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Genetically Encoded FRET Probe for PKC Activity Based on Pleckstrin

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Cell fate and cellular functions are regulated by modulation of protein activity. Protein phosphorylation and dephosphorylation provide one of several systems that tightly control protein activity. Protein kinase C (PKC) with all its isoforms is one of the most extensively studied phosphorylating enzymes in this respect.[1] Therefore, fluorescent methods that allow monitoring this and other enzyme activities are important tools in cell biology[2] and drug discovery. Here we introduce a genetically encoded reporter for protein kinase C activity based on Förster resonance energy transfer (FRET).

Genetically encoded reporter molecules have been introduced for intracellular signaling molecules such as calcium, cAMP, and cGMP as well as enzyme activities.[2−5] In the most widely used approach for measuring enzyme activity, a substrate sequence for the enzyme of interest and a concomitant recognition or binding domain is enclosed by two FRET-capable fluorescent proteins. Upon enzyme activation, the substrate sequence becomes modified (e.g., phosphorylated[6−9] or methylated[10]) and the recognition domain (e.g., Src-homology 2 domain or chromodomain, respectively) binds intramolecularly to the modified substrate sequence. This invokes a structural rearrangement of the protein that includes the fluorophores. The alteration of the fluorophore conformation or distance to each other results in a change of FRET.

In contrast to the above-described design, our PKC probe (KCP-1) relies on the altered intramolecular interaction of two neighboring protein domains due to phosphorylation of an intervening substrate sequence (Figure 1a). The approach does not require additional binding domains to recognize the modified substrate loop. This eliminates the possibility of unwanted interaction of the recognition domain with endogenous ligands and reduces the possibility of interference of the substrate loop with extramolecular domains. A molecule that exhibits conformational changes upon PKC phosphorylation is pleckstrin (or p47), the major substrate for PKC in platelets.[11] Pleckstrin consists of a DEP (Dishevelled, Egl-10, pleckstrin) domain flanked by two pleckstrin homology (PH) domains. The PKC recognition sequence is positioned in the 14 amino acid loop, located between the first PH domain and the DEP domain. The two serines Ser-113 and Ser-117 and threonine Thr-114 are phosphorylated by PKC. We employ a truncated version (PH-DEP) of pleckstrin that undergoes a conformational change upon phosphorylation, as was confirmed by NMR studies (unpublished results). Enhanced yellow (EYFP) and green (GFP^2)[12] fluorescent protein were attached to the N- and C-termini, respectively. GFP^2 is more photostable compared to wild-type GFP and has a predominant absorption band at 390 nm. This allows for excitation with a 405 nm laser line without direct excitation of EYFP, in contrast to enhanced GFP (488 nm) or enhanced cyan fluorescent protein (ECFP, 458 nm). Since the emission spectrum of GFP^2 largely overlaps with the excitation spectrum of EYFP, the two fluorophores form an excellent FRET pair.[13] We also selected the GFP^2/EYFP pair over the classical ECFP/EYFP pair due to higher brightness and FRET efficiency and the lack of direct acceptor excitation at 405 nm.

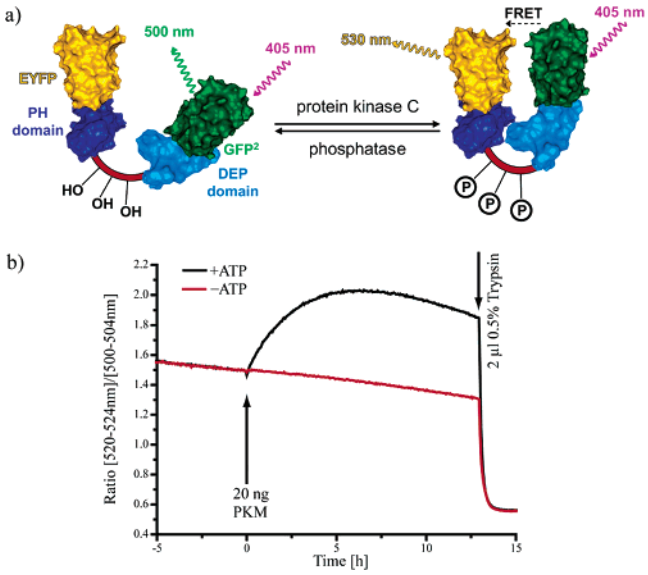

Figure 1. (a) Proposed function of the PKC probe KCP-1: Upon phosphorylation, an intramolecular conformational change alters the orientation of two protein domains, thus increasing the FRET efficiency of the two fluorophores. (b) KCP-1 was phosphorylated by the catalytic subunit of PKC (PKM) in the presence of ATP in vitro, leading to an increase in emission ratio, a measure for FRET efficiency. Subsequent addition of trypsin cleaved the probe, resulting in a total loss of FRET.

We prepared KCP-1 in a mammalian and a bacterial expression vector using standard molecular biology methods. For in vitro analysis, the protein was expressed in and purified from *E. coli*. KCP-1 was phosphorylated in vitro in the presence of ATP and PKM, the catalytic subunit of PKC (Figure 1b). This experiment revealed that the unphosphorylated probe has an intermediate FRET efficiency compared to fully phosphorylated and trypsin-digested KCP-1. Similar experiments with protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) showed no FRET changes (Supporting Information).

In addition, we tested KCP-1 for enzyme specificity with a radioassay using different PKC subtypes, namely, PKCδ, PKCϵ, PKCη, PKCθ, and PKCζ. We observed only minor differences, with PKCθ being the most potent isoform (Supporting Information).

To investigate the capabilities of KCP-1 for monitoring PKC activity in living cells, we expressed the probe in HeLa cells and N1E-115 neuroblasts, respectively. Activation of PKC with phorbol ester (TPA) in HeLa or N1E-115 cells led to a fast increase of FRET, reaching a maximum after about 5 min (cell images are shown in the Supporting Information). Addition of Gö6976,[14] a specific inhibitor for the calcium-dependent kinase isoforms PKCα and PKCβ, did not affect the FRET signal. Subsequent treatment of the cells with Gö6983,[14] an inhibitor for all PKC members, completely abolished the effect of TPA (Figure 2a, data of HeLa

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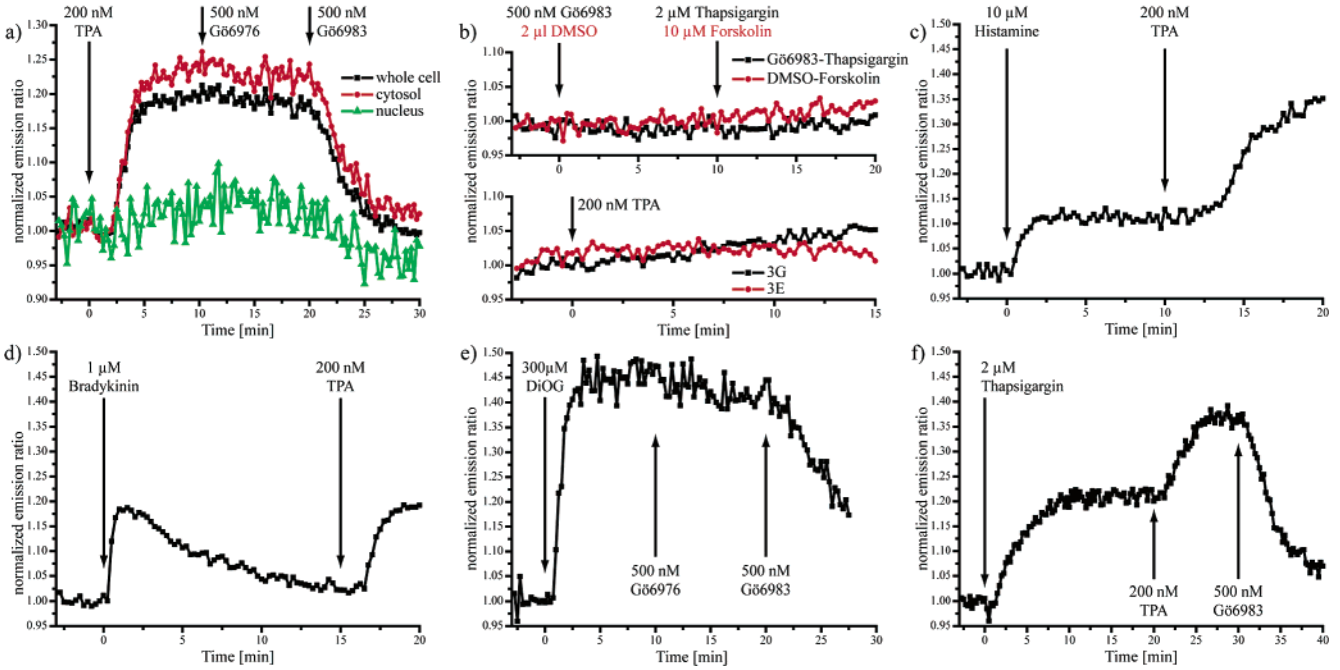

Figure 2. (a) KCP-1 reports the activation of PKC by phorbol ester (TPA) in N1E-115 neuroblastoma cells. PKC activity was observed in the cytosol but not in the nucleus. Addition of the inhibitor Gö6983 reversed PKC activity, reflecting phosphatase activity. (b) Activation of PKA or CaMKII does not result in FRET change (upper panel). Mutating serins and threonins in the substrate loop to glycins (3G) or glutamates (3E) renders the probe insensitive toward TPA treatment of the cells (lower panel). (c) HeLa cells treated with histamine followed by TPA show the difference between physiological and artificial stimulation. (d) Stimulation of N1E-115 cells with bradykinin led to a transient activation of PKC. (e) A supramaximal dose of di-*O*-octanoyl glycerol (DiOG) was sufficient to activate PKC to maximum levels. (f) Administration of thapsigargin failed to fully activate PKC.

cells not shown, unless otherwise stated). This experiment suggests that PKCα and/or PKCβ did not contribute significantly to the phosphorylation of KCP-1; instead, other PKCs seem to be involved. The FRET changes occurred predominantly in the cytosol, while the nucleus was excluded from enzyme activity (see cell images in Supporting Information). The probe is not modified by PKA or CaMKII, since stimulation of PKA with forskolin or activation of CaMKII by thapsigargin in the presence of Gö6983 did not change FRET (Figure 2b, upper panel). When the three phosphorylation sites were mutated to three glutamates or glycins, respectively, no FRET change upon TPA treatment was observed (Figure 2b, lower panel).

To test KCP-1 under more physiologically relevant conditions, we stimulated HeLa cells with histamine. The probe showed smaller FRET changes than with TPA (Figure 2c). When N1E-115 cells were stimulated with bradykinin, a fast and intense increase in FRET was observed. FRET reached a maximum after 2 min, followed by a slow decrease in FRET within 5−10 min (Figure 2d). Bradykinin induces Ins(1,4,5)P_3-mediated release of calcium from internal stores and generates the second messenger diacylglycerol (DAG), both known activators of PKC. Interestingly, the addition of di-*O*-octanoyl glycerol (DiOG), a short-chain DAG derivative, resulted in a full and sustained activation of PKC (Figure 2e), while thapsigargin, an ATPase inhibitor that elevates calcium levels, showed only an intermediate increase (Figure 2f). The contribution and synergism of the two messengers will be studied with KCP-1 in the future.

In conclusion, we generated a new reporter for protein kinase C activity. In contrast to the only other genetically encoded PKC activity probe,[9] our construct does not rely on a general binding domain but performs entirely via conformational rearrangement after phosphorylation. It responds fast to stimuli triggering PKC and is capable of detecting physiologically relevant PKC activity modulations.

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Supporting Information Available: Experimental protocols for cloning, reporter expression, phosphorylation assays, and cell imaging (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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