Oxysterol-binding protein related-proteins (ORPs) 5 and 8 regulate calcium signaling at specific cell compartments

Ilari Pulli, Taru Lassila, Guoping Pan, Daoguang Yan, Vesa M. Olkkonen, Kid Törnquist

Åbo Akademi University, Tykistökatu 6A, 20520 Turku, Finland
The Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Department of Biotechnology, College of Life Science and Technology, Jinan University, Guangzhou, 510632, China
Minerva Foundation Institut For Medical Research, Biomedicum Helsinki, 00290 Helsinki, Finland
Department of Anatomy, Faculty of Medicine, FI-00014 University of Helsinki, Finland

ARTICLE INFO

Keywords:
ORP5
ORP8
Caveolae
Mitochondria
Endoplasmic reticulum
Calcium

ABSTRACT

Oxysterol-binding protein related-protein 5 and 8 (ORP5/8) localize to the membrane contact sites (MCS) of the endoplasmic reticulum (ER) and the mitochondria, as well as to the ER-plasma membrane (PM) MCS. The MCS are emerging as important regulators of cell signaling events, including calcium (Ca²⁺) signaling. ORP5/8 have been shown to interact with phosphatidylinositol-4,5-bisphosphate (PIP2) in the PM, and to modulate mitochondrial respiration and morphology. PIP2 is the direct precursor of inositol trisphosphate (IP3), a key second messenger responsible for Ca²⁺ release from the intracellular Ca²⁺ stores. Further, mitochondrial respiration is linked to Ca²⁺ transfer from the ER to the mitochondria. Hence, we asked whether ORP5/8 would affect Ca²⁺ signaling in these cell compartments, and employed genetically engineered aequorin Ca²⁺ probes to investigate the effect of ORP5/8 in the regulation of mitochondrial and caveolar Ca²⁺. Our results show that ORP5/8 overexpression leads to increased mitochondrial matrix Ca²⁺ as well as to increased Ca²⁺ concentration at the caveolar subdomains of the PM during histamine stimulation, while having no effect on the cytoplasmic Ca²⁺. Also, we found that ORP5/8 overexpression increases cell proliferation. Our results show that ORP5/8 regulate Ca²⁺ signaling at specific MCS foci. These local ORP5/8-mediated Ca²⁺ signaling events are likely to play roles in processes such as mitochondrial respiration and cell proliferation.

1. Introduction

Members of the oxysterol-binding protein related-protein (OSBP-related, or ORP) family are involved in the regulation of lipid transport at membrane contact sites (MCS) [1]. MCS are crucial for inter-organellar communication and lipid transfer as well as for the regulation of second messenger molecules, including the calcium ion (Ca²⁺) [2]. ORPs, including ORP5 and ORP8 (ORP5/8), are involved in oncogenesis, possibly through the regulation of membrane lipids and the related downstream signaling events [3]. ORP5/8 have been characterized as countertransporters of phosphatidylinositol-4-phosphate (P14P) and phosphatidylserine at the interface of the endoplasmic reticulum (ER) and the plasma membrane (PM) [4]. ORP5/8 were also shown to bind to phosphatidylinositol-4,5-bisphosphate (PIP2) and modulate its transport from the PM to the ER [5]. Also, it was shown that ORP8 may not bind to P14P and PIP2 with high affinity [6]. Interestingly, it was recently reported that ORP8 is not strongly associated with the PM in resting conditions but becomes recruited to the PM upon induction of PIP2 production [7]. Moreover, ORP5/8 were recently found to localize at the ER-mitochondria contact sites where they modulate mitochondrial morphology and function [8]. Approximately 80% of the ORP5 and ORP8 sequences overlap, they both insert into the ER membrane through a C-terminal transmembrane (TM) domain, contain a lipid transfer domain (ORD) and interact with the PM through a pleckstrin homology (PH) domain [5,9]. The mitochondrial interactions of ORP5/8 depend on the ORD domain that binds to the mitochondrial outer membrane protein PTPIP51 [8].

Ca²⁺ is a ubiquitous second messenger that regulates several cellular functions including proliferation, migration and respiration. Intracellular Ca²⁺ concentrations are tightly regulated and multiple organelles and cellular compartments are involved in the handling and sensing of Ca²⁺ signals. Ca²⁺ is stored in the ER from where it can be released to the cytosol upon different stimuli, such as activation of the inositol trisphosphate receptor (IP₃R) [10]. ER Ca²⁺ release can be directed towards the mitochondria through the molecular interactions at contact sites designated as mitochondria-associated membranes...
Mitochondria take up Ca\textsuperscript{2+} through the mitochondrial calcium uniporter (MCU) to activate the respiratory enzymes, whereas mitochondrial Ca\textsuperscript{2+} overloading can lead to apoptosis [11,12]. MCU is tightly controlled by accessory proteins, the threshold Ca\textsuperscript{2+} concentration for MCU activation is high, and hence, ER-mitochondria tethering is essential for the generation of high Ca\textsuperscript{2+} micromodains that allow for MCU activation [12–17]. Also, local, spatiotemporally restricted Ca\textsuperscript{2+} signaling events may take place at cytoplasmic leaflets of the PM and at the lipid-rich PM compartments, caveolae, where many molecules related to Ca\textsuperscript{2+} signaling, such as PIP\textsubscript{2}, have been shown to localize [18,19]. Importantly, MAMS and caveolae-ER contacts are implicated in oncogenesis, and Ca\textsuperscript{2+} transfer trough the IP\textsubscript{3}R to the mitochondria is essential for cancer cell metabolism [20,21]. Further, ORP5/8 reside at the MAMS, which have been recognized to play a role in cardiovascular pathophysiology in part through disturbed mitochondrial Ca\textsuperscript{2+} signaling [22]. Also, the possible involvement of ORP5/8 in Ca\textsuperscript{2+} signaling has been recently reviewed [23].

Considering that ORP5/8 are involved in the physical interactions and lipid transfer between the ER, the PM and the mitochondria, we hypothesized that this might also affect the Ca\textsuperscript{2+} handling at these membrane interfaces. Further, ORP5/8 modulate the PM levels of PIP\textsubscript{2} [5], which is the substrate for phospholipase C (PLC) that hydrolyzes PIP\textsubscript{2} to IP\textsubscript{3} and diacylglycerol (DAG). IP\textsubscript{3} binds to and activates the IP\textsubscript{3}R in the ER, and DAG activates many of the PM Ca\textsuperscript{2+} channels. The ORP5/8 mediated modulation of PIP\textsubscript{2} transport might thus affect intracellular Ca\textsuperscript{2+} signaling. Therefore, we set out to characterize the importance of ORP5/8 in regulating Ca\textsuperscript{2+} specifically in the mitochondria, at the caveolar compartments of the PM, and in the overall cytoplasm.

2. Methods

2.1. Cell culture and plasmid and siRNA transfections

HeLa cells were used in all experiments of this study. The cells were routinely cultured in DMEM (#D6046, Sigma-Aldrich) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). The cells were grown in a humidified incubator at 37 °C and 5% CO\textsubscript{2}. The GFP-ORP5 expression plasmid is from professor Paolo Pinton (University of Ferrara, Italy).

2.2. Intracellular calcium measurements by employing aequorin

The luminescent Ca\textsuperscript{2+} binding recombinant protein, aequorin, was employed to measure Ca\textsuperscript{2+} concentrations in the cytoplasm, in the mitochondria, and at the caveolae as previously described in [24,25]. Briefly, 5000–10,000 HeLa cells were seeded on to 96-well plates. The cells were grown overnight and transfected with the desired aequorin plasmids. Western blot analysis

Cell lysates were prepared by washing the cells three times with PBS, whereas L-aemml sample buffer was added to the samples. The lysates were boiled for 5 min and separated by SDS-PAGE, whereafter the proteins were transferred to a nitrocellulose membrane. The membranes were subjected to the desired primary or secondary antibodies in a phosphate buffered saline (PBS) solution containing 1% bovine serum albumin. The ORP5 antibodies were from Sigma-Aldrich and the ORP8 antibodies are described in [26]. HSC70 antibody was purchased from Enzo life sciences. The dilutions for the primary antibodies were 1:1000 and 1:3000 for the secondary antibodies (anti-rabbit HRP-conjugated antibody, BioRad; anti-rat HRP-conjugated antibody, Cell Signaling Technology). The protein bands were visualized by chemiluminescence (ECL, Perkin Elmer). Anti-Xpress and Anti-Xpress-HRP antibodies (Invitrogen) were employed for the detection of epitope-tagged ORP5/8 constructs that were used in the IP\textsubscript{3} production measurements.

2.5. Measurement of IP\textsubscript{3} production

IP\textsubscript{3} was measured using the HitHunter IP\textsubscript{3} Fluorescence Polarization Assay Kits (DiscoverRx Tech, Fremont, CA, USA). Briefly, 10,000 cells transfected with pcDNA4 HisMax C, ORP5 or ORP8 vector in 96-well plates (Corning, USA) were treated with 10μM histamine for the designated times, and the cellular reaction was terminated by adding 0.2 N perchloric acid. The plate was shaken at 650 r.p.m. for 5 min. Then 20 ul mixture buffer from 96-well plates were transferred into black 384-well plates (Greiner, Germany). The IP\textsubscript{3} tracer was subsequently added to each well, and the IP\textsubscript{3} binding protein was added to the plate. The polarized fluorescence from the IP\textsubscript{3} tracer was read on a Microplate Reader (CLARIOstar, BMG LABTECH). The IP\textsubscript{3} concentration was calculated from the IP\textsubscript{3} standard curve.

2.6. Proliferation assay

Fifty-thousand HeLa cells were plated on 6-well plates and grown.... CaCl\textsubscript{2} or, alternatively, 150μM EGTA, when Ca\textsuperscript{2+} free buffer was used). The cells were stimulated with 100μM histamine in Ca\textsuperscript{2+} containing or in Ca\textsuperscript{2+} free HBSS depending on the experimental setup. The cells were primed for store-operated calcium entry (SOCE) by treating the cells with 1μM thapsigargin (TG) for five minutes in the presence of 150μM EGTA whereafter the SOCE was induced by perfusing the cells with HBSS buffer containing 1μM TG and 1mM Ca\textsuperscript{2+}. At the end of each experiment, the cells were lysed with 100μM digitonin in the presence of 10 mM Ca\textsuperscript{2+} to obtain the maximal light production values of the sample. The obtained luminescence raw data values were analysed and transformed as described in [24]. The cytoplasmic and mitochondria targeting aequorin constructs were a kind gift from professor Paolo Pinton (University of Ferrara, Italy).
overnight. Thereafter the cells were transfected with plasmid constructs carrying GFP, GFP-ORP5 or GFP-ORP8, respectively. The cells were grown for 48 h and subjected to 3H-thymidine (0.4 µCi/ml) for the final four hours. Thereafter the cells were washed three times in ice-cold PBS on ice, followed by a 10-min incubation in 5% trichlor acetic acid, and then incubated for 10 min with 0.1 M NaOH. Then the suspension was mixed 1:5 with Optiphase Hisafe 3 scintillation liquid (PerkinElmer). Radioactivity was measured using a Wallac 1410 liquid scintillation counter. 3H-thymidine was from PerkinElmer.

2.7. Confocal microscopy

The cells were plated on poly-lysine coated coverslips, grown overnight and transfected with plasmid constructs carrying GFP, GFP-ORP5 or GFP-ORP8, respectively. The cells were then grown overnight, washed in PBS, fixed using 4% paraformaldehyde and mounted with Mowiol mounting medium. Microscopy was conducted by employing a Leica SP5 (Wetzlar, Germany) confocal microscope.

2.8. Statistics

The results are shown as the mean ± S.E.M. Statistical analysis of the data was conducted by unpaired Student’s t-test when two means were compared or by One-Way Anova with Tukey post hoc test when three or more means were compared. The GraphPad Prism S program (GraphPad Software Inc., San Diego, CA) was used for the statistical analyses. A P-value below 0.05 was considered statistically significant.

3. Results

To assess the importance of ORP5/8 in regulating Ca2+ in intracellular compartments, we employed the luminescent calcium indicator, aequorin. Upon transient expression, wild-type aequorin localizes to the cytosol, but aequorin has been genetically engineered to target different cell compartments such as the mitochondria and the caveolae [24,25]. First, we set out to measure cytoplasmic and mitochondrial Ca2+ upon overexpression of GFP-ORP5 or −ORP8 in the presence of extracellular Ca2+. To induce the intracellular Ca2+ fluxes we used the G-protein coupled receptor (GPCR) agonist, histamine. Our results show that the cytoplasmic Ca2+ was not affected in cells overexpressing either ORP5 or ORP8 when stimulated with 100 µM histamine, whereas ORP5 overexpression resulted in increased mitochondrial Ca2+ uptake upon histamine stimulation (Fig. 1A). To further explore the possible involvement of ORP8 in the ER-mitochondrial Ca2+ flux, we excluded the extracellular Ca2+ from the experimental setting. In this condition, ORP8 overexpression significantly increased mitochondrial Ca2+ uptake upon stimulation with histamine (Fig. 1B). Treating the cells with 10 µM BAPTA-AM for 1 h completely abolished the mitochondrial Ca2+ responses (Supplementary Fig. 1A). Overexpression of the GFP-ORP5/8 constructs was confirmed by microscopy (Fig. 1C). However, knock-down of ORP5 and −8 separately or simultaneously did not affect the stimulation-evoked mitochondrial Ca2+ uptake (Fig. 2A). Efficient knock-down of ORP5/8 was confirmed by western blotting (Fig. 2B).

As mentioned above, ORP5 and −8 bind to PIP2 and are localized to the ER-PM contact sites, and caveolae are enriched in PIP2 [5,19]. Therefore, we tested whether the caveolar microdomain of Ca2+ might be affected by ORP5/8 overexpression or silencing. We found that ORP8 overexpression slightly but significantly increased the histamine-induced Ca2+ concentration at the caveolae ([Ca2+]cav) in the presence of extracellular Ca2+ (Fig. 3A) This effect was more pronounced in the absence of extracellular Ca2+ (Fig. 3B). Also ORP5 overexpression significantly augmented the histamine-induced [Ca2+]cav (Fig. 3C). In contrast, knockdown of ORP5/8 did not affect [Ca2+]cav (Fig. 3D).

Caveolae may harbour molecules that are involved in the regulation of the store-operated Ca2+ entry (SOCE) [18]. Hence, we investigated whether ORP5/8 would affect [Ca2+]cav during thapsigargin (TG)-induced SOCE. We found that overexpression of ORP5 or −8 was without an effect on SOCE at the caveolar microdomain (Fig. 4A&B). Interestingly, cytoplasmic Ca2+ was slightly but significantly inhibited by ORP5 overexpression during TG-induced SOCE (Fig. 4C). Further, ORP5 overexpression inhibited the cytosolic Ca2+ flux in an experimental setting where Ca2+ was present in the extracellular milieu during the TG treatment (Supplementary Fig. 1B).

Even though we could not detect an effect of ORP5 or −8 on the agonist-induced cytoplasmic Ca2+, we found it interesting to test whether the overexpression of these proteins might modify the release of IP3 by PLCs. Therefore, the cellular IP3 concentration was measured upon histamine stimulation. We found that ORP5 overexpression significantly augmented IP3 production whereas ORP8 overexpression was without an effect (Fig. 5).

ORP proteins have been implicated in the control of cell proliferation. It has been shown that ORP4 silencing halts cell proliferation and that ORP4L modulates proliferation through Ca2+ and the nuclear factor of activated T cells (NFAT) pathway [27,28]. Also, it is well established that intracellular Ca2+ plays key roles in controlling cell proliferation [10]. Hence, we used the 3H-thymidine incorporation assay to quantify the proliferation of GFP, GFP-ORP5 or GFP-ORP8 transfected HeLa cells. Interestingly, we found that the ORP5/8 overexpressing cells showed a significant increase in proliferation as compared to the GFP-transfected controls (Fig. 6).

4. Discussion

In this study we present evidence that elevated cellular levels of the OSBP-related proteins ORP5 or −8 modify Ca2+ homeostasis in an organelle-specific fashion. Experiments with caveoleae- or mitochondria-targeted aequorin Ca2+ probes demonstrated that while neither ORP5 nor −8 overexpression had a significant effect on the cytoplasmic Ca2+ concentration in histamine-stimulated cells, ORP5 and −8 elevated the Ca2+ concentrations both in the mitochondrial matrix as well as at the caveolar sub-compartments of the PM. The effect of ORP5 on mitochondrial Ca2+ was more pronounced than that of ORP8, the effect of which was only detectable when extracellular Ca2+ was chelated. Modulation of mitochondrial calcium by ORP5/8 is consistent with the reported disturbance of mitochondrial respiratory function observed in ORP5/8-depleted cells [8], considering that mitochondrial calcium, as well as the proximity of mitochondria to the intracellular Ca2+ stores, are crucial for the activity of key mitochondrial machineries responsible for oxidative energy production [21,29,30].

In contrast to what has been reported for ORP4L in T-cell acute lymphoblastic leukemia cells [31], we detected no significant changes in the cytoplasmic Ca2+ levels during histamine-stimulation in cells overexpressing ORP5/8. This finding underlines the role of ORP5/8 as regulators and coordinators of local Ca2+ signaling events specifically at the MCS at the ER-mitochondria and at the ER-PM/caveolae interfaces. Previously, ORP4L has been reported to augment IP3 production in T-cells [31]. In our study, we found that ORP5 overexpression leads to increased cellular IP3 levels during histamine stimulation, whereas ORP8 overexpression did not affect the histamine-induced IP3 generation. Hence, ORP5 may modulate the detected local Ca2+ signaling events in part through IP3-dependent mechanisms whereas the effect of ORP8 is attributed to other factors. We find it possible that ORP5 overexpression augments mitochondrial Ca2+ uptake in part through increased apposition of ER and mitochondria, and that the resulting increase in mitochondrial Ca2+ buffering capacity masks the cytoplasmic Ca2+ effects that would otherwise be expected due to the ORP5-mediated increase in the IP3 production. Further, we speculate that ORP5-mediated interactions at the ER-caveolae MCS may allow for the generation of local caveolar Ca2+ hotspots at these sites even in the presence of high mitochondrial Ca2+ buffering.

As noted above, the effect of ORP8 overexpression on mitochondrial
Fig. 1. Mitochondrial, but not cytosolic, $[\text{Ca}^{2+}]$ is increased by ORP5/8 overexpression upon 100 μM histamine stimulation. A) Representative traces and quantification of cytoplasmic and mitochondrial Ca$^{2+}$ fluxes during histamine stimulation in the presence of 1 mM Ca$^{2+}$. B) Mitochondrial Ca$^{2+}$ flux during histamine stimulation in the presence of 150 μM EGTA. C) Confocal microscopy images showing HeLa cells expressing GFP, GFP-ORP5 and GFP-ORP8 constructs, respectively. The bars represent the mean ± SEM of the change in the calcium concentration during the experiment (maximal value – basal value). *, p < 0.05; ns = not significant. N = 3–6.
calcium was only detectable in the absence of extracellular Ca\(^{2+}\). This may be explained by the previous finding that ORP8 is more evenly distributed throughout the ER membranes whereas ORP5 is more specifically localized to the MAM MCS [4,8]. Thus, the effect of ORP8 overexpression on mitochondrial Ca\(^{2+}\) uptake was unmasked when the extracellular Ca\(^{2+}\) was chelated and the IP3-releaseable ER Ca\(^{2+}\) was the primary source of Ca\(^{2+}\). The data suggests that ORP5/8, and ORP5 in particular, has the ability to organize and expand an ER-mitochondrial membrane contact domain at which Ca\(^{2+}\) transport is active. The physical association of ORP5/8 with PTPIP51 is consistent with this notion, considering that the mitochondrial outer membrane protein PTPIP51 in a complex with the ER protein VAPB is also reported to control the ER-mitochondria associations [8,32]. Further, ORP5/8 are involved in the modulation of mitochondrial morphology and the integrity of the mitochondrial network, key aspects which are known to affect the regulation of mitochondrial Ca\(^{2+}\) [8,33].

It has been reported that ER and PM can form junctions at the caveolae [34]. Also, the interaction of IP3R1 and −3 with the Ca\(^{2+}\)-conducting transient receptor potential canonical cation channels 1 and 3 (TRPC1, −3), respectively, is coordinated at the caveolae [35,36]. Further, caveolae are considered as important signaling hubs that control a wide range of cellular events such as migration, adhesion and invasion, and these processes are modulated by Ca\(^{2+}\) signals [18,34]. Our results showing that ORP5/8 modulate Ca\(^{2+}\) at the caveolae may thus in part offer new mechanistic insight to the previously reported ORP5/8-mediated regulation of invasion and migration [3,37].

We did not observe disturbances in mitochondrial or caveolar calcium upon siRNA-mediated knock-down of ORP5, −8 or both. This is consistent with the previous observation that inter-organelle MCSs rarely depend on a single tethering component. For example, in yeast the abolishment of the ER-PM contact sites required the genetic disruption of six MCS components [38]. We also find it possible that knocking down the ORPs involved in the vital MCS calcium transport processes will lead to compensatory responses in order to maintain organelle Ca\(^{2+}\) homeostasis, which may lead to a dampening of the effect of ORP5/8 knock-down on mitochondrial or caveolar Ca\(^{2+}\). Also, the partial knock-down of ORP5/8 proteins achieved by siRNA treatment may not be sufficient to induce significant effects in the robust Ca\(^{2+}\) fluxes that were studied. Further, we found that ORP5/8 overexpression did not affect SOCE at the caveolar microdomain of the plasma membrane. However, cytoplasmic Ca\(^{2+}\) was slightly reduced in ORP5 overexpressing cells during TG-induced SOCE. Interestingly, SOCE proteins STIM1 and Orai1 are translocated from the PIP2-
rich PM domains to the PIP2-poor PM domains upon ER Ca2+ depletion, whereas ORP5/8, and especially ORP5, interact with PIP2 at the PM [5,7,39]. Intriguingly, we find that ORP5 augments histamine-induced Ca2+ fluxes whereas cytoplasmic Ca2+ during SOCE is reduced upon ORP5 overexpression. These results are not contradictory as histamine-induced Ca2+ fluxes and SOCE recruit different proteins and signaling pathways, even if these pathways are interconnected (e.g. SOCE following the IP3-mediated Ca2+ release). Hence, there are kinetic differences in the activation of the histamine-induced Ca2+ fluxes and SOCE, SOCE being activated through STIM and ORAI oligomerization in response to the IP3-mediated ER emptying [40,41].

We also found that ORP5/8 overexpression increases the proliferation of HeLa cells. This is in line with the similar findings regarding ORP4L [28]. Further, ORP5 overexpression is associated with tumor cell invasion [3] and proliferation (unpublished observations disclosed in ref. [3] and published during the revision of the present article [42]). In contrast to our finding, ORP8 has been linked to inhibition of cell growth in gastric cancer cells through induction of ER stress and to initiation of apoptosis in hepatocellular cancer cells through the Fas-pathway [43,44]. Interestingly, Ca2+ is essential for the initiation of Fas-mediated apoptosis [45], and ER stress, as well as the regulation of apoptotic events are associated with ER-mitochondria Ca2+ transfer [46]. On the other hand, Ca2+ handling at the ER-mitochondria interfaces is involved in the regulation of energy metabolism and cell proliferation [47]. Therefore, we find it possible that ORP8-mediated changes in mitochondrial Ca2+ homeostasis may modulate cell fate in a context-dependent manner.

In conclusion, our results provide evidence for the importance of ORP5/8 in regulating Ca2+ in the mitochondrial matrix as well as at the caveolar sub-compartments of the PM. These findings corroborate the role of ORP5/8 as MCS proteins facilitating organelle interactions at specific membrane interfaces.
Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants to I.P. from the Magnus Ehrnrooths stiftelse, Finsk-Norska medicinska stiftelsen, Tor, Joe och Pentti Borgs Minnesfond, Oscar Öflunds stiftelse, Waldemar Von Frenckells stiftelse, K. Albin Johanssons stiftelse, Ida Montins stiftelse and Svenska Kulturfonden. V.M.O. was supported by the Academy of Finland (grant 285223), the Sigrid Juselius Foundation, the Finnish Foundation for Cardiovascular Research and the Magnus Ehrnrooths stiftelse. K.T. was supported by the Sigrid Juselius Foundation and the Liv och Hälsa Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jece.2018.03.001.

References


