Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments

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Abstract

A regulatable fusion protein was constructed for controlling the localization of plasmid products. A ligand-inducible nuclear localization signal, nuclear export signal (NES) and a truncated form of the ligand binding domain of the progesterone receptor were attached to the desired protein. Enhanced green fluorescent protein (EGFP) was used as a model protein and its trafficking between the nucleus and cytoplasm was studied using fluorescence microscopy in response to the ligand, mifepristone. It was found that the protein trafficking into the nucleus was dose dependent with ligand concentration. Increasing the ligand dose from 1 to 100 nM enhanced import and reduced the rate of export of the fusion protein from the nucleus to the cytoplasm. This study demonstrates the feasibility of using an export signal and a ligand-inducible nuclear import signal as a bi-directional on/off switch with potential use for controlled targeting of therapeutic proteins to subcellular compartments.

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1. Introduction

Gene therapy has been cited as the future of human medicine [1] and is a rapidly evolving field with significant room for improvement. The development of tailored, novel gene products will improve the management of human diseases with the next era of gene therapy witnessing a complete manipulation of the delivered gene products in the targeted cells. The bi-directional protein switch described in this paper provides a step in this direction by allowing the scientist to control the function of the therapeutic gene product by changing its subcellular localization. The major goal of the project is to greatly expand the

Abbreviations: CRT, calreticulin; DBD, DNA binding domain; DMEM, Dulbecco’s modified eagle’s medium; LBD, ligand binding domain; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; MAPKK, mitogen activated protein kinase; MFP, mifepristone; NCoR, nuclear receptor co-repressor; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; PBS, phosphate-buffered saline; PR, progesterone receptor; PRB, progesterone receptor B isoform; ROI, region of interest.

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range of applicability of gene therapy by directly modulating the protein transport and compartmentalization mechanisms. Delivery of gene therapy products to specific sites of action can be controlled by removing the natural signals (import and export) of the gene of interest and replacing them with regulatable signals. This allows gene therapy to be directed by the health care practitioners rather than the endogenous gene. In the case of the endogenous gene, regulation of localization may be dependent on factors in the cell that are poorly defined, difficult to control, or malfunctioning.

Eukaryotic cells are highly organized structures that use compartmentalization to regulate biological activity by defining locations of molecules, pathways and processes [2]. Proteins and other vital molecules have specific sites of localization and function in subcellular compartments, enabling the cell to control vital cellular processes and functions. Protein trafficking between the nucleus and the cytoplasm occurs mainly through nuclear pore complexes (NPCs) in the nuclear envelope. These passive diffusion channels have an effective diameter of 9 nm and allow for passage of proteins of less than 45–60 kDa [3,4]. Transport of larger macromolecules occurs via amino acid signals which are recognized by specific import and export receptors [5].

Import receptors recognize and bind to certain amino acid sequences called nuclear localization signals (NLS). Classical NLSs can be monopartite and contain a single stretch of basic amino acids, like the signal found in SV-40 large T-antigen (PKKKRKV) [6]. Bipartite and tripartite nuclear localization signals have also been identified in proteins like nucleoplasmin and steroid hormone receptors [7,8]. Proteins containing nuclear localization signals are imported into the nucleus by importin α/β heterodimer. Importin α contains the NLS binding site while importin β mediates the translocation through the nuclear pore [5,9].

The classical export receptor CRM1 recognizes and binds to leucine-rich nuclear export signals (NESs) [10,11]. Classical NESs are typically 8–11 amino acid sequences with hydrophobic residues. A common consensus NES is Lx_{1−3}Lx_{2−3}LxJ (where L = Leu X = spacer J = Leu, Val or Ile) [12]. CRM1 also functions as an export receptor for RNA [5]. A non-classical NES is recognized by calreticulin (CRT) a multifunctional calcium binding protein that resides in the ER lumen. CRT has been shown to recognize a KVFFKR amino acid motif in steroid receptors, and may export steroid receptors such as the glucocorticoid receptor under stress conditions [13].

The selective movement of macromolecules across the nuclear envelope can be exploited for novel drug therapy by blocking or facilitating the localization of the delivered protein to its active compartment via signal sequences. This paper demonstrates the feasibility of using ligand-inducible nuclear import and export signals as a bi-directional on/off switch for controlled targeting of proteins to the nucleus and cytoplasm. The localization and rate of delivery of these proteins can be manipulated by using naturally occurring signal sequences that are regulated by an external stimulus such as a ligand.

A protein capable of bi-directional trafficking was constructed by tagging it with a strong NES, a ligand-inducible NLS [8], and a mifepristone-specific ligand binding domain (LBD) [14]. The protein chosen for proof of concept was enhanced green fluorescent protein (EGFP), which can easily be tracked in living cells [15–17]. NESs with different strengths were initially tested and optimized. In the “off” or unliganded state, the controlled targeting system with the protein of interest (EGFP) is directed to the cytoplasm by virtue of its nuclear export signal (NES). The targeting system is turned “on” when ligand is added. This sends the protein to the nucleus due to the ligand-inducible NLS and interaction with the LBD. The system can be turned “off” again by removing the ligand, resulting in the export of the protein back to the cytoplasm. The protein can be cycled multiple times as needed.

Trafficking was studied using fluorescence microscopy. A recent paper showed that fluorescence microscopy is a valid method of quantitation of trafficking [18]. It was shown that, despite the heterogeneity of cells, trends and conclusions could be derived from cellular microscopic data. Many groups have used confocal/fluorescence microscopy for studying and quantitating protein dynamics [19,20]. Live cell imaging with fluorescence microscopy allows for the tracking of movement of the fusion protein over time. Live cell imaging is a better method for studying translocation as it can overcome any
fixation artifacts [21]. In the current study, confocal microscopy of fixed cells was performed as well and showed protein localization trends similar to fluorescence microscopy (data not shown).

2. Material and methods

2.1. Cell line

1471.1 cells (murine adenocarcinoma cells that do not express endogenous progesterone receptor) were used for these studies. 1471.1 cells were maintained in Dulbecco’s modified eagle’s medium (DMEM; GIBCO BRL, Grand Island, NY) with 10% FBS (Hyclone Laboratories, Logan, UT), penicillin streptomycin (100 U/ml, GIBCO BRL), gentamycin (0.5 mg/ml, Hyclone) and l-glutamine (2 mM, Hyclone) in a 5 % CO2 incubator at 37 °C.

2.2. Subcloning and construction of plasmids

PCR was performed on pEGFP-PRB [12] to extract out the 246 amino acid ligand binding domain (LBD) fragment (645–891) using the primers: 5'-GGTACCGTCAGAGTTGTGAGAGCACTGGA-3' and 5'-GGATCCCAAGAGTACAGATGAAGTGTTTG-3' with KpnI and BamHI sticky ends. This fragment was inserted into the multiple cloning site of EGFP-C1 (BD Biosciences Clontech, Palo Alto, CA; encodes enhanced green fluorescent protein and contains the CMV promoter/enhancer) digested with the enzymes KpnI and BamHI to create the plasmid EGFP-LBD. PCR was performed on pEGFP-PRB to obtain the 43 amino acid inducible nuclear localization signal (NLSi) using the primers: 5'-AATTCTGCAATGGAAGGGCAAGCAGCAA-3' and 5'-GTCGACTCGACCTCCAAGGACCATGCC-3' with restriction sites for the enzymes EcoRI and SalI. The plasmid EGFP-LBD was then digested with EcoRI and SalI and ligated to the insert NLSi to make pEGFP-NLSi-LBD.

EGFP-15 Pep-NLSi-LBD (which contains the 15 amino acid peptide), was constructed by inserting the oligonucleotide 5'-CCGGATGTGGGAGCTGTAAGGTCTTCTTTAAGAGGGAAGGACACACTTTACTATC-3' and its complementary strand encoding for the 15 amino acid peptide in the PR DNA binding domain into the vector EGFP-NLS-LBD, which had been digested with BspE1 and XhoI.

EGFP-MAPKK-NLSi-LBD was subcloned by inserting the oligonucleotide 5'-CCGGACTTCAAAAAACTTGAGAAGACTGAGCTGTC-3' and its complementary strand encoding the strong nuclear export signal protein from MAPKK into the construct EGFP-NLSi-LBD digested with BspE1 and XhoI.

EGFP-NES-NLSi-LBD was subcloned by inserting the cDNA encoding the PRNES (KVFFKR), which has cohesive ends to enzymes BspE1 and XhoI and ligated to EGFP-NLS-LBD cut with BspE1 and XhoI.

EGFP-DBD-NLSi-LBD was constructed by performing PCR on EGFP-PRB to extract the 63 amino acid fragment encoding for the DNA binding domain of PRB with BspE1 and XhoI restriction sites and was ligated to EGFP-NLS-LBD cut with BspE1 and XhoI.

EGFP-HIV-NLSi-LBD was subcloned by inserting the oligomers encoding the HIV NES 5'-CCGGACTTCACAACCTCTCTTGAGAGACTTACTCTTTC-3' and complementary strand: 5'-TCGAGAAAGAGTAAGTCTCTCAAGGAAGGAGTTGAAGT-3' into EGFP-NLS-LBD cut with BspE1 and XhoI.

EGFP-Adeno-NLSi-LBD was subcloned using primers 5'-CCGGACTTTATCCTGAGAGACTTAATGAAATTCTTACTTC-3' and its complementary strand 5'-TCGAGAAGTAAGAATTCTTCTAAGCTCTCAAGATAAAAGT-3' and ligated to EGFP-NLS-LBD cut with BspE1 and XhoI.

EGFP-p53-NLSi-LBD was constructed similarly using 5'-CCGGAATGTTTAAATGAGGCTCTTGAGCTTAAACATC-3' and the complementary strand 5'-TCGAGAAGTAAGAATTCTTCTAAGCTCTCAAGATAAAAGT-3' and ligated to EGFP-NLS-LBD cut with BspE1 and XhoI.

2.3. Transient transfections

Transient transfections of 1471.1 cells were carried out by electroporation as previously described [12]. Briefly, 5 × 10^6 cells were transfected with 2 μg of
each of the desired plasmid(s) and 8 µg of carrier DNA, pGL3 basic (Promega, Madison, WI), to make 10 µg total DNA. Transfections were performed using an Electrosquare porator ECM 830 Electroporation system (BTX, San Diego, CA) at a voltage of 140 V for 10 ms with three pulses in a total of 100-µl cold plain DMEM. After a 5–10-min recovery on ice, the electroporated cells were diluted with complete phenol red-free DMEM (10% FBS charcoal/dextran treated, Hyclone, l-glutamine, pen-strep and gentamicin) and plated on a clear coverglass (Corning no. 1, 22 mm²) in six well plates or into live cell chambers (Lab-tek chamber slide system, 2 ml, Nalge NUNC International, Naperville, IL). These cells were then incubated in a 5% CO₂ incubator at 37 °C for 18–24 h.

2.4. Microscopy

Approximately 24 h after electroporation, protein expression of NES-NLS-LBD plasmids was viewed by fluorescence microscopy in fixed or living cells. An Olympus IX701F inverted fluorescence microscope (Scientific Instrument Co, Aurora, CO) with high-quality narrow band GFP filter (excitation filter set HQ480/20; emission filter set HQ510/20 nm; with beamsplitter Q4951p) from Chroma Technology (Brattleboro, VT) was utilized. Cells were photographed at a magnification of 60 × using an F-View Monochrome CCD camera. To minimize photo-bleaching of the EGFP chromophore, cells were imaged using neutral-density filters that transmit 25% of the total light, and short exposure times of 500 ms were used.

In addition, a Zeiss (Thornwood, NY) LSM 510 confocal imaging system with an Axioplan 2 microscope (100 × plan-apo objective, NA = 1.4, oil) and an argon laser was used for imaging fixed cells. A laser line with excitation of 488-nm and a 505-nm-long pass emission filter was used. For fixed cell microscopy, the coverslips were rinsed with PBS, incubated with 4% paraformaldehyde for 20 min, sealed with clear nail polish and inverted onto microscope slides.

For live cell imaging a Nevtek ASI 400 Air Stream Incubator (Burnsville, VA) with variable temperature control was used to maintain the microscope stage and the live cell chambers at 37 °C. Cells were photographed using a F-View Monochrome CCD camera on an IX701F microscope (mentioned above) at time points 0, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120 min for import studies of the various constructs. Export studies were conducted over a period of 24 h using the same conditions.

2.5. Ligand induction and removal studies

Cells transfected with a protein switch plasmid were induced with ligand 24 h post transfection. Mifepristone in final concentrations of 1, 10 and 100 nM were used for induction of import. Prior to microscopy, transfected cells were either fixed with 4% paraformaldehyde or plated in live cell chambers. For removal of ligand, cells were washed five times with PBS, rinsed with DMEM plain media and then fresh media was added. Cells were fixed for the primary studies to test the feasibility of the system using confocal microscopy. These results were then corroborated using live cell fluorescence microscopy to avoid any potential artifacts due to fixation.

2.6. Preliminary studies using fixed cells

To determine if the protein construct was capable of undergoing regulated import and export, experiments were performed on fixed cells. Two milliliters of transfected cells were plated on glass coverslips in four 6 well plates. Cells in the first plate were fixed after a 24-h incubation period to be used as a control (off state). Cells in plates 2, 3 and 4 were induced with 10 nM MFP. 2 h after incubation, cells in plate 2 were fixed with 4% paraformaldehyde and examined for import of fusion protein. Wells in plates 3 and 4 were washed five times with phosphate buffer saline (Mediatech, Herndon, VA) and then rinsed and incubated with phenol red-free media. After an 8-h incubation, cells in plate 3 were fixed with paraformaldehyde and examined for export of the fusion protein. If the protein was capable of import, on ligand induction, and export on ligand removal, cells in plate 4 were re-induced for two h with 10 nM MFP. Constructs that were capable of bidirectional trafficking were chosen for live cell import and export studies.

2.7. Import studies on living cells

Time lapse studies on live cells were carried out to test the kinetics of import with increasing ligand con-
2.8. Export studies on living cells

Export studies after hormone washout were carried out in live cells. These studies were performed to determine if the export rate of fusion proteins was dependent on MFP induction doses. One milliliter of electroporated cells was plated in live cell chambers with 2 ml of phenol red-free DMEM. Twenty-four hours later, media was removed and 2 ml of fresh media was added. Cells were kept in the incubator for 30 min and ligand was added to make the final concentration 1 or 10 nM. After incubating for 2 h, the ligand was removed by washing five times with PBS without calcium or magnesium followed by a rinse with phenol red-free DMEM. Two milliliters of fresh complete phenol red-free DMEM was then added and the chamber was kept in the incubator at 37 °C for the desired periods. The export was studied by detecting GFP fluorescence under the microscope at time points of 8, 12 and 24 h after ligand withdrawal and washing.

2.9. Quantitation of EGFP fluorescence in the nucleus and cytoplasm

The amount of fluorescence in the nuclear and cytoplasmic compartments was quantitated as previously described [18]. All images were analyzed by analySIS® software (Soft Imaging System, Lake-wood). This software allows one to trace an outline around a region of interest (ROI), then the pixel (fluorescence) intensity in that ROI is calculated. After tracing, the intensities were calculated in the total cell area (total intensity) and in the nucleus (nuclear intensity). Cytoplasmic fluorescence was calculated by subtracting the nuclear intensity from the total intensity. The fluorescence measurements were then normalized to area by calculating the average nuclear and cytoplasmic intensities. These average values were obtained by dividing the intensities by the area of the nucleus and cytoplasm, respectively (Eq. (1) and (2)). Calculating the average intensity overcomes variations due to differences in the shape and spread of the cells.

To calculate the relative intensity of the nucleus to the cytoplasm, the average nuclear intensity (nuclear intensity divided by nuclear area) was calculated for each cell, and divided by the average cytoplasmic intensity (intensity per area), and normalized to the first relative intensity value (before induction with ligand) (Eq. (3) and (4)). This denotes the actual difference in the fluorescence intensity due to import of the fluorescent protein on induction with ligand.

\[
\text{avg.N.I.} = \frac{\text{N.I.}}{\text{N.A.}} \quad (1)
\]

\[
\text{avg.C.I.} = \frac{\text{T.I.} - \text{N.I.}}{\text{T.A.} - \text{N.A.}} \quad (2)
\]

\[
\text{Rel.I.}_{T0} = \frac{a\text{N.I.}_{T0}}{a\text{C.I.}_{T0}} \quad (3)
\]

\[
\text{Rel.I.}_{Tn} = \frac{a\text{N.I.}_{Tn}}{a\text{C.I.}_{Tn}} \frac{\text{Rel.I.}_{T0}}{(4)}
\]

where, N.I. = nuclear intensity; N.A. = nuclear area; T.I. = total fluorescence intensity; T.A. = total area of the cell; C.I. = cytoplasmic intensity; Rel.I.\(_{T0}\) = Relative intensity measured either at time T0 (before ligand induction) or at the time T (after ligand induction); Rel.I.\(_{Tn}\) = Relative intensity measured at time Tn minus relative intensity at T0 to analyze the actual increase in relative fluorescence after ligand induction.

2.10. Statistical analysis

The results of import and export studies of MAPKK and HIV NES constructs carried out in living cells were analyzed quantitatively. For all experiments, 10 cells were chosen for analysis (n = 10). The relationship between time and nuclear intensity for each dose tested was fitted to a two parameter exponential relationship using nonlinear regression (Sigma Plot, Chicago, IL). The model parameters were the maximum relative cellular fluorescence intensity and a rate constant that defined the time until transport reached steady state. Differences in the rate constant as a function of ligand concentration were assessed using Kruskal–Wallis ANOVA and Student–Newman–Kuels test to isolate individ-
ual differences. Friedman repeated measures ANOVA was used to determine differences in export over time with the Student–Newman–Kuels test to isolate individual differences. For either test, a $p$ value $< 0.05$ was considered significant.

### 3. Results

#### 3.1. The initial localization of the construct depends on the strength of the NES

As shown in Figs. 1 and 2, protein switch constructs showed variable degrees of localization in the cytoplasm during the protein switch off state. In the off state, the protein should localize in the cytoplasm (inactive compartment). This is achieved by including a strong NES in the protein construct. NESs recognized either by CRM1 or CRT were tested (Table 1). The classical NESs that function via the CRM1-mediated export pathway have been classified according to their strength and rate of export [22,23] (Table 1); however, KVFFKR containing motifs are recognized by the non-classical exporter calreticulin (CRT). The relative strength of the NESs recognized by calreticulin versus the classical nuclear export signals is not yet known.

MAPKK-NLS$_1$-LBD (Fig. 2a), Pep-NLS$_2$-LBD (Fig. 1b), HIV-NLS$_3$-LBD (Fig. 2b) and PRB NES2-NLS$_3$-LBD (Fig. 2c) mainly localized in the cytoplasm, prior to addition of ligand. Proteins with other weaker NESs like the export signal from adenovirus (Fig. 2d), p53 (Fig. 2e), DNA binding domain of PRB (Fig. 1a) and a minimal putative NES (KVFFKR) of PRB, recognized by calreticulin (Fig. 1c) did not cause predominant cytoplasmic localization of the EGFP protein. MAPKK, being the strongest nuclear export signal used in these studies caused complete cytoplasmic localization of the fusion protein. Protein constructs with HIV NES and PRB NES were more cytoplasmic compared to the Pep-NES. In summary, stronger nuclear export signals directed the protein to be more localized in the cytoplasm in the unliganded or off state.

#### 3.2. Constructs are capable of subcellular trafficking

As shown in Fig. 3, the constructs that localized mainly in the cytoplasm in the off state were studied for shuttling capacity. Plasmids containing MAPKK NES (Fig. 3A), HIV NES (Fig. 3B), 15 amino acid peptide (Fig. 3C) with the KVFFKR motif present in the DNA binding domain of progesterone receptor,

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**Table 1**

Relative strengths of the nuclear export signals used in the constructs

<table>
<thead>
<tr>
<th>NES from protein</th>
<th>Export signal seq</th>
<th>Strength</th>
<th>Rate of export (speed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPKK</td>
<td>LQKKLLEELEL</td>
<td>strong</td>
<td>fast (5–10 min)</td>
</tr>
<tr>
<td>HIV-1 rev NES</td>
<td>LQLPPLERLTL</td>
<td>medium</td>
<td>medium (10–20 min)</td>
</tr>
<tr>
<td>Adeno type 5 E1B-55K p53</td>
<td>LYPERLRLRIL</td>
<td>weak</td>
<td>slow (30–60 min)</td>
</tr>
<tr>
<td>PRDBD complete</td>
<td>KVFFKR</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PR DBD 6 aa</td>
<td>KVFFKR</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PR 15 aa pep</td>
<td>KVFFKRAMEQGHNYL</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PRB NES2</td>
<td>LHDLVKLQHL</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

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![Fig. 1. Constructs with non-classical NES (KVFFKR) motif recognized by calreticulin.](image)
and a putative classical NES (PRB NES2) (Fig. 3D) [12] were tested for nuclear import by inducing with a dose of 10 nM MFP. The off state was re-introduced by washing off ligand. The protein translocated back into the cytoplasm within 8 h of ligand removal. Reinduction with the ligand caused protein import into the nucleus. This shows that the localization of the chimeric protein with the import and export signals can be regulated via the ligand. The amount of protein localized in the nucleus on induction also depended on the strength of the NES. If the NES was too strong the import into the nucleus was lower. The construct with MAPKK NES showed more cytoplasmic localization in the uninduced state compared to the constructs containing HIV NES/Pep NES or PRB NES 2. All the four constructs showed that the fusion protein could be switched on or off via nucleocytoplasmic trafficking.

3.3. Nuclear localization of the fusion protein can only be directed by mifepristone (MFP)

The LBD attached to the protein switch construct has been shown to be specific to low concentrations of mifepristone in earlier studies [24]. The LBD used in these constructs is a 42 amino acid truncated version of the LBD from wt PRB, (amino acids 645–891). It was important to determine whether the protein switch constructs were truly non-responsive to natural progesterone and synthetic progestin (R5020). The effects of progesterone (Fig. 4A) and R5020 (Fig. 4B) in doses as high as 1000 nM were studied, and was found that neither drug could mediate the nuclear localization of these constructs. Normally, doses of R5020 or progesterone of 100 nM or higher can cause nuclear localization of wt PRB in as little as 30 min [21]. Therefore, both endogenous progesterone and a synthetic progestin such as R5020 do not have any effect on the import of the protein construct. One possible concern with MFP is that it can antagonize the inherent progesterone and glucocorticoid receptors found in mammalian cells. However, the concentration of MFP required for this antagonism is in the micromolar range [25,26]. Therefore, the nanomolar concentrations of MFP required for the protein switch will not cause any general endogenous antagonism.

3.4. Dependence of import characteristics on ligand concentration

Using live cell microscopy, pictures of nuclear localization of the fusion protein containing EGFP
A. MAPKK NES

Fig. 3. Off–on–off–on cycles of induction of constructs into the nucleus. Initially, no ligand is present (a). With the addition of ligand (b) the constructs translocate to the nucleus. The removal of ligand (c) results in translocation back to the cytoplasm within 8 h, and addition of ligand (d) causes translocation back to the nucleus.

B. HIV NES

C. 15aa peptide with KVFFKR motif

D. PRNES2

Fig. 3. Off–on–off–on cycles of induction of constructs into the nucleus. Initially, no ligand is present (a). With the addition of ligand (b) the constructs translocate to the nucleus. The removal of ligand (c) results in translocation back to the cytoplasm within 8 h, and addition of ligand (d) causes translocation back to the nucleus.
Fig. 4. MAPKK NES construct was tested for its response to progesterone and synthetic progestin R5020, by induction with 100 and 1000 nM of each for 2 h and studying import.

Fig. 5. Representative time dependent import and export studies of HIV NES constructs induced with 10 nM MFP. Only a few time points of the time lapse experiments are shown. Maximum relative import was achieved as early as 40 min after ligand induction. Export of most of this protein occurred after 8 h of ligand withdrawal (LW = ligand withdrawal).
Fig. 6. Import of MAPKK and HIV NES constructs with 1, 10, and 100 nM MFP. Import of the two proteins induced with 10, 100 or 1000 nM was studied for 2 h. Data is reported as mean ± S.D., n = 10. A p < 0.05 was obtained for the difference in rates between MAPKK NES induced with doses of 1 and 100 nM. A statistically significant difference (p < 0.05) was obtained for all the three doses tested for HIV NES (L = ligand, LW = ligand withdrawal).
were taken at time points 0, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 120 min to determine the kinetics of nuclear localization of constructs with MAPKK and HIV export signals. The import was studied over a period of 2 h, since after 2 h no further increases in import were seen (data not shown). The effects of MFP in doses of 1, 10, and 100 nM were studied and a representative example of 10 nM is shown in Fig. 5A. Relative intensity in the nucleus was plotted as a function of time for doses of 1, 10, and 100 nM MFP for the MAPKK and HIV NES constructs (Fig. 6A and B). The maximum relative intensities for the MAPKK NES construct were 0.11 at 1 nM, 0.24 at 10 nM, and 0.27 at a dose of 100 nM MFP. Maximum relative intensities for the HIV NES construct were 0.20 at 1 nM, 0.31 at 10 nM, and 0.38 at a dose of 100 nM of MFP, which were higher than the MAPKK constructs indicating that import is slower for a protein with a stronger NES.

The rate of nuclear import for both MAPKK NES and HIV NES constructs was MFP dose dependant. This was indicated by the steepness of the import curves for 1, 10 and a 100 nM in Fig. 6A and B. The amount of protein entering the nucleus was also dose-dependent as shown by the increase in maximum relative intensity on induction with 1 and 100 nM MFP. The import rate constants for MAPKK constructs were $0.139 \pm 0.0140$, $0.107 \pm 0.00765$, $0.421 \pm 0.267$ for the three doses, respectively. There was a significant difference in the rate constants for the higher doses ($p<0.05$, Kruskal–Wallis test with SNK correction for multiple comparison). The constructs containing the HIV NES also showed a significant difference in import rate constant. These import rates were $0.0547 \pm 0.00138$, $0.226 \pm 0.00815$ and $0.609 \pm 0.129$ for the three doses, respectively, which were all significantly different ($p<0.05$, Kruskal–Wallis test with SNK correction).

**A. MAPKK NES**

**1nM MAPKK export**

**10nM MAPKK export**

**B. HIV NES**

**1nM HIV export**

**10nM HIV export**

Fig. 7. Live cell export studies were conducted for MAPKK NES and for construct HIV NES induced with MFP 1 and 10 nM. Export of the two proteins was studied over a period of 24 h. Data is reported as mean ± S.D., $n=10$. No statistical difference ($p>0.05$) was found for the rate of export between 8, 12 and 24 h time points (L = ligand, LW = ligand withdrawal).
for multiple comparison). The constructs containing MAPKK NES showed an effect at the higher doses as the import rate constant with the dose of 100 nM was higher than that of 10 and 1 nM, but there was no significant difference found between the doses of 10 and 1 nM. Results also indicated that there was a 9-fold increase in the rate constant between the doses of 100 nM and 1 nM for HIV, while the MAPKK fusion proteins showed an 8-fold increase between the two doses. Thus, the strength of the NES played a role in determining the balance between the nuclear import brought about by the NLS and the inherent cytoplasmic localization due to the NES.

3.5. Dose of the ligand and time dependence of protein localization to the cytoplasm after washing

After inducing the cells for 2 h with MFP at a concentration of 1 or 10 nM, the export of MAPKK NES and HIV NES constructs were studied using live cell microscopy. Export was observed at time points of 8, 16 and 24 h (Fig. 7A and B) after hormone washout. Friedman repeated measures ANOVA was used to determine differences in export over time. While there was a significant change in export at the 8-h time point after washout \((p < 0.05)\), it was found that there were no significant differences in the cytoplasmic localization of the construct, between the 8-, 12-, and 24-h time points \((p > 0.05, \text{Friedman ANOVA})\). Thus, export is essentially complete by 8 h. These results demonstrate that high concentrations of the ligand lead to reduction in the rate of export of the protein. This study was carried out for only for 1 and 10 nM as these might be more relevant for clinical applications as compared to higher doses [25,26].

4. Discussion

This paper demonstrates the feasibility of bi-directional trafficking of a model protein. The constructs described here contain a nuclear export signal to cause the cytoplasmic localization of the protein in the off state, a nuclear import signal (NLS) that is responsive to ligand induction and a ligand binding domain (LBD) that confers responsiveness to MFP in the on state. The removal of the ligand results in the cytoplasmic or off state of the protein. The NES-NLS-LBD plasmid is transcribed into mRNA and translated into protein in the cytoplasm. In the absence of MFP, the export signal in the protein dominates and results in the cytoplasmic localization due to interaction with the export receptors (CRM1 or CRT). Induction with MFP causes the ligand binding domain and the ligand-inducible nuclear localization signals to take over. This results in a conformational change in the protein that allows it to translocate to the nucleus via the nuclear import machinery involving nuclear import receptors. Removal of ligand triggers protein export to the cytoplasm via its export receptor.

There have been reports of other systems that are capable of inducing gene expression of plasmids. In these cases, the inducing agent binds to response elements on DNA to promote gene expression. The inducing agents include heavy metal ions, heat shock, isopropyl beta-D-thiogalactoside (β Gal) and steroid hormones [27–29]. Other inducible systems include those controlled by antibiotics like tetracycline [30–32], novobiocin [33], rapamycin [34]. Ecdysone receptor-based gene regulation system has also been described [35,36]. Other types of induction systems have also been described [37,38].

Recently in a review by Lee and Hannick [39], it was mentioned that the “ability to sequester a protein or transcription factor in the cytoplasm, to allow its transport into the nucleus in response to specific stimuli, and then to remove it from the nucleus once the stimulus is removed, provides a powerful mechanism for the temporal regulation of gene expression” Similarly, the ability to change the subcellular localization of a therapeutic protein from a compartment where the protein is active to one where it becomes inactive has potential use in this era when gene therapy is at the brink of becoming a major player in therapeutics.

Researchers in the past have exploited signal sequences for unidirectional targeted delivery of genes or gene therapy products (proteins) [40]. However, specific, bidirectional control of proteins is the next phase in gene therapy. Optimally, bidirectional targeting of gene products would direct these proteins in a controlled manner by use of an external stimulus to a specific subcellular compartment where they are exclusively active, and removed from the compartment when the stimulus is removed. A regulatable fusion protein with the activation
domain of a steroid receptor co-repressor, NCoR, is currently being constructed. This can be used for controlled transcriptional repression of progesterone receptor B depending on its subcellular localization. On localization in the nucleus, it can repress progesterone-mediated gene transcription and removal from the nucleus on ligand withdrawal can abolish the repression.

There are many proteins in a cell whose functions are directed by means of regulated traffic between the active and inactive compartment. For example, steroid hormone receptors and many other transcription factors only exert their genomic effects when directed to the nucleus of cells. Once in the nucleus, these receptors can interact with chromatin and the transcription machinery, ultimately resulting in gene activation [41]. Localization controllable versions of proteins via a protein switch could be used to correct for mislocalization of endogenous proteins. For example in chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia, BCR-ABL proteins are expressed. BCR-ABL proteins have tyrosine kinase activity that leads to oncogenesis [27,42]. BCR-ABL proteins are oncogenic in the cytoplasmic compartment only, and must multimerize in order to be active. Introduction of ligand regulatable mutant versions of BCR-ABL for gene therapy should allow the clinician to direct the wild-type BCR-ABL proteins to the nucleus, resulting in loss of oncogenic activity. The use of the protein switch in this situation is the focus of future studies.

Other tumor suppressors that mislocalize to the cytoplasm in cancer cells include p53 and INI1 [43]. Similarly, the mislocalization of cell cycle inhibitors can be detrimental. Relocalization of a cell cycle inhibitor to the “wrong” compartment can lead to tumor progression. This occurs with the cell cycle inhibitor p21^WAF-1^ which, normally localizes in the nucleus where it exerts its inhibitory action. Mislocalization to the cytoplasm leads to tumor progression [44]. In addition to applications in cancer, controlled delivery of gene therapy products will be useful in chronic disease states that exhibit transient symptoms.

The protein switch described here can also function as a safety switch to control gene therapy with the inactive state being the default mechanism. The gene is introduced via gene therapy, but becomes active only when external ligand is added. At the first sign of negative side effects, gene therapy can be easily terminated by removing the protein from the active compartment. Another use of the protein switch is to reduce side effects due to long-term exposure of therapeutic agent when the protein is not continually activated. Pulsatile delivery of gene therapy products to the nucleus could reduce toxicity (analogous to intermittent dosing of highly toxic cancer chemotherapeutic agents). Chronic activation of target genes by the therapeutic gene product has the potential to activate other signal transduction pathways which may lead to detrimental results. Similarly, if chronic activation of the target gene triggered by the therapeutic agent is avoided, the likelihood of tolerance decreases.

Thus, the protein switch has numerous applications in the field of gene therapy where the control of the gene therapy product is beneficial in maintaining the safety and efficacy of the therapeutic gene.

5. Conclusions

A protein switch responsive to nanomolar doses of mifepristone (MFP) was created. This regulatable fusion protein was tagged with nuclear localization and export signals that caused the translocation of the protein between the active and inactive compartment in response to ligand induction. EGFP was used as a model protein for bi-directional trafficking. Stronger nuclear export signals tagged to EGFP caused predominant cytoplasmic localization as seen in Fig. 2a–c. Induction and removal of MFP caused on/off cycles of the MAKK NES, HIV NES, Pep NES and PRB NES2 protein constructs shown in Fig. 3A–D. These protein constructs were unresponsive to high doses of progesterone and synthetic progestin (R5020) and could only be induced with MFP (Fig. 4). Import rates and amounts of protein in the nucleus were dependent on MFP dose. Nuclear import occurred within 20–120 min depending on the dose of MFP used. Export of most of the protein into the cytoplasm occurred within a period of 8 h. Thus, a fusion protein capable of controlled trafficking between the active and inactive compartment that will be useful in regulatable gene therapy was constructed. In summary, the protein switch described here will allow for a higher level of regulation of gene therapy products by
exploiting and harnessing known import and export signals for use in drug delivery.

References


