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Involvement of β3A Subunit of Adaptor Protein-3 in Intracellular Trafficking of Receptor-like Protein Tyrosine Phosphatase PCP-2

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Abstract PCP-2 is a human receptor-like protein tyrosine phosphatase and a member of the MAM domain family cloned in human pancreatic adenocarcinoma cells. Previous studies showed that PCP-2 directly interacted with β-catenin through the juxtamembrane domain, dephosphorylated β-catenin and played an important role in the regulation of cell adhesion. Recent study showed that PCP-2 was also involved in the repression of β-catenin-induced transcriptional activity. Here we describe the interactions of PCP-2 with the β3A subunit of adaptor protein (AP)-3 and sorting nexin (SNX) 3. These protein complexes were detected using the yeast two-hybrid assay with the juxtamembrane and membrane-proximal catalytic domain of PCP-2 as "bait". Both AP-3 and SNX3 are molecules involved in intracellular trafficking of membrane receptors. The association between the β3A subunit of AP-3 and PCP-2 was further confirmed in mammalian cells. Our results suggested a possible mechanism of intracellular trafficking of PCP-2 mediated by AP-3 and SNX3 which might participate in the regulation of PCP-2 functions.

Keywords PCP-2; yeast two-hybrid; adaptor protein-3

It is common knowledge that tyrosine phosphorylation plays a vital role in regulating cell growth, differentiation, development and survival. The phosphorylation level of tyrosine is balanced by coordinative actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [1]. PTPs are divided into two subfamilies according to their cellular distributions: transmembrane receptor-like PTPs and cytosolic PTPs. PCP-2, one of the human receptor-like PTPs (RPTPs), was first identified in human pancreatic adenocarcinoma cells by Wang *et al.* [2]. PCP-2 contains two intracellular phosphatase catalytic domains and extracellular domains including an MAM domain, an immunoglobulin-like domain and four fibronectin III-like repeats. Between the intracellular and extracellular domains, there is a long juxtamembrane segment that displays homology to the conserved intracellular domain of the cadherin family. A previous study showed that PCP-2 was endogenously expressed at the cell surface and co-localized with E-cadherin and β-catenin at sites of cell-cell contact [2]. Our further investigations revealed that PCP-2 interacted physically with β-catenin through its juxtamembrane domain and significantly reduced the phosphorylation level of β-catenin, thus playing a potential role in cell adhesion [2–4]. Aerne and Ish-Horowicz reported that PCP-2 played a critical role in regulating the normal functioning of the somitogenesis clock in zebrafish [5]. Recently, a novel role of PCP-2 was described by Yan *et al.*, that PCP-2 was involved in repression of β-catenin-induced transcriptional activity [6].

Trafficking of proteins within cytoplasm involves formation of transport vesicles that are wrapped by various kinds of cytosolic protein coats [7–9]. Adaptor protein (AP)-1, AP-2, AP-3 and AP-4 are reported to be components of these protein coats and play different roles in protein trafficking. AP-1, AP-3, and AP-4 are related to the intracellular sorting process of transmembrane proteins

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through the *trans*-Golgi network (TGN) and/or endosomes, whereas AP-2 is involved in the rapid internalization of endocytic receptors [10–13]. AP-3 is composed of four subunits named δ, β, μ, and σ. Both β and μ subunits have two isoforms of ubiquitously expressed isoform (β3A and μ3A) and neuronal-specific isoform (β3B/β-NAP and μ3B) [14–16]. Each of the four subunits displays different functions. For example, the σ subunit is necessary for the AP-3 complex to keep its integrated function; the δ subunit is supposed to be associated with some regulating factors of protein coat assembly; and the μ subunit is responsible for recognizing tyrosine-based or leucine-based sorting signals contained within the cytoplasmic tails of membrane proteins [17–22]. However, the exact role of the β3A subunit remains unclear till now, although the β subunits of AP-1 and AP-2 were reported to bind a leucine-based sorting motif [23–25].

The sorting nexins (SNXs), a family of cytoplasmic and membrane-associated proteins regulating membrane traffic, are required to form a complex machinery guiding cellular trafficking of cell surface receptors and mediating downstream signaling [26,27]. Generally, each SNX protein contains one PX domain and several coiled-coil domains, and SNX9 also has an additional SH3 domain [28]. It was shown that both the PX domain of SNX1, which recognized phosphatidylinositol-3-phosphate, and its coiled-coil domains, which participated in protein-protein interactions, were necessary for its proper function [29]. However, SNX3 only consists of one PX domain and lacks the coiled-coil domain, which might weaken or diminish its association with other proteins. Studies showed that SNX1, SNX2, SNX4, and SNX6 could form heteromeric complexes with another sorting nexin, but SNX3 could not [27]. Also, SNX3 did not interact with any of the receptors binding to other SNXs, which made it quite different to other members of sorting nexins. The relationship between its unique structure and uncommon function remains to be illustrated by further investigations.

In this paper, we provide evidence that PCP-2 interacts with protein trafficking molecules, the β3A subunit of AP-3 and SNX3, using the yeast two-hybrid system. We further confirmed the interaction between PCP-2 and the β3A subunit of AP-3 in mammalian cells. Although we failed to detect the interaction between PCP-2 and SNX3 in COS-7 cells, such a possibility could not be completely excluded due to the limitations of the co-immunoprecipitation assay. This study documents the involvement of APs and SNXs in intracellular trafficking of receptor-like tyrosine phosphatases, thus adding another interaction partner to the protein trafficking molecules and presenting a possible pathway in which the function of RPTPs is regulated.

Materials and Methods

Yeast two-hybrid assay

The Matchmaker two-hybrid system (Clontech, Mountain View, USA) was used to carry out an interaction trap assay. The juxtamembrane and membrane-proximal catalytic domain of PCP-2 (bp 2437–3606) was amplified using the following set of primers: 5′-CGGAATTCCCGGTGAACATGACCAAG-3′ (forward) and 5′-GCGTCGACGGAGGAATTACTCTGAGG-3′ (reverse). The polymerase chain reaction (PCR) product was then subcloned into pGBKT7 vector (Clontech) using *Eco*RI and *Sal*I restriction sites to create the "bait" construct. The "bait" plasmid was transformed into yeast strain AH109, tested for no transcriptional activation and then mated with a pre-transformed human brain cDNA library in yeast strain Y187 according to the manufacturer's instructions. Positive colonies that grew on minimal medium lacking leucine, tryptophan, histidine and adenine to keep selective pressure of auxotrophic markers were restreaked on new plates containing the same medium and passed through the β-gal colony-lift filter assay. Blue colonies were selected to isolate "prey" plasmids. Each "prey" plasmid was then co-transformed with the "bait" construct into AH109 and plated on the minimal medium mentioned above. Negative controls were carried out to confirm the binding specificity.

Plasmids and antibodies

Full-length PCP-2 was cloned into pRK5S vector as described previously [2]. The plasmid encoding the full-length of the β3A subunit of AP-3 was a kind gift from Dr. BONIFACINO (National Institutes of Health, Bethesda, USA). The entire open reading frame of SNX3 was cloned from human spleen using reverse transcription PCR with primers: 5′-CGGAATTCGGCAGCAGCTACAGCGAAATG-3′ (forward) and 5′-CGGGATCCGGCATGTCTTATTTTAGATGGAGTATAGC-3′ (reverse). The PCR product was subcloned into pcDNA3.1-Myc/His vector (Invitrogen, Shanghai, China) in frame with the Myc tag using *Eco*RI and *Bam*HI restriction sites. The mouse monoclonal antibody anti-Myc clone 9E10 was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The polyclonal antibody of β3C1 against the C-terminal of the β3A subunit of AP-3 was also provided by Dr. BONIFACINO [15]. The JM-Ab antibody was generated to detect PCP-2 as described elsewhere [3].

Cell culture and transfection

COS-7 cells [purchased from American Type Culture Collection (ATCC), Manassas, USA] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. PC-12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 5% horse serum. Other cells were grown in their corresponding medium as indicated by ATCC. Transient transfection of COS-7 cells was carried out using Lipofectamine reagent (Gibico BRL, Grand Island, USA) according to the manufacturer's instructions. Thirty-six to forty-eight hours after transfection, cells were washed three times with phosphate-buffered saline and lysed.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM orthovanadate, 10 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride]. After centrifugation at 13,000 *g* for 15 min at 4 °C, the supernatants were transferred to new tubes and divided into several parts with equal amounts of total protein. Each part of the supernatants was incubated with appropriate antibodies at 4 °C for 3 h with rotation. Then Protein A–Sepharose was added and the incubation was prolonged for an additional 3 h. Sepharose beads were collected by centrifugation and washed four times with lysis buffer. Bound proteins were eluted from the beads with SDS sample buffer and separated on SDS-polyacrylamide gels. For subsequent Western blot analysis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by electroblotting, blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 overnight at 4 °C, then probed with appropriate primary antibodies. Blots were developed using horseradish peroxidase-conjugated secondary antibody (Calbiochem, San Diego, USA) in combination with an ECL system (Amersham, Uppsala, Sweden). The nitrocellulose sheets were stripped for reprobing by incubation in 68 mM Tris-HCl (pH 6.8), 2% SDS, and 0.1% β-mercaptoethanol at 50 ºC for 1 h.

Results

Yeast two-hybrid screening

Our previous studies revealed that PCP-2 directly interacted with β-catenin through its juxtamembrane domain. In order to identify more candidate partner proteins interacting with PCP-2, a yeast two-hybrid assay was carried out with the juxtamembrane and membrane-proximal catalytic domain of PCP-2 as the "bait" protein. A pre-transformed human brain cDNA library was assayed and totally 6.2×10^6 transformants were screened. Five hundred and thirty-seven colonies were found to grow on the minimal medium lacking leucine, tryptophan, histidine, and adenine. These colonies were restreaked on new plates containing the same medium with X-gal and the β-gal colony-lift filter assay was carried out to eliminate false positive colonies. Totally 41 blue positive colonies were selected for further verification. Library plasmids were isolated from these colonies and co-transformed with the PCP-2 "bait" construct into AH109 to retest the protein-protein interactions. As a negative control, library plasmids were also co-transformed with pGBKT7 vector (**Fig. 1**). Finally, 12 library plasmids were selected for sequencing. One of these plasmids encodes the β3A subunit of human AP-3 complex from amino acid 946 to 1078, and another encodes the SNX3 from amino acid 16 to 127.

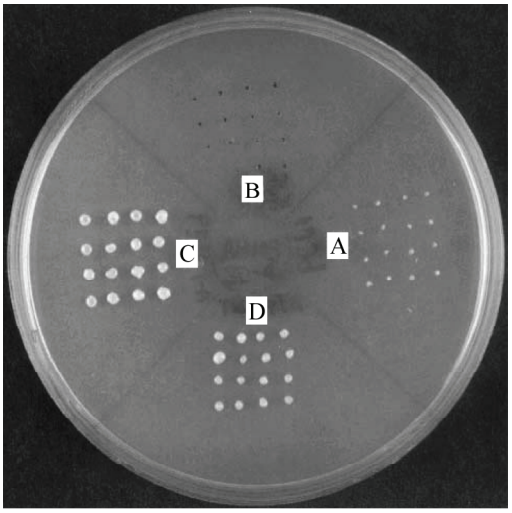

Fig. 1 PCP-2 interacted with β3A subunit of adaptor protein (AP)-3 and sorting nexin (SNX)3 in yeast

Yeast strain AH109 was co-transformed with PCP-2 "bait" plasmid and the "prey" plasmid of either the β3A subunit of AP-3 or SNX3, which were obtained from screening a pre-transformed human brain cDNA library. Transformants were grown on minimal medium lacking leucine, tryptophan, histidine and adenine, then restreaked regularly on a new plate containing the same medium. (A) The pGBKT7 vector was co-transformed with the β3A subunit of AP-3. (B) The pGBKT7 vector was co-transformed with SNX3. (C) PCP-2 was co-transformed with the β3A subunit of AP-3. (D) PCP-2 was co-transformed with SNX3. Positive colonies were robust and grew to more than 2 mm in diameter. Negative colonies were small or did not grow.

Endogenous expression of β3A subunit of AP-3

The expression pattern of human β3A subunit of AP-3 mRNA in various tissues and cell lines has been shown to be ubiquitous [15]. We further examined its endogenous protein expression in cell lines of 293, BHK-21, NIH3T3, WRL68, HepG2, SK-HEP-1 and PC-12, and the results were consistent with previous studies. As shown in **Fig. 2**, a single band of β3A subunit of approximately 140 kDa was detected in all the cell lines examined. The endogenous expression pattern of PCP-2 was also examined in the same cell lines. A 180 kDa band of PCP-2 was observed in PC-12 cells but not in other cell lines examined. Thus the β3A subunit and PCP-2 were co-expressed endogenously in PC-12 cells, which might facilitate the study of their interactions in mammalian cells.

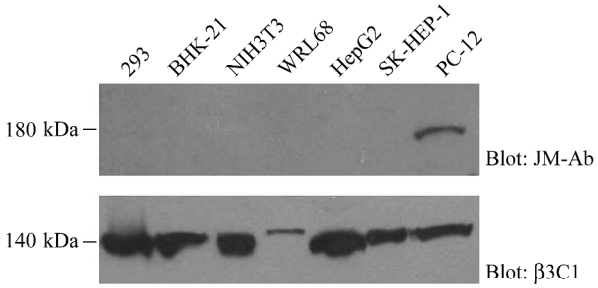

Fig. 2 Endogenous expression of β3A subunit of adaptor protein-3 and PCP-2

Total protein (50 μg) from cell lysates of 293, BHK-21, NIH3T3, WRL68, HepG2, SK-HEP-1, and PC-12 cells were separated and probed with β3C1 (anti-β3A) and JM-Ab (anti-PCP-2) antibodies in succession. The positions of protein markers are indicated on the left. A single band of β3A subunit at approximately 140 kDa was observed in all the cell lines, and the 180 kDa band of PCP-2 was only detected in PC-12 cells.

PCP-2 interacts with β3A subunit of AP-3 in COS-7

The juxtamembrane and membrane-proximal catalytic domain of PCP-2 was found to interact with the C-terminal of β3A subunit of AP-3 in the yeast two-hybrid assay. The co-immunoprecipitation experiment was carried out subsequently using PC-12 cells to confirm their possible interactions. However, we were unable to detect PCP-2 in anti-β3A subunit immunoprecipitation in PC-12 cells, nor could we find the β3A subunit in anti-PCP-2 immunoprecipitation (data not shown). Considering that AP-3 might be responsible for the trafficking of quite a lot of transmembrane proteins at the same time, the level of endogenous PCP-2/β3A subunit complex in PC-12 cells might be too low to be detected by this experiment.

Two plasmids, one containing full-length PCP-2 and the other containing full-length β3A subunit, were co-transfected into COS-7. Immunoprecipitation was carried out using antibodies of either anti-PCP-2 (JM-Ab) or anti-β3A (β3C1). The precipitates were separated on 8% SDS-polyacrylamide gels, assayed by Western blot, and probed with JM-Ab and β3C1 antibodies in succession (**Fig. 3**). The association between the β3A subunit and PCP-2 was confirmed by detecting PCP-2 in the anti-β3A immunoprecipitation. However, the β3A subunit was not detected in the anti-PCP-2 immunoprecipitation. It is possible that the binding site of the β3A subunit in PCP-2 molecules was hindered by the JM-Ab antibody, or the amount of binding complexes immunoprecipitated by JM-Ab antibody was under the limitation of detection.

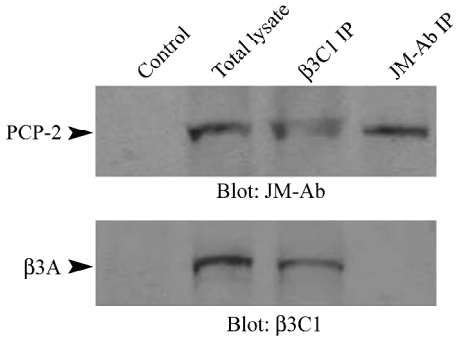

Fig. 3 Interaction between PCP-2 and β3A subunit of adaptor protein (AP)-3

Expression vectors containing full-length PCP-2 and the β3A subunit of AP-3 were co-transfected into COS-7 cells. Total lysates as well as anti-PCP-2 (JM-Ab) and anti-β3A (β3C1) immunoprecipitation (IP) were split by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Western blot analysis. As indicated, PCP-2 was co-immunoprecipitated by anti-β3A antibody. As a negative control, the irrelevant antibody did not precipitate either PCP-2 or the β3A subunit of AP-3.

SNX3 is a putative interactor of PCP-2

SNX3 was supposed to be associated with PCP-2 from the yeast two-hybrid assays. As SNX3 was reported to be highly expressed in human peripheral leukocytes, spleen, heart, and skeletal muscle [27], we cloned the entire open reading frame of SNX3 from human spleen into pcDNA3.1-Myc/His. Then the Myc-tagged SNX3 and full-length PCP-2 were co-transfected into COS-7 cells and immunoprecipitation was carried out using anti-Myc antibody

or JM-Ab antibody. But neither the Myc-tagged SNX3 nor PCP-2 could be detected in the complex immunoprecipitated by another antibody (data not shown). Consistently, several groups also failed to confirm the interactions between SNX3 and other receptors in mammalian cells [27,30]. Possible reasons could be that SNX3 bound weakly or transiently to the receptors or it was rapidly degraded after binding. We suggest that SNX3 was a putative interaction partner of PCP-2.

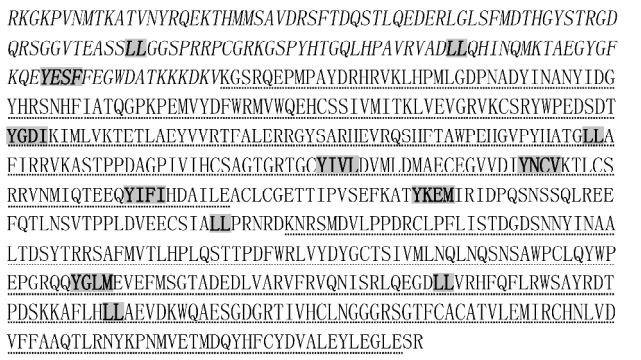

Fig. 4 Putative sorting signals recognized by adaptor protein complexes within cytoplasmic domain of PCP-2

The juxtamembrane domain of PCP-2 is shown in italics and two phosphatase domains are indicated by dotted underlining. Both tyrosine-based sorting signals YXX∅ (Y is tyrosine, X is any amino acid, and ∅ is an amino acid with a bulky hydrophobic side chain) and dileucine-based sorting signals (both shown in shaded and bold letters) are found in the juxtamembrane and two phosphatase catalytic domains of PCP-2.

Discussion

It was reported that receptor-like protein tyrosine phosphatase PCP-2 plays an important role in regulating cell adhesion through direct interaction with β-catenin [3, 4]. In this study, we described the interaction between PCP-2 and the β3A subunit of AP-3 both in yeast and in mammalian cells. We also discovered the association between PCP-2 and SNX3 in yeast two-hybrid assay. Both AP-3 and SNX3 were involved in the trafficking of proteins within cytoplasm. Our investigation might provide a new clue on the mechanism of RPTPs trafficking *in vivo*.

Trafficking of proteins at different stages of the secretory and endocytic pathways is mediated by various protein coats which choose the cargo by interacting with the cytoplasmic domains of selected transmembrane proteins [7]. Among these protein coats, clathrin is the best characterized. The coats also contain protein complexes of adaptors (AP). The members of the AP family can identify similar sorting signals within the cytoplasmic domains of transmembrane proteins such as tyrosine-based sorting signals, which were indicated to bind to the μ subunits, and dileucine-based sorting signals, which might interact with the β subunits [13,24,25]. Some membrane proteins such as mannose 6-phosphate receptor contain more than one sorting motif in their cytoplasmic tails [31]. This redundancy might be helpful in enhancing the binding affinity and specificity by exposing different sorting signals in different circumstances. As a transmembrane receptor, PCP-2 also contains both tyrosine-based sorting signals and dileucine-based sorting signals within its cytoplasmic domain (**Fig. 4**).

The intracellular localization of AP-3 was at or near the TGN and on endosomes. It is supposed that AP-3 mediates protein trafficking between the TGN and endosomes/lysosomes [14,32]. However, whether AP-3 does or does not interact with clathrin during trafficking is still under discussion [33,34]. It was shown that AP-3 might play a role in the normal trafficking of CD63, LAMP-I, LAMP-II, and tyrosinase [35–38]. Moreover, AP-3 is responsible for targeting P-selectin to secretory granules in endothelial cells, thus regulating the expression of P-selectin at the cell surface [39]. In human, the mutations in β3A subunit of AP-3 alter the trafficking of integral membrane proteins and cause Hermansky–Pudlak syndrome, a disorder of oculocutaneous albinism, absent platelet dense bodies, and neutropenia [40]. The access of CD1 to lysosomes was also disrupted in AP-3 deficient cells derived from Hermansky-Pudlak syndrome patients, resulting in a failure of antigen presentation [41]. Faundez and Kelly showed that casein kinase I selectively phosphorylated the β3A subunit at the hinge domain and inhibition of this kinase hindered the recruitment of AP-3 to endosomes [42]. Thus the activity of AP-3 was supposed to be regulated by a model that phosphorylation of AP-3 changes its conformation and activates its function in coating. However, it was shown that the β3A subunit existed as a serine-phosphorylated protein under basal conditions [15]. So PCP-2 is not likely to contribute to the phosphorylation regulation of the β3A subunit but rather to act as a membrane protein being identified by the β3A subunit and then trafficked by AP-3.

The SNX family is hypothesized to play a role in the intracellular trafficking of plasma membrane receptors. Previous studies suggested a possible role for SNX in altering the number of membrane receptors expressed on the cell surface, which might subsequently regulate their signal transduction [43–45] . In this study, we identified a

new putative interaction partner of SNX3 through yeast two-hybrid assay, PCP-2, a member of receptor-like protein tyrosine phosphatases. Our ongoing studies will address whether SNX3 is involved in the intracellular trafficking of PCP-2 and regulates the expression level of PCP-2 on the cell surface, and whether PCP-2 might influence the structure and function of SNX3 by altering its phosphorylation state.

Interestingly, SNX might relate to APs at least in one case. Lundmark and Carlsson showed that SNX9 selectively bound to the C-terminal appendage domain of the β subunit of AP-2 and might assist AP-2 at the plasma membrane, serving as a link between cell-surface receptors and the AP-2-coated vesicles [46]. In this paper, we provided evidence that both the β3A subunit of AP-3 and SNX3 interact with PCP-2, suggesting a pathway of intracellular trafficking of PCP-2 mediated by the β3A subunit of AP-3 and SNX3 which might regulate the amount of PCP-2 at the cell surface. The detailed mechanism of PCP-2 trafficking within cytoplasm and the association, if any, between the β3A subunit of AP-3 and SNX3 need to be further investigated.

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