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A New Fluorescent Biosensor for Inositol Trisphosphate

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An intracellular second messenger D-*myo*-inositol-1,4,5-trisphosphate (IP_3) controls the cellular Ca^{2+} concentration, thereby regulating many critical cellular functions.[1] While HPLC[2] and capillary electrophoretic[3] analyses provide sensitive methods to extract and analyze the concentration of IP_3, a fluorescent sensor would be an ideal tool[4] for the convenient measurements of the IP_3 concentration. A synthetic chemical approach has been reported to design an in vitro chemosensor for IP_3 by using a cleft-like receptor possessing guanidinium groups.[5] An alternative framework for the design of IP_3 biosensors is a natural protein domain with high affinity and specificity to IP_3. One of such candidates is the pleckstrin homology (PH) domain, a ~120 amino acid protein module identified in several key regulatory proteins.[6] The PH domain of phospholipase C (PLC) δ_1 binds IP_3 with a submicromolar affinity and associates with lipid vesicles containing phosphatidylinositol-4,5-bisphosphate, but only weakly binds other inositol phosphates and phosphatidylinositol-4-phosphate.[7] By using these characteristics, modification of the native cysteine residue of the PH domain with a thiol reactive fluorophore gave a PH domain−fluorophore conjugate,[8] and a fusion protein of PLCδ_1 PH domain and the green fluorescent protein was used to visualize the cellular distribution of phosphoinositides.[9] Because a variety of optical sensors for small ligands has been successfully constructed by the site-specific incorporation of fluorophores to proteins,[10] we have reasoned that a structure-based approach could afford an optical sensor with high specificity by using the PH domain. We report here that a biosensor designed from a natural protein domain optically responds to the concentration change of IP_3 and binds IP_3 with higher specificity than the parent PH domain does.

The three-dimensional structure of the PH domain from PLCδ_1 and IP_3 complex[11] (Figure 1) was used to identify locations where a single cysteine mutation could be introduced for the covalent attachment of environmentally sensitive fluorophores without greatly affecting the affinity to the ligand. Modeling studies indicated Arg56, Val58, and Asn106 as possible labeling sites (Figure 1). These three residues do not contribute to IP_3 binding, and the β-carbons of these amino acids are located 8−9.7 Å away from the C2 carbon of bound IP_3, implying that the fluorophore introduced at each site would snugly fit in the binding pocket. Formation of such a pseudocomplex would prevent the semispecific ligand binding, thereby increasing the ligand selectivity of fluorophore-labeled PH domain.

Two invariant cysteine residues (positions 48 and 96) of the PH domain from rat PLCδ_1 were first mutated to serine residues,

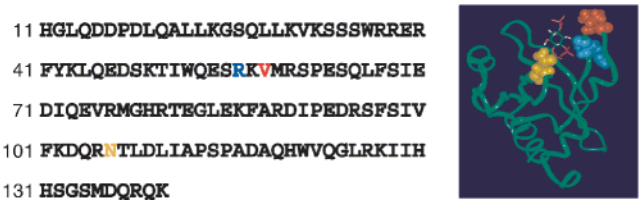

Figure 1. Amino acid sequence for the PLCδ_1 PH domain (left) shows the positions mutated to cysteine at 56R (blue), 58V (red), and 106N (yellow). Numbering corrresponds to rat PLCδ_1, and constructs used here contain N-terminal Met. A schematic illustration shows the structure of PLCδ_1 PH domain−IP_3 complex[11] (right). Positions labeled by fluorophores at 56R, 58V, and 106N are indicated by CPK representation in blue, red, and yellow, respectively, and IP_3 is shown by a wire-frame model.

followed by an introduction of unique cysteine near the IP_3 binding site. Three mutant PH domains with a unique cysteine residue were constructed by a PCR-based site-directed mutagenesis at these three sites to give PH56, PH58, and PH106. Four thiol reactive fluorophores, 5-iodoacetamidofluorescein (5F), 6-iodoacetamidofluorescein (6F), 6-bromoacetyl-2-dimethyl-aminonaphthalene (DAN), and 2-[4′-(2′′-iodoacetamido)phenyl]aminonaphthalene-6-sulfonic acid (ANS) were attached at each of these positions, and successive purification with a gel filtration followed by a cation-exchange chromatography gave 12 different fluorophore-labeled PH domains. CD spectra of these PH domains were indistinguishable from that of the wild type, indicating that the secondary structure was not perturbed by the introduction of fluorophore.[7b,12]

Each fluorophore-labeled PH domain was tested for IP_3 binding by monitoring the fluorescent emission spectra of a 100 nM solution. Distinct changes of emission spectra were observed for all the PH106 derivatives as typically shown for 6F106 and DAN106 in Figure 2. Among the sensors responding to the change of IP_3 concentration, only 6F106 showed an increase in the intensity of fluorescence emission upon addition of IP_3 (Figure 2a). The emission intensity of DAN derivatives at positions 56 and 106 (DAN56 and DAN106) decreased significantly with increasing concentration of IP_3, and the emission maxima were shifted from 505 to 525 nm and 490 to 535 nm, respectively, for DAN106 (Figure 2b) and DAN56. The intensity of emission spectra for DAN106 in the absence of IP_3 was 4 times greater than that of DAN56. The dimethylaminonaphthalene fluorophore of DAN is sensitive to the polarity of the environment and shows a significant blue shift in nonpolar relative to polar environments.[13] The observed changes in the emission maxima of DAN 56 and DAN106 indicate that the attached DAN fluorophore is placed in the binding pocket in the absence of IP_3 and is exposed to the solvent upon IP_3 binding. All the PH58 derivatives and both the 6F and 5F derivatives of PH56 showed little spectral change in response to the concentration

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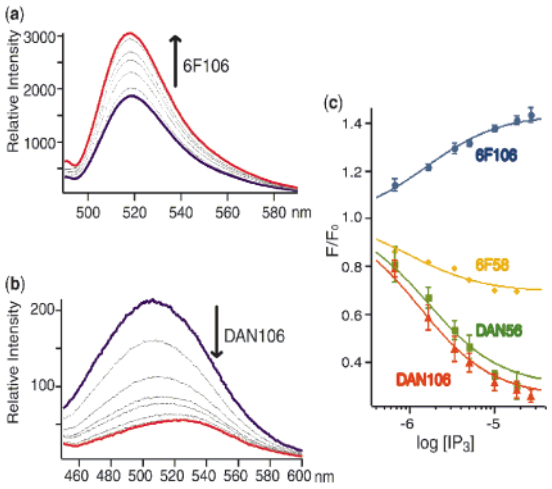

Figure 2. Emission spectra of the fluorophore-labeled PH domains 6F106 (a) and DAN106 (b) with increasing amount of IP_3. The initial spectra are shown in blue and the final spectra in red. Arrows indicate the increase (a) and decrease (b) in the spectral intensity with addition of IP_3 (0.33−25.5 μM). (c) Binding curves for DAN56 (green), 6F58 (brown), DAN106 (red), and 6F106 (blue) were determined by fluorescent titrations. The experimental data points of relative intensity (F/F_o) is plotted against the IP_3 concentration (log [IP_3]).

Table 1. Dissociation Constants (K_d) and Fluorescence Change Ratios of IP_3 Sensor in 10 mM Phosphate Buffer (pH 7.0) Containing 50 mM NaCl and 0.005% Tween 20 at 25 °C

PH domain	conjugate	fluorescence change	K_d (μM)
PH106	DAN	0.30	2.21 ± 0.17^a
	6F	1.59	2.14 ± 0.34^a
	6F	−	2.83 ± 0.27^b
PH56	DAN	0.24	2.37 ± 0.21^a
PH58	6F	0.70	0.90 ± 0.09^a
	DAN	0.68	0.99 ± 0.10^a

[a] Obtained by the fluorescent titration. [b] Obtained by the surface plasmon resonance binding assay.

of IP_3. Even modified with the same fluorophore 6F, the changes in the fluorescence are highly dependent on the attachment position, as typically documented by the increasing fluorescence for 6F106 and the decreasing one for 6F58.

The binding constants were determined by monitoring the changes in fluorescence emission upon addition of IP_3 to a 100 nM solution of the fluorophore-labeled PH domain. The resulting curves were analyzed by fitting to a standard binding isotherm,[14] and were shown in Figure 2c. Although the affinity of sensors to IP_3 varied both with the labeled position and with fluorophore, the equilibrium dissociation constants obtained by the fluorescent titrations were comparable to those obtained by the surface plasmon resonance binding study using the immobilized IP_3[15] (Table 1). These results suggest that a combinatorial approach with various fluorophore and with attachment positions is necessary to optimize the sensitivity of the biosensor.

The selectivity of biosensor to various IP_3 derivatives was next analyzed. DAN106 discriminated L-IP_3 and D-*myo*-inositol-1,3,4,5-tetrakis-phosphate (IP_4) from D-IP_3 more efficiently than nonlabeled PH106 did. DAN106 revealed selectivity to IP at least similar to that of the parent PH106. Since PH106 binds IP_3 with 3 times greater affinity, the high selectivity of DAN106 might be accomplished at the expense of affinity to IP_3 (Table 2). As clearly shown by the shift of emission maxima in the absence or presence of IP_3, the fluorophore of DAN106 forms a pseudocomplex in the IP_3 binding pocket in the absence of IP_3. It is quite possible that stablity of such a pseudocomplex modulates the selectivity of DAN106.

Table 2. Dissociation Constants (K_d) of DAN106 and Unlabeled PH106 to Inositol Phosphate Derivatives in 10 mM Phosphate Buffer (pH 7.0) Containing 50 mM NaCl and 0.005% Tween 20 at 25 °C

ligand	DAN106 K_d (μM)	S^a	PH106 K_d (μM)[b]	S^a
D-IP_3	2.21 ± 0.17		0.73 ± 0.03	
L-IP_3	28.18 ± 0.20	12.8	2.63 ± 0.67	3.6
IP_4	63.68 ± 6.33	29.0	13.0 ± 3.0	17.8
IP	>100		>100	

[a] *S* represents the ratio to the dissociation constant for D-IP_3. [b] Obtained by competition assay using respective DAN106−inositol derivative complexes.

In summary, proper placement of an environmentally sensitive fluorophore within the PH domain successfully afforded a variety of IP_3 biosensors that enables the monitoring of the IP_3 concentration at various wavelengths. The sensor targets IP_3 with a micromolar dissociation constant and with a selectivity to IP_3 over other inositol derivatives higher than the parent PH domain. The strategy opens a possibility for the design of optical sensors with higher ligand selectivity than naturally occurring receptors or ligand binding sites of proteins. It would be interesting to see whether these sensors could monitor IP_3 in vivo, which is currently underway.

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Supporting Information Available: Experimental details for synthesis and fluorescent titrations with IP_3 of fluorophore-labeled PH56, PH58, and PH106 derivatives (PDF). This material is available free of charge via the Internet at http://pubs.asc.org.

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