Optimizing the protein switch: Altering nuclear import and export signals, and ligand binding domain

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Abstract

Ligand regulated localization controllable protein constructs were optimized in this study. Several constructs were made from a classical nuclear export signal (HIV-rev, MAPKK, or progesterone receptor) in combination with a SV40 T-antigen type nuclear import signal. Different ligand binding domains (LBDs from glucocorticoid receptor or progesterone receptor) were also tested for their ability to impart control over localization of proteins. This study was designed to create constructs which are cytoplasmic in the absence of ligand and nuclear in the presence of ligand, and also to regulate the amount of protein translocating to the nucleus on ligand induction. The balance between the strengths of import and export signals was critical for overall localization of proteins. The amount of protein entering the nucleus was also affected by the dose of ligand (10–100 nM). However, the overall import characteristics were determined by the strengths of localization signals and the inherent localization properties of the LBD used. This study established that the amount of protein present in a particular compartment can be regulated by the use of localization signals of various strengths. These optimized localization controllable protein constructs can be used to correct for diseases due to aberrant localization of proteins.

Keywords: Protein switch; Controlled localization; Nuclear export signals; Nuclear import signals; Ligand binding domain; Protein mislocalization

1. Introduction

Intracellular localization of genes and gene products plays an important role in maintaining normal cellular functions. Molecules involved in regulation of the cell cycle–splicing factors, proteosomes, transcription factors, protein kinases, cell cycle inhibitors, chromatin assembly proteins, and numerous other regulatory proteins–undergo changes in intracellular localization during various phases of the cell cycle [1–6]. Studies have shown that mislocalization of these proteins and other tumor suppressors can lead to cancer. Over 10 known tumor suppressors are regulated via nucleo-cytoplasmic shuttling, including p53, p73, Beclin, BRCA1, APC, VHL, PML, Smad4, p130, and INI1/HSNF5 [7]. Malfunctioning of shuttling can cause disease; conversely, manipulation of shuttling could be utilized to treat disease. In our previous study by Kanwal et al. [8] a bi-directional protein construct called the “protein switch” was devised, responsive to an external ligand to control intracellular localization of protein. Our current study describes an improved and optimized ligand inducible protein construct which could be used to correct for mislocalization of endogenous proteins involved in certain diseases. The engineered construct containing the mislocalized protein has controlled localization, and can overcome the aberrant localization of the endogenous malfunctioning protein. Aside from the compartmentalization application presented in this research, ligand induced localization controllable protein constructs can also be used for independent regulation of genes, artificial control of transcription (via transcription factors), engineering of enzymes, gene therapy applications, and biosensor arrays. This ability to regulate and control localization of genes and gene products has the potential to make gene therapy safer and more efficient. It would also expand the range of gene therapy applications.
by allowing customized disease management and a greater control over the therapy.

In eukaryotes, DNA replication and RNA synthesis occur in the nucleus, while protein synthesis occurs in the cytoplasm [9]. All nuclear proteins, such as histones and transcription factors are synthesized in the cytoplasm and are transported back to the nucleus, whereas, transfer RNA, messenger RNA, and ribosomal RNA are transcribed in the nucleus and are transported to the cytoplasm for translation. Compartmentalization of these macromolecules allows for regulation of these cellular events [10]. Contents of the nucleus and cytoplasm are separated by a double membrane called the nuclear envelope [9,10]. Nucleo-cytoplasmic transport of proteins and other macromolecules occurs through nuclear pore complexes present in the nuclear envelope. These pores (∼9 nm diameter) allow for passive diffusion of small molecules less than 45–60 kDa in size [9,11–13]. Large macromolecules are transported by an active process utilizing specific transport signals [10] which consist of specific amino acid sequences recognized by different receptors to shuttle proteins to their active compartments.

Signal sequences that are recognized by import receptors and facilitate movement of molecules from the cytoplasm to the nucleus are called nuclear localization signals (NLS). There are three different classes of NLSs. Classical NLSs are either monopartite, single stretches of basic amino acids (PKKKRK), or bipartite (two short sequences with a spacer) basic amino acid stretches (KRPAATKKAQAAKXKLDK) [8,10,13–16]. The mono- and bipartite import signal sequences, from SV40 T-antigen and nucleoplasm in respectively, are considered to be prototypes of classical nuclear localization signals [10,14,17]. The third class of NLSs, for example, the yeast homeodomain-containing protein Mata-2, has polar residues interspersed with non-polar residues (MNKIPKDLINPQ) [13]. The sequences flanking the NLSs are often important for localization [15,16]. In addition to these three classes, tripartite signals (three short sequences with a spacer) have been identified in some steroid hormone receptors, encompassing a SV40 T-antigen like monopartite signal within the signal sequence [18]. All NLS sequences are recognized by the importin α/β heterodimer [8,10,13]. Importin α recognizes the NLS sequence and binds to it via its NLS binding site, while importin β mediates translocation of this importin α-NLS cargo complex through the nuclear pore complex to the nucleus [8–10,19]. SV40 T-antigen type classical NLSs have already been studied for their relative strengths based on their interaction with importin α [16]. These classical NLSs with varying import strengths are used in our research. There have been many studies using nuclear import signals to facilitate the movement of macromolecules into the nucleus of cells [20–22]. Others have used NLSs to facilitate import of plasmid DNA into the nucleus of cells [23,24].

Opposing nuclear import, the cell uses leucine-rich nuclear export signals (NES) that actively transport proteins from the nucleus to the cytoplasm. These sequences are recognized by the classical export receptor CRM1 (chromosome region maintenance), also called exportin 1 [10,25,26]. Classical NESs are ∼10 amino acids in length with hydrophobic residues, especially leucines. As a common consensus a NES sequence is LX (1–3) LX (2–3) LXJ where L is leucine, X is a spacer and J is either leucine, valine or isoleucine [8,27–30].

Previously we devised localization controllable protein constructs containing a nuclear export signal (NES), a nuclear import/localization signal (NLS), and a ligand binding domain (LBD) to confer responsiveness to an external ligand [8]. EGFP was used as a gene of interest to track the localization of a protein construct using fluorescence microscopy [8,30–34]. In the absence of external ligand, the protein construct was mostly present in the cytoplasm due to the dominant effect of the NES. Upon ligand addition, the protein construct translocated to the nucleus via the LBD and the NLS. It was shown that the rate and amount of protein translocating to the nucleus correlated with the dose of ligand [8]. The purpose of our current work was to optimize the protein switch to make it more cytoplasmic in the “off” state (unliganded) and more nuclear in the “on” state (ligand). The utility of the protein switch constructs lies in the ability to keep the attached therapeutic protein in one cellular compartment where it is inactive. Upon addition of ligand, the therapeutic protein can move to another compartment where it has activity. The optimization process leads to improved control of protein location, and better control of protein function. The effect of the critical balance between NES and NLS strengths on the localization of constructs was tested. Based on our previously published observation indicating that NLSi is a weak import signal [35], the NLSi used in our prototype constructs [8] was replaced with stronger classical NLSs. Various NESs were also tested in combination with NLSs. Ligand binding domains from progesterone receptor (PR) and glucocorticoid receptor (GR) were also tested to make protein constructs responsive to different ligands.

2. Materials and methods

Protein constructs were made by cloning a nuclear export signal (NES), a nuclear localization/import signal (NLS), and a ligand binding domain (LBD) in pEGFP-C1 mammalian expression vector. Classical NESs, HIV-rev protein, MAPKK and PRNES2 were used to make constructs [8,10]. These were used in various combinations with classical SV40 T-antigen type NLSs of diverse strengths [16]. Two different ligand binding domains—a truncated version of wtPR LBD and a GR LBD—were used to regulate localization with an external ligand. Mifepristone was used to regulate protein constructs with PR LBD, and dexmethasone was used for constructs containing GR LBD [8,36,37].

2.1. Cell line and culture conditions

The murine adenocarcinoma cell line 1471.1 (which does not express endogenous progesterone receptor) was used in this research. Cells were grown as monolayers in 175 cm² flasks
containing DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin (100 U/ml, GIBCO BRL), 0.1% gentamicin (0.5 mg/ml, Hyclone), and 1% l-glutamine (2 nM, Hyclone). Cells were maintained in a 5% CO₂ incubator at 37 °C.

2.2. Transient transfections

Transient transfections of 1471.1 cells were carried out by electroporation as previously defined [8,30]. Transfected cells were plated on a clear cover glass (Corning no. 1, 22 mm²) in six well plates or plated into live cell chambers (Lab-tek chamber slide system, 2 ml, Nalge NUNC International, Naperville, IL) containing complete phenol red-free DMEM with 10% FBS (charcoal/dextran treated, Hyclone), 1% penicillin-streptomycin (100 U/ml, GIBCO BRL), 0.1% gentamicin (0.5 mg/ml, Hyclone), and 1% l-glutamine (2 nM, Hyclone), and maintained in a 5% CO₂ incubator at 37 °C for 18–24 h.

2.3. Microscopy

Approximately 18–24 h after electroporation, localization of protein constructs was viewed by fluorescence microscopy [8]. An Olympus IX701F inverted fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quantity narrow band GFP filter (excitation HQ480/20 nm, emission HQ510/20 nm, with beam splitter Q4951p) from Chroma Technology (Brattleboro, VT) was used. Cells were photographed at a magnification of 40× using an F-view monochrome CCD camera. To minimize photobleaching of EGFP chromophore, cells were imaged using neutral density filters at short (500 ms) exposure times. An air stream incubator (Nevtek ASI 400, Burnsville, VA) with temperature control was used to maintain the microscope stage at 37 °C.

2.4. Construction of plasmids

Different plasmid constructs were created by changing the sequence of NES, NLS, and LBD, which hereafter follow the pattern NES-NLS-LBD. The plasmids HIV-NLSi-PRLBD and MAPKK-NLSi-PRLBD, from our previous study [8], were used for construction of new plasmids.

HIV-SV40-PRLBD and MAPKK-SV40-PRLBD were constructed by removing NLSi using restriction enzymes EcoRI and SalI and inserting it in HIV-NLSi-PRLBD already digested with EcoRI and SalI (to remove NLSi).

HIV-SV40A1-PRLBD and HIV-SV40A7-PRLBD were constructed by removing NLSi using EcoRI and SalI from HIV-NLSi-PRLBD and replacing it with oligonucleotide 5′-AATTCATGCCCCAAAAGAAAAGAAGAAAGTTGCAAG-3′ and its complementary strand which encodes SV40A1, truncated version of classical NLS from SV40 T-antigen protein, and oligonucleotide 5′-AATTCATGCCCCAAAAGAAAAGAAGAAAGTTGCAAG-3′ and its complementary strand which encodes SV40A7, a truncated version of NLS from SV40 T-antigen protein, respectively [16] (see Table 1 for NLS amino acid sequence).

MAPKK-CDC-PRLBD, MAPKK-PKC-PRLBD and MAPKK-SV40A4-PRLBD were constructed by removing NLSi using EcoRI and SalI from MAPKK-NLSi-PRLBD and replacing it with oligonucleotides and their complementary strands encoding NLSs for the respective plasmids. Oligonucleotide 5′-AATTCATGCCCCAAAAGAAAAGAAGAAAGTTGCAAG-3′ and its complementary strand which encodes the classical NLS from CDC2 protein (see Table 1 for NLS amino acid sequence).

MAPKK-Myca8-PRLBD was constructed by removing NLSi out of MAPKK-NLSi-PRLBD using restriction enzymes EcoRI and SalI and replacing it with oligonucleotide 5′-AATTCATGCCCCAAAAGAAAAGAAGAAAGTTGCAAG-3′ and its complementary strand which encodes the classical type NLS sequence from Myca8 protein (see Table 1 for NLS amino acid sequence).

Table 1

<table>
<thead>
<tr>
<th>Type of signal/ domain</th>
<th>Amino acid sequence (or numbering)</th>
<th>Strength of signal (Kd nM [16], if known)</th>
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<tbody>
<tr>
<td>Nuclear export signals (NES)</td>
<td></td>
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<tr>
<td>MAPKK</td>
<td>LQKLEELEL [28]</td>
<td>Strong</td>
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<tr>
<td>HIV</td>
<td>LQPPLERLTL [28]</td>
<td>Medium</td>
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<tr>
<td>PRB NES2</td>
<td>LHDLVKQLHH [30]</td>
<td>n/a</td>
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<tr>
<td>Nuclear import signals (NLS)</td>
<td></td>
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</tr>
<tr>
<td>SV40</td>
<td>SPKKRKKEVE [16]</td>
<td>9±4 strong</td>
</tr>
<tr>
<td>SV40A1</td>
<td>SAKKKRKEVE [16]</td>
<td>36±2 medium</td>
</tr>
<tr>
<td>SV40A7</td>
<td>SPKKKKKEAE [16]</td>
<td>53±4 medium</td>
</tr>
<tr>
<td>MycA8</td>
<td>PAAKVKADE [16]</td>
<td>85±7 medium</td>
</tr>
<tr>
<td>SV40A4</td>
<td>SPKKARKKEVE [16]</td>
<td>335±7 weak</td>
</tr>
<tr>
<td>CDC2</td>
<td>GVVKJGIRKTGG [41]</td>
<td>n/a weak</td>
</tr>
<tr>
<td>PKC</td>
<td>FVVHHRKHE [41]</td>
<td>n/a weak</td>
</tr>
<tr>
<td>NLSi</td>
<td>RAMEQHNYLGACRNDICVSDKIKR- RKNCPCACRLRKCQAGVMVGG</td>
<td>n/a weak</td>
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</table>

HIV-Myca8-PRLBD was constructed by removing Myca8 NLS from MAPKK-Myca8-PRLBD using EcoRI and SalI and inserting it in HIV-NLSi-PRLBD already digested with EcoRI and SalI (to remove NLSi).

PRNES2-SV40-PRLBD and PRNES2-Myca8-PRLBD were constructed by replacing MAPKK NLS from MAPKK- SV40-PRLBD and MAPKK-Myca8-PRLBD using restriction enzymes BspEI and Xhol by the oligonucleotide 5′-
CGGGACTACAGCTTCTCTGATGAAACAG-3’ and its complementary strand which encodes the classical consensus NES present in progesterone receptor (PRNES2) [8] (see Table 1 for NES amino acid sequence).

HIV-MycA8-MycA8-PRLBD was constructed by inserting the oligonucleotide 5′-TCGGAGACCAGCAGCAGAAAAAGATAAAACGAGCAGGAGGG-3’ and its complementary strand which encodes the classical type NLS from MycA8 protein into the construct HIV-MycA8-PRLBD previously digested with XhoI and EcoRI.

HIV-HIV-MycA8-MycA8-PRLBD was constructed by replacing HIV NES from HIV-MycA8-MycA8-PRLBD with the oligonucleotide 5′-CCGGACTCAACTGCTCCCTGTGAGCCCTAACTTGGGAGCACTCCAACTGCTCCCTTGAGGCAGCTAACTTGGTC-3′ and its complementary strand which encodes the two repeats of the classical consensus NES present in progesterone receptor (PRNES2) [8] (see Table 1 for NES amino acid sequence).

2.5. Protein translocation studies

All protein constructs were induced with ligand 24 h after transfection. Mifepristone and dexamethasone were used as ligands for constructs with PR and GR LBD respectively. Based on our previous studies showing maximum import occurring within one hour of ligand induction [14], constructs were induced with 10 nM dose of ligand for 1 h. Pictures of live cells were taken using a fluorescence microscope after one hour. As controls, cells were also photographed without inducing with ligand.

2.6. Protein import studies

As previously, import kinetics of protein constructs was studied by conducting time lapse experiments at three different concentrations: 1 nM, 10 nM and 100 nM. Cells (2.5 ml) were plated in live cell chambers after transfection by electroporation [8]. After 18–24 h of transfection, media was removed, cells were washed three times with PBS without calcium, and 2.5 ml of fresh media were added. Cells were incubated at 37 °C for 45 min prior to induction. Pictures were taken without ligand, and also with ligand induction, at time 0, 2, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min.

2.7. Quantitation of protein translocation

Fluorescence microscopy was used to study and quantify the amount of protein present in a cellular compartment as done previously by us and others [8,31,39,40]. Fluorescence intensity of a particular compartment relates to the amount of protein present in that compartment; therefore, quantitation of protein in the nucleus and cytoplasm was carried out by measuring the fluorescence intensity of EGFP, tagged to the protein construct, as previously described [8,30]. All the images were analyzed using analySIS® software (Soft Imaging System, Lakewood, CO). The nuclear and cytoplasmic intensity values were divided by the area values of the nucleus and cytoplasm to obtain average nuclear and cytoplasmic intensities, respectively, to normalize for differences in cell shape and size. Relative nuclear intensity was calculated by dividing the average nuclear intensity with average cytoplasmic intensity. Percentage nuclear increase (Fig. 3A), the increase in the nuclear intensity (which indicates the increase in the total amount of protein present in the nucleus) on ligand induction, and change in relative nuclear intensity (Fig. 3B) on ligand induction, were calculated as below to compare different versions of protein constructs.

\[
\text{Average Nuclear Intensity} = \left( \frac{\text{NI}}{\text{NA}} \right)
\]

\[
\text{Average Cytoplasmic Intensity} = \left( \frac{\text{CI}}{\text{CA}} \right)
\]

\[
\text{Percentage Nuclear} = \left( \frac{\text{ANI}}{\text{ANI} + \text{ACI}} \right) 	imes 100\%
\]

\[
\text{Relative Nuclear Intensity} = \left( \frac{\text{ANI}}{\text{ACI}} \right)
\]

\[
\text{Relative Nuclear Intensity Change (percentage)} = \left( \frac{\text{RNI} - \text{RNIo}}{\text{RNIo}} \right) \times 100\%
\]

Where NI=nuclear intensity, NA=area of nucleus, CI=cytoplasmic intensity, CA=area of cytoplasm, ANI=average nuclear intensity, ACI=average cytoplasmic intensity, PN=percentage of total protein present in the nucleus 1 h. after ligand induction, PNo=percentage of total protein present in the nucleus without ligand induction, RNI=relative nuclear intensity 1 h. after ligand induction, RNIo=relative nuclear intensity without ligand induction.

2.8. Curve fitting and statistical analysis

Ligand induction and import studies for all constructs were repeated in triplicate (n=3), and 10 cells representative of the whole population were analyzed in each experiment. Statistical differences between the relative nuclear intensity values were resolved using unpaired t-test with Welch’s correction. One way ANOVA with Tukey’s Multiple Comparison Post test was used to assess differences between RNI and PNI values for all constructs. The relationship between time and relative nuclear intensity for each tested dose was fitted to a monoexponential function.
function using non-linear regression (GraphPad Prism, San Diego, CA) using the following equation:

\[ Y = Y_0 + Y_{\text{max}} \times (1 - \exp (-K^*t)) \]

Where \( Y \) = relative nuclear intensity, \( t \) = time, \( Y_0 \) = relative nuclear intensity in the absence of ligand, \( Y_{\text{max}} \) = relative nuclear intensity in the presence of ligand at steady state, \( K^* \) = rate of nuclear import. Differences in the rate of nuclear import (\( K^* \)) and relative nuclear intensity in the presence of ligand at steady state (\( Y_{\text{max}} \)) were also compared between constructs using one way ANOVA with Tukey’s multiple comparison. A \( p \) value < 0.05 was considered significant.

3. Results and discussion

In our previous study [8], we established that intracellular localization of an exogenous protein can be controlled by using a protein construct containing NES, NLS and LBD. This construct is present in the cytoplasm in the absence of ligand and translocates to the nucleus on addition of ligand. In this study we aimed to rationally design constructs which were more cytoplasmic in the absence of ligand and more nuclear in the presence of ligand compared to the prototype devised in our previous study. We demonstrated that the amount of protein present in a particular compartment can be regulated by using NES and NLS of different strengths and in different combinations. It was also seen that localization controllable protein constructs can be made responsive to more than one combination. It was also seen that localization controllable protein in the nucleus on ligand induction is affected by the amount of protein present in the nucleus before ligand induction. Accordingly, the percentage increase in protein present in the nucleus on ligand induction (Fig. 2, 1st pair of columns) due to the strong SV40 NLS compared to the weak NES of MycA8 [16]. The difference between HIV-SV40-PRLBD and HIV-NLSi-PRLBD was statistically significant. On ligand induction, the nuclear intensity in the absence of ligand was more for HIV-SV40-PRLBD (Fig. 2 column 2, 1st pair of columns) than HIV-NLSi-PRLBD (Fig. 2, 2nd pair of columns), due to the stronger binding with importin \( \alpha \) and hence greater nuclear import [16]. Though NLSi does not have a published value for its interaction with importin \( \alpha \), it has been reported to have very weak import activity [35]. NLSi has not been directly tested in comparison to SV40 NLS or MycA8 NLS, though. From relative nuclear intensity values in Fig. 2, it is observed that the amount of protein present in the nucleus even without ligand induction was more for HIV-SV40-PRLBD (3rd pair of columns) than HIV-NLSi-PRLBD (1st pair of columns), due to the strong SV40 NLS. The other hand, HIV-MycA8-PRLBD constructs had the least amount of protein in the nucleus in the absence of ligand, compared to the other two constructs (Fig. 2, 2nd pair of columns), because of relatively weaker nuclear import activity of MycA8 NLS versus SV40 NLS [16]. The difference between HIV-MycA8-PRLBD and HIV-NLSi-PRLBD in the absence of ligand was not statistically significant. On ligand induction, the relative nuclear intensity for HIV-MycA8-PRLBD (Fig. 2, 1st pair of columns) protein construct was also less than HIV-SV40-PRLBD (Fig. 2 column 3, 3rd pair of columns) due to the lower strength of MycA8 NLS.

The nuclear intensity, and hence the amount of protein present in the nucleus on ligand induction is affected by the amount of protein present in the nucleus before ligand induction. Accordingly, the percentage increase in protein present in the nucleus on ligand induction for HIV-SV40-PRLBD (column 3) while a 44% increase for HIV-MycA8-PRLBD (column 2). The change in relative nuclear intensity values was 140% and 109% for these two constructs respectively (Fig. 3B, column 2 vs column 3). HIV-SV40A7-PRLBD constructs tested did not show a significant change in localization on ligand induction (data not shown).

3.1. Changing the strength of NLS regulates the amount of protein translocated to the nucleus

Since nuclear localization signals vary in strength, the effect of changing the NLS sequence in the presence and absence of ligand was studied [16]. Starting from one of the original plasmids, HIV-NLSi-PRLBD [8], other constructs were cloned containing constant NES and LBD sequences and substituting NLSs of different strengths (one “medium” strength NLS from MycA8 [16], and a “strong” NLS from SV40 [16] and others [16,41]; see Table 2). Percentage nuclear increase and change in relative nuclear intensity values were calculated (as described in Materials and methods) to compare different protein constructs. As shown in Fig. 2, HIV-SV40-PRLBD (3rd pair of columns) had the maximum relative nuclear intensity on ligand induction with 10 nM mifepristone, and HIV-NLSi-PRLBD (1st pair of columns) the least. It was also observed that the difference in relative nuclear intensities in the presence and absence of ligand was greater for HIV-SV40-PRLBD than HIV-NLSi-PRLBD. The difference in relative nuclear intensity values for these constructs was presumably due to the variation in strengths of NLSs used. Studies have shown that SV40 NLS is one of strongest NLSs known, as suggested by its low Kd (dissociation) value for interaction with importin \( \alpha \) [16]. The lower the Kd value of a NLS, the stronger the binding with importin \( \alpha \) and hence greater nuclear import [16]. Though NLSi does not have a published value for its interaction with importin \( \alpha \), it has been reported to have very weak import activity [35]. NLSi has not been directly tested in comparison to SV40 NLS or MycA8 NLS, though. From relative nuclear intensity values in Fig. 2, it is observed that the amount of protein present in the nucleus even without ligand induction was more for HIV-SV40-PRLBD (3rd pair of columns) than HIV-NLSi-PRLBD (1st pair of columns), due to the strong SV40 NLS. The other hand, HIV-MycA8-PRLBD constructs had the least amount of protein in the nucleus in the absence of ligand, compared to the other two constructs (Fig. 2, 2nd pair of columns), because of relatively weaker nuclear import activity of MycA8 NLS versus SV40 NLS [16]. The difference between HIV-MycA8-PRLBD and HIV-NLSi-PRLBD in the absence of ligand was not statistically significant. On ligand induction, the relative nuclear intensity for HIV-MycA8-PRLBD (Fig. 2, 1st pair of columns) protein construct was also less than HIV-SV40-PRLBD (Fig. 2 column 3, 3rd pair of columns) due to the lower strength of MycA8 NLS.

The nuclear intensity, and hence the amount of protein present in the nucleus on ligand induction affected by the amount of protein present in the nucleus before ligand induction. According to the percentage nuclear increase (PNI, Fig. 3A) and the change in relative nuclear intensity (RNI, Fig. 3B), on ligand induction, were calculated to determine the best protein construct (the least amount of protein in the nucleus coupled with the highest protein translocation to the nucleus on ligand induction). It can be seen from Fig. 3A that there was a 39% increase in amount of protein present in the nucleus on ligand induction for HIV-SV40-PRLBD (column 3) while a 44% increase for HIV-MycA8-PRLBD (column 2). The change in relative nuclear intensity values was 140% and 109% for these two constructs respectively (Fig. 3B, column 3 and 2). The change in PNI and RNI values for HIV-NLSi-PRLBD were only 17% (Fig. 3A, column 1) and 38% (Fig. 3B, column 1), respectively. Both new constructs, HIV-MycA8-PRLBD (Fig. 3A column 2) and HIV-SV40-PRLBD (Fig. 3A column 3), showed significant improvement (\( p<0.05 \)) in the amount of protein present in the nucleus on ligand induction over HIV-NLSi-PRLBD (Fig. 3A column 1); however, HIV-MycA8-PRLBD and HIV-SV40- PRLBD were not significantly different from each other (compare Fig. 3A column 2 vs column 3). HIV-SV40A1-PRLBD and HIV-SV40A7-PRLBD constructs tested did not show a significant change in localization on ligand induction (data not shown).
These results suggest that changing the strength of NLS in the protein construct considerably changes the amount of protein in the nucleus in the presence and absence of ligand.

3.2. Changing the strength of the NES affects the localization of protein constructs

To investigate the affect of the NES on the localization of protein constructs, the NES in the prototype HIV-NLSi-PRLBD version was changed. In one set, HIV NES was replaced by NES from MAPKK protein to make MAPKK-NLSi-PRLBD, MAPKK-MycA8-PRLBD and MAPKK-SV40-PRLBD [8] (Table 2). In another set of constructs, PRNES2 [14] was used to make PRNES2-MycA8-PRLBD and PRNES2-SV40-PRLBD (Table 2). As seen in Fig. 1 and Fig. 2 (columns 6 and 4) only the MAPKK-MycA8-PRLBD and PRNES2-MycA8-PRLBD constructs showed a significant change ($p<0.05$) in the localization of protein from the cytoplasm to nucleus on ligand induction. The change in relative nuclear intensity for MAPKK-NLSi-PRLBD, MAPKK-MycA8-PRLBD and MAPKK-SV40-PRLBD was 16%, 36% and 19%, respectively (Fig. 3B, columns 5, 6 and 7), compared to over 100% change with the HIV NES constructs (Fig. 3B, columns 2 and 3). The percentage nuclear increase for these MAPKK constructs was also low, at 8%, 17% and 8% respectively (Fig. 3A, columns 5, 6 and 7). As mentioned above, of the three constructs containing MAPKK NES, only MAPKK-
MycA8-PRLBD showed a significant difference in the amount of protein in the presence and absence of ligand; however, the magnitude of the change in PNI and RNI was low. This is observed because MAPKK is a much stronger NES than HIV, and due to its greater export strength it presumably prevents movement of protein to the nucleus from the cytoplasm on ligand induction. Other MAPKK versions of the protein construct with weaker NLSs—MAPKK-CDC-PRLBD, MAPKK-PKC-PRLBD and MAPKK-SV40A4-PRLBD—also did not show significant change in nuclear import on ligand induction (our unpublished data).

The PRNES2-MycA8-PRLBD construct showed a significant change in localization on ligand induction, except with a different extent of localization, presumably since PRNES2 is a weaker NES than MAPKK NES. There was a 40% increase in the amount of protein in the nucleus (Fig. 3A, column 4) and a relative nuclear intensity change of 120% (Fig. 3B, column 4) on ligand induction. There was no significant difference observed between this construct (PRNES2-MycA8-PRLBD) and the construct containing the HIV NES (HIVNES-MycA8-PRLBD) (Fig. 3A and B, columns 4 and 2), presumably due to comparative strengths of PRNES2 and HIV NES [8]. The PRNES2-SV40-PRLBD construct did not induce well and show any change in localization (our unpublished observation). Since PRNES2-SV40-PRLBD is already quite nuclear to begin with, the import machinery may already be saturated, even without ligand. Adding ligand does not cause more to go into the nucleus. In this particular construct, the balance between the NES and the NLS is tipped to the NLS in the unliganded state, so import does not occur with ligand.

Overall, these findings indicate that the strength of NES impacts the effectiveness of protein constructs by influencing the initial localization of the construct in the absence of ligand, and the overall balance of NES-NLS strengths [8].

### 3.3. Protein constructs can be made responsive to other ligands

The ligand binding domain provides control over the localization. Protein constructs described above contain a truncated version of PR LBD not responsive to endogenous PR, but responsive to antagonist mifepristone [8,42]. To confer responsiveness to a different ligand, protein constructs were created using the glucocorticoid receptor LBD (C656G GRLBD). These constructs were designed to be responsive to dexamethasone (dex) [36]. GR LBD with a point mutation C656G was used in these constructs because this mutated GR LBD is 9-fold more sensitive to dex [36,38]. The benefit of this particular system is that low doses of dex can be used to cause nuclear induction, and these sub-nanomolar levels of dex will not activate endogenous GRs [38]. Constructs containing C656G GR LBD, HIV-MycA8-GRLBD and HIV-SV40-GRLBD, showed change in localization on ligand induction similar to constructs containing PR LBD (Fig. 1). Upon inducing the constructs with 10 nM dexamethasone, as observed in Fig. 2, pairs of columns 8 and 9, there was a significant difference ($p<0.05$) in relative nuclear intensity values compared to uninduced. The increase in amount of protein present in the nucleus was 33% for HIV-MycA8-GRLBD constructs (Fig. 3A, column 8) and 35% for HIV-SV40-GRLBD constructs (Fig. 3A, column 9). The change in relative nuclear intensity values was 90% and 91%, respectively, for these two constructs (Fig. 3B, columns 8 and 9). To conclude, protein constructs for controlled intracellular localization of proteins can be customized with different ligand

### Table 2

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<tr>
<th>NES</th>
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<tr>
<td>HIV</td>
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Fig. 2. Change in relative nuclear intensity of protein constructs on induction with 10 nM ligand for 1 h. This graph depicts the change in nuclear intensity of protein constructs on induction with 10 nM dose of ligand for 1 h. Relative nuclear intensity for each construct was calculated by dividing the average nuclear intensity with average cytoplasmic intensity. A significant difference ($p<0.05$) was observed in relative nuclear intensity values before and after ligand induction for most of the constructs. Mifepristone was used as a ligand for constructs with PR LBD and dexamethasone for constructs with GR LBD. (* significant difference between drug and no drug, $p<0.05$).
binding domains to confer responsiveness to a range of ligands. Responsiveness of protein switch to different ligands will provide greater flexibility and ease of therapy by utilizing already available and tested non-controversial and non-toxic ligands.

3.4. Import of protein constructs depends on NES, NLS, LBD and dose of ligand

To further study the affect of the balance between NES and NLS on protein localization, import characteristics of some of the protein constructs were studied at different ligand doses. Import of HIV-SV40-PRLBD, HIV-MycA8-PRLBD, PRNES2-MycA8-PRLBD, HIV-SV40-GRLBD, and HIV-MycA8-GRLBD was studied over a period of 2 h at three different ligand doses (1 nM, 10 nM, and 100 nM). Of all the constructs made in this study, the above mentioned five were chosen to study nuclear import characteristics because of their greater percentage nuclear increase on ligand induction (Fig. 3). Import was studied for 2 h because it was determined in our previous study [8] that no change in import is observed after 2 h. Mifepristone and dexamethasone were used as ligands for protein constructs with PR and GR LBD, respectively. Import studies were carried out by using live cell fluorescence microscopy and photography of cells at time points 0, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min. A representative set of pictures for import studies over 1 h at 10 nM mifepristone dose for HIV-SV40-PRLBD and HIV-MycA8-PRLBD is shown in Fig. 4. All the pictures were analyzed to calculate relative nuclear intensity (described in Materials and methods). To study the import kinetics of protein constructs, relative nuclear intensity was plotted as a function of time (Fig. 5). The rate and extent of nuclear import for all the constructs depend on dose of the ligand as well as the NES and NLS used. As seen in Fig. 5 and Table 3, the steepness of import curves relates to the rate of import. Import rate constant of all the tested constructs, except HIV-SV40-PRLBD, showed dose-dependence. A significant increase (p<0.05) in import rate constant was observed from 1 nM to 10 nM and 1 nM to 100 nM for both HIV-MycA8-PRLBD and PRNES2-MycA8-PRLBD (Table 3). There was no significant difference observed at the higher doses (between 10 nM and 100 nM) due to saturation of nuclear import (Table 3). The PR LBD used in these constructs is taken from the B isoform of the PR receptor (PRB). In our earlier studies we have shown that the PR LBD alone localizes both in the cytoplasm and the nucleus in the absence of ligand [43] and the import of PRB gets saturated around a 10 nM dose of the ligand [31,35]. Analogously, the significant difference in import rate constants (Table 3) of the protein constructs that is observed may be due to

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**Fig. 3.** Comparison of various protein constructs based on ligand induction studies. A) Percentage increase in the amount of protein in the nucleus on ligand induction for each construct is shown in this graph. The amount of protein in the nucleus was calculated in the presence and absence of ligand and the percentage increase value was calculated. A 10 nM dose of mifepristone and dexamethasone were used as ligand for protein constructs with PR and GR LBD, respectively. B) Change in relative nuclear intensity on ligand induction for protein constructs is depicted in this graph for different protein constructs. Relative nuclear intensity was calculated in the presence and absence of ligand, and percentage change was calculated. A 10 nM dose of mifepristone and dexamethasone were used as ligand for protein constructs with PR and GR LBD, respectively.
faster translocation of PR LBD containing constructs to the nucleus at lower doses of the ligand, and no significant difference is observed at higher doses (10 nM and 100 nM) due to saturation of nuclear import. It was observed that import rate constants of HIV-SV40-PRLBD construct did not show any dose-dependent response presumably due to a much higher strength of SV40 NLS coupled with inherent localization and nuclear import properties of PR LBD.

Constructs containing GR LBD (HIV-MycA8-GRLBD and HIV-SV40-GRLBD) showed a significant change in import rate constant between 1 nM and 100 nM, and 10 nM and 100 nM, but not between 1 and 10 nM (Table 3). The GR LBD used in these constructs was obtained from C656G mutant version of GR which shows dose-dependent nuclear import [38,44]. Consistent with previous studies, the GR LBD used in these constructs imparts dose-dependent nuclear import characteristics. However, a significant difference was not observed in the import rate constants at 1 nM and 10 nM doses due to greater variance within the data at 1 nM (large standard error and noise; data not shown). A dramatic change in nuclear intensity was not observed at 1 nM for these constructs due to poor signal strength. The import kinetic studies suggest that the rate of nuclear import on ligand induction depends on the dose of the ligand used, and is also influenced by the NES-NLS balance as well as the inherent localization characteristics of the LBD used.

3.5. Multiple NLS and NES render the protein construct unresponsive to external ligand

In order to study the effect of multiple localization signals on nuclear import of protein constructs a second MycA8 NLS was added to HIV-MycA8-PRLBD, resulting in HIV-MycA8-MycA8-PRLBD. It was observed that this construct with two NLSs was mostly nuclear even in the absence of ligand, and localization was not altered by addition of ligand (Fig. 6). Addition of another export signal (HIV-HIV-MycA8-MycA8-PRLBD) also did not alter the nuclear localization of the constructs. No significant difference was observed in the amount of protein present in the nucleus in the presence and absence of ligand for these constructs with multiple localization signals.
To summarize, various protein constructs were made by using classical NESs—HIV, MAPKK and PRNES2—in combination with SV40 T-antigen type NLSs of various strengths (Table 2). Most of the constructs tested showed a significant change in localization on ligand induction. All of the new constructs reported in this study showed a significant improvement in control of localization over our previous prototype constructs. New constructs containing stronger NLSs than the one used in our previous study (NLSi) showed a much greater increase in the amount of protein in the nucleus on ligand induction, signifying that the strength of NLS used influences the overall translocation of the construct, shifting the localization of constructs to the nucleus. It was seen that stronger NLSs, due to their greater interaction with import receptors [16], tend to shift the protein to the nucleus even in the absence of ligand. Similarly, the strength of NES also influences the localization of protein constructs, however, in the opposite direction. In all the constructs used in this study containing MAPKK NES, addition of ligand did not cause a significant change in localization from the cytoplasm to the nucleus. This indicated that a strong NES, such as MAPKK, tends to keep the constructs mostly in the cytoplasm in the absence of ligand. We also observed that addition of multiple repeats of localization signals tends to shift the balance of localization towards one compartment to a

Fig. 5. Import studies for various protein constructs at different ligand doses. These graphs depict the change in relative nuclear intensity with time after inducing the protein switch with 1, 10 and 100 nM ligand. The import was studied for 2 h. Data is reported as mean±S.D., n=3 with 10 cells analyzed in each experiment. The steepness of the curves suggests that there were differences in the import rate constant for different constructs at different doses. See Table 3 for rate constants.
dexamethasone were used as ligand for protein constructs with PR and GR LBD, PRLBD, and at higher dose for constructs with GRLBD. Mifepristone and (p except HIV-SV40-PRLBD showed dose-dependence. A significant difference characteristics of protein constructs studied for import. All the protein constructs three different doses. These values give a comparative analysis of import
Import rate constant values were calculated for different protein constructs at

greater degree, making it unresponsive to the ligand. The construct containing “medium” strength HIV NES, “medium” strength MycA8 NLS and PR LBD showed most favorable results with the least amount of protein present in the nucleus in the absence of ligand, and the greatest translocation to the nucleus with ligand. Hence, ultimately it is the balance of NES and NLS strengths that determines the overall localization of protein constructs in the presence and absence of ligand. This balance can be manipulated to control the amount of protein translocation to a compartment. It was also observed that the rate of nuclear import of the protein constructs depends on the dose of the ligand used, as well as the inherent import characteristics of the LBD used. These protein constructs can however be made responsive to more than one ligand. In our study, we successfully tested two sets of constructs containing truncated PR LBD and mutated C656G GR LBD, responsive to mifepristone and dexamethasone, respectively. The ability to substitute LBDs to make localization controllable protein constructs provides the advantage of using various non-toxic, previously tested ligands, thus allowing creation of customized protein constructs.

Intracellular transport and localization of proteins is significant for normal functioning of cells, and any deviation from this highly regulated transport system results in diseases which may range from metabolic disorder to cancer [3,7,45–48]. The constructs described here exploit nuclear localization and nuclear export signals to optimize a system for controlling intracellular localization of proteins. Control over intracellular protein localization may render a protein inactive in one compartment and active in another; thus manipulating the localization of a protein using an external ligand would provide more control over the therapy. An endogenous gene, with native localization signals intact, may still not localize to its correct compartment in the cell on transfection, due to malfunctioning of transport, mutations or other cellular events that are difficult to control [8]. However, attaching the gene with localization controlled protein constructs would render the ability to target the gene (and hence protein) to its desired compartment with greater control and assurance. Changing the localization of a protein by utilizing signal sequences to treat diseases is an advancement over current gene therapy. Many diseases are caused due to mislocalization of proteins. Various types of cancers, ranging from breast cancer to certain leukemias involve mislocalization of proteins [49]. Most of these cancers involve mislocalization between the nucleus and the cytoplasm. In our recent review we stressed the need of exploring modes for controlling intracellular localization of proteins [49]. Localization controllable protein constructs with a range of NES-NLS combinations studied in this paper is one such approach, exploiting intracellular localization for treatment of diseases. A protein which is mislocalized in a disease state can be directed to its “normal” compartment by using localization controllable protein constructs. These constructs can also be used in states where there is inadequate production of a certain protein by using a construct with appropriate NES-NLS balance to regulate the amount of protein translocating to a compartment.

### 4. Conclusions

The optimization of localization controllable protein constructs was carried out in this study. Classical NESs from HIV-rev, PR and MAPKK proteins were used in combination with SV40 T-antigen NLSs of various strengths, along with a LBD to make the constructs. The overall localization of the constructs was influenced by the individual strengths of the localization signals used, as well as the critical balance between the NES-NLS strengths. A strong NES, such as MAPKK, tends to keep the constructs cytoplasmic and did not show any significant change in localization with ligand. On the other hand, a strong localization signal such as SV40 NLS, caused translocation of the protein constructs to the nucleus even in the absence of ligand. Using multiple repeats of localization signals also tend to shift localization of the constructs towards one compartment. Thus, the NES-NLS used in a protein construct can be manipulated to regulate the amount of protein translocating to a compartment.

![HIV-MycA8-MycA8-PRLBD (no ligand)](image1)

![HIV-MycA8-MycA8-PRLBD (10nM MFP)](image2)

![HIV-HIV-MycA8-MycA8-PRLBD (no ligand)](image3)

![HIV-HIV-MycA8-MycA8-PRLBD (10nM MFP)](image4)

**Fig. 6.** Effect of multiple signals on localization of constructs. The addition of two NLSs made the constructs completely nuclear even in the absence of ligand. No change in localization was observed on adding a second NES to these constructs. Significant difference was not observed in localization of these constructs in the presence and absence of ligand.
compartment. In this study, protein constructs were made by using two different LBDs responsive to their respective ligand providing a means of making customized constructs. The optimized versions of localization controllable constructs also showed a significant change in import rate at various ligand doses. It was observed that the change in rate of nuclear import was affected by the type of LBD used. The optimized localized protein constructs discussed in this study provide a means for regulating the amount of protein present in a compartment, and protein translocation across various intracellular compartments, and pave the way for treating diseases which are caused due to mislocalization of proteins.

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References


