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Engineering Ca^{2+}/calmodulin-mediated modulation of protein translocation by overlapping binding and signaling peptide sequences

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ABSTRACT

Protein translocation is used by cells to regulate protein activity in time and space. Synthetic systems have studied the effect of second messengers and exogenous chemicals on translocation, and have used translocation-based sensors to monitor unrelated pathways such as caspase activity. We have created a synthetic Ca^{2+}-inducible protein using calmodulin binding peptides that selectively reveal nuclear localization and export signals in low Ca^{2+} (0 μM) and high Ca^{2+} (10 μM) environments, respectively. Experiments in live cells showed that our construct translocates between the nucleolus and plasma membrane with time constants of approximately 2 h. Further, a single amino acid mutation (Cys20Ala) in our construct prevented translocation to the plasma membrane and instead targeted it the mitochondria as predicted by bioinformatic analysis. Lastly, we studied the effect of cell line, Ca^{2+} concentration, chemical inhibitors, and cell morphology on translocation and found these conditions affected the rate, extent and direction of translocation. Our work demonstrates the feasibility of engineering Ca^{2+}/calmodulin-mediated modulation of protein translocation and suggests that more natural analogs may exist.

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1. Introduction

Protein translocation is a dynamic cellular process used to regulate the activity of a wide variety of proteins with a high degree of spatiotemporal control. In turn, these proteins can then regulate diverse and complex cellular events such as apoptosis, adhesion, migration and proliferation. There are at least two important considerations in any translocation event: the cellular compartments between which the protein translocates and the signal or cellular machinery that induces the translocation. A common translocation pattern is from the cytoplasm to the nucleus mediated by a protein's nuclear localization signal (NLS) and importin proteins [1], as is the case in a wide variety of transcriptional regulators (such as Stat [2], Smad [3], NF-κβ [4] and NF-AT [5]). Conversely, translocation from the nucleus to cytoplasm, mediated by the nuclear export signal (NES) and exportin proteins [1] is responsible for the regulation of proteins such as tumor suppressor p53 [6] and mRNA [7]. Other common modes of protein translocation include cytoplasm to plasma membrane (such as the Rho GTPases after lipid modification and GTP binding [8]), endosomal vesicles to plasma membrane (such as GLUT4 glucose transporter in response to insulin [9]) and cytoplasm to mitochondria (such as Bid after cleavage to tBid by caspase-8 [10]).

The regulation of protein translocation is achieved through a variety of mechanisms including phosphorylation [1,7,11], cleavage by proteases [10], lipid modification [8,12], intramolecular masking [1,5,6] and intermolecular masking [5,13–15]. Intermolecular masking is a potentially powerful tool for manipulating protein translocation because it utilizes native second messenger systems (such as cAMP or Ca^{2+}) and has the potential to be reversible as the concentration of the second messenger can be tightly regulated. There are many cellular examples of intermolecular masking that controls nuclear import and export such as calcineurin blocking the NES of NF-AT at resting Ca^{2+} [1,5], mRNA blocking the NLS of the HIV protein Rev [16] and hormone ligand blocking the NES of androgen receptor [17].

The characteristics of protein translocation (the rate, extent and direction of translocation) can be affected by the protein–protein interaction that mediates the translocation as well as the environment. Translocation kinetics are governed by multiple factors and determine how rapidly a protein will respond to a localization signal. For example, binding interactions between karyopherins and NLS or NES containing proteins, combined with the specific nuclear pore complex geometry, results in rapid import and export of proteins on the time scale of seconds to minutes. However, caspase-mediated apoptosis initiated by Bid recruitment to the mitochondria, which does not have a specific translocation mech-

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anism or particular morphological constraint, has a time scale of minutes to hours [10]. The morphology of different cellular environments can vary widely, from long and narrow processes of neuronal axons, extended and flattened morphology of lamellipodia or the short and very thin extensions of filopodia.

To explore the design potential of controlling the protein localization by selectively exposing signaling peptides inside living cells, we developed a Ca^{2+}-inducible protein that translocates from the nucleolus to the plasma membrane given a sustained Ca^{2+} stimulus. We accomplished this by designing overlapping peptides that selectively expose NLS and NES sequences depending on the intracellular Ca^{2+} concentration. Furthermore, we characterized the effect on protein translocation from environmental variables such as intracellular Ca^{2+}, cell morphology and differences between cell types. The feasibility of this design suggests that more natural analogs may exist.

2. Results

2.1. Conceptual design of a Ca^{2+}-inducible protein translocation system

Conceptually, a Ca^{2+}-inducible protein translocation system can be created if a protein has a tendency to be in one cellular compartment when Ca^{2+} is low, translocate into another compartment when Ca^{2+} is high, and then return to the original compartment when Ca^{2+} is low again. To accomplish this, we based our design on proteins such as p53 [6], Rev [16] and NF-AT4 [5] which reversibly translocate between the nucleus and cytoplasm by selectively revealing NES and NLS peptides. Specifically, NF-AT4 transduces an intracellular Ca^{2+} signal into a subcellular localization by selective binding to calcineurin [5].

In theory, we could control when NES and NLS peptides are revealed by overlapping [18] them with calmodulin (CaM) binding peptides (CBP's) with different characteristic responses to Ca^{2+}. For example, IQ motif peptides tend to bind CaM when Ca^{2+} is low and dissociate when Ca^{2+} is high [19], while 1–14 motif peptides bind CaM when Ca^{2+} is high and dissociate when Ca^{2+} is low [20,21]. We chose to use the IQ motif from mouse myosin V (IQp) [19] and the 1–14 peptide from skeletal muscle myosin light chain kinase (MLCKp) [22]. We searched the online database NESbase1.0 for NES peptides [23] and performed a manual literature search for NLS peptides. We chose to overlap *I*Qp with the N*E*S from actin [24] to form the IE peptide and the N*L*S from mouse p54 [25] with *M*LCKp to form the LM peptide because those overlaps could be made with minimal changes to key hydrophobic and basic residues within the CBPs (Fig. 1A and B). We chose to modify the CBP rather than the NES or NLS because of CaM's robust binding peptide recognition. When this synthetic peptide is fused to a fluorescent protein like the YFP Venus [26], the translocation can be monitored with a high degree of spatiotemporal accuracy. The functionality of NLS and NES signal sequences were verified by fusing them N-terminally to Venus and observing the subcellular fluorescence distribution (Fig. 1C and E, respectively) compared to the distribution of Venus (Fig. 1D). The nuclear import and export of Venus is not complete because the size of the fusion protein allows for some passive diffusion through the nuclear pore complex [27,28].

2.2. The synthetic protein translocates from the nucleolus to the plasma membrane

The IE-LM-Venus construct translocated from the nucleolus to the plasma membrane in a high Ca^{2+} (10 μM) environment sustained for approximately 3 h (Fig. 2). Stimulation with 1 μM ionomycin in PBS supplemented with 10 μM Ca^{2+} was

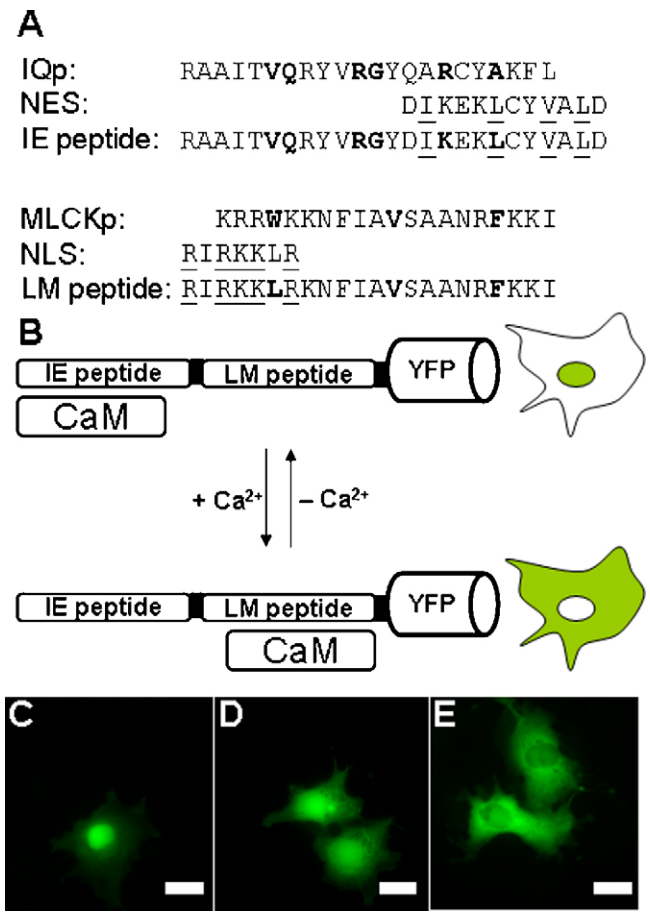

Fig. 1. Schematic of the peptide overlaps and proposed Ca^{2+}–CaM dependent localization. (A) The original sequences for the four functional peptides are shown with key CaM anchoring residues bolded and key NES or NLS interacting residues underlined. (B) In low Ca^{2+}, CaM will preferentially bind the IQp portion of the IE peptide, allowing the NLS of the LM peptide to interact with importins. In high Ca^{2+}, CaM will preferentially bind the MLCKp portion of the LM peptide, allowing the NES of the IE peptide to interact with exportins. (C–E) COS-7 cells transfected with NLS-Venus, Venus and NES-Venus, respectively. Scale bars in C–E are 30 μm.

sufficient to induce translocation from the nucleolus to the plasma membrane. After approximately 1 h, some fluorescence was noticeable on the plasma membrane, while after 2 h there was noticeable dimming of the nucleolus in some cells, and net translocation appeared to slow and stop in the third hour (Fig. 2A–F).

We analyzed the rate of nuclear export by plotting a profile of the fluorescence intensity across a cell (Fig. 2G, using the white line from Fig. 2A, C and E). We then formed a ratio of the fluorescence intensity at the nucleolus to the fluorescence intensity at the plasma membrane and plotted that ratio over time (Fig. 2H). The fluorescence intensity distribution plot (Fig. 2G) shows that at the beginning of the experiment, there is one strong peak in the middle of the cell, representing intense fluorescence localization in the nucleolus. There were three trends depicted in the plots: first, the peak representing the nucleolus (at approximately 70 pixels) faded until it was indistinguishable from the cytoplasm; second, the intensity in the cytoplasm increased evenly throughout the cell; third, two peaks arose on either side of the cell (at 25 and 100 pixels, respectively) which became brighter, representing the plasma membrane. The cell morphology did change somewhat during the experiment, which was noticeable because the rightmost peak representing the plasma membrane slowly shifted from approximately 100 to 85 pixels in 3 h. The magnitude of this change was compara-

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