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Improved strategies for the delivery of GFP-based Ca^{2+} sensors into the mitochondrial matrix

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Abstract

The role of mitochondria in Ca^{2+} handling has acquired renewed interest in recent years in the field of cell signaling. Detailed studies of Ca^{2+} dynamics in this organelle at the single cell level have been hampered by technical problems in the available Ca^{2+} probes. Some of the latest generation GFP-based Ca^{2+} probes (Camgaroos, Cameleons and Pericams) show great potential to address this issue. Our data show that the choice of targeting sequence influences not only the overall efficiency of subcellular localization of the probes, but also their functional characteristics within the matrix. In particular, we here show that the use of a tandemly duplicated mitochondrial targeting sequence is capable of improving the delivery efficacy of all tested probes into the organelle's matrix, in particular that of Cameleon, a GFP-based Ca^{2+} probe that is otherwise largely mistargeted to the cytosol. The devised strategy should be generally applicable to other proteins that are characterized by poor targeting. Last, but not least, we also demonstrate that if the targeting sequence is not removed from the imported protein, the fluorescent properties and the Ca^{2+} affinity of the probe can be grossly affected.

Keywords: GFP; Ca^{2+} probes; Mitochondrial targeting

1. Introduction

Calcium is undoubtedly one of the most versatile players in cell signaling. The importance of this second messenger and its handling by mitochondria has regained the focus of attention of many researchers in recent years, as clearly demonstrated by the dramatic increase in publications in this area. One of the major limitations for the detailed study of this organelle's role in signal transduction and calcium homeostasis is the availability of reliable probes for the specific measurement of changes in $[Ca^{2+}]$ within the mitochondrial matrix ($[Ca^{2+}]_m$) at the single cell level. Studies using targeted aequorin [1,2], valuable as they have unquestionably been, have been carried out primarily at the cell population level (and thus are limited in terms of spatial resolution), while the use of this probe at the single cell level has intrinsic limitations. Conversely, all the probes that can be used at the single cell level (e.g., rhod-2 or GFP-based Ca^{2+} indicators) exhibit acknowledged limitations, although they have been rapidly adopted by several laboratories [3]. As far as the genetically encoded Ca^{2+} indicators are concerned, they are affected by two severe problems: partial mis-targeting (in extreme cases, incorrect localization) and, in some cases, reduced response efficiency when compared to cytosolic versions.

Genetically encoded probes are generally touted as far superior to chemical probes due to their ability to be targeted exclusively to specific subcellular locations. This is indeed an enormous advantage and relies ultimately on the addition of a targeting signal to the protein probe of interest. The theoretical basis for this methodology was originally proposed by Blobel and Sabatini over 30 years ago [4], gained credibility through the groundbreaking work of

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Blobel and Dobberstein during the mid 1970s [5,6], and is widely known as the signal hypothesis (for an overview, see [7]). Nowadays, scientists interested in directing a protein to a specific subcellular location have a plethora of targeting sequences to choose from; the success of this methodology is so high that targeting sequences have come to be considered as a form of "magic bullet" that will guide any given protein into a chosen subcellular location. A simple fluorescent beacon such as the green fluorescent protein (GFP), exemplifies the power of genetic engineering techniques in this context: inclusion of different sequences can target this protein efficiently and specifically to mitochondria, nucleus, ER, Golgi, etc. [8]. Unfortunately, this deceptively simple strategy can be plagued by unsuspected pitfalls, as the present generation of genetically engineered $[Ca^{2+}]_m$ probes clearly demonstrates.

In the present work, we present an improved molecular engineering approach for the efficient delivery of Ca^{2+}-sensing probes into the mitochondrial matrix. The devised strategy has yielded sensors with increased targeting efficiency and has also led to the discovery of a series of unpredicted effects of the targeting sequences on the functions of the probes within the mitochondrial matrix.

2. Materials and methods

2.1. Constructs

Generally, the prefix mt- denotes a mitochondrial version of any given probe, without specifying the identity of the targeting peptide (this concept applies also to the references listed). However, given the nature of the present work, the exact mitochondrial targeting peptide is indicated in each probe: mt4- refers to the first 12 amino acids of subunit IV of yeast cytochrome *c* oxidase (COX) [9], and mt8- refers to the first 36 amino acids of subunit VIII of human COX [8]; duplication of the signal peptide is indicated accordingly (i.e., 2mt8- refers to a duplicated COX VIII signal peptide); cDNA encoding the signal peptides is fused, in frame, with cDNA encoding the Ca^{2+} probe. The probes used in this study are listed in Table 1. Cytosolic and nuclear ratiometric Pericams [10] are indicated by the epithets cytPR and nuPR, respectively. Details of all constructs are available upon request.

2.2. Cell cultures and transfection

HeLa cells were grown in DMEM containing 10% FCS, supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml), in a humidified atmosphere containing 5% CO_2. For transient transfections, cells were seeded onto glass coverslips (24-mm diameter); transfections were performed at 50–70% confluence using the calcium phosphate method, with a total of 10 μg of DNA.

Table 1
List of probes used in this study and respective epithets

Core probe	References	Epithet
Camgaroo-2	[18]	mt8CG2
	This study	2mt8CG2
Ratiometric Pericam	[3,10]	mt4PR
	This study	mt8PR
	This study	2mt8PR
Yellow-Cameleon-2	[13]	mt8YC2
	This study	2mt8YC2
	This study	2mt8YC2.1[a]
	This study	2mt8YC2.3[a]
	This study	2mt8YC2.12[a]

Camgaroo-2 was originally provided by R.Y. Tsien, yellow-Cameleon-2 by N. Demaurex, while ratiometric Pericam probes are originally from A. Miyawaki.

[a] In probes 2mt8YC2.1, 2mt8YC2.3 and 2mt8YC2.12, the YFP moiety of 2mt8YC2 has been substituted by EYFP-V86L/Q69K [19], "citrine" [18] and "Venus" [20], respectively.

2.3. Cell imaging

Cells expressing fluorescent probes were observed 48–72 h after transfection (see text for details) on an inverted fluorescence microscope (Zeiss Axioplan), with an oil immersion objective (63×, N.A. 1.40). Excitation light at appropriate wavelengths was produced by a monochromator (Polychrome II, TILL Photonics, Martinsried, Germany): 440 nm for Cameleons, 500 nm for Camgaroos, and 415 nm and 490 nm for ratiometric Pericams. Dichroic beamsplitters were 455DRLP, 525DRLP, and 505DRLP, respectively. Emission filters were 480DF30 (for CFP) and 545DF35 (for YFP) in the case of Cameleons, HQ520LP in the case of Camgaroos, and 535RDF45 in the case of PRs; when using Cameleons, the emission filters were alternated using a filter wheel (Lambda 10-2, Sutter Instruments, San Rafael, CA, USA). Filters and dichroic beamsplitters were purchased from Omega Optical and Chroma Technologies (Brattleboro, VT, USA). Images were acquired using a cooled CCD camera (Imago, TILL Photonics) attached to a 12-bit frame grabber. Synchronization of the monochromator and CCD camera was performed through a control unit ran by TILLvisION v.4.0 (TILL Photonics); this software was also used for image analysis. Additional image analyses employed the public domain ImageJ program (developed at the U.S. National Institutes of Health by Wayne Rasband and available on the Internet at http://rsb.info.nih.gov/ij/). For time-course experiments, the mean fluorescence intensity was determined over regions covering >50% of the total nuclear or mitochondrial area. For studies of localization efficiency, confocal planes were obtained using a Nipkow disk confocal microscopy system (UltraVIEW; Perkin-Elmer, USA) equipped with a 488/543 nm Ar laser (Spectra Physics, USA) and a digital camera (Orca; Hamamatsu, Japan); the microscope (an inverted Nikon TE200) was equipped with a 60× oil immersion objective (NA 1.4; Nikon) and the 488 nm laser line was used for excitation. Regions of interest (ROIs) were selected