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Synthetic Self-Localizing Ligands That Control the Spatial Location of Proteins in Living Cells

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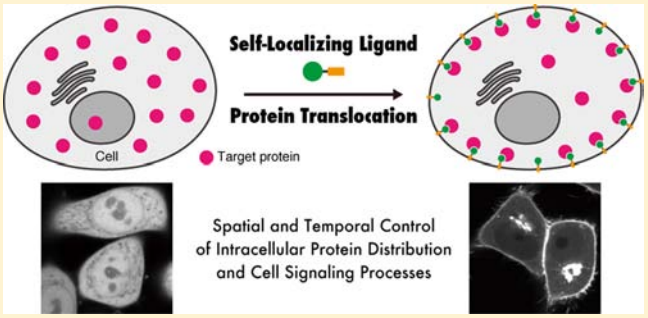
Supporting Information

ABSTRACT: Small-molecule ligands that control the spatial location of proteins in living cells would be valuable tools for regulating biological systems. However, the creation of such molecules remains almost unexplored because of the lack of a design methodology. Here we introduce a conceptually new type of synthetic ligands, self-localizing ligands (SLLs), which spontaneously localize to specific subcellular regions in mammalian cells. We show that SLLs bind their target proteins and relocate (tether) them rapidly from the cytoplasm to their targeting sites, thus serving as synthetic protein translocators. SLL-induced protein translocation enables us to manipulate diverse synthetic/endogenous signaling pathways. The method is also applicable to reversible protein translocation and allows control of multiple proteins at different times and locations in the same cell. These results demonstrate the usefulness of SLLs in the spatial (and temporal) control of intracellular protein distribution and biological processes, opening a new direction in the design of small-molecule tools or drugs for cell regulation.



INTRODUCTION

Cells are spatially organized molecular systems that contain various organelles, cytoskeletal structures, and other membrane domains inside the plasma membrane.[1] The location of proteins in the cell is critical to their cellular functions. In addition, upon cell stimulation, many proteins change their location dynamically, for example, from the cytoplasm to the surface of a particular organelle. Such protein translocation is a fundamental mechanism underlying the spatial regulation of cell signaling processes.[2]

Controlling the intracellular location of proteins with synthetic small molecules would be a powerful chemical approach for artificially regulating cell function. Yet, methods for this purpose are very limited. Chemically inducible dimerization (CID) is the only general approach currently available.[3] The best-characterized CID system uses the small-molecule rapamycin, which mediates dimerization of FK506-binding protein 12 (FKBP12) and the FKBP-rapamycin binding domain (FRB). In this technique, FRB (or FKBP12) is localized to a specific cellular site using a targeting sequence, whereas the partner FKBP12 (or FRB) is expressed in the cytoplasm as a fusion with a protein of interest (POI). Upon rapamycin addition, the POI is moved to the target site through the formation of a ternary FKBP12-rapamycin-FRB complex. The CID system is versatile and has been successfully used for various biological applications.[3c,d] However, it is also associated with several limitations. First, this method requires the introduction of at least two fusion proteins into a cell to control a single POI. Second, although new abscisic acid[4] and gibberellin[5]-based dimerizers have recently been developed, expanding the repertoire of orthogonal chemical dimerizers is quite difficult. In experimental biology, there is a growing need for the ability to control multiple molecular processes at different times and locations in the same cell. Third, induced protein translocation is essentially irreversible with current CID systems.[3c,d,6,7] Finally, perhaps the most crucial limitation is its inapplicability to control endogenous proteins: POIs must always be conjugated with a dimerization domain.

From the viewpoint of chemistry, it is of great importance to establish a molecular approach to manipulate the spatial location of a single POI with a single ligand without relying

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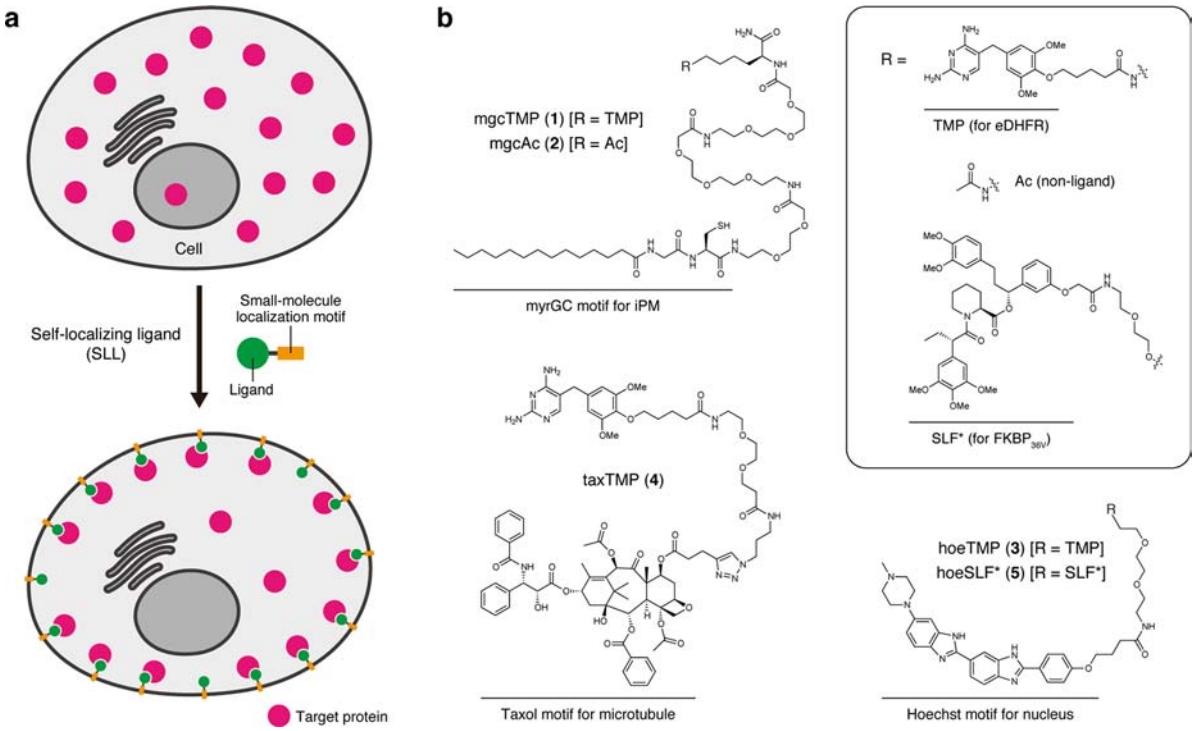

Figure 1. Spatial control of intracellular protein location by SLLs. (a) Schematic illustration of the strategy (in the case of iPM targeting). SLLs are synthetic ligands modified with small-molecule localization motifs that determine the site of localization. The SLL molecule can relocate its target protein from the cytoplasm to the assigned region after entering the cell. (b) Structures of SLLs used in this study: **1**, **3**, and **4** for eDHFR; **5** for FKBP12 (F36V) mutant; **2**, control compound for **1**.

on the expression of a second protein. Such a technique would overcome many of the limitations of the CID approach and may also provide a new direction for small molecule-based cell regulation. This goal requires the development of synthetic small molecules capable of controlling intracellular protein location in their own right. However, despite its potential, the creation of such molecules remains almost unexplored because of the lack of a design methodology.

Here we introduce a novel class of synthetic ligands, self-localizing ligands (SLLs), which spontaneously localize to specific organelles or subcellular regions in living mammalian cells. In particular, in this work we developed several SLLs for the inner leaflet of the plasma membrane (iPM), the nucleus, and the cytoskeleton based on small-molecule ligands for *Escherichia coli* dihydrofolate reductase (eDHFR) and (engineered and native) human FKBP12. We show that SLLs bind their target proteins and relocate (tether) them rapidly from the cytoplasm to their targeting sites, thus acting as synthetic protein translocators. The SLL tools were able to induce protein translocation in a reversible manner, manipulate diverse synthetic and natural signaling pathways, and simultaneously control the location of distinct proteins at different times and locations in the same cell. We also show that the SLL method can be used to translocate an endogenous protein in intact cells. These results demonstrate that conferring a self-localizing ability on small-molecule ligands is a promising strategy to generate synthetic small molecules that allow the spatial regulation of protein distribution and biological processes in living cells.

RESULTS AND DISCUSSION

Basic Strategy and SLL Design. To develop a general design strategy for synthetic protein translocators, we focused on the mechanism of phorbol myristate acetate (PMA), a small-molecule activator of protein kinase C (PKC).[8] PMA is a unique molecule with two functionalities. The phorbol moiety serves as a specific ligand to PKC, whereas the myristate group enables localization of the PMA molecule in the plasma membrane. As such, PMA can bind PKC and tether it from the cytoplasm to the iPM, resulting in PKC translocation and activation. Inspired by this, we reasoned that if a POI-selective ligand has the ability to localize at a specific intracellular region[9] (in a way that the ligand is still accessible by POI), the ligand itself would spatially relocate the POI from the cytoplasm to its targeting site in a "single ligand-single protein" manner (Figure 1a).[10] We adopt a modular approach[11] for designing such SLLs (Figure 1b). A ligand of interest is linked to a small-molecule localization motif that can bind to a specific component in a targeted organelle or membrane region. The modular design allows us to construct a variety of SLLs by interchanging the ligand molecule and the localization motif. To test this idea, we first chose trimethoprim (TMP) and eDHFR as a candidate ligand and protein pair.[12] Neither of the molecules has any intracellular localization property, and thus, both diffuse readily in the cytoplasm. TMP binds to eDHFR with high (nanomolar) affinity and exhibits >1000-fold higher selectivity to this protein over mammalian counterparts.[12] Therefore, TMP-based SLLs allow specific control of eDHFR-fusion proteins with minimal background binding to endogenous DHFR in mammalian cells.

Inducible eDHFR Translocation by iPM-Localizing TMP. The iPM is an important domain for cell signaling. We thus targeted this region first and sought to generate a TMP-based SLL that induces iPM translocation of an eDHFR fusion protein in living cells. A previous study demonstrated that a myristoyl-Gly-Cys (myrGC) lipopeptide derived from the N-

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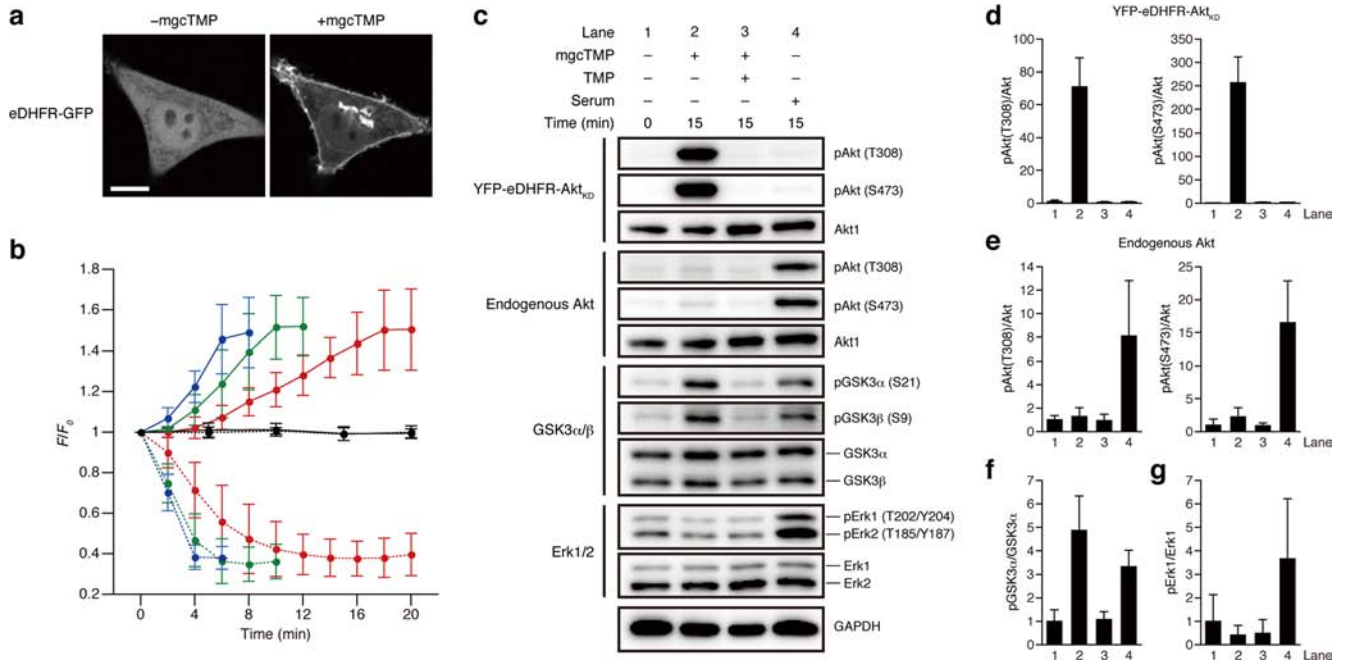

Figure 2. mgcTMP-mediated protein translocation and activation of the synthetic Akt pathway. (a) Inducible translocation of eDHFR-GFP by mgcTMP. Confocal fluorescence images of HeLa cells expressing eDHFR-GFP were obtained before and 20 min after the addition of 5 μM mgcTMP. Scale bar, 10 μm. (b) Time course and dose dependence of eDHFR-GFP translocation. eDHFR-GFP translocation was induced by 5 μM (red), 10 μM (green), or 20 μM (blue) mgcTMP or by 5 μM free TMP (black). Relative fluorescence signal intensities (F/F_0) in the iPM (solid line) and cytoplasm (dashed line) of cells were plotted as a function of time after the ligand addition. Data are represented as mean ± s.d. (n = 10 cells). (c) Immunoblot analysis of the synthetic Akt activity. NIH3T3 cells expressing YFP-eDHFR-Akt$_{KD}$ were serum-starved and incubated for 15 min under the following conditions: lane 1, none; lane 2, 5 μM mgcTMP; lane 3, 5 μM mgcTMP and 50 μM TMP; lane 4, 10% fetal bovine serum (FBS). Cell lysates were immunoblotted with the indicated antibodies. (d–g) Quantification of phosphorylation. Lane numbers correspond to the conditions given in panel c. Phosphorylation of each protein was normalized to its corresponding total protein. Data are represented as mean values relative to the mean of the no-treatment condition (lane 1) from three independent experiments. Error bars represent s.d. For GSK3β and Erk2, see Supporting Information, Figure S2d,e.

terminus of Src-family proteins is membrane-permeant and localizes to the iPM (and partially at the Golgi) in cells.[13] Accordingly, we designed an iPM-localizing TMP in a modular manner by connecting a myrGC localization motif to the TMP ligand through a flexible spacer (mgcTMP, **1**) (Figure 1b and see Supporting Information for synthesis). To assess its localization and translocation properties, we used human epithelial HeLa cells expressing eDHFR fused to green fluorescent protein (GFP) (eDHFR-GFP). Confocal fluorescence imaging showed that eDHFR-GFP was distributed throughout the cytoplasm at rest and moved to the iPM within 20 min upon addition of 5 μM mgcTMP (Figure 2a,b and Figure S1a,b). The partial accumulation at the Golgi (Figure S1c) is consistent with the localization properties of the myrGC motif.[13] The addition of free TMP instead of mgcTMP had no effect on the spatial distribution of eDHFR-GFP (Figure 2b and Figure S1d), indicating that the myrGC motif is responsible for the induced iPM translocation. The presence of excess free TMP blocked the mgcTMP-induced translocation (Figure S1e). Also, no translocation occurred when an mgcTMP analogue lacking the TMP ligand (mgcAc, **2**, Figure 1b) was used (Figure S1f,g). These results verify that eDHFR-GFP is recruited to the iPM by its direct binding to the ligand moiety of mgcTMP. The eDHFR-GFP translocation was accelerated in a dose-dependent manner and was completed in 8 min at 20 μM mgcTMP (Figure 2b). Thus, the mgcTMP-based system induces rapid protein translocation on a time scale of minutes. Overall, we demonstrated that a synthetic ligand with self-localization ability can control the spatial location of its target protein in living cells.

mgcTMP-Mediated Control of Synthetic and Endogenous Signaling Pathways. Cells induce various types of signaling by directing specific proteins to the iPM.[1a,2] We therefore investigated whether mgcTMP-induced protein translocation can be used to artificially manipulate biological signaling in living cells. We began by targeting the Akt signaling pathway. Akt is a protein kinase involved in diverse cellular processes such as cell survival, proliferation, metabolism, and migration.[14] Upon growth factor stimulation, Akt is recruited to the iPM through binding of its N-terminal pleckstrin homology (PH) domain to phosphatidylinositol 3,4,5-triphosphate (PIP_3) produced by phosphoinositide 3-kinase (PI3K). Subsequently, Akt is activated by phosphorylation at Thr308 and Ser473 by PDK1 and the mTOR-rictor complex, respectively.[15] To build a synthetic signaling system in which the Akt pathway is selectively activated by mgcTMP, we linked yellow fluorescent protein (YFP)-tagged eDHFR (YFP-eDHFR) to an Akt kinase domain lacking the PH domain[16] (YFP-eDHFR-Akt$_{KD}$). We expressed this protein in mouse fibroblast NIH3T3 cells. Treatment of cells with 5 μM mgcTMP for 20 min led to efficient translocation of YFP-eDHFR-Akt$_{KD}$ from the cytoplasm to the iPM (Figure S2a,b). The translocation rate (Figure S2c) was comparable to that observed with eDHFR-GFP, indicating that both the N- and C-termini of eDHFR can be fused to various (and relatively large) proteins without affecting mgcTMP-mediated translocation. Furthermore, immunoblot analysis revealed that YFP-eDHFR-Akt$_{KD}$ underwent

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phosphorylation at both Thr308 and Ser473 upon addition of mgcTMP (Figure 2c,d, lane 2). The phosphorylation levels of endogenous GSK3α and β, downstream substrates of Akt, also significantly increased (Figure 2c,f and Figure S2d, lane 2). Excess free TMP blocked the translocation and phosphorylation of YFP-eDHFR-Akt$_{KD}$ (Figure 2c,d, lane 3, and Figure S2f). These results demonstrate that mgcTMP stimulates the Akt pathway by recruiting YFP-eDHFR-Akt$_{KD}$ to the iPM. Moreover, serum treatment induced phosphorylation of endogenous Akt but not YFP-eDHFR-Akt$_{KD}$ because of the lack of the PH domain (Figure 2c–e, lane 4). Addition of mgcTMP did not activate endogenous Akt or other signaling proteins, such as Erk1/2 (Figure 2c,g and Figure S2e, lane 2). Therefore, this system allows the specific, inducible activation of the synthetic Akt pathway in living cells using mgcTMP as an input, independent of extracellular (upstream) stimuli.

To further test the usefulness of the mgcTMP-induced spatial protein translocation system, we applied this method to two other signaling molecules: the Rho-family small GTPase Rac[17] and the lipid kinase PI3K.[18] First, we attempted to activate endogenous Rac by recruiting Tiam1, a guanine nucleotide exchange factor for Rac, to the iPM.[19] We fused YFP-eDHFR to the DH-PH domain of Tiam1 (YFP-eDHFR-Tiam1$_{DH\text{-}PH}$) and expressed this protein in NIH3T3 cells. The addition of mgcTMP translocated YFP-eDHFR-Tiam1$_{DH\text{-}PH}$ to the iPM and induced lamellipodia in most transfected cells (ca. 70%) (Figure S3a–d). No obvious changes in cell morphology were observed when the translocation was inhibited by free TMP or when YFP-eDHFR (lacking the Tiam1$_{DH\text{-}PH}$ domain) was recruited to the iPM (Figure S3d). The activation of endogenous Rac was confirmed using a fluorescent biosensor, PakGBD-mCherry,[20] which binds to the active form of Rac (Figure S3e,f). Next, we constructed a synthetic PI3K (mCherry-eDHFR-p85$_{iSH}$) by combining mCherry-tagged eDHFR (mCherry-eDHFR) with the inter-Src homology 2 domain from p85 (p85$_{iSH}$), which forms a complex with the endogenous p110 subunit of PI3K in cells.[21] The addition of mgcTMP resulted in the production of PIP_3 in mCherry-eDHFR-p85$_{iSH}$-expressing HeLa cells but not in mCherry-eDHFR-expressing cells, as monitored by the PIP_3 indicator AktPH-GFP[22] (Figure S4a,b). Immunoblotting revealed the subsequent activation of endogenous Akt (Figure S4c,d). Therefore, the mgcTMP-based eDHFR translocation system is applicable to the conditional control of various iPM-organized signaling pathways involving proteins and lipids.

Self-localizing TMPs for Other Organelles and Reversible Protein Translocation. To demonstrate the generality of the SLL approach to other cellular regions, we next designed and synthesized nucleus- and cytoskeleton-localizing TMPs. The former was generated using the DNA-binding Hoechst dye[23] as the localization motif (hoeTMP, **3**) (Figure 1b). The latter was based on the tubulin-binding drug taxol[24] (taxTMP, **4**) (Figure 1b). Addition of hoeTMP efficiently relocalized eDHFR-GFP from the cytoplasm to the interior of the nucleus (Figure 3a and Figure S5a–e). Likewise, eDHFR-GFP was assembled on the microtubule structure upon addition of taxTMP (Figure 3b and Figure S6a–d). It was shown that once translocated, eDHFR-GFP remained in the nucleus or on the microtubule for at least 24 h, even after washing the cells (Figure S5f and Figure S6e). However, it was possible to readily return the protein to the original location upon the addition of excess free TMP (Figure S7). Thus, reversible small-molecule control of protein localization can be readily achieved with the SLL system.

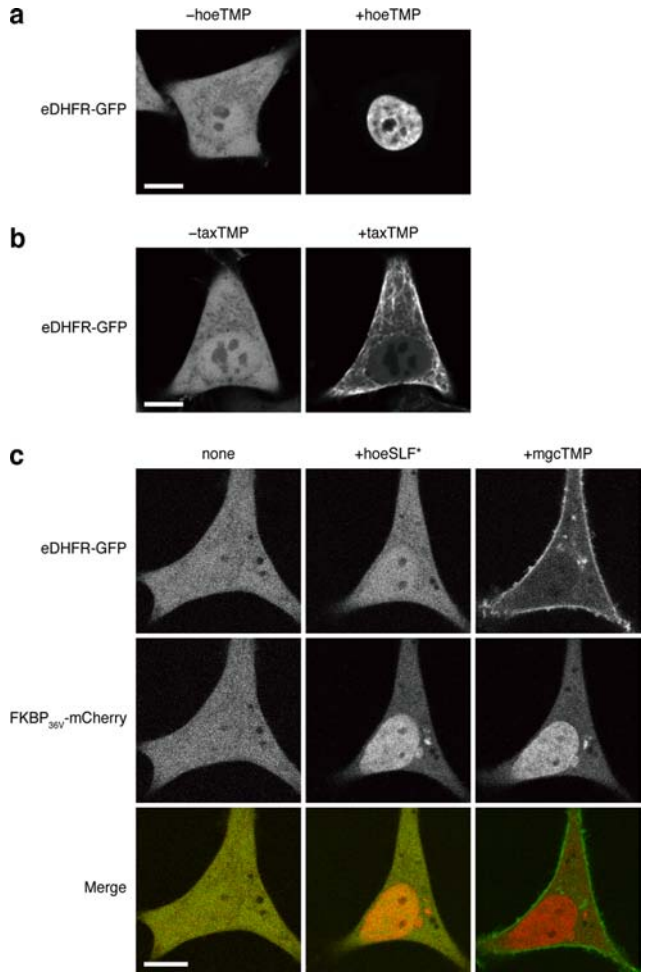

Figure 3. Protein translocation to various organelles. (a) Nuclear translocation by hoeTMP. Confocal fluorescence images of HeLa cells expressing eDHFR-GFP were obtained before and 90 min after the addition of 5 μM hoeTMP. (b) Microtubule translocation by taxTMP. Images were obtained before and 60 min after the addition of 5 μM taxTMP. (c) Simultaneous control of two distinct proteins in the same cell with orthogonal SLLs. Confocal fluorescence images of HeLa cells coexpressing eDHFR-GFP and FKBP$_{36V}$-mCherry were obtained before (left), 90 min after the first addition of 2.5 μM hoeSLF* (center), and 20 min after the second addition of 2.5 μM mgcTMP (right). Top, GFP fluorescence; middle, mCherry fluorescence; bottom, merged images. For translocation using hoeTMP and taxTMP, see Supporting Information, Figure S9. Scale bar, 10 μm.

Orthogonal Control of Multiple Proteins in the Same Cell. Inducible translocation of a protein distinct from eDHFR can be achieved by replacing the ligand moiety. We designed hoeSLF* (**5**) by linking SLF*, a specific synthetic ligand for the F36V mutant of FKBP12 (FKBP$_{36V}$),[25] to the Hoechst motif (Figure 1b). SLF* has a high (subnanomolar) affinity to FKBP$_{36V}$ and 1000-fold selectivity to this protein over wild-type FKBP12,[25a] allowing hoeSLF* to preferentially bind to FKBP$_{36V}$-fusion proteins in cells. The addition of hoeSLF* induced the nucleus translocation of an FKBP$_{36V}$-mCherry fusion protein (FKBP$_{36V}$-mCherry) (Figure S8). Because TMP–eDHFR and SLF*–FKBP$_{36V}$ pairs are orthogonal to each other, we were able to control the two distinct proteins simultaneously and independently in the same cell. When

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hoeSLF* and one of the self-localizing TMPs were added sequentially, $FKBP_{36V}$-mCherry and eDHFR-GFP moved to the nucleus and its expected site, respectively (Figure 3c and Figure S9). Similarly, TMP-based SLLs were orthogonal to the rapamycin CID-based translocation system (Figure S10).

SLL-Based Spatial Control of an Endogenous Protein. Finally, we investigated whether the SLL approach is applicable to control the spatial location of an endogenous protein in cells. As a pilot study, here we targeted native (wild-type) FKBP12 (nFKBP), a cytoplasmic protein, and sought to relocate it to the nucleus. Accordingly, we synthesized hoeSLF (**6**) consisting of the SLF (no asterisk) ligand for nFKBP[25a] and the Hoechst motif (Figure S11a). SLF specifically binds to nFKBP with high (K_D of 20 nM) affinity.[25a] Immunoblotting of cell fractions indicated that nFKBP was predominantly localized in the cytoplasm in intact HeLa cells, whereas the majority of the protein moved to the nucleus upon addition of hoeSLF (Figure S11b−d). The hoeSLF-induced translocation did not occur in the presence of a competitive ligand (rapamycin), verifying that nFKBP was directed to the nucleus through its direct binding to hoeSLF. We therefore demonstrated that the intracellular distribution of a specific endogenous protein can be manipulated by the SLL method.

CONCLUSIONS AND OUTLOOK

In this work, we have established a unique design methodology for synthetic ligands that control spatial protein location in living mammalian cells. A simple, diffusible ligand can be converted to a synthetic protein translocator by endowing it with self-localization ability. In the SLL system, the small-molecule localization motif determines the subcellular destination and tethers the binding protein to the target site. The SLL molecules could manipulate intracellular processes in diverse ways, such as inducible (reversible) protein translocation, conditional activation of synthetic and natural signaling pathways, and simultaneous control of multiple proteins at different times and locations in the same cell. No significant cytotoxicity was observed for all SLLs reported herein under the conditions used (Figure S12). The SLL-induced protein relocation technique will become a powerful new chemical approach for probing the role of protein localization and spatiotemporally regulated signaling in cell biology.

The modular design is a chief advantage of our SLL system. It allows us to generate various SLLs for different proteins by simply exchanging the ligand moiety. In addition to eDHFR and $FKBP_{36V}$ used in this work, other orthogonal protein labeling techniques, for example, SNAP-tag[26] and HaloTag,[27] are now established. Therefore, by developing SLLs for these protein tags using corresponding ligands/substrates, we will be able to construct an integrated synthetic cell wherein the location of diverse engineered (tag-fusion) proteins can be flexibly controlled. Such a system enables more sophisticated modulation of cellular functions based on protein location control, providing an attractive new platform for synthetic biology.

Another notable feature of the SLL approach is its applicability to endogenous proteins. The use of appropriate ligands may allow us to create novel SLL tools or drugs that regulate cell behavior by altering the intracellular location of specific endogenous proteins. Although there has been significant progress in the development of bioactive small molecules, the majority of these molecules are inhibitors of enzyme activity or protein−protein interactions.[28] Finding signaling activators is more challenging.[29] Because many cell signaling processes are triggered by protein translocation, we envision that the SLL strategy can be a key component in expanding the repertoire of small molecule-based signaling activators.[30,31]

Further identification of small-molecule localization motifs targeting a variety of intracellular locations is definitely required to exploit the full potential of the SLL methodology. For example, organelle-staining dyes,[32] natural products,[33] or short (lipidated) peptide segments found in natural membrane-associated proteins[34] would be an important source for this purpose. Such an effort is currently underway.

In closing, we believe that the concept of SLL (self-localizing ligand) described herein opens a new direction in the design of small-molecule tools or drugs for the study and control of living biological systems.

ASSOCIATED CONTENT

Supporting Information

Figures S1−S12 and detailed experimental procedures for chemical synthesis, plasmid construction, and cell biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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