A Novel Phosphatidylserine-binding Peptide Motif Defined by an Anti-idiotypic Monoclonal Antibody

LOCALIZATION OF PHOSPHATIDYLSERINE-SPECIFIC BINDING SITES ON PROTEIN KINASE C AND PHOSPHATIDYLSERINE DECARBOXYLASE*

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A monoclonal anti-idiotypic antibody, Id8F7, previously shown to bind to a phosphatidylserine (PS)-specific binding site on protein kinase C (PKC) has been used to identify a 12-amino acid consensus sequence shared by PKC and phosphatidylserine decarboxylase (PSD). The 14-amino acid synthetic peptide derived from the corresponding region of PSD (amino acids 227–240) bound effectively and specifically to PS, and that derived from rat PKCγ (amino acids 227–240) bound weakly but specifically to PS. Analysis of binding of Id8F7 to various synthetic peptides revealed that the consensus sequence motif, FXFXXLXXXXXXRX, is responsible for the interaction with both Id8F7 and PS. The results suggest that the conserved amino acid residues represent a basic structural motif for the specific interaction with PS, and the corresponding regions of PKC and PSD form the PS-specific binding sites of these enzymes.

Although it is generally accepted that phospholipids in membranes are essential for the catalytic activity of many membrane-bound enzymes, it is still unclear how phospholipids regulate the activity of the enzymes. Phosphatidylserine (PS)1 in membranes is known to be an essential cofactor for the activation of protein kinase C (PKC) (1–3) and blood coagulation (4–6). Recent analyses have shown that it regulates the activity of various enzymes such as c-Raf-1 protein kinase (7), nitric oxide synthase (8), Na+K+-ATPase (9), Dynamin GTPase (10), and diacylglycerol kinase (11) and acts as a ligand in recognition of apoptotic cells (12, 13). PKC is a family of phospholipid-dependent kinases, and its enzymatic activity is allosterically regulated by 1,2-diacyl-sn-glycero-3-phospho-l-serine (PS) (14, 15). Analyses of the interaction of conventional PKC (cPKC) with membrane lipids support a two-step model for the activation of cPKC, which includes initial binding to membranes that does not require PS since cPKC binds various acidic phospholipids in a Ca2+-dependent manner and allosterically activates by specific interaction with PS and diacylglycerol (15). The initial binding of cPKC to membranes is believed to be mediated by the Ca2+-dependent phospholipid-binding (CaLB) domain (amino acids 186–233 of PKCγ), a sequence motif that is also found in other cytosolic proteins such as cytosolic phospholipase A2, GTPase-activating protein, and synaptotagmin (16, 17). However, it has been difficult to validate the presence of the PS-specific binding site on PKC, since the enzyme interacts with multiple phospholipid molecules during activation. Our previous studies showed that the binding to PKC of the monoclonal anti-idiotypic antibody Id8F7 raised against the combining site of the PS-specific antibody is inhibited by PS but not by other phospholipids including the synthetic PS analogue, 1,2-diacyl-sn-glycero-3-phospho-l-serine (18, 19). The binding of Id8F7 to PKC is significantly enhanced by the presence of diacylglycerol and is independent of the presence of Ca2+ (19). These observations indicate that Id8F7 binds to the PS-specific binding site, which is involved in the allosteric activation of PKC. In this study, we first mapped the Id8F7 binding site within the C2 region of rat PKCγ, and then we identified a 12-amino acid peptide motif responsible for the specific interaction with PS.

EXPERIMENTAL PROCEDURES

Materials—Among 34 anti-idiotypic monoclonal antibodies raised against the combining site of the PS-specific monoclonal antibody PS4A7, one clone, Id8F7 (IgM, κ), which showed an extensive cross-reaction with PKC, was used in this study (19). PKCγ was purified to homogeneity as described previously (19). Isopropyl-β-D-thiogalactopyranoside, phenylmethylsulfonyl fluoride, Triton X-100, and 2-mercaptoethanol were purchased from Nacalai Tesque (Kyoto, Japan). Leupeptin and bovine serum albumin were purchased from Sigma. Other materials used are as described previously (10).

Construction of PKC Fragment Expression Vectors—Recombinant fragments of rat PKCγ, PKC1–173 (amino acids 1–173 of the C1 region of the PKC) and PKC161–373 (amino acids 161–373 of the C2 region of the PKC), were expressed by plasmids referred to as pTB967 and pTB968, respectively (20). PKC161–220 and PKC220–296 fragments were expressed as fused proteins with maltose binding protein. The DNA fragments encoding PKC161–220 and PKC220–296 were obtained from pTB761, which contains the full coding sequence of rat PKCγ. The 2.9-kilobase EcoRI fragment excised out of pTB761 was digested with Hinfl and bluntned by DNA polymerase I (large fragment) (Takara, Kyoto, Japan), followed by digestion with PsiI. The 452-bp PsiI-Hinfl-filled fragment (nucleotides 345–798) and 232-bp Hinfl-filled-PsiI (nucleotides 798–1026) fragment were isolated. To express the PKC161–220, the PsiI-Hinfl-filled fragment was cloned in between the HindIII site bluntned with DNA polymerase I (large fragment) and the PsiI site of pMALc2 vector (New England Biolabs Inc., Beverly, MA). In order to remove the region overlapping PKC1–161, the result-

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†The abbreviations used are: PS, phosphatidylserine; PKC, protein kinase C; cPKC, conventional PKC; CaLB, Ca2+-dependent phospholipid binding; bp, base pair(s); HB, hypotonic buffer; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; PSD, PS decarboxylase; CHO, Chinese hamster ovary.
ant plasmid was digested with XmnI and AatII (nucleotide 617) and was blunted by T4 DNA polymerase I, followed by self-ligation. To express the PKC161–296, the HinfI-filIed-PstI fragment was cloned in between the XmnI site and PstI site of pMAlC2 vector. Each expression plasmid was introduced to Escherichia coli strain TB1 (New England Biolabs).

The transformant cells were cultured at 37°C and were induced by addition of isopropyl-β-D-thiogalactopyranoside to the final concentration of 0.5 mM. The cells were harvested by centrifugation after 3 h of incubation, and the cell pellets were resuspended in hypotonic buffer (HB; 20 mM Tris, 5 mM EDTA, 5 mM EGTA, 20 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, pH 7.5). PKC1–173 and PKC161–373 were purified by consecutive washing with HB containing 1% Triton X-100 and 6 and 8 mM urea. The final precipitates were dissolved in HB containing 6 mM guanidine. PKC161–220 and PKC220–296 were resuspended in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 50 μg/ml phenylmethylsulfonyl fluoride and purified to homogeneity by affinity chromatography with amylase resin (New England Biolabs). Expressed PKC fragments were at least 95% pure as judged by SDS-polyacrylamide gel electrophoresis.

To confirm the amino acid sequences of the recombinant fragments, N-terminal amino acid sequences of each polypeptide were identified by amino acid sequencer (Applied Biosystems amino acid sequencer model 477A) after purification.

Identification of Id8F7 Binding Site on Rat PKCy—The binding of PKCy to PKC fragments was examined by ELISA (19). PKCy (10 ng/well) or PKC fragments (50 ng/well) were coated onto the microtiter plate, and the binding of Id8F7 (5 μg/ml) was determined using biotinated anti-mouse IgM (Zymed Laboratory, San Francisco, CA) and peroxidase-conjugated streptavidin (Zymed). One hundred μg/ml of the PKC fragment, PKC220–296, which showed a strong reactivity with Id8F7, was digested with either Lys-C or Asp-N endoproteinase (Boehringer Mannheim, Germany) in Tris-buffered saline containing 1 mM urea. After digestion for 2 h at 37°C, the digested fragments were separated on reverse phase HPLC with an ODS-250T column (Tosoh, Japan) using a linear gradient containing 0.1% trifluoroacetic acid. An aliquot of each fraction was coated onto microtiter wells, and the binding of Id8F7 to the plate-coated fragments was examined by ELISA as described above.

The N-terminal amino acid sequences of the Id8F7-reactive peptide fragments were determined by the amino acid sequencer as described above. The synthetic peptides derived from the consensus sequence of PKC and PC desacetylase (PDS) 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 the peptides (22). The peptides were purified by reverse-phase HPLC with an ODS 120 column (Tosoh Co.). The sequence and molecular mass of each peptide were confirmed using the amino acid sequencer as described above. The synthetic peptides resulted in the identification of a single reactive peptide with the amino acid sequence, ATLNPVWNETFVFNLKPGDVER (single-letter amino acid code, from amino acid 217). The analysis using the endoproteinase Asp-N identified two Id8F7-reactive peptide fragments, DPYVKKLKLIPDPRN–– (from amino acid 193) and DPRNLTKQKTDTVKA–– (from amino acid 203). These results suggest that the Id8F7 binding site is either located in the CaLB domain (amino acids 186–242 of rat PKCy) (17) or spanned to amino acids outside the CaLB domain.

Identification of Binding Domain on PKCy—The binding of PKC fragments to phospholipids was examined by ELISA (19). The wells of the microtiter plate were coated with 50 pmol/well of each phospholipid (18) and incubated with 5 μg/ml of the PKC fragments. The binding of the fragments to plate-coated phospholipids was determined using rabbit anti-maltose binding protein, IgG (New England Biolabs), and peroxidase-conjugated antibodies (Cappel, West Chester, PA). The binding of the synthetic peptides to phospholipids was determined by ELISA. In brief, alkaline phosphatase was activated with N-succinimidyl-3-(2-pyridyl)thiopropionate (Pharmacia Biotech Inc.) (molar ratio of alkaline phosphatase to N-succinimidyl-3-(2-pyridyl)thiopropionate of 1:8) followed by conjugation with the synthetic peptides via their cysteine residue (molar ratio of the peptides to the alkaline phosphatase of 36:1).

According to this protocol, an average of three peptides was coupled to the alkaline phosphatase. The free peptides were removed by gel filtration with the PD-10 column (Pharmacia, Uppsala, Sweden), and the alkaline phosphatase-conjugated peptides (10 μg/ml) were incubated with the plate-coated phospholipids as described above. The binding of the peptides was determined by addition of 0.25 mM 4-methylumbelliferyl phosphate substrate solution (18).

RESULTS AND DISCUSSION

To identify the PS-specific binding site on PKCy, we mapped the Id8F7 binding site using recombinant and synthetic PKCy fragments. The fragments covering the C1 region (amino acids 1–173, PKC1–173) and the C2 region (amino acids 161–373, PKC161–373) of PKCy were expressed (Fig. 1A) (20) and examined for reactivity with Id8F7. Id8F7 bound effectively to PKC161–373 but not to PKC1–173 (Fig. 1B). Partial proteolytic digestion of PKC161–373 with endoproteinase Lys-C followed by separation and sequence analysis of the Id8F7-reactive peptides resulted in the identification of a single reactive peptide with the amino acid sequence, ATLNPVWNETFVFNLKPGDVER (17) or spanned to amino acids outside the CalB domain.

We performed computer-aided similarity searching (SDC-GENETYX program, Software Development, Tokyo) for amino acid sequences in PKC220–296 and PDS from Chinese hamster ovary (CHO) cell (23), the only example of the enzyme that has been shown to specifically recognize the molecular structure of PS (23, 24). The analysis revealed significant homology between the primary sequences of PKCy (amino acids 227–238) and PDS (amino acids 351–362) (Fig. 3A). The consensus sequence is conserved among the cPKC family from different species (1). We synthesized various peptides derived from the
AGQKIRFG bound effectively and specifically to PS, and the examined peptide derived from PSD (FNFRLK) was examined by ELISA. Among the synthetic peptides, alkaline phosphatase, and its binding to plate-coated phospholipids. In this assay, each peptide was conjugated with the peptidemotif with the consensus F

To assess the functional role of the consensus sequence, we examined the binding of each synthetic peptide to various phospholipids. The binding of the fragments to plate-coated phospholipids was determined by rabbit anti-maltose binding protein IgG and peroxidase-conjugated anti-rabbit IgG. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; CL, cardiolipin.

Deletion of the F

The present study provides the first structural information of polypeptides responsible for the specific interaction with PS. Since the 14-amino acid synthetic peptides derived from PS decarboxylase bound effectively and specifically to PS, the corresponding region may form the substrate recognition site of the enzyme. The results using the synthetic peptides suggest that the conserved amino acid residues among PKC and CHO cell PSD, FVFNLKXXXXKXR amino acid sequence is conserved in the yeast PSD (amino acids 475–486, FKFDVRVKDVKV) but not in the E. coli enzyme. PSD is a pyruvoyl-dependent enzyme, and the post-translational cleavage of a proenzyme (m-subunit) is believed to form the pyruvoyl prosthetic group at the N terminus of the α subunit, which is composed of the C-terminal 32 amino acids of the yeast enzyme (amino acids 534–577 with high homology to the C2 regions of PKC (amino acids 211–254 of rat PKCγ) and the two C2 regions of synaptotagmin (29). Trotter et al. (28, 29) suggested that this C2 homology domain represents a phospholipid-binding motif of these proteins. The present consensus sequence motif of rat PKCγ (amino acids 227–238) resides in the C2 homology domain, and the similar sequence motif is also identified in PSD2 (amino acids 561–572) but not in synaptotagmin. Since PKC (14, 15) and PSD2 (29) were shown to interact specifically with PS but synaptotagmin binds nonspecifically to various acidic phospholipids (16), the consensus sequence between PKC and PSD, FXFXI(V/L/K)RXXDQ(K), is likely to mediate the specific interaction of these proteins with PS.

Fig. 2. Binding of PKC recombinant fragments to PS. The binding of PKC161–220 and PKC220–296 to various phospholipids was tested by ELISA. The wells of the microtiter plate were coated with 50 pmol/well of the phospholipids and incubated with 5 μg/ml of the PKC fragments. The binding of the fragments to plate-coated phospholipids was determined by rabbit anti-maltose binding protein IgG and peroxidase-conjugated streptavidin.

FIG. 3. Identification of Id8F7-binding peptide sequence on PKC and PSD. A, comparison of amino acid sequences shared by rat PKC family and PSD from CHO cells. Conserved amino acid residues are indicated by boldface letters, with residue numbers on the right. Searching for similar amino acid sequences was performed using the SDC-GENETYX program (Software Development, Tokyo). B, binding of Id8F7 to the synthetic peptides derived from the consensus sequence of PKC and PSD. The synthetic peptides (100 ng/well) were coated onto the microtiter plate by incubating for 12 h at 37 °C and incubated with Id8F7 (5 mg/ml). Binding of Id8F7 was determined using biotinylated anti-mouse IgM and peroxidase-conjugated streptavidin.

Fig. 4. Specific binding of 14 amino acids synthetic peptides to PS. Synthetic peptides, FNFRLKAGQKIRFGC from PSD (A) and FVFNLKPGDVERRLC from PKCy (B), were conjugated to alkaline phosphatase as described under “Experimental Procedures.” The alkaline phosphatase-conjugated peptides (10 μg/ml) were incubated with the plate-coated phospholipids (50 pmol/well for A and B and 5 pmol/well for C), and the binding of the peptides was determined by the addition of 0.25 mM 4-methylumbelliferyl phosphate substrate solution.

namic acids 475–486, FKFDVRVKDVKV) but not in the E. coli enzyme. PSD is a pyruvoyl-dependent enzyme, and the post-translational cleavage of a proenzyme (m-subunit) is believed to form the pyruvoyl prosthetic group at the N terminus of the α subunit, which is composed of the C-terminal 32 amino acids of the yeast enzyme (23, 26, 27). It is noteworthy here that the consensus sequences in yeast and mammalian PSD are located in the α subunit of the enzyme, which are close to the catalytic carbonyl group of the enzyme. Recently, Trotter et al. (28, 29) isolated the structural gene encoding the second PSD gene (PSD2) from yeast. The PSD2 amino acid sequence contains a region of about 40 residues (from 534 to 577 with high homology to the C2 regions of PKC (amino acids 211–254 of rat PKCγ) and the two C2 regions of synaptotagmin (29). Trotter et al. (29) suggested that this C2 homology domain represents a phospholipid-binding motif of these proteins. The present consensus sequence motif of rat PKCγ (amino acids 227–238) resides in the C2 homology domain, and the similar sequence motif is also identified in PSD2 (amino acids 561–572) but not in synaptotagmin. Since PKC (14, 15) and PSD2 (29) were shown to interact specifically with PS but synaptotagmin binds nonspecifically to various acidic phospholipids (16), the consensus sequence between PKC and PSD, FXFXI(V/L/K)RXXDQ(K), is likely to mediate the specific interaction of these proteins with PS.

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ing showed that various cellular proteins have a homologous sequence motif; typical examples are DNA topoisomerase II α (amino acids 1022–1033) (30, 31), spectrin α (amino acids 1598–1609) (32), calmodulin-sensitive adenylate cyclase (amino acids 148–159) (33), complement receptor type II α (amino acids 381–392) (34), urinary fatty acid binding protein (amino acids 73–86) (35). Dengue virus non-structural protein NS2A (amino acids 1332–1343) (36), and RNA polymerase β subunit (amino acids 2108–2119) (37). Among these proteins, rat DNA topoisomerase II α sequence motif; typical examples are DNA topoisomerase ing showed that various cellular proteins have a homologous sequence motif; typical examples are DNA topoisomerase II α (amino acids 1022–1033) (30, 31), spectrin α (amino acids 1598–1609) (32), calmodulin-sensitive adenylate cyclase (amino acids 148–159) (33), complement receptor type II α (amino acids 381–392) (34), urinary fatty acid binding protein (amino acids 73–86) (35). 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