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(Pro)renin receptor: subcellular localizations and functions

Gabin Sihn[1], Celine Burckle[2], Anthony Rousselle[1], Tatiana Reimer[1], Michael Bader[1]

[1]Max-Delbrueck-Center for Molecular Medicine (MDC), Robert-Roessle-Strasse 10, D-13125 Berlin-Buch, Germany, [2]INSERM U983, Tour Lavoisier, Hopital Necker-Enfants Malades, 149 rue de Sevres, Paris, France

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1. ABSTRACT

Since its first report in 1996, the concept of the so-called (Pro)renin receptor ((P)RR/ATP6ap2) has dramactically evolved from a receptor mediating cellular effects of (pro)renin, to a protein with more basic and potentially essential intracellular functions. Among the arguments urging to reconsider the role of (P)RR was the observation that its localization appears mainly intracellular, although this does not preclude potential functions at the cell surface. However, despite about 10 years of research boosted by the generation of genetically modified animal models, the basic mechanisms of action of this protein at the cellular level remain elusive. This review aims at discussing the functions described for (P)RR in relation to its subcellular localization(s).

2. INTRODUCTION

In the old-fashioned view of blood pressure control in vertebrates, the renin-angiotensin system (RAS) was conceived as a circulating regulatory system. Renin, the enzyme controlling the rate-limiting degradation of angiotensinogen into vasoactive peptides, was believed to have a biological significance only in its proteolytically activated form. Its precursor prorenin was considered to be devoid of any function. However, these two dogmas have recently been challenged by the description of so-called tissular/local RAS (which refer to the presence of some or all components of the RAS within tissues, where they can initiate actions at the cellular level), as well as the discovery of new components with unexpected functions (1). In this regard, the discovery of the (pro)renin receptor

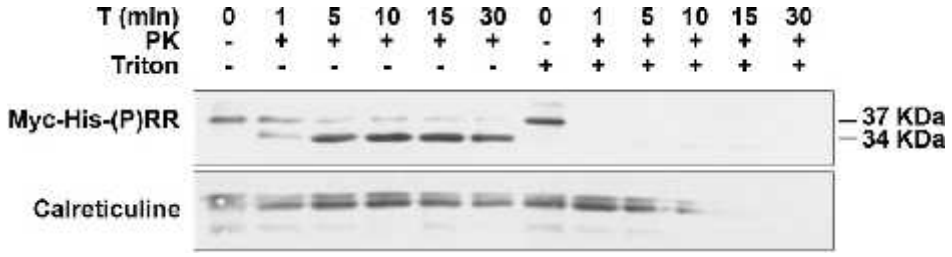

Figure 1. (P)RR topology. Microsomes were prepared from human embryonic kidney cells (HEK293) transfected with a N-terminal-tagged (P)RR construct, in which a Myc-His tag was inserted just behind the signal peptide. Microsomes were subjected to proteinase K (PK) digestion up to 30 min. The tagged construct, visualized with an anti-myc antibody, was detectable after PK digestion, indicating that the N-terminal part of the protein was kept preserved inside the microsomes. A shift from 37 to 34 kDa was observed, consistent with the loss of the short (22-26 aa) cytosolic tail at the C-terminus. The ER-resident protein calreticuline was used as a positive control. To check PK efficiency, microsomes were permeabilized with 1% Triton X-100 and subsequent digestion of (P)RR and calreticuline was monitored.

((P)RR) epitomized this modernized conception of the RAS. Described for the first time in 1996 for its ability to bind renin at the surface of mesangial cells *in vitro* and later reported to have high affinity for prorenin as well (2, 3), the (P)RR stood out from the other (pro)renin binding sites described before (for review, see (4)) by its properties of a real receptor. Upon binding of (pro)renin, the (P)RR did not internalize (3, 5) but rather induced a dual cellular response by 1) a non-proteolytic, catalytic activation of both renin forms for the angiotensinogen-to-angiotensin I conversion, and 2) an angiotensin-independent, intracellular signaling pathway via Mitogen-activated protein kinase (MAPK) phosphorylation (3). Thus, it provided the field with an unexpected functional relevance for prorenin, and a novel mechanism for the actions of (pro)renin at the molecular level.

However, accumulating evidence suggests the possibility that this function as a receptor may actually be secondary to more fundamental cellular functions. First, it has to be remembered that even before its first cloning (3), the (P)RR had been identified as a possible accessory subunit of the multi-protein complex Vacuolar H+-ATPase (V-ATPase). Hence (P)RR got an additional denomination, ATPase, H+ transporting, lysosomal accessory protein 2 (Atp6ap2) (6). In support of a functional link between them, zebrafish mutants for atp6ap2 phenocopy those for various subunits of the V-ATPase (7, 8) (G. Sihn, unpublished data). On the contrary, the comparison of the gene deletions for (P)RR and the various RAS components in the mouse failed to show any similarity, thus not confirming a functional link between (P)RR and (pro)renin *in vivo* (see chapter 4). Secondly, it has become evident that (P)RR mainly resides within intracellular organelles, and the proportion of the protein at the cell surface is a matter of debate. In the absence of (pro)renin internalization, the relevance of its function as a cell surface receptor needs to be re-evaluated, and other intracellular functions are now being considered (see below).

Nevertheless, with regards to the increasing amount of research devoted to (P)RR over the past ten years, it is surprising that the actual subcellular distribution of this protein has not been clarified yet. This may be a key issue as it could confirm or invalidate some of the cellular mechanisms of action described so far. This paper aims at summarizing the literature and presenting novel data on the subcellular localization of (P)RR and its implications concerning the functions of the protein.

3. (P)RR STRUCTURE AND SUBCELLULAR LOCALIZATION

(Pro)renin receptor ((P)RR/ATP6ap2) was discovered in human mesangial cells *in vitro* and cloned in 2002 (3). The human *ATP6AP2* gene, located on the X chromosome at locus p11.4, codes for a protein with 350-amino acids and a predicted mass of around 37 kDa. Analysis of the amino acid sequence revealed two hydrophobic domains predictive of a signal peptide and a transmembrane domain and suggested a type I-transmembrane protein. We used proteinase K digestion assays on membrane preparations *in vitro* (Figure 1) and confirmed the type I structure with an N-terminal signal peptide, an extracellular domain likely to be involved in (pro)renin binding, a single transmembrane domain and a short cytoplasmic domain. In addition, shedding events have been described, which allow the generation of shorter forms upon the action of the proteases furin and/or ADAM19 within the Golgi/trans-Golgi apparatus (9, 10): 1) a soluble (P)RR of 28-29 kDa corresponding to the first 278 amino acids, and 2) a segment including the transmembrane domain and the C-terminal tail and roughly corresponding to the so-called M8-9 fragment (see below). So far, the question of the respective functions of these shorter forms as well as whether they are trafficked and localized in a similar way in the cell, is still unclear (see below).

In their seminal articles, Nguyen *et al.* provided evidence for cell surface expression of (P)RR (2, 3). When transfecting human fetal mesangial cell lines with the (P)RR cDNA *in vitro*, they were able to localize the protein at the plasma membrane. Consistent with its role as a receptor, binding of (pro)renin was reported to trigger cellular effects such as hyperplasia and fibrinolytic activity (2), as well as angiotensin-dependent and -independent molecular responses (3). (P)RR at the plasma membrane

has also been reported in other cell types, like in primary neurons in culture (11) or A-type intercalated cells of the rat kidney collecting ducts, which express it in the microvilli at their apical surface (12).

However, accumulating evidence suggests localization and/or functions within intracellular compartments: 1) the M8-9 fragment was localized in the membrane of chromaffin granules, an organelle related to synaptic vesicle (6), and in synaptic vesicles themselves (13); and 2) the full-length (P)RR appeared to be identical to the yet unpublished protein CAPER (Genbank entry AY038990) which stands for endoplasmic reticulum localized type I transmembrane adaptor precursor C, thus suggesting location in the endoplasmic reticulum (ER).

At the molecular level, examination of the amino acid sequence revealed two theoretical targeting signals within the cytoplasmic tail, both compatible with such locations: a tyrosin-based motif Y^{335}DSI and a C-terminal dibasic motif (K^{346}IRMD) (14). The tyrosine-based sorting signal, YxxØ (where x is any amino acid, and Ø is a large hydrophobic amino acid), is used for protein sorting to endosomes and lysosomes through interaction with adaptor protein (AP) complexes (15). The final C-terminal motif is reminiscent of the conventional dibasic signals K(x)Kxx and R(x)Rxx and is optimally localized for constituting an ER retention/retrieval signal. However it is an uncommon sequence in vertebrates. Nevertheless, the replacement of the K(x)Rxx sequence by R(x)Rxx in a tagged version of the full length receptor, was shown to affect its main localization in the ER, with a relocation partially overlapping the lysosomal compartment, suggesting that the KIRMD motif is functional (16). Correspondingly, a fusion protein consisting of the human (P)RR with a flag inserted between the signal peptide and the extracellular domain and expressed in insect cells, was also reported to localize in the ER mainly (17). Very interestingly, in both studies, shorter forms of (P)RR presented distribution patterns that differed from the full length receptor. A tagged form of the M8-9 segment had a predominant lysosomal homing (16). On the contrary, a short form lacking the transmembrane and the intracellular domains displayed a punctuate pattern within cell organelles which poorly overlapped the Golgi complex or the lysosomes (17). The implication of these observations will be further discussed at the end of the chapter.

That (P)RR is mainly localized in the ER is also supported by data obtained in our group. We studied the subcellular distribution of (P)RR in human embryonic kidney cells using a (P)RR fusion protein labeled with the green fluorescent protein (GFP) at its N-terminus (Figure 2). Immunofluorescence with various organelle markers showed a colocalization of the tagged protein with the ER marker TRAPα (Figure 2D-F) but no or little colocalization with Golgi (Mannosidase II, Figure 2G-I) and lysosomal/endolysosomal (Figure 2J-L) markers.

In addition to the ER, other intracellular localizations have also been reported in the literature. In A-type intercalated cells of the kidney collecting duct, Advani *et al.* could detect (P)RR in structures reminiscent of the Golgi apparatus, where it colocalized with the B1/2 subunit of the V-ATPase as revealed by confocal immunofluorescence (12). Cousin *et al.*, while describing the cleavage of the native (P)RR by furin in human glomerular epithelial cells, described the full length protein to be mainly in the trans-Golgi, where the cleavage by furin was reportedly taking place (9). Yoshikawa *et al.*, while describing the cleavage of a tagged-(P)RR by ADAM19 in vascular smooth muscle cells, reported a main localization in the Golgi and the ER and confirmed that the generation of shorter forms of (P)RR was taking place in the Golgi (10).

Such discrepancies about (P)RR subcellular distribution could be attributed at least in part, to differences between the cell types analyzed in these various studies. In addition, it is important to underline that most of these data have been obtained with histo/cytochemical techniques, which could also account for part of the differences due to the drawbacks of these methods. Indeed, immunochemistry/fluorescence is highly dependent on the reliability of the antibodies as well as on the epitope they target. In addition, overexpression of constructs in cells after transient transfection may lead to missorting of the encoded proteins. Finally, addition of tags at both the N- or C-terminus of a protein (such as the construct used in Figure 2) may lead to protein misfolding as well as affect sequences important for protein trafficking (see chapter 1), thereby further increasing the risk of missorting.

We tackled this problem by using a biochemical approach with endogenously expressed untagged (P)RR. We first performed cell surface biotinylation on mouse embryonic stem cells *in vitro* (Figure 3A). In this technique, cell surface proteins are biotinylated and, after cell lysis, are purified on a streptavidin column (bound fraction) while the intracellular proteins are recovered in the eluate. Using a specific antibody against (P)RR, we were able to show that only a small fraction of the total cellular content of (P)RR was in the bound fraction, the vast majority being found in the eluate. By using knockout embryonic stem (ES) cells as control we could verify that the 39kDa band in the western blots is indeed (P)RR. These data provide strong support to other publications describing a mainly intracellular localization for (P)RR (16, 17). However, it is important to stress that the anti-(P)RR antibody used in this experiment has been raised against the N-terminal part of the protein, which is subject to withdrawal upon shedding by proteases like furin and/or ADAM19 (9, 10). As the cell supernatant was not included in our analysis, it is likely that at least part of the receptors at the cell surface could not be detected with our antibody. Nevertheless, a small but detectable portion of full-length (P)RR appears to reside on the cell surface, substantiating its postulated receptor function. We then examined (P)RR location using the percoll gradient fractionation method with extracts from ES cells and kidney of mice. (P)RR was indeed highly concentrated in a mixed membrane compartment comprising ER and endosomes, but absent in the highest density fraction corresponding to lysosomes (Figure 3B-C). Moreover, an additional smaller band

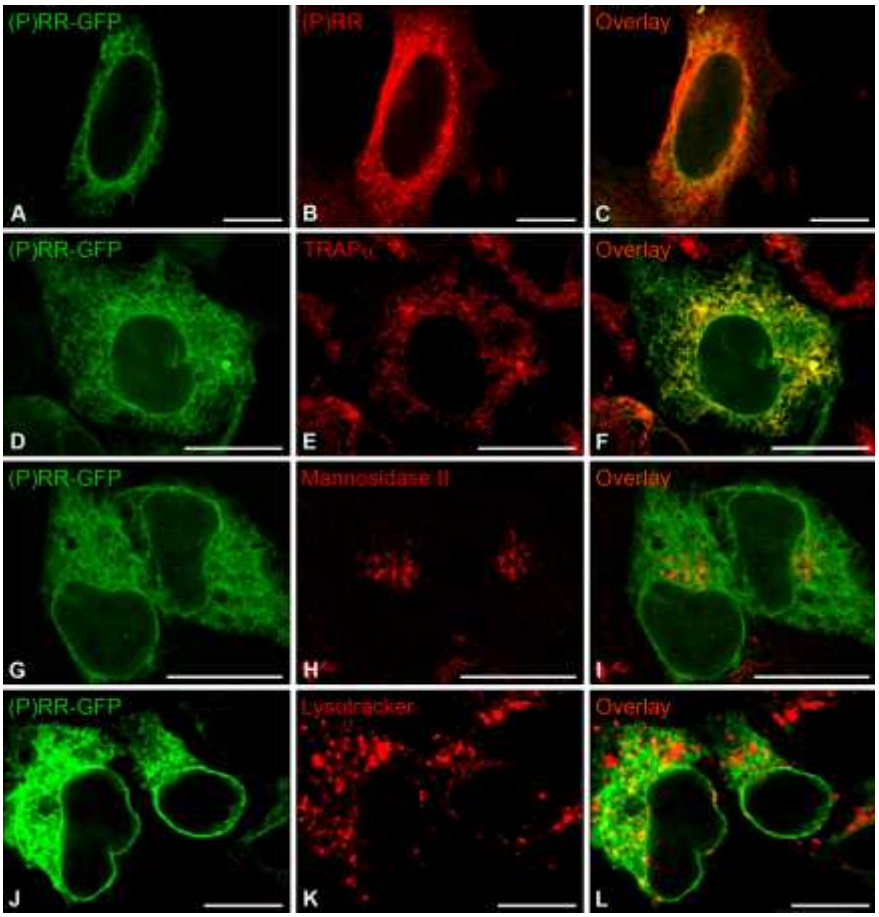

Figure 2. Subcellular distribution of (P)RR in HEK293 cells. A-C: Colocalization of a transiently transfected GFP-tagged (P)RR (revealed with an anti-GFP antibody) with the endogenous (P)RR (revealed with a specific antibody, kind gift from G. Nguyen), ensuring that (P)RR-GFP can be used to assess (P)RR trafficking. D-L: immunofluorescence showing colocalization of (P)RR-GFP with the ER marker TRAPα but no or little colocalization with Golgi (Mannosidase II) and lysosomal/endolysosomal (Lysotracker) markers. Scale bars: 5 µm (A-C), 10 µm (D-F and J-L) and 15 µm (G-I).

detected in the ER-endosome fraction of kidney preparations suggested processing of (P)RR in tissues (Figure 3C). Similar subcellular distribution and processing was also observed in brain extracts (data not shown). Together with our immunofluorescence results (Figure 2), these data support a predominant intracellular localization, and favor the ER as the main homing compartment of (P)RR, in accordance to the abovementioned literature and the CAPER denomination.

However as mentioned above, shorter forms of (P)RR distribute in a different manner as the full length form (16, 17). Notably, the M8-9 segment reportedly locates preferentially to the lysosomal compartment, despite the presence of the ER-retention signal KIRMD (16). This may suggest that this motif is necessary, although not sufficient, for targeting the receptor to the ER. The limitation due to the use of tagged proteins in these 2 studies needs to be kept in mind. Nonetheless, they give some credit to the hypothesis that the various forms and/or domains of (P)RR may have different subcellular distributions and/or functions.

4. ROLE OF (P)RR AS A CELL SURFACE RECEPTOR?

Can the functions described for (P)RR to date help to define in a more precise manner its subcellular localization? Above all, it is important to underline that although several biological functions have been proposed, most of them need further confirmation and it remains to be determined whether they reflect the versatility of a multi-functional protein (in regard to the hypothesis that the different domains may have different functions and localizations), or whether they represent diverse outcomes of one single molecular mechanism.

Already the original description by Nguyen *et al.* shaped the concept of a cell surface receptor. According to the authors, (P)RR was able to bind (pro)renin at the cell surface *in vitro*, and to mediate cellular responses by at least two mechanisms: a non-proteolytic activation of (pro)renin leading to increased generation of angiotensin peptides, and the induction of an angiotensin-independent intracellular signaling through ERK1/2 phosphorylation (2,

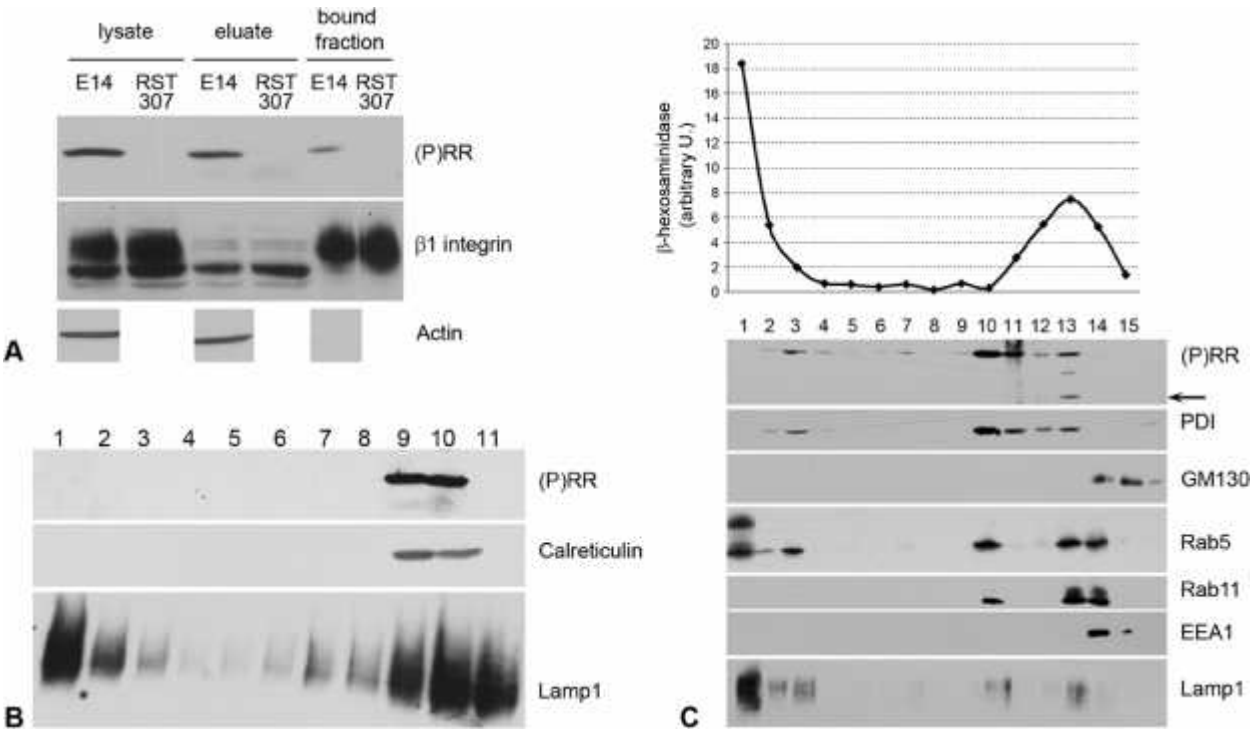

Figure 3. Subcellular localization of (P)RR. A: Cell surface biotinylation on E14 mouse embryonic stem (ES) cells, revealing the respective (P)RR fractions at the cell surface (bound fraction) and in the intracellular compartment (eluate) compared to the whole cell lysate; RST307 ES cells (knockout for (P)RR) were used as a negative control; β1-integrin and actin were used as positive controls for the cell surface and the intracellular fractions, respectively. B: Percoll gradient fractionation of E14 ES cell extracts, showing colocalization of (P)RR with the ER marker calreticulin; colocalization with the lysosome marker Lamp1 is not evident. C: Percoll gradient fractionation of mouse kidney confirming colocalization with PDI (ER resident protein marker), but no or only partial colocalization with cycling endosomal (Rab5 and Rab11), cis-Golgi (GM130), early and late endosomal (EEA1) and lysosomal (Lamp1 and β-hexosaminidase activity) markers. The arrow marks a smaller band which possibly represents the cleavage product of (P)RR.

3). The finding that (pro)renin was not internalized upon binding (3, 5), suggested that (P)RR function as a receptor was taking place at the cell surface. Alternative pathways in response to renin binding were also reported: the group of Unger proposed that upon activation by renin, (P)RR interacts with the Promyelocytic leukaemia zinc finger protein 1 (PLZF) (identified in a yeast 2-hybrid screening) by its short cytoplasmic tail and stimulates PLZF translocation into the nucleus where it can modulate transcriptional activity (16). It is however not clear whether (pro)renin binding takes place at the cell surface as they described (P)RR to be mainly intracellular. Also, Advani *et al.*, in an attempt to reconcile both aspects of the protein as a receptor and an accessory subunit of the V-ATPase, proposed a functional link between (P)RR and the proton pump: by either knocking down (P)RR expression or blocking the V-ATPase function with bafilomycin in MDCK cells *in vitro*, they were able to impair the intracellular induction of ERK 1/2 phosphorylation upon (pro)renin binding (12). Yet, their study was not totally conclusive as both (P)RR downregulation and bafilomycin may only indirectly impair the response to (pro)renin by affecting other functions like intracellular trafficking.

Thus, thirteen years after the first description of (P)RR, its function as a receptor is still a matter of debate. The ability of (P)RR to interact with its putative ligands and elicit cellular responses has mainly been confirmed *in vitro* and in cell culture (5, 18-22) (for review see (23)). *In vivo*, its relevance lacks substantial evidence, like in the study of Kaneshiro *et al.*, in which no direct role of (pro)renin can be ascertained concerning the increased ERK phosphorylation and cyclooxygenase-2 upregulation observed in the renal cortex of transgenic rats overexpressing the human (P)RR (24). In fact, experiments conducted in rodent models using a decoy peptide designed to block prorenin/(P)RR interaction, led to contradictory and inconsistent conclusions about the involvement of (P)RR in cardiovascular pathophysiology (19, 25-31) (for review see (23)), and the specificity and stability of the decoy peptide *in vivo* has yet to be proven (30, 32). More importantly, the link between (P)RR and (pro)renin is not supported by the comparison of the knockouts in the mouse. Complete (P)RR gene deletion leads to embryonic lethality (23), and the first tissue-specific knockouts reported to date die soon after birth due to massive tissue degeneration and defective cellular autophagy (see chapter 5). These phenotypes do not correlate with those of the

knockouts of the various RAS components, which mainly display cardiovascular and metabolic syndromes (33-35).

Another important function described recently for (P)RR is its role in Wnt signaling. Cruciat *et al.* were the first to characterize it in Xenopus as a coreceptor in the canonical Wnt signaling pathway, interacting by its extracellular domain with the seven transmembrane receptor Frizzled 8 (Fz8) and the low-density lipoprotein receptor-related protein 6 (LRP6) (36). Later, the involvement of (P)RR in the non-canonical Wnt signaling pathway was also demonstrated in two studies studying the Planar Cell Polarity (PCP) process in Drosophila (37, 38). This role of (P)RR in both canonical and non-canonical Wnt pathways were reported to be independent of the RAS. Again in these studies, the subcellular localization of (P)RR in relation to its function is unclear. Buechling *et al.* were the only ones to assess this question by immunofluorescence and showed it mainly at the plasma membrane of Drosophila ovarian cells in culture, although not exclusively since a significant amount of the receptor was also located in a perinuclear compartment (37). Cruciat *et al.* (36) did not report on (P)RR subcellular distribution, but as the interaction of Wnt ligands with their receptors occur at the plasma membrane, one can hypothesize the same for (P)RR.

However, the molecular mechanisms proposed in these studies do not suggest that the function of (P)RR is restricted to the plasma membrane. Cruciat *et al.* showed that (P)RR (directly or indirectly) interacts with two subunits of the integral (membrane) domain of the V-ATPase, which suggests that (P)RR is also localized within the intracellular organelles in which the V-ATPase function takes place (36). The V-ATPase is a multi-subunit complex involved in organelle acidification, thereby promoting vesicle trafficking and notably endocytosis (39). A possibility would be that (P)RR actually shuttles between the plasma membrane and some intracellular vesicles such as the so-called signalosomes (40), and serves as an adaptor bridging canonical Wnt signaling to endocytosis for optimal activation. Similarly, both Buechling and Hermle reported that in (P)RR mutant flies, Fz is retained in intracellular vesicles, suggesting that (P)RR might be required for Fz targeting to the plasma membrane (37, 38). Thus, besides being plasma membrane-associated, Fz and (P)RR might be mutually required for plasma membrane targeting and subsequently efficient Wnt-PCP signaling. Thus, (P)RR might regulate protein trafficking *e.g.* from the post Golgi network to the plasma membrane, or recycling between the plasma membrane and the endosomal compartment.

5. ROLE OF (P)RR IN INTRACELLULAR VESICLE TRAFFICKING?

Is an intracellular localization of (P)RR incompatible with a function as a receptor for (pro)renin? In view of the fact that (pro)renin needs to transit through intracellular vesicles before being exocytosed, it cannot be excluded that the two interact at that level in certain (renin-producting) cell types.

However, there is more evidence for an interaction of (P)RR with the V-ATPase, suggested as early as 1998 (6). Recent reports of the first tissue-specific deletions of (P)RR in the mouse, strengthen the hypothesis that the receptor may influence the V-ATPase function by shedding light on a potential role in autophagy. Autophagy is a general term for a process of self-degradation of cellular components, during which these components are engulfed into autophagosomes that ultimately fuse to lysosomes (41-44). Mice with a specific deletion of (P)RR in cardiomyocytes developed heart failure and died within 3 weeks of age (45). The cellular mechanism proposed was an impaired vesicular acidification leading to the accumulation of numerous autophagic vacuoles containing undigested cellular components. In addition, gene deletion in mouse embryonic fibroblasts in culture revealed a downregulation of V0 subunits of the V-ATPase, essential for autophagy due to its crucial role in the acidification of lysosomes and their fusion with autophagosomes (13, 39, 46). A similar impairment in autophagy has been observed in yeast after ablation of some assembly proteins and the loss of one V0 subunit (47, 48). Similar results were described for podocyte-specific (P)RR knockout mice, which developed congenital nephrotic syndrome and died within 3 to 4 weeks of age (49, 50). In addition to blocking autophagy, disruption of pH homeostasis altered cytoskeleton arrangement and podocyte morphology and led to necrosis. In view of the fact that the M8-9 fragment has been described as an accessory subunit Atp6ap2 of the V-ATPase (6), these three articles (45, 49, 50) were the first to suggest a molecular mechanism by which (P)RR can act on V-ATPase function, by controlling its assembly and stability. However, it is important to note that the molecular mechanism is not really proven yet. The disruption of the V-ATPase complex and the impairment in vesicle acidification and autophagy may not be the result of a direct effect of (P)RR on the V-ATPase but the indirect consequence of other cellular defects.

6. CONCLUSION

It is interesting that although various functions have been proposed for (P)RR such as being a receptor for (pro)renin, a co-receptor for Wnt signaling, or a subunit for the V-ATPase, all the phenotypes observed when (P)RR expression is impaired can be explained at least in part, by dysregulations of basic cellular functions such as intracellular trafficking. A better understanding of how this protein transits and distributes inside the cell may prove useful to confirm already described functions, or instead give hints for unraveling unexpected ones.

7. ACKNOWLEDGEMENT

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Send correspondence to: Michael Bader, Max-Delbrueck-Center for Molecular Medicine, Robert-Roessle-Strasse 10, D-13125 Berlin-Buch, Germany, Tel: 49 30 9406 2193, Fax: 49 30 9406 2110, E-mail: mbader@mdc-berlin.de