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Engineering Ca²⁺/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 Ca²⁺-inducible protein using calmodulin binding peptides that selectively reveal nuclear localization and export signals in low $Ca^{2+}(0 \mu M)$ and high $Ca^{2+}(10 \mu M)$ 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, Ca²⁺ 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 Ca²⁺ [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 Ca²⁺-inducible protein translocation system

Conceptually, a Ca²⁺-inducible protein translocation system can be created if a protein has a tendency to be in one cellular compartment when Ca²⁺ is low, translocate into another compartment when Ca²⁺ is high, and then return to the original compartment when Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 NESbase 1.0 for NES peptides [23] and performed a manual literature search for NLS peptides. We chose to overlap IQp with the NES from actin [24] to form the IE peptide and the NLS from mouse p54 [25] with MLCKp 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 μ M) environment sustained for approximately 3 h (Fig. 2). Stimulation with 1 μ M ionomycin in PBS supplemented with 10 μ M Ca^{2+} was

A	
IQp:	RAAIT VQ RYV RG YQA R CY A KFL
NÉS:	DIKEKLCYVALD
IE peptide:	RAAIT VQ RYV RG YDIKEKLCYVALD
MLCKp:	KRRWKKNFIA V SAANR F KKI
NLS:	RIRKKLR
LM peptide:	RIRKK L RKNFIA V SAANR F KKI
В	
E peptide	
	V
	+ Ca^{2+} - Ca^{2+}
E peptide	CaM
C	

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 Ca²⁺, CaM will preferentially bind the IQp portion of the IE peptide, allowing the NLS of the LM peptide to interact with importins. In high Ca²⁺, CaM will preferentially bind the MLCKp portion of the IE peptide, allowing the NES of the LM peptide to interact with exporting 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 μ m.

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-



Fig. 2. Synthetic protein translocation and translocation analysis. (A–F) Time course of COS-7 cells transfected with the IE-LM-Venus construct and stimulated with ionomycin over 3 h. The white line indicates the fluorescence intensity profile used to generate (G) which shows the change in fluorescence distribution over 3 h (black, dark grey, light grey and outlined markers indicate 0, 1, 2 and 3 h after stimulus, respectively). (H) The intensity ratio of nucleolus to leftmost plasma membrane over time, superimposed with the line of best fit for the export phase of translocation. Error bars are the reading error. Scale bars in A–F are 30 µm.

ble to a Venus transfected cell exposed to 1 μM ionomycin for 3 h (data not shown).

The average rate of translocation of the IE-LM-Venus construct was $122 \pm 21 \min(n=6 \text{ cells})$. To make comparisons between cells, the fluorescence ratio time course was normalized to range between 0 and 1 using the maximum ratio in a given trial as 1 and the 0 ratio as 0. In the first 20 min, we noticed a transient increase in the intensity ratio, which indicated the construct moved into the nucleolus. We therefore considered translocation to occur in two stages: an initial transient phase, followed by a sustained export phase. We fit the export phase to an exponential decay of the form $e^{-t/\tau}$ where τ is the characteristic time constant. The time constant for the particular analysis presented here (Fig. 2) was 104 min. The time constants ranged from 102 to 150 min (mean 122 ± 21 min, n = 6). The quality of these fits was very good as the r^2 value ranged from 0.86 to 0.95.

The rate of translocation from the nucleolus to the plasma membrane was not a function of distance from the nucleolus to the plasma membrane under normal morphological conditions. To investigate whether the rate of translocation within a cell was a function of the location on the plasma membrane where we measured the fluorescence intensity, we selected four locations on the plasma membrane for the cell that had already been analyzed (bottom cell, Fig. 2A–F). We performed the same analysis using these four points, all of which were different distances from the nucleolus. The four time constants were 98.3, 101, 104 and 116 min (mean 105 ± 8 min). There was no correlation between the distance between the membrane and nucleolus and the observed time constant, however, it could be that our method of analysis was not sensitive enough to detect differences in translocation within the cytoplasm. Given the variability in the time constants between cells, the relative variability within this cell was small.

Next, we verified that the mechanism of translocation was consistent with our model: that high Ca²⁺ causes CaM to switch binding from the IE to the LM portion of the peptide construct and that the construct is actively exported to the cytoplasm. We treated cells with the CaM inhibitor calmidazolium (CDZ) (Fig. 3A–C) or with the exportin CRM1 inhibitor leptomycin-B (LMB) (Fig. 3D–F) and stimulated them as before. The CaM inhibitor was able to completely prevent translocation, suggesting that CaM binding to our protein is the mechanism that initiates translocation. While one may have expected CDZ treatment to cause some export, since the NES and NLS are equally exposed, this would likely have taken many hours considering that even with the NLS covered (high Ca²⁺ condition) export still required 2–3 h.

LMB was not able to totally arrest protein translocation, but rather had the effect of reducing the rate and extent of translocation. Translocation in cells treated with LMB showed that the apparent rate of nuclear export had slowed to $156 \pm 23 \min (n=4)$, which is significantly less than the case without the export inhibitor (p=0.045). As well, the intensity ratio values after translocation (before normalizing) were significantly higher (p < 0.001) for cells treated with LMB (6.78 ± 1.13 , n=4) than without (1.20 ± 0.33) , n=6). This suggests that not only did LMB slow the rate of translocation, but it also significantly reduced the extent of translocation. It was not surprising that translocation was not totally prevented, because the predicted molecular mass of the IE-LM-Venus protein is approximately 28 kDa, which would allow for some passive diffusion through the nuclear pore complex [27,28].

We found that a single amino acid was necessary for targeting IE-LM-Venus to the plasma membrane, and that when it was mutated (Cys20Ala), the mutant construct targeted the mitochondria. To investigate the mechanism of plasma membrane association, which was not immediately apparent from our design, we checked the predicted localization of our peptide (IE-LM) using two localization prediction tools, PSORT [29] and LOCtree [30], however neither suggested membrane localization. We suspected that the peptide may have been lipid modified in some way which was driving its association with the plasma membrane, however our peptide lacked the consensus motifs required for prenylation and myristoylation. We wanted to investigate the potential of palmitoylation on Cys20, however cells treated with the palmitoylation inhibitor 2bromopalmitate were not viable, even at concentrations well below those reported in the literature [12].

As an indirect test of this hypothesis, we mutated the peptide (Cys20Ala) to remove the potential palmitoylation site and created mIE-LM-Venus. This resulted in an unexpected fluorescence distribution between the nucleolus and what appeared to be the mitochondria (Fig. 3G). We returned the mutated sequence to PSORT and LOCtree which now both predicted mitochondrial localization. To confirm this new hypothesis, we co-transfected cells with the mIE-LM-Venus construct and with pKillerRed-dmito, which would act as a mitochondrial marker [31] and found that



Fig. 3. The mechanism of protein translocation. (A–C) COS-7 cells exposed to CaM inhibitor CDZ before (A) and after (B) stimulation with ionomycin, with the ratio time course (C). (D–F) Cells exposed to export in inhibitor LMB before (D) and after (E) stimulation with ionomycin, with the ratio time course (F). (G and H) Cells co-transfected with mIE-LM-Venus and pKillerRed-dmito and then merged in I. Error bars are the reading error. Scale bars are 30 μ m.

they co-localized (Fig. 3G–I). We observed no plasma membrane association at anytime during translocation out of the nucleolus with the mIE-LM-Venus construct. We therefore suspect that the Cys20 residue was modified, likely by palmitoylation, which drove the membrane association, whereas when this functionality was removed in the mutated peptide, the construct was targeted to the mitochondria.

As a further test of the robustness of translocation, we found that the IE-LM-Venus construct translocated in HeLa and NIH3T3 cells, and that variations on the IE-LM-Venus construct could translocate in COS-7 cells (Fig. 4). We found that the rate of nucleolus to plasma membrane translocation in HeLa cells was $197 \pm 9 \min(n=4)$ which was significantly slower (p < 0.001) than translocation in COS-7 cells (Fig. 4A–C). We observed after approximately 1 h a punctuate fluorescence appeared in the cytoplasm concomitantly with the decrease in fluorescence in the nucleolus. This cytoplasmic fluorescence may have been responsible for the absence of increased fluorescence on the membrane and the resultant slower apparent rate of translocation. We observed an average rate of nucleolus to plasma membrane translocation in NIH3T3 cells of $103 \pm 33 \min(n=3)$ which was not significantly different than the rate observed for COS-7 (p=0.396) (Fig. 4D–F).

When the construct was modified to IE-LM-mRFP, translocation was comparable to IE-LM-Venus, however we did not observe any translocation when we used the tetrameric RFP dsRed in IE-LM-dsRed (Fig. 4G–I and J–L, respectively). The translocation of IE-LM-mRFP appeared to occur more slowly than IE-LM-Ven (144 \pm 16 min, *n* = 4), but the difference was not significant (*p* = 0.098) (Fig. 4G–I). The tetrameric construct IE-LM-dsRed did not translocate during our experiment (Fig. 4J–L). This was not surprising given that its predicted molecular mass would be significantly above the passive diffusion cut-off for the nuclear pore complex, and as shown above, passive diffusion is responsible for a large contribution to the translocation out of the nucleus.

2.3. Protein translocation can be modified by Ca^{2+} concentration

The rate, extent and direction of translocation can be influenced by controlling the intracellular Ca²⁺ concentration: very low intracellular Ca²⁺ can reverse translocation into the nucleolus, while low and moderate Ca²⁺ result in two-stage translocation that is a combination of import and export processes (Fig. 5). The rate and extent of protein translocation in our system should be dependent on the intracellular Ca²⁺ concentration: when Ca²⁺ is very low (below the K_D for Ca²⁺–CaM of about 1 μ M [20]) nuclear import should be favoured whereas when the Ca²⁺ concentration is near the Ca²⁺–CaM K_D , export should occur, but presumably more slowly and less completely than in the case of saturating Ca²⁺. We used a Ca²⁺ calibration buffer kit to hold the extracellular concentration at a desired value (0, 0.1 or 1.0 μ M) and again used 1 μ M ionomycin to stimulate the cells.

In the case of very low extracellular Ca^{2+} (0 μ M), we observed reverse translocation from the plasma membrane and cytoplasm into the nucleolus (Fig. 5A–E). In some transfected cells we observed an initial distribution of fluorescence on the plasma membrane and in the nucleolus. We believe that this was the result of competition for the peptide immediately after translation where in some cells, a subpopulation of proteins was lipid modified and membrane targeted before being imported to the nucleus and nucleolus. These cells represented an opportunity to investigate



Fig. 4. Protein translocation in other cell lines and with construct variants. HeLa and NIH3T3 cells transfected with IE-LM-Venus and COS-7 cells transfected with IE-LM-mRFP and IE-LM-dsRed, before (A, D, G and J, respectively) and after (B, E, H, and K, respectively) ionomycin stimulus with ratio time courses and lines of best fit (C, F, I, and L, respectively). Error bars are the reading error. Scale bars are 10 μ m for A and B, 15 μ m for D and E and 30 μ m for G, H, J and K.



Fig. 5. Protein translocation under different Ca²⁺ concentrations. (A–D) Representative example of translocation in COS-7 cells from the plasma membrane to the nucleolus in very low (0 μ M) Ca²⁺ environment at 0, 15, 30 and 60 min, respectively, (E) the matching ratio time course. (F) Representative example of translocation in low (0.1 μ M) Ca²⁺ environment. (G) The intensity ratio of three cells over 2 h after stimulus. The curves have been fit as discussed in the text. Error bars are the reading error. Scale bars in A–D are 15 μ m.

reversibility of translocation to see if translocation could be redirected from the plasma membrane to the nucleolus. The intensity ratio time course was prepared as before and the import was modeled as an exponential increase towards an asymptote $(1 - c_0 e^{-t/\tau})$ followed by a plateau. In this experiment, since the ratio increased rather than decreased we needed to include a new parameter, c_0 , that reflected the ratio at the beginning of the experiment. We found import τ values ranged from 15.4 to 26.0 min (21.8 ± 4.8 min, n = 4) and excellent qualities of fit with r^2 ranging between 0.87 and 0.98.

For the low and moderate extracellular Ca²⁺ cases (0.1 and $1.0 \,\mu$ M, respectively) we found that the translocation behaviour appeared to be a combination of the high and very low Ca²⁺ cases: there was initially an increase in the localization ratio to a brief plateau and then a decrease towards a new steady state (Fig. 5F and G). We suspected that this was actually a sum of the two processes that we had observed previously: a relatively fast nuclear import process, with a time constant on the order of 20–25 min, and a slower nuclear export process with a time constant of approximately 2 h. Therefore, we considered these curves in two parts: the phase where the intensity ratio was increasing (nuclear import) and the phase where the intensity ratio was decreasing (nuclear export). The nuclear import phase of translocation was fit to exponential increase toward an asymptote, as modeled above, with τ values ranging from 4.1 to 14.6 min $(10.6 \pm 5.7 \text{ min}, n = 3)$ and excellent qualities of fit with r^2 ranging from 0.92 to 0.98. The nuclear export phase of translocation was fit to a decaying exponential, using the model developed above, with τ values ranging from 118 to 121 min (120 \pm 1 min, n = 3) and good qualities of fit with r^2 ranging from 0.86 to 0.92. The import and export characteristics were very consistent between cells at this low Ca²⁺ concentration.

For the moderate Ca^{2+} case, there was more variability between cells: in some cells we noticed that there was efficient nuclear export, almost comparable to the high Ca^{2+} case of the previous section, while in others there was net nuclear import similar to some of the cells under the low Ca^{2+} condition (Fig. 5G). As with the low Ca^{2+} concentration case above, we wanted to fit the apparent nuclear import and export phases of the translocation in order to quantify the rate of translocation in each case. We found that τ values for the import phase ranged from 11.5 to 30.0 min (19.8 ± 9.3 min, n=4) with r^2 ranging from 0.87 to 0.99. For the export phase, τ ranged considerably from 45 to 186 min (119 ± 59 min, n=4) with r^2 ranging from 0.89 to 0.97. Unlike the low Ca^{2+} condition, there was considerable variability in translocation rate between cells for the moderate Ca^{2+} condition.

2.4. Protein translocation can be modified by cell morphology

Specific cell morphologies can alter the translocation characteristics of our construct (Fig. 6). We wanted to investigate the effect that altered cell morphology would have on protein translocation because the morphology of the environment is known to affect protein translocation by restricting the surface area available for diffusion [32] or by creating an entropically favourable or unfavourable process [33]. We have observed in our lab that a truncated version of RhoA (tRhoA) that lacks the C-terminal prenylation site, when fused to a fluorescent protein, causes long membrane extensions in COS-7 cells (Fig. 6A). To investigate the behaviour of our translocation sensor in this environment, we co-transfected the IE-LM-Venus construct with tRhoA fused to a cyan fluorescent protein (tRhoA-CFP) [34,35].

The IE-LM-Venus construct translocated to the plasma membrane in the body of co-transfected cells at a similar rate and extent as in normally transfected cells, as is shown for a representative cell (Fig. 6). However, the translocation to the membrane of the cellular extension was generally slower and less complete than that of the cell body. There did not appear to be any correlation between the distance along the extension and the change in translocation rate with respect to the body. Rather, the narrowest part of the extension appeared to act as a partition that separated the two translocation behaviours. In this case there was a significant (p = 0.019) difference in the average τ value for the cell body of $82 \pm 8 \min (n=3 \text{ randomly selected spots on the body plasma$



Fig. 6. Protein translocation under extended cell morphology. (A and B) COS-7 cells co-transfected with tRhoA-CFP and IE-LM-Venus showing tRhoA-CFP (white arrows show the membrane extension), and IE-LM-Venus, respectively, before Ca^{2+} stimulus. (C) The same cells showing IE-LM-Venus localization after 2 h of Ca^{2+} stimulus. (D) Time course analysis of this cell showing the intensity ratio between the nucleolus and cell body plasma membrane (circles, black line) or membrane extension (squares, grey line). Error bars are the reading error. Scale bars in A–C are 30 μ m.

membrane) compared to the average τ value in the extension of $112 \pm 11 \text{ min} (n=3)$. The fits for these data were very good, with r^2 ranging from 0.89 to 0.92.

3. Discussion

We have demonstrated that a synthetic, engineered protein is capable of translocating from the nucleolus to the plasma membrane in response to a sustained Ca²⁺ stimulus. This is the only synthetic protein, to our knowledge, which translocates between such extreme boundaries of the cell, and while a few native proteins have been found in both the membrane and nucleolar compartments (such as RhoA [36] and phosphoionisitol-4 kinase [37]) it is unclear whether they actively translocate between these compartments. Such an extreme translocation event enables our construct to potentially report on multiple cellular phenomena at each stage of its translocation such as nuclear export, diffusion through the cytoplasm and membrane insertion. However, a drawback of our system is the relative slowness of translocation compared to some nuclear import and export processes (timescale of minutes rather than 1–2 h). While the use of a different NES with a faster kinetic profile, such as from HIV Rev or RanBP1 [1,11], may improve this, the current design is limited by the requirement that the NES sequence overlap with the IQ peptide sequence in such a way as to minimize the need for changes in the amino acids of either peptide. The current design may be improved upon in the future by finding a better combination of NES and IQ peptide pairs that allows for faster nuclear export while maintaining minimal changes to the peptides' primary sequences.

An unexpected outcome of these experiments was the finding that a single amino acid substitution was able to control the subcellular targeting of our protein construct. Our protein originally translocated between the nucleolus and the plasma membrane, which we determined was linked to the presence of a single cysteine residue that had likely been lipid modified at some stage of translocation. However when we performed an alanine mutation to this site we observed mitochondrial targeting, which was supported by bioinformatic analysis. While most cells showed a dual distribution between nucleolus and mitochondria, a few cells (Fig. 3G–I) showed only mitochondrial distribution. We took this to be a consequence of the mitochondrial targeting sequence outcompeting the NLS in some cells, similar to how in some cells we observed membrane targeting as well as nucleolar localization before ionomycin stimulation. This result highlights how protein localization must be tightly regulated by cells in order to preserve proper protein function. The ability to rationally target a translocating protein construct to different subcellular locations is an attractive functionality, given that spatial localization is often an important regulator of protein function. In the current design the price for dynamic movement between multiple compartments appears to be the reduced speed of overall translocation.

Our protein construct was able to monitor translocation under a variety of conditions. We found that the rate and extent of protein translocation could be affected by the intracellular Ca²⁺ concentration. For Ca²⁺ concentrations that were greater than the K_D of Ca²⁺–CaM (about 1 μ M), the protein was efficiently exported from the nucleus while for concentrations less than 1 μ M, the protein was further imported into the nucleus. We took this as an indirect test of the reversibility of our system, that the protein translocation could be directed from the plasma membrane into the nucleolus. The process of our construct being removed from the membrane is consistent with our hypothesis of palmitoylation because it is possible for proteins to be de-palmitoylated [38]. We could not test reversibility of translocation directly because of the long time constants involved.

We found a great deal of cell to cell variability in the translocation characteristics for Ca^{2+} concentrations that were approximately 1 μ M. In some cells we observed efficient export,

similar to the higher concentration condition, while in other cells we observed minimal export, similar to some of the lower concentration experiments. We suspected that since the Ca²⁺ concentration was so close to the Ca²⁺–CaM K_D that CaM was being activated unreliably between cells for the purposes of interacting with our construct.

We were also able to apply our synthetic translocation system to study the effect of cellular morphology on protein translocation in the context of translocation through a long cellular extension. We found that within the main cell body there was no correlation between translocation and the distance from the nucleolus and the plasma membrane, however, protein translocation was significantly slower to the end of a cellular extension with a narrow neck. This has implications for protein translocation in cell types with elongated morphologies such as neurons and some kind of epithelial cells. These results are in accord with our previous work which showed that the morphology of a translocation conduit between two compartments can affect the rate and extent of translocation [32].

A potential future application of our work is the creation of an inducible protein cargo shuttle. This work has shown that it is possible to control the cellular translocation of a fluorescent protein, and so it should be possible to generalize this work to other proteins of similar size. One limitation of the system as it stands currently is the slowness of translocation. However, modifications to incorporate an NES with a faster kinetic profile would allow the dynamic regulation of constitutively active proteins by shuttling them from a non-native compartment (sequestered) to a native compartment (active). In theory our design could be easily adapted to import proteins to the nucleus in response to Ca²⁺ by overlapping an NES with the MLCKp and an NLS with the IQp. This opens up the possibility of using Ca²⁺-mediated protein translocation to regulate the activity of synthetic fusion proteins for cell fate control.

4. Conclusion

We have engineered a synthetic translocation system using overlapping NLS, NES and CaM binding peptides that can be activated by a sustained Ca²⁺ signal. Ca²⁺ is an excellent stimulus because it is a robust signal with that can be delivered with a high degree of temporal resolution and can be reversible. We have shown that our system can report on differing translocation environments between cell lines, under the effects of different chemical inhibitors, with a range of intracellular Ca²⁺ concentrations, and under extreme cell morphological conditions. In particular we found that Ca^{2+} concentration ranging from 0 to 10 μ M was able to direct translocation further into or out of the nucleoli. We found that long cellular extensions acted as bottlenecks for translocation with a detectible difference in the rate of translocation on either side of the neck of the extension. Our experiments focused on Ca²⁺-activated nuclear to cytoplasmic translocation, however the generality of our design approach allows for Ca²⁺-activated cytoplasmic to nuclear translocation, or in general, translocation between any two compartments that could be regulated by intermolecular masking.

5. Materials and methods

5.1. Plasmid construction

The IE-LM gene was purchased from Invitrogen (Carlsbad, CA, USA) and inserted into pCfvtx; all subsequent cloning to replace Venus with mRFP and DsRed was done as previously described [39]. RhoA Q63L was from Addgene plasmid 12968.

5.2. Cell culture, transfection and chemicals used

All experiments were performed with COS-7 cells, except where otherwise noted. Cell lines were a generous gift from Dr. M. Ikura. Cells were cultured in a 37 °C incubator with 5% CO₂ in high glucose DMEM supplemented with 10% fetal bovine serum (Invitrogen). Cells were passaged at 90% confluency using trypsin EDTA buffer (Sigma–Aldrich, St. Louis, MO, USA). Lipofectamine 2000 reagent was used for transfections according to the manufacturer's protocol (Invitrogen). LMB (Bioshop, Burlington, ON, Canada) was used at 1 nM; 2-bromopalmitate was used at 100 μ M to 2 mM (Sigma) and CDZ was used at 10 μ M (Sigma). Cells were incubated with LMB for 30 min before ionomycin stimulation and with CDZ for 1 h before ionomycin stimulation. Ionomycin (Sigma) was used at 1 μ M and was added at time 0 for Figs. 2–6. The Ca²⁺-calibration kit was from Biotium (Hayward, CA, USA). The pKillerRed-dmito plasmid was a generous gift from Dr. G. Zheng.

5.3. Imaging and data analysis

Imaging was performed using an inverted IX81 microscope with Lambda DG4 xenon lamp source and QuantEM 512SC CCD camera with a $60 \times oil$ immersion objective (Olympus, Markham, ON, Canada). Filter excitation (EX) and emission (EM) bandpass specifications were as follows (in nm): for CFP EX: 438/24, EM: 482/32; for YFP EX: 500/24, EM: 542/27; for RFP–EX: 580/20, EM: 630/60 (Semrock, Rochester, NY, USA). Image acquisition was done using QED In Vivo and processing (image merging and intensity ratio calculations) was performed using ImagePro Analyzer (Media Cybernetics, Bethesda, MD, USA).

Fluorescence localization relative to the nucleus and nucleoli before and after Ca^{2+} stimulation was confirmed by observing the transfected cells under bright field conditions, where the nucleus and nucleoli are clearly visible at the stated magnification. There were no cases where there was disagreement between the fluorescent protein localization and the cellular structure observed under bright field. The location of the plasma membrane edge for the purpose of measuring the fluorescence intensity was also determined by overlaying the bright field image with the filtered fluorescent image. In cases where there was some ambiguity (due to overlapping or complex boundaries), the cell edge was estimated by determining the location of greatest change in fluorescence intensity occurred between the image background and cytoplasm fluorescence (refer to Fig. 2G for an intensity plot that could be used to determine this boundary).

Error is the standard deviation unless stated otherwise. Reading errors in forming the intensity ratios were due to the determining the peak values to use for the intensity of the nucleolus and plasma membrane caused by the change in fluorescence intensity over 1 pixel width. Lines of best fit were calculated by minimizing the sum of square differences between the data and a model curve using a linear regression algorithm. Significance values were calculated using the independent Student's *t*-test.

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