Published in final edited form as:

J Control Release. 2007 July 31; 120(3): 220–232.

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

Mudit Kakar¹, James R. Davis¹, Steve E. Kern^{1,2}, and Carol S. Lim^{1,*}

¹*Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 421 Wakara Way # 318, Salt Lake City, UT 84108, USA*

²*Department of Anesthesiology, University of Utah, Salt Lake City UT 84132, USA*

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 (10-100nM). 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

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, proteasomes, 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;

*Corresponding author. Tel: 801-587-9711 Fax: 801-585-3614, E-mail address: carol.lim@pharm.utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

conversely, manipulation of shuttling could be utilized to treat disease. In our previous study (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 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 (diameter ~9nm) 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 (KRPAATKKAGQAKKKKLDK) [8,10,13-16]. The mono- and bipartite import signal sequences, from SV40 T-antigen and nucleoplasmin 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 Mat α -2, has polar residues interspersed with non-polar residues (MNKIPIKDLLNPQ) [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]. We have shown in our previous study that classical NESs from HIV-rev protein and MAPKK (mitogen activated protein kinase kinase) protein can individually confer export to an enhanced green fluorescent protein (EGFP)-containing construct [8].

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 (with 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.

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 dexamethasone was used for constructs containing GR LBD [8,36,37].

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 175cm² flasks containing DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin (100U/ml, GIBCO BRL), 0.1% gentamicin (0.5mg/ml, Hyclone), and 1% L-glutamine (2mM, Hyclone). Cells were maintained in a 5% CO₂ incubator at 37 °C.

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, 22mm²) 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 (100U/ml, GIBCO BRL),

0.1% gentamycin (0.5mg/ml, Hyclone), and 1% L-glutamine (2nM, Hyclone), and maintained in a 5% CO₂ incubator at 37 °C for 18-24 hours.

Microscopy

Approximately 18-24 hours 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 HQ480/20nm, emission HQ510/20nm, with beam splitter Q4951p) from Chroma Technology (Brattleboro, VT) was used. Cells were photographed at a magnification of 40x using an F-View Monochrome CCD camera. To minimize photobleaching of EGFP chromophore, cells were imaged using neutral density filters at short (500ms) exposure times. An air stream incubator (Nevtek ASI 400, Burnsville, VA) with temperature control was used to maintain the microscope stage at 37°C.

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 *Sall* and replacing it with oligonucleotide 5'-AATTCTAGCCCAAAGAAGAAGAGAAAAGTAGAAG -3' and its complementary strand which encodes the classical NLS from SV40 T-antigen 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 *Sall* and replacing it with oligonucleotide 5'-AATTCTCCAGCAGCAAAAAGAGTAAAAGCAGACGAAG -3' and its complementary strand which encodes the classical type NLS sequence from MycA8 protein (see table 1 for NLS amino acid sequence).

HIV-MycA8-PRLBD was constructed by removing MycA8 NLS from MAPKK-MycA8-PRLBD using *EcoRI* and *Sall* and inserting it in HIV-NLSi-PRLBD already digested with *EcoRI* and *Sall* (to remove NLSi).

HIV-SV40A1-PRLBD and HIV-SV40A7-PRLBD were constructed by removing NLSi using *EcoRI* and *Sall* from HIV-NLSi-PRLBD and replacing it with oligonucleotide 5'-AATTCTAGCCCAAAGAAGAAGAGAAAAGTCGAAG -3' and its complementary strand which encodes SV40A1, a truncated version of classical NLS from SV40 T-antigen protein, and oligonucleotide 5'-AATTCTAGCCCAAAGAAGAAGAGAAAAGCAGAAG -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 *Sall* from MAPKK-NLSi-PRLBD and replacing it with oligonucleotides and their complementary strands encoding NLSs for the respective plasmids. Oligonucleotide 5'-AATTCTGGAGTAGTATACAAAGGAAGACACAAAACA ACAGGAG -3' and its complementary strand which encodes the NLS from CDC2HS; 5'-AATTCTTTTCGTAGTACACAAAAGATGCCACGAAG -3' and its complementary which strand encodes the NLS from protein kinase C (PKC); and 5'-AATTCTAGCCCAAAAAAAGCAAGAAAAGTAGAAG -3' and its complementary strand

which encodes the truncated NLS (SV40A4) from SV40 T-antigen protein were used to make these constructs (see table 1 for NLS amino acid sequence).

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 *XhoI* by the oligonucleotide 5' – CCGGACTACACGACCTAGTAAAACAACTACACCTAGC -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'-TCGAGGACCAGCAGCAAAAAGAGTAAAAGCAGACGAAGGG -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'-CCGGACTCCAACTGCCTCCCTTGGAGCGCCTAACTTGGGAGCACTCCAACTGCC TCCCTTGGAGCGCCTAACTTTGTC-3' and its complementary strand which encodes the two repeats of the classical consensus NESs from HIV-Rev protein, using enzymes *BspEI* and *XhoI*.

HIV-SV40-GRLBD and HIV-MycA8-GRLBD were constructed by replacing PR LBD with GR LBD (C656G GR LBD [38]) in the constructs HIV-SV40-PRLBD and HIV-MycA8-PRLBD respectively, using restriction enzymes *KpnI* and *BamHI*. The 248 amino acid long C656G GR LBD was taken out of full length GFP-C656G GR by performing PCR using the primers 5' –AGGGTACCCTCACCCCTACCTTG -3' and 5'-CGCGCGGATCCTTTTGTGATGAAACAG -3' with *KpnI* and *BamHI* ends, respectively.

Protein translocation studies

All protein constructs were induced with ligand 24 hours 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 10nM dose of ligand for one hour. Pictures of live cells were taken using a fluorescence microscope after one hour. As controls, cells were also photographed without inducing with ligand.

Protein Import Studies

As previously, import kinetics of protein constructs was studied by conducting time lapse experiments at three different concentrations: 1nM, 10nM and 100nM. Cells (2.5ml) were plated in live cell chambers after transfection by electroporation [8]. After 18-24 hours 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 minutes 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 minutes.

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 (figure 3a) is the increase in the nuclear intensity (which indicates the increase in the total amount of protein present in the nucleus) on ligand induction. Change in relative nuclear intensity (figure 3b) on ligand induction, are calculated as below to compare different versions of protein constructs.

$$\text{Average Nuclear Intensity} = \left(\frac{\text{NI}}{\text{NA}} \right) \quad \text{Average Cytoplasmic Intensity} = \left(\frac{\text{CI}}{\text{CA}} \right)$$

$$\text{Percentage Nuclear} = \left(\frac{\text{ANI}}{\text{ANI} + \text{ACI}} \right) 100 \quad \text{Percentage Nuclear Increase} = \left(\frac{\text{PN} - \text{PNo}}{\text{PNo}} \right) \times 100\%$$

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

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

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 1hr. after ligand induction, PNo = percentage of total protein present in the nucleus without ligand induction, RNI = relative nuclear intensity 1hr. after ligand induction, RNIo = relative nuclear intensity without ligand induction.

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 using non-linear regression (GraphPad Prism, San Diego, CA) using the following equation: $Y = Y_0 + Y_{\max} * (1 - \exp(-K * t))$

Where Y = relative nuclear intensity, t = time, Y₀ = relative nuclear intensity in the absence of ligand, Y_{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 presences of ligand at steady state (Y_{max}) were also compared between constructs using one way ANOVA with Tukey's multiple comparison. A p value <0.05 was considered significant.

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 ligand presence 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 ligand, and also show a certain degree of dose-responsiveness before saturation.

As seen in figure 1, the addition of ligand caused a change in localization of the protein constructs. Most of these tested protein constructs showed a significant change ($p < 0.05$) in localization from the cytoplasm to the nucleus on ligand induction. However, as noted in figure 2 this change in amount of protein in the nucleus varied for each protein construct. The localization of protein depends on the strengths of NES and NLS, while the LBD provides a means to externally control this localization with a ligand. In the absence of a ligand, protein constructs are mostly cytoplasmic due to a dominant NES, while on addition of ligand, changes in conformation of LBD make the constructs translocate to the nucleus via a now dominant NLS. The amount of protein entering the nucleus depends on the critical balance between the strengths of NES and NLS.

Changing the strength of NLS regulates the amount of protein transported 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 methods) to compare different protein constructs. As shown in figure 2, HIV-SV40-PRLBD (3rd pair of columns) had the maximum relative nuclear intensity on ligand induction with 10nM 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 K_d (dissociation) value for interaction with importin α [16]. The lower the K_d value of a NLS, the stronger the binding with importin α and hence greater nuclear import [16]. Though NLSi does not have a published value for its interaction with importin α , 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 figure 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. On 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 (figure 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 (figure 2, 2nd pair of columns) protein construct was also less than HIV-SV40-PRLBD (figure 2, 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 nuclear increase (PNI, figure 3A) and the change in relative nuclear intensity (RNI, figure 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 figure 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 (figure 3B, column 3 and 2). The change in PNI and RNI values for HIV-NLSi-PRLBD were only 17% (figure 3A, column 1) and 38% (figure 3B, column 1), respectively. Both new constructs, HIV-MycA8-PRLBD (figure 3A column 2) and HIV-SV40-PRLBD (figure 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 (figure 3A column 1); however, HIV-MycA8-PRLBD and HIV-SV40-PRLBD were not significantly different from each other (compare figure 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.

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 figure 1(d and f) and figure 2(columns 6 and 4) only the MAPKK-MycA8-PRLBD and PRNES2-MycA8-PRLBD constructs showed a significant change ($p < 0.05$) in 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 (figure 3b, columns 5, 6 and 7), compared to over 100% change with the HIV NES constructs (figure 3b, columns 2 and 3). The percentage nuclear increase for these MAPKK constructs was also low, at 8%, 17% and 8% respectively (figure 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 (figure 3A, column 4) and a relative nuclear intensity change of 120% (figure 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) (figure 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].

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 (figure 1, j and i). Upon inducing the constructs with 10nM dexamethasone, as observed in figure 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 (figure 3a, column 8) and 35% for HIV-SV40-GRLBD constructs (figure 3a, column 9). The change in relative nuclear intensity values was 90% and 91%, respectively, for these two constructs (figure 3b, columns 8 and 9). To conclude, protein constructs for controlled intracellular localization of proteins can be customized with different ligand 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.

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 hours at three different ligand doses (1nM, 10nM, and 100nM). 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 (figure 3). Import was studied for 2 hours because it was determined in our previous study [8] that no change in import is observed after 2 hours. Mifepristone and dexamethasone were used as ligands for 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 hour at 10nM mifepristone dose for HIV-SV40-PRLBD and HIV-MycA8-PRLBD is shown in figure 4. All the pictures were analyzed to calculate relative nuclear intensity (described in methods). To study the import kinetics of protein constructs, relative nuclear intensity was plotted as a function of time (figure 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 figure 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 1nM to 10nM and 1nM to 100nM for both HIV-MycA8-PRLBD and PRNES2-MycA8-PRLBD (table 3). There was no significant difference observed at the higher doses (between 10nM and 100nM) 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 10nM 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 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 (10nM and 100nM) 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 1nM and 100nM, and 10nM and 100nM, but not between 1 and 10nM (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 1nM and 10nM doses due to greater variance within the data at 1nM (large standard error and noise; data not shown). A dramatic change in nuclear intensity was not observed at 1nM 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.

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 (figure 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 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 mifipristone 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 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.

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. 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 localization controllable protein constructs discussed in this study provide a mean 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.

Acknowledgements

This work was supported by NIH DK070060 grant. The authors would also like to acknowledge Sandra Dittmar for her technical work.

References

1. Amsterdam A, Pitzer F, Baumeister W. Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cell cycle control. *Proc Natl Acad Sci U S A* 1993;90(1):99–103. [PubMed: 8380501]
2. Gui JF, Lane WS, Fu XD. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* 1994;369(6482):678–682. [PubMed: 8208298]
3. Keeshan K, Cotter TG, McKenna SL. Bcr-Abl upregulates cytosolic p21WAF-1/CIP-1 by a phosphoinositide-3-kinase (PI3K)-independent pathway. *Br J Haematol* 2003;123(1):34–44. [PubMed: 14510940]
4. Marheineke K, Krude T. Nucleosome assembly activity and intracellular localization of human CAF-1 changes during the cell division cycle. *J Biol Chem* 1998;273(24):15279–15286. [PubMed: 9614144]
5. Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. *Cell* 1991;66(4):743–758. [PubMed: 1652372]
6. Vriz S, Lemaitre JM, Leibovici M, Thierry N, Mechali M. Comparative analysis of the intracellular localization of c-Myc, c-Fos, and replicative proteins during cell cycle progression. *Mol Cell Biol* 1992;12(8):3548–3555. [PubMed: 1352852]
7. Fabbro M, Henderson BR. Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. *Exp Cell Res* 2003;282(2):59–69. [PubMed: 12531692]
8. Kanwal C, Mu S, Kern SE, Lim CS. Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments. *J Control Release* 2004;98(3):379–393. [PubMed: 15312994]
9. Nigg EA. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* 1997;386(6627):779–787. [PubMed: 9126736]
10. Gorlich D, Kutay U. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 1999;15:607–660. [PubMed: 10611974]
11. Lyman SK, Guan T, Bednenko J, Wodrich H, Gerace L. Influence of cargo size on Ran and energy requirements for nuclear protein import. *J Cell Biol* 2002;159(1):55–67. [PubMed: 12370244]
12. Turpin P, Ossareh-Nazari B, Dargemont C. Nuclear transport and transcriptional regulation. *FEBS Lett* 1999;452(12):82–86. [PubMed: 10376683]
13. Chan CK, Jans DA. Using nuclear targeting signals to enhance non-viral gene transfer. *Immunol Cell Biol* 2002;80(2):119–130. [PubMed: 11940112]
14. Kalderon D, Roberts BL, Richardson WD, Smith AE. A short amino acid sequence able to specify nuclear location. *Cell* 1984;39(3 Pt 2):499–509. [PubMed: 6096007]
15. Efthymiadis A, Shao H, Hubner S, Jans DA. Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) in vivo and in vitro. A comparison with the SV40 large T-antigen NLS. *J Biol Chem* 1997;272(35):22134–22139. [PubMed: 9268357]
16. Hodel MR, Corbett AH, Hodel AE. Dissection of a nuclear localization signal. *J Biol Chem* 2001;276(2):1317–1325. [PubMed: 11038364]
17. Robbins J, Dilworth SM, Laskey RA, Dingwall C. Two interdependent basic domains in nucleoplasmic nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* 1991;64(3):615–623. [PubMed: 1991323]

18. Ylikomi T, Bocquel MT, Berry M, Gronemeyer H, Chambon P. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *Embo J* 1992;11(10):3681–3694. [PubMed: 1327748]
19. Mattaj JW, Englmeier L. Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* 1998;67:265–306. [PubMed: 9759490]
20. Brokx RD, Bisland SK, Gariepy J. Designing peptide-based scaffolds as drug delivery vehicles. *J Control Release* 2002;78(13):115–123. [PubMed: 11772453]
21. Rojanasakul Y, Luo Q, Ye J, Antonini J, Toledo D. Cellular delivery of functional peptides to block cytokine gene expression. *J Control Release* 2000;65(12):13–17. [PubMed: 10699265]
22. Talsma SS, Babensee JE, Murthy N, Williams IR. Development and in vitro validation of a targeted delivery vehicle for DNA vaccines. *J Control Release* 2006;112(2):271–279. [PubMed: 16549219]
23. Eguchi A, Furusawa H, Yamamoto A, Akuta T, Hasegawa M, Okahata Y, Nakanishi M. Optimization of nuclear localization signal for nuclear transport of DNA-encapsulating particles. *J Control Release* 2005;104(3):507–519. [PubMed: 15911050]
24. Nagasaki T, Kawazu T, Tachibana T, Tamagaki S, Shinkai S. Enhanced nuclear import and transfection efficiency of plasmid DNA using streptavidin-fused importin- β . *J Control Release* 2005;103(1):199–207. [PubMed: 15710511]
25. Fornerod M, Ohno M, Yoshida M, Mattaj JW. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 1997;90(6):1051–1060. [PubMed: 9323133]
26. Macara IG. Transport into and out of the nucleus. *Microbiol Mol Biol Rev* 2001;65(4):570–594. [PubMed: 11729264]table of contents
27. Bogerd HP, Fridell RA, Benson RE, Hua J, Cullen BR. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. *Mol Cell Biol* 1996;16(8):4207–4214. [PubMed: 8754820]
28. Henderson BR, Eleftheriou A. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp Cell Res* 2000;256(1):213–224. [PubMed: 10739668]
29. Ikuta T, Eguchi H, Tachibana T, Yoneda Y, Kawajiri K. Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J Biol Chem* 1998;273(5):2895–2904. [PubMed: 9446600]
30. Kanwal C, Li H, Lim CS. Model system to study classical nuclear export signals. *AAPS PharmSci* 2002;4(3)
31. Li H, Yan G, Kern SE, Lim CS. Correlation Among Agonist Dose, Rate of Import, and Transcriptional Activity of Liganded Progesterone Receptor B Isoform in Living Cells. *Pharm Res* 2003;20(10):1574–1580. [PubMed: 14620510]
32. Lim CS, Baumann CT, Htun H, Xian W, Irie M, Smith CL, Hager GL. Differential localization and activity of the A- and B-forms of the human progesterone receptor using green fluorescent protein chimeras. *Mol Endocrinol* 1999;13(3):366–375. [PubMed: 10076994]
33. Siegel RM, Chan FK, Zacharias DA, Swofford R, Holmes KL, Tsien RY, Lenardo MJ. Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of the green fluorescent protein. *Sci STKE* 2000;2000(38):L1.
34. Tsien RY. The green fluorescent protein. *Annu Rev Biochem* 1998;67:509–544. [PubMed: 9759496]
35. Li H, Fidler ML, Lim CS. Effect of initial subcellular localization of progesterone receptor on import kinetics and transcriptional activity. *Mol Pharm* 2005;2(6):509–518. [PubMed: 16323958]
36. Chakraborti PK, Garabedian MJ, Yamamoto KR, Simons SS Jr. Creation of “super” glucocorticoid receptors by point mutations in the steroid binding domain. *J Biol Chem* 1991;266(33):22075–22078. [PubMed: 1939229]
37. Wang Y, O'Malley BW Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. *Proc Natl Acad Sci U S A* 1994;91(17):8180–8184. [PubMed: 8058776]
38. Htun H, Barsony J, Renyi I, Gould DL, Hager GL. Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc Natl Acad Sci U S A* 1996;93(10):4845–4850. [PubMed: 8643491]
39. Htun H, Holth LT, Walker D, Davie JR, Hager GL. Direct visualization of the human estrogen receptor α reveals a role for ligand in the nuclear distribution of the receptor. *Mol Biol Cell* 1999;10(2):471–486. [PubMed: 9950689]

40. Schaufele F, Chang CY, Liu W, Baxter JD, Nordeen SK, Wan Y, Day RN, McDonnell DP. Temporally distinct and ligand-specific recruitment of nuclear receptor-interacting peptides and cofactors to subnuclear domains containing the estrogen receptor. *Mol Endocrinol* 2000;14(12):2024–2039. [PubMed: 11117532]
41. Boulikas T. Nuclear import of protein kinases and cyclins. *J Cell Biochem* 1996;60(1):61–82. [PubMed: 8825417]
42. Vegeto E, Allan GF, Schrader WT, Tsai MJ, McDonnell DP, O'Malley BW. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 1992;69(4):703–713. [PubMed: 1586949]
43. Kakar KC, Davis M JR, Li H, Lim CS. Geldanamycin, an Inhibitor of Hsp90, Blocks Cytoplasmic Retention of Progesterone Receptors and Glucocorticoid Receptors via Their Respective Ligand Binding Domains. *The AAPS Journal* 2006;8(4):E718–E728. [PubMed: 17233535]
44. Ray DW, Suen CS, Brass A, Soden J, White A. Structure/function of the human glucocorticoid receptor: tyrosine 735 is important for transactivation. *Mol Endocrinol* 1999;13(11):1855–1863. [PubMed: 10551779]
45. Edwards SW, Tan CM, Limbird LE. Localization of G-protein-coupled receptors in health and disease. *Trends Pharmacol Sci* 2000;21(8):304–308. [PubMed: 10918636]
46. Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu Rev Genet* 1990;24:133–170. [PubMed: 2088165]
47. Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell* 1993;73(7):1251–1254. [PubMed: 7686820]
48. Wurzer G, Mosgoeller W, Chabicosky M, Cerni C, Wesierska-Gadek J. Nuclear Ras: Unexpected subcellular distribution of oncogenic forms. *J Cell Biochem* 2001;81(S36):1–11. [PubMed: 11180393]
49. Davis JR, Kakar M, Lim CS. Controlling protein compartmentalization to overcome disease. *Pharm Res* 2007;24(1):17–27. [PubMed: 16969692]
50. Guiochon-Mantel A, Lescop P, Christin-Maitre S, Loosfelt H, Perrot-Applanat M, Milgrom E. Nucleocytoplasmic shuttling of the progesterone receptor. *Embo J* 1991;10(12):3851–3859. [PubMed: 1935904]

Figure 1. Change in protein localization on ligand induction

Change in localization of protein is observed after induction with ligand for 1hr. A 10nM dose of mifepristone (MFP) and dexamethasone (DEX) is used as ligands for constructs with PR and GR LBD respectively.

Figure 2. Change in relative nuclear intensity of protein constructs on induction with 10nM ligand for one hour

This graph depicts the change in nuclear intensity of protein constructs on induction with 10nM dose of ligand for 1 hr. 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, $p < 0.05$)

Figure 3. Comparison of various protein constructs based on ligand induction studies

3a. 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 10nM dose of mifepristone and dexamethasone were used as ligand for protein constructs with PR and GR LBD, respectively.

3b. 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 10nM dose of mifepristone and dexamethasone were used as ligand for protein constructs with PR and GR LBD, respectively.

A. HIV-SV40-PRLBD (10nM MFP) **B. HIV-MycA8-PRLBD (10nM MFP)** **Figure 4. Representative time dependent import studies for protein constructs**

Representative time dependent import studies for HIV-SV40-PRLBD and HIV-MycA8-PRLBD constructs with 10nM MFP. Only a few time points of the time lapse experiments are shown. It can be observed that there is a movement of protein from the nucleus to the cytoplasm over time on ligand induction.

Figure 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 100nM ligand. The import was studied for 2 hours. Data is reported as mean $\pm$ 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.

HIV-MycA8-MycA8-PRLBD
(no ligand)

HIV-MycA8-MycA8-PRLBD
(10nM MFP)

HIV-HIV-MycA8-MycA8-PRLBD
(no ligand)

HIV-HIV-MycA8-MycA8-PRLBD
(10nM MFP)

Figure 6. Effect of multiple signals on localization of constructs

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.

Table 1

Nuclear export signals, nuclear import signals, and ligand binding domains used in this study.

Type of signal/domain	Amino acid sequence (or numbering)	Strength of signal (K_d nM [16], if known)
Nuclear export signals (NES):		
MAPKK	LQKKLELEL [28]	strong
HIV	LQLPLERLTL [28]	medium
PRB NES2	LHDLVKQLHL [30]	n/a
Nuclear import signals (NLS):		
SV40	SPKKKRKVE [16]	9±4 strong
SV40A1	SAKKKRKVE [16]	36±2 medium
SV40A7	SPKKKRKAE [16]	53±4 medium
MycA8	PAAKRVKADE [16]	85±7 medium
SV40A4	SPKKARKVE [16]	335±7 weak
CDC2	GVVYKGRHKTG [41]	n/a weak
PKC	FVVHKRCHE [41]	n/a weak
NLSi	RAMEGOHNYLCAGRND CIVDKIRRKNC PACLRKCCQAGMVLGG [50]	n/a weak
Ligand binding domains (LBD):		
PR LBD	645-891aa [30]	
GR LBD	547-795aa [38]	

Table 2

Combinations of NES, NLS, and LBD used in this study.

NES	NLS	LBD
HIV	NLSi	PRLBD
HIV	SV40	PRLBD
HIV	MycA8	PRLBD
HIV	SV40A1	PRLBD
HIV	SV40A7	PRLBD
MAPKK	NLSi	PRLBD
MAPKK	SV40	PRLBD
MAPKK	MycA8	PRLBD
MAPKK	SV40A4	PRLBD
MAPKK	PKC	PRLBD
MAPKK	CDC	PRLBD
PRB NES2	SV40	PRLBD
PRB NES2	MycA8	PRLBD
HIV	SV40	GRLBD
HIV	MycA8	GRLBD
HIV	MycA8 $\times$ 2	PRLBD
HIV $\times$ 2	MycA8 $\times$ 2	PRLBD

Table 3Import Rate Constant of Protein Constructs ($K \text{ min}^{-1}$)

Protein Construct	1nM	10nM	100nM
HIV-MycA8-PRLBD	0.01303	0.09078	0.08117
HIV-SV40-PRLBD	0.05815	0.06449	0.1337
PRNES2-MycA8-PRLBD	0.01703	0.08724	0.07535
HIV-MycA8-GRLBD	0.0008745	0.03602	0.07960
HIV-SV40-GRLBD	0.01638	0.03208	0.1149

Import rate constant values were calculated for different protein constructs at three different doses. These values give a comparative analysis of import characteristics of protein constructs studied for import. All the protein constructs except HIV-SV40-PRLBD showed dose-dependence. A significant difference ($p < 0.05$) in import rate constant was observed at lower dose for constructs with PRLBD, and at higher dose for constructs with GRLBD. Mifepristone and dexamethasone were used as ligand for protein constructs with PR and GR LBD, respectively.