Published in final edited form as:
Biochem Biophys Res Commun. 2009 August 14; 386(1): 197–201. doi:10.1016/j.bbrc.2009.06.010.

Efficient Identification of Phosphatidylserine-Binding Proteins by ORF Phage Display

Nora B. Caberoy[a,†], **Yixiong Zhou**[a,†,§], **Gabriela Alvarado**[a], **Xianqun Fan**[b], and **Wei Li**[a,*]

[a]Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Miami, FL 33136, USA.

[b]Department of Ophthalmology, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Abstract

To efficiently elucidate the biological roles of phosphatidylserine (PS), we developed open-reading-frame (ORF) phage display to identify PS-binding proteins. The procedure of phage panning was optimized with a phage clone expressing MFG-E8, a well-known PS-binding protein. Three rounds of phage panning with ORF phage display cDNA library resulted in ~300-fold enrichment in PS-binding activity. A total of 17 PS-binding phage clones were identified. Unlike phage display with conventional cDNA libraries, all 17 PS-binding clones are ORFs encoding 13 real proteins. Sequence analysis revealed that all identified PS-specific phage clones had dimeric basic amino acid residues. GST fusion proteins were expressed for 3 PS-binding proteins and verified for their binding activity to PS liposomes, but not phosphatidylcholine liposomes. These results elucidate previously unknown PS-binding proteins and demonstrated that ORF phage display is a versatile technology capable of efficiently identifying binding proteins for non-protein molecules like PS.

Keywords

Phage display; phosphatidylserine; phosphatidylserine-binding protein; ORF phage display; phosphatidylcholine

Introduction

Phospholipids not only serve as major structural components of cellular membranes, but also function as signaling molecules regulating both intracellular and extracellular biological processes. For example, phosphatidylserine (PS) regulates a number of events, including protein kinase C (PKC) activation, phagocytic removal of apoptotic cells and blood coagulation [1]. In healthy cells, PS is predominantly confined to the inner layer of the plasma membrane, regulating an array of signaling proteins, such as PKC, phospholipase C (PLC), synaptotagmin

*Corresponding author:, Wei Li, Ph.D., Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, 1638 NW 10th Avenue, Miami, FL33136, Tel: 305-326-6445, Fax: 305-547-3667, E-mail: E-mail: wli@med.miami.edu.
†Both authors contributed equally.
§Current address: Department of Ophthalmology, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

[1]. During apoptosis, PS flips across the plasma membrane, displays on the surface of apoptotic cells and serves as eat-me signals to facilitate phagocytic removal of apoptotic cells by binding to phagocytic receptors or bridging molecules, including Gas6, MFG-E8 (also called lactadherin) and PS receptors [1–3]. PS on activated platelets stimulates the assembly of the coagulation system by binding to factor V, VII, VIII, IX, X, etc [1]. PS binding to these proteins is delicately regulated by different mechanisms. For example, PS binds to PKC and PLCδ through Ca^{2+}-dependent C2 domains, whereas PS binding to Gas6, protein S, factor VII, IX, X and prothrombin is mediated by post-translationally modified γ-carboxyglutamic acid (Gla) domain [1]. Other proteins, such as MFG-E8, can directly bind to PS through a Ca^{2+}-independent discoidin-like C2 domain [1].

Because of its importance, the search for proteins specifically interacting with PS is of considerable interest and should help define its regulatory roles. However, PS-binding proteins were traditionally identified on a case-by-case basis with daunting challenges [2,3]. Recently, several groups reported the identification of PS-binding peptides from random peptide libraries by phage display [4,5]. Similar to annexin V, these peptides are valuable imaging probes to detect apoptotic cells for clinical diagnosis or basic research. However, unnatural peptides identified from random peptide libraries have minimal biological implications in protein networks.

In this study we efficiently identified a panel of PS-binding proteins from a cDNA library by phage display. However, unlike random peptide libraries encoding short peptides or antibody (Ab) libraries, cDNA repertoires with unpredictable reading frames and stop codons may interfere with phage capsid expression when fused to the N-terminus of the capsid protein [6]. To circumvent the problem, phage display vectors, such as pJuFo phagemid [6] and T7 phage vector [7], were developed with C-terminal display. However, C-terminal display cannot ensure that the cDNA library is expressed in the correct reading frames. Unlike yeast two-hybrid system, out-of-frame phage clones encoding unnatural short peptides tend to outgrow clones with open reading frames (ORFs) through multiple rounds of liquid-based selection and amplification. Consequently, most identified phage clones are non-ORFs encoding unnatural short peptides. For example, only ~6% (8/130) of clones identified from a conventional C-terminal cDNA library of T7 phage display encoded real proteins [8]. Another study showed <10% (24/243) of identified ORF clones [9]. Thus, it is far less efficient to identify phage clones from cDNA libraries than from Ab libraries or random peptide libraries. As a result, a previous study identified PS-binding peptides from a phage display random peptide library, delineated a PS-binding motif, analyzed *Drosophila* genes for the possible match to the motif, identified and verified a new PS-binding protein [10]. This complicated process illustrated the challenge to identify a PS-binding protein.

Here we describe an efficient, convenient and versatile technology of ORF phage display to directly identify unknown PS-binding proteins. Rather than using a conventional cDNA library, we constructed an ORF phage display cDNA library with most phage clones in ORFs. Because of our poor understanding of Ca^{2+}-independent PS-binding proteins, we designed a phage panning scheme to efficiently identify PS-binding proteins from the ORF library in the absence of calcium. All 17 identified PS-binding phage clones were ORFs encoding 13 different proteins. Identified PS-binding proteins were independently validated by their specific binding to PS liposomes, but not to phosphatidylcholine (PC) liposomes.

Materials and Methods

ORF phage display cDNA library

Total RNA was extracted from 4 C57BL/6 mouse embryos at E18 using TRIzol reagent (Invitrogen). mRNA was purified with Oligotex mRNA purification kit (Qiagen). Orientation-

directed cDNA repertoire was generated by random priming method. The first-strand cDNA was generated by SuperScript II reverse transcriptase (Invitrogen) with the first-strand tagged primer (5'-GCGCCGCGACCNNNNNNNNN-3'). The second-strand cDNA was generated by Klenow (3'→5' exo$^-$) with the second-strand tagged primer (5'-GGCCGGCCTCCNNNNNNNNN-3'). The cDNA library was amplified by frame-shift PCR for 30 cycles with 3 forward and 3 reverse primers (Forward primers: 5'-AACTAGATAAGAATAGCGGCCGCGCGCCGCGACC-3', 5'-AACTAGATAAGAATAGCGGCCGCAGCGCCGCGACC-3', 5'-AACTAGATAAGAATAGCGGCCGCAAGCGCCGCGACC-3'; Reverse primers: 5'-AATGATCTCGAGGGCCGGCCTCC-3', 5'-AATGATCTCGAGTGGCCGGCCTCC-3', 5'-AATGATCTCGAGTAGGCCGGCCTCC-3'). The PCR product between 300 bp-1.5 kb was purified on 2% agarose gel, digested with NotI and XhoI, and ligated into T7Bio phage vector [11] at NotI and XhoI. After packaging using T7 phage package extract (Novagen), the original phage library titer was ~1×10^8 pfu. The library was amplified once on bacterial plates, bound to streptavidin-coated paramagnetic beads (Promega), eluted, and amplified to generate ORF library. All procedures for T7 phage display were described previously [7,11].

Phage selection

Phage selection was carried out as previously described [7,11] with the following modifications. ELISA plates (Corning Life Science) were coated with PS (10 nmol/100 μl methanol/well). After evaporation of the solvent, the plates were blocked with 1% polyvinyl alcohol in phosphate buffered saline (PBS) for 1 h. The phage library (~1×10^{10} pfu) was incubated with the immobilized PS for 1 h at room temperature. The plates were washed with PBS for 6 min × 8. Bound phages were eluted, amplified and used as input for the next round of selection. Total eluted phages were quantified by plaque assay. After 3 rounds of selection, enriched phages were selected twice with immobilized streptavidin, as previously described [11]. Unless otherwise indicated, no detergent like Tween-20 was used in phage panning and binding. Individual phage clones were randomly picked from phage plates, amplified, analyzed for their binding activity to immobilized PS or mock coated wells, and quantified for the bound phages by plaque assay.

Recombinant proteins

The cDNA coding sequences of Akap12(345D-522G), pinin(187Q-306E) and serum response factor binding protein 1 (Srfbp1)(110V-264E) were amplified by PCR from identified phage clones (Table 1), cloned into pGEX-2T plasmid (GE Healthcare) and verified by sequencing. GST fusion proteins were expressed in BL21(DE3) bacteria and purified using glutathione columns, as described previously [12].

Liposome binding assay

PS and PC liposomes were prepared as previously described [13]. Liposomes (50 nmol) were incubated with GST or GST fusion proteins (~10 μg) in 0.5 ml PBS at 4°C for 1 h, washed four times with ice-cold PBS by centrifugation at 230,000 × g for 30 min at 4°C as previously described [14], and analyzed by Western blot using anti-GST monoclonal Ab (mAb) (AnaSpec), as previously described [12].

Results and discussion

Optimization of PS binding conditions

Give that PS is a phospholipid, detergents in the binding or washing buffer may strip off non-covalently immobilized PS on ELISA plates. However, non-ionic detergents like Tween-20 were widely used to reduce non-specific binding in phage panning, including the studies to

identify PS-binding peptides [4,5,10]. To characterize the effect of Tween-20 on phage binding to immobilized PS, we analyzed PS binding with a phage clone displaying MFG-E8, a well-characterized PS-binding protein [15]. MFG-E8-phage showed higher PS-binding activity than the control phage without cDNA insert in the absence of Twee-20 (Fig. 1A). However, the binding activity was significantly reduced by the washing buffer containing 0.1% of Tween-20, suggesting that PS-binding assay or phage panning is optimal in the absence of Tween-20. As a result, phage panning and phage binding assay in this study were carried out without any detergent.

PS-binding proteins identified by ORF phage display

We immobilized PS on ELISA plates and performed 3 rounds of phage panning with the ORF phage display library, followed by 2 rounds of selection with immobilized streptavidin to eliminate non-ORF phage clones. The selection resulted in ~300-fold increase in PS-binding activity (Fig. 1B). We randomly picked and analyzed 62 clones for phage plates, analyzed their binding activity to immobilized PS vs. mock coated wells, identified and sequenced 17 PS-binding phage clones. Sequence analysis revealed that all 17 sequenced clones had ORF cDNA inserts, encoding 13 different proteins (Table 1). Three identical clones were identified for an unknown protein (5830404H04Rik). Two non-redundant clones with overlapping amino acid sequences were identified for Srfbp1 or another unknown protein (2500003M10Rik).

A previous study reported that Akap12 [A kinase (PRKA) anchor protein 12, gravin or SSeCKs] binds to protein kinase Cα (PKCα) or PKC in a PS-dependent manner [16,17]. A total of 3 proteins were identified with unknown function (Table 1). Their PS-binding activity may help delineate their functional roles. Sequence analysis revealed that 5830404H04Rik with the highest binding activity to PS shares 74% identity with human C2 calcium-dependent domain containing 2 (NM_015500). C2 domain is found in PKC, PLCδ and synaptotagmin, and is responsible for their Ca^{2+}-dependent phospholipid-binding activity [18]. Some isoforms of C2 domains do not appear to contain Ca^{2+}-binding sites [18].

Characterization of PS-binding proteins

The PS-binding activity of all identified phage clones was analyzed. All identified phage clones exhibited various PS-binding activity, ranging from ~12-fold to ~29,430-fold higher binding activity to immobilized PS than to blank well control, whereas the control phage showed no binding activity (Table 1). Their binding specificity to different phospholipids was also analyzed in a similar manner. All except one clone preferentially bound to immobilized PS, but not to PC. Fibrillarin was the only clone with better binding activity to PC than to PS. The unknown protein (5830404H04Rik) had the highest binding activity and specificity to PS. Consequently, this protein has the potential to be used as a detecting reagent or imaging probe for PS [4,5]. However, further characterization of its binding specificity to different phospholipids and comparison of its PS-binding affinity with other well-established PS-detecting reagent, such as annexin V, are necessary.

As the phage panning was performed in the absence of calcium, it is likely that identified PS-binding proteins were Ca^{2+}-independent. This was verified by their similar PS-binding activity in the presence and absence of calcium (Fig. 2A). All 4 tested phage clones bound to PS in the presence and absence of 2 mM $CaCl_2$ with no significant difference.

PS-binding motif

Given that PS is an anionic phospholipid, previously identified PS-binding domains were rich in basic amino acid residues, such as arginine (R) and lysine (K) [1]. For example, a study reported identification of PS-binding short peptides rich in RR sequences from a random peptide library by phage display [10]. However, adjacent peptides also seem to be important

for the binding activity [1]. Sequence analysis of the 17 identified PS-binding protein domains revealed that all identified proteins except fibrillarin had at least one RR, KK or RK motif (Fig. 3). The absence of RR sequence in fibrillarin may explain its minimal PS-binding specificity (Table 1). However, no obvious correlation between the abundance of RR, KK or RK motif and PS-binding activity was revealed, implicating that the surrounding motifs, such as hydrophobic side chains and an amphipathic helix, may also play an important role in PS-binding, as previously suggested [1].

Independent verification of PS-binding proteins

To independently verify the identified PS-binding proteins, GST fusion proteins of $Akap12_{345–522}$, $pinin_{187–306}$ and serum response factor binding protein 1 ($Srfbp_{1110–264}$) were expressed in *E. coli* and purified with glutathione columns. All 3 GST fusion proteins were co-purified with GST, which was likely a product of premature termination of translation (Fig. 2B). The fusion proteins were incubated with PS and PC liposomes, and washed by ultracentrifugation. The liposome pellets were analyzed for the liposome-binding proteins by Western blot using anti-GST Ab. The result showed that all 3 fusion proteins, but not GST control, bound to PS liposomes (Fig. 2B). In contrast, neither GST nor the fusion proteins bound to PC liposomes, suggesting that all 3 identified protein domains specifically bound to PS liposomes.

Although beyond the scope of this paper, it is important to verify the identified PS-binding proteins in a physiological context by demonstrating their binding to PS-rich membranes and possible functional roles in biological processes. Despite MFG-E8-phage was used as a positive control to verify the selection strategy, no known PS-binding protein except Akap12 was identified from the library in this study. One of the reasons is that some proteins, such as Gas6 and protein S, require Gla domain [1]. As *E. coli* lacks the posttranslational carboxylation of glutamic acid residues, the proteins displayed on phage surface cannot have the Gla domain. Consequently, the system is incapable of identifying these PS-binding proteins. Other proteins, such as PKC, PLCδ and synaptotagmin, bind to PS in a Ca^{2+}-dependent manner [1]. Although this study focused on Ca^{2+}-independent PS-binding proteins, modification of phage panning conditions, such as binding in the presence of Ca^{2+} and elution with EDTA, will enable the identification of Ca^{2+}-dependent PS-binding proteins.

ORF phage display

The key to improve the high percentage of ORFs in identified phage clones is the quality of ORF cDNA library. The ORF phage display cDNA library was generated based on the fact that ORF cDNA has no stop codon, whereas non-ORF has high frequency of stop codons. Database analysis revealed that ~96% of 200-bp non-ORF cDNAs have at least one stop codon [19]. This number drastically increases to 99.6% for non-ORF cDNAs with 300 bp. The engineered phages with ORF cDNA inserts were capable of expressing the C-terminal biotinylation tag that was biotinylated by *E. coli* BirA ligase [11,20]. Biotinylated phages were enriched by binding to immobilized streptavidin to generate ORF cDNA library, whereas non-ORF phages without biotinylation were eliminated. Because short non-ORF inserts may also lack stop codon, leading to biotinylation of cognate phages, the size distribution of the cDNA insert was critical for the quality of the ORF library. PCR analysis revealed that >75% of the ORF phage library clones had the cDNA inserts longer than 300 bp (not shown). Immunoblotting analysis with horseradish peroxidase (HRP)-conjugated streptavidin detected that >90% of the streptavidin-enriched library phage clones were biotinylated (not shown), suggesting that most cDNA inserts were ORFs. As most of the cDNA inserts (~62%) in the library were between 300 −500 bp, identified phage clones encoded protein domains with 38 to 228 amino acid residues, and most of them were around 100 residues (Fig. 3).

ORF phage display is a versatile technology. We have recently used it to identify proteins with different binding activities or functions. For example, we identified 15 new proteins with specific binding activity to a bait protein of tubby (unpublished data, Caberoy et al.), including 10 of them independently verified by yeast two-hybrid assay and protein pull-down assay. In another case, we used retinal pigment epithelium (RPE) cells, one of the most active phagocytes in the body, for a cell phagocytosis-based phage selection. In the absence of knowledge of phagocytic receptors, we identified 9 putative eat-me signals and further characterized two proteins capable of stimulating RPE phagocytosis thorugh a well-known phagocytic receptor (unpublished data Caberoy et al.). While most phage clones identified from conventional cDNA libraries were non-ORFs [8,9], nearly all phage clones identified in our 3 studies with protein bait (tubby), non-protein molecule (PS) or multimolecular bait (RPE cells) were ORFs encoding real proteins. Unlike unnatural short peptides identified from random peptide libraries, ORF phage display can identify real endogenous proteins with functional implications in protein networks. This strategy should be broadly applicable to different bait molecules, including proteins, non-protein molecules or multimolecular baits for efficient elucidation of protein biological networks, disease mechanisms or therapeutic targets.

Acknowledgements

This project was supported by NIH R01EY016211, P30-EY014801 and Research to Prevent Blindness. NBC is a recipient of a Fight for Sight postdoctoral fellowship. YZ is supported by a pre-doctoral fellowship from China Scholarship Council. We thank Dr. X. Jiang for technical assistance, and Dr. A Hackam for the critical reading of the manuscript.

References

1. Stace CL, Ktistakis NT. Phosphatidic acid- and phosphatidylserine-binding proteins. Biochim Biophys Acta 2006;1761:913–926. [PubMed: 16624617]
2. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature 2007;450:435–439. [PubMed: 17960135]
3. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature 2007;450:430–434. [PubMed: 17960134]
4. Laumonier C, Segers J, Laurent S, Michel A, Coppee F, Belayew A, Elst LV, Muller RN, A new peptidic vector for molecular apoptosis imagingof. identified by phage display technology. J Biomol Screen 2006;11:537–545. [PubMed: 16760366]
5. Thapa N, Kim S, So IS, Lee BH, Kwon IC, Choi K, Kim IS. Discovery of a phosphatidylserine-recognizing peptide and its utility in molecular imaging of tumour apoptosis. J Cell Mol Med 2008;12:1649–1660. [PubMed: 18363834]
6. Paschke M. Phage display systems and their applications. Appl Microbiol Biotechnol 2006;70:2–11. [PubMed: 16365766]
7. Zhang Y, Davis JL, Li W. Identification of tribbles homolog 2 as an autoantigen in autoimmune uveitis by phage display. Mol Immunol 2005;42:1275–1281. [PubMed: 15950723]
8. Lin HS, Talwar HS, Tarca AL, Ionan A, Chatterjee M, Ye B, Wojciechowski J, Mohapatra S, Basson MD, Yoo GH, Peshek B, Lonardo F, Pan CJ, Folbe AJ, Draghici S, Abrams J, Tainsky MA. Autoantibody approach for serum-based detection of head and neck cancer. Cancer Epidemiol Biomarkers Prev 2007;16:2396–2405. [PubMed: 18006929]
9. Kalnina Z, Silina K, Meistere I, Zayakin P, Rivosh A, Abols A, Leja M, Minenkova O, Schadendorf D, Line A. Evaluation of T7 and lambda phage display systems for survey of autoantibody profiles in cancer patients. J Immunol Methods 2008;334:37–50. [PubMed: 18314130]
10. Nakai Y, Nomura Y, Sato T, Shiratsuchi A, Nakanishi Y. Isolation of a Drosophila gene coding for a protein containing a novel phosphatidylserine-binding motif. J Biochem 2005;137:593–599. [PubMed: 15944412]

11. Caberoy NB, Zhou Y, Li W. Can phage display be used as a tool to functionally identify endogenous eat-me signals in phagocytosis? J Biomol Screen. 2009In press
12. Li W, Handschumacher RE. Identification of two calcineurin B-binding proteins: tubulin and heat shock protein 60. Biochim Biophys Acta 2002;1599:72–81. [PubMed: 12479407]
13. Lee G, Pollard HB. Highly sensitive and stable phosphatidylserine liposome aggregation assay for annexins. Anal Biochem 1997;252:160–164. [PubMed: 9324954]
14. Gustincich S, Vatta P, Goruppi S, Wolf M, Saccone S, Valle GDella, Baggiolini M, Schneider C. The human serum deprivation response gene (SDPR) maps to 2q32-q33 and codes for a phosphatidylserine-binding protein. Genomics 1999;57:120–129. [PubMed: 10191091]
15. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature 2002;417:182–187. [PubMed: 12000961]
16. Lin X, Tombler E, Nelson PJ, Ross M, Gelman IH. A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. J Biol Chem 1996;271:28430–28438. [PubMed: 8910468]
17. Nauert JB, Klauck TM, Langeberg LK, Scott JD, Gravin anautoantigenrecognizedbyserumfrom, patients myastheniagravis. is a kinase scaffold protein. Curr Biol 1997;7:52–62. [PubMed: 9000000]
18. Rizo J, Sudhof TC. C2-domains structure and function of a universal Ca2+-binding domain. J Biol Chem 1998;273:15879–15882. [PubMed: 9632630]
19. Garufi G, Minenkova O, Passo CLo, Pernice I, Felici F. Display libraries on bacteriophage lambda capsid. Biotechnol Annu Rev 2005;11:153–190. [PubMed: 16216777]
20. Ansuini H, Cicchini C, Nicosia A, Tripodi M, Cortese R, Luzzago A. Biotin-tagged cDNA expression libraries displayed on lambda phage: a new tool for the selection of natural protein ligands. Nucleic Acids Res 2002;30:e78. [PubMed: 12140340]

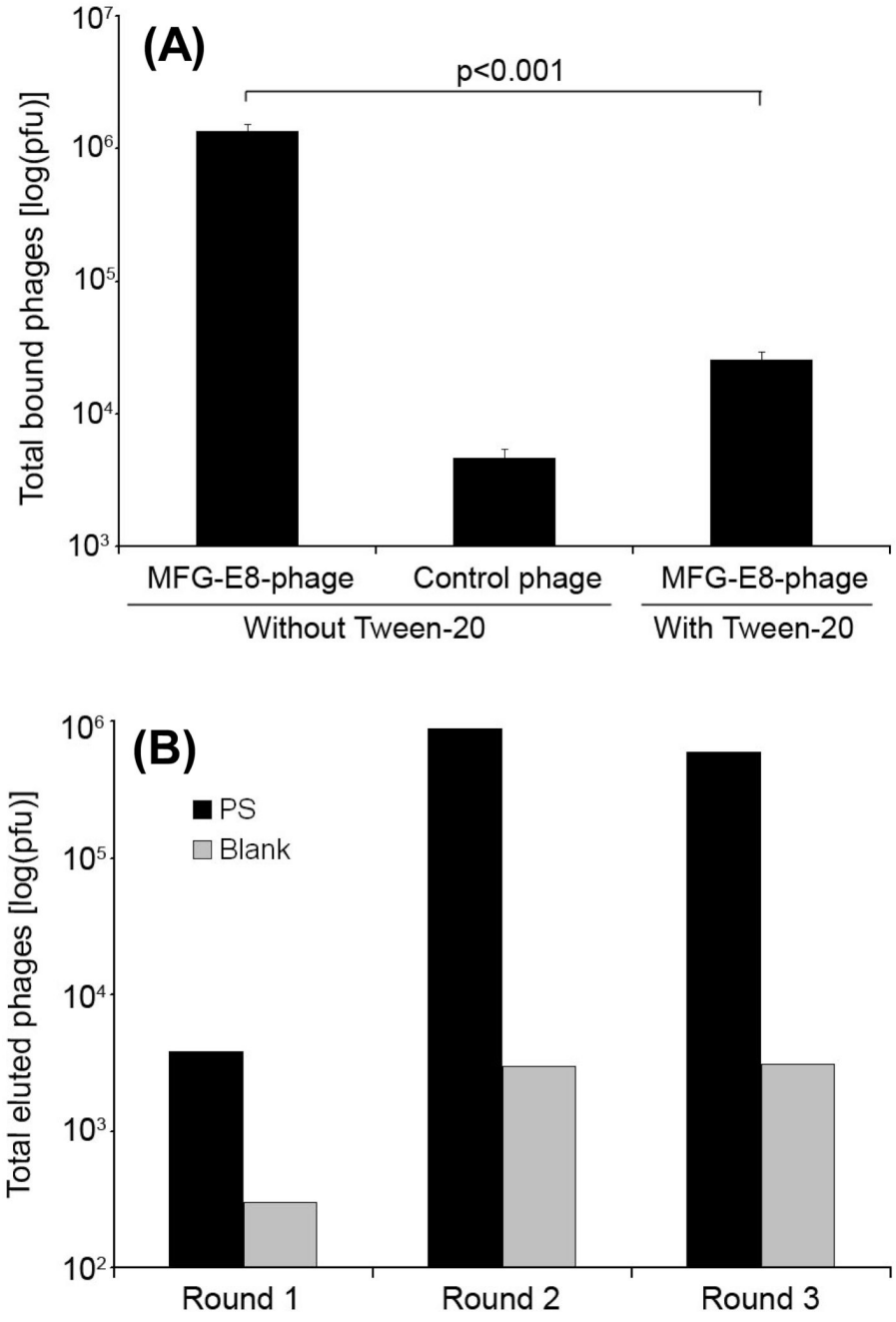

Fig. 1.

(A) Optimization of phage binding to immobilized PS. MFG-E8-phage bound to immobilized PS in the absence of Tween-20, and washed with PBS in the presence or absence of 0.1% of Tween-20. Tween-20 in the washing buffer significantly reduced MFG-E8-phage binding to PS (± S.D., n=3, Student's t-test). **(B)** PS-specific phage enrichment. The ORF phage library was selected by 3 rounds of phage panning with immobilized PS. Bound phages were eluted and quantified by plaque assay. Phage binding to mock coated wells was analyzed to monitor possible enrichment of plate-binding phages.

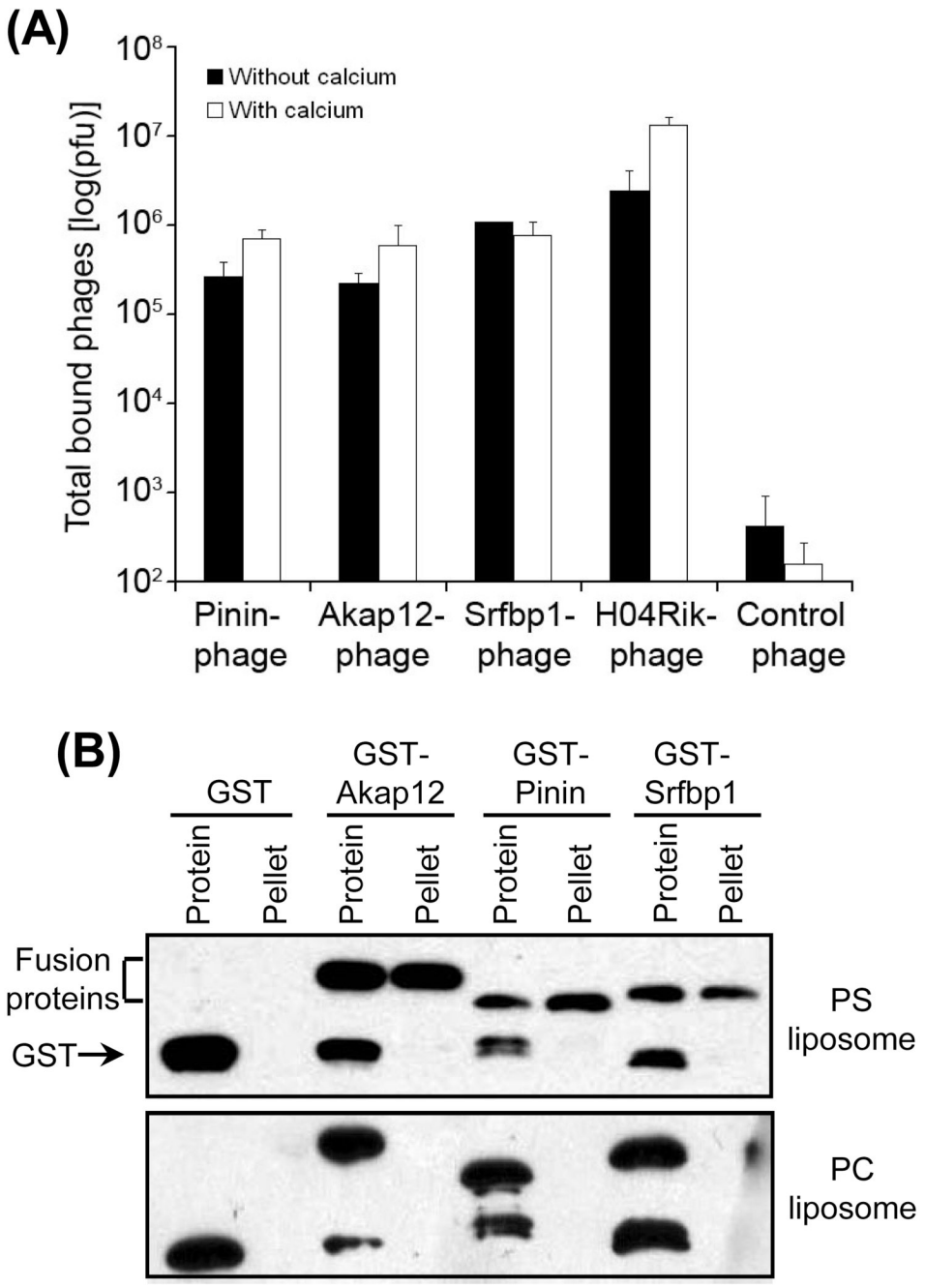

Fig. 2.

(**A**) Ca^{2+}-independent PS-binding activity. Phage clones of pinin, Akap12, Srfbp1 and an unknown protein (5830404H04Rik) were analyzed for their binding activity to immobilized PS in the presence or absence of 2 mM $CaCl_2$. Bound phage were eluted and quantified by plaque assay (±S.D., n=3). **(B)** Independent validation of PS-binding activity of Akap12, pinin and Srfbp1 by liposome binding assay. Purified GST fusion proteins of $Akap12_{345–522}$, $pinin_{187–306}$ and $Srfbp1_{110–264}$ were incubated with PS or PC liposomes. After washing by ultracentrifugation, liposome pellets were analyzed by Western blot using anti-GST mAb. All 3 proteins specifically bound to PS liposomes, but not PC liposomes. GST was included as a negative control.

```
Pinin:          QVENERRELFEERRAKQTELRLLEQKVELAQLQEEWNEHNAKIIKYIRTKTKPHLFYIPGRMCPATQKLIEESQRKMNALFE
                GRRIEFAEQINKMEARPRRQSMKEKEHQVVRNEEQKAE
Vti1b:          EEKKKLVRDFDEKQQEANETLAEMEEELRYAPLTFRNPMMSKLRNYRKDLAKLHREVRSTPLT
Fibrillarin:    MKPGFSPRGGGFGGRGGFGDRGGRGGGRGGRGGFGGGRGGFGGGG
Akap12:         DQARLSADYEKVELPLEDQVGDLEALSEKCAPLATEVFDEKTEAHQEVVAEVHVSTVEKMTKGQGGAEVEGDVVVEGSGESL
                PPEKLAETQEVPQEAEPVEELMKTKEVCVSGGDHTQLTLSPEEKMLPKHPEGIVSEVEMLSSQERIKVQGSPLKKLFSSSGL
                KKLSGKKQKGKRG
5830404H04Rik:  PEENEPAQTLPALKPRENDLDSWELEKESPVASWSGPALQEPDGDELSESSLSTSELGAMKKHKGGLLRKGAKLFFRRRHQQ
                KDPGLSQSHNDLV
Col11a2:        PGQKGETGIPGASGPIGPGGPPGLPGPSGPKGAKGATGPAGPKGEKGVQGPPGHPGPPGEVIQPLPIQMPKKTRRSVDGSKL
                IQDEEAVPTGGAPGSPAGLEEIFGSLDSLREEIEQMRRPA
Mylk:           KYMARRKWQKTGNAVRAIGRLSSMAMISGLSGRKSSTGSPTSPINAEKLESEDDVSQAFLEAVAEEKPHVKPYFSKTIRDLE
                VVEGSAARFDCKIEGYPDPEVVWFKDDQSIRESRHFQIDYDEDGNCSLIISDVCGDDDAKYTCKAVNSLGEATCTAELIVET
                MEEG
Srfbp1:         VARLAGHPLLKKKIDVLKAAVQAFKDARQSAPAAESSESTSGEGRYKDIARSKDDASESQHPERTVVSEQKAKDTNTAAKNA
                GSGSKEKLAKTEQAPRAGTTPGSQGKPSGKGAGVNSEHQGAPAPGDSNQGKASTKTPADSVCEPANNGVSEEEE
Prpf40a:        EESSKQEECTTASTAPVPTTEIPTTMSTMAAAEAAAAVVAAAAAAAAAAANANTSTTPTNTVGSVPVAPEPEVTSIVATAVDN
                ENTVTVSTEEQAQLANTTAIQDLSGDISSNTGEEPAKQETVSDFTPKKEEEESQPAKKTYTWNTKEEAKQAFKELLK
C2cd2l:         RSPSKVEVTEKMTTVLSESSGPSNASHSSSRESHLSNGLDPVAETAIRQLTEPSGRAAKKTPTKRSTLIISGVSKVPIAQDE
                LALSLGYAASLEASMQDDAGTSGGPSSPPSDPSATSPGPVDALSSPTSVQEADETTRSDISERPSVDDVESETGSTGALETR
                SLKDHKVSFLRSGTKLIFRRRPRQKEAGLSQSHDDLSNTTATPSVRKKAGSFSRRLIKRFSFKS
2500003M10Rik:  MLKNKQPMPVNIRASMQQQQQLASARNRRLAQQMENRPSVQAALKLKQ-SLKQRLGKSNIQARLGRPIGALARGAIGGRGLP
                IIQRGLPRGGLRGGR
Akap8l:         TKKKKRKQGGSPDEPDSKATRTDCSDNSDSDNDEGTEG
1200014J11Rik:  DKMRSRDASEDKSSEKNKKDKQEDSSDDDETEEGEVEDENSSDVELDTLSQVEEESLLRNDLRPANQLAKGNRLFMRFATKD
                DQKELGAARRSQYYMKYGNPNYGGMKGILSNSWKRRYHSRRIQRDVIKKRALIGDDVGLTSYKHRHSGLVNVPEEPIEE
```

Fig. 3.

Amino acid sequences of identified PS-binding proteins. Basic amino acid residues of arginine (R) and lysine (K) are highlighted with shadow gray. All PS-binding proteins except fibrillarin have dimeric basic amino acid residues of RR, KK or RK. Fibrillarin is the only identified protein without PS-binding specificity (Table 1).

Table 1

PS-binding proteins

Protein	Accession number	Matched aa residues	Freq. [a]	Binding specificity [b]	
				PS	PC
ORF matched to protein coding sequence					
Pinin (Pnn)	NM_008891	187Q-306E	1	~354X	~3X
Vesicle transport through interaction with t-SNAREs 1B homolog (Vti1b)	NM_016800	39E-101T	1	~37X	~1X
Fibrillarin (Fbl)	NM_007991	1M-45G	1	~24X	~480X
A kinase (PRKA) anchor protein (gravin) 12 (Akap12)	NM_031185	345D-522G	1	~1,067X	~2X
RIKEN cDNA 5830404H04 gene (5830404H04Rik)	NM_174847	560P-654V	3	~29,430X	~7X
Procollagen, type XI, alpha 2 (Col11a2)	NM_009926	1354P-1475A	1	~321X	~6X
Myosin, light polypeptide kinase (Mylk)	NM_139300	1769K-1936G	1	~88X	~1X
Serum response factor binding protein 1 (Srfbp1)	NM_026040	110V-264E	2 [c]	~577X	~4X
PRP40 pre-mRNA processing factor 40 homolog A (yeast) (Prpf40a)	NM_018785	243E-401K	1	~666X	~1X
C2 calcium-dependent domain containing 2-like (C2cd2l)	NM_027909	467R-694S	1	~725X	~8X
RIKEN cDNA 2500003M10 gene (2500003M10Rik)	NM_023215	1M-96R	2 [c]	~1,367X	~4X
A kinase (PRKA) anchor protein 8-like (Akap8l)	NM_017476	274T-311G	1	~248X	~7X
RIKEN cDNA 1200014J11 gene (1200014J11Rik)	NM_025818	186K-345E	1	~12X	~1X
Control phage with no cDNA insert				~1X	~1X
Non-ORF			0		

[a] Clone frequency. All identified PS-binding phage clones encodes proteins with ORFs.

[b] Binding specificity is the pfu ratio of clonal phage binding to PS or PC versus to the mock coated wells. Phage binding activities were representatives of three independent experiments.

[c] Independent phage clones with overlapping, but not identical, amino acid sequences. Three redundant clones for 5830404H04Rik (NM_174847) were identical.