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Both the PH domain and N-terminal region of oxysterol-binding protein related protein 8S are required for localization to PM-ER contact sites

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ABSTRACT

Oxysterol-binding protein-related proteins are implicated in the sensing and transporting lipids at the membrane contact sites. One of the members of the mammalian ORP family, ORP8, is thought to transport lipids through directly tethering both ER and PM membranes. Targeting to PM is thought to be mediated by N-terminal pleckstrin homology domain via binding to phosphoinositides. Sequence alignments and NMR structural determination revealed that the PH domain of ORP8 is atypical and contains an insertion of 20 amino acids in an unstructured loop region that may potentially block interactions with ligands. Using standard lipid-protein overlay assays or liposomal binding assays we could not detect binding of a recombinant version of the PH domain. Examination of a series of deletion constructs demonstrated that both the N-terminal polybasic region and the PH domain are required for proper targeting of the short splice variant ORP8S to the PM-ER contact site in Chinese hamster ovary cells.

1. Introduction

The non-vesicular movement of lipids is thought to occur at membrane contact sites (MCS) [1]. MCS are subcellular locations where the endoplasmic reticulum (ER) and another organelle come in close apposition ≈10–30 nm [2,3]. The ER comprises an extensively branched network of membranes whose large structure helps to facilitate the establishment of MCS with other organelles including the Golgi apparatus, mitochondria, peroxisomes and the plasma membrane (PM) [4]. Additionally, the ER is the site where most phospholipids are synthesized, and thus MCS can aid in the redistribution of new phospholipids to other organelles. Importantly, due to their hydrophobic nature, the spontaneous movement of phospholipids between organelles is insignificant in the context of cell biology [4,5]. Instead, a variety of lipid transport proteins (LTP) have evolved to mediate the these non-vesicular transport pathways [3].

One family of LTPs is named after the human oxysterol binding protein (OSBP) and referred to as OSBP-related proteins (ORPs) in humans and OSBP-homologs (Osh) in yeast [6,7]. The ORP/Osh family is characterized by a highly conserved lipid-binding domain that can transfer a variety of lipids including cholesterol, phosphatidylserine (PS) and phosphatidylinositol 4-phosphate (PI4P) depending on the isoform [8–10]. The ORP/Osh proteins can be further classified based on the mechanism used to localize to the ER. Several of the ORPs contain an FFAT (2 phenylalanines in an acidic tract) motif which allows them to bind to an ER-resident protein Vamp-associated protein (VAP) [11]. Alternatively, the human ORP5 and ORP8, contain a transmembrane domain at the C-terminal end of the protein which keeps it “anchored” to the ER [12]. Finally, the third subtype of ORP proteins possesses neither an FFAT-motif or an ER-tail anchor. How these proteins localize to their proper subcellular location is currently unknown.

ORP5 and ORP8 are thought to tether to both the ER and PM and mediate the exchange of PS for PI4P [13]. ORP5/8 possess homologous PH domains near the N-terminus of the protein that are required for the protein to interact with the PM. In many proteins, PH domains facilitate the interactions of proteins with their target

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membranes through binding of phosphoinositides. However, ≈10% of all PH domains in the human genome display significant binding to phosphoinositides [14].

ORP8 is expressed as two isoforms, a full-length (ORP8L) that contains 889 amino acids and as a shorter splice variant of (ORP8S) that lacks the first 42 amino acids due to missing exon in the 5′ region. Previous results demonstrated that ORP8L was found throughout the ER, while ORP8S was found enriched in peripheral ER in areas that would likely contain ER-PM contact sites [13]. The difference between ORP8L and 8S is an additional 42 amino acids located at the N-terminal (Nt) end of the ORP8L. These additional amino acids provide a net negative charge (−5) to the Nt region (1–141) of the ORP8L which is reflected in its isoelectric point of pI = 5.04 [13]. Conversely, the Nt region of ORP8S (43–141) has a slightly basic isoelectric point of pI = 8.08. Thus, the lower pI of ORP8L may produce a repulsive force between the Nt of ORP8L and the negatively charged inner leaflet of the PM and thus giving ORP8L a diffuse ER localization whereas the Nt ORP8S should not have that effect. Indeed the basic nature may promote interactions between the Nt of ORP8S and anionic phospholipids. Whether the Nt of ORP8S was acting a positive contributor to the localization of the protein or simply was not replying the protein from the PM is unclear.

Due to the importance of the PH domain and N-terminal region of ORP8 in targeting the PM, we investigated the structure-function of this domain in more detail. While the PH domain shows a minimal affinity for lipids *in vitro*, we found that both the PH domain and the adjacent N-terminal region are required for the proper localization of ORP8.

2. Material and methods

2.1. Reagents

Reduced glutathione and other fine chemicals were purchased from Sigma. Phusion DNA polymerase and restriction enzymes were purchased from Thermo Fisher Scientific. Bulk phospholipids lipids were purchased from Avanti Polar Lipids while phosphoinositides and recombinant PH-PLCδ were purchased from Echleon Biosciences.

2.2. DNA constructs

The GST-LACT-C2, GFP-K-Ras tail and GFP-Sec61 plasmids used in this study were previously described. To generate the mCherry-ORP8L, mCherry-ORP8S and mCherry-ORP8S$^{\Delta N\Delta PH}$ (268–889), polymerase chain reaction was used using the primer pairs ORP8L, ORP8S, and ORP8S$^{\Delta N\Delta PH}$ (268–889), respectively (Supplemental Table 1). PCR primers contained the relevant restriction enzyme sites and thus the PCR products were digested with either BglII and PstI (ORP8L, ORP8S$^{\Delta N\ \Delta PH}$) or BglII and KpnI (ORP8S) and ligated into pmCherry-C1. pGEX-OSBP-PH, ORP8-PH, and PLC$_\delta$-PH were constructed by PCR amplification, restriction digestion with BglII and XhoI, and insertion of the PCR products into pGEX-6p1 plasmids. Deletion constructs, ORP8S$^{\Delta PH}$(43–889, Δ142-280) and ORP8S$^{\Delta N}$ (142–889) were generated using Phusion Site-Directed Mutagenesis Kit according to manufacturer's instruction. Briefly, 5′ phosphorylated primer pairs were designed which could hybridize to regions on either side of the deletion area. Primers for ORP8S$^{\Delta PH}$ were (Fw 5′CATGACC-TGAGCGTTTCATCA3′, Rev 5′ACTGAGCAGCTCCTTTGTGG3′) and for ORP8S$^{\Delta N}$ were (Fw 5′-ACAATCACAGATCCTTCTGTTATTGT3′ Rev 5′AGATCTGAGTCCGGA-CTTGTACAG3′) and mCherry-ORP8S and ORP8L were used as PCR template, respectively. PCR amplification mix was treated with DpnI to digest template plasmid, and the linear plasmids of interest were ligated and transformed into DH5α *E. coli.*

2.2.1. GST fusion protein purification

Plasmids were transformed into chemically competent *E. coli* BL21 (DE3) Rosetta cells (Novagen) and protein expression was induced by the addition of 0.5 mM IPTG to exponential-phase cells. After 3 h at 37 °C, cells were lysed with B-PER reagent (Thermo Fisher Scientific) and purified using glutathione agarose (Thermo Fisher Scientific) using standard PBS and glutathione elution buffers for GST fusions proteins as previously described [15]. SDS/PAGE analysis and Coomassie Blue staining of the purified protein preparations gave an estimated purity of >90% for each construct.

2.2.2. Structure-based multiple sequence alignments

Protein sequences of the indicted PH domains were retrieved from National Center for Biotechnology Information (NCBI). Multiple sequence alignments were performed by using Profile Multiple Alignment with predicted Local Structures and 3D constraints (PROMALS3D) [16].

2.2.3. NMR and X-ray structure analysis

The NMR resolved PH domain of ORP8 and X-ray structures of other PH domains were obtained from Protein Data Base (PDB). PDB identification codes were for ORP8 PH (1V88), AKT PH in complex with inositol 1,3,4,5-*tetrakis*phosphate (1UNQ), and PLCδ PH in complex with inositol 1,4,5-*tris*phosphate (1MAI).

2.2.4. Liposome preparation

Each liposome contained 2% dansyl-PE. Specific compositions used in the indicated liposomes were: PC liposome (98% DOPC) PS liposome (78% PC, 20% PS) and the indicated PIP$_x$ liposome (75% PC, 20% PS, 3% PIP$_x$). Bulk phospholipid dissolved in either chloroform: methanol (1:1, v/v) or chloroform:methanol:water (20:9:1, v/v) were aliquoted to glass vials and dried under a stream of nitrogen gas. Large multilamellar vesicles (MLVs) were formed by the rehydration of dried phospholipids in aqueous Hepes-buffered saline at 37 °C for 1 h, followed by 20 min of sonication in a water bath. Small unilamellar vesicles (SUVs) were prepared by extruding MLVs through 100 nm diameter pore of polycarbonate membranes following manufacturers suggested protocol (Avestin, Ottawa, Canada).

2.2.5. In vitro lipid-protein binding assay

GST fusion proteins were incubated with phospholipids immobilized on nitrocellulose membrane (PIP Strips, Echelon Biosciences) according to the manufacturer's instruction. Briefly, PIP strips were blocked in PBS-T (0.1% v/v Tween-20) + 3% delipidated BSA for 1 h at room temperature. Next, 0.5 μg/mL of GST protein was added in PBS-T+3% BSA and incubated for 1 h at RT. Following the incubation period, the membranes were washed in PBS-T five times for 10 min each. The membranes were then incubated with anti-GST antibody for 1hr, washed and incubated with goat anti-mouse HRP-conjugated secondary antibody. SuperSignal chemiluminescence substrate (Thermo Fisher Scientific) was used for visualization and a ChemiDoc Touch (Bio-Rad) was used to capture the images.

2.2.6. Liposome-protein fluorescence resonance energy transfer (FRET) assay

Binding of GST fusion proteins to liposomes was measured by FRET between donor tryptophan residues in the protein to acceptor dansyl-phosphatidylethanolamine (dansyl-PE) in the liposomes as previously described [17,18] using a Synergy Neo multi-mode reader (Biotek, Winooski, VT). Binding of recombinant proteins to the liposomes was monitored by the increase of the dansyl acceptor