FULL PAPER

Flipping the Switch on Clathrin-Mediated Endocytosis using Thermally Responsive Protein Microdomains

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A ubiquitous approach to studying protein function is to knock down activity (gene deletions, siRNA, small molecule inhibitors, etc.) and to study the cellular effects. Using a new methodology, this article describes how to rapidly and specifically switch off cellular pathways using thermally responsive protein polymers. A small increase in temperature stimulates cytosolic elastin-like polypeptides (ELPs) to assemble microdomains. It is hypothesized that ELPs fused to a key effector in a target macromolecular complex will sequester the complex within these microdomains, which will bring the pathway to a halt. To test this hypothesis, ELPs are fused to clathrin-light chain (CLC), a protein associated with clathrin-mediated endocytosis. Prior to thermal stimulation, the ELP fusion is soluble and clathrin-mediated endocytosis remains "on". Increasing the temperature induces the assembly of ELP fusion proteins into organelle-sized microdomains that switches clathrin-mediated endocytosis "off". These microdomains can be thermally activated and inactivated within minutes, are reversible, do not require exogenous chemical stimulation, and are specific for components trafficked within the clathrin-mediated endocytosis pathway. This temperature-triggered cell switch system represents a new platform for the temporal manipulation of trafficking mechanisms in normal and disease cell models and has applications for manipulating other intracellular pathways.

1. Introduction

The disciplines of synthetic biology,[1] chemical genetics,[2] and gene switches[3] all rely on one fundamental and unifying concept: that control of protein-protein interactions elucidates underlying mechanisms fundamental to cell biology. Within the past 50 years, an array of molecular tools have been developed to deliberately control target protein activities.[4] Temperature sensitive mutants and chemical dimerizers[5] allow scientists to manipulate endogenous proteins, while tetracycline-inducible promoters[6] and riboregulators[7] allow for the exogenous control of genetic transcription. These inducible systems have been engineered to respond to endogenous proteins, exogenous small molecules[8] as well as environmental sensors such as pH,[9] light,[10] heat,[11] and glucose.[12] Unfortunately, major barriers for the synthetic control of biological processes persist. The majority of existing methodologies control protein activity at the genetic level, by placing the desired proteins for study under the control of inducible promoters. While these systems are robust and afford high levels of induction control, they lack the temporal sensitivity to rapidly modulate biologic processes.[5] For example, standard tetracycline-inducible systems as well as RNAi knockdown require 24 to 48 hours between input and measurable output of the system. This lag time can confound data due to compensatory mechanisms and off-target effects.[13–16] In addition, other limitations of current programmable biologic systems include leaky promoters, delayed reversibility,[17] and labor-intensive mutant screens and system optimization.

Herein, we describe a rapid, reversible post-translational protein switch that addresses these problems. We propose to confer temperature sensitivity to a target process by fusing one of its key effector proteins to an environmentally responsive protein polymer at the genetic level.[11] This thermally sensitive elastin-like polypeptide (ELP) fusion is soluble at 31 °C and below, which allows the target protein complexes to assemble and disassemble as they would in a normal cell. However, at 37 °C this ELP fusion phase separates, forming genetically engineered protein microdomains (GEPMs)[11,18] enriched in the effector protein. The fusion of the effector protein to the ELP causes other components of the target complex to be sequestered in assembled microdomains. This inhibits their normal protein interactions, effectively shutting "off" the target pathway. Additionally, the reversible nature of ELP phase separation allows the rapid return to the "on" state with a temperature decrease. This strategy is conceivably adaptable to a variety of target protein complexes.

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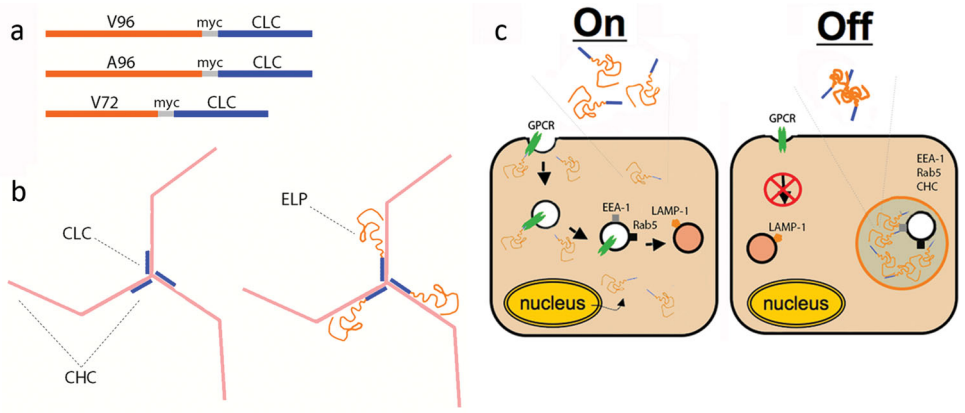

Figure 1. Triggered microdomain assembly in the cytosol inhibits clathrin-mediated endocytosis. a) Genes encoding three thermally responsive ELPs (V96, A96, and V72) were fused to the clathrin-light chain (CLC) gene. A myc epitope was incorporated between the ELP and CLC genes to enable immunofluorescence detection of expressed proteins, called V96-CLC, A96-CLC, and V72-CLC. b) Schematic of clathrin triskelia composed of clathrin heavy chain (CHC) and CLC with and without an ELP tag. c) The permissive state, or "on" state, for clathrin-mediated endocytosis exists when the ELP fusion polymer remains soluble. Upon heat stimulation, the ELP fusion protein reversibly phase separates, forming a genetically engineered protein microdomain. Assembly brings the fused CLC into the microdomain, along with other critical target proteins (EEA-1, Rab5, CHC) associated with clathrin-mediated endocytosis. This shuts "off" the internalization pathway, which can be visualized by internalization of a G-protein coupled receptor (GPCR).

As a proof of concept, we constructed a protein switch to exogenously control the clathrin-mediated endocytosis pathway, a highly characterized mechanism for cellular internalization of G-protein coupled receptors (GPCRs).[19] Upon receptor stimulation by ligand, the clathrin-light chain (CLC) and clathrin-heavy chain (CHC) are recruited from the cytosol to the plasma membrane to form a basket-like triskelion around the budding vesicle. This clathrin-coated vesicle selectively sorts the receptor and cargo to clathrin-coated pits that bud and traffic to early endosomes for further sorting. We chose the CLC for fusion with our thermally responsive polypeptide for the following reasons: i) it is essential for triskelion formation[19]; ii) its absence inhibits internalization of GPCRs[20]; and iii) it has been reported in fusion to green fluorescent protein without loss of activity[21] (**Figure 1**). Triskelion formation requires clathrin light and heavy chain interactions at the C-terminus, leaving the N-terminus accessible to the cytosol.[22] Based on this information, we posited that the addition of a 38 kD soluble ELP to the N-terminus of CLC would not disrupt function (Figure 1A).

To demonstrate the potential for this platform, this manuscript describes fusions between ELP and CLC and their ability to inhibit receptor internalization upon formation of microdomains. When cooled, the ELP microdomains dissolve, CLC fusion proteins return to a diffuse cytosolic distribution, and receptor internalization returns to its previous level.

2. Results

2.1. Design and Characterization of a Thermally Responsive Switch for Clathrin-Mediated Endocytosis

Intracellular microdomain assembly depends on the transition temperature of the free ELP.[11] Therefore, we hypothesized that microdomain assembly and clathrin-mediated endocytosis shutdown can be rationally designed based on prior ELP characterization. For an ELP of the motif [Val-Pro-Gly-**Xaa**-Gly]$_n$, as polymer chain length, n, increases, the assembly temperature decreases. Based on previous intracellular ELP characterization,[11] three polymers were selected for fusion with CLC (Table S1, Supporting Information). Near physiological temperatures, V72-CLC and V96-CLC (Xaa = Val, n = 72 and 96 respectively) phase separate, while A96-CLC (Xaa = Ala, n = 96) remains soluble (**Figure 2**A). After a 45 min incubation, the longer V96-CLC polymer transitioned fully at 37 °C, while V72-CLC only reaches maximal microdomain assembly at 42 °C (Figure 2B). Both transition temperatures are ≈5 °C higher than for intracellular green fluorescent protein fusions to V72 and V96.[11] The kinetics for V96-CLC assembly revealed that a 45 min incubation at 37 °C was sufficient to observe microdomains in more than 80% of transfected Chinese hamster ovary (CHO) cells (Figure 2C), and this finding was reconfirmed in HEK293 cells (Figure S1A, Supporting Information). Additionally, V96-CLC cells incubated below 31 °C for times in excess of 45 min did not exhibit ELP phase separation, indicating an absence of a time limitation (data not shown). At the temperatures explored, no microdomain assembly was observed for A96-CLC; therefore, this fusion protein served as a control for overexpression of a non-switchable CLC fusion protein.

2.2. Cytosolic Microdomains Colocalize with Early Markers of Clathrin-Mediated Endocytosis

Using confocal laser scanning microscopy and indirect immunofluorescence, we assessed the intracellular effect of microdomain formation on proteins involved in clathrin-mediated endocytosis. An antigenic *myc* epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was inserted between ELP and CLC to serve two functions: i) detect transfected cells; and ii) characterize the formation of microdomains. A96-CLC, which does not assemble microdomains at 25 or 37 °C, did not co-localize

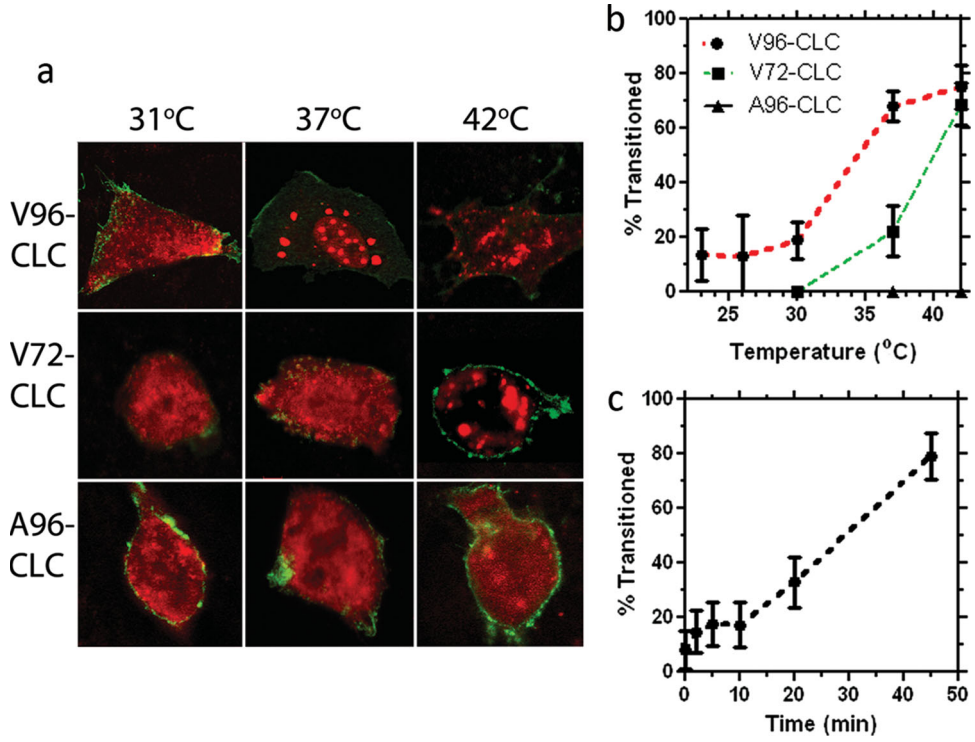

Figure 2. CLC microdomain assembly is tunable and rapid. ELPs fused to CLC were expressed in CHO cells, incubated at various temperatures, fixed, and stained for the myc epitope (red). a) Hydrophobicity and chain length were used to control microdomain formation. Hydrophobic ELPs V96-CLC and V72-CLC (red) co-expressed with AngIIR (green) resulted in microdomain formation at physiologic temperatures while the more hydrophilic ELP A96-CLC (red) did not. b) The temperature of microdomain assembly depends on ELP length. V72-CLC and V96-CLC reach maximum assembly at 42 and 37 °C respectively. A96-CLC does not assemble at the temperatures evaluated. c) Kinetics of microdomain formation in cells transfected with V96-CLC were assessed after shifting to 37 °C. Cells were pre-incubated at 4 °C to ensure uniform solubilization prior to heating. Within 45 minutes, 80% of cells expressing V96-CLC formed microdomains. Mean ± 95% confidence interval ($n = 3$).

with CHC (**Figure 3**A). By contrast, V96-CLC assembled microdomains at 37 °C (Figure 3B). When soluble at 25 °C, colocalization of CHC with V96-CLC remains low, whereas they were strikingly colocalized after microdomain formation (67.0%) (**Table 1**). Next, total CLC was assessed for colocalization with microdomains, which includes signal from both endogenous and expressed fusion proteins. Similarly to CHC, significantly more CLC colocalized with V96-CLC at 37 °C (40.8%) relative to room temperature (9.7%). A summary of co-localization studies performed in CHO cells can be found in Table 1, and similar results were confirmed using HEK293 (Figure S2, Supporting Information). These results strongly suggest that during microdomain formation, V96-CLC binds and sequesters other proteins associated with endocytosis. To probe this further, Rab5, an adaptor protein found on clathrin-coated vesicles and EEA-1, a protein involved in vesicle docking to early endosomes, were assessed for colocalization with V96-CLC. While the colocalization of Rab5 and EEA-1 with V96-CLC microdomains was not as robust as seen with CHC, there remained a significant increase in overlap of the fluorescence signal upon microdomain formation at 37 °C (Table 1). However, this pattern was not observed with LAMP-1, a lysosomal marker (Figure 3C). LAMP-1 colocalized at the same background percentage when microdomains formed as in their absence. This suggests that only proteins associated with clathrin-coated endocytotic vesicles incorporate into these microdomains.

To further investigate their morphology, structured illumination fluorescence microscopy was used to dissect a microdomain (Figure 3D). This revealed that V96-CLC microdomains are either hollow or impermeable to antibody probes. The V96-CLC appears to form a corona, surrounding a core enriched in CHC, which is consistent with observations made using confocal microscopy (Figure 3B). Thus, it appears that the microdomains are not homogenous features in the cytosol, but adopt a structure of some complexity.

2.3. Temperature Triggered Pathway Modulation in Mammalian Cells

To validate that V96-CLC microdomain formation knocks down clathrin-mediated endocytosis, CHO cells were transfected with V96-CLC and one of two receptors, either the angiotensin II receptor (AngIIR) or M1 muscarinic receptor. G-protein coupled receptors internalize via clathrin-mediated endocytosis; moreover, their agonist-dependent endocytosis is strongly affected by CLC knockdown.[20] We used a single cell-based method to quantify the amount of cargo endocytosed. This method allows us to measure the amount of internalized receptor with respect to the total amount of receptor, internal and surface-associated, using confocal microscopy (**Figure 4**A). Examination of AngIIR using antibodies

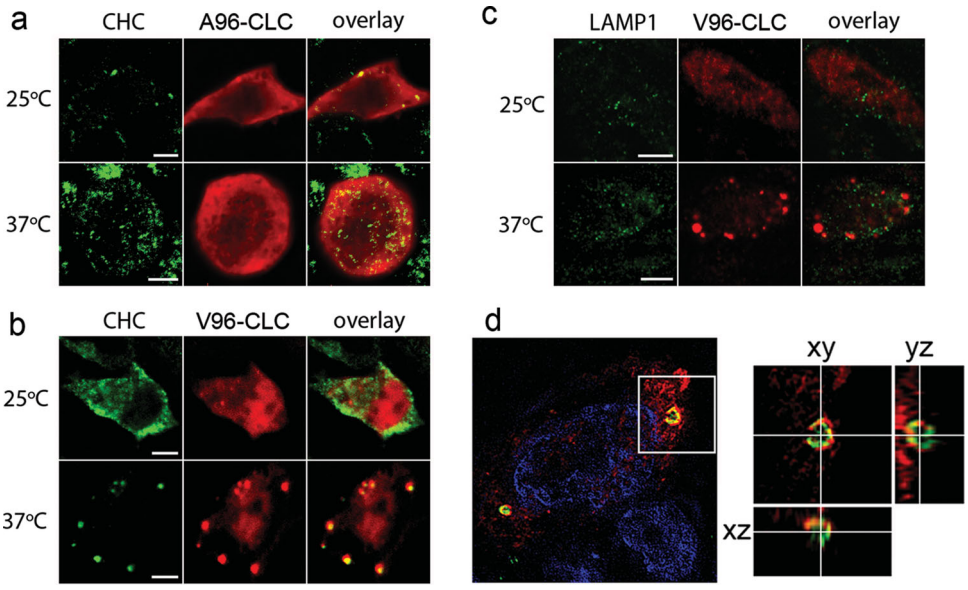

Figure 3. ELP-CLC microdomains are colocalized with clathrin heavy chain and not lysosomal markers. CHO cells were labeled to detect a) A96-CLC or b) V96-CLC using an anti-myc primary antibody (red) and the endogenous clathrin heavy chain (CHC) using an anti-clathrin-heavy chain antibody (green), and imaged using confocal laser scanning microscopy. At room temperature, the soluble ELP appears diffuse throughout the cytoplasm. At 37 °C CHC colocalized with V96-CLC microdomains. c) By contrast, CLC-V96 microdomains were not colocalized with the lysosomal marker, LAMP1. d) Super resolution optical microscopy images of V96-CLC microdomains were reconstructed using structured illumination by an OMX microscope. The nucleus was stained with DAPI (blue), and primary anti-CHC (green) and anti-myc (red) antibodies were used to visualize the three dimensional structure of the microdomain.

established observable receptor internalization after a 30 min agonist stimulation. We found that 60% of AngIIR was internalized after stimulation with 100 nM angiotensin, consistent with previously published results.[20] At 31 °C, cells transfected with V96-CLC internalized AngIIR similarly to cells expressing only AngIIR (Figure 4B). However, at 37 °C, when V96-CLC forms microdomains, the degree of AngIIR internalization decreased significantly relative to cells expressing only AngIIR ($p < 0.001$). This effect was even more pronounced at 42 °C ($p < 0.001$) (Figure 4C). Similar observations were confirmed using the M1 muscarinic receptor (Figure S3, Supporting Information).

To compare the effectiveness of microdomain-mediated knockdown of endocytosis relative to chemical inhibitors, we assayed receptor internalization in the presence or absence of monodansylcadaverine and dynasore. Monodansylcadaverine inhibits clathrin-mediated endocytosis while dynasore inhibits both clathrin-mediated and clathrin-independent/lipid raft endocytosis pathways.[19] AngIIR transfected cells pretreated with dynasore and monodansylcadaverine exhibited reduced internalization of AngIIR (**Figure 5**A). In this model, pretreatment with monodansylcadaverine and dynasore had the same effect on AngIIR internalization as cells expressing V96-CLC microdomains or un-stimulated cells. Interestingly,

Table 1. CLC microdomains colocalize with markers of early endocytosis.

ELP fusion	Antibody target	Marker	Percent colocalization at		Significance p value
			23 °C [%][a]	37 °C [%][a]	
V96-CLC (assembles at 37 °C)	CLC	Clathrin coated vesicles	9.7 ± 16.2	40.8 ± 22.4	8.9×10^{-7}
	CHC	Clathrin coated vesicles	9.7 ± 8.2	67.0 ± 21.3	4.7×10^{-14}
	EEA-1	Early endosomes	9.1 ± 8.5	42.6 ± 27.7	1.7×10^{-8}
	Rab5	Early endosomes	10.4 ± 10.5	30.2 ± 23.1	8.3×10^{-5}
	LAMP-1	Lysosomes	8.3 ± 6.3	10.1 ± 8.9	n.s.
A96-CLC (not assembled at 37 °C)	CLC	Clathrin coated vesicles	10.1 ± 4.3	10.0 ± 5.9	n.s.
	CHC	Clathrin coated vesicles	20.9 ± 9.6	16.2 ± 9.8	n.s.

[a]CHO cells were transfected with an ELP fusion protein, incubated at 23 or 37 °C, fixed, stained using an anti-myc antibody for the ELP and an antibody against the indicated target protein, imaged using confocal laser scanning fluorescence microscopy and quantified for colocalization. Mean ± SD ($n = 3$), n.s. not significant, $p > 0.05$.

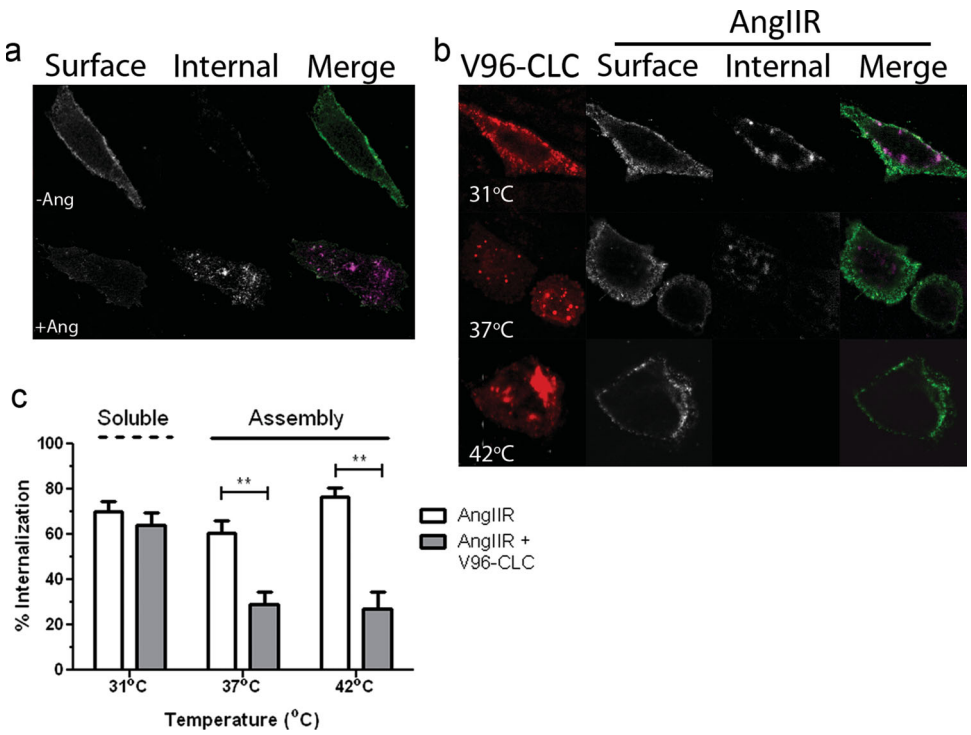

Figure 4. AngiotensinII receptor internalization is significantly reduced by microdomain assembly. CHO cells were transfected with AngIIR, stimulated with (+Ang) or without (-Ang) angiotensin (100 nM) for 30 min, and fixed. a) Surface AngIIR was labeled by immunofluorescence (green), the cell membrane was permeabilized, and internalized AngIIR was then labeled (purple). Bar = 5 μm. b) Cells were then co-transfected with both V96-CLC and AngIIR, incubated with angiotensin above and below the microdomain assembly temperature, and stained for AngIIR internalization. V96-CLC expressing cells were identified by anti-myc antibodies (red). Microdomains were observed at 37 and 42 °C, at which temperature receptor internalization was significantly decreased. Bar = 5 μm. c) AngIIR internalization with stimulation was quantified using image analysis at different temperatures for cells with and without V96-CLC. At 37 and 42 °C there was a significant decrease in receptor internalization for cells with V96-CLC microdomains compared to those without (**$p < 0.0001$). At 31 °C, V96-CLC remains soluble and does not affect receptor internalization. Mean ±95% confidence interval ($n = 3$).

pretreatment using chemical inhibitors of clathrin-mediated endocytosis had no added effect on cells containing V96-CLC microdomains. Similar results were confirmed using the M1 muscarinic receptor (Figure S4, Supporting Information). Finally, knockdown of AngIIR internalization by CLC microdomains had no apparent effect on uptake of a marker for caveolin-mediated internalization (Figure S5, Supporting Information).

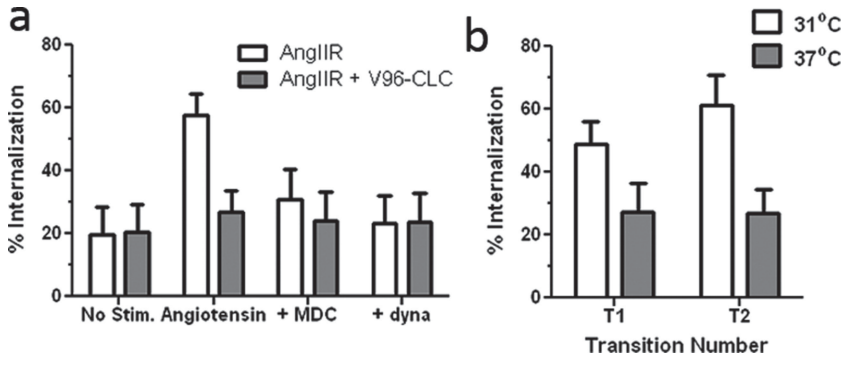

Figure 5. CLC-V96 microdomain assembly blocks clathrin-dependent internalization as effectively as chemical inhibition and is reversible. CHO cells were co-transfected with AngIIR with or without V96-CLC. Differential AngIIR internalization with and without angiotensin stimulation was quantified using immunofluorescence. a) When stimulated cells were pre-incubated with either monodansylcadaverine (MDC) or dynasore (dyna), they yielded a similar reduction of internalization relative to un-stimulated cells. b) AngIIR continues to be internalized and be inhibited at 31 and 37 °C, respectively, after 2 rounds (T1, T2) of heating and cooling, which indicates the reversibility of this methodology. Mean ± 95% confidence interval ($n = 3$).

2.4. Reversibility of Microdomain-Mediated Knockdown of Endocytosis

Since ELP phase separation is reversible in vitro,[11] we assessed whether reversibility was conferred to microdomain-mediated knockdown of clathrin-dependent endocytosis (Figure 5B). Cells expressing both AngIIR and V96-CLC were preincubated at 4 °C with the AngIIR labeling antibody (Figure 5B), as described in our knockdown experiment (Figure 4). Cells (T1) were then incubated at 37 °C for 45 min to induce intracellular microdomain formation. Two subsets of these cells were incubated at room temperature for 45 min, to allow their microdomains to solubilize. Among the resolubilized cells (T2), one set was incubated at 31 °C to assess receptor internalization upon

stimulation, and the second set was returned to 37 °C. These reheated cells assembled microdomains for a second time, which re-inhibited internalization. The results demonstrate that two rounds of heating and cooling causes no loss of AngIIR internalization activity at 31 °C. When reheated to 37 °C, the microdomains once again functionally block AngIIR internalization (Figure 5B).

3. Discussion

Life scientists need better tools to elucidate a vast array of still unknown molecular mechanisms. Herein, we have described a powerful new approach using a protein polymer system that expands our arsenal of tools to probe cellular function in a rational, immediate, and reversible manner.

Control of protein function is typically approached using one of two strategies. The first targets protein transcription and translation prior to protein production.[3] The tetracycline system places a gene of interest downstream of an inducible promoter, and gene activation or inactivation occurs upon addition of a stimulus. However, the most significant drawback to targeting proteins prior to synthesis is the extended time required for the input to result in the desired output.

The second, less-utilized method targets protein activity after the protein has been synthesized. This approach allows for a higher level of temporal control compared to methods targeting the transcriptional/translational machinery. Variations of the FK506 molecule have been adopted for just this purpose. Addition of a FK506 analogue stimulates the dimerization or oligomerization of a protein tagged to the receptor of the FK506 analogue.[21,23] This system's output can be observed rapidly, yet its inactivation requires the addition of a competing analogue or is entirely irreversible. Also, dimerization may increase, decrease, or leave the targeted protein pathway entirely unaffected. Although there are many variations on this chemically inducible system for the rapid control of protein interaction, there has until now been no technology that can rapidly drive proteins to associate and lose function that is both rapid and reversible. Our system, designed using genetically encodable protein polymers—ELPs—avoids the need for the addition of small molecules to control the system. To demonstrate this technology, we fused an effector gene for clathrin-light chain with that of a thermally responsive ELP, V96. V96 is a protein polymer that, when expressed intracellularly, forms discreet microdomains above 35 °C, but is solubilized into individual polymers at room temperature.

The assembly of V96-CLC polymers into microdomains at 37 °C was visualized using confocal fluorescence microscopy. The addition of V96 to CLC conferred the phase transition properties of the V96 polymer onto CLC. We were surprised to observe that additional proteins were localized inside these microdomains. Clathrin-heavy chain, EEA-1, and Rab5, all markers of early endocytosis formed puncta along with V96-CLC. However, Rab5 and EEA-1, proteins involved in cargo sorting, associated with V96-CLC to a lesser degree than CHC. We hypothesize that since Rab5 and EEA1 do not directly bind CLC, but interact via adaptor proteins, not as many associate in the V96-CLC microdomains. Markers of late stage endocytosis, such as LAMP1, do not enter microdomains at all, further supporting this hypothesis (Figure 2C).

Validation of clathrin-mediated endocytosis knockdown upon V96-CLC microdomain formation was performed by measuring internalization rates of 2 G-protein coupled receptors: AngIIR and the M1 muscarinic receptor. Agonist binding to these receptors stimulates internalization, a process confirmed in the literature to occur by clathrin-mediated endocytosis.[24,25] AngIIR internalization was reduced by 50% at 37 °C and this effect was more pronounced at 42 °C, when slightly more thermal energy results in higher internalization of AngIIR. Furthermore, microdomain-mediated inactivation of clathrin-mediated endocytosis appears to be as effective as two chemical suppressants of this process, dynasore and monodansylcadaverine.

In contrast to other intracellular switches, the assembly of ELP microdomains is rapidly reversible.[11,18] After two cycles of heating and cooling, AngIIR continues to be internalized at 31 °C and to be impaired for internalization at 37 °C. The degree of internalization is consistent with that seen after the first round of heating and cooling, indicating that V96-CLC cycles between soluble and insoluble aggregate and turns clathrin-mediated endocytosis "on" and "off".

4. Conclusion

This report demonstrates that sequestration of a key effector protein, CLC, within an organelle-like microdomain can inhibit the clathrin-mediated cell trafficking pathway. Although these results validate our proof-of-concept study, a more thorough understanding of the mechanisms of microdomain formation is necessary for the broad adoption of genetically engineered protein microdomains as intracellular switches. For example, residual internalization occuring even after microdomain formation may be due to endogenous CLC still present in the cell. While previous studies have shown that overexpressed CLC dominates endogenous CLC and that the effect of endogenous CLC is negligible,[21] we believe that the complete elimination of endogenous CLC may augment the effect of these microdomains. Regardless of these limitations, the apparent success of this approach opens the door for applications in the controlled suppression of other cell signaling/trafficking pathways.

5. Experimental Section

Vector Construction: The CLC gene was inserted into the IRES-dsRED2 (Clontech, Mountain View, CA) vector using PCR extension. The following primers were used to amplify CLC: Forward (TAGCCTGACCTCGAGatggctgagctgg) and Reverse (TCATCAGAATTCTCATCAcaccagcggggcctg). The amplified sequence was digested with NheI/EcoRI and ligated into IRES-dsRED2 that had been digested with the same enzymes. ELP genes, carried by a pET25b(+) plasmid (Clontech), were generated using recursive directional ligation as previously described.[11] DNA sequences encoding ELPs were ligated upstream of CLC after digesting the ELP plasmid with BseRI/ AcuI and the CLC plasmid with BcgI. For plasmids containing the myc epitope, myc was inserted between ELP and CLC using XhoI/ BlpI enzymes. The final open reading frames and encoded proteins are summarized in Table S1, Supporting Information, after confirmation using DNA sequencing (Retrogen, San Diego, CA).

Immunofluorescence: For fluorophore colocalization assays, cells were cooled at 4 °C for 45 min and incubated at specified temperatures for 45 min prior to fixation. Cells were washed with 50 mM ammonium chloride before a 10 min permeabilization step using 0.1% Triton-X in phosphate buffered saline (PBS). After washing 2× with PBS, cells were incubated for 1 hour at 37 °C with primary antibodies, washed, and incubated for another hour with secondary antibodies. Cells were incubated with DAPI prior to mounting. Primary antibodies included chicken anti-myc (Abcam, San Francisco, CA, ab19233), mouse anti-myc (Invitrogen, Carlsbad, CA, R950–25), mouse anti-CLC (Abcam, ab24579), mouse-anti CHC (X22 hybridoma courtesy of Prof. Okamoto,[26] rabbit anti-HA (Cell Signaling, Danvers, MA, C29F4), mouse anti-EEA-1 (Abnova, Taipei, Taiwan, H00008411-M03), LAMP1 (Abcam, ab24170), and Rab5 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-46692). All secondary antibodies were purchased from Invitrogen and included Alexa 568 goat anti-chicken (A11041), Alexa 488 goat anti-mouse (A11001), Alexa 488 goat anti-rabbit (A11008), Alexa 633 goat anti-rabbit (A21070). Confocal images were captured on a Zeiss LSM 510 Meta NLO equipped with Argon, red HeNe, and green HeNe lasers. Three-dimensional SIM images were acquired using a 60× oil-objective lens on a GE DeltaVision OMX system equipped with 3 sCMOS cameras and 405, 448, and 568 excitation lines.

Receptor Internalization Assays: To assess receptor internalization, CHO cells were plated on glass coverslips and transfected using Turbofect (Thermo Scientific, Waltham, MA) the following day with AngIIR (N-terminal HA tagged) +/– V96-CLC (myc tagged between V96 and CLC). Due to ELP's transition's dependence on concentration,[27] transfected cells were given 48 hours to reach maximum ELP-CLC expression level prior to assay. 48 hours after transfection, cells were incubated at 4 °C for 45 min with rabbit anti-HA antibody (IgG) (C29F4, Cell Signaling, Boston, MA) to label the receptor at the plasma membrane. After washing, cells were incubated at 37 °C for 45 min to allow for maximal ELP transition. For studies assessing the chemical inhibition of receptor internalization, cells were treated with dynasore (50 μM) or monodansylcadaverine (300 μM). Angiotensin II (100 nM, Sigma-Aldrich, St. Louis, MO) was added for 30 min to stimulate internalization of AngIIR. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed 1× with 50 mM ammonium chloride, and 1× with PBS before a 1 hr incubation at room temperature with ≈5 μg mL^{-1} Alexa488 goat anti-rabbit antibody (Invitrogen). Cells were then incubated overnight at 4 °C with ≈2 μg mL^{-1} unlabeled goat anti-rabbit to block unlabeled surface antibodies from subsequent probes. Cells were washed 2× with PBS and permeabilized with 0.1% Triton-X. After a PBS wash, cells were incubated for 1 hr at room temperature with ≈5 μg mL^{-1} Alexa633 donkey anti-rabbit (Invitrogen). For cells expressing ELP CLC fusions, immediately following permeabilization, cells were incubated at room temperature for 1 hr with mouse monoclonal anti-myc antibody (Invitrogen) before probing with Alexa633 donkey anti-rabbit (Invitrogen) and Alexa565 goat anti-mouse (Invitrogen). Cells were washed in PBS before mounting.

Quantification of Receptor Internalization in Single Cells: To determine the amount of surface receptor internalized after stimulation, we used a single cell-based method. This technique quantifies the amount of fluorescence emitted by the internalized receptor relative to the total (internal and external) and presents it as a percentage of internalized receptor $\frac{\text{internal}}{\text{internal}+\text{external}}\times 100$. After a pre-incubation at 4 °C for 45 min with rabbit anti-HA antibody (Cell Signaling, C29F4), cells were stimulated with agonist at various time points and conditions prior to PFA fixation. Prior to permeabilization with Triton-X-100, cells were incubated for 1 hour with Alexa488 anti-rabbit secondary antibody. Once permeabilized, the internalized receptor was stained with Alexa633 anti-rabbit secondary antibody. Each experiment contained stimulated and un-stimulated control cells, which were used to determine acquisition parameters on the confocal microscope. A 63× oil lens was used for these experiments with an optical section of 4 μm. For each treatment parameter, 60 to 100 cells were quantified for fluorescence using Zeiss LSM 510 Image Browser. Cells expressing high levels of receptor (beyond the dynamic range) were excluded from the analysis.

Statistics: Unless otherwise noted, data are presented as means and 95% confidence intervals. The significance of changes in receptor colocalization were determined a Student's t-test. The inhibition of receptor internalization due to ELP addition to CLC was first analyzed using 2-way ANOVA for all groups. When a significant difference was found (p <0.05), a post hoc analysis for each temperature and treatment was obtained using the Bonferroni t-test. Student's t-tests were performed between temperatures when comparing colocalization coefficients of antibodies.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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