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Rapid and Efficient Induction of an Endogenous Cell Signaling Event by Subcellular Targeting of a Synthetic Ligand

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It is now well-recognized that the spatial localization of proteins at the subcellular level is essential in regulating cell signaling.[1] On the other hand, research efforts aimed at developing synthetic modulators of intracellular signaling processes have usually focused on the affinity and specificity of ligands to their target proteins, neglecting the localization of the molecules in cells. In this communication, we show that controlling the subcellular locations of ligand molecules can be a key strategy in manipulating endogenous cell signaling pathways.

Triggering the activation of specific signaling proteins and pathways with synthetic molecules has emerged as a powerful approach for investigating biological systems.[2] In the last two decades, significant progress has been made in the construction of engineered proteins whose activity[3] or stability[4] can be turned on by a small molecule to initiate their downstream events in living cells. Although extremely useful, the protein-engineering-based approaches control the activity of modified proteins (not their natural counterparts) that are exogenously expressed in cells, often at artificially high levels. Clearly, the development of tools that allow a particular "endogenous" signaling pathway to be activated without modifying any protein participant should be of considerable importance. However, the rational design of small agonist molecules remains extremely difficult. In most cases, synthetic ligands that bind target proteins function not as activators but as inhibitors. Consequently, despite numerous efforts, including the screening of diverse chemical/natural product libraries, the number of synthetic molecules capable of activating specific endogenous biological processes is still very limited.

Protein translocation from the cytosol to the inner leaflet of the plasma membrane (PM) is a fundamental mechanism of eukaryotic cell signaling.[1,5] Many proteins are known to activate their corresponding downstream pathways upon localization at the PM. Importantly, this process is mostly mediated by protein−protein interactions. For example, phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), and Grb2 are recruited to the PM by recognition of a specific phosphorylated sequence within the stimulated growth factor receptor embedded in the cell membrane.[1,5] We thus reasoned that if a synthetic ligand of an endogenous protein of interest is precisely placed at the PM, the localized ligand would induce the translocation of the protein to the PM, resulting in the activation of its downstream cascade (Figure 1A).[6] To test this strategy, here we targeted the endogenous PI3K pathway. PI3K is a lipid kinase that catalyzes the phosphorylation of phosphatidylinositide-4,5-bisphosphate, producing a lipid second messenger, phosphatidylinositide-3,4,5-trisphosphate (PIP_3).[7] PIP_3 is involved in the regulation of diverse cellular functions, such as cell survival, motility, and neurite outgrowth. As a specific ligand of PI3K, a doubly phosphorylated peptide derived from the platelet-derived growth factor receptor (PDGFR) was chosen.[8] In order to control the intracellular locales of the peptide ligand, the use of bacterial dihydrofolate reductase (DHFR) and its strong interaction with trimethoprim (TMP) was selected.[9,10] By directing DHFR to the PM via fusion with a membrane localization sequence, a peptide containing TMP can be targeted to the PM by complexation with the DHFR. Accordingly, synthetic peptide ligand **1** incorporating TMP and Alexa Fluor 647 (for fluorescent visualization of the peptide) was synthesized (Figure 1B). In addition, cytosol (CYT)- and PM-targeted forms of DHFR were used (Figure 1C and Figure S1 in the Supporting Information). The activation of the PI3K pathway was monitored in live cells using the green fluorescent protein (GFP)-fused Akt pleckstrin homology (PH) domain (GFP-PHAkt), a fluorescent indicator for PIP_3,[11] on a confocal laser scanning microscope (CLSM).

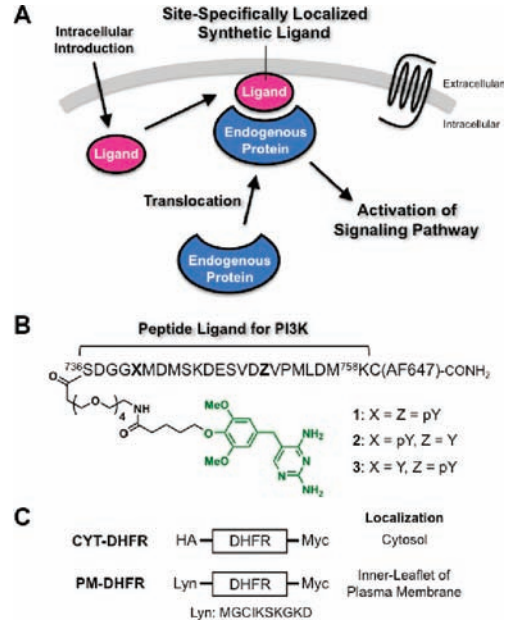

Figure 1. Subcellular targeting of synthetic ligands leads to the activation of endogenous cell signaling pathways. (A) Schematic illustration of the concept. (B) Peptide ligands used in this study. The sequence was derived from residues 736−758 of PDGFR.[8] TMP is shown in green. AF647 is Alexa Fluor 647 C_2-maleimide. (C) DHFR constructs used for controlling the subcellular localization of the peptide ligands. Lyn is a myristoylation/palmitoylation (PM-targeting) sequence from the protein lyn.

Stimulation of Chinese hamster ovary (CHO-K1) cells with platelet-derived growth factor (PDGF) led to the activation of the PI3K pathway and the efficient generation of PIP_3 within 1 min (Figure S2). With this information in hand, we investigated whether it was possible to induce the activation of the PI3K pathway using

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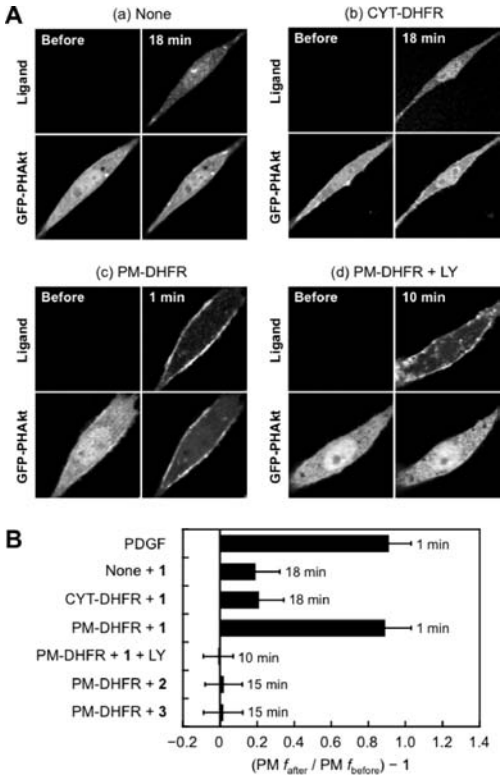

Figure 2. Triggering the activation of the endogenous PI3K pathway in single cells. (A) CLSM images of CHO-K1 cells before and after the microinjection of peptide ligand **1**. Cells were transfected to express (a) GFP-PHAkt alone, (b) GFP-PHAkt and CYT-DHFR, and (c, d) GFP-PHAkt and PM-DHFR. In (d), cells were pretreated with LY. Alexa Fluor 647 fluorescence (top) and GFP fluorescence images (bottom) are shown in each panel. (B) Quantitative analysis of the formation of PIP_3. The fraction of the PM fluorescence in the cell (PM *f*) was determined by the following equation: PM $f = F_{PM}/(F_{PM} + F_{CYT})$, where F_{PM} and F_{CYT} are the fluorescence intensities for the PM and cytosol regions, respectively. Increments of PIP_3 by simulation were estimated as (PM f_{after}/PM f_{before}) − 1. Incubation times are shown beside the bars.

the synthetic ligand **1**. **1** was microinjected into cells expressing no or CYT-DHFR. In both cases, **1** was evenly distributed throughout the cells, as observed by the fluorescence of the peptide [Figure 2A(a,b)]. Only slight increases of PIP_3 were observed (also see Figure 2B) following prolonged incubation (18 min). The results indicate that the agonist activity of the peptide ligand is very weak when it is diffusing in the cytosol. Synthetic ligand **1** was then microinjected into the cells expressing PM-DHFR. As shown in Figure 2A(c), immediately following the injection of the ligand into the cell, specific localization of the peptide at the PM was observed. At the same time, generation of a significant amount of PIP_3 was unambiguously detected. The fluorescence signals from the peptide and GFP-PHAkt on the PM merged, indicating that the production of PIP_3 occurred in the region where the peptide resides. The recruitment of endogenous PI3K to the PM was also verified by immunofluorescence staining (Figure S2). No visible production of PIP_3 was observed when the same experiment was carried out after the cells were treated with the PI3K inhibitor LY294002 (LY) [Figure 2A(d)]. These results clearly demonstrate that the PM-localized ligand **1** activated the endogenous PI3K pathway. Moreover, quantitative analysis revealed that the efficiency of PIP_3 production by the PM-anchored ligand was comparable to the efficiency when the cells were stimulated with PDGF (Figure 2B). The time required for the accumulation of PIP_3 was also very short (<1 min).

Since the present approach provides a flexible route for altering the phosphorylation state of the peptide ligand, we next examined whether both or either one of two phosphotyrosine residues in **1** is required for the PI3K activation in a live-cell context. Interestingly, both of the monophosphorylated ligands **2** and **3** were localized to the PM but failed to induce PIP_3 production (Figure 2B and Figure S2). Consequently, double phosphorylation is essential for the activation of the PI3K pathway by the PM-anchored ligand.

In summary, we have demonstrated that it is feasible to rapidly and efficiently activate an endogenous signaling pathway by placing a synthetic ligand at a specific location within a cell. The strategy should be applicable to other endogenous proteins and pathways through the choice of appropriate ligand molecules. More significantly, this proof-of-principle study highlights the importance of controlling the subcellular locales of molecules in the design of new synthetic modulators of intracellular biological events. There might be a number of compounds (not only activators but also inhibitors) that have been dismissed but may acquire potent biological activities when they are endowed with subcellular-targeting functions. Our next challenge is to develop cell-permeable carriers capable of delivering cargo ligands to specifically defined regions or organelles inside cells.

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Supporting Information Available: Figures S1 and S2 and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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