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Model System to Study Classical Nuclear Export Signals

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ABSTRACT Signal-mediated protein transport through the nuclear pore complex is of considerable interest in the field of molecular pharmaceutics. Nuclear localization signals can be used to target genes/antisense delivery systems to the nucleus. [1] Studying nuclear export is useful in enhancing the expression and the efficiency of action of these therapeutic agents. The mechanism of nuclear import has been well studied and most of the proteins participating in this mechanism have been identified. The subject of nuclear export is still in the initial stages, and there is a considerable amount of uncertainty in this area. Two main export receptors identified so far are Exportin 1 (Crm1) and Calreticulin. Crm1 recognizes certain leucine-rich amino acid sequences in the proteins it exports called classical nuclear export signals. This paper describes a model system to study, identify, and establish these classical nuclear export signals using green fluorescent protein (GFP). Two putative export signals in the human progesterone receptor (PR) and the strongest nuclear export signal known (from mitogen activated protein kinase kinase [MAPKK]) were studied using this model system.

KEYWORDS: nuclear export, green fluorescent protein (GFP), progesterone receptor.

INTRODUCTION Peptidic nuclear localization signals (NLSs) are pharmaceutically relevant as they are highly utilized in gene and other types of drug delivery. In native proteins, NLSs are peptide sequences encoded by DNA that direct the protein to the nucleus of the cell. In the case of gene delivery, NLS peptide sequences are fused to pieces of DNA to be delivered to the nucleus, [2] or fused to cationic lipids or polymers that act as targeting agents or carriers for DNA delivery [3] to enhance delivery to the nucleus. Less well studied by the pharmaceutical field are nuclear export signals (NESs). Recently, however, NESs have been used as cytosolic targeting agents for oligonucleotide delivery. NESs fused to oligonucleotides can successfully target cytosolic mRNA. [4]

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Theoretically, NES fusions are useful for targeting of ribozymes, translational arrest, and disruption of stable mRNA structures, all of which occur in the cytoplasm. Additionally, in native proteins, NESs are critical for replication of proteins such as the retrovirus HIV. The potential utility of NESs as targeting agents and as pharmaceutical targets themselves has not yet been realized. Besides describing the barrier to nuclear import and export, the nuclear pore complex, classical nuclear import and export, and a new, nonclassical export pathway, this paper describes a model system to study, identify, and establish classical nuclear export signals using green fluorescent protein (GFP). Last, a search for an export signal for the hPR (Human progesterone receptor) using this model system is also described. Human PR is shown to contain a nonclassical NES and follows a novel export pathway that may be common to other steroid hormone receptors.

Nuclear Pore Complex

Eukaryotic cells are divided into 2 functionally distinct membrane-bound compartments, the nucleus and the cytoplasm. The nucleus is enclosed in a double membrane structure called the nuclear envelope (NE), which is perforated by nuclear pores that allow the bidirectional trafficking of macromolecules between the nucleus and the cytosol. These nuclear pores are formed by a large elaborate structure called the nuclear pore complex with a molecular mass of about 125 Mda, [5] composed of nearly 50 to100 different proteins called nucleoporins. The passive diffusion channel of the nuclear pore complex (NPC) is estimated to be around 9 nm allowing proteins smaller than 40 kd to passively diffuse through. Proteins greater than 40 kd to 60 kd usually undergo active nucleocytoplasmic transport, or facilitated nuclear transport across the nuclear pore complex. [6] This transport is a selective process that occurs because of the presence of certain specific transport signals in these macromolecules. [7]

Active transport requires energy and can take molecules with or against a concentration gradient. Facilitated diffusion, on the other hand, utilizes membrane protein channels to take molecules across the NPC, and can only take molecules down (not against) a concentration gradient. [8,9,10] For classical active transport, energy is usually provided by ATP (Adenosine triphosphate). [11] For import and export, however, energy is provided by Ran GTP [Ran Guanosine triphosphate]. [12] Some groups

refer to import/export of some proteins such as steroid receptors as facilitated diffusion instead. [13] Import receptor and export receptor mediated transport can transfer cargoes against a concentration gradient, which requires energy. This energy originates from the chemical potential of the Ran GTP gradient, which in turn is maintained by proteins like Ran GAP (Ran GTPase -activating protein) and Ran GEF (RCC1) (guanine nucleotide exchange factor). This gradient of Ran GTP and GDP (Guanosine diphosphate) upholds the directionality of transport by these transport receptors. The facilitated translocation process does not lead to translocation of cargoes against a gradient and thus is not directly coupled to nucleotide hydrolysis. These import and export processes require Ran GTP and it is not known whether the energy produced by GTP hydrolysis is used for protein translocation for the recycling of transport factors.

Nuclear Import and Export

Nuclear import of proteins occurs because of the presence of nuclear localization signals (NLSs), which are recognized by certain import receptors. The classical NLS contains a short stretch of amino acids (typically 4-8 amino acids) rich in positively charged amino acids like lysine and arginine. [14] Another type of nuclear import signal (called the bipartite type) is composed of 2 basic stretches separated by a 10 amino acid spacer. [15] These proteins containing NLSs bind to import receptors (importins a, ß) and are translocated into the nucleus. Binding of Ran GTP to the import receptor then separates this complex, discharging the protein in the nucleus.

Nuclear export of proteins, on the other hand, is thought to be brought about mainly by NES. These NESs are leucine-rich sequences of amino acids recognized by a soluble export receptor, known as Exportin1 or Crm 1. A possible consensus nuclear export sequence is $LX_{1-3}LX_{2-3}LXL$, where L = leucine and X = amino acid; the last leucine can be replaced by conservative substitutions (isoleucine, valine, etc) based on Bogerd et al [16] and Ikuta et al. [17] Nuclear export via leucine-rich nuclear export sequences occurs in a similar fashion as nuclear import, by the formation of a trimeric complex between the protein carrying the NES, Exportin 1 and Ran GTP in the nucleus. This trimeric complex is then transported out of the nucleus into the cytoplasm, where it dissociates and releases the protein in the cytosol. [18]

Conventionally, nuclear export has been studied via microinjection studies, wherein the protein whose export is being tested is injected into the nucleus and then its export out of the nucleus is followed. However, lately GFP technology has been widely used to study intracellular distribution and movement of a variety of different proteins. [19] These proteins labeled with GFP can be visualized directly in living cells and also retain their intrinsic cellular functions/activities. [20] A red shifted variant of GFP called enhanced green fluorescent protein (EGFP) shows increased translation efficiency and brighter luminescence in mammalian systems and is being used extensively. [21] The simplicity of using GFP is taken advantage of in this export model by tagging the putative nuclear export signals to EGFP and then observing their translocation using fluorescence microscopy.

For the purposes of this study, we were interested in testing putative NESs in the human PR, a steroid hormone receptor. Steroid hormone receptors are ligand-dependent transcription factors that regulate the expression of certain target genes in response to agonists and antagonists. [22] Most of these receptors are said to be in dynamic equilibrium between the nucleus and the cytoplasm and tend to accumulate within these compartments due to limitations in the overall rates of import and export. [23] The nuclear import of these receptors is caused by NLSs, but the mechanism of their nuclear export is still controversial. It is known that multiple pathways exist for protein import and export, and several hypotheses have been made regarding the rationale behind the nuclear export of steroid receptors, but there have been no definitive conclusions made. One hypothesis for the cytoplasmic localization of steroid receptors, which is currently under study, is that nuclear export signals may be involved in the export. PR is a member of the nuclear receptor super family and exists as 2 isoforms, A and B. PRA is a 164-amino acid truncated form of PRB and is completely localized in the nucleus as compared with PRB, which is present in both the nucleus and the cytoplasm. There were 2 putative NESs found in PRB, 1 was present in the N-terminal domain of PRB and absent in PRA, and the other was found in the region common to both the A and B isoforms.

MATERIALS AND METHODS

Plasmid constructions

pEGFP-PRB and pEGFP-PRA:

For pEGFP-PRB, oligonucleotides 5'-GTCCTCGAGCGTTGACTGAGCTGAAGGCAAAGGG-3' (changed the *Mlu I* start site of pGFP-PRB to *Xho I*) and 5'-ACGCGTCGTCCTTGGGCTCGGCGTCGGGCGGG-3' (contains an *Mlu I* site) were used to PCR [Polymerase chain reaction] out a 1130-base pair (bp) fragment. After digestion with *Xho I* and *Mlu I* , the resulting 1130-bp fragment was isolated via gel purification and used below. For pEGFP-PRA, oligonucleotides 5'-GTCCTCGAGCGTTGAGCCGGTCCGGGTGCAAGGTTGG-3' (changed the *Mlu I* start site of pGFP-PRA to *Xho*

I) and 5'-ACGCGTCGTCCTTGGGCTCGGCGTCGGGCGGG-3' (contains a *Mlu I* site) were used to PCR out a 630-bp fragment. After digestion with *Xho I* and *Mlu I* , the resulting 630-bp fragment was isolated and used below.

pGFP-PRB was digested to completion with *Kpn I* and partially digested with *Mlu I* . The longest *Mlu I-Kpn I* fragment was inserted into pGL3 basic (Promega, Madison, WI) cut with *Mlu I* and *Kpn I* , resulting in the plasmid pGL3-PR5. pGL3-PR5 was then cut with *Xho I* and *Mlu I* and ligated with either the 1130-bp PCR fragment (for PRB) or the 630-bp PCR fragment (for PRA) resulting in the plasmids pGL3-PRB and pGL3-PRA, respectively. pGL3-PRB and pGL3-PRA were cut with *Xho I* and *Kpn I* and inserted separately into a *Xho I-Kpn I* cut EGFP-C1 vector, resulting in pEGFP-PRB and pEGFP-PRA plasmids.

pEGFP-EGFP

pEGFP-C1 (Clontech, Palo Alto, California) was digested with *AgeI* and *XmaI* to give a 790-bp EGFP fragment. pEGFP-C1 was digested with *AgeI* and CIP treated, and then the 790-bp fragment was inserted into it, resulting in the plasmid pEGFP-EGFP.

pEGFP-PRNES1

To create pEGFP-PRNES1, oligonucleotides 5'-TCGAGGGCTGCTGGACAGTGTCTTGGACACTCTGTTGGCGGGTAC-3' and 5'-CCGCCAACAGAGTGTCCAAGACACTGTCCAGCAGCCC-3' encoding the first putative NES in PR were synthesized (DNA and peptide resource, University of Utah, Salt Lake City) with *Xho I* and *Kpn I* ends. After annealing, the oligonucleotides were ligated into a *Xho I-Kpn I* digested pEGFP-C1 (Clontech) vector using T4 DNA ligase, such that the insert was downstream to the C-terminus of the EGFP.

To construct the chimera pEGFP-EGFP-PR NES1, pEGFP-PRNES1 was digested with restriction enzyme *Age I* and CIP treated. The vector pEGFP-C1 was also cut with *Xma I* and *Age I* and the resulting 790-bp fragment was ligated to *Age I* digested plasmid above.

The pEGFP and pEGFP-EGFP chimeras containing the second PRNES2 were also constructed similarly using the oligos 5'-TCGAGCTCTGCACGACCTGGTCAAGCAGCTGCACCTGGGTAC-3' and 5'-CCAGGTGCAGCTGCTTGACCAGGTCGTGCAGAGC-3' encoding the second putative NES in PR.

pEGFP-PRA-PRNES1 was constructed by digesting pEGFP-PRA with *BspEI* and *BglII* , collecting the larger fragment, and ligating the oligonucleotide 5'-GATCTCGCCAACAGAGTGTCCAAGACACTG TCC AGC AGT-3' 5'-CCGGACTGCTGGACAGTGTCTTGGACACTCTGTTGGCGA-3' encoding for PRNES1 with cohesive ends.

pEGFP-NF1-PRNES1 was constructed by digesting the vector pEGFP-NF1 [7] with *XhoI* and *KpnI* and inserting a sequence encoding for PR-NES1 5'-TCGAGGGCTGCTGGACAGTG TCTTGGACACTCTGTTGGCGGGTAC-3' 5'-CCGCCAACAGAGTGTCCAAGACACTGTCCAGCAGCCC-3' with cohesive ends with the digested vector.

pEGFP-NF1-MAPKKNES, pEGFP-MAPKKNES, and pEGFP-EGFP-MAPKKNES were constructed with a similar ligation scheme as above except that MAPKKNES 5'-TCGAGGCT TCAAAAAAAACTTGAAGAACTTGAACTTGACGGTAC-3' and 5'-CCGTCAAGTTCAAGTTCTTCAAGTTTTTTTTGAAGCCC-3' was ligated instead of PRNES. pEGFP-PRA-MAPKKNES was constructed by digesting pEGFP-PRA with *BglII* and *BspEI* , collecting the larger fragment and inserting the oligo 5'-CCGGACTTCAAAAAAAACTTGAACTTGAACTTGACA-3' 5'-GATCTGTCAAGTTCAAGTTCTTCAAGTTTTTTTTGAAGT-3'.

Cell Lines and Cell Culture

For this study 1471.1, a C-127 derived murine mammary tumor line was used. [24] Cells were grown in DMEM (Dulbecco's modified eagle's medium; GIBCO BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), penicillin streptomycin (100 U/mL and 100ug/ml, Hyclone), gentamycin (0.5 mg/mL, GIBCO BRL) and L-glutamine (2 mM, Hyclone) in a 5% CO_2 incubator (VWR, model 2300, West Chester, PA) at 37°C.

Transfections

1471.1 cells were split 1:5 the day before tranfections and were harvested at nearly 70% to 80% confluency. Cells (5 x 10^6) were transfected with 2 µg of the following plasmids pEGFP-C1, pEGFP-EGFP, pEGFP-PRNES1, pEGFP EGFP-PRNES1, pEGFP-PRA-PRNES1, pEGFP-NF1-PRNES1, pEGFP-PRNES2, or pEGFP EGFP-PRNES2 and 8 µg of pGL3 basic as carrier DNA. Transfections were performed using an Electrosquare porator ECM 830 Electroporation system (BTX, San Diego, CA) at a voltage of 135 V, 10 milliseconds, and 3 pulses in a total of 100 µL cold plain DMEM. After a 5- to 10-minute recovery on ice, the electroporated cells were diluted with phenol red-free DMEM [10% FBS charcoal/dextran treated (Hyclone), L-glutamine, penicillin-streptomycin, and gentamycin] and plated on a clear cover glass (no. 1, 22 mm^2, Corning, Action, MA) in 6-well plates. These cells were then incubated in a 5% CO_2 incubator (VWR, model 2300, Pittsburg, U.S.A) at 37°C for 18 hours to 24 hours. The cells were then rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde and inverted onto microscope slides and sealed with clear nail polish. Cells near the edges of the slides were avoided.

Microscopy

Fixed cells were viewed using a Zeiss Axioplan Confocal Microscope (LSM 510) (Thornwood, New York) using an argon laser line (excitation 488 nm) and an emission

filter (LP505). All images (see **Figures 1 - 6**) were obtained from cells transfected with plasmid constructs as indicated in the figure legends.

RESULTS AND DISCUSSION Most earlier experiments on nuclear export involve techniques such as microinjection and indirect immunofluorescence. Microinjecting requires specialized equipment, cells large enough to microinject, and purification of test proteins. Indirect immunofluorescence can also be problematic, requiring the use of antibodies, fixation, etc, which may introduce artifacts into the system.

A simpler model system, which can be used easily to identify nuclear export signals, was designed. The strongest nuclear export signal known is that from mitogen activated protein kinase kinase (MAPKK). [25] This NES was tested for its ability to bring about the cytoplasmic localization of a protein that is not localized preferentially either in the nucleus or the cytoplasm. EGFP, which is easily detected by fluorescence microscopy, seemed ideal for this purpose. EGFP does not contain NESs or NLSs, so it is an ideal "neutral" protein, which localizes everywhere (nucleus and cytoplasm) in cells. MAPKKNES was tagged to EGFP and the localization of the EGFP-MAPKKNES protein was examined using confocal microscopy. While mostly cytoplasmic, some EGFP-MAPKKNES was present in the nucleus of some cells (**Figure 1**), indicating that EGFP-MAPKKNES might be small enough to passively diffuse through the NPC . Indeed, the overall size of EGFP-MAPKKNES is only about 28.5 kd, possibly small enough for passive passage through the NPC. In other words, export may occur via an NES, but influx back into the nucleus could theoretically occur passively due to the small overall size of the protein. As seen in **Figure 1** , there is some EGFP-MAPKKNES localizing in the cytoplasm. The overall distribution of this protein is mainly cytoplasmic, but the small amount in the nucleus of some cells could be explained by passive entry into the nucleus. If EGFP-MAPKKNES was a bit larger, we hypothesize that this passive reentry into the nucleus could have been prevented. To solve this problem, constructs with 2 EGFP motifs were prepared and their localization tested (nearly 55.5 kd including the NES). In this case, EGFP-EGFP-MAPKKNES (**Figure 1**) was found to be more definitively cytoplasmic.

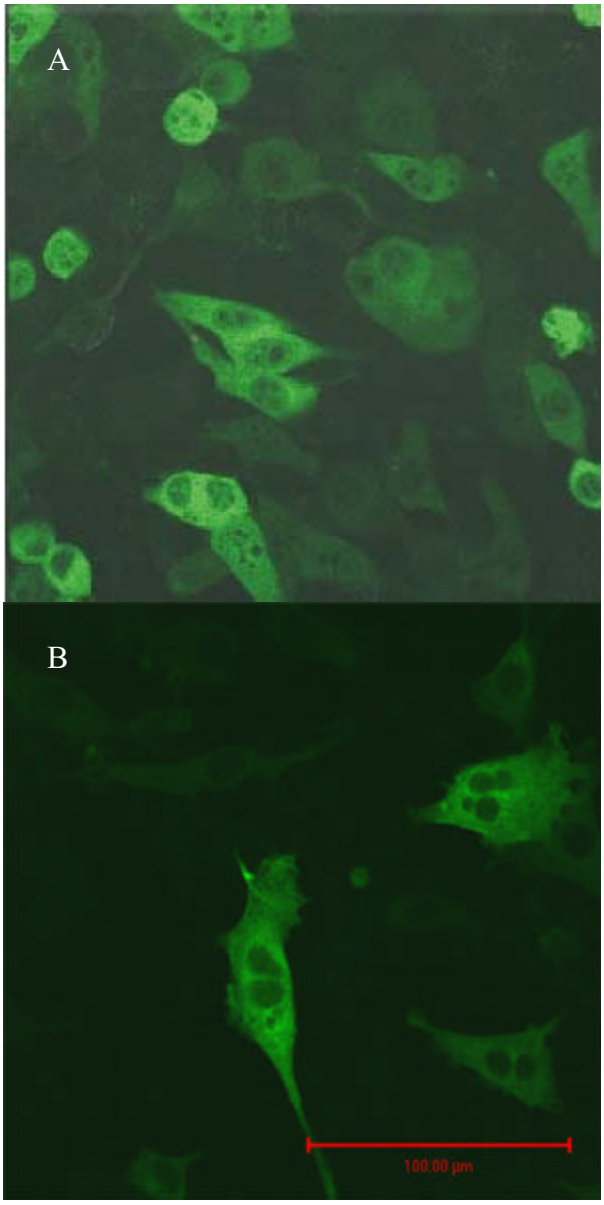

Figure 1. A) EGFP-MAPKKNES was mainly cytoplasmic in most cells, which indicates the functionality of the NES. However, it was also present in the nucleus and cytoplasm of some cells indicating that the chimera might be small enough to diffuse through the NPC. B) To overcome this problem a double motif of EGFP: EGFP-EGFP-MAPKKNES was constructed and transfected. It was found to be completely cytoplasmic showing that MAPKKNES acts as true NES, and that the presence of the extra EGFP motif made the construct too large to diffuse through the NPC. Both are flourescent images.

To determine whether a nuclear export signal can bring about the cytoplasmic localization of a nuclear protein, EGFP-tagged nuclear proteins were constructed with an NES. In the first case, EGFP-tagged PRA (a predominantly nuclear protein) was subcloned to MAPKKNES (total size of protein, 28.5 kd). Also, another nuclear protein, EGFP-tagged NF1, which is a nuclear transcription factor, was subcloned to MAPKKNES (total size of protein, 84 kd). EGFP-NF1 itself is completely nuclear. [19] Upon transfection of these constructs,

cytoplasmic localization of the nuclear proteins was seen (**Figure 2**), indicating that the strongest known NES to date is capable of exporting normally nuclear proteins (PRA and NF1) into the cytoplasm. These experiments were testing the relative strengths of the nuclear localization signals (of PRA and NF1) and the export signal (MAPKKNES) (**Figure 3**).

alanine in this position is a fairly benign substitution. There are presently no studies that show that an alanine in this last position is detrimental to nuclear export. As shown in **Figures 3** and **4** , NES1 or NES2 attached to EGFP or EGFP-EGFP show that these NESs do not function as export signals. When attached to nuclear proteins such as PRA or NF1, NES1 has no impact on the export of these proteins (**Figure 6**).

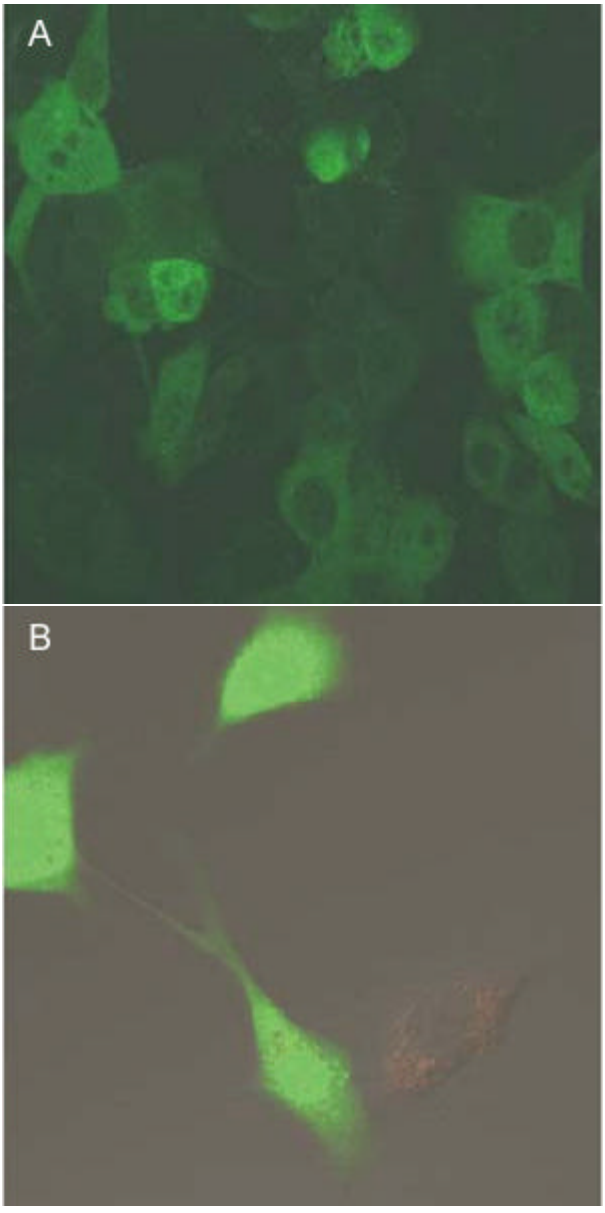

Figure 2. A) EGFP-PRA-MAPKKNES was seen to localize in the cytoplasm in most of the cells, showing that the MAPKKNES is strong enough to overcome the NLS of PRA and bring it to the cytoplasm (see Figure 3 for comparison to EGFP-PRA). Flourescent image. B) Another nuclear protein, NF-1, a transcription factor was fused to MAPKKNES. EGFP-NF1-MAPKKNES was nuclear and cytoplasmic, indicating that MAPKKNES was strong enough to overcome NF1's NLS to some extent. Flourescent image superimposed over differential interference contrast (DIC) image.

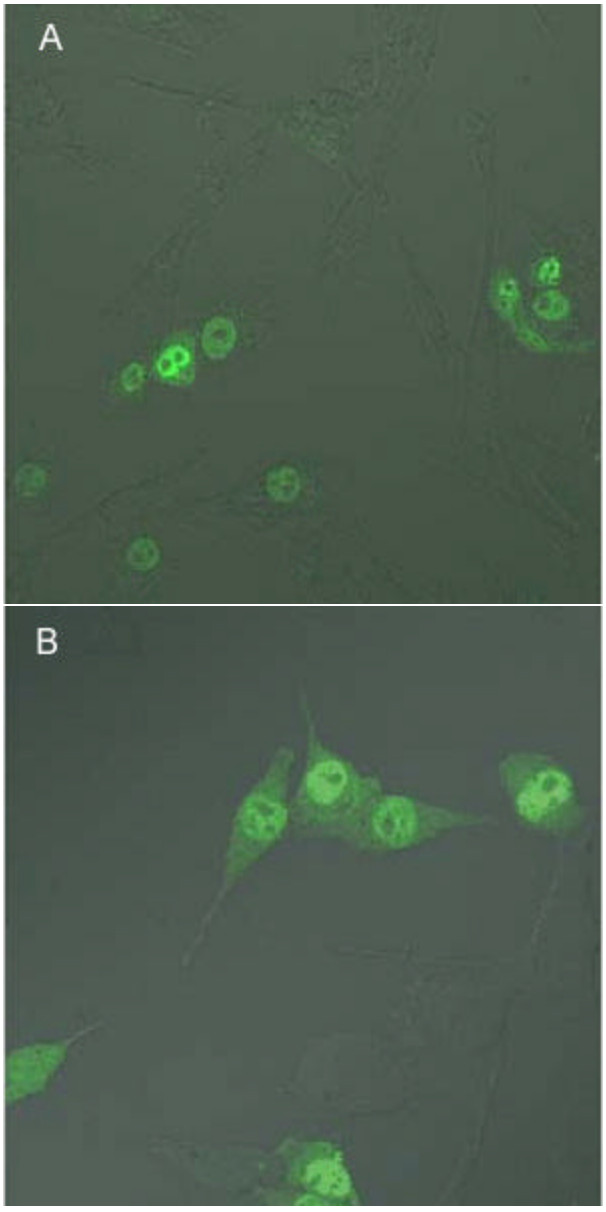

Figure 3. This Figure shows the subcellular localization of PRA and B. A) EGFP-PRA is a predominantly nuclear protein with very little cytoplasmic localization while in B) EGFP-PRB is found to localize both in the nucleus and the cytoplasm. Both are flourescent images superimposed over differential interference contrast iamges.

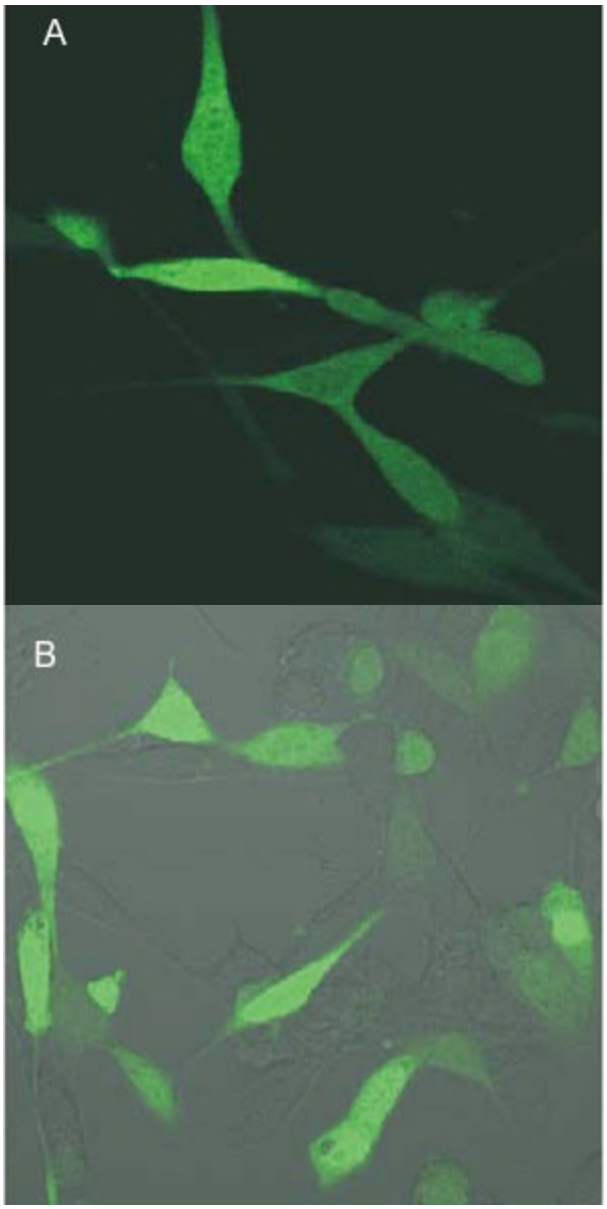

Figure 4. A) EGFP-PRNES1 did not seem to have a preferential localization and the accumulation pattern was identical to that of EGFP alone (both nuclear and cytoplasmic), showing that NES 1 did not have any effect. Flourescent image. B) On attaching another EGFP motif; EGFP-EGFP-PRNES1 still localized both in the nucleus and the cytoplasm further indicating that NES1 probably does not act like a true NES (compare the localization of EGFP-EGFP-PRNES1 with EGFP-EGFP [Figure 7]). Flourescent image superimposed over differential interference contrast (DIC) image.

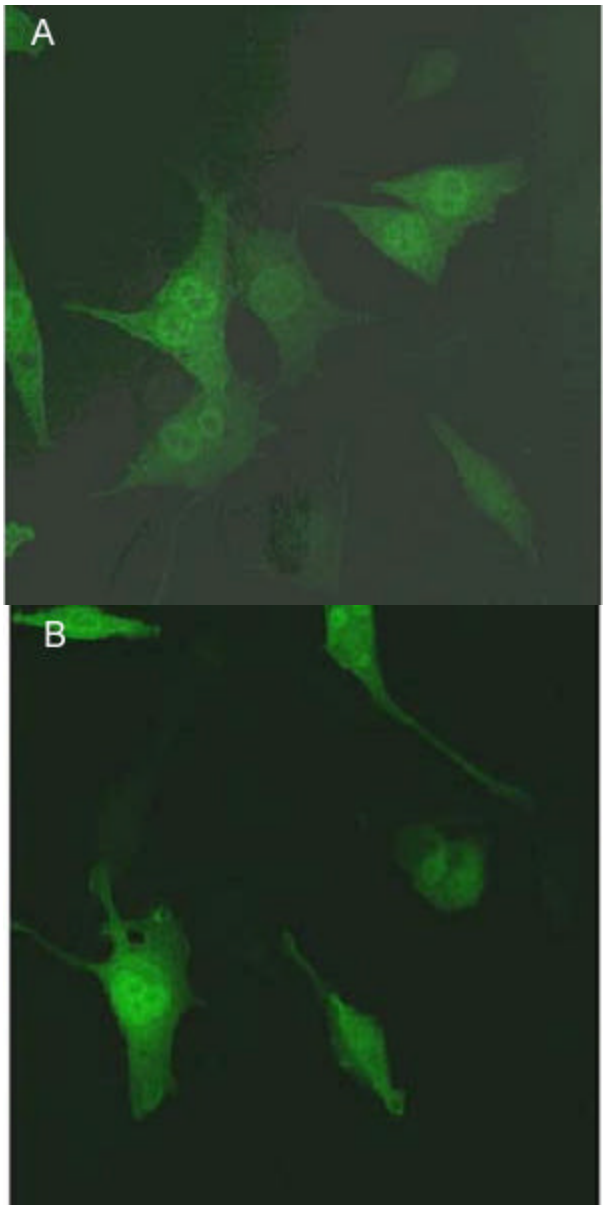

Figure 5. A) EGFP-PR-NES2 in spite of being completely homologous to the consensus sequence of a classical NES does not seem to cause the cytoplasmic localization of EGFP as seen with MAPKKNES suggesting that NES2 also does not act as a true NES. Flourescent image superimposed over differential interference contrast (DIC) image. B) Similarly, EGFP-EGFP-PRNES2 also showed the same localization pattern as EGFP NES2, being present in both the nucleus and cytoplasm, confirming that NES2 does not act as an export signal. Flourescent image.

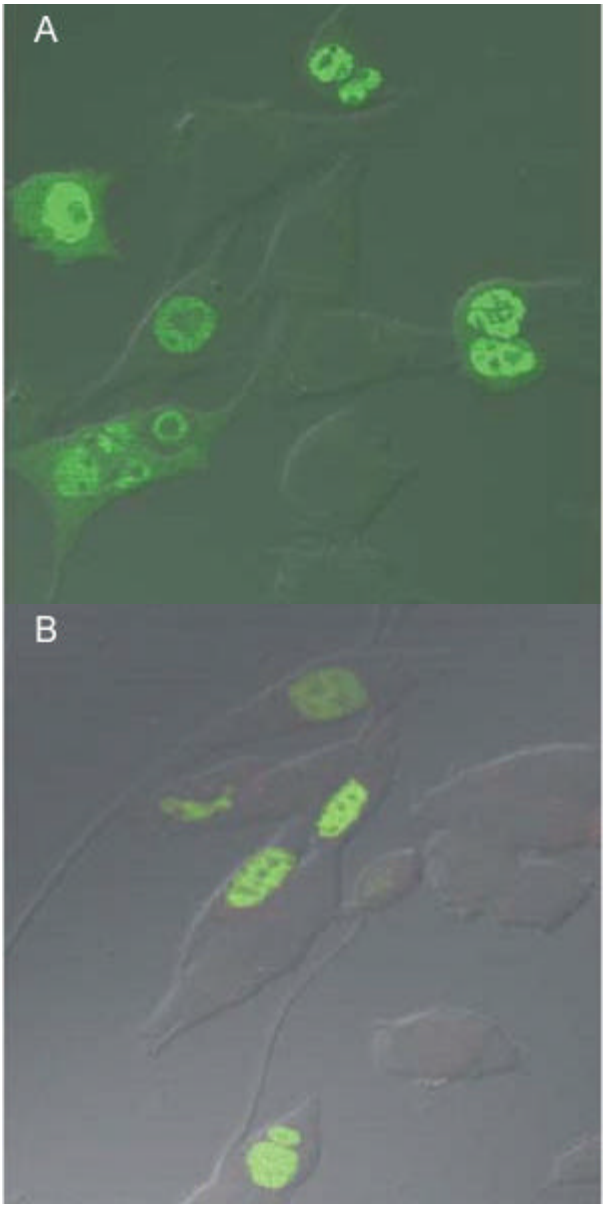

Figure 6. A) EGFP-PRA-PRNES1 was present mostly in the nucleus showing that NES1 was not able to bring it to the cytoplasm and similarly for the other nuclear protein, on fusing NF-1 with NES1. B) EGFP-NF1-PRNES1 was completely nuclear and on comparison with EGFP-NF1-MAPKKNES (Figure 4) suggests that NES 1 is not a true NES. Both are flourescent images superimposed over differential interference contrast (DIC) images.

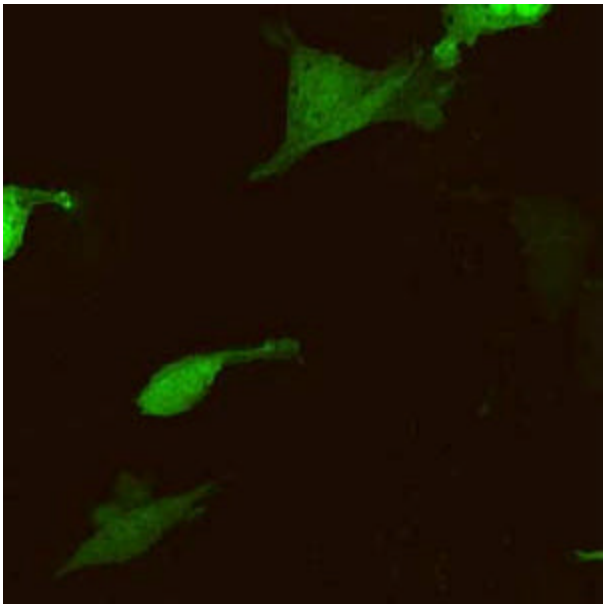

Figure 7. EGFP-EGFP (control) localizes both in the nucleus and the cytoplasm showing that in spite of being larger than the passive diffusional limit, without an NES it localizes in both nuclear and cytoplasmic compartments.

Our experiments showed that neither NES1 nor NES2 functioned independently as a true NES. Recently published data [26] further confirm our findings that classical NESs that mediate export via the Exportin1 receptor are not involved in the export of the progesterone receptor. While the consensus sequence suggested by Henderson and others is a starting point ($LX_{1-3}LX_{2-3}LXJ$), the authors do show that some motifs that fit the NES consensus do not function as NESs. [25] The spacing and the nature of the "wobble" amino acids (X) are currently hard to interpret. While there does not seem to be any preference in these amino acids, apparently this must not be the case. There are only a handful of known NESs to date that both fit the above consensus and do indeed function as NESs. As more NESs that fit the consensus are found, a pattern for the "X" amino acids may emerge.

The fact that NESs do not neatly fit into 1 consensus underscores the need for a model system to test NESs. Henderson and others present a similar system; however, in their system, relative NES strengths are compared with GFP-REV, with REV containing its own NLSs. EGFP-EGFP is perhaps a more "neutral" system. EGFP has been shown, in many cases, to be innocuous when subcloned onto other proteins of interest. Therefore, the utility of this model system is that any putative classical NES can be tested for its ability to act as an export signal.

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