Glycine Transporter Dimers

**EVIDENCE FOR OCCURRENCE IN THE PLASMA MEMBRANE***

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Different Na+/Cl−-dependent neurotransmitter transporters of the SLC6a family have been shown to form dimers or oligomers in both intracellular compartments and at the cell surface. In contrast, the glycine transporters (GlyTs) GlyT1 and -2 have been reported to exist as monomers in the plasma membrane based on hydrodynamic and native gel electrophoretic studies. Here, we used cysteine substitution and oxidative cross-linking to show that of GlyT1 and GlyT2 also form dimeric complexes within the plasma membrane. GlyT oligomerization at the cell surface was confirmed for both GlyT1 and GlyT2 by fluorescence resonance energy transfer microscopy. Endoglycosidase treatment and surface biotinylation further revealed that complex-glycosylated GlyTs form dimers located at the cell surface. Furthermore, substitution of tryptophan 469 of GlyT2 by an arginine generated a transporter deficient in dimerization that was retained intracellularly. Based on these results and GlyT structures modeled by using the crystal structure of the bacterial homolog LeuTAa, as a template, residues located within the extracellular loop 3 and at the beginning of transmembrane domain 6 are proposed to contribute to the dimerization interface of GlyTs.

After presynaptic release and postsynaptic receptor activation, neurotransmitters have to be rapidly removed from the synaptic cleft in order to allow synaptic transmission to proceed with high spatial and temporal resolution. This is achieved by neurotransmitter transporters located in the plasma membrane of nerve terminals and adjacent glia cells. The family of Na+/Cl−-dependent neurotransmitter transporters (SLC6a) includes transporters for γ-aminobutyric acid (GAT1 to -3),5 glycine (GlyT1 and -2), dopamine (DAT), serotonin (SERT), and norepinephrine (NET) (1–3). All of these transporters display significant sequence similarity and share a common membrane topology with 12 transmembrane segments (TMs), a large second extracellular loop (EL2) connecting TM3 and TM4, and cytoplasmic N- and C-terminal regions. Although the functional unit of Na+/Cl−-dependent transporters is thought to be a monomer, there is increasing evidence that these transporters form dimers or even higher oligomers within the plasma membrane (4). Oxidative treatment of the DAT produces dimers and tetramers due to intermolecular disulfide bond formation between two cysteine residues, Cys243 and Cys306, located within TM4 and at the end of EL3, respectively (5, 6). Similarly, chemical cross-linking and co-isolation of differentially tagged transporter polypeptides has shown that the recombinant SERT forms oligomers in human embryonic kidney (HEK) 293 cells (7, 8). Furthermore, for the DAT, D 3 H, and SERT it has been shown that co-expression of the transporter polypeptides fused to cyan fluorescent protein (CFP) with the respective yellow fluorescent protein (YFP)-tagged protein results in a Förster resonance energy transfer (FRET) signal, suggesting that at least dimers of these transporters exist (9, 10). A possible oligomerization interface has been identified within TM2 of GAT1, where both the disruption of a leucine heptad motif and substitution of polar amino acid residues caused intracellular retention of the transporter and a loss of the FRET signal (11, 12). Also, x-ray crystallography of a bacterial ortholog of the SLC6a transporter family, LeuTAa, revealed that this transporter forms homodimers (13). Together, all of these data support the idea of Na+/Cl−-dependent neurotransmitter transporters being oligomeric proteins.

There is, however, one reported exception from this rule, the glycine transporters GlyT1 and GlyT2 (14–16). These transporters mediate the uptake of the inhibitory neurotransmitter

5 The abbreviations used are: GAT, γ-aminobutyric acid transporter; CFP, cyan fluorescent protein; CRFR1, corticotropine-releasing factor receptor 1; CuP, copper-α-phenanthroline; DAT, dopamine transporter; EL, extracellular loop; FRET, fluorescence resonance energy transfer; FRETc, corrected FRET; GlyT, glycine transporter; HEK 293T cells, human embryonic kidney 293T cells; His, histidyl; NET, norepinephrine transporter; PBS, phosphate-buffered saline; SERT, serotonin transporter; TM, transmembrane domain; YFP, yellow fluorescent protein; WT, wild type; NTA, nitrolotriacetic acid.
glycine from the extracellular space into glial cells (GlyT1) and
glycinergic neurons (GlyT2), respectively (3). Studies on GlyT-
deficient mice have shown that, after birth, GlyT1 is essential
for removing glycine from postsynaptic glycine receptors (17),
whereas GlyT2 is required for the reuptake of glycine into the
presynaptic terminal (18). Additionally, GlyT1 has been shown
to be involved in the regulation of glutamatergic neurotrans-
mapping by controlling the occupancy of the glycine binding site
of the N-methyl-D-aspartate subtype of glutamate receptors
(19–21). Evidence for a predominantly monomeric state of
these transporters came from two independent studies that
failed to detect oligomeric GlyTs within the plasma membrane.
Both hydrodynamic analysis of GlyT1 solubilized from rat spi-
nal cord (15) and affinity purification of surface-labeled recom-
binant GlyT1 and GlyT2 followed by blue native PAGE (14)
provided evidence for GlyTs being monomers at the cell sur-
face. Dimers and oligomers were only detected in intracellular
membranes and suggested to represent overexpression artifacts (14). Here, we used a combination of mutagenesis and
cysteine-mediated cross-linking as well as FRET analysis in liv-
ing cells to reexamine whether GlyTs form oligomers. Our data
indicate that these transporters are dimers not only in intracel-
lar compartments but also in the plasma membrane of HEK
293T cells.

EXPERIMENTAL PROCEDURES

Homology Modeling—The crystal structure of the bacterial
leucine transporter LeuTAs (Protein Data Bank code 2A65) was
used as a template to build a homology model of GlyT2. To this
end, the amino acid sequence of GlyT2 (accession no. Q761V0)
(22) was truncated at its N and C termini (residues 1–192 and
747–799, respectively), and the large EL2 (residues 313–364)
was deleted. The remaining sequence was aligned with LeuTAs
according to Yamashita et al. (13). Three-dimensional models
(10 structures) of GlyT1 and GlyT2 were built from the aligned
sequences on a Silicon Graphics Octane R12000 work station
using the MODELLER program (23). The models resulting in
the lowest root mean square deviation as compared with the
original LeuTAs structure were retained for analysis without
further refinement. Dimers of GlyT2 were created by juxtapos-
ting two transporter molecules using Thr664 as an anchoring
point. Figures were generated using PyMOL software (Delano
Scientific, Palo Alto, CA).

cDNA Constructs and Heterologous Expression—An expres-
sion construct for the human GlyT1c was kindly provided by
Dr. Katherine Fisher (Groton Laboratories, Pfizer, NY). The
GlyT2 cDNA was isolated from mouse brain stem mRNA using
standard cloning techniques. N-terminal heptahistidyl (His),
FLAG, and Myc tags were added by PCR-based mutagenesis.
After subcloning into the pcDNA3.1+ vector (Invitrogen), the
respective substitutions were introduced by using the QuikChange
site-directed mutagenesis kit (Stratagene, La Jolla, CA).
For fluorescence analysis, the coding regions of GlyT1 and
GlyT2 were subcloned by PCR into pcEFP-C1 or pEYFP-C1
(Clontech-Takara Bio Europe, Saint-Germain-en-Laye,
France) to create CFP- or YFP-tagged GlyT1 or GlyT2, respec-
tively. All constructs were verified by sequencing, and all sur-
face-expressed transporters were shown to be functional upon

heterologous expression in HEK 293T cells as revealed by
[3H]glycine uptake measurements (data not shown). An
expression construct for the human DAT (24) was kindly pro-
vided by Dr. Marc G. Caron (Duke University, Durham, NC),
and a membrane-bound form of YFP (25) was kindly provided
by Viacheslav Nikolaev (University of Würüzburg, Germany).
HEK 293T cells were grown in modified Eagle’s medium sup-
glemented with glutamine (2 mm), 10% (v/v) fetal calf serum,
penicillin (50 units/ml), streptomycin (50 μg/ml), and 50 μM
β-mercaptoethanol (all reagents from Invitrogen) at 37 °C in a
humidified 5% CO2 atmosphere. Cells were seeded on the day
prior to transfection into 6-well plates, and transfection was
performed at 70–85% confluence using the Polyfect transfec-
tion reagent (Qiagen, Hilden, Germany) according to the man-
ufacturer’s protocol. For GlyT1 expression, GripTite 293 cell
lines (Invitrogen) stably expressing GlyT1, GlyT1L343C, and
GlyT1C116A/L343C were generated and seeded 48 h before the
experiment.

Oxidative Cross-linking—Cross-linking experiments were
performed 48–72 h after transfection. Adherent cells were
washed twice with phosphate-buffered saline (PBS) or HEPES
buffered salt solution (130 mM NaCl, 1.3 mM KCl, 1.2 mM
MgSO4, 1.2 mM KH2PO4, 2 mM CaCl2, 10 mM glucose, 10 mM
NaOH/HEPES, pH 7.4) and incubated with 0.1–0.5 mM CuSO4
and 0.8–2 mM o-phenanthroline (CuP) in PBS or HEPES buff-
ered salt solution for 10 min at room temperature. The reaction
was stopped by removal of the reagent and incubation of the
cells with 10 mM N-ethylmaleimide in PBS for 15–20 min.

Western Blotting—Cells were lysed at 4 °C in lysis buffer con-
taining 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100,
0.25% (w/v) deoxycholate, 0.1% (w/v) SDS, 1 mM Pefabloc SC
(Roche Applied Science), and 50 mM HEPES/Tris, pH 7.4. After
centrifugation for 15 min at 16,000 g, the protein content in
the supernatant was determined using the Bio-Rad Protein
assay (Bio-Rad, Munich, Germany), and 10 μg of supernatant
protein were mixed with the appropriate volume of 4× nonre-
ducing loading buffer (Invitrogen) prior to separation by SDS-
PAGE on 3–8% Tris acetate gels (Invitrogen). Proteins were
transferred to either nitrocellulose (Whatman, Dassel, Ger-
many) or polyvinylidene fluoride (Amersham Biosciences)
membranes. The membranes were blocked in Tris-buffered
saline supplemented with 0.1% (w/v) Tween 20 and 5% (w/v)
nonfat milk powder for at least 30 min prior to a 30–min
incubation with primary antibodies directed against either the
N terminus of GlyT2 (polyclonal rabbit) (18), DAT (polyclonal
rabbit, Chemicon, Temecula, CA), the Myc epitope (polyclonal
rabbit, Abcam, Cambridge, UK) (all diluted 1:2000 in blocking
buffer), or the FLAG epitope (monoclonal mouse; Sigma; 4
μg/ml). After washing with Tris-buffered saline containing
0.5% (w/v) Tween 20, membranes were incubated with horse-
radish peroxidase-conjugated goat anti-rabbit antibodies
(Invitrogen) and detected with the SuperSignal kit (Pierce).

Affinity Purification of His-tagged GlyT2—HEK 293T cells
were transfected with the indicated constructs and, 48 h later,
lysed in 100 mM sodium phosphate buffer, pH 8.0, containing
0.2% (w/v) dodecylmaltoside and 1 mM Pefabloc SC. The lysates
were centrifuged at 16,000 × g for 15 min, and 190 μg of super-
natant protein were incubated under continuous agitation with

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GlyTs Form Oligomers in the Plasma Membrane

Ni\(^{2+}\)-NTA-agarose beads (Qiagen, Hilden, Germany) in the aforementioned lysis buffer supplemented with 10 mM imidazole for 1 h at 4 °C. After washing with lysis buffer containing 30 mM imidazole and 0.075% (w/v) dodecymaltoside, agarose-bound proteins were eluted with 2 × SDS loading buffer (Invitrogen) for 10 min at 25 °C.

Functional Characterization of GlyT2—His-GlyT2\(_{\text{WT}}\) or His-GlyT2\(_{\text{T464C}}\) was expressed in *Xenopus laevis* oocytes as described previously (14). Glycine (30 μM)-induced currents were monitored using the two-electrode voltage clamp technique as described previously (26). The oocytes were treated with 5 mM CuP for 15 min, and glycine-induced currents were measured again. To analyze the efficacy of CuP-induced cross-linking, His-tagged proteins were isolated from detergent extracts of these oocytes as described (14) and subjected to Western blotting. Dimers of 200 kDa were only seen after CuP treatment of His-GlyT2\(_{\text{T464C}}\) but not of untreated or His-GlyT2\(_{\text{WT}}\)-expressing cells (data not shown).

Surface Biotinylation—Surface biotinylation of transfected HEK 293T cells with 1 mM NHS-SS-biotin (Pierce) was performed essentially as described (27). After biotinylation, cell lysates (50 μg of protein) were incubated with streptavidinagarose beads (60 μl; Pierce) for 3 h at 4 °C. The beads were then washed three times with lysis buffer, and bound biotinylated proteins were eluted by a 30-min incubation at 4 °C with 30 mM glycine (30 mM CuP) for 15 min, and glycine-induced currents were measured again. To analyze the efficacy of CuP-induced cross-linking, His-tagged proteins were isolated from detergent extracts of these oocytes as described (14) and subjected to Western blotting. Dimers of 200 kDa were only seen after CuP treatment of His-GlyT2\(_{\text{T464C}}\) but not of untreated or His-GlyT2\(_{\text{WT}}\)-expressing cells (data not shown).

Fluorescence Resonance Energy Transfer—FRET signals (28) were measured with an epifluorescence microscope (Carl Zeiss Axiovert 200) using the “three-filter method” according to Xia and Liu (29). Images were taken from HEK 293T cells maintained and transfected as described previously (30) using a ×63 oil objective and a LUDL filter wheel that allows for rapid exchange of filters. The system was equipped with the following fluorescence filters: CFP filter (λ\(_{\text{excitation}}\) = 436 nm, dichroism = 455 nm, emission = 480 nm), YFP filter (λ\(_{\text{excitation}}\) = 500 nm, dichroism = 515 nm, emission = 535 nm), and FRET filter (λ\(_{\text{excitation}}\) = 436 nm, dichroism = 455 nm, emission = 535 nm). The acquisition of the images was done with MetaMorph version 4.6. (Molecular Devices Corp., Downingtown, PA). Background fluorescence was subtracted from all images, and fluorescence intensity was measured at the plasma membrane and in cytosolic regions in all images. To calculate a normalized FRET signal (\(N_{\text{FRET}}\)), we used the following equation,

\[
N_{\text{FRET}} = \frac{l_{\text{FRET}} - a \times l_{\text{YFP}} - b \times l_{\text{CFP}}}{\sqrt{l_{\text{YFP}} \times l_{\text{CFP}}}} \quad \text{(Eq. 1)}
\]

where \(a\) and \(b\) represent the bleed-through values for YFP and CFP. All \(N_{\text{FRET}}\) values are expressed as means ± S.E. Corrected FRET (FRET\(_{\text{c}}\)) images were obtained according to Ref. 31. Briefly, after background subtraction from all three images, CFP and YFP images were multiplied with their corresponding bleed-through value. The following equation was used for the calculation of FRET\(_{\text{c}}\) images, FRET\(_{\text{c}}\) = FRET – (\(b \times \text{CFP}\)) – (\(a \times \text{YFP}\)).

Confocal Microscopy—GlyT cell surface expression was visualized by confocal microscopy using a Zeiss Axiovert 200-LSM 510 confocal microscope (argon laser, 30 milliwatts; helium/neon laser, 1 milliwatt) equipped with an oil immersion objective (Zeiss Plan-Neofluar ×40/1.3). In brief, HEK 293 cells transfected with the indicated construct were seeded onto glass coverslips and examined 1 day later. In co-expression experiments, fluorescent protein-tagged constructs were detected with a band pass filter (475–525 nm) using the 458-nm (for CFP, at 30–45% input power) or 488 nm (for YFP, at 8–10% input power) laser lines. Plasma membranes were visualized after the addition of 20 μl of trypan blue (0.05% (w/v) in PBS) at an excitation wavelength of 543 nm with a long pass filter (585 nm). CFP and trypan blue images were captured sequentially, and overlay images were produced with Zeiss imaging software as described (32).

RESULTS

For the DAT, residue Cys\(_{306}\), located at the end of EL3 between TM5 and TM6, has been shown to be essential for intermolecular disulfide bond formation upon oxidative treatment (5). Sequence comparison revealed that in the GlyTs, leucine and threonine residues are found at the homologous positions (Leu\(_{343}\) in GlyT1c and Thr\(_{464}\) in GlyT2; Fig. 1A). To assess whether these residues reside at the surfaces of the respective transporter proteins and might be close to a possible dimerization interface, we generated a model of GlyT2 by using the crystal structure of LeuT\(_{Aa}\) as a template (13). Regions that did not display significant homology to the LeuT\(_{Aa}\) protein, like the intracellular N and C termini and the large EL2 between TM3 and TM4, were deleted from the GlyT2 sequence to optimize sequence alignment. The resulting GlyT2 model showed a root mean square deviation of 3.62 Å from the LeuT\(_{Aa}\) structure when 498 of 504 pairwise Cα atoms were aligned (root mean square deviation of 1.157 Å for 398 Cα atoms). In this model, the side chain of Thr\(_{464}\) was located on the surface of the transporter above the helix formed by TM11 (Fig. 1, B and C). Thus, Thr\(_{464}\) might be close to a possible dimerization interface. Apposition of two monomers at this interface created a GlyT2 dimer, in which the two Thr\(_{464}\) residues are in close proximity (Fig. 1, B–E). This dimeric model suggests that, in addition to interactions between the EL3 regions of the monomers, side chains of TM11 could contribute to dimer stabilization. Likewise, in a similar model of GlyT1, Leu\(_{343}\), the amino acid residue corresponding to Thr\(_{464}\) in GlyT2 and Cys\(_{306}\) in DAT, is predicted to be localized at the surface of the transporter protein (data not shown).

To establish whether Thr\(_{464}\) is indeed located at a dimerization interface of GlyT2, we replaced this residue by a cysteine in order to examine whether GlyT2 oligomers can be stabilized by intermolecular disulfide cross-linking, as reported for the DAT (5). As controls, we generated two GlyT2 expression constructs, in which residues Lys\(_{462}\) and Thr\(_{557}\) were substituted by cysteines. According to our model, both residues are unlikely to face the proposed dimerization interface (Fig. 1, B–E). HEK 293T cells were transfected with these three cysteine-substituted GlyT2 constructs and analyzed for \(^{3}H\)glycine uptake activity and subcellular localization of GlyT2 immunoreactiv-
GlyTs Form Oligomers in the Plasma Membrane

The GlyT2T464C Mutant Efficiently Forms Dimers—To determine whether position Thr464 is close to a potential dimerization interface of GlyT2, HEK 293T cells transiently expressing GlyT2WT, the GlyT2T464C mutant, or, as a control, DATWT were treated with the oxidizing agent CuP, followed by detergent extraction, nonreducing SDS-PAGE, and Western blot analysis. With DATWT-expressing cells, CuP treatment resulted in the appearance of a distinct band at 170 kDa in addition to immunoreactive bands at 85 and 57 kDa (Fig. 2A), which represent the mature and immature monomeric forms of this transporter (5). The 170 kDa band corresponds in size to the previously described DATWT dimer (5), thereby confirming a close proximity of the Cys306 residues at the dimer interface. Consistent with previous findings (5), the formation of DAT dimers by CuP was completely abolished when Cys306 was replaced by an alanine (DATC306A; Fig. 2A).

In contrast to these results obtained with DATWT, CuP treatment of cells expressing GlyT2WT did not result in the formation of SDS-resistant GlyT2 oligomers (Fig. 2B, left). However, CuP treatment of GlyT2T464C-expressing cells generated a major GlyT2-immunoreactive adduct of 200 kDa. This was accompanied by a strong decrease in the intensity of the 90 kDa band, which represents the fully glycosylated GlyT2 monomer (Fig. 2B, left). These results are consistent with GlyT2T464C forming a dimer that is cross-linked upon oxidative treatment. To exclude the possibility that this 200-kDa adduct represents an unspecific aggregate induced by CuP treatment, detergent extracts from CuP-treated cells expressing GlyT2T464C were supplemented with β-mercaptoethanol. This reducing treatment resulted in a strong reduction of the 200 kDa band and an increase in the intensity of the mature monomer band at 90 kDa indicative of cleavage of the disulfide cross-link (Fig. 2B, right). Notably, in contrast to what was found with GlyT2T464C, CuP treatment of GlyT2T557C, or GlyT2K462C-expressing cells failed to produce the 200-kDa GlyT2 immunoreactive adduct (Fig. 2C). Thus, disulfide-mediated cross-linking of the GlyT2T464C mutant is position-specific.

The functional consequences of CuP cross-linking were analyzed by measuring glycine-induced currents by two-electrode voltage clamp in Xenopus oocytes expressing His-GlyT2WT or His-GlyT2T464C before and after treatment with CuP. Application of 30 μM glycine induced currents of 76 ± 8 pA for GlyT2WT and 39 ± 5 pA for GlyT2T464C (n = 6). The smaller current monitored for the GlyT2T464C mutant most likely reflects a slightly reduced expression also seen in Western blots prepared from detergent extracts from the oocytes (data not shown). After treatment with CuP, the currents recorded from the same oocytes were not significantly reduced as compared with the currents measured before cross-linking (reduction of 18 ± 13% for His-GlyT2WT and of 15 ± 12% for His-GlyT2T464C, as compared with the untreated controls; p ≥ 0.5). Similar to what was seen in HEK 293T cells, treatment of His-GlyT2T464C but not His-GlyT2WT-expressing oocytes with
CuP resulted in the appearance of a prominent GlyT2-immunoactive band at 200 kDa (data not shown). Together, these results are consistent with cross-linking of GlyT2 dimers by CuP treatment not interfering with transporter function.

Very similar findings were also obtained with GlyT1. CuP treatment of GlyT1 WT stably expressing cells did not result in the appearance of an immunoreactive band of higher molecular weight. However, treatment of a GlyT1L343C-expressing cell line produced a prominent immunoreactive band at 190 kDa. To exclude the possibility that this dimer formation might involve oxidation of the extracellular cysteine Cys116, we repeated this experiment with a GlyT1C116A/L343C double mutant. Again efficient cross-linkage of the 190-kDa adduct was seen. Thus, Cys116 is not involved in oxidative cross-linking (Fig. 2D). The slight increase in cross-linking efficacy seen in cells expressing the GlyT1C116A/L343C double mutant most likely reflects differences in expression levels between the stable and thus presumably intracellularly localized, GlyT2 protein (14). In contrast, the immunoreactive band at 200 kDa generated upon CuP treatment of GlyT2 T464C was insensitive to treatment with endoglycosidase H (Fig. 3A). Thus, this cross-linked GlyT2 adduct did not contain core-glycosylated proteins. Incubation of the lysates from these cells with peptide-N-glycosidase F reduced the apparent molecular masses of all major immunoreactive bands to 140 and 60 kDa, respectively (Fig. 3A). Together, these data support our conclusion that the GlyT2 complex is derived from immature, cell lines used. Taken together, these data demonstrate that the apparent molecular weight of both GlyTs increase by a factor of about 2 upon treatment with CuP when residues homologous to Cys306 within the DAT are substituted by cysteines.

Transmembrane Transporters Cross-linked by CuP Are Found in the Plasma Membrane—To determine the subcellular localization of the cross-linked GlyT2 T464C protein, we first examined its glycosylation status. Previous studies have shown that complex-glycosylated GlyTs are predominantly localized in the plasma membrane, whereas immature core-glycosylated or nonglycosylated transporter forms are retained in intracellular compartments (34, 35). When detergent extracts prepared from CuP-treated HEK 293T cells expressing GlyT2 WT or the GlyT2 T464C mutant were incubated with endoglycosidase H, an enzyme that selectively removes core glycosylations from proteins, the apparent molecular mass of the band at 90 kDa did not change, but that of the immunoreactive 65 kDa band was reduced to 60 kDa (Fig. 3A). This confirms that the 65-kDa polypeptide represents the core-glycosylated form of GlyT2 (35). In addition, treatment with endoglycosidase H reduced the molecular mass of the 155 kDa immunoreactive band to about 140 kDa, indicating that this GlyT2 complex is derived from immature,
required for efficient elution from the streptavidin-agarose beads (see “Experimental Procedures”).

**GlyT Oligomerization Monitored by FRET**—To further corroborate the existence of GlyT dimers in the plasma membrane by an independent technique, we employed FRET microscopy (28) using the three-filter method according to Xia and Liu (29) that allows us to quantitatively monitor protein oligomerization. Visualization of protein oligomerization in intact cells was achieved by the method of Sorkin et al. (31), which generates FRET images. Cytosolic proteins known to lack or only sparsely form oligomeric complexes, enhanced CFP and enhanced YFP, were used as background controls. To provide a reference for membrane protein oligomerization, we co-expressed CFP- and YFP-tagged versions of the human DAT (C-DAT and Y-DAT, respectively), which has been shown to homo-oligomerize by both FRET microscopy and biochemical approaches (5, 10, 36). In addition, we used SERT tagged with CFP and YFP on its cytoplasmic N and C termini, respectively (C-SERT-Y) (i.e. a transporter construct predicted to produce strong homotypic FRET signals) (37).

Expression of C-SERT-Y and coexpression of C-DAT and Y-DAT resulted in enriched plasma membrane fluorescence (Fig. 4A and supplemental Fig. S1, respectively) and, as expected, robust \( N_{\text{FRET}} \) signals (0.712 ± 0.024 and 0.262 ± 0.017, respectively; Fig. 4C). Similarly, expression of fluorescently labeled GlyTs resulted in predominant plasma membrane fluorescence (Fig. 4A and supplemental Fig. S1). \( N_{\text{FRET}} \) values obtained with C-GlyT2 and Y-GlyT2 (0.268 ± 0.019; Fig. 4C) were similar to those found with C-DAT and Y-DAT. This confirms that GlyT2 forms oligomers in living cells. Although co-expression of C-GlyT1 and Y-GlyT1 produced a somewhat lower \( N_{\text{FRET}} \) value (0.206 ± 0.030; Fig. 4C), this value was significantly higher than that obtained with different membrane proteins employed as controls (\( p < 0.001 \); analysis of variance with Bonferroni correction). In accordance with the data shown in Fig. 3 and observations made with other members of the \( \text{Na}^+/\text{Cl}^- \)-dependent neurotransmitter transporter family (9, 10), we also detected GlyT oligomers by FRET at intracellular locations (Fig. 4A; “cytosolic” \( N_{\text{FRET}} \) values: GlyT1, 0.196 ± 0.012; GlyT2, 0.283 ± 0.016). To validate our FRET data, we also employed a membrane-bound form of YFP that predominantly inserts into the plasma membrane (25); co-expression with C-GlyT2 yielded an \( N_{\text{FRET}} \) value of 0.152 ± 0.018 (Fig. 4C and supplemental Fig. S1).

Apparently, this membrane-attached YFP can serve as an acceptor for CFP fluorophores attached to proteins integral to the plasma membrane; indeed, some spurious FRET signal was faintly visible upon co-expression with C-GlyT2 (Fig. 4B and supplemental Fig. S1). Therefore, we co-expressed both GlyT1 and GlyT2 with another unrelated membrane protein, the G-protein-coupled receptor for dopamine subtype 2, which had already served as a negative control in FRET studies on SERT and GAT1 (9). Similar to what was observed upon co-expression with the corticotropin-releasing factor receptor (CRFR1-Y; see Fig. 7D), co-expression of either C-GlyT1 or C-GlyT2 with dopamine subtype 2-Y resulted in significantly lower plasma membrane \( N_{\text{FRET}} \) values as compared with those obtained upon coexpressing CFP-

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**FIGURE 3.** The CuP-induced GlyT2 adduct is complex-glycosylated and localized at the cell surface. A, detergent extracts of CuP (+)-treated HEK 293T cells transfected with His-GlyT2WT or His-GlyT2T464C were incubated with endoglycosidase H (Endo H) or peptide:N-glycosidase F (PNGase F), as indicated, and analyzed by Western blotting using a GlyT2-specific antibody. B, HEK 293T cells transfected with His-GlyT2WT or His-GlyT2T464C were treated with CuP (+), washed, and subjected to surface biotinylation. Aliquots of the detergent extracts prepared from these cells (total) as well as the eluates from the streptavidin-agarose beads containing the biotinylated proteins (surface) were analyzed by Western blotting analysis with GlyT2-specific antibodies. Different transporter forms are indicated as follows. ●, immature monomer; ○, deglycosylated monomer; ●, mature monomer; ○○, immature dimer; ○○○, deglycosylated dimer; ●●, mature dimer.

down approach. Western blot analysis of the bound biotinylated protein fraction revealed that the bands at 90 and 200 kDa representing mature monomeric transporter and the cross-linked putative GlyT2T464C homodimer, respectively, were enriched within the surface fraction. Thus, a major fraction of the CuP-cross-linked GlyT2T464C protein is localized in the plasma membrane. The apparently increased 90 kDa/200 kDa ratio seen in the GlyT2T464C eluate might reflect cleavage of some of the cross-linked dimer during affinity isolation, due to inclusion of a low concentration of dithiothreitol being
and YFP-tagged GlyTs (0.137 ± 0.012 and 0.152 ± 0.015, respectively; p < 0.001; see Fig. 4, A–C, and supplemental Fig. S1). Together, these results confirm that both GlyTs oligomerize in intracellular compartments as well as at the plasma membrane.

Dimer Formation Revealed by Co-isolation of Differently Tagged GlyT2 Polypeptide—To prove that the putative GlyT2T\(^{464C}\) dimer of 200 kDa contains two GlyT2 polypeptides, we used a co-isolation approach. To this end, we first singly transfected plasmids encoding His-GlyT2\(^{464C}\), Myc-GlyT2\(^{WT}\), and Myc-GlyT2\(^{464C}\) into HEK 293T cells. Western blot analysis of detergent extracts prepared from the transfected cells with a GlyT2-specific antibody demonstrated that all of these tagged GlyT2 proteins were expressed at comparable levels (Fig. 5A, lanes C1, L1, and L2) and formed the previously described immunoreactive bands of 65 kDa corresponding to the core-glycosylated protein of 90 kDa, representing the mature monomer, and, in varying amounts, the 155-kDa dimer of the core-glycosylated GlyT2 (14). Upon coexpression of the His- and Myc-tagged transporters, no major changes in expression were detected (Fig. 5A, lanes L3 and L4). After CuP treatment, GlyT2 adducts of 200 kDa were only seen in cells transfected with Myc- and/or His-GlyT2\(^{464C}\) mutant constructs (Fig. 5A, lanes C2 and L6–L8), whereas samples from CuP-treated Myc-GlyT2\(^{WT}\)-expressing cells failed to produce this band (lane L5). Reprobing of the blots with a Myc-specific antibody revealed that both Myc-tagged transporters were expressed at similar levels when expressed individually (lanes L9 and L10) or together with His-GlyT2\(^{464C}\) (lanes L11 and L12). Upon CuP treatment, Myc immunoreactivity was incorporated into the 200 kDa band only in cells expressing the Myc-GlyT2\(^{464C}\) mutant (Fig. 5, lanes L14 and L16) but not Myc-GlyT2\(^{WT}\) (lanes L13 and L15). Therefore, the GlyT2-immunoreactive 200 kDa band seen in lane L7 represents a GlyT2 dimer consisting exclusively of His-GlyT2\(^{464C}\).

Subsequently, we used Ni\(^{2+}\)-NTA-agarose beads to isolate His-GlyT2 and associated proteins from the detergent extracts of the single- and double-transfected cells. The agarose-bound proteins were then eluted and subjected to Western blot analysis. Fig. 5B shows that GlyT2 protein was efficiently affinity-isolated from detergent extracts prepared from His-GlyT2-expressing cells (lanes E3, E4, E7, and E8). No GlyT2 immunoreactivity was found in samples isolated from cells transfected only with Myc-tagged GlyT2 constructs (lanes E1, E2, E5, and E6), consistent with a specific binding of the His tag to the Ni\(^{2+}\)-NTA-agarose. Reprobing of the blot membranes with a Myc-specific antibody revealed that affinity purification on Ni\(^{2+}\)-NTA-agarose co-isolated core-glycosylated monomeric and dimeric Myc-tagged GlyT2 protein (65 and 155 kDa) from cells expressing both His-GlyT2\(^{464C}\) and Myc-GlyT2\(^{WT}\) or Myc-GlyT2\(^{464C}\) and that this co-isolation was independent of CuP treatment (Fig. 5B, lanes E11, E12, E15, and E16). This result confirms that core-glycosylated GlyT2 forms stable oligomers intracellularly (see also Ref. 14 and Fig. 3), thus allowing the co-isolation of Myc-GlyT2 protein. Furthermore, incorporation of Myc immunoreactivity into the 155-kDa band suggests that this band is indeed a GlyT2 dimer, which contains both His-GlyT2\(^{464C}\) and Myc-GlyT2 protein.

Upon cotransfection of the His-GlyT2\(^{464C}\) mutant with Myc-GlyT2\(^{WT}\) followed by CuP treatment, Myc immunoreactivity was not found at 200 kDa (Fig. 5B, lane E15), although...
some reactive 200-kDa complex had been isolated (compare lanes L7 and E7). An additional Myc-immunoreactive band at 220 kDa (lane E15, *) did not react with the GlyT2 antibody and thus represents an unknown cross-reactive protein complex presumably derived from endogenous HEK 293T cell proteins. Incorporation of Myc immunoreactivity into the cross-linked 200-kDa GlyT2 complex was only observed when His-GlyT2T464C and Myc-GlyT2T464C were coexpressed (Fig. 5, lane E16). These data prove that the band at 200 kDa represents a GlyT2 dimer, which is covalently stabilized by the CuP-induced disulfide bond. Since dimer formation of complex-glycosylated GlyT2 proteins required the presence of the T464C substitution in both tagged proteins, disulfide bonding apparently involves Cys464 exclusively, consistent with transporter dimerization involving identical interfaces.

**FIGURE 5.** The 200-kDa CuP-induced GlyT2 adduct is a homodimer. HEK 293T cells cotransfected with His-GlyT2T464C and either Myc-GlyT2WT or Myc-GlyT2T464C were treated with CuP (+) or PBS (−) as indicated. A, aliquots of detergent extracts prepared from the treated cells were sequentially analyzed by Western blotting using the GlyT2-specific antibody (lanes C1, C2, L1–L8) and, after removal of bound antibody, an anti-Myc antibody (lanes L9–L16). Note the presence of mature GlyT2 dimers in lanes L6–L8 and L14 and L16, respectively. B, aliquots of the extracts used in A were affinity-purified on Ni²⁺-NTA-agarose. Blots prepared from the eluates were first probed with the GlyT2 antibody (lanes E1–E8) and, after removal of the bound antibodies, retained with a Myc-specific antibody (lanes E9–E16). Note that a Myc-immunoreactive 200-kDa adduct was isolated only after CuP treatment of cells expressing both His-GlyT2T464C and Myc-GlyT2T464C (lane E16). The different transporter forms are indicated: ⋄, immature monomer; ●, mature monomer; ●●, immature dimer; ●●●, mature dimer. An asterisk indicates an unspecific band detected with the anti-Myc antibody.

GlyT2 Can Form Hetero-oligomers with Other Members of the SLC6a Family—Given the high degree of overall homology between the different transporter subtypes in the SLC6a family, we surmised that they might also form hetero-oligomeric complexes. Hence, we searched for resonance energy transfer between GlyT2 and DAT or GAT1. Co-expression of C-DAT with Y-GlyT2 resulted in a FRET signal at the plasma membrane level (Fig. 6A; \( N_{\text{FRET}} = 0.25 \pm 0.02 \)), which was comparable with that detected with homotypic GlyT2- or DAT-expressing cells (\( N_{\text{FRET}} = 0.31 \pm 0.03 \) and 0.26 ± 0.02, respectively; Fig. 6B). Similar results were obtained with cells expressing C-GAT1 and Y-GlyT2 (\( N_{\text{FRET}} = 0.28 \pm 0.01 \); Fig. 6, A and B), confirming that GlyT2 forms hetero-oligomers with other members of the SLC6a family.

To further investigate whether this oligomerization is mediated via an interface close to threonine 464 in GlyT2, we performed co-isolation experiments with cells expressing both GlyT2T464C and/or His-DATWT and/or His-DATWT and/or GAT1. Co-expression of C-DAT and Y-GlyT2 (Fig. 6C, lanes L2, L3, L5, and L6), although coexpression with His-DATWT resulted in a reduction of GlyT2T464C expression. Similarly, DAT immunoreactivity was only detected in lanes loaded with detergent extracts from His-DATWT-transfected cells (Fig. 6C, lanes L7, L8, L10, and L11). Dimers of the expected sizes were detected after treatment of the cells with CuP (Fig. 6C, lanes L5, L6, L10, and L11). However, coexpression of His-DATWT and GlyT2T464C failed to produce additional GlyT2- and DAT-immunoreactive bands, demonstrating that the predominant dimers formed were homodimers consisting of His-DATWT or GlyT2T464C. These detergent extracts were subsequently subjected to Ni²⁺-NTA-agarose affinity purification of His-tagged proteins. Western blots of the respective eluates showed that DAT immunoreactivity was efficiently enriched in samples from His-DATWT-expressing cells (Fig. 6C, lanes L7, L8, and L11). GlyT2 immunoreactivity was only detected in eluates from cells co-expressing His-DATWT and GlyT2T464C (Fig. 6C, lanes L2 and L3).
GlyTs Form Oligomers in the Plasma Membrane

**FIGURE 6. Heteromer formation of GlyT2 with DAT and GAT1.** A, HEK 293T cells were transiently transfected with cDNAs encoding CFP- or YFP-tagged proteins, as indicated. Two days after transfection, FRET, values were obtained. A look-up table of the color code used is given in the upper right. N_{FRET} values (percentage) normalized to C-GlyT2/Y-GlyT2-expressing cells (100%) are given for cells expressing C-ECFP and EYFP (n = 16), C-GATWt and Y-GlyT2 (n = 34), C-DATWt and Y-GlyT2 (n = 34), C-DATWt and Y-GlyT2 (n = 30), and C-GlyT2 and Y-GlyT2 (n = 18), respectively. Note that N_{FRET} values obtained from C-GlyT2/Y-GlyT2-expressing cells were not significantly different statistically from that of cells expressing C-GATWt/Y-GlyT2 or C-DATWt/Y-GlyT2 (p > 0.05). Data represent means ± S.E. of analysis of variance with post hoc Bonferroni’s test for multiple comparison. C, HEK 293T cells were transfected with His-DATWt, GlyT2, or both constructs and treated with CuP (+) or PBS (−), and detergent extracts prepared from these cells were analyzed by Western blotting with the GlyT2 antibody (lanes L1–L6), followed, after removal of the bound antibodies, by an anti-DAT antibody (lanes L7–L12). Aliquots of the detergent extracts were subjected to Ni2+NTA-based purification of His-tagged proteins. The eluates from the beads were sequentially analyzed by Western blotting using first the anti-GlyT2 (lanes E1–E6) and then the anti-DAT antibody (lanes E7–E12). Note that in the eluates, GlyT2 immunoreactivity was only found in fractions derived from His-DATWt and GlyT2-expressing cells and that both the mature monomer and the cross-linked dimer were present in these fractions. Different transporter forms are indicated. ○, immature monomer; ●, mature monomer; ●●, immature dimer; ●●●, mature dimer. Filled symbols, GlyT2; open symbols, DAT.

E5), whereas the other lanes were devoid of specific signals, indicating that co-isolation was dependent on His-DATWt. Thus, these results confirm the data obtained by FRET microscopy. Surprisingly, both lanes showed a band of −90 kDa, resembling the mature GlyT2^{T464C} monomer, whereas immature forms of the transporter (i.e. the intracellular immature monomer and its dimer) were excluded from this fraction. In addition to this band, eluates prepared from GlyT2^{T464C}/His-DATWt-expressing cells produced another GlyT2-immunoreactive band at −200 kDa after treatment with CuP. Its position corresponds to that of the mature GlyT2 dimer (Fig. 6C, lane E5) and is clearly distinct from the DAT-immunoreactive bands in the same sample (Fig. 6C, lane E11). Taken together, these data reveal that the mature GlyT2^{T464C} protein can form a dimer or higher order oligomer with His-DATWT. However, these heteromeric GlyT2^{T464C}-DATWT complexes form only with low efficacy and cannot be cross-linked by CuP, suggesting that their structure differs from that of the respective homodimers.

A Mutation Impairing Dimerization Results in Intracellular Retention of GlyT2—In order to identify residues other than Thr464 that might contribute to the putative dimer interface of GlyT2, we examined our structural model for neighboring residues. A candidate residue likely to contribute to the dimerization interface was found to be residue Trp469, located after the end of EL3 at the beginning of TM6. In the model shown in Fig. 1, this residue is predicted to contact the neighboring transporter monomer. We therefore generated a double mutant C-GlyT2^{T464C}/W469R, in which the hydrophobic tryptophan residue had been replaced by a charged arginine side chain. This substitution caused intracellular retention of mutant GlyT2, as observed by confocal microscopy employing trypan blue to specifically stain the plasma membrane of living cells (Fig. 7A). In contrast, GlyT2s containing the single substitution T464C or T457K were expressed at the cell surface to an extent indistinguishable from that seen with GlyT2WT (not shown, but see Fig. 7B). Both C-GlyT2^{T464C} and C-GlyT2^{T457K} colocalized with Y-GlyT2WT at the plasma membrane upon co-expression,
whereas C-GlyT2T464C/W469R colocalized only weakly in intracellular compartments (Fig. 7B). Importantly, this double mutant was incapable of retaining Y-GlyT2WT intracellularly. In analogy to retention assays employed with SERT (37) and DAT (38), this served as the first indication that this double mutant dimerizes only poorly. Subsequently, we co-expressed C-GlyT2T464C/W469R with Y-GlyT2WT and performed FRET microscopy. Fig. 7, C and D, shows clearly that the FRET signal in intracellular compartments was greatly reduced ($N_{\text{FRET}}$) as compared with that observed in C-GlyT2WT and Y-GlyT2WT (not shown, but see Fig. 4) but similar to that found in C-GlyT2WT and CRFR1-Y co-expressing cells (Fig. 7C). Although massive intracellular accumulation of the two proteins was observed, we failed to detect a specific intracellular FRET signal in these cells ($N_{\text{FRET}}$) as compared with $N_{\text{FRET}}$ of C-GlyT2WT- and Y-GlyT2WT-expressing cells (Fig. 7E). This result further indicates that the GlyT2T464C/W469R mutant is unable to oligomerize and is retained intracellularly.

To biochemically prove that GlyT2T464C/W469R is indeed unable to dimerize, a Myc-tagged version of the mutant protein was co-expressed with His-GlyT2WT. Western blot analysis of detergent extracts prepared from cells expressing His-GlyT2WT, Myc-GlyT2T464C/W469R, or both constructs...
GlyTs Form Oligomers in the Plasma Membrane

FIGURE 8. GlyT2T464C/W469R does not form complexes with GlyT2T464C. A, HEK 293T cells were transfected with His-GlyT2T464C, Myc-GlyT2T464C/W469R, or both constructs. After 48 h, the cells were treated with CuP (+) or PBS (−). Detergent extracts prepared from these cells were sequentially analyzed by Western blotting with first the anti-GlyT2 antibody and then anti-Myc antibody. B, aliquots of the detergent extracts were subjected to Ni²⁺-NTA affinity purification of His-tagged proteins. Western blots of the eluates were analyzed by using first anti-GlyT2 and then anti-Myc antibodies. Note that no Myc immunoreactivity was found in the eluates prepared from cells co-expressing His-GlyT2T464C and Myc-GlyT2T464C/W469R, although purification of His-GlyT2T464C was successful, as indicated by GlyT2 immunoreactivity (lower panel, left). The different transporter forms are indicated as follows. ●, immature monomer; ●, mature monomer; ●●, immature dimer; ●●●, mature dimer.

revealed strong GlyT2 immunoreactivity in all three samples (Fig. 8A, lanes L1–L3). Upon CuP treatment, a strong GlyT2-immunoreactive band at 200 kDa was seen in His-GlyT2T464C-expressing (lanes L5 and L6) but not Myc-GlyT2T464C/W469R-expressing cells (lane L4). Reprobing of these blots with a Myc-specific antibody revealed immunoreactive bands in all lanes loaded with extracts prepared from Myc-GlyT2T464C/W469R-expressing cells (Fig. 8, lanes L7, L8, L10, and L11). Consistent with our findings using confocal microscopy, we failed to detect any mature forms of the transporter; only the intracellular monomer or its dimer could be detected in these lanes. Aliquots of these extracts were then subjected to Ni²⁺-NTA affinity purification of His-GlyT2T464C. Probing Western blots prepared from these eluates with the GlyT2 antibody revealed immunoreactive bands for the intracellular monomer and its dimer, the mature monomer, and, in samples prepared from CuP-treated cells, also the mature dimer, in the His-GlyT2T464C-containing samples (Fig. 8B, lanes E2, E3, E5, and E6). Reprobing of these Western blots with Myc-specific antibodies consistently failed to produce any specific band (Fig. 8B, lanes E7–E12), indicating that even in intracellular compartments, Myc-GlyT2T464C/W469R does not interact with His-GlyT2T464C. We therefore conclude that EL3 and the beginning of TM6 (i.e. the region containing the crucial residues Thr⁴⁶⁴ and Trp⁴⁶⁹) is important for GlyT2 dimerization.

within EL3, the beginning of TM6, and possibly TM11. Indeed, upon cysteine substitution and oxidizing treatment, GlyT2T464C and GlyT1L343C were efficiently cross-linked to 200- and 190-kDa adducts, respectively. In contrast, neither GlyT1WT nor GlyT2WT nor other cysteine-substituted GlyT2 proteins (GlyT2K462C and GlyT2T557C) produced the 200-kDa band upon CuP treatment. Furthermore, in the case of GlyT1, a double mutant (GlyT1C116A/L343C), in which a second extracellular cysteine at position 116 had been replaced by alanine, was found to be as efficiently cross-linked as GlyT1L343C. All of these data are consistent with a high positional specificity of cysteine-mediated cross-linking at an interface formed by EL3. Cross-linking did not significantly affect transporter function, as demonstrated by recordings from GlyT2T464C-expressing oocytes prior to and after CuP treatment. This is consistent with the idea that the dimerization interface does not undergo major structural rearrangements during the transport cycle. Also, reduction of the cross-linked transporter dimer with β-mercaptoethanol resulted in a cleavage of the 200-kDa adduct, indicating that CuP treatment did not induce aggregation of the GlyT2T464C mutant.

The differential sensitivity to endoglycosidase H and peptide N-glycosidase F treatment as well as our surface biotinylation experiments indicated that the CuP-cross-linked cysteine-substituted GlyT2 adducts of 200 kDa correspond to dimers com-

**DISCUSSION**

In this study, we used two independent approaches, cysteine-mediated cross-linking and FRET microscopy, to show that upon heterologous expression both GlyT2 and GlyT1 form dimers in the plasma membrane. Comparison with other members of the SLC6a family suggests that GlyTs oligomerize via an interface that is conserved between SLC6a members.

Based on oxidative cross-linking data obtained with the DAT and GlyT models generated by using the crystal structure of the bacterial homolog LeuTAs as a template, we focused on EL3 linking TM5 and TM6 as contributing to a potential oligomerization interface. This loop is located at the outer surface of the transporter monomer above TM11 and, in the DAT, contains residue Cys⁴⁰⁶, which is responsible for oxidative cross-linking of the transporter dimer (6). Here, modeling predicted the GlyT positions homologous to Cys⁴⁰⁶ of the DAT, Thr⁴⁶⁴ in GlyT2, and Leu⁴⁴³ in GlyT1c to similarly face a conserved dimerization interface of the transporters that might involve residues
posed of complex-glycosylated, mature transporter polypeptides located in the plasma membrane. Since detection of these 200 kDa GlyT2 dimers by SDS-PAGE was stringently dependent on CuP cross-linking, its constituent monomers appeared to be only loosely associated. This is consistent with blue native PAGE experiments in which we failed to detect electrophoresis-resistant GlyT oligomers at the cell surface of both oocytes and HEK 293 cells (see Ref. 14). Also, in sedimentation studies, detergent-solubilized GlyT1 behaves as a monomeric protein (40). In line with our previous data (14), we found dimers of immature core-glycosylated transporters that were localized intracellularly, as revealed by their inaccessibility to surface biotinylation. Notably, these intracellular GlyT complexes displayed partial resistance toward dissociation by SDS, and CuP treatment did not increase their abundance. This may in part be due to inefficient permeation of CuP into the cytosol. Alternatively, a different orientation of the EL3 cysteine in the immature GlyT2 polypeptide might impede cross-linking. The differences noted in the SDS resistance of mature versus immature GlyT2 complexes might even reflect distinct steps in intracellular transporter processing. The complex carbohydrate side chains that are added and modified posttranslationally might weaken the interactions between dimerization interfaces and thereby result in a detergent-sensitive dimer upon plasma membrane incorporation. Indeed, evidence for a role of glycosylation in transporter processing and stability has been obtained in studies showing reduced transport activity upon mutation of the transporters’ N-glycosylation sites (41, 42).

Direct support for the existence of GlyT oligomers in living cells comes from our FRET data. Consistent with our biochemical results, a specific FRET signal was seen upon co-expression of CFP- and YFP-tagged GlyT1 or GlyT2 polypeptides in both the plasma membrane and intracellular compartments. During revision of this manuscript, a very similar result with GlyT1 has been reported by others (43). Differences found in FRET signal intensities between the two transporters (see Fig. 4) may reflect differences in expression levels and/or membrane targeting as well as different orientations of the fluorophores, due to the different sizes of the N-terminal domains to which the fluorophores were attached.

That the CuP-induced GlyT complexes are indeed homodimers was proven here by co-isolation of the differentially tagged transporter polypeptides. This and its size of about 200 kDa indicate that the cross-linked GlyT2 dimer contains no other additional polypeptides. Since our co-isolation protocol produced dimers only when both monomers carried the T464C substitution, we conclude that this particular cysteine is required and sufficient for complex stabilization by intermolecular disulfide bonding. Our results indicate that the transporter monomers dimerize via an interface that is conserved between the GlyTs and the DAT (5, 6) and possibly also other SLC6a family members. For the DAT, it has been reported that a second cysteine, located within TM4, also allows intermolecular cross-linking. This cysteine is conserved between DAT and the GlyTs (Cys393 in GlyT2 and Cys220 in GlyT1, respectively).

However, under our experimental conditions, we consistently failed to detect the formation of any higher molecular weight adducts with the respective wild-type constructs. This indicates that within the GlyTs, this cysteine is not accessible for intermolecular cross-linking.

In addition to the homodimers described above, heteromer formation between GlyT2 and DAT1, or DAT, was seen by both FRET analysis and co-purification of GlyT2 T464C immunoreactivity with His-DATWT on Ni2+-NTA-agarose. However, this co-isolation was completely independent of CuP cross-linking, since we failed to detect any cross-linked heterodimers in eluates from Ni2+-NTA columns. Furthermore, we only found the fully glycosylated GlyT2 polypeptide in these eluates, whereas immature transporter forms were excluded from this fraction; this suggests that heterodimerization occurs at the plasma membrane. Together with the observation that even the cross-linked GlyT2 T464C dimer was co-isolated with His-DATWT, we conclude that CuP-induced intermolecular cross-linking is selective for each SLC6a transporter subtype. This conclusion implies that the interaction of His-DATWT with the complex glycosylated GlyT2 monomer and dimer involves interaction sites distinct from that mediating dimer formation. It is tempting to speculate that such interaction sites could allow the formation of higher order transporter oligomers at sites of high transporter density, such as the presynaptic terminal membrane.

Based on our modeling data, we propose that the SLC6a transporters dimerize via an interface to which the EL3 region and the extracellular end of TM6 contribute. Support for this proposal comes from our analysis of the GlyT2 T464C/W469R double mutant, which carries a positive charge instead of a tryptophan at the extracellular end of TM6 (see Fig. 1, D and E). This mutant transporter protein was found to be deficient in dimerization as shown by (i) the low of FRET signal resulting upon co-expression with GlyT2WT, (ii) impaired co-isolation with the GlyT2 T464C mutant, and (iii) the inability to retain GlyT2WT intracellularly. We also tried to identify determinants for dimerization within TM11, which according to our model is closest to the dimerization interface. However, substitutions of aromatic residues within TM11 by branched aliphatic amino acids failed to reduce CuP cross-linking of the respective mutants,7 indicating that these side chains are not specific determinants of transporter oligomerization. Taken together, our data suggest that GlyTs dimerize via an interface involving EL3 and the extracellular end of TM6. Disruption of this interface results in dimerization-deficient transporter protein that is retained intracellularly.

In addition to the dimerization interface defined by EL3 and the extracellular end of TM6, other putative oligomerization domains have been described in earlier studies of other SLC6a family members. First, a leucine heptad motif within TM2 of GAT1 has been found to be essential for both plasma membrane insertion and oligomerization of the transporter (11). According to our structural models, these leucines as well as glutamate and tyrosine residues that also have been proposed to

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6 I. Bartholomäus and A. Nicke, unpublished data.

7 V. Eulenburg, unpublished data.
be important for oligomerization (12) are at least partially buried within the transporter structure and thus unlikely to directly participate in oligomer formation (see also Ref. 4). Presumably, substitutions at these positions lead to structural changes that indirectly disturb the dimerization interface. Similarly, the glycophorin A homology motif, which has been identified in TM6 of the DAT in close proximity to EL3 (38), is predicted to lie within the central core of the transporter close to the substrate binding site and thus is unlikely to directly contribute to monomer interactions. Presumably, these mutations affect folding or proper tertiary structure acquisition and thus cause a loss of function and/or intracellular retention of the transporters. Also, it is noteworthy that LeuT \( \text{AA} \) has been crystallized in dimeric structural form. In this bacterial homolog, a homomeric dimerization interface is formed by TM9 and TM12 (i.e. a region that, based on retention assays with partial transporter constructs, has also been suggested to serve as a dimerization domain in the human SERT) (37).

Oligomerization of SLC6a transporter proteins has been proposed to be important for both posttranslational processing and transporter function at the plasma membrane (4, 8). In different studies, truncated or mutated transporter fragments, which are retained intracellularly, have been shown to exert dominant negative effects on co-expressed full-length transporters. For example, the expression of SERT fragments containing TM1 and -2, TM5 and -6, or TM11 and -12 resulted in intracellular retention of the co-expressed full-length SERT (37). In the case of the NET, a heterozygous mutation (A457P), which are retained intracellularly, have been shown to exert dominant negative effects on co-expressed full-length transporters. Also, it is noteworthy that LeuT \( \text{AA} \) has been crystallized in dimeric structural form. In this bacterial homolog, a homomeric dimerization interface is formed by TM9 and TM12 (i.e. a region that, based on retention assays with partial transporter constructs, has also been suggested to serve as a dimerization domain in the human SERT) (37).

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GlyTs Form Oligomers in the Plasma Membrane

Supplemental Material:

Suppl. Fig. S1. HEK 293 cells were transiently transfected with cDNAs encoding CFP- or YFP-tagged proteins as indicated. Two days after transfection, epifluorescence microscopy was performed; the first and second columns show images obtained with CFP and YFP filter sets, the third column displays a corrected and inverted FRET image (FRETc). A look-up table of the colour code used is presented in the upper right panel. A quantification of respective FRETc images (\(N_{\text{FRET}}\) values) is presented in Fig. 4C.
Glycine Transporter Dimers: EVIDENCE FOR OCCURRENCE IN THE PLASMA MEMBRANE

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