Identification and molecular characterization of BP75, a novel bromodomain-containing protein

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Abstract We here describe the identification and characterization of a novel bromodomain-containing protein, the bromodomain protein of 75 kDa (BP75). Initially, we identified BP75 in a two-hybrid screening for proteins that interact with the first PDZ (acronym for post-synaptic density protein PSD-95, *Drosophila* discs large tumor suppressor DlgA and the tight junction protein ZO-1) domain in protein tyrosine phosphatase-BAS-like (PTP-BL). We found that BP75 is expressed ubiquitously and show that both BP75 and a PTP-BL deletion mutant consisting of the first PDZ domain are located mainly in the nucleus, although cytoplasmic localization is also evident. Full-length PTP-BL, on the contrary, is predominantly localized in the cytoplasm, although some basal nuclear staining is observed. The described molecular interaction may reflect a mechanism of coupling submembraneous signalling events and nuclear events.

Key words: Protein-protein interaction; Protein tyrosine phosphatase; Bromodomain; Signalling

1. Introduction

The accuracy of the signal transduction process, whereby extracellular signals evoke a specific cellular response, largely relies on mechanisms that regulate steps in the transmembrane transmission and the integration or divergence of signalling pathways within the cells' cytosol and nucleus. An important mechanism to achieve specificity in signalling is the use of scaffold, anchoring and adapter proteins [1]. Such proteins are considered organizers of multiprotein complexes, creating 'signallosomes' with specialized functions at restricted microcompartments [1]. Protein modules like src homology type 2, src homology type 3, WW, LIM, WD, phosphotyrosine binding, pleckstrin homology and PDZ (acronym for post-synaptic density protein PSD-95, *Drosophila* discs large tumor suppressor DlgA and the tight junction protein ZO-1) domains form the basal building blocks and play an important role in the architecture of signal transduction complexes [2].

The mouse protein tyrosine phosphatase-BAS-like (PTP-BL) [3] or RIP [4] and the human homologue of this protein named PTP-BAS [5], PTPL1 [6], PTP1E [7] or FAP-1 [8] have several characteristics (Fig. 1) that make them potential organizers of signal transduction complexes. PTP-BL harbors a FERM domain [9], a domain commonly found in cytoskeleton-associated proteins like ezrin, radixin, moesin, merlin and talin [10], that directs the putative multiprotein complex to its highly restricted submembraneous site (Cuppen et al., submitted). Furthermore, PTP-BL contains five PDZ domains, small (about 90 amino acids (aa)) protein modules [11] that have now been identified in many members of a diverse group of proteins [12] and can mediate protein-protein interactions [13,14]. PDZ domains associate with transmembrane proteins [15], cytoskeletal components [16] and signal transduction enzymes [14]. The five PDZ domains in PTP-BL may thus enable the formation of large multiprotein complexes in which the phosphotyrosine phosphatase action of PTP-BL itself can be combined with potential downstream substrates, competitor signalling proteins and putative upstream regulatory molecules. Indeed, proteins that interact with PDZ domains of PTP-BL have already been described. The GTPase-activating protein for Rho, phosphatase-associated Rho GTPase-1, interacts with PDZ-IV [17], the small adaptor protein RIL, that is an in vitro substrate for PTP-BL, interacts with both PDZ-II and IV [18] and B-type ephrins can bind to PDZ-IV [19]. The Fas receptor has also been described to bind to PDZ-II in the human homologue of PTP-BL [8,20], but this interaction could not be confirmed in mouse [21]. Recently, PDZ-I was found to interact with IκBα [22], suggesting a regulatory role for PTP-BL in transcriptional control via NF-κB.

We here report on the identification of yet another protein, the novel bromodomain-containing protein of about 75 kDa (BP75), that interacts with PDZ-I in PTP-BL. The bromodomain, originally described as an evolutionary highly conserved 80 aa domain in human, *Drosophila* and yeast proteins [23], is now defined as a conserved sequence of approximately 110 aa that is found in over 40 proteins [24]. Many of these proteins are involved in transcriptional control and recently, structural data from the bromodomain of the histone-acetyltransferase (HAT) co-activator P/CAF provided evidence that bromodomains can bind acetylated lysines [25]. Bromodomains may thus be important determinants for targetting proteins or protein complexes to specific chromosomal sites by binding to acetylated histones and thereby regulating transcriptional activity. Our results suggest that BP75 may have a role in linking signalling events in the PTP-BL multiprotein complex and the nucleus.

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Abbreviations: BP75, bromodomain protein of 75 kDa; BYC, acronym for BP75, YN92 and C01H6.7; PTP-BL, protein tyrosine phosphatase-BAS-like; PDZ, acronym for post-synaptic density protein PSD-95, *Drosophila* discs large tumor suppressor DlgA and the tight junction protein ZO-1

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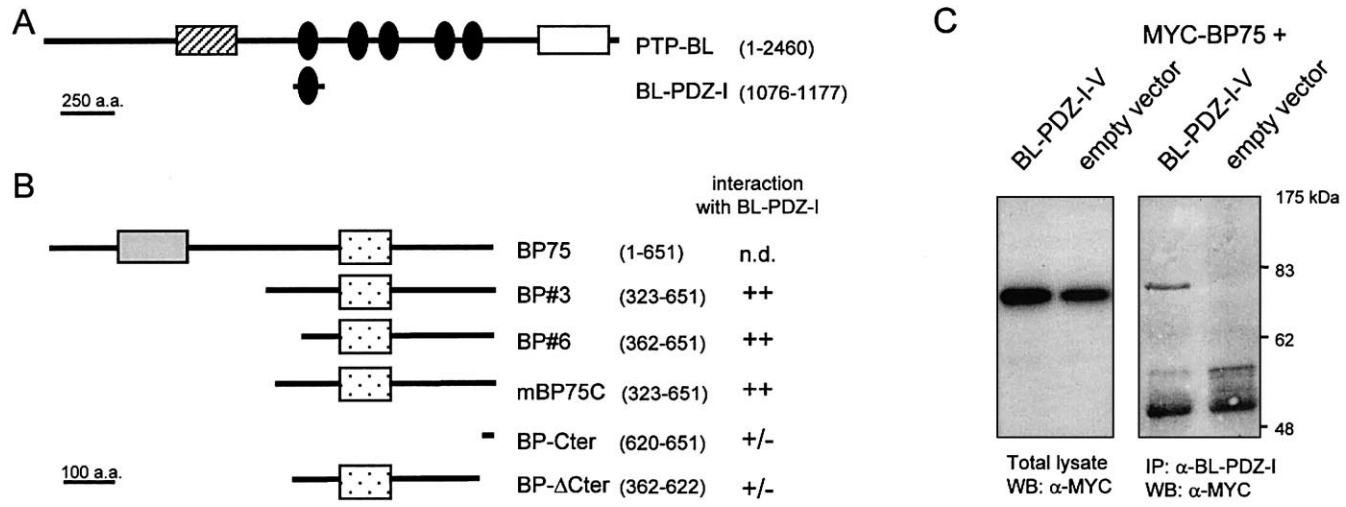

Fig. 1. The C-terminal half of BP75 interacts with the first PDZ motif in PTP-BL. Schematic representation of the protein segments as they are used in the two-hybrid interaction trap. Depicted are (A) the PDZ-I segment of PTP-BL used as bait and (B) the BP75 segments retrieved from the HeLa cDNA library (BP#3 and BP#6) and used as deletion constructs. The numbers corresponding to the first and last aa positions in the PTP-BL (Z32740) and BP75 (AF084259) segments are shown in parentheses, followed by a quantitative interpretation of the two-hybrid interaction results with PDZ-I. ++, strong interaction; ±, very weak interaction. Band 4.1-like domain, hatched box; PTPase, open box; PDZ domains, black ovals; bromodomain, gray box; BYC homology domain (see text), stippled box. BP75 co-precipitates with PDZ domains of PTP-BL (C). COS-1 cells were co-transfected with a construct encoding cMyc epitope-tagged BP75 and a construct encoding the five PDZ domains of PTP-BL (BL-PDZ-I–V) or empty vector as a control. Western blotting using an α-cMyc antibody detected a 75 kDa protein in the total cell lysates (left panel). After immunoprecipitation using a polyclonal antiserum directed against the first PDZ domain of PTP-BL (α-BL-PDZ-I), co-precipitation of cMyc epitope-tagged BP75 with the five PDZ domains of PTP-BL is shown (right panel). No co-precipitation of BP75 is observed in lysates of COS cells co-transfected with empty vector.

2. Materials and methods

2.1. Two-hybrid interaction trap

Plasmid DNAs, the yeast strain EGY48 and the HeLa cell cDNA library used for the interaction trap assay were kindly provided by Dr Roger Brent and colleagues (Massachusetts General Hospital, Boston, MA, USA) and used as described [26]. The construction of the PTP-BL PDZ, RIL-PDZ, nNOS PDZ and SAP-PDZ baits has been described previously [18,27]. For two-hybrid interaction assays, plasmids were introduced in yeast strain EGY48 (*MAT*a *trp1 ura3 his3 LEU2*::pLexAop6-LEU2) containing the plasmid pSH18-34, which includes the reporter *lac*Z gene, and interactions were validated by growth and blue coloring on minimal agar plates lacking histidine, tryptophan, uracil and leucine, which contain 2% galactose, 1% raffinose and 80 μg/ml X-gal, buffered at pH 7.0. Comparison of candidate prey cDNA sequences with database entries was done using the BLAST program [28]. Mouse BP75 prey constructs containing the fragment corresponding to the shortest human prey insert (mBP75C, aa 323–651), the C-terminus of BP75 (BP-Cter, aa 620–651) and the C-terminal half of BP75 lacking the C-terminus (BP-ΔCter, aa 362–622) were generated by PCR using specific primers with either *Eco*RI or *Xho*I restriction enzyme recognition sites, using mouse full-length BP75 cDNA (AF084259) as a template. The resulting fragments were cloned in-frame in an *Eco*RI and *Xho*I-digested pJG4-5 prey vector [26]. All constructs generated by PCR were checked for absence of mutations by DNA sequencing.

2.2. Isolation and characterization of mouse BP75 cDNAs

The 1.2 kb BP#3 insert was isolated, labelled radioactively by random priming and used to screen a mouse brain λ-ZAPII cDNA phage library (Stratagene, La Jolla, CA, USA) following standard procedures. Resulting phages were plaque-purified and inserts were rescued as pBluescript SK^- plasmids according to the manufacturer's protocols. Nucleotide sequences were determined by double-stranded DNA dideoxy sequencing using T3 and T7 sequencing primers on rescued plasmids and subclones derived thereof. Sequences were aligned with Multalin [29] using the Blosum62 comparison table and annotated with Genedoc (shareware, version 2.2.005 [30]).

2.3. RNA expression studies

Total RNA from several mouse (strain C57BL/6) tissues was prepared as described previously [21]. Fifteen μg RNA was loaded on a 1% formamide agarose gel and after electrophoresis, the RNA was transferred to a nylon membrane (Hybond-N, Amersham) according to standard procedures. Complete mouse BP75, PTP-BL and GAPDH cDNAs were radioactively labelled by random priming and used as probes for hybridization as described [21]. RNA in situ hybridization on C57BL/6 mouse embryo cryosections was according to previously published protocols [3]. To generate 'sense' and 'antisense' RNA probes, the mouse BP75 cDNA pBluescript SK-based plasmid was linearized with *Xho*I and *Not*I, respectively, and used for in vitro transcription by T7 or T3 RNA polymerase, respectively. Both probes were labelled with [α-^{35}S]UTP (400 Ci/mmol, Amersham) to a specific activity of $>10^9$ dpm/μg.

2.4. Expression plasmid constructions

Restriction fragments encoding aa stretches 1056–1284 and 1002–2018 of PTP-BL (Z32740) encompassing PDZ-I and PDZ-I-V, respectively, were cloned into a modified version of the eukaryotic expression vector pSG5 [31] that contains an initiator AUG codon at the multiple cloning site. A construct for the expression of an epitope-tagged version of mouse BP75 was generated as follows. Using a subcloned *Sma*I fragment of BP75 as template, the 5′-end of the BP75 open reading frame was PCR-amplified with a specific oligonucleotide (5′-CGAATTCATGGGCAAGAAGCACAAG-3′, introducing an *Eco*RI site directly in front of the start codon) and the vector-specific T7 oligonucleotide. The resulting 0.2 kb fragment was digested with *Eco*RI and *Xma*I and used together with a 1.9 kb *Xma*I-*Xho*I restriction fragment of BP75 in a three-way ligation into the *Eco*RI-*Xho*I-digested pMyc vector. pMyc is a pCDNA3-derived vector in which at the 5′-end of the pCDNA3 (Invitrogen) multiple cloning site, a synthetic DNA fragment was introduced that entails an initiator AUG codon followed by the cMyc epitope tag and an *Eco*RI site. Full-length PTP-BL [3] was subcloned as a *Not*I-*Xho*I restriction fragment in pCDNA3.

2.5. Immunoprecipitation and Western blotting

COS-1 cells were cultured in DMEM/10% fetal calf serum (FCS). For each assay, 1.5×10^6 cells were electroporated at 0.3 kV and 125 μF using the Bio-Rad GenePulser with a 4 mm electroporation cuvette, in 200 μl of phosphate-buffered saline (PBS) containing 10 μg of supercoiled plasmid DNA. Cells were plated on a 10 cm dish and

cultured for 24 h in DMEM/10% FCS. Cells were washed with cold PBS and lysed on a plate with 550 μl ice-cold RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% DOC, 1 mM PMSF and protease inhibitor cocktail; Boehringer). After a 1 h incubation on ice, the lysate was centrifuged for 10 min at 10 000×*g* at 4°C and proteins in 50 μl of the lysate were separated for further analysis. After addition of the polyclonal antibody α-BL-PDZ-I (affinity-purified, 50× diluted; [18]) and overnight rotation at 4°C, 50 μl of protein A-Sepharose CL-4B (Pharmacia) was added and incubation was prolonged overnight. The Sepharose beads with immunobound proteins were washed four times with 1 ml RIPA lysis buffer and boiled for 5 min in 50 μl of 2× sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Fifteen μl of lysates and immunobound proteins was resolved on 8% polyacrylamide gels and transferred to nitrocellulose membranes by Western blotting. Blots were blocked using 5% non-fat dry milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20 (TBST). Cell culture supernatant of the α-cMyc hybridoma 9E10 [32] was used to detect co-precipitation of epitope-tagged BP75. Incubations with primary and secondary antibodies (1 h, 10 000× diluted peroxidase-conjugated goat anti-mouse IgG, Pierce) and subsequent washes were done in TBST at room temperature. Labelled bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris-HCl, pH 8.5, 1.25 mM *p*-coumaric acid (Sigma), 0.2 mM luminol (Sigma) and 0.009% H_2O_2).

2.6. Cell transfection

Mouse embryonic fibroblasts (NIH 3T3) and MDCK type II cells (a kind gift of K. Ekroos, Heidelberg, Germany) were cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% FCS (Zellbiologische Produkte, St. Salvator, Germany). Prior to transfection, cells were seeded onto 14 mm diameter glass coverslips (Menzel-Glaser, Germany) in 24 well cell culture plates. Transfection mix was made by adding 0.3 μg supercoiled plasmid DNA to 2 μg DAC-30 (Eurogentec, Seraing, Belgium) in 150 μl OptiMEM (Gibco BRL, Gaithersburg, MD, USA) and incubating for 30 min at room temperature. After replacing the culture medium on the cells (at 30–50% confluency) with 150 μl fresh DMEM containing 10% FCS, the transfection mix (150 μl) was added to the cells. Following a 5 h incubation at 37°C, the medium was replaced with fresh DMEM containing 10% FCS and incubation was prolonged overnight. Cells were subsequently used for immunofluorescence.

2.7. Immunofluorescence and microscopy

All solutions are prepared in PBS and incubations were performed at room temperature in the presence of 1% goat serum. Cells were fixed for 20 min in 2% paraformaldehyde, permeabilized with 0.5% NP-40 and then, free aldehyde groups were quenched for 10 min in 0.1 M glycine. Fifty μl of a 1:2 dilution of monoclonal mouse α-cMyc (hybridoma culture supernatant, 9E10; [32]) and a 1:50 dilution of affinity-purified polyclonal rabbit α-BL-PDZ-I (directed against the first PDZ domain of PTP-BL; [18]) was used for the 1 h incubation with primary antibody. After thoroughly rinsing in PBS, cells were incubated for 5 min with the second antibodies, 50 μl of a 1:75 dilution Texas red-conjugated AffiniPure goat anti-mouse IgG and a 1:75 dilution fluorescein-conjugated AffiniPure goat anti-rabbit IgG (both from Jackson ImmunoResearch Laboratories, West Grove, PA, USA). In cases where DNA was stained with propidium iodine (5 μg/ml), the fluorescein-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as second antibody. Finally, the coverslips were rinsed in PBS and water and mounted on slides by inversion over 5 μl Mowiol mountant (Sigma Chemical). Cells were examined using a confocal laser scanning microscope (MRC1000, Bio-Rad).

3. Results

3.1. BP75 interacts with the first PDZ motif in PTP-BL

Several candidate proteins interacting with the first PDZ domain of PTP-BL (Fig. 1A) were identified in a yeast two-hybrid interaction trap screening of a HeLa cell cDNA library. Four clones out of 0.9×10^6 transformants remained positive after re-introduction into freshly prepared yeast cells containing appropriate bait and reporter plasmids. Two clones, BP#3 and BP#6 with inserts of 1.2 and 1.1 kb, respectively, were found to represent independent cDNAs from the same messenger RNA (Fig. 1B). Comparison of the aa sequences of their predicted protein products with database entries did not reveal any similarity to known proteins. Furthermore, nucleotide database searches with the BP#3 cDNA insert showed only homology to expressed sequence tags from various tissues, indicating that BP#3 encodes a novel ubiquitously expressed protein. Since PTP-BL is of mouse origin and the interacting clones were obtained from a human cDNA library, we isolated mouse cDNAs that are homologous to BP#3 (see below) and confirmed the two-hybrid interaction using the cDNA segment encoding the C-terminal half of the mouse protein (mBP75C, aa 323–651, Fig. 1B). Since several PDZ motifs have been described to specifically recognize short C-terminal peptides [33,34], we tested deletion mutants of mBP75 (Fig. 1B) to delineate the interaction interface. Deletion of the C-terminus (mBP-ΔCter, aa 362–622) almost completely abolished the interaction, but presence of the C-terminus proper (mBP-Cter, aa 620–651) is not sufficient for a strong interaction (Fig. 1B). None of the mBP75 constructs interacts with an empty bait or with other PDZ domains of PTP-BL (not shown). Furthermore, no interaction was observed with the PDZ domain of RIL, nNOS or the second PDZ domain of SAP90 (not shown).

To obtain independent evidence for the interaction between BP75 and PTP-BL, we co-transfected mammalian epithelial cells (COS-1) with an expression construct encoding Myc epitope-tagged full-length BP75 and a construct encoding the five PDZ domains of PTP-BL (BL-PDZ-I–V) or, as a control, the empty expression vector. Specific co-precipitation of anti-Myc detectable full-length BP75 was demonstrated for BL-PDZ-I–V (Fig. 1C) when using an antibody against BL-PDZ-I [18], confirming the results obtained in the yeast two-hybrid interaction trap.

3.2. Isolation and characterization of mouse BP75 cDNA clones

Sequence analysis of BP#3 and BP#6 cDNA inserts revealed that only partial sequence information of the encoded protein was obtained. To acquire additional sequence data and at the same time have a more complete tool to study the biological function of the encoded protein, we searched for full-length cDNAs by screening a mouse fetal brain cDNA library using the human BP#3 cDNA insert as a probe. Two clones, with inserts of 1.8 and 2.4 kb, respectively, were obtained. The 2361 bp insert of the longest cDNA (GenBank accession number AF084259) most probably represents a full-length cDNA since Northern blot analysis revealed a transcript of about 2.5 kb in length (Fig. 3A). The smaller cDNA was found to be identical to the last 1804 bp of this clone. Sequence analysis revealed a polyadenylation sequence located 18 bp upstream of the poly-A tract and an open reading frame that specifies a 651 aa polypeptide (Fig. 2A). The predicted protein was found to contain a bromodomain (see below) and has a molecular mass of about 75 kDa and therefore, we termed it BP75. BP75 has overall homology with only a single protein present in the databases, the hypothetical *Caenorhabditis elegans* protein C01H6.7 (Z71258; [35]). Sequence alignment shows that there are two domains of high homology between these proteins (Fig. 2A). The first domain

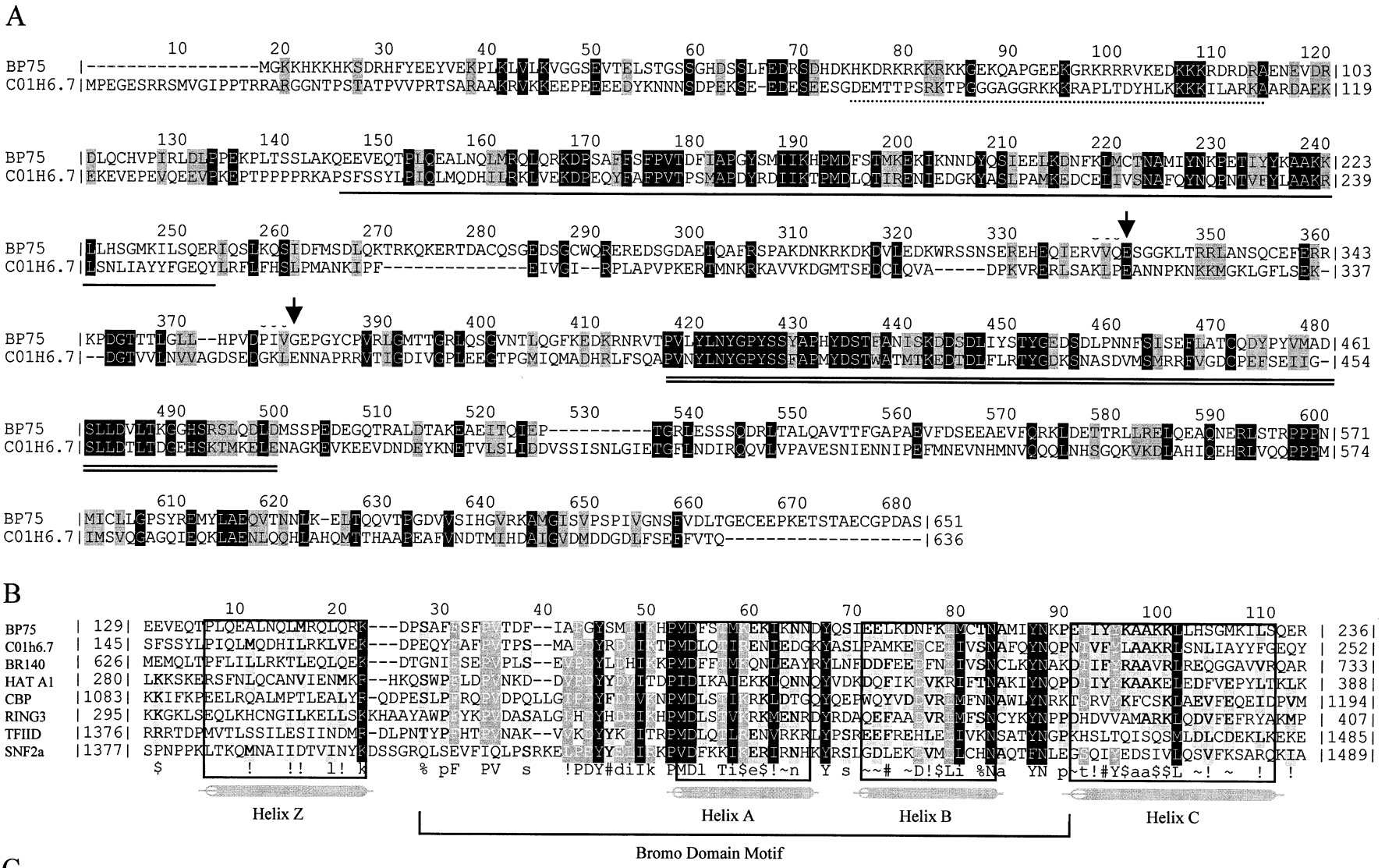
A
BP75
C01H6.7
B
BP75
C01h6.7
BR140
HAT A1
CBP
RING3
TFIID
SNF2a
Helix Z
Helix A
Helix B
Helix C
Bromo Domain Motif
C
BP75
C01h6.7
YN92

Fig. 2. Sequence comparison of the BP75 protein. (A) The complete BP75 protein sequence (AF084259) was aligned with the predicted protein C01H6.7 from the *C. elegans* contig C01H6 (Z71258). Identical aa are shown on a black background and similar aa are indicated on a gray background. The bromodomain and BYC domain are single and double-underlined, respectively, and the predicted nuclear localization signals are indicated by a stippled line. The vertical arrows indicate the start of the clones identified in the two-hybrid interaction trap (BP#3 and BP#6, respectively). (B) The bromodomain of BP75 is shown, aligned with bromodomains from different proteins. The original 80 aa bromodomain motif [23] and the secondary structure as predicted from a multiple alignment of 37 proteins [24] are indicated at the bottom. The aligned proteins are the hypothetical *C. elegans* protein C01H6.7 (Z71258), human peregrin or BR140 (JC2069), *Tetrahymena thermophila* HAT A1 (U47321), mouse CREB binding protein (CBP, P45481), mouse RING3 (D89801), human transcription initiation factor TFIID, 250 kDa subunit (TAFII-250, P21675) and human SNF2α (S45251). The numbers on the left and right of the peptides indicate the corresponding N- and C-terminal aa positions in the database entry. Symbols used in the consensus sequence: $, positive charge (K,R); !, (M,L,I,V); %, (S,T); #, (Y,F). (C) Multiple alignment of the BYC domain of BP75 (AF084259), C01H6.7 (Z71258) and YN92 (P53749).
←

was found to encode a bromodomain (Fig. 2B; [23]). The evolutionary highly conserved bromodomains (about 110 aa) have now been identified in more than 40 different proteins, many of which are involved in transcriptional activation [24]. Database comparison and sequence alignment (Fig. 2B) reveal that the BP75 bromodomain displays the highest similarity to the bromodomain in C01H6.7 and a human protein called peregrin or BR140 (JC2069; [36]). The second stretch of high homology between BP75 and C01H6.7 of about 80 aa has no clear similarity with any of the known bromodomain-containing proteins nor any other known protein except for a yeast protein called YN92 (Fig. 2C). The sequence of this segment has 70 and 34% similarity between BP75 and C01H6.7 and YN92, respectively. Therefore, we will refer to this segment as BYC domain, for its presence in BP75, YN92 and C01H6.7. YN92 is a putative transcription regulatory protein from yeast that contains a DNA binding Zn(2)-Cys(6) fungal-type binuclear cluster domain [37] for which no homology exists in BP75. Analysis of BP75 using the PROSITE protein motif database predicts two consensus tyrosine phosphorylation sites (aa 14 and 430), two partially overlapping bipartite nuclear localization signals (aa 66–83 and 80–97; [38]) and several potential protein kinase C and casein kinase II phosphorylation sites. This suggests a nuclear localization for BP75 and indicates that the protein may be post-translational modified by both serine/threonine and tyrosine phosphorylation.

3.3. BP75 expression pattern

The observed interaction between BP75 and PTP-BL can only be of biological significance when both genes are co-expressed in at least some tissues. Therefore, the BP75 cDNA insert (Fig. 3A, upper panel) and full-length PTP-BL cDNA (Fig. 3A, second panel) were used to probe a Northern blot of total RNA from several different mouse tissues. A BP75 transcript of about 2.5 kb is highly expressed in all tissues examined, with the highest levels in lung, stomach and thymus. The 8.5 kb PTP-BL transcript has a more restricted expression pattern and is most abundant in lung, kidney, testis, stomach and skin. To further delineate the BP75 expression pattern, we used an antisense BP75 RNA probe for in situ hybridization on mouse embryo cryosections. BP75 expression is found throughout development in all embryonic stages (10.5–18.5 dpc) analyzed (not shown). In 16.5 dpc embryos, BP75 is ubiquitously expressed, with the highest expression levels in thymus, lung, liver, colon and the spinal cord (Fig. 3B, middle

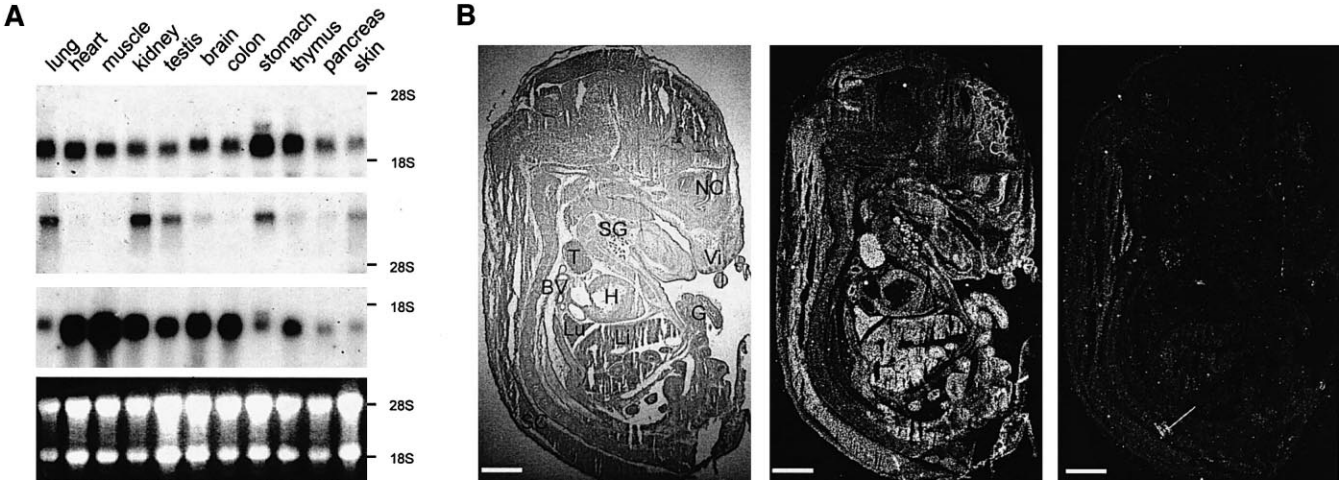

Fig. 3. Tissue distribution of BP75 and PTP-BL. (A) Northern blot analysis of total RNA from mouse tissues for BP75 (upper panel) and PTP-BL (second panel). Expression analysis of the housekeeping gene GAPDH (third panel) was used as a control. As another control for RNA loading, rRNA bands in total RNA on an ethidium bromide-stained gel are shown (bottom panel). BP75 is expressed ubiquitously in all tissues examined, whereas PTP-BL is highly expressed in lung, kidney, testis, stomach and skin and to a lower level in brain and thymus. (B) BP75 RNA in situ hybridization on 16.5 dpc mouse embryo sections. A bright field image (left panel) and dark field images using an antisense BP75 RNA probe (middle panel) and a sense BP75 probe (right panel) on 16.5 dpc mouse embryo cryosections are shown. BP75 is expressed throughout the whole embryo with the highest expressions in thymus, lung, liver, colon and the spinal cord. Furthermore, a high expression is also observed in salivary glands, vibrisae and skin. T, thymus; lu, lung; li, liver; g, gut; sc, spinal cord; sg, salivary glands; vi, vibrisae; h, heart; nc, nasal cavity. Bars, 1 mm.

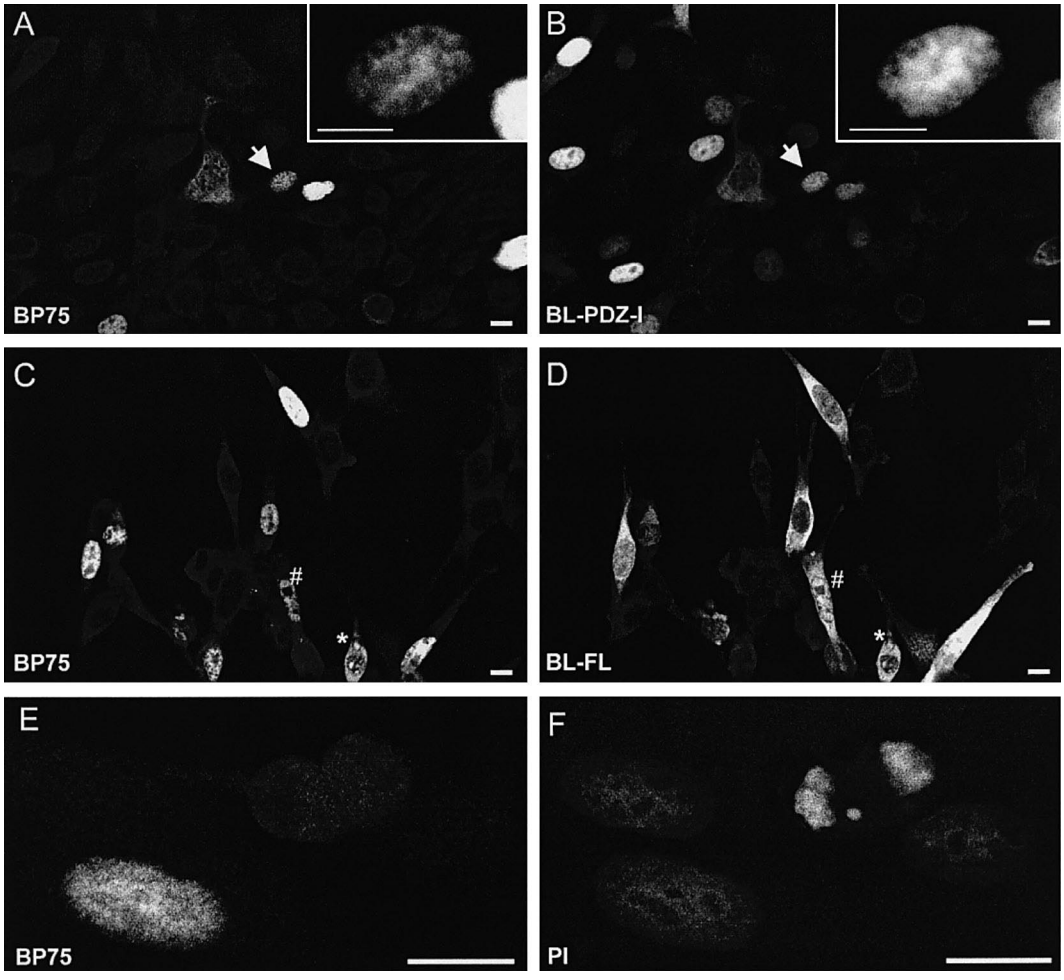

Fig. 4. BP75 and PTP-BL subcellular localization. cMyc-tagged BP75 was transiently co-expressed with the first PDZ domain of PTP-BL (A, B) or with full-length PTP-BL (C, D) in NIH 3T3. Cells were double-labelled for BP75 (A, C) and PTP-BL PDZ-I (B) or full-length PTP-BL (D). The inserts in (A) and (B) show an enlargement of the cell indicated with an arrow. In cells co-expressing BP75 and PTP-BL, both cytoplasmic (*) and nuclear (#) co-localization is observed (C, D). In mitotic cells, only weak BP75 staining can be observed (E) and excludes the area of the chromosomes as detected by propidium iodine staining (F). Bars, 10 μm.

panel). Significant expression is also found in salivary glands, vibrissae and skin. No positive signal was obtained when a BP75 sense RNA probe was used (Fig. 3B, right panel).

3.4. BP75 and PTP-BL protein localization

To study the subcellular distribution of both interactors, full-length cMyc epitope-tagged BP75 was expressed transiently in NIH 3T3 fibroblasts together with either the first PTP-BL PDZ domain or full-length PTP-BL. Using fluorescent double-labelling, we found that BP75 localizes predominantly to the nucleus (Fig. 4A,C), as does BL-PDZ-I (Fig. 4B), although cytoplasmic staining is also evident in some cells. There is an interesting correlation between the BP75 and BL-PDZ-I nuclear and cytoplasmic location in double-transfected cells. In cells where BP75 is located in the cytoplasm, BL-PDZ-I is also cytoplasmically localized. Furthermore, at a higher magnification, a typical distribution pattern that is indicative for a nucleoplasmic localization becomes obvious for both BP75 (Fig. 4A, insert) and BL-PDZ-I (Fig. 4B, insert). It is interesting to note that PTP-BL PDZ-I is the only domain out of seven PDZ domains tested by transient expression that localizes to the nucleus (E.C., unpublished observations). The BP75 localization hardly overlaps with that of the complete PTP-BL protein, although both cytoplasmic (Fig. 4C,D, cell indicated by #) and nuclear (Fig. 4C,D, indicated by *) co-localization can occasionally be observed. Hardly any mitotic cells could be found that express BP75. However, the few cells observed showed very faint cytosolic staining, largely excluding the area of the chromosomes (Fig. 4E,F). These observations suggest that BP75 is degraded before or during mitosis.

4. Discussion

We report here on the identification of two independent clones encoding the C-terminal part of a novel bromodomain-containing protein, BP75, that specifically interact with the first PDZ domain of the protein tyrosine phosphatase PTP-BL. It is becoming increasingly clear that PDZ domains, as they occur in PTP-BL and a variety of other proteins [12], are protein-protein interaction modules that generally bind to short C-terminal peptides [33,34]. In line with this, we found that deletion of the last 29 aa of BP75 almost completely abolished any detectable interaction. In contrast, the last 30 aa of BP75 alone are not sufficient for a strong interaction. Moreover, the last three residues of BP75 (-DAS*) do not

resemble any of the known consensus motifs for PDZ recognition [33] and screening of a two-hybrid random peptide library to identify a consensus C-terminal peptide target sequence for PTP-BL PDZ-I did not result in interacting peptides (E.C., M.v.H., unpublished work), whereas the same approach was successful for the neuronal NOS PDZ domain [27]. Taken together, this argues against a simple model in which PDZ domains uniquely engage in C-terminal peptide binding. Perhaps additional protein segments are necessary for a strong interaction to occur. Interestingly, secondary structure prediction suggests the presence of coiled-coil elements in the C-terminal half of BP75 (aa 534–565) and perhaps dimerization via these structures may be a prerequisite for interaction with BL-PDZ-I. Alternatively, BL-PDZ-I may belong to a subset of PDZ domains that is not capable of recognizing C-terminal peptides. Indeed, evidence is accumulating that internal peptide sequences may equally well serve as targets for recognition by PDZ domains [16,18,39,40] and recently, a molecular basis for internal peptide recognition was provided by the structure of the nNOS-syntrophin complex [41]. In line with this, Maekawa and coworkers found that IκBα associates with PDZ-I in PTP-BAS through a stretch of N-terminal ankyrin repeats [22].

The interaction identified in the yeast two-hybrid interaction trap was confirmed in an independent assay under conditions that better resemble the in vivo situation. Complete BP75 can be co-immunoprecipitated from transfected mammalian cells with a protein segment encompassing the five PDZ domains of PTP-BL (Fig. 1C). Unfortunately, we were unable to show an interaction between full-length proteins due to the very low PTP-BL protein levels that were obtained in our transfection studies (E.C., unpublished observations). Northern blot and RNA in situ hybridization analysis show an ubiquitous expression of BP75 mRNA (Fig. 3), suggesting a structural or general function for the protein. In contrast, PTP-BL expression is restricted to epithelial tissues (Fig. 3, [3,18]), restraining the biological function for the observed interaction.

Transfection studies, as shown in Fig. 4, reveal BP75 to have predominantly a nuclear location. This is in keeping with the presence of the bromodomain [23,24] and the bipartite nuclear localization signals in the product specified by the full-length mouse cDNA. Bromodomains have been proposed to mediate protein-protein interactions in the nucleus, thereby influencing the assembly and/or activity of multiprotein complexes [23,24]. Indeed, GCN5, a putative transcriptional adapter in humans and yeast, was found to bind to DNA-dependent protein kinase holoenzyme via its bromodomain, resulting in phosphorylation of GCN5 and inhibition of its HAT activity [42]. Furthermore, it was recently shown that the bromodomain of yeast Gcn5p can interact in vitro with the N-terminus of histone H4 [43] and the solution structure of the bromodomain of the HAT co-activator P/CAF (p300/CBP-associated factor) revealed specific recognition of acetylated lysines by this domain [25]. These observations suggest that bromodomain-containing proteins may be important for the assembly and activity of multiprotein complexes at specific chromosomal sites, thereby regulating chromatin remodelling and transcriptional activation. Interestingly, we identified a novel domain in BP75, the BYC domain (the conserved segment in BP75, YN92 and C01H6.7), with similarity to the putative transcriptional regulatory protein YN92 from yeast. Although the role for this domain is open for further study, the presence of both the bromo and the BYC domain suggests that BP75 may have a role in regulation of the chromatin structure and/or transcriptional activity.

Is there compatibility between the behavior and putative role of BP75 and that of PTP-BL? In contrast to BP75 and the isolated BL-PDZ-I domain, full-length PTP-BL protein is essentially exluded from the nucleus (Fig. 7, [18]; E.C., unpublished observations), although in some cells, a nuclear localization can be observed in our experimental set-up. Interestingly, also low levels of endogenous PTP-BL have been found in nuclei of mouse keratinocytes (Derick G. Wansink, unpublished work), leaving open the possibility of BP75 and PTP-BL to physically interact. It is also possible that BP75 and/or PTP-BL shuttle between the cytosol and the nucleus, coupling signal transduction processes at the cell cortex to nuclear events or vice versa. In this light, it is interesting to note that protein 4.1, which contains a FERM domain like PTP-BL, is also located both at the cell membrane and in the nucleus [44]. Furthermore, several other submembraneous proteins like zyxin, ZO-1, β-catenin and plakophilins 2a and 2b are also found located in the nucleus [45–49]. Conversely, Br140, another primarily nuclear bromodomain-containing protein, was initially identified due to its co-purification with an integrin [36]. Shuttling of proteins or protein complexes between compartments may therefore form an important pathway for coupling submembraneous signalling events to nuclear activity. PTP-BL may organize the different components necessary for such a mechanism via its PDZ motifs and/or regulate the characteristics of associated proteins by its catalytic activity. Clearly, understanding the function of BP75 awaits a better understanding of PTP-BL as a potential scaffold protein and therefore, it will be essential to obtain more knowledge about the biological significance of PTP-BL for example by using knock-out mouse models.

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