CHAPTER I

Steroid receptors and components of nuclear import machinery exit nucleus via exportin-1/CRM-1 independent pathway
INTRODUCTION

For more than two decades the members of nuclear receptor superfamily have been shown to function as ligand-activated transcription factors. In response to their cognate ligands nuclear receptors modulate expression of a large number of genes and attribute to profound effects not only on normal physiology but also with immense relevance in a number of human diseases (Pratt and Toft, 1997; Cheng and Balk, 2003; Huang et al., 2010). Typically, nuclear receptor superfamily consists of classical steroid receptors including, androgen receptor (AR), glucocorticoid receptor (GR), estrogen receptor (ER), progesterone receptor (PR), mineralocorticoid receptor (MR); non-steroid receptors, including receptors for thyroid hormone, retinoic acid, vitamin D and numerous other orphan receptors whose cognate ligands are not identical as yet (Nuclear Receptor Nomenclature Committee 1999; Escriva et al, 2000; Thornton and De Salle, 2000). These nuclear receptors are structurally characterized by three distinct domains, i.e., an amino-terminal transactivation domain, a central DNA-binding domain and a carboxy-terminal ligand binding domain. Since these ligand-activated transcription factors share major structural and functional similarities with each other, the information obtained from one can be generally applicable to other receptors in the superfamily.

The ability to translocate and localize proteins to specific compartments is fundamental to organization and functioning of all living cells. Subcellular localization and dynamic movements of transcription factors appears to be one of the major strategies of regulating their transcriptional activity (DeFranco, 2002; Black and Paschal, 2004). The confinement of transcription and translation process into distinct compartments is considered an ideal strategy for modulating these dynamic events more effectively (Black and Paschal, 2004). Different classes of soluble transport receptors mediate translocation of macromolecules between the cytoplasm and the nucleus in conjunction with other immobile components associated the
translocation machinery. Some of the well-characterized soluble components of nuclear import machinery mediating inward journey to the nucleus include members of importin-α/β protein family, the Mex67/Tap family, small nuclear factor-2 (NFT2)/P10, Ran-GTP, transportins, Kap95p homologues to importin-β and other less-characterized or unknown import factor (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Kataoka et al., 1999; Weis, 2002; Davis et al., 2006; Cook et al., 2007). All soluble nuclear import factors can bidirectionally transverse the nuclear pore complex through specific interactions with the immobile components of nuclear pore complex. After the delivery of the nuclear localization signal (NLS)-bearing substrate into the nucleus, the import factors must return to the cytoplasm in order to transport the next NLS-bearing substrate into the nucleus. For the outward journey from the nucleus, the best-studied and commonly used nuclear export processes are mediated by classical leucine-rich nuclear export signals (NES) that specify recognition by the exportin-1/CRM-1 nuclear export receptor (Ossareh-Nazari et al., 1997; Fornerod et al., 1997). Though in lesser instances, CAS (Kutay et al., 1997), calreticulin (Holaska et al., 2001), Msn5 (Kaffman et al., 1998) and other alternative mechanisms are also reported to be involved in nuclear export processes. However, the proteins known to exit nucleus via exportin pathway far exceed in number when compared with the ones following alternate nuclear export pathways. The discovery of this exportin-1 dependent pathway is attributed to a potent and specific nuclear export inhibitor leptomycin B (LMB) that helped establishing this and other nuclear export pathways (Ossareh-Nazari et al., 1997; Wolff et al., 1997; Tyagi et al., 1998). The figure 8 demonstrates the mechanism of action of leptomycin B. However, as a whole, the general understanding of nuclear export mechanisms utilized by steroid receptors and import/export factors, navigating between the two compartments, as components of large nuclear import/export machinery is still fragmentary.
Several groups have shown that nuclear receptors continuously shuttle between the cytoplasm and the nucleus and the steady state localization of a nucleocytoplasmic shuttling protein is a consequence of a fine balance between operational strengths of 'nuclear localization signal' (NLS) and 'nuclear export signal' (NES) (Black et al., 2001; DeFranco, 2002). Now it is increasingly becoming evident that the regulation of gene expression by nuclear receptors is modulated mainly through the subcellular compartmentalization of liganded or unliganded receptors. Transcription modulation by steroid receptors is a function that is imparted in response to interaction with their cognate ligand(s). When in ligand-bound state all steroid receptors are nuclear. However, in the absence of hormone, steroid receptors (SRs) are reported to show varied subcellular localization. Steroid receptors are distinguished from other members of the nuclear receptor superfamily by their association with a chaperone protein complex. In absence of ligands, steroid receptors are anchored to a number of heat shock proteins (hsp70, hsp90) and immunophilins, (Pratt and Toft, 1997; Ylikomi, 1998; Hache et al, 1999). Such an association between chaperone proteins and unliganded steroid receptors is believed to create a receptor conformation having high competency to bind steroid and is also a control point in
steroid signalling. For example, in case of AR and GR, the two receptors are apparent in the cytoplasm in complex with chaperone proteins capable of high-affinity binding to androgens and glucocorticoids respectively. However, unliganded ER and PR in most cell types, have been localized predominantly in the nucleus with only a fraction of receptor visible in the cytoplasm. Unliganded MR, in most instances, is reported uniformly distributed between the cytoplasmic and nuclear compartments. Hormone binding leads to a conformational change in the receptor resulting in its dissociation from the chaperone or co-repressor proteins, translocation into nuclear compartment and ultimately resulting in binding of the receptor as a homodimer to cognate sites in the steroid-responsive genes. In recent years, most of the steroid receptors tagged to GFP are providing exciting results from live cell imaging.

In this section of the study, to derive results from living cells, green fluorescent protein (GFP)-tagged human androgen receptor (GFP-AR) and human glucocorticoid receptor (GFP-GR) chimera were used. Another chimeric protein RelA-GFP (p65-GFP) was used in control experiments whose nuclear export is established to occur via exportin-dependent pathway and can be efficiently inhibited by LMB (Tyagi et al. 2000; Birbach et al., 2002). Therefore, Rel A-GFP served as our working model, and together with the application of LMB, we investigated if steroid receptors and the general components of nuclear import machinery exit the nuclear compartment utilizing the exportin-1 dependent or exportin-1 independent pathway.

RESULTS

Exportin-1 dependent nucleo-cytoplasmic shuttling of p65-GFP (RelA-GFP) is inhibited by leptomycin B

Studies on mechanism of nucleo-cytoplasmic shuttling of p65/p50 (NFκB) and IκB have recently been reported and the molecular events involved in their shuttling are now beginning to be well characterized
(Malek et al., 2001; Birbach et al., 2002). In the present study we have used RelA-GFP as a well-characterized protein whose exportin-1 dependent nucleocytoplasmic trafficking and inhibition of its nuclear export by LMB is well documented (Johnson et al., 1999; Huang et al., 2000; Birbach et al., 2002). For optimization of our experimental conditions, initially RelA-GFP was transiently transfected and expressed in COS-1 cells. We observed adequate expression of this chimeric protein after 24 hours of transfection. The typical subcellular distribution pattern of RelA-GFP in living cells is shown in figure 9. The transfected RelA-GFP protein showed varied subcellular localization pattern with significant number of cells showing either N=C or N>C localization pattern (Figure 9 untreated). This can be explained on the basis of the involvement of another important protein named IκB. It is believed that when IκB is present inside the cell at subsaturation levels, it alters this varied localization pattern of RelA to a predominantly cytoplasmic localization. In our experiments with living cells, when RelA-GFP was co-transfected with super-repressor IκB we observed its dynamic shift to cytoplasm (Figure 9, +IκB) in agreement with the recent results reported by Birbach et al. (2002). Alongside, in another set of experimental plates with similar cotransfection the cells were treated with LMB (20ng/ml) which is a specific inhibitor of nuclear export of proteins bearing leucine-rich nuclear export signals. Within a time span of about 30 minutes more than 95% of the cells expressing RelA-GFP were observed to have protein localized predominantly in the nuclear compartment (Figure 9, +IκB+LMB). Similar results were observed when COS-1 cells were transfected with RelA-GFP alone (without IκB) and treated with LMB (Figure 9, +LMB). It is demonstrated that both NFκB and IκB could accumulate in the nucleus after addition of the nuclear export blocking agent LMB (Johnson et al., 1999; Huang et al., 2000; Birbach et al., 2002). These observations not only emphasize the efficient working situation in our living cell system but also suggest that RelA-GFP is indeed exported via exportin-1 dependent pathway. It may also be envisaged that similar to RelA-GFP a number of other related proteins will
Figure 9: Exportin-1 dependent nuclear export of RelA-GFP is inhibited by LMB. COS-1 cells were transfected with relA-GFP alone or along with IκBα and the cells were allowed to express the protein for 24 hours. RelA-GFP expressed alone is seen uniformly distributed between nucleus and cytoplasm (Figure 9, untreated), or predominantly localized in the cytoplasm when expressed with IκBα (Figure 9, +IκB). In both the situations treatment with LMB (20ng/ml) for 4 hours arrested the shuttling relA-GFP into the nuclei of the cells indicating involvement of exportin-1 dependent nuclear export pathway and the effectiveness of LMB inhibition (Figure 9, +LMB and +IκB+LMB). The fluorescent live cell images were recorded with upright fluorescence microscope. The final images were prepared and labeled in Microsoft PowerPoint. Figures on the right panel show hoechst staining performed to visualize the nuclei of the corresponding cells on the left panel.
also be similarly trapped in the nuclear compartment that are constantly shuttling via exportin-1 nuclear export pathway. This exportin-1 dependent pathway now appears to be used by most of the proteins that are constantly shuttling between the cytoplasmic and nuclear compartment.

**Exportin-1 independent nuclear export of steroid receptors, AR and GR**

The two steroid receptors, androgen receptor (AR) and glucocorticoid receptor (GR) are the members of nuclear receptor superfamily that function as transcription factors. Unliganded AR and GR exist in cytoplasm with their NLS buried in a heteromeric complex consisting minimally of a dimer of hsp90, a 23-kDa acidic protein (p23), and FK506-binding immunophilin (Pratt et al., 1999; Defranco, 2002). Upon ligand binding, AR and GR interactions with chaperone proteins are dramatically changed resulting in exposure of NLS that activates their nuclear translocation process. Nuclear import of AR and GR involve recognition of nuclear localization signal (NLS) by importins, which mediate translocation into nucleus in the presence of their respective hormones, androgen and glucocorticoid (Defranco, 2002). Nuclear receptors have been reported to have different NES in DBD. A pair of phenylalanines amino acid is reported to be more important in a 15 amino acid long NES (KVFFKRAVEGQHNYL) present in the DBD of GR (Black et al., 2001).

After establishing the RelA working conditions and LMB sensitivity for nuclear export inhibition, to check the effect of LMB on human AR and GR we carried out the experiments with GFP-AR and GFP-GR in COS-1 cells. For this purpose, GFP-AR and GFP-GR were transiently transfected in COS-1 cells and allowed to express both the receptors in unliganded situation. After expression, cells were treated with LMB (20ng/ml) for 4h. Our results demonstrated that in unliganded conditions both the receptors were cytoplasmic. Contrary to RelA-GFP where RelA-GFP was arrested in the nucleus, LMB treatment did not affect
Figure 10: Leptomycin B does not alter the localization of GFP-AR and GFP-GR. COS-1 cells were transfected with 500ng of GFP-AR and GFP-GR as described under 'Materials and Methods'. Following the transfection period, cells were treated with vehicle alone (DMSO:ethanol, 1:1) and LMB (20ng/ml) for 4 hours. GFP-AR and GFP-GR localized in the cytoplasm of cells treated with vehicle alone (DMSO:ethanol, 1:1) as well as with LMB (20ng/ml). The fluorescent live cell images were recorded and the final images were prepared and labeled in Microsoft PowerPoint. Figures on the right panel show hoechst staining performed to visualize the nuclei of the corresponding cells on the left panel.
the localization of both the steroid receptors (Figure 10).

From the previous reports, it is known that steroid receptors (AR, GR and PR) after ligand-mediated translocation can also be exported from the nucleus following stepwise hormone withdrawal (Tyagi et al., 1998; Tyagi et al., 2000; Defranco, 2002). It is known that AR can be exported to the cytoplasmic compartment after androgen withdrawal for 12 h in the presence of cycloheximide (Tyagi et al, 2000). In this perspective, we assessed export of human AR and GR in COS-1 cells. COS-1 cells were transfected with 500ng of human GFP-AR and GFP-GR as described under 'Materials and Methods'. Following the transfection period, cells were treated with vehicle alone (DMSO:ethanol, 1:1) and with hormone DHT ($10^{-8}$ M) and Dex ($10^{-7}$ M). The receptors were translocated into the nuclear compartment within 60 minutes. After 2 h of hormone treatment and nuclear import of receptors, LMB (20ng/ml) was added to the cells for 4 h to arrest both the steroid receptors into nucleus. Cycloheximide (100 $\mu$g/ml medium) was included to prevent de novo synthesis of proteins. After 4 h of incubation with LMB, the hormones containing medium was replaced with steroid-free medium. The cells expressing GFP-AR and GFP-GR were then incubated for 12 h in the steroid-free medium containing cycloheximide (100 $\mu$g/ml) followed by changing the medium and cycloheximide at different time intervals (4, 8, and 12 h) in the presence and absence of LMB (20ng/ml).

The GFP-AR and GFP-GR were cytoplasmic in unliganded conditions. They translocated to the nucleus when treated with their ligands DHT ($10^{-8}$M) and Dex ($10^{-7}$M) respectively (Figure 11). The GFP-AR and GFP-GR was exported from the nucleus to the cytoplasm after hormone withdrawal in absence of LMB. The rate of the export of both the receptors from nucleus to cytoplasm is same in the presence or absence of LMB (Figure 11). These results indicate that LMB does not have any effect on the nuclear export of GFP-AR and GFP-GR and these two receptors are not exported from nucleus to cytoplasm by exportin-1 dependent pathway. So, in conclusion, they follow the exportin-1 independent export pathway for their nuclear export.
Figure 11: Exportin-1/CRM-1 independent nuclear export of GFP-AR and GFP-GR. COS-1 cells were transfected with 500ng of GFP-AR and GFP-GR as described under 'Materials and Methods'. Following the transfection period, cells were treated with vehicle alone (DMSO:Ethanol, 1:1) and with hormone DHT (10\(^{-8}\) M) and Dex (10\(^{-7}\) M) respectively. The receptors were translocated into the nuclear compartment within 60 minutes. After 2 h of hormone treatment and nuclear import of receptors, LMB (20ng/ml) was added to the cells for 4 h to arrest both the receptors into nucleus. Cycloheximide (100 μg/ml medium) was included to prevent de novo synthesis of proteins. After 4 h of incubation with LMB, the hormones containing medium was replaced with steroid free medium. The cells expressing GFP-AR and GFP-GR are then incubated for 12 h in the steroid-free medium containing cycloheximide (100 μg/ml) followed by changing the medium and cycloheximide at different time intervals (4, 8, and 12 h) in the presence and absence of LMB (20ng/ml). After completion of 12h, the live cell images were recorded by florescence microscope.
Leptomycin B does not arrest or impede shuttling of the components of nuclear import machinery

Nuclear translocation of steroid receptors is mediated by number of import factors. Similar to steroid receptors the import factors also follow the dynamic import/export pathways to support the continuous translocation cycles of steroid receptors. At this point there is a logical question that needs to be asked. Are these components of the nuclear import machinery being exported via classical exportin-1 dependent or they follow exportin-1 independent nuclear export pathway? The results from relA-GFP and steroids receptors studies can serve as the basis to answer this query. Unlike a number of proteins including relA-GFP, the cytoplasmic localization of AR and GR remain unaltered upon treatment with leptomycin B. This finding was exploited in the present study since it creates a favorable situation where unliganded AR and GR can be retained in the cytoplasm while all the shuttling proteins that exit the nucleus via exportin-1 dependent pathway will be arrested in the nucleus upon treatment with LMB. This trap will also work for the shuttling components of nuclear import machinery if they exit nucleus via exportin-1 dependent pathway. If so, the unliganded AR and GR that remain localized in the cytoplasm after treatment with LMB will not be able to enter the nucleus upon treatment with their ligands. The AR and GR failure to translocate will be evident only if one or more of the critical components of the nuclear import machinery are arrested in the nuclear compartment rendering them incompetent to make next round of nuclear import. On the contrary, if all the critical components of the nuclear import machinery remain un-arrested in the nucleus following LMB treatment, hormone-induced nuclear translocation of AR and GR should remain unaffected upon treatment with hormone.

In this context, GFP-AR and GFP-GR were transfected and expressed in COS-1 cells. We observed adequate expression of these chimeric proteins after 24 hours of transfection. In the absence of their natural ligands DHT and Dex, more than 90 % of the cells showed
their localization to be predominantly cytoplasmic (Figure 12A, 12B and 13). After 60 min of treatment with their ligands DHT and Dex, the receptors were translocated into the nucleus (Figure 12A, 12B). After ligands treatment, more than 90 percent cells were observed to be predominantly nuclear (Figure 13). When unliganded GFP-AR and GFP-GR expressing cells were treated with LMB alone (20 ng/ml), the localization of the receptors remained unaltered (showing more than 90% cells with predominantly cytoplasmic) that was similar to unliganded GFP-GR transfected cells (Figure 12A, 12B and 13). In similar control experiments this concentration of LMB was effective in inhibiting the exportin-1 dependent pathway as revealed by our observations with RelA-GFP experiments (Figure 9). In additional experiments, GFP-AR and GFP-GR transfected cells were pretreated with LMB to trap all the proteins shuttling via exportin-1 pathway. When these LMB pretreated cells were challenged with DHT and Dex, both GFP-AR and GFP-GR efficiently translocated into the nuclear compartment (Figure 12A, 12B and 13). In terms of kinetics, the rate of translocation of GFP-AR and GFP-GR in cells pretreated or untreated with LMB were same. Similar results were obtained when LMB was used for pretreatment at higher concentration (40ng/ml) or for prolonged periods of time (overnight). To rule out the possibility that the de novo synthesized import machinery may get involved in nuclear import of GFP-AR and GFP-GR in LMB pretreated cells, cycloheximide (100μg/ml) was included in the cell medium prior to such treatments.

This implies that the shuttling components of nuclear import machinery could not be trapped in the nucleus that could impede or inhibit the nuclear translocation of GFP-AR and GFP-GR. Hence, all the shuttling soluble members of nuclear import machinery exit the nucleus via exportin-1 independent nuclear export pathway.
Figure 12: Pre-treatment of cells with LMB does not arrest the components of import machinery involved in translocation of GFP-AR and GFP-GR from cytoplasm to nucleus. COS-1 cells were transfected with 500ng of GFP-AR and GFP-GR as described under 'Materials and Methods'. GFP-AR and GFP-GR were localized in the cytoplasm of control cells treated with vehicle alone (DMSO:ethanol, 1:1) (Figure 12A and 12B). When treated with hormone DHT (10^{-8} M) and Dex (10^{-7} M) the receptors were translocated into the nuclear compartment within 60 minutes. Treatment with LMB alone (20ng/ml) for 4 hours did not have any effect on localization of GFP-AR and GFP-GR. Cells pre-treated with LMB for 4 hours and then treated with hormone DHT and Dex were still capable of translocating the receptor into the nucleus. During the course of these treatments, cycloheximide (100 μg/ml medium) was included to prevent de novo synthesis of proteins. The live cell were observed and imaged with fluorescence microscope. The final images were prepared and labeled in Microsoft PowerPoint. Figures on the right panels show the hoechst staining to visualize the nuclei of the corresponding cells on the left panel.
Figure 13: Shuttling components of nuclear import machinery are not arrested in nucleus by exportin-1 inhibitor LMB. COS-1 cells were transfected either with GFP-AR, GFP-GR or cotransfected with RelA-GFP and IκBα expression vectors as described under 'Materials and Methods'. After 24 h of transfection, the cells were (pre-)treated with LMB for 4 hrs (RelA+I+L / AR+L / AR+L+H / GR+L / GR+L+H). After the LMB pre-treatment the GFP-AR and GFP-GR transfected cells (AR+L+H) and (GR+L+H) were challenged with hormone DHT and Dex respectively. The AR+H and GR+H served as control where the cells were challenged with hormone alone without pre-treatment with LMB. AR / AR+L and GR / GR+L served as additional controls where treatment with or without LMB has no effect on GFP-AR and GFP-GR localization. (RelA+I) shows cytoplasmic localization of RelA-GFP in absence of LMB treatment. On the contrary (RelA+I+L) shows nuclear accumulation of RelA-GFP in presence of LMB suggesting inhibition of exportin-1 pathway. The sub-cellular localization of the GFP-tagged proteins was determined in at least 100 cells for each experimental condition (n=4) and the percent of cells showing predominantly nuclear localization under different experimental conditions are indicated. Fluorescence was considered nuclear (N) when it was exclusively in the nucleus or cytoplasmic (C) when present exclusively in the cytoplasm. When the protein was present primarily in the nucleus or primarily in the cytoplasm, it was considered N>C and C>N respectively. When the fluorescence was uniformly divided between the nucleus and the cytoplasm it was classified as N=C. For graphical presentation N + N>C were grouped as predominantly nuclear while C + C>N were grouped as predominantly cytoplasmic for receptor localization. Symbol on the graph: RelA = RelA-GFP, I = IκBα, AR = GFP-AR, GR = GFP-GR, L = leptomycin B, H = hormone DHT and dexamethasone.
Three principal nuclear import factors exit nucleus via exportin-1 independent pathway

Since the sequence and functional similarities of NLS of steroid receptors resembles with that of other well-characterized NLS (e.g., nucleoplasmin and SV40 large T antigen), it is presumed that steroid receptors utilize the classical import pathway with the involvement of importin-α and importin-β. The stepwise nuclear import process involves NLS recognition, nuclear pore docking, translocation through the pore and cargo release from the inner side of the pore (Macara, 2001; Weis, 2003; Davis et al., 2006; Cook et al., 2007). The NLS receptor complex is now well characterized and consists of NLS-bearing proteins, importin-α (karyopherin-α) and importin-β (karyopherin-β) that form a nuclear import competent heterotrimeric complex. In simplest terms, importin-α recognizes the NLS of a putative nuclear protein, while importin-β mediates docking interaction onto the nuclear membrane/pore. With the participation of several other key players in the soluble phase and immobile components of nuclear pore complex the event of nuclear translocation is carried out to completion (Macara, 2001; Lyman et al., 2002; Weis, 2002; Shank and Paschal, 2005; Cook et al., 2007).

In our study we showed that mobile component of import machinery exit the nucleus via the exportin-1 independent pathway though, with an indirect approach to examine export pathway. Therefore, to further substantiate our observations directly in live cells we used three fluorescent protein tagged import factors, YFP-importin-α and YFP-importin-β and Ran-GFP. All the three import factors were transiently expressed in COS-1 cells. They showed varied distribution patterns ranging mainly between N=C to N>C. The subcellular distribution profile of these import factors was comparable to that of RelA-GFP (expressed in absence super-repressor IκB-α) and was therefore, included as a control in these experiments. The results presented in figure 14 show the distribution pattern of fluorescent protein tagged importin-α, importin-β and Ran prior to LMB treatment and after LMB treatment.
Figure 14: Nucleocytoplasmic shuttling of three principal nuclear import factors is not inhibited by LMB. COS-I cells were transiently transfected with 500 ng of YFP-importin-α, YFP-importin-β, Ran-GFP and the control relA-GFP plasmids as described under ‘Materials and Methods’. Following the transfection period, cells were treated with vehicle alone (DMSO:ethanol, 1:1) and LMB (20 ng/ml) for 4 hours. LMB treatment did not alter the subcellular localization of any of the three import factors. Under these conditions nucleocytoplasmic shuttling of relA-GFP was inhibited by LMB as evident by its nuclear accumulation. During the course of these treatments cycloheximide (100 μg/ml medium) was included to prevent de novo synthesis of proteins. The fluorescent live cell images were recorded with an epifluorescence microscope. The final images were prepared and labeled in Microsoft PowerPoint. On the right side of each YFP or GFP images hoechst staining reveals the corresponding nuclei.

We observed that in all the cases except RelA-GFP the protein distribution remained unaffected by LMB treatment. These results suggest that importin-α, importin-β and Ran do not follow the exportin-1 dependent pathway for their export.
DISCUSSION

Nucleocytoplasmic shuttling and dynamic movements of steroid receptors are important steps in accomplishing their transcriptional regulatory functions. Ligand binding has profound influence on the subcellular distribution of steroid receptors. In the presence of their cognate ligands, GR, AR, TR and PR rapidly translocate to the nuclear compartment (Tyagi et al., 2000; DeFranco, 2002). However, hormone withdrawal from cells induces return of GR and AR to the cytoplasmic compartment (Tyagi et al., 2000). The balance of nuclear import and export could be an important mechanism for regulating the transactivation potential of steroid receptors. Nuclear import of steroid receptors involves recognition of nuclear localization signal (NLS) by adapter molecules like importin-α and importin-β, which mediate the translocation into nucleus (DeFranco, 2002). Moreover, these import factors also have to shuttle between nucleus and cytoplasm to carry out the subsequent cycles of translocation. Although the mechanism of nuclear import of steroid receptors is well documented however, but how these steroid receptors and their import factors are exported to the cytoplasm was virtually unexplored (DeFranco, 2001). The best-characterized nuclear export pathway uses exportin-1/CRM-1, which recognizes specific leucine-rich NES present in cargo proteins. Discovery of potent exportin-1/CRM-1 inhibitor LMB has facilitated the identification of many transcription factors which utilizes this pathway for their export. As steroid receptors and importins do not contain such leucine-rich NES, it was reasonable to hypothesize that these proteins may not follow the classical CRM-1/exportin-1 pathway for their export. In the present study, we confirmed that there was no effect of LMB on subcellular localization of AR and GR as well as their shuttling components like importin-α and importin-β. Hence, based on the observation made in the present study we conclude that the steroid receptors and the shuttling soluble members of nuclear import machinery exit the nucleus via exportin-1 independent nuclear export pathway.
So, one interesting query arrived from this study is what are the factors involved in export mechanism of steroid receptors? A study has suggested that calcium binding protein, calreticulin is involved in export of steroid receptors (Holaska et al., 2001). It will be interesting to know whether the component of import machinery also follow the similar mechanism? Overall, the present study should initiate a search for the existence of alternative nuclear export pathways that may be involved in nuclear exit of soluble phase of central nuclear import machinery.

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