Tax1-binding protein 1 is expressed in the retina and interacts with the GABA_C receptor ρ 1 subunit

Melanie ULRICH, Silke SEEGER, Cord-Michael BECKER and Ralf ENZ¹

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

Macromolecular signalling complexes that link neurotransmitter receptors to functionally and structurally associated proteins play an important role in the regulation of neurotransmission. Thus the identification of proteins binding to neurotransmitter receptors describes molecular mechanisms of synaptic signal transduction. To identify interacting proteins of GABA_C (where GABA is γ -aminobutyric acid) receptors in the retina, we used antibodies specific for GABA_C receptor ρ 1–3 subunits. Analysis of immunoprecipitated proteins by MALDI–TOF MS (matrix-assisted laser-desorption ionization–time-of-flight MS) identified the liver regeneration-related protein 2 that is identical with amino acids 253–813 of the Tax1BP1 (Tax1-binding protein 1). A C-terminal region of Tax1BP1 bound to an intracellular domain

of the ρ 1 subunit, but not to other subunits of GABA_C, GABA_A or glycine receptors. Confocal laser-scanning microscopy demonstrated co-localization of Tax1BP1 and ρ 1 in clusters at the cell membrane of transfected cells. Furthermore, Tax1BP1 and GABA_C receptors were co-expressed in both synaptic layers of the retina, indicating that Tax1BP1 is a component of GABA_C receptor-containing signal complexes.

Key words: γ -aminobutyric acid (GABA), immunoprecipitation, matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF), protein interaction, synaptic signalling complex, Tax1-binding protein 1 (Tax1BP1).

INTRODUCTION

Neuronal excitability is regulated by a delicate balance between excitatory and inhibitory neurotransmission. GABA (γ -aminobutyric acid) is the most important inhibitory neurotransmitter receptor in the CNS (central nervous system). The molecule binds to three GABA receptor types, termed A, B and C. Although GABA_A and GABA_C receptors form ligand-gated ion channels, GABA_B receptors couple with G-protein-associated signal cascades [1,2]. GABA_C receptors are insensitive to the classical GABA_A receptor antagonist bicuculline, do not respond to anaesthetics such as barbiturates and benzodiazepines and have a faster kinetic when compared with GABA_A receptors. Within the CNS, GABA_C receptors are highly expressed in the retina where they contribute to the sharpening of the visual image [3].

Mammalian GABA_C receptors are composed of three ρ subunits (ρ 1–3) that assemble into pentameric homo- and hetero-oligomeric receptor complexes [4]. In the retina, ρ subunits are concentrated in discrete clusters at bipolar cells axon terminal systems, while they are more diffusively expressed at the dendrites of these neurons [5–7]. In analogy to the nicotinic acetylcholine receptor, it is assumed that each ρ subunit is anchored in the membrane by four transmembrane domains (TM1–4). N- and C-termini are on the extracellular side of the membrane and one extra- and two intracellular loops connect the four TMS. The intracellular loop between TM3 and TM4 contains consensus sequences for enzymes and phosphorylation of this region was demonstrated in [8]. In recent years, several proteins binding to the TM3–4 loop of different ρ subunits were identified, including proteins of the ZIP family that interact with PKC (protein kinase C), GLYT-1 (glycine transporter-1), CRABP-I (cellular

retinoic acid-binding protein-I) and MAP-1B (microtubule-associated protein 1B) [9–12]. However, MAP-1B knockout mice had a normal retina [13] and the physiological consequences of the other protein interactions have not been elucidated either.

Using antibodies specific for the extracellular N-termini of ρ subunits [5], we immunoprecipitated GABA_C receptors from solubilized retinal proteins and identified the Tax1BP-1 (Tax1-binding protein 1) as a new interactor of this receptor class. Tax1BP1 was identified by different groups in yeast two-hybrid screens against the human T-cell leukaemia virus type I Tax protein, the zinc-finger protein A20 and TRAF-6 (tumour-necrosis-factor-receptor-associated factor 6) [14–18]. Depending on the originally assigned function of Tax1BP1, the protein is also designated T6BP and TXBP151. In the retina, Tax1BP1 is co-expressed with GABA_C receptors in synaptic layers and binds specifically to the TM3–4 loop of the ρ 1 subunit, but not to other subunits of GABA_C, GABA_A or glycine receptors. Based on our data we suggest that Tax1BP1 is a component of GABA_C receptor-containing signal complexes in the retina.

EXPERIMENTAL

Preparation of membrane proteins and immunoprecipitation

Six retinas of adult rat were homogenized in 1.5 ml of buffer I (150 mM NaCl and 50 mM Tris/HCl, pH 7.5) containing 1 % Nonidet P40 (Sigma–Aldrich, Taufkirchen, Germany), 0.5 % sodium deoxycholate, 10 mg/ml DNaseI and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany) using a Teflon glass homogenizer. The sample was sonicated with 3 bursts for 10 s at 300 W and incubated for 30 min under slow

Abbreviations used: CNS, central nervous system; CRABP-I, cellular retinoic acid-binding protein-I; DTAF, 5-(4,6-dichlorotriazinyl)aminofluorescein; GABA, γ -aminobutyric acid; GCL, ganglion cell layer; GLYT-1, glycine transporter-1; GST, glutathione S-transferase; HEK-293 cells, human embryonic kidney cells; INL, inner nuclear layer; IPL, inner plexiform layer; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAP, microtubule-associated protein; NCBI, National Center for Biotechnology Information; ONL, outer nuclear layer; OPL, outer plexiform layer; PKC, protein kinase C; RhoGAP, Rho GTPase-activating protein; RT, reverse transcriptase; Tax1BP1, Tax1-binding protein 1; TM, transmembrane domain; TRAF-6, tumour-necrosis-factor-receptor-associated factor 6.

¹ To whom correspondence should be addressed (email ralf.enz@biochem.uni-erlangen.de).

agitation. Subsequently, ultracentrifugation was carried out at 100 000 *g* for 1 h at 4°C. The supernatant was precleared for 2 h with 25 µl of Protein A-conjugated agarose (Roche Diagnostics). After sedimentation of the beads, the supernatant was incubated with 50 µl of GABA_C receptor-specific antibodies [5] for 2 h under slow agitation, supplemented with 25 µl of fresh Protein A-conjugated agarose and agitated for another 16 h. Thereafter, the beads were sedimented, washed twice in 1 ml of buffer I and twice in buffer II (500 mM NaCl and 50 mM Tris/HCl, pH 7.5) containing 0.1 % Nonidet P40, 0.05 % sodium deoxycholate and the protease inhibitor cocktail. Finally, bound proteins were eluted from the beads by boiling in 30 µl of SDS sample buffer for 10 min. All protein preparation steps were carried out on ice or at 4°C.

Peptide mass 'fingerprinting'

The identification of immunoprecipitated proteins by MS was described previously [19]. Briefly, precipitated proteins were separated on 10–15 % protein gels, visualized with SyproRubyTM and bands of interest were excised. Gel bands were destained, dried and resuspended in 50 µl of 25 mM NH₄HCO₃ containing 0.2 µg/µl sequencing grade trypsin for the enzymatic digestion of the purified proteins. The generated peptide fragments were eluted from the gel, eluates were collected and dried and peptides were resuspended in 10 µl of water containing 0.1 % trifluoroacetic acid. Subsequently, peptide solutions were diluted 1:10 to 1:50 with water and 0.5 µl of diluted samples were mixed with 0.5 µl of 3-hydroxy- α -cyanocinnamic acid matrix, dotted on to a steel target and air-dried. MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) analysis was performed on an Autoflex (Bruker Daltonics, Bremen, Germany) in the reflector mode, using a nitrogen laser (337 nm) for sample desorption and an acceleration with 19 kV after a delay of 3500 ns. Masses obtained by MALDI-TOF MS were used for a peptide mass 'fingerprint' search using the search program MS-Fit and the NCBI (National Center for Biotechnology Information) protein database.

Pull-down techniques

Coding sequences for the rat TM3–4 loops of the ρ 1–3 subunits, of the α 1, β 2, γ 2 subunits of the GABA_A receptor and of the α 1 subunit of the glycine receptor were ligated in-frame to the coding sequence of GST (glutathione S-transferase) in pET-41. The coding sequences of the liver regeneration-related protein 2 and of Tax1BP1 were tagged with a T7 epitope by cloning in pET-21. DNA sequencing verified the identity of all constructs, as well as of the plasmids used in yeast two-hybrid experiments and for expression in HEK-293 cells (human embryonic kidney cells), as described below. Plasmids were transformed in *Escherichia coli* BL21(DE3)pLysS and protein expression was induced by adding 1 mM IPTG (isopropyl β -D-thiogalactoside; Sigma-Aldrich). Fusion proteins were purified under native conditions and immobilized to glutathione-Sepharose as described in [20]. Similar concentrations of immobilized fusion proteins were incubated with the cytosolic fraction of *E. coli* expressing T7-tagged proteins for 1 h at 4°C under slow agitation, followed by three washes (4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl and 0.1 % Triton X-100, pH 7.3). Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE and analysed by Western blotting using a monoclonal anti-T7 immune serum and the enhanced chemiluminescence system (ECL[®]; Amersham, Braunschweig, Germany). Unless otherwise stated, all reagents were purchased from Novagen (Madison, WI, U.S.A.).

To analyse the interaction between GABA_C receptors and Tax1BP1 in the retina, rat retinae were homogenized in buffer I as described above and cell debris was removed from the extract by centrifugation at 20 000 *g* for 30 min. The resulting protein preparation was incubated overnight at 4°C with 30 µl of a 50 % (v/v) suspension of Protein A-agarose (Amersham) that was pre-incubated with antibodies specific for GABA_C receptors (50 µl; [5]) or for mGluR2 (metabotropic glutamate receptor type 2; 5 µg; Upstate Biotechnology, Lake Placid, NY, U.S.A.) as control. Precipitates were washed four times with 500 µl of 150 mM NaCl, 50 mM Tris/HCl and 0.1 % Triton X-100 (pH 7.5), bound proteins were eluted from the beads by boiling in 20 µl of SDS sample buffer and Tax1BP1 was detected on Western blots using a specific immune serum (sc-15274; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Yeast two-hybrid techniques

The coding sequence of the TM3–4 loop of the rat ρ 1 subunit was fused to the activation domain of Gal4 in the bait vector pGBKT7-BD. Coding sequences of Tax1BP1 or of N-terminal deletions were fused to the DNA binding domain of Gal4 in the prey vector pGADT7. Yeast AH109 cells were first transformed with the ρ 1 bait vector and the resulting bait strain was subsequently transformed with 1 µg of prey vector constructs. Transformed yeast cells were plated on media selecting for His3 reporter gene activation and growing yeast colonies were then transferred to plates containing ONPG (*o*-nitrophenyl β -D-galactopyranoside; Sigma-Aldrich) to test β -galactosidase reporter gene activation. All reagents including the plasmid for the negative control were parts of the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA, U.S.A.).

Cell culture and immunocytochemistry

Coding sequences of the GABA_C receptor ρ 1 subunit and of the T7-tagged Tax1BP1 were ligated in the eukaryotic expression vectors pRC/CMV and pRK7 to allow protein expression under the control of the CMV (cytomegalovirus) promoter. Human embryonic kidney cells (HEK-293 cells, ATCC no. CRL 1573) were grown in 3 cm dishes on glass coverslips coated with poly(L-lysine) to approx. 80 % confluency and transfected with 1 µg of each plasmid using the calcium phosphate method [21]. After 2 days, the cells were washed three times with PBS, fixed in 4 % (v/v) formaldehyde/PBS for 10–15 min on ice, washed again as above and blocked with 10 % (v/v) fetal calf serum containing 2 % (w/v) BSA and 0.1 % Triton X-100 for 10 min. Subsequently, cells were incubated for 2 h with GABA_C receptor-specific antibodies from rabbit (1:100) [5] and an anti-T7 serum from mouse to detect Tax1BP1 (1:3000; Novagen) diluted in PBS containing 1 % fetal calf serum and 0.1 % Triton X-100. After washing, Texas Red (1:500; red fluorescence) and DTAF [5-(4,6-dichlorotriazinyl)amino fluorescein; 1:300; green fluorescence] conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (Dianova, Hamburg, Germany) were applied for 45 min to reveal the binding sites of the primary antibodies. The cells were extensively washed and mounted in Mowiol (Sigma-Aldrich). Labelled proteins were examined with a Leica inverted microscope (DMIRB; Bensheim, Germany) equipped with a cooled MicroMax CCD camera (charge-coupled-device camera; Princeton Instruments, Stanford, CA, U.S.A.) or a Leica confocal microscope (TCS SL). Fluorescence profiles were determined with the beta 4.0.2 version of the data acquisition and analysis software from Scion Corporation (Frederick, MD, U.S.A.).

Figure 1 MALDI-TOF analysis of immunoprecipitated proteins using GABA_C receptor-specific antibodies

The spectra show relative peak intensities of peptide masses analysed by MALDI-TOF MS. Peaks derived from peptides that matched the theoretical trypsin digests of proteins in the NCBI protein database are indicated by stars and the identified proteins are labelled in the upper corner of each spectrum. BSA served as a positive control and the indicated negative control was generated from a gel fragment not containing protein.

Isolation of RNA and RT (reverse transcriptase)-PCR

Total RNA was extracted from adult rat tissues following a method described in [22], reverse transcribed and amplified as described previously [20]. The following primers were used for PCR amplification: Tax1BP1 sense, 5'-AGGGAGAAGGAAGTTACAAAG-

3'; Tax1BP1 antisense, 5'-CTAGTCGAAGTTGAGAACATTC-3'; EF1 α sense, 5'-GTCTGCCCCAGAAAGCTCAG-3'; EF1 α antisense, 5'-AATGGTCTCAAAATTCTGTGAC-3'. Five microlitres of each PCR product were separated on a 1.5% agarose gel and stained with ethidium bromide. Controls were treated as above without adding cDNA or RT and showed no PCR products. Both PCR products were identified by DNA sequencing.

Immunohistochemistry

Adult mice were anaesthetized with halothane and decapitated. Retinal cryostat sections with a thickness of 12 μ m were prepared as described in [23] and incubated with a mixture of antibodies specifically recognizing Tax1BP1 (1:100; Santa Cruz Biotechnology) and GABA_C receptor ρ 1–3 subunits (1:100; [5]). The binding sites of the primary antibodies were revealed by the secondary antibodies Alexa Fluor[®] 594 (red fluorescence) and Alexa Fluor[®] 488 (green fluorescence) donkey anti-goat or donkey anti-rabbit IgG (H + L) conjugates respectively (1:500; Molecular Probes, Eugene, OR, U.S.A.). The sections were examined as described above.

RESULTS

Identification of GABA_C receptor-associated proteins

In order to identify new binding partners of GABA_C receptors in the retina, detergent extracts from retinal membranes were immunoprecipitated by using antibodies specifically recognizing the extracellular N-termini of the GABA_C receptors ρ 1–3 subunits [5]. Precipitated proteins were separated on SDS gels, stained with SyproRuby[™] (PerkinElmer, Norwalk, CT, U.S.A.) and visualized under UV light. Protein bands were excised from the SDS gel, digested with trypsin and the mass of the generated peptides was determined by MALDI-TOF MS. The resulting peptide mass 'fingerprints' (Figure 1) were aligned to theoretical trypsin digests of proteins listed in the NCBI database and yielded several potential GABA_C receptor interactors (Table 1). The highest coverage of identified peptides showed the protein sequence of the rat liver regeneration-related protein 2. Importantly, among the precipitated proteins was the GABA_C receptor ρ 2 subunit, proving that we isolated GABA_C receptor-associated protein complexes.

Tax1BP1 binds to the TM3–4 loop of the ρ 1 subunit *in vitro*

Up to date, all interacting proteins that bind to acetylcholine-type neurotransmitter receptors interact with the intracellular TM3–4 loops of their subunits. Because also GABA_C receptors are part of this receptor family, the candidates listed in Table 1 were tested for their binding affinities to the intracellular TM3–4 loops of ρ subunits. In GST pull-down assays, we identified the liver regeneration-related protein 2 as an interactor of these domains

Table 1 Potential GABA_C receptor binding partners identified by immunoprecipitation with GABA_C receptor-specific antibodies and subsequent MALDI-TOF analysis

The liver regeneration-related protein 2 is identical with amino acids 253–813 of Tax1BP1. Two peptides of the liver regeneration-related protein 2 with *m/z* values larger than 3300 are not labelled in Figure 1(A).

Candidate	NCBI accession number	Molecular mass (kDa)	Matched peptides	Coverage (%)
Liver regeneration-related protein 2	21314404	64.59	15	41
GABA _C receptor ρ 2 subunit	6679921	54.00	7	17
BCR (B-cell receptor) protein	29179429	44.82	5	14
Dynamin-1 like protein	26252094	68.70	4	10
piUS protein	11067377	49.52	4	9
fez-like protein	11125691	48.94	4	7

Figure 2 Specific binding between a C-terminal region of Tax1BP1 and the TM3-4 loop of the GABA_C receptor ρ 1 subunit

(A) GST and GST-fusion proteins were immobilized on glutathione-Sepharose and incubated with T7-tagged liver regeneration-related protein 2 (LRP2) or Tax1BP1. Bound proteins were detected on Western blots using a monoclonal anti-T7 immune serum as indicated. Protein concentrations of coated Sepharose beads are shown on Coomassie-stained SDS/PAGE (arrowheads in the lower panels). (B) Immunoprecipitations were performed with antibodies specifically recognizing GABA_C receptors or the metabotropic glutamate receptor type 2 as indicated, and bound Tax1BP1 was detected on a Western blot. (C) Schematic representation of the domain structure of Tax1BP1 (T1) and of N-terminal deletion constructs (T2, T3). Encoded amino acids are given as numbers in parentheses. T1–3 constructs were individually tested in yeast cells for their ability to bind the TM3-4 loop of the ρ 1 subunit. Interactions were monitored by the ability of transformants to grow on selective media, indicated by '+' or '-'. Relative affinities were quantified and visualized as arbitrary β -galactosidase units (β -Gal; horizontal bars). Each value represents the mean for three yeast clones. Error bars are $\pm$ S.E. [CC, coiled-coil; HLH, helix-loop-helix; (-), negative control.]

(Figure 2A, top panel). The cDNA sequence of the liver regeneration-related protein 2 was cloned from regenerated liver of rat and directly submitted to the NCBI database (accession number AAM46928). A corresponding protein sequence with the mass of 64.6 kDa was translated, but no further data about this protein was available in the literature. In addition, the predicted amino acid sequence does not start with a methionine, suggesting that N-terminal sequences are missing. By searching the NCBI databases we found that amino acids 253–813 of the Tax1BP1 (accession number NP.001004199) are identical with the liver regeneration-related protein 2. Interestingly, the 89.5 kDa protein Tax1BP1 is cleaved by caspases, resulting in a prominent proteolysis product

of approx. 65 kDa [14]. Therefore we assumed that cleavage of an N-terminal region of Tax1BP1 generated a 65 kDa proteolytic fragment that we immunoprecipitated together with GABA_C receptors. To test this possibility, we analysed Tax1BP1 for its ability to bind the TM3-4 loops of ρ subunits and found a specific interaction with the ρ 1 subunit (Figure 2A, middle panel). Intracellular TM3-4 loops of other GABA_C receptors ρ subunits, or of the related GABA_A and glycine receptors did not bind to Tax1BP1 (Figure 2A, right panel). From these results we concluded that Tax1BP1 specifically interacts with the ρ 1 TM3-4 loop and thus we focused on Tax1BP1 for all future experiments.

Tax1BP1 and the TM3-4 loop of the ρ 1 subunit interact *in vivo*

To analyse if this interaction also occurs *in vivo*, we incubated solubilized retinal proteins with Protein A-agarose that was pre-conjugated with antibodies recognizing GABA_C receptors or the metabotropic glutamate receptor type 2 as negative control. Sedimentation of the agarose beads specifically co-precipitated Tax1BP1 with GABA_C receptors, indicating that both proteins can interact in retinal cells (Figure 2B).

Furthermore, we transformed the coding sequences of Tax1BP1 and the ρ 1 TM3-4 loop, fused to the activation and DNA binding domains of the Gal4 transcription factor, into yeast cells. Binding between Tax1BP1 and the ρ 1 TM3-4 loop was demonstrated by the ability of transformants to grow on selective media upon activation of the His3 reporter gene (Figure 2C, construct T1). Tax1BP1 is composed of 813 amino acids and contains three coiled-coil and two helix-loop-helix domains (Figure 2C). To map the ρ 1 interacting region in Tax1BP1, we generated the N-terminal deletion constructs T2 and T3 that were tested for their ability to bind the TM3-4 loop of the ρ 1 subunit as above. To our surprise, the C-terminal 220 amino acids of construct T3 were sufficient for the binding, while none of the defined protein domains was required for the interaction with the ρ 1 TM3-4 loop (Figure 2C).

We estimated the relative binding strengths between the different Tax1BP1 constructs and the ρ 1 TM3-4 loop using a quantitative β -galactosidase assay. The results are presented as arbitrary β -galactosidase units (horizontal columns in Figure 2C). Compared with the wild-type Tax1BP1 (T1), construct T3 showed a small, statistically not significant reduction in binding strength, indicating that the C-terminal 220 amino acids of Tax1BP1 are the main region that mediated the binding to the ρ 1 TM3-4 loop. These results indicated that Tax1BP1 interacts with the ρ 1 TM3-4 loop *in vivo* and that its C-terminal 220 amino acids play an important role in this interaction.

Tax1BP1 and the ρ 1 subunit are co-localized at the cell membrane

In a next step we analysed the subcellular localization of Tax1BP1 and the GABA_C receptor ρ 1 subunit in mammalian cells. First, eukaryotic expression plasmids for TAX1BP1 and the ρ 1 subunit were separately transfected in HEK-293 cells. The localization of the expressed proteins was analysed using antibodies recognizing the extracellular ρ 1 N-terminus or the T7 epitope fused to the N-terminus of Tax1BP1. Inspection of the subcellular distribution of both proteins by conventional light microscopy demonstrated a comparable localization of Tax1BP1 and the GABA_C receptors ρ 1 subunit (Figure 3A). Specific signals were detected in cytosolic compartments surrounding the nucleus and in or near the outer cell membrane. Negative controls that were incubated with secondary antibodies only showed no signals (Figure 3B).

To compare the distribution of the binding partners, both proteins were co-expressed in the same cells and analysed as

Figure 3 Tax1BP1 is present in clusters of the outer cell membrane that contain the GABA_A receptor $\rho 1$ subunit

(A) HEK-293 cells transiently transfected with T7-tagged Tax1BP1 or GABA_A receptor $\rho 1$ subunits were incubated with specific primary antibodies, as indicated. Immunoreactivity was detected by secondary antibodies coupled with DTAF (green) or Texas Red (red) using conventional light microscopy. (B) No signals were visible when cells were incubated with secondary antibodies only. The lower panel shows a phase-contrast image of the cells. (C) Tax1BP1 and GABA_A receptor $\rho 1$ subunits were co-expressed in the same cell and the distribution of expressed proteins was analysed as in (A). Co-localization of Tax1BP1 and $\rho 1$ at the outer cell membrane can be seen in the merge of the two stainings, resulting in yellow signals. Arrows point to regions of different cytoplasmic localization of both proteins, while arrowheads indicate co-localization in cellular processes. (D) Confocal micrographs of a single z-stack of a HEK-293 cell co-transfected as in (C). The lower panel shows the merge of both upper panels and yellow signals indicate a co-localization of Tax1BP1 and $\rho 1$ subunits. Regions marked by arrows are shown in higher magnification in (E) and the boxed area is enlarged in (F). Arrowheads in (D) and (F) point to clusters at the outer cell membrane containing Tax1BP1 and $\rho 1$ subunits. In (E) and (F), fluorescence profiles were measured along the arrows and relative signal intensities are compared in the diagrams. Scale bars, 10 μ m.

above. Co-localization between Tax1BP1 and the $\rho 1$ subunit was clearly detected as yellow label in several regions of the outer cell membrane of HEK-293 cells (Figure 3C). In addition, specific signals for both proteins were superimposed in cellular processes (arrowheads in Figure 3C). Tax1BP1 and the $\rho 1$ subunit were also observed in the cytoplasm of transfected HEK-293 cells, but mostly at different localizations (arrows in Figure 3C). This is consistent with Tax1BP1 being a cytosolic protein that uses a different machinery for protein biosynthesis than the membrane targeted $\rho 1$ subunit. Indeed, when the same experiment was performed with non-permeabilized cells, the intracellular staining for Tax1BP1 disappeared completely and the $\rho 1$ subunit was detected at the membrane only, proving the cytosolic localization of Tax1BP1 (results not shown).

To verify our findings, we analysed co-transfected HEK-293 cells by confocal laser-scanning microscopy. Signals of a single z-stack specific for Tax1BP1 and the $\rho 1$ subunit demonstrated that both proteins were co-localized in several regions of the outer cell membrane, including cellular processes (Figure 3D). Tax1BP1 and the $\rho 1$ subunit were also present in the cytoplasm. However, as evident from a large amount of red and green label in the lower panel of Figure 3(D), the two proteins showed nearly

no intracellular co-localization, consistent with our data from Figures 3(A)–3(C).

The GABA_A receptor-specific antibody recognizes the extracellular N-terminus of the $\rho 1$ subunit, while Tax1BP1 binds to its intracellular TM3–4 loop and thus is present within the cytosol. As a result, red label arising from the binding of antibodies to the extracellular $\rho 1$ N-terminus is located at the outer surface of the cell membrane, while green signals represent Tax1BP1 inside the cell. Thus in the event of co-localization, red and green signals will only partially overlap generating a yellow region between both colours. Indeed, we observed the described pattern of red-yellow-green at several regions along the cell membrane (arrows in Figure 3D), which can be best seen in higher power views for the three indicated areas (Figure 3E). The spatial distribution of red and green signals across the outer cell membrane was measured (arrows in Figure 3E) and the fluorescence profiles were compared in the lower panels. Clearly, red and green signals labelling the positions of the $\rho 1$ subunit and Tax1BP1 relative to the membrane overlap in a middle region, visible in yellow.

The $\rho 1$ subunit did not appear homogeneously distributed at the cell surface, but was rather concentrated in clusters along the outer cell membrane (arrowheads in Figure 3D). Importantly,

Figure 4 Tax1BP1 is expressed in synaptic layers of the retina

(A) DNA fragments amplified by RT-PCR from cDNA, obtained after reverse transcription of total RNA isolated from different tissues, as indicated. Amplification of fragments for EF1 α ensured similar cDNA concentrations in all samples. Control reactions without the addition of cDNA (H₂O), or RT (below dashed lines) resulted in the absence of PCR products. (B) Retinal layers are indicated using phase contrast (IS, inner segments of photoreceptors). (C) Fluorescent micrograph of a vertical cryostat section through the adult mouse retina incubated with Tax1BP1-specific antibodies. Tax1BP1 was strongly expressed in both synaptic layers, the OPL and the IPL and in cell bodies of the GCL. In contrast, weak signals are present in the INL and no label was detected in the ONL. Inner segments of photoreceptors located in the upper part of the micrograph show a faint autofluorescence. Scale bar, 20 μ m.

Tax1BP1-specific signals were also present in these clusters. To compare the spatial distribution of these red and green signals in detail, the boxed area of Figure 3(D) was magnified and signal intensities were measured along the arrow drawn in Figure 3(F). The fluorescence profile shown in the inset unequivocally demonstrated that Tax1BP1 is present in clusters of the ρ 1 subunit. Based on these findings we concluded that Tax1BP1 and the ρ 1 subunit are present at the same subcellular compartments, which is a prerequisite for their physical interaction.

Tax1BP1 is expressed in the mammalian retina and is co-localized with GABA_C receptors

From the results presented so far we postulate a protein interaction between Tax1BP1 and the ρ 1 subunit of GABA_C receptors. Because GABA_C receptors are mainly expressed in the retina, we tested if Tax1BP1 is also present in this tissue. Semi-quantitative RT-PCR experiments detected a robust expression of Tax1BP1 in the retina (Figure 4A). In addition, similar transcript levels were observed for other neuronal or non-neuronal tissues. PCR products of about equal intensity for the housekeeping gene EF1 α indicated that comparable amounts of cDNA were used for each sample.

The distribution of GABA_C receptor ρ subunits in the retina has been extensively studied [5–7]. Therefore we analysed the retinal localization of Tax1BP1 and compared it with the expression pattern of GABA_C receptors. Upon staining vertical cryostat sections of the adult mouse retina with antibodies specific for Tax1BP1, label was present in both synaptic layers, the OPL (outer plexiform layer) and IPL (inner plexiform layer) (Figure 4C). No signals were detected in the ONL (outer nuclear layer) containing cell bodies of photoreceptors, and only faint label was observed in the INL (inner nuclear layer), where the somata of bipolar, horizontal and amacrine cells are located. In contrast, strong staining was present in the GCL (ganglion cell layer). A weak autofluorescence of the inner segments of photoreceptors is visible at the upper border of the micrograph.

Next, we compared the expression of Tax1BP1 and GABA_C receptors by double-labelling retinal sections with specific antibodies for both proteins. Tax1BP1 and GABA_C receptors showed a comparable expression in both synaptic layers of the retina (Figure 5A). Indeed, the merge of the two panels demonstrated a good co-localization of both proteins in the OPL and in the IPL (Figures 5B and 5C). In contrast, Tax1BP1 expressed in the GCL did not co-localize with GABA_C receptors, indicating that Tax1BP1 and GABA_C receptors are co-expressed in a subset of retinal neurons.

DISCUSSION

Macromolecular signalling complexes that physically link neurotransmitter receptors, ion channels, enzymes and scaffold proteins are important regulators for the neurotransmission in the CNS. In the present study, we identified Tax1BP1 as a new binding partner of GABA_C receptors. Antibodies that specifically recognize the extracellular N-termini of GABA_C receptor ρ subunits immunoprecipitated the liver regeneration-related protein 2 with a size of approx. 65 kDa that is a shorter variant of the 89.5 kDa protein Tax1BP1. The sequence of the NCBI database entry for the liver regeneration-related protein 2 (accession number AAM46928) does not start with a methionine residue, suggesting that N-terminal sequences are missing. Interestingly, Tax1BP1 is cleaved by members of the caspase3-like subfamily, e.g. caspase 6, resulting in a prominent proteolysis product of approx. 65 kDa [14]. Based on these data, we assume that an N-terminal region of Tax1BP1 was cleaved by caspases, generating a 65 kDa proteolytic fragment that bound to the immunoprecipitated GABA_C receptors and that we detected by MS. This assumption is supported by our finding that the C-terminal 220 amino acids of Tax1BP1 being identical with the liver regeneration-related protein 2 are sufficient for the interaction with the ρ 1 subunit. Furthermore, all peptides of the peptide mass ‘fingerprints’ that led to the identification of the liver regeneration-related protein 2 are also present in Tax1BP1.

GST pull-down and yeast two-hybrid experiments demonstrated a direct interaction between the intracellular TM3–4 loop of the ρ 1 subunit with the C-terminal 220 amino acids of Tax1BP1. In contrast, the same domains of the ρ 2 and ρ 3 subunits did not bind to Tax1BP1. This was surprising, because the mass ‘fingerprints’ of our initial immunoprecipitation identified only the ρ 2 subunit. An explanation for this discrepancy could be that (i) native GABA_C receptors are hetero-oligomeric protein complexes mostly composed of ρ 1 and ρ 2 subunits [24] and that (ii) the retinal expression level of the ρ 2 subunit is much higher than that of ρ 1 [25,26]. Thus, although we identified ρ 2 but not ρ 1 in our immunoprecipitate, both subunits are co-assembled in hetero-oligomeric GABA_C receptor pentamers, enabling ρ 1

Figure 5 Tax1BP1 and GABA_C receptors are co-expressed in synaptic layers of the retina

(A) Micrographs of vertical cryostat sections through an adult mouse retina double-immunolabelled for Tax1BP1 (red) and GABA_C receptors (green) demonstrate a similar distribution of both proteins in the synaptic layers of the retina. This can be best seen as yellow colour in the merge of the two stainings shown in higher magnification in (B). In contrast, cell bodies located in the GCL are labelled for Tax1BP1 but do not express GABA_C receptors. Regions of extensive co-localization in the OPL are indicated by arrowheads, while the boxed areas in the IPL are enlarged in (C). Scale bars, 20 μ m.

to bind Tax1BP1, while the higher expressed ρ 2 was detected in our mass 'fingerprints'. Alternatively, the proteolytic 65 kDa fragment of Tax1BP1 could simply fold into a three-dimensional structure different from the 89.5 kDa Tax1BP1, resulting in different binding characteristics for ρ subunits. Indeed, we found that the shorter fragment interacted with all ρ subunits, including ρ 2.

Tax1BP1 is an anti-apoptotic protein that is constitutively expressed in human cells and that binds to the Tax protein of the human T-cell leukaemia virus type 1. Tax is localized in the nucleus and in the cytoplasm of infected cells. There, it interacts with different host proteins such as Tax1BP1 in order to regulate viral and cellular transcription, signal transduction pathways

of the host cell and apoptosis [27,28]. Tax1BP1 also interacts with A20, a Cys2/Cys2 zinc-finger protein that is induced by inflammatory stimuli and inhibits apoptosis [14]. Binding of Tax1BP1 to the C-terminal zinc-finger domains of A20 mediated its anti-apoptotic activity [29]. Furthermore, Tax1BP1 is associated with the Rho GTPase-activating protein RhoGAP [30].

Because Tax1BP1 is a TRAF-6-binding protein it is also designated T6BP [18]. The interaction between Tax1BP and TRAF-6 is mediated by coiled-coil domains of Tax1BP1 that bind to the N-terminal ring- and zinc-finger domains of TRAF-6 [18]. Because Tax1BP uses its C-terminal 220 amino acids to interact with the ρ 1 TM3–4 loop, its coiled-coil domains are free to bind TRAF-6, enabling a simultaneous association of the three proteins. In addition to binding Tax1BP1, TRAF-6 interacts with members of the PKC- ζ interacting protein family that are termed p62 or ZIP1-3 [31]. Interestingly, as Tax1BP1, also ZIP proteins bind to the TM3–4 loops of GABA_C receptor ρ subunits and it was proposed that ZIP proteins function as a linker between GABA_C receptors and PKC- ζ [11]. Similar to Tax1BP1, ZIP proteins use different domains to bind GABA_C receptors, TRAF-6 and PKC- ζ and thus could serve as a scaffold between these proteins [11,31]. This hypothesis is supported by our finding that Tax1BP1 and the ZIP-binding PKC- ζ are co-localized with GABA_C receptors in the retina.

Tax1BP1 and its binding partners A20, RhoGAP, TRAF-6 and proteins of the ZIP family are associated with intracellular signal cascades that are regulated by interleukin-1, nerve growth factors, tumour necrosis factors and ubiquitin, effecting e.g. gene expression and apoptosis [18,29,31]. Recently, CRABP was described as a binding partner of the GABA_C receptor ρ 1 subunit in the retina [12]. The authors suggest that this interaction could serve as a link between the GABA signalling pathway and the control of gene expression in neurons. However, an influence of GABA_C receptors-mediated signalling on the above-mentioned factors such as interleukin-1 or tumour necrosis factor is not known. Interestingly, GABA_C receptor ρ 1 and ρ 2 subunits were expressed normally in apoptotic retinas of an animal model for secondary angle closure glaucoma [32].

Besides being expressed in the retina, GABA_C receptor ρ subunits were also found in other brain regions such as the hippocampus, cerebellum and the superior colliculus, as well as in the spinal cord [33–36]. Furthermore, ρ subunits were detected in secretory cells of the pituitary, in neurons of the gastrointestinal tract and in a neuroendocrine tumour cell line of the gut [37–40]. These findings suggest that in addition to regulating neurotransmission, GABA_C receptors are associated with secretory pathways. This idea is strengthened by the described binding between ρ 1 and Tax1BP1, because Tax1 itself interacts with several secretory pathways [41].

The lowest sequence similarity between ρ subunits is found in their intracellular TM3–4 loops. The same holds true when the TM3–4 loops of GABA_C receptors are compared with subunits of GABA_A and glycine receptors. Because of this sequence heterogeneity, these domains offer an ideal molecular mechanism for the diverse regulation of different receptor types, simply by specifically interacting with a large variety of intracellular proteins. Thus it was not surprising that also the immunoprecipitated Tax1BP1 bound to this domain. Tax1BP used a C-terminal region to interact with the ρ 1 subunit, leaving its coiled-coil and helix–loop–helix domains accessible for other protein interactions as well as for dimerization [18]. By this means one can imagine a picture in which the intracellular TM3–4 loop of a GABA_C receptor ρ 1 subunit binds to a C-terminal region of Tax1BP1. Dimerization of Tax1BP1 via its second coiled-coil domain could cross-link two GABA_C receptors. If the cross-linked GABA_C

receptors contain more than one $\rho 1$ subunit, additional Tax1BP1 proteins can bind and dimerize, forming connections to other GABA_C receptors which would generate large clusters at synaptic sites. In addition, the first and third coiled-coil domains and the helix–loop–helix regions of Tax1BP1 are still accessible for other protein interactions, thus enabling the formation of multimeric signal complexes at GABA_C receptor-expressing synapses in the retina.

We thank Melanie Rose and Angela Graulich for continuous support in staining retinal cryostat sections and Nadja Schröder for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

- Bormann, J. (2000) The 'ABC' of GABA receptors. *Trends Pharmacol. Sci.* **21**, 16–19
- Rudolph, U., Crestani, F. and Möhler, H. (2001) GABA(A) receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol. Sci.* **22**, 188–194
- Roska, B. and Werblin, F. (2001) Vertical interactions across ten parallel, stacked representations in the mammalian retina. *Nature* **410**, 583–587
- Enz, R. (2001) GABA_C receptors: a molecular view. *Biol. Chem.* **382**, 1111–1122
- Enz, R., Brandstätter, J. H., Wässle, H. and Bormann, J. (1996) Immunocytochemical localization of the GABA_C receptor rho subunits in the mammalian retina. *J. Neurosci.* **16**, 4479–4490
- Fletcher, E. L., Koulen, P. and Wässle, H. (1998) GABA_A and GABA_C receptors on mammalian rod bipolar cells. *J. Comp. Neurol.* **396**, 351–365
- Koulen, P., Brandstätter, J. H., Enz, R., Bormann, J. and Wässle, H. (1998) Synaptic clustering of GABA_C receptor rho-subunits in the rat retina. *Eur. J. Neurosci.* **10**, 115–127
- Sedelnikova, A. and Weiss, D. S. (2002) Phosphorylation of the recombinant rho1 GABA receptor. *Int. J. Dev. Neurosci.* **20**, 237–246
- Hanley, J. G., Jones, E. M. and Moss, S. J. (2000) GABA receptor rho1 subunit interacts with a novel splice variant of the glycine transporter, GLYT-1. *J. Biol. Chem.* **275**, 840–846
- Hanley, J. G., Koulen, P., Bedford, F., Gordon-Weeks, P. R. and Moss, S. J. (1999) The protein MAP-1B links GABA_C receptors to the cytoskeleton at retinal synapses. *Nature* **397**, 66–69
- Croci, C., Brandstätter, J. H. and Enz, R. (2003) ZIP3, a new splice variant of the PKC- ξ -interacting protein family, binds to GABA_C receptors, PKC- ξ and Kv β 2. *J. Biol. Chem.* **278**, 6128–6135
- Song, X. Q., Meng, F., Ramsey, D. J., Rippes, H. and Qian, H. (2005) The GABA rho1 subunit interacts with a cellular retinoic acid binding protein in mammalian retina. *Neuroscience* **136**, 467–475
- Meixner, A., Haverkamp, S., Wässle, H., Fuhrer, S., Thalhammer, J., Kropf, N., Bittner, R. E., Lassmann, H., Wiche, G. and Probst, F. (2000) MAP1B is required for axon guidance and is involved in the development of the central and peripheral nervous system. *J. Cell Biol.* **151**, 1169–1178
- De Valck, D., Jin, D. Y., Heynink, K., Van de Craen, M., Contreras, R., Fiers, W., Jeang, K. T. and Beyaert, R. (1999) The zinc finger protein A20 interacts with a novel anti-apoptotic protein which is cleaved by specific caspases. *Oncogene* **18**, 4182–4190
- Gachon, F., Peleraux, A., Thebault, S., Dick, J., Lemasson, I., Devaux, C. and Mesnard, J. M. (1998) CREB-2, a cellular CRE-dependent transcription repressor, functions in association with Tax as an activator of the human T-cell leukemia virus type 1 promoter. *J. Virol.* **72**, 8332–8337
- Jin, D. Y., Teramoto, H., Giam, C. Z., Chun, R. F., Gutkind, J. S. and Jeang, K. T. (1997) A human suppressor of c-Jun N-terminal kinase 1 activation by tumor necrosis factor alpha. *J. Biol. Chem.* **272**, 25816–25823
- Wu, K., Bottazzi, M. E., de la Fuente, C., Deng, L., Gitlin, S. D., Maddukuri, A., Dadgar, S., Li, H., Vertes, A., Pumfery, A. et al. (2004) Protein profile of tax-associated complexes. *J. Biol. Chem.* **279**, 495–508
- Ling, L. and Goeddel, D. V. (2000) T6BP, a TRAF-6-interacting protein involved in IL-1 signaling. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9567–9572
- Seeber, S., Humeny, A., Herkert, M., Rau, T., Eschenhagen, T. and Becker, C. M. (2004) Formation of molecular complexes by N-methyl-D-aspartate receptor subunit NR2B and ryanodine receptor 2 in neonatal rat myocard. *J. Biol. Chem.* **279**, 21062–21068
- Croci, C., Sticht, H., Brandstätter, J. H. and Enz, R. (2003) Group I metabotropic glutamate receptors bind to protein phosphatase 1C. Mapping and modeling of interacting sequences. *J. Biol. Chem.* **278**, 50682–50690
- Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745–2752
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162**, 156–159
- 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
- Zhang, D., Pan, Z. H., Zhang, X., Brideau, A. D. and Lipton, S. A. (1995) Cloning of a gamma-aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11756–11760
- Enz, R., Brandstätter, J. H., Hartveit, E., Wässle, H. and Bormann, J. (1995) Expression of GABA receptor rho 1 and rho 2 subunits in the retina and brain of the rat. *Eur. J. Neurosci.* **7**, 1495–1501
- Enz, R. and Cutting, G. R. (1999) GABA_C receptor rho subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. *Eur. J. Neurosci.* **11**, 41–50
- Grassmann, R., Aboud, M. and Jeang, K. T. (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* **24**, 5976–5985
- Jeang, K. T., Giam, C. Z., Majone, F. and Aboud, M. (2004) Life, death, and tax: role of HTLV-1 oncoprotein in genetic instability and cellular transformation. *J. Biol. Chem.* **279**, 31991–31994
- Klinkenberg, M., Van Huffel, S., Heynink, K. and Beyaert, R. (2001) Functional redundancy of the zinc fingers of A20 for inhibition of NF- κ B activation and protein–protein interactions. *FEBS Lett.* **498**, 93–97
- Nagaraja, G. M. and Kandpal, R. P. (2004) Chromosome 13q12 encoded Rho GTPase activating protein suppresses growth of breast carcinoma cells, and yeast two-hybrid screen shows its interaction with several proteins. *Biochem. Biophys. Res. Commun.* **313**, 654–665
- Geetha, T. and Wooten, M. W. (2002) Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett.* **512**, 19–24
- Dyka, F. M., May, C. A. and Enz, R. (2004) Metabotropic glutamate receptors are differentially regulated under elevated intraocular pressure. *J. Neurochem.* **90**, 190–202
- Rozzo, A., Armellini, M., Franzot, J., Chiaruttini, C., Nistri, A. and Tongiorgi, E. (2002) Expression and dendritic mRNA localization of GABA_C receptor rho1 and rho2 subunits in developing rat brain and spinal cord. *Eur. J. Neurosci.* **15**, 1747–1758
- Alakuijala, A., Palgi, M., Wegelius, K., Schmidt, M., Enz, R., Paulin, L., Saarna, M. and Pasternack, M. (2005) GABA receptor rho subunit expression in the developing rat brain. *Brain Res. Dev. Brain Res.* **154**, 15–23
- Boue-Grabot, E., Roudbaraki, M., Bascles, L., Tramu, G., Bloch, B. and Garret, M. (1998) Expression of GABA receptor rho subunits in rat brain. *J. Neurochem.* **70**, 899–907
- Zheng, W., Xie, W., Zhang, J., Strong, J. A., Wang, L., Yu, L., Xu, M. and Lu, L. (2003) Function of gamma-aminobutyric acid receptor/channel rho 1 subunits in spinal cord. *J. Biol. Chem.* **278**, 48321–48329
- Fletcher, E. L., Clark, M. J., Senior, P. and Furness, J. B. (2001) Gene expression and localization of GABA_C receptors in neurons of the rat gastrointestinal tract. *Neuroscience* **107**, 181–189
- Boue-Grabot, E., Taupignon, A., Tramu, G. and Garret, M. (2000) Molecular and electrophysiological evidence for a GABA_C receptor in thyrotropin-secreting cells. *Endocrinology* **141**, 1627–1632
- Jansen, A., Hoepfner, M., Herzig, K. H., Riecken, E. O. and Scherubel, H. (2000) GABA_C receptors in neuroendocrine gut cells: a new GABA-binding site in the gut. *Pflügers Arch.* **441**, 294–300
- Gamel-Didelon, K., Kunz, L., Fohr, K. J., Gratzl, M. and Mayerhofer, A. (2003) Molecular and physiological evidence for functional γ -aminobutyric acid (GABA)-C receptors in growth hormone-secreting cells. *J. Biol. Chem.* **278**, 20192–20195
- Alefantis, T., Jain, P., Ahuja, J., Mostoller, K. and Wigdahl, B. (2005) HTLV-1 Tax nucleocytoplasmic shuttling, interaction with the secretory pathway, extracellular signaling, and implications for neurologic disease. *J. Biomed. Sci.* **12**, 961–974

Received 10 July 2006/7 September 2006; accepted 25 September 2006

Published as BJ Immediate Publication 25 September 2006, doi:10.1042/BJ20061036