

Identification of Alix-type and Non-Alix-type ALG-2-binding Sites in Human Phospholipid Scramblase 3

DIFFERENTIAL BINDING TO AN ALTERNATIVELY SPLICED ISOFORM AND AMINO ACID-SUBSTITUTED MUTANTS^{*[5]}

Received for publication, January 28, 2008 Published, JBC Papers in Press, February 6, 2008, DOI 10.1074/jbc.M800717200

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ALG-2, a prototypic member of the penta-EF-hand protein family, interacts with Alix at its C-terminal Pro-rich region containing four tandem PXY repeats. Human phospholipid scramblase 3 (PLSCR3) has a similar sequence (ABS-1) in its N-terminal region. In the present study, we found that ALG-2 interacts with PLSCR3 expressed in HEK293 cells in a Ca^{2+} -dependent manner by co-immunoprecipitation, pulldown with glutathione *S*-transferase (GST) fused ALG-2 and an overlay assay using biotin-labeled ALG-2. The GST fusion protein of an alternatively spliced isoform of ALG-2, GST-ALG-2^{ΔGF122}, pulled down green fluorescent protein (GFP)-fused PLSCR3 but not GFP Alix. Deletion of a region containing ABS-1 was not sufficient to abrogate the binding. A second ALG-2-binding site (ABS-2) was essential for interaction with ALG-2^{ΔGF122}. Real-time interaction analyses with a surface plasmon resonance biosensor using synthetic oligopeptides and recombinant proteins corroborated direct Ca^{2+} -dependent binding of ABS-1 to ALG-2 and that of ABS-2 to ALG-2 as well as to ALG-2^{ΔGF122}. The sequence of ABS-2 contains multiple prolines and two phenylalanines, among which Phe⁴⁹ was found to be critical, because its substitution with Ala or Tyr caused a loss of binding ability by pull-down assays using oligopeptide-immobilized beads. ALG-2-interacting proteins were classified into two groups based on binding ability to ALG-2^{ΔGF122}: (i) isoform-non-interactive (ABS-1) types, including Alix, annexin A7, annexin A11, and TSG101 and (ii) isoform-interactive (ABS-2) types including PLSCR3, PLSCR4 and Sec31A. GST-pulldown assays using single amino acid-substituted ALG-2 mutants revealed differences in binding specificities between the two groups, suggesting structural flexibility in ALG-2-ligand complex formation.

ALG-2 is a 22-kDa calcium-binding protein possessing five serially repetitive EF-hand motifs, and it belongs to the penta-

EF-hand (PEF)² protein family (1, 2). Based on differences in the primary structures of EF1, mammalian PEF proteins are classified into two groups: group I (ALG-2 and peflin) and group II (typical calpains, including the small subunit, sorcin and grancalcin) (2). Homologs of group I PEF proteins have been found not only in vertebrates but also widely in eukaryotes, including lower animals, plants, fungi, and protists (3). On the other hand, group II PEF proteins are restricted to the animal kingdom and are thought to have diverged during the evolution of animals (4). ALG-2 forms a homodimer or a heterodimer with peflin through their EF5 regions (5, 6). Despite the original report of a pro-apoptotic function of ALG-2 in T cell hybridomas (7), ALG-2-deficient mice develop normally with no obvious abnormalities in the immune system (8). Nonetheless, potential physiological roles of ALG-2 in regulation of ER-stress-induced apoptosis (9), neuronal cell death during development (10), and cancer (11, 12) have been reported. Alix (also named AIP1) was the first protein identified as an ALG-2-interacting protein (13, 14). This cytoplasmic 95-kDa protein is now recognized as a multifunctional protein involved in various cellular functions, including endosomal sorting, retrovirus budding, actin cytoskeleton assembly, signal transduction, and apoptosis (see Refs. 15–18 for reviews).

We and others previously identified an ALG-2-binding site in the C-terminal proline-rich region in Alix (19, 20). Results of analyses of amino acid-substituted mutants and deletion mutants of Alix suggested that a potential polyproline II-helix containing four tandem PXY repeats is an ALG-2-interacting core motif (801-PPYPTYPGYPGY-812), in which Ala substitutions of either all four Pro or Tyr residues and Phe substitutions of all four Tyr residues abolished the binding ability (19). Generally, Pro-rich regions often serve as domains for either specific protein-protein interaction or rapid but nonspecific interaction via their sticky arms extending out from the rest of the protein molecules (21, 22). Both sorcin and ALG-2 bind to the presumably extended or disordered N-terminal Pro-rich regions of annexins A7 and A11 (23–25). While exact binding

^{*} This work was supported by a Grant-in-Aid for Scientific Research (B) (to M. M.) and a Grant-in-Aid for Young Scientists (B) (to H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, references, Table S1, and Figs. S1 and S2.

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² The abbreviations used are: PEF, penta-EF-hand; ABS, ALG-2-binding site; bio-, biotin-labeled; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; GFP, green fluorescent protein; GST, glutathione *S*-transferase; PLSCR, phospholipid scramblase; SPR, surface plasmon resonance; pAb, polyclonal antibody; mAb, monoclonal antibody; Fc1, -2, -3, Flow cell nos. 1–3; TBS, Tris-buffered saline; CaM, calmodulin.

sites for ALG-2 have not been determined yet, the N-terminal ~30 residues that are rich in Pro, Gly and Tyr but have no charged residues in annexin A11 are sufficient for binding to sorcin (23). Neither annexin A7 nor annexin A11, however, possesses the four tandem PXY repeats found in Alix. Instead, both annexins have GYPP repeats in sorcin-binding regions. One conspicuous difference between sorcin and ALG-2 for annexin A7 binding is the requirement of N-terminal non-PEF regions: the N-terminal domain of sorcin is essential (26), but that of ALG-2 is dispensable (25). ALG-2 binding motifs remain to be clarified.

In this study, we found that phospholipid scramblase 3 (PLSCR3) is a novel ALG-2-interacting protein, and we identified two binding sites, designated ABS (ALG-2-binding site)-1 and ABS-2, in its N-terminal Pro-rich region. Although ABS-1 resembles the Alix PXY-repeat motif, ABS-2 does not conform to the canonical sequence and does not contain Tyr. ABS-2 possesses a unique binding property: it interacts with an alternatively spliced ALG-2 isoform, ALG-2^{ΔGF122}, and other single amino acid-substituted ALG-2 mutants, with which ABS-1 and Alix do not interact. An ABS-2-oligopeptide-immobilized column has been proved to be useful for rapid one-step affinity purification of untagged recombinant ALG-2 proteins, including ALG-2^{ΔGF122}. Furthermore, we demonstrated that ALG-2-interacting proteins can be classified into two groups based on the binding ability to ALG-2^{ΔGF122}. Differences in binding specificities to other ALG-2 mutants suggest structural flexibility in ALG-2-ligand complex formation.

EXPERIMENTAL PROCEDURES

Materials—Untagged, biotin-tagged, and phosphorylated synthetic oligopeptides were obtained from Biosynthesis (Lewisville, TX). Rabbit anti-GFP polyclonal antibody (pAb) and anti-apoptosis inducing factor pAb were purchased from Abcam (#ab1998, Cambridge, UK). Mouse monoclonal antibodies (mAbs) of anti-GFP (B2) and anti-annexin VII (A-1) and goat anti-annexin XI pAb (N-17) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs of anti- α -tubulin (clone DM1A), anti-glyceraldehyde-3-phosphate dehydrogenase, and anti-Lamp-1 were from Sigma, Chemicon, and Pharmingen, respectively. Anti-TSG101 mAb (4A10) and anti-Sec31A mAb (clone 32) were from Gene Tex (San Antonio, TX) and BD Transduction Laboratories (San Diego, CA), respectively. Streptavidin-peroxidase was obtained from Rockland Immunochemicals (Philadelphia, PA). Preparations of rabbit anti-ALG-2 pAb and anti-Alix pAb were described previously (27, 28). Anti-PLSCR3 antiserum was raised in rabbits using GST-fused PLSCR3 N-terminal Pro-rich region protein as antigen. Sulfosuccinimidyl *N*-(*d*-biotinyl)-6-aminohexanoate was purchased from Dojin (Kumamoto, Japan).

Plasmid Constructions—Human cDNAs for PLSCR1, PLSCR3, and PLSCR4 were cloned from a human skeletal muscle cDNA library (Clontech) (for PLSCR3) or a Human Fetus Marathon-ReadyTM cDNA library (Clontech) (for PLSCR1 and PLSCR4) by the PCR method using a proofreading thermostable *Pfu*Turbo DNA polymerase (Stratagene) (see supplemental materials). pGFP-Alix was described previously (29). Alanine-substituted mutants of GST-fused human ALG-2 at

various amino acid residues were created by the PCR-based mutagenesis method (see supplemental materials). Construction of an *Escherichia coli* expression plasmid of N-terminally truncated ALG-2 that lacks the hydrophobic and Pro/Gly-rich region, pET3d-des3-23ALG-2 (previous name: pET3d-ALG-2 Δ N23), was described previously (25), and the produced protein was re-named des3-23ALG-2 in this study. Construction of a plasmid for GST-fused protein of an alternatively spliced ALG-2 isoform that lacks Gly¹²¹-Phe¹²² (designated GST-ALG-2^{ΔGF122}) was described previously (30). Other constructs are described in the supplemental materials.

Establishment of PLSCR3-expressing Cell Line—A PLSCR3 cDNA fragment was inserted into the BamHI site of pIRES1neo (Clontech), and the resultant expression vector was used for transfection of HEK293 subcloned cells designated YS14 (28). G418-resistant cells were isolated by cylinder cloning and further screened for constant expression of PLSCR3 by Western blotting using anti-PLSCR3 antibody. Subcellular fractionation by differential centrifugation was performed essentially as described previously (28).

GST-ALG-2 Pulldown, Bio-ALG-2 Overlay, and Co-immunoprecipitation—Pulldown assays using GST-ALG-2 and its mutant proteins were performed as described previously (28, 30) using the cleared lysate of HEK293 cells untransfected or transfected with respective expression vectors of GFP-fused proteins. An overlay assay using bio-ALG-2 was carried out as described previously (30) using the immunoprecipitates of anti-GFP pAb from the cleared lysate of HEK293 cells that had been transfected with pGFP-PLSCR3 or an empty pEGFP vector. Alternatively, immunoprecipitation was performed in the presence or absence of Ca²⁺, and immunoprecipitates were subjected to Western blotting with anti-ALG-2 pAb essentially as described previously (19). Specific conditions for incubation with antibodies are described in the supplemental materials.

Pulldown Assay of des3-23ALG-2 with Mutant Oligopeptide-immobilized Sepharose Beads—Each synthetic oligopeptide (0.2 mg) dissolved in 0.1 ml of the coupling buffer (0.2 M NaHCO₃, pH 8.3, 0.5 M NaCl) was immobilized to a 0.2-ml bed volume of NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare/Amersham Biosciences) according to the manufacturer's instructions. A binding assay was performed by incubating 10 μ l of beads in a total volume of 50 μ l in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM 2-mercaptoethanol, 0.5 mM CaCl₂, 0.5% Triton X-100) containing 10 μ g each of des3-23ALG-2, bovine serum albumin (BSA), ovalbumin, and α -lactalbumin by mixing with MicroMixer E-36 (Taitec, Japan) at room temperature for 30 min. Then the mixture was centrifuged at 3000 rpm for 3 min, and pelleted beads were washed with 0.4 ml of binding buffer three times. The unbound fraction (first supernatant) and bound fraction (beads) were subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (CBB) and relative amounts of proteins were estimated by densitometric analysis using the free imaging software (Scion Image Beta 4.02) available from Scion.

Affinity Purification of Recombinant ALG-2 Proteins—Lys-tagged ABS-2 oligopeptide (k-ABS-2: kQVPAPAPGFALFP-SPGPVA; k, extraneous Lys for facilitating cross-linking) was

Alix-type and Non-Alix-type ALG-2-binding Sites in PLSCR3

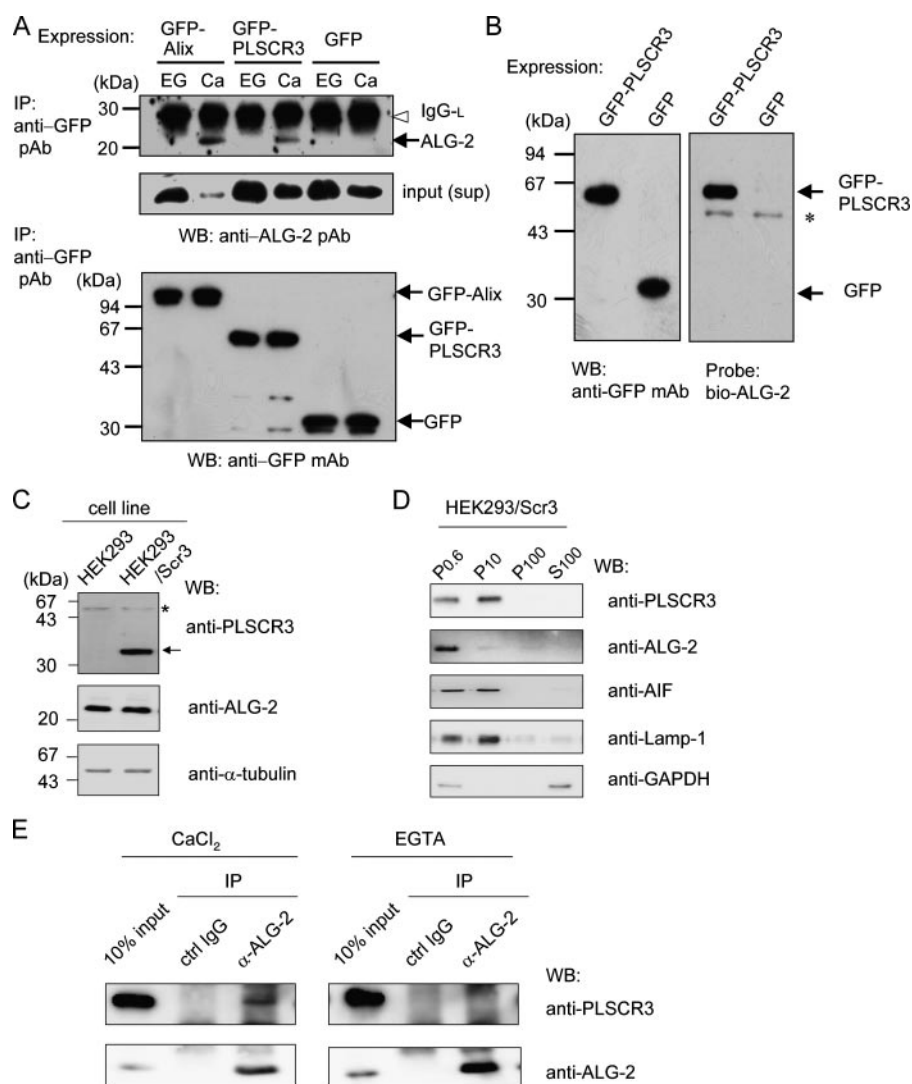


FIGURE 2. Ca^{2+} -dependent interaction of ALG-2 with PLSCR3. *A*, co-immunoprecipitation assay. HEK293 cells transiently transfected with respective expression vectors of GFP-fused proteins were lysed in the presence of $10 \mu\text{M}$ Ca^{2+} (Ca) or 2 mM EGTA (EG). After centrifugation, cleared lysates (input) were immunoprecipitated with anti-GFP pAb. The lysates and immunoprecipitates (IP) were analyzed by Western blotting (WB) with anti-ALG-2 pAb or with anti-GFP mAb. *B*, overlay assay with biotin-labeled ALG-2. HEK293 cells transiently transfected with expression vectors of GFP-PLSCR3 or GFP were lysed and immunoprecipitated with anti-GFP pAb. The immunoprecipitates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membrane was then probed either with bio-ALG-2 in the presence of $100 \mu\text{M}$ Ca^{2+} (right panel) or with anti-GFP mAb (left panel). An asterisk indicates nonspecific binding of bio-ALG-2 to rabbit immunoglobulin heavy chains. *C*, establishment of a PLSCR3-expressing cell line. Parental HEK293 subclone YS14 cells were transfected with untagged PLSCR3-expressing vector (pIRES1neo-PLSCR3), and G418-resistant clones were selected. Total cell lysates were subjected to Western blotting using anti-PLSCR3 pAb, anti-ALG-2 pAb, and anti- α -tubulin mAb. Arrow, PLSCR3; asterisk, nonspecific bands. *D*, subcellular fractionation of PLSCR3. HEK293/Scr3 cells were lysed in the lysis buffer (10 mM HEPES-NaOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 5 mM 2-mercaptoethanol containing $10 \mu\text{M}$ Ca^{2+} and protease inhibitors) fractionated by differential successive centrifugations at 4°C . Pellets of $600 \times g$ ($\text{P}_{0.6}$), $10,000 \times g$ (P_{10}), $100,000 \times g$ (P_{100}), and the final supernatant of $100,000 \times g$ (S_{100}) were subjected to Western blotting with antiserum against PLSCR3, and with purified antibodies against ALG-2, apoptosis inducing factor (AIF), Lamp-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. *E*, co-immunoprecipitation assay. PLSCR3 was extracted from the P_{10} fraction by brief sonication with the lysis buffer without 2-mercaptoethanol but supplemented with 142.5 mM KCl, detergent (0.2% Nonidet P-40) and either $100 \mu\text{M}$ CaCl_2 or 2 mM EGTA. After centrifugation at $10,000 \times g$ for 10 min at 4°C , the supernatant was subjected to immunoprecipitation with anti-ALG-2 pAb or control rabbit IgG, and the immunoprecipitates were analyzed by Western blotting using antiserum against PLSCR3 and anti-ALG-2 pAb.

P20073), homeobox protein Nkx-2.5 (25.7, P52952), NFATc4 (25.2, Q14934), EYA2 (25.2, O00167), TSG101 (24.8, Q99816), and SCAMP4 (24.8, Q969E2). In this study, we selected PLSCR3 as the most possible candidate protein for *in vitro*

space protein), Lamp-1 (late endosome/lysosome protein), and glyceraldehyde-3-phosphate dehydrogenase (cytosolic protein) in the $600 \times g$ pellets ($\text{P}_{0.6}$) in addition to their expected fractions of either $10,000 \times g$ pellets (P_{10}) or $100,000 \times g$ superna-

ALG-2 binding analyses because of the absence of a gap in the aligned PXY-repeat sequence (Fig. 1B).

Analyses of Interaction between ALG-2 and PLSCR3—We performed a co-immunoprecipitation assay by overexpressing GFP-fused PLSCR3, GFP-Alix (positive control), and GFP (negative control), respectively, in HEK293 cells. The expressed proteins were immunoprecipitated with anti-GFP pAb. As shown in Fig. 2A, specific bands corresponding to ALG-2 were detected with anti-ALG-2 pAb in the immunoprecipitates of GFP-Alix and GFP-PLSCR3 in the presence of $10 \mu\text{M}$ Ca^{2+} but not in the presence of a Ca^{2+} -chelator, EGTA (2 mM). No ALG-2 bands were detected in the immunoprecipitates of GFP in either condition. In a separate experiment, we performed an overlay assay using biotin-labeled ALG-2 (bio-ALG-2) as a probe. As shown in Fig. 2B, the immunoprecipitate of GFP-PLSCR3, but not that of GFP, presented a specific band, suggesting a direct physical interaction between ALG-2 and PLSCR3.

To investigate whether ALG-2 associates with endogenous PLSCR3, human lymphoma cell lines such as Raji and Jurkat and rat fat cells were first analyzed for expression of PLSCR3. Endogenous PLSCR3 protein, however, was not detected with our rabbit anti-PLSCR3 antiserum. Then, we established a PLSCR3-expressing HEK293 cell line (HEK293/Scr3) as shown in Fig. 2C. Using this stable cell line as an alternative source of endogenous PLSCR3, we performed subcellular fractionation by the differential centrifugation method to obtain PLSCR3-enriched fraction. Fig. 2D shows a representative result of the fractionation in the presence of $10 \mu\text{M}$ CaCl_2 under incomplete cell disruption condition as indicated by the presence of anti-apoptosis inducing factor (mitochondrial inter-membrane

tant (S_{100}). In agreement with previous studies (5, 28), ALG-2 was recovered mostly in the $P_{0.6}$ fraction when the lysis buffer containing Ca^{2+} was used, but faint bands corresponding to ALG-2 were detected in the P_{10} and P_{100} fractions. PLSCR3 was detected more in the P_{10} fraction than in the $P_{0.6}$ fraction. PLSCR3 was extracted from the P_{10} fraction with the buffer-containing detergent (0.2% Nonidet P-40) and either 100 μ M $CaCl_2$ or 2 mM EGTA, and then the supernatant was subjected to a co-immunoprecipitation assay using anti-ALG-2 pAb for immunoprecipitation and anti-PLSCR3 pAb for Western blotting. As shown in Fig. 2E, PLSCR3 was detected in the immunoprecipitates by anti-ALG-2 pAb (α -ALG-2) but not by control IgG.

Two ALG-2-binding Sites in the Pro-rich Region of PLSCR3—To further analyze the interaction between human ALG-2 and PLSCR3, GST-pulldown assays were performed using GST-fused ALG-2 of wild-type and previously constructed mutants. ALG-2^{E47A/114A} is a calcium-binding defective mutant (34), which was shown to bind neither Alix (19) nor TSG101 (30). In Western blot analysis using anti-GFP mAb, a GFP-PLSCR3 band was not detected in the pelleted bead fraction (pulldown products) of GST-ALG-2^{E47A/114A} in the presence or absence of Ca^{2+} in the binding buffer (Fig. 3A). ALG-2 ^{Δ GF122} corresponds to a naturally occurring alternatively spliced isoform of human ALG-2 lacking Gly¹²¹Phe¹²², which was first reported in mice (35). Consistent with the previous report that the isoform did not interact with Alix by the yeast two-hybrid assay (35), GST-ALG-2 ^{Δ GF122} did not pull down GFP-Alix, but, surprisingly, it pulled down GFP-PLSCR3 (Fig. 3B). Because the initially predicted ABS in the Pro-rich region of PLSCR3 has a PXY repeat and resembles the Alix-ABS, we speculated that PLSCR3 contains an additional ABS that is capable of binding to ALG-2 ^{Δ GF122}. To corroborate this hypothesis, we constructed a deletion mutant of GFP-PLSCR3 lacking residues 12–27 in the Pro-rich region (designated GFP-PLSCR3 Δ ABS-1) and performed the pulldown assay. Not only GST-ALG-2 but also GST-ALG-2 ^{Δ GF122} efficiently pulled down GFP-PLSCR3 Δ ABS-1 (Fig. 3C). Next, a mutant lacking residues 43–58 (designated GFP-PLSCR3 Δ ABS-2) was constructed because of the notable presence of two phenylalanine residues surrounded by proline residues. GFP-PLSCR3 Δ ABS-2 was pulled down by GST-ALG-2 but not by GST-ALG-2 ^{Δ GF122}. When both ABS-1 and ABS-2 were deleted, the mutant (designated GFP-PLSCR3 Δ ABS-1/2) was not essentially pulled down anymore.

Affinity Purification of ALG-2 Proteins with an ABS-2 Oligopeptide-immobilized Column—The nature of Ca^{2+} -dependent specific interaction of PEF proteins with target peptide sequences has been utilized for affinity purification of recombinant PEF domain proteins of the calpain large subunit and the small subunit (previous name: calmodulin-like domain) using calpastatin subdomain oligopeptides that were immobilized to Sepharose beads (31, 32). A similar approach was taken to purify untagged recombinant ALG-2 proteins expressed in *E. coli*. A Lys-tagged synthetic oligopeptide of ABS-2 (k-ABS-2: kQVPAPAPGFALFPSPGPVA) (Table 1) was immobilized to NHS-activated Sepharose beads. The bacterial cleared lysate, prepared in the presence of non-ionic detergent Triton X-100 (final concentration of 0.1%), was applied to the column in the

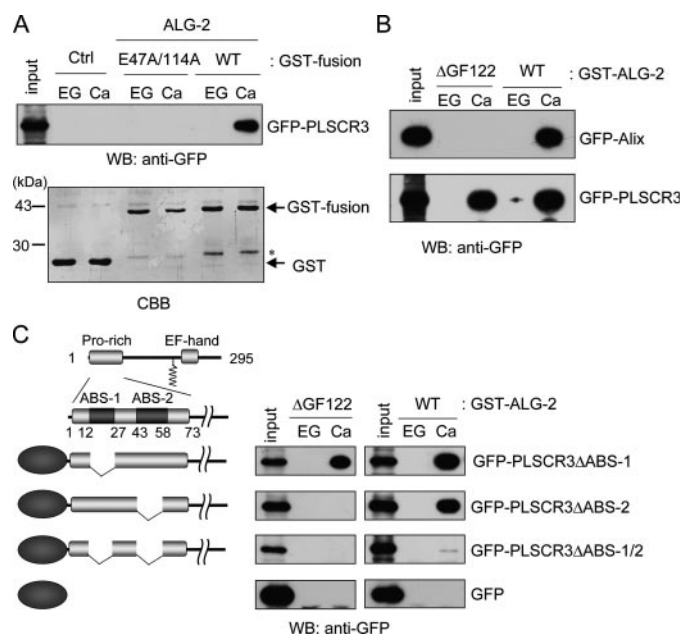


FIGURE 3. Identification of two independent ALG-2-binding sites (ABS-1 and ABS-2) by GST-ALG-2 pulldown assays. HEK293 cells transiently transfected with respective expression vectors of GFP-fused proteins were lysed. After centrifugation, cleared lysates (input) were adjusted to 100 μ M Ca^{2+} (Ca) or 1 mM EGTA (EG) and incubated with glutathione-Sepharose beads that carried GST, GST-fused wild-type ALG-2 (WT), or its mutants as indicated. After the beads had been pelleted by centrifugation and washed, bead-bound proteins (pulldown products) were analyzed by Western blotting with anti-GFP mAb. A, requirement of Ca^{2+} -binding avidity of ALG-2 for the interaction with PLSCR3. ALG-2^{E47A/114A} is a Ca^{2+} -binding-defective mutant. The pulldown products were analyzed by Western blotting (WB, upper panel) or visualized by staining with Coomassie Brilliant Blue R-250 (CBB, lower panel) to confirm recovery of GST or GST fusion proteins bound to the beads. An asterisk indicates degraded GST fusion proteins. B, interaction of GST-ALG-2 ^{Δ GF122} with GFP-PLSCR3 but not with GFP-Alix. ALG-2 ^{Δ GF122} is an alternatively spliced isoform of ALG-2 that lacks two amino acid residues, Gly¹²¹ and Phe¹²², compared with a longer form of ALG-2. C, two independent ALG-2-binding sites in PLSCR3 Pro-rich region. Schematic structures of GFP-fused PLSCR3 and its deletion constructs of ABS-1 and ABS-2 (see Fig. 1) in Pro-rich region are represented on the left. PLSCR3 Δ ABS-1/2 has deletion of both ABS-1 and ABS-2.

presence of 0.1 mM free Ca^{2+} . Inclusion of Triton X-100 was necessary to avoid Ca^{2+} -dependent precipitation of ALG-2 (27). After extensive washing with buffer in the presence of Ca^{2+} but absence of Triton X-100, the ALG-2 proteins adsorbed to the column were eluted with the buffer containing EGTA. Fig. 4 shows a representative result of purification of the human ALG-2 PEF domain protein (des3-23ALG-2). The eluted ALG-2 proteins were sufficiently pure for further interaction analyses *in vitro*. Because of the high protein concentrations of eluted ALG-2 proteins (up to 3 mg/ml), inclusion of a higher concentration of a Ca^{2+} chelator (5 mM EGTA) was found to be better to avoid appearance of turbidity of the ALG-2 solution, particularly in the case of full-length ALG-2 (data not shown). The oligopeptide-immobilized affinity column chromatography was also an effective method to purify ALG-2 ^{Δ GF122} and des3-23ALG-2 ^{Δ GF122} (Fig. 4C).

Real-time Interaction Analysis with an SPR Biosensor—Purified ALG-2 proteins were used for SPR real-time analyses of interactions with N-terminally biotinylated PLSCR3 oligopeptides of ABS-1 (bio-ABS-1) and ABS-2 (bio-ABS-2) that were captured by each flow cell on an SA-immobilized sensor chip.

TABLE 1

Sequences of the synthetic ABS-2 oligopeptides used for preparation of immobilized Sepharose beads

The lowercase letter k denotes a lysine tag to facilitate immobilization efficiency. Closed and open circles indicate conserved proline and aromatic residues, respectively, shown in Fig. 1. The asterisk indicates the critical phenylalanine identified in this study. Boldface letters indicate substituted residues.

Oligopeptides	Sequences
	41 45 47 49 52 53 61
k-ABS-2	kQVPAPAPGFALFPSPGPVA
k-ABS-2-f2a2	kQVPAPAPGAALAPSPGPVA
k-ABS-2-p2a2	kQVPAPAAAGFALFASPSPGPVA
k-ABS-2-5del	kQ--APAPGFALFPSPG
k-ABS-2-5del-p4a4	kQ--AAAAGFALFASAG
k-ABS-2-5del-L51A	kQ--APAPGFAAFPSPG
k-ABS-2-5del-F52A	kQ--APAPGFALAPSPG
k-ABS-2-5del-F49A	kQ--APAPGAALFPSPG
k-ABS-2-5del-F49Y	kQ--APAPGYALFPSPG
k-ABS-2-5del-F49W	kQ--APAPGWALFPSPG
k-ABS-2-5del-F49L	kQ--APAPGLALFPSPG

As shown in the overlaid sensorgrams (Fig. 5, A and B), both full-length ALG-2 and des3-23ALG-2 interacted with bio-ABS-1 and bio-ABS-2 in a similar pattern of association (analyte injection phase) and dissociation (buffer washing phase) but with 1.5–2-fold higher resonance signals for full-length ALG-2. In contrast, resonance signals of the binding of ALG-2^{ΔGF122} and des3-23ALG-2^{ΔGF122} (dotted lines) to bio-ABS-1 were extremely low (Fig. 5A), whereas significant binding of these proteins to bio-ABS-2 was observed (Fig. 5B).

Ca²⁺ concentration dependence was investigated using the SPR biosensor (Fig. 5, C and D). The Ca²⁺ concentrations that gave 50% maximal binding, [Ca²⁺]_{1/2}, were estimated from the respective hyperbolic curves for binding to bio-ABS-1 ([Ca²⁺]_{1/2}: ALG-2, 5.1 μM; des3-23ALG-2, 6.7 μM) and to bio-ABS-2 ([Ca²⁺]_{1/2}: ALG-2, 3.7 μM; des3-23ALG-2, 4.7 μM; ALG-2^{ΔGF122}, 9.4 μM; des3-23ALG-2^{ΔGF122}, 13 μM). Zinc ions are known to bind some EF-hand proteins such as S₁₀₀ family members (36). Dependence of ALG-2 binding to bio-ABS-1 and -2 on other divalent metal ions was investigated using the biosensor (supplemental Fig. S1). Although Mg²⁺ was essentially non-effective for binding to both bio-ABS-1 and -2, Zn²⁺ was found to be effective. Because concentrations of Zn²⁺ one to two orders of magnitude higher than those of Ca²⁺ are required, there may be no biological significance of Zn²⁺-dependent binding of ALG-2 to target peptides *in vivo*. Knowledge of the formation of an ALG-2/Zn²⁺ complex, however, may be useful for future physicochemical analyses of ALG-2 *in vitro*. The kinetic parameters (association rate, *k*_{ass}; dissociation rate, *k*_{diss}; dissociation constant, *K*_D) of des3-23ALG-2 binding in the presence of 50 μM Ca²⁺ were estimated by the global fitting curves for ABS-1, *k*_{ass} = 1.4 × 10⁶ M⁻¹ s⁻¹, *k*_{diss} = 5.7 ×

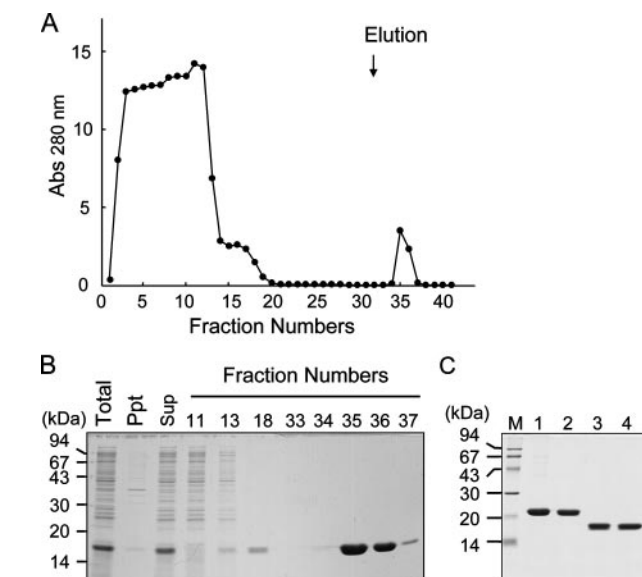


FIGURE 4. Affinity purification of recombinant ALG-2 proteins using an ABS-2 oligopeptide-immobilized column. A, lysine-tagged synthetic oligopeptide of ABS-2 (k-ABS-2) was immobilized to NHS-activated Sepharose beads. The recombinant PEF domain of ALG-2 (des3-23ALG-2) was expressed in *E. coli*. The bacterial cleared lysate, prepared in the presence of non-ionic detergent Triton X-100 (final concentration of 0.1%), was applied to the column in the presence of 0.1 mM free Ca²⁺ at the flow rate of 0.27 ml/min. After extensive washing with a buffer in the presence of Ca²⁺ but absence of Triton X-100 at the flow rate of 0.54 ml/min, the des3-23ALG-2 protein adsorbed to the column was eluted with a buffer containing 1 mM EGTA. A, each fraction was monitored by absorbance at 280 nm. Fraction volume: flow-through and wash, 1 ml; elution, 0.5 ml. B, proteins were analyzed by SDS-PAGE using a 12.5% gel, which was stained with CBB after electrophoresis. Total, total cell lysate; Ppt, pellet fraction after centrifugation of the bacterial lysate; Sup, cleared lysate. C, SDS-PAGE analysis of purified recombinant ALG-2 proteins. ALG-2 wild-type and N-terminal deletion mutant proteins were affinity-purified and subjected SDS-PAGE followed by CBB staining. M, molecular mass marker proteins; lane 1, ALG-2; lane 2, ALG-2^{ΔGF122}; lane 3, des3-23ALG-2; and lane 4, des3-23ALG-2^{ΔGF122}.

10⁻² s⁻¹, and *K*_D = 4.0 × 10⁻⁸ M and by those for ABS-2, *k*_{ass} = 2.3 × 10⁶ M⁻¹ s⁻¹, *k*_{diss} = 5.7 × 10⁻² s⁻¹, and *K*_D = 2.5 × 10⁻⁸ M, respectively (supplemental Fig. S2).

Critical Residues in ABS-2 for ALG-2 Binding—We investigated whether an analogous hypothesis of the polyproline II-helix-based PXY-repeat motif in Alix-ABS is also applicable to PLSCR3 ABS-2 with a modified motif containing Phe. Lys-tagged synthetic oligopeptides with various amino acid substitutions (Table 1) were immobilized to NHS-activated Sepharose beads, which were used for pulldown assay of the recombinant human ALG-2 PEF domain protein (des3-23ALG-2) from the mixture containing standard proteins (BSA; OVA, ovalbumin; and α-LA, α-lactalbumin) in the presence of Ca²⁺. As shown in Fig. 6, >70% of the input des3-23ALG-2 protein was recovered in the bound fraction (panel A, lanes B; panel B, closed bars) of wild-type (WT) k-ABS-2-immobilized beads, whereas standard proteins remained in the supernatant (panel A, U). The amount of des3-23ALG-2 recovered in the bead fraction in the presence of 5 mM EGTA was <3% (data not shown). As expected, double substitutions of Phe⁴⁹ and Phe⁵² with Ala (k-ABS-2-f2a2) abolished the binding capacity. Pro⁴⁷ and Pro⁵³, positioned proximal to both Phe residues, are conserved among Alix-ABS, PLSCR3 ABS-1, and PLSCR3 ABS-2 (Fig. 1B). Unexpectedly, however, double sub-

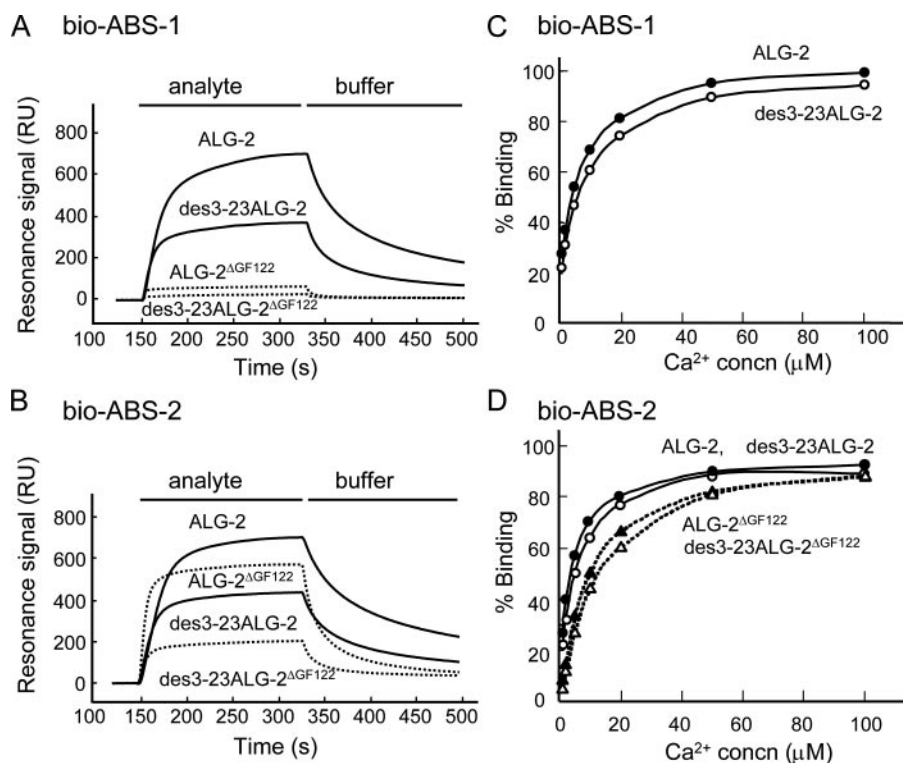


FIGURE 5. Real-time interaction analyses of ALG-2 binding to ABS-1 and ABS-2 using an SPR biosensor. Overlaid sensorgrams represent specific binding of ALG-2 (solid line), ALG-2 Δ GF122 (dotted line), des3-23ALG-2 (solid line), or des3-23ALG-2 Δ GF122 (dotted line) to immobilized bio-ABS-1 oligopeptide (A) in flow cell 2 (Fc2) and bio-ABS-2 (B) in flow cell 3 (Fc3) using the running buffer HBS-CP (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.005% surfactant P20). The non-immobilized surface in flow cell 1 (Fc1) was used as a reference surface to subtract effects of the buffer and salts used for dissolving solutes on resonance signals recorded for Fc2 and Fc3. Each protein sample (analyte, 100 nM) was injected at 150 s and kept flowing over the sensor chip surface for 180 s, and then washed with the running buffer. Sensor chip surfaces were regenerated by washing with the buffer containing 10 mM EDTA. Ca^{2+} concentration dependence of ALG-2 proteins to bio-ABS-1 (C) and bio-ABS-2 (D) was analyzed with the SPR biosensor using the running buffer HBS-P (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.005% surfactant P20) containing varying concentrations of $CaCl_2$. Resonance signals expressed in resonance unit (RU) values were measured at the end of analyte injection and used for a saturation plot of Ca^{2+} concentration versus RUs. Symbols indicate percentages of RU values at each Ca^{2+} concentration (closed circles, ALG-2; open circles, des3-23ALG-2; closed triangles, ALG-2 Δ GF122; open triangles, des3-23ALG-2 Δ GF122). The maximum binding value (B_{max}) of each hyperbolic curve was determined by a Lineweaver-Burk plot and then used as 100% maximal binding activity. Based on these B_{max} values, the original data were transformed, expressed as a percentage of maximal binding activity, and re-plotted as shown.

stitutions of these Pro residues with Ala (k-ABS-2-p2a2) did not affect the binding capacity. Trimming two and three residues from the N terminus (leaving Lys and Gln as a linker for immobilization) and from the C terminus, respectively, did not significantly influence the binding (k-ABS-2-5del). Using the trimmed sequence, a mutant with substitutions of all four Pro residues with Ala (k-ABS-2-5del-p4a4) was found to significantly reduce the binding capacity. Although substitution of either Leu⁵¹ or Phe⁵² with Ala (k-ABS-2-5del-L51A, -F52A) barely affected the binding capacity, substitution of Phe⁴⁹ with Ala, Tyr, or Leu (k-ABS-2-5del-F49A, -F49Y, and -F49L) abolished or significantly reduced the binding capacity. On the other hand, substitution of Phe⁴⁹ with Trp (k-ABS-2-5del-F49W) did not affect the binding capacity.

Binding of ALG-2 to Other PLSCR Isoforms—There are four phospholipid scramblase isoforms encoded by different genes in the human genome. Each phospholipid scramblase (PLSCR1 to -4) has a Pro-rich region (70–110 amino acids) at its N terminus except for PLSCR2, which is expressed specifically in the testis (37). Although there is no obvious tandem PXY repeat-

like sequence in PLSCR1 and PLSCR4, their Pro-rich regions contain multiple Tyr residues. Western blotting of the GST-ALG-2 pull-down products revealed barely detectable bands for GFP-PLSCR1 (Fig. 7A). On the other hand, clear bands were detected for GFP-PLSCR4 by pull-down assay with GST-ALG-2 as well as GST-ALG-2 Δ GF122.

Different Binding Specificities to ALG-2 Mutants—Binding of Ca^{2+} to ALG-2 induces conformational changes and exposure of hydrophobic surfaces (27, 34, 35, 38). We investigated whether hydrophobic interactions with specific residues in ALG-2 are equally used among various ALG-2-interacting proteins. Various GFP-fused PLSCR constructs (Fig. 7A) and endogenous ALG-2-interacting proteins (Fig. 7B) were subjected to GST-pulldown assays using ALG-2 mutants whose aromatic residues were substituted with Ala. The mutants of amino acid residues positioned in the region from EF3 to EF5 (Y91A, W95A, and Y180A) lost binding abilities to Alix, annexin A7, annexin A11, and GFP-PLSCR3 Δ ABS-2 but not to GFP-PLSCR3, GFP-PLSCR3 Δ ABS-1, GFP-PLSCR4, and Sec31A. TSG101 exhibited reduced binding efficiencies against mutants of W95A and Y180A, among which the W95A

mutant had a more profound effect. Mutants in the EF1-EF2 linker region (W57A and F60A) showed different results: W57A, no or little effect on the tested proteins except GFP-PLSCR3 Δ ABS-1 and annexin A7 with a significant reduction in binding; F60A, complete loss of binding to GFP-PLSCR3 Δ ABS-1, GFP-PLSCR3 Δ ABS-2, Alix, annexin A7, annexin A11, and Sec31A.

Reduced Binding of ALG-2 to Thr²¹-phosphorylated ABS-1—PLSCR3 is phosphorylated by protein kinase C- δ , and phosphorylation at Thr²¹ in ABS-1 correlates with AD198 (protein kinase C- δ activator)-induced apoptosis (39). Although substitution of Thr²¹ to Ala reduced AD198-induced apoptosis, the phosphomimetic mutant PLSCR3(T21D) induced apoptosis without the protein kinase C- δ activator (40). We investigated whether phosphorylation of ABS-1 at Thr²¹ influences ALG-2 binding. First, we attempted pulldown assays by immobilizing an ABS-1 oligopeptide (KGYAPSPPPYPVTPGYPEPA) to NHS-activated Sepharose beads, but the ALG-2-binding efficiency was poor. Instead, we employed an enzyme-linked immunosorbent assay-like method using commercially avail-

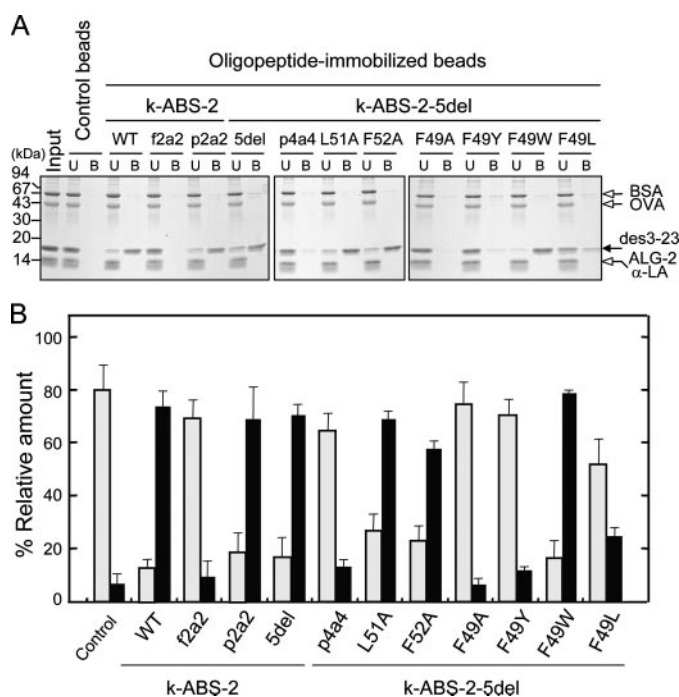


FIGURE 6. Pulldown assay of des3-23ALG-2 with mutant oligopeptide-immobilized beads. *A*, after Sepharose beads carrying ABS-2 or various mutant oligopeptides (Table 1) had been incubated with des3-23ALG-2 in the presence of CaCl_2 as described under "Experimental Procedures," the pelleted beads were washed with binding buffer three times. The unbound fraction (supernatant, U) and bound fraction (beads, B) were subjected to SDS-PAGE, followed by staining with CBB. *B*, relative amounts of proteins compared with input des3-23ALG-2 (100%) were estimated by densitometric analyses of the stained gels.

able covalent-coupling 96-well plates. After varying amounts of the ABS-1 oligopeptide and its Thr²¹-phosphorylated oligopeptide (ABS-1-pT) were immobilized to the plates, binding to bio-ALG-2 was measured by reactivity of SA-peroxidase. Measured values of binding to bio-ALG-2 increased in proportion to the amounts of the respective oligopeptides used for immobilization but reached a plateau (Fig. 8). The phosphorylated ABS-1 oligopeptide exhibited ~70% reduction in bio-ALG-2 binding in comparison with the unphosphorylated oligopeptide.

DISCUSSION

ALG-2 is present both in the cytosol and nuclear fraction (5, 41), and it translocates to membranous fractions including endosomes and endoplasmic reticulum exit sites in a Ca^{2+} -dependent manner (5, 28, 30, 42, 43). This Ca^{2+} -dependent behavior of ALG-2 as a Ca^{2+} sensor is dependent on interaction with various intracellular proteins. In the present study, we found that phospholipid scramblase 3 (PLSCR3) is a novel ALG-2-interacting protein. PLSCR3 has a Pro-rich region at its N terminus, clustered cysteine residues for potential fatty acid linkage, one unit of EF-hand, and a putative transmembrane region at the C terminus (37). By analyses of PLSCR3 deletion mutants (Fig. 3) and *in vitro* binding analyses using recombinant ALG-2 proteins as well as synthetic oligopeptides (Figs. 5, 6, and 8), we identified two ALG-2-binding sites: ABS-1 and ABS-2 (Fig. 1). ABS-1 has interrupted PXY repeats, and a similar sequence is present between Alix and PLSCR3 (PPYPXXPGYP). On the other hand, ABS-2 does not conform to the

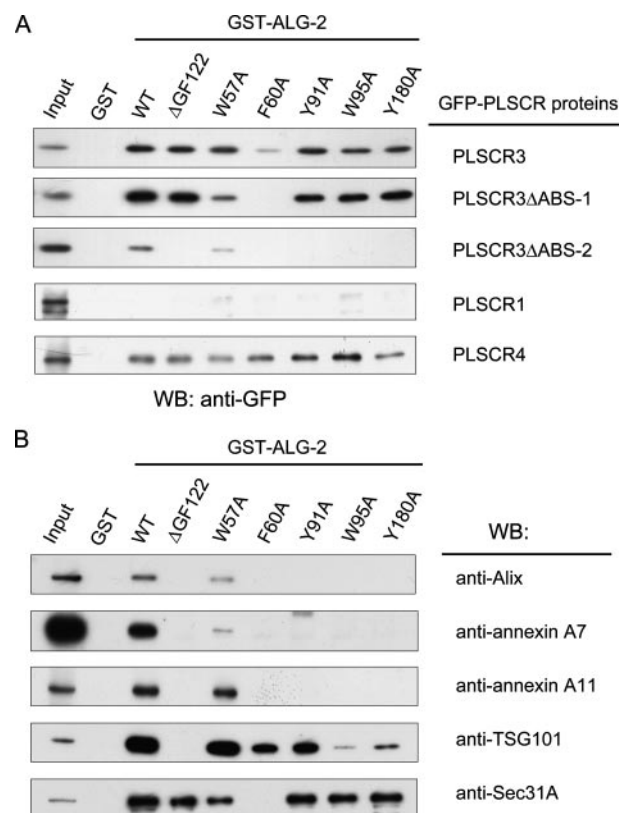


FIGURE 7. Effects of point mutations of ALG-2 on the binding to GFP-PLSCRs and other interacting proteins. GST, GST-ALG-2, GST-ALG2^{ΔGF122}, and indicated amino acid-substituted mutant proteins were used for pull-down assays of GFP-fused constructs of PLSCR3 and its ABS deletion mutants as well as GFP-PLSCR1 and -PLSCR4 as described in the legend to Fig. 3 using plasmid-transfected HEK293 cells (*A*). For analyses of endogenous proteins (*B*), untransfected HEK293 cells were used. Pulldown products were analyzed by Western blotting (WB) using anti-GFP mAb (*A*) and using specific antibodies as indicated (*B*). The volumes of analyzed cleared lysates (*input*) correspond to 11% (*A*) or 13% (*B*) of the lysate volumes used for pulldown product analyses by Western blotting.

canonical sequence but contains six Pro and two Phe residues. Although Phe⁴⁹ is essential and cannot be substituted with Tyr or Ala, Phe⁵¹ is substitutable with Ala (Fig. 6). Because Trp can replace Phe⁴⁹, a large non-polar side chain seems to be required for the strong interaction. ABS-2 possesses a unique property in that it binds the alternatively spliced ALG-2 isoform (lacking the two residues Gly¹²¹-Phe¹²², designated ALG-2^{ΔGF122}) in contrast to the lack of binding ability of ABS-1 to this isoform (Figs. 3 and 5). The results of our GST-pulldown assays using GST-ALG-2^{ΔGF122} (Fig. 7) agree with results of previous studies showing that Alix does not associate efficiently either with the alternatively spliced ALG-2 isoform (35) or with the Phe¹²²-deletion mutant (38). In addition to our previous finding that GST-ALG-2^{ΔGF122} does not bind TSG101 (30), we have found that it has no capacity of binding to annexin A7 and annexin A11 (Fig. 7). Interestingly, the W95A mutant also does not interact with PLSCR3 ABS-1, Alix, annexin A7, or annexin A11 or interacts barely with TSG101. There is a good correlation between ALG-2^{ΔGF122} and W95A for loss of binding to these proteins. Alix-type ABS (ABS-1) should have a common motif, composed of Pro/Gly and Tyr for ALG-2 binding. Future mutational analyses in combination with three-dimensional structural analyses of ALG-2-oligopeptide complexes will clarify the

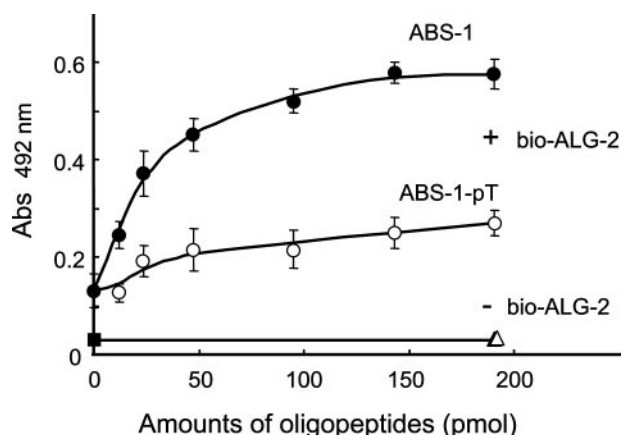


FIGURE 8. Effects of phosphorylation of Thr²¹ in ABS-1 on ALG-2 binding. Varying amounts (11–183 pmol) of ABS-1 (KGYAPSPPPYPVTPGYPEPA) and Thr²¹-phosphorylated ABS-1 (KGYAPSPPPYPV-pT-PGYPEPA, ABS-1-pT) oligopeptides were immobilized to a 96-well plate. The immobilized oligopeptides were probed with 12 pmol of bio-ALG-2 as described under “Experimental Procedures.” The relative amounts of bound bio-ALG-2 were estimated by the colorimetric method using SA-peroxidase, and the reaction products were measured with a plate reader at 492 nm. Values are expressed as means $\pm$ S.D. ($n = 4$). Closed circles, ABS-1; open circles, ABS-1-pT. Streptavidin-peroxidase reactions without bio-ALG-2 were carried out to estimate background measurements of the plate assay. Closed square, no oligopeptide; closed triangle, ABS-1; open triangle, ABS-1-pT.

exact binding motif of ABS-1 type. Interestingly, Sec31A binds GST-ALG-2 ^{Δ GF122} (28) (Fig. 7), suggesting the presence of ABS-2 type. The Pro-rich region of Sec31A, however, contains multiple Tyr residues in addition to Pro, Gly, and Phe. Therefore, we cannot exclude the possibility that Sec31A has both types of ABS as in the case of PLSCR3.

The adverse effect of phosphorylation at Thr²¹ in ABS-1 on ALG-2 binding (Fig. 8) suggests that the physical association between ALG-2 and ABS-1 is dependent on hydrophobic interactions. Jia *et al.* elucidated the three-dimensional structure of des1-20ALG-2 (elastase-treated ALG-2) in a Ca²⁺-loaded form by x-ray crystallography (44). Unexpectedly, the N-terminal clipped Gly/Pro-rich peptide (8-PGPGGGPGPA-17) was retained in a hydrophobic pocket formed by residues positioned from EF3 to EF5, and the peptide was estimated to form a polyproline II helix. We investigated the possibility of binding of PLSCR3, Alix, and other ALG-2-interacting proteins to this hydrophobic pocket by GST-pulldown assays using various ALG-2 mutants whose aromatic residues were substituted with Ala (Fig. 7). Loss or significant reduction of binding abilities in these mutants (Y91A, W95A, and Y180A) suggests that Alix, annexin A7, annexin A11, and PLSCR3 ABS-1 bind to the same hydrophobic pocket in which the N-terminal peptide of ALG-2 is held regardless of no apparent sequence similarity between these sequences and the N-terminal peptide except for the presence of multiple Pro residues. On the other hand, these point mutations did not affect the binding to ABS-2, indicating that ABS-1 binds to a hydrophobic pocket different from the one for ABS-2. Interestingly, the mutants of F60A and Y91A retain significant binding abilities to TSG101, suggesting that TSG101 contacts with ALG-2 at a surface sharing with other isoform-non-interactive (ABS-1) type proteins, but the contact surface is not exactly the same.

Among the four PLSCRs, only PLSCR1 and PLSCR3 are well characterized. PLSCR1 was originally implicated in remodeling

of the trans-bilayer distribution of plasma membrane phospholipids (45, 46). Although its role in membrane phospholipid scrambling is controversial, increasing evidence shows that PLSCR1 has biological significance in cell signaling, maturation, and apoptosis by interacting with various proteins (Ref. 47 and references therein), and it also functions as a DNA-binding transcription factor (48). Expression of PLSCR3 is required for normal adipocyte and/or macrophage maturation or function (49). PLSCR3 transports cardiolipin from the inner to the outer mitochondrial membrane (50), and it facilitates mitochondrial targeting of N-terminally truncated Bid, which interacts with cardiolipin and dissociates from the N-terminal cleaved peptide (51). The results of the GST-pulldown assays suggest that ALG-2 interacts also with PLSCR4 but barely with PLSCR1 (Fig. 7). Binding specificities to the ALG-2 isoform ALG-2 ^{Δ GF122} and mutants are similar between PLSCR3 and PLSCR4 except for significant reduction in binding of PLSCR3 to F60A. Because no PLSCR3 ABS-2-like sequence is found in the Pro-rich region of PLSCR4, presence of a different type of non-Alix-type ABS is suggested in this region.

Flexibility of binding motifs is well characterized in calmodulin (CaM), which interacts with a large number of proteins to regulate their biological functions in response to calcium stimuli. CaM-binding sites are grouped into several classes and subclasses based on structural and sequence information such as the IQ motif in myosin V, basic 1-8-14 motif in skeletal muscle myosin light chain kinase and basic 1-5-10 motifs in CaMK1 and CaMKII (52). Some IQ class proteins do not require Ca²⁺ for CaM binding (53). As demonstrated in the present study (Fig. 7), the Alix-type (ABS-1) and non-Alix-type (ABS-2) ALG-2-binding sites in PLSCR3 displayed differences in binding to the alternatively spliced ALG-2 isoform and mutants. Similarly, Alix, annexin A7, annexin A11, TSG101, and Sec31A exhibited different binding specificities to ALG-2 mutants. Other ALG-2-interacting proteins such as RNA-binding protein RBM22 (41) and HD-PTP (54) do not have sequences similar to either ABS-1 or ABS-2 in their Pro-rich regions. As in the case of PLSCR4, these proteins may have a non-Alix-type ABS that interacts with ALG-2 ^{Δ GF122}. On the other hand, ASK-1 (55) and Raf-1 (56) are also known to associate with ALG-2, but they do not possess conspicuous Pro-rich regions (57). Thus, ALG-2 may present flexible binding surfaces to various proteins with broad specificities and different affinities. More systematic analyses using, for example, an array of mutant ALG-2 protein chips, will be required to categorize ABS of all known and unknown ALG-2-interacting proteins in more detail. Although the biological significance of the Ca²⁺-dependent interaction between ALG-2 and PLSCR3 remains to be clarified, our finding of a non-Alix-type ALG-2-binding site has broadened the structural view of ALG-2-interacting proteins and the recognition mechanisms. We observed Zn²⁺-dependent as well as Ca²⁺-dependent association of ALG-2 to the PLSCR3 peptides (supplemental Fig. S1). While this report was in preparation, Vernarecci *et al.* also reported that the yeast homolog of ALG-2, Pef1p, binds both Ca²⁺ and Zn²⁺ and that the metal ion induces conformational change of the protein (58). Although the biological significance of Zn²⁺ binding to the PEF proteins remains unknown, the heavy metal ion may be useful for future

x-ray crystal structure analyses as an alternative condition when crystallization of ALG-2-target peptide complex in the presence of Ca²⁺ results in failure.

Acknowledgments—We thank Y. Nakano and Y. Sano for earlier works related to this study and Dr. K. Hitomi for valuable suggestions. We also thank Dr. P. J. Sims for valuable suggestions and providing antibody that was used at the early stage in our study.

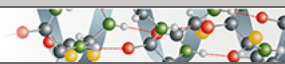
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Kakiuchi, Tatsutoshi Inuzuka, Haruna
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J. Biol. Chem. 2008, 283:9623-9632.
doi: 10.1074/jbc.M800717200 originally published online February 6, 2008

Access the most updated version of this article at doi: [10.1074/jbc.M800717200](https://doi.org/10.1074/jbc.M800717200)

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