particle cryo-electron microscopy (Cryo-EM) [22]. The structure of VGLUT2 adopts an outward-open conformation with the central cavity facing the synaptic vesicle lumen.

DgoT belongs to a large family of bacterial transporters, with several in *E. coli* alone, that have not previously been characterized in terms of function. The *DgoT* gene belongs to an operon involved in the transport and metabolism of galactonic acid [35], and we found that DgoT functions as a galactonate transporter with high apparent affinity (K_m ~18 μM) on the *E. coli* inner membrane. DgoT does not recognize the epimer gluconate that differs only at the 4′- stereo centre, indicating high specificity [21]. In the absence of a direct assay for binding, we do not know the actual affinity of either DgoT or the VGLUTs, but the VGLUTs transport glutamate with ~100-fold lower apparent affinity (K_m ~ 3 mM) and still exhibit high specificity: they do not recognize aspartate which differs by a single methylene group in the sidechain [8].

The structures of DgoT with and without substrate and of VGLUT2 help us to understand substrate recognition by the SLC17 family. DgoT and the VGLUTs use different strategies to balance affinity and specificity (Figure 2). DgoT engages every functional group on D-galactonate with highly specific H-bounds and ionic interactions (Figure 2c). The large number of bonds presumably summates to confer high selectivity, with even small (e.g., stereochemical) changes in the substrate impairing these interactions. In contrast, glutamate has fewer functional groups. Taking advantage of the highly conserved sequence at the substrate binding site and the substrate-bound structure of DgoT, we docked glutamate into the apo structure of VGLUT2. Both possible orientations of glutamate suggest that the VGLUTs engage primarily the two carboxyl groups at the ends of the molecule (Figure 2d, e). The shorter aspartate cannot simultaneously engage residues on both ends, and is thus not recognized or transported. In light of this distance requirement, Pi is not likely to interact or translocate in the same manner, consistent with earlier studies implicating different residues in Pi and glutamate flux [24]. On the other hand, the two anions may compete for an overlapping site [25,26].

Comparison of the binding sites across the SLC17 family suggests that the anionic group of the substrate interacts with the highly conserved TM1 arginine (R47 in DgoT, R88 in VGLUT2) [12,21,24]. However, the rest of the binding site shows much more variation, to accommodate the diversity of organic anion substrates for SLC17 (Figure 2a, b). For example, the VGLUTs contain an important arginine residue (R322 of VGLUT2) in TM7 that may recognize the second carboxyl group in glutamate. Indeed, this arginine is required for glutamate transport [22] and is not conserved in other SLC17 proteins that recognize substrates with a single carboxyl group (Figure 2b). The structures of DgoT and VGLUT2

now allow homology modelling and computational docking of putative ligands for other SLC17 transporters. Sialin and VNUT are of particular interest due to their potential as therapeutic targets. Docking and simulation of sialin shows that the carboxyl group of an inhibitor (Fmoc-Cys[Coum-7-OH]-OH) interacts with the highly conserved R57, Y119 and Y306 in sialin (equivalent of R88 on TM1, Y135 on TM2 and Y327 on TM7 of VGLUT2) [36].

H^+ coupling vs. allosteric regulation—The structures also reveal clusters of polar residues within both N- and C-domains (Figure 3). The N-domain cluster includes a glutamate in TM4 that appears to form a charge pair with the arginine in TM1 [21,22]. This would provide a mechanism for coupling flux to H^+: protonation of the TM4 glutamate releases the TM1 arginine to bind substrate. Indeed, E133 in TM4 is crucial for transport by DgoT [21], but its role in the VGLUTs remains unknown. Presumably, the liberation of R47 from E133 in DgoT enables recognition of substrate and translocation across the membrane with reformation of the E133-R47 charge pair promoting orientation of the empty carrier back to the periplasm, but the mechanism connecting changes in protonation state to changes in structure of the helical bundles remains unclear.

The N-domain polar pocket also contains the TM4 arginine (R184 in VGLUT2) that mediates allosteric activation of the VGLUT-associated Cl^- conductance by lumenal Cl^- [12]. This arginine is highly conserved through the SLC17 family but sialin and DgoT show no requirement for activation by lumenal Cl^-. The physiological role of allosteric regulation by lumenal Cl^- for VGLUTs could be specific to the dynamic ionic environment of the recycling synaptic vesicles but remains to be further investigated. The C-domain contains a smaller set of polar residues unique to the VGLUTs that center around R322 in TM7. These likely play a role in coupling interaction of the second carboxyl in glutamate with a conformational change in the C-domain bundle.

Alternating access—Although DgoT and the VGLUTs transport in opposite directions driven by different forces, the structures in 3 distinct conformations along the transport cycle now help to understand the underlying mechanism. The N-domain including the highly conserved arginine in TM1 does not change much through the cycle whereas the C-domain rotates around the central substrate binding site to allow alternating access. TM7 and TM10, which contain 7 substrate-binding residues in DgoT, play a particularly important role in this conformational change (Figure 4). For the VGLUTs (Figure 4, left to right), glutamate binding from the cytoplasm triggers rotation of the C-domain around the substrate, with bending of TM7 and TM10 on the cytoplasmic side. TM10 then seals the pocket from the cytoplasm while the lumenal half of TM7 rotates away from the substrate, enabling access to the lumen for release. After glutamate discharge, the transporter reorients to the cytoplasm without substrate, resulting in net flux. This occluded/apo form appears difficult to capture, suggesting a short-lived transition state. DgoT effectively proceeds through the cycle in reverse (Figure 4, right to left). In the outwardly oriented conformation, protonation of E133 liberates R47 to bind the carboxyl of periplasmic D-galactonate. In response to substrate binding, the periplasmic gate then closes through interaction of the upper residues in TM1 and TM7, reorienting DgoT to the cytoplasm. After discharge of substrate and H^+,

R47 reforms a charge pair with E133 and the empty transporter reorients to the periplasm. The extensively studied lac permease (LacY) of *E. coli* also recognizes substrate with both N- and C-domains, and relies on protonation of a critical glutamate for ionic coupling [37]. However, protonation of the critical glutamate in LacY contributes directly to substrate recognition [37], rather than through liberation of another residue (e.g., R47 in DgoT) to recognize substrate. The allosteric regulation of VGLUTs by pH may involve the same residues that couple substrate to H^+ in DgoT and sialin, but this remains to be determined, and we know very little about the mechanisms underlying allosteric regulation of any transporter.

Conclusion and perspectives

The recent structures of DgoT and VGLUT2 provide a basis to understand substrate recognition and ionic coupling by the SLC17 family of organic anion transporters. The high specificity of DgoT for D-galactonate is achieved through multiple residues recognizing essentially all functional groups of D-galactonate whereas the VGLUTs rely primarily on recognition of the two carboxyl groups of glutamate, which may account for the high K_m. Nonetheless, the requirements for recognition (including stereochemistry in DgoT and side chain length in VGLUTs) account for the high specificity of both bacterial and mammalian transporters. High sequence conservation around the substrate binding site suggests that SLC17 family transporters use the N-domain surrounding the highly conserved TM1 arginine to engage anionic groups on the substrate while other residues in the binding site accommodate different features. In addition, the highly conserved glutamate in TM4 may confer the coupling of substrate flux to H^+ transport, at least in the case of DgoT: protonation of E133 should release R47 in TM1 to recognize the carboxyl of galactonate. However, the role of this glutamate (E191) remains unclear in the VGLUTs. A glutamate is conserved in the same position of sialin but changes to glutamine in the Na^+ coupled Pi transporters (SLC17A1–4) and VNUT. The role of the TM4 glutamate thus appears to have evolved with the driving force and regulation of transport. We do not know where Pi binds, how Cl^- permeates and how these diverse functions are coordinated with diverse, changing physiological conditions.

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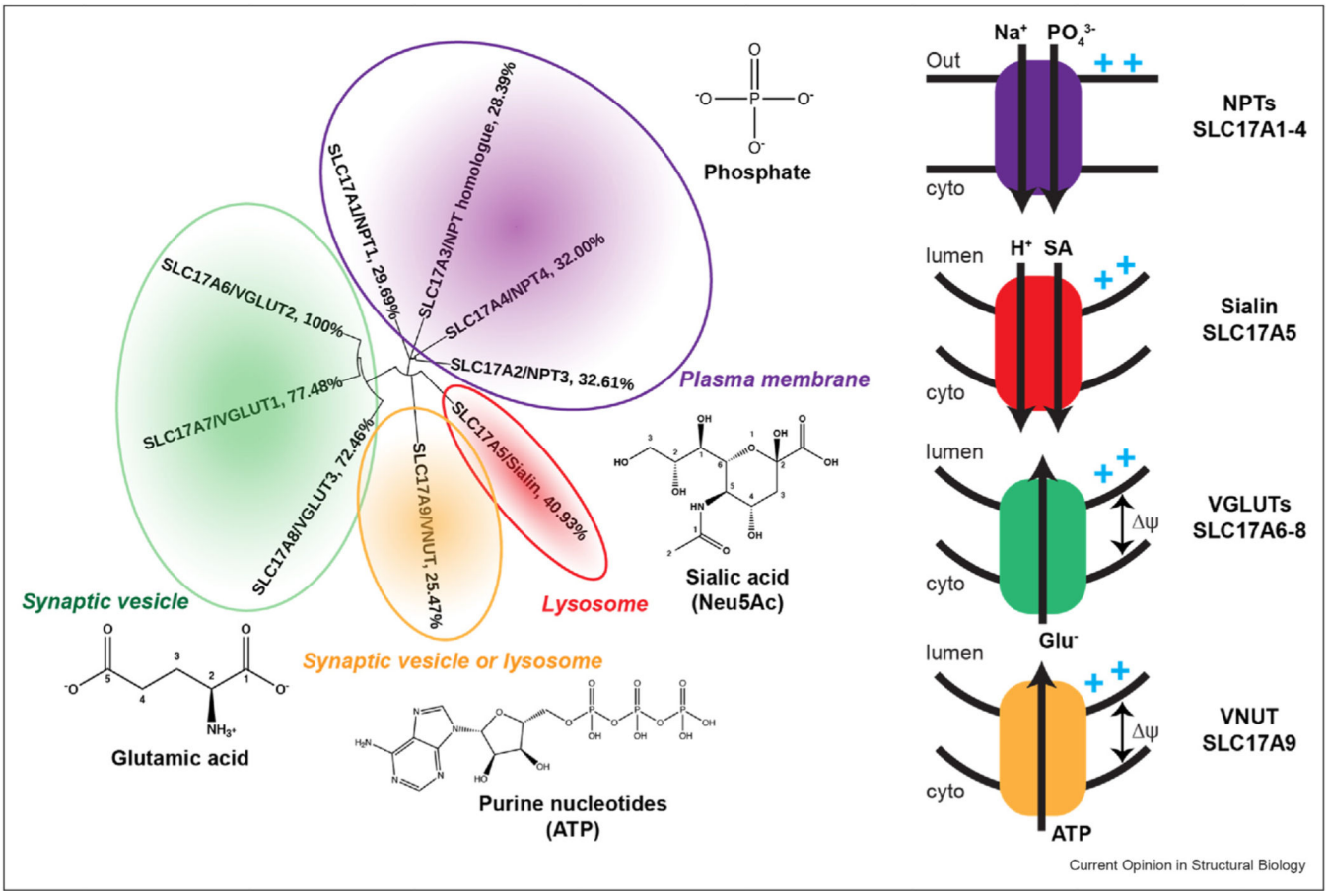

Figure 1. Mammalian members of SLC17 use distinct mechanisms to transport organic anions.

Nine mammalian members of SLC17, grouped by localization (plasma membrane, lysosome and synaptic vesicles) and substrates (inorganic phosphate, sialic acid, ATP and glutamate). Number indicates sequence conservation to human VGLUT2. Diagrams on the right indicate the driving force and mechanism of ionic coupling: Na^+ cotransport for the NPTs, H^+ cotransport for sialin and membrane potential ($\Delta\Psi$) for the VGLUTs and VNUT. Since members of SLC17 reside on either the cell surface or an organelle, we use inward to indicate directed to the cytoplasm in both cases and outward to indicate facing the external solution or vesicle lumen.

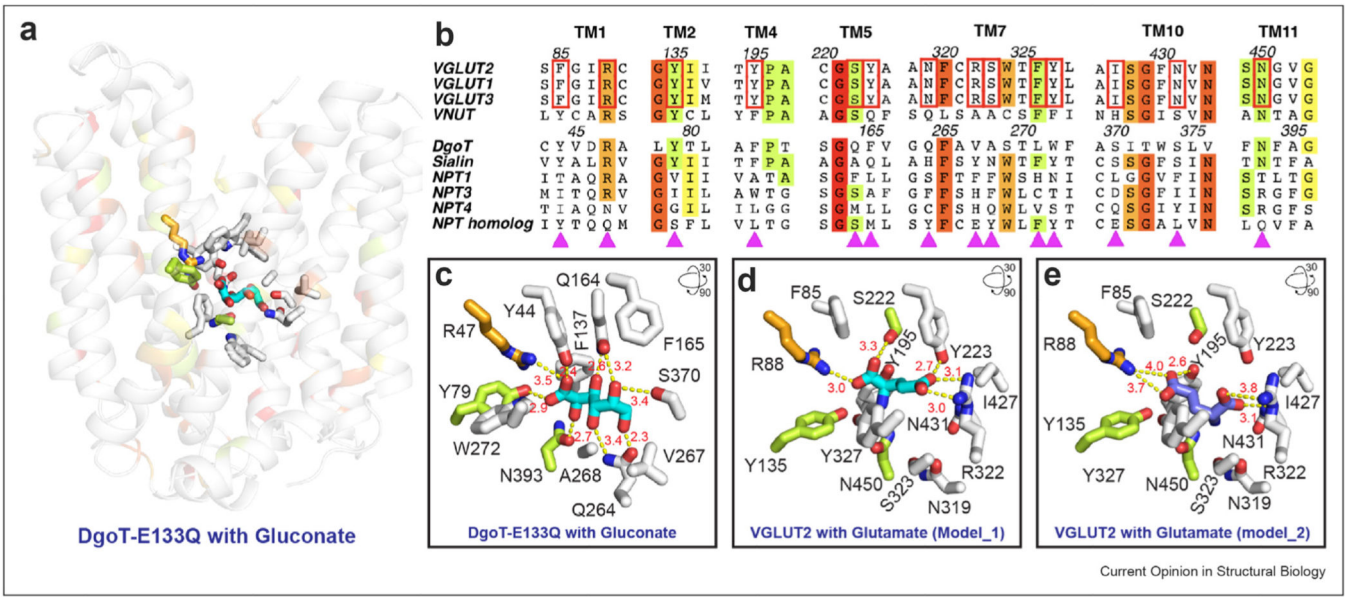

Figure 2. Structural basis of substrate specificity by the SLC17 family.

(**a**) Crystal structure of DgoT E133Q in an outwardly oriented, partially occluded state with D-galactonate shown in cyan. Structure is coloured based on sequence conservation shown in (**b**). (**b**) Sequence alignment of *E. coli* DgoT and mammalian SLC17 family members focusing on residues involved in substrate recognition (indicated as magenta triangles). Highlight color indicates the extent of conservation, with red the most conserved and white the least. The red boxes indicate residues predicted to interact with substrate in the VGLUTs. (**c–e**) Structures of the substrate recognition site for DgoT E133Q with D-galactonate (**c**) and VGLUT2 with glutamate docked in the binding site in two possible orientations (**d, e**). D-galactonate interacts with many residues in DgoT, whereas glutamate is predicted to have an overlapping but smaller set of interactions with VGLUT.

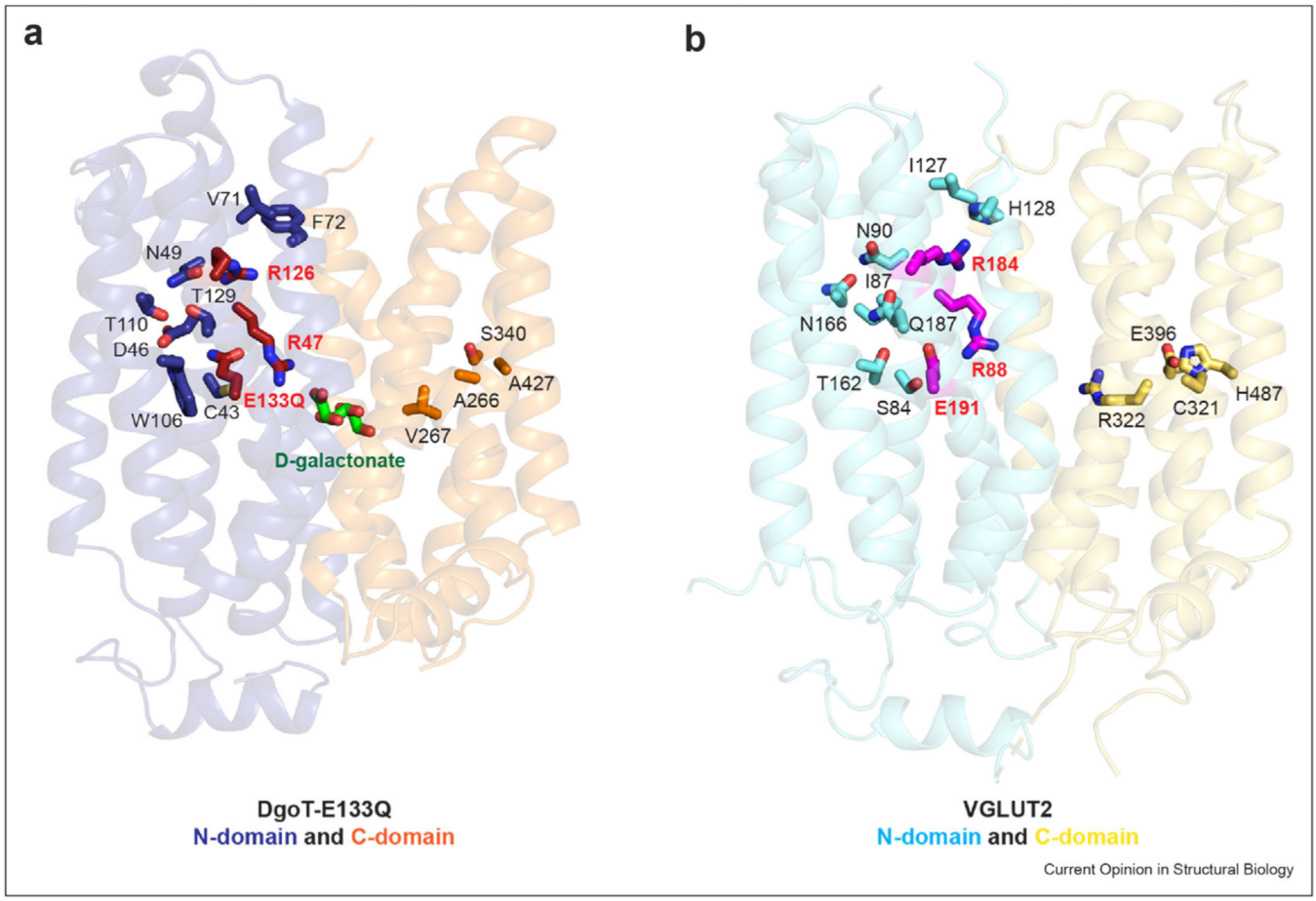

Figure 3. Clusters of polar residues within the N- and C-domains of DgoT and VGLUT2.

(**a**) Outwardly oriented conformation of DgoT E133Q with D-galactonate (green) bound. Residues of the N-terminal polar pocket are shown in blue, those of the C-domain pocket in orange. E133 in TM4 makes an electrostatic interaction with Arg47 of TM1 which is disrupted by protonation or the E133Q mutation. (**b**) The lumenally oriented conformation of VGLUT2 shows a similar arrangement of E191 and R88, with Arg184 in TM4 involved in allosteric activation by lumenal Cl^- [12]. Critical residues from the N-domain are in magenta, the other N-domain residues in light blue and the C-domain in yellow. Critical, highly conserved residues (TM1 arginine, TM4 glutamate and arginine) are labelled in red.

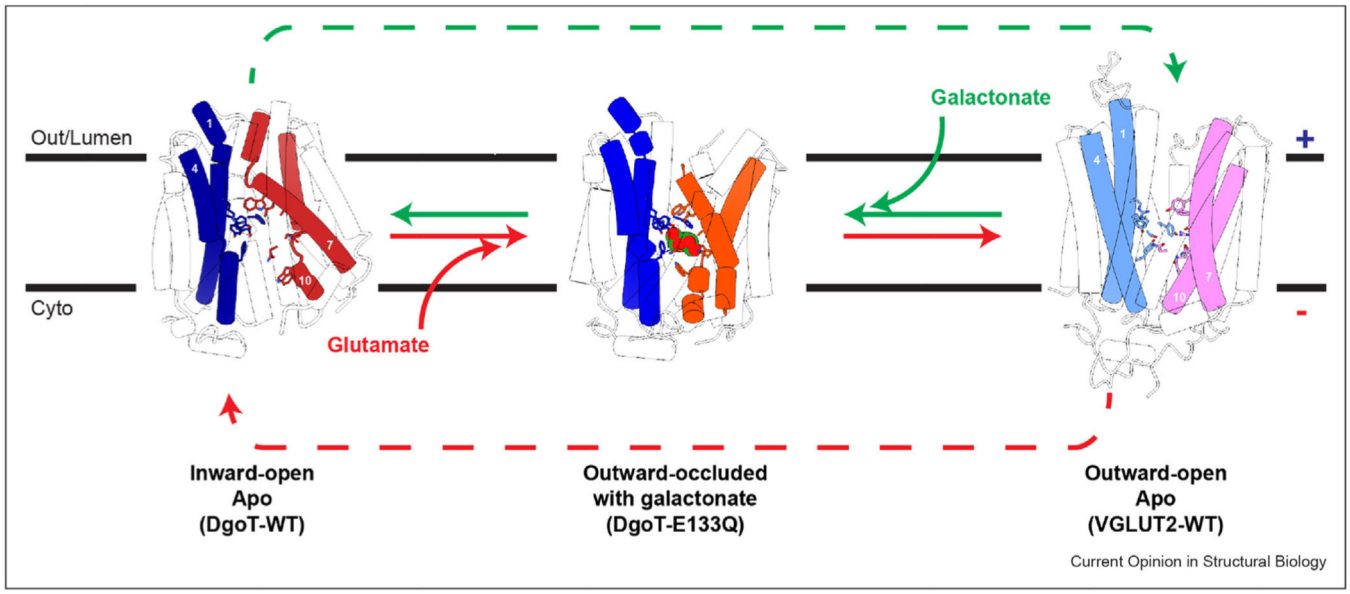

Figure 4. Conformational changes involved in transport by the SLC17 family.

Left, the inwardly oriented structure of WT DgoT, with TM1 and 7 sealing the main cavity from the periplasm. Middle, with substrate (galactonate) binding in the partially occluded structure of DgoT E133Q with TM7 and 10 rearrange to close the cytoplasmic gate. Right, the lumen-open structure of WT VGLUT2 shows TM7 bent back to enable substrate access from the lumen. DgoT and the VGLUTs undergo similar conformational changes during the transport cycle but in opposite directions, with glutamate binding to the VGLUTs from the cytoplasm and galactonate binding from the periplasm.