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Identification of a tubulin binding motif on the $P2X_2$ receptor

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Abstract

To isolate proteins interacting with P2X receptors, GST fusion proteins containing the intracellular C terminal tail of $P2X_2$, $P2X_5$, or $P2X_7$ were used as bait to screen detergent extracts of rat brain synaptosomes. By SDS–PAGE combined with mass spectrometry, two interacting proteins were identified: βIII tubulin and myelin basic protein. While myelin basic protein bound to all three P2X subunits, βIII tubulin interacted exclusively with the $P2X_2$ subunit. The tubulin binding domain could be confined to a proline-rich segment (amino acids 371–412) of the $P2X_2$ subunit. Our results suggest a role for microtubules in the cellular localisation of the $P2X_2$ receptor.
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Keywords: β-Tubulin; GST fusion protein; Myelin basic protein; P2X receptor isoforms

1. Introduction

The colocalisation of ion transport proteins and ion channels with cytoskeletal structures provides a frame for the specific spatial localisation of transmembrane proteins at the plasma membrane. Ankyrin, for example, links actin and spectrin to the Na,K-pump, thus locating this enzyme at the basolateral surface of epithelial cells [1]. The spectrin cytoskeleton is also associated with Na^+ channels in epithelial and non-epithelial cells [2]. In the nervous system, efficient signalling depends critically on the precise positioning of ligand-gated ion channels (LGICs) in high density in the cell membrane at sites postsynaptic to nerve terminals releasing that neurotransmitter. Among the intracellular proteins which are enriched in the postsynaptic density and provide a link to the cytoskeleton are gephyrin [3], rapsyn [4], and GABARAP [5]. The tubulin binding protein gephyrin, for example, is crucial for the clustering and postsynaptic localisation of inhibitory glycine receptors at synapses of the spinal cord by linking the heteropentameric αβ glycine receptor complex through the β subunit with microtubules [6,7]. In addition, gephyrin mediates the postsynaptic clustering of $GABA_A$ receptors in the brain [8,9]. Likewise, GABARAP, a $17\cdot10^3$ M_r protein, has been shown to associate with $GABA_A$ receptors and to bind to microtubules [5,10] by serving as a linker between $GABA_A$ and gephyrin [11]. In contrast to gephyrin, however, GABARAP appears to play a role in intracellular transport of $GABA_A$ receptors rather than in receptor anchoring at the synapse [11,12].

P2X receptors for the neurotransmitter ATP are LGICs that play a role in fast synaptic transmission

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between neurons, from neurons to smooth muscle, and in ATP-mediated lysis of antigen presenting cells (for reviews, see Refs. [13,14]). P2X receptors feature a trimeric architecture [15] distinct from the pentameric architecture of the glycine receptor family and the $GABA_A$ receptor family mentioned above, which by themselves are assigned to the same class of the nicotinic receptor superfamily. The seven isoforms that constitute the P2X receptor family share a common membrane topology with two membrane-spanning regions, which contribute to the wall of the ion channel [16]. Both N and C terminal tails are intracellularly located. The C terminal tail is the most divergent region among the seven P2X isoforms ranging in length between 27 ($P2X_6$) and 239 ($P2X_7$) amino acids. Moreover, the C terminal tails show no apparent sequence homology [17], suggesting that they may carry isoform-specific information important for subcellular targeting and membrane clustering of the receptor.

In this study, we aimed to identify proteins that are involved in the intracellular transport and localisation of P2X receptors in the brain. Using GST–P2X fusion proteins as bait, we identified myelin basic protein (MBP) and βIII tubulin as binding partners of P2X subunits.

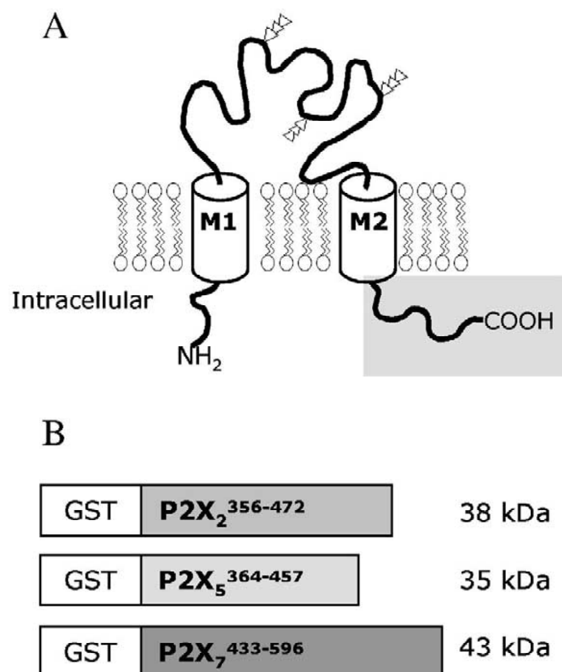

Fig. 1. Membrane topology of P2X subunits and design of the GST–P2X fusion proteins. (A) P2X subunits consist of a short intracellular N-terminal tail, two transmembrane segments (M1 and M2) connected by a large glycosylated extracellular loop (triangles, N-linked oligosaccharide side chains), and an intracellular C terminal tail. Both sequence and length of the C terminal tail (shadowed box) vary considerably among the various P2X isoforms. (B) The fusion proteins used comprise the glutathione *S*-transferase fused N-terminally to the C-terminal tail of one of three P2X isoforms, $P2X_2$, $P2X_5$, and $P2X_7$. Superscripts refer to the amino acid sequence of the corresponding full length P2X subunit. The total mass of the fusion protein including the GST portion is indicated in 10^3 M_r.

2. Experimental

2.1. Subcloning of P2X cDNAs in vector pGEX-2T

DNA manipulations were carried out using standard protocols. cDNAs encoding the C-terminal intracellular tails of the rat P2X isoforms $P2X_2$ (amino acids 356–472), $P2X_5$ (amino acids 364–457), and $P2X_7$ (amino acids 433–596) were amplified by PCR from plasmids comprising the complete coding sequences of the respective P2X subunits. The PCR primers used contained unique restriction sites for *Bam* HI and *Eco* RI at the 5′ end and downstream of the stop codon, respectively, to allow for in frame insertion of the PCR products into the bacterial expression vector pGEX-2T (Amersham Biosciences), generating GST–$P2X_2^{356\text{-}472}$, GST–$P2X_5^{364\text{-}457}$ and GST–$P2X_7^{433\text{-}596}$ (Fig. 1B). The superscripts indicate the range of amino acid sequence of the corresponding full length P2X isoform incorporated in the fusion protein. Using the same approach, a series of GST–$P2X_2$ fusion proteins was generated encompassing amino acids 356–412 (GST–$P2X_2^{356\text{-}412}$), 406–472 (GST–$P2X_2^{406\text{-}472}$), 371–412 (GST–$P2X_2^{371\text{-}412}$), and 371–472 (GST–$P2X_2^{371\text{-}472}$) of the full length $P2X_2$ subunit. To generate GST–$P2X_2^{356\text{-}381}$, a stop codon was inserted by site-directed mutagenesis into the GST–$P2X_2^{356\text{-}412}$ plasmid using the QuikChange™ muta-

genesis kit (Stratagene). All insertions and mutations were verified by DNA sequencing.

2.2. Purification of GST–P2X fusion proteins

pGEX-2T constructs were expressed in BL21 *Escherichia coli* cells to produce fusion proteins comprising the glutathione *S*-transferase fused N-terminally to the C-terminal tail of one of the various P2X isoforms. Small-scale overnight cultures, grown at 37 °C, were diluted 1:50 to 1 liter LB medium and induced for 3 h with 0.2 m*M* isopropyl β-D-thiogalactopyranoside. Bacteria were harvested by centrifugation, and lysed by sonication (six times for 30 s; Sonifier 450, Branson) in 10 ml of lysis buffer (20 m*M* Tris–HCl, pH 7.4, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT) supplemented with 1% Triton X-100 (Roche Molecular Biochemicals, membrane research grade) and 1 m*M* PMSF. The lysate was cleared by centrifugation (10 min at 17 000 *g* and 4 °C), adsorbed to 0.5 ml glutathione-Sepharose 4B (Amersham Biosciences) for 1 h at 4 °C, and washed three times with lysis buffer. Finally, the beads were resuspended in fresh washing buffer containing 25% of sterile glycerol (v/v) and stored in aliquots at −80 °C.

For overlay assays, GST fusion proteins were eluted from the glutathione-Sepharose beads by a 1-h incubation at room temperature with end-over-end mixing in 50 m*M* Tris–HCl, pH 8.0 containing 10 m*M* reduced glutathione (Merck, Darmstadt, Germany). The GST fusion proteins were then passed through a HiTrap desalting column (Amersham Biosciences) equilibrated with sodium phosphate buffer (100 m*M* PO_4^{3-}, pH 8.0) to remove glutathione. GST fusion proteins were stored in aliquots at −80 °C.

For determination of protein concentrations, the various GST fusion proteins were released from equal aliquots of glutathione beads by boiling in SDS–PAGE loading buffer and resolved on linear SDS–polyacrylamide gels (12.5% acrylamide) in parallel with known amounts of bovine serum albumin (2.5–15 μg per lane) as standards. After staining the gel with Coomassie Blue, the concentrations of the various GST fusion proteins were estimated by visual comparison of the intensity of the protein bands.

2.3. Preparation of detergent extracts of rat brain synaptosomes

Sprague–Dawley rats were anaesthetised with isoflurane (Abbot) and killed by decapitation. Brains were rapidly removed, dropped into ice-cold isolation buffer (0.32 *M* sucrose, 10 m*M* Tris–HCl, pH 7.4), and homogenised with a Potter homogeniser. A crude mitochondrial/synaptosomal P2 pellet was prepared as described previously [18]. When indicated, the P2 synaptosomal fraction was purified by Ficoll density gradient centrifugation (Ficoll™ PM 400, Amersham Biosciences) [19]. Synaptosomes (purified or not) were resuspended in isolation buffer and diluted with sodium phosphate buffer (100 m*M* PO_4^{3-}, pH 8.0) supplemented with protease inhibitors (50 μ*M* leupeptin, 10 μ*M* antipain, 5 μ*M* pepstatin A, 100 μ*M* Pefabloc SC) and 0.1% Triton X-100 (v/v) or 1% digitonin (w/v) as indicated in the figure legends. After 10 min of incubation at 4 °C by end-over-end mixing, the detergent extract was centrifuged for 10 min at 15 000 *g* and 4 °C. The resulting supernatant was used for the pull-down experiments and for overlay assays.

2.4. In vitro protein interaction assay

The synaptosomal detergent extract (4 ml) from one rat brain was pre-cleared by 30 min of end-over-end mixing at 4 °C with about 200 μg of GST protein alone, immobilised on glutathione-Sepharose. The beads were discarded, and 1 ml of the supernatant (about 3 mg of synaptosomal proteins) was incubated with end-over-end mixing for 1 h at 4 °C with about 50 μg of one of the various GST–P2X fusion proteins, immobilised on glutathione-Sepharose. As a control, the same quantity of immobilised GST–P2X fusion protein was incubated with phosphate buffer alone. Beads were then washed three times with sodium phosphate buffer containing protease inhibitors. Proteins that remained bound to the beads were eluted by boiling with SDS–PAGE loading buffer, resolved by tricine–SDS–PAGE appropriate for resolution of low mass polypeptides

[20], and detected by Coomassie Blue staining or immunoblotting.

2.5. Immunoblot analyses

Proteins were transferred on a PVDF membrane (Millipore) in 25 m*M* Tris, 192 m*M* glycine, 20% methanol for 4 h at 100 mA. Membranes were blocked by overnight incubation with TBST (10 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.05% Tween 20) containing 1% (w/v) non-fat dry milk, and 1% (w/v) BSA, and then incubated for 1 h with anti-βIII tubulin (1:400, Sigma) or anti-MBP (1:400 BD PharMingen), followed by horseradish peroxidase conjugated goat anti-mouse IgG (1:5000, Amersham Biosciences). Finally, the proteins were detected by enhanced chemiluminescence (ECL; Amersham Biosciences).

2.6. Mass spectrometric analysis

Coomassie Blue-stained protein bands were cut out, digested with trypsin for 12 h at 37 °C in the gel matrix, and processed for matrix-assisted laser desorption ionisation–mass spectrometry (MALDI–MS) as described previously [21]. MALDI/Time of Flight (TOF) analysis was carried out on a Bruker Biflex mass spectrometer. The computer program Mass Spectrometry-Digest (Peter Baker and Karl Clauser, UCSF Mass Spectrometry Facility) was used for computer-assisted comparison of the tryptic peptide mapping data with the expected set of peptides.

2.7. GST–$P2X_2^{356-472}$ overlay

Overlay assays were carried out essentially as described [22]. Triton X-100 extract of synaptosomes was supplemented with reducing SDS–PAGE loading buffer, boiled for 2 min, and then resolved on an SDS–polyacrylamide gel (10% acrylamide). After semi-wet protein transfer, the PVDF membrane was blocked by overnight incubation with Blotto (150 m*M* NaCl, 50 m*M* phosphate, pH 7.5, 10% of non-fat dry milk), washed, and incubated for 1 h in buffer B [22] containing 3% BSA, 1 m*M* DTT, and 4.5 μg/ml of one of the GST–P2X fusion proteins. The membrane was washed three times with buffer B containing 3% BSA, and then incubated with an antibody against GST (Amersham Biosciences; 1:10 000) in buffer B, 3% BSA. Subsequently, the membrane was washed twice with buffer C (10 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl), four times with buffer D (0.2% SDS, 0.5% Triton X-100, 0.5% BSA, 0.9% NaCl, pH 7.0), and twice with buffer C. Bound antibody against GST was labelled by incubation with [^{125}I]protein A (Amersham Biosciences; 30 000 cpm/ml) for 1 h in buffer C, followed by washing (2× buffer C, 5× buffer D, 3× buffer C) and visualisation of bound radioactivity by Phosphor-Imaging.

3. Results

3.1. Tubulin binds specifically to the $P2X_2$ subunit

The members of the P2X receptor family share a common membrane topology with two membrane spanning segments linked by a large, glycosylated ectodomain (Fig. 1A). The first transmembrane segment is preceded by an intracellular N-terminal domain, which has a similar length of 24–31 amino acids. In contrast, the intracellular C terminal tails varies considerably between the various P2X isoforms both in length (ranging from 30 to 239 amino acids) and sequence. For a search of putative binding proteins, we focused on the three P2X subunits that possess the longest C terminal domains within the P2X family, $P2X_2$, $P2X_5$ and $P2X_7$, assuming that they have the highest probability to be involved in protein–protein interactions. The different masses of the GST–P2X fusion proteins reflect the individual length of the C terminal tail of the corresponding P2X isoforms (Fig. 1B).

To identify possible interacting proteins, the GST fusion proteins incorporating the C terminal domains of the rat $P2X_2$, $P2X_5$ and $P2X_7$ isoforms were incubated with a digitonin extract of rat brain synaptosomes. The presence of $P2X_7$ receptors in rat brain synaptosomes has recently been shown by a functional assay [23]. After washing and centrifugation, bound proteins were eluted from the glutathione-Sepharose beads and resolved by tricine–SDS–PAGE followed by Coomassie Blue staining (Fig. 2A). A protein band of approximately $55 \cdot 10^3$

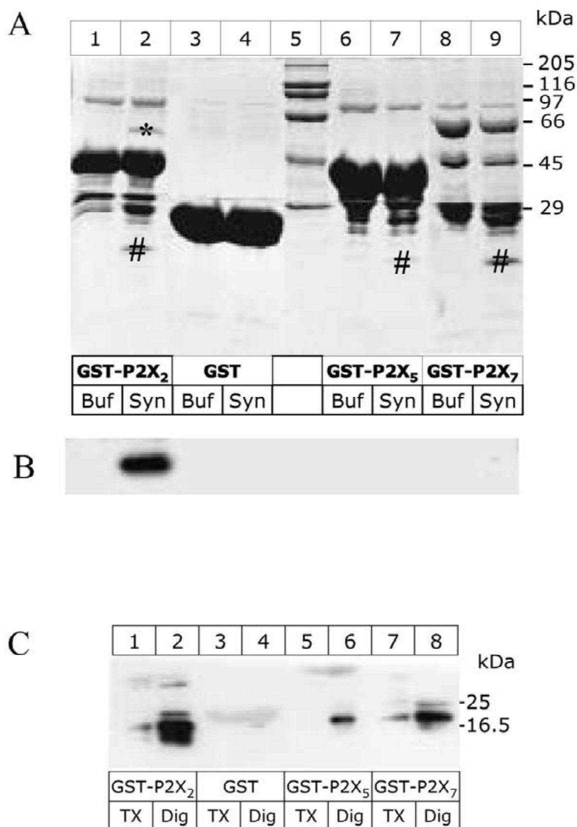

Fig. 2. Pull-down of rat brain proteins with GST–P2X fusion proteins. Proteins were pulled down from a digitonin extract of crude rat brain synaptosomes (Syn) as described under the Experimental section using the indicated glutathione-Sepharose-immobilised GST–P2X fusion proteins. Samples treated identically, yet incubated solely with sodium phosphate buffer (buf) instead of brain proteins served as controls. (A) Shown is a Coomassie Blue-stained tricine–SDS–polyacrylamide gel of the proteins eluted from the glutathione-Sepharose beads. *~$55 \cdot 10^3$ M_r protein band identified as βIII tubulin by MALDI/TOF; #~$20 \cdot 10^3$ M_r protein band identified as MBP by MALDI/TOF. (B) Immunoblot analysis of the same samples as in (A) with antibody to βIII tubulin, indicating that βIII tubulin was isolated solely by GST–P2X$_2$. (C) Proteins were pulled down with the indicated GST–P2X fusion proteins from synaptosomal Triton X-100 extracts (TX) or digitonin extracts (Dig) and probed with an antibody against MBP followed by ECL.

M_r was detected in the samples incubated with GST–P2X$_2^{356\text{-}472}$ and brain detergent extract (lane 2). In contrast, this band was not isolated when the brain detergent extract was incubated with GST alone (lane 4), with GST–P2X$_5^{364\text{-}457}$ (lane 7) or GST–P2X$_7^{433\text{-}596}$ (lane 9). The $55 \cdot 10^3$ M_r band was excised from the gel, digested with trypsin in the gel matrix, and identified by MALDI/TOF mass spectrometry as βIII tubulin (accession number: P04691), a brain-specific β tubulin isoform. These results were corroborated by immunoblot analyses, which clearly demonstrate that tubulin was pulled-down with GST–P2X$_2^{356\text{-}472}$, but not with the two other GST–P2X fusion proteins nor with GST alone (Fig. 2B).

3.2. All three GST–P2X fusion proteins bind MBP

An additional protein band of about $20 \cdot 10^3$ M_r was observed to occur with all three GST–P2X fusion proteins and identified by MALDI/TOF mass spectrometry to constitute myelin basic protein (MBP, accession number: CAA10807). Consistent with this result, probing of an immunoblot with an antibody against MBP-labelled protein bands of ~$20 \cdot 10^3$ M_r in the same samples (Fig. 2C), supporting the view that MBP interacts with the three fusion proteins GST–P2X$_2^{356\text{-}472}$, GST–P2X$_5^{364\text{-}457}$ and GST–P2X$_7^{433\text{-}596}$. The antibody against MBP recognised several protein bands of similar masses; this can be attributed to the occurrence of four major MBP isoforms of 21.5, 18.5, 17 and $14 \cdot 10^3$ M_r in rat generated by alternative splicing of a single primary MBP transcript [24]. The amount of MBP was greatly reduced when Triton X-100 instead of digitonin was used for protein extraction (Fig. 2C), indicating that the interaction was more stable in the presence of a mild detergent.

To examine whether MBP isolated through the GST–P2X fusion proteins originates predominantly or exclusively from the large amount of myelin present in the crude P2 synaptosome preparation, we removed myelin from the synaptosomes by Ficoll density gradient centrifugation [19]. A pull-down experiment with a digitonin extract of the purified synaptosomes, followed by immunoblot analysis with the antibody against MBP showed that MBP could be isolated in significant amounts with GST–P2X$_2^{356\text{-}472}$ even when contaminating myelin was greatly reduced (results not shown). This suggests that the interaction between P2X receptors and MBP is not an artefact due to the contamination of synaptosomes with myelin, but occurs with MBP present in the nerve terminals.

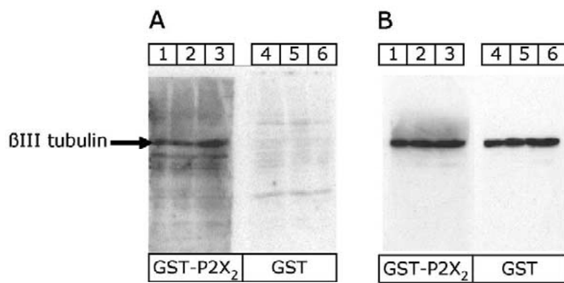

Fig. 3. Overlay assay with GST–P2X$_2^{356\text{-}472}$. Synaptosomal proteins (lane 1, 25 μg; lane 2, 50 μg; lane 3, 75 μg) solubilised with Triton X-100 were resolved on a SDS–polyacrylamide gel (10% acrylamide) and then transferred on a PVDF membrane. (A) Synaptosomal proteins were overlaid with GST–P2X$_2^{356\text{-}472}$ or GST alone as indicated and then probed by a GST antibody and [^{125}I]protein A. Radioactivity was detected by PhosphorImaging. (B) The same membrane as in (A) was probed with the antibody against anti-βIII tubulin to confirm that the lanes contained equal amounts of βIII tubulin.

3.3. The P2X$_2$ subunit binds tubulin directly

To examine whether tubulin binds directly to the C terminus of the P2X$_2$ subunit or requires a linker protein, we carried out an overlay assay. A Triton X-100 (0.1%) extract of synaptosomes was resolved by SDS–PAGE, transferred to a PVDF membrane, and overlaid with GST–P2X$_2^{356\text{-}472}$. Binding of the fusion protein was then revealed with a GST antibody and [^{125}I]protein A. The protein that became labelled corresponded in molecular mass to tubulin (Fig. 3A, lanes 1–3). The identity of this protein band with βIII tubulin was verified by immunoblot analysis of the same membrane, which led to the labelling of exactly the same protein band (Fig. 3B, lanes 1–6). GST alone did not exhibit any binding (Fig. 3A, lanes 4–6). These results suggest that βIII tubulin binds directly to the C terminal tail of the P2X$_2$ receptor subunit.

3.4. Tubulin binding is mediated by a proline-rich segment of the P2X$_2$ C terminal tail

In an attempt to map the βIII tubulin binding motif of the P2X$_2$ subunit, we generated four additional GST–P2X$_2$ fusion proteins containing various portions of the intracellular C terminal domain of the P2X$_2$ subunit. A sequence comparison of the P2X$_2$ portions of the proteins is shown in Fig. 4A. These GST–P2X$_2$ fusion proteins were used in pull-down experiments with a Triton X-100 extract of synaptosomes, followed by immunoblot analysis with the antibody against βIII tubulin. βIII tubulin was isolated with a fusion protein including the first half of the P2X$_2$ C terminal tail (Fig. 4B; construct B, amino acids 356–412), but not at all with the

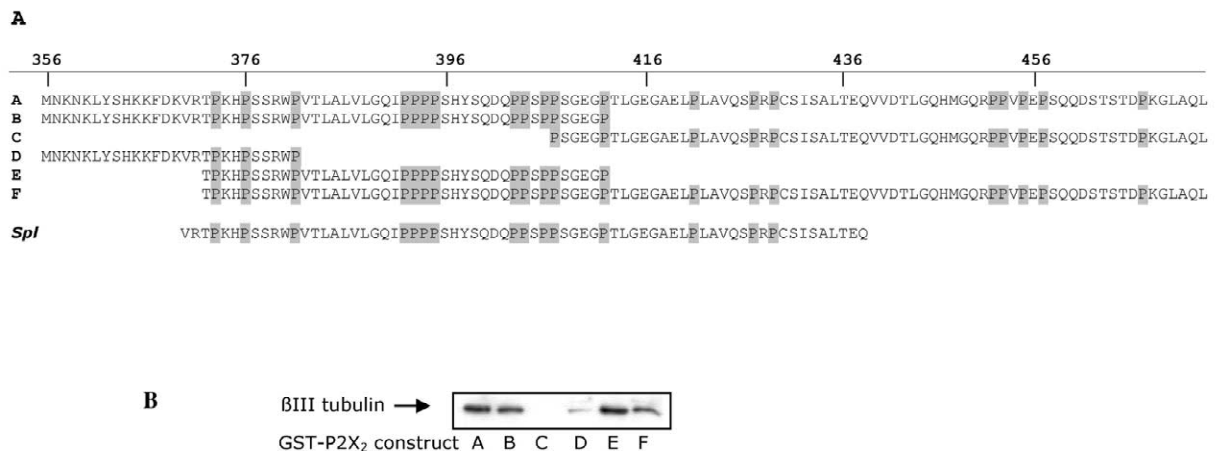

Fig. 4. Mapping of the tubulin binding domain of P2X$_2$. (A) Sequence comparison of the GST–P2X fusion proteins generated to map the tubulin binding domain. Proline residues are shown shadowed. *Spl*, 69 amino acid sequence lacking in the P2X$_{2B}$ subunit due to splicing. Residue numbers corresponding to the full length P2X$_2$ subunits are presented on the top. (B) Immunoblot detection of βIII tubulin pulled down with the different GST–P2X$_2$ fusion proteins from a digitonin extract of rat synaptosomes.

second half of the C terminus (construct C, amino acids 406–472). We therefore first considered a highly positively charged region of fusion protein B (amino acids 356–381 of the $P2X_2$ subunit, construct D) as a possible tubulin binding motif similar to that of GABARAP [5]. However, fusion protein D comprising the basic portion of construct B interacted only weakly with tubulin (Fig. 4B). In contrast, fusion protein E comprising the second half of construct B (amino acids 371–412) bound tubulin as efficiently as the full length $P2X_2$ C terminal tail (construct A) or construct F, lacking the basic domain, but including all C terminal proline residues (amino acids 371–472).

4. Discussion

4.1. βIII tubulin as a binding partner of the $P2X_2$ subunit

By affinity chromatography with GST–P2X fusion proteins combined with MALDI–TOF mass spectrometry analysis, we have identified βIII tubulin, a nervous system-specific isoform of tubulin, as a major brain protein interacting with the C terminal domain of the $P2X_2$ subunit. The interaction was confirmed by immunoblot analysis using a βIII tubulin-specific antibody. βIII tubulin did not interact with $P2X_5$ or $P2X_7$ subunits, suggesting that the $P2X_2$–βIII tubulin interaction is isoform-specific.

Tubulin is already known to interact with a variety of receptors including the metabotropic glutamate receptor type 1α [25] and two LGICs, the inhibitory glycine receptor [6] and the inhibitory $GABA_A$ receptor [5]. However, while cross-bridging linker proteins such as gephyrin and GABARAP are required for the binding of glycine and $GABA_A$ receptors to tubulin [8,9], the binding of tubulin to the $P2X_2$ subunit occurs directly, as deduced from the direct $P2X_2$–tubulin interaction detected in the overlay assay.

The potential consequences of the tubulin–$P2X_2$ receptor interaction for the distribution of $P2X_2$ receptors in neurons are unknown at present. Since microtubules are essential for diverse functions such as cell division, cytoplasmic organisation, and vesicle trafficking, a role in intracellular transport of vesicles carrying $P2X_2$ receptors, in the regulation of receptor density by endo- and exocytosis as well as in anchoring $P2X_2$ receptors at specific sites of the plasma membrane is possible. Also, a more regulatory function of the $P2X_2$–tubulin interaction may be considered, since there is increasing evidence for a functional relationship between tubulin and various signalling molecules. It has been shown for example that βIII tubulin couples to and is phosphorylated by βARK, a G-protein coupled receptor kinase associated with the β adrenergic receptor [26]. This raises the intriguing possibility that tubulin by itself could act as a cross-linking bridge bringing together receptors and modifying enzymes to participate in complex regulatory interactions.

4.2. Localisation of the tubulin binding motif on the $P2X_2$ subunit

By using GST fusion proteins that include various portions of the C terminal tail of the $P2X_2$ subunit, we were able to confine the binding domain of $P2X_2$ for tubulin to a 42 amino acid long region, ranging from amino acid 371 to 412. This region contains a total of six serine residues and 12 proline residues, some of which may be interpreted to constitute binding motifs for SH3 domains, at least when based on the minimal consensus motif, PxxP [27]. In contrast, both the $P2X_5$ and $P2X_7$ subunits do not possess domains rich in proline and serine residues, which may explain their inability to interact with tubulin. SH3 domains play a critical role in a variety of biological processes, particularly in signalling pathways, by mediating the assembly of large multiprotein complexes. The epithelial Na^+ channel ENaC, for example, is located at the apical membrane by binding through the proline-rich sequence at the C terminal tail of the pore forming α subunit of the $(\alpha)_2\beta\gamma$ receptor to the SH3 domain of α-spectrin [28]. Microtubule-associated proteins (MAPs) such as MAP2, MAP4, and Tau also have a proline-rich region in their microtubule-binding domain, which stimulates tubulin polymerisation and stabilises microtubules inside cells [29].

Interestingly, the tubulin binding sequence identified in the present study overlaps to a significant extent with a 69 amino acid long sequence (V^{370}–Q^{438}; Fig. 4A), which is lacking in a splice variant

isoform (designated $P2X_{2B}$) of the $P2X_2$ subunit and which has already been suggested to be involved in tethering $P2X_2$ receptors to unknown cytoskeletal elements [30,31]. This deletion does not correspond to an entire exon, but results from splicing of a fragment directly out of exon 11. The $P2X_{2B}$ receptor is known to desensitise significantly faster than the receptor consisting of full-length $P2X_2$ subunits. In view of the present results, the faster desensitisation may be attributed to the failure of the $P2X_{2B}$ subunit to interact with tubulin due to a lack of the tubulin binding domain. Support for a role of a cytoplasmic factor in the control of desensitisation comes from patch clamp experiments, showing that the slowly desensitising $P2X_2$ receptor begins to desensitise rapidly when analysed on excised patches in the presence of extracellular Ca^{2+}, suggesting that essential cytoplasmic elements are washed out by this procedure [32].

Altogether, the direct and specific association of βIII tubulin with the $P2X_2$ receptor may play an important physiological role in receptor desensitisation or in the targeting and segregation of $P2X_2$ receptors to specialised regions of neurons by anchoring the receptor to the microtubule cytoskeleton.

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