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The TAX1 binding protein 1 is expressed in the retina and interacts with the GABA_C receptor ρ 1 subunit

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Running Title: Tax1BP1 binds to GABA_C receptors

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Abstract

Macromolecular signaling 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 receptors in the retina, we used antibodies specific for GABA_C receptor ρ 1-3 subunits. Analysis of immunoprecipitated proteins by MALDI-TOF mass spectrometry identified the liver regeneration-related protein 2 that is identical to amino acids 253-813 of the Tax1 binding protein Tax1BP1. 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.

Keywords: Tax1BP1, synaptic signaling complex, protein interaction, immunoprecipitation, MALDI-TOF.

Introduction

Neuronal excitability is regulated by a delicate balance between excitatory and inhibitory neurotransmission. γ -Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter receptor in the central nervous system (CNS). The molecule binds to three GABA receptor types, termed A, B and C. While GABA_A and GABA_C receptors form ligand gated ion channels, GABA_B receptors couple to G-protein associated signal cascades [1, 2]. GABA_C receptors are insensitive to the classical GABA_A receptor antagonist bicuculline, do not respond to anesthetics such as barbiturates and benzodiazepines and have a faster kinetic when compared to 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 heterooligomeric 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 transmembrane domains. The intracellular loop between TM3 and TM4 contains consensus sequences for enzymes and phosphorylation of this region was demonstrated [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, the glycine transporter GLYT-1, the cellular retinoic acid binding protein CRABP I and the microtubuli associated protein MAB-1B [9-12]. However, MAP-1B knock-out 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 Tax1 binding protein 1 (Tax1BP1) as a new interactor of this receptor class. Tax1BP1 was identified by different groups in yeast two-hybrid screens against the human T-cell leukemia virus type I Tax1 protein, the zinc-finger protein A20 and the tumor necrosis factor associated factor TRAF6 [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 specifically bound to the TM3-4 loop of the $\rho 1$ 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 retinæ of adult rat were homogenized in 1.5ml of buffer I (150mM NaCl, 50mM Tris-HCl, pH=7.5) containing 1% Nonidet P-40 (Sigma-Aldrich, Taufkirchen, Germany), 0.5% sodium deoxycholate, 10mg/ml DNaseI and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany) using a glass/Teflon homogenizer. The sample was sonificated with 3 bursts for 10s at 300W and incubated for 30min under slow agitation. Subsequently, ultracentrifugation was carried out at 100.000xg for 1h. The supernatant was pre-cleared for 2h with 25µl of protein A conjugated Agarose (Roche Diagnostics). After sedimentation of the beads, the supernatant was incubated with 50µl of GABA_C receptor specific antibodies [5] for 2h under slow agitation, subjected with 25µl of fresh protein A conjugated Agarose and agitated for another 16h. Thereafter, the beads were sedimented, washed 2 times in 1ml of buffer I and 2 times in buffer II (500mM NaCl, 50mM Tris-HCl, pH=7.5) containing 0.1% Nonidet P-40, 0.05% sodium deoxycholate and the protease inhibitor cocktail. Finally, bound proteins were eluted from the beads by boiling in 30µl SDS sample buffer for 10min. All protein preparation steps were carried out on ice or at 4°C.

Peptide mass fingerprinting: The identification of immunoprecipitated proteins by mass spectrometry 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 50µl of 25mM NH₄HCO₃ containing 0.2µg/µl sequencing grade trypsin for the enzymatic digestion of the purified proteins. The generated peptide fragments were eluted from the gel, eluates were collected, dried and peptides were resuspended in 10µl of H₂O containing 0.1% trifluoroacetic acid. Subsequently, peptide solutions were diluted 1:10 to 1:50 with H₂O and 0.5µl of diluted samples were mixed with 0.5µl of 3-hydroxy- α -cyanocinnamic acid matrix, dotted onto a steel target and air-dried. MALDI-TOF analysis was performed on an Autoflex (Bruker Daltonics, Bremen, Germany) in the reflector mode, using a nitrogen laser (337nm) for sample desorption and an acceleration with 19kV after a delay of 3500ns. Masses obtained by MALDI-TOF-MS were used for a peptide mass fingerprint search using the search program MS-Fit and the NCBI protein data base.

Pull-down techniques. Coding sequences for the rat TM3-4 loops of the $\rho 1$ -3 subunits, of the $\alpha 1$, $\beta 2$, $\gamma 2$ subunits of the GABA_A receptor and of the $\alpha 1$ subunit of the glycine receptor of were ligated in-frame to the coding sequence of glutathione-S-transferase (GST) 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, as described below. Plasmids were transformed in *E.coli* BL21(DE3)pLysS and protein expression was induced by adding 1mM isopropyl-beta-D-thiogalactoside (IPTG; Sigma-Aldrich). Fusion proteins were purified under native conditions and immobilized to glutathione Sepharose as described [20]. Similar concentrations of immobilized fusion proteins were incubated with the cytosolic fraction of *E.coli* expressing T7-tagged proteins for 1h at 4°C under slow agitation, followed by 3 washes (4.3mM Na₂HPO₄, 1.47mM KH₂PO₄, 137mM NaCl, 2.7mM KCl, 0.1% Triton X-100, pH=7.3). Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE and analyzed by Western blotting using a monoclonal anti-T7 immuneserum and the enhanced chemoluminescence system (ECL; Amersham, Braunschweig, Germany). Unless otherwise stated, all reagents were purchased from Novagen (Madison, WI).

To analyze 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 20.000xg for 30min. The resulting protein preparation was incubated over night at 4°C with 30μl of a 50% (v/v) suspension of protein A-Agarose (Amersham) that was preincubated with antibodies specific for GABA_C receptors (50μl; [5]) or for mGluR2 (5μg; Upstate Biotechnology, Lake Placid, NY) as control. Precipitates were washed four times with 500μl of 150mM NaCl, 50mM Tris-HCl, 0.1% Triton X-100, pH=7.5, bound proteins were eluted from the beads by boiling in 20μl SDS sample buffer and Tax1BP1 was detected on Western Blots using a specific immuneserum (sc-15274; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA).

Yeast two-hybrid techniques. The coding sequence of the TM3-4 loop of the rat $\rho 1$ 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 p1 bait vector and the resulting bait strain was subsequently transformed with 1µg 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 o-Nitrophenyl-β-D-galactopyranoside (ONPG; 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).

Cell culture and immunocytochemistry. Coding sequences of the GABA_C receptor p1 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 promotor. Human embryonic kidney cells (HEK-293 cells, ATCC # CRL 1573) were grown in 3cm dishes on glass cover slips coated with poly-L-lysine to about 80% confluency and transfected with 1µg of each plasmid using the calcium phosphate method [21]. After 2 days, the cells were washed 3 times with PBS, fixed in 4% formaldehyde/PBS for 10-15min on ice, washed again as above and blocked with 10% fetal calf serum containing 2% BSA and 0.1% Triton X-100 for 10min. Subsequently, cells were incubated for 2h 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 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF; 1:300; green fluorescence) conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (Dianova, Hamburg, Germany) were applied for 45min to reveal the binding sites of the primary antibodies. The cells were extensively washed and mounted in Mowiol (Sigma-Aldrich). Labeled proteins were examined with an Leica inverted microscope (DMIRB; Bensheim, Germany) equipped with a cooled MicroMax CCD camera (Princeton Instruments, Stanford, CA) 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 (Scion Corporation, Frederick, MD).

Isolation of RNA and RT-PCR. Total RNA was extracted from adult rat tissues following a method of [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'-GTCTGCCCAGAAAGCTCAG-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 reverse transcriptase and showed no PCR products. Both PCR products were identified by DNA sequencing.

Immunohistochemistry. Adult mice were anesthetized with halothane and decapitated. Retinal cryostat sections with a thickness of 12 μ m were prepared as described [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 AlexaTM 594 (red fluorescence) and AlexaTM 488 (green fluorescence) donkey anti-goat or donkey anti-rabbit IgG (H+L) conjugates, respectively (1:500; Molecular Probes, Eugene, Oregon, USA). 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 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 SyproRubyTM 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 mass spectrometry. The resulting peptide mass fingerprints (Fig. 1) were aligned to theoretical trypsin digests of proteins listed in the NCBI data base and yielded several potential GABA_C receptor interactors (Tab. 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 Tab. 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 (Fig. 2A, upper 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 Tax1 binding protein 1 (Tax1BP1; accession number NP_001004199) are identical to the liver regeneration-related protein 2. Interestingly, the 89.5 kDa protein Tax1BP1 is cleaved by caspases resulting in a prominent proteolysis product of about 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 analyzed Tax1BP1 for its ability to bind the

TM3-4 loops of ρ subunits and found a specific interaction with the $\rho 1$ subunit (Fig. 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 (Fig. 2A, right panel). From this data we concluded that Tax1BP1 specifically interacts with the $\rho 1$ TM3-4 loop and thus, we focused on Tax1BP1 for all future experiments.

Tax1BP1 and the TM3-4 loop of the $\rho 1$ subunit interact *in-vivo*

To analyze, 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 (Fig. 2B).

Furthermore, we transformed the coding sequences of Tax1BP1 and the $\rho 1$ TM3-4 loop, fused to the activation and DNA binding domains of the Gal4 transcription factor, into yeast cells. Binding between Tax1BP1 and the $\rho 1$ TM3-4 loop was demonstrated by the ability of transformants to grow on selective media upon activation of the His3 reporter gene (Fig. 2C, construct T1). Tax1BP1 is composed of 813 amino acids and contains three coiled-coil and two helix-loop-helix domains (Fig. 2C). To map the $\rho 1$ 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 $\rho 1$ 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 $\rho 1$ TM3-4 loop (Fig. 2C).

We estimated the relative binding strengths between the different Tax1BP1 constructs and the $\rho 1$ TM3-4 loop using a quantitative β -galactosidase assay. The results are presented as arbitrary β -galactosidase units (horizontal columns in Fig. 2C). Compared to 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 $\rho 1$ TM3-4 loop. These results indicated that Tax1BP1 interacts with the $\rho 1$ TM3-4 loop *in-vivo* and that its C-terminal 220 amino acids play an important role in this interaction.

Tax1BP1 and the $\rho 1$ subunit are co-localized at the cell membrane

In a next step we analyzed the subcellular localization of Tax1BP and the GABA_C receptor $\rho 1$ subunit in mammalian cells. First, eukaryotic expression plasmids for TAX1BP1 and the $\rho 1$ subunit were separately transfected in HEK-293 cells. The localization of the expressed proteins was analyzed using antibodies recognizing the extracellular $\rho 1$ 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 $\rho 1$ subunit (Fig. 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 (Fig. 3B).

To compare the distribution of the binding partners, both proteins were co-expressed in the same cells and analyzed as 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 (Fig. 3C). In addition, specific signals for both proteins were superimposed in cellular processes (arrowheads in Fig. 3C). Tax1BP1 and the $\rho 1$ subunit were also observed in the cytoplasm of transfected HEK-293 cells, however mostly at different localizations (arrows in Fig. 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 (data not shown).

To verify our findings, we analyzed 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 (Fig. 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 Fig. 3D, The two proteins showed nearly no intracellular co-localization, consistent with our data from Fig. 3A-C.

The GABA_C 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 colors. Indeed, we observed the described pattern of red-yellow-green at several regions along the cell membrane (arrows in Fig. 3D), which can be best seen in higher power views for the three indicated areas (Fig. 3E). The spatial distribution of red and green signals across the outer cell membrane was measured (arrows in Fig. 3E) and the fluorescence profiles were compared in the lower panels. Clearly, red and green signals labeling 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 Fig. 3D). Importantly, 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 Fig. 3D area was magnified and signal intensities were measured along the arrow drawn in Fig. 3F. The fluorescence profile shown in the inset unequivocally demonstrated that Tax1BP1 is present in clusters of the $\rho 1$ subunit. Based on these findings we concluded that Tax1BP1 and the $\rho 1$ subunit are present at the same sub-cellular 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 data presented so far we postulate a protein interaction between Tax1BP1 and the $\rho 1$ 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 (Fig. 4A). In addition, similar transcript levels were observed for other neuronal or non-neuronal tissues. PCR products of about equal intensity for the house keeping 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 analyzed 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 outer (OPL) and the inner (IPL) plexiform layer (Fig. 4C). No signals were detected in the outer nuclear layer (ONL) containing cell bodies of photoreceptors, and only faint label was observed in the inner nuclear layer (INL), where the somata of bipolar, horizontal and amacrine cells are located. In contrast, strong staining was present in the ganglion cell layer (GCL). 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-labeling retinal sections with specific antibodies for both proteins. Tax1BP1 and GABA_C receptors showed a comparable expression in both synaptic layers of the retina (Fig. 5A). Indeed, the merge of the two panels demonstrated a good co-localization of both proteins in the OPL and in the IPL (Fig. 5B, C). 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 signaling complexes that physically link neurotransmitter receptors, ion channels, enzymes and scaffold proteins are important regulators for the neurotransmission in the CNS. In this 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 about 65 kDa that is a shorter variant of the 89.5 kDa protein Tax1BP1. The sequence of the NCBI data base entry for the liver regeneration-related protein 2 (accession number AAM46928) does not start with a methionine, 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 about 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 mass spectrometry. This assumption is supported by our finding that the C-terminal 220 amino acids of Tax1BP1 being identical to 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 Tax1BP. In contrast, the same domains of the ρ 2 and ρ 3 subunits did not bind to Tax1BP1. This was surprising, because the mass finger prints of our initial immunoprecipitation identified only the ρ 2 subunit. An explanation for this discrepancy could be that (i) native GABA_C receptors are heterooligomeric 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 heterooligomeric GABA_C receptor pentamers, enabling ρ 1 to bind Tax1BP1, while the higher expressed ρ 2 was detected in our mass finger prints. Alternatively, the proteolytic 65 kDa fragment of Tax1BP1 could simply fold into a 3-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 leukemia 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 TRAF6 binding protein it is also designated T6BP [18]. The interaction between Tax1BP and TRAF6 is mediated by coiled-coil domains of Tax1BP1 that binds to the N-terminal ring- and zinc-finger domains of TRAF6 [18]. Because Tax1BP uses its C-terminal 220 amino acids to interact with the $\rho 1$ TM3-4 loop, its coiled-coil domains are free to bind TRAF6, enabling a simultaneous association of the three proteins. In addition to bind Tax1BP1, TRAF6 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, TRAF6 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, TRAF6 and proteins of the ZIP family are associated with intracellular signal cascades that are regulated by interleukin-1, nerve growth factors, tumor necrosis factors and ubiquitin, effecting e.g. gene expression and apoptosis [18, 29, 31]. Recently, the cellular retinoic acid binding protein CRABP was described as a binding partner of the GABA_C receptor $\rho 1$ subunit in the retina [12]. The authors suggest that this interaction could serve as a link between the GABA signaling pathway and the control of gene expression in neurons. However, an influence of GABA_C receptors mediated signaling on the above mentioned factors such as interleukin-1 or tumor necrosis factor is not known.

Interestingly, GABA_C receptor $\rho 1$ and $\rho 2$ subunits were expressed normally in apoptotic retinæ 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 tumor cell line of the gut [37-40]. These findings suggest that in addition to regulate neurotransmission, GABA_C receptors are associated with secretory pathways. This idea is strengthened by the described binding between $\rho 1$ 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 $\rho 1$ 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 $\rho 1$ 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.

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Figure Legends

Fig. 1: MALDI-TOF analysis of immunoprecipitated proteins using GABA_C receptor specific antibodies. The spectra show relative peak intensities of peptide masses analyzed by MALDI-TOF mass spectrometry. Peaks derived from peptides that matched the theoretical trypsin digests of proteins in the NCBI protein data base are indicated by stars and the identified proteins are labeled in the upper corner of each spectrum. Bovine serum albumin (BSA) served as a positive control and the indicated negative control was generated from a gel fragment not containing protein.

Fig. 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 Commassie-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 of 3 yeast clones. Error bars are \pm SE. (cc – coiled-coil, HLH – helix-loop-helix; (-) – negative control)

Fig. 3: Tax1BP1 is present in clusters of the outer cell membrane that contain the GABA_C receptor ρ 1 subunit. (A) HEK-293 cells transiently transfected with T7-tagged Tax1BP1 or GABA_C receptor ρ 1 subunits were incubated with specific primary antibodies, as indicated. Immunoreactivity was detected by secondary antibodies coupled to 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_C receptor $\rho 1$ subunits were co-expressed in the same cell and the distribution of expressed proteins was analyzed 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 are 10 μm .

Fig. 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 reverse transcriptase (below dashed lines) resulted in the absence of PCR products. **(B)** Retinal layers are indicated using phase contrast (IS - inner segments of photoreceptors, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer). **(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. The scale bar is 20 μm .

Fig. 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-immunolabeled 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 color in the merge of the two stainings shown in higher magnification in (**B**). In contrast, cell bodies located in the GCL are labeled 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 are 20 μ m.

Tab.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 to amino acids 253-813 of Tax1BP1. Two peptides of the liver regeneration-related protein 2 with m/z values larger than 3300 are not labeled in Fig. 1A.

candidate	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 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 1

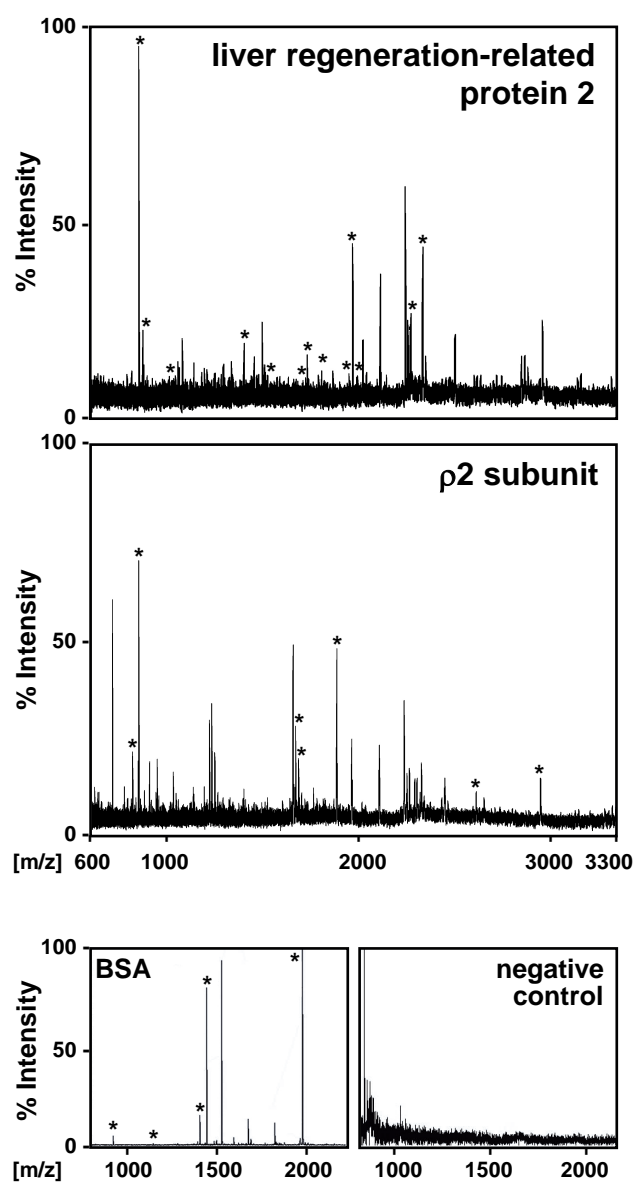




Figure 3

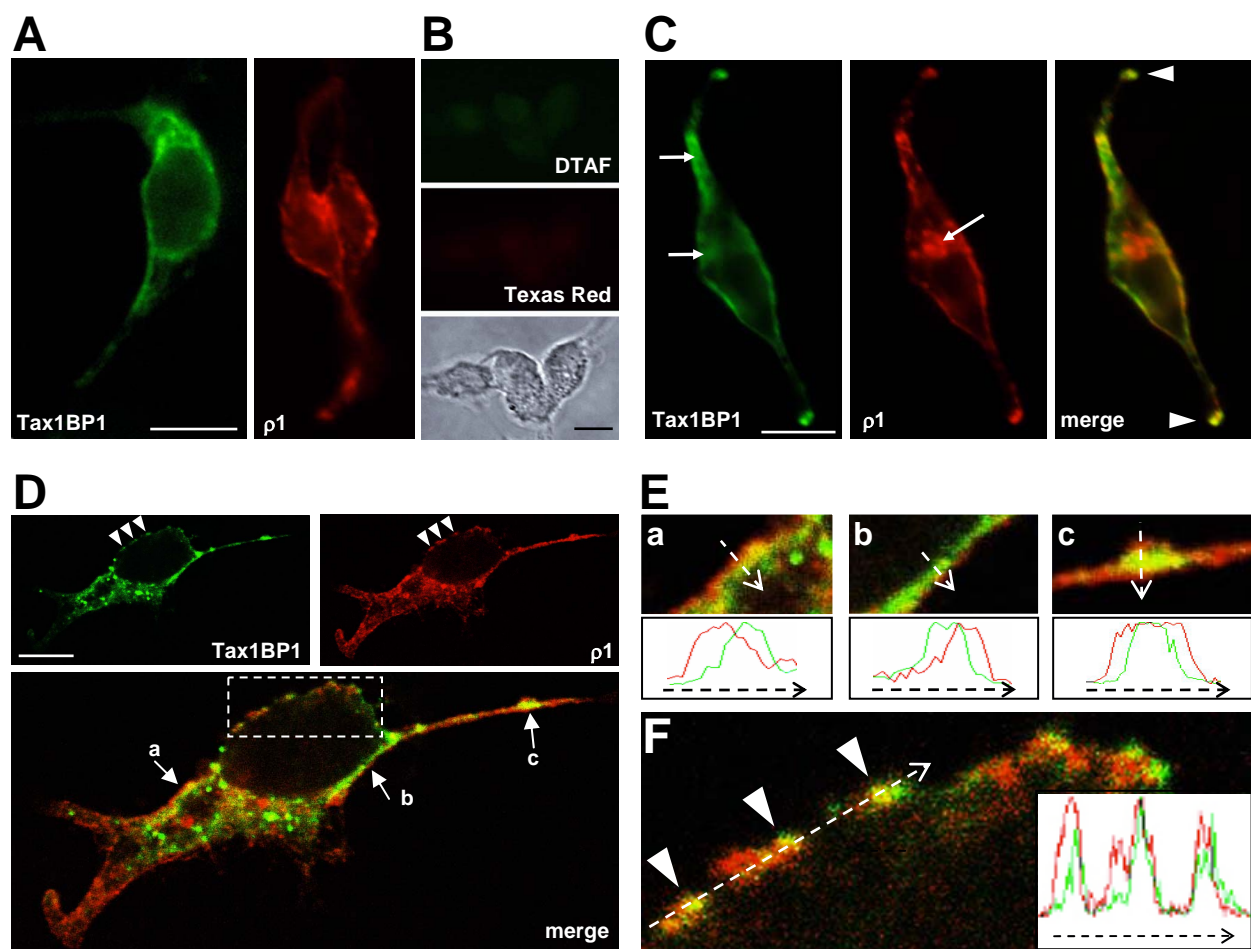


Figure 4

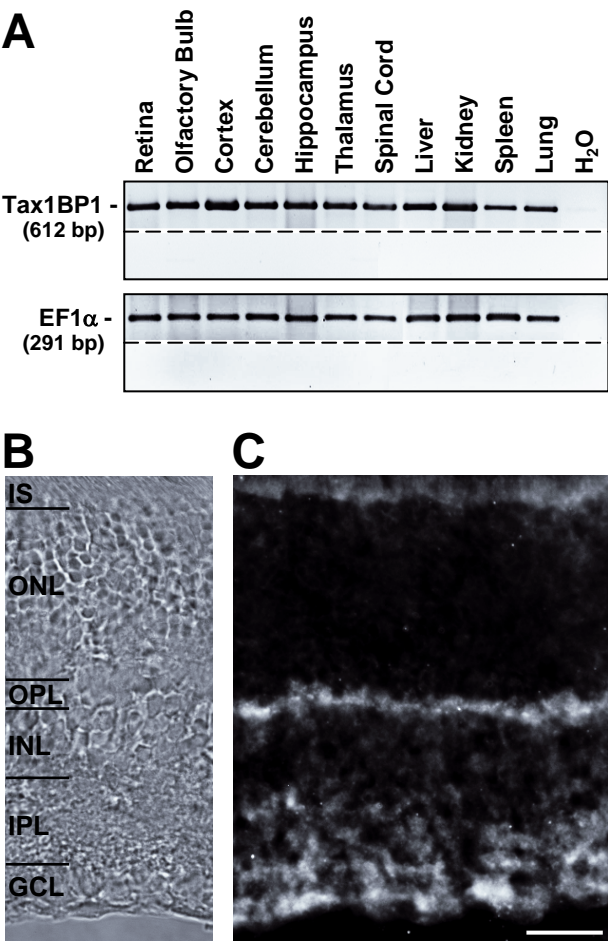


Figure 5

