Biochemical and Biophysical Research Communications 454 (2014) 25–29

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Using a collection of MUPP1 domains to investigate the similarities of neurotransmitter transporters C-terminal PDZ motifs

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ARTICLE INFO

Article history:
Received 29 September 2014
Available online 13 October 2014

Keywords:
Neurotransmitter transporters
PDZ motif
PDZ domain
MUPP1

ABSTRACT

A ubiquitous feature of neurotransmitter transporters is the presence of short C-terminal PDZ binding motifs acting as important trafficking elements. Depending on their very C-terminal sequences, PDZ binding motifs are usually divided into at least three groups; however this classification has recently been questioned. To introduce a 3D aspect into transporter's PDZ motif similarities, we compared their interactions with the natural collection of all 13 PDZ domains of the largest PDZ binding protein MUPP1. The GABA, glycine and serotonin transporters showed unique binding preferences scattered over one or several MUPP1 domains. On the contrary, the dopamine and norepinephrine transporter PDZ motifs did not show any significant affinity to MUPP1 domains. Interestingly, despite their terminal sequence diversity all three GABA transporter PDZ motifs interacted with MUPP1 domain 7. These results indicate that similarities in binding schemes of individual transporter groups might exist. Results also suggest the existence of variable PDZ binding modes, allowing several transporters to interact with identical PDZ domains and potentially share interaction partners *in vivo*.

1. Introduction

The sodium/chloride dependent neurotransmitter transporters are membrane proteins, which regulate concentration of important neurotransmitters in the vicinity of brain nerve terminals and surrounding glial cells [1–4]. This implies that the transporter proteins are critically involved in shaping the neurotransmission in CNS and their improper function might cause serious neurological illnesses [5–8]. The activity of neurotransmitter transporters could be influenced either *via* specific inhibitors affecting their permeation pathway [9,10] or by influencing their total number in the membrane [11–13]. One of the most important signaling elements, which regulate the transporters surface expression, are PDZ binding motifs located at the very end of their C-terminal domains. These motifs interact with PDZ domains of regulatory and clustering proteins [14–18] to control the activity and localization of transporters.

The PDZ domains are most common protein interaction modules abundant in animals, yet scarce in yeast, bacteria and plants [19,20]. These domains were originally identified as series of GLGF repeats in proteins. Their original names GLGF and DHR were finally agreed to be replaced with name PDZ, consisting of starting letters of the first three discovered proteins containing these domain, namely postsynaptic **P**SD95 protein, Drosophila **D**isc-large protein and tight-junction **Z**O-1 protein [21]. Except for few cases [22], all PDZ domains exhibit identical canonical binding mode engaging the C-terminal peptide ligand into the binding groove of PDZ domain through main chain hydrogen bonds. The mode of interaction is such that the ligand adopts an extended β-strand conformation by interacting in an antiparallel fashion with a PDZ β-strand [23,24].

The MUPP1 is PDZ signaling protein, which was originally identified as a protein interacting with the C-terminus of the serotonin 5-hydroxytryptamine type 2C (5-HT2C) receptor [25]. MUPP1 is the largest known PDZ signaling protein containing 13 PDZ domains. Since all its domains reside on the same molecule, it is likely that they possess non-redundant specificity serving for binding of diverse protein ligands. In this work we separated DNA coding regions of MUPP1 PDZ domains and fused them in frame with GST protein. Subsequently, we used collection of these 13 PDZ fusion proteins immobilized on glutathione Sepharose matrix for probing their interaction with transporters minimal PDZ binding motifs also fused with small cellulose binding protein (CBD). Interestingly, this simple pull-down assay revealed that groups of transporters might have similarities in PDZ interactions that are not apparent from their primary amino acid sequences.

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http://dx.doi.org/10.1016/j.bbrc.2014.10.011
0006-291X/

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by Microsynth AG (Balgach, Switzerland). Tris (Hydroxymethyl) aminomethane (Tris) free base, hydroxyethyl piperazineethanesulfonic acid (HEPES) free acid, all molecular biology grade, were from Merck Millipore (Darmstadt, Germany). All other chemicals used were of the per analysis or molecular biology grade.

2.2. Preparation of GST and CBD fusion proteins

Mouse MUPP1 cDNA plasmid pCAGGS-MUPP1-myc [26] was obtained from Prof. Makoto Adachi (Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan). MUPP1 numbering used here follows the Uniprot reference protein sequence: Mouse Mpdz 17475; NM_010820; Q8VBX6. For the purposes of the pull down assays the EcoRI/SalI PCR fragments encompassing the amino-acids coding regions of MUPP1 PDZ domains 1 (aa 133–232), 2 (aa 231–347), 3 (aa 351–466), 4 (aa 533–631), 5 (aa 667–782), 6 (aa 990–1081), 7 (aa 1130–1235), 8 (aa 1332–1429), 9 (aa 1464–1560), 10 (aa 1598–1699), 11 (aa 1702–1812), 12 (aa 1839–1941) and 13 (aa 1953–2055) were inserted into pGEX-5X-1 plasmid (GE Healthcare, Freiburg, Germany) in frame with the GST coding sequence. Complementary DNA oligonucleotides corresponding to the last 7 amino acids of transporters flanked the EcoRI and SalI restriction sites were designed and synthesized for transporters GAT1, GAT2, GAT3, GlyT1, GlyT2, SERT, DAT, NET as well as for the mutated NET motifs: NET-E corresponding to mutation W614E in position −3 and NET-Y corresponding to mutation A616Y in position −1 (the last transporter amino acid is in PDZ terminology marked as the position 0). Oligonucleotides were synthesized, annealed and inserted into pET34b(+) plasmid (Novagen, Merck, Darmstadt, Germany) in frame with cellulose binding protein (CBD). Correct insertion was verified by sequencing.

2.3. Overexpression and isolation of GST and CBD fusion proteins and pull-down assay

The GST fusion protein plasmids were transformed into BL21 (DE3) cells (Novagen; Merck, Darmstadt, Germany) in the presence of ampicillin. A small (20 ml) inoculum of the cells was grown overnight and 6 ml from this culture was transferred into fresh LB flask containing 250 ml LB. The cells were further grown for two hours at 37 °C and following the culture was cooled to 18 °C, fusion proteins were induced overnight with addition of IPTG to final 0.3 mM concentration in cooled shaker at 180 rpm and 18 °C. *Escherichia coli* cells were centrifuged, resuspended in ice cold homogenization solution containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 10 mM EDTA and 1% Triton X100. Cells were then open with sonication on ice and homogenate was centrifuged at 9000×*g* for 15 min at 4 °C. Supernatant was mixed with 0.8 ml (50% V/V) of glutathione resin (GE Healthcare, Freiburg, Germany) for 30 min at room temperature, washed with homogenization buffer containing Triton X100 reduced to 0.1%. Finally, resins with immobilized proteins were stored until use at −80 °C.

The CBD–PDZ motifs fusion proteins plasmids were transformed into *E. coli* BL21 (DE3) in the presence of kanamycin and proteins were induced as above except that 2.5 ml of overnight inoculum was transferred to 100 ml LB. The cells were grown for two hours at 37 °C and following the culture was cooled to 18 °C, fusion proteins were induced overnight with addition of IPTG to final 0.3 mM concentration in cooled shaker at 180 rpm and 18 °C. Induced cells were centrifuged washed with 50 ml of 0.9% NaCl resuspended in 30 ml of interaction solution 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X100, sonicated, centrifuged 15 min at 9000×*g*. Supernatants (15 ml) were preadsorbed by adding small amount of glutathione-Sepharose beads and slowly mixing the content by rotation for 30 min at room temperature. Following centrifugation, one ml aliquot of precleared extract was mixed with 0.03 ml of glutathione-Sepharose beads with immobilized PDZ domain fusion protein. Mixture was left to interact in rotator for 1 h at room temperature. In order to check the presence and integrity of fusion protein, an additional aliquot of supernatant was mixed with 0.04 ml of microcrystalline cellulose (50% V/V) and mixed at room temperature 20 min. Following washing GST fusion proteins were eluted with 10 mM glutathione together with the interacting proteins. Cellulose immobilized proteins were eluted by 1 min boiling with SDS sample buffer. Proteins were resolved on 12% PAGE and visualized with coomassie staining.

3. Results and discussion

The PDZ interaction motifs of neurotransmitter transporters belong to a typical short C-terminal motifs [13–17]. The binding capacity of these motifs strongly depends on the last 3 amino-acids in their C-terminus [24,27]. Consequently, the sequence alignment is not very reliable method to study their similarity and binding predictions. The classification of these motifs into various classes, having a characteristic amino-acids in specific positions [24,28,29] also usually do not guarantee binding to identical PDZ domain. Large number of *in silico* methods predicting PDZ mediated interactions has been developed recently. Even though these methods are helpful, the flexibility of binding modes causes that experimental verification still represents the only way to ensure that PDZ motif binds to investigated PDZ domain [30].

To study 3D properties of PDZ interaction motifs by using the bench methods it will be helpful to have several PDZ interaction probes with variable geometry of the PDZ interaction cavity. One possibility to obtain such probes is to explore some of the natural PDZ domains, such as the collection of 13 domains of the largest PDZ binding protein MUPP1. Because MUPP1 is suppose to bring together several signaling proteins, it is likely that these domains possess diverse specificity.

In this article we picked up the most typical members of neurotransmitter transporters and investigated the interaction of their minimal PDZ binding motifs with individual MUPP1 domains. Since in many cases these proteins are not colocalized *in vivo*, a physiological role of such interaction was not a question investigated here. Similarly not all here identified interactions will probably be observed with solubilized intact transporters. The reasons for that might be for example the interference of simultaneously solubilized non-physiological high affinity PDZ ligand. Solubilization might also remove the important proteins interacting with the upstream transporter C-terminus and possibly narrowing the conformation of PDZ motifs in certain interactions *in vivo*. For these reasons here identified PDZ interactions of transporter motifs only reflect their theoretical potential and flexibility of PDZ interaction schemes. Finally one cannot exclude also the physiological function when colocalization of MUPP1 with certain transporters is possible.

To study the similarities of neurotransmitter transporters PDZ interactions we used here the simple pull-down assay employing two well-established GST and CBD fusion proteins. GST fusion protein belongs to most frequently used protein in interaction studies because of its high expression, low background and relative high-affinity to glutathione-Sepharose beads. Commercial vector

pGEX-5X-1 was used for the fusion of all thirteen individual domains in frame with GST. The PCR primers were designed in such a way that all previously identified important PDZ domain elements were present in inserted coding regions (Fig. 1). Even though we did not observed significant production of inclusion bodies during 37 °C overexpression, all GST fusion proteins were isolated following the induction at lowered (18 °C) temperature in order to give the proteins more time for proper folding.

A contribution to binding affinity and specificity of short linear PDZ motifs seems to decrease rapidly upstream of the main terminal tripeptide and it is minimal upstream of the first 7 C-terminal amino acid residues [31]. For these reasons we decided to insert short linkers coding for the last 7 C-terminal amino acid of investigated transporters in frame with small 17 kDa cellulose binding protein (CBD). These fusion proteins containing PDZ motifs of neurotransmitter transporters were subsequently overexpressed in *E. coli* and interacted with GST-PDZ domains fusion proteins immobilized on glutathione-Sepharose beads. The disadvantage of CBD is its frequent creation of inclusion bodies, which however can be overcame using low temperature induction procedure. Induction of proteins at 18 °C resulted in soluble expression for most of the constructs, except for norepinephrine and dopamine fusion proteins, which also were present as inclusion bodies. These two proteins were however still sufficiently expressed in soluble form as we were able to recover them from the soluble supernatant using affinity binding to microcrystalline cellulose. Our results further showed that the amount of protein recovered in our pull-down assay is high enough to be detected with coomassie dye staining (Fig. 2).

From Figs. 2 and 3 it is clear that while interaction promiscuity of PDZ motifs vary between one (SERT), two (GAT3, GlyT2), three (GAT4, GlyT1) and five (GAT1) MUPP1 domains, dopamine and norepinephrine transporters do not show significant affinity to MUPP1 domains. Interestingly, a very promiscuous PDZ motif of GAT1 likely suggests its high flexibility. The reason for that might be the pleiotropic functions of GABA, which might requires the interactions with many partners. The results also suggest that dopamine and norepinephrine transporters PDZ motif might somehow differ from that of serotonin transporter and GABA and glycine transporters. This is supported also by the nature of their amino-acid sequences upstream of core terminal tripeptide (Fig. 3). Since upstream positions are probably not directly involved in binding of motif into PDZ carboxylate binding loop, these amino acids might influence the conformation geometry of terminal tripeptide. Even though these tripeptides are quite different in investigated transporters, 7AA motifs interacting with MUPP1 domains contain at least one or two negatively charged amino acids in position (−3)–(−5). Non interacting transporter motifs present in the C-terminus of DAT and NET transporters do not contain negative amino-acids in these positions, instead they contain positive amino acids doublet in position −4, −5 and set of hydrophobic amino-acids in the rest of the very C-terminal end. At this time, it is difficult to say if these differences represents certain rules, however the introduction of glutamic acid into position −3 of norepinephrine transporter PDZ motif indeed resulted in interaction of modified motif with MUPP1 domain 3 (Figs. 2 and 3). On the contrary, the interruption of hydrophobic triplet on NETC motif with polar tyrosine in position −2, which mimic the GAT1 motif did not resulted in binding of mutated motif to any of the MUPP1 domains (Figs. 2 and 3). Above might suggest that amino-acids upstream of transporters terminal tripeptide significantly influence binding specificity of PDZ motifs.

MUPP1 domains also had variable capacity to interact with transporter motifs (Figs. 2 and 3). While MUPP1 domains 1, 6, 8, 9 and 12 did not interacted with any of the transporter motifs, other MUPP1 domains were able to bind one (domains 2, 5, 11), two (domains 4, 10, 13) three (domain 3) or four transporter PDZ motifs (domains 7). How up to four diverse PDZ transporter motifs can target domain 7 is not clear. Since PDZ domains are very compact and do not change the conformation during the interaction, PDZ motifs are likely to be the highly flexible parts during the interaction. These results also suggest that transporter motifs might possess a multiple modes of interaction. The ability of various transporter PDZ motifs to interact with identical domain might be advantageous under certain circumstances. Such circumstances could be for example the transient or stable interaction of several transporters with the identical regulatory/clustering protein during a general process of trafficking and clustering. In this way cell does not have to made separate regulatory protein for each transporter.

Neurotransmitter transporters create family of membrane proteins and despite of their sequence diversity and differences in compartmentalization dictated by the substrates, the common feature of many of them is their presynaptic localization. This feature might cause a possible existence of common anchoring schemes. Such features might not be readily identifiable using linear peptide sequences because of possible existence of several potentially disordered 3D conformers. In our experiments the monoamine transporters differ significantly from glycine, GABA and serotonin transporter, concerning of their ability to interact with MUPP1 domains. Previous immunohistochemical localization of neurotransmitter transporters indeed revealed interesting and previously not appreciated differences between individual members. While immunoreactivity of GABA [32] glycine [33] and serotonin [34] transporters was found almost exclusively in neuronal and glial processes, the immunoreactivity of norepinephrine [35] and dopamine [36] transporters were also observed in cell bodies. This is in concert with our results and it suggests that monoamine

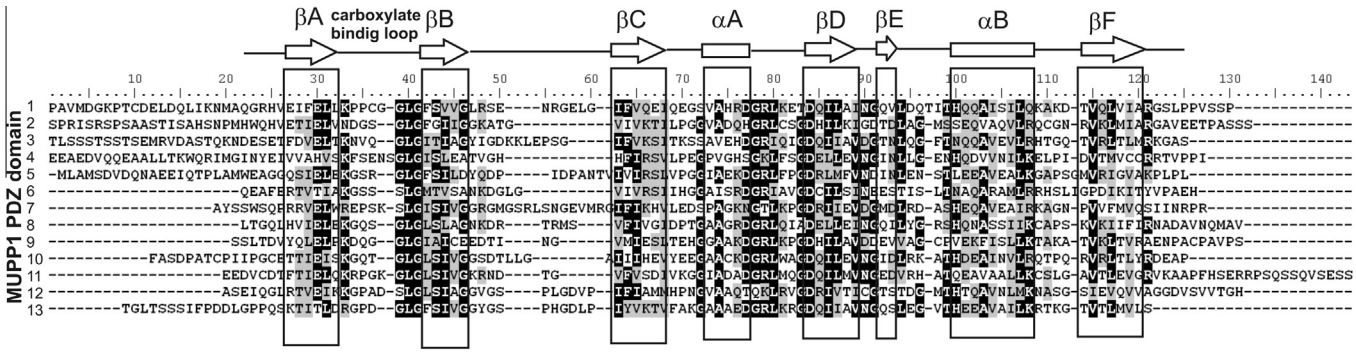

Fig. 1. Schematic representation of MUPP1 domains fused with GST protein. Arrows and boxes indicate the presence of all important structural elements of PDZ domains.

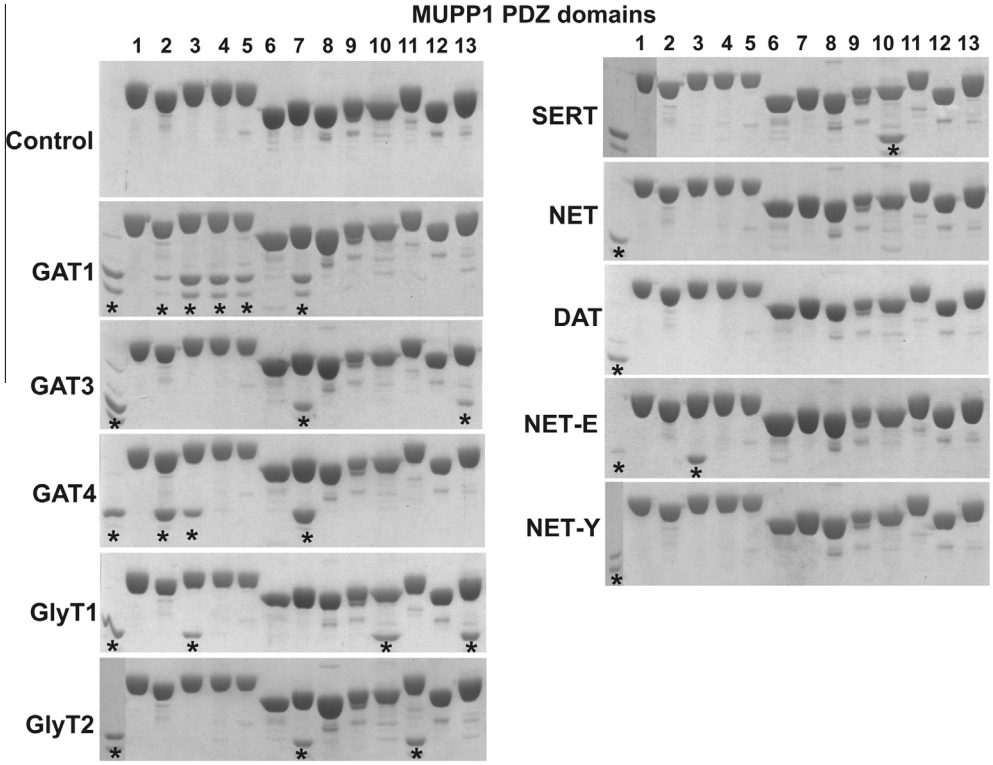

Fig. 2. Interaction of CBD–PDZ transporter motifs with GST-MUPP1 PDZ domains. Immobilized GST fusion proteins containing PDZ domains of MUPP1 were interacted with *E. coli* extracts containing over-expressed CBD fusion proteins bearing a neurotransmitter transporter PDZ binding motifs. Following washing proteins were eluted with glutathione, resolved in 12% PAGE and stained with coomassie dye. Asterisk in the first line indicates position of CBD-PDZ-motif fusion proteins recovered from *E. coli* extracts using affinity binding to microcrystalline cellulose and subsequent thermal elution with SDS sample buffer. The asterisks in lines 1–13 show the presence of interacting PDZ motif.

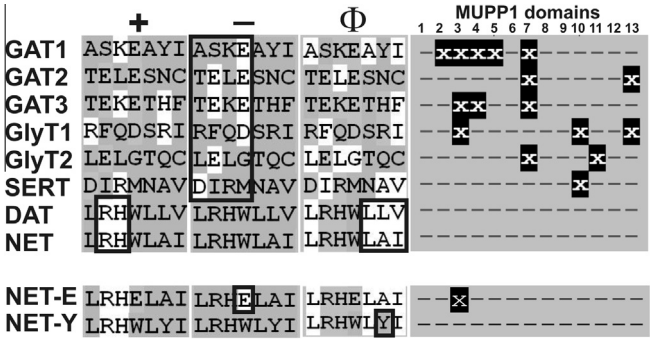

Fig. 3. Alignment of the last seven amino-acids of investigated neurotransmitter transporters indicating distribution of hydrophobic (ϕ), positive (+) and negative (−) amino-acids which is showed in the left upper part of the figure. The NET-E and NET-Y in left lower part of the figure indicate NET PDZ motifs mutated in positions −1 and −3 (the last amino acids is defined as position 0). Crossed fields on the right site of the figure shows the interaction of indicated PDZ motifs with domains of MUPP1.

transporters NET and DAT might indeed possess specific information for PDZ interaction scheme distinct from GABA glycine and serotonin transporters. This localization can be observed only in brain tissues, which indicates that in native brain the regulation of transporters trafficking is far more complex than in frequently used cell line models.

Acknowledgments

This work was supported by the Slovak grant agency VEGA, grant 2/0086/13.

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