The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress

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Alterations of protein folding or Ca²⁺ levels within the endoplasmic reticulum (ER) result in the unfolded-protein response (UPR), a process considered as an endogenous inducer of inflammation. Thereby, understanding how genetic factors modify UPR is particularly relevant in chronic inflammatory diseases such as asthma. Here we identified that ORMDL3, the only genetic risk factor recently associated to asthma in a genome wide study, alters ER-mediated Ca²⁺ homeostasis and facilitates the UPR. Heterologous expression of human ER-resident transmembrane ORMDL3 protein increased resting cytosolic Ca²⁺ levels and reduced ER-mediated Ca²⁺ signaling, an effect reverted by co-expression with the sarco-endoplasmic reticulum Ca²⁺ pump (SERCA). Increased ORMDL3 expression also promoted stronger activation of UPR transducing molecules and target genes while siRNA-mediated knock-down of endogenous ORMDL3 potentiated ER Ca²⁺ release and attenuated the UPR. In conclusion, our findings are consistent with a model in which ORMDL3 binds and inhibits SERCA resulting in a reduced ER Ca²⁺ concentration and increased UPR. Thus, we provide a first insight into the molecular mechanism explaining the association of ORMDL3 with proinflammatory diseases.

INTRODUCTION

The ER is essential for the generation of intracellular Ca²⁺ signals, functioning as a regulated Ca²⁺ store (1,2). There are Ca²⁺ release channels (e.g. inositol trisphosphate receptor and ryanodine receptor) that control the exit of Ca²⁺ from the ER into the cytoplasm and pumps [sarco-endoplasmic reticulum Ca²⁺ ATPase (SERCA)] that return Ca²⁺ to the ER. In this sense, the activity of SERCA determines the rate of removal of cytosolic Ca²⁺, shaping the Ca²⁺ signal and helping to maintain low cytosolic Ca²⁺ levels. SERCA activity also influences Ca²⁺-dependent cellular responses by determining the ER [Ca²⁺] that is available for release in response to the next stimulus (2). Consistent with the key role of SERCA in Ca²⁺ homeostasis, several pathological conditions presenting ER Ca²⁺ dysregulation are associated with SERCA dysfunction (3), including asthma (4) and Alzheimer disease (5,6), although in the latter case ER Ca²⁺ dysregulation has been also attributed to a Ca²⁺ leak via presenilin (7) or via the ryanodine receptor (8).

The assembly and folding of numerous proteins also occurs at the ER, a process that requires appropriate ER Ca²⁺ levels (9–11). Alterations of protein folding or Ca²⁺ levels within the ER result in the unfolded-protein response (UPR) (12–16). The UPR involves different signaling pathways that sense conditions of ER stress and trigger the subsequent cellular homeostatic response (17). Although the UPR has been characterized in different cell models, it is particularly relevant in cells from the immune system where it is related to lymphocyte development (18) as well as the generation and function of antibody-secreting plasma cells (19). The UPR can also trigger the activation of NF-κB and JNK (20–22), key molecules in the onset of inflammation. Together, the UPR has been considered a condition closely related to the inflammatory

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response of specialized cells (17), either through the differentiation of inflammatory cells or the production of molecules involved in the onset of inflammation (23).

Recently, the ORMDL3 gene, coding for the ER-resident transmembrane protein ORMDL3 (24), has been associated with asthma (25–29), a chronic inflammatory disease of the airways (30,31). ORMDL3 shows a wide distribution in different tissues, being particularly high its expression in cells participating in the inflammatory response (24,25). A common C/T polymorphism (rs7216389) controlling ORMDL3 expression was associated to asthma (25), but neither the function of ORMDL3 nor the underlying mechanism for its association with asthma is known at present. Therefore, considering that: (i) there is a close relationship between UPR and inflammation; (ii) different transmembrane proteins of the ER participate in the sensing and initiation of the UPR; (iii) perturbation of ER calcium stores induces the UPR and (iv) diminished SERCA expression contributes to airway remodeling (4); we examined whether ORMDL3 may modulate ER-mediated Ca\(^{2+}\) signals and/or participate in the UPR, thereby providing a plausible pathophysiological mechanism explaining its association to asthma.

### RESULTS AND DISCUSSION

#### Expression and topology of ORMDL3

Computer-based protein sequence analysis of the ER-resident human ORMDL3 protein originally predicted four membrane-spanning domains with different hydrophobicity scores (24). To obtain more detailed topological information, we used a fluorescence protease protection assay (32). Three versions of ORMDL3 tagged with fluorescence proteins (FP) were generated: at the N-terminus; C-terminus and amino acid 79 of the protein (Fig. 1A). HEK293 cells were transfected with different combinations of ORMDL3-CFP or ORMDL3-YFP, ER targeting sequence of calreticulin fused to Discosoma sp. red fluorescent protein, STIM1-YFP and TRPV4-CFP were treated with 20 μM digitonin. Images were taken before (B) and after (C) application of trypsin (2 μm). The FP color combination was selected to code cytosolic fluorescent markers in blue and intrareticular fluorescent markers in yellow for transmembrane proteins and red for globular proteins. (D) Schematic structure cartoon based on predictions obtained using TMHMM-2 (www.cbs.dtu.dk/services/TMHMM-2.0), PSIPRED V2.3 (bioinf.cs.ucl.ac.uk/psipred) and the topology experiments shown in a-c. Scale bars 10 μm.
tagged at amino acid 79, suggesting that the central region of ORM DL3 faces the ER lumen. Based on these results and the fact that only two domains, of around 20 amino acids each (Supplementary Material, Fig. S1), show considerable hydrophobicity scores (>80% probability), we proposed a topology model for ORM DL3 consisting of two transmembrane domains, with N- and C-terminus facing the cytoplasm and a large loop within the ER lumen (Fig. 1D).

**Impact of ORMDL3 on cellular Ca\(^{2+}\) homeostasis**

The impact of heterologous expression of ORMDL3 on ER-mediated intracellular Ca\(^{2+}\) signals was evaluated in HEK293 cells. Cytosolic Ca\(^{2+}\) signals (using fura-2 calcium-sensitive dye) were recorded from GFP- and ORMDL3-transfected cells in response to carbachol, a muscarinic agonist that triggers the release of Ca\(^{2+}\) from inositol trisphosphate (IP\(_3\))-sensitive signals.
ER (Fig. 2A). The Ca\(^{2+}\) signal generated, which mainly reflects the amount of Ca\(^{2+}\) stored in the ER, was quantified by calculating the mean area under the curve (inset). ORMDL3 overexpression also slowed the decay rate of cytosolic Ca\(^{2+}\) clearance following carbachol stimulation (Fig. 2B), indicative of decreased SERCA activity (5,35–37), as this Ca\(^{2+}\) pump is in charge of replenishing the stores with Ca\(^{2+}\) pumped from the cytosol into the ER (38). This observation was further confirmed by direct measurements of intra-ER Ca\(^{2+}\) levels using a low-affinity Ca\(^{2+}\)-sensitive dye (mag fura-2) trapped within the ER in cells permeabilized with digitonin (39). Higher resting mag fura-2 ratios were observed in GFP- (Fig. 2C) than ORMDL3-transfected HEK293 cells (Fig. 2D). Mean basal ratios are shown in Fig. 2E. When permeabilized cells were stimulated with 5 \(\mu\)M IP\(_3\), the ratio decreased rapidly in both GFP- and ORMDL3-transfected cells. Upon IP\(_3\) removal, ratio increased, indicating reuptake of Ca\(^{2+}\) into the IP\(_3\)-sensitive store (Fig. 2C and D), but with a slower kinetics in ORMDL3-cells. Exponential fitting of Ca\(^{2+}\) reuptake gave mean time constants of 15 \(\pm\) 2 min \((n = 16)\) and 26 \(\pm\) 5 min \((n = 18)\) for GFP- and ORMDL3-expressing cells \((P < 0.05)\), respectively (Fig. 2F). Addition of the SERCA inhibitor thapsigargin (TG) (3,38) prevented Ca\(^{2+}\) reuptake into the intracellular organelle following a second IP\(_3\) challenge (Fig. 2C and D). This approach using mag fura-2 that only reports on Ca\(^{2+}\) signal within the ER, minimized the effect of Ca\(^{2+}\) extrusion through the plasma membrane on calcium clearance that may be present in the experiment shown in Fig. 2B. In other words, while in Figure 2B, time constant of Ca\(^{2+}\) clearance from the cytosol may be influenced by both Ca\(^{2+}\) reuptake into the ER and Ca\(^{2+}\) extrusion through the plasma membrane, data shown in Figure 2F only refers to the time constants of Ca\(^{2+}\) reuptake into the ER.

The effect of other stimuli and knockdown of endogenous ORMDL3 with siRNA was also tested. ORMDL3 overexpression induced significantly higher basal eIF2\(_\alpha\) response while knocking down ORMDL3 overexpression induced significantly lower basal eIF2\(_\alpha\) response while knocking down ORMDL3 levels and release from the ER; slower Ca\(^{2+}\) reuptake into the ER and cytosolic Ca\(^{2+}\) Clearance; and higher basal cytosolic Ca\(^{2+}\) concentrations. Besides, co-localization and co-immunoprecipitation of both ORMDL3 and SERCA suggest a physical interaction between both proteins. Together, these results obtained from ORMDL3 overexpressing cells are consistent with the inhibition of the SERCA pump that contributes to maintain low cytosolic Ca\(^{2+}\) concentrations by pumping Ca\(^{2+}\) from the cytosol into the ER (3,38). Although at present we cannot fully discard that ORMDL3 may also present certain channel activity, as reported for other modulators of SERCA (7).

In our attempt to identify ORMDL3 functional motifs, we deleted the last nine amino acids of ORMDL3 that contain a putative ER retention sequence (Fig. 5C). ORMDL3-Δ145–153 showed cellular distribution similar to ORMDL3-WT (Supplementary Material, Fig. S3) but lost the inhibitory effect on ER-mediated Ca\(^{2+}\) signaling, evaluated by exposing cells to 5 \(\mu\)M carbachol (Fig. 5C and D). Interestingly, ORMDL3-Δ145–153 did not lose the ability to immunoprecipitate with SERCA (results not shown), suggesting a separation between the ORMDL3 domains involved in functional and physical interaction with SERCA.

**ORMDL3 interacts with and modulates SERCA activity**

ORMDL3-GFP co-localized with endogenous SERCA2b in HEK293 cells (Fig. 4A) and SERCA2b (either native or overexpressed) co-immunoprecipitated ORMDL3-GFP in HEK293 cells (Fig. 4B). Expression of the proteins of interest was probed by western blotting in the same cell lysates (input) used for immunoprecipitation (Supplementary Material, Fig. S2). These results suggested that the modulatory effect of ORMDL3 upon SERCA may involve a direct association. As with other SERCA-interacting proteins that regulate pump activity (40–42), co-immunoprecipitation of ORMDL3 and SERCA2b was stronger at 5 mM Ca\(^{2+}\).

Next, we attempted reverting ORMDL3-induced phenotype by co-expressing SERCA and ORMDL3. Cells overexpressing ORMDL3 and stimulated with 1 \(\mu\)M TG—that also promotes passive release of Ca\(^{2+}\) from ER—showed reduced Ca\(^{2+}\) release from the ER, a phenotype that was reverted by co-expressing SERCA2b (Fig. 5A and B). Basal cytosolic [Ca\(^{2+}\)] was also returned to control conditions in cells co-expressing SERCA2b and ORMDL3 (61 \(\pm\) 1 nm, \(n = 80\); \(P = 0.1\) versus GFP transfected cells).

Our results showed: lower ER Ca\(^{2+}\) levels and release from the ER; slower Ca\(^{2+}\) reuptake into the ER and cytosolic Ca\(^{2+}\) Clearance; and higher basal cytosolic Ca\(^{2+}\) concentrations.

**ORMDL3 facilitates UPR**

A decrease in ER Ca\(^{2+}\) (using SERCA inhibitors) triggers the UPR, characterized by activation of signaling molecules and, ultimately, increased transcriptional activation of immediate-early genes and others directly related with the onset of inflammation (17). UPR signaling pathways at the ER usually follow the activation of one or more of the known protein sensors: pancreatic endoplasmic reticulum kinase (PERK), inositol-requiring 1 \(\alpha\) (IRE1\(_\alpha\)) or activating transcription factor 6 (ATF6). The levels of ER stress were evaluated by analyzing the phosphorylation of eukaryotic initiation factor 2 \(\alpha\) (eIF2\(_\alpha\)), an early marker of UPR downstream of PERK activation (15,17). ORMDL3 overexpression induced significantly higher basal eIF2\(_\alpha\).
phosphorylation, without changing total eIF2α, whereas ORM DL3 knock-down with siRNA significantly reduced the phosphorylated eIF2α levels (Fig. 5E and F). Overexpression of ORM DL3-Δ145–153 did not significantly affect eIF2α phosphorylation (Fig. 4E and F). Therefore, expression of functional ORM DL3 correlated with the level of phosphorylated eIF2α. We also evaluated the influence of ORM DL3 on two UPR target genes downstream of eIF2α, BIP and EGR-1 (15, 43).

Similar to what we observed with eIF2α, overexpression of ORM DL3 augmented BIP while overexpression of ORM DL3-Δ145–153 did not significantly affect BIP expression (Fig. 5G). Loss of ORM DL3—with siRNA—also attenuated BIP expression (Supplementary Material, Fig. S4A). Similar results were obtained when evaluated EGR-1 transcription (Supplementary Material, Fig. S4B). Typically, ER stress activates different UPR signaling pathways (22), although preferences may exist.

Figure 3. Silencing ORM DL3 in HEK293 cells increases ER Ca2+ signals. (A) Conventional (top) and quantitative RT–PCR (bottom) demonstrating down-regulation of ORM DL3 in HEK293 cells transfected with ORM DL3 siRNA, relative to control siRNA transfected cells (H2O: no template control). (B) Genetic down-regulation of ORM DL3 expression with ORM DL3 siRNA (filled triangle) increases ER Ca2+ response to cyclopiazonic acid (CPA, 10 μM) while ORM DL3 overexpression (open circle) lowers ER Ca2+ response compared with GFP transfected cells (filled circle). (C) Average of areas under the curves derived from the experiment in (B). Mean response to control siRNA (not plotted in B, for the sake of clarity) is also included. (D) Mean values of basal cytosolic [Ca2+]i. (E) ORM DL3 siRNA (filled circle) increases ER Ca2+ response to ionomycin (1 μM) while ORM DL3 overexpression (open circle) lowers ER Ca2+ response compared to GFP transfected cells (filled circle). Inset, mean average of areas under the curves. Data are expressed as the mean ± SEM (N values shown for each bar). *P < 0.05 (versus GFP transfected cells), one way ANOVA and Tukey post hoc for comparison of multiple conditions or Student’s t-test for comparison of two conditions.
cells, ORMDL3 overexpression in Jurkat cells decreased the Ca\(^{2+}\) response to the SERCA inhibitor CPA (30 \(\mu\)M) (Fig. 6A). Phosphorylation of eIF2\(\alpha\) was also evaluated in Jurkat cells transfected with GFP or ORMDL3-CFP. Due to the low transfection rate of Jurkat cells, eIF2\(\alpha\)-P was analyzed on individual cells by immunofluorescence confocal microscopy (Fig. 6B). Mean normalized levels of eIF2\(\alpha\)-P and percentage of responding Jurkat cells are clearly increased in ORMDL3-transfected cells, compared with GFP transfected Jurkat cells (Fig. 6C and D).

Conclusions

Our data reports on the involvement of ORMDL3 on ER-mediated Ca\(^{2+}\) signaling and facilitation of ER-mediated inflammatory responses. Besides, our study increases the understanding of the cellular and molecular mechanisms underlying the reported association of ORMDL3 with inflammatory diseases such as asthma (25) and Crohn’s disease (44). Interestingly, the increased risk of asthma conferred by ORMDL3 variants has been recently associated to tobacco smoke (29), being this environmental disease modifier an inducer of UPR (45). In conclusion, our observation offers a novel target for the study of ER Ca\(^{2+}\) signaling and its impact on disease pathophysiology.

MATERIALS AND METHODS

Plasmids and cell transfection

Human ORMDL3 and pig SERCA2b expression vectors were a kind gift from Drs R. Gonzalez-Duarte (University of Barcelona) and M. Brini (University of Padova), respectively; pDsRed-ER was obtained from CLONTECH. ORMDL3 tagged with GFP at the N- and C-terminus was generated by subcloning ORMDL3 cDNA into pCDNA3-CFP and pECFP-C1 vectors, respectively. ORMDL3 tagged with YFP at amino acid position 79 and ORMDL3\(-145\)–153 (deletion from amino acid 145 to the C-terminus) were generated by PCR. All constructs were verified by sequencing (Big Dye 3.1, AmpiPrism, Applied Biosystems).

HEK293 cells were transiently transfected with ExGen500 (Fermentas MBI), following manufacturer instructions. Jurkat cells were transfected in 24-well plates using 1 \(\mu\)g plasmid DNA and 2 \(\mu\)l TransIT-Jurkat Transfection Reagent (Mirus Bio Corporation) per well.
Measurement of intracellular $[\text{Ca}^{2+}]$

Cytosolic $\text{Ca}^{2+}$ signal was determined at RT in cells loaded with 4.5 $\mu$M fura-2-AM (20 min) as previously described (46). Cytosolic $[\text{Ca}^{2+}]$ increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation.
(fura-2 ratio 340/380). Absolute basal Ca$^{2+}$ concentration was obtained from the fluorescence ratios using an on-cell calibration protocol (47). All experiments were carried out at room temperature and cells were bathed in an isotonic solution containing (in mM): 140 NaCl, 5 KCl, 1.2 CaCl$_2$, 0.5 MgCl$_2$, 5 glucose, 10 HEPES (300 mosmol/l, pH 7.4 with Tris). Ca$^{2+}$-free solutions were obtained by replacing CaCl$_2$ with equal amount of MgCl$_2$ plus 0.5 mM EGTA.

To measure free [Ca$^{2+}$] inside the ER cells were incubated 45 min at 37°C with the low-affinity Ca$^{2+}$ dye mag fura-2-AM (5 μM) in isotonic medium containing 0.02% pluronic F-127 (39). To wash out the cytosolic dye, plasma membrane was permeabilized in ATP-free intracellular-like media (in mM: 120 KCl, 25 NaCl, 0.1 MgCl$_2$, 0.75 CaCl$_2$, 0.5 EGTA (~100 mM [Ca$^{2+}$]) and 10 HEPES; pH 7.2 with KOH, 300 mOsm) containing 8 μM digitonin. Following permeabilization, digitonin was removed and 1 mM ATP added to the intracellular solution for at least 15 min, until stable mag fura-2 340/380 ratio levels were obtained, which are proportional to [Ca$^{2+}$]$_{ER}$.

Fluorescence protease protection assay

Twenty-four hours after transfection, live cells were permeabilized with 20 μM digitonin at RT for 3 min. After permeabilization, we incubated the cells with 2 mM trypsin for 2 min. The subcellular localization of tagged proteins was analyzed before and after trypsin treatment under a 40 × 1.32 Oil Ph3 CS objective, LCS Leica Confocal software and Argen (488 nm, JDS Uniphase Corporation) and HeNe (555 and 633 nm, JDS Uniphase Corporation and LASOS Lasertechnik GmbH, respectively) lasers using an inverted Leica SP2 Confocal Microscope, as previously described (32). Images were taken at room temperature and were not further processed except to adjust brightness, contrast and color balance.

Expression knock-down and quantitative RT–PCR analysis

Cells were seeded in 6-well plates at 90% confluency and exposed to 100 pmoles of ORM DL3 siRNA (5’-TAAGTACGACCAGATCCATT-3’) (Qiagen) or control siRNA (5’-AATTTCCGAACTGTGCACGT-3’) (Qiagen) diluted into 100 μl serum-free medium. Cells were transfected by Lipofectamine 2000 (Invitrogen) procedure following the manufacturer’s instructions, as described previously (48). RNA extraction (Nucleospin RNA II kit, Macherey-Nagel) was carried out 48 h after transfection and RT–PCR was performed as described previously (48) using SuperScrp-RT system (Invitrogen) and aliquots of 1 μg cDNA were used as template for quantitative PCR. Quantitative RT–PCR was performed on an ABI Prism 7900HT (Applied Biosystems) with SYBR-Green (SYBR-Green Power PCR Master Mix, Applied Biosystems) and ORMDL3 QuantiTect Primer Assay (Qiagen). Other primers used included: BIP 5’-CGGGCAAAGATGTCAGGAAAG-3’ and 5’-TCTTGGAAACGGCTTCAATGAG-3’; EGR-1 5’-CAGCACCCCTCAAACCCTCAG-3’ and 5’-AGGGCCAGTATAAGGTGATG-3’. Beta-actin or ribosomal protein Rpl19 served as an internal normalization standard (20). PCR conditions for ORM DL3 and EGR-1 were: 95°C for 5 min; 95°C for 30 s; 60°C for 30 s, 72°C for 30 s; 72°C for 5 min; with 40 cycles of amplification. PCR conditions for BIP were: 95°C for 5 min; 95°C for 30 s; 55°C for 30 s, 72°C for 1 min; 78°C for 10 s; 72°C for 5 min, with 40 cycles of amplification.

Immunoprecipitation assay and immunodetection

Co-immunoprecipitation experiments were run as previously described (46). HEK293 cells were transiently transfected with human ORMDL3-GFP (or ORMDL3:ORMDL3-GFP ratio 1:1) and pig SERCA2b plasmids were lysated with immunoprecipitation buffer (0.5% Triton plus protease inhibitor cocktail in nominal free Ca$^{2+}$ HBS or HBS containing 1 mM TG, 5 mM Ca$^{2+}$ or 5 mM EGTA) and centrifuged at 100 000g to collect total protein in supernatant. Then 1000 μg total protein were incubated at 4°C overnight with anti-SERCA2b antibody (Abcam). After 2 h incubation with 30 μl of G protein (Amersham) at RT, immunocomplexes were washed with immunoprecipitation buffer five times and boiled for 5 min with loading buffer. Co-immunoprecipitation of ORM DL3 was detected with anti-GFP antibody (1:1000, mouse monoclonal antibody, Clontech Laboratories, Inc.). For western blotting of eIF2α, 50 μg of total protein obtained from HEK293 cells 48 h after transfection with GFP, ORM DL3, ORM DL3-A145–153 or ORM DL3 siRNA were separated on 4–10% gradient polyacrylamide gel and transferred to PVDF membranes. Primary antibodies used were mouse anti-eIF2α (1:500) and rabbit anti-phosphoS51-eIF2α (1:500) from Abcam. Secondary antibodies used were horse-radish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:3000, GE Healthcare). Immunoreactive signal was detected by SuperSignal West Pico Chemiluminiscent substrate (Pierce) and visualized by Molecular Imager Chemidoc XRS system (Biorad).

For immunodetection of phosphorylated eIF2α, 24 h after transfection of Jurkat cells with ORM DL3-CFP or pCFP vectors, cells were attached for 30 min to poly-L-Lysine coated coverslips, fixed with 4% PFA, permeabilized with 0.1% Triton x-100 in PBS and blocked with 1% BSA, 2% FBS, 0.05% Triton in PBS. Samples were stained for phospho-eIF2α using anti-phosphoS51-eIF2α antibody (1:50 in blocking solution). Secondary antibody was a goat anti-rabbit Alexa Fluor555 (Molecular Probes). Images were acquired using an inverted Leica SP2 Confocal Microscope with a 40 × 1.32 Oil Ph3 CS objective and analyzed using ImageJ software. Intensity/area ratio of every transfected cell was normalized to the intensity/area mean of the surrounding non transfected cells in the same image. Endogenous SERCA immunodetection was carried out following the same procedure using SERCA 2b antibody (1:250, Genetex).

Statistics

All data were expressed as means ± SEM of N (number of cells analyzed) or n (number of experiments carried out). Statistical analysis was performed with Student’s unpaired tests, or one-way analysis of variance (ANOVA) using SigmaPlot or OriginPro software. Bonferroni’s or Tukey’s tests were
used for post hoc comparison of means. The criterion for a significant difference was a final value of $P < 0.05$.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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