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Localization of the Phosphatidylserine-Binding Site of Glyceraldehyde-3-Phosphate Dehydrogenase Responsible for Membrane Fusion[1]

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In this study, we demonstrated that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a phosphatidylserine (PS)-binding protein and localized the putative PS-binding site involved in the membrane fusion induced by this enzyme. In an attempt to identify the PS-binding proteins, we raised polyclonal antibodies against a 15-amino-acid synthetic peptide (amino acid residues 390-403 of phosphatidylserine decarboxylase), which was shown to bind specifically to PS. One polyclonal antibody, designated aPSD-2, crossreacted with GAPDH, and its binding to GAPDH was inhibited by PS but not by other phospholipids such as phosphatidylethanolamine and phosphatidylinositol. Kinetic analysis of GAPDH binding to phospholipid membranes by measuring surface plasmon resonance showed that GAPDH associated with the phospholipid membrane containing PS rapidly ($k_{on}=2.8\times10^4$ $M^{-1}\cdot s^{-1}$) and dissociated extremely slowly ($k_{off}=5.9\times10^{-5}$ s^{-1}), giving a low dissociation constant ($K_D=2.6$ nM). GAPDH bound less effectively to membranes without PS with a dissociation constants of 0.2 μM. GAPDH-induced vesicle fusion was also inhibited by aPSD-2, suggesting that this antibody recognizes the putative PS-binding site on GAPDH involved in the enzyme-induced membrane fusion. Chemical fragmentation of GAPDH with cyanogen bromide followed by separation and sequence analysis of the reactive peptide resulted in the identification of a single reactive peptide with the sequence of amino acid residues 45-103 of GAPDH. Analysis of aPSD-2 binding to synthetic peptides derived from the corresponding region localized the antibody-binding site to amino acid residues 70-94 of GAPDH. Both the 25-amino-acid synthetic peptide (amino acid residues 70-94 of GAPDH) and polyclonal antibody raised against this peptide inhibited GAPDH-induced membrane fusion, suggesting that these amino acid residues play a crucial role in this membrane fusion process.

Key words: glyceraldehyde-3-phosphate dehydrogenase, membrane fusion, phosphatidylethanolamine, phosphatidylserine, surface plasmon resonance.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.2.12; GAPDH) is a glycolytic enzyme that catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate (*1-3*). It is a tetramer composed of identical subunits with a molecular mass of 37 kDa (*1, 4*). The activity of this enzyme is regulated by its association with cell membranes, possibly through interacting with the anion transporter protein AE-1 (band 3 of erythrocyte membrane protein) (*5-7*). Recent studies have shown that GAPDH is a multi-functional protein that binds to DNA, causing activation of transcription as well as DNA replication (*8-10*), and that induces membrane fusion (*11*). In a study of the fusogenic activity of GAPDH, Glaser and Gross (*11*) isolated a protein from brain supernatant that induced vesicle fusion and identified this protein as GAPDH. They showed that an isoform of GAPDH catalyzed vesicle fusion and that the isoforms which mediated dehydrogenase activity and membrane fusion activity represent separate and distinct GAPDH isoforms. The fusion catalyzed by GAPDH was highly dependent on the presence of plasmenylethanolamine (pl-PE) in the vesicles, and fusion was not induced when phosphatidylethanolamine (PE) was incorporated into the vesicles instead of pl-PE. GAPDH also showed a strict requirement for phosphatidyl-

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aPSD, a rabbit polyclonal antibody raised against a 15-amino-acid synthetic peptide (FNFRLKAGQKIRFGC, amino acids residues 390-403) derived from phosphatidylserine decarboxylase; PSD, phosphatidylserine decarboxylase; PS, phosphatidylserine; PE, phosphatidylethanolamine; pl-PE, plasmenylethanolamine; PI, phosphatidylinositol; PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; CHO cell, Chinese hamster ovary cell; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; mAb, monoclonal antibody; k_{on}, association rate constant; k_{off}, dissociation rate constant; K_D, dissociation constant; CNBr, cyanogen bromide; BSA, bovine serum albumin.

serine (PS) for vesicle fusion. The most rapid GAPDH-induced membrane fusion occurred when the vesicles contained 6 mol% of PS, although this fusion process was independent of the presence of Ca^{2+}, which has been shown to induce fusion of membranes containing PS (*12, 13*). Although these results suggest that GAPDH interacts with membrane phospholipids such as PS and/or pl-PE, there is, to the best of our knowledge, no information available about the protein structure responsible for the interactions with these phospholipids. The molecular natures of the phospholipids, which always form multimolecular complexes such as bilayers and micelles, and stick nonspecifically to proteins through hydrophobic and electrostatic interactions, has hampered the detailed analysis of phospholipid-protein interactions.

We have employed an immunochemical approach to study the molecular mechanisms responsible for the specific lipid-protein interactions (*14-18*). As the use of anti-idiotypic antibodies directed against combining sites of antibodies revealed that the ligand-binding sites of the receptor molecules and the combining sites of antibody molecules of equivalent specificities were structurally similar (*19-21*), phospholipid-binding sites of cellular proteins could be identified using anti-idiotypic antibodies against the combining sites of anti-phospholipid antibodies. In a previous study, we showed that an anti-idiotypic monoclonal antibody (mAb) against the combining site of a PS-specific mAb cross-reacted extensively with protein kinase C and localized the PS-binding site on the enzyme (*16, 17*). We identified a conserved 12-amino-acid peptide motif between protein kinase C and phosphatidylserine decarboxylase (PSD) and demonstrated that a 15-amino-acid synthetic peptide (FNFRLKAGQKIRFGC) (*22, 23*) derived from the α-subunit of PSD bound effectively and specifically to PS (*17*). In this study, we raised polyclonal antibodies against the synthetic peptide derived from PSD and found that one antibody, designated aPSD-2, cross-reacted with GAPDH. aPSD-2 binding to GAPDH was inhibited specifically by PS, and this antibody also inhibited GAPDH-induced membrane fusion. These results suggest that this antibody binds to the PS-binding site of GAPDH involved in membrane fusion. We mapped the aPSD-2 binding site of GAPDH to 25 amino acid residues and analyzed the role of this region in GAPDH-induced membrane fusion.

EXPERIMENTAL PROCEDURES

Polyclonal Antibody Production—Synthetic peptides were synthesized using an automated peptide synthesizer (Advanced Chemtech, model 396 MPS) and an extra cysteine residue was added to the C-terminal end of each peptide (*16*). The peptides were purified by reverse-phase HPLC with a Puresil C18 column (Waters, Milford, MA) using a 5-60% (v/v) acetonitrile linear gradient containing 0.1% (v/v) trifluoroacetic acid. The sequence and molecular mass of each peptide were confirmed using an amino acid sequencer and a JMS-HX110 double focusing mass spectrometer equipped with a fast atom bombardment ion source and a JMA-DA5000 data system (JEOL, Tokyo), respectively. Polyclonal antibodies against a 15-amino-acid synthetic peptide (FNFRLKAGQKIRFGC, amino acid residues 390-403) (*22, 23*) derived from CHO cell PSD was generated in Japanese White female rabbits. The peptide was conjugated with maleimide-activated keyhole lympet hemocyanin (KLH) (Pierce Chemical, Rochford, IL) according to the manufacturer's protocol. Three rabbits were immunized subcutaneously with 150 μg synthetic peptide-KLH conjugate in Freund's complete adjuvant, followed by three injections of the antigen in Freund's incomplete adjuvant at 2-wk intervals. Immunoglobulin G (IgG) was isolated from the preimmune and immune sera of the rabbits by ammonium sulfate precipitation and affinity chromatography on a protein G-Sepharose (Pharmacia Biotec) column. One polyclonal antibody, designated aPSD-2, was further purified by affinity chromatography on the immobilized synthetic peptide coupled to 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP)-activated Cellulofine according to the manufacturer's protocol (Seikagaku, Tokyo). A polyclonal antibody against a 25-amino-acid synthetic peptide (KPITIFQERDPVKIKWGDAGAEYVC, amino acid residues 70-94 of rat GAPDH; access #: Swiss-prot P04797) was produced and purified in the same manner.

Measurement of Antibody Binding to GAPDH—Immunoblotting analysis: Proteins from various sources were separated by SDS-PAGE using a 10% (w/v) acrylamide gel and transferred to nitrocellulose membranes at 4°C using the protein transfer system (Bio-Rad, Hercules, CA), as described previously (*24*). Each nitrocellulose membrane with blotted protein was blocked by incubation with Tris-buffered saline (TBS, 10 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl) containing 30 mg/ml bovine serum albumin (3% BSA-TBS) at room temperature for 2 h with gentle shaking, then incubated with the polyclonal antibody aPSD-2 (2 μg/ml) in TBS containing 10 mg/ml BSA (1% BSA-TBS) for 2 h at room temperature, followed by incubation with biotinylated anti-rabbit IgG (Vector, CA) and peroxidase-conjugated streptavidin (Zymed). The antibody bound to the membrane was detected by color development using 4-chloro-1-naphthol as the substrate. GAPDH was subjected to two-dimensional polyacrylamide gel electrophoresis (PAGE), performed according to the method of O'Farrell (*25*) with the following modification (*26*). For the first isoelectric focusing separation, 20 μg of purified GAPDH from rabbit muscle was focused for 3 h at 500 V, followed by focusing for 1 h at 700 V in gels containing 3.8% acrylamide, 0.2% bis-acrylamide, 8 M urea, 1% Biolyte 7/9® Ampholyte (Bio-Rad, Hercules, CA), and 1% Biolyte 3/10® Ampholyte and 2% CHAPS (Dojindo, Kumamoto). After isoelectric focusing, the proteins were subjected to SDS-PAGE using 10% (w/v) acrylamide gel and the protein bands were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane followed by immunoblotting analysis, as described above.

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA was performed at room temperature as described previously (*17*). In brief, the wells of microtiter plates (Immulon 2, Dynatech Laboratories, Alexandria, VA) were coated with either GAPDH or the required peptide, and aPSD-2 binding was evaluated using biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin, followed by color development with *o*-phenylenediamine as the substrate. The effects of various phospholipid vesicles on aPSD-2 binding to GAPDH were examined by inhibition assay of ELISA. The GAPDH-coated wells were preincubated with phos-

pholipid vesicles for 1 h at room temperature, then aPSD-2 binding to GAPDH was evaluated as described above. The phospholipid vesicles were prepared as described previously (*14*). In brief, dried lipid films containing various phospholipids (2 μmol) were swollen in TBS and sonicated (Branson Sonifier model 250D) for 10 min on ice. The vesicles were composed entirely of PC only or a combination of PC and another phospholipid at a molar ratio of 3:1. PS from bovine brain, PI and PE from bovine liver, and PC were purchased from Avanti Polar-Lipids. pl-PE was purchased from Serdary Research Lab. and cholesterol from Sigma Chemicals.

Measurement of GAPDH-Induced Vesicle Fusion—GAPDH-induced vesicle fusion was studied by measuring lipid mixing using octadecylrhodamine as described by Glaser and Gross (*11*). Phospholipid vesicles composed of PC (27 mol%), pl-PE (27 mol%), PS (6 mol%), and cholesterol (40 mol%) were prepared as described above. Separate populations of vesicles containing, in addition, 4 mol% octadecylrhodamine were also prepared. Equimolar amounts of labeled and unlabeled vesicles were suspended in assay buffer [100 mM sodium chloride, 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.0, and 0.1 mM EGTA] to a final concentration of 40 μM, and the reaction was started by addition of GAPDH. The 100% fusion level was assessed by quantifying the octadecylrhodamine fluorescence of the liposomes comprised of the mixture which would result if all the vesicles fused. The fluorescence intensity was monitored at 590 nm after excitation at 560 nm utilizing a Hitachi spectrofluorometer model 2000F equipped with a stirring apparatus at 37°C.

Kinetic Analysis of GAPDH-Binding to Immobilized Phospholipid Membranes—GAPDH binding to phospholipid membranes was measured using a BIAcore™ system instrument (Pharmacia Biosensor AB, Uppsala, Sweden), an optical biosensor based on the principles of surface plasmon resonance (*27*). The sensor chip HPA has a surface composed of long-chain alkanethiol molecules in a flat quasi-crystalline hydrophobic layer (Pharmacia Biosensor AB), and formation of lipid bilayers by fusion of phospholipid vesicles with the alkanethiol monolayer on the sensor chip was performed according to the manufacturer's protocol. Small unilamellar vesicles composed entirely of PC or a combination of PC and another phospholipid at a molar ratio of 1:1 were prepared in 20 mM 4-(2-hydroxyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0, containing 150 mM NaCl, as described above. To immobilize the phospholipid layer on the sensor chip, the sensor chip was washed with 40 mM octyl glucoside, and vesicles containing 0.5 mM phospholipid were injected into the BIAcore system at a flow rate of 5 μl/min at 25°C. The analyte binding assay was performed repeatedly after washing the surface with 30 mM NaOH using 50 μl at a flow rate of 20 μl/min. The binding kinetics were analyzed according to the manual of the software Biaevaluation 2.1. The resonance unit is an arbitrary unit used by the BIAcore system, and there is a linear relationship between the mass of protein bound to the sensor chip and the resonance unit recorded (*28, 29*).

Localization of the aPSD-2-Binding Site on GAPDH—Rabbit muscle GAPDH (1 mg) was subjected to CNBr fragmentation after reduction and *S*-carboxymethylation of the cystein/cystine residues. The resulting peptide fragments were separated by reverse-phase HPLC with a PEGASIL-300 C4 column (Senshu, Tokyo) using a 5-80% (v/v) acetonitrile linear gradient containing 0.1% (v/v) trifluoroacetic acid. An aliquot of each fraction was coated onto microtiter wells, and aPSD-2 binding to the fragment-coated wells was evaluated by ELISA, as described above. The reactive peptide fractions were separated by discontinuous SDS-PAGE according to the method of Schägger and van Jagow (*30*). The aPSD-2-reactive peptide band was identified after transferring the peptide to a PVDF filter membrane (Mini Probott Membranes, Applied Biosystems) followed by immunoblotting analysis, as described above. The reactive peptide band was excised from the PVDF filter and subjected to N-terminal amino acid sequence analysis with an amino acid sequencer (Applied Biosystems model 477A). Synthetic peptides derived from amino acid residues 45-94 of rabbit muscle GAPDH were synthesized and purified by reverse-phase HPLC. Microtiter plate wells were coated with peptides (100 ng/well) by incubation for 16 h at 37°C, then incubated with aPSD-2 (2 μg/ml), and aPSD-2 binding was evaluated by ELISA, as described above.

RESULTS AND DISCUSSION

Binding of Anti-PS Motif to GAPDH—PSD is a pyruvoyl-dependent enzyme which strictly recognizes the molecular structure of PS and catalyzes the conversion of PS to PE (*31, 32*). In a previous study, we showed that a

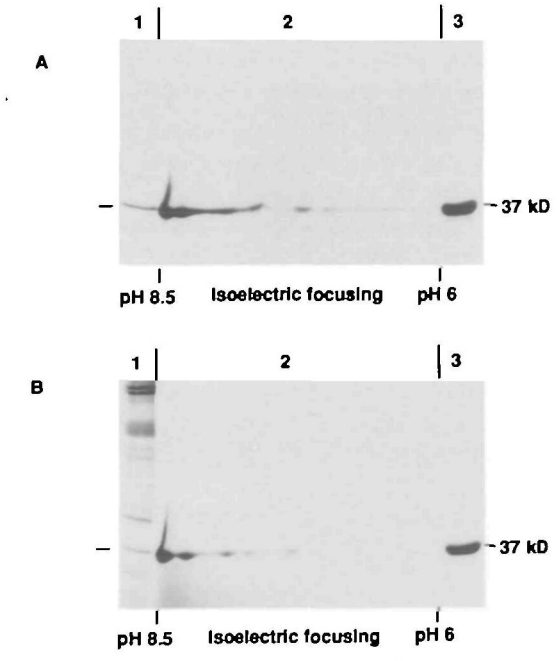


Fig. 1. **Binding of aPSD to GAPDH.** GAPDH from rabbit muscle (16 μg) was separated by isoelectric focusing and subjected to SDS-PAGE using 10% (w/v) acrylamide gels (lane 2). In SDS-PAGE, erythrocyte membrane proteins (14 μg) (lane 1), and GAPDH (8 μg) (lane 3) were loaded onto the same polyacrylamide gel. The proteins were either transferred to nitrocellulose membranes and subjected to immunoblotting analysis with 2 μg/ml aPSD-2 (panel A) or stained with Coomassie Brilliant Blue (panel B). In panel A, bound aPSD-2 was detected with biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin.

15-amino-acid synthetic peptide (FNFRLKAGQKIRFGC) derived from the α-subunit of PSD bound specifically to PS and proposed that the consensus sequence between PSD and protein kinase C, FXF(I/V/L)(K/R)XX(D/Q)K, mediates the specific interactions of these proteins with PS (*17*).

To search for cellular PS-binding proteins with similar peptide motifs, we raised polyclonal antibodies against the above 15-amino-acid synthetic peptide derived from CHO cell PSD and analyzed their binding with various cellular proteins. Immunoblotting analysis of human erythrocyte membranes showed that one polyclonal antibody, designated aPSD-2, bound to a 37-kDa band corresponding to band 6 (GAPDH) of erythrocyte membranes, and immunoblotting after two-dimensional electrophoresis of rabbit muscle GAPDH showed this antibody bound to all GAPDH isoforms (Fig. 1, A and B). The ELISA showed that aPSD-2 binding to GAPDH was inhibited by vesicles containing PS but not by those containing other phospholipids such as PE and PI (Fig. 2). These results suggest that aPSD-2 bound to a region of GAPDH which may be involved in the interaction with PS.

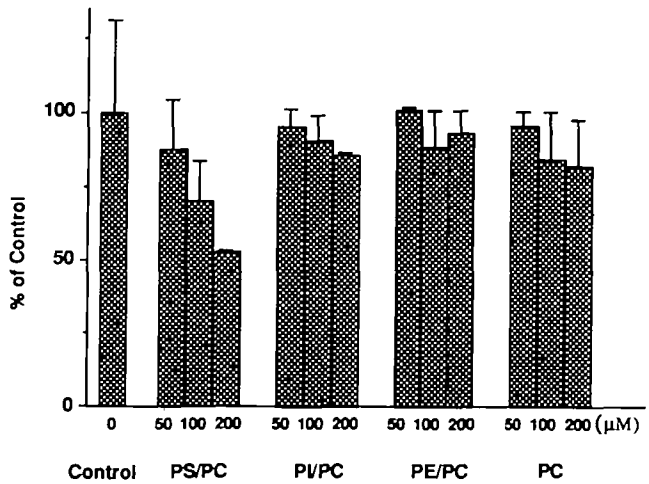


Fig. 2. **Effects of various phospholipid vesicles on aPSD-2 binding to GAPDH.** The wells of microtiter plates were coated with 4 μg/ml GAPDH, preincubated with vesicles containing PS, PI, PE, or PC, then incubated with aPSD-2 (2 μg/ml). aPSD-2 bound to GAPDH coated on the plates was detected with biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin. The results represent the mean value±SD of three different experiments.

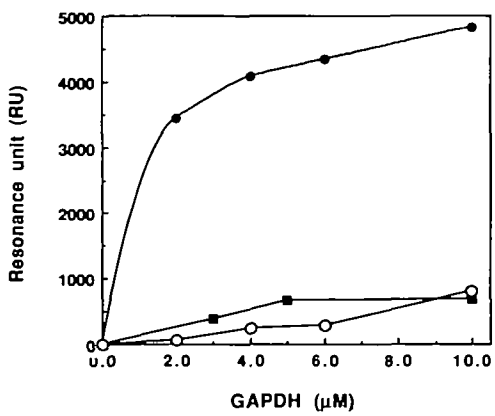


Fig. 3. **GAPDH-binding to immobilized phospholipid membranes.** The alkanethiol monolayers associated with the thin gold film on the sensor chips were coated with phospholipid vesicles, and GAPDH binding to the immobilized phospholipid membranes was examined by measuring the surface plasmon resonances. The vesicles were composed of either 50 mol% PC and 50 mol% PS (●), 50 mol% pl-PE and 50 mol% PC (■), or 100 mol% PC (○). The resonance unit is an arbitrary unit used by the BIAcore system and there is a linear relationship between the mass of the protein bound to the sensor chip and the resonance unit observed (*28, 29*). Each value represents the means of two different experiments.

Kinetic Analysis of GAPDH Binding to Phospholipid Membranes—We analyzed the interaction between GAPDH and phospholipid membranes by measuring the binding of GAPDH to phospholipid membranes directly using the BIAcore™ system, an optical biosensor based on the principles of surface plasmon resonance (*28*). The alkanethiol/gold surface of the sensor chip was coated with phospholipid vesicles of various phospholipid compositions, and kinetic analysis of the interactions between the phospholipid membranes and GAPDH at various concentrations (0.2-10 μM) was performed. GAPDH binding to phospholipid membranes composed of PC was enhanced in the presence of PS, whereas incorporation of either pl-PE or PE (data not shown) into the membrane surfaces had no effect on GAPDH binding (Fig. 3). Kinetic analysis of GAPDH binding to phospholipid membranes composed of PS/PC (1:1) showed that GAPDH bound rapidly ($k_{on}=2.8\pm1.9\times10^4$ $M^{-1}\cdot s^{-1}$) and dissociated extremely slowly ($k_{off}=5.9\pm1.9\times10^{-5}$ s^{-1}), giving a low dissociation constant ($K_D=2.6\pm1.2$ nM; Table I). GAPDH bound less effectively to membranes composed of either PC only or PE/PC (1:1) with a dissociation constants of 0.2 and 0.4 μM, respectively. The resonance unit is an arbitrary unit used in the BIAcore system, and there is a linear relationship between

TABLE I. **Kinetics of GAPDH binding to phospholipid membranes.** Kinetic analysis of GAPDH binding to immobilized phospholipid membranes was performed using BIAcore™ system instrument as described in experimental procedures.

Lipid composition	k_{on} ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})	K_D (M)
PC	3.5±2.7 (×10³)	7.2±3.8 (×10⁻⁴)	2.4±0.8 (×10⁻⁷)
pl-PE/PC	3.1±1.7 (×10³)	8.6±0.4 (×10⁻⁴)	4.1±2.4 (×10⁻⁷)
PS/PC	2.8±1.9 (×10⁴)	5.9±1.9 (×10⁻⁵)	2.6±1.2 (×10⁻⁹)

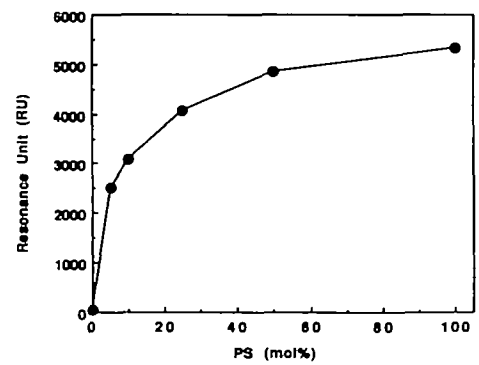


Fig. 4. **PS-dependent binding of GAPDH to immobilized phospholipid membranes.** The sensor chips were coated with phospholipid vesicles composed of PC and various amounts of PS, and GAPDH binding to the immobilized phospholipid membranes was examined by measuring the surface plasmon resonances. Each value represents the means of two different experiments.

the mass of protein bound to the sensor tip and the resonance unit observed (*28, 29*). As shown in Fig. 4, the amounts of GAPDH bound to membrane surfaces was increased by PS in a concentration-dependent manner and reached a plateau when the membrane contained 50 mol% PS.

The low dissociation was attributable to the extremely slow dissociation of GAPDH from the PS-containing membrane ($k_{off} = 5.9 \times 10^{-5}\ s^{-1}$). GAPDH has been shown to be present as a tetrameric form in solution, and this oligomeric state of the enzyme may lead to multivalent interactions with membrane surfaces, resulting in slow enzyme dissociation from membrane surfaces. Heat denaturation of GAPDH (100°C for 10 min) abolished GAPDH binding to the membranes, suggesting that the conformational structure of the enzyme is essential for binding (data not shown). These results demonstrate clearly that GAPDH is a PS-binding protein and the region recognized by aPSD-2 is involved in the interaction with PS.

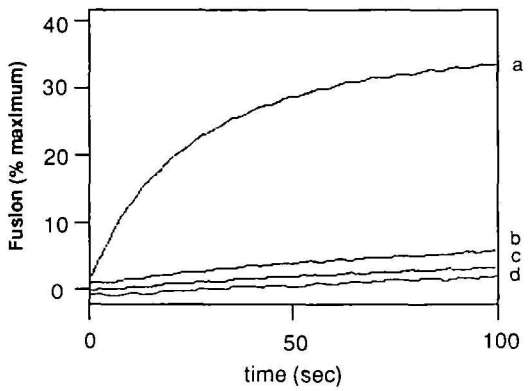
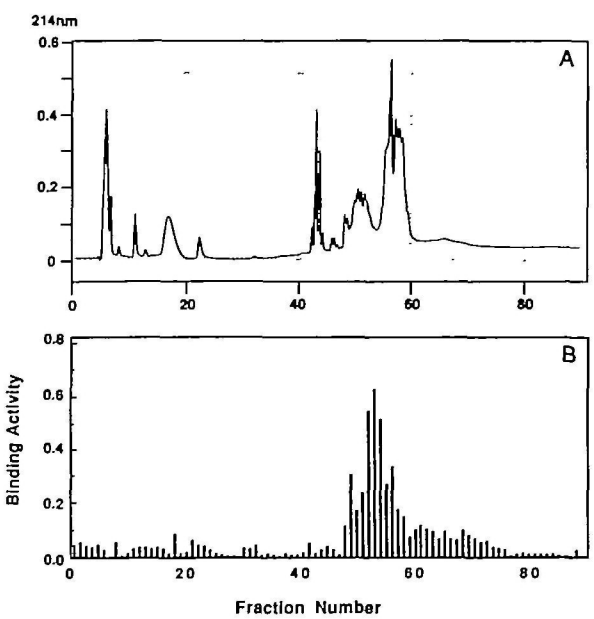


Fig. 5. **Inhibition of GAPDH-induced vesicle fusion by aPSD-2.** Vesicles composed of PC (27 mol%), pl-PE (27 mol%), PS (6 mol%), and cholesterol (40 mol%) and the same amount of identical vesicles also containing 4 mol% octadecylrhodamine were mixed with GAPDH (final concentration of 3 μg/ml), and vesicle fusion was assessed by monitoring the increased fluorescence intensity resulting from the dequenching of octadecylrhodamine. The effect of aPSD-2 on GAPDH-induced vesicle fusion was studied by preincubating GAPDH (5 μg/ml) with various amounts of either aPSD-2 or control rabbit IgG for 60 min at 22°C, adding the resulting mixture to the vesicle solution and assessing vesicle fusion, as described above. a: 16.7 μg/ml control IgG, b: 4 μg/ml aPSD-2, c: 8 μg/ml aPSD-2, d: 16.7 μg/ml aPSD.

Effect of the Anti-PS Motif on GAPDH-Induced Membrane Fusion—Recently, GAPDH was shown to induce vesicle fusion, and this action was strictly dependent on the presence of both pl-PE and PS in the vesicles (*11*). To determine whether the aPSD-2 binding site is involved in membrane fusion, we examined the effect of this antibody on GAPDH-induced membrane fusion. Vesicle fusion was assessed by measuring lipid mixing using octadecylrhodamine, and rabbit muscle GAPDH induced membrane fusion only when both pl-PE and PS were incorporated into the vesicles (data not shown), a result consistent with those reported previously (*11*). GAPDH-induced vesicle fusion was inhibited by aPSD-2, whereas IgG purified from preimmune serum had no effect, suggesting that the antibody-binding site is involved in membrane fusion, as well as the interaction of GAPDH with PS (Fig. 5).

Localization of the PS-Binding Region of GAPDH and Its Role in Membrane Fusion—To identify the PS-binding site on GAPDH, we mapped the aPSD-2-binding site after chemical fragmentation of rabbit muscle GAPDH with CNBr. The resulting peptides were separated by reverse-phase HPLC and examined for reactivity with aPSD-2 (Fig. 6, A and B). The main reactivity was recovered in fractions 52–54, and immunoblotting analysis showed that aPSD-2 bound specifically to a 6,500-Da peptide fragment eluted in fractions 52–54 (Fig. 6, C and D). Edman degradation of this peptide resulted in a single amino acid sequence (FQYDSTHGKFHGTVKAENGK, single-letter amino acid code, from amino acid residue 45) (*33*). On the basis of its molecular mass, this 6,500-Da CNBr-fragment corresponds to amino acids 45–103 of GAPDH.

To localize the aPSD-2-binding site more precisely, we



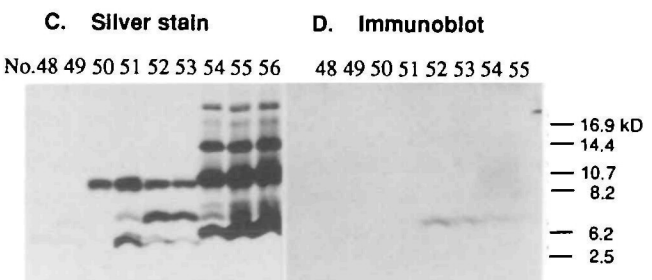


Fig. 6. **Localization of the aPSD-2-binding site on GAPDH.** Peptide fragments after chemical fragmentation of GAPDH with CNBr were separated by reverse-phase HPLC (A). Microtiter plates were coated with aliquots of each fraction, and the reactivities with aPSD-2 were examined by ELISA (B). The aPSD-2-reactive fractions were subjected to discontinuous SDS-PAGE (*30*), followed by either staining the peptide bands with silver nitrate (C) or immunoblotting analysis with aPSD-2 (D).

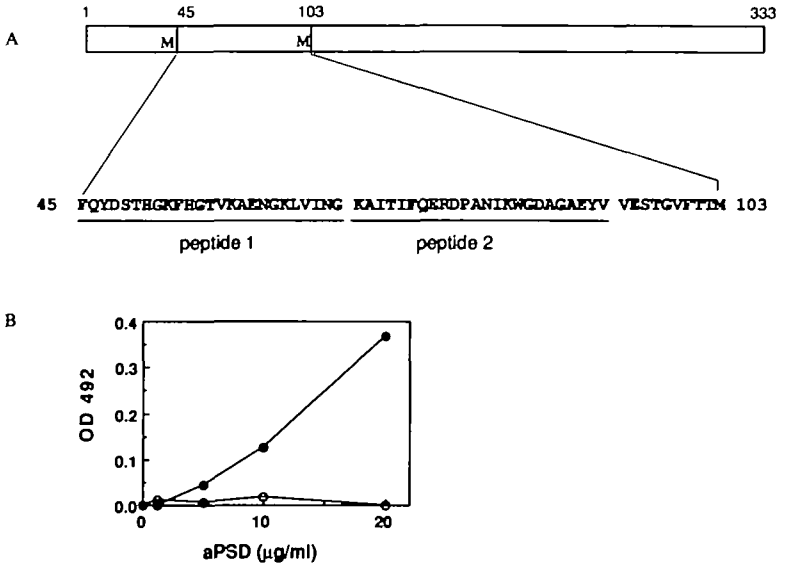


Fig. 7. **Identification of the aPSD-2-binding site on GAPDH.** A: Schematic representation of the putative aPSD-2-binding site on GAPDH. B: Binding of aPSD-2 to synthetic peptides derived from amino acid residues 45-103 of GAPDH, GAPDH45 (○), and GAPDH70 (●), synthetic peptides covering amino acid residues 45-69 and 70-94, respectively. Microtiter plates were coated with each peptide fragment (2 μg/ml), and aPSD-2 binding was evaluated using biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin. Each value represents the mean of two different experiments.

synthesized peptides covering amino acid residues 45-69 (GAPDH45) and 70-94 (GAPDH70) of rabbit GAPDH and their reactivities with aPSD-2 were examined by ELISA. As shown in Fig. 7B, aPSD-2 bound to GAPDH70 but not significantly to GAPDH45, further supporting that the binding site of aPSD-2 is localized to amino acid residues 70-94. As GAPDH bound to membrane surfaces containing PS and aPSD-2 binding to GAPDH was inhibited specifically by PS, it is likely that amino acid residues 70-94 form a putative PS-binding site of GAPDH.

Amino acid residues 70-94 are located in the NAD-binding domain of GAPDH, and the interaction between the GAPDH and NAD caused conformational changes of the enzyme (*3*). We examined the effects of β-NAD and DL-glyceraldehyde 3-phosphate on GAPDH binding to a PS-containing membrane surface using the surface plasmon resonance measurement technique as described above. Neither β-NAD nor DL-glyceraldehyde 3-phosphate had a significant effect on the binding, suggesting that the PS-binding domain is not related to the domains which mediate dehydrogenase activity (data not shown).

We assessed the functional role of amino acid residues 70-94 in GAPDH-induced membrane fusion by raising a polyclonal antibody against the synthetic peptide GAPDH70-94 derived from rat GAPDH (access #: Swiss-prot P04797) and studying its effect on GAPDH-induced membrane fusion. The polyclonal antibody inhibited membrane fusion in a concentration-dependent manner, whereas control IgG purified from the preimmune serum had no significant effect (Fig. 8A). GAPDH70 was also found to inhibit membrane fusion in a concentration-dependent manner (Fig. 8B). These results indicate that amino acid residues 70-94 are involved in the GAPDH-induced membrane fusion, as well as the interaction with PS.

Glaser and Gross (*34*) demonstrated that pl-PE facilitates greatly both calcium- and GAPDH-induced fusion, suggesting that the propensity of pl-PE to adopt an inverted hexagonal phase plays a critical role in the membrane fusion (*35, 36*). Membrane fusion requires the merging of the lipid bilayers, which is achieved by close contact between adjacent lipid bilayers. Although it is not clear

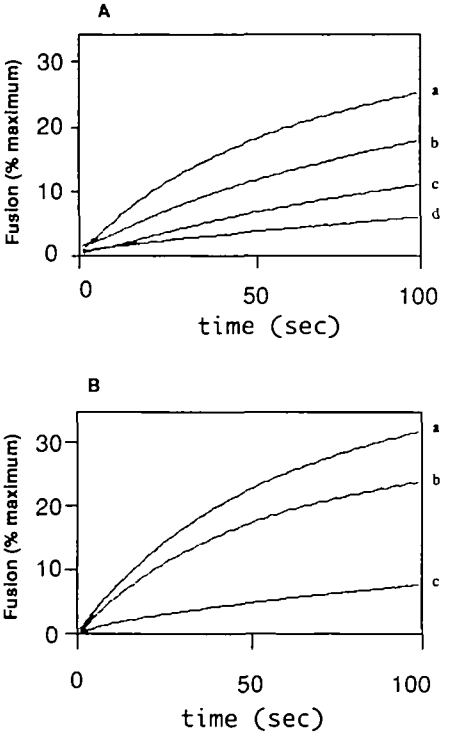


Fig. 8. **Inhibition of GAPDH-induced vesicle fusion by the synthetic peptide GAPDH70 and polyclonal antibody raised against the GAPDH70.** GAPDH-induced vesicle fusion was assessed by measuring lipid mixing using octadecylrhodamine, as described in the legend to Fig. 5. A: GAPDH was preincubated with the polyclonal antibody raised against GAPDH70 for 60 min at 22°C, and the GAPDH fusion activity was examined. Antibodies used are: control IgG (a, 15 μg/ml and anti-GAPDH70; b, 3.6 μg/ml; c, 7.2 μg/ml; d, 14.5 μg/ml). B: Vesicle fusion in the presence of various concentrations of the synthetic peptide GAPDH70 covering amino acid residues 70-94 of GAPDH was measured. a, 0 μg/ml; b, 10 μg/ml; c, 20 μg/ml.

whether the oligomeric state of GAPDH is essential for its ability to induce vesicle fusion, it is possible that multivalent interactions between GAPDH and vesicles lead to close contact between vesicle surfaces, resulting in enhancement of membrane fusion.

GAPDH has been shown to associate with the cytoplasmic faces of cell membranes (*37*). Although the association of GAPDH with erythrocyte membranes has been shown to be mediated by its interaction with the band 3 anion transporter AE1 (*6, 7*), it is possible that the interaction of GAPDH with PS on cellular membranes may participate in their association. Recent studies have shown that GAPDH

is taken up selectively by isolated rat liver lysosomes (*38, 39*), and GAPDH may associate with lysosomal membranes by interacting with PS, which has been suggested to reside on the cytoplasmic faces of lysosomal membranes (*40*). Further investigations using genetic engineering techniques, such as determining the effects of replacing amino acids of the PS-binding site on the enzyme function and making yeast DNA transformants carrying mutated GAPDH genes, are required to elucidate the *in vivo* roles and functions of the putative PS-binding site and GAPDH-PS interaction.

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