Dissecting Clathrin-mediated Endocytosis

Studying Protein Dynamics in Genome-edited Mammalian Cells

Clathrin-mediated endocytosis (CME) is one type of receptor-mediated endocytosis (RME) and defines a process by which cells selectively internalize materials from their plasma membrane and surrounding environment. CME is important for many essential cell functions such as nutrient uptake, transmembrane signaling, cell-to-cell communication and regulation of membrane receptor composition. The dynamic process is highly orchestrated with more than 60 proteins contributing to the temporal and spatial events in a tightly coordinated manner, starting from ligand binding to the cell surface and progressing through degradation or recycling of membrane receptors from the late-stage endosomes [1,2].

Early studies of CME in budding yeast exploited the ability to create fluorescent fusion proteins at endogenous genomic loci and retain physiological protein expression levels [3,4]. Live-cell imaging studies on the dynamics of the tagged fusion proteins demonstrated the CME process in yeast to be highly regular and efficient [5]. These experiments were initially extended to mammalian cells by ectopically expressing fusion proteins from the same genes (e.g., clathrin, dynamin). Notably, this resulted in significantly higher protein levels than typically found in the untransfected cells and yielded observations that CME was significantly less efficient and more heterogeneous than previously determined in yeast [6,7]. These findings raised the important possibility that the CME process may be fundamentally different in mammalian cells or, alternatively, that the native biology of CME dynamics may be perturbed by the altered ratios of specific proteins due to ectopic overexpression.

A relatively new technology allowed these possibilities to be addressed with a series of well-designed genome engineering studies. Zinc finger nucleases (ZFNs) are DNA restriction enzymes that create double strand DNA breaks at highly specific sites in genomic DNA of mammalian cells. The sequence specificity for the ZFN cut site is achieved through the complement of zinc finger protein modules chosen for the DNA binding domains of each ZFN heterodimer [8]. A desired sequence
Modification can be engineered at a defined site on a gene locus by introducing a specific donor plasmid, which the cell uses as the template for homologous recombination-mediated repair of an adjacent, ZFN-mediated double stranded break.

Applying this approach to integrate a fluorescent reporter sequence into a target gene, it is possible to create fluorescent fusion proteins expressed from endogenous gene loci and regulated by the native promoter.

In contrast to an overexpression system, this results in protein expression levels similar to those found in the parental cell lines not treated with the ZFNs. Therefore ZFN technology was exploited to develop cell lines expressing fluorescent fusion proteins for clathrin light chain A-red fluorescent protein (CLTA-RFP) and dynamin-2-green fluorescent protein (DNM2-GFP), in addition to a cell line expressing both fusion proteins [9]. These tools facilitated the design of experimental studies to directly compare CME dynamics of endogenously tagged proteins to those of ectopically overexpressed counterparts (fig. 1).

**Experimental Design**

Human melanoma (SK-MEL-2) cells from the American Type Culture Collection were used to create engineered cell line [9]. Briefly, stable, overexpressing cell lines for both CLTA-RFP and DNM2-GFP were generated by transfecting specifically designed plasmids into the cells with Lipofectamine 2000 (Life Technologies), selecting positive clones expressing the fluorescent proteins with fluorescence-activated cell sorting (FACS) and amplification by dilution cloning.

SK-MEL-2 cell lines expressing endogenously-coded fusion proteins (CLTA-RFP and DNM2-GFP) were created with ZFNs designed for targeted integration at the C-terminus near the stop codon of the proteins [9]. Positive clones were selected with FACS and single cell clones established by expansion following limiting dilution.
Following co-transfection of ZFNs and donors for both genes, one cell line generated expressed both fusion proteins.

CME was stimulated by the addition of labeled transferrin to cultured cells and incubated for 45 minutes. Subsequently, the cells were fixed in paraformaldehyde and transferrin uptake measured in an image analysis system to determine the mean fluorescence intensity of individual cells. Particle detection and tracking were performed using Imaris 7.1 (Bitplane) following background subtraction [9].

**Results and Discussion**

The studies in the SK-MEL-2 cells were designed to examine the process of CME with fluorescent fusion proteins expressed from their native loci. Specifically, the goal was to determine how the spatiotemporal events of CME in these cells compared to previous reports that utilized ectopically overexpressed fusion proteins and described the mammalian CME process as more heterogeneous and inefficient. Initial results in the genome-edited cells demonstrated the ability to tag CLTA with RFP on either one allele or all alleles. In each case, fluorescence imaging confirmed that CLTA-RFP co-localized with clathrin heavy chain. Functional analysis (tab. 1) showed the heterozygous-tagged cells (one allele) maintained robust transferrin uptake and exhibited a short fluorescence lifetime (44 +/- 3.1 sec), whereas cells overexpressing the same CLTA-RFP fusion yielded decreased endocytosis and a significantly longer fluorescence lifetime (63 +/- 2.9 sec). Interestingly, cells with all alleles tagged using ZFNs yielded an intermediate lifetime (52 +/- 2.0 sec), sustained modest transferrin uptake, and showed large, stable clathrin-coated structures at the plasma membrane similar to those observed in cells overexpressing the fusion protein. These data demonstrated that CME in mammalian cells is more dynamic than first thought and that increased levels of fluorescent fusion proteins result in decreased CME dynamics and endocytic function.

Similar studies were conducted in SK-MEL-2 cells to examine the effects of overexpression on DMN2 dynamics. DNM2-GFP cell lines with one or all alleles tagged using ZFNs were compared to the same cell line with the same fluorescent fusion protein ectopically overexpressed. The results confirmed that the latter cell line had dramatically reduced endocytic function and bright, irregular and highly stable dynamin structures that make analysis of DNM2 membrane dynamics impossible. Similar to experiments using CLTA-RFP, the genome-edited DNM2-GFP cell lines were highly dynamic and exhibited average lifetimes of 24 +/- 1.0 sec (single-tagged allele) and 36 +/- 2.1 sec (all alleles tagged). Thus, for both CLTA-RFP and DNM2-GFP cell lines, CME dynamics were found to be highly sensitive to the level of protein expression and number of alleles tagged.
Early CME studies in yeast benefited from the ability to label multiple genes with different fluorescent tags and thus define spatiotemporal associations of proteins. To accomplish this in the SK-MEL-2 cells, a tagged cell line having one allele of CLTA-RFP and all alleles of DNM2-GFP was isolated. The average fluorescence lifetimes were consistent with those observed in the single-tagged cell lines (45 +/- 1.0 sec for CLTA-RFP and 32 +/- 3.3 sec for DNM2-GFP). Fluorescence imaging also confirmed that DNM2 was heavily recruited to the plasma membrane during the final stages of clathrin-coated pit maturation. Notably, the dual-tagged cell line afforded the opportunity to simultaneously measure the dynamics of clathrin and dynamin and quantitate the efficiency of dynamin recruitment during the disappearance of clathrin puncta. Most surprising was the finding that most of the events (91%) in the SK-MEL-2 cells were productive (ended with the bright dynamin peak), whereas cells overexpressing both CLTA-RFP and DNM2-GFP displayed abundant, large stationary structures with both dynamin and clathrin associated.

The results of these studies have significantly advanced our understanding of the mechanism and regulation of CME, particularly that it is more similar to the process in yeast than initially believed. Equally important, however, are the insights gained regarding the impact of protein overexpression and allele modification on biological processes involving dynamic multimeric complexes. The ability to create fluorescent fusion proteins from their endogenous gene loci using ZFN-mediated genome editing will advance our studies of protein dynamics to a new level of understanding previously unappreciated due to technical limitations.

References


Authors
David G. Drubin, UC Berkeley, Bradley T. Keller, Sigma Life Science, Ryan Ferrell, Harris D. McKinney

Contact

**Harris D. McKinney**
55 West Wacker Drive
60601 Chicago, IL
USA
Phone: +1 312-506-5200