The neuronal glycine transporter 2 interacts with the PDZ domain protein syntenin-1

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The glycine transporter subtype 2 (GlyT2) is localized at glycineric axon terminals where it mediates the re-uptake of glycine from the extracellular space. In this study, we used the yeast two-hybrid system to search for proteins that interact with the cytoplasmic carboxy terminal tail region of GlyT2. Screening of a rat brain cDNA library identified the PDZ domain protein syntenin-1 as an intracellular binding partner of GlyT2. In pull-down experiments, the interaction between GlyT2 and syntenin-1 was found to involve the C-terminal amino acid residues of GlyT2 and the PDZ2 domain of syntenin-1. Syntenin-1 is widely expressed in brain and co-localizes with GlyT2 in brainstem sections. Furthermore, syntenin-1 binds syntaxin 1A, which is known to regulate the plasma membrane insertion of GlyT2. Thus, syntenin-1 may be an in vivo binding partner of GlyT2 that regulates its trafficking and/or presynaptic localization in glycineric neurons. © 2004 Elsevier Inc. All rights reserved.

Introduction

The amino acid glycine serves as a principal neurotransmitter at many inhibitory synapses in spinal cord and brainstem (Betz, 1992) and, in addition, as coagonist of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors (Kemp and Leeson, 1993). Termination of glycine neurotransmission is thought to involve the rapid uptake of released glycine into presynaptic terminals and surrounding glial cells. Specific plasma membrane transporters mediate this process. So far, two types of glycine transporters have been cloned, glycine transporter subtype 1 (GlyT1) and glycine transporter subtype 2 (GlyT2) (reviewed in Gomez a et al., 2003a; Lopez-Corcuera et al., 2001). Both proteins are members of the family of Na+/Cl⁻-dependent neurotransmitter transporters characterized by 12 transmembrane segments and cytoplasmic N- and C-terminal tail regions (Nelson, 1998; Schloss et al., 1994).

In situ hybridization and immunohistochemistry have shown that GlyT1 is localized in glial cells throughout the central nervous system (CNS), whereas GlyT2 is found in neurons of the spinal cord and brainstem (Adams et al., 1995; Jursky and Nelson, 1995; Luque et al., 1995; Zafra et al., 1995). The restricted expression of GlyT2 matches that of strychnine-sensitive inhibitory glycine receptors; thus, GlyT2 constitutes a specific marker of inhibitory glycineric nerve terminals. Recent studies with knockout mice have shown that GlyT1 activity is essential for the removal of glycine from the synaptic cleft of inhibitory synapses, whereas GlyT2 is needed for efficient reloading of synaptic vesicles with glycine (Gomez a et al., 2003b,c). Consistent with this functional assignment, immunoelectron microscopy revealed an enrichment of GlyT2 in the presynaptic nerve terminal membrane surrounding the neurotransmitter release sites (Mahendrasingam et al., 2003; Spike et al., 1997).

During the past decade, an increasing number of proteins containing PDZ (for postsynaptic density protein-95, discs large, zona occludens-1) domains has been shown to interact with the carboxy terminal regions of different membrane proteins (Fanning and Anderson, 1999; Harris and Lim, 2001; Sheng and Sala, 2001) and suggested to regulate the synaptic distribution and functional properties of ion channels and neurotransmitter receptors. Recently, Torres et al. (2001) have identified a PDZ domain protein, PICK1, as a binding partner of the human dopamine transporter (DAT), and shown that this interaction modulates transporter function. PICK1 (for protein interacting with C kinase 1) is a protein kinase C (PKC) substrate, as is MacMARCKS (for macrophage myristoylated alanine-rich C‘ kinase substrate), a plasma membrane-associated protein which interacts with the serotonin transporter (SERT) (Jess et al., 2002). Notably, different Na+/Cl⁻-dependent neurotransmitter transporters including GlyT1 are known to undergo acute down-
regulation in response to PKC activation (Corey et al., 1994; Sato et al., 1995). This down-regulation involves redistribution of the transporter proteins from the plasma membrane to intracellular compartments (Blakely and Bauman, 2000). Thus, transporter interacting proteins like PICK1 and MacMARCKS may be implicated in the regulation of both transporter function and trafficking. In addition, such proteins may be crucial for the nerve terminal localization of these membrane proteins, as demonstrated in case of PICK1 for the presynaptic metabotropic glutamate receptor 7 (Boudin et al., 2000).

Here, we used the yeast two-hybrid system to search for novel binding partners of GlyT2 and identified the PDZ protein syntenin-1, which had originally been identified as a syndecan binding protein (Grootjans et al., 1997), as a specifically interacting protein. Furthermore, we present evidence that syntenin-1 can interact with syntaxin 1A, a SNARE (for soluble NSF attachment protein receptor) complex component that has been implicated in the trafficking of neurotransmitter transporters including GlyT2 (Geerlings et al., 2001). Our data are consistent with a role of syntenin-1 in GlyT2 trafficking and/or presynaptic localization.

### Results

#### Identification of syntenin-1 as a GlyT2 binding protein

To identify intracellular brain proteins that bind GlyT2, we performed a yeast two-hybrid screen with the cytoplasmic C-terminal domain of GlyT2 as bait. Screening of a rat brain cDNA library led to the isolation of eight independent clones including an in-frame rat syntenin-1 cDNA (GenBank accession no. AJ292243). Syntenin-1 is a member of the large family of PDZ domain-containing proteins and possesses two tandem PDZ domains separated by a short linker. As GlyT2 carries a putative PDZ binding motif (TQC) at the end of its C-terminal region, it seemed possible that the interaction between GlyT2 and syntenin-1 involves the C terminus of GlyT2 and a PDZ domain of syntenin-1. To test this hypothesis, we generated deletion mutants of the GlyT2 C-terminal region (GlyT2C) and examined their binding to full-length syntenin-1 in both yeast-two hybrid assays and pull-down experiments with glutathione S-transferase (GST) fusion proteins (Fig. 1). Although the interaction of GlyT2C mutants with syntenin-1 was still detectable in the yeast-two hybrid assay, it was significantly reduced in...
comparison to the unmodified construct (Fig. 1A). In GST pull-down assays using enhanced green fluorescent protein (EGFP)-tagged GlyT2C and GlyT2CΔTQC, an even more obvious difference was obtained. Deletion of the last three amino acid residues of the cytoplasmic tail of GlyT2 completely abolished binding to GST–syntenin-1 (Fig. 1B). Similarly, pull-down assays using detergent-solubilized full-length constructs of GlyT2 produced the same result (Fig. 1C). Thus, the last three amino acids of GlyT2 are necessary for its interaction with syntenin-1.

To explore whether other members of the Na⁺/Cl⁻/Ca²⁺-dependent transporter family may interact with syntenin-1, we generated bait constructs encoding the C-terminal sequences of the rat DAT and SERT proteins (DATC, SERTC) and examined their ability to interact with syntenin-1 in the yeast-two hybrid system. No interaction could be detected in these experiments (Fig. 1A). Human DAT has been shown to bind the PDZ domain containing protein PICK1, consistent with the presence of a PDZ binding motif (LKV) at its C-terminal end (Torres et al., 2001). The rat DAT and SERT proteins, however, do not contain a consensus PDZ binding motif, and thus, in this organism, syntenin-1 specifically interacts with GlyT2 but not the other transporter family members examined.

Binding of syntenin-1 to the C terminus of GlyT2 appeared to have no effect on transporter function. When determining [³H]glycine uptake kinetics into human embryonic kidney (HEK) 293 cells that were either co-transfected with a GlyT2 expression plasmid (GlyT2–pRC/CMV) and an empty pEGFP-C2 expression vector (Clontech), or with GlyT2–pRC/CMV and an EGFP–syntenin-1 expression plasmid, no significant differences in apparent transport affinity and maximal uptake were found upon syntenin-1 co-transfection. Three independent experiments revealed $K_m$ values of 118 ± 31 and 147 ± 9 $\mu$M (mean ± SD, $n = 3$) for control and EGFP–syntenin-1 co-transfected cells, respectively. Maximal uptake rates ($V_{\text{max}}$) of syntenin-1 co-transfected cells ranged between 97% and 107% of control. Similar results were obtained upon co-expression of a syntenin-1 construct tagged with a c-myc epitope (data not shown).

Both PDZ domains of syntenin-1 contribute to GlyT2 binding

Because the C terminus of GlyT2 carries a PDZ domain binding motif that is necessary for its interaction with syntenin-1, PDZ domains of syntenin-1 are possibly involved in this binding. To map the location of the GlyT2 binding site, we generated GST-fused deletion mutants of syntenin-1 and examined their binding to the C-terminal region of GlyT2 by GST pull-down. Among the three deletion constructs tested, GST–PDZ1 + PDZ2 (Fig. 2A) was found to bind EGFP–GlyT2C, while the constructs containing only one PDZ domain did not. This result indicates that both PDZ domains of syntenin-1 are required for GlyT2 binding. A similar binding mode involving both PDZ domains has previously been reported for the interaction of syntenin-1 with syndecan (Grootjans et al., 1997; Zimmermann et al., 2001) and neurofascin (Koroll et al., 2001).

Each of the two PDZ domains of syntenin-1 has a different affinity for syndecan and seems to function either as “accessory” (PDZ1) or “major” (PDZ2) syndecan binding domain (Grootjans et al., 2000). To test whether the PDZ domains of syntenin-1 might differently contribute to the GlyT2 interaction, we employed point mutants of full-length syntenin-1 in which the last glycine residue within the carboxylate-binding loop of each PDZ domain was substituted by either glutamate (G128E in PDZ1) or aspartate (G212D in PDZ2). Such mutations are known to disrupt the interaction with the ligand’s carboxylate group (Ponting et al., 1997). Compared to the binding of wild-type syntenin-1, the PDZ1 mutant (PDZ1*) did not show any detectable reduction in binding to GlyT2C. However, mutations of the second (PDZ2*) or both (PDZ1*2*) PDZ domains abolished the interaction with GlyT2C in the yeast-two hybrid assay completely (Fig. 2B). We therefore conclude that binding of syntenin-1 to GlyT2C requires
PDZ2, and that PDZ2 must be linked to PDZ1 for displaying binding activity.

Syntenin-2 is another member of the syntenin family and exists in two alternatively spliced variants, 2α and 2β, which differ in the lengths of their N-terminal regions (Koroll et al., 2001). The amino acid sequences of the tandem PDZ domains are the same in both syntenin-2 variants and exhibit ~70% identity to those of syntenin-1. However, our yeast-two hybrid assay did not show any detectable interaction of GlyT2C with syntenin-2α or -2β, indicating that GlyT2 interacts selectively with syntenin-1 (Fig. 2B).

Regional and subcellular localization of syntenin-1 in the rat CNS

To examine whether syntenin-1 and GlyT2 might interact in vivo, we determined the regional distribution of syntenin-1 in the rat CNS and compared it to that of GlyT2. On Western blots, our anti-syntenin-1 antibody #30 recognized a ca. 36 kDa protein in all CNS regions examined (Fig. 3A). This size corresponds to the apparent molecular mass of syntenin-1 determined in previous studies (Koroll et al., 2001; Zimmermann et al., 2001). GlyT2 is predominantly expressed in brainstem, cerebellum, and spinal cord. We found syntenin-1 immunoreactivity in all these CNS regions.

To characterize the subcellular localization of syntenin-1, we fractionated homogenates of brainstem and spinal cord according to a standard procedure (Li et al., 1996) and analyzed the fractions with antibodies directed against syntenin-1, GlyT2, PSD-95, and synaptophysin (Fig. 3B). As expected, PSD-95 and synaptophysin were enriched in the LP1 and LP2 fractions, respectively. Consistent with the observation that GlyT2 is localized in presynaptic terminals (Zafra et al., 1995), GlyT2 immunoreactivity was found in the LP1 fraction. In addition, significant levels of GlyT2 immunoreactivity were also detected in the LP2 sample (see Geerlings et al., 2001). Syntenin-1 was found in the crude synaptosome fraction (P2) and, like GlyT2, present in the LP1 and LP2 fractions. However, some syntenin-1 immunoreactivity was also seen in the soluble S3 fraction. These results indicate that syntenin-1 is likely to be associated with synaptic membranes but also present in other subcellular compartments.

In conclusion, both GlyT2 and syntenin-1 are found in brainstem, cerebellum, and spinal cord and may be associated with membrane structures of inhibitory synapses. In support of this hypothesis, endogenous GlyT2 solubilized from a P2 membrane fraction isolated from brainstem and spinal cord was found to bind to GST–syntenin-1 in a pull-down assay (Fig. 3C). Thus, GlyT2 and syntenin-1 could be co-localized at glycineergic synapses.

Co-localization of syntenin-1 and GlyT2

To more precisely define the sites of syntenin-1 expression in brain, we performed in situ hybridization and immunohistochemistry on sections from adult rat brain. In situ hybridization analysis showed a wide distribution of syntenin-1 transcripts throughout the rat CNS (Figs. 4A, B). Intense labeling was observed in the granule cell layer of the dentate gyrus and in the pyramidal cell layer of the Ammon’s horn. Moderate levels of mRNA expression were found in the olfactory bulb, piriform cortex, cerebral cortex, cerebellum, and, importantly, several regions of brainstem and spinal cord. All labeling was abolished when an excess of unlabeled oligonucleotide was added to the hybridization mix (Fig. 4C).

Fig. 3. Regional and subcellular localization of syntenin-1 in the rat CNS. (A) Western blot analysis showing a widespread expression of syntenin-1 in the CNS. Thirty micrograms of the respective protein extracts from brain and lung were loaded per lane. The anti-syntenin-1 antibody #30 recognized a band of 36 kDa, consistent with the calculated molecular mass of rat syntenin-1 (32.4 kDa). An asterisk indicates a non-specific band. (B) Subcellular fractionations of brainstem and spinal cord were performed as described under Experimental methods. PSD-95 and synaptophysin immunoreactivities were used as markers for synaptic membrane and synaptic vesicle fractions, respectively. Both syntenin-1 and GlyT2 were enriched in pellets P2 and P3 and present in the synaptic membrane (LP1) and synaptic vesicle (LP2) fractions. Note additional syntenin-1 immunoreactivity in the soluble fraction (S3). (C) GST pull-down assays with brainstem and spinal cord extracts show that native GlyT2 interacts with bacterially produced syntenin-1.
Because the syntenin-1 antibody #30 stained some non-specific bands in Western blots (Fig. 3A), we used another polyclonal syntenin-1 antibody #29 for immunohistochemistry. On Western blots, antibody #29 revealed a single band migrating at 36 kDa in both rat cerebral cortex and brainstem extracts (Fig. 5A, lanes 3 and 4). This corresponds to the previously defined molecular mass of syntenin-1 (Grootjans et al., 1997). In lysates from 293T cells transfected with the myc-syntenin-1 cDNA (Fig. 5A, lane 2), this antibody reacted with a slightly larger polypeptide due to the presence of the additional epitope tag. The staining of all these immunoreactive bands was completely blocked when antibody #29 was preincubated with GST–syntenin-1 before blotting (Fig. 5A, lanes 5–7).

On tissue sections, antibody #29 showed syntenin-1 immunoreactivity in various neuron populations of the adult rat brain. In the forebrain, strong staining was found in the olfactory bulb (data not shown), hippocampus (Figs. 5B, D), and cerebral cortex (Fig. 5E). In hippocampus, the dentate gyrus, in particular its molecular layer, was more intensely stained than the Ammon’s horn (Fig. 5B). In the granule cell layer of the dentate gyrus, where strong syntenin mRNA signals had been detected by in situ hybridization, syntenin-1 staining was not seen around cell bodies (Fig. 5D). In pyramidal neurons of the cerebral cortex, intense immunostaining was observed in both cell bodies and dendrites (Fig. 5E). All immunostaining was completely abolished when antibody #29 was preincubated with 30 μg of GST–syntenin-1 (Fig. 5C). In general, these results are consistent with the expression pattern of syntenin-1 mRNA defined by in situ hybridization.

We also compared the localization of syntenin-1 with that of GlyT2 in lower brainstem (Fig. 5F). Overlapping distributions of syntenin-1 and GlyT2 immunoreactivities were observed in the hypoglossal nucleus, cochlear nucleus, and some areas of the reticular formation. However, other regions did not display co-localization. For example, we found intense syntenin-1 but only little GlyT2 staining in the inferior olivary complex (IO). On the other hand, strong GlyT2 immunoreactivity was seen in the spinal trigeminal nucleus (Sp5), whereas syntenin-1 could not be detected.

To explore whether GlyT2 and syntenin-1 are co-localized in the same neuronal structures in vivo, we performed confocal laser-scanning microscopy on the ventral cochlear nucleus, a region known to be rich in glycinegic synapses (Altschuler et al., 1986) (Fig. 5G). Consistent with previous reports (Friauf et al., 1999; Zafra et al., 1995), punctate GlyT2 immunoreactivity (green) was observed in the neuropil and around the cell bodies of cochlear neurons, which corresponded to glycinegic axon terminals. Syntenin-1 also showed a punctate staining around cochlear neurons (red), and many of these punctate structures co-localized with GlyT2 (yellow). In addition, we found weak syntenin-1 immunofluorescence in the cytoplasm of cochlear neurons. This is in agreement with our biochemical data that revealed syntenin-1 in the soluble S3 fraction. Upon preincubation of antibody #29 with GST–syntenin-1, both the punctate pattern of syntenin-1 immunoreactivity and the more diffuse cytoplasmic staining were abolished (data not shown).

To corroborate the apparent co-localizations of GlyT2 and syntenin-1 biochemically, we performed co-immunoprecipitation experiments using a solubilized P2 fraction prepared from adult rat brainstem and spinal cord (Fig. 5H). Anti-syntenin-1 antibody #29 precipitated GlyT2 from the extract, whereas a control serum did not. These data lead us to conclude that syntenin-1 is an in vivo binding partner of GlyT2.

**Syntenin-1 binds to syntaxin 1A**

Geerlings et al. (2001) have demonstrated that GlyT2 is present in synaptic vesicles, and that the SNARE protein syntaxin 1A is involved in its trafficking to the plasma membrane. Similarly, syntaxin 1A regulates the surface expression of the GABA transporter subtype 1 (GAT1), and a direct interaction between syntaxin 1A and the N-terminal region of GAT1 has been shown to be crucial for this regulation (Deken et al., 2000). However, whether binding of syntaxin 1A to the N terminus of GlyT2 occurs in vivo and is required for GlyT2 trafficking, and/or whether additional factors are involved, has not been examined. To test the hypothesis that syntenin-1 may participate in syntaxin 1A-mediated GlyT2 trafficking, we examined the ability of syntenin-1 to form a complex with both endogenous syntaxin 1A present in rat brain and recombinant squid syntaxin, which is highly homologous to rat syntaxin 1A (O’Connor et al., 1997). First, immobilized GST–syntenin-1 was incubated with a solubi-
lized P2 fraction from rat brain, and the proteins bound were analyzed by Western blotting using an anti-syntaxin 1A antibody. When the protein sample was boiled before SDS-PAGE, the anti-syntaxin 1A antibody reacted only with a 35-kDa band (not shown), which corresponds to the molecular mass of syntaxin 1A (Yoshida et al., 1992). Without boiling, however, several bands of higher molecular mass were in addition stained (Fig. 6A, arrows). We considered the possibility that these SDS-resistant high-molecular mass bands might represent syntaxin 1A-containing SNARE complexes (Söllner et al., 1993) and therefore analyzed the bound proteins after boiling by SDS-PAGE and Western blotting with antibodies specific for the SNARE components syntaxin 1A, SNAP-25 (for synaptosomal associated protein of 25 kDa) and synaptobrevin-2 (Pellegrini et al., 1995). Fig. 6B shows that not only syntaxin 1A, but also SNAP-25 and synaptobrevin-2 were included in the GST-syntaxin-1 precipitates, indicating that syntaxin-1 indeed interacts with the fully assembled SDS-resistant SNARE complex. To examine whether the N-terminal region of GlyT2 also may bind SNARE components, the same pull-down assay was repeated using GST–GlyT2N (Fig. 6). We found that some SNARE immunoreactivity was retained on GST–GlyT2N (Fig. 6B), but the intensities of the syntaxin-1A and SNAP25 bands recovered were much weaker with GST–GlyT2N than with GST–syntaxin-1 (Fig. 6B), and synaptobrevin-2 could not be detected. Also, some syntaxin 1A was even bound by immobilized GST. We therefore conclude that the N terminus of GlyT2 binds syntaxin-1A (and associated SNAREs) only with low affinity.
To demonstrate that the interaction of syntenin-1 with syntaxin is direct, and to map the binding site of syntaxin on syntenin-1, we generated recombinant His-tagged squid syntaxin and examined its binding to GST–syntenin-1 and its deletion constructs (Fig. 6C). His-tagged syntaxin was precipitated with the GST syntenin-1, GST–PDZ1 + PDZ2 and GST–PDZ2 fusion proteins, but not with GST–PDZ1 or GST alone (Fig. 6C). Consistent with the data shown in Fig. 6B, His-tagged syntaxin 1A binds to GST–PDZ2 as well as to GST–PDZ1 + PDZ2. In addition, a direct interaction of syntaxin 1A with GlyT2N is seen. These results indicate that syntaxins interact directly with syntenin-1, and that the syntaxin binding site lies in the PDZ2 domain and/or the C-terminal tail region of syntenin-1. Additional competitive GST pull-down assay showed that binding of GlyT2C to syntenin-1 was reduced in the presence of His-tagged syntaxin in a concentration-dependent manner (Fig. 6D). Hence, the residues of syntenin-1 involved in syntaxin binding may at least partially overlap with determinants of GlyT2 binding. Consequently, GlyT2 and syntaxin 1A binding to syntenin-1 may be mutually exclusive, indicating that syntenin-1 is unlikely to function as an adaptor molecule that mediates the interaction between syntaxin 1A and the C-terminal region of GlyT2.

Discussion

In this paper, we report a novel interaction of syntenin-1 with the neuronal glycine transporter subtype, GlyT2. Syntenin-1 has been originally isolated by yeast-two hybrid screening using the cytoplasmic tail of syndecan as a bait (Grootjans et al., 1997). Subsequently, syntenin-1 has been found to bind the cytoplasmic C termini of a number of membrane proteins, for example, B-ephrins (Lin et al., 1999; Torres et al., 1998),...
neurexin (Grootjans et al., 2000), pro-TGFα (Fernandez-Larrea et al., 1999), neurofascin (Koroll et al., 2001), tyrosine phosphatase η (Iuliano et al., 2001), interleukin-5 receptor α (Geijsen et al., 2001), as well as AMPA, kainate, and metabotropic glutamate receptors (Hirbec et al., 2002, 2003). These diverse interactions have been attributed to recognition by syntenin’s two PDZ domains. PDZ binding motifs can be classified into at least three distinct classes: class I PDZ domains recognize the motif (S/T) – X – (V/I) – COOH, class II PDZ domains the motif Φ–X–Φ–COOH, and class III PDZ domains the motif X–X–C–COOH (where Φ represents a bulky hydrophobic amino acid, and X any amino acid residue) (Harris and Lim, 2001). The reported catalogue of syntenin-1 binding partners suggests that syntenin-1, like PICK1 (El Far et al., 2000), has the ability to recognize both class I and II binding motifs. GlyT2 has a class III PDZ binding sequence (TQC) at the C terminus, and we found that these three amino acid residues are necessary for its interaction with syntenin-1. Consistent with selective PDZ domain-mediated binding, the tail regions of both rat DAT and SERT, which do not contain C-terminal PDZ binding motifs, did not display detectable binding to syntenin-1 in the yeast two-hybrid assay, although human DAT, a type II PDZ binding motif containing protein, binds PICK1 via its carboxy terminal tail sequence (Torres et al., 2001). In a preliminary report, an interaction of syntenin-1 with human DAT has indeed been observed (Torres et al., 2002). Very recently, Kang et al. (2003b) have obtained crystal structures of the PDZ2 domain of syntenin-1 in complexes with different C-terminal binding peptides. This disclosed that this PDZ2 domain interacts with different target sequences by combinatorial occupation of three distinct subsites displaying high affinity for hydrophobic residues at the C-terminal positions 0, –1, and –2. The degenerate specificity of syntenin-1’s PDZ domain interactions thus can at least in part be attributed to versatile multisite binding pockets.

Consistent with previous reports (Geijsen et al., 2001; Grootjans et al., 1997; Iuliano et al., 2001; Jannatipour et al., 2001; Koroll et al., 2001), we found that both PDZ domains of syntenin-1 are required for binding to GlyT2. Yeast two-hybrid assays with point mutations within each PDZ domain of syntenin-1 showed that the functional integrity of the carboxylate-binding loop of PDZ2 but not of PDZ1 is essential for GlyT2 binding. These results raise the question of how PDZ1 contributes to the interaction of syntenin-1 with GlyT2. One possible explanation could be that the two PDZ domains of syntenin-1 cooperate and require a pair of linked ligand peptides for high-affinity binding. Indeed, Grootjans et al. (2000) have proposed that syntenin-1 recognizes suitable pairs of ligand peptides in high-affinity supramolecular complexes. However, this explanation is difficult to reconcile with the requirement of PDZ1 observed in our GST pull-down assay, because GlyT2C is unlikely to form a heterologous ligand pair in HEK cell lysates although in Xenopus oocytes, GlyT2 dimers are found in intracellular compartments (Horiuchi et al., 2001). Alternatively, PDZ1 may be required to create a fully folded PDZ2 domain. This idea receives support from a structural analysis of the PDZ domains of the glutamate receptor-interacting protein, GRIP (Zhang et al., 2001). Here, two tandemly arranged PDZ repeats (PDZ4 and 5) are necessary for the effective binding of GRIP to GluR2 (Dong et al., 1997). Using NMR and circular dichroism spectroscopy, Zhang et al. (2001) have demonstrated that the PDZ4 and PDZ5 domains of GRIP directly contact each other, and that this contact provides for a mutual chaperoning effect between these two PDZ domains. This chaperoning effect is only observed when both PDZ domains are covalently connected. The similar structural features of the paired syntenin-1 PDZ domains suggest that the PDZ1 domain of syntenin-1 may be essential for proper folding of PDZ2. Recent studies on the stability of PDZ domain constructs of syntenin-1 have suggested that the two PDZ domains are structurally associated and undergo cooperative denaturation (Kang et al., 2003a). Moreover, the overall structural integrity of syntenin-1 may be required for its homo-dimerization, which has been proposed to constitute a prerequisite for binding of class I ligands by PDZ2 (Koroll et al., 2001).

The physiological functions of syntenin-1 binding to the cytoplasmic tails of specific membrane proteins presently remain largely enigmatic. For pro-TGFα, PDZ-domain-mediated binding of syntenin-1 has been reported to promote the transport of this protein through the secretory pathway (Fernandez-Larrea et al., 1999). In the case of the interleukin-5 (IL-5) receptor-α, syntenin-1 serves as an adaptor protein that is required for IL-5-mediated transcriptional activation by Sox4 (Geijsen et al., 2001). However, for the majority of the membrane proteins known to interact, functional consequences of syntenin-1 binding have not been reported. Similarly, here we were unable to detect an effect of syntenin-1 co-expression on recombinant GlyT2 activity. Neither the apparent substrate affinity (K_m) of glycine transport nor the cell surface expression of GlyT2 as reflected by maximal glycine transport velocity (B_max) were changed in syntenin-1 co-transfected HEK 293 cells as compared to controls. This suggests that syntenin-1 interactions may be relevant for processes that occur only in neuronal cells, such as axonal targeting, recruitment of specific regulatory components, or a selective localization/anchoring at specialized sites. Our data on the distribution of syntenin-1 in the mammalian CNS lend some support to this idea. We found at both the mRNA and protein levels, that syntenin-1 is broadly expressed in the brain. Our immunohistochemical data indicate that syntenin-1 is predominantly synthesized in neurons, but its subcellular distribution appears to differ between neuronal subpopulations. For example, in the cerebral cortex, both the cell bodies and dendrites of pyramidal cells were diffusely stained with the syntenin-1 antibody, whereas no staining was seen in the cytoplasm of granule cells in the dentate gyrus which contain high levels of syntenin-1 mRNA. In the cochlear nucleus, a structure known to harbour many glycinergic interneurons (Altschuler et al., 1986), syntenin-1 immunoreactivity co-localized to a large extent with punctate GlyT2 immunostaining, consistent with a presynaptic interaction of these binding partners. Also, upon subcellular fractionation, syntenin-1 was found in the GlyT2-containing LP1 fraction in addition to LP2 membranes and the cytosolic supernatant. Furthermore, a syntenin-1 antibody co-immunoprecipitated GlyT2 from a detergent extract of brainstem and spinal cord membranes. Thus, syntenin-1 is likely to interact in vivo with this transporter protein in addition to several other binding partners. Previous work has identified syntenin-1 as a major component of early apical recycling endosomes (Fialka et al., 1999), as a functional component of the early secretory pathway (Fernandez-Larrea et al., 1999), and as a cell adhesion and microfilament-associated protein (Zimmermann et al., 2001). In addition, syntenin-1 is a binding partner for a number of other membrane proteins localized at pre- and post-synaptic sites (Grootjans et al., 1997;
Hirbec et al., 2002, 2003; Koroll et al., 2001; Lin et al., 1999; Torres et al., 1998). These multiple associations of syntenin-1 could explain its broad distribution in the brain and the diversity of its subcellular localizations found in neurons.

GlyT2 is enriched at presynaptic terminals of glycineric synapses and thus ideally positioned to mediate the re-uptake of released glycine into the presynaptic cytoplasm (Gomez et al., 2002c; Roux and Supplisson, 2000). Presently, it is unknown how this transporter is targeted to the presynaptic terminal and anchored around neurotransmitter release sites. Deken et al. (2000) have demonstrated that syntaxin 1A is involved in the trafficking of GaT1 to the cell membrane, and that direct binding of syntaxin 1A to the N-terminal region of GaT1 is crucial for this process. Similarly, Geerlings et al. (2001) have suggested that SNARE complexes participate in plasma membrane trafficking of GlyT2. Here, we found that the N terminus of GlyT2 binds syntaxin 1A (and complexed SNAREs such as SNAP-25) with comparatively low affinity. This is consistent with transient binding of SNARE complexes to the N terminus of GlyT2 being important for the transport of GlyT2 to the plasma membrane, as proposed for GaT1 trafficking. In addition, syntenin-1 was found to bind syntaxin 1A as well as SDS-resistant SNARE complexes more avidly than GlyT2N. However, competitive GST pull-down assays revealed that syntenin-1 cannot bind syntaxin 1A and GlyT2 simultaneously. This suggests that syntenin-1 does not directly participate in syntaxin 1A-mediated GlyT2 trafficking but acts at a step following membrane fusion. Interestingly, syntaxin 1A was capable of binding to a deletion construct of syntenin-1 that encompassed the isolated PDZ2 domain and the C-terminal region only. This differs significantly from the interaction of the other known syntenin-1 binding partners, which possess PDZ binding motifs. We therefore speculate that binding of syntaxin 1A to syntenin-1 may have a special function, such as negatively regulating interactions mediated by its PDZ2 domain. Syntenin-1 is known to interact with both PIP2 and the actin-binding protein merlin through its PDZ1 domain (Jannatipour et al., 2001; Kang et al., 2003a; Zimmermann et al., 2002). This suggests mechanisms by which GlyT2 could be localized to specialized membrane microdomains. GlyT2 may be targeted via syntenin-1 and PIP2 to lipid rafts in the nerve terminal membrane or even to discrete subdomains of intracellular compartments. Alternatively, syntenin-1 bound GlyT2 may be anchored to the actin cytoskeleton via merlin. Syntaxin 1A may regulate these localizations of GlyT2 by competitively binding to syntenin-1, and thereby impairing its interaction with GlyT2, thus allowing for facilitated internalization, or fusion with other compartments such as endosomes, of GlyT2 containing vesicles upon PKC activation or other stimuli. Further studies will be required to examine these hypotheses.

**Experimental methods**

**Yeast two-hybrid screening**

The DNA sequence encoding the C-terminal region of GlyT2 (amino acids 738–799) was amplified by PCR from a plasmid encoding the full-length rat GlyT2 cDNA. The amplified fragment was subcloned in frame into the inducible pGilda bait vector and verified by DNA sequencing. Yeast two-hybrid screening was carried out with the DupLex-A two-hybrid system kit (Origene Technologies). The bait construct (GlyT2C) was used to screen 10^8 independent recombinant clones of a rat brain cDNA library in pJG4-5 transformed into the yeast strain EGY48. Out of 72 blue colonies picked from the original galactose-induced selection plates (–Ura, –Trp, –Leu, –His), 8 contained an in-frame insertion of the full-length rat syntenin-1 cDNA. These plasmids allowed specific and reproducible growth on selective medium as well as β-galactosidase induction upon isolation and retransformation with the bait construct.

The specificity of the interaction between syntenin-1 and GlyT2C was analyzed by two-hybrid assay. To this end, we generated deletion mutants of GlyT2C lacking the very last (GlyT2CΔC) and the three last (GlyT2CΔTQC) amino acids. For testing the interaction of syntenin-1 with the rat DAT and SERT C-termini, cDNA fragments encompassing the C-terminal regions of DAT (amino acids 577–619; DATC) and SERT (amino acids 595–630; SERTC) were amplified by reverse transcriptase (RT)-mediated polymerase chain reaction (PCR) from rat brain total RNA, and pGilda–DATC and pGilda–SERTC plasmids were generated for yeast two-hybrid analysis. Constructs encoding syntenin-2α and -2β as well as the PDZ point mutants of syntenin-1 have been described previously (Koroll et al., 2001).

**Bacterial recombinant fusion proteins**

An EcoRI–XhoI fragment containing the entire coding region of rat syntenin-1 was excised from the pJG4-5 plasmid obtained by yeast two-hybrid assay and ligated into pGEX-5X-1 (Amersham Pharmacia Biotech). Deletion constructs of syntenin-1 were amplified by PCR and subcloned into pGEX-5X-1. These recombinant plasmids as well as insertless pGEX-5X-1 were transfected into Escherichia coli BL21 according to the manufacturer’s instructions. BL21 cells were lysed by passage through a French press in phosphate-buffered saline (PBS), centrifuged at 20,000 × g for 30 min, and the supernatants were collected and kept at −80°C until use. A GST fusion protein encompassing amino acid residues 1–201 of the N-terminal region of GlyT2 was also prepared following the same method. A recombinant squid His-syntenin protein was generated as described previously (O’Connor et al., 1997).

**Antibodies**

Two polyclonal antibodies (#29 and #30) against rat syntenin-1 (1:500) (Koroll et al., 2001) and a polyclonal antibody against the N-terminal region of GlyT2 (1:2000) (Jursky and Nelson, 1995) were employed as described previously. Other antibodies used in this study were as follows: anti-GlyT2 polyclonal antibody (1:1000; Chemicon); anti-GFP polyclonal antibody (1:200; Clontech); anti-PSD-95 monoclonal antibody (1:1000; Affinity BioReagents); anti-synaptophysin monoclonal antibody (1:1000; Roche Diagnostics); anti-syntenin 1A monoclonal antibody 6D2 (1:10,000) (Yoshida et al., 1992); anti-SNAP25 monoclonal antibody (1:5000; Affiniti Research); and anti-synaptobrevin-2 polyclonal antibody (1:5000) (Hunt et al., 1994).

**Transfection of 293T cells and GST pull-down**

Human 293T cells were grown in MEM supplemented with 10% (v/v) fetal calf serum and antibiotics. At approximately 70% confluence, 293T cells in 10-cm dishes were transfected with 10 μg of expression vector DNAs using the calcium phosphate method.
For GST pull-down assays, we inserted the full-length GlyT2 cDNA and a construct in which the last three codons had been deleted (GlyT2ΔTQC) in frame into the pEGFP-C2 vector (Clontech) and generated EGFP-tagged GlyT2 and EGFP-tagged GlyT2ΔTQC. The EGFP-tagged carboxy terminus of GlyT2 (EGFP–GlyT2C) and EGFP-tagged GlyT2C lacking the last three amino acids (EGFP–GlyT2CΔTQC) were generated by ligation of the corresponding fragments into the pEGFP-C2 vector. Myc-tagged syntenin-1 cDNA was generated by PCR and inserted between the EcoRI and XhoI sites of the pcDNA3.1 vector (Invitrogen). Two days after transfection, cells were collected and incubated for 1 h in lysis buffer: [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1.5 mM MgCl2, 4 mM EDTA, 10% (v/v) glycerol, complete protease inhibitor cocktail (Roche Diagnostics) containing 1% (v/v) Triton X-100, or 2% (w/v) cholic acid, respectively]. Cell lysates were cleared by centrifugation at 15,000 rpm for 30 min. For pull-down assays, 30 μl of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) were incubated with GST fusion proteins for 1 h, washed three times with binding buffer (25 mM NaCl, 20 mM Tris, pH 7.5, 0.1 mM DTT) and incubated with the cleared lysates for 3 h. After incubation, the beads were washed four times in washing buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1.5 mM MgCl2, 5 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, or 2% (w/v) cholic acid, respectively]. All these procedures were performed at 4°C. Proteins retained on the beads were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Schleicher & Schüll). The membrane was blocked with 5% (w/v) nonfat dry milk powder in PBS for 60 min followed by a 12-h incubation with primary antibodies. After washing, bound Igs were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL system (Pierce).

Subcellular fractionation and co-immunoprecipitation of endogenous proteins

Subcellular fractions of adult rat brainstem and spinal cord were prepared as described by Li et al. (1996). In brief, adult brainstem and spinal cord were homogenized in 4 mM Hepes, pH 7.4, containing 0.32 M sucrose, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors, using a Teflon potter homogenizer. Homogenates were centrifuged at 800 g for 10 min to yield supernatant (S1). The S1 was centrifuged for 10 min at 15,000 g to give crude synaptosomal fraction (S2). The S2 was further centrifuged at 165,000 g for 1 h to yield pellet P1. The supernatant was centrifuged at 25,000 g for 20 min, and the supernatant was further centrifuged at 165,000 g for 60 min to obtain crude synaptosomal fraction (S3). The S2 pellet was resuspended in homogenization buffer and represents a crude synaptosome fraction. The crude synaptosome fraction was lysed by adding 10 vol of ice-cold water containing protease inhibitors followed by homogenization with a glass–Teflon homogenizer. The synaptic membrane fraction (LP1) was collected from this homogenate by centrifugation at 25,000 × g for 20 min, and the supernatant was further centrifuged at 165,000 × g for 60 min to give crude synaptic vesicle pellet (LP2) and supernatant (LS2) fractions.

In situ hybridization

Adult male rat brains were removed and quickly frozen in dry ice. Frozen sections (18 μm thick) were cut on a cryostat and kept at −20°C until use. The syntenin-1 mRNA-specific oligonucleotide 5’-GGTTGGCCGTCACAGACAGGCATGT TTTCACATTT, which corresponds to nucleotide positions 273–312 of the rat syntenin-1 cDNA (accession number AF248548), was labeled using [35S]dATP and incubated with the sections in hybridization buffer [4 × SSC, 50% deionized formamide, 0.12 M phosphate buffer, pH 7.2]. Denhardt’s solution, 2.5% (w/v) tRNA, 10% (w/v) dextran sulfate) for 24 h at 42°C. After hybridization, the sections were rinsed in 1× SSC (pH 7.2) for 10 min, followed by three washes in 1× SSC at 55°C for 20 min, each. The sections were then dehydrated through a graded ethanol series (70–100%) and exposed to BIOMAX MR film (Kodak) for 1 week. Control sections were processed using the same procedure, except that a 300-fold excess of non-labeled probe was added to the hybridization buffer.

Immunohistochemistry

Wistar male rats (200 g) were deeply anesthetized and intracardially perfused with 4% (w/v) paraformaldehyde in PBS. Brains were further fixed in the same fixative overnight and then cryoprotected in 30% (w/v) sucrose in PBS. Frozen sections (18 μm thick) were cut on a cryostat, blocked 3 h at room temperature in PBS containing 0.3% (v/v) Triton X-100, 1% (w/v) goat serum and 1% (w/v) bovine albumin (buffer A), and incubated with anti-syntenin-1 antibody #29 in buffer A for 2 days at 4°C. Sections were then incubated overnight at 4°C with biotinylated anti-rabbit antibody (Vector Laboratories) followed by incubation with VECTASTAIN ABC reagent (Vector Laboratories) and finally visualized with diaminobenzidine (DAB) as a substrate. In case of confocal microscopic analysis, sections were incubated with anti-syntenin-1 antibody #29 and anti-GlyT2 antibody (Chemicon), and visualized with Alexa anti-rabbit-594 and anti-guinea pig-488 secondary antibodies, respectively. Confocal microscopy was performed using a confocal laser-scanning microscope Leica TCS-SP equipped with the image software Leica-TCS-NT (version 1.6.551).

[3H]Glycine uptake measurements

293T cells were seeded in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum, glutamine, and antibiotics at a density of 1.8 × 10^6 cells per 10 cm culture dish and, after 1 day, co-transfected with the plasmids “GlyT2-pRC/CMV”, which contained the coding region of the GlyT2 cDNA in the expression vector pRC/CMV (Invitrogen), “GEP-syntenin” containing the coding region of syntenin-1 in frame within peEGF-C2 (Clontech), or the parental peEGF-C2 vector as control. The day after transfection, cells were seeded onto poly-L-lysine-coated 48-well plates at a density of 0.05–0.1 × 10^6 cells per well. [3H]Glycine uptake assays were performed 1–2 days after plating as described previously (Scholze et al., 2000; Sitte et al., 2002), except that incubations were with 50 nM (0.15 μCi) [3H]glycine and various concentrations of unlabelled glycine for 4 min. Non-GlyT2-mediated [3H]glycine uptake was estimated in parallel with non-transfected 293T cells. For protein determinations, wells were treated as above, lysed in 50 μl NP40 buffer [0.5% (w/v) NP40; 30 mM Tris/HCl; 140 mM NaCl; final pH = 7.5], and protein was measured using the Bradford method. Curve fitting was done using the Prism4 software (GraphPad, San
Diego, CA). $V_{\text{max}}$ and $K_m$ values were calculated using the equation $Y = V_{\text{max}}X/(K_m + X)$, where $Y = V$ and $X$ = substrate concentration.

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References


