Vesicular glutamate transporters (VGLUTs) are responsible for the vesicular storage of \(L\)-glutamate and play an essential role in glutamatergic signal transmission in the central nervous system. The molecular mechanism of the transport remains unknown. Here, we established a novel in vitro assay procedure, which includes purification of wild and mutant VGLUT2 and their reconstitution with purified bacterial \(F_0F_1\)-ATPase (F-ATPase) into liposomes. Upon the addition of ATP, the proteoliposomes facilitated \(L\)-glutamate uptake in a membrane potential (\(\Delta\psi\))-dependent fashion. The ATP-dependent \(L\)-glutamate uptake exhibited an absolute requirement for \(-4 \text{ mM} \text{ Cl}^-\), was sensitive to Evans blue, but was insensitive to \(\text{d,L-aspartate}\). VGLUT2s with mutations in the transmembrane-located residues Arg\(^{184}\), His\(^{238}\), and Glu\(^{191}\) showed a dramatic loss in \(L\)-glutamate transport activity, whereas \(Na^+\)-dependent inorganic phosphate (\(P_i\)) uptake remained comparable to that of the wild type. Furthermore, \(P_i\) transport did not require \(\text{Cl}^-\) and was not inhibited by Evans blue. Thus, VGLUT2 appears to possess two intrinsic transport machineries that are independent of each other: a \(\Delta\psi\)-dependent \(L\)-glutamate uptake and a \(Na^+\)-dependent \(P_i\) uptake.

Vesicular storage and subsequent exocytosis of \(L\)-glutamate is the major pathway for excitatory signal transmission in the central nervous system (1–3). Vesicular glutamate transporters (VGLUTs)\(^2\) are essential for the vesicular storage of \(L\)-glutamate through active transport of \(L\)-glutamate into synaptic vesicles at the expense of \(\Delta\mu\text{H}^+\) established by vacuolar \(H^+\)-ATPase (V-ATPase) (1). There are three isoforms of VGLUT, denoted VGLUT1, VGLUT2, and VGLUT3 on the basis of the order of their discovery (2, 4–6). VGLUT1 and VGLUT2 show a complementary expression pattern in essentially all known glutamatergic neurons, suggesting that the two VGLUTs are involved in glutamatergic neurotransmission (7–9). In fact, VGLUT1 knock-out mice exhibit a loss of secretion of \(L\)-glutamate and glutamatergic neurotransmission in neurons that normally express VGLUT1 (4, 10). In contrast, VGLUT3 is expressed in neurons that are usually classified as non-glutamatergic neurons and astrocytes suggesting the involvement of VGLUT3 in a novel mode of \(L\)-glutamate signaling (11–13). VGLUTs are also expressed in peripheral non-neuronal cells, associated with a wide variety of secretory vesicles and are responsible for glutamate-mediated regulation in various cellular processes (5).

VGLUTs belong to the SLC17/type I anion transport family, one of the major facilitator superfamilies (MFS), and are not related to other neurotransmitter transporters such as vesicular acetylcholine transporter and vesicular monoamine transporter (2, 14). VGLUT exhibits unique transport properties when compared with other vesicular neurotransmitter transporters. For one, VGLUT is activated by low concentrations of \(\text{Cl}^-\) (\(-4 \text{ mM} \) through a putative \(\text{Cl}^-\) binding site (15–18). Furthermore, VGLUT requires membrane potential (positive inside) as a driving force, transports \(L\)-glutamate electrophoretically, shows strict substrate recognition and does not recognize \(\text{d,L-aspartate}\) as a substrate (2, 5, 15–18). VGLUT also exhibits \(Na^+\)-driven \(P_i\) transport when expressed in oocytes, like \(Na-P_i\) co-transporter 1 (NPT1), another SLC17/type 1 family (19–20). However, no information on the transport mechanism and mechanistic relationship between the transport of \(L\)-glutamate and \(P_i\) is currently available. The amino acid residues involved in substrate recognition, transport, and regulation also have not been identified.

In general, site-directed mutagenesis is a powerful tool for analyzing the structure and function relationship of transporter proteins. Recombinant VGLUT activity can be assessed as ATP-dependent \(L\)-glutamate uptake in membrane vesicle fractions and/or in detergent-permeabilized cells (2, 3, 5). However, VGLUT activity measured by this procedure is too low to allow quantitative evaluation of the effect of mutations. Moreover, it is frequently observed that mutations disturb targeting of the protein to the correct cellular organelle. For instance, sialin, another member of the SLC17 family, is a lysosomal sialic acid/H\(^+\) co-transporter and targeted to the plasma membrane when mutations are introduced into its dileucine motif (21, 22). Because the VGLUT assay procedure is based on the assumption that the transporter is targeted to acidic organelles and that endogenous ATP fully energizes V-ATPase to drive the uptake, it is not certain whether this assay is valid for mutant VGLUT.
Consequently, information on mutant VGLUTs has yet to be published.

In the present work, we developed a procedure to assess the activity of wild and mutant VGLUT with purified and thus, well-defined components. This procedure includes mutagenesis, expression of wild and mutant VGLUT2 in insect cells, purification, and co-reconstitution with bacterial F-ATPase. F-ATPase is a well-defined electrogenic proton pump with a pharmacological profile distinct from that of V-ATPase (23). F-ATPase is a well-defined component. This procedure includes mutagenesis, and co-reconstitution with bacterial F-ATPase.

EXPERIMENTAL PROCEDURES

Expression—Recombinant baculoviruses containing wild type and mutant rat VGLUT2 cDNA (24) were constructed using the Bac-N-Blue and Bac-to-Bac baculovirus expression systems (Invitrogen) according to the manufacturer’s protocols. For the Bac-N-Blue system, VGLUT2 cDNA was amplified by PCR with the primers (5’-TTACAAACCATTGCGATCCGCTAAAACAGAAAAG-3’ and 5’-GCTTGAATTCGAAATACTCTCGTTG3’) and ligated into the NcoI and EcoRI sites of pBlueBacHis2b. Resultant plasmid and Bac-N-Blue DNA were used to transfect sf9 cells to generate recombinant baculovirus. For the Bac-to-Bac system, rat VGLUT2 cDNA was amplified by PCR using the primers (5’-CACCATTGGAGCTCGTAAAAACAGATT-3’ and 5’-TTGGTATGAATAACATCGTCCGTCTC3’) and cloned into a pENTR/D-TOPO vector. VGLUT2 cDNA was transferred from the pENTR/D-TOPO vector to a destination vector and named pDEST10-VGLUT2. The resulting VGLUT2 gene encoded an N-terminal His tag. DH10Bac cells carrying bacmid DNA were transformed with pDEST10-VGLUT2. Recombinant bacmid was isolated from DH10Bac cells and used for transfection of sf9 cells to generate recombinant baculoviruses. Sf9 or High Five cells were used for expression of VGLUT2 protein. Sf9 cells (2 x 10^7 cells/10-cm dish) were grown in complete TNM-FH medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.25 mg/ml Fungizone and 100 mg/ml penicillin-streptomycin at 27 °C. Alternatively, High Five cells (1 x 10^7 cells/10-cm dish) were grown in Express Five medium (Invitrogen) supplemented with 2 mM L-glutamine, 10 mg/ml gentamicin at 27 °C. Cells were infected by recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 2 and cultured a further 72 h for sf9 and 48 h for High Five. Afterward, the cells were harvested for membrane preparation.

Mutagenesis—Mutations were introduced to pDEST10-VGLUT2 by PCR. The sequence was confirmed by nucleotide sequencing. The following primers were used: R88A, 5’-GTTCAGGCGATACCAAGG-3’; H128A, 5’-CCCCCGCAATCATC-CCACAGTGT-3’; R184A, 5’-CAATATTTGCAACAAAGATG-ACGCA-3’; R184K, 5’-CAATATTTGCAACAAAGATGACGCA-3’; R184E, 5’-CAATATTTGCAACAAAGATGACGCCA-3’; E191D, 5’-GTGACCCGTCACCCTACCCA-3’; E191Q, 5’-GGTTACGCGCTTGGACACGT-3’; E191A, 5’-AGGTAGCCGGCCCAAGTTC-3’; and R322A, 5’-AAGTCCACATC-TCGGCCAGAAGTTT-3’.

Purification—Insect cells (1 x 10^7 cells/dish) were suspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 0.1 M potassium acetate, 10% glycerol, 0.5 mM dithiothreitol, 1 mg/ml pepstatin A, and 1 mg/ml leupeptin and disrupted by sonication with a TOMY UD200 tip sonifier. Cell lysates were centrifuged at 700 x g for 10 min to remove debris, and the resultant supernatant was centrifuged at 160,000 x g for 1 h. The pellet (membrane fraction) was suspended in buffer containing 20 mM MOPS-Tris, pH 7.0, 10% glycerol, 1 mg/ml pepstatin A, and 1 mg/ml leupeptin (~1.5 mg protein/ml). The membrane fraction was solubilized by addition of octyl glucoside (2% final) to suspension. After centrifugation at 260,000 x g for 30 min, the supernatant was added to 1 ml of Ni-NTA Superflow resin (QIagen) and incubated for 4 h at 4 °C with shaking. The resin was poured into a column and washed with 10 ml 20 mM MOPS-Tris, pH 7.0, 5 mM imidazole, 10% glycerol and 1% octyl glucoside. VGLUT2 was eluted from the resin with 3 ml of the same buffer containing 60 mM imidazole. The eluate containing purified VGLUT2 was stored at −80 °C. Bacterial F-ATPase was expressed in Escherichia coli cells strain DK8/pBWU13 and purified by glyceral density gradient centrifugation as described previously (25).

Reconstitution—Co-reconstitution of purified recombinant VGLUT2 and bacterial F-ATPase into liposomes was carried out by the freeze-thaw method described in Ref. 25. In brief, 10 μg of VGLUT2 was mixed with 90 μg of F-ATPase and prepared liposomes (0.5 mg lipid; see below), frozen at −80 °C and left at this temperature for at least 5 min. The mixture was thawed quickly by holding the tube in the hands and diluted 60-fold with reconstitution buffer (20 mM MOPS-Tris, pH 7.0, 0.5 mM dithiothreitol, 0.1 mM potassium acetate and 5 mM magnesium acetate). Reconstituted proteoliposomes were pelleted by centrifugation at 160,000 x g for 1 h at 4 °C and suspended in 0.4 ml of 20 mM MOPS-Tris, pH 7.0 containing 4 mM KCl, 0.1 mM potassium acetate and 5 mM magnesium acetate. Proteoliposomes for phosphate transport assay were prepared without F-ATPase. Liposomes were prepared as follows: soybean lecithin (20 mg; Sigma Type IIS) was suspended in 2 ml 20 mM MOPS-NaOH, pH 7.0, containing 0.5 mM dithiothreitol. The mixture was sonicated in a bath-type sonicator until clear, divided into small aliquots, and stored at −80 °C until use.

Protease Treatment—Reconstituted proteoliposomes (50 μg/ml) were incubated for 1 h at 27 °C in a reaction mixture containing 20 mM MOPS-Tris, pH 7.0, 100 mM potassium acetate, 5 mM magnesium acetate, and 0.5 μg/ml trypsin. Digestion was terminated by the adding of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The reaction mixture was analyzed on a dot blot probed with antibodies specific for the first loop region (G86-G134) of VGLUT2 (26).

Transport Assay—Reconstituted proteoliposomes (5 μg of total protein) were suspended in 20 mM MOPS-Tris, pH 7.5, 5 mM magnesium acetate, 4 mM KCl, and 0.1 mM potassium acetate and incubated for 3 min at 27 °C. ATP was added to give a final concentration of 2 mM and the mixture incubated for a further
**RESULTS**

**Expression, Purification, and Reconstitution of VGLUT2**—We employed a baculovirus overexpression system for the expression and purification of wild and mutant VGLUT2. Upon infection, the High Five cells expressed His-tagged VGLUT2 as revealed by Western blot analysis (Fig. 1A). Maximum expression was obtained 48–72 h after infection at an m.o.i. of 1–2. The membrane vesicles prepared from insect cells did not show any ATP-dependent l-glutamate uptake under the standard assay conditions described under “Experimental Procedures.” After solubilization of the membrane fraction with octyl glucoside, VGLUT2 was purified to near homogeneity by Ni-NTA column chromatography (Fig. 1B, lane 4). The extent of enrichment over membranes, which was roughly estimated by Western blot analysis, was 4.0-fold for Sf9 cells and 5.5-fold for High Five cells with recoveries of ~3 and ~50%, respectively. Hence, we used High Five cells for purification of VGLUT2. Purified VGLUT2 was incorporated into liposomes with purified bacterial F-ATPase using the freeze/thaw/dilution procedure. The resultant proteoliposomes contained VGLUT2 as well as F-ATPase (Fig. 1B). Almost 50% of the cytoplasmic region of VGLUT2 incorporated in proteoliposomes was accessible to digestion with excess trypsin, indicating that about half of the VGLUT2 was oriented with the cytoplasmic face directed to the outside of the vesicle.

**F-ATPase-coupled l-Glutamate Uptake**—Because VGLUT utilizes Δψ as the driving force of l-glutamate uptake, generation of a stable Δψ is required for the in vitro transport assay. We used F-ATPase as a generator of Δψ. Upon the addition of ATP, the proteoliposomes facilitated l-glutamate uptake in a time-dependent manner (Fig. 2A). Omission of VGLUT reduced the uptake to a very low background level, indicating that VGLUT2 was an obligatory factor for the l-glutamate uptake (Table 1). Omission of F-ATPase reduced l-glutamate uptake to the same extent as omitting ATP, suggesting that l-glutamate uptake was the sum of active and passive transport through VGLUT (Table 1). Because the internal volume of the reconstituted proteoliposomes corresponded to 0.034% of total reaction mixture, the concentration gradient of l-glutamate across liposomal membrane was around 7-fold under steady state conditions. Establishment of the l-glutamate concentration gradient was also confirmed by the addition of 1 μM CCCP: this proton conductor caused rapid release of internal l-glutamate accumulated in proteoliposomes (supplemental Fig. S1).

The l-glutamate uptake was inhibited by azide, a F-ATPase inhibitor, but not by bafilomycin A1, a V-ATPase inhibitor, indicating a successful switch from the primary H+-pump of V-ATPase to F-ATPase (Table 1). Both ATP-dependent and independent l-glutamate uptake exhibited saturation kinetics (Fig. 2B). The apparent $K_m$ and $V_{max}$ values of ATP-dependent l-glutamate uptake were 4.8 mM and 120 nmol/mg/min for l-glutamate, respectively (Fig. 2B). The $K_m$ and $V_{max}$ of the purified system were ~4- and 14-fold higher than l-glutamate uptake in synaptic vesicles, respectively (32). The reason for the increased $K_m$ value is considered under “Discussion.” The puri-
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**TABLE 1**

Reconstitution and the effects of various ligands on L-glutamate uptake

L-Glutamate uptake was measured in the presence or absence of various ligands as described under "Experimental Procedures." Reconstituted vesicles were preincubated with these ligands for 2 min before addition of ATP. L-Glutamate uptake at 5 min was determined in the presence of 100 μM L-glutamate. L-Glutamate uptake of the complete reaction was 18.9 ± 1.6 (nmol/mg total protein/5 min). For control reactions without F-ATPase or VGLUT2, the same volume of proteoliposome mixture as the complete sample was used.

| System                             | L-Glutamate uptake (%) |
|------------------------------------|------------------------|
| Complete                           | 100.0 ± 8.4            |
| -VGLUT2                            | 5.9 ± 0.6              |
| -F-ATPase                          | 18.4 ± 2.2             |
| -ATP                               | 22.1 ± 2.9             |
| Complete + 1 mM NaN₃               | 14.1 ± 2.7             |
| Complete + 1 μM Bafilomycin A1      | 85.3 ± 3.5             |
| Complete + 1 μM Evans blue          | 21.3 ± 2.9             |
| Complete + 10 mM DL-aspartate       | 98.1 ± 3.7             |
| Complete + 1 mM N-ethylmaleimide    | 93.2 ± 7.2             |
| Complete + 1 μM CCCP               | 27.0 ± 5.3             |
| Complete + 2 mM (NH₄)₂SO₄           | 95.5 ± 9.3             |
| Complete + 2 μM valinomycin         | 46.8 ± 4.1             |
| Complete + 2 μM nigericin           | 72.6 ± 3.0             |
| Complete + 2 μM nigericin + 2 μM nigericin | 18.0 ± 1.6 |

**FIGURE 3. Chloride dependence of L-glutamate uptake.** L-Glutamate uptake at 5 min in the presence of ATP (closed circles) and absence of ATP (open circles) was measured at various Cl⁻ (as KCl) concentrations. Part of the potassium acetate in the reaction mixture was replaced with the indicated concentration of KCl. Total concentration of potassium was held constant at 100 mM. Fluorescence changes of oxonol-V and acridine orange as indicators of Δψ (closed triangles) and ΔpH (open triangles), respectively, were measured and plotted as relative fluorescence changes compared with controls in which KCl (Δψ) was absent or with controls containing 100 mM KCl (ΔpH). One hundred percent activity corresponded to 11.0% and 9.0% fluorescence quenching of oxonol-V and acridine orange, respectively.

**Mutational Analysis**—Recent analyses of structure-function relationship of membrane transporters have pointed out the functional importance of the conserved charged amino acid residues in transmembrane domains. The topological model of VGLUT2 predicts twelve transmembrane spanning helices (26); several charged amino acid residues have been identified that are conserved in these putative transmembrane regions of the SLC17 or VGLUT families (19, 20, 35, 36). We focused on five amino acid residues, Arg<sup>184</sup> and Glu<sup>191</sup> in transmembrane segment 4 (TM4), Arg<sup>88</sup> in TM1, His<sup>278</sup> in TM2, and Arg<sup>322</sup> in TM7 and introduced mutations in these amino acid residues (Fig. 4). Mutated VGLUTs were purified to near homogeneity and reconstituted into liposomes by applying the methods used for the wild type protein (Fig. 5, inset). L-Glutamate uptake activity was assayed after incorporation into liposomes.

Arg<sup>184</sup> is conserved in all the members of the SLC17 family (Fig. 4). Replacement of this residue with alanine resulted in an almost complete loss of ATP-dependent L-glutamate transport activity (Fig. 5). Even at L-glutamate concentrations up to 5 mM, ATP-dependent transport activity of the R184A mutant was not greatly improved (Table 2). Similar to the R184A, mutant VGLUTs R184E and R184K were also severely affected in their activity (Fig. 5). Interestingly, R184E mutant showed lower ATP-independent activity than other mutants. Because the

fied VGLUT2 can be stored at −80 °C for at least a few months without loss of the transport activity.

**Properties of L-Glutamate Uptake**—Unique properties of VGLUT have been elucidated during the past decade, such as the requirement for Δψ as the driving force, insensitivity to DL-aspartate, sensitivity to Evans blue, and the dependence on Cl⁻ (1, 15–18). However, these properties were obtained with synaptic vesicle fractions that contained many other proteins that could interfere with interpretation of the data. We, therefore, reevaluated the basic properties of VGLUT2 (Table 1). The ATP-dependent L-glutamate uptake was sensitive to carbonylcyanide 3-chlorophenylhydrazone (CCCP), indicating that ΔμH<sup>+</sup> drove the uptake. Valinomycin reduced the L-glutamate uptake to 47% of the control, while nigericin in the presence of K⁺ showed limited effect. A combination of valinomycin and nigericin abolished the activity. Furthermore, ammonium sulfate, a dissipator of transmembrane pH gradient (ΔpH) but not of Δψ, did not affect the L-glutamate uptake. These results indicated that Δψ was a much stronger driving force for uptake than was ΔpH. High concentrations of DL-aspartate did not affect the L-glutamate uptake. Evans blue, a known inhibitor of VGLUT2 (33, 34), prevented L-glutamate uptake. Furthermore, preincubation of VGLUT2 with 1 mM N-ethylmaleimide failed to inhibit L-glutamate uptake, suggesting that VGLUT2 does not have N-ethylmaleimide-accessible cysteine residues that are important for the transport activity.

**Requirement for Cl⁻**—The most remarkable feature of VGLUT is its biphasic dependence on Cl⁻ for L-glutamate uptake in synaptic vesicles (15–18): ATP-dependent L-glutamate uptake is stimulated by low concentrations of Cl⁻ (~4 mM) and inhibited at higher concentrations of Cl⁻. In our purified system, an almost absolute requirement of Cl⁻ was observed; essentially no ATP-dependent L-glutamate uptake is observed in the absence of Cl⁻. Then, the ATP-dependent uptake activity appeared upon the addition of Cl⁻ and was maximal at ~4 mM, gradually decreased with increasing concentrations of Cl⁻ (Fig. 3). Under these conditions, the ATP-dependent generation of Δψ as detected by oxonol-V fluorescence, was maximal in the absence of Cl⁻ and continuously decreased with increasing Cl⁻ concentrations, while ΔpH, as detected by acridine orange fluorescence, is negligible in the absence of Cl⁻ and increases with Cl⁻ concentration. Thus, VGLUT2 obligatory requires Cl⁻ for the L-glutamate uptake, and the biphasic Cl⁻ effect is because of a combination of activation of VGLUT2 by Cl⁻ and decreased driving force by higher Cl⁻ concentrations.
ATP-independent activity corresponded to the downhill transport of L-glutamate through VGLUT2, this mutation critically affected both the active and passive transport capability.

Substitution of His128 in TM2 also resulted in loss of activity. In contrast to Arg184, this residue is conserved only in members of the VGLUT family, suggesting a specific role for L-glutamate transport. Replacement of Arg88 in TM1, which is conserved in VGLUTs, NPT1, NPT3, and sialin (19, 20, 35, 36), with alanine decreased the ATP-dependent uptake activity to 74% of the wild type. Replacement of Glu191 in TM4, which is conserved in VGLUTs and sialin, with aspartate, glutamine, and alanine, decreased the ATP-dependent activity to 39, 27, and 7% of the wild type, respectively (Fig. 5). L-Glutamate uptake in alanine mutant was also assayed at L-glutamate concentrations up to 5 mM. Because the L-glutamate uptake activity of the mutants R88A and R322A were similar to that of the wild type, these arginine residues were not required for transport activity (Fig. 5).

VGLUT-mediated Pi transport—Na+/Pi-dependent Pi transport activity might be another remarkable feature of VGLUTs, because this activity appeared upon expression of VGLUT in Xenopus oocytes (19, 20). We tested whether proteoliposomes containing wild type VGLUT2 exhibit Na+-dependent Pi transport activity. When a Na+/Pi gradient was imposed in proteoliposomes containing wild-type VGLUT2, Pi uptake was observed with $K_m$ and $V_{max}$ values of 10.1 mM and 127 nmol/mg/min, respectively (Fig. 6, A and B), indicating that Na+-driven Pi transport activity is intrinsic property of VGLUT2. We then characterized the properties of the Na+-dependent Pi uptake. The activity was not inhibited by excess amounts of D,L-aspartate, L-glutamate, or even by Evans blue (Table 3). Cl− at up to 100 mM did not affect Na+-driven Pi uptake activity (Fig. 6C). More significantly, none of the mutations had a deleterious effect on Na+-dependent Pi uptake (Fig. 6D).

**DISCUSSION**

The lack of an efficient in vitro assay system prevents the study of the molecular mechanism of vesicular L-glutamate transport. In the present study, we established an in vitro assay system with the purified protein that enabled us to analyze the structure-function relationship of VGLUT.

We observed significant L-glutamate uptake even in the absence of ATP in our assay system. Because the ATP-independent fraction of L-glutamate transport was negligible when VGLUT2 was omitted from proteoliposomes, this fraction may correspond to the passive downhill movement of L-glutamate.
Transport Mechanism of VGLUT

**FIGURE 6.** $P_i$ uptake by VGLUT2 in the reconstituted liposomes. **A**, time course of $P_i$ uptake for wild-type VGLUT2. The reaction was started by adding reconstituted proteoliposomes to a mixture containing 100 $\mu$M $[^{32}P]P_i$ phosphate in the presence of 100 mM sodium acetate (closed circles) or potassium acetate (open circles). **B**, dose dependence of $P_i$ uptake at 1 min by wild-type VGLUT2 in the presence of 100 mM sodium acetate (closed circles) or potassium acetate (open circles). **C**, effects of Cl$^-$ on $P_i$ uptake. $P_i$ uptake was measured at various concentrations of Cl$^-$ ion. Part of the sodium acetate was replaced by the indicated amount of NaCl. **D**, effect of mutations on $P_i$ uptake. $P_i$ uptake by the mutants shown was measured in 100 mM $[^{32}P]P_i$ phosphate in the presence of 100 mM sodium acetate (filled bars) or potassium acetate (open bars).

**TABLE 3**
Reconstitution and the effects of various ligands on Na$^+$-dependent $P_i$ uptake

| System                  | $P_i$ uptake |
|-------------------------|--------------|
| Complete                | 100.0 ± 4.3  |
| -VGLUT2                 | 17.0 ± 4.9   |
| -Na$^+$                 | 47.0 ± 2.3   |
| Complete + 10 mM d,l-aspartate | 94.8 ± 3.1   |
| Complete + 10 mM l-glutamate | 101.7 ± 4.0  |
| Complete + 1$\mu$M Evans blue | 107.7 ± 10.0 |

$P_i$ uptake was measured in the presence or absence of various substances as described under “Experimental Procedures.” For experiments without Na$^+$, uptake was measured in 100 mM potassium acetate. The specific activity of the complete reaction was 6.85 ± 0.30 (nmol/mg/min). For the control experiment without VGLUT2, the same amount of liposomes was added to the reaction.

through VGLUT2. The decreased ATP-independent activity of mutant VGLUT2s also supports this notion. Based on this assumption, the ratio of passive downhill and active transports may correspond to the L-glutamate gradient across the membrane vesicles. This value was 5 ~ 10 and is in good agreement with the L-glutamate gradient across synaptic vesicle membranes (37).

We confirmed that some features of VGLUT observed in synaptic vesicles are indeed intrinsic to VGLUT. These include sensitivity to Evans blue and insensitivity to d,l-aspartate. On the other hand, two significant differences appeared in the reconstituted systems: the higher $K_m$ value for L-glutamate and the obligatory requirement for Cl$^-$. We obtained 4-fold higher $K_m$ for L-glutamate transport in reconstituted system in comparison with that in synaptic vesicles. A major determinant of $K_m$ is the equilibrium of L-glutamate binding to VGLUT. Because L-glutamate is an anion at physiological pH, the common structure of acidic phospholipids in the liposomes and the strength of $\Delta\psi$ could have affected the local concentration and kinetic energy of L-glutamate near the vesicle surface. Thus, the higher $K_m$ value in the proteoliposomes may be explained by a shift in the L-glutamate binding equilibrium because of these factors. The vesicle diameter and kinetic differences between V and F-ATPases may have also affected the apparent $K_m$ through altered surface potential of the liposome. As for the obligatory requirement for Cl$^-$, VGLUT2 exhibited essentially no ATP-dependent transport in the absence of Cl$^-$ but a maximum at round 4 mM in the reconstituted systems. Because $\Delta\psi$ was not affected at this concentration, the results strongly suggest that Cl$^-$ directly interacts with VGLUT2 and regulates L-glutamate transport activity. Thus, VGLUT2 is a Cl$^-$-dependent transporter. Because Cl$^-$ activated active transport without affecting the passive downhill movement of L-glutamate through VGLUT2, Cl$^-$ may be involved in the active transport process.

Identification of amino acid residues is crucial to understand the molecular mechanism of the transporters. Our results clearly showed that Arg$^{184}$ was the most critical residue in VGLUT2. The almost complete loss of L-glutamate transport activity by the substitution of arginine with lysine indicated that the precise location of a guanidino group is required for L-glutamate transport. Because only Arg$^{184}$ is conserved in all members of SLC17 organic anion transporters, this amino acid residue is a good candidate for the recognition of the carboxyl group of anions. Additionally, replacement of His$^{128}$ with alanine resulted in a similar loss in activity. This residue is conserved only in members of the VGLUT subfamily and thus, may play a specific role in the transport of L-glutamate. The large decrease of activity by the substitution of Glu$^{191}$ with alanine or glutamine suggests that precise location of a negative charge at this position. A large reduction of L-glutamate transport by replacement with aspartate suggests that precise location of negative charge of this residue is required for full activity. Because passive L-glutamate transport was less sensitive to these mutations, especially with regard to residues His$^{128}$ and Glu$^{191}$, the major role of these three residues on L-glutamate transport seems to be specific to active transport.

Recent progress in the crystallography of the bacterial glycerc-3-phosphate transporter and lactose permease revealed the common structure of MFS-type transporters (29, 38, 39). Because SLC17 belongs to the MFS-type transporters, the structure of VGLUT2 is expected to be similar to that of this
transporter group. Thus, based on the structure of glycerol-3-phosphate transporter, we constructed a structural model of VGLUT2. As shown in Fig. 7, Arg^{184}, His^{128}, and Glu^{191}, which are important for L-glutamate transport as described above, are all located near the bottom of central cavity. Because these amino acid residues are present in the same area as the substrate binding site of glycerol-3-phosphate transporter or lactose permease (29, 38, 39), these residues may participate in L-glutamate binding through hydrogen bonds or salt bridges. From the viewpoint of an alternating access model, which was proposed as the transport mechanism of bacterial MFS transporters (38, 39), a conformation change may make the substrate binding site accessible to either one side or the other side of membrane. It is likely that VGLUTs have a similar mechanism for transporting L-glutamate. Because most of the conserved residues in the putative substrate binding site of VGLUT2 are basic in nature (Fig. 7), binding of the L-glutamate anion and/or Cl^- may stabilize the overall positively charged substrate binding site. Such ionic interactions may promote closure and reorientation of the substrate binding site of VGLUT2, leading to the translocation of L-glutamate.

Na^- dependent P_i transport is another intrinsic property of VGLUT2. Because Arg^{184} is essential for L-glutamate transport, we expected that this amino acid residue is also important for P_i transport. Unexpectedly, however, mutations in the amino acid residue did not affect Na^- driven P_i transport at all (Fig. 6). His^{128} and Glu^{191} were also not required for P_i transport (Fig. 6). These results indicate that amino acid residues responsible for the transport of L-glutamate are different, at least in part, from those for P_i transport. In addition to these observations, the L-glutamate and P_i transports differ in their characteristics as to requirement for Cl^- and sensitivities to D,L-aspartate, L-glutamate, and Evans blue. Thus, Na^- driven P_i transport differed from L-glutamate transport in terms of substrate, driving force, Cl^- activation, sensitivity to inhibitors and required amino acid residues. Based on these observations, we propose that VGLUT2 contains two independent transport machineries inside a molecule. Although we currently have no information on amino acid residues essential to P_i transport, it is likely that charged or hydrophilic residues in the transmembrane domain would participate in this process just as for L-glutamate transport. Such a study is under in progress in our laboratory.

In conclusion, we have presented evidence that VGLUT2 contains two independent machineries for the Cl^- dependent uptake of L-glutamate and the Na^- dependent uptake of P_i. Systematic mutational analysis combined with biochemical studies in our reconstituted system provided for the first time structural and functional insights on VGLUT.

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