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Fluorescence correlation spectroscopy analysis of the hydrophobic interactions of protein 4.1 with phosphatidyl serine liposomes

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Abstract

Fluorescence correlation spectroscopy (FCS) was applied to examine the interactions between a protein and a membrane lipid. The protein 4.1–phosphatidyl serine (PS) interactions served as the model system to demonstrate the membrane lipid–protein interactions. This protein was labeled with rhodamine and its interactions with PS-liposomes were measured by FCS. The present results clearly demonstrated that a small protein molecule, protein 4.1, interacts specifically with a large particle, a PS-liposome. This interaction appears to be hydrophobic and not electrostatic, since the bound protein 4.1 did not dissociate in solution and was specifically released from PS-liposomes by treatment with phospholipase A_2 (PLA_2). In the present study, using FCS we could demonstrate that the serine residue of PS is required for protein 4.1 to bind to PS-liposomes and that the bound protein 4.1 is closely associated with the fatty acid of the PS molecule in the liposomes.

Keywords: Fluorescence correlation spectroscopy (FCS); Fluorescence autocorrelation functions (FAF); Diffusion time; Protein 4.1; Phosphatidyl serine (PS); Phospholipase A_2 (PLA_2)

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1. Introduction

Fluorescence correlation spectroscopy (FCS) is based on a fluctuation analysis of fluorescent intensity to detect and characterize fluorophores at the single molecule level in solution. In bioscience, FCS facilitates the measurement of two important physical parameters: the average number of molecules in the detection volume and the translational diffusion time of the molecules, through the open volume of detection [1–3]. This paper is primarily concerned with the diffusion aspects of FCS measurement, in that diffusional motion reflects the molecular weight and the shape of the fluorescent species. FCS allows the analysis of the binding interactions of biomolecules leading to the formation of a complex between a small fluorescent molecule and a much larger molecule, by measuring the changes in the diffusional behavior of the small molecules in a homogeneous solution. Detailed theoretical explanations and potential applications have appeared in the literature [1–5]. FCS was developed in the mid-1970s, but has only recently been applied to nucleic acid research [6–8], protein aggregation [9], ligand binding to membrane-solution interfaces [10], ligand–protein interactions [11], and protein–protein interactions [12,13]. These applications have been made possible by the technical advances in confocal optical systems and the development of high quantum-efficiency detectors during the past 10 years [3].

Protein–liposome binding interactions have been studied only to a limited degree by this method [14]. The hydrophobicity of many proteins induces their adsorption onto the surface of the sample chamber, and consequently the number of molecules cannot be accurately determined, even using FCS. The labeling of proteins is a disadvantage in FCS, owing to the complicated sample preparation procedure, which may alter the properties of the proteins. However, FCS is very promising for studying the binding interactions of proteins in a homogeneous solution. In the present study, we used FCS with a biological system to analyze the binding between protein 4.1 and phosphatidyl serine (PS) liposomes.

Protein 4.1 is required to maintain the membrane mechanical properties of deformability and stability of red blood cells [15,16]. Purified protein 4.1 is a globular protein of approximately 80kDa, as determined by SDS-polyacrylamide gel electrophoresis [17]. This protein interacts with spectrin and actin [18,19] and two important transmembrane proteins, band 3 [20–22] and glycophorin C [23,24] in red blood cells, as well as with CD44 (cluster of differentiation 44) in keratinocytes [24]. All of these interactions of protein 4.1 were previously shown to be modulated by Ca^{2+} and calmodulin [25,26] and also possibly by phosphorylation. The interactions of this protein with membrane lipids, particularly PS located in the inner leaflet of the lipid bilayer [27,28], are not well understood. In the present study, FCS was applied to the analysis of the interactions between protein 4.1 and a PS-liposome, which were found to be hydrophobic.

2. Methods

2.1. Preparation of rhodamine-labeled protein

Protein 4.1 (0.24 mg/ml) was purified from human erythrocytes by the method of Tyler et al. [17] with minor modifications, and was dialyzed against 0.1 M phosphate buffer, pH 9.0. One milligram of rhodamine [tetramethylrhodamine-6-isothiocyanate (TRITC); Molecular Probes, OR, USA] was dissolved in 330 μl dimethylformamide (DMF). Protein 4.1 was mixed with rhodamine at a molar ratio of 1:2, and the solution was incubated at 4°C overnight. Rhodamine-labeled protein 4.1 (rhodamine-4.1) was separated from unbound rhodamine using a column (1.0×10 cm) of Calmodulin Sepharose-4B (Pharmacia Biotech, Uppsala, Sweden). After a dilution with five volumes of 10 mM Tris–HCl (pH 7.5) 0.1 M NaCl, and 0.2 mM $CaCl_2$, the mixture was loaded onto the column. The unbound rhodamine was eluted with the same buffer containing 5% ethanol. After a buffer wash, the rhodamine-4.1 was eluted with buffer containing 0.6 M NaCl instead of 0.1 M NaCl. The protein concentration of rhodamine-4.1 (0.059 mg/ml) was measured [29] and then the fluorescence intensity, with exci-

tation at 554 nm and emission at 573 nm, was determined with a fluorescence spectrophotometer (Hitachi F-3010, Japan). The rhodamine to protein 4.1 ratio was calculated to be one.

3. Preparation of liposomes

Phospholipids [PS, phosphatidyl choline (PC) or a mixture of PS and PC] were dissolved in chloroform and were evaporated under reduced pressure in a rotary evaporator. To the phospholipid film thus obtained, the binding buffer was added to make a phospholipid concentration of 1 mg/ml, and then the solution was sonicated using a bath type sonicator (Branson Sonifier 450) for 1 h at room temperature, under a nitrogen atmosphere to prevent oxidation. The phospholipid concentration in the liposomes was measured according to Bartlett [30].

4. Incubation of rhodamine-4.1 with liposomes

Rhodamine-4.1 (0.059 mg/ml) was diluted fivefold with 10 mM Tris–HCl buffer (pH 7.5) 0.1 M NaCl, and 0.2 mM $CaCl_2$. To 10 μl of this solution, 2 μl of liposomes (1 mg/ml) containing various molar ratios of PS and PC were added to study the interactions of rhodamine-4.1 with liposomes by FCS. In some experiments, 2-μl PS-liposome aliquots were sequentially added to determine whether the bound rhodamine-4.1 was redistributed among the added PS-liposomes. The nature of the protein 4.1–PS interaction was examined by the addition of 2 M KCl or 1 mg/ml of calmodulin and 100 μM Ca^{2+} to the complex of rhodamine-4.1 and PS-liposomes. The effects of various phospholipases were also examined by treatment of rhodamine-4.1-bound PS-liposomes with 10 U of phospholipase A_2 (PLA_2) or phospholipase C (PLC) for 1 h at 25°C in the presence of 100 μM Ca^{2+}.

5. FCS measurement and data analysis

FCS measurements were carried out using a ConfoCor spectrometer (Carl Zeiss, Jena GmbH, Jena, Germany) equipped with a CW Ar^+ laser (excitation at 514.5 nm). The sample was excited with the laser beam at 50 μW power, focused by a water immersion objective (C-Apochromat, 40×, 1.2 NA; Carl Zeiss). Fluorescence from the focused detection volume was separated by a dichroic mirror (> 510 nm) and an emission filter (~ 530–610 nm), and was detected by an avalanche photodiode (SPCM-200-PQ, EG&G, Quebec, Canada). The pinhole diameter at the image plane in front of the photodiode was adjusted to 30 μm for confocal detection. All measurements at 60 s were performed on Lab-Tek chambered coverglass slides with eight wells and an ~ 140 μm thick coverslide on the bottom (Nalge Nunc International, Naperville, IL, USA). Fluorescence autocorrelation functions (FAF), $G(\tau)$, were determined with a digital correlator (ALV 5000/E, ALV GmbH, Germany) and were fitted with the FCS Access Fit software (EVOTEC BioSystems GmbH, Hamburg, Germany) by one- or two-component model as follows:

$$G(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2} = 1 + \frac{1}{N}\left[\frac{1-y}{\left(1+\frac{\tau}{\tau_{free}}\right)\left(1+s^2\frac{\tau}{\tau_{free}}\right)^{1/2}} + \frac{y}{\left(1+\frac{\tau}{\tau_{bound}}\right)\left(1+s^2\frac{\tau}{\tau_{bound}}\right)^{1/2}}\right]$$

where y is the bound ratio of labeled molecule, N is the number of labeled molecules in the detection volume element defined by a radius w_0 and a length $2z_0$, s is the structure parameter representing the ratio of the radius to one-half length of the detection volume, $s = w_0/z_0$ [4,5], and τ_{free} and τ_{bound} are the translational diffusion times of the free and the bound labeled molecules through the detection volume respectively. The diffusion coefficient of rhodamine 6G, D (2.8×10^{-10} m^2/s), was used as an authentic value for determination of the parameters, w_0 and s, prior to

the experiments with $D = w_0^2/4\ \tau_{free}$. The structure parameter, s, and the detection volume evaluated by FCS were $\sim 0.1–0.2$ and $\sim 0.4–0.6$ fl, respectively.

6. Results and discussion

As a first step in the FCS experiments, the binding of quickly diffusing molecules (rhodamine-4.1) to slowly diffusing particules, such as liposomes, was measured based on the change in diffusion time in the binding buffer. Fig. 1 shows the normalized FAFs of four different fluorescent molecules or particles in the binding buffer: rhodamine 6G, rhodamine-4.1, and its complex form in the presence of PS-liposomes or PC-liposomes. The FAF of rhodamine-4.1 in the presence of PS-liposomes was shifted to the right, due to binding to the much higher molecular weight structure of the PS-liposome. The diffusion time of rhodamine-4.1 in the presence of PC-liposomes exhibited no significant change. It is thus apparent that rhodamine-4.1 binds to PS-liposomes, but does not bind to PC-liposomes.

The molecular weight of rhodamine-4.1 and its complex form with the PS-liposome could be calculated, based on the average diffusion times obtained by one-component fit to $MW_{fm} = (\tau_{fm}/\tau_{rho})^3 \times MW_{rho}$, assuming the molecule to be spherical in shape; MW_{fm} and τ_{fm} are the calculated molecular weight and the measured average diffusion time of the fluorescent molecules. MW_{rho} and τ_{rho} are the molecular weight (0.479 kDa) and the measured diffusion time of rhodamine, respectively. The diffusion time of rhodamine-4.1 was found to be 0.24–0.38 ms corresponding to a calculated molecular weight of 75–381 kDa. The diffusion time and the calculated molecular weight of the complex form with PS-liposomes under conditions of saturation ($y = 1$) were approximately 1.4 ms and 23 400 kDa, respectively.

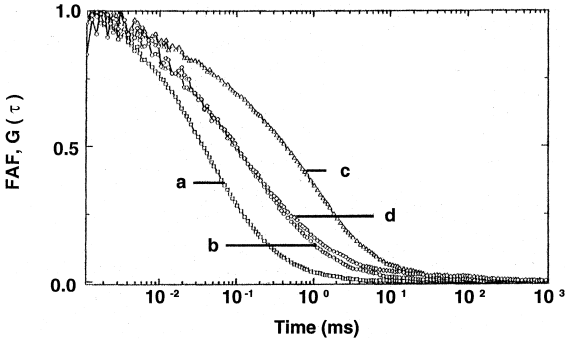

Fig. 1. Measurements of the interactions between rhodamine-4.1 and PS- or PC-liposomes by FCS. Rhodamine-4.1 was incubated with PS- or PC- liposomes as described in Section 2. The FAFs of (a) rhodamine 6G alone, (b) rhodamine-4.1, (c) rhodamine-4.1 in the presence of PS-liposomes, and (d) rhodamine-4.1 in the presence of PC-liposomes are plotted against the correlation time on a log scale.

To confirm whether this binding depends on the PS content, rhodamine-4.1 was mixed with liposomes containing various PS/PC ratios. As shown in Fig. 2a, the FAF of rhodamine-4.1 shifted to the right, with the extent depending on the PS/PC ratio. The amount of bound rhodamine-4.1 increased proportionally to the amount of PS (Fig. 2b), and protein 4.1 is thus shown to bind specifically to PS and not to PC. These results suggest that the head group of PS, the serine residue, is required for protein 4.1 to bind to PS.

The interaction of protein 4.1 with PS was studied by examining the conditions under which bound protein 4.1 could be eluted from PS-liposomes. High salt concentrations (2 M KCl), known to dissociate protein 4.1 from its protein interactions within the red cell membranes, were initially used, but there was absolutely no release of rhodamine-4.1 from the PS-liposomes (data not shown). Under the same conditions, most of the protein 4.1 was released when it was bound to band 3 or glycophorin C. The interactions between protein 4.1 and PS thus appear to be hydrophobic and not electrostatic.

PS-liposomes were added to a mixture of rhodamine-4.1 and PS liposomes to see whether the bound rhodamine-4.1 is released from the previously added PS-liposomes and becomes redistributed among both the previously and newly added PS-liposomes. As shown in Fig. 3a, the addition of up to fivefold more PS-liposomes caused no significant change in the FAFs. The calculated number of particles (PS-liposomes) bound to rhodamine-4.1 did not change, although

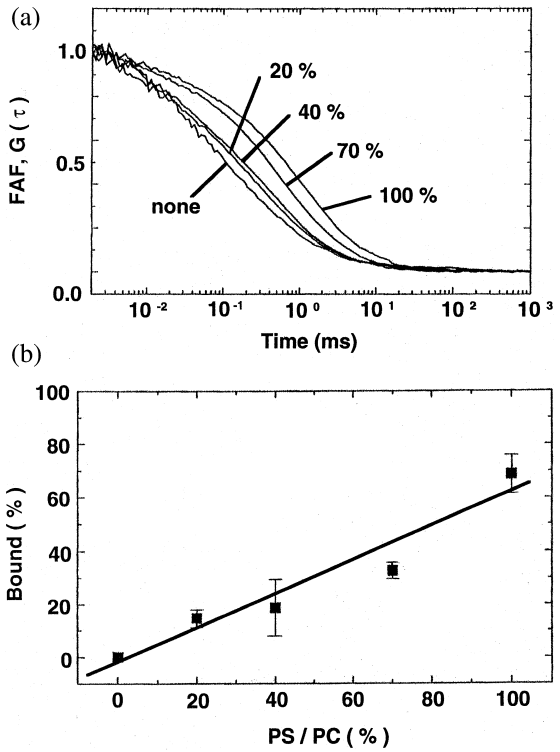

Fig. 2. Interactions of rhodamine-4.1 with liposomes containing various PS/PC ratios. In (a), the FAFs of rhodamine-4.1 with liposomes containing 0–100% of PS are plotted against the correlation time on a log scale. In (b), the amounts of bound rhodamine-4.1 are plotted against the PS content in the liposomes.

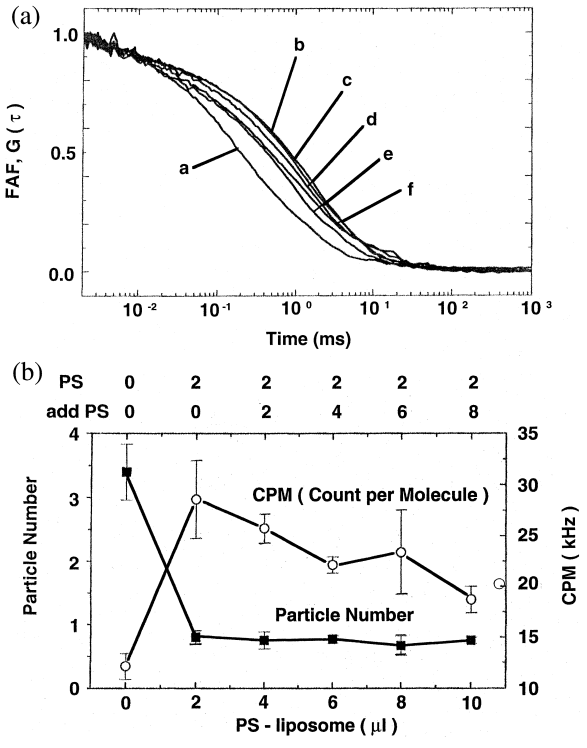

Fig. 3. Binding of rhodamine-4.1 with sequentially added PS-liposomes. In (a), the FAFs of rhodamine-4.1 without a and with sequentially added b 2, c 4, d 6, e 8, and f 10 μl of PS-liposomes are plotted against the correlation time on a log scale. In (b), the calculated number of particles (PS-liposomes) bound to rhodamine-4.1 and the counts per molecule are plotted against the amounts of added PS-liposomes.

the counts per molecule slightly decreased with an increase in the total number of PS-liposomes (Fig. 3b). It is thus evident that once protein 4.1 binds to PS-liposomes, it does not dissociate in solution. This hydrophobic nature of the protein 4.1–PS interactions is inconsistent with a previous report, which suggested the possibility of electrostatic interactions, based on the results of a centrifugation analysis [28].

The bound rhodamine-4.1 was specifically released from PS-liposomes by treatment with PLA_2, while PLC had no effect. As shown in Fig. 4, the FAF of rhodamine-4.1 in the presence of PS-liposomes was shifted to the right and back to the original level of the unbound protein by PLA_2 treatment. The PLA_2-catalyzed hydrolysis of the ester bonds between the *sn*-2 acyl chain (fatty acid) and the glycerol in the PS molecule released rhodamine-4.1 from PS, suggesting that protein 4.1 is closely associated with the fatty acid of the PS molecule in the liposome. This finding was confirmed by the fact that the hydrolytic cleavage of the head group of PS, the phospho-serine residue, by PLC did not affect the rhodamine 4.1–PS interaction. Preliminary biochemical analytical results have revealed that the released protein 4.1 actually contains fatty acids (An and Takakuwa, unpublished data). Taken together, the present results imply that the serine residue allows the protein 4.1 molecule to bind to PS-liposomes and that once protein 4.1 gets into PS-liposomes, it associates hydrophobically with fatty acids of phospholipids.

The effects of calmodulin and Ca^{2+}, which are

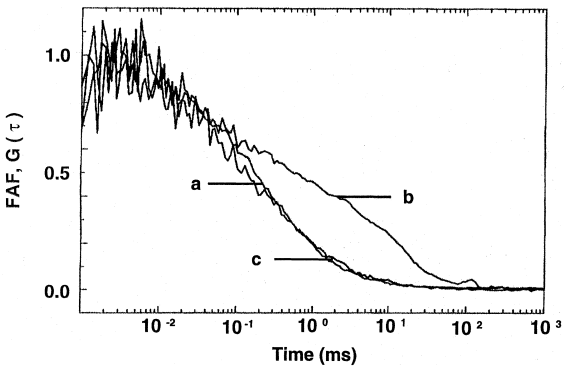

Fig. 4. Dissociation of rhodamine-4.1 from PS-liposomes by PLA_2 treatment. PS-liposomes with bound rhodamine-4.1 were treated with PLA_2, as described in Section 2. The FAFs of (a) rhodamine-4.1, (b) rhodamine-4.1 with PS-liposomes, and (c) rhodamine-4.1 with PS-liposomes treated by PLA_2 are plotted against the correlation time on a log scale.

known to dissociate protein 4.1 from its protein interactions within the red cell membranes, were also examined. As shown in Fig. 5, the FAF of rhodamine-4.1 in the presence of PS-liposomes was shifted to the right (line b) and was not back to the original level of the unbound protein (line a) by addition of 1 mg/ml of calmodulin (line c) and by both calmodulin and 100-μM Ca^{2+} (line d). These results indicate absolutely no release of the rhodamine-4.1 from the PS-liposomes by calmodulin and Ca^{2+}.

In the present study, FCS was used to examine the interactions between a membrane lipid and a protein. The protein 4.1–PS interactions served as the model system to demonstrate the membrane lipid-protein interactions. This protein was labeled with rhodamine and its interactions with PS-liposomes were measured by FCS. The present results are useful for analyzing protein interactions with biological membrane lipids, since PS is located in the inner leaflet of the lipid bilayer. Using this method, the protein 4.1 interactions with PS present on the surface of the inside-out vesicles prepared from red blood cells are now being examined by the authors.

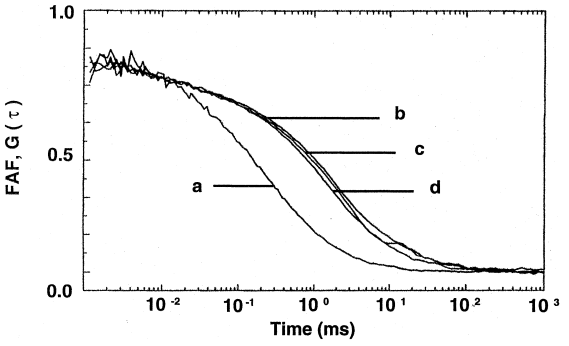

Fig. 5. No effect of calmodulin/Ca^{2+} on rhodamine-4.1–PS-liposome interactions. Calmodulin and Ca^{2+} were added to the complex of rhodamine-4.1 and PS liposomes, as described in Section 2. The FAFs of (a) rhodamine-4.1, (b) rhodamine-4.1 with PS-liposomes, (c, b) with calmodulin, and (d, c) with Ca^{2+} are plotted against correlation time on a log scale.

7. Nomenclature

FCS:	Fluorescence correlation spectroscopy
Rhodamine-4.1:	Rhodamine-labeled protein 4.1
PS:	Phosphatidyl serine
PLA_2:	Phospholipase A_2
PC:	Phosphatidyl choline
PLC:	Phospholipase C
FAF:	Fluorescence autocorrelation function

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