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A Dual Parameter FRET Probe for Measuring PKC and PKA Activity in Living Cells

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Genetically encoded reporter molecules are the method of choice to observe real-time intracellular events with spatial resolution. For live cell applications, some probes are based on a translocating protein that usually moves from the cytosol to the membrane or vice versa.[1,2] When a fluorophore is attached, clearance of the protein from or translocation to the cytosol is easily observed. Other reporters are composed of a sensor unit fused to one or two fluorescent proteins.[3,4] For kinases and phosphatases, the sensor unit often consists of a substrate sequence and corresponding recognition domain that binds intramolecularly to the phosphorylated but not the unphosphorylated substrate. The binding event is accompanied by a conformational change that alters the distance and/or orientation between the two terminal fluorophores, yielding a detectable change in FRET efficiency. Probes developed on this basis were used to monitor various kinase activities as well as histone modifications.[5−7] We recently developed a FRET probe for protein kinase C (PKC) based on pleckstrin, termed KCP-1.[8] This approach differed from those described above because it lacked a recognition sequence, thus making the probe more versatile for further engineering. Although pleckstrin is the major substrate for PKC in platelets, it is virtually absent from cells not of hemopoietic origin.[9,10] Therefore, little interference with signaling pathways in other cells is expected. Upon phosphorylation, pleckstrin's N-terminal pleckstrin homology (PH) domain 1 and the DEP (*D*ishevelled, *E*gl-10, *p*leckstrin) domain change conformation relative to each other. A 14 amino acid loop connecting the two domains has three PKC-sensitive phosphorylation sites (Ser-113, Thr-114, and Ser-117). The phosphorylation of these residues increased the emission ratio of enhanced yellow (EYFP) to green (GFP[2]) fluorescent proteins, which are attached to the N- and C-termini, respectively. This construct successfully monitored changes in PKC-driven phosphorylation activity in living cells. Here we explore possibilities for engineering a probe sensitive to protein kinase A (PKA), based on KCP-1.

Since KCP-1 does not require a particular recognition domain to sense phosphorylation, we reasoned that it should be possible to alter its substrate specificity, rendering the probe sensitive to other kinases. However, direct replacement of the substrate loop (RK-STRRSIRL) by protein kinase A consensus sequences, including Kemptide (RKSLRRASLG) and a variety of other peptides, was only partially successful. These constructs were either still sensitive to PKC or were neither a substrate for PKA nor PKC. Only one substrate loop variant (RKSLRRASLL) reacted to PKA as well as PKC activation (unpublished data). However, the response was only on the order of 10%. Selectivity for PKA was only observed in the nucleus, where KCP-1 is not phosphorylated by TPA-stimulated PKC.

In an effort to determine the function of the regions flanking the PH and DEP domains, we eliminated an 18 amino acid stretch between the DEP domain and the C-terminal GFP[2], generating a probe termed KCP-2. Surprisingly, the probe was still sensitive to phorbol ester-induced PKC phosphorylation, but responded with a 30−40% decrease in emission ratio, opposite to that observed for KCP-1 (Figure 1A). The result suggested that, in KCP-2, phosphorylation by PKC led to an increase in the distance between the two fluorophores and hence very different conformational changes for KCP-1 and KCP-2 (Figure 1B). Results obtained with KCP-1 could be explained by a double conformational change. On the basis of NMR studies, a nonfluorophore-tagged version of KCP-1 adopted a closed conformation which opened after phosphorylation (unpublished data). This effect was apparently overcome by interaction between the strongly negatively charged C-terminal loop and the body of the molecule (Figure 1C). To support this model, we prepared several mutants, in which one or more acidic residues in the C-terminal loop were replaced by uncharged amino acids (Glu → Gln, Asp → Asn). While all mutants gave relatively small responses, four showed a response in cells that resembled that of KCP-2, indicating that crucial interactions were disrupted between parts of the molecule (Figure S1A). Other experiments showed that 10 amino acid extensions between the C-terminal loop and GFP[2] did not significantly alter the performance of KCP-1 (Figure S1B).

Intrigued by the sensitivity to single acidic amino acids in the C-terminal loop, we introduced a 7 amino acid PKA consensus sequence (Kemptide) between the loop and the fluorescent protein. The resulting probe (KCAP-1) continued to respond to treatment with phorbol ester in N1E-115 neuroblastoma cells. However, when agents that elevated intracellular cAMP levels (forskolin, IBMX, cAMP derivatives, prostaglandin E_1) were subsequently added, the probe responded with a significant drop in emission ratio (Figures 2A and S4). This cAMP response was independent of PKC activity because cells not receiving phorbol ester responded in a similar way. Subsequent treatment with phorbol ester also progressed with normal amplitude, and the signal was sensitive to the PKC inhibitor Gö6983, which reduced emission ratios back to the cAMP-induced levels (Figure 2B). In addition, PKA activity could be separated from PKC activity by pretreatment with PKC inhibitor (Figure 2C). Mutants lacking the serine in the Kemptide sequence did not respond to forskolin/IBMX treatment (Figure S5), which defines the PKA phosphorylation site of KCAP-1. A KCAP-1 mutant (EE227-8QQ) with fewer negative charges in the C-terminal loop responded to PKC with a negative FRET change (Figure S6), similar to KCP-2 and KCP-1$^{EE227-8QQ}$, suggesting that the conformational changes in KCP-1 and KCAP-1 are based on related mechanisms.

KCAP-1 was expressed in and purified from *E. coli* for in vitro analysis. KCAP-1 was phosphorylated by PKC or PKA in vitro in the presence of [^{32}P]-ATP (Figure S7). KCAP-1 was also phosphorylated by other related kinases. While PKB was ineffective, CaMKII marginally phosphorylated KCAP-1. Aurora A and B kinase[11] were both active. Whether KCAP-1 is able to monitor Aurora kinase activity in living cells remains to be explored in the

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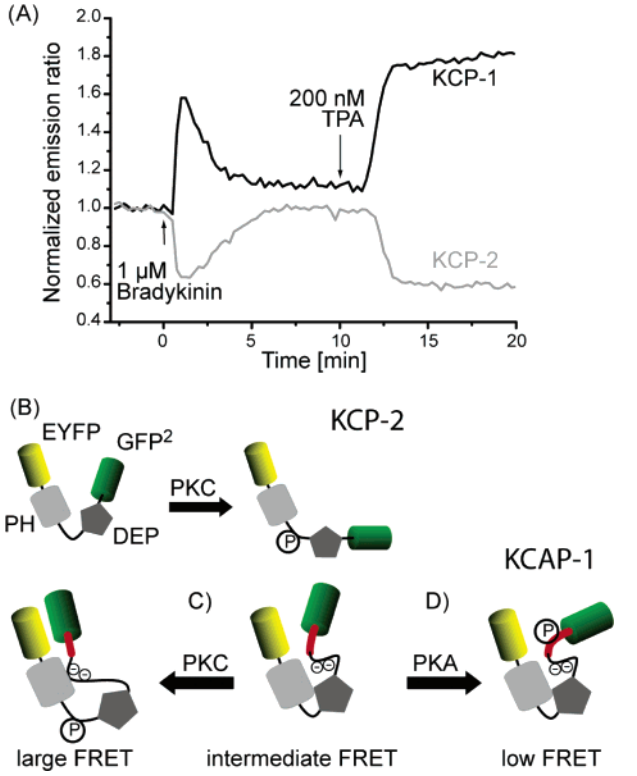

Figure 1. (A) Increases in FRET efficiency in response to bradykinin- or TPA-induced PKC activation are exhibited by KCP-1 in N1E cells. In contrast, the truncated probe KCP-2 shows a decrease in emission ratio. (B) NMR experiments with nonfluorophore-labeled PH-DEP suggest that KCP-2 stretches upon phosphorylation, thereby lowering the FRET efficiency between the fluorophores. Hypothetical mechanism of KCAP-1: (C) Phosphorylation by PKC results in a FRET increase as is observed for KCP-1. (D) The introduction of additional negative charges by PKA phosphorylation leads to reduced FRET efficiency, presumably due to disturbance of ionic interactions. Hence, by introducing a PKA consensus sequence, the probe, KCAP-1, monitors both PKC and PKA activity. The two effects seem to be independent of each other. This hints toward an intermediate interaction of the acidic loop with the C-terminal part of the molecule. Note that changes affecting the orientation of the fluorophore's dipole moments also contribute to the FRET changes.

future. Due to the distinct localization of Aurora kinases and their exclusive appearance during mitosis, a cross reaction with PKA is unlikely.

Finally, we replaced the three PKC-sensitive amino acids with three alanines (KAP-1) or three glutamates (KAP-2). Both sensors were fully capable of monitoring forskolin-induced PKA activity in N1E-115 cells (Figure 2D) and HeLa cells (data not shown). Neither construct exhibited a response to PKC activation in vivo nor were they a substrate for the catalytic subunit of PKC in vitro (Figure S7B).

In summary, we generated new genetically encoded reporters for protein kinase A activity and, to our knowledge, the first genetically encoded dual parameter FRET probe. The PKA sensitivity appears to rely entirely on charge interaction between the C-terminal tail and the main body of the sensor. Due to this generic nature of action, any substrate sequence that alters the number of charges in the C-terminal loop after enzyme activity should be a candidate for a reporter. This could include other kinases and phosphatases as well as acetylases and deacetylases. Dual parameter probes will be valuable tools for analyzing complex intracellular signaling pathways. Particularly in multiparameter imaging, these probes have the advantage of occupying only half

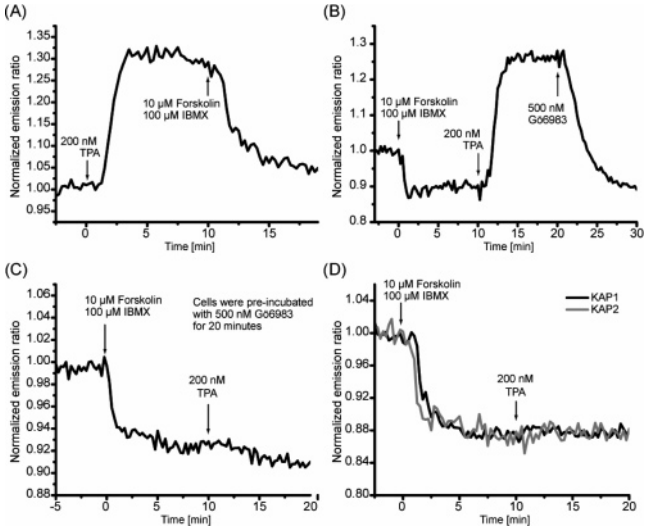

Figure 2. (A) KCAP-1 reported the activation of PKC by phorbol ester (TPA) in N1E-115 neuroblastoma cells. Subsequent treatment with forskolin/IBMX resulted in a drop of emission ratio. (B) Addition of forskolin/IBMX prior to phorbol ester reduced emission ratio, followed by a full TPA response. Subsequent addition of the PKC inhibitor Gö6983 abolished only the PKC response. (C) Pretreatment of cells with Gö6983 resulted in a "pure" protein kinase A response. (D) Replacement of the PKC-sensitive phosphorylation sites by alanines or glutamates yielded two probes (KAP-1 and KAP-2, respectively), sensitive to PKA but not PKC.

of the optical spectra compared to two single FRET probes.[12] In addition, it is tricky to express two single probes at similar levels in the same cell.

Problems could occur when PKA and PKC are activated simultaneously. In this case, the use of specific kinase inhibitors will be required to dissect the signaling event.

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

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