



Diacylglycerol kinase η colocalizes and interacts with apoptosis signal-regulating kinase 3 in response to osmotic shock

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ABSTRACT

Diacylglycerol kinase (DGK) η translocates from the cytoplasm to punctate vehicles via osmotic shock. Apoptosis signal-regulating kinase (ASK) 3 (MAP kinase kinase kinase (MAPKKK) 15) is also reported to respond to osmotic shock. Therefore, in the present study, we examined the subcellular localization of DGK η and ASK3 expressed in COS-7 cells under osmotic stress. We found that DGK η was almost completely colocalized with ASK3 in punctate structures in response to osmotic shock. In contrast, DGK δ , which is closely related to DGK η structurally, was not colocalized with ASK3, and DGK η failed to colocalize with another MAPKKK, C-Raf, even under osmotic stress. The structures in which DGK η and ASK3 localized were not stained with stress granule makers. Notably, DGK η strongly interacted with ASK3 in an osmotic shock-dependent manner. These results indicate that DGK η and ASK3 undergo osmotic shock-dependent colocalization and associate with each other in specialized structures.

1. Introduction

Diacylglycerol kinase (DGK) converts diacylglycerol to phosphatidic acid [1–5]. Both diacylglycerol [6–8] and phosphatidic acid [9–11] are known to serve as second messengers controlling many key enzymes. Therefore, DGK plays a critical role in the regulation of a wide variety of physiological and pathological phenomena.

DGK consists of ten isozymes (α , β , γ , δ , ϵ , ζ , η , θ , ι , κ) [1–5]. These DGK isozymes are divided into five groups (type I – V) according to their structural characteristics. DGK η belongs to the type II DGK group, which also contains DGK δ and κ [12,13]. Moreover, splice variants of DGK η exist, *i.e.*, DGK η 1 – 4 [13–15]. DGK η 1, which is a main alternative splicing product of the DGK η gene [13,16], has a pleckstrin homology domain at its N-terminal and a catalytic domain that is divided into two subdomains.

Several genome-wide association studies showed that the DGK η gene is associated with the etiology of bipolar disorder (BPD) [17–19]. Moreover, the DGK η gene is located within the BPD linkage region on 13q14 [20,21]. Notably, we recently generated DGK η -knockout (KO) mice and analyzed their phenotypes. Intriguingly, the DGK η -KO mice showed lithium-sensitive BPD (mania)-like behaviors [22,23]. Moreover we recently demonstrated that DGK η deficiency caused hyperactivity of the dopaminergic system [24]. Therefore, DGK η is attracting much

attention as a crucial BPD-associated gene [25]. In addition, we reported that DGK η interacted with C-Raf and B-Raf (mitogen-activated kinase (MAPK) kinase kinase (MAPKKK)) in response to epidermal growth factor (EGF) stimulation and regulated the Raf–MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK pathway [26]. On the other hand, stress stimulation (osmotic shock) induced translocation of DGK η to nonionic detergent-resistant membranes [27] in punctate structures [13,28]. However, the exact nature of these punctate structures remains unclear.

Apoptosis signal-regulating kinase (ASK) 1 was identified as a MAPKKK that activates the MKK4/7–JNK and the MKK3/6–p38 pathways resulting in apoptosis [29]. ASK2 and ASK3 were subsequently found as members of the ASK family [30,31]. The ASK family was shown to be involved in a wide range of stress signaling pathways (oxidative, osmotic and endoplasmic reticulum stresses) and several diseases including cancer [32,33]. ASK3 (also known as MAPKKK15) is known to respond to osmotic stress and regulate p38 activity and is essential to regulate bidirectional cell volume under both hypoosmotic and hypo-osmotic stresses [34–36].

In the present study, we examined the subcellular localization of DGK η 1 and ASK3, and their interaction in response to osmotic shock. Intriguingly, we found that DGK η 1 and ASK3 strongly interacted with one another, and moreover, were almost completely colocalized in punctate structures specialized for these enzymes in an osmotic stress-

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Abbreviations:

ASK	apoptosis signal-regulating kinase
BPD	bipolar disorder
DGK	diacylglycerol kinase
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
G3BP1	Ras GTPase-activating protein SH3-domain-binding protein
KO	knockout
MAPK	mitogen-activated kinase
MAPKKK	MAPK kinase kinase
MEK	MAPK/ERK kinase
TIAR	T-cell intracellular antigen 1 related protein

dependent manner.

2. Materials and methods

2.1. cDNA constructs

pAcGFP-C1-human DGK η 1 [27] and p3 × FLAG-CMV-DGK η 1 [13]

were generated as described previously. cDNA encoding human DGK η 1 was excised from pAcGFP-C1-human DGK η 1 with SalI and BamHI and inserted into the SalI/BamHI site of the pDsRed-monomer-C1 vector (Takara-Clontech, Kusatsu, Japan). cDNA encoding human DGK δ 2 [37] was excised from pAcGFP-C1-human DGK δ 2 [37] with EcoRI and SalI and inserted into the EcoRI/SalI site of the pDsRed-monomer-C1 vector. EGFP-tagged human ASK3 was generated as described previously [36].

2.2. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (Biological Industries (Beit-Haemek, Israel)), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Wako Pure Chemical Industries) at 37 °C in an atmosphere containing 5% CO₂. Cells were transiently transfected using PolyFect reagent (Qiagen, Hilden, Germany) as described by the manufacturer.

2.3. Confocal microscopy

After 24 h of transfection with DsRed-monomer-DGK η 1 and/or EGFP-ASK3, COS-7 cells were incubated in DMEM with or without 500 mM sorbitol for 30 min. The cells were fixed in 3.7% paraformaldehyde. The coverslips were mounted using Vectashield (Vector Laboratories,

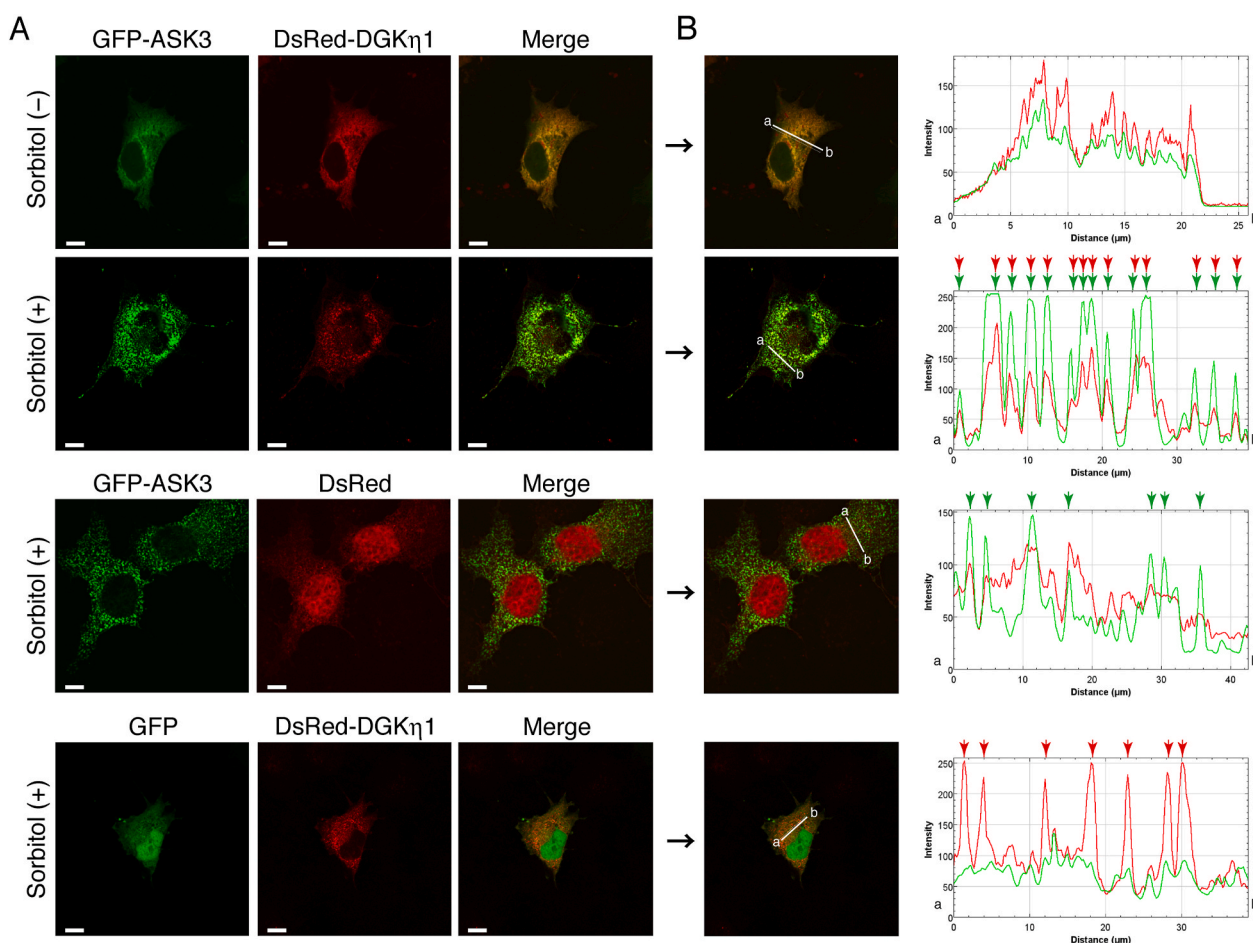


Fig. 1. Subcellular localization of DGK η 1 and ASK3 in COS-7 cells in response to osmotic shock. (A) Either pDsRed-monomer alone or pDsRed-monomer-DGK η 1 was cotransfected with either pEGFP alone or pEGFP-ASK3 in COS-7 cells as indicated. After 24 h of transfection, the cells were incubated in the presence or absence of 500 mM sorbitol for 30 min. After osmotic shock, the cells were fixed and imaged. Representative data from three independent experiments are shown. Scale bars, 20 μ m. (B) The distribution of DsRed-monomer-DGK η 1 and EGFP-ASK3 were quantified using ImageJ software. Obvious punctate structures in the presence of 500 mM sorbitol are indicated with arrows. Green, EGFP; Red, DsRed-monomer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

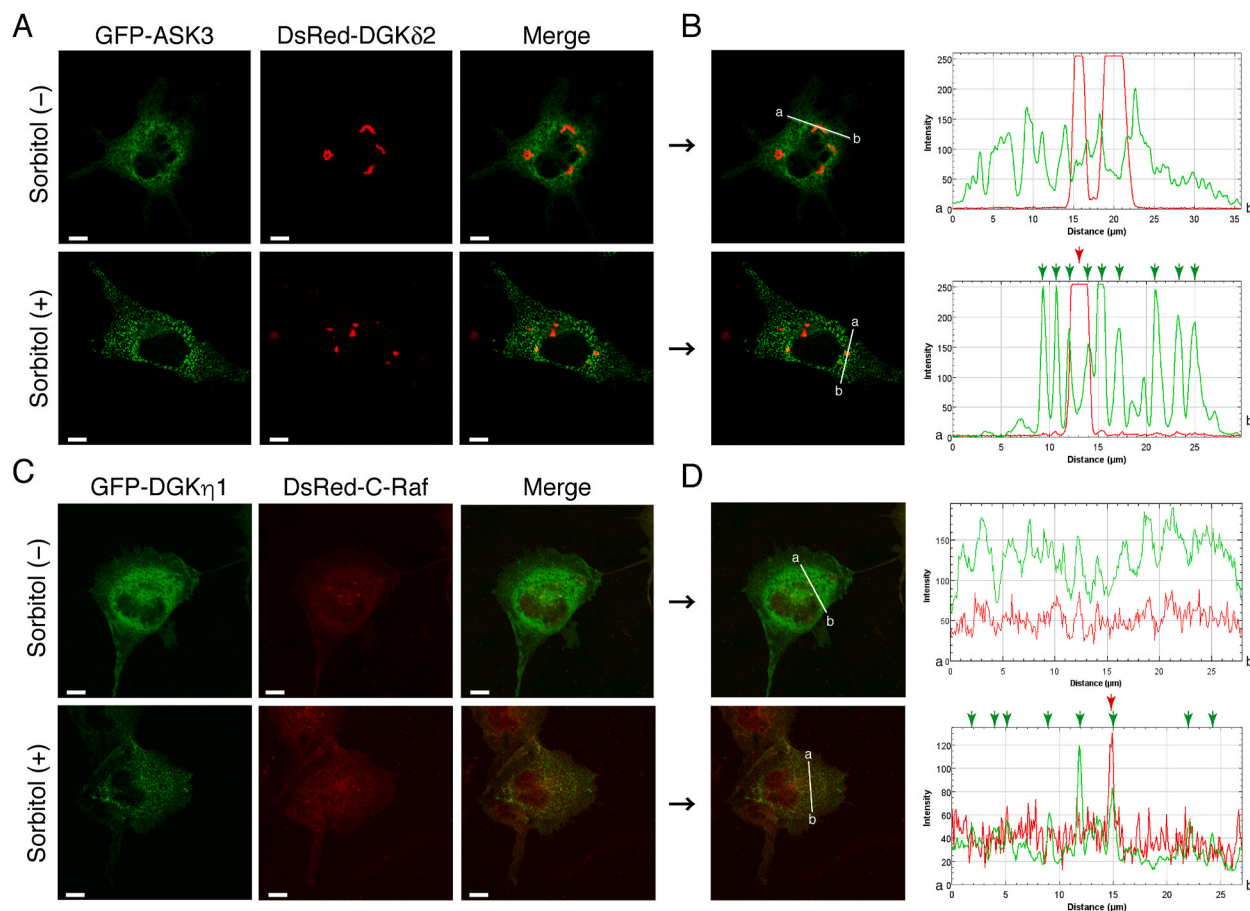


Fig. 2. Subcellular localization of ASK3, DGK δ 2, DGK η 1 and C-Raf in COS-7 cells in response to osmotic shock. (A) pDsRed-monomer-DGK δ 2 was cotransfected with pEGFP-ASK3 in COS-7 cells. (C) DsRed-monomer-C-Raf was cotransfected with pEGFP-DGK η 1 in COS-7 cells. After 24 h of transfection, the cells were incubated in the presence or absence of 500 mM sorbitol for 30 min. After the osmotic shock, the cells were fixed and imaged. Representative data from three independent experiments are shown. Scale bars, 20 μ m. (B, D) The distribution of DsRed-monomer-DGK δ 2 and EGFP-ASK3 (B) and DsRed-monomer-C-Raf and EGFP-DGK η 1 (D) was quantified using ImageJ software. Obvious punctate structures in the presence of 500 mM sorbitol are indicated with arrows. Green, EGFP; Red, DsRed-monomer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Burlingame, CA, USA). Fluorescence imaging was performed using an Olympus FV1000-D (IX81) confocal laser scanning microscope (Olympus, Tokyo, Japan). Images were acquired using FV-10 ASW software (Olympus).

To observe stress granule markers, Ras GTPase-activating protein SH3-domain-binding protein (G3BP1) and T-cell intracellular antigen 1 related protein (TIAR), cells were fixed and then permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1% bovine serum albumin. Coverslips were incubated with an anti-G3BP1 (Cat. #: 611126, BD Biosciences, Franklin Lakes, NJ, USA) or anti-TIAR (Cat. #: 610352, BD Biosciences) mouse monoclonal antibody for 1 h and then incubated with Alexa 594-conjugated anti-mouse IgG (Molecular Probe) for 1 h.

2.4. Immunoprecipitation

COS-7 cells (100 mm dish) were lysed in 500 μ L of HEPES buffer (50 mM, pH 7.2) containing 100 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40 and cOmplete™ protease inhibitor cocktail (EDTA-free, Sigma-Aldrich). Cell lysates were subjected to immunoprecipitation with anti-FLAG monoclonal antibody (Cat. #: F1804, Sigma-Aldrich) and Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) as described previously [38,39]. The immunoprecipitates were boiled in SDS sample buffer.

2.5. Western blotting

COS-7 cell lysates (20 μ g) and immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis. Western blotting was performed as previously described [40,41] using an anti-GFP (Cat. #: sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FLAG or anti C-Raf (Cat. #: 610152, BD Biosciences) antibody along with a peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibody.

3. Results

3.1. DGK η selectively colocalizes with ASK3 in response to osmotic shock

We previously reported that DGK η translocated from the cytoplasm to punctate structures in an osmotic shock-dependent manner [13,27]. ASK3 is also known to act in response to osmotic stress [35,36]. Therefore, we first examined whether DGK η colocalizes with ASK3 in an osmotic shock-dependent fashion. For this purpose, we utilized DGK η 1, which is a primary alternative splicing product of the DGK η gene [13, 16]. When DsRed-monomer-tagged DGK η 1 was coexpressed with EGFP-tagged ASK3 in COS-7 cells, they each showed cytoplasmic distribution and were partially colocalized (Fig. 1A). When the cells were exposed to osmotic shock (500 mM sorbitol for 30 min), DsRed-monomer-DGK η 1 and EGFP-ASK3 were markedly and exclusively colocalized at punctate structures (Fig. 1A). Densitometric

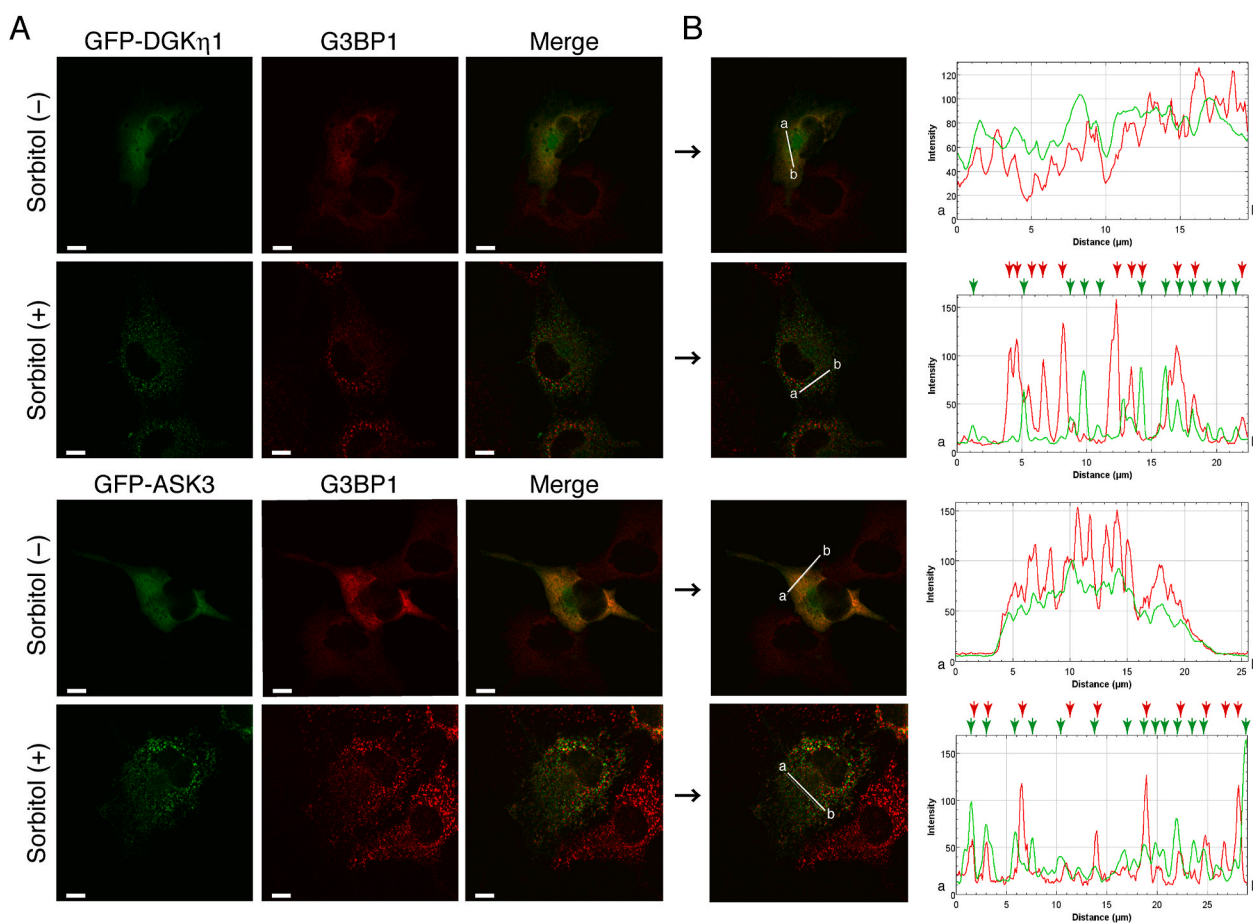


Fig. 3. Subcellular localization of DGK η 1, ASK3 and a stress granule marker (G3BP1) in COS-7 cells. (A) pEGFP-DGK η 1 or pEGFP-ASK3 was transfected into COS-7 cells. After 24 h of transfection, the cells were incubated in the presence or absence of 500 mM sorbitol for 30 min and then were stained with mouse monoclonal anti-G3BP1 (stress granule marker) and Alexa Fluor 594-conjugated anti-mouse IgG antibodies. Representative data from three independent experiments are shown. Scale bars, 20 μ m. (B) The distribution of EGFP-DGK η 1, EGFP-ASK3 and G3BP1 was quantified using ImageJ software. Obvious punctate structures in the presence of 500 mM sorbitol are indicated with arrows. Green, EGFP; Red, DsRed-monomer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analysis of confocal microscopy exhibits that DGK η 1 and ASK3 were almost perfectly colocalized at punctate structures in osmotic-shocked COS-7 cells (Fig. 1B). However, DsRed-monomer-DGK η 1 did not colocalize with EGFP alone, even in the presence of osmotic stress (Fig. 1). Moreover, EGFP-ASK3 failed to codistribute with DsRed-monomer alone under the same conditions (Fig. 1).

DGK δ also belongs to the type II DGKs, and its structure is closely related to that of DGK η [42]. Thus, we determined next if DGK δ 2, which is a main alternative splicing product of the DGK δ gene [43], is colocalized with ASK3. However, as shown in Fig. 2, DsRed-monomer-DGK δ 2 was not colocalized with EGFP-ASK3, even in the presence of osmotic shock (Fig. 2).

We previously reported that DGK η 1 was colocalized and interacted with C-Raf in an EGF stimulation-dependent manner in HeLa cells [26]. However, AcGFP-DGK η 1 did not exhibit marked colocalization with DsRed-monomer-C-Raf, even under osmotic stress (Fig. 2). Collectively, these results indicate that DGK η 1 was selectively colocalized with ASK3 in response to osmotic shock.

3.2. Punctate structures where DGK η and ASK3 are located are distinct from stress granules

Stress granules, which are dense aggregations composed of proteins and RNAs in the cytosol, appear when the cell is under stress, including osmotic shock [44]. Therefore, we investigated whether DGK η and ASK3

colocalize in stress granules. As shown in Fig. 3, Ras GTPase-activating protein SH3-domain-binding protein (G3BP1), a stress granule marker [45], was not located at punctate structures where DGK η 1 existed. In addition to DGK η 1, ASK3 also failed to codistribute with G3BP1. Moreover, another stress granule marker, T-cell intracellular antigen 1 related protein (TIAR) [46], also failed to colocalize with DGK η 1 (Suppl. Fig. 1). These results strongly suggest that DGK η 1 and ASK3 translocated from the cytoplasm to their specialized structures but not to general stress granules.

3.3. DGK η interacts with ASK3 in response to osmotic shock

We next examined whether DGK η 1 interacts with ASK3. To test this, we performed immunoprecipitation using COS-7 cells expressing 3 \times FLAG-tagged DGK η 1 and EGFP-tagged ASK3. EGFP-ASK3 was not detected in the immunoprecipitant of 3 \times FLAG-DGK η 1 using an anti-FLAG antibody in the absence of osmotic shock (Fig. 4). However, 3 \times FLAG-DGK η 1, but not 3 \times FLAG alone, strongly coimmunoprecipitated with EGFP-ASK3 in the presence of osmotic stress (Fig. 4), indicating that DGK η 1 interacts with ASK3 in an osmotic shock-dependent manner.

We previously reported that DGK η interacted with C-Raf in response to EGF [26]. Therefore, we determined next whether 3 \times FLAG-tagged DGK η 1 interacts with endogenous C-Raf in osmotic-shocked COS-7 cells. However, C-Raf was only slightly coimmunoprecipitated with 3 \times

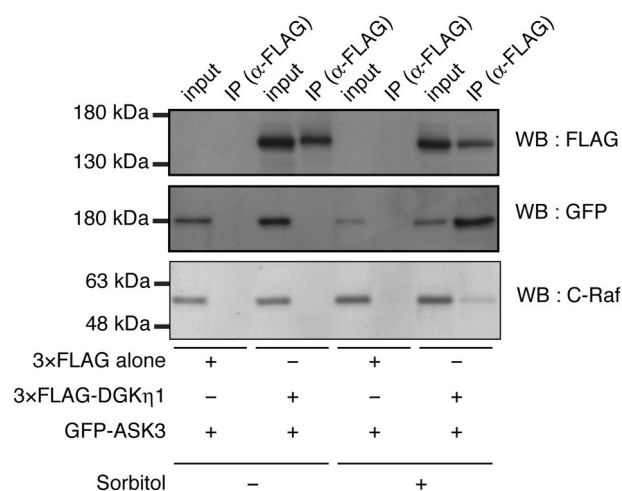


Fig. 4. DGK η 1 interacts with ASK3 in response to osmotic shock. pEGFP-tagged ASK3 was cotransfected with either p3 \times FLAG vector alone or p3 \times FLAG-tagged DGK η 1 in COS-7 cells for 24 h and then treated with or without 500 mM sorbitol for 30 min. 3 \times FLAG-DGK η 1 was immunoprecipitated by the anti-FLAG antibody. Immunoprecipitated 3 \times FLAG-DGK η 1 and EGFP-ASK3 and endogenous C-Raf were analyzed by Western blotting with anti-FLAG, anti-GFP and anti-C-Raf antibodies, respectively. Representative data from three independent experiments are shown.

FLAG-DGK η 1 in response to osmotic shock (Fig. 4). These results indicated that DGK η 1 is associated with C-Raf in addition to ASK3 under osmotic stress conditions.

Altogether, the results obtained in the present study indicate that DGK η 1 and ASK3 selectively colocalize and associate with each other in specialized structures in response to osmotic stress.

4. Discussion

In the present study, we revealed for the first time the osmotic stress-dependent colocalization and interaction between DGK η 1 and ASK3. The lipid kinase DGK metabolizes signal lipids, diacylglycerol and phosphatidic acid, which each regulate diverse physiological and pathological events [1–5,25,47]. ASK3, which is a MAPKKK, controls bidirectional cell volume and blood pressure in response to hypo- and hyperosmotic shocks [35,36]. Although DGK η 1 and ASK3 have been independently studied, it is interesting that these physiologically important enzymes colocalize and interact with each other under osmotic stress conditions (Figs. 1 and 4), implying a functional linkage between them.

We found colocalization of DGK η 1 with ASK3 at punctate structures in an osmotic shock-dependent manner (Fig. 1). Stress granules are dense aggregations in the cytosol and appear under stressful conditions, including osmotic shock [44]. However, the stress granule markers G3BP1 and TIAR failed to colocalize with DGK η 1 and ASK3. Therefore, it is likely that the punctate structures where DGK η 1 and ASK3 exist are not canonical stress granules, and consequently, are unique structures specialized for these enzymes that have not yet been identified. We previously showed that early endosome antigen 1 and clathrin partially colocalized with DGK η 1 under osmotic stress [27]. Thus, the unknown structures may partly be a kind of clathrin-coated endosome-like vesicle. Recently, Watanabe et al. reported that ASK3 forms condensates, which are membraneless unlike the classic organelles surrounded with lipid bilayers, under hyperosmotic stress [48]. Therefore, it is possible that DGK η 1 is also translocated to liquid-like condensates through the interaction with ASK3 in response to osmotic shock. Because DGK η (DGK η 2) forms oligomers via its sterile α domain [13], DGK η may contribute to the formation of ASK3 condensates.

DGK η 1 and ASK3 were distributed in the cytoplasm in unstimulated

cells. However, they did not interact with each other in unstimulated cells (Fig. 4). Therefore, it is likely that DGK η 1 and ASK3 are not markedly colocalized in resting cells. DGK η 1 was located in punctate structures without ASK3 expression, and *vice versa* (Fig. 1). Therefore, it is likely that DGK η 1 and ASK3 do not recruit each other.

DGK δ [42], a closely related isozyme of DGK η , did not distribute to the punctate structures where ASK3 and DGK η 1 were localized, indicating that the subcellular localization of these isozymes is distinctly regulated. The subcellular localization of DGK δ was reported to be regulated by phosphorylation induced by phorbol 12-myristate 13-acetate and conventional protein kinase C [38,49].

DGK η 1 interacted with C-Raf in response to EGF stimulation and associated with ASK3 under osmotic stress [26]. Both C-Raf and ASK3 are MAPKKKs. Thus, DGK η 1 likely interacts with distinct MAPKKKs in differentially stimulated cells. ERK (MAPK) acts downstream of DGK η 1-C-Raf (MAPKKK) in EGF-stimulated cells [26]. In contrast, JNK and P38 [35] function downstream of ASK3. ASK3 also regulates WNK1-SPAK/OSR1 signaling [36]. However, it remains unclear whether DGK η 1 utilizes these pathways under osmotic stress.

The colocalization and interaction between DGK η 1 and ASK3 (Figs. 1 and 4) imply their functional linkage. Because ASK3 (NCBI, <https://www.ncbi.nlm.nih.gov/gene/389840>) and DGK η (NCBI, <https://www.ncbi.nlm.nih.gov/gene/160851>) are broadly expressed in many tissues, they can also work together in a broad range of tissues. The ASK3 gene is reportedly related to neurodegenerative diseases (Alzheimer's disease) [30]. Interestingly, genome wide association studies (GWAS Central, <https://www.gwascentral.org>) also suggested that DGK η , which is highly expressed in the brain [16], is associated with neurodegenerative diseases, such as Alzheimer's disease. In addition, DGK η is implicated in BPD [22,23]. The etiology of BPD is correlated with chronic stress [50]. Thus, stress may influence the pathogenesis of neurodegenerative diseases and BPD through DGK η and ASK3.

In the present study, for the first time, we identified the colocalization and interaction of DGK η and ASK3 in punctate granules that are specialized for DGK η and ASK3, in response to osmotic shock. The exclusive colocalization and interaction of DGK η and ASK3 imply their functional linkage, although it needs to be explored further. It will be interesting to determine the identity of the structures and the functional linkage between DGK η and ASK3.

Declaration of competing interest

The authors declare no conflicts of interest associated with the content of this article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.101006>.

Author contributions

Y.S. primarily designed and conducted the experiments, analyzed the data and wrote the manuscript. M.A. designed and conducted the experiments and analyzed the data. D.T. designed the study and revised it critically for important intellectual content. F.S. conceived the research and wrote the manuscript. All authors revised the manuscript and approved its final version.

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