Rat protein tyrosine phosphatase η physically interacts with the PDZ domains of syntenin

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Abstract The tyrosine phosphatase r-PTPη is able to suppress the malignant phenotype of rat thyroid tumorigenic cell lines. To identify r-PTPη interacting proteins, a yeast two-hybrid screening was performed and an insert corresponding to the full-length syntenin cDNA was isolated. It encodes a protein containing two PDZ domains that mediates the binding of syntenin to proteins such as syndecan, proTGF-α, β-ephrins and neurofascin. We show that r-PTPη is able to interact with syntenin also in mammalian cells, and although syntenin is a tyrosine-phosphorylated protein it is not a substrate of r-PTPη. The integrity of both PDZ domains of syntenin and the carboxy-terminal region of r-PTPη are required for the interaction between syntenin and r-PTPη. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Tyrosine phosphatase; Thyroid; Syntenin; PDZ domain

1. Introduction

The r-PTPη gene encoding a receptor type tyrosine phosphatase was isolated by our group from a normal rat thyroid cell line, PC Cl 3 [1]. The r-PTPη protein contains a unique intracellular catalytic domain, a short transmembrane domain, and an extracellular region containing eight fibronectin type III-like repeats. Its expression is positively regulated by thyrotropin through the protein kinase A pathway, and is negatively regulated by protein kinase C activation [2]. Moreover, r-PTPη gene expression is reduced in several thyroid oncogene-transformed cells, and absent in highly malignant thyroid cells [1]. The expression of its human homolog, DEP-1/HPTPη, is increased in dense cell culture in comparison to sparse cell culture [3]. DEP-1/HPTPη plays a relevant role in signal transduction of activated T lymphocytes [4,5] and it is associated to memory of B lymphocytes [6]. The mouse homolog of r-PTPη induces terminal differentiation in erythroleukemia cells [7].

A possible role of this phosphatase as a tumor suppressor has been recently envisaged by our group. We demonstrated a dramatic reduction of DEP-1/HPTPη in human thyroid carcinomas compared to normal thyroid tissues and that the transfection of r-PTPη gene in tumorigenic rat thyroid cells suppresses the malignant phenotype. Tumor suppressor activity of r-PTPη is mediated by $p27^{Kip1}$ protein stabilization, through the inhibition of mitogen-activated protein kinase activation [8]. These observations indicate a role of r-PTPη in signal transduction processes that promote malignant growth suppression and cell differentiation. However, the cellular pathways in which r-PTPη is involved still need to be defined. Therefore, the search for proteins interacting with r-PTPη may represent an important tool for the definition of its biological functions. Some DEP-1/HPTPη interacting proteins were detected by co-precipitation and in vitro binding experiments; one of them was identified as a 64 kDa serine/threonine kinase, but the functional role of this interaction was not investigated [9]. Recently, it has been demonstrated that DEP-1/HPTPη is able to de-phosphorylate PDGFβ receptor at site-specific tyrosines [10].

To identify molecular partners of r-PTPη, we have performed a two-hybrid screening. Here, we demonstrate that r-PTPη is able to interact with syntenin, firstly described as a syndecan binding protein [11]. Syntenin contains two PDZ (PSD-95, Dlg, ZO-1) domains, which are protein recognition modules that bind specific peptides on target proteins [12]. PDZ family proteins are involved in protein targeting on the inner surface of the plasma membrane and in assembly of multiprotein complexes [13]. We show that the r-PTPη binds with its carboxy-terminal region to the PDZ domains of syntenin.

2. Materials and methods

2.1. Yeast two-hybrid screening

The intracellular gene fragment of C1118S mutant of r-PTPη was amplified by PCR with *Pfu* polymerase (Stratagene) and cloned in frame at *Eco*RI and *Pst*I restriction sites of the pBridge vector (Clontech). Yeast strain PJ69-2A (Clontech) was transformed with the pBridge-PTPη recombinant vector and mated following the manufacturer's instructions with the strain Y187 pretransformed with a HeLa cDNA library cloned in pGADGH vector (Clontech). Positive clones were selected on quadruple drop-out agar plates (−Leu, −Trp, −His, −Ade) and assayed for β-galactosidase activity. To assess the specificity of the interaction, positive library recombinant vectors were used to co-transform PJ69-2A with pBridge-PTPη, pBridge empty vector or pBridge-HMGI(Y) (a recombinant pBridge vector in which the full-length of high mobility group I-Y protein was cloned). Trans-

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formants were plated on double drop-out agar plates. At least five co-transformed colonies for each transformation were streaked on quadruple drop-out agar plates and yeast growth was controlled after 2 days. The cDNA inserts from the specific clones were sequenced using the dideoxy termination method.

Mutant forms of r-PTPη intracellular region and syntenin were generated by PCR with *Pfu* polymerase. All constructs containing r-PTPη and syntenin mutant forms were sequenced to confirm the correct synthesis.

Interaction assay with mutant gene forms was performed by co-transformation of PJ69-2A followed by growth verification of transformants in quadruple drop-out agar plates.

2.2. Expression vectors

Full-length sequence of r-PTPη was subcloned at an *Eco*RI site of pcDNA3 expression vector (Invitrogen). A mutant form of r-PTPη, lacking the last four amino acids, was generated by PCR and subcloned at *Eco*RI and *Sal*I of pcDNA3.

The pHA-tagged syntenin was generated by PCR amplification of the full-length of syntenin. The PCR fragment was cloned into an *Eco*RI site, blunted by Klenow polymerase, of pCEFL-HA mammalian expression vector. All recombinant constructs were sequenced to confirm the correct synthesis.

2.3. Antibodies and immunoprecipitations

Antibodies used were: polyclonal rabbit antibodies generated against a DEP-1 peptide [9], polyclonal rabbit antibodies generated against the intracellular region of r-PTPη fused to GST [8], anti-HA antibodies (Roche) and anti-phosphotyrosine (anti-pTyr) antibodies (Santa Cruz Biotechnology).

HEK 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected by the calcium phosphate method. Cells were lysed 48 h after transfection in a buffer (1% Nonidet P-40, 50 mM Tris–HCl, 150 mM NaCl) with complete protease inhibitors (Roche) and 1 mM sodium vanadate. Proteins were quantified by a modified Bradford assay (Bio-Rad). 500 μg of proteins were subjected to immunoprecipitation with 5 μg of anti-DEP-1 or 2 μg of anti-HA antibodies and 40 μl of protein A-agarose (Roche). Immunoprecipitates were washed three times in HNTG buffer (20 mM HEPES, pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl). Proteins were separated by SDS–PAGE and transferred to Immobilon-P membrane (Millipore). The filter was incubated with the appropriate antibody and the detection of proteins was performed with the enhanced chemiluminescence system (Amersham).

3. Results

3.1. Two-hybrid screening of r-PTPη interacting proteins: isolation of the syntenin gene

In order to identify the proteins that associate with r-PTPη protein, we performed a two-hybrid screening of a cDNA HeLa library using the cytoplasmic region of r-PTPη as a bait. The sequence of three positive clones revealed that they contained identical inserts corresponding to the full-length of syntenin cDNA. Specificity of the interaction between r-PTPη and syntenin was confirmed by two-hybrid assay as described in Section 2.

Syntenin is a 32 kDa protein containing an amino-terminal region rich in serines, threonines and tyrosines and a carboxy-terminal region with two PDZ domains which are protein–protein interaction modules [12,13]. Syntenin has been isolated as a syndecan binding protein [11]; afterwards, its interaction with class B ephrins, proTGF-α, neurexin and neurofascin has been demonstrated [14–17]. Transcriptional induction of syntenin was observed in human umbilical arterial endothelial cells stimulated with tumor necrosis factor [18]. Recently, the rat homolog of syntenin human gene has been cloned. Rat syntenin protein shows 91% identity with human syntenin [17].

3.2. Mapping of r-PTPη–syntenin interaction

To define the minimal regions required for syntenin–r-PTPη interaction, deletion constructs of r-PTPη and syntenin were generated and used in two-hybrid assays. All the previously described molecular partners associate with syntenin only if both syntenin's PDZ domains are present [14–17]. Moreover, the binding occurs by the carboxy-terminal regions of syntenin interacting proteins. These regions possess characteristic peptides defined as PDZ binding peptides and essential for the interaction with the proteins containing PDZ domains [12].

As shown in Fig. 1A, also for the interaction with r-PTPη, both PDZ domains of syntenin are required. The intracellular region of r-PTPη is able to bind the PDZ containing region but not the N-terminal region of syntenin. Furthermore, syntenin interacts with the C-terminal region but not with the juxtamembrane region of r-PTPη (Fig. 1B). These results suggest that the carboxy-terminal region of r-PTPη contains a PDZ binding peptide. Consistently, r-PTPη has an alanine at position 0 and a tyrosine at position −2; these amino acids confer the features of binding site for PDZ domains to the carboxy-terminal peptide of r-PTPη, as assessed in previous studies [12]. Thus, we made a mutant bait containing the cytoplasmic r-PTPη region deleted of the C-terminal four amino acids. This bait fails to interact with syntenin in a two-hybrid assay. In addition, a bait containing the C-termi-

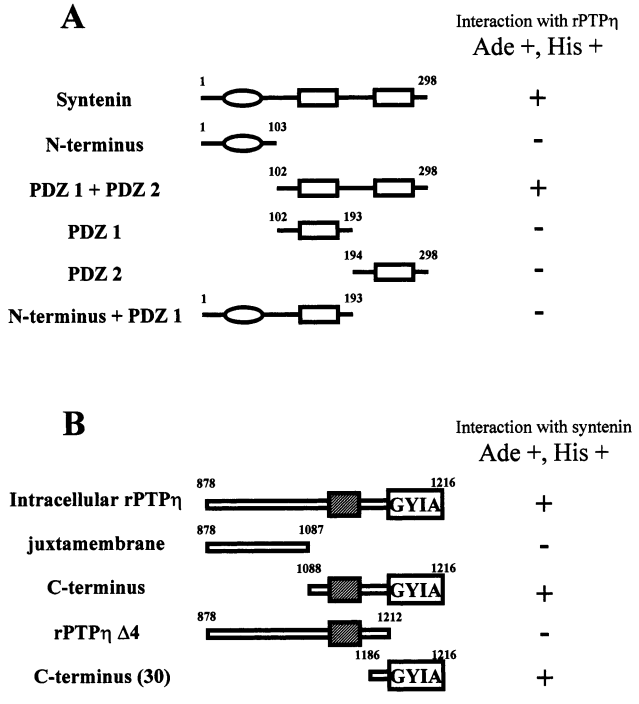

Fig. 1. Mapping of the interaction domains of syntenin and r-PTPη by yeast two-hybrid assay. Interactions were scored as growth of co-transformants on −Ade and −His plates. A: Full-length and different deleted versions of syntenin fused with a GAL4 activation domain were co-transformed with a vector containing the intracellular region of r-PTPη fused to a GAL4 binding domain (pBridge-r-PTPη). B: The intracellular region and different deleted versions of r-PTPη fused with a GAL4 binding domain were co-transformed with a vector containing the full-length of syntenin fused to a GAL4 activation domain (pGADGH-syntenin). Ellipses represent the N-terminal region of syntenin, open boxes represent the PDZ domains of syntenin, dashed boxes represent the catalytic domain of r-PTPη and the GYIA is the C-terminal sequence of r-PTPη.

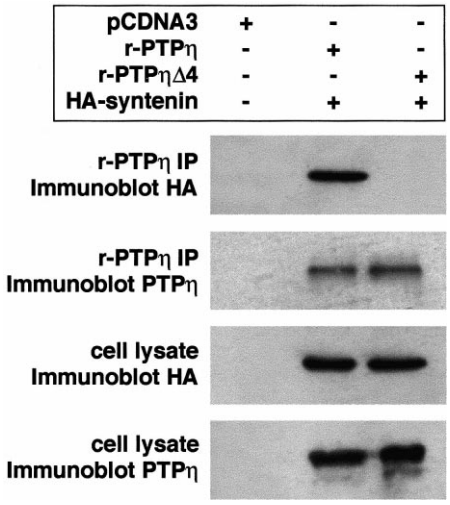

Fig. 2. HEK 293T cells were transfected with the expression vectors indicated. Cell lysates were immunoprecipitated with anti-DEP antibodies that recognize the extracellular region of r-PTPη. Cell lysates and immunoprecipitates (IP) were blotted and then hybridized with antibodies specific for the intracellular region of r-PTPη or HA epitope.

nal 30 amino acids of r-PTPη (lacking the catalytic domain) is still able to interact with syntenin.

3.3. Immunoprecipitation studies

Co-immunoprecipitation experiments were performed to investigate whether r-PTPη is able to interact in mammalian cells with syntenin. HEK 293T cells were co-transfected with the full-length of the r-PTPη gene or the r-PTPη gene deleted of the last four codons (r-PTPηΔ4) and the full-length syntenin cDNA tagged with an HA epitope. Subsequently, cell lysates were immunoprecipitated with the antibodies raised versus the extracellular region of the phosphatase. The immunoprecipitates were then immunoblotted and hybridized with an anti-HA antibody. As shown in Fig. 2, co-immunoprecipitation was observed with r-PTPη full-length but not with r-PTPηΔ4, indicating that also in mammalian cells r-PTPη associates with syntenin and that the interaction depends on the phosphatase C-terminal region.

We then tried to demonstrate a possible role of r-PTPη in the regulation of syntenin activity. Human syntenin, in the amino-terminal region, has five tyrosines that may represent a potential target for tyrosine kinases. To investigate whether syntenin is a tyrosine-phosphorylated protein, we transfected HA-syntenin in HEK 293T and immunoprecipitated the cell lysates with anti-HA antibodies. The immunoprecipitates were blotted and hybridized with anti-pTyr antibodies. As shown in Fig. 3A, syntenin is a tyrosine-phosphorylated protein. We then checked whether tyrosine phosphorylation of syntenin was affected by the over-expression of r-PTPη and we found that in HEK 293T cells, the expression of r-PTPη is not able to reduce tyrosine phosphorylation levels of syntenin (Fig. 3A). The phosphatase activity was intact as assessed by comparing the pattern of phosphorylated proteins in cells transfected or not with r-PTPη ([8] and data not shown). This result seems to exclude syntenin as a substrate of r-PTPη.

In order to investigate whether r-PTPη binds the phosphorylated or the unphosphorylated form of syntenin, we co-transfected HEK 293T cells with HA-syntenin or pCEFL-HA empty vector and r-PTPη expression vector. We performed an immunoprecipitation with specific antibodies against the extracellular region of r-PTPη. The immunoprecipitated proteins were blotted and incubated with anti-pTyr antibodies. At the molecular weight corresponding to that of syntenin, a very weak tyrosine-phosphorylated band was detected in the immunoprecipitated sample containing HA-syntenin (Fig. 3B). In contrast, the incubation with anti-HA antibodies showed an intense band corresponding to the syntenin protein (Fig. 3B). These data suggest that r-PTPη interacts preferentially with the unphosphorylated form of syntenin.

4. Discussion

In this study we provide evidences that the r-PTPη phosphatase associates with syntenin, a protein containing two PDZ domains. By using the two-hybrid system we showed that syntenin binding occurs at the carboxy-terminus of

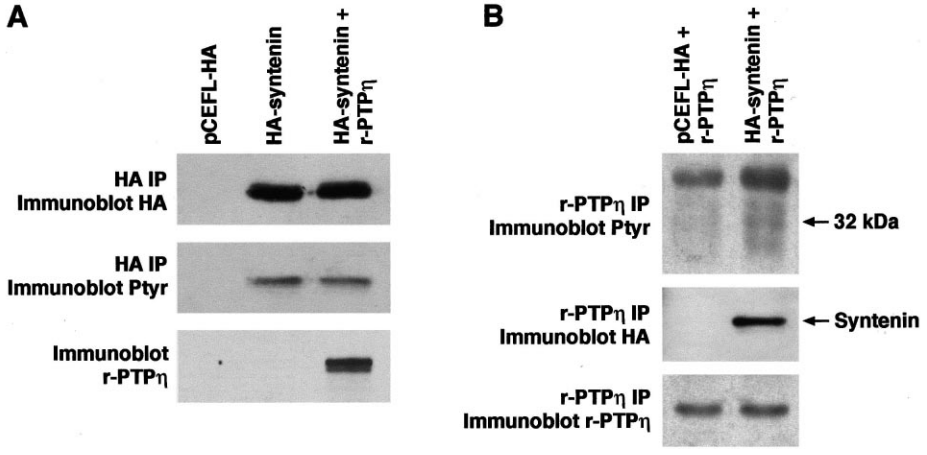

Fig. 3. A: HEK 293T cells were transfected with pCEFL-HA-syntenin in the presence of pcDNA3 vector or pcDNA3-r-PTPη. Cell lysates were immunoprecipitated with anti-HA antibodies, blotted and then hybridized with anti-HA or anti-pTyr antibodies. Cell lysates were blotted and then incubated with anti-r-PTPη antibodies to demonstrate the phosphatase over-expression. B: HEK 293T cells were transfected with pcDNA3-r-PTPη in the presence of pCEFL-HA empty vector or pCEFL-HA-syntenin. Cell lysates were immunoprecipitated with anti-DEP antibodies, blotted, and then incubated with anti-pTyr or anti-HA or anti-r-PTPη antibodies.

r-PTPη protein. Both PDZ domains of syntenin are required for the interaction. In other studies it has been demonstrated [14–17] that the binding of syntenin to its molecular partners depends on the integrity of both PDZ domains.

The carboxy-terminus of r-PTPη belongs to the class II PDZ binding peptides because it contains an aromatic amino acid (tyrosine) at the −2 position and an hydrophobic amino acid (alanine) at the 0 position [12]. Deletion of the four carboxy-terminal amino acids abolishes r-PTPη binding to syntenin. In addition, to further confirm the specificity of the interaction and to demonstrate that it is independent from phosphatase catalytic domain, we found that the C-terminal 30 amino acidic region of r-PTPη is still able to interact with syntenin. Deletion of the N-terminal region of syntenin does not impair r-PTPη interaction with syntenin. This finding is in accordance with the presence of a PDZ class II carboxy-terminal peptide in r-PTPη. In fact, the presence of the N-terminal region of syntenin does not influence the interaction with all syntenin interacting proteins belonging to the PDZ class II such as ephrin B2, syndecan and neurexin [11,14,17].

Co-immunoprecipitation studies confirmed that the interaction between r-PTPη and syntenin depends on the carboxy-terminal region of r-PTPη. Interestingly, the C-terminal four amino acids of r-PTPη are identical to those of human PTPη, suggesting that the interaction with syntenin could occur also in humans. Moreover, we found that syntenin is a tyrosine-phosphorylated protein, but the over-expression of r-PTPη does not modify tyrosine phosphorylation levels of syntenin. These findings indicated that syntenin is not a substrate of r-PTPη. Moreover, we found that r-PTPη binds preferentially the unphosphorylated form of syntenin, and this could explain the inability of r-PTPη to decrease the phosphorylation level of syntenin.

In this study, we analyzed the molecular aspects of the interaction between r-PTPη and syntenin. Since syntenin is not a substrate of r-PTPη, it is hard to speculate a regulatory activity of r-PTPη on it. On the other hand, syntenin could regulate r-PTPη activity in different ways according to its established functions. Syntenin may transport and position r-PTPη in the cell as occurs for proTGF-α [15], and in light of the syntenin localization either in plasma membrane or in intracellular vesicular compartment [19]. Alternatively, syntenin may enhance r-PTPη action recruiting other syntenin binding proteins as substrates, because syntenin can interact simultaneously with two PDZ binding peptides [16] or dimerize forming complexes that contained different proteins [17]. Therefore, modulation of r-PTPη activity by syntenin may be important in the inhibition of the transformed cell growth mediated by r-PTPη.

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