Different binding motifs in metabotropic glutamate receptor type 7b for filamin A, protein phosphatase 1C, protein interacting with protein kinase C (PICK) 1 and syntenin allow the formation of multimeric protein complexes

Ralf ENZ[1] and Cristina CROCI

Emil-Fischer Zentrum, Institut für Biochemie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Fahrstrasse 17, D-91054 Erlangen, Germany

Metabotropic glutamate receptor (mGluR) type 7-mediated neurotransmission depends critically on its regulation by associated molecules, such as kinases, phosphatases and structural proteins. The splice variants mGluR7a and mGluR7b are defined by different intracellular C-termini, and simultaneous or exclusive binding of interacting proteins to these domains modulates mGluR7-mediated signalling. However, molecular determinants defining binding regions for associated proteins within mGluR7 C-termini are mostly unknown. In the present study, we have mapped the binding domains of four proteins [filamin A, protein phosphatase (PP) 1C, protein interacting with protein kinase C (PICK) 1 and syntenin] interacting with the mGluR7b variant, and show that the alternatively spliced distal part of the mGluR7b C-terminus was sufficient for the interactions. By individual substitution of all mGluR7b isoform-specific amino acids with alanine and construction of a series of deletion constructs, residues important for the interactions were identified and binding regions could be defined. Interestingly, mGluR7b contains an unusual PP1C-binding motif, located at the N-terminus of the binding domains for PICK1 and syntenin. Consistently, binding of PP1C and PICK1 or PP1C and syntenin to mGluR7b was not competitive. Furthermore, PICK1, but not PP1C, interacted physically with syntenin. Our results represent a molecular description of the binding mechanisms of four mGluR7-associated proteins, and indicate the formation of ternary protein complexes composed of mGluR7b, PP1C, PICK1 and syntenin.

Key words: alanine scan, binding domain, metabotropic glutamate receptor, synaptic-signalling complex.

INTRODUCTION

In the mammalian central nervous system, glutamate mediates excitational neurotransmission via ion-channel-associated (ionotropic) and G-protein-coupled (metabotropic) receptors. Whereas ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type, kainate-type and *N*-methyl-D-aspartate-type glutamate receptors mediate fast synaptic transmission, metabotropic glutamate receptors (mGluRs) modulate neuronal excitability and development, synaptic plasticity, transmitter release and memory function using a variety of intracellular second-messenger systems [1,2]. To date, eight different members of the mGluR family have been cloned, which are subdivided into three groups, based on sequence similarity, associated second-messenger systems and pharmacological properties [3,4]. The mGluR type 7 is a member of group III mGluRs (mGluR4, mGluR6, mGluR7 and mGluR8), which are negatively coupled with adenylate cyclase, and are primarily located presynaptically in the active zone where they might function as autoreceptors [5–13]. Specific subcellular localization of mGluR7 is of particular interest. The protein is differently concentrated at the same synaptic terminal of hippocampal pyramidal cells, depending on the nature of the postsynaptic neuron [12,13]. Similarly, at bipolar cell axon termini of the retina, expression of mGluR7 is restricted to one side of the active zone of ribbon synapses [14]. Based on the specific presynaptic localization, mGluR7 could inhibit synapses firing above a certain frequency, thus functioning as a low-pass filter [12]. In agreement with this hypothesis, genetic studies revealed that mice lacking expression of mGluR7 show behavioural deficits and late-onset epilepsy [15].

Two splice variants exist for mGluR7 (mGluR7a and mGluR7b), which differ in the final amino acids of their intracellular C-termini. Recently, several proteins interacting with these C-termini have been identified [16]. For example, calmodulin binds to an identical region of both variants in a Ca^{2+}-dependent manner and competes at its binding site with the $\beta\gamma$-subunit of a heterotrimeric G-protein, directly affecting mGluR7-associated second-messenger cascades and transmitter release [17,18]. Protein kinase C (PKC) regulates the formation of the mGluR7–Ca^{2+}–calmodulin complex by phosphorylation of a conserved serine residue (S862) on the receptor [19]. Another mGluR7-binding protein is protein interacting with PKC (PICK) 1, originally identified for its interactions with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors and activated PKCα [20,21]. PICK1 binds to the C-termini of mGluR7a and mGluR7b, where it is involved in their phosphorylation by PKCα [22–24]. Furthermore, recent studies [25–28] identified glutamate receptor-interacting protein 1, syntenin, α-tubulin, filamin A and the catalytic γ-subunits of protein phosphatase (PP) 1C (PP1γ1/PP1γ2) as mGluR7-binding partners.

mGluR7-mediated neurotransmission depends critically on its regulation by associated molecules, such as enzymes, scaffold proteins and synaptic anchor proteins. As shown for the Ca^{2+}-dependent competition between calmodulin and G-proteins, simultaneous or exclusive binding of these interacting proteins

Abbreviations used: β-gal, β-galactosidase; GST, glutathione S-transferase; mGluR, metabotropic glutamate receptor; PKC, protein kinase C; PICK, protein interacting with PKC; PP, protein phosphatase; for brevity the one-letter system for amino acids has been used, e.g. V909 means Val^{909}.

[1] To whom correspondence should be addressed (e-mail ralf.enz@biochem.uni-erlangen.de).

could directly influence mGluR7-mediated neurotransmission [18,29]. In addition, mGluR7-associated proteins might interact with each other. Currently, molecular determinants of mGluR7 C-termini, defining binding regions of associated proteins, are largely unknown. In the present study, we mapped the binding domains of four proteins interacting with the mGluR7b splice variant (filamin A, PP1γ1, PICK1 and syntenin), and identified an unusual PP1γ1-binding motif. Furthermore, we analysed the binding characteristics among PP1γ1, PICK1 and syntenin, and demonstrated simultaneous interaction of PP1γ1 and PICK1 or PP1γ1 and syntenin with the final 23 amino acids of the mGluR7b C-terminus.

EXPERIMENTAL

Materials

Yeast two-hybrid bait and prey vectors were kindly provided by Dr Jeremy Nathans (The Johns Hopkins University, Baltimore, MD, U.S.A.). The yeast strain L40 was obtained from Invitrogen (Groningen, The Netherlands). Lysozyme, isopropyl β-D-thiogalactoside, *o*-nitrophenyl β-D-galactopyranoside and basic chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany). A protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany) and Coomassie Brilliant Blue R-250 was from Serva (Heidelberg, Germany). The *Escherichia coli* expression vectors pET21 and pET41, the BL21(DE3)pLysS *E. coli* strain, the BugBuster GST-Bind-Purification Kit (where GST stands for glutathione S-transferase) and a monoclonal anti-T7 antibody were from Novagen (Madison, WI, U.S.A.). The enhanced chemiluminescence (ECL®) system was obtained from Amersham International (Braunschweig, Germany).

Yeast two-hybrid techniques

The C-terminal domains of mGluR7 were PCR-amplified from rat brain cDNA and subcloned into the bait vector pBTM116. The complete coding regions of rat PP1γ1, PICK1, syntenin and repeats 21–24 of bovine filamin A (amino acids 2231–2647 [27]) were also generated by PCR and ligated into the prey vector pVP16. Mutation and deletion constructs of mGluR7b were generated by PCR cloning techniques in pBTM116. An N-terminal fragment of the shaker β1-subunit (amino acids 1–227) inserted into bait and prey vectors was used for controls. All the generated constructs were sequenced [30] using an automated DNA sequencer (AbiPrism 377; Applied Biosystems, Foster City, CA, U.S.A.). Protein–protein interactions were tested in the yeast strain L40 as described previously [26]. In brief, individual yeast strains were generated expressing the prey constructs, using the lithium acetate method for plasmid transformation in yeast [31]. Binding between proteins was analysed by transforming the prey strains with pBTM116 constructs, monitored by the activation of *His3* and β-galactosidase (β-gal) reporter genes on selection plates, supplemented with or without 3 mM 3-amino-1,2,4-triazole. Transactivation of the constructs was tested using the shaker β1-control vectors. Semi-quantitative intensities of protein–protein interactions were calculated in accordance with the 'Yeast Protocols Handbook' from Clontech (Palo Alto, CA, U.S.A.) using *o*-nitrophenyl β-D-galactopyranoside as a substrate. Values represent the means $\pm$ S.E.M. of the reporter gene activity from 3 to 6 yeast colonies.

GST pull-down assays

The C-termini of mGluR6 (amino acids 840–871), mGluR7a (amino acids 851–915), mGluR7b (amino acids 851–922) and splice-specific regions mGluR7-sp (amino acids 851–899), mGluR7a-sp (amino acids 900–915) and mGluR7b-sp (amino acids 900–922), were amplified by reverse transcriptase–PCR from retina or total RNA from brain of rat and ligated in frame to the coding sequence of GST of the pET-41 vector. The coding sequences of filamin A (repeats 21–24), PP1γ1, PICK1 and syntenin were tagged with a T7 epitope by cloning into pET-21. Plasmids were transformed in *E. coli* BL21(DE3)pLysS and the protein expression was induced by adding 1 mM isopropyl β-D-thiogalactoside. Fusion proteins were purified under native conditions from frozen bacteria pellets by incubating for 30 min in ice-cold lysis buffer (50 mM NaH_2PO_4/300 mM NaCl; pH 8.0), containing 25 units/ml benzonase, 1 mg/ml lysozyme and a cocktail of protease inhibitors, and subsequent sonication (6 bursts for 10 s at 300 W). In an alternative experiment, the Bug-Buster GST-Bind-Purification Kit from Novagen was also used. GST-fusion proteins were immobilized under native conditions to glutathione–Sepharose beads. Protein concentrations of the beads were estimated from Coomassie Blue-stained SDS/PAGE. Approximately 5–10 μl of these beads was incubated with the cytosolic fractions of *E. coli* expressing filamin A, PP1γ1, PICK1 or syntenin (corresponding to approx. 0.1 g wet weight of the bacteria pellet) for 1 h at 4 °C under slow agitation, followed by three washes with 0.5 ml of GST-washing buffer (4.3 mM Na_2HPO_4/1.47 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl; pH 7.3). To obtain comparable conditions in competition experiments, *E. coli* protein extracts of similar protein concentrations, as measured at 280 nm, were used. For all samples, the total volume was adjusted to 600 μl (defined as 100 %) by adding protein extract of non-transformed *E. coli* BL21(DE3)pLysS. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE and visualized with Coomassie Brilliant Blue R-250 or detected by Western-blotting analysis using a monoclonal anti-T7 antibody and the ECL®.

RESULTS

Filamin A, PP1γ1, PICK1 and syntenin bind to the isoform-specific domain of mGluR7b

To analyse in detail the binding of four proteins shown previously to interact with mGluR7b, cDNA sequences coding for complete or splice-specific protein domains of both mGluR7 isoforms (Figure 1A) were cloned into the yeast two-hybrid bait vector and tested individually against filamin A (repeats 21–24), PP1γ1, PICK1 and syntenin. Interactions were monitored by the ability of transformed yeast cells to grow on selective media on activation of the *His3* and β-gal reporter genes. Consistent with previous reports [25–27], filamin A, PICK1 and syntenin bound to both splice variants, whereas PP1γ1 interacted selectively with the mGluR7b isoform (Figure 1B). Dissecting the mGluR7 C-termini in the proximal (identical) and the distal (splice-specific) regions demonstrated the splice-specific domains to contain sufficient information for the protein interactions. Only filamin A failed to interact with the distal domain of mGluR7a, but instead showed weak binding to the proximal part of the C-terminus, indicating that binding motifs might be present in both the proximal and the distal C-terminal regions of the mGluR7 isoforms [27].

The relative intensity of the protein–protein interactions was estimated using a semiquantitative β-gal assay and is expressed in arbitrary β-gal units (black horizontal columns in Figure 1B). Except for the interaction between filamin A and mGluR7a, the complete mGluR7 C-termini as well as their splice-specific distal

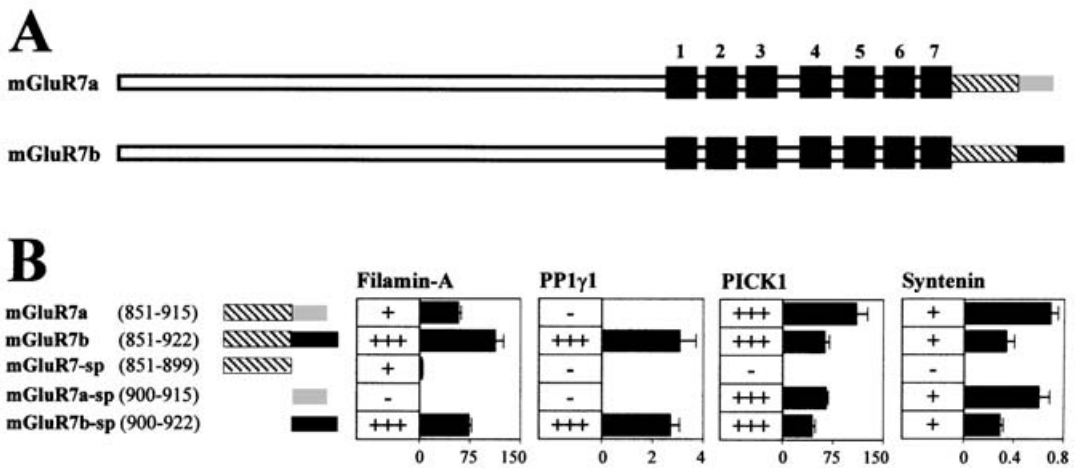

Figure 1 Binding characteristics of mGluR7b-associated proteins

(**A**) Schematic representation of the two mGluR splice variants. Numbered black squares represent transmembrane regions 1–7, hatched boxes indicate identical parts of the C-terminus, and grey and black boxes represent splice-specific regions. (**B**) Complete mGluR7 C-termini (mGluR7a, mGluR7b), their proximal (mGluR7-sp) or distal regions (mGluR7a-sp, mGluR7b-sp) were individually tested in binary two-hybrid experiments for their ability to interact with filamin A, PP1γ1, PICK1 and syntenin. The interaction was monitored by the ability of transformants to grow on selective media with or without the addition of 3 mM 3-amino-1,2,4-triazole, indicated by (+) and (+ + +) respectively, on activation of the *His3* and β-gal reporter genes; (−) indicates no growth of yeast colonies. C-termini are drawn to scale and the corresponding amino acid numbers are given in parentheses. The relative strength of interactions was quantified and is visualized as arbitrary β-gal units (horizontal black columns). Each value represents the mean ± S.E.M. from 3–6 yeast clones. Note the different scales used on the *x*-axis.

domains revealed similar binding affinities for each of the tested proteins, indicating that interacting domains are located within the splice-specific distal parts of the mGluR7a and mGluR7b C-termini. The binding affinity of syntenin was approx. 1–2 orders of magnitude lower compared with those of the other mGluR7-binding partners. Indeed, syntenin only showed binding to mGluR7 C-termini when a competitive inhibitor of the *His3* gene (3-amino-1,2,4-triazole), used to increase the stringency of the binding assay, was omitted from the selection plates. The numerical values of the binding affinities are summarized in the upper part of Table 1. Although calmodulin, α-tubulin and glutamate receptor-interacting protein 1 were reported to bind mGluR7 C-termini, these proteins were not included in the present study, since their binding sites have already been located to the identical proximal part of the mGluR7 C-termini, or no binding was observed for mGluR7b [25,28,29].

To confirm the binding characteristics of proteins interacting with mGluR7b, GST pull-down assays using immobilized C-terminal domains fused with GST were performed for filamin A, PP1γ1, PICK1 and syntenin. Loaded glutathione–Sepharose beads were incubated with *E. coli* protein extracts and bound proteins were analysed on Western blots. The binding pattern of the analysed interactions was identical with that obtained from the yeast two-hybrid experiments (Figure 2), with some variations in the relative binding affinities. Most importantly, the splice-specific distal part of mGluR7b was sufficient to interact with all tested binding partners. Since interactions of these proteins with the C-terminus of mGluR6 have not been reported in the literature, we used this domain as a negative control for the assay and, consistently, no binding was detected.

Alanine scan of the splice-specific C-terminal domain of mGluR7b

To identify individual residues involved in the protein–protein interactions, we mapped the binding sites for filamin A, PP1γ1, PICK1 and syntenin within the distal splice-specific region of the mGluR7b C-terminus. To assess the contribution of each

Table 1 Relative strength of interactions quantified as arbitrary β-gal units

Each value represents the means ± S.E.M. from 3 to 6 yeast clones.

	Interaction (units)			
mGluR7	Filamin A	PP1γ1	PICK1	Syntenin
mGluR7a	58.74 ± 3.06	–	110.54 ± 15.76	0.70 ± 0.05
mGluR7b	113.39 ± 11.38	3.10 ± 0.60	63.71 ± 5.91	0.34 ± 0.06
mGluR7-sp	5.26 ± 0.11	–	–	–
mGluR7a-sp	–	–	65.82 ± 1.87	0.61 ± 0.08
mGluR7b-sp	74.58 ± 2.86	2.73 ± 0.33	45.39 ± 3.46	0.29 ± 0.02
N900A	62.04 ± 9.31	2.76 ± 0.47	53.57 ± 5.38	0.14 ± 0.02
C901A	78.81 ± 6.69	2.53 ± 0.11	48.29 ± 2.22	0.15 ± 0.01
I902A	52.64 ± 2.62	2.91 ± 0.13	37.31 ± 2.57	0.14 ± 0.02
P903A	37.19 ± 1.87	2.10 ± 0.10	46.98 ± 8.58	0.14 ± 0.02
P904A	38.67 ± 6.00	2.48 ± 0.09	48.19 ± 7.48	0.18 ± 0.03
V905A	80.74 ± 23.22	1.68 ± 0.07	37.05 ± 4.73	0.10 ± 0.03
R906A	13.62 ± 0.46	1.62 ± 0.33	37.04 ± 7.84	0.15 ± 0.00
K907A	47.58 ± 5.16	1.65 ± 0.20	43.55 ± 10.84	0.14 ± 0.04
S908A	59.63 ± 5.46	1.31 ± 0.28	39.73 ± 4.32	0.19 ± 0.04
V909A	–	2.11 ± 0.38	50.67 ± 11.11	0.24 ± 0.08
Q910A	35.81 ± 4.06	1.93 ± 0.15	34.58 ± 7.52	0.20 ± 0.03
K911A	81.33 ± 8.36	–	47.74 ± 2.77	0.28 ± 0.06
S912A	26.48 ± 2.93	1.73 ± 0.49	34.16 ± 2.49	0.17 ± 0.02
V913A	–	–	37.64 ± 5.42	0.24 ± 0.03
T914A	90.46 ± 5.22	–	54.41 ± 4.52	0.23 ± 0.03
W915A	–	–	–	–
Y916A	–	6.58 ± 0.11	–	–
T917A	51.54 ± 0.97	3.79 ± 0.70	28.89 ± 1.28	0.20 ± 0.04
I918A	–	3.66 ± 0.27	31.55 ± 3.12	0.15 ± 0.05
P919A	88.96 ± 6.51	2.34 ± 0.30	75.92 ± 10.12	0.31 ± 0.04
P920A	21.55 ± 3.29	1.97 ± 0.19	68.05 ± 9.49	0.18 ± 0.02
T921A	89.28 ± 1.00	2.45 ± 0.14	71.46 ± 3.50	0.12 ± 0.00
V922A	82.22 ± 1.47	2.34 ± 0.17	–	–

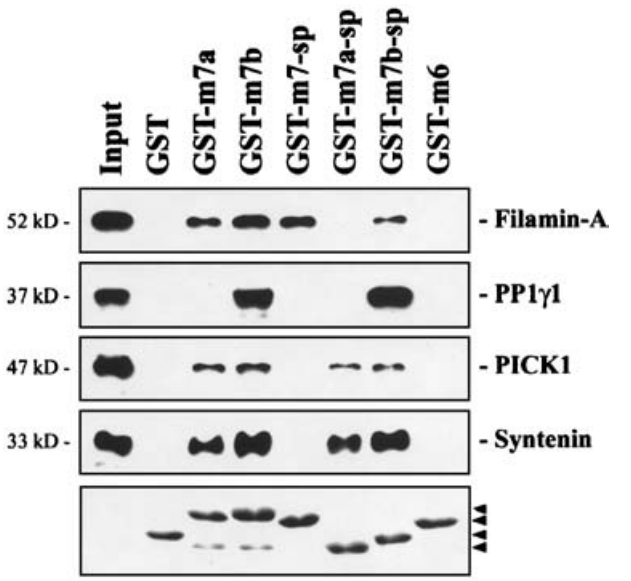

Figure 2 The mGluR7b splice-specific domain interacts with filamin A, PP1γ1, PICK1 and syntenin

GST and GST-fusion proteins were immobilized on glutathione–Sepharose beads and incubated with T7-tagged filamin A, PP1γ1, PICK1 or syntenin purified from *E. coli* as indicated. Bound proteins were detected on Western blots (upper panels) using a monoclonal anti-T7 immunoserum. The calculated molecular masses of proteins are indicated on the left-hand side in kDa. Bottom panel: equal protein concentration of the coated Sepharose beads is shown on Coomassie Blue-stained SDS/PAGE (arrowheads).

individual amino acid of the mGluR7b C-terminus, one residue at a time was replaced by alanine, assuming that the amino acid side chains rather than the peptide backbone form the specific binding surfaces for the interaction partners.

The filamin-binding domain on mGluR7b extended over ten residues from V909 to I918 (Figure 3A). Interestingly, not all amino acids within this domain were involved in the formation of the binding surface for filamin A: only five residues seemed to be critical for the interaction. In contrast, PP1γ1 required a stretch of five amino acids within mGluR7b, which was organized in two blocks separated by S912. Finally, three amino acids mediated the interaction of mGluR7b with PICK1 and syntenin, namely W915, Y916 and the C-terminal amino acid of mGluR7b, namely V922.

To estimate the relative binding affinities of the generated mutants for filamin A, PP1γ1, PICK1 and syntenin, a semi-quantitative β-gal assay was performed (black horizontal columns in Figure 3A). Most of the interacting alanine mutants revealed affinities similar to the wild-type mGluR7b C-terminus, indicating that the corresponding amino acids did not contribute substantially to the binding surface for the interaction partners. Mutations at some positions decreased or increased the protein–protein interactions by approx. 2–4-fold (e.g. filamin A, V905 and R906; PP1γ1, Y916), pointing to the possibility that these residues, although not needed for the interaction in the first place, might influence the binding strength. The numerical values of all the binding affinities calculated are given in relative β-gal units in Table 1.

To confirm the major determinants of the binding domains analysed in yeast cells, GST pull-down assays were performed for filamin A, PP1γ1, PICK1 and syntenin. Glutathione–Sepharose beads were coated with mGluR7b constructs and incubated with *E. coli* protein extracts containing the four binding partners, as indicated (Figure 3B). In agreement with the results of the yeast cell assay, no binding could be observed on deletion of the first or last amino acid of every binding region. Furthermore, construct V922A was capable of binding filamin A and PP1γ1, but not PICK1 or syntenin.

Identification of mGluR7b binding domains for filamin A, PP1γ1, PICK1 and syntenin

Having identified amino acids within the mGluR7b C-terminus that mediate the interactions with filamin A, PP1γ1, PICK1 and syntenin, the present study was extended by testing a series of deletion mutants. The alanine scan revealed that all amino acids forming interacting surfaces are located distal to V909. To ensure that no binding domains are formed proximal to V909, we introduced a stop codon at position 910 in the mGluR7b C-terminus. As expected, this construct was not capable of interacting with any of the mGluR7b-binding partners tested (Figure 4A). Furthermore, construct W915Stop, which contained five more amino acids, did not reveal detectable interactions with filamin A, PP1γ1, PICK1 or syntenin, indicating that all binding motifs include amino acids between W915 and V922. As shown in Figure 3, all four mGluR7b-binding partners failed to interact when W915 was mutated into alanine. Thus we deleted this amino acid (Δ915W) and could not observe any binding, which was consistent with our results from the alanine scan, and indicated that W915 is present in all four binding motifs (Figure 4A). Finally, deleting two characteristic proline residues of mGluR7b (Δ919P/920P), not present in mGluR7a, had no effect on the binding characteristic of mGluR7b, again in agreement with the results obtained from the alanine scan.

Amino acids of the mGluR7b C-terminus mediating binding to filamin A, PP1γ1, PICK1 and syntenin were always separated by non-participating residues (Figure 3). To determine whether these amino acids are needed to space the interacting residues correctly, residues located within the identified binding domains but not directly involved in the interactions were deleted (Figure 4A). Filamin A was still capable of interacting with the mGluR7b C-terminus after deletion of T914 and T917 (Δ914T/917T), and also after the deletion of all non-interacting amino acids present within its binding domain (ΔQKS-T-T). In contrast, on deletion of S912 (Δ912S), binding to PP1γ1 was completely prevented, indicating that S912, which is not required to interact directly with PP1γ1 in the first place, might be involved in forming a correct three-dimensional structure of the PP1γ1-binding domain. Finally, deleting all amino acids separating Y916 and V922 (ΔTIPPT) had no effect on the binding of mGluR7b to PICK1 and syntenin.

As the last step, we tested whether amino acids flanking the identified binding domains did participate in the interactions. Deletion of two proline residues (Δ919P/920P) directly C-terminal to the filamin A-binding domain did not influence this interaction (Figure 4A). Similarly, deletion of amino acids at the N- and C-terminal sides of the PP1γ1-binding motif (ΔSVQ, ΔYTIPPT) did not disturb the affinity of mGluR7b for PP1γ1 and, finally, deletion of T914 and T917 (Δ914T/917T) did not alter the binding between mGluR7b and PICK1 or syntenin. The relative strength of all protein–protein interactions shown in Figure 4(A) was quantified as described above and resulted in values similar to those in Table 1 (results not shown).

Results of the binding assays in yeast cells were again confirmed by performing GST pull-down experiments. As in the two-hybrid experiments, none of the proteins was capable of interacting with the construct W915Stop (Figure 4B). Furthermore, deleting those amino acids located within the proposed binding domains that were not directly involved in the interactions resulted in the same binding pattern as observed in yeast cells.

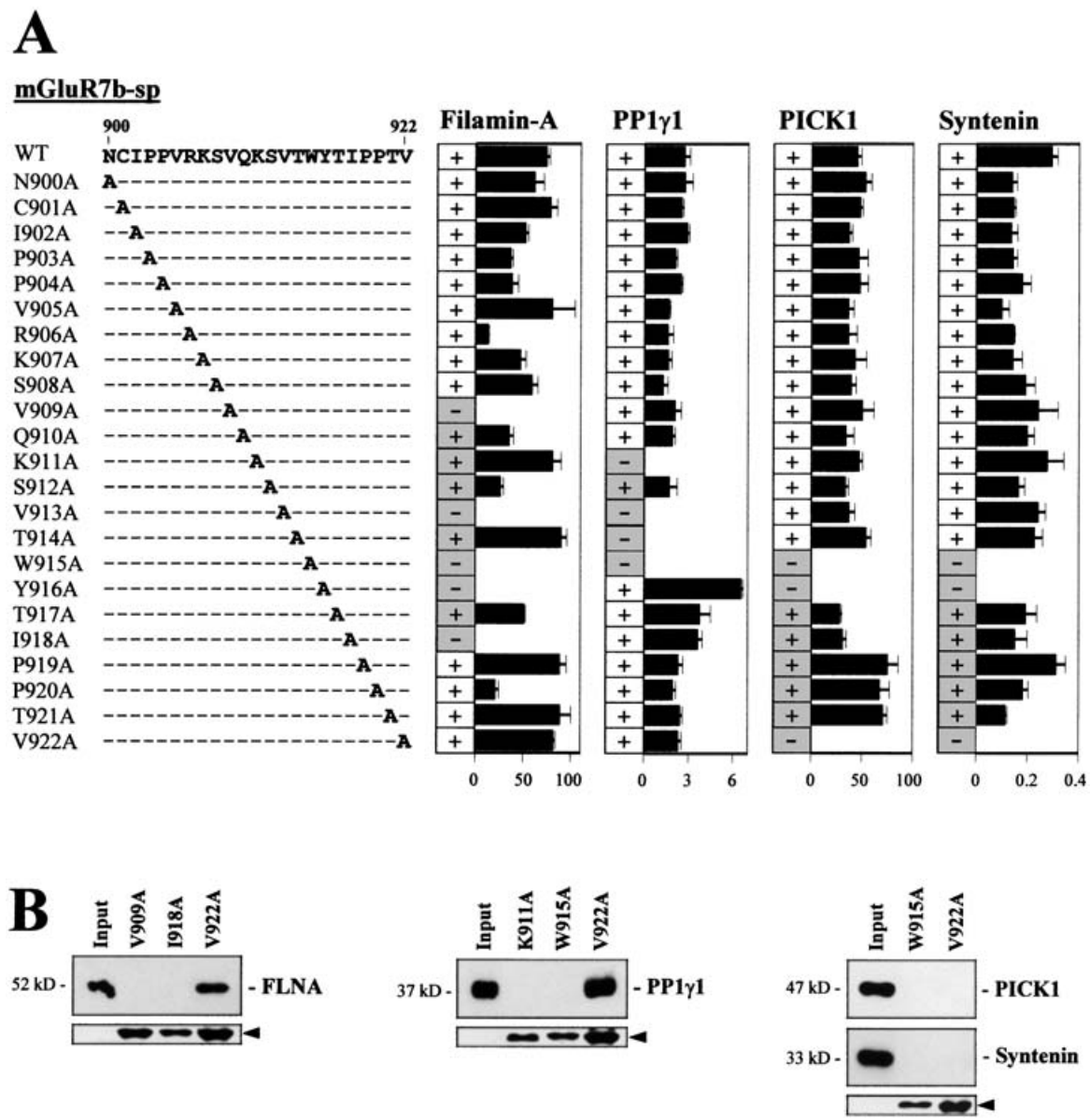

Figure 3 Mapping of filamin A-, PP1γ1-, PICK1- and syntenin-binding domains within the splice-specific part of the mGluR7b C-terminus

(**A**) All amino acids of the splice-specific distal part of the mGluR7b C-terminus were independently substituted with alanine and binding of the resulting mutants for filamin A, PP1γ1, PICK1 and syntenin was analysed in binary yeast two-hybrid experiments. Protein–protein interaction was monitored and the affinities were calculated as described in Figure 1. Regions important for the interactions are highlighted in grey. WT, wild-type. (**B**) GST-fusion proteins of mutation constructs were immobilized on glutathione–Sepharose beads and incubated with T7-tagged filamin A, PP1γ1, PICK1 or syntenin purified from *E. coli* as indicated. Bound proteins were detected as described in Figure 2. Protein concentrations of coated Sepharose beads are indicated on Coomassie Blue-stained SDS/PAGE (arrowheads). Calculated protein molecular masses are indicated on the left-hand side in kDa.

Based on the results presented above, we could now define amino acids important for the interactions between mGluR7b and filamin A, PP1γ1, PICK1 and syntenin (Figure 5). Except for the PP1γ1-binding domain, important amino acids are located at different positions within the mGluR7b C-terminus, separated by residues not needed for the protein–protein interactions in the first place.

Formation of ternary protein complexes between mGluR7b, PP1γ1, PICK1 and syntenin

W915 is the only amino acid common to all the four identified binding motifs (Figure 5), and its mutation to alanine or deletion disrupted all protein–protein interactions (Figures 3 and 4). To elucidate further the role of W915 in the protein–protein interactions, we focused on the binding domains for PP1γ1 and PICK1/syntenin, since they overlap only at W915 (Figure 5). If W915 interacted directly with PP1γ1, PICK1 or syntenin, binding of PP1γ1 and PICK1 or PP1γ1 and syntenin should be competitive. On the other hand, W915 might not directly contact the mGluR7b-binding partners, but rather serve as a structural amino acid shaping the three-dimensional binding surfaces in its neighbourhood. Then, the binding of PP1γ1 and PICK1 or PP1γ1 and syntenin might occur simultaneously. To study these possibilities, competition experiments for the binding of PP1γ1, PICK1 and syntenin to the splice-specific distal part

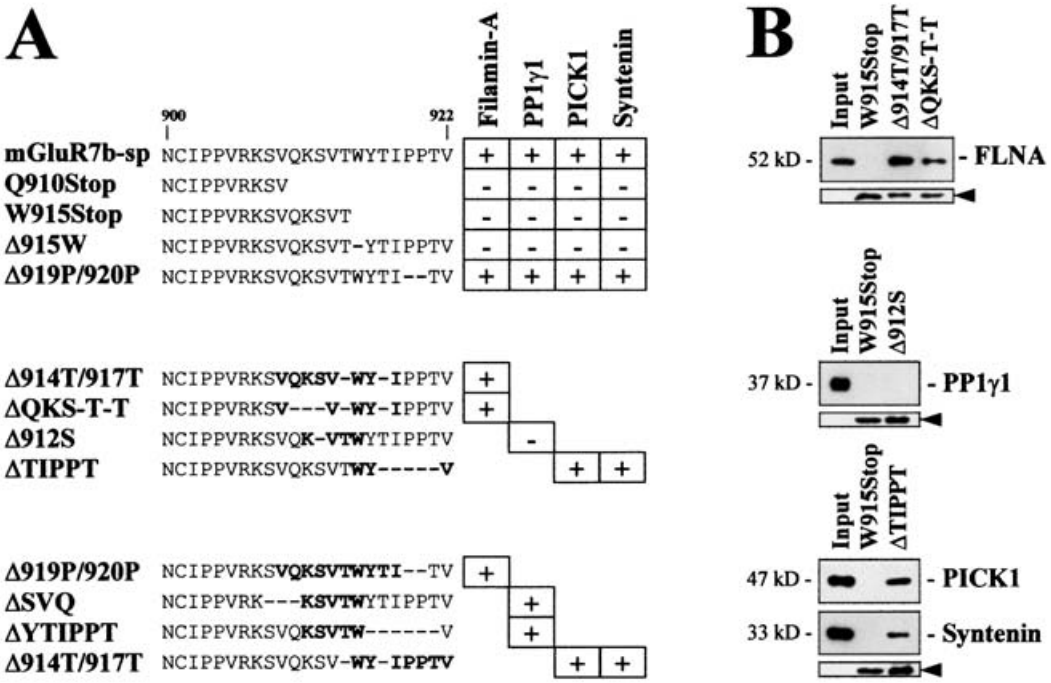

Figure 4 Identification of amino acids mediating the binding between mGluR7b and filamin A, PP1γ1, PICK1 and syntenin

(**A**) Deletion constructs of the splice-specific part of the mGluR7b C-terminus were analysed for binding to filamin A, PP1γ1, PICK1 and syntenin in yeast cells. Interactions were analysed as described in Figure 1. Amino acids located in regions needed for the interaction, as identified from the alanine scan, are marked with boldface characters. (**B**) GST-fusion proteins of deletion constructs were immobilized on glutathione–Sepharose beads and incubated with T7-tagged filamin A (FNA), PP1γ1, PICK1 or syntenin purified from *E. coli* as indicated. Bound proteins were detected as described in Figure 2. Protein concentrations of coated Sepharose beads are indicated on Coomassie Blue-stained SDS/PAGE (arrowheads). Calculated protein molecular masses are indicated on the left-hand side in kDa.

of the mGluR7b C-terminus were performed. For comparison, *E. coli* extracts with similar protein concentrations were used in all experiments and a total volume of 600 μl (defined as 100 % in Figure 6) was adjusted with protein extracts of non-transformed *E. coli*.

In a first step, the binding capacity of the GST–mGluR7b-sp-coated glutathione–Sepharose beads was tested by incubating a constant volume of beads with increasing amounts of protein extracts obtained from *E. coli* expressing PP1γ1 or PICK1. Under the conditions used, 50–100 % of the total volume (600 μl) was needed to occupy all binding places with PP1γ1 or PICK1 (Figure 6A). Detection of a non-bound protein in the flow-through

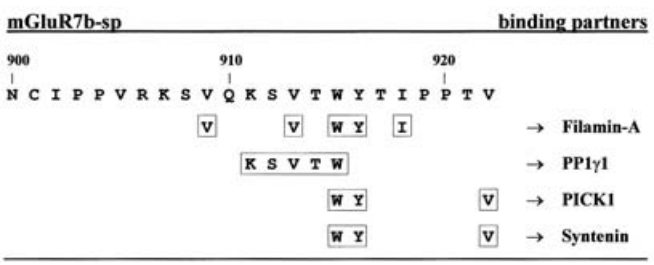

Figure 5 Summary of identified binding sites for mGluR7b-associated proteins

Graphical overview of the identified binding regions in mGluR7b interacting with filamin A, PP1γ1, PICK1 and syntenin. Amino acids that were found in the present study to be crucial for the interactions are boxed.

of the 100 % samples indicated that the GST–mGluR7b-sp-coated beads were indeed saturated with PP1γ1 or PICK1. Thus, for competition between PP1γ1 and PICK1, GST–mGluR7b-sp-loaded beads were first incubated with 100 % PP1γ1 extract, which completely occupied all mGluR7-binding sites (see Figure 6A), and subsequently mixed with increasing quantities of PICK1 protein extract. High concentrations of PICK1 were not capable of reducing the binding of PP1γ1 to mGluR7b-sp significantly (Figure 6B, upper panel), indicating that the two proteins might use different binding sites within the mGluR7b C-terminus. A similar experiment was performed for PP1γ1 and syntenin (Figure 6B, middle panel), showing again simultaneous binding of the two proteins to mGluR7b. Importantly, PP1γ1 did not bind directly to PICK1 or syntenin, as determined by GST pull-down experiments (Figure 6C). Thus although the binding sites of PP1γ1 and PICK1/syntenin overlap at position W915, their binding was not exclusive, indicating that W915 is not physically contacted at the same time by the proteins. These results point to the formation of trimeric protein complexes, composed of mGluR7b and PP1γ1 together with PICK1 or syntenin.

PICK1 and syntenin, but not PP1γ1, have been reported to dimerize [20,32] and, indeed, we found self-assembly of syntenin but not of PP1γ1 in GST pull-down experiments (Figure 6C). Furthermore, we could demonstrate direct binding between PICK1 and syntenin (Figure 6C), possibly via the C-terminus of syntenin forming a type II PDZ-binding motif [16] that interacts with the PDZ domain of PICK1. In the presence of mGluR7b, PICK1 and syntenin still interacted with each other, as indicated by the formation of a trimeric protein complex with the receptor (Figure 6B, lower panel).

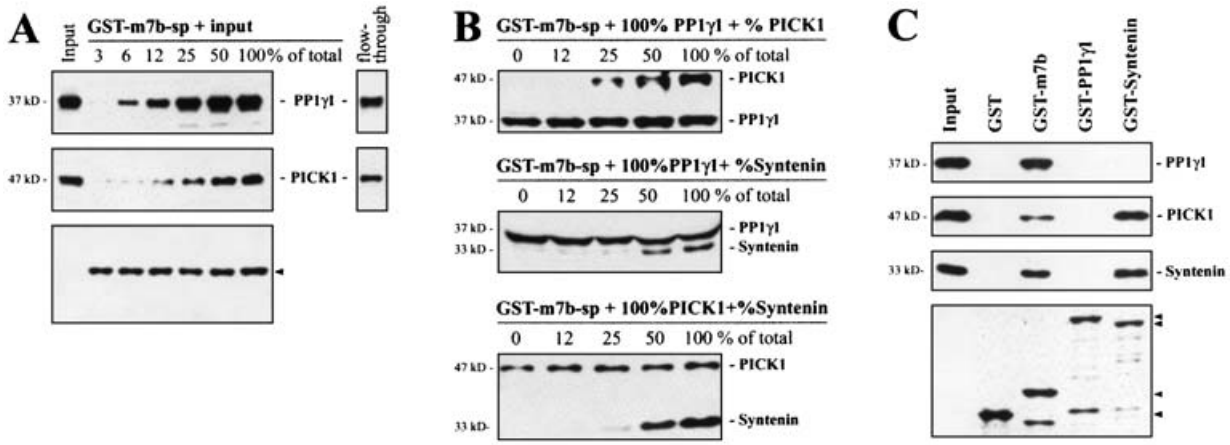

Figure 6 Simultaneous binding of PP1γ1, PICK1 and syntenin to mGluR7b

(**A**) Equal amounts of GST–mGluR7b-sp fusion proteins immobilized on glutathione–Sepharose beads, as indicated by the arrowhead on the right-hand side of the Coomassie Blue-stained gel in the lower panel, were incubated with increasing volumes of recombinant PP1γ1- or PICK1-containing protein extracts. Bound proteins were detected on Western blots using a monoclonal anti-T7 immunoserum (flow-through = non-bound proteins of the 100 % samples). (**B**) Constant amounts of GST–mGluR7b-sp-coated glutathione–Sepharose beads completely saturated with PP1γ1 or PICK1 (defined as 100 %) were incubated with increasing volumes of PICK1- or syntenin-containing protein extracts as indicated. The volumes were adjusted to 600 μl (defined as 100 % of the total volume) by adding protein extracts of non-transformed *E. coli*. Bound proteins were detected as in (**A**). (**C**) GST and GST-fusion proteins immobilized on glutathione–Sepharose beads were incubated with T7-tagged PP1γ1, PICK1 or syntenin as indicated and bound proteins were detected as in (**A**). Coomassie Blue-stained SDS/PAGE demonstrates equal protein concentrations in the coated Sepharose beads (arrowheads). Calculated molecular masses of the proteins are indicated on the left-hand side in kDa.

DISCUSSION

Macromolecular signalling complexes containing ion channels, neurotransmitter receptors, kinases, phosphatases and structural proteins represent important factors in controlling neuronal excitability. Therefore, mGluR7-mediated neurotransmission depends critically on its interaction with associated molecules [18,29]. To identify molecular determinants defining binding regions in mGluR7 C-termini, we mapped the binding domains of four proteins (filamin A, PP1γ1, PICK1 and syntenin) interacting with the mGluR7b variant. The splice-specific distal part of mGluR7b contained sufficient information for such interactions, and amino acids forming the binding domains were identified by an alanine scan and a series of deletion constructs. Finally, simultaneous association of PP1γ1 and PICK1 or PP1γ1 and syntenin with mGluR7b, as well as direct binding between PICK1 and syntenin, indicated the formation of trimeric protein complexes.

Significantly, amino acid W915 was common to all the four identified binding motifs (Figure 5). Furthermore, the binding sites of PP1γ1 and PICK1/syntenin were located in different regions of the mGluR7b C-terminus, overlapping at residue W915. We showed in competition experiments between PP1γ1 and PICK1/syntenin that PP1γ1 and PICK1 as well as PP1γ1 and syntenin did bind simultaneously to mGluR7b, forming trimeric protein complexes (Figure 6B). Importantly, neither PICK1 nor syntenin interacted directly with PP1γ1. Simultaneous binding of PP1γ1 and PICK1/syntenin to the mGluR7b C-terminus would require W915 to be contacted on opposite sites of the aromatic side chain, which sterically seems to be rather unlikely. Alternatively, in a probably more convincing model, W915 would not be contacted by any of the mGluR7b-interacting proteins, but instead could form a hydrophobic interaction with a distinct region of the mGluR7b C-terminus, thereby defining its three-dimensional structure. In this way, W915 would determine the correct orientation of binding surfaces for interacting proteins proximal and distal to its position. This model would be consistent with our finding that mutation or deletion of W915 disrupts the interaction between mGluR7b and filamin A, PP1γ1, PICK1 and syntenin, regardless of the location of their binding sites (Figures 3 and 4).

Five amino acids of mGluR7b were identified to be necessary for interaction with filamin A, including Y916 (Figure 5), according to previous results showing that the aromatic system but not the hydroxy group of Y916 was important for binding mGluR7b [27]. Interestingly, the five residues forming the filamin A-binding domain are not adjacent to each other, but are grouped in four blocks. Thus the complete binding domain includes ten amino acids within the mGluR7b C-terminus. Surprisingly, amino acids within this binding domain, which were not directly involved in the interaction with filamin A, could be deleted without interfering with the mGluR7b–filamin A binding. Thus these residues were neither contacting filamin A directly nor needed to place interacting amino acids in a correct position.

Secondary-structure prediction of the mGluR7b C-terminus using the Chou–Fasman and Robson–Garnier algorithms pointed to a mixed conformation, containing random coils between residues N900 and K911, a β-sheet for S912 and T917 and again random coils between I918 and V922. Thus a possible binding mechanism that would describe the interaction between mGluR7b and filamin A could be described by a binding surface formed by all the five identified amino acids arranged adjacent to each other, with intermediate residues looping back from the binding surface. This model is consistent with our findings that deletion of the intermediate amino acids did not affect the interaction between mGluR7b and filamin A (Figure 4). However, as mentioned before, W915 might not contribute to the filamin A-binding surface of mGluR7b, but instead position proximally and distally located interacting residues in the correct orientation.

Deletion of S912 in the PP1γ1-binding domain of mGluR7b, the only amino acid not directly needed for this protein–protein interaction, completely abolished the association of the two binding partners (Figure 4A). Therefore the PP1γ1-binding site is represented by one block of five amino acids within the mGluR7b C-terminus. Deletion of residues at the N- or C-terminal end of this domain (ΔSVQ or ΔYTIPPT) did not influence the binding,

which defined the PP1γ1-binding domain within the mGluR7b C-terminus as the motif 'KSVTW' (amino acids 911–915), with the amino acid at position 912 being variable, tolerating at least an alanine residue (Figure 3A). The identified binding motif is similar but not identical to the proposed PP1γ-binding motif '(R/K)-(V/I)-X-F' (X, any amino acid), found in many, but not all, proteins interacting with PP1γ [33].

Significantly, a putative targeting subunit for PP1 (p99) also binds to PP1γ1 via the 'KSVTW' motif [34]. When we replaced the tryptophan in the last position of the motif by alanine, the ability of p99 to bind PP1γ1 was abolished, in agreement with our findings on mutation or deletion of W915 in mGluR7b (Figures 3A and 4). In contrast, changing the same tryptophan into phenylalanine did not influence the interaction with PP1γ1, indicating that an aromatic system is needed in the last position of the 'KSVTW' motif. Indeed, phenylalanine is present as the last amino acid in the original defined PP1γ-binding motif '(R/K)-(V/I)-X-F' [33]. When valine or phenylalanine of this motif was substituted by alanine, interaction with PP1γ1 was abolished, similar to our results, where mutation of V913 or W915 into alanine prevented mGluR7b from binding PP1γ1 (Figure 3A). Furthermore, a secondary structure prediction for the mGluR7b C-terminus resulted in a β-sheet conformation for the sequence 'SVTW' (amino acids 912–915). Interestingly, co-crystallization of PP1γ1 with a 13-residue peptide derived from the PP1γ1 regulatory G_M-subunit including the sequence 'RRVSFA' (amino acids 64–69 of G_M), which matches the original PP1γ1 consensus motif '(R/K)-(V/I)-X-F', demonstrated the formation of a β-sheet between S67 and A69 [33]. Furthermore, we could show that this β-sheet contacts a β-sheet of PP1γ1 on binding. Finally, preliminary results from our laboratory indicate that the mGluR type 5 binds to PP1γ1, and consistently, the motif 'KSVTW' (amino acids 880–884) is present at its C-terminus (results not shown).

The binding domains for PICK1 and syntenin were found to be identical, consisting of three amino acids grouped in two blocks (Figure 5). Importantly, the final amino acid of the mGluR7b C-terminus (V922) was essential for the interactions. V922 is part of the C-terminal sequence 'PTV', which is a consensus sequence for type II PDZ-binding motifs (Φ-X-Φ, where Φ stands for a hydrophobic residue and X for any amino acid) [16], and both PICK1 and syntenin carry PDZ domains that recognize type II PDZ-binding motifs [25]. Similar to mGluR7b, the interaction between the mGluR7a variant and PICK1 is also mediated by the C-terminal amino acid of mGluR7a (I915) [23–25,35]. The last 15 amino acids (P901–I915), but not the last seven residues (S909–I915), of mGluR7a were sufficient to bind PICK1 [23,25], indicating that amino acids proximal to S909 were needed for the interaction. These results point to the existence of at least two binding pockets in type II PDZ domains, one contacting the very C-terminal amino acids of the ligand, and a second region binding to residues between the -6 and -14 positions. Indeed, co-crystallization of the type II PDZ domain of the membrane-associated guanylate kinase CASK with its ligand demonstrated the existence of a second hydrophobic binding pocket [36]. Consistent with this finding, we identified two aromatic amino acids located at the -6 and -7 positions (W915 and Y916) of mGluR7b as important determinants for the interaction of mGluR7b with PICK1, whereas residues located between Y916 and V922 were not needed for the binding (Figure 4). In this deletion construct (ΔTIPPT), a type II PDZ domain consensus motif was restored by the C-terminal amino acid sequence 'WYV', which might explain the observed interaction of this construct with PICK1 and syntenin.

Based on these findings, we assume the following binding mechanism between mGluR7b and PICK1/syntenin. The three amino acids of the mGluR7b C-terminus would fit into one of the two proposed binding pockets in the type II PDZ domains of PICK1 and syntenin. The second binding region might form a hydrophobic contact (see [36]) with the aromatic system of Y916 located at the -6 position, which in turn is placed in its correct orientation by W915. As pointed out before, W915 quite probably does not contact mGluR7b-binding proteins, but rather ensures the correct orientation of proximally and distally located interacting amino acids. Since deletion of residues T917–T921 in the mGluR7b C-terminus did not prevent the protein–protein interactions, both binding regions should be located adjacent to each other. Therefore in the wild-type situation, amino acids of mGluR7b contacting these two binding pockets are quite probably located close to each other, with the five intermediate residues (T917–T921) looping back from the binding surface. In summary, ligands of type II PDZ domains seem to have a similar architecture for their binding motif, consisting of the extreme C-terminal amino acids and hydrophobic residues located around the -6 position.

Binding affinities for the mGluR7b-syntenin interaction differed substantially between the yeast assays and GST pull-down experiments. Although in yeast cells the binding strength of syntenin was approx. 1–2 orders of magnitude lower compared with those of the other mGluR7-binding partners (Figure 1B), GST pull-down experiments showed relatively higher protein concentrations interacting with mGluR7b (Figure 2). This discrepancy would have been caused quite probably by the different fusion partners of syntenin used in the experiments. For expression in yeast cells, syntenin was fused at the C-terminus of the activation domain of the GAL4 transcription factor, which consists of 113 amino acids. In contrast, for GST pull-down assays, syntenin was expressed in *E. coli* with a N-terminal T7 tag of 11 residues. Quite probably, the relatively large GAL4 activation domain interfered with the correct folding of syntenin or sterically hindered the binding between syntenin and mGluR7b in yeast cells. However, different protein modification mechanisms present in yeast and *E. coli* might have caused the different binding affinities between mGluR7b and syntenin in the two assay systems.

We identified a direct interaction between PICK1 and syntenin, both proteins containing PDZ domains. The C-terminus of syntenin ends with the amino acid sequence PEV, thereby forming a type II PDZ-binding motif (Φ-X-Φ [16]). Since the PDZ domain of PICK1 has been shown to interact with type II PDZ-binding motifs [23], the observed interaction between PICK1 and syntenin quite probably occurred via the C-terminus of syntenin fitting into the PDZ domain of PICK1. In the presence of mGluR7b, PICK1 and syntenin still interacted with each other, as indicated by the formation of a trimeric protein complex (Figure 6B).

In summary, the present study identified molecular determinants governing the interaction of mGluR7b with four of its binding partners. We cannot rule out the possibility that substitution with alanine could be tolerated at positions involved in the protein–protein interactions. Furthermore, the protein backbone might be a part of the binding surface, and this would not have been detected under our experimental conditions. Thus the amino acids identified in the present study represent a minimal set of molecular determinants needed for binding. Since mGluR7b-mediated signal transduction depends largely on its association with regulatory proteins, the identification of binding motifs presents a molecular basis for the understanding of mGluR7b activity. Particularly, the simultaneous but not exclusive binding of PP1γ1, PICK1 and syntenin could have direct functional

consequences at mGluR7b-expressing synapses in the central nervous system.

We thank Jeremy Nathans (The Johns Hopkins University, Baltimore, MD, U.S.A.) for providing yeast two-hybrid plasmids, Erika Jung-Körner, Ines Walter and Nadja Schröder for excellent technical assistance, Adaling Ogilvie, Cord-Michael Becker and Heinrich Sticht for helpful discussions, and Hans-Georg Breitinger and Stefan Stamm for a critical reading of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (EN 349 and SFB 539).

REFERENCES

1 Hollmann, M. and Heinemann, S. (1994) Cloned glutamate receptors. Annu. Rev. Neurosci. **17**, 31–108
2 Nakanishi, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S. and Okada, M. (1998) Glutamate receptors: brain function and signal transduction. Brain Res. Brain Res. Rev. **26**, 230–235
3 Pin, J. P. and Duvoisin, R. (1995) Neurotransmitter receptors. I. The metabotropic glutamate receptors: structure and functions. Neuropharmacology **34**, 1–26
4 Conn, P. J. and Pin, J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol. Toxicol. **37**, 205–237
5 Nakajima, Y., Iwakabe, H., Akazawa, C., Nawa, H., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1993) Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. J. Biol. Chem. **268**, 11868–11873
6 Okamoto, N., Hori, S., Akazawa, C., Hayashi, Y., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1994) Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. J. Biol. Chem. **269**, 1231–1236
7 Saugstad, J. A., Kinzie, J. M., Mulvihill, E. R., Segerson, T. P. and Westbrook, G. L. (1994) Cloning and expression of a new member of the L-2-amino-4-phosphonobutyric acid-sensitive class of metabotropic glutamate receptors. Mol. Pharmacol. **45**, 367–372
8 Duvoisin, R. M., Zhang, C. and Ramonell, K. (1995) A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. J. Neurosci. **15**, 3075–3083
9 Macek, T. A., Winder, D. G., Gereau, VI, R. W., Ladd, C. O. and Conn, P. J. (1996) Differential involvement of group II and group III mGluRs as autoreceptors at lateral and medial perforant path synapses. J. Neurophysiol. **76**, 3798–3806
10 Takahashi, T., Forsythe, I. D., Tsujimoto, T., Barnes-Davies, M. and Onodera, K. (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. Science **274**, 594–597
11 Li, H., Ohishi, H., Kinoshita, A., Shigemoto, R., Nomura, S. and Mizuno, N. (1997) Localization of a metabotropic glutamate receptor, mGluR7, in axon terminals of presumed nociceptive, primary afferent fibers in the superficial layers of the spinal dorsal horn: an electron microscope study in the rat. Neurosci. Lett. **223**, 153–156
12 Shigemoto, R., Kulik, A., Roberts, J. D., Ohishi, H., Nusser, Z., Kaneko, T. and Somogyi, P. (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature (London) **381**, 523–525
13 Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P. J., Neki, A., Abe, T., Nakanishi, S. et al. (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J. Neurosci. **17**, 7503–7522
14 Brandstätter, J. H., Koulen, P., Kuhn, R., van der Putten, H. and Wässle, H. (1996) Compartmental localization of a metabotropic glutamate receptor (mGluR7): two different active sites at a retinal synapse. J. Neurosci. **16**, 4749–4756
15 Masugi, M., Yokoi, M., Shigemoto, R., Muguruma, K., Watanabe, Y., Sansig, G., van der Putten, H. and Nakanishi, S. (1999) Metabotropic glutamate receptor subtype 7 ablation causes deficit in fear response and conditioned taste aversion. J. Neurosci. **19**, 955–963
16 Dev, K. K., Nakanishi, S. and Henley, J. M. (2001) Regulation of mglu(7) receptors by proteins that interact with the intracellular C-terminus. Trends Pharmacol. Sci. **22**, 355–361
17 Nakajima, Y., Yamamoto, T., Nakayama, T. and Nakanishi, S. (1999) A relationship between protein kinase C phosphorylation and calmodulin binding to the metabotropic glutamate receptor subtype 7. J. Biol. Chem. **274**, 27573–27577
18 O'Connor, V., El Far, O., Bofil-Cardona, E., Nanoff, C., Freissmuth, M., Karschin, A., Airas, J. M., Betz, H. and Boehm, S. (1999) Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. Science **286**, 1180–1184
19 Airas, J. M., Betz, H. and El Far, O. (2001) PKC phosphorylation of a conserved serine residue in the C-terminus of group III metabotropic glutamate receptors inhibits calmodulin binding. FEBS Lett. **494**, 60–63
20 Staudinger, J., Lu, J. and Olson, E. N. (1997) Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-α. J. Biol. Chem. **272**, 32019–32024
21 Xia, J., Zhang, X., Staudinger, J. and Huganir, R. L. (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron **22**, 179–187
22 Boudin, H., Doan, A., Xia, J., Shigemoto, R., Huganir, R. L., Worley, P. and Craig, A. M. (2000) Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron **28**, 485–497
23 Dev, K. K., Nakajima, Y., Kitano, J., Braithwaite, S. P., Henley, J. M. and Nakanishi, S. (2000) PICK1 interacts with and regulates PKC phosphorylation of mGLUR7. J. Neurosci. **20**, 7252–7257
24 El Far, O., Airas, J., Wischmeyer, E., Nehring, R. B., Karschin, A. and Betz, H. (2000) Interaction of the C-terminal tail region of the metabotropic glutamate receptor 7 with the protein kinase C substrate PICK1. Eur. J. Neurosci. **12**, 4215–4221
25 Hirbec, H., Perestenko, O., Nishimune, A., Meyer, G., Nakanishi, S., Henley, J. M. and Dev, K. K. (2002) The PDZ proteins PICK1, GRIP, and syntenin bind multiple glutamate receptor subtypes. Analysis of PDZ binding motifs. J. Biol. Chem. **277**, 15221–15224
26 Enz, R. (2002) The metabotropic glutamate receptor mGluR7b binds to the catalytic γ-subunit of protein phosphatase 1. J. Neurochem. **81**, 1130–1140
27 Enz, R. (2002) The actin-binding protein Filamin-A interacts with the metabotropic glutamate receptor type 7. FEBS Lett. **514**, 184–188
28 Saugstad, J. A., Yang, S., Pohl, J., Hall, R. A. and Conn, P. J. (2002) Interaction between metabotropic glutamate receptor 7 and α tubulin. J. Neurochem. **80**, 980–988
29 El Far, O., Bofill-Cardona, E., Airas, J. M., O'Connor, V., Boehm, S., Freissmuth, M., Nanoff, C. and Betz, H. (2001) Mapping of calmodulin and G$\beta\gamma$ binding domains within the C-terminal region of the metabotropic glutamate receptor 7A. J. Biol. Chem. **276**, 30662–30669
30 Sanger, F. (1977) Determination of nucleotide sequences in DNA. Science **214**, 1205–1210
31 Schiestl, R. H. and Gietz, R. D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16**, 339–346
32 Koroll, M., Rathjen, F. G. and Volkmer, H. (2001) The neural cell recognition molecule neurofascin interacts with syntenin-1 but not with syntenin-2, both of which reveal self-associating activity. J. Biol. Chem. **276**, 10646–10654
33 Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P. and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. EMBO J. **16**, 1876–1887
34 Kreivi, J. P., Trinkle-Mulcahy, L., Lyon, C. E., Morrice, N. A., Cohen, P. and Lamond, A. I. (1997) Purification and characterisation of p99, a nuclear modulator of protein phosphatase 1 activity. FEBS Lett. **420**, 57–62
35 Perroy, J., El Far, O., Bertaso, F., Pin, J. P., Betz, H., Bockaert, J. and Fagni, L. (2002) PICK1 is required for the control of synaptic transmission by the metabotropic glutamate receptor 7. EMBO J. **21**, 2990–2999
36 Daniels, D. L., Cohen, A. R., Anderson, J. M. and Brunger, A. T. (1998) Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition. Nat. Struct. Biol. **5**, 317–325

Received 8 November 2002/3 February 2003; accepted 5 February 2003
Published as BJ Immediate Publication 5 February 2003, DOI 10.1042/BJ20021750