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Use of calpain for native GlyT2 N-terminal region separation and its potential use in transporter N-terminus interaction studies

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Unusual feature of glycine transporter GlyT2 is its N-terminus, which is at least three times longer than the N-terminal sequences found in other family members. There is a continuing effort to find the proteins interacting with this extended domain. We previously described the existence of calpain cleavage sites located in the vicinity of the first transmembrane transporter segment. Here we report that these sites could be explored for isolation of native protein fragments corresponding to major part of the GlyT2 N-terminal sequences. Simple hypoosmotic lysis of rat spinal cord synaptosomes in presence of high calcium with subsequent high-speed centrifugation allows to separate the GlyT2 N-terminal immunoreactivity from membrane bound transporter skeleton. Such peptides could have advantage for interaction studies because in contrast to recombinant analogues they represent the original folding and potential modification. Crosslinking experiments indicate that in these conditions the GlyT2 N-terminal fragment released by calpain is not associated with any other protein.

Key words: glycine, transporter, GlyT2, calpain, protein, interaction.

The amino acid glycine is, except of its metabolic functions, also important neurotransmitter. In caudal brain parts glycine inhibits neuronal activity acting on strychnine sensitive glycine receptor (BETZ, 1992). Function of glycine in rostral brain is modulation of excitatory neurotransmission, namely glycine here acts as coagonist of NMDA receptor (JOHNSON & ASCHER, 1987). Functional studies and localization show that substantial role in regulation of glycine neurotransmitter pools is played by the glycine transporters GlyT1 and GlyT2 (JURSKY & NELSON 1996; POYATOS et al., 1997; SUPPLISSON & BERGMAN 1997; BERGER et al. 1998; BREGERON et al. 1998). Both these proteins belong to family of sodium dependent neurotransmitter transporters (NELSON 1998; JURSKY & BALIOVA 2002). They exhibit 12-transmembrane topology with their N- and C-terminal regions oriented into the cytoplasm (ANDROUTSELLIS-THEOTOKIS & RUDNICK, 2002). Role of these regions are currently not well understood, however, it is of consider-

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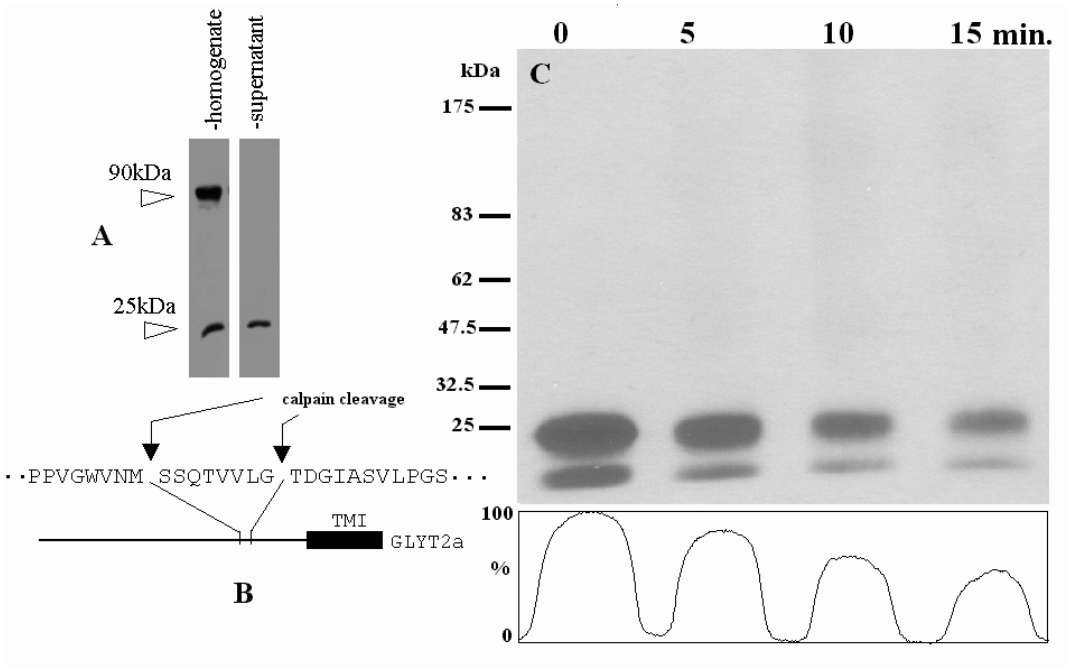

Figure 1. (**A**) Separation of GlyT2 N-terminal peptide from the transporter skeleton. Rat spinal cord synaptosomes isolated according to Baliova et al. (2004) were suspended in high calcium hypotonic buffer (10mM Hepes/KOH/5mM-$CaCl_2$/pH-7.5) and GlyT2 N-terminus was cleaved of with endogenous calpain activity for 30 min at 37 °C. Synaptosomes were peleted at 105,000 *g* for 30 min. Supernatant and sediment samples were loaded on 7/12% PAG and transferred to immobilon using standard Western blotting technique. Position of GlyT2 and its released N-terminal fragment was detected using anti-GlyT2N antibodies (Baliova et al., 2004). (**B**) Position of the major cleavage sites on the glycine transporter GlyT2 N-terminus. (**C**) Glutaraldehyde crosslinking of the calpain released GlyT2 N-terminal peptide. Supernatant from high-speed centrifugation (Fig. 1A) was supplemented with NaCl to 150 mM, 1mM $MgCl_2$ and incubated for 1 hour at room temperature. Supernatant was then crosslinked with 0.1% glutaraldehyde for different times at room temperature. Crosslinking reaction was stopped with addition of SDS sample buffer. Samples were boiled, resolved on 4-12% PAG, transferred to immobilon and probed with antibodies raised against the N-terminal domain of GlyT2. Quantification of the immunoreactive bands was made with Scion Image software (lower part of Fig. 1C).

able interest to understand the regulation of neurotransmitter transporters with proteins that directly bind to their cytosolic regions. SNARE protein syntaxin 1A was amongst the first discovered proteins that bind to all transporters (Quick et al 1997; Geerlings, 2000). Syntaxin 1A interacts with N-terminal region close to the first transmembrane domain and plays important role in transporters trafficking. Experiments with GABA transporter GAT1 showed that this interaction also leads to alosteric interference with the transport mechanism (Hansra, 2004). Comparison of neurotransmitter transporter molecules shows that the size of cytoplasmic domains is approximately the same with few exceptions. Glycine transporter GlyT2 N-terminus is about three-times longer when compared with the N-terminal domains of other transporters (Liu et al., 1993) and homology search does not show any significant similarity with other protein sequences. The length of this domain, which comprises 201 amino acid residues, should give high chance for interaction with intracellular proteins and links to certain yet not identified regulation machinery. One of the interactions uncovered recently in our laboratory is the interaction of GlyT2 N-terminal domain with calcium dependent protease calpain (Baliova et al., 2004). This interaction leads to transporter truncation in conditions of calpain activation. Most abundant 25 kDa peptide fragment liberated by calpain according to calculated molecular weight should represent major part of native GlyT2 N-terminus (Fig. 1B). Since all our recent efforts with pull down essays and two-

hybrid screens were unsuccessful, we decided to explore calpain liberated native GlyT2 N-terminus to screen for its interaction partners. Such peptide has the advantage of native folding and potential modification (Baliova & Jursky, 2004). Frequently used approach in protein interaction studies is the crosslinking of the proteins in native environment where proteins interact with each other in their physiological contents. Aggregates are then detected in a mixture of proteins by antibodies against one of the interacting partners. This approach is however difficult to use in proteins with high molecular weight since increase in size of crosslinked complexes is not apparent if interacting partner is to small. Additionally large complexes are frequently limited by performance of resolving techniques. Calpain-produced GlyT2 N-terminal peptide, in contrast to intact GlyT2, possesses advantage of small interacting protein. In reality, calpain cleavage is never quantitative and remaining intact transporter creates interference on Western blot during detection of potential interactions (Fig. 1A). We have developed an approach that overcomes this interference. To obtain clean peptide we performed high-speed centrifugation of lysed synaptosomes where the N-terminal domain was cleaved of *via* the endogenous calpain activity. Membrane bound truncated transporters skeleton together with immunologically interfering uncleaved GlyT2 remained in the sediment. Obtained supernatant contained immunologically pure N-terminal peptide ready for crosslinking or overlay assays (Fig. 1A). To visualize the position of the N-terminal GlyT2 fragment and its crosslinked intermediates in complex mixture of spinal cord proteins we used polyclonal antibody directed against this domain. Figure 1C shows that under these conditions glutaraldehyde crosslinking of N-terminal GlyT2 N-peptide does not create any crosslinked aggregates and is likely in free form without interacting partner. Results in these experiments might be affected by the fact that the GlyT2 N-terminus/interacting partner might also be a target of calpain proteolysis or epitopes might be inaccessible after crosslinking. This technique can be, however, further improved, scaled up for preparative immunoprecipitation and complexes could be resolved by MALDI-TOF spectrophotometry. In addition membranes can be crosslinked and subsequently cleaved with externally added calpain. In this case the possibility of interacting artifact is greatly limited. Calpain usually cleaves between protein domain boundaries (Perrin & Huttenlocher, 2002; Goll et al. 2003). This approach thus can also be applied for interaction screening in other proteins containing calpain cleavage sites.

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