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Rapidly Reversible Manipulation of Molecular Activities Using Dual Chemical Dimerizers

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Abstract

The rapid, inducible, reversible modulation of molecular activities by dual CID systems. Rapamycin (Rapa) treatment induces relocation of FRB-POI to the GAI-FKBP-C2(LACT)-labeled plasma membrane and activates POI-dependent signaling event. A subsequent GA₃-AM treatment induces second relocation of a whole GAI-FKBP-C2(LACT)/rapamycin/FRB-POI

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complex from the plasma membrane to the Tom20-GID1-labeled mitochondria and leads to termination of POI-dependent signaling.

Keywords

Chemically inducible dimerization; reversible perturbation; small GTPase; phospholipid; cell signaling

Rapid, local, specific, and reversible are the four main characteristics of cellular signaling events. With these features, cells spatio-temporally choreograph dynamic signaling. In particular, reversibility allows cells to adjust the duration of a signaling event and efficiently utilize their finite resources. This is especially exemplified by small GTPases and phosphatidylinositol lipids that trigger diverse cellular processes including proliferation, transformation, migration, and apoptosis.^[1] To generate the precise command for each function, these signaling molecules are tightly regulated by a pair of enzymes that switches their activity on or off: guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins for small GTPases, and phosphatidylinositol kinases or phosphatases for phosphatidylinositol lipids.^[1b, 2]

The chemically inducible dimerization (CID) technique has been widely used to rapidly manipulate molecular activities.^[3] In a CID system, a chemical dimerizer such as rapamycin (Scheme 1a) induces dimerization of two proteins: FK506 binding protein (FKBP) and the FKBP12-rapamycin binding protein (FRB). By prelocalizing FKBP to the plasma membrane and FRB-fused protein of interest (FRB-POI) to the cytosol, rapamycin-induced dimerization results in relocation of cytosolic FRB-POI to the plasma membrane (left and middle panels in Scheme 1b).^[4] The accumulation of POI at the plasma membrane subsequently triggers a biological effect that is pertinent to the specific POI molecule of choice. The entire action can be induced on a timescale of seconds in intact living cells. Due to rapid, local and specific induction of signaling, the CID technique has proven powerful and versatile as an experimental perturbation tool. In order to fulfill the fourth nature of signaling, namely reversibility, one may think of washing rapamycin out to dissociate the dimerized complex. However, the clearance rate of rapamycin from cells is extremely slow.^[5] In addition, the binding affinity between rapamycin and FKBP is extremely high (200 pM).^[3b, 3c, 6] As expected, once rapamycin-induced manipulation is turned on, it is challenging to turn it off on a timescale comparable to that of the onset.^[3b, 3c, 7]

Nevertheless, the rapamycin dissociation kinetics should be a function of experimental conditions such as the washout protocol, cell type, dimerizer concentration, and protein configuration and expression level of both FKBP and FRB constructs. Therefore, we began to evaluate the reversibility of CID using a series of CID probes previously developed in our laboratory. Specifically, we co-transfected COS-7 cells with fluorescently tagged FKBP and FRB proteins that each reside in a distinct compartment within the cell: the CFP-FRB is cytosolic while the YFP-FKBP is expressed at the plasma membrane once fused with the Lyn N-terminal signal sequence (Lyn-YFP-FKBP). We then added rapamycin to induce relocation of CFP-FRB to the plasma membrane (Figure S1a). To evaluate dissociation kinetics of the induced dimerization complex, we then washed away the rapamycin-containing media ten times. Each washout efficiency was over 95%, theoretically diluting extracellular rapamycin by a quadrillion times. Even three hours after the extensive washout procedure, CFP-FRB primarily remained at the plasma membrane (Figure S1b). We then took a more proactive approach by using chemical competitors of rapamycin.^[8] In one study, FK506 reversed rapamycin-induced dimerization reasonably well on a timescale of 10 min in HEK-293T cells.^[9] To test the efficiency of this competition strategy in our

dimerization system, we first treated cells with rapamycin to induce the rapid relocation of CFP-FRB from cytosol to the Lyn-YFP-FKBP-expressing plasma membrane. Subsequently, we added increasing concentrations of FK506 to compete with the interaction between FKBP and rapamycin. However, even one-hour treatment with FK506 failed to dislodge CFP-FRB from the plasma membrane (Figure S2). Other rapamycin competitors such as FK506-M and synthetic ligand of FKBP also resulted in little to no effect (data not shown). Taken together, neither extensive washout nor excessive competitors could reverse the rapamycin-induced dimerization on a timescale of seconds, highlighting the inherent limitations of these approaches.

We therefore explored a different strategy to induce rapid reversal of dimerization-induced cellular effects. We reasoned that sequestration of a whole dimerized complex containing POI from the plasma membrane could deactivate the POI-triggered effect. We have recently developed a novel CID system that is completely orthogonal to the existing rapamycin system.^[4b] This new system employs GA₃-AM as a chemical dimerizer (Scheme 1a) that induces rapid dimerization of GAIs (N-terminal 92 amino acids from gibberellin insensitive) and GID1 (gibberellin insensitive dwarf 1).^[4b] It may be possible to induce relocation of POI to the plasma membrane using rapamycin, and then relocate the rapamycin-induced dimerization complex as a whole to other places in cells such as mitochondria using GA₃-AM (middle and right panels, Scheme 1b). With this double relocation strategy, it is crucial to design a target mechanism of FKBP to the plasma membrane such that FKBP is localized to the plasma membrane firmly enough to accumulate the FRB-POI for induction of biological effects, but loosely enough to be dissociated from the plasma membrane, a delicate criterion the Lyn sequence as a membrane anchor does not satisfy. We therefore chose lipid-binding domains such as a C2 domain from lactadherin (C2(LACT)).^[10] The C2(LACT) binds to phosphatidylserine, and primarily localizes to the inner leaflet of the plasma membrane, yet shuttles between membrane and cytosol with a biased retention at the membrane.^[11]

We first evaluated C2(LACT) as a targeting motif for the double relocation strategy. To this end, we introduced three constructs in the COS-7 cells: CFP-FRB, GAIs-YFP-FKBP-C2(LACT), and Tom20-mCherry-GID1 (Tom20 is a mitochondria transmembrane protein). These fusion proteins localized to cytosol, plasma membrane, and mitochondria, respectively (Figure 1a). Rapamycin addition induced rapid accumulation of CFP-FRB at the plasma membrane (Figure 1a and Movie S1). Upon subsequent addition of a second dimerizer, GA₃-AM, both CFP and YFP fluorescence decreased at the plasma membrane with their concomitant increase at the mitochondria (Figure 1a,b and Movie S1), indicating that the rapamycin-induced dimerization complex (i.e., CFP-FRB and GAIs-YFP-FKBP-C2(LACT)) has been successfully sequestered to the mitochondria via GA₃-AM-induced dimerization with Tom20-mCherry-GID1. The kinetics of CFP-FRB relocations were quantified by calculating the time required for half-maximal accumulation ($t_{1/2}$) at the plasma membrane (26.7 ± 4.6 sec for the initial relocation) and mitochondria (97.8 ± 17.3 sec for the second relocation) (Figure 1b). While the kinetics of initial relocation to the plasma membrane reflects rapamycin-induced dimerization alone, the second one away from the plasma membrane is a function of two factors: dissociation of C2(LACT) from the plasma membrane and dimerization of GAI and GID1. In control experiments, we replaced one of the chemical dimerizers with DMSO and observed a single relocation event of CFP-FRB (Figure S3a,b,c). We also confirmed that the FRB domain is indispensable for the dual relocation strategy (Figure S3d,e). These results clearly demonstrate that dual CID systems can rapidly sequester the initial dimerized complex as a whole.

To test whether this double relocation strategy can be used to rapidly turn on and then off molecular activity, we first chose Rac small GTPase as a model. Previously, we have shown

that the recruitment of a Rac specific GEF, Tiam1, to the plasma membrane activates endogenous Rac and induces membrane ruffling in a “sustained” manner.^[4a, 12] For “transient” Rac activation, we generated CFP-FRB-Tiam1 which was co-transfected into cells with GAIs-YFP-FKBP-C2(LACT), Tom20-mCherry-GID1 and mCherry CAAX (a plasma membrane marker). Rapamycin treatment triggered accumulation of CFP-FRB-Tiam1 at the plasma membrane (Figure 2a and Movie S2). Upon subsequent addition of GA₃-AM, the rapamycin-induced dimerization complex (i.e., CFP-FRB-Tiam1 and GAIs-YFP-FKBP-C2(LACT)) was rapidly recruited from the plasma membrane to the mitochondria (Figure 2a and Movie S2). A quantitative analysis of mCherry fluorescence at the plasma membrane region indicated that initial dimerization significantly increased membrane ruffling and the subsequent one decreased this ruffling to the basal level (Figure 2b,c,d). These results clearly demonstrate that the double relocation strategy can be used to rapidly turn on and off the Rac signaling pathway.

We next determined whether the same strategy could be applied to other signaling molecules. We have previously shown that recruitment of an inter SH2 domain (iSH) from a regulatory PI3K subunit, p85, to the plasma membrane activates phosphatidylinositol 3-kinase (PI3K) and produces phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), resulting in membrane ruffling.^[13] We thus constructed CFP-FRB-iSH which was co-transfected with Lyn-mCherry-FKBP and YFP-labeled PH domain from AKT (YFP-PH(AKT) as a PIP₃ biosensor). Upon rapamycin addition, CFP-FRB-iSH accumulated at the plasma membrane, which was followed by accumulation of YFP-PH(AKT) at the plasma membrane and “sustained” induction of membrane ruffles (Figure S4 and Movie S3). We then co-transfected CFP-FRB-iSH with GAIs-YFP-FKBP-C2(LACT), Tom20-mCherry-GID1, and mCherry-CAAX for reversible experiments. After rapamycin-induced dimerization of FKBP and FRB fusion proteins, we added GA₃-AM which triggered the relocation of the dimerized complex of CFP-FRB-iSH and GAIs-YFP-FKBP-C2(LACT) from the plasma membrane to the mitochondria (Figure 3a). A quantitative analysis of mCherry fluorescence at the plasma membrane indicated that the first dimerization induced membrane ruffling while the second dimerization abolished it (Figure 3b,c,d, Movie S4). To rule out the possibility that GA₃-AM treatment and/or a sudden decrease in the concentration of GAIs-YFP-FKBP-C2(LACT) at the plasma membrane affected the membrane ruffling, we performed the following control experiment. Cells were transfected with GAIs-YFP-FKBP-C2(LACT), Tom20-mCherry-GID1 and mCherry-CAAX, and exposed to epidermal growth factor to induce membrane ruffling. We then administered GA₃-AM to trigger relocation of GAIs-YFP-FKBP-C2(LACT) from the plasma membrane to mitochondria. However, this procedure did not affect the once formed membrane ruffles (Figure S5 and Movie S5).

There are many perturbation tools available with an emphasis on different aspects of signaling events. For example, Neddermann et al. established a eukaryotic gene-expression system based on engineered bacterial quorum-sensing.^[14] Kinzel et al. developed an orthogonal estrogen-receptor-based gene switch system responsive to ligands.^[15] Shimizu-Sato et al. developed a light-switchable gene promoter system.^[16] With a distinct contrast to these techniques, the double relocation strategy turns on and off signaling events on a timescale of seconds in living intact cells. In addition, this strategy could be applied to different intracellular locations. We used C2(LACT) for plasma membrane targeting in this study. Alternatively, a PH domain from FAPP (PH(FAPP)) can be used for Golgi apparatus targeting. PH(FAPP) binds to phosphatidylinositol 4-phosphate at the Golgi and still diffuses in the cytosol.^[12] Using PH(FAPP) in replacement of C2(LACT), it will be possible to reversibly manipulate molecular activity at the Golgi. In summary, the present technique offers a powerful tool to gain deeper insights into the spatio-temporal dynamics of cell signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Double relocation of cytosolic protein by dual CID systems. (a) GAIs-YFP-FKBP-C2(LACT) (green), CFP-FRB (blue), and Tom20-mCherry-GID1 (red) were expressed in COS-7 cells. Rapamycin (Rapa; 100 nM) treatment induced relocation of CFP-FRB to the plasma membrane. The subsequent GA₃-AM (10 μ M) treatment induced relocation of the GAI-YFP-FKBP-C2(LACT)/rapamycin/CFP-FRB complex to the mitochondria. Insets indicate enlarged views of boxed regions with a higher contrast. Rapamycin-induced relocation of CFP-FRB to plasma membrane is highlighted by red arrowheads. Scale bar: 10 μ m. (b) The fluorescence intensity of CFP-FRB in the regions of interest was measured at indicated time points. The values indicate fluorescence intensity divided by initial fluorescence. Error bars represent the S.E.M (n=10 cells from three independent experiments).

Figure 2.

Rapidly reversible induction of Rac-dependent membrane ruffling (a) GAIs-YFP-FKBP-C2(LACT) (green), CFP-FRB-Tiam1 (blue), Tom20-mCherry-GID1 (red), and mCherry-CAAX (also red) were expressed in COS7 cells. After serum starvation for 2 hours, addition of rapamycin (Rapa; 100 nM) induced relocation of CFP-FRB-Tiam1 to the plasma membrane which was followed by relocation of the chemically dimerized complex (i.e., GAIs-YFP-FKBP-C2(LACT)/rapamycin/CFP-FRB-Tiam1) as a whole from the plasma membrane to the mitochondria induced by GA₃-AM treatment (10 μM). Arrows indicate membrane ruffles. (b) Video frames of enlarged views of the boxed regions in (a) illustrates the reversible induction of Rac-dependent membrane ruffling. (c) Ruffling activity was quantified by measuring the fluorescence intensity of mCherry-CAAX at the cell periphery. (d) The fluorescence intensity of mCherry-CAAX at indicated time points in (c). ** p<0.01. Error bars represent the S.E.M (n=8 cells from three independent experiments). The values indicate fluorescence change divided by initial fluorescence. Scale bar: 10 μm.

Figure 3.

Rapidly reversible induction of PI3K-dependent membrane ruffling (a) GAIs-YFP-FKBP-C2(LACT) (green), CFP-FRB-iSH (blue), Tom20-mCherry-GID1 (red), and mCherry-CAAX (also red) were expressed in COS7 cells. Rapamycin treatment (Rapa; 100 nM) induced relocation of CFP-FRB-iSH to the plasma membrane which was followed by relocation of the chemically dimerized complex (i.e., GAIs-YFP-FKBP-C2(LACT)/rapamycin/CFP-FRB-iSH) as a whole from the plasma membrane to the mitochondria induced by GA₃-AM treatment (10 μ M). Arrows indicate membrane ruffles. (b) Video frames of enlarged views of the boxed regions in (a) illustrate the reversible induction of PI3K-dependent membrane ruffling. (c) Ruffling activity was quantified by measuring the fluorescence intensity of mCherry-CAAX at the cell periphery. (d) The fluorescence intensity of mCherry-CAAX at indicated time points in (c). * $p < 0.05$. Error bars represent the S.E.M (n=5 cells from two independent experiments). The values indicate fluorescence change divided by initial fluorescence. Scale bar: 10 μ m.

Scheme 1.

Schematic of the rapid, local, specific and reversible modulation of molecular activities by dual CID systems. (a) Structure of dimerizers rapamycin (Rapa) and GA₃-AM used in this study. (b) Rapamycin (Rapa) binds to FKBP and traps FRB, relocating CFP-FRB-POI from cytosol to the GAls-YFP-FKBP-C2(LACT)-labelled plasma membrane (left to middle panels). This activates POI-dependent signaling event at the plasma membrane (indicated by an “ON” sign). A subsequent addition of GA₃-AM induces dimerization between GAls and GID1, resulting in relocation of the GAls-YFP-FKBP-C2(LACT)/rapamycin/CFP-FRB-POI complex as a whole from the plasma membrane to the Tom20-mCh-GID1-labeled mitochondria. This leads to termination of POI-dependent signaling event on the plasma membrane (indicated by an “OFF” sign).