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The role of cholesterol in the association of endoplasmic reticulum membranes with mitochondria

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ABSTRACT

The unique endoplasmic reticulum (ER) subdomain termed the mitochondria-associated ER membrane (MAM) engages the physical connection between the ER and the mitochondrial outer membrane and plays a role in regulating IP_3 receptor-mediated Ca^{2+} influx and the phospholipid transport between the two organelles. The MAM contains certain signaling and membrane-tethering proteins but also lipids including cholesterol. The biophysical role of lipids at the MAM, specifically in the physical interaction between the MAM of the ER and mitochondria, remains not totally clarified. Here we employed the in vitro membrane association assay to investigate the role of cholesterol in the association between MAMs and mitochondria. The purified MAMs and mitochondria were mixed in vitro in a test tube and then the physical association of the two subcellular organelles was quantified indirectly by measuring the presence of the MAM-specific protein sigma-1 receptors in the mitochondria fraction. Purified MAMs contained free cholesterol approximately 7 times higher than that in microsomes. We found that depletion of cholesterol in MAMs with methyl-β-cyclodextrin (MβC) significantly increases the association between MAMs and mitochondria, whereas MβC saturated with cholesterol does not change the association. ^{14}C-Serine pulse-labeling demonstrated that the treatment of living cells with MβC decreases the level of *de novo* synthesized ^{14}C-phosphatidylserine (PtSer) and concomitantly increases greatly the synthesis of ^{14}C-phosphatidylethanolamine (PtEt). Apparently, cholesterol depletion increased the PtSer transport from MAMs to mitochondria. Our findings suggest that cholesterol is an important substrate in regulating the association between MAMs of the ER and mitochondria.

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1. Introduction

The coordination of complex intracellular transports allows organelles to develop physical membrane-to-membrane interactions between organelles. As shown by a number of studies, the endoplasmic reticulum (ER) membranes associate with mitochondria, Golgi intermediate compartments, peroxisomes, and plasma membranes to facilitate transport of proteins, lipids, and Ca^{2+} [1–8]. The association between ER and mitochondria membranes recently receives growing attentions mainly because of the vast importance of the association in regulation of Ca^{2+} signaling, mitochondrial bioenergetics, apoptosis, and lipid metabolism [5–7,9]. Ca^{2+} transferred directly from ER to mitochondria via the membrane contact is known to activate the tricarboxylic acid cycle, whereas overloading of mitochondrial Ca^{2+} causes apoptosis [9,10]. Phosphatidylserine (PtSer) synthesized at the ER directly moves to mitochondria *via* the physical membrane contact for its carboxylation to form phosphatidylethanolamine (PtEt) [5,6]. Particularly in hepatocytes, PtEt synthesized in mitochondria is transported back to the ER via the membrane contacts for its subsequent methylation to form phosphatidylcholine (PtChol) [5,6]. Thus, the interface between ER and mitochondria serves as a center place for the phospholipid biosynthesis.

The unique subdomain of the ER that associates with mitochondria is termed 'the mitochondria-associated ER membrane (MAM) [6].' The MAM accommodates specific proteins, such as Ca^{2+} signaling proteins (e.g., IP_3 receptor), molecular chaperones (e.g., sigma-1 receptor chaperone, BiP, calreticulin), Bcl-2 family proteins (e.g.,

Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; HTLC, high performance thin-layer chromatography; MAM, mitochondria-associated ER membrane; MβC, methyl-β-cyclodextrin; MβC-Chol, MβC conjugated with cholesterol; PtChol, phosphatidylcholine; PtEt, phosphatidylethanolamine; PtSer, phosphatidylserine; SM, sphingomyelin.

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Bcl-2), ubiquitin ligases (e.g., AMFR/gp78), membrane tethering/vesicular transport proteins (e.g., mitofusion-2, PACS-2), and lipid synthases (e.g., PtSer synthase, acetyl-CoA: cholesterol acyltransferase) [11–13]. However, the mechanism regulating the protein recruitment to the MAM remains unknown. The association of MAMs with mitochondria is highly dynamic. Elevation of cytoplasmic Ca^{2+} is shown to cause the reversible rapid dissociation of MAMs from mitochondria [14]. The molecular mechanism regulating the dynamics of the MAM–mitochondrion association is also largely unknown.

We previously found that, in contrast to the bulk of ER membranes, the MAM is highly enriched with cholesterol and ceramides, thus containing lipid raft-like microdomains [15]. We also found that depletion of cholesterol or ceramide causes the relocation of the MAM-enriched proteins sigma-1 receptors and IP_3 receptors from MAMs to the bulk of ER membrane [15]. Those results suggest that lipids may be important in promoting formation of specialized protein assemblies at the MAM and might relate to the association of MAMs with mitochondria. To support the hypothesis, we decided to focus on cholesterol and examined specifically whether depletion of cholesterol in purified MAMs may affect their association with mitochondria. To the best of our knowledge, this is the first report showing that cholesterol affects the physical association between isolated MAMs and mitochondrial membranes.

2. Materials and methods

2.1. Reagents

Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA). Antibodies were from the following sources: anti-ATP synthase inhibitor and anti-cytochrome *c* oxidase subunit I were from Invitrogen. Anti-IP_3 receptor type-3 and anti-BiP were from BD Biosciences (San Jose, CA). Anti-sigma-1 receptor antibodies were developed as described previously [16]. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were maintained in minimal essential medium-α Glutamax containing 10% (v/v) heat-inactivated fetal bovine serum at 37 °C with 5% CO_2. CHO cells were treated with methyl-β-cyclodextrin (MβC) or cholesterol-conjugated MβC (MβC-Chol) at 5 mM in culture medium without serum. MβC-Chol was prepared by rotating 1 ml of MβC solution (100 mM) with a cholesterol film (4 mg, dried under N_2) overnight at room temperature.

2.3. MAM preparation

The MAM fraction was prepared as described previously [17] with minor modifications. Briefly, CHO cells in two 15-cm dishes (100% confluency) were homogenized by a glass Dounce homogenizer with homogenization buffer (0.25 M sucrose, 10 mM Hepes/KOH, pH 7.4). The homogenate was centrifuged at 600*g*. The pellet (P1) contains nuclei and unbroken cells. The supernatant was centrifuged at 10,300*g* for 20 min to pellet the crude mitochondrial fraction. The supernatant was centrifuged at 100,000*g* for 1 h to yield P3 microsomal and cytosolic fractions. The crude mitochondrial fraction in 0.5 ml of isolation medium (250 mM mannitol, 5 mM Hepes/KOH at pH 7.4, and 0.5 mM EGTA/KOH) was layered on a Percoll solution [225 mM mannitol, 25 mM Hepes/KOH at pH 7.4, 1 mM EGTA/KOH, and 30% (v/v) Percoll (GE Healthcare, Buckinghamshire, UK)] followed by a centrifugation at 95,000*g* for 30 min in an SW 55Ti rotor. Purified mitochondrial and MAM fractions were washed three times successively with the isolation medium, 50 mM phosphate buffer (pH 7.2) containing 0.25 M sucrose and 5 mM β-mercaptoethanol, and saline. Protein concentrations in each fraction were measured by a Micro BCA Assay (Thermo Fisher Scientific, Rockford, IL).

2.4. MAM–mitochondria association assay and Western Blotting

The MAM–mitochondria association assay reported by de Brito and Scorrano [13] was modified. MAMs were centrifuged at 6300*g* for 10 min, and the supernatant was immediately used in the assay. Twenty microgram of MAMs and mitochondria were mixed in reaction buffer (RB) [10 mM Tris (pH 7.4), 150 mM KCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM sodium succinate], and incubated at 37 °C for 1–30 min. In some experiments, MAMs were incubated with 5 mM of MβC or MβC-Chol with protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO) at 37 °C for 30 min. The membranes treated were washed to remove MβC or MβC-Chol prior to the assay. After the incubation of the mixture of two membranes, mitochondria and MAMs associating with mitochondria were pelleted by a centrifugation at 6300*g* for 10 min. The supernatant was collected as a source of unbound MAMs (note that MAMs not associated with mitochondria can sediment only at 100,000*g* or higher). The level of MAM-specific protein sigma-1 receptors in the pellet and the supernatant was measured by Western blotting according to the protocol described before [16]. Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and Kodak Image Station 440CF (Carestream, Rochester, NY).

2.5. Free cholesterol measurement

Cholesterol in 50 μg of mitochondria, MAMs, and microsome membranes were extracted by a Bligh & Dyer lipid extract method. Total lipid extracts were dissolved in100 μl of isopropanol by incubating at 37 °C for 10 min with vortex. Free cholesterol was measured by a Free Cholesterol E kit (Wako Chemicals USA, Richmond VA).

2.6. High performance thin-layer chromatography (HTLC)

CHO cells were cultured in 6-cm dishes, and treated with MβC or MβC-Chol for 2 h in culture medium without serum. Medium was replaced with 1 ml of fresh culture medium containing 0.3 μCi/ml of ^{14}C-serine with MβC or MβC-Chol. After 1 h of pulse-labeling, cells were harvested and total lipids were extracted as described above. Lipids were resolved by a HTLC with the $CHCl_3$–methanol–H_2O mixture (13:5:0.8). Lipids were visualized by direct autoradiography.

3. Results and discussion

Three previous studies have examined the effect of MβC that extracts cholesterol from membranes on the function of the MAM. One study found that the MβC treatment normalizes overloading of mitochondrial Ca^{2+} that is caused by the pathological accumulation of GM_1 ganglioside in fibroblasts of lysosomal β-galactosidase knockout mice [18]. The MβC treatment was also shown to alleviate targeting of sigma-1 receptors or human cytomegalovirus protein UL37exon 1 to lipid microdomains at the MAM [15,19]. Further a recent study using confocal microscopy showed that the treatment of CHO cells with MβC does not significantly change the colocalization co-efficiency between green fluorescent proteins

and DsRed proteins respectively expressed in ER and mitochondria [15]. However, any of currently available data do not provide direct and sufficient evidence supporting the possibility that depletion of cholesterol changes the physical association of the two subcellular membranes. Further, microscopic observations appear to have a certain limitation in precisely determining the physical membrane association. Therefore, we here employed an in vitro assay [13] to monitor the direct physical association between MAMs and mitochondria in vitro, and examined the effect of the MβC treatment on the association.

The principal of the assay is based on the unique property of the MAM–mitochondria association that can be reconstituted rapidly and energy-independently in a test tube [5,6]. Simply mixing purified MAMs with mitochondria promotes the interaction of the two distinct membranes. MAMs associated with mitochondria can be separated by a low-speed centrifugation as free MAMs stay exclusively in the supernatant. Accordingly, we first prepared MAMs and mitochondria membranes from CHO cells, and verified respective purity (Fig. 1A). Immunoblotting confirmed that mitochondrial proteins are present mostly in the prepared mitochondrial fraction (Fig. 1A). Sigma-1 receptors, the ER proteins localized at MAMs [15,16], were highly enriched in the MAM fraction (Fig. 1A). Sigma-1 receptors were also detected in the mitochondria fraction, but the level is significantly lower than that in the MAM fraction. When the MAM alone was centrifuged at 6300*g* where the MAM stays in the supernatant, sigma-1 receptors were detected solely in the supernatant (Fig. 1B). In contrast, when MAMs were incubated together with purified mitochondria in the test tube, sigma-1 receptors was now detected in the pellet after the centrifugation with a concomitant decrease of its level in the supernatant (Fig. 1B). Similar results were obtained when membranes were prepared from the rat liver (Fig. 1B). Co-sedimentation of MAMs with mitochondria was observed as early as 1 min after the incubation of MAMs with mitochondria, and continued to increase up to 30 min (data not shown). These results clearly demonstrate the validity of the MAM–mitochondria association assay with accompanying sigma-1 receptor immunoblotting for the monitoring of the physical association of the two membranes in vitro.

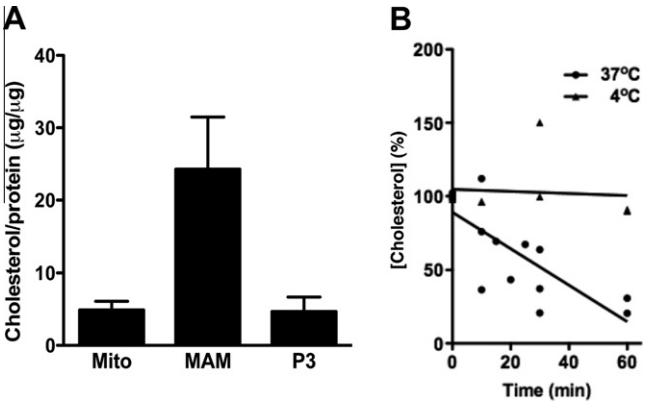

Fig. 2. Depletion of cholesterol in MAMs with MβC. (A) The level of free cholesterol in isolated mitochondria, MAMs, and microsomal membranes, $N = 3$, mean ± SEM. (B) Time-dependent effect of MβC in depleting membrane cholesterol. The crude mitochondrial fraction (containing both mitochondria and MAMs) were incubated with 5 mM of M®C at 37 or 4 °C for indicated periods of time.

The level of cholesterol in the MAM and its alteration after the MβC treatment were examined. The MAM contains 5–7 times higher levels of cholesterol when compared with mitochondria or microsomes (Fig. 2A). This result supports a previous finding showing that MAMs are highly enriched with lipid raft-like microdomains [15], and suggests the potential significance of examining the role of cholesterol in the association between MAMs and mitochondria. When MAMs were incubated with MβC at 37 °C, the cholesterol level was time-dependently decreased and the cholesterol level reached approximately 50% of the original in 30 min (Fig. 2B). In contrast, at 4 °C, MβC was ineffective in causing a reduction of the membrane cholesterol (Fig. 2B).

The MAM–mitochondria association assay was then conducted with MAMs pre-incubated with MβC for 30 min at 37 °C. Depletion

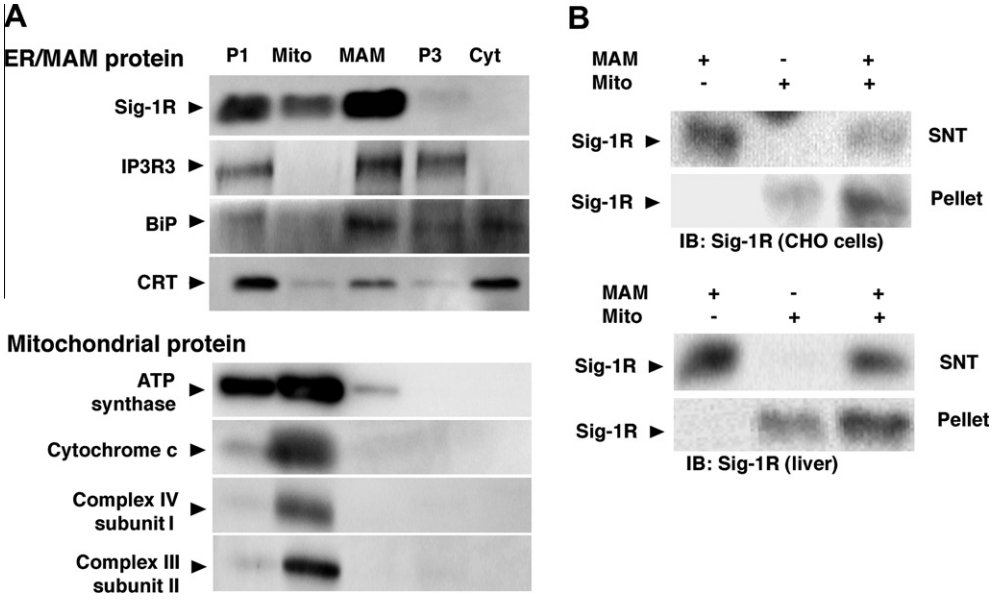

Fig. 1. The in vitro MAM–mitochondria association assay. (A) MAM and mitochondrial membranes from CHO cells. P1, nuclear; Mito, mitochondrial; P3, microsomal; Cyt, cytosolic fractions. Sig-1R, sigma-1 receptor; IP3R3, IP_3 receptor type-3; CRT, calreticulin. Levels of respective organelle markers were measured by immunoblotting. Note that ER chaperones involved in the ER-to-Golgi vesicle transport (i.e., BiP, CRT) are also detected in the cytoplasmic fraction. (B) In vitro association of MAMs with purified mitochondria. MAMs and mitochondria membranes were prepared from CHO cells or rat livers. In the MAM(+) – Mito(+) sample, the two membranes were incubated together for 30 min. Pellets and supernatants (SNT) were obtained by a centrifugation at 6300*g*. The level of MAM-enriched protein sigma-1 receptors (Sig-1R) was measured by immunoblotting.

of cholesterol in MAMs significantly increased MAMs co-sedimented with mitochondria (Fig. 3). The treatment with MβC did not cause sedimentation of MAMs in the absence of mitochondria (data not shown). The pre-incubation of MAMs with MβC conjugated with cholesterol no longer potentiated the co-sedimentation of MAMs with mitochondria (Fig. 3). These findings suggest that the effect of MβC is specific in depleting cholesterol from the membrane and that the depletion of cholesterol in MAMs facilitates the association between MAMs and mitochondria.

Since cholesterol at the MAM plays a role in formation of lipid microdomains [15], which leads to the formation of protein assemblies at the loci [15], we originally speculated that cholesterol may promote the association between MAMs and mitochondria, and therefore depletion of cholesterol at MAMs might reduce their association with mitochondria. However, here we found that, in contrast to our speculation, depletion of cholesterol increased the membrane association. Although details of the underlying mechanism is unknown at present, our finding may suggest the existence of two distinct systems at the MAM in regulating its association with mitochondria: one that is to promote the membrane association via specific membrane-tethering proteins such as mitofusin-2 [13] and the other that is used to restrict the association wherein cholesterol may play a role. Probably, the overwhelmed membrane association between ER and mitochondria is unfavorable for the proper cellular functioning. Thus the latter mechanism serves as a brake in a coordinated manner with the membrane-tethering mechanism to fine-tune the ER–mitochondria association. In fact, a manipulation causing a tighter association between MAM and mitochondrial membranes has been shown to promote apoptosis due to overloading of mitochondrial Ca^{2+} [20]. Further, in the most types of cells, the ER associates with only a limited portion of mitochondria (mostly 10–20% of the entire mitochondria surface) [20,21]. Therefore, the two systems at the MAM may create a yin-yang effect to regulate number and/or size of the contact sites. Our data suggest that certain lipids such as cholesterol might have a novel biophysical action limiting coalescence of two distinct intracellular membranes.

How cholesterol alters the association of MAM with mitochondrial membranes is unknown at present. Depletion of cholesterol in the MAM might change the membrane integration or the structure of proteins involved in the membrane tethering and/or dissociation. Alternatively, since the MAM–mitochondria association can be partially achieved (approximately 50%) with co-incubation of proteinase K-treated MAMs and mitochondria [22], a protein-independent mechanism regulating the membrane association may exist. Membrane lipids *per se* may thus be active substrates in regulating the MAM–mitochondria association.

Since above experiments were all performed with isolated membrane preparations, the result may not necessarily reflect the in vivo process. To provide the physiological insight of our finding, we examined whether the MβC treatment alters the MAM-to-mitochondria phospholipid transport in living cells. When transported from MAMs to mitochondria, PtSer is rapidly converted to PtEt, where the PtEt synthesis predominantly depends on activity of the intermembrane transport of PtSer *via* the physical membrane contacts [5,6,22]. Therefore, monitoring the synthesis of PtSer and PtEt helps indirectly assess the degree of the MAM–mitochondria association [22,23]. Accordingly, CHO cells were pulse-labeled with ^{14}C-serine for 1 h, permitting the near maximal velocity in the PtSer transport as well as the mitochondrial PtEt synthesis in CHO cells [23]. The level of synthesized ^{14}C-PtSer and ^{14}C-PtEt were then measured. Although the MβC treatment of living CHO cells is shown to initially deplete cholesterol on the cell surface, an extended treatment (e.g., 2 h) can decrease cholesterol in subcellular membranes including MAMs [15]. Therefore, cells were pretreated with MβC or MβC-Chol for 2 h prior to being pulse-labeled with ^{14}C-serine. The treatment with MβC slightly decreased the level of ^{14}C-PtSer synthesized during the 1-h incubation with ^{14}C-serine, but significantly increased the synthesis of ^{14}C-PtEt (Fig. 4). This result indicates that the MβC treatment does not significantly compromise the PtSer synthesis at the ER, but promoted the availability of PtSer for the biosynthesis of PtEt in mitochondria.

Albeit MβC appears to slightly increase the ^{14}C-PtChol synthesis (Fig. 4), the data may not be conclusive because of the inherited low level of PtChol derived from serine in CHO cells. The result is likely due to low expression of the PtEt *N*-methyltransferase activity in CHO cells [23]. In addition, in an agreement with a previous report showing that cholesterol depletion increases the *de novo* synthesis of the sphingosine backbone [24], we found that the MβC treatment increases the synthesis of sphingomyelin (Fig. 4). Details of this mechanism however remain unknown.

When MβC was pre-conjugated or saturated with cholesterol, the MβC effect on the *de novo* synthesis of phospholipids disappeared. This result suggests that the effect of MβC on the phospholipid synthesis is mainly due to its cholesterol-depleting action. Because the physical association of the two organelles is important in the PtSer transport and the subsequent PtEt biosynthesis [5,6,22], this finding with MβC-saturated with cholesterol further supports our notion that cholesterol negatively regulates the membrane association between MAMs and mitochondria.

Although cholesterol and sphingolipids are synthesized at the ER, they are actively transported by the vesicular or non-vesicular transport to Golgi and other organelles [25]. Thus, the bulk of ER

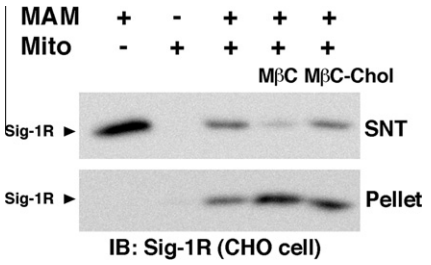

Fig. 3. Effect of cholesterol depletion on the association between MAMs and mitochondria. MAMs were incubated with MβC or MβC-Chol at 37 °C for 30 min. The MAM–mitochondria association assay was then performed as described in Fig. 1. Mito, mitochondria; SNT, supernatant. Sigma-1 receptors (Sig-1R) were measured by immunoblotting.

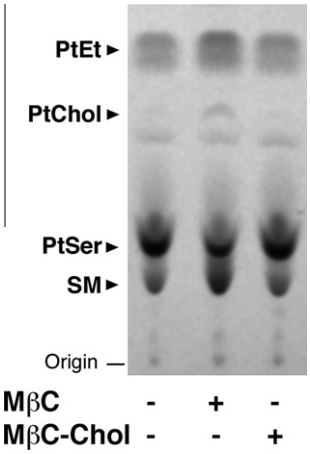

Fig. 4. Effect of cholesterol depletion on the *de novo* biosynthesis of PtSer and PtEt in CHO cells. CHO cells were treated with 5 mM of MβC or MβC-Chol for 2 h prior to ^{14}C-serine pulse-labeling for 1 h. Total lipids were extracted and visualized by HPTLC followed by autoradiography. SM, sphingomyelin.

membranes is composed mostly of phospholipids [25]. In contrast, the MAM of the ER contains the exceptionally high level of cholesterol [15]. Cholesterol at the MAM therefore leads to formation of lipid microdomains that recruit and compartmentalize signaling proteins at the ER–mitochondria interface [15]. Extending from the facts that at the plasma membrane lipid rafts also control signal transductions at connections between the plasma membrane and ER membrane [26,27], we suggest that intracellular lipid microdomains, including those at the ER, might be universally utilized by cells to regulate docking of distinct organelle membranes or substrates. In summary, our results showing the potentiation of biological membrane associations induced by cholesterol depletion may provide a new insight into the role of lipids in the dynamics of membrane association and dissociation in the biological system.

Acknowledgments

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