14-3-3 protein and ATRAP bind to the soluble class IIB phosphatidylinositol transfer protein RdgBβ at distinct sites

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Abstract
PITPs (phosphatidylinositol transfer proteins) are characterized by the presence of the PITP domain whose biochemical properties of binding and transferring PI (phosphatidylinositol) are well studied. Despite their wide-spread expression in both unicellular and multicellular organisms, they remain functionally uncharacterized. An emerging theme is that individual PITPs play highly specific roles in either membrane trafficking or signal transduction. To identify specific roles for PITPs, identification of interacting molecules would shed light on their molecular function. In the present paper, we describe binding partners for the class IIB PITP RdgBβ (retinal degeneration type Bβ). RdgBβ is a soluble PITP but is unique in that it contains a region of disorder at its C-terminus following its defining N-terminal PITP domain. The C-terminus of RdgBβ is phosphorylated at two serine residues, Ser774 and Ser799, which form a docking site for 14-3-3 proteins. Binding to 14-3-3 proteins protects RdgBβ from degradation that occurs at the proteasome after ubiquitination. In addition to binding 14-3-3, the PITP domain of RdgBβ interacts with the Ang II (angiotensin II)-associated protein ATRAP (Ang II receptor-associated protein). ATRAP is also an interacting partner for the AT1R (Ang II type 1 receptor). We present a model whereby RdgBβ functions by being recruited to the membrane by ATRAP and release of 14-3-3 from the C-terminus allows the disordered region to bind a second membrane to create a membrane bridge for lipid transfer, possibly under the control of Ang II.

Introduction
Eukaryotic cells are characterized by membrane-bounded organelles. These organelles perform specific functions and their identity is defined by their complement of lipids and proteins. The plasma membrane is a special case because it provides a boundary to the entire cell. Most membranes, particularly the plasma membrane, are not homogeneous entities, but are organized into domains of distinct lipid and protein composition. Although lipids are synthesized in specific organelles, they are redistributed within the cell and the membrane composition of the different organelles does not reflect their lipid biosynthetic capacity. For example, phosphatidylinerine is synthesized at the ER (endoplasmic reticulum), but is highly abundant in the plasma membrane. To achieve this redistribution, different mechanisms exist. The movement of lipids can occur through vesicular pathways, which allow the transport of mixtures of phospholipids, or by lipid transfer proteins, which facilitate the transport of specific lipids. Soluble lipid transfer proteins that sequester hydrophobic molecules can, in principle, redistribute specific lipids to different membrane compartments.

PI (phosphatidylinositol) constitutes 5–7% of the total cellular phospholipid and is a precursor for at least seven phosphorylated derivatives. These phosphorylated forms have essential roles in cell signalling and membrane trafficking, and function at distant locations with the plasma membrane being a major site. The main consumption of PI [in the form of PI(4,5)P2 (phosphatidylinositol 4,5-biphosphate)] occurs during PLC (phospholipase C)-mediated hydrolysis to generate DAG (diacylglycerol) and myo-inositol trisphosphate. Thus proper distribution of PI between cellular membranes is complicated by the fact that different membrane compartments contain PI kinases that phosphorylate PI after transfer. Synthesis of PI from the precursor lipid, PA (phosphatidic acid), requires two enzymatic steps: the conversion of PA into CDP–DAG by CDS (CDP–DAG synthase) and the conversion of CDP–DAG into PI by PIS (PI synthase). CDS and PIS are both localized at the ER, making that organelle the site of PI synthesis [1]. In humans, a single gene codes for PIS; the protein is a 24 kDa integral membrane protein with four transmembrane domains. Two CDS genes are present in the human genome, one located on chromosome 4 and the other on chromosome 20. The encoded proteins have a molecular
mass of 53 kDa: one is associated with mitochondria for cardiolipin (diphosphatidylglycerol) biosynthesis and the other at the ER. A recent study suggests that PIS is also present on a mobile membrane compartment derived from the ER that makes close contact, but does not fuse, with the plasma membrane [2]. However, this mobile compartment is devoid of CDS. If PI is synthesized in these mobile membrane compartments, the question that remains unanswered is how PI is moved from these sites of synthesis to the plasma membrane. Is the transfer mediated by lipid transporters or by a vesicular route? In principle, protein-mediated transfer of PI can be accomplished by members of the PITP (phosphatidylinositol transfer protein) family. A PITP can bind a molecule of PI or PC (phosphatidylcholine) and facilitate their exchange between membrane compartments. Class I PITPs (PITPα and PITPβ) are best characterized [3] and, in the present paper, we review a new member of the soluble PITPs, RdgBβ (retinal degeneration type Bβ), which may be involved in PI transfer during signalling by Ang II (angiotensin II).

The first PITP (PITPα) was purified from bovine brain as a 35 kDa soluble protein that bound to either a molecule of PI or PC [4]. The lipid cargo could be easily exchanged such that when the protein was exposed to vesicles containing PI, the bound PC was replaced by PI and vice versa. However, the affinity for PI is greater than the affinity for PC such that PI exchange for PC is less favourable compared with PC exchange for PI. PITPα is a single domain protein, but the PITP domain is also found together with other domains (Figure 1A). It can be present with the DDHD, LNS2 (lipin/Ned1/Smp2), PH (pleckstrin homology) and OSBP (oxysterol-binding protein) domains and with the FFAT motif. The DDHD domain is found in PA-PLA1 (phospholipase A₁) [5] and the Sec23-interacting protein p125 [6] and LNS2 domain is found in PA phosphatases, also known as lipins [7], yet the function of these two domains remains unknown. PH domains are quite diverse and often bind phosphoinositides, whereas the OSBP domain binds sterols. The FFAT motif binds to the integral membrane protein VAP [VAMP (vesicle-associated membrane protein)-associated protein], which is localized at the ER [8]. The presence of these additional domains in PITPs suggests that these proteins can be regulated through interactions with other proteins and lipids, and that lipid exchange must be under stringent control.

Studies in model organisms indicate that individual members of the PITP family work in specific signal transduction and membrane trafficking pathways. In Drosophila melanogaster (Dm), three PITP are present: a single domain class I PITP, RdgBβ and RdgBα. The single domain PITP, Giotto, is required for spermatocyte cytokinesis and regulates the activity of Rab11 and PI4Kβ (PI 4-kinase β) [9–11]. The multi-domain Dm–RdgBα participates in the Gα/PLCβ signalling pathway in phototransduction. It localizes to a sub-compartment of the ER, the sub-microvillar cisternae, which is in close proximity with the microvillar plasma membrane of Drosophila photoreceptors cells [12,13]. Loss-of-function mutants in Dm–RdgBα are characterized by abnormal termination of the light response and profound loss of the electroretinogram amplitude shortly after initial light exposure. In addition, the rhabdomeric membranes become vesiculated leading to photoreceptor cell degeneration, giving this protein its name: RdgB. In Caenorhabditis elegans (Ce), there are also three PITP proteins: two single domain Class I PITPs and RdgBα. Ce–RdgBα is required in the sensory neurons for synaptic transmission and behavioural plasticity [14]. The nervous system of C. elegans consists of 302 neurons, and Ce–RdgBα acts in the gustatory neuron ASER at the presynapse. ASER mediates the attraction behaviour to salt as well as avoidance behaviour after pre-exposure to salt under starvation conditions. In PITP mutants,
attraction behaviour is reduced and conditioned repulsion is eliminated. The phenotype of RdgBβ loss-of-function mutants in Drosophila and in C. elegans can be restored by expression of the PITP domain alone, indicating that this domain is central to the biological activity of the protein [15,16]. Ce-RdgBα is also found in neurons of the visual and olfactory organs as well as indicating that it may have additional roles in this organism. In zebrafish, there are four soluble PITPs: PITPα, PITPβ, PITPβ-like and RdgBβ. PITPα is required for axonal outgrowth of motor neurons [17], whereas PITPβ is required for the biogenesis/maintenance of the double-cone photoreceptor cells outer segments in the developing eye [14]. In mice, PITPα is also required for axonal outgrowth and the C-terminus of PITPα interacts with the receptor for netrin-1, DCC (deleted in colon cancer). DCC and neogenin are closely related integral membrane receptor proteins whose cytosolic region contains a domain, P3, that interacts with PITPα [18]. Consequently, PITPα specifically participates in netrin-1-induced PLCγ activation and neurite outgrowth [17–19].

To identify the specific molecular pathways that RdgBβ functions in, we focused on the binding partners for the mammalian PITP protein RdgBβ. RdgBβ is encoded by the PITPPNC1 gene, is a 38 kDa protein and consists of 332 amino acid residues [20]. A short splice variant (RdgBβ-sp2) was subsequently identified that is 268 amino acids in length [21]. RdgBβ belongs to the class II family of PITPs whose lipid-binding properties are not well characterized [22]. Studies with class I PITPs, PITPα and PITPβ, indicate that these two PITPs bind PI and PC. Residues for binding the myo-inositol ring of PI are well-characterized: four residues have been identified (Thr59, Lys61, Asn90 and Glu86, mouse PITPα numbering) (Figure 1B), and mutation of any of these residues is sufficient to lead to loss of PI binding in class I PITPs [23]. These residues are conserved in RdgBβ and the protein has been shown to transfer PI in in vitro assays [20,24]. However, much higher concentrations of protein are required to demonstrate the in vitro PI transfer activity compared with PITPαβ. In addition to binding the myo-inositol ring, four residues (Gln52, Thr97, Thr114 and Lys195) have been identified that make contact with the phosphate moiety. Again, these residues are conserved in RdgBβ.

RdgBβ binds to 14-3-3 proteins

RdgBβ, like PITPα and PITPβ, is a soluble PITP but is unique in that it contains a region of disorder at its C-terminus following its defining N-terminal PITP domain (Figure 2B). Intrinsically disordered proteins commonly have signalling and regulatory functions since disordered segments can permit flexible interactions with several proteins [25–27]. In addition, disordered proteins are subject to tight regulation and targeted protein degradation. Overall, these regions have a much higher frequency of phosphorylation than ordered regions. The C-terminus of RdgBβ is phosphorylated at two serine residues, Ser274 and Ser299 [28–30], which form a docking site for 14-3-3 proteins. 14-3-3 proteins are homodimeric molecules that adopt double horseshoe-shaped structure. Each subunit of the dimer binds one phosphoserine/threonine residue ([31,32] and see also Figure 2). The distance between the two phosphorylated serine residues in RdgBβ is sufficient for one 14-3-3 dimer to bind a single RdgBβ molecule. There are seven 14-3-3 proteins in the human genome, although the isoform that RdgBβ binds has not yet been identified. Phosphorylation of both serine residues is essential for 14-3-3 binding as mutation of either serine residue is sufficient to impair 14-3-3 binding. The consensus sites for binding 14-3-3 are similar to the consensus sequences for phosphorylation by the basophilic protein kinases, PKA (protein kinase A), PKB (protein kinase B) and PKC (protein kinase C). With the use of protein kinase inhibitors, PKC emerges as the most likely candidate responsible for phosphorylating one or both of these residues. The phosphatases responsible for dephosphorylation remain to be identified.

Binding of 14-3-3 to RdgBβ could affect the localization of RdgBβ, regulate its transfer activity and also affect its stability [33]. The short splice form of RdgBβ, RdgBβ-sp2, which lacks the 14-3-3-binding site is found in the nucleus and the cytosol. In contrast, the long form is excluded from the nucleus [21]. The release of 14-3-3 from the RdgBβ C-terminus could provide the signal for entry into the nucleus. The C-terminus of RdgBβ is also the region most likely to form the lid that regulates the entry or exit of the lipid from the hydrophobic cavity (Figure 2B). Thus binding of 14-3-3 to the C-terminus could have major consequences for the lipid exchange function of RdgBβ. One possibility is that the C-terminus is immobilized when bound to 14-3-3 so that the
G-helix of the PITP domain (coloured red in Figure 2B) would be unable to move, prohibiting the opening of the cavity [34]. Phosphorylation would keep RdgBβ in an inactive state and dephosphorylation would be required for RdgBβ to function as an exchange protein. The recombinant RdgBβ made in Escherichia coli, and therefore unphosphorylated, does possess in vitro PI transfer activity. Transfer activity of the RdgBβ–14-3-3 complex obtained from mammalian cells overexpressing RdgBβ has been tested, but no activity was observed. Since the amount of RdgBβ protein required for the in vitro transfer assay is relatively high, the lack of activity of the RdgBβ–14-3-3 complex is likely to be due to insufficient quantities of the material used in the assay. Further studies will be required to address this question conclusively.

The C-terminus of RdgBβ harbours two PEST sequences, which are regions rich in proline, glutamic acid, serine and threonine. PEST sequences are most common in rapidly degraded proteins [35,36]. Binding of 14-3-3 to the C-terminus of RdgBβ could shield the PEST sequences and provide protection from degradation [33]. This certainly seems to be the case. Binding of 14-3-3 proteins doubles the t1/2 of RdgBβ from 2 to 4 h. It is noted that the t1/2 of the wild-type RdgBβ protein is quite short compared with another PITP, PITPβ, suggesting that the protein could be transcriptionally regulated. Thus in human dermal fibroblasts when stimulated to proliferate with serum, or with other growth factors including EGF (epidermal growth factor), FGF (fibroblast growth factor) and PDGF (platelet-derived growth factor), increased expression of the PITPNC1 transcripts is observed as is increased expression of 14-3-3γ [37]. Increased transcription of the PITPNC1 gene is also observed when human prostate cancer cells (LNCaP cells) are treated with methyleseolic acid [38]. It is interesting to note that intrinsically disordered proteins are in general tightly regulated and that dosage-sensitive genes encode proteins with disordered segments. RdgBβ shows all the hallmarks of intrinsically disordered proteins [39,40]. RdgBβ can be ubiquitylated, a common mechanism by which intracellular proteins are degraded involving the 26S proteasome. Whether RdgBβ is a dosage-sensitive gene remains to be explored.

**RdgBβ binds to ATRAP (Ang II receptor-associated protein)**

In addition to binding 14-3-3 proteins, RdgBβ also binds ATRAP [24]. ATRAP interacts with the PITP domain of RdgBβ, a site distinct from the 14-3-3-binding site. For this interaction to be observed, treatment of cells with PMA for 4–16 h is required. The significance of this chronic stimulation is unknown. ATRAP is an integral membrane protein of 18 kDa, which is composed of three transmembrane domains and an extended hydrophilic cytoplasmic C-terminal tail (108–159 amino acids) (Figure 2). ATRAP was originally identified in a yeast two-hybrid screen as an interacting partner of the AT1R (Ang II type 1 receptor), and ATRAP residues 110–120 amino acids are required for this interaction [41]. A short splice form of 152 amino acids is also present; this lacks residues 116–121, which is part of the region that interacts with the receptor [41]. Both splice variants of ATRAP are widely expressed in cultured cell lines including HL60 cells and COS-7 cells (S. Cockroft and M. Li, unpublished work). The widespread expression of ATRAP suggests that its function is unlikely to be restricted to AT1R signalling. Recently, a fusion of ATRAP with BRAF was found as a causative oncogene in gastric cancer in humans [42].

The function of ATRAP has been explored both at the cellular level as well as the level of the organism. ATRAP apparently regulates the trafficking of the AT1R and is reported to enhance AT1R internalization with a consequent reduction in PLC activation, transcription and cell proliferation [43]. However, ATRAP also binds to several other proteins including CAML (calcium modulating cyclophilin ligand) [44] and RACK1 (receptor for activated protein C kinase 1) [45]. CAML regulates NFAT (nuclear factor of activated T-cells) and studies suggest that ATRAP can inactivate the NFAT pathway, irrespective of its effects on AT1R trafficking [44,46].

The effects of ATRAP expression in the cardiovascular and renal systems has been explored using mouse models. Transgenic mice overexpressing ATRAP are protected against Ang II-induced cardiac hypertrophy [47] and after an inflammatory vascular injury [48]. On the other hand, ATRAP-deficient mice have increased arterial BP (blood pressure) and an expanded PV (plasma volume) [49]. Essentially, ATRAP protects against cardiac hypertrophy and hypertension and it is worthy of note that transient pre-hypertensive treatment that prolongs anti-hypertensive and cardioprotective effects is likely to be beneficial owing to enhanced ATRAP expression [50].

In view of these effects of ATRAP in cardiac tissue, it is interesting to observe that RdgBβ is highly expressed in rat heart [24]. Antibodies against RdgBβ are available, but are unable to detect RdgBβ in lysates from tissues or cultured cell lines that we have examined so far; this is very likely to be due to the poor sensitivity of the antibodies, both commercial and made in-house [24]. Therefore we partially concentrated the proteins by separating the proteins by size-exclusion chromatography. The highest amounts of RdgBβ were found in rat heart cytosol partially co-purifying with 14-3-3 proteins, suggesting that only part of the RdgBβ proteins are present in a complex with 14-3-3. Additionally, RdgBβ proteins have been identified in several phosphorotemic screens, for example in the analysis of human embryonic stem cells [30] and developing mouse brain [28]. Future studies will have to address the function of RdgBβ by using both animal models and studies in cells where RdgBβ levels can be manipulated by RNAi (RNA interference). Homologues of RdgBβ and ATRAP are found in *Drosophila*, *C. elegans* and zebrafish, three model systems that are in common use and amenable to genetic manipulation.

RdgBβ is a cytosolic PI-binding protein and shows relatively weak transfer activity in vitro compared with the
ubiquitously expressed PITPα and PITPβ. ATRAP is an integral membrane protein that localizes in a perinuclear location in HEK-293 cells (human embryonic kidney cells) cells when overexpressed. ATRAP appears to be associated with the ER, Golgi and endosomes [43] ATRAP localization in cardiomyocytes is not known and this would provide clues to its real function in these cells. Estimates suggest that less than 10% of ATRAP is associated with the AT1R. A priority is to identify additional binding partners for RdgBβ, and this will facilitate in developing this model.

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References


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