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Isolation of a *Drosophila* Gene Coding for a Protein Containing a Novel Phosphatidylserine-Binding Motif

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To elucidate the molecular basis of the binding of proteins to the membrane phospholipid phosphatidylserine (PS), we characterized PS-binding peptides isolated from a phage display library. Amino acid sequences deduced from the nucleotide sequences of over 60 phage clones isolated revealed that there was no common primary structure among these peptides, but all peptides were rich in basic amino acid residues. In particular, 15 clones encoded peptides that contained contiguous arginine residues. Characterization of two such peptides in more detail showed that they bound to PS, and to a much lower extent to other phospholipids, including phosphatidylinositol, phosphatidylethanolamine, and phosphatidylcholine. Unlike other Ca^{2+}-dependent PS-binding proteins, these peptides did not require Ca^{2+} for binding to PS, and the addition of Ca^{2+} did not alter the phospholipid specificity. Substitution of one of the two RR sequences in one peptide by alanine had no effect, but that of both sequences completely abolished the activity. Furthermore, we identified a *Drosophila* gene coding for a presumed nuclear protein that shares an amino acid sequence, including a RR residue, with one of the two PS-binding peptides. This protein bound to PS partly depending on the presence of the RR residue. These results allowed us to conclude that an amino acid sequence including contiguous arginine residues is a novel motif that defines Ca^{2+}-independent PS-binding activity.

Key words: apoptosis, phage display library, phospholipid, protein–lipid interaction.

Abbreviations: GFP, green fluorescence protein; PS, phosphatidylserine; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PC, phosphatidylcholine.

Membrane phospholipids play important roles not only as major structural components of biological membranes but also as molecules that transmit signals both in and out of cells. Among such functional phospholipids, phosphatidylserine (PS) is involved in a variety of cellular events, including blood coagulation, phagocytic elimination of aged or pathological red blood cells, intracellular membrane fusion, and activation of protein kinase C (reviewed in Ref. *1*). More recently, PS was shown to serve as an 'eat-me' signal for apoptotic cells to be recognized and engulfed by phagocytic cells (reviewed in Ref. *2*). Phospholipids are unevenly distributed in the two layers of the plasma membrane, and PS is one of the phospholipids that are mostly confined to the inner layer (reviewed in Ref. *1*). The distribution of membrane phospholipids is determined by the action of multiple transporters responsible for the active movement of phospholipids across the two membrane layers (reviewed in Ref. *3*). The presence of PS in the inner layer is important for the activation of cytoplasmic protein kinase C and the regulation of intracellular membrane fusion. In contrast, PS is found in the outer layer in sickle or aged red cells, activated platelets, and apoptotic cells, and regulates cell-to-cell recognition or the activity of plasma proteins. The activity of phospholipid transporters is altered in these cells, and this presumably causes the equal distribution of all phospholipids in the plasma membrane bilayer (reviewed in Ref. *4*). The consequences of this change should be as follows: phospholipids that are normally restricted to the inner layer become exposed on the cell surface, and those restricted to the outer layer are internalized. At the present time, it is unclear how the activity of phospholipid transporters is altered in such circumstances.

PS exerts its function through specific interaction with PS-binding proteins. These proteins include coagulation factors V, VIIIa, and Xa in the activation of blood coagulation; membrane-bound PS receptors or PS-binding soluble proteins in the regulation of phagocytosis of unwanted cells (reviewed in Ref. *5*); and cytoplasmic proteins such as protein kinase C (reviewed in Ref. *6*) and synaptotagmin I (*7*) in the regulation of signaling pathways and membrane fusion, respectively. Previous studies have revealed the presence of primary sequences responsible for the binding to PS, such as the C2 domain in protein kinase C (*8*–*10*), synaptotagmin I (*7*), and factor VIII (*11*), and the γ-carboxyglutamic acid domain in factor Xa (*12*) and Gas6 (*13*), the latter of which is presumably involved in phagocyte recognition of apoptotic cells (*14*). Many PS-binding proteins require Ca^{2+}, and this ion has been shown to bridge PS and protein kinase C (*8*, *10*). However, nothing is known about the molecular

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basis of the interaction between PS and Ca^{2+}-independent PS-binding proteins. The present study was undertaken to obtain some clues about this issue as well as to obtain PS-binding small peptides as a research tool.

MATERIALS AND METHODS

Construction and Screening of a Phage Display Library—A phage library displaying 15-amino-acid peptides was constructed using the fUSE-5 vector (kindly provided by G. Smith of the University of Missouri) according to the procedure developed by Smith and colleagues (*15*). In brief, DNAs coding for peptides with a random 15-amino-acid sequence were generated by PCR using a synthetic DNA, 5′-ACTCGGCCGACGGGGCT$(NNM)_{15}$GGGGCCG-CTGGGGCCGAA, in which 'N' and 'M' denote any of the four bases and G or T, respectively, as a template, and 5′-ACTCGGCCGACGGGGC and 5′-TTCGGCCCCAGCGG-CCCC as primers. The amplified DNA was digested with *Bgl*I and then ligated to fUSE-5 that had been cleaved with *Sfi*I. *E. coli* strain MC1061 was electroporated with the resulting DNA and cultured in NZY medium. Phages present in the culture medium were precipitated with polyethylene glycol, suspended in Tris-buffered saline (TBS; 50 mM Tris-HCl (pH 7.5) and 0.15 M NaCl), and then used for screening. To determine the phage titer, *E. coli* $K91Kan^R$ was infected with an aliquot of phages, and then spread on NZY agar plates containing tetracycline (40 μg/ml) and kanamycin (100 μg/ml). The phage titer was calculated based on the number of colonies appearing on the plates and expressed as tetracycline-transducing units. Phage clones that bound to PS were selected by means of a panning method using PS-coated plastic plates. PS (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in ethanol was added to 96-well plates (0.3 μg per well) (MS-8496F; Sumitomo Bakelite, Tokyo, Japan), and the plates were left to dry at room temperature, followed by blocking with 3% (w/v) bovine serum albumin in TBS for 1 h at room temperature. The original phage library (1×10^9 tetracycline-transducing units in 0.1 ml TBS) was added to each well, and the plates were incubated for 1 h at room temperature. The wells were washed with 0.2 ml of phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20, and bound phages were eluted by incubation with 0.1 ml of 0.1 M glycine-HCl (pH 2.2) containing bovine serum albumin (1 mg/ml) and 0.05% (w/v) Tween 20 for 15 min at room temperature. The eluates were immediately neutralized with 1 M Tris-HCl (pH 9.5), and a 10-μl aliquot was used to infect $K91Kan^R$. The cells were incubated with NZY medium, and the amplified phages were recovered and subjected to second-round screening as described above. To determine the nucleotide sequences of DNAs of selected phage clones, DNA was extracted from $K91Kan^R$ infected with phages and sequenced using primer 5′-TGAATTTTCTGTATGAGG and a commercial kit (Big Dye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA).

Preparation of Bacterially Expressed Proteins Encoded by Selected Phage Clones—The region of phage DNA encoding a putative PS-binding peptide was inserted into the pGEX-KG vector by PCR, according to the procedure described previously (*16*), so that the peptide was fused to the carboxyl terminus of glutathione S-transferase (GST). The resulting DNA was introduced into *E. coli* BL21, and the cells were cultured for 4 h at 25°C in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside to induce the expression of the peptide-encoding DNA. The cells were lysed, and the GST-fusion protein was purified to homogeneity by affinity chromatography using glutathione Sepharose 4B (Amersham Biosciences, Uppsala, Sweden).

Phospholipid-Binding Assay—The binding of GST-fusion proteins to phospholipids was examined by means of a solid-phase assay as described previously (*17*). In brief, plastic 96-well plates (MS-8496F) coated with various phospholipids (Avanti Polar Lipids) were supplemented with proteins, washed, and subsequently reacted with anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham Biosciences). The amount of the secondary antibody remaining in wells was determined by means of a coloring reaction involving *o*-phenylenediamine, followed by measurement of A_{490}. For assays with a synthetic peptide labeled with biotin at the amino terminus, washed plates were supplemented with horseradish peroxidase–conjugated streptavidin (Zymed Laboratories, San Francisco, CA, USA), and the amount of bound peptide was determined as described above.

Isolation of Drosophila cDNA and Analysis of Encoded Proteins—Two *Drosophila* cDNAs corresponding to gene CG7824 were obtained by reverse transcription-mediated PCR using RNA of l(2)mbn cells, a cell line derived from *Drosophila* larval hemocytes. The CG7824 proteins fused to GST were prepared using the pGEX-KG vector and analyzed for the ability to bind to phospholipids as described above. A cDNA fragment coding for CG7824S protein (see RESULTS) was generated by PCR and subcloned with the pEGFP-N3 vector (Clontech Laboratories, Palo Alto, CA, USA). A DNA fragment corresponding to CG7824S protein fused to green fluorescence protein (GFP) was then isolated and inserted into the pAc5.1 vector (Invitrogen, Carlsbad, CA, USA). The pAc5.1 vector expressing GFP alone was similarly constructed. S2 cells, a *Drosophila* cell line, were maintained at 25°C in Schneider's medium containing 10% (v/v) heat-inactivated fetal bovine serum and transfected with DNA using Cellfectin (Invitrogen). These cells were stained with Hoechst 33342 and then examined for the localization of the GFP-fusion protein by fluorescence microscopy.

RESULTS

Isolation and Characterization of Peptides with PS-Binding Activity—A phage library displaying peptides with random 15-amino-acid sequences was subjected to selection based on the ability to bind to PS-coated plastic plates in the absence of Ca^{2+}, as described under "MATERIALS AND METHODS." To determine the proportion of phages that bound to PS-coated plates, the phage titer was determined, and compared before and after the screening. We found that the proportion of bound phages increased on the second-round screening but remained the same in the next selection (data not shown). The recovery after the third-round screening was as low as

	LHLHRRQMRP
	QHRRRSRLLMQKLPN
#44	RSRRMTRRARARAA
#52	RRTTHSPSTKLRPYP
	PSRRKQ
	SRRSPYY
	QRRLTRTA
#58	TSSRRLIRLMRRTQY
	TPRNIMLRRPHPRRP
	IRRNPS
#67	PPKTRRRRQRNTKNT
	RRISTN
	RRQQ
	RRIIP
	RRHNTTTY

Fig. 1. **Amino acid sequences of peptides containing contiguous arginine residues.** Amino acid sequences were deduced from the nucleotide sequences of the DNAs of 93 phage clones, 15 sequences that include contiguous arginine residues being shown (the amino terminus is towards the left). Basic amino acid residues are boxed. The peptides analyzed as to the binding to phospholipids in this study are numbered.

0.2%, but this value was close to that observed by other investigators who successfully isolated ganglioside-binding peptides using the same method (*18*). We therefore decided not to perform further screening, but instead to determine the nucleotide sequences of the DNA inserts of phage clones obtained on the second-round screening. Of the 93 clones sequenced, no two clones shared the same sequence, and the amino acid sequences deduced from the nucleotide sequences did not match those of any natural proteins registered in data bases. Sixty-five clones coded for peptides that are rich in basic amino acid residues, and 15 peptides possessed contiguous arginine residues (Fig. 1).

We then examined whether or not peptides encoded by DNAs of the selected phages actually bind to PS. For this purpose, we chose four peptides, designated as #44, #52, #58, and #67, and all containing contiguous arginine residues (see Fig. 1), and prepared GST-fusion proteins containing their sequences. The resultant proteins were subjected to a solid-phase binding assay as described under "MATERIALS AND METHODS," and this assay revealed that three of them bound to PS more effectively than GST alone (Fig. 2A). Furthermore, a synthetic peptide corresponding to peptide #44 showed PS-binding activity but apparently no affinity for another phospholipid, phosphatidylcholine (PC) (Fig. 2B), indicating that such peptides obtained from the phage display library actually possess PS-binding activity. We decided to further characterize peptides #44 and #67, which appeared to possess higher activity than the other peptides. GST-fusion proteins containing either peptide #44 or #67 were examined for binding to various phospholipids, including phosphatidylethanolamine, phosphatidylinositol, PC, and PS. Peptide #44 appeared to strictly bind to PS, whereas peptide #67 showed some affinity for phosphatidylinositol as well as PS (Fig. 3, A and B). The presence of Ca^{2+} did not affect either the level of PS-binding activity or the phospholipid specificity of either peptide, except for an inhibitory effect at higher concentrations (Fig. 3, C and D). These results indicate that both peptides #44 and #67 possess Ca^{2+}-independent PS-binding activity, and that peptide #44 specifically binds to PS.

Identification of the Motif Defining the PS-Binding Activity—We next determined the amino acid residues responsible for the PS-binding activity of peptide #44. For this purpose, GST-fusion proteins containing altered sequences of peptide #44, in which one or both of the RR sequences at amino acid positions 3-4 and 7-8 with

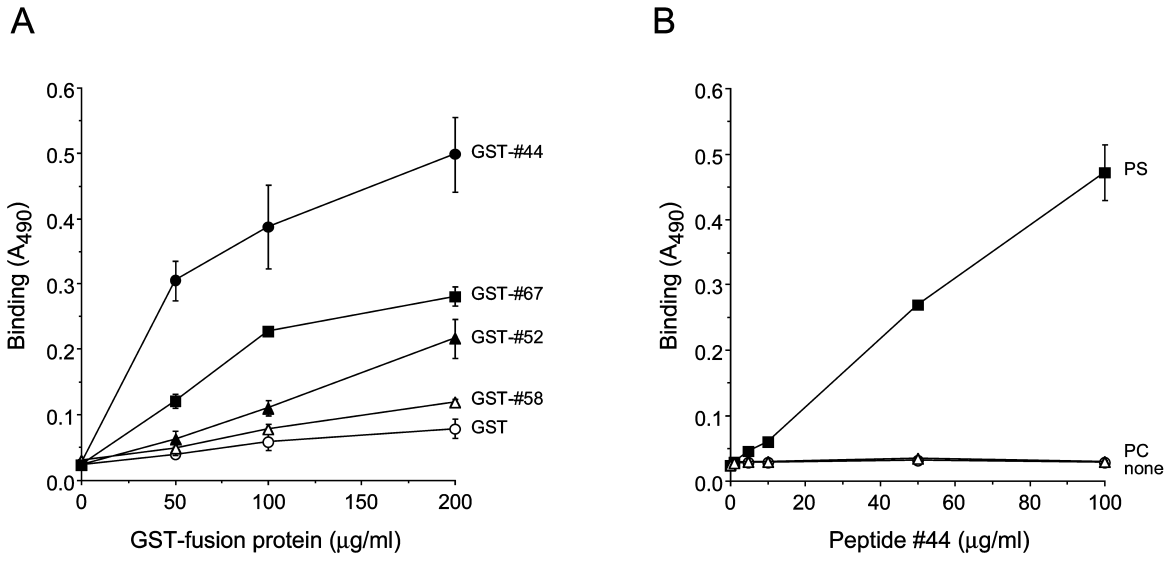

Fig. 2. **PS-binding assay of GST-fusion proteins.** A: Four GST-fusion proteins and GST alone were examined as to binding to PS by means of a solid-phase assay. The numbers following 'GST' correspond to those in Fig. 1. A representative of three independent experiments with similar results is shown with means and SD (n = 3). B: A synthetic peptide corresponding to peptide #44 was examined as to binding to PS and PC. 'none' (circles) represents the results of an assay with no added phospholipid. The data for one of two experiments with similar results are shown with means and SD (n = 3).

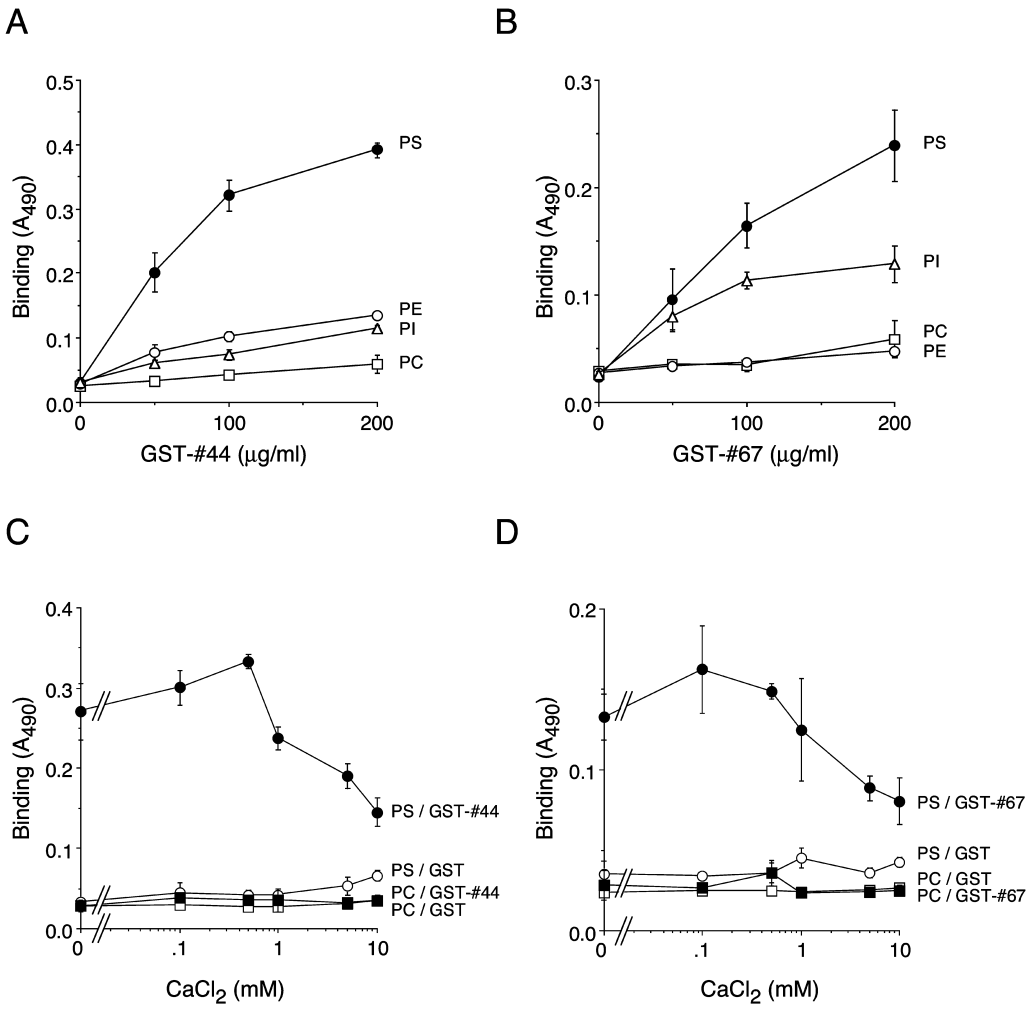

Fig. 3. **Phospholipid specificity and Ca^{2+} requirement of peptides #44 and #67.** GST-fusion proteins containing peptide #44 (A) or #67 (B) were examined as to binding to PS, phosphatidylethanolamine (PE), phosphatidylinositol (PI), or PC. Representatives of two (A) and three (B) independent experiments with similar results are shown with means and SD (n = 3). A phospholipid-binding assay with PS or PC was conducted with GST-fusion proteins containing peptide #44 (C) or #67 (D) in the presence of various concentrations of $CaCl_2$. The data for one of two experiments with similar results are shown with means and SD (n = 3).

respect to the amino terminus as position 1 were substituted by alanine, were prepared, and their activity was examined. The fusion proteins with substitution in either of the two regions retained the ability to bind to PS, but substitution in both regions completely abolished the activity (Fig. 4A). This indicates that the presence of a single RR sequence is sufficient for the PS-binding activity of peptide #44 and probably of other peptides selected through the screening as well. However, other parts of the peptide also seem to be needed for the activity, because peptide #58, which contains two RR sequences, did not effectively bind to PS (see Fig. 2A). We finally determined the activity of a GST-fusion protein that contains a tandem repeat of the sequence of peptide #44. As shown in Fig. 4B, the multimerized protein exhibited much higher PS-binding activity than the original one, indicating that this sequence synergistically increases the affinity of a protein for PS.

Isolation and Characterization of a Drosophila Protein Possessing the PS-Binding Motif—In order to identify natural proteins that possess the putative PS-binding motif, we performed a BLAST search with the GenBank data base using the sequence of peptide #44 as a query and found an as-yet uncharacterized *Drosophila* gene designated as CG7824. This gene codes for two mostly overlapping proteins (tentatively named CG7824S and CG7824L) that contain a sequence identical to the C-terminal half of peptide #44 including contiguous arginine residues (Fig. 5). We then prepared bacterially expressed proteins that consisted of either CG7824 protein fused to GST and tested them for the binding to phospholipids. Both GST-fusion proteins possessed the ability to bind to PS, and their preferences for phospholipids were basically the same; they bound to PS and phosphatidylethanolamine, less effectively to phosphatidylinositol, but not to PC (Fig. 6, A and B). This specificity seemed to be different from that observed for peptide #44, which almost exclusively bound to PS (see Fig. 3A). We next examined whether or not the RR sequence present within the putative PS-binding motif is required for the PS-binding activity of CG7824S protein. For this purpose, the RR sequence in the GST-fusion protein was substi-

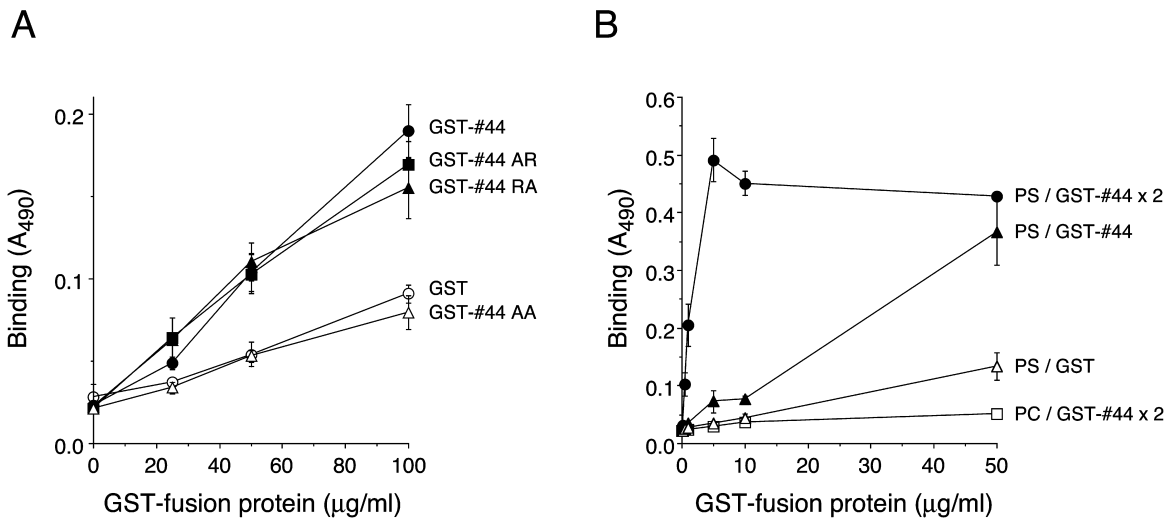

Fig. 4. **PS-binding assay with GST-fusion proteins containing altered sequences of peptide #44.** A: GST-fusion proteins were generated, in which one (GST-#44AR and GST-#44RA) or both (GST-#44AA) of the two RR sequences of peptide #44 are substituted by alanine. These proteins together with the wild-type protein (GST-#44) and GST alone were examined as to binding to PS. A representative of three independent experiments with similar results is shown with means and SD (n = 3). GST-#44AR, the RR sequence at amino acid positions 3-4 was changed to AA; GST-#44RA, the RR sequence at amino acid positions 7-8 was changed to AA. B: GST-fusion protein containing a tandem repeat of the sequence of peptide #44 (GST-#44 × 2) was generated and examined as to binding to PS or PC together with the wild-type protein (GST-#44) and GST alone. The data for one of two experiments with similar results are shown with means and SD (n = 3).

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CG7824L  MSSIWTKRKDSFCNNENIRAQKFEDVIVIDDNDRGDSLR S   40

CG7824L  VKSEPPEEKPRLSPQDANQKRAVKEEKEMSRRARARAKKI  80
CG7824S                MTMDANQKRAVKEEKEMSRRARARAKKI  28

CG7824L  RNTEARAAKSVVTRGAGKKARKRKKQNKAALKKASELEPK  120
CG7824S  RNTEARAAKSVVTRGAGKKARKRKKQNKAALKKASELEPK  68

CG7824L  KAKGSKEASAHKKAKGPKNTSPPMAAKPNLAKSDNLTMAL  160
CG7824S  KAKGSKEASAHKKAKGPKNTSPPMAAKPNLAKSDNLTMAL  108

CG7824L  VDELHEDGRLLNERWQEVEAQLAYMVTDRVMAMPGGPVPS  200
CG7824S  VDELHEDGRLLNERWQEVEAQLAYMVTDRVMAMPGGPVP S  148

CG7824L  FDSSDVVRGHRVIRCEDGFSKVFLSDCVAAISNSWHDMR I  240
CG7824S  FDSSDVVRGHRVIRCEDGFSKVFLSDCVAAISNSWHDMRI  188

CG7824L  KLVHVSDVPFAPQARIWLPTGQTDHNRIMICLRAQNLNV D  280
CG7824S  KLVHVSDVPFAPQARIWLPTGQTDHNRIMICLRAQNLNV D  228

CG7824L  MSDWSILRAEEVMEISQSFLLLINRRCIPQLDAVDYKLRY  320
CG7824S  MSDWSILRAEEVMEISQSFLLLINRRCIPQLDAVDYKLRY  268

CG7824L  GIRMAQIQLILSEADDLPSEFDNYHPFCHFPH          352
CG7824S  GIRMAQIQLILSEADDLPSEFDNYHPFCHFPH          300
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Fig. 5. **Amino acid sequences of CG7824 proteins.** The amino acid sequences of two proteins (CG7824S and CG7824L) deduced from the nucleotide sequence of *Drosophila* gene CG7824 are shown. The sequence identical to a part of peptide #44 is underlined. The numerals indicate the positions of amino acid residues, the amino terminus being numbered 1.

tuted by alanine, and the resulting protein was analyzed for the activity. We found that the GST-fusion protein with the altered amino acid sequence exhibited lower activity than the original protein (Fig. 6C). This indicates that the RR sequence is partly responsible for the PS-binding activity of CG7824S protein.

We then determined the intracellular localization of CG7824S protein. *Drosophila* S2 cells were transiently transfected with DNA expressing CG7824S protein fused to GFP or GFP alone, and then examined them by fluorescence microscopy. GFP alone appeared to be distributed throughout the cells, whereas CG7824S-GFP was

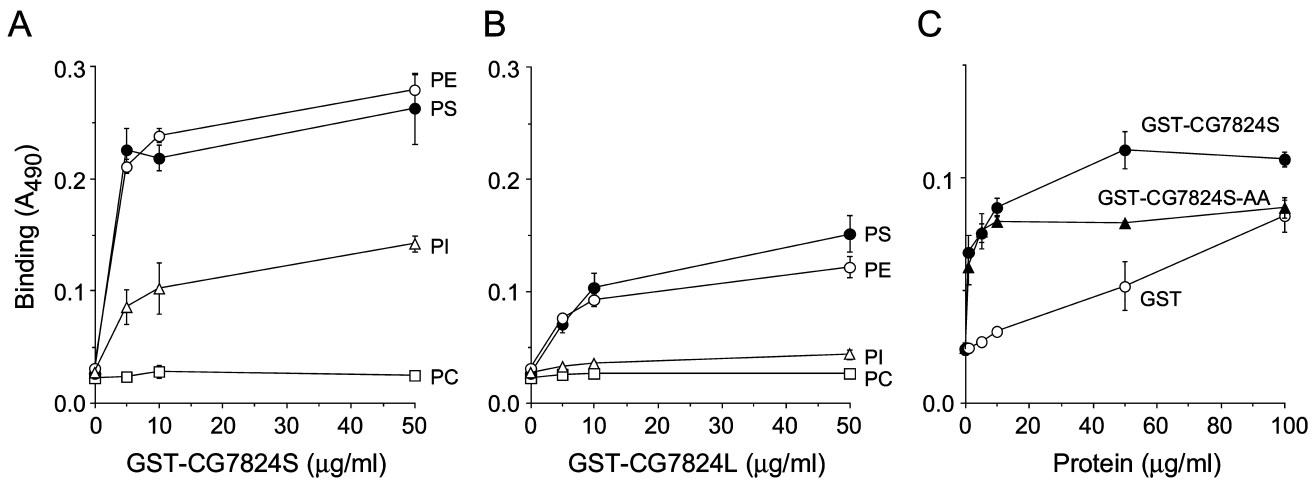

Fig. 6. **Binding of CG7824 proteins to phospholipids.** GST-fusion proteins containing CG7824S (A) and CG7824L (B) proteins were examined as to binding to various phospholipids. In (C), GST-fused CG7824S protein with contiguous arginine residues substituted by alanine (GST-CG7824S-AA) was analyzed as to binding to PS. Representatives of two independent experiments with similar results are shown with means and SD (n = 3). PE, phosphatidylethanolamine; PI, phosphatidylinositol.

restricted to a particular region within the nucleus (Fig. 7). This suggested that CG7824S protein is a nuclear protein.

DISCUSSION

We have screened a phage display library in a search for peptides that bind to PS in the absence of Ca^{2+}. Many peptides with distinct amino acid sequences were found, and at least one of them appeared to specifically bind to PS irrespective of the presence of Ca^{2+}. This peptide, designated as #44, required contiguous arginine residues for its ability to bind to PS. A number of PS-binding proteins have been reported, many of which bind to PS only in the presence of Ca^{2+}. Studies on a couple of Ca^{2+}-dependent PS-binding proteins revealed that Ca^{2+} bridges PS and these proteins (*8*, *10*). It may be reasonably anticipated that Ca^{2+} electrostatically binds to PS, an anionic phospholipid. We speculate that the contiguous arginine residues of peptide #44 directly interact with PS, as Ca^{2+} does. Alternatively, contiguous arginine residues might help the peptide form the structure necessary for PS-binding activity, because two arginine residues are sterically close to each other in the PS-binding domain of annexin V, a Ca^{2+}-dependent PS-binding protein (*19*). Determination of the structure of peptide #44 bound by PS is necessary to resolve this issue.

The identification of a novel PS-binding motif should help us isolate new proteins that exert functions through binding to PS. In fact, a data base search allowed us to identify a *Drosophila* gene that codes for two mostly overlapping proteins containing a sequence identical to a part of peptide #44 including contiguous arginine residues. We showed that the two proteins encoded by this gene effectively bound to PS, partly depending on the contiguous arginine residues. This indicates that the motif containing contiguous arginine residues endows a natural protein with the ability to bind to PS. However, unlike peptide #44, these proteins appeared to exhibit affinity with another phospholipid phosphatidylethanolamine, the extent being similar to that with PS. This suggests that amino acid sequences neighboring contiguous arginine

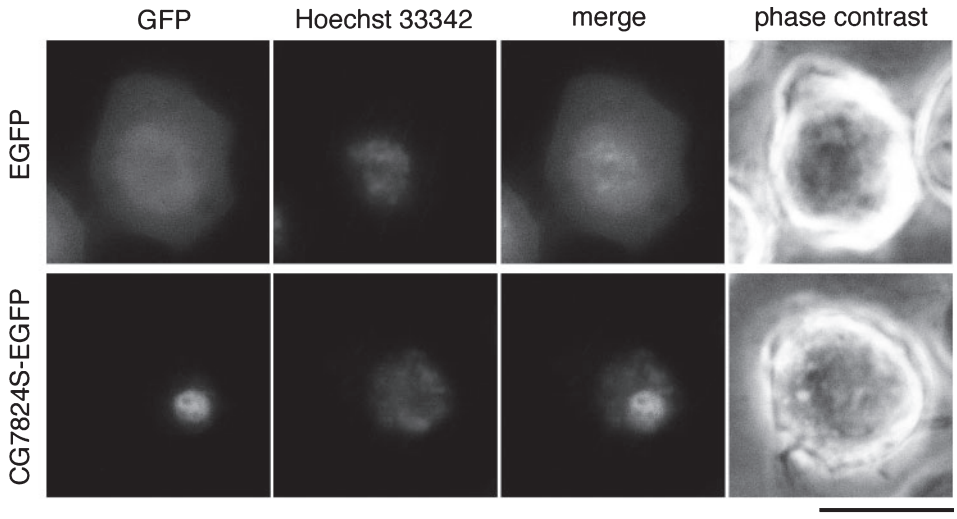

Fig. 7. **Intracellular distribution of CG7824S protein fused to GFP.** *Drosophila* S2 cells were transiently transfected with DNA expressing GFP alone (EGFP) or CG7824S protein fused to GFP (CG7824S-EGFP), stained with DNA-binding fluorochrome Hoechst 33342, and then examined under a fluorescence/phase contrast microscope. Fluorescence and phase contrast views of the same microscopic fields are shown in each row. Bar, 10 μm.

residues are important for defining the target specificity. One of the two proteins appeared to be localized in the nucleus, but their biological roles need to be clarified. Finally, these small peptides with Ca^{2+}-independent PS-binding activity could be useful for *in vivo* detection of PS-exposing cells or for modulating PS-mediated biological events outside or inside cells.

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