The protein kinase C-related kinase PRK2 interacts with the protein tyrosine phosphatase PTP-BL via a novel PDZ domain binding motif

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Abstract Protein tyrosine phosphatase-basophil like (PTP-BL) is a large non-transmembrane protein tyrosine phosphatase implicated in the modulation of the cytoskeleton. Here we describe a novel interaction of PTP-BL with the protein kinase C-related kinase 2 (PRK2), a serine/threonine kinase regulated by the G-protein rho. This interaction is mediated by the PSD-95, *Drosophila* discs large, zonula occludens (PDZ)3 domain of PTP-BL and the extreme C-terminus of PRK2 as shown by yeast two-hybrid assays and coimmunoprecipitation experiments from transfected HeLa cells. In particular, we demonstrate that a conserved C-terminal cysteine of PRK2 is indispensable for the interaction with PTP-BL. In HeLa cells we demonstrate colocalization of both proteins in lamellipodia like structures. Interaction of PTP-BL with the rho effector kinase PRK2 gives further evidence for a possible function of PTP-BL in the regulation of the actin cytoskeleton.

Key words: Rho; Cytoskeleton; Actin; Signaling; Protein/protein interaction

1. Introduction

Protein tyrosine phosphatase-basophil like (PTP-BL), its human homologs are also known as PTPl1 [1], hPTP1e [2], FAP-1 [3], and PTP-BAS [4], is a large non-transmembrane protein tyrosine phosphatase. This phosphatase is structured into functional modules consisting of an N-terminal four point one, ezrin, radixin, moesin (FERM) domain followed by five different PSD-95, *Drosophila* discs large, zonula occludens (PDZ) domains and a C-terminal located catalytic domain. A number of proteins have been described to interact with one or two of the five PDZ domains of PTP-BL. The IkappaBα protein [5] and the bromodomain protein BP75 [6] interact with PDZ1, Fas [3], the zyxin family member ZRP-1/TRIP6 [7,8] and the tumor suppressor protein APC [9] interact with PDZ2, ephrinB [10], the rho GTPase activating protein (GAP) PARG [11] interact with PDZ4, and the protein RIL is able to interact with PDZ2 and PDZ4 [12]. However, to date no interacting proteins for PDZ3 and PDZ5 have been described.

Here we describe that the protein kinase C-related kinase (PRK)2 interacts selectively with the PDZ3 domain of PTP-BL. This interaction depends on the last three amino acids of PRK2. Moreover, in contrast to many other known binding sequences for PDZ domains, a C-terminal cysteine is indispensable for this interaction establishing a novel binding motif for PDZ domains.

2. Materials and methods

2.1. Plasmids and antibodies

Full-length PRK2 was cloned from the clone isolated by two-hybrid screening. The missing part of PRK2 was amplified from an expressed sequence tag clone (M045304) using primers PAKC (5′-GATGGTACCGTCCATACCGGAGCGCAATG) and PAKD (5′-ACTCGTTTGCTCATGAAAGATG) (fragment I). Full-length PRK2 was generated using fragment I and a *Bam*HI/*Eco*RI restriction fragment from pGBT9 containing the cDNA of PRK2 isolated by two-hybrid screening in a SOE (splicing by overlapping ends) PCR using primers PAKC and PAKB (5′-TAGGGATCCGCTGAAGTACAATGAGGAGCCAC). Full-length PRK2 was subcloned using *Kpn*I and *Bam*HI sites into pcDNA3. To increase expression of PRK2 the 3′-untranslated region was shortened by exchanging a *Bst*EII/*Bam*HI fragment with a corresponding PCR fragment ending with the translational stop codon (primers PAKB and PAKT (TAGGGATCCTTAACACCAATCAGCAATGTAGTC)). K/R mutant was generated by SOE-PCR using mutant primers PAKK (5′-AGATGTTTGCTATAAGAGCCTTAAAGAAAGGAGATATTG) and PAKL (5′-CCTTTCTTTAAGGCTCTTATAGCAAACATCTCATTTGTG-3′). The PRK2 construct lacking the last three amino acids was generated by replacing a *Bst*EII/*Bam*HI fragment with the corresponding PCR fragment using primers PAKA (5′-GATGAATTCCCAACACTAAGTCCACGGTCAAAG-3′) and PAKU (5′-TAGGGATCCTTAAGCAATGTAGTCAAAATCTCTG-3′), similar the C/S mutation was introduced by replacing a *Bst*EII/*Bam*HI fragment with a PCR fragment using primers PAKA and PAKR (5′-TAGGGATCCTTAAGACCAATCAGCAATGTAGTCAAAATC-3′). All constructs were subcloned into pTRE2 (Clontech).

The coding region of each PDZ domain was amplified by PCR from plasmid pcDNA3-PTP-BL and subcloned into pGEX2-T (Amersham Pharmacia Biotech). PDZ1–5 was amplified from pcDNA3-PTP-BL and subcloned in frame into pcDNA3 encoding for enhanced green fluorescence protein (EGFP), generating an N-terminal EGFP tagged PDZ1–5 fragment. Monoclonal anti-PRK2 antibody was purchased from Transduction Laboratories, anti-PTP-BL has been described before [9].

2.2. Yeast two-hybrid system

Yeast strain Hf7c (Clontech) was transformed sequentially with pGBT9-PDZ1–5 coding for PDZ1 to PDZ5 of PTP-BL and a HeLa

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Abbreviations: EGFP, enhanced green fluorescence protein; FERM, four point one, ezrin, radixin, moesin domain; GAP, GTPase activating protein; PDZ, PSD-95, *Drosophila* discs large, zonula occludens domain; PRK2, protein kinase C-related kinase 2; PTP-BL, protein tyrosine phosphatase-basophil like

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cDNA library (Clontech). Cotransformants were selected on minimal agar plates lacking leucine, tryptophan and histidine. For β-galactosidase assays, cotransformants were plated on plates lacking leucine and tryptophan, filter-lifts were performed and incubated with 560 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). For identification of the interacting PDZ domain and for characterizing the binding region in PRK2, Hf7c cells were simultaneously transformed with the indicated expression constructs. Interaction was determined by replating on minimal plates lacking leucine, tryptophan and histidine. β-Galactosidase assay was performed as described above.

2.3. Transient expression, pull-down assay and immunoprecipitation

Transfection of HeLa cells was performed using Polyfect (Qiagen) according to the instructions of the manufacturer. HeLa cells were transfected with expression vectors for the indicated PRK2 constructs. 48 h after transfection cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 0.27 M sucrose, 0.1% β-mercaptoethanol, 1×complete protease inhibitor cocktail (Roche)). After centrifugation at 20 000×*g* for 30 min the supernatant was divided into five equal parts and incubated with 5 μg glutathione-*S*-transferase (GST)-PDZ domain fusion protein bound to glutathione-Sepharose beads. After incubation at 4°C for 3 h, beads were collected by centrifugation and washed four times with lysis buffer. Bound proteins were eluted with 1×Laemmli buffer and separated on a 8% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an anti-PRK2 antibody. Blots were developed using the ECL system (Pharmacia Amersham Biotech). For coimmunoprecipitation experiments, HeLa cells were cotransfected with an expression vector for PDZ1–5-EGFP and the indicated PRK2 expression construct. Lysates were prepared as described above. Equal amounts of lysate were incubated with 4 μg of anti-PRK2 antibody and incubated at 4°C for 1 h with rotation. After addition of 50 μl protein A-Sepharose the incubation was prolonged for an additional 3 h. Sepharose beads were collected by centrifugation, and bound proteins were processed as described above.

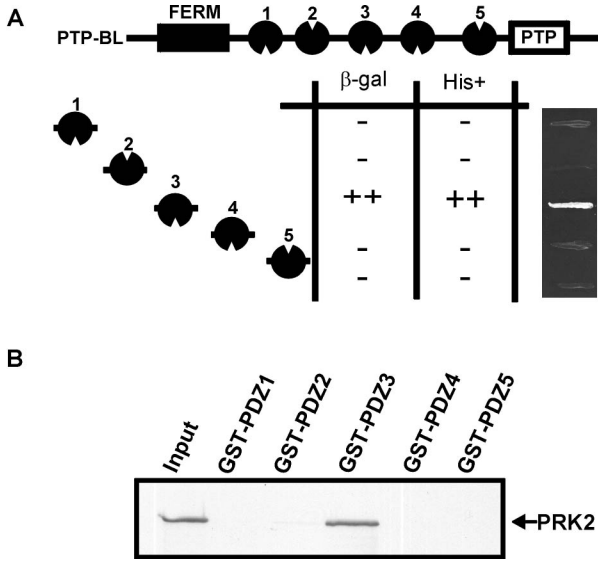

Fig. 1. PRK2 interacts selectively with the PDZ3 domain of PTP-BL. A: HF7c cells were cotransformed with an expression construct for PRK2 (amino acids 301–984) and for the PDZ domains of PTP-BL. Interaction was monitored by histidine prototrophy and β-galactosidase activity. B: HeLa cells were transiently transfected with an expression vector for full-length PRK2, cells were lysed and equal amounts of lysates were incubated with the indicated GST PDZ domain fusion proteins immobilized on glutathione-Sepharose. Bound proteins were separated by SDS–PAGE and transferred to nitrocellulose. The blot was developed using an anti-PRK2 antibody.

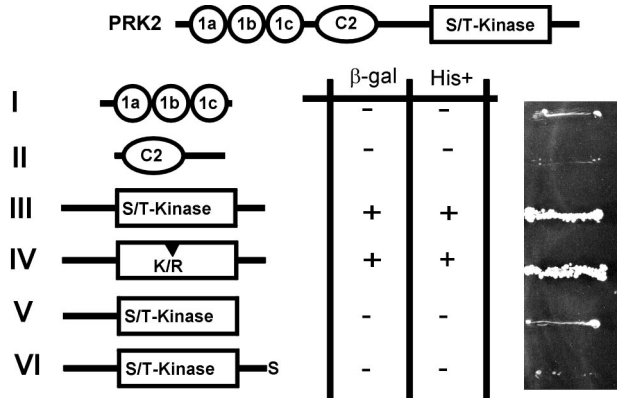

Fig. 2. The C-terminus of PRK2 is necessary for interaction with PTP-BL. HF7c cells were cotransformed with expression vectors for PDZ1–5 of PTP-BL and the indicated fragments of PRK2 subcloned into pGADGH. Interaction is indicated by histidine prototrophy and β-galactosidase activity.

2.4. Immunocytochemistry

HeLa cells were fixed with 4% paraformaldehyde containing 4% sucrose for 15 min on ice. After incubation with 0.25% Triton X-100 in PBS (65 mM NaCl, 2.5 mM KCl, 1.2 mM $NaHCO_3$, 2 mM $KHCO_3$, pH 7.4) for 15 min, cells were washed extensively with PBS. Unspecific binding was blocked by 0.5% bovine serum albumin in PBS/0.1% Triton X-100 for 15 min at room temperature. Cells were incubated for 1 h with affinity purified anti-PRK2 (1:30) and anti-PTP-BL (1:30) antibody. First antibodies were detected using an ALEXA488 (Molecular Probes) conjugated anti-rabbit or a Cy3 conjugated anti-mouse antibody. After each incubation the coverslips were washed four times for 8 min with PBS. The coverslips were mounted using Immunoflor (ICN Biomedicals) to prevent bleaching.

3. Results

3.1. PRK2 interacts selectively with the PDZ3 domain of PTP-BL

To identify new interacting proteins for the protein tyrosine phosphatase PTP-BL, we applied the yeast two-hybrid system. Using all five PDZ domains as a bait, we screened a HeLa cDNA library. Among a number of positive clones one clone encoded part of the protein kinase PRK2 [13]. We decided to analyze this interaction in more detail, because PTP-BL is implicated in the modulation of the cytoskeleton and PRK2 is a known rho effector kinase possibly involved in the modulation of the actin cytoskeleton [14,15]. First we identified the interacting PDZ domain of PTP-BL. Each PDZ domain was subcloned into the yeast expression vector pGADGH and tested for interaction with PRK2 by cotransformation into yeast. There was only interaction detectable with the PDZ3 domain, indicated by histidine prototrophy and β-galactosidase activity (Fig. 1A). To establish additional evidence for this interaction, we performed pull-down experiments from lysates of HeLa cells transfected with an expression vector for full-length PRK2. Using fusion proteins of each PDZ domain fused to GST, we observed only coprecipitation of PRK2 with the GST-PDZ3 fusion protein (Fig. 1B).

3.2. The C-terminus of PRK2 is essential for interaction with PTP-BL

Next we mapped the region in PRK2 responsible for binding to PDZ3 of PTP-BL. PRK2 consists of an N-terminal rho binding site (hr1a, hr1b, hr1c), a C2 domain, involved in lipid

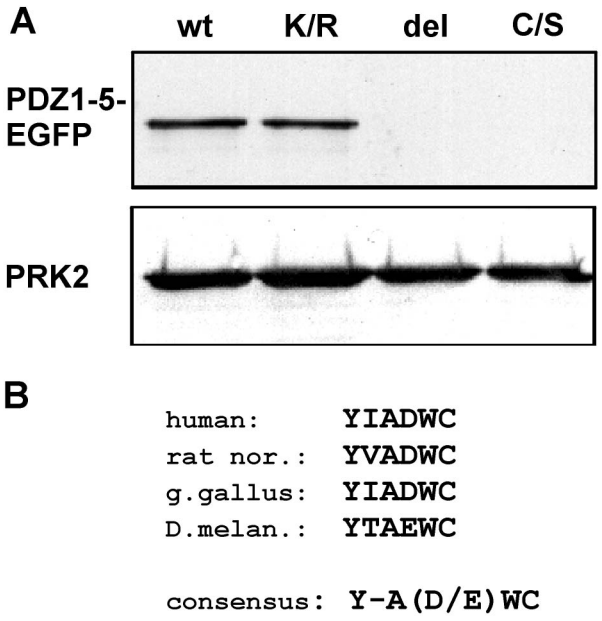

Fig. 3. Coimmunoprecipitation of PRK2 and PDZ1–5 from transfected HeLa cells. A: HeLa cells were transiently transfected with expression vectors for the indicated PRK2 constructs (wt, wild-type; del, deletion of the last three amino acids; C/S, exchange of the C-terminal cysteine to serine) and PDZ1–5-EGFP. Cells were lysed and lysates were subjected to immunoprecipitation using anti-PRK2 antibody. Bound proteins were separated by SDS–PAGE and transferred to nitrocellulose. The blot was developed using anti-PTP-BL antibody, the blot was stripped and redetected using anti-PRK2 antibody. B: Comparison of the C-termini of PRK2 homologs of the indicated species and deduced consensus sequence; for comparison the following expressed sequence tags (ESTs) were used: *Rattus norvegicus*, BF404373, *Gallus gallus*, AJ395750, *Drosophila melanogaster* AA699065, human full-length cDNA of PRK2, U33052.

binding and a kinase domain followed by a short C-terminal tail. PRK2 was divided into three fragments corresponding to these domains and tested for interaction with a construct encoding PDZ1 to PDZ5 (PDZ1–5) in the yeast two-hybrid system. Only fragment III containing the kinase domain and the C-terminus of the protein was able to interact with PDZ1–5 (Fig. 2). To analyze this interaction with respect to the phosphorylation status of PRK2 we performed a point mutation K686 to R described to reduce the autophosphorylation of PRK1, a close related family member of PRK2 [16]. In addition we deleted the last three amino acids in construct III. Point mutation of K to R did not alter binding of PRK2 to PDZ1–5, but deletion of the last three amino acids lead to a complete abolishment of the interaction. Moreover, mutation of the C-terminal cysteine to serine in fragment III was sufficient to disrupt the interaction completely (Fig. 2). To prove these results independent from the two-hybrid system, we generated full-length PRK2 mutants corresponding to our two-hybrid constructs. Lysates of HeLa cells cotransfected with expression vectors for PRK2 mutants and PDZ1–5-EGFP were subjected to immunoprecipitation using a monoclonal antibody to PRK2. Immunoprecipitates were separated on SDS–polyacrylamide gels, transferred to nitrocellulose and probed with an antibody recognizing PDZ1–5. In line with our two-hybrid assays only wild-type PRK2 or PRK2 K/R was able to precipitate PDZ1–5 but not PRK2 devoid of the last three amino acids or carrying a mutation in the C-terminal cysteine (C/S) (Fig. 3A). Using expressed sequence tags (ESTs) from different species we compared the amino acid sequences of the C-termini of PRK2 homologs and deduced the consensus sequence -YX(D/E)WC showing, that the C-terminal cysteine is absolutely conserved (Fig. 3B).

3.3. PRK2 and PTP-BL colocalize in HeLa cells

To give a first evidence for a possible physiological relevance of the interaction, we performed double immunocytochemistry on cultured HeLa cells. We observed strong colocalization of both proteins in lamellipodia like structures and the nuclei of the cells (Fig. 4).

In conclusion we demonstrate a specific interaction of the serine/threonine kinase PRK2 with the third PDZ domain of PTP-BL. This interaction depends on the C-terminus of PRK2, which contains an unusual PDZ binding motif with an invariable C-terminal cysteine.

4. Discussion

Here we describe a novel interaction of the rho effector kinase PRK2 with the PDZ3 domain of PTP-BL. This interaction is highly specific since no interaction could be detected with the other four PDZ domains of PTP-BL as demonstrated by yeast two-hybrid analysis and pull-down experiments using the respective GST-PDZ domain fusion proteins. Moreover, the interaction depends on the presence of a C-terminal cysteine in PRK2. Deletion of the last three amino acids or a conservative exchange of the C-terminal cysteine to serine

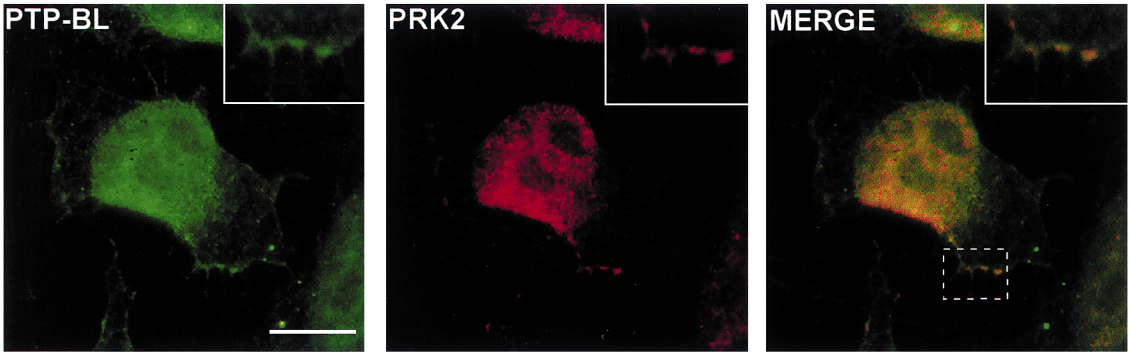

Fig. 4. Colocalization of PRK2 and PTP-BL in HeLa cells. HeLa cells were stained for endogenous hPTP-BL (green) and PRK2 (red), the insert shows an enlarged region of prominent colocalization, bar = 10 μm.

leads to a complete abolishment of the interaction. Interestingly, a comparison of the C-termini of PRK2 homologs from different species indicates a strong conservation of the last four amino acids including the C-terminal cysteine suggesting an important role in PRK2 function. Previously characterized PDZ domains show a preference for large hydrophobic amino acids like valine, leucine or isoleucine in the C-terminal position (position 0) [17]. Explanation for this specificity was given by the existence of a hydrophobic pocket within the PDZ domain, which can only be filled in an energetically favored way by large hydrophobic amino acids [18–20]. However, the necessity of a C-terminal cysteine in position 0 as described here for the PRK2/PTP-BL interaction suggests an additional selection mechanism of PDZ domains for the last amino acid of bound peptides. Interestingly Borrell-Pages et al. described recently an invariable C-terminal cysteine for binding of the L6A protein to the PDZ domain of SITAC (similar to TAC-IP18) [21], however they identified a C-terminal consensus sequence -Y-X-C-COOH essential for binding, which differs to ours in −2 position, where PRK2 shows conservation of an acidic amino acid. Our finding suggests that there are multiple consensus binding sequences ending with an invariable C-terminal cysteine indicating that this might be a more general mode of peptide/PDZ domain interaction.

The physiological relevance of the interaction between PRK2 and PTP-BL is underlined by their colocalization in lamellipodia like structures in HeLa cells. These structures are known to be regions of large actin turnover. Moreover, this interaction is intriguing with respect to a possible function of PTP-BL. PTP-BL interacts via its PDZ4 domain with the rhoGAP PARG [11]; we demonstrate that the adjacent PDZ domain interacts with the rho effector PRK2. Thus, PTP-BL might function as a scaffold for proteins involved in rho/rac signal transduction and thereby regulating the cytoskeleton.

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