Anti-idiotypic monoclonal antibody recognizes a consensus recognition site for phosphatidylserine in phosphatidylserine-specific monoclonal antibody and protein kinase C

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Abstract
In order to elucidate the molecular mechanisms responsible for the specific lipid–protein interactions, we have undertaken structural and idiotypic analyses of a monoclonal antibody, PS4A7, which binds specifically to phosphatidylserine (PS). Here we showed that one of the anti-idiotypic monoclonal antibodies raised against PS4A7 cross-reacted extensively with protein kinase C (PKC) and inhibited the activation of the enzymatic activity. The binding of the anti-idiotypic antibody to PKC was inhibited specifically by PS, but not by other phospholipids including 1,2-diacyl-sn-glycerol-3-phospho-o-serine or 1,2-diacyl-sn-glycerol-3-phospho-L-homoserine. In contrast, the binding of the anti-idiotypic mAb to the enzyme was significantly enhanced in the presence of either diacylglycerol or sphingosine. These findings indicate that the PS-specific monoclonal antibody and PKC share a consensus structure which is responsible for the specific interaction with PS and both diacylglycerol and sphingosine may induce a similar conformational change which exposes the PS-specific binding site of the enzyme.

Key words: Lipid–protein interaction; Phosphatidylserine; Protein kinase C; Anti-phospholipid antibody; Anti-idiotypic antibody; Diacylglycerol

1. Introduction
Phosphatidylserine (PS) is a major anionic lipid component of mammalian plasma membranes. Recent analyses have shown that PS contributes to many regulatory processes of biological responses. The well-known functions of PS are its ability to regulate the enzymatic activity of PKC [1–9] and to promote blood coagulation [10–12]. Some other proteins have been shown to interact specifically with PS. Typical examples are amino-phospholipid translocase [13], synaptotagmin [14], aducin [15], synapsin I [16], putative receptor molecules on cell surfaces [17–20] and PS-specific phospholipase [21]. Although these proteins are supposed to interact with PS through their specific binding sites, no information has been available about the molecular mechanisms underlying the specific lipid–protein interactions.

PKC is a family of PS-dependent kinases that binds to the plasma membrane in response to receptor-mediated generation of diacylglycerol and Ca2+ [1–3]. Membrane-association of PKC is mediated by the interaction with multiple acidic phospholipids in the presence of Ca2+, while the enzymatic activity displays the strict structural requirement for 1,2-diacyl-sn-glycerol-3-phospho-L-serine (PS) [4–7]. Although these observations suggest the existence of specific binding site for PS, it has been very difficult to validate the presence of a PS-specific binding site on the PKC, since the enzyme interacts with multiple phospholipid molecules during activation [8,9].

In order to elucidate the molecular mechanisms responsible for the specific lipid–protein interactions, we have undertaken structural and idiotypic analyses of monoclonal antibodies (mAbs), which show the strict specificity to a certain phospholipid [22–26]. One mAb, named PS4A7, bound specifically to PS, and distinguished the stereo-specific configuration of the serine residue in PS [22], showing a reactivity profile similar to that reported for the activation of PKC. This mAb is expected to provide valuable information about specific lipid–protein interactions and be a structural template for the production of an anti-idiotypic antibody which can cross-react with the cellular PS-binding molecules.
Our previous analyses have shown that polyclonal anti-idiotypic antibody raised against a PC-specific mAb cross-reacted extensively with a PC-specific lipid transfer protein from bovine liver, which supports the hypothesis that they share a consensus recognition site for PC, and that part of the anti-idiotypic antibody may carry the internal image of the PC molecule [24]. In this study we established a series of anti-idiotypic mAbs against the PS-specific mAb and studied the interaction between the anti-idiotypic mAb and PKC.

2. Materials and methods

2.1. Establishment of anti-idiotypic mAb

The PS-specific mAb, PS4A7, was produced by the direct immunization of 10 mM ~-hydroxyethylpiperazone. Each of each lipids were swollen in 10 mM ~-hydroxyethylpiperazone. The anti-idiotypic mAbs were produced by the method as described previously [27]. The anti-idiotypic mAbs were purified to homogeneity and the binding of the anti-idiotypic antibody (2 &mu/ml) to PKC was examined by ELISA as described previously [27]. In brief, BALB/c mice were intraperitoneally immunized with 50 &mu g PS4A7-KLH conjugate in complete Freund's adjuvant, and received an additional 50 &mu g conjugate in incomplete Freund's adjuvant 2 weeks later. Two weeks after the second injection, the mice received 50 &mu g PS4A7-KLH conjugate in saline, and fusion with P3-X63-Ag.653 cells was performed 3 days later. Hybridomas were cultured in a synthetic medium (GIT medium, Nippon Seiyaku Co. Ltd., Osaka, Japan) without a serum supplement. The hybridoma supernatants were screened for antibodies that bound to PS4A7, but not to an unrelated mouse IgM. In order to obtain anti-idiotypic mAbs directed against the plate-coated PKCs was examined as described previously [27]. BALB/c mice were immunized with PS4A7 conjugated to keyhole limpet hemocyanin and the hybridoma supernatants were screened for antibodies that bound to PS4A7 but not to MO1, an unrelated mouse IgM. In order to obtain anti-idiotypic mAbs directed against the combining site of PS4A7. We established 34 anti-idiotypic mAbs against the PS-specific mAb and studied the interaction between the anti-idiotypic mAb and PKC.

2.3. Inhibition of the binding of anti-idiotypic mAb to PKC by various phospholipids

The effect of various lipids on the binding of the anti-idiotypic mAb to PKC was examined as follows. The dried lipid films containing 2.0 &mu mol of each lipid were swollen in 10 mM N-2-hydroxyethylpiperazine- N-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 150 mM NaCl and was sonicated by the Branson Sonifier for 10 mm at 0°C under the nitrogen flow. PKC (1 &mu g/ml) was coated onto microtiter plates and was preincubated with various concentrations of the lipid suspensions for 1 h at room temperature. Then the binding of the anti-idiotypic antibody (2 &mu g/ml) to PKC was examined by ELISA as described above. Various phospholipids and synthetic PS analogs were prepared as described previously [22]. Sphingosine from bovine brain and 1,2-dioleoylglycerol were purchased from Serdary Research Laboratories. Inc. (Ontario, Canada).

2.4. Inhibition of the activation of the enzymatic activity of PKC by anti-idiotypic mAb

Enzymatic activity of PKC was determined by measuring the incorporation of 32P from [gamma-32P]ATP into a synthetic substrate peptide (RKRTLRL) as substrate [29]. The reaction mixture (in 75 &mu l of 50 mM Tris-HCl buffer, pH 7.5) contains 1 mM Ca-acetate, 15 mM Mg-acetate, 2.5 mM dithiothreitol, 0.2 &mu g/ml phorbol 12-myristate acetate, 50 &mu M ATP, 0.25 &muCi of [gamma-32P]ATP, and 0.01% Triton X-100 mixed micelle containing 8 mol% of PS. After incubation at 25°C for 15 min, the reaction was stopped by addition of a solution (25 &mu l) containing 0.1 M ATP, 0.1 M EDTA. Aliquots (75 &mu l) were spotted onto Whatman P81 filter paper, washed by 5% acetic acid solution, and prepared for liquid scintillation counting [30]. In the inhibition assay, PKC (1 ng) was preincubated with various concentrations of the anti-idiotypic antibody for 60 min at 4°C and the residual enzymatic activity was measured as described above.

3. Results and discussion

A monoclonal antibody PS4A7 [22] binds to 1,2-diacetyl-sn-glycero-3-phospho-l-serine (PS) but neither to 1,2-diacetyl-sn-glycero-3-phospho-D-serine nor 1,2-diacetyl-sn-glycero-phospho L-homoserine, showing a similar phospholipid specificity to that observed in the activation of PKC [5]. We choose this mAb as a potential structural template for the production of anti-idiotypic mAbs according to the method as described previously [27]. BALB/c mice were immunized with PS4A7 conjugated to keyhole limpet hemocyanin and the hybridoma supernatants were screened for antibodies that bound to PS4A7 but not to MO1, an unrelated mouse IgM. In order to obtain anti-idiotypic mAbs directed against the combining site of PS4A7, the clones were further selected according to their ability to inhibit the binding of PS4A7 to PS. We established 34 anti-idiotypic mAbs against the combining site of PS4A7 from 3 fusions and then examined the reactivity of the anti-idiotypic mAbs to PKC. Three subspecies of PKC were purified to homogeneity and the binding of the anti-idiotypic mAbs to the plate-coated PKCs was examined by ELISA. Among the anti-idiotypic mAbs established, one named Id8F7 bound significantly to the three subspecies of PKC (Fig. 1) and effectively inhibited the acti-
Fig. 2. Effect of Id8F7 on the activation of enzymatic activity of PKC. PKC (1 ng) was preincubated with either Id8F7 or a control monoclonal antibody (anti-phospholipase A2) for 60 min at 4°C, then the enzymatic activity was measured. The anti-idiotypic mAb inhibited the activation of the enzymatic activity of the three subspecies of PKC equally, and the result obtained with PKCa is shown.

Fig. 3. Inhibition of the binding of anti-idiotypic mAb to PKC by PS. Effect of natural phospholipids (a) and synthetic PS analogs (b) on the binding of Id8F7 to PKC was analyzed by ELISA. Purified protein kinase Cα (1 μg/ml) was coated onto the microtiter plates and was preincubated with various concentrations of phospholipid vesicles for 1 h at room temperature. Then the Id8F7 (2 μg/ml) was added to each well and Id8F7 bound was detected with biotinylated anti-mouse IgM and peroxidase-conjugated streptavidin.

cylation of the enzymatic activity (Fig. 2). Neither Ca\(^{2+}\) nor EDTA had an appreciable effect on the binding of Id8F7 to PKC (data not shown).

In order to study whether or not the recognition site of Id8F7 on PKC is related to the PS binding site of the enzyme, we examined the effect of various lipids on the binding of Id8F7 to PKC. As shown in Fig. 3a, the binding of Id8F7 to PKC was specifically inhibited by PS, but not by other phospholipids such as phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine. The inhibitory activity of PS is strictly dependent on the structure of PS, since the synthetic PS analogs such as 1,2-diacyl-sn-glycero-3-phospho-D-serine and 1,2-diacyl-sn-glycero-phospho-L-homoserine showed no significant inhibitory effect on the binding (Fig. 3b). The results clearly indicate that the anti-idiotypic mAb recognized a common structure between PS-specific mAb and PKC, which is involved in the specific interaction with PS.

The functional consequences of the binding of anti-idiotypic mAb to the enzyme could be manifested as both selective inhibition and stimulation of the enzymatic activity. Many studies have shown that not all antibodies that bind to receptors trigger the effector function; some antibody may actually inhibit the action of ligand, as would an antagonist [31]. Since the antibody-combining site is larger than the small ligand such as PS, the interactions between the anti-idiotypic mAb and the enzyme may sterically inhibit the activation of the enzymatic activity of PKC.

In contrast to PS, preincubation of PKC with either 1,2-dioleoyl-glycerol or sphingosine remarkably enhanced the binding of Id8F7 to PKC. (Fig. 4). The diacylglycerol binding site has been mapped to the cystein rich regions in the C1 region [32] and the interaction with diacylglycerol was suggested to enhance the affinity of PKC for PS [7]. Although the concentrations of the lipids required for the enhancement of the binding was quite high compared to those required for the activation of the enzymatic activity [4], it is likely that the interaction of PKC with diacylglycerol caused the conformational change of the enzyme to expose the specific binding site for PS and resulted in the enhanced binding of the anti-idiotypic antibody to the enzyme. Since sphingosine is a potent inhibitor of the diacylglycerol binding to PKC [33], sphingosine may interact with the same site in PKC, inducing a similar conformational change. It should be stressed here that the conformational change induced by sphingosine should be different in some sense from that induced by diacylglycerol, since sphingosine inhibits the enzymatic activity of PKC whereas diacylglycerol stimulates the activity.
Concerning the phospholipid-binding domain in PKC. Clark et al. [34] reported a sequence homology among the Ca\(^{2+}\)-dependent phospholipid-binding proteins such as the cytosolic phospholipase A2 (cPLA2), GTPase activating protein (GAP) and PKC. The region was tentatively identified as a Ca\(^{2+}\)-dependent phospholipid-binding (CaLB) domain, since an amino-terminal fragment of cPLA2 (140 amino acid residues), which contains the CaLB domain, translocates to natural membrane vesicle in the presence of Ca\(^{2+}\) [34,35]. The putative CaLB domain in PKC was located to the amino acid residues 186-238 [34] in the C2 domain. Recent analyses, however, have shown that the activities of PKC (δ, ε, ζ, η, and φ) which lack the C2 domain are not affected by Ca\(^{2+}\), while they still require PS for activation [36]. The observations suggest that these PKC might have another recognition site for PS. In the present study, the binding of the anti-idiotype mAb to PKC was not influenced at all by free Ca\(^{2+}\) or chelating agent such as EDTA, implying that the anti-idiotype mAb might recognize an additional recognition site for PS. Our current studies are directed toward identifying the recognition site of the anti-idiotypic mAb in PKC.

The present analyses using the anti-idiotypic antibody clearly demonstrated that the anti-idiotypic mAb recognized a consensus structure between the PS specific mAb and PKC, a structure which may be responsible for the specific interaction with PS. The observations indicated that there is a distinct and specific recognition site for PS on PKC. Our recent work showed that the Id8F7 also cross-reacted extensively with blood coagulation factor V and VIII, suggesting that the structure, which is recognized by the anti-idiotype antibody, may form a consensus structural motif responsible for the specific interaction with PS (unpublished observation). The anti-idiotypic antibody will provide a useful tool to identify the PS specific recognition site and also to purify the cellular PS-binding proteins which have been difficult to isolate by the conventional biochemical techniques.

References
