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Research Paper

Signal Sequences for Targeting of Gene Therapy Products to Subcellular Compartments: The Role Of CRM1 in Nucleocytoplasmic Shuttling of the Protein Switch

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Purpose. The purpose of this study was to understand the mechanism of nuclear export of the protein switch, used for controlled intracellular delivery of gene products, by studying the involvement of classical export receptor CRM1.
Method. Transient transfections of protein switch constructs, isolated nuclear export and import signals were carried out. Effect of leptomycin B (inhibitor of export receptor) and geldanamycin (inhibitor of Hsp90) on localization of these constructs was studied using fluorescence microscopy. Putative nuclear export signals in the glucocorticoid and progesterone receptor ligand binding domains were identified and studied.
Results. It was observed that treatment with leptomycin B caused nuclear accumulation of the protein switch constructs. However, geldanamycin did not have any pronounced effect on the localization. The isolated nuclear export signal from glucocorticoid receptor localized mostly in the cytoplasm, while its mutated version was present everywhere.
Conclusion. The localization controlled protein switch constructs are exported out of the nucleus by the classical CRM1 receptors. The ligand binding domain of these protein switch constructs plays an important role in maintaining these constructs in the cytoplasm in the absence of ligand, as well the re-export back to the cytoplasm from the nucleus after ligand washout.

KEY WORDS: controlled localization; CRM1; export signal; import signal; ligand binding domain; signal sequences.

INTRODUCTION

Signal Sequences

Signal sequences have been utilized for precise drug delivery, targeting drugs to specific cellular compartments. Known signal sequences are in part defined by their ability to confer localization to a particular compartment, even when taken out of context of the whole protein. Perhaps the best known example is the use of nuclear import signals (NLSs) for delivery of drugs (such as DNA or proteins) to the nucleus (1). The prototype NLS comes from SV40 large T antigen and consists of a short stretch of basic residues (PKKKRKV) (2,3). Zanta *et al*. used the NLS sequence PKKKRKVEDPYC and irreversibly linked this single NLS to one end of a gene to enhance delivery of DNA by 10 to 1,000 fold (in fibroblasts, hepatocytes, and epithelial carcinoma cells) (4). On the other hand, nuclear export signals (NESs) are leucine rich and can be used to direct proteins/DNA to the cytoplasm of cells. For nuclear export signals, we have previously noted a leucine-rich "consensus" sequence of $LX_{1-3}LX_{2-3}LXJ$, where L = leu, X is any residue, and J = leu, val, or ile (5). However, since exceptions to this exist, this "consensus" may unnecessarily eliminate NESs that do not exactly fit this sequence. A more detailed examination of NES signals has been conducted by la Cour *et al*. which takes into account spacing and positioning of hydrophobic residues (6). These authors have made a NES database and prediction server publicly available (NetNES 1.1). This database compiled 67 high-confidence NESs validated in the literature experimentally. NESs have been attached to oligonucleotides for successful delivery to the cytoplasm (7). Similarly, the M9 shuttling (import/export) signal sequence attached to cationic peptides have been used as a delivery system for plasmid DNA (8). More recently, Tat peptides (derived from HIV-1 transactivating factor Tat), also known as "cell penetrating

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ABBREVIATIONS: CRM1, Chromosome maintenance region 1 protein, also known as exportin 1; EGFP, Enhanced green fluorescent protein; GA, Geldanamycin; GR, glucocorticoid receptor; Hsp90, Heat shock protein 90; LBD, Ligand binding domain; LMB, Leptomycin B; NES, Nuclear export signal; NLS, Nuclear import signal; PR, Progesterone receptor.

peptides" or "protein transduction domains" (9) have been used for translocation across the cell plasma membrane into the cytoplasm (10). Tat peptides, like NLSs, are basic in nature but slightly longer; the prototype sequence is RKKRRQRRR (9).

Besides the nucleus and cytoplasm, other subcellular compartments can be targeted as well *via* conserved amino acid sequences or motifs (for a review, see (11)). Proteins can also be targeted to the *nucleolus* (12) *via* a RRRANNRRR motif; the *mitochondria via* an amino acid amphipathic alpha helix with positive residues clustered on one side, and polar residues clustered on the other side (13,14); the *trans-golgi* network (15) *via* a signal consisting of a di-leucine motif followed by two acidic clusters; *lysosomes and endosomes* (16) *via* tyrosine-based sorting signals that conform to the NPXY or YXX consensus motifs or di-leucine-based [DE]XXXL[LI] and DXXLL consensus motifs; *peroxisomes via* the SKL signal sequence at the C-terminus (13); the *endoplasmic reticulum via* the well-known KDEL endoplasmic retention signal (17,18) and others.

Signal Sequences: Functional Purpose

Proteins are directed to particular organelles for a functional purpose. For example, steroid hormone receptors only exert their genomic effects when directed to the nucleus of cells (19). Besides certain proteins that only function in the nucleus, there are other proteins that function in other cellular compartments. If these proteins are not in their "correct" compartments, disease may ensue. Correction of this mislocalization could be used for therapy of these diseases. We have previously identified a number of proteins whose mislocalization causes disease (particularly cancer) (11) and illustrate here one example for each organelle listed above.

FOXO protein, a member of the Forkhead family of transcription factors, normally localizes in the nucleus of cells. When mislocalized to the cytoplasm, FOXO cannot activate the tumor suppressor PTEN, resulting in cancer (20). The mislocalization of the transcription factor NF-κB in the converse direction, from the cytoplasm to the nucleus, has been implicated in cancers as well (21,22). The protein nucleophosmin's mislocalization from the nucleolus to the cytoplasm has been implicated in acute myelogenous leukemia (23). Mislocalization of the copper transporting protein ATP7B from the trans-golgi network to the endoplasmic reticulum leads to Wilson's disease (fatal if untreated) (24,25). In late-onset familial and sporadic Alzheimer's disease, ApoE4 protein mislocalizes from the ER to the mitochondria, resulting in increased amyloid fibril formation (26,27). In Barth syndrome, tafazzin protein is mislocalized from the inner and outer mitochondrial membranes, to the mitochondrial matrix (28). Recent work has implicated defects in lysosomal localization of TRPML3 protein in mucolipidosis type IV (29). Lastly, the mislocalization of an aminotransferase protein from the peroxisome (to the mitochondria) leads to primary hyperoxaluria (30).

Using Signal Sequences for Therapy

The next logical phase in gene therapy is the controlled targeting and delivery of plasmid (gene) products. At present, most gene therapy strategies are concerned with *delivering* the gene of interest encoded by a plasmid, into the cell. Once the plasmid gets into the nucleus, the cell takes over, the protein is expressed and transported it to its final destination. What happens once the plasmid is expressed (after delivery into the cell) has not been fully explored. In standard gene therapy, once the plasmid is delivered to the nucleus of cells, the protein that gets expressed presumably contains signals encoded in its amino acid sequence that direct it to the correct compartment.

Signals have been routinely used for unidirectional targeting of a drug (be it an oligonucleotide, polymer or plasmid) to a final compartment (1). We have previously described a bidirectional protein switch whose subcellular localization is controlled by ligand (5). The protein switch consists of an NES, an NLS, and ligand binding domain (LBD). LBD confers responsiveness to ligand. The addition of ligand causes translocation of the protein switch to the nucleus, just as addition of ligand to steroid receptors causes nuclear translocation (19,31,32). Removal of ligand causes the protein to translocate back to the nucleus. The protein switch is constructed as a plasmid encoding the corresponding DNA. EGFP is added as a marker to follow the expression of the plasmid in living cells, but any other protein could be substituted.

As shown in Fig. 1, once expressed in cells, the NES in the protein switch predominates, and the protein localizes in the cytoplasm of cells. Upon addition of ligand, the NLS predominates, and the protein translocates to the nucleus. Removal of ligand causes the protein to come back out to the cytoplasm (5). Leucine-rich NESs are recognized by CRM1 (exportin 1), the classical export receptor (33,34). The formation of a CRM1-cargo complex requires RanGTP, and the export by CRM1 is saturable (34–36). On the other hand, NLSs are imported into the nucleus by the importin α/β

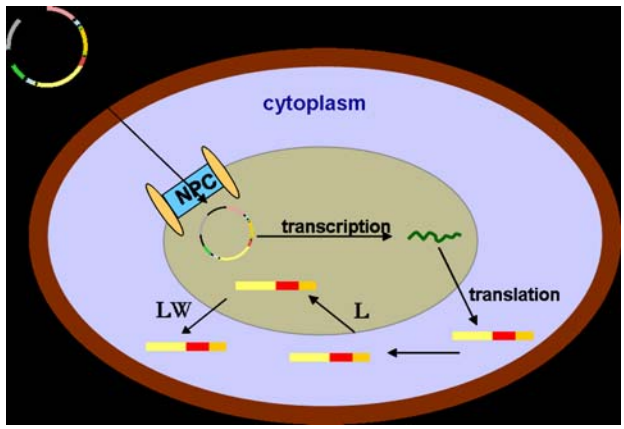

Fig. 1. The protein switch: NES-NLS-LBD system. The ligand directs the translocation of the fusion protein, which contains the gene of interest. The NES-NLS-LBD plasmid (*circle*) gets transcribed into mRNA (*squiggle*), and translated into protein (*rectangle*) in the cytoplasm. In the absence of ligand, the export signal in the protein dominates, which retains the protein in the cytoplasm by interaction with the export receptor, CRM1. In the presence of ligand, a change in the conformation of the LBD occurs that allows translocation to the nucleus. Removal of ligand from the external environment by washing leads to diffusion of ligand out of the nucleus of the cell following the concentration gradient. The removal of ligand results in protein export to the cytoplasm again

heterodimer. Importin α contains the NLS binding site, whereas importin β mediates the translocation through the nuclear pore (34,37). Ran, a small GTPase, which converts from RanGDP to RanGTP in the nucleus, plays a critical role in both import and export. Importins respond to the RanGTP-GDP gradient which allows for the transport of cargo proteins to the nucleus (34).

The utility of the protein switch is to control where the protein goes (which subcellular compartment) as well as to control the amount of protein delivered to that compartment. Localization controllable versions of proteins would be used to correct for mislocalization of endogenous proteins. As the engineered protein switch version of the protein has controlled localization and therefore activity, this can overcome the aberrant localization of the endogenous, malfunctioning protein.

This protein switch system has wide applicability to the study of many basic biomedical problems, especially disease states involved in complex cascades or signal transduction pathways. Ligand-controllable proteins can be utilized in a number of exciting new and useful ways: to independently regulate genes, for artificial control of transcription, for gene therapy applications, and for biosensor arrays (38).

The purpose of this study was to delineate the export mechanism of the protein switch. Two possible mechanisms of export of the protein switch were studied: export by CRM1, the classical export receptor (33,34), and export by Hsp90, a chaperone protein involved in cytoplasmic retention of steroid receptors (39,40). Export by CRM1 is likely since the protein switch contains a known NES, which is targeted for export by CRM1. Export by Hsp90 was also a possibility, since Hsp90 interaction with steroid receptors occurs in part *via* the LBD (41), which is also present in the protein switch.

Using inhibitors of CRM1 (leptomycin B, which specifically binds to CRM1 (42)) and Hsp90 (geldanamycin, known to tightly bind to Hsp90 complexes (43)), we show that export of the protein switch is mediated primarily by CRM1. Interestingly, we also have identified potential NESs in the ligand binding domain of the protein switch that may also contribute to its cytoplasmic localization.

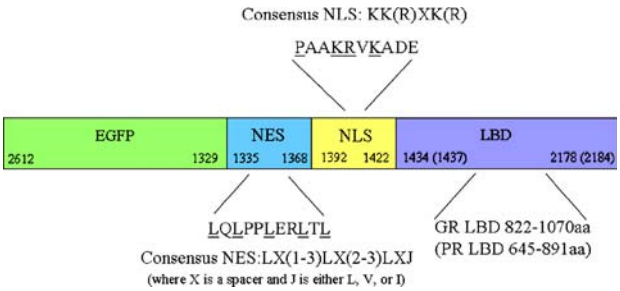

Fig. 2. Protein switch construction. The protein switch consists of NES, NLS, and LBD attached to EGFP (or any protein of interest). Numbering in boxes refers to base pairs. The NES is from HIV rev protein (50); the NLS is from Myc A8 protein (3), and the LBD is either the rat C656G version of the glucocorticoid receptor LBD (responsive to dexamethasone) (45) or a truncated progesterone receptor LBD (responsive to mifepristone) (44). GR LBD and PR LBD numbering obtained from NCBI Entrez Protein accession number AAB07866 and NP000917, respectively

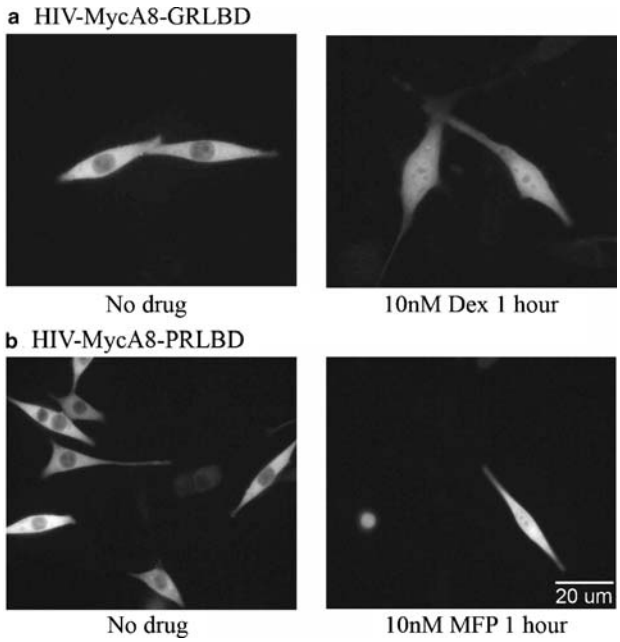

Fig. 3. Change in localization of protein switch with ligand induction. **a** HIV-MycA8-GRLBD constructs were either untreated (no drug) or treated with a 10 nM dose of dexamethasone for one hour. **b** HIV-MycA8-PRLBD constructs were either untreated (no drug) or treated with a 10 nM dose of mifepristone for one hour. All experiments were repeated in triplicates (n=3) and ten cells were analyzed for each time-point in each experiment

METHODS

Protein switch constructs were made by cloning a NES from HIV-rev protein, a classical SV40 T-antigen type NLS from MycA8, and a steroid receptor LBD in pEGFP-C1 mammalian expression vector (5). Two different constructs were made by utilizing a truncated version of wild type progesterone receptor LBD (44) and mutated version of GR LBD (C656G) responsive to mifepristone and dexamethasone, respectively (45,46). See Fig. 2 for a schematic.

Cell Culture

The murine adenocarcinoma cell line 1471.1 was used to study localization of protein switch constructs as described previously (5). Monolayers of cells were grown in 175 cm^2 flasks containing DMEM (GIBCO BRL, Grand Island, NY) fortified with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% penicillin–streptomycin (10 U/ml, GIBCO BRL), 0.1% gentamycin (0.5 mg/ml, Hyclone), and 1% L-glutamine (2 nM, Hyclone). Cells were maintained in a 5% CO_2 incubator at 37°C.

Transient Transfections

Transient transfections of 1471.1 cells with protein switch plasmid DNA were carried out using electroporation as previously described (5,40,47). Transfected cells were plated on either a coverglass (Corning no. 1, 22 mm^2), in six-well plates or plated directly into live cell chambers (Lab-tek II chamber cover glass system, Nalge Nunc International, Naperville, IL) containing phenol-free DMEM fortified with

10% charcoal/dextran treated (hormone-free) fetal bovine serum (Hyclone) and antibiotics as described above. Cells were maintained in a 5% CO_2 incubator at 37°C overnight before observing localization.

Microscopy

Approximately 18–24 h after transfections, localization of protein constructs was observed as before (5,40,47) using an Olympus 1X701F inverted fluorescence microscope (Scientific Instrument Company, Aurora, CO) with a high-quality narrow band GFP filter (excitation HQ480/20 nm, emission HQ 510/20 nm, Chroma Technology, Battlebolo, VT). Photographs of cells were taken at 40x magnification using a F-View Monochrome CCD camera. Temperature of microscope stage was maintained at 37°C by using an air stream incubator (Nevtek ASI400, Burnsville, VA).

Plasmid Construction

HIV-MycA8-PRLBD protein switch plasmid was constructed by replacing the NLSi from HIV-NLSi-PRLBD (constructed in our previous study (5)) with oligonucleotide 5′-AATTCTCCAGCAGCAAAAAGAGTAAAAGCA

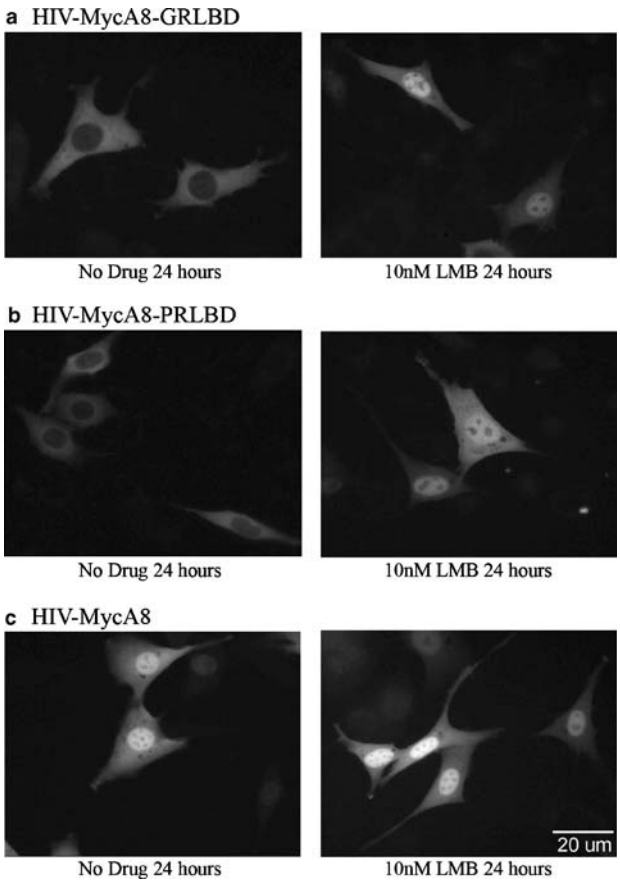

Fig. 4. Effect of LMB, an inhibitor of CRM1, on localization of the protein switch. A 10 nM dose for 24 h was used to study the effect of LMB on localization of protein switch constructs. *Left panels*, no LMB; *right panels*, with LMB. **a** HIV-MycA8-GRLBD, **b** HIV-MycA8-PRLBD, and **c** HIV-MycA8 (no LBD). All experiments were repeated in triplicates (n=3) and ten cells were analyzed for each time-point in each experiment

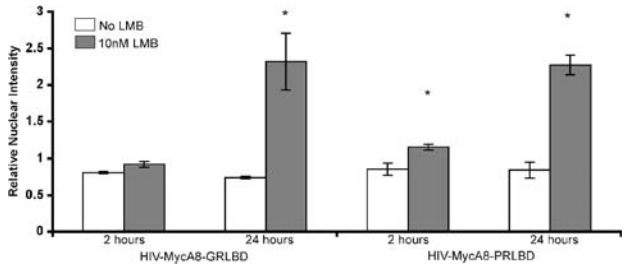

Fig. 5. Quantitation of nuclear intensity in Fig. 4. Change in relative nuclear intensity of cells transfected with protein switch constructs in the presence and absence of 10 nM LMB at 2 and 24 h time points. For each bar (experimental group), ten cells were analyzed. Statistical differences indicated by * (p<0.05) using one-way ANOVA with Tukey's Multiple Comparison, comparing no LMB to 10 nM LMB for each pair

GACGAAG-3′ and its complimentary strand, which encodes the classical NLS sequence from MycA8 protein, at the *EcoRI* and *SalI* restriction enzyme sites. HIV-MycA8-GRLBD protein switch plasmid was constructed by substituting the truncated wt PR LBD from HIV-MycA8-PRLBD plasmid with the C656G GR LBD at the *KpnI* and *BamHI* restriction enzyme sites. The C656G GR LBD used to make this plasmid was extracted out of pCI-nGFP-C656 (45) by performing PCR using the primers 5′-AGGGTACCCT CACCCCTACCTTG-3′ and 5′-CGCGCGGATCCTTTTT GATGAAACAG-3′ with *KpnI* and *BamHI* ends respectively.

HIV-MycA8 plasmid was constructed by inserting an oligonucleotide 5′-CCGGACTTCAACTTCCTCCTCTT GAGAGACTTACTCTTCCAGCAGCAAAAAGAG TAAAAGCAGACGAAA-3′ and its complimentary strand, which encodes for HIV NES and MycA8 NLS at the BspEI and BglII sites. GRNES1 plasmid was constructed by inserting oligonucleotide 5′-CTAGGCTTGAGAAACTTA CACCTC-3′ and its complimentary strand, which encodes for NES 1 from GR, at the *BspEI* and *XhoI* sites.

Localization Studies

Both the protein contructs—HIV-MycA8-PRLBD and HIV-MycA8-GRLBD—were induced with ligand about 18–24 h after transfection. Prior to induction media was replaced with fresh phenol red-free complete DMEM.

To show the change in localization with ligand induction, cells were induced with a 10 uM dose of ligand for 1–2 h, optimized in our previous study (5). Mifepristone and dexamethasone were used as ligands for protein switch constructs containing PR LBD and GR LBD, respectively. Photographs of live cells were taken using the fluorescence microscope after 1 h. Cells were also photographed without ligand as control.

To study the effect of leptomycin B (LMB, Sigma-Aldrich, St. Louis, MO), transfected cells were treated with a 10 nM dose of LMB. Photographs were taken at 2 and 24 h time points. As controls, cells were also untreated and photographed.

Effect of geldanamycin (InvivoGen, San Diego, CA) was studied by treating the cells with high dose geldanamycin (GA) for a short time (25 uM/20 min.) and low dose GA for a long time (0.9 uM/24 h); doses were selected based on our

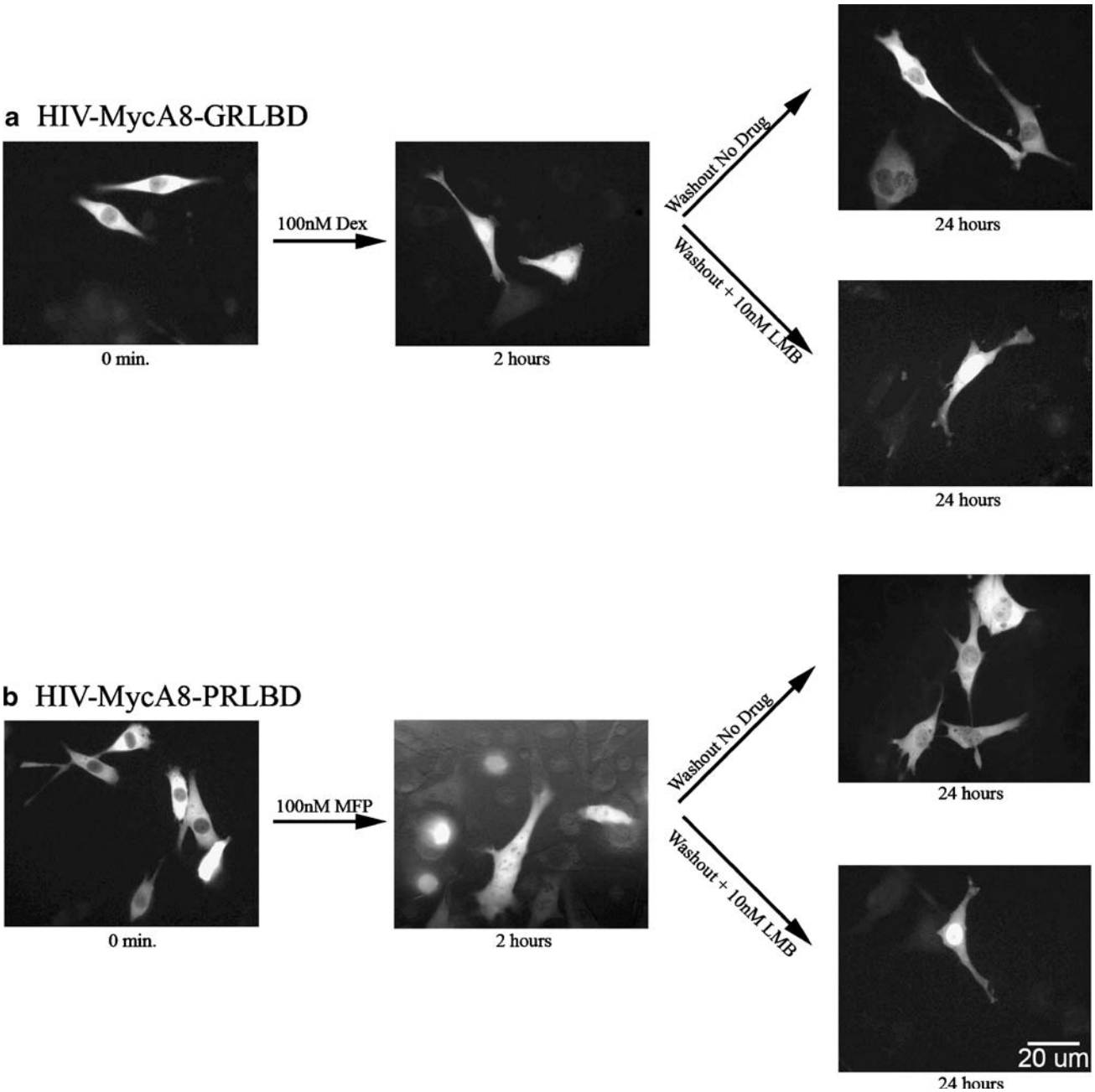

Fig. 6. Effect of LMB after ligand washout. The protein switch constructs were induced with 100 nM dose of respective ligands, Dex. for HIV-MycA8-GRLBD and MFP for HIV-MycA8-PRLBD for 2 h, followed by washout of ligand and treatment with 10 nM LMB

previous studies (40). Untreated cells (treated only with vehicle, DMSO) served as controls.

Washout Studies

About 18–24 h after transfection, media on the cells was changed to fresh phenol red-free DMEM containing charcoal stripped FBS. Time zero photographs were taken for all cells. Cells transfected with HIV-MycA8-PRLBD or HIV-MycA8-GRLBD were induced with 100 nM dose of mifepristone or dexamethasone, respectively. Two hours after ligand treatment, photographs of cells were taken, and cells were washed ten times with phosphate buffered saline containing calcium/magnesium. Following the washout, the media was replenished. Cells transfected with either protein switch constructs were treated with 10 uM LMB or left untreated as control, and photographed after 2 h (data not shown) and 24 h.

Data Analysis and Statistics

Quantitation of protein switch constructs present in the nucleus and cytoplasm was carried out by fluorescence intensity of the EGFP, tagged to the protein constructs, as described previously (5,40). All photographed cells were analyzed using analySIS® software (Soft Imaging System, Lakewood, CO). The nuclear and cytoplasmic intensity values were divided by their respective area values to normalize for differences in cell shape and size. Relative

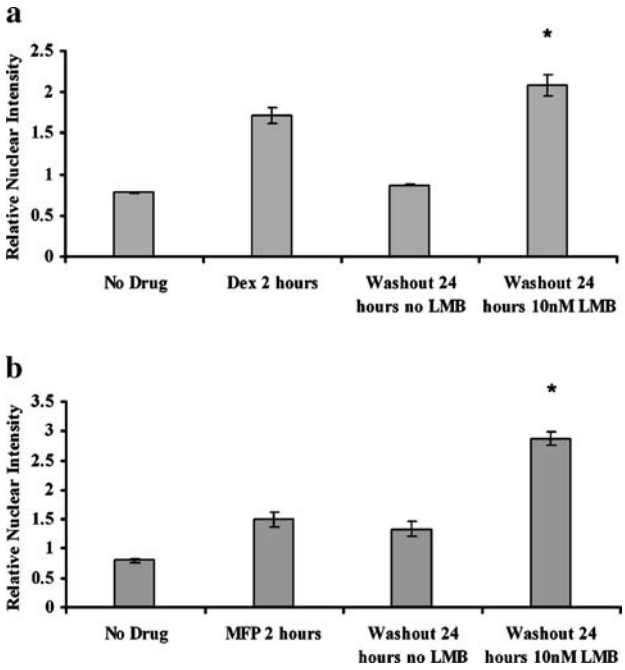

Fig. 7. Quantitation of nuclear intensity in Fig. 6. The effect of LMB on export of the protein switch constructs from the nucleus to the cytoplasm after ligand washout. **a** HIV-MycA8-GRLBD, and **b** HIV-MycA8-PRLBD. For both, cells were photographed first with no drug, followed by a 2 h ligand induction, then ligand washout followed by either no treatment or 24 h treatment with 10 nM LMB. *Asterisks* indicate significant ($p<0.05$) inhibition of nuclear export with LMB after ligand washout at the 24 h time point, compared to no LMB

nuclear intensity was calculated by dividing the average nuclear intensity with average cytoplasmic intensity. One-way ANOVA with Tukey's Multiple Comparison Post test was used to assess statistical differences between the relative nuclear intensity values in various experiments. All experiments were repeated in triplicates ($n=3$) and ten cells were analyzed for each time-point in each experiment.

RESULTS

Two protein switches, each containing HIV NES, Myc A8 NLS, but with different LBDs were used here based on optimization studies (48). Optimization studies were aimed at maximizing the amount of protein in the cytoplasm in the unliganded state, and maximizing the amount of nuclear translocation upon ligand addition. One of these protein switches contained the C656G GR LBD and the other, truncated PR LBD (see Fig. 2).

In this study, two ligand binding domains were separately utilized: a truncated version of progesterone receptor LBD (responsive only to antagonist mifepristone, or MFP) (44), and the C656G point mutation of the rat glucocorticoid receptor (GR) LBD (responsive to 10-fold lower doses of dexamethasone compared to wild type) (45). The ligand binding domains of steroid receptors can function out of context (ie, not in the full length receptor) and can be swapped out with LBD's of other steroids and retain their unique ligand binding abilities (49).

As shown in Fig. 3, both protein switch constructs, when expressed in cells, are cytoplasmic in the off state (no ligand added), but translocate to the nucleus when their respective ligands are added. A significant change ($p<0.05$) in localization is seen from the cytoplasm to the nucleus with ligand induction, for both the HIV-MycA8-GRLBD and the HIV-MycA8-PRLBD.

In the protein switch, a classical leucine-rich nuclear export signal from HIV rev protein (50) was rationally included to confer localization to the cytoplasm. To confirm that the mechanism of export of the protein switch was *via* CRM1, the classical export receptor which recognizes leucine rich NESs (33), the nuclear export inhibitor leptomycin B (LMB) was used. LMB is a streptomyces metabolite that directly binds to CRM1 to inhibit its export function (42). Figure 4a and b show that the addition of LMB to cells expressing either the HIV-MycA8-GRLBD or the HIV-MycA8-PRLBD protein switch causes the protein switch to accumulate into the nucleus. As seen in Fig. 5, the cells expressing HIV-MycA8-PRLBD and treated with LMB showed a significant increase ($p<0.05$) in the relative nuclear intensity at both 2 and 24 h time points compared to the cells left untreated. Similarly, the cells expressing HIV-MycA8-GRLBD also showed a significant increase ($p<0.05$) in the relative nuclear intensity on LMB treatment at the 24 h time point. For this construct, although there was a change observed in the relative nuclear intensity at the 2 h time-point, it was not significant. These results suggest that blocking the export receptor CRM1 does not allow the protein switch to fully come back out to cytoplasm.

Interestingly, we observed that the constructs containing only the HIV NES and MycA8 NLS, and no LBD were mostly nuclear (Fig. 4c). Addition of LMB did not cause any significant change in localization of this construct. In this isolated construct, the NLS dictates the localization (dominates over the single HIV NES), and there are no other signals present due to lack of a LBD. This observation suggested a strong role of the LBD in the cytoplasmic localization of the protein switch constructs in the absence of the ligand. Several putative NESs may exist in both PR and GR LBD that contribute to cytoplasmic localization.

Next, ligand washout studies with LMB were carried out to study the involvement of CRM1 in re-export of the protein switch to the cytoplasm (after ligand induction). We have previously shown that after ligand washout, the protein switch relocalizes back to the cytoplasm (5). Addition of LMB immediately after ligand washout does not allow CRM1 to export the protein switch back out into the cytoplasm. When export is blocked, the protein switch stays trapped in the nucleus (Fig. 6a and b, bottom right panels). When there is no LMB present, the protein switch can get exported to the cytoplasm (Fig. 6a and b, top right panels).

As seen in Fig. 7, there was a significant ($p<0.05$) inhibition of nuclear export with LMB after ligand washout at the 24 h time point, compared to cells not treated with LMB. Hence CRM1 is also involved in the re-export of the protein switch after ligand washout, as expected.

Since the initial (unliganded) cytoplasmic localization of the protein switch is not entirely determined by the HIV NES (Fig. 4c), we suspected that other NESs may exist in both the PR LBD and the GR LBD that contribute to

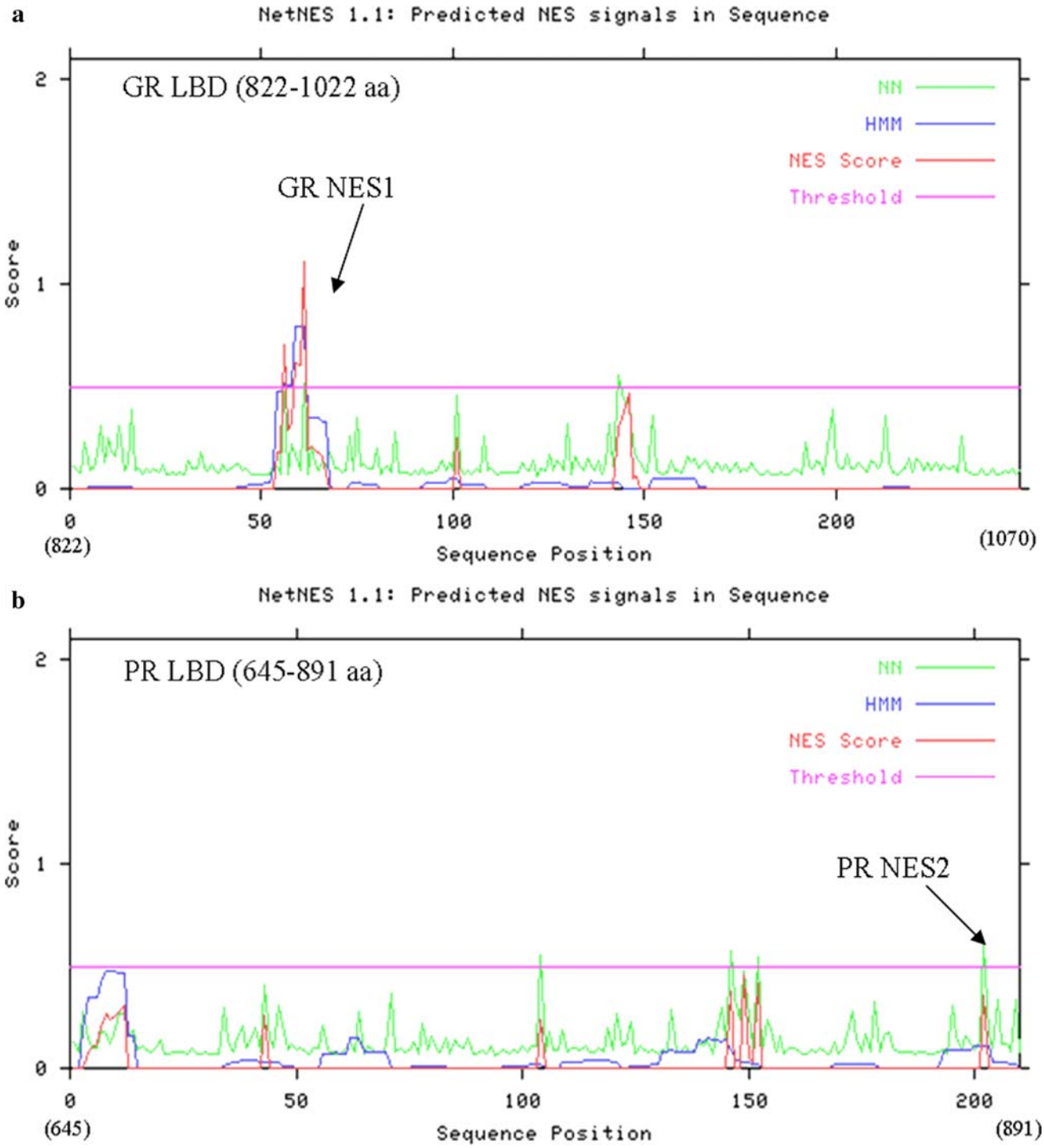

Fig. 8. Putative NES in GR and PR LBDs recognized *via* NetNES 1.1 (6). **a** C656G GR LBD sequences (residues 822–1,070 from NCBI Entrez Protein accession number AAB07866) and **b** PR LBD sequences (residues 645–891 from accession number NP000917) were inputted into the NetNES 1.1 server, and graphs were generated directly from this server (6). NN=neural network (*green*); HMM=hidden Markov model (*blue*); NES score (*red*); threshold is indicated in *magenta* (6,51). Location of GR NES1 and PR NES 2 indicated on graphs

cytoplasmic localization. In our previous work, we have shown that the isolated GR LBD is found in the cytoplasm of cells; protein switches with either GR LBD or PR LBD localize in the cytoplasm when no ligand is added (48). Upon closer manual inspection of the GR and PR LBDs, NESs can be identified using our previously defined "consensus" NES sequence, $LX_{1-3}LX_{2-3}LXJ$, where L = leu, X is any residue, and J = leu, val, or ile (5). In the GR LBD, the sequence LGLRNLHL fits the consensus (denoted GR NES1), and in the PR LBD, LHDLVKQLHL fits the consensus (denoted PR NES2). To confirm these sequences, GR LBD and PR LBD sequences were inputted into the NetNES 1.1 server, designed to identify putative NESs (6). NetNES is a NES prediction server made available through Center for Biolog-

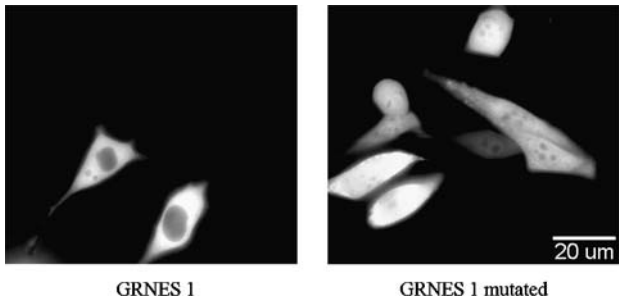

Fig. 9. Localization of GRNES 1. GR NES1 (LGLRNLHL) tagged to EGFP shows cytoplasmic localization, *left panel*. GR NES1 mutated (AGALRNLHA) tagged to EGFP, shows nuclear and cytoplasmic localization, *right panel*

ical Sequence Analysis, Technical University of Denmark. The prediction server utilizes NES data collected based on presence of hydrophobic residues, flaking sequences, secondary structure, flexibility and accessibility of leucine rich regions. NetNES 1.1 takes into account all known NESs, and is a machine learning prediction method that may represent a significant improvement over the generally used consensus patterns, using neural networks and hidden Markov models (51). Interestingly, as shown in Fig. 8, both GR NES1 and PR NES2 appear as potential NESs. The GR NES1 is at the beginning of the LBD (Fig. 8a), while PR NES2 is at the end of the LBD (Fig. 8b). Based on the NES scores (in red) and the threshold, GR NES1 is more likely a stronger NES than PR NES2.

Indeed, we have tested both GR NES1 (Fig. 9) and PR NES2 (52) out of context. When tagged to EGFP, GR NES1 acts as an export signal in isolation (Fig. 9, left panel); when critical leucine residues in GR NES1 are mutated to alanines (LGLRNLHL with leucines mutated to AGALRNLHA), it no longer localizes in the cytoplasm, as expected (Fig. 9, right panel). PR NES2 was previously found to be both nuclear and cytoplasmic (52), thus does not appear to be a stand-alone NES. There is reason to believe that PR NES2 may act as a weak NES, though, since it can confer export of a protein switch containing MycA8 NLS and PR LBD (48). PR NES2, although weak, when combined with other possible NESs in the LBD, may tip the balance of this protein switch to export (cytoplasmic localization).

In addition to CRM1's role in export of the protein switch, the role of Hsp90 in cytoplasmic retention of the protein switch was also tested. Hsp90 has long been implicated in the cytoplasmic retention of steroid receptors and is known to interact with steroid receptors *via* their LBDs (39,40). Therefore, to test the effects of Hsp90 on the protein switch, geldanamycin (GA), a benzoquinoid ansamycin, that specifically inhibits Hsp90, was used (53). Using either high dose GA/short duration (25 uM/20 min; Fig. 10) or low dose GA/long duration (0.9 nM/24 h) (data not shown), exposure to GA had no effect on the localization of the protein switch. There was no significant difference in localization with or without GA ($p>0.05$). If Hsp90 played a major role in the export of the unliganded protein switch, addition of GA would have pushed the protein switch into the nucleus, but this was not the case.

Although GA was found to have an effect on the localization of the GR LBD in isolation (40), we believe that in the protein switch construct, the addition of the extra NES dictates the final localization of the protein.

DISCUSSION

Signal sequences can be used for controlled localization of proteins to the nucleus, cytoplasm, and other subcellular compartments. Our recently developed protein switch constructs utilize an import signal, an export signal, and ligand binding domain for controlled localization. In this study, the mechanism of export of our protein switch was determined to occur *via* the general export receptor, CRM1, also known as exportin 1. The addition of LMB, a specific inhibitor of CRM1, to cells expressing the protein switch causes the protein switch to accumulate into the nucleus. Blocking the export receptor CRM1 does not allow the protein switch to fully come back out to cytoplasm.

Interestingly, the LBDs used here may contain previously unrecognized NESs, as determined by manual inspection, and correlated with a program (NetNES 1.1) designed to identify NESs in eukaryotic proteins. Although not all putative export signals in the LBD were experimentally analyzed here, one NES in the GR LBD does act as a NES out of context, and may contribute to the overall cytoplasmic localization of the HIV-MycA8-GRLBD protein switch. The putative NES in PR LBD studied here, while unable to act as an export signal out of context, may serve as a weak NES and still contribute to the overall cytoplasmic localization of HIV-MycA8-PRLBD.

Ligand binding domains of steroid receptors may contain multiple NESs that work in concert but not alone. Upon ligand binding, a conformation may occur resulting in

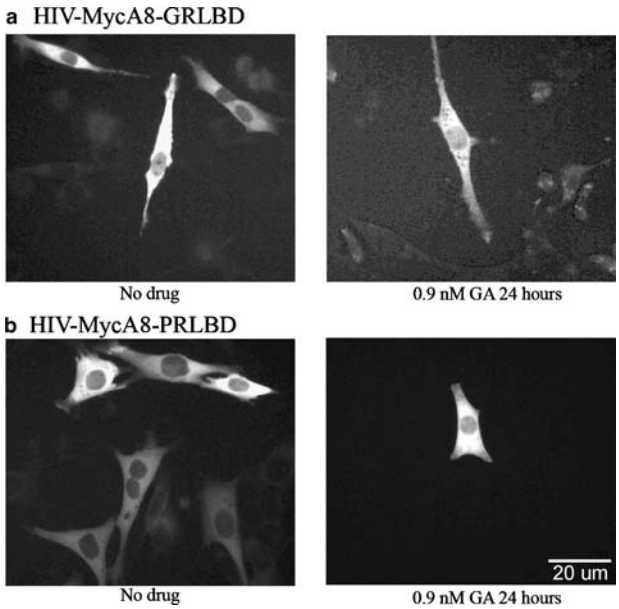

Fig. 10. Effect of GA, an inhibitor of Hsp90, on localization of the protein switch. Protein switch constructs were either untreated (*left panels*) or treated with 0.9 uM GA for 24 h. **a** HIV-MycA8-GR LBD, **b** HIV-MycA8-PR LBD. All experiments were repeated in triplicates ($n=3$) and ten cells were analyzed for each time-point in each experiment

masking of these NESs—not just in the protein switch but in steroid receptors as well. Nuclear export signals are still not well understood, and a general consensus based on primary amino acid sequence may not be sufficient. The concept of masking/unmasking signal sequences *via* ligand binding domains or by other domains with the ability to undergo conformational changes provide an extra level of sophistication for targeting of proteins to subcellular compartments.

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