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Novel tyrosine phosphorylated and cardiolipin-binding protein CLPABP functions as mitochondrial RNA granule

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Abstract

We identified a new protein containing the pleckstrin homology (PH) domain through tyrosine phosphoproteomics using epidermal growth factor-stimulated cells. The tandem PH domains of this protein can bind to mitochondria-specific phospholipid, cardiolipin or its dehydro product, phosphatidic acid; therefore, we have designated this protein as *c*ardiolipin and *p*hosphatidic *a*cid-*b*inding *p*rotein (CLPABP). In this study, we show that CLPABP is localized on the tubulin network and the mitochondrial surface in the granular form along with other proteins and RNA. The affinity of CLPABP to mitochondria is elevated depending on the extent of tyrosine phosphorylation. The CLPABP complex contains various proteins related to cytoplasmic mRNA metabolism. The unique subcellular localization of CLPABP requires its PH domains and a multifunctional protein, SF2p32, as its binding protein. The CLPABP granule also contains the cytochrome *c* transcript, which may be mediated by the RNA-binding protein HuR. Immunofluorescence staining reveals that the CLPABP granule is colocalized with cytochrome *c* and various ribosomal proteins that are present in the CLPABP complex. Therefore, the CLPABP RNA–protein complex may play a role in transporting cytochrome *c* mRNA and its translated product to the mitochondria.

Keywords: RNA granule; Mitochondria; PH domain; Tyrosine phosphorylation; Cytochrome *c*

1. Introduction

In eukaryotic cells, after the introns are excluded, the processed mRNA is translocated from the nucleus to the cytoplasm [1–4]. To complete this process, a large functional protein complex termed the spliceosome associates itself with the mRNA in the nucleus [5,6], and some of its components such as serine/arginine-rich (SR)-family proteins are exported to the cytoplasm along with the mRNA [7]. Other SR-family proteins are separated from the mRNA and imported into the nucleus. This shuttling mechanism is regulated by the phosphorylation of these proteins by SR-protein kinase (SRPK) [8,9]. Cytoplasmic mRNA is known to exist along with various proteins that function in controlling translation and degradation, which is important for the regulation of eukaryotic gene expression [10]. These protein–mRNA complexes are probably localized in the granular form at certain foci in mammalian cells and are known as RNA granules [11]. Major RNA granules, including the stress granule [12] and the processing body (P-body) [13,14] in the somatic cells and the neuronal granule in neurons [15], are classified based on their protein components and functions [11]. Various proteins in each RNA granule, such as ribosomal subunits, translation factors, decay enzymes, helicases, scaffold proteins, and RNA-binding proteins, have been identified and are categorized as either independent or overlapping factors [11,16]. Although the independent function of each RNA granule remains debated, all the RNA granules can repress mRNA translation by suppressing their protein components [11,13]. On the whole, the regulation of each mRNA in

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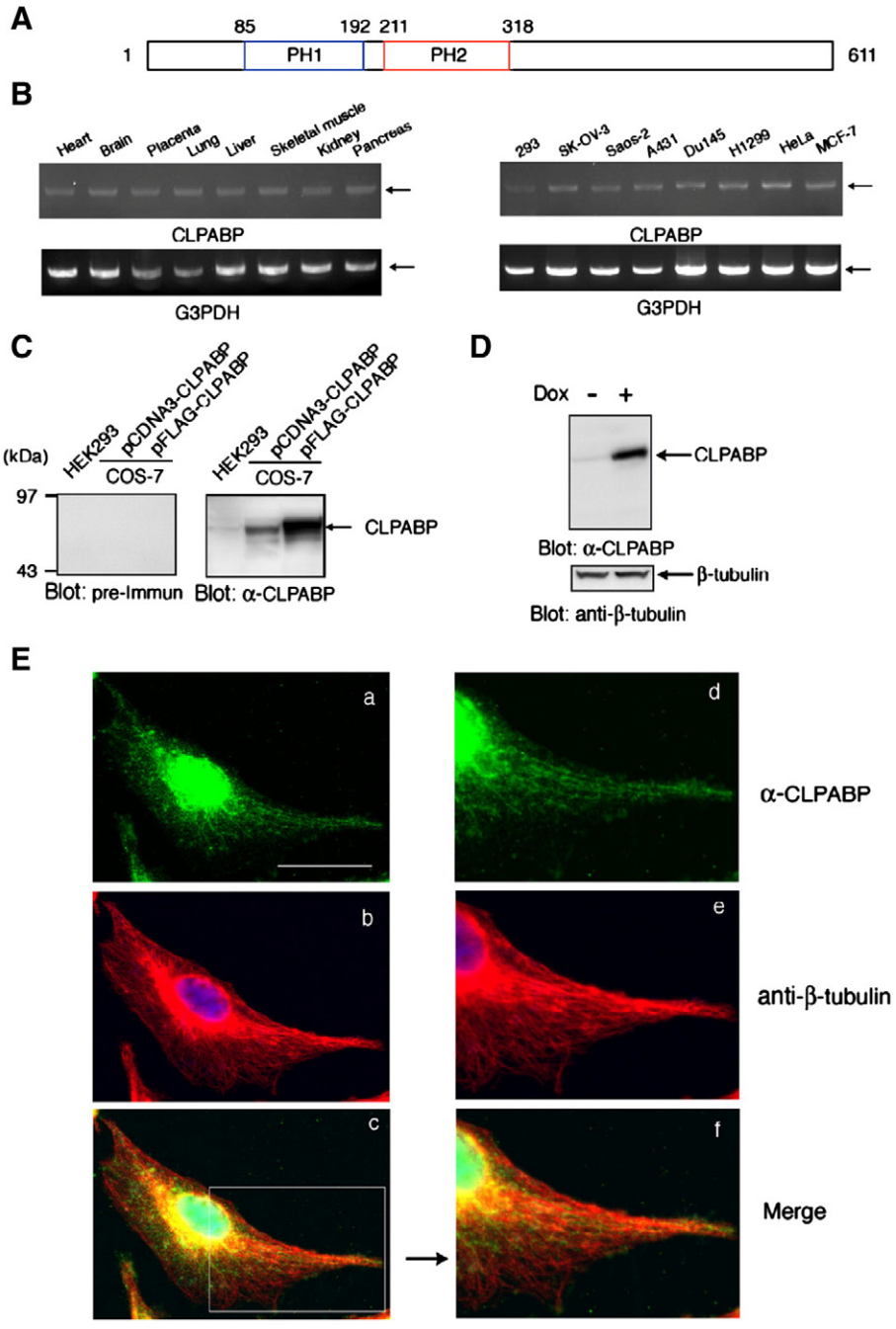


Fig. 1. Primary structure, expression, and subcellular localization of CLPABP. (A) Schematic representation of the primary structure of CLPABP. The number of amino acid residues on the boundaries is indicated. The locations of the two PH domains are indicated as PH1 and PH2. (B) CLPABP mRNA expression in various human tissues (left) and cultured cells (right). Quantitative PCR was performed; the amplified DNA fragments of the expected size (355 bp) derived from CLPABP cDNAs are indicated by arrows in the upper panel. G3PDH was used as a loading control (lower panels). The number of PCR cycles for all the samples was 30. (C) Detection of CLPABP by immunoblotting using a CLPABP-specific antibody (right panel) and preimmune rabbit serum (left panel). The WCL of HEK293 cells (left), COS-7 cells transfected with the expression vector for pcDNA3-CLPABP (center), and N-terminal FLAG-tagged CLPABP (right) were used. Immunoblotting analysis was carried out using an anti (α)-CLPABP antibody. The positions of the protein are indicated by arrows. (D) Induction of CLPABP expression in T-REX™-293 cells by adding DOX. A comparison of the expression levels of CLPABP (upper panel) with (right) or without (left) DOX treatment for 24 h and the expression levels of β-tubulin in each cell line as a control are shown (lower panel). (E) Endogenous CLPABP is localized on the microtubule in HeLa cells. The subcellular localization of CLPABP was traced by immunofluorescence using anti-CLPABP (a and d) and anti-β-tubulin antibodies (b and e); the nucleus was stained with Hechest33342 (b and e), and the merged images are shown (c and f). The magnified images in the white box (c) are shown (d–f). The scale bars represent 10 μm.

mammalian cells—from its generation to its decay following translation—is a complicated process. Recently, we analyzed novel molecules that were identified through tyrosine phosphoproteomics upon epidermal-growth-factor (EGF) stimulation, and we reported two molecules as the novel regulators of EGF-receptor homeostasis [17,18]. In this report, we analyzed another molecule containing two pleckstrin homology (PH) domains and discovered that this protein functions in the mitochondrial RNA

granule. In mammalian cells, it is localized as a granule-like structure on the mitochondrial surface and is stabilized by its association with tubulin. The RNA granule assumes various shapes and sizes while attached to the mitochondrial surface, and some of its vesicles merge with cytochrome *c*. Both the PH domains of this protein can bind to mitochondria-specific phospholipids, cardiolipin (CL) or its dehydro product, phosphatidic acid (PA); therefore, we designated this protein as CLPABP (*CL*-and *PA*-*b*inding *p*rotein).

The CLPABP complex includes various proteins related to mRNA metabolism, such as SR-family, ribosome-subunit, RNA-binding, and RNA-stabilizing proteins. Furthermore, it contains cytochrome *c* mRNA. Our results suggest that CLPABP may conform to the mitochondrial RNA granule for carrying mRNA of some mitochondrial proteins for facilitating its recruitment.

2. Materials and methods

2.1. Cell culture and transfection

COS-7, HeLa, HEK293, 293T, and T-REX™-293 (Invitrogen) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin. Plasmid transfection into COS-7 or 293T cells was carried out by electroporation using Gene-Pulser (Bio-Rad). To establish a stable T-REX™-293 cell line, transfection was carried out using Lipofectamine 2000 (Invitrogen). Prior to EGF stimulation, the cells were serum-starved for 16 h, and 100 ng/ml EGF (Sigma) dissolved in a serum-free medium was added. Alternatively, the cultured cells were treated with 0.5 mM H_2O_2.

2.2. cDNA cloning and vector construction

CLPABP cDNA was purchased from the RZPD German Resource Center for Genome Research. To clone this cDNA into an expression vector, polymerase chain reaction (PCR) was performed using 2 primers—(5′-GTTTAAGCTTATGGGGAACAGCCACTGTGTC-3′ and 5′-GAAAGAATTCTCAGATCCACTGCACAAGCCC-3′), which were designed based on the sequences (gi|14149786) provided in the NCBI database. The amplified products were first subcloned into a TOPO™ TA cloning vector, namely, pCR®2.1-TOPO (Invitrogen), and subsequently sequenced. The CLPABP cDNA was inserted into a pcDNA3 vector (Stratagene) or into the in-frame expression vector pCMV-FLAG6a (Sigma), and the constructs were expressed as non-tagged or N-terminal FLAG-tagged proteins, respectively. The CLPABP cDNA deletion mutants were generated by digesting the cDNA with suitable restriction enzymes, followed by PCR amplification, and the constructs were subcloned into a pCMV-FLAG6-series vector. Point mutations or deletions were introduced by using the QuickChange kit (Stratagene) according to the manufacturer's protocol. All the nucleotide sequences were determined and verified using an ABI prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI Prism 3100-Avant genetic analyzer.

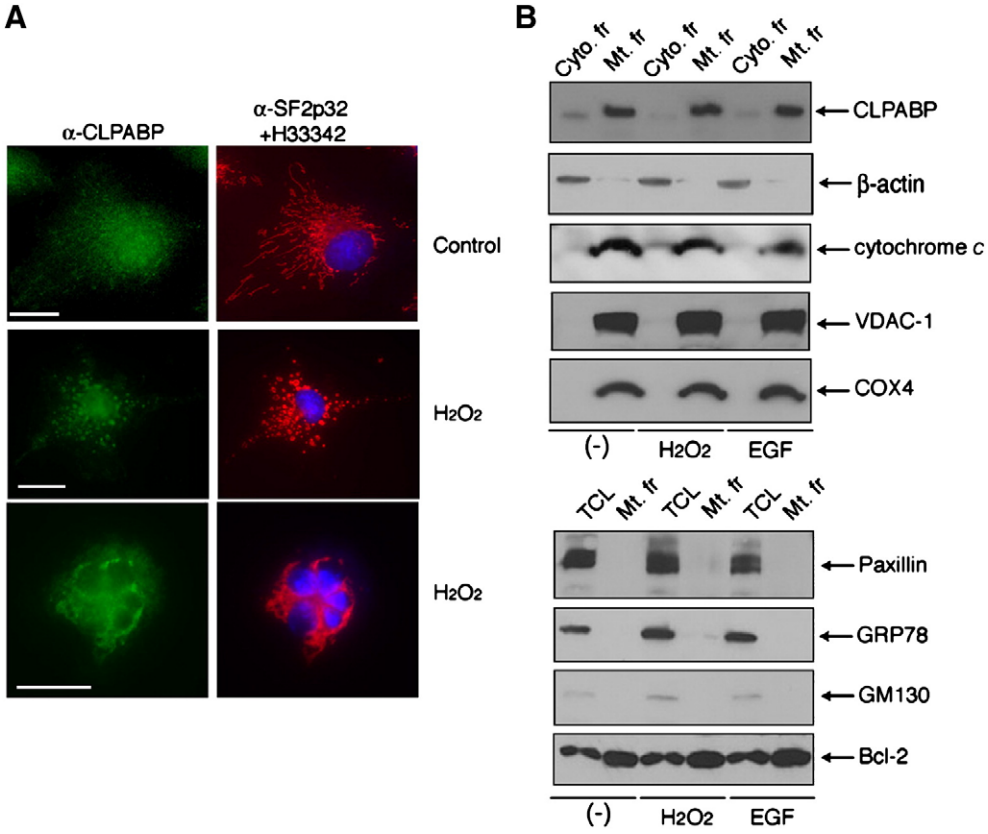


Fig. 2. CLPABP is localized on the mitochondria, and its distribution is altered in response to cellular stimuli. The subcellular localization of endogenous CLPABP was traced by immunofluorescence staining with the anti-CLPABP antibody (green); mitochondria were stained with the anti-SF2p32 antibody (red). The nucleus was stained with Hechest33342 (blue). The scale bars represent 10 μ m. (A) The accumulation of endogenous CLPABP in the mitochondria due to oxidative stress. COS-7 cells were not treated (upper panel) or treated with (middle and lower panels) 0.5 mM H_2O_2 for 90 min. An image of a typical apoptotic cell is shown in the lower panels. (B) Biochemical analysis of the subcellular distribution of endogenous CLPABP. HeLa cells were treated with either EGF or H_2O_2. The proteins from the total cell lysate (TCL), the cytosolic (Cyto. fr) and mitochondrial (Mito. fr) fractions of each cell line (20 μg each) were used as indicated, and immunoblotting analyses were carried out using the indicated antibodies.

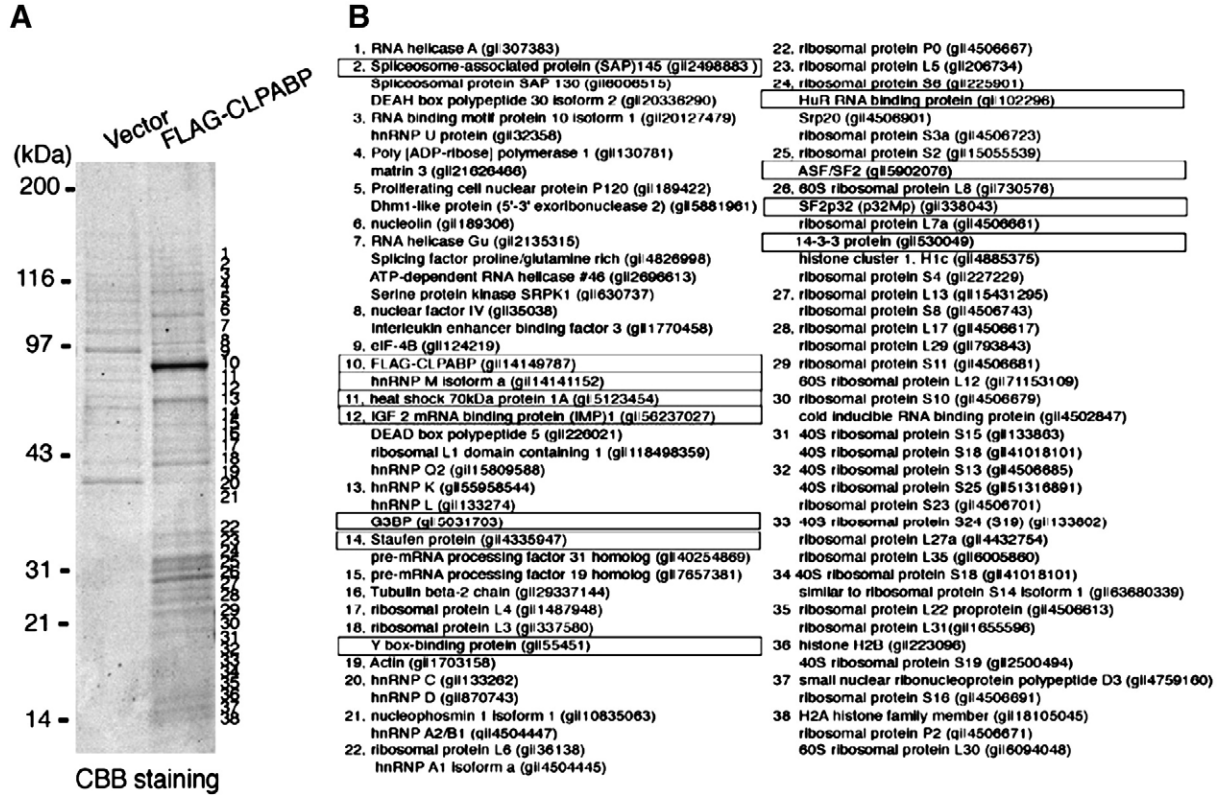


Fig. 3. Identification of CLPABP-binding proteins. (A) The purification of the CLPABP complex. FLAG-CLPABP was purified from HEK293T cells transfected with the expression plasmid (right lane). As a control, mock-transfected HEK293T cells transfected with the empty vector were used (left lane). The affinity-purified protein eluted using the FLAG peptide was analyzed by SDS-PAGE. All the visible bands indicated by numbers were subjected to in-gel digestion, and the peptides were analyzed by LC/MS/MS. (B) The names of proteins identified from the numbered gel pieces (A) and their gene identification (gi) numbers are indicated. The existence of boxed molecules in the complex was confirmed by immunoblotting in Fig. 4.

2.3. Antibodies

An anti-CLPABP rabbit polyclonal antibody was raised against a CLPABP fragment containing residues 500–611, which was bacterially produced as a glutathione S-transferase (GST) fusion protein by using the pGEX4T vector (Amersham Biosciences). This antibody was purified by using HiTrap *N*-hydroxysuccinimide-activated sepharose columns (Amersham Biosciences) coupled with an immunizing antigen. An anti-IMP rabbit polyclonal antibody was raised against a 15-amino acid peptide corresponding to the C-terminal of the protein. The following other antibodies were obtained commercially from various companies: anti-FLAG M2, anti-β-actin, and anti-β-tubulin (Sigma); anti-phosphotyrosine (4G10, Upstate Biotech Inc.); anti-SF2p32, anti-14-3-3ε, anti-cytochrome *c*, anti-SAP145, anti-hnRNP M3/4, anti-Y-box, anti-ASF/SF2, anti-COX4, and anti-HuR (sc-23885, sc-17784, sc-7159, sc-14279, sc-20001, sc-18057, sc-28724, sc-20694, sc-58348, and sc-5261, respectively; Santa Cruz Biotechnology); anti-GST (MBL); anti-myc (9E10, Roche); anti-paxillin, anti-GRP78, anti-GM130, anti-Bcl-2 and anti-Hsp70 (BD Transduction); anti-G3BP (Abcam); anti-VDAC1 (Calbiochem); and anti-Staufen (Chemicon).

2.4. Semiquantitative reverse transcriptase (RT)-PCR

To assess the relative expression levels of the CLPABP transcripts, RT-PCR was performed on each panel of eight different human culture cell and tissue cDNAs (human tissue and cell line MTC panel, Clontech) by using the primers 5′-GTTTGGATCCTCTGTGCCTGCCTCTGACCCT-3′ and 5′-GAAAGAATTCTCAGATCCACTGCACAAGCCC-3′. The normalized cDNA was amplified under the following conditions: denaturation at 94 °C for 1 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and extension at 72 °C for 5 min. The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide.

2.5. Cloning of the T-REX™-293 stable cell line

Full-length CLPABP cDNA was inserted into a pcDNA4 vector (Invitrogen). T-REX™-293 cells were then transfected with either the empty vector or the resulting hybrid. Zeocin™-resistant cells were isolated in the presence of 0.1 mg/ml Zeocin™ (Invitrogen) and cloned. The CLPABP expression was induced by the addition of 1 μg/ml doxycycline (DOX) (Clontech) in the medium.

2.6. Small interference RNA (siRNA)

Synthetic siRNA duplexes were used for the knockdown of CLPABP expression in the HeLa cells. The targeted human CLPABP sequence was 5′-AAGGAGAAGCAGATCCGCTCCTT-3′ (nucleotides 832–854). The siRNA for green fluorescence protein (GFP) was used as a control. The HeLa cells were transfected with siRNA by using oligofectamine (Invitrogen).

2.7. 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 DTT, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and a complete protease inhibitor cocktail (Roche) to produce a whole-cell lysate (WCL). For the immunoprecipitation experiments, the WCL 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 (Amersham Biosciences) was added, and 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 boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto an Immobilon-P membrane (Millipore). Immunoblot analysis was carried out using primary

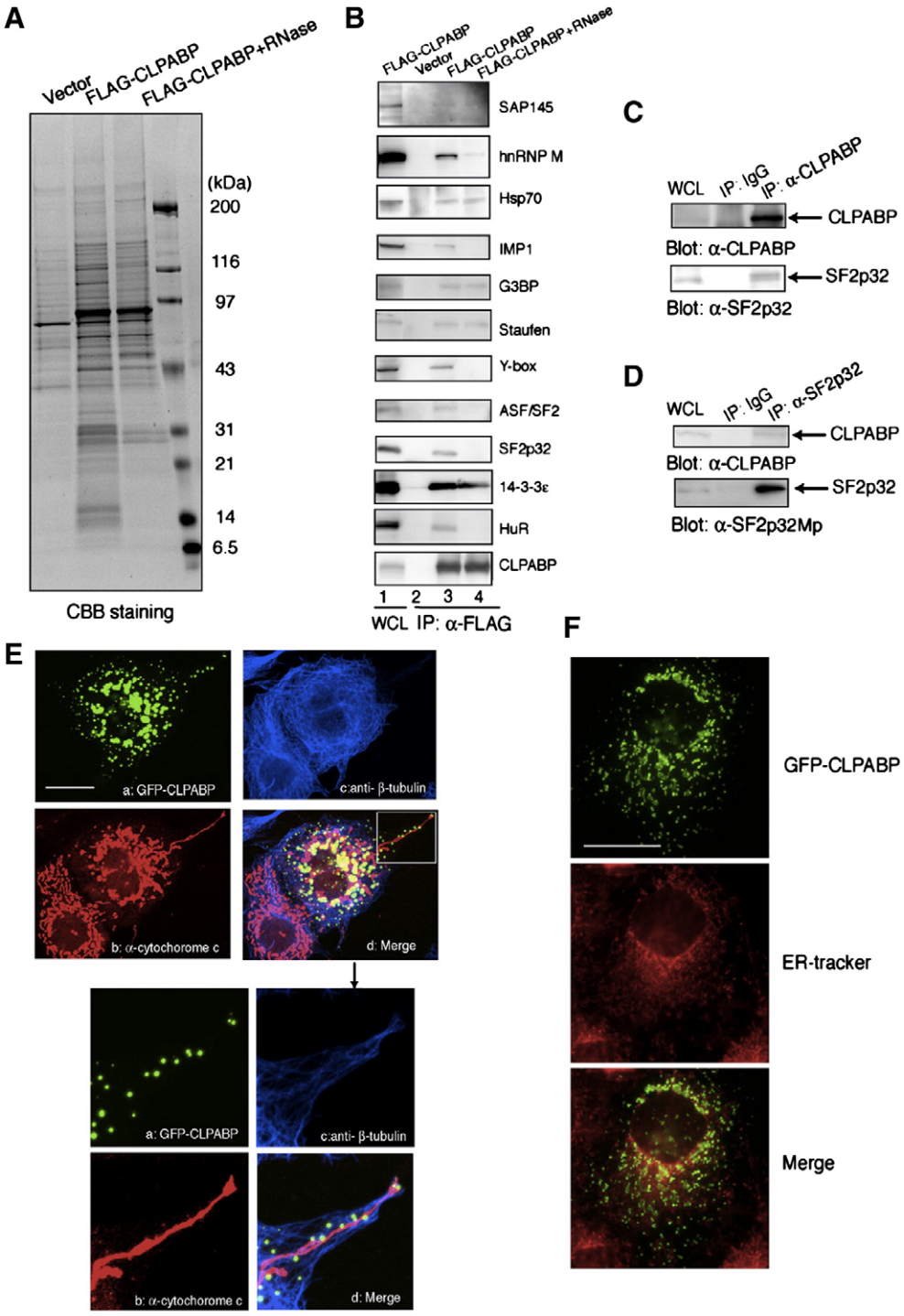


Fig. 4. Purification of CLPABP complex with or without RNase treatment. (A) In this assay, 10 μg/ml of RNase was added to the lysate prior to the purification step (lane 3). Each purified CLPABP complex was visualized as shown in Fig. 3. (B) The binding of FLAG-CLPABP to the indicated proteins was confirmed by immunoblotting. The WCL of HEK293T cells expressing FLAG-CLPABP was applied to lane 1. Coimmunoprecipitation studies were carried out with the anti-FLAG antibody using the lysates of HEK293T cells transfected with the empty vector (lane 2) or the FLAG-CLPABP expression plasmid (lane 3, 4). The WCL was treated with RNase A (10 μg/ml) prior to immunoprecipitation (lane 3). Immunoblot analysis was carried out using the anti-CLPABP antibody (bottom panel) or the indicated antibodies. (C) Association between endogenous CLPABP and SF2p32 *in vivo*. Coimmunoprecipitation experiments were carried out with control IgG and anti-CLPABP antibodies using the HeLa cell lysates. Immunoblot analysis was carried out using anti-CLPABP (upper panel) and anti-SF2p32 antibodies (lower panel). (D) Reciprocal coimmunoprecipitation studies were carried out using the HeLa cell lysates. (E) Subcellular localization of GFP-CLPABP. COS-7 cells were transfected with the expression vector encoding GFP-CLPABP (a). The cells were processed for coimmunofluorescence staining 36 h after transfection using the anti-cytochrome *c* (b) and anti-β-tubulin antibodies (c). Merged images of the panels are shown (panel d). The magnified images in the white box (E) are shown in four panels below. (F) Partial colocalization of CLPABP in the ER. Coimmunofluorescence staining was performed as that in (E). The ER was stained with ER-Tracker dye (middle). Merged images of the panels are shown in the bottom panel.

antibodies, as described in the figure legends. Immunoreactive bands were visualized using horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG and ECL reagent (Amersham Biosciences).

2.8. Purification of FLAG-CLPABP and its binding proteins

Then, 293T cells (1×10^8 cells) transiently expressing FLAG-tagged CLPABP were lysed with the lysis buffer. The lysate was centrifuged, and the supernatant was incubated with a FLAG-affinity gel (bed volume: 50 μl) for 2 h. The gel was applied to an empty mini column (Bio-Rad) and washed several times with the lysis buffer. FLAG-CLPABP was eluted with the FLAG peptide and resolved by performing SDS-PAGE. All the bands that were visualized by Coomassie brilliant blue (CBB) staining were subjected to mass spectrometry (MS/MS) analysis following in-gel digestion with trypsin. The in-gel digestion and mass spectrometric analysis were performed as described previously [19].

2.9. Lipid-binding assay

Membrane lipid strips spotted with 15 different lipids were obtained from Echelon Biosciences Inc. The strips were blocked with 3% fatty-acid-free bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T; 20 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) and incubated with GST-fused protein (0.5 μg/ml) for 3 h. After washing, the membranes were incubated with an anti-GST antibody for 2 h, followed by additional washing and incubation with an HRP-conjugated antibody. After the final washing, lipid-bound GST-fusion proteins were detected by ECL.

2.10. Fluorescence and confocal microscopy analysis

To express GFP-fused CLPABP in the cultured cells, the full-length CLPABP cDNA was inserted into a pEGFP-C3 vector (Clontech) in frame. Transfected and nontransfected cells were fixed with 5% formaldehyde in PBS for 10 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and washed with PBS once again. Following a blocking step with 3% bovine serum albumin in PBS for 30 min, the primary antibodies, as illustrated in the figures, were applied for 1 h. After washing with PBS, the cells were incubated with the appropriate secondary antibodies conjugated with Alexa fluorescent dyes (Molecular Probes) for 45 min. If necessary, the cells were treated with Mito-or ER-Tracker (Molecular Probes), and the nuclei were simultaneously stained with 2 μM Hoechst 33342 (Molecular Probes). Finally, the cells were rinsed three times with PBS and mounted onto microscope slides with ProLong Antifade reagents (Molecular Probes). Images were captured using a Zeiss Axiovert 200 fluorescence microscope or an LSM 510 Meta laser scanning confocal microscope with a 40, 63, and 100×1.0 numerical aperture PlanApo objective (Zeiss Axioskop, Carl Zeiss Inc.). The figures were prepared using Adobe Photoshop.

2.11. Preparation of mitochondrial fraction

For CLPABP and cytochrome *c* redistribution studies, the mitochondrial fraction was prepared from HeLa cells with or without cellular stimulation, as described previously [20].

2.12. Detection of apoptotic cells

Apoptotic cells were quantified by counting the COS-7 cells that expressed GFP or GFP-CLPABP under a fluorescence microscope following Annexin V-Alexa594 (Molecular Probes) staining. More than approximately 100 GFP-positive cells were counted. Three independent sets of experiments were carried out. The error bars show the standard deviation.

2.13. RNA preparation

RNA was extracted from the immunoprecipitated CLPABP complex with either the anti-FLAG or anti-CLPABP antibody using the Trizol reagent, as instructed by the manufacturer (Invitrogen). The following primers were used for RT-PCR of the cytochrome *c* (full-length) and G3PDH transcripts: cytochrome *c*, 5′-GTTTAAGCTTTGGGTGATGTTGAGAAA-3′ and 5′-TGAAGGTCG-GAGTCAACGGATTTGGT-3′, and G3PDH, 5′-TGAAGGTCGGAGT-CAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

3. Results

3.1. Primary structure, expression, and subcellular localization of CLPABP

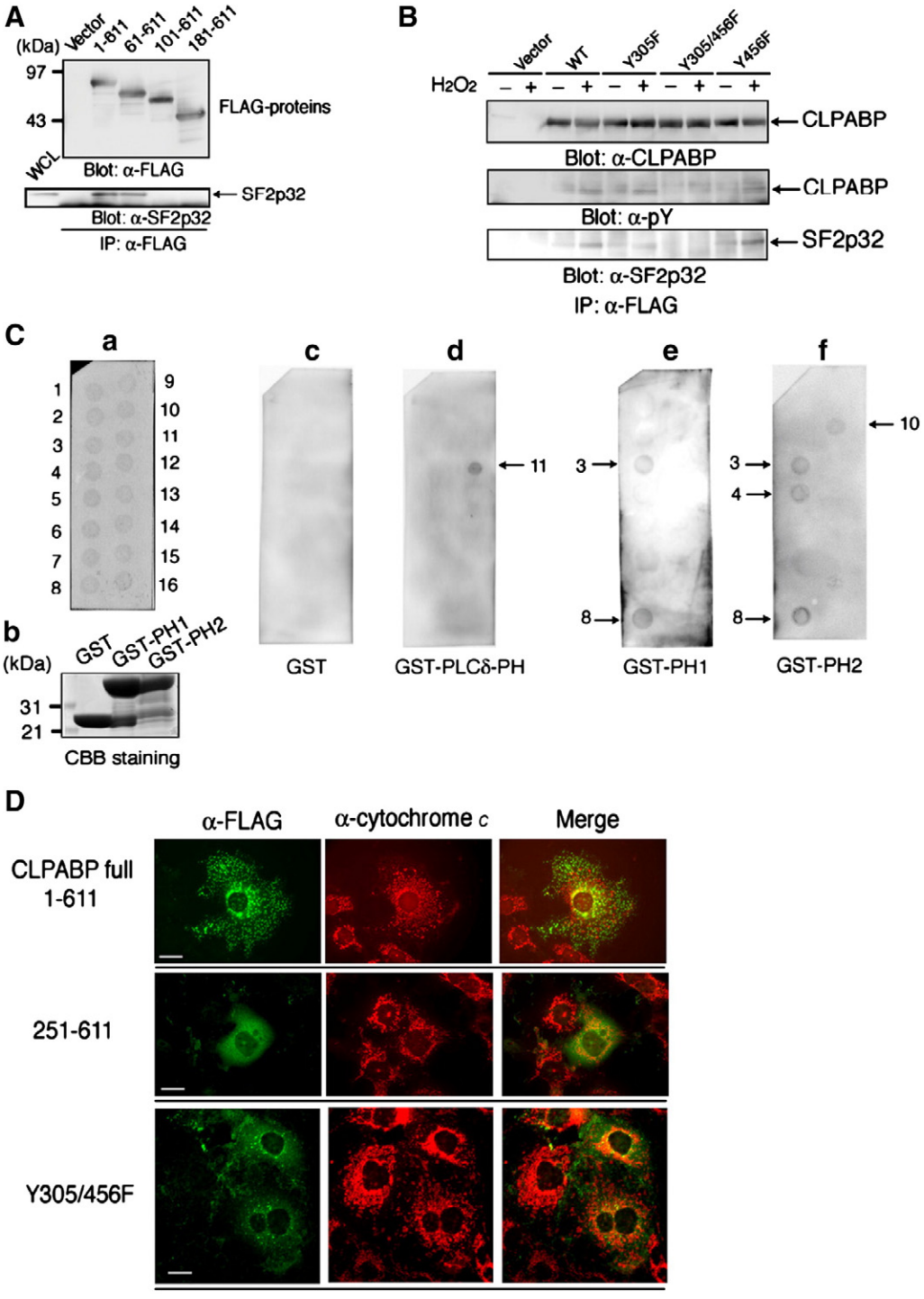
Previously, we identified more than 150 proteins through phosphoproteomic studies using the lysate of EGF-stimulated A431 cells [17,18]. In the present study, we focused on a newly identified molecule, CLPABP, that contains two PH domains in its N-terminal half, as shown in Fig. 1A. The CLPABP protein comprises 611 amino acid residues, and its cDNA has been cloned in human full-length cDNA sequencing projects in Germany and has been stored as DKFZP434H2010 (gi|14149786). According to the genome database, the homologous genes of human CLPABP have been cloned from zebra fish, frog, and several species of mammals but not from worms and fly species. Semiquantitative RT-PCR confirmed that the CLPABP gene expression is essentially ubiquitous in human tissues and cultured cells (Fig. 1B). The specific antibody produced by immunizing the GST-fused C-terminal region of CLPABP was effective for the detection of endogenous and exogenous CLPABP in the WCL analysis, whereas preimmune serum was not (Fig. 1C). Furthermore, we established cell lines that inducibly expressed exogenous CLPABP: using this specific antibody, we determined whether CLPABP expression in the WCL was elevated due to the DOX treatment or not (Fig. 1D). The PH domain is well established as a lipid-binding domain, and various proteins related to growth factor receptor-mediated signal transduction possess this functional domain for their localization at the plasma membrane. However, immunofluorescence microscopy of HeLa cells revealed that CLPABP was subcellularly localized on the microtubule in the form of small granules (Fig. 1E).

3.2. CLPABP is localized on mitochondria

The recruitment of a PH-domain-containing protein to the plasma membrane is dependent on cellular stimuli such as treatment with growth factors [21]. Therefore, we observed the subcellular distribution of CLPABP in response to EGF treatment and oxidative stress. CLPABP was not translocated to the plasma membrane following these stimulations; however, it accumulated on the mitochondria (Fig. 2A). Biochemical observations revealed that CLPABP was included in the mitochondrial fraction, and small amounts of CLPABP in the cytosolic fraction (but not in the mitochondrial fraction) were reduced with EGF stimulation and oxidative stress (Fig. 2B). These results suggest that the localization and function of CLPABP might differ from those of typical PH-domain-containing proteins that play a role as downstream molecules for the EGF receptor.

3.3. Identification of proteins interacting with CLPABP

To gain insights into the functions of CLPABP, we analyzed the proteins that coprecipitated with it by performing immunoaffinity purification. HEK293T cells transfected with the expression plasmid for FLAG-tagged CLPABP were lysed with buffer containing 1% Triton X-100, and immunoprecipitation was carried out using an anti-FLAG affinity gel. Proteins eluted along with the FLAG peptide were analyzed by SDS-PAGE (Fig. 3A). In addition to CLPABP, other observed protein bands were also analyzed by tandem mass spectrometry (LC/MS/MS) in order to identify their amino acid sequences. The identified proteins were almost related to RNA processing (Fig. 3B), and representative proteins could be confirmed by their existences in the FLAG-CLPABP complex with or without RNase treatment (Fig. 4A, B). Although many proteins were associated with FLAG-CLPABP, SF2Ap32 (also known as a p32 mitochondrial matrix protein, hyaluronan-binding protein, or gC1Q-R) may play a central role in the mitochondrial localization of CLPABP. In addition to SF2Ap32, ASF/SF2 was also included



in the CLPABP complex; the regulation of the ternary complex comprising these three proteins depended on the presence of RNA (Fig. 4B). The association of SF2Ap32 with CLPABP in HeLa cells was further confirmed by an immunoprecipitation assay using specific antibodies. Endogenous SF2Ap32 was detected in the immune complex that was precipitated by using the anti-CLPABP antibody. These results were confirmed by reciprocal immunoprecipitation experiments performed using the anti-SF2Ap32 antibody (Fig. 4C, D). It is well known that mitochondrial morphology and distribution were coordinated with a tubulin cytoskeleton [22]. Consistent with the biochemical results suggesting the presence of α-tubulin in the CLPABP complex, GFP-fused CLPABP was colocalized not only on tubular mitochondria but also on the small cytochrome *c* granules contained in the tubulin network (Fig. 4E). Other organelle markers (golgi, early endosomes, and lysosomes) were not merged with the vesicles (data not shown). However, some of the granules were localized close to the endoplasmic reticulum (ER) (Fig. 4F).

3.4. Detection of CLPABP domains essential for its binding with other proteins

To analyze the binding mechanisms of CLPABP and SF2p32 in detail, several constructs were expressed in COS-7 cells, and the interaction was assessed by immunoprecipitation (Fig. 5A). The obtained results revealed that the N-terminal region (amino acid residues: 61–100) of CLPABP is required for its binding to endogenous SF2p32. Moreover, its binding affinity is upregulated when the tyrosine phosphorylation of CLPABP is induced by oxidative stress (Fig. 5B). Next, we constructed truncated mutants (data not shown) and several point mutants of CLPABP that were replaced at potentially phosphorylated tyrosine residues (Y) by phenylalanine (F). A double mutant of Y305/456F almost abolished the tyrosine phosphorylation upon H_2O_2 stimulation. Furthermore, this mutant was hardly bound to SF2p32 (Fig. 5B). Therefore, the interaction of these proteins may be regulated by the phosphorylation of the two tyrosine residues (Y305 and Y456) of CLPABP.

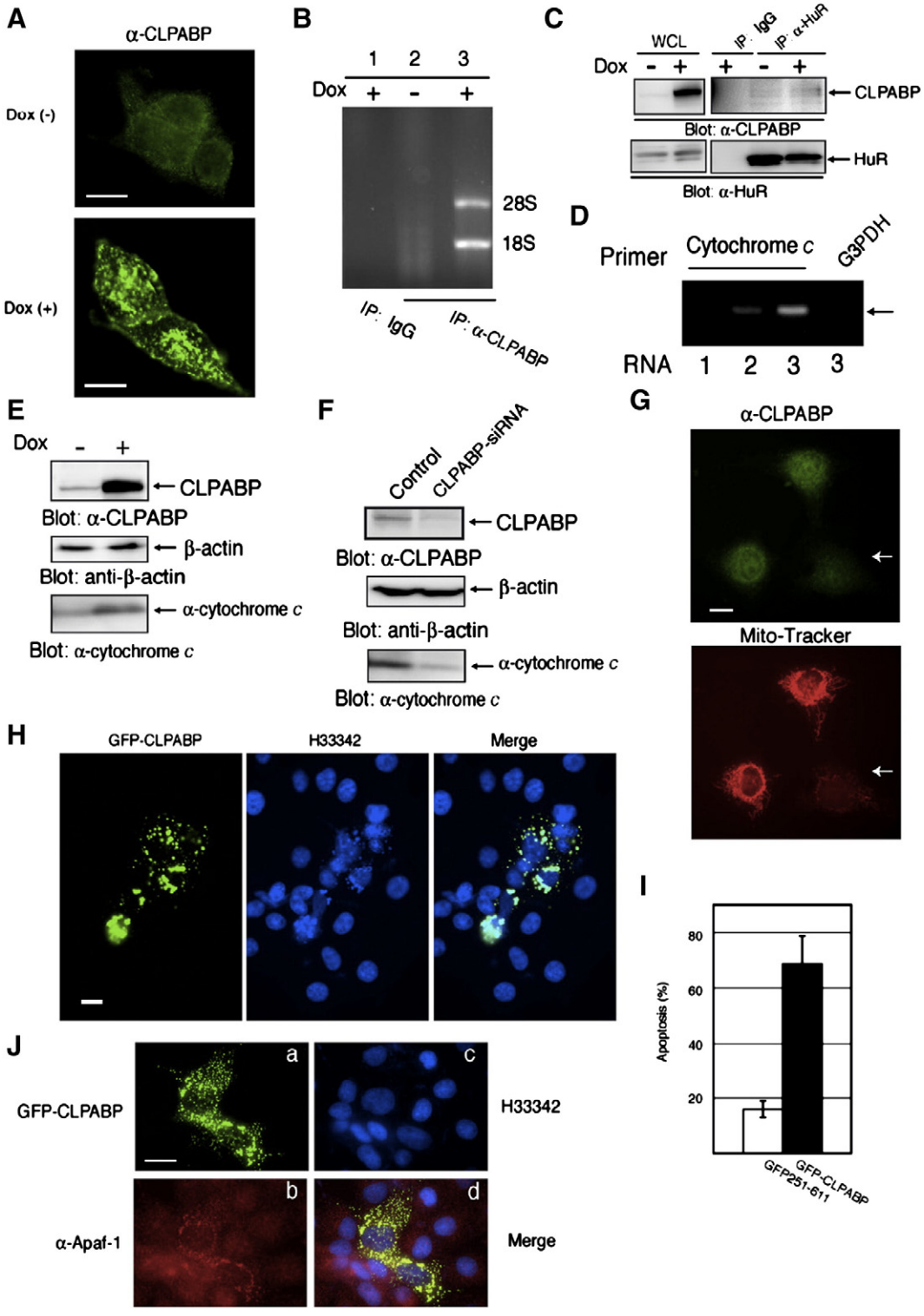
The two PH domains are the most typical functional regions in CLPABP. The PH domain is known as a lipid-binding domain that functions in the recruitment of the protein to the plasma membrane or the surface of some organelles related to endocytosis [21]. To screen the lipids that are bound to the PH domain of CLPABP, we performed an overlay assay using GST-fused PH domains (PH1 and PH2) as a bait (Fig. 5C(b)) and membrane lipid strips (Fig. 5C(a)). GST was not bound to any of the spotted lipids, and a positive control of the GST-PH domain derived from PLCγ recognized phosphatidylinositol 4,5-diphosphates (PI 4,5-P_2) (Fig. 5C(c, d)). However, the mitochondria-specific lipid, CL and its hydrolysis product, PA formed by phospholipase D (PLD) were observed to bind both the PH domains (Fig. 5C(e, f)). In addition, phosphatidylserine (PS) and PI4-P could bind to the C-terminal PH domain (PH2) (Fig. 5C (f)). These results suggest that the N-terminal region of CLPABP containing the PH domains may be necessary for its unique subcellular localization. Therefore, we analyzed the localization of each CLPABP derivative in the cells (Fig. 5D). The two truncated mutants containing amino acid residues 1–450 and 61–611 resembled the wild-type CLPABP. The mutants lacked the SF2Ap32 binding site (101–611), and the first PH domain (181–611) still exhibited the vesicular form around the mitochondria; however, the intensity of the immunoreactive signal in the cytosolic fraction increased dramatically (data not shown). Vesicle formation and mitochondrial localization was abolished in the shortest mutant containing amino acid residues 251–611 and lacking the N-terminal region of PH2 and in a double mutant of Y305/456F. Therefore, we conclude that the SF2Ap32-binding site and two PH domains of CLPABP are essential for its unique localization in cells, which is regulated by tyrosine phosphorylation.

3.5. Function of CLPABP as cargo protein for cytochrome c mRNA

To effectively analyze the functions of CLPABP, we attempted to establish a stable cell line overexpressing this protein. The cell lines obtained by using a constitutively active promoter did not express recombinant CLPABP for cell toxicity. Alternatively, T-REX™-293 cell lines obtained on using a tetracycline-inducible promoter expressed high amounts of CLPABP when a tetracycline analogue DOX was added. After induction of the CLPABP expression, a large number of CLPABP granules were detected in the cells (Fig. 6A). Next, we attempted to obtain RNA from the immune complex formed with the CLPABP

Fig. 5. Identification of the SF2p32-binding site in CLPABP, and the roles of its PH domains. (A) Binding of FLAG-CLPABP to endogenous SF2p32. Coimmunoprecipitation studies were carried out with the anti-FLAG antibody using the lysates of COS-7 cells transfected with the empty vector or the expression plasmid of FLAG-CLPABP derivatives. The number of amino acid residues of each derivative is shown. Immunoblotting analysis was carried out using the anti-FLAG (upper panel) or anti-SF2p32 antibodies (lower panel). (B) Effects of tyrosine phosphorylation of CLPABP induced by oxidative stress on its binding to SF2p32. Coimmunoprecipitation studies were carried out with the anti-FLAG antibody using the lysates of COS-7 cells transfected with the empty vector or the expression plasmid of FLAG-CLPABP derivatives that replaced tyrosine residues (Y) by phenyl alanine (F) were treated with (+) or without (−) 0.5 mM H_2O_2 for 30 min. The number of each derivative are tyrosine residues IB analysis as carried out using the anti-FLAG (upper panel), anti-pY (middle panel), or anti-SF2p32 antibody (lower panel). (C) The screening of the lipids bound to the PH domains of CLPABP. (a) Nitrocellulose strips spotted with the lipids as follows: 1, triglyceride; 2, diacylgycerol; 3, phosphatidic acid; 4, phosphatidylserine; 5, phosphatidylethanolamine; 6, phosphatidylcholine; 7, phosphatidylglycerol; 8, cardiolipin; 9, PtdIns; 10, PtdIns(4)P; 11, PtdIns(4,5)P_2; 12, PtdIns(3,4,5)P_3; 13, cholesterol; 14, sphingomyelin; 15, 3-sulfogalactosylceramide; and 16, solvent blank. Purified GST-PH1, 2 of CLPABP and control GST were stained with CBB, as shown in (b). The strip was incubated with GST (c), GST-PLCγ-PH (d), GST-PH1 (e), or GST-PH2 (f). The signal was detected by overlay assay using the anti-GST antibody. Bound lipids are indicated by arrows and numbers. (D) The domain effects of CLPABP on its subcellular localization. COS-7 cells were transfected with the expression vector encoding FLAG-CLPABP derivatives. The cells were processed for coimmunofluorescence staining, 36 h after the transfection using anti-FLAG (green) and anti-cytochrome *c* (red) antibodies. The merged image of the panels is shown in the right panel. The scale bars represent 10 μm.

antibody. RNA was extracted not only from the cells wherein CLPABP expression was induced but also from the control cells that expressed the protein at an endogenous level (Fig. 6B). We investigated a crucial factor according to which mRNA transcripts were included in the CLPABP complex. Recently, an RNA-binding protein, HuR, has been identified to function as a translational regulatory factor of cytochrome *c* by binding to the 3′-UTR sequence of its mRNA [23]. Since HuR is one of the factors associated with the CLPABP complex, endogenous HuR and CLPABP interacted within the cells (Fig. 6C). Thus, we attempted to detect cytochrome *c* mRNA in the CLPABP complex by performing RT-PCR. Expectedly, the mRNA of cytochrome *c* (but not of G3PDH) was amplified from the RNA extracted from the CLPABP complex (Fig. 6D). Furthermore,



the protein expression levels of cytochrome *c* were elevated in the cells overexpressing CLPABP (Fig. 6E). On the other hand, in cells where the CLPABP expression was suppressed by RNA interference, the cytochrome *c* level was decreased (Fig. 6F) and mitochondrial defects were observed (Fig. 6G). Therefore, CLPABP might play a role in the general maintenance of mitochondria to sustain the expression level of cytochrome *c* in the cells. The CLPABP complex contained many ribosomal proteins. Therefore, it is possible that cytochrome *c* translation may have occurred. The fluorescence microscopy analyses revealed that the cytoplasmic small dot signals of cytochrome *c* existed at the same location as that of GFP-CLPABP; this prompted us to investigate the relationship between CLPABP and the apoptosome of apoptotic cells. The COS-7 cells that expressed recombinant CLPABP but not the N-terminal deletion mutant exhibited an apoptotic morphology (Fig. 6H, I). Therefore, we analyzed the relationship between CLPABP and Apaf-1, one of the central components of the apoptosome [24]. Apaf-1 was diffusively localized in the cytosol of normal cells; however, two days after transfection, it aggregated in the vesicle of GFP-CLPABP (Fig. 6J). We demonstrated that small cytochrome *c* granules were present in the CLPABP vesicle (Fig. 3D). Therefore, translated cytochrome *c* may be associated with Apaf-1 in the CLPABP complex.

4. Discussion

In the present study, we demonstrated that CLPABP is localized on the microtubule and mitochondria as a large protein-RNA complex. CLPABP accumulates in the mitochondria in the granular form; this unique subcellular localization occurs via phosphorylation of its tyrosine residues in response to EGF stimulation or oxidative stress. We identified certain CLPABP-binding proteins; among these, SF2Ap32 may be important for the recruitment of CLPABP into the mitochondria. However, SF2Ap32 has not been analyzed in detail with regard to its mitochondrial function. Although SF2Ap32 has recently been identified as a mitochondrial matrix protein, it was originally identified as a protein that binds and regulates ASF/SF2, a member of the SR-protein family [25,26]. The binding of ASF/SF2 to RNA is known to be controlled by its phosphorylation and association with SF2Ap32 [7,8,27]. In addition to SF2Ap32, ASF/SF2, other SR proteins, Srp20, and SRPK, a specific kinase [28] for SR proteins, were also included in the CLPABP complex. SR proteins play roles as export adaptors of mRNA, mediators of mRNA stability, and regulators of mRNA translation [26]. Thus, these events may have occurred in the CLPABP complex.

Our CLPABP-deletion analysis revealed that SF2Ap32 binding is indispensable for the unique localization of CLPABP on the surface of mitochondria; the 40 amino acid N-terminal region of CLPABP, which has no similarity with other proteins, plays a crucial role in the binding of CLPABP to SF2Ap32 (Fig. 3A, B). Furthermore, the interaction between SF2Ap32 and CLPABP is regulated by the tyrosine phosphorylation of CLPABP induced by oxidative stress.

Although various types of PH domains have been studied with regard to their wide range of lipid specificities [21], the PH domains of CLPABP possess a unique ability to bind to CL, PA, PS, and PI4-P (Fig. 4D). The PH domain of Cool/βPix that functions downstream in growth factor receptor signaling exhibits a slight similarity to the PH domains of CLPABP [29]. However, Cool/βPix is not reported to be localized on the mitochondria. To construct an effector mutant for further functional studies on CLPABP in the future, mutational and structural analyses based on the information involving other PH domains is required.

We hypothesized the mechanism by which CL and SF2Ap32, which are typically lodged within the mitochondria, come in contact with CLPABP. Data obtained from recent studies on CL metabolism may provide some insights into this mechanism [30–32]. The negatively charged lipid CL is mainly present in the inner membrane of the mitochondria. However, CL has been shown to translocate from the inner membrane to the outer membrane and is also known to leak outward from the mitochondria during apoptosis [33]. Most recently, it has been reported that the hydrolysis of CL to PA in the outer leaflet of the outer mitochondrial membrane by PLD plays a crucial role in mitochondrial fusion [34]. Frequently, the mitochondria are morphologically altered by fission and fusion [35]; therefore, it is possible that less-abundant CL is normally localized on the mitochondrial surface. Thus, CLPABP may recognize

Fig. 6. CLPABP complex as RNA granules. (A) Induction of CLPABP expression in T-REX™-293 cells by adding DOX. Cells were processed for coimmunofluorescence staining for two days with (lower) or without (upper) DOX treatment by using the anti-CLPABP antibody. The scale bars represent 10 μm. (B) The CLPABP complex containing RNA. The extraction of RNA from the immunoprecipitants using control IgG (lane 1) or the anti-CLPABP antibody (lane 2, 3). Immunoprecipitation was performed using the WCL of the untreated (lane 2) and DOX-treated cells. (C) Association between endogenous CLPABP and HuR *in vivo*. Coimmunoprecipitation experiments were carried out with control IgG and anti-HuR antibodies using the indicated cell lysates. Immunoblot analysis was carried out using anti-CLPABP (upper panel) and anti-HuR antibodies (lower panel). (D) The full-length mRNA of cytochrome *c* is present in the CLPABP complex. RT-PCR was carried out using primers for cytochrome *c* (three left lanes) or G3PDH (lane to the extreme right). The purified RNA in (B) was used as a template, and the sample numbers are comparable to (B). (E) Comparison of the expression levels of CLPABP (upper panel) and cytochrome *c* (lower panel) with (right lane) or without (left lane) DOX treatment for 48 h; the expression levels of β-actin in each cell line are shown as a control (middle lane). (F) CLPABP knockdown experiment using siRNA in HeLa cells. Immunoblot analyses were carried out using the indicated antibodies, and the expression levels of β-actin are shown as a control (middle lane). (G) Effect of siRNA-induced suppression of CLPABP expression on the mitochondrial morphology. HeLa cells were transfected with siRNA for CLPABP. Representative cell images of staining with the anti-CLPABP antibody (upper panel) and with Mito-Tracker (lower) are shown. Cells with reduced CLPABP expression are indicated by arrows. The scale bars represent 10 μm. (H) Effect of CLPABP expression on apoptosis. The induction of apoptosis in COS-7 cells due to GFP-CLPABP overexpression 60 h after transfection. The GFP-CLPABP images are shown in the left panel; Hoechst 33342 (center panel) was used to stain the nucleus. (I) Graph showing the percentage of apoptosis induced by expressing GFP-CLPABP251-611 as a control (white) and GFP-CLPABP (black). (J) Co-localization of GFP-CLPABP and endogenous Apaf-1. COS-7 cells were transfected with the expression vector encoding GFP-CLPABP (a). The cells were processed for coimmunofluorescence staining at 48 h after transfection using anti-Apaf-1 (b); Hechest33342 was used to stain the nucleus (c). Merged images of the panels are shown (d). The scale bars represent 10 μm.

and determine its position for localization depending on the amount of CL present on the surface of the mitochondrial outer membrane.

In this study, we investigated many common components of major RNA granules such as the stress granule [12] or the P-body [13]. Although the relevance of each molecule in the role of the CLPABP complex is unclear, it is interesting that the complex contains cytochrome *c* mRNA. Kawai et al. have demonstrated that HuR binds to the 3′-UTR sequence of cytochrome *c* mRNA to control mRNA stabilization and translation. In addition to the SR-family proteins, the CLPABP complex also contains HuR and hnRNPA1, which are also known as nuclear shuttling proteins [36,37]. Therefore, the CLPABP complex may play a role in carrying cytochrome *c* mRNA to the mitochondria via HuR.

The translational process involving ribosomal subunits may be active in the CLPABP-RNA granule as compared to the stress granule or the P-body. The CLPABP granule was partially colocalized at the ER; many ribosomal proteins were included in the CLPABP complex, and the granule was successfully stained with the anti-cytochrome *c* antibody due to cytochrome *c* production therein. Furthermore, the apoptosome that is known to be an oligomer of cytochrome *c* and Apaf-1 was generated, and apoptosis occurred in the cells that overexpressed CLPABP.

Cytochrome *c* is crucial for mitochondrial metabolism and apoptotic events and therefore should be effectively translocated into the mitochondria following the translational process in the cytoplasm. We propose that CLPABP may play a role in this translocation by forming a novel type of RNA granule that carries the cytochrome *c* mRNA to the mitochondria, which facilitates the mitochondrial recruitment of the polypeptide of cytochrome *c*. Here, GFP-CLPABP could be transported along the tubulin network, and endogenous CLPABP was colocalized with mitochondria in the neuronal tubule of NGF-stimulated PC12 cells (data not shown). Therefore, CLPABP may function to transport cytochrome *c* mRNA into neurons along with kinesin motor proteins [38].

In contrast to the conventional types of RNA granules, some proteins in the CLPABP-RNA granule were identified as functional molecules responsible for the specific localization of mRNA within cells. Zip-code-binding protein (ZBP) 1, a human IGF-II mRNA-binding protein (IMP) orthologue, functions in carrying the β-actin mRNA to appropriate sites within the vertebrate cells [39]; this process is regulated by tyrosine phosphorylation [40]. Similarly, tyrosine-phosphorylated CLPABP may function to transport the mRNA of mitochondrial-specific proteins; DNA microarray analysis is necessary in order to detect the entire spectrum of mRNA transported by the CLPABP complex.

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