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Functional relationship between CABIT, SAM and 14-3-3 binding domains of GAREM1 that play a role in its subcellular localization

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ABSTRACT

GAREM1 (Grb2-associated regulator of Erk/MAPK1) is an adaptor protein that is involved in the epidermal growth factor (EGF) pathway. The nuclear localization of GAREM1 depends on the nuclear localization sequence (NLS), which is located at the N-terminal CABIT (cysteine-containing, all in Themis) domain. Here, we identified 14-3-3ε as a GAREM-binding protein, and its binding site is closely located to the NLS. This 14-3-3 binding site was of the atypical type and independent of GAREM phosphorylation. Moreover, the binding of 14-3-3 had an effect on the nuclear localization of GAREM1. Unexpectedly, we observed that the CABIT domain had intramolecular association with the C-terminal SAM (sterile alpha motif) domain. This association might be inhibited by binding of 14-3-3 at the CABIT domain. Our results demonstrate that the mechanism underlying the nuclear localization of GAREM1 depends on its NLS in the CABIT domain, which is controlled by the binding of 14-3-3 and the C-terminal SAM domain. We suggest that the interplay between 14-3-3, SAM domain and CABIT domain might be responsible for the distribution of GAREM1 in mammalian cells.

1. Introduction

Adaptor proteins have several protein or phospholipid binding domains and these proteins play a crucial role as mediators of signaling pathways from growth factors or cytokine receptors at the plasma membrane to the nucleus [1,2]. Numerous studies of representative adaptor proteins such as Grb2 (growth factor receptor-bound protein 2), Gab (Grb2-associated-binding protein) and IRS1 (insulin receptor substrate 1) have revealed the significance of their role in physiological functions such as cell growth and differentiation [3]. Furthermore, it has been shown that their underlying molecular mechanisms are mainly dependent on the protein–protein or protein–phospholipid interaction mediated by SH2 (Src-homology 2), SH3, pleckstrin homology, and phosphotyrosine binding domains [4–6]. For example, in the cells stimulated by growth factors, adaptor proteins form a complex with phopholipids at the plasma membrane as well as tyrosine phosphorylated growth factor receptor and mediate the growth factor-induced signaling transduction pathway.

The GAREM family of proteins that include the ubiquitously expressed GAREM1 and brain-specific type, GAREM2, have been recently categorized as novel adaptor proteins [7,8]. The binding of GAREM to SH3 domain of Grb2 is mediated through its proline-rich motif, and to SH2 domain of Shp2 (Src homology 2-containing protein tyrosine phosphatase 2) via its tyrosine phosphorylated ITIM (immunoreceptor tyrosine-based inhibitory motifs). Consequently, the expression of GAREM has an effect on regulating the activation of Erk. In addition to these functional motifs, there are two more important domains on GAREM1 known as the CABIT domain at the N-terminus and SAM domain at the C-terminus. The CABIT domain was originally known to be a conserved region, commonly found among the Themis family of proteins, which play a vital role in T-cell development [9,10]. On the other hand, the SAM domain is known to mediate protein–protein interactions by forming homo- and heterotypic oligomers [11,12]. However, the roles of both of these domains in the functional aspects of GAREM are still unclear. Previously, GAREM1 has been shown to possess a nuclear localization sequence (NLS) at the N-terminus of the CABIT domain, which contributed to its nuclear localization. In contrast, GAREM2 does not possess an NLS and is distributed in the cytosol [8].

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In this study, we have identified 14-3-3ε as a novel interacting partner for GAREM. We have also shown that an atypical 14-3-3 binding site is located close to the NLS in GAREM1. Here, we hypothesize that the intramolecular association of the CABIT and SAM domain, which is in turn affected by 14-3-3 binding, might control the subcellular localization of GAREM1.

2. Materials and methods

2.1. Cell culture, transfection and reagents

COS-7 and human and embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 units/ml of penicillin. Transfection was carried out by electroporation using the Gene-Pulser system (Bio-Rad).

2.2. cDNA cloning and vector constructions

FLAG-tagged expression plasmids of the GAREM family and its derivatives were created as described previously [7,8]. GAREM1 cDNA was subcloned into the pCMV-3Tag-2 vector (Stratagene) to be expressed as an N-terminal 3 × myc-tagged protein. Point mutations were introduced using the QuikChange Kit (Stratagene), and mutations were verified by DNA sequencing.

2.3. Antibodies

An anti-GAREM1 and −GAREM2 rabbit polyclonal antibodies have been previously described [7,8]. The following antibodies were obtained commercially from various companies: anti-FLAG M2 (Sigma); anti-14-3-3ε, (Santa Cruz Biotechnology); and anti-myc (9E10, Roche).

2.4. Immunoprecipitation and immunoblot analysis

The following procedures were carried out at 0–4 °C. The transfected cells were lysed in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and a complete protease inhibitor mixture (Roche Applied Science) to produce a total cell lysate (TCL). For the immunoprecipitation experiments, the total cell lysate was centrifuged, and the supernatant was incubated for 2 h with either the primary antibody or an anti-FLAG affinity gel (Sigma). Protein G-Sepharose (GE Healthcare) was added. For the *in vitro* binding assay by GST (Glutathione *S*-transferase) pulldown, bacterially expressed GST fusion protein was immobilized on glutathione-Sepharose (GE Healthcare). Then the resulting mixture was rotated at 4 °C for 1 h. The beads were subsequently washed three times with the lysis buffer. The processed samples were analyzed as described previously [13]. GST-SAM domain containing residues 781–875 was bacterially produced by subcloning into the pGEX4T vector (GE Healthcare) to express as GST fusion protein [13].

2.5. Fluorescence microscopy analysis

Transfected cells were fixed and observed as described previously [8].

3. Results

3.1. Identification of 14-3-3ε as a GAREM-interacting protein

To gain insights into the functions of each GAREM subtype, it is important to understand its binding partners. To this end, we analyzed the proteins that were observed to co-precipitate along with GAREM1 by performing immunoaffinity purification. HEK293T cells transfected with the expression plasmid for FLAG-tagged GAREM1 were lysed, and immunoprecipitation was

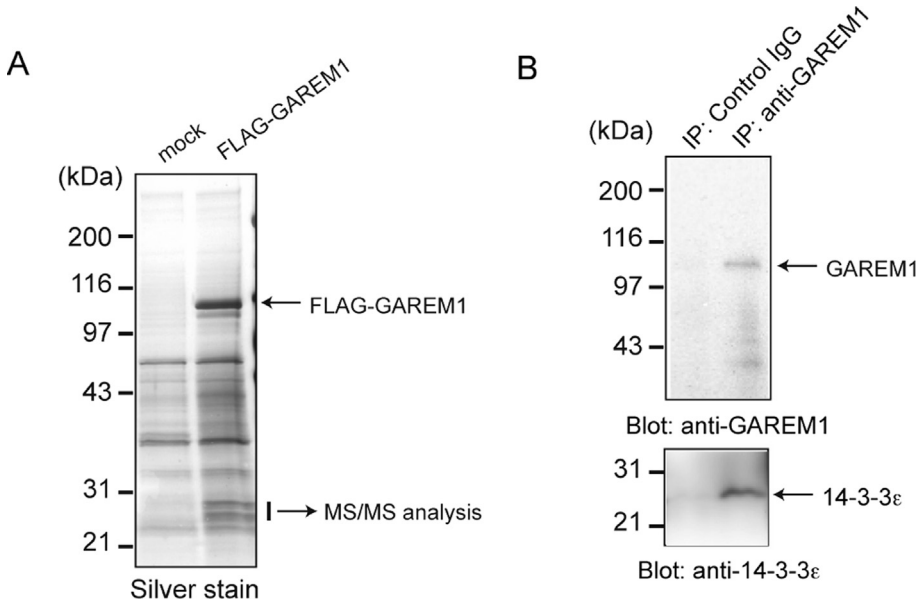

Fig. 1. Identification of 14-3-3ε as a GAREM1 specific-binding protein. (A) Purification of the GAREM1 complex. FLAG-tagged GAREM1 from 293T cells transfected with the expression plasmid (right lane). Mock-transfected 293T cells (left lane). Affinity-purified protein eluted with FLAG peptide analyzed by SDS-PAGE. Purified GAREM1 and co-precipitated bands (30 kDa) visualized by silver staining are indicated by arrows and were in-gel digested; peptides were analyzed by mass spectrometry. (B) Interaction between endogenous GAREM1 and 14-3-3ε. Co-immunoprecipitation studies performed using the anti-GAREM1 antibody and lysates of HeLa cells. Immunoblotting was performed using anti-GAREM1 (upper panel) or anti-14-3-3ε antibodies (lower panel).

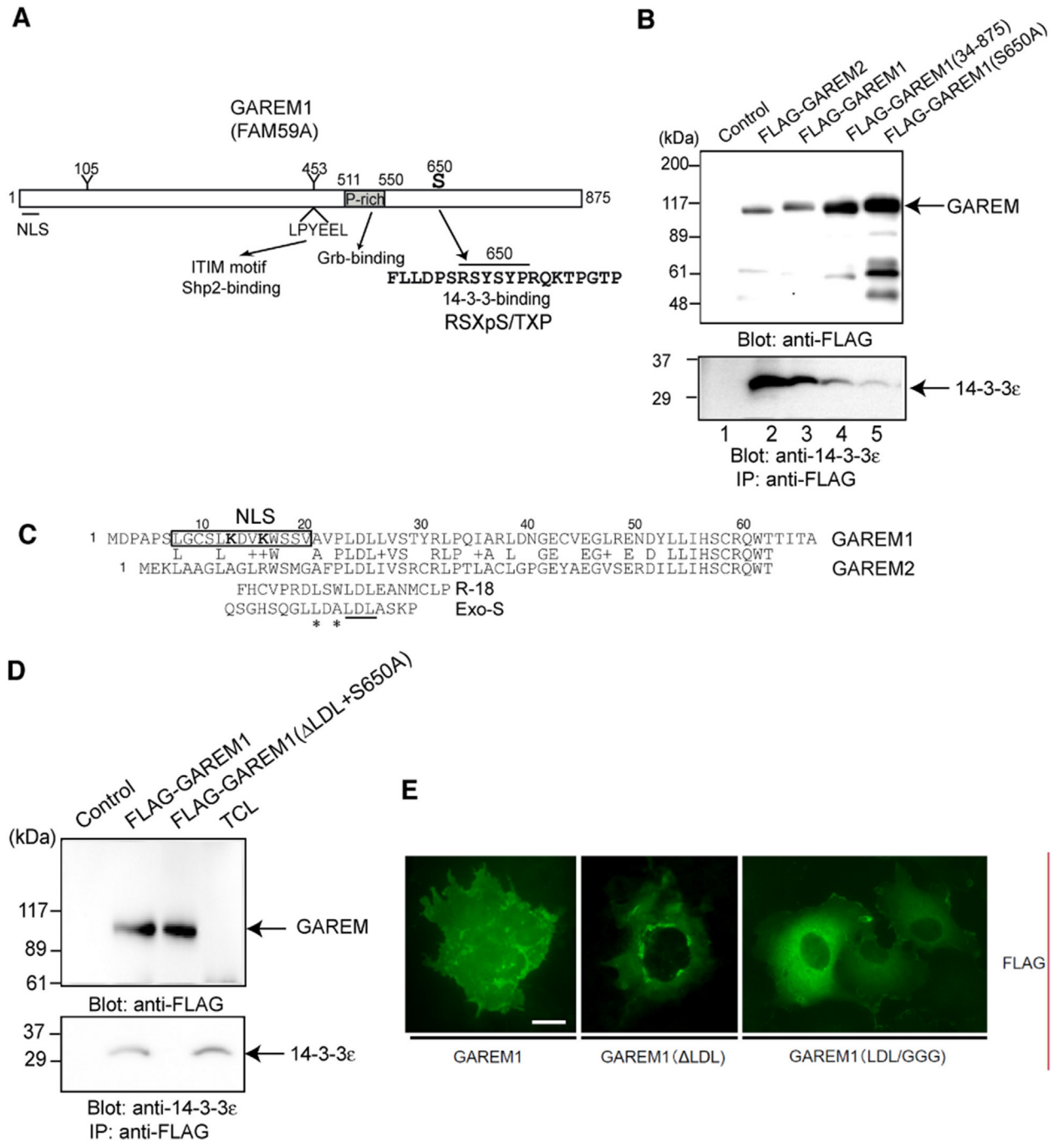

Fig. 2. Identification of the binding domain of GAREM1 to 14-3-3 ε. (A) Schematic representations of GAREM1 primary structure. Representative tyrosine residues and the surrounding amino acid sequence in GAREM1 are indicated by numbers. Amino acid sequences of the proline-rich region of GAREM1. Proline-rich regions that may bind the SH3 domain are indicated in the box. Amino acid sequence surrounding the phosphorylation site, Y453, is $LP_{phospho}YEEL$; this site is a good match to the consensus sequence of the Shp binding site, which is also known as ITIM. In addition, the amino acid sequence surrounding the phosphorylation site, S650, is $RSY_{phospho}SXP$; this site is a good match to the consensus sequence of the 14-3-3 family binding site. (B) The N-terminal region and S650 in GAREM2 enable binding to 14-3-3ε. Co-immunoprecipitation studies were performed with the anti-FLAG antibody using the COS-7 cell lysates transfected with plasmids carrying FLAG-GAREM1 derivatives. Immunoblotting was performed using anti-FLAG (upper panel) or anti-14-3-3ε antibodies (lower panel). (C) Amino acid sequences of the N-terminal region of GAREM1 (upper) and GAREM2 (lower) are indicated as numbers. Lysine 12 and lysine15 in the putative nuclear localization sequence of GAREM1 (box) are indicated in bold. The peptide sequences of non-phosphorylated atypical motif of 14-3-3-binding including R-18 and Exo-S are aligned with the GAREM N-terminal sequences. Each LDL sequence of these molecules is conserved (underlined), and the surrounding amino acid residues character is similar (asterisk). (D) The N-terminal LDL motif and S650 in GAREM2 enable binding to 14-3-3ε. Co-immunoprecipitation and immunoblotting was performed as described in (B). (E) Nuclear localization sequence of GAREM1 is dependent on the N-terminal 14-3-3-binding motif. All recombinant proteins were expressed with the N-terminal FLAG-tagged form. The results of the immunofluorescence staining using anti-FLAG antibody are indicated in the panels. Numbers indicate amino acid residues. deltaLDL: Deletion mutant of full length GAREM1 lacking three amino acids from L24 to L26. LDL/GGG: Triple mutant of full length GAREM with L24, D25 and L26 replacements with glycine. The scale bars represent 10 μm.

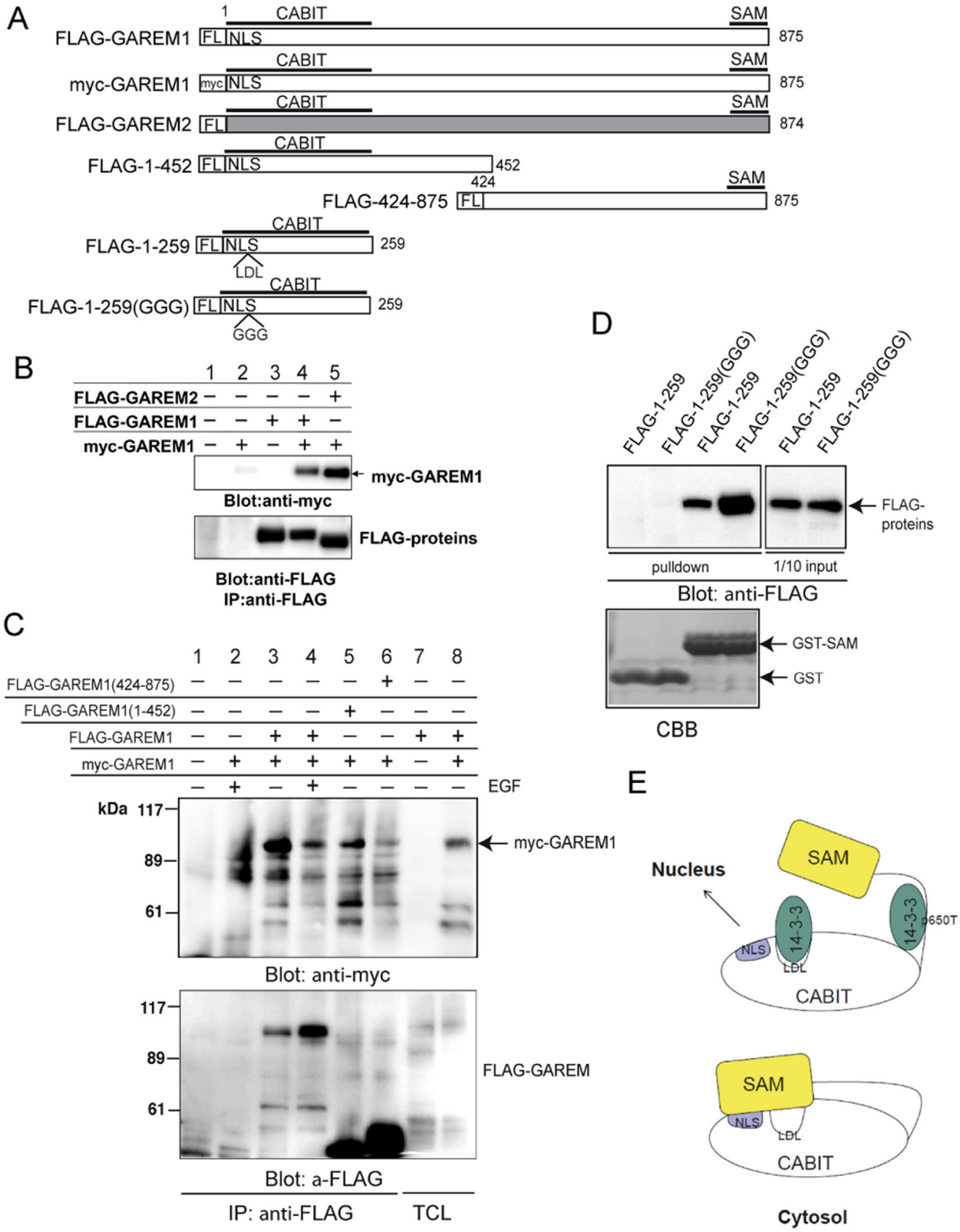

Fig. 3. Interaction between the CABIT domain and SAM domain. (A) Schematic representation of the expression constructs of the GAREM family derivatives used in this study. CABIT and SAM domains are indicated by underlined text. (B) Homo- or hetro-dimerization of GAREM molecules. Co-immunoprecipitation studies were carried out using the cell lysates from COS-7 cells with (+) or without (−)-overexpressing myc−GAREM1 and FLAG−GAREM1 or −GAREM2. Each FLAG-tagged molecule was immunoprecipitated with an anti-FLAG antibody. Immunoblot analysis was carried out using an anti-myc antibody (upper panel) or an anti-FLAG antibody (lower panel). (C) Mapping of the interaction sites in GAREM1. Co-immunoprecipitation studies were performed using the cell lysates from COS-7 cells with (+) or without (−)-overexpressing myc-GAREM1 and each derivative molecule of FLAG-GAREM. Immunoprecipitation and immunoblotting were carried out as described above. (D) *In vitro* interaction between the CABIT and SAM domains of GAREM1 was confirmed by GST pulldown assays. GST fusion proteins used in this assay were visualized by Coomassie Brilliant Blue (CBB) staining (lower panel). Immunoblotting was used to determine the amount of FLAG-1-259 or FLAG-1-259(GGG) (upper panel) bound to GST fusion proteins by using anti-FLAG antibody. The total lysate (1/10 used in this assay) from COS-7 cells expressing FLAGproteins is shown in the right lane. (E) Putative model of the intramolecular association and dissociation of the CABIT and SAM domain in GAREM1. The relationship between CABIT, NLS, SAM and 14-3-3 (white, blue, yellow, and green respectively) is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carried out using an anti-FLAG affinity gel (Fig. 1A). The proteins that eluted along with the FLAG peptide were analyzed by tandem mass spectrometry (LC/MS/MS). Using this method, we identified 14-3-3ε as a GAREM1-binding protein and also confirmed the association of endogenous GAREM1 with 14-3-3ε in HeLa cells (Fig. 1B).

3.2. Mapping of 14-3-3 binding domain in GAREM1

According to motif screening by Scansite (http://scansite.mit.edu/), GAREM1 and GAREM2 have conserved phosphorylation dependent 14-3-3-binding sites. The sequences neighboring the serine 650 (S650) residue of GAREM1 and threonine 588 (T588) residue of GAREM2 are RSYSYP and RALTEP, respectively, which are good matches for consensus binding sites of the 14-3-3 family member, RXXphosphoS/TXP (where *X* is any amino acid residue) (Fig. 2A) [14].

Both GAREM1 and GAREM2 were able to bind to 14-3-3ε in COS-7 cells confirmed by immunoprecipitation (Fig. 2 lane 2, 3). Substitution of S650 of GAREM1 with alanine (S650A) reduced the binding ability to 14-3-3ε, but did not eliminate it (Fig. 2 lane 5). This result suggested that other 14-3-3 binding sites might exist on GAREM1.

In our previous study, we identified the N-terminal (amino acid residues 7–20) nuclear localization sequence (NLS) in GAREM1 by creating various truncated mutants [8]. Serendipitously, we also found that a mutant with 33 amino acid residues deleted from its N-terminus has diminished ability to bind to 14-3-3 (Fig. 2 lane 4). Interestingly, sequence conservation was observed between GAREM1 and proteins containing non-phosphorylated 14-3-3 binding motifs such as bacterial Exoenzyme S (ExoS) [15] and R-18 [16] (Fig. 2C). Specifically, leucine 24 (L24), aspartate 25 (D25) and leucine 26 (L26) (LDL motif) on GAREM1 completely matched with the LDL motifs of the other sequences. It was also seen that the neighboring amino acids of the LDL motifs of GAREM1 and the aligned sequences were similar. Subsequently, we generated the deletion or substitution mutants of these three amino acids and analyzed their biochemical properties. Consequently, the mutant with deleted LDL motif and substituted S650A was unable to bind to 14-3-3ε (Fig. 2D). Although this mutant retained its NLS, there was a marked reduction in its nuclear localization (Fig. 2E). When all of the amino acids of the LDL motif were substituted with glycine, the protein remained in the cytosol, suggesting that the nuclear localization was completely abrogated. This might be due to the effect of 14-3-3 binding on the nuclear localization of GAREM1.

3.3. Intramolecular association of the CABIT and SAM domains in the GAREM family

GAREM mainly consists of an N-terminal CABIT domain, an internal proline-rich region, and a C-terminal SAM domain (Fig. 3A). As the SAM domain has been reported to mediate protein–protein interactions by forming homo- and heterotypic oligomers, we tried to confirm the possibility of SAM–SAM interaction of the GAREM family. We observed both homodimeric association of FLAG-tagged GAREM1 and myc-tagged GAREM1 (Fig. 3B lane 4) as well as heterodimeric association of FLAG-tagged GAREM2 and myc-tagged GAREM1 (Fig. 3B lane 5) in COS-7 cells. The N-terminal truncated mutant of GAREM1(424-875) could bind to the myc-tagged full length GAREM1 (Fig. 3C lane 6). Surprisingly, the mutant containing the CABIT domain (GAREM1(1-452)) also bound to the myc-tagged full length GAREM1 (Fig. 3B lane 4). Furthermore, the GST pulldown experiment showed that GST-SAM bound to the CABIT domain, and the mutant CABIT domain without 14-3-3 binding ability showed enhanced binding to GST-SAM (Fig. 3D). These results suggested that the CABIT and SAM domains in GAREM1 might be mutually interacting through an intramolecular interaction. As a result, the binding of GAREM1 to 14-3-3 might be inhibited. As the N-terminal NLS and 14-3-3 binding site are located in the CABIT domain, the CABIT-SAM interaction might affect the functioning of the NLS as well as 14-3-3 binding. A functional model including the characterization of each domain and interaction relationship is shown in Fig. 3E.

4. Discussion

GAREM1 and GAREM2 are composed of a CABIT domain, proline-rich region, SAM domain, as well as tyrosine residues, which can be phosphorylated through growth factor stimulation. Among these functional domains and hot spots for post-translational modifications on GAREM1, proline-rich regions and tyrosine phosphorylations have been established to play important roles in the binding to Grb2 and Shp2, respectively. On the other hand, the CABIT and SAM domains are yet to be elucidated. The CABIT domain has been known to be a conserved region found in the Themis protein family and two tandem CABIT domains occur at the N-terminus of all mammalian Themis subtypes. Recently, the distinct functional involvement of each of the CABIT domains of the Themis family proteins on T-cell development in mouse immunological organs have been reported [17]. However, the molecular mechanism and binding partners of the CABIT domain are not yet known. The GAREM family has structural similarity with the Themis members. The CABIT domain of GAREM1 and the second CABIT domain of Themis contain NLS. However, the Themis family of proteins does not possess the SAM domain that is found at the C-terminal portion of GAREM.

In this study, we identified 14-3-3ε as a GAREM binding protein. We show that the atypical non-phosphorylated 14-3-3 binding site near the NLS in the CABIT domain plays a role in the subcellular distribution of GAREM1. Furthermore, we propose that the interaction of the CABIT-SAM domains contributes to form a closed conformation of GAREM1 and consequently, mask the NLS leading to abolition of nuclear translocation of GAREM1 (Fig. 3E). This closed conformation of GAREM1 might be opened by 14-3-3 binding to the N-terminal LDL site in the CABIT domain. We observed that the CABIT mutant, which was unable to bind to 14-3-3, had enhanced association with GST-SAM (Fig. 3D). This finding supports our speculation.

In general, the 14-3-3 domains bind to the target protein in a phosphorylation dependent manner, and regulate the function or subcellular localization of its target protein [18]. It has been reported that the nuclear localization of hTERT was inhibited by binding to 14-3-3θ [19]. However, the N-terminal 14-3-3 binding site of GAREM is dependent on a conserved amino-acid motif, which induces constitutive binding. It is possible that the heterogeneity in the structure of GAREM1 arises from the associative and dissociative interactions of the CABIT-SAM domains *in vivo*. The associated form of the CABIT-SAM domains cannot bind to 14-3-3 due to masking of the NLS and as a result, the function of the NLS is suppressed and the protein mainly remains in the cytosol. On the other hand, the GAREM1 protein with dissociated CABIT-SAM domains binds to 14-3-3 and can be localized to the nucleus due to a functional NLS. Our preliminary results show that tyrosine phosphorylation of GAREM might play a role in altering the ratio of the associative and dissociative forms CABIT-SAM domains. Furthermore, both domains might have an effect on the activation of Erk in the EGF-stimulated cells (unpublished observations).

The physiological role as well as the exact function of each of the GAREM subtypes remain unknown. Through our current study as well as future work involving GAREM knock-out mice, we aim to

elucidate the role of this protein through the analysis of structural–functional relationship of intramolecular CABIT-SAM domains.

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Transparency document

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