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Specific binding of human C-reactive protein towards supported monolayers of binary and engineered phospholipids

Tatsuro Goda*, Yuji Miyahara

Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (TMDU), 2-3-10 Kanda-Surugadai, Chiyoda, Tokyo 101-0062, Japan

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ABSTRACT

Circulating C-reactive protein (CRP) recognizes altered plasma membranes and activates complements systems in the acute phase of inflammation and infection in human. We have shown previously the calcium-independent adsorption of CRP toward 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phosphocholine (POPC) and lysophosphatidylcholine (LPC) on supported phospholipid monolayers. Here, we extended our study to other phospholipids and additives to elucidate the pattern recognition of CRP using a surface plasmon resonance biosensor. Surface density and lateral fluidity depended on the type of phospholipids in the monolayers as characterized by SPR and fluorescence recovery after photobleaching measurements. CRP recognized 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phospho-(1′-rac-glycerol) (POPG) in the supported POPC monolayers without calcium at pH 7.4 and 5.5. As opposed to LPC, CRP did not recognize 3-*sn*-lysophosphatidylethanolamine in the POPC monolayers in calcium-free conditions. While, the addition of 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phosphoethanolamine (POPE) or sphingomyelin to supported POPC monolayers blocked CRP adsorption. Calcium-dependent CRP binding was observed only at pH 5.5 on supported monolayers of engineered phospholipids with inverted headgroups relative to POPC. The complement 1q (C1q) protein recognized the active form of CRP on the supported phospholipid monolayers. The discovery of CRP recognition with these phospholipids aids our understanding of the activation dynamics of CRP with phospholipid-based biomaterials when used during the acute phase.

1. Introduction

Innate immunity is the first line of host defense system against foreign challenge or tissue damage. The acute-phase reactant human C-reactive protein (CRP) in circulation increases by hundreds fold within 1 day from the onset of inflammation and infection and recruits complement molecules for the clearance of pathogens or cell debris [1,2]. The interaction of systemic CRP with altered plasma membranes determines the physiological roles of CRP [3–5]. For example, CRP adsorption onto a ligand in plasma membranes or lipoproteins leads to both pro-inflammatory and anti-inflammatory effects, depending on the ligand type and the ionic microenvironments [6–9]. The latest studies have revealed that structural transition of CRP from the original isopentamer to its protomer following target recognition triggers pro-inflammatory cascades [10–18]. Although CRP is beneficial in numerous physiological functions, it has some harmful effects especially in post-reproductive-age diseases such as atherothrombosis, autoimmune and other chronic inflammatory conditions [19]. Nowadays, a minor elevation in the systemic CRP level under non-acute conditions is recognized as a risk marker for cardiovascular diseases [16,20–22].

Fundamental understanding of the CRP interaction with various phospholipids is important, as many phospholipid-based materials from natural and synthetic origins are used in bioengineering researches, such as drug delivery systems (DDS), cell therapy, and biomedical devices. Phosphatidylcholine, phosphatidylserine, and phosphatidylinositol, have been used in combination with cholesterol and charged amphiphiles such as stearylamine or phosphatidic acid for making liposomes [23]. Recently, synthetic phospholipids with inverted headgroups have gained attention due to their unique physicochemical properties that make them amenable for applications in DDS [24–28]. Amphipathic polymers bearing the zwitterionic phosphorylcholine group were discovered to enter the cytoplasm across otherwise impermeable lipid bilayer barriers of plasma membranes [29,30]. Phospholipid-mimicking polymers have been developed for producing biologically inert functions to biomedical devices in *in vivo* situations [31,32]. Despite the prevalence of phospholipid-based biomaterials in nanomedicine, the interaction of these materials with CRP which

* Corresponding author.
E-mail address: goda.bsr@tmd.ac.jp (T. Goda).

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is implicated in complement activation remains to be completely elucidated. The potential risk of phospholipid-based biomaterials for CRP-related complement activations and inflammation in post-reproductive-age diseases may not come into the open. This is because CRP level in non-acute phase is quite low for activating complement and inflammation. In addition, *in vivo* safety validation of these biomaterials are mostly performed using model animals such as mouse and rat where CRP is not an acute phase reactant [1,2,33].

To understand CRP/phospholipid interplay on molecular basis, we have been conducting researches using model biointerfaces and sensitive biosensors. Traditionally, CRP is known to recognize the zwitterionic phosphorylcholine headgroup in the presence of calcium [34]. To our knowledge, both calcium- and pH- dependent binding of human CRP onto phospholipid-mimetic materials was noted [9,35]. The amount of complement component C1q bound onto the active form of CRP on model surfaces was also enhanced under weakly acidic pH conditions, a common signature of inflammation site [36]. Moreover, we newly identified a calcium-independent interaction of CRP with a bioactive phospholipid, lysophosphatidylcholine (LPC), in model surfaces [37]. The glycerophosphate motif in LPC was responsible for the specific binding of CRP in the absence of calcium ions. To extend our study, we have examined the interaction of CRP with a series of bioactive or synthetic phospholipids that are frequently used in biomedical applications [23–28]. Surface density and lateral diffusivity of lipid molecules in supported monolayers were characterized to see those effects on the binding properties. Then, the complex formation between CRP on model surfaces with C1q was investigated. Surface plasmon resonance (SPR) was employed as a label-free biosensor for determining the binding kinetics and the amount of CRP adsorbed [38].

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoethanolamine (POPE), sphingomyelin (SM) from chicken egg yolk, 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-(1′-rac-glycerol) (POPG), 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate (DOCP), and 1-palmitoyl-2-(6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)-*sn-glycero*-3-phosphocholine (NBD-PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Tetradecanethiol, L-α-lysophosphatidylcholine (LPC) from soybean (Acyl chain length of 16:0 and 18:0), 3-*sn*-lysophosphatidylethanolamine (LPE) from egg yolk (acyl chain length of 16:0 and 18:0), cholesterol (3β-hydroxy-5-cholesten; Chol), CRP from human plasma, complement component C1q from human serum and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). D,L-α-phosphatidylcholine, dipalmitoyl (D,L-DPPC) was obtained from Santa-Cruz Biotechnology, Inc. (Dallas, TX). dodecyltrimethoxysilane was obtained from TCI Co. (Tokyo, Japan). All the other reagents of extra-pure grade were purchased from commercial sources and used as received. Chemical structure of phospholipids are shown in Fig. S1.

2.2. Preparation of supported phospholipid monolayers

Phospholipids in chloroform were mixed at desired compositions, followed by drying using a rotary evaporator under vacuum at 37 °C for 2 h. An aqueous buffer solution was then added to the dry lipid film to give a final concentration of 5 mM. The mixture underwent five freeze-thaw cycles using liquid nitrogen. Thereafter, the phospholipid suspension was passed 20 times through a polycarbonate filter with 100 nm pores using a mini-extruder kit (Avanti Polar Lipids). The extrusion process yields a suspension of unilamellar phospholipid vesicles with a homogenous size distribution.

A gold SPR sensor chip was soaked in 10 mM tetradecanethiol in ethanol for 2 h to form self-assembled monolayer (SAM) on gold, followed by rinsing with ethanol and water. The supported monolayer of phospholipids was formed on the SAM using the Biacore X100 (GE Healthcare Japan, Tokyo, Japan). The sensor chip was cleaned using 0.5 wt% sodium dodecyl sulfate (SDS) for 60 s, followed by flushing through buffer solution to obtain a stable baseline. The suspension of unilamellar phospholipid vesicle in a buffer solution (0.75 mM) was then injected under a flow rate of 5 or 30 μL min^{-1} for 18 min at 25.0 °C, followed by rinsing with 1 mM NaOH for 60 s or buffer solution for 60 min to remove any loosely adsorbed lipids from the surface. The lateral density (γ) of the phospholipids in the monolayers was determined by the SPR signal (ΔSPR) as:

$$(1)\gamma = \Delta SPR \cdot N_A / Mw$$

where N_A and Mw represent Avogadro's number and the molecular weight, respectively. The amount of phospholipids adsorbed was calculated based on the conversion of 1 resonance unit (R.U.) as 1 pg mm^{-2} [39].

2.3. FRAP measurements

A glass coverslip (No. 1S, Matsunami Glass, Osaka, Japan) was rinsed by ethanol and water for 5 min each. Then, the cleaned coverslip was silanized in a vapor of 10.0 vol% solution of dodecyltrimethoxysilane with toluene for 3 h at 100 °C [40]. The surface modified was washed by ethanol and water, followed by dried in air. Supported monolayers of phospholipids containing 0.5 mol% NBD-PC was prepared on dodecyltrimethoxysilane SAM by the vesicle fusion process. The lateral fluidity of supported monolayer was determined by fluorescence recovery after photobleaching (FRAP) measurements using a Nikon Eclipse Ti inverted microscope with a confocal laser scanning system (Tokyo, Japan). NBD-PC was excited with the 488 nm line of a diode laser (Nikon Lu−N4), and emission was detected at 525 nm using a standard PMT detector (Nikon C2-DU3). A 60 × water immersion objective lens (1.2 NA, Nikon) was used. The samples were measured at seven random locations. Initial intensities were recorded for 30 s. The bleaching was performed at a defined spot with the nominal radius (r_n) of 25 μm for 1 s at 100% laser power, and the recovery signal after photobleaching was recorded for 6 min. NIS-Elements software (Nikon) was used for determining the fluorescent intensity at the nominal bleach region of interest (ROI), the half time of recovery ($\tau_{1/2}$), and the spreading radius of photobleach profile (r_e) [41]. The following equation was used to determine bleaching depth parameter (K) and r_e from the fluorescence intensity profile across the bleach ROI from post bleach images:

$$f(x) = 1 - Kexp(-2x^2/r_e^2) \quad (2)$$

The diffusion coefficient (D_{FRAP}) was determined by the following equation [41]:

$$D_{FRAP} = (r_n^2 + r_e^2)/8\tau_{1/2} \quad (3)$$

2.4. CRP binding kinetics using SPR

The kinetic rate constants of CRP adsorption onto supported phospholipid monolayers was assessed using a Biacore X100 at