Triggered Assembly of Functional Protein Microdomains in the Cytosol

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ABSTRACT

The ability to precisely control or alter cellular machinery remains challenging. If it were possible to alter cytosolic processes inside living cells, it would prove to be a great tool in understanding trafficking and complex signaling pathways in specific diseases. To address this issue, we report that Elastin-like polypeptides (ELPs), which belong to a class of environmentally responsive protein polymers, have the ability to not only assemble but also to control specific cytosolic machinery inside living cells. To test this hypothesis, ELP fusion proteins were cloned in mammalian vector and transfected in mammalian cells. At low temperatures, ELP fusion proteins remain soluble. However, at physiological temperatures, ELPs phase separate which causes the fusion protein to assemble into microdomains. This assembly prevents the functional protein domain's inherent ability to carry out signaling pathways.

Keywords: elastin-like polypeptides, transition temperature, microdomains, assembly, clathrin-mediated endocytosis.

1 INTRODUCTION

Biology is unparalleled in the replication of complex structures with diverse functions on the molecular, cellular, and even ecological scale. Engineering matches this control at large scales; however, our ability to design functional structures that operate inside cells remains primitive. By using biological materials to assemble structures, process information, and harness energy, the emerging field of synthetic biology may bridge this gap between current technology and that needed to study and intervene in disease. To explore functional nano-biomaterials, our work focuses on genetically-engineered protein polymers. Previously, we reported how ELPs can reversibly phase separate in respond to controlled protein-mediated dimerization [1]. Now, we report that ELPs can assemble within the cytosol [2, 3], as well as rapidly intervene the biology of transport processes such as clathrin-mediated endocytosis [4].

2 HYPOTHESIS

While there are multiple ways to control protein activity (gene deletion, siRNA, drugs, *etc.*), few approaches are fast and reversible. Elastin-like polypeptides (ELPs) are protein polymers, of the sequence $(VPG\mathbf{X}G)_l$ that have potential applications as reversible switches. ELPs self-associate above a transition temperature (T_t), which is a function of the identity of **X** and *l*. Utilizing the temperature responsive behavior of ELPs, we hypothesized that ELP fusion proteins on phase separation would trigger assembly of the attached functional protein domain. This assembly would prevent the functional protein to participate in its cellular process, thus inhibiting it. To demonstrate the above two hypothesis, we have used three examples of ELP fusion proteins: GFP-ELP, RFP-ELP and CLC-ELP where we have shown assembly of Green fluorescent protein (GFP), Red fluorescent protein (RFP) (**Fig. 1a**) at physiological

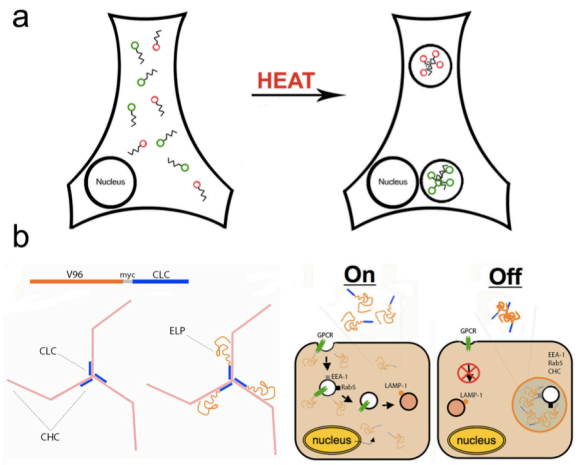

Figure 1: Tunable assembly of ELP fusion proteins. (a) Before heating, GFP-ELP (green) and RFP-ELP (red) remains soluble in the cytosol. After heating, protein polymers assemble into respective microdomains. (b) The permissive state, or 'ON' state, for clathrin mediated endocytosis exists when the ELP-CLC fusion polymer is soluble. Upon heat stimulation, the ELP fusion undergoes a thermodynamically reversible phase separation into an ELP microdomain. Assembly brings the fused CLC into the microdomain, along with markers of CME, and specifically shuts 'OFF' the internalization pathway.

temperatures; and sequestration of clathrin light chain (CLC) which switches off clathrin mediated endocytosis (**Fig. 1b**).

3 RESULTS AND DISCUSSION

To test their ability to associate inside live mammalian cells, we characterized the biophysics of intracellular ELP fusion proteins. After probing fusions of various lengths and sequence, $(VPGVG)_{96}$ ELP, abbreviated as V96 was selected as the model ELP such that the cytosolic phase separation was tuned to occur within a few degrees of 37 °C. Given our hypothesis that ELP fusion proteins on phase separation would cause assembly of the appended functional protein domain, we first tested this concept using Green fluorescent protein (GFP) cloned genetically to V96

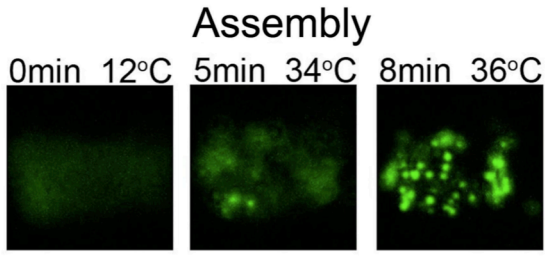

Figure 2: GFP-V96 cytosolic assembly in Hek293 cells is rapid. The fluorescent fusion protein remains soluble at low temperatures, however, it assembles into distinct microdomains at physiological temperature, as distinct punctae.

(**Fig. 2**). NT-GFP-pcDNA3.1 vector was modified using restriction cut sites such that ELP is genetically attached downstream of GFP. The GFP-ELP vector was transfected in Hek293 cells. Cells were ramped from 10 to 40 °C. The intracellular phase separation of GFP-ELP occurs at 34.6 ± 1.4 °C. As shown in the figure, GFP-ELP remains soluble

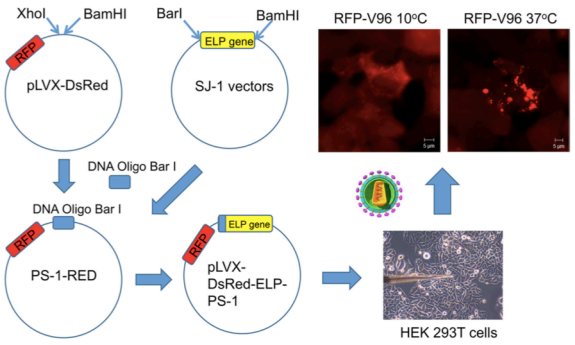

Figure 3: Characterization of a stable RFP-ELP lentiviral construct. The fluorescent fusion protein RFP-ELP remains soluble at low temperature, but at physiological temperatures, the fusion protein assembles into distinct microdomains in the cytosol.

at low temperature, however, above the transition temperature, GFP-ELP rapidly assembles which causes GFP to assemble into distinct microdomain puncta. Similarly, assembly of Red fluorescent protein (RFP) was induced inside cells when cloned genetically to V96 (**Fig. 3**). A gene encoding a soluble monoblock V96 ELP was inserted into downstream of RFP sequence in a lentiviral vector pLVX-DsRed. The genetically engineered lentiviral vector was transfected and packaged in Hek293T cells. The stable cell line that expressed RFP-ELP fusion protein was screened and established. As shown in the figure, RFP-RLP is soluble at 10 °C. When heated above its transition temperature, RFP-ELP assembles into distinct red puncta. To further test the microdomains ability to control or alter cellular processes, clathrin light chain was cloned genetically downstream of the V96 ELP sequence in a mammalian vector with a small myc epitope tag in between the two genes for immunofluorescence detection. When CLC-ELP is soluble, the clathrin-mediated endocytosis remains permissive. Upon heat stimulation, ELP phase separates into a microdomain. This assembly brings fused CLC into the microdomain, along with internalization markers of CLC and shuts off clathrin-mediated

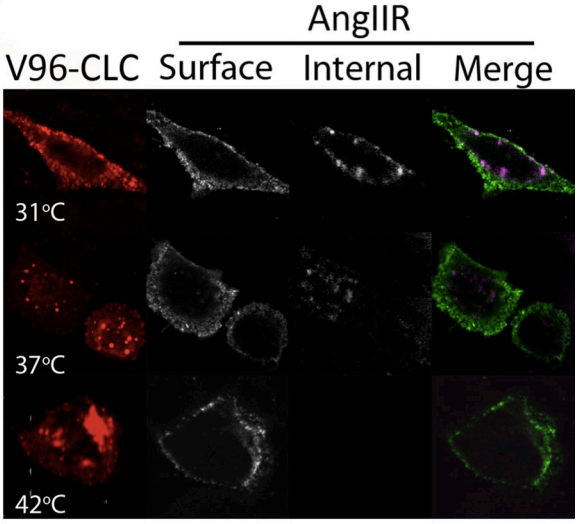

Figure 4: Angiotensin II Receptor Internalization is knocked down by ELP assembly. CHO Cells were co-transfected with both V96-CLC and AngIIR, incubated with angiotensin above (37, 42 °C) and below (31 °C) the microdomain assembly temperature, and stained for AngIIR internalization (purple). Surface AngIIR was stained prior to permeabilizing the cell (green). V96-CLC expressing cells were identified by anti-myc antibodies (red). Microdomains of V96-CLC were observed at 37 and 42 °C, at which temperatures receptor internalization decreased indicating inhibition of clathrin-mediated endocytosis.

endocytosis. When co-expressed in CHO cells, the ELP-CLC fusion protein permits clathrin-mediated endocytosis of angiotensin II receptor (AngIIR); however, above the

cytosolic transition temperature, AngIIR internalization was blocked (**Fig. 4**).

The inhibition of clathrin-mediated endocytosis was further validated by co-localization of V96-CLC with early and late markers of internalization such as clathrin-heavy chain (CHC), Rab5; and lysosomal-associating protein-1

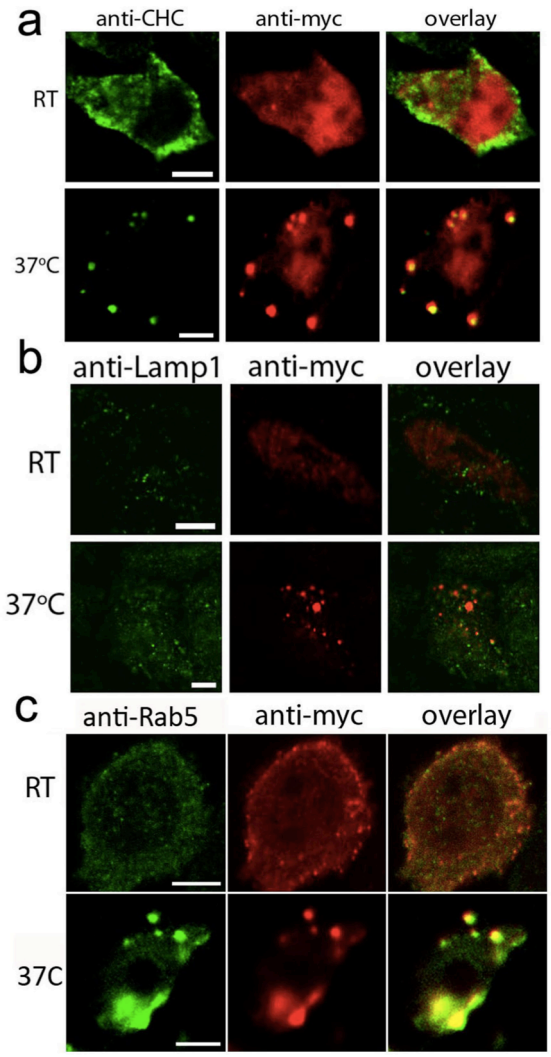

Figure 5: Co-localization of V96-CLC with markers of internalization. At room temperature (RT), when the ELP is soluble, V96-myc tagged CLC moderately colocalizes with markers of internalization. However, when V96 sequesters CLC in cytosolic microdomains, early markers of internalization also associate with these vesicles, but not late markers of internalization. V96-CLC associates highly with CHC and Rab5, and not at all with LAMP1.

(LAMP1) (**Fig. 5**). We observed that when V96-CLC phase separates, it was highly co-localized with early markers of internalization such as CHC and Rab5 but not at all with late marker of internalization such as LAMP1.

4 CONCLUSIONS

Via genetic engineering, it is possible to express ELP fusion proteins in the cytosol of eukaryotes. When induced to phase separate, these ELPs form distinct microdomains that remove the cellular machinery required for transport processes such as clathrin-mediated trafficking. We have successfully shown microdomain assembly of three ELP fusion proteins: GFP-ELP, RFP-ELP, and ELP-CLC, the latter inhibiting clathrin-mediated endocytosis. With additional study, this technology may become a tool for the controlled manipulation of biological signaling and trafficking pathways.

5 ACHNOWLEDGEMENTS

The National Institute of Health R21EB012281 to J.A.M., and P30 CA014089 to the Norris Comprehensive Cancer Center.

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