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Fluorescent-Based Orthogonal Sensing Methods for Double Evaluation in PKC Ligands Screening

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Protein kinase C (PKC) is proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. To find drug lead compounds, development of screening methods, which would be applicable to high-throughput screening, is critical. In this study, we developed two screening methods based on fluorescent labeling to PKC ligand binding domain (C1 domain) and DAG-lactone.

Keywords: DAG-lactone, liagnd-screening, fluorescent labeling, PKC C1 domain

Introduction

Protein kinase C (PKC) is a family of enzymes for phosphorylation, which is specific for Ser and Thr residues. PKC family comprises at least 11 isozymes, which play fundamental roles in signaling pathways that regulate cell cycle progression, differentiation and apoptosis [1]. PKC has also been proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. In PKC activation that depends on such as a diacylglycerol (DAG) and phorbol ester (tumor promoter), C1 domain plays a critical role in these ligands binding. To evaluate ligand binding to C1 domain, two different approaches utilizing fluorescent sensing were developed instead of conventional method using radioisotopes. The first method utilizes the synthetic δC1b domain derivatives bearing a dansyl group, which is environmentally responsive (Fig. 1, left). The second method involves competitive ligand

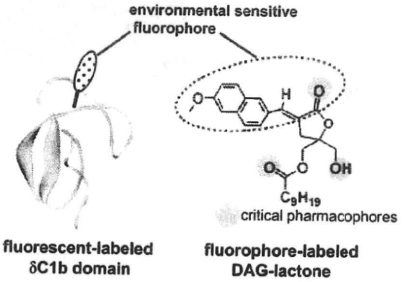

Fig. 1. Orthogonal fluorescent screening tools.

replacement by a fluorophore-labeled DAG-lactone (Fig. 1, right). DAG-lactone is a synthetic PKC ligand based on DAG. To reduce the entropic penalty of DAG, the glycerol backbone is constrained by forming a lactone ring [2]. In this study, the fluorescent screening tools, differently labeled on the acceptor and the ligand, were applied for evaluations of binding affinity of PKC ligands.

Results and Discussion

For the C1b domain of PKCδ (δC1b) (221-281), amino acids at three positions near the binding pocket were selected as insertion sites of fluorescent dye (Ser 240, Thr 242) [3]. A dansyl group was adopted as a fluorescent dye based on its sensitivity to environmental change and small molecular size. To introduce the dansyl group by Fmoc-SPPS, Fmoc-Lys(dansyl-Gly)-OH was prepared. Each fluorescent-labled δC1b domain analog was successfully synthesized. Ligand bindings of these fluorescent analogs were evaluated by utilizing [^{3}H]-phorbol 12,13-dibutylate (PDBu). The two δC1b analogs, T242K(dansyl-G) and S240K(dansyl-G), showed similar binding affinity with that of wild-type. In titration experiments of ligands, the increase of fluorescent intensity and the shift of fluorescence emission maxima showed reasonable correspondence to the binding affinity of ligands for PKC evaluated by the RI method (Fig. 2a).

As a new probe of PKC ligands binding assay based on the competitive inhibition, a fluorophore-labeled DAG-lactone was synthesized. The lactone has environmentally sensitive fluorophore on the position which is not necessary for binding to PKC C1 domain. The binding affinity of a synthetic fluorophore-labeled DAG-lactone for the PKCδ was evaluated by utilizing [^{3}H]-PDBu. The environmental sensitivity of the synthetic DAG-lactone derivative was confirmed by fluorescent measurement in various solvents. The fluorescent intensity of the fluorophore-labeled DAG-lactone was increased by binding to PKC, which indicates the hydrophobic environment of the binding pocket. According to an increase in the concentration of a test compound, the fluorescence intensity was decreased, indicating replacement of the fluorophore-labeled DAG-lactone. The fluorescent spectra in titration of PDBu are shown in Fig. 2b. The IC_{50} values were obtained from the curve-fitting of titrations of known compounds. The fluorescent-based inhibition assay showed a positive correlation between IC_{50} values and K_i values in the RI assay.

In summary, novel screening tools for PKC ligands based on fluorescent-labeling of C1b domain and DAG-lactone have been successfully developed. The combinational use of these fluorescent-labeling methods would lead to detailed and reliable evaluation of ligand compounds for PKC ligands, wchich does not require washing steps.

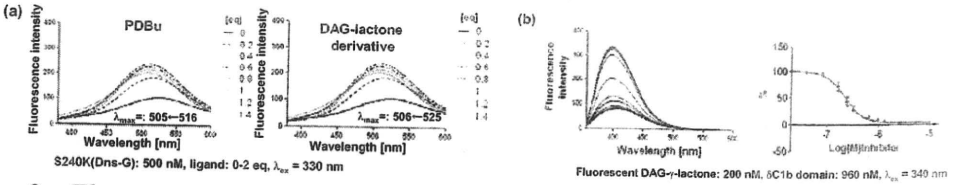

Fig. 2. Fluorescent titration of fluorescent-labeled δC1b (a) and fluorophore -labeled DAG-lactone (b).

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