Characterization of Novel Export Sequences in the Thyroid Hormone Receptor

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Characterization of Novel Export Sequences in the Thyroid Hormone Receptor

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honors in Biology from the College of William and Mary in Virginia

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Abstract

Characterization of Novel Export Sequences in the Thyroid Hormone Receptor

Thyroid hormone receptor α1 (TRα1) is a nuclear receptor that is functionally dependent on its nucleocytoplasmic shuttling for gene regulation. Some sequence motifs responsible for import and export of the receptor have been previously identified, but its full range of nuclear export signal (NES) motifs has not been fully defined. We have previously determined the minimal, transferable sequence for a conserved NES in helix 12 of the receptor but the helix 3/helix 6 (H3/H6) bipartite NES, or monopartite NESs, was not fully characterized. Similarly, prior studies show that TRα1 also possesses a CRM1/calreticulin-dependent export pathway; however, the NES responsible for this mechanism is not yet defined. A candidate sequence located from residues 188-206 of the ligand binding domain has shown a potential role in export regulation. To study the role of these potential NES sequences in TRα1 nuclear shuttling, we performed transient transfection of mCherry-tagged fusion proteins in HeLa cells followed by scoring of the intracellular distribution by fluorescence microscopy. This was accomplished by designing protein constructs containing mCherry-maltose binding protein (MBP) (to create a fusion protein too large to diffuse through the nuclear pore complexes), the TRα1 hinge region NLS (to allow nuclear import), and each NES motif of interest excluding their overlapping residues. These motifs are from helix 3 residues 209-236 and helix 6 residues 239-265 (NES-H3 and NES-H6, respectively). A third fusion protein, H3/H6-219-254, was designed to include central residues from each helix (residues 219-254) to determine if the functional NES lies individual export function of localizing the mCherry-MBP-Hinge nuclear protein to the cytosol. The combined H3/H6-219-254 fragment and the CRM1-dependent candidate sequence LBD-188-206 did not show significant cytosolic localization when analyzed using quantitative and semi-quantitative scoring. These results suggest that the two helices each contain independently acting NES motifs. Furthermore, the inability of the overlapping residue sequence H3/H6 to shuttle proteins to the cytosol indicates that the functional regions of these sequences may lie towards the peripheral ends of each motif. Further work was done on NES-H3 and NES-H6 interaction with specific exportins. Prior studies show TRα1 localization is influenced by exportins 4, 5, and 7. To better understand the mechanism of NES-H3 and NES-H6-directed shuttling, we performed “RFP-trap” coimmunoprecipitation assays to determine which exportins interact specifically with these signals. However, results of these assays were inconclusive, and indicate an area for future work in analyzing TRα1-exportin interactions within the context of the full-length receptor. Ultimately, these multiple export signals emphasize the meticulous balance between nuclear import and export of TRα1 for its crucial role in gene regulation and metabolism.
**INTRODUCTION**

Nucleocytoplasmic shuttling is important in regulating the effects of transcription factors on target gene expression. Nuclear receptors that function in response to ligand stimulation are also subject to this level of functional regulation. Nuclear import of the thyroid hormone receptor is required for its subsequent ability to bind DNA and affect transcription, and nuclear export is equally critical in attenuation of this binding. Consequently, a full understanding of factors mediating this translocation both into and out of the nucleus is necessary for applications in biological systems and human health. While the importins involved in this shuttling have been well-characterized in both knockdown assays and coimmunoprecipitation with TRα1 showing direct protein-importin interaction, export of TRα1 is not completely characterized (Roggero et al, 2016). Leptomycin B (LMB) treatment to specifically inhibit the export protein CRM1, overexpression, and knockdown assays have identified at least three export proteins crucial to this shuttling (Grespin et al., 2008; Subramanian et al, 2015). Therefore, the specific amino acid sequence necessary for TRα1 recognition by exportin proteins is an important functional detail in characterizing this crucial pathway. Consequently, identification of export signal sequences responsible for subcellular trafficking is important for elucidating specific mechanisms of thyroid hormone nuclear export.

**Thyroid Hormone**

The thyroid gland is an integral part of the endocrine system responsible for regulation of homeostasis, metabolism, growth, and development in tissues throughout the body. Consequently, disorders of the thyroid are a pervasive issue of public health, and mechanisms of thyroid function are an equally significant field of study. These important roles are carried out
initially through stimulation of the hypothalamic-pituitary-thyroid axis (HPT) and are subsequently controlled through feedback inhibition. Starting from stimulation of the paraventricular nucleus of the hypothalamus, thyrotropin releasing hormone (TRH) stimulates TRH receptors in thyrotopes of the anterior pituitary (Sugrue et al., 2010). These cells in the pituitary then synthesize and secrete thyroid stimulating hormone (TSH) which targets TSH receptors in the thyroid follicular cells of the thyroid gland. There are many genes regulated by activation of TSH receptors that are necessary for thyroid hormone production (Yen et al., 2006). Following expression of these gene products, thyroid follicular cells are able to synthesize thyroid hormones (TH), T₃ and T₄, through iodination of the glycoprotein thyroglobulin. Once TH is synthesized, it is stored in the lumen of the thyroid gland until secretion of TH to the circulation. Control of the HPT axis is mediated by peripheral circulation of TH that acts as an inhibitor to both the pituitary and the hypothalamus (Chiamolera and Wondisford, 2009) (Figure 1).

While both T₄ and T₃ are secreted by the thyroid, only T₃ is the biologically active form of thyroid hormone with a far greater binding capability to TR. However, the majority of circulating TH released from the thyroid gland is in the T₄ form (Gereben et al, 2008). Therefore, deiodinases D1 and D2 are necessary for removing the 5’ iodine in intracellular conversion of T₄ to T₃ for intracellular use (Bianco and Kim, 2006). Depending on the target tissue, this can occur in the brain, pituitary, brown adipose tissue, liver, or kidney (Larsen and Berry, 1995). Uptake of T₃ and T₄ from the bloodstream into the cell, though originally thought to be via passive diffusion, is mediated by membrane transporters with binding sites for both T₃ and T₄ (Abe, et al., 2002). Three tissue-specific transporters, OATP1C1, MCT8, and MCT10, have been identified that possess high affinity to iodothyronines (Visser et al., 2008). Once TH is within the
Figure 1: Overview of the HPT axis for production of thyroid hormones T3 and T4

The hypothalamus stimulates the pituitary through signaling with thyrotropin releasing hormone (TRH). The anterior pituitary then produces thyrotropin (TSH) which then stimulates the thyroid gland to produce thyroid hormone in both T3 and T4 forms. This hormone is released to circulation and delivered to peripheral tissues where it is converted to T3. This circulating T3 acts as an inhibitor to both the pituitary gland and the hypothalamus resulting in down regulation of TRH and TSH. Consequently, low circulating levels of T3 allow for more production of TRH and TSH.
target cell, it is able to bind a thyroid hormone receptor (TR) which can bind thyroid hormone response elements (TRE) and then affect gene transcription (Figure 2). This transcriptional effect of T3 is responsible for the ubiquitous role of T3 in regulation of important biological systems. The wide range of functional roles include influencing energy metabolism, neurodevelopment, skeletal development, and cardiovascular homeostasis (Williams et al., 2008; Klieverik et al., 2009; Gogakos et al, 2010; Galli et al., 2008). Consequently, the mechanism of TH action on the subcellular level is an important field of study relevant to several body systems.

**Thyroid Hormone Receptors**

*Structure of Thyroid Hormone Receptor*

Once inside the cell, T3 is able to carry out its physiological effects through binding one of the TRs. This receptor is expressed in two subtypes, TRα and TRβ, which have both distinct, and cooperative cellular actions (Brent, 2012). TRα expression is predominant in brain and heart tissue and is expressed first in development while TRβ expression is higher in the kidney, liver, lungs, and is specific to retina, inner ear, and sensory system development (Wallis, 2010; Williams, 2000; Nunez, 2008). Both subtypes are expressed in skeletal and brown adipose tissue (Riberio et al., 2010). These human subtypes are transcribed from the two genes, TRHA and TRHB, on chromosomes 17 and 3 respectively. Additionally, each of these subtypes has three isoforms resulting in a total of six TRs. In humans, TRα has three mRNA splice variants, TRα1 (the focus of this thesis), TRα2, and TRα3, and the TRHB gene encodes three isoforms, TRβ1, TRβ2, TRβ3 (Mitsuhashi et al. 1988; Williams, 2000).
Figure 2: Overview of Thyroid Hormone Action

Circulating T₃ and T₄ are able to enter the cell through transmembrane transporters. Once inside, T₄ is converted to T₃ via a deiodinase D1 and D2. TH can enter the nucleus, bind DNA, and affect transcription of thyroid hormone-target genes.
TRs are members of the nuclear receptor superfamily which include transcription factors such as the progesterone receptors, estrogen receptors, and glucocorticoid receptors. These receptors share four classical domains necessary for their roles of binding ligand and acting as transcription factors. These domains include: an N-terminal transactivation domain (A/B), DNA-binding domain (DBD), a hinge region, ligand-binding domain (LBD), and a variable C-terminal domain. Among these domains, the DNA binding domain is the most highly conserved across the nuclear receptor family whereas the LBD is highly variable among nuclear receptors (NRs) (Sonoda, 2008).

The most N-terminal A/B domain is highly variable among TR variants. The transactivation region (AF-1) of the A/B domain is involved in binding dimers or co-repressors in the absence of ligand. Variation between the AF-1 regions of TR variants can account for selective binding regulation of TREs and preferential dimerization among isoforms. The A/B domain of TRα1, in particular, preferentially forms a heterodimer with RXR which allows for its primary transcriptional function (Hollenberg et al, 1995). This domain in TRα1 also contains a unique nuclear localization signal (NLS), termed NLS-2, not identified in TRβ1 (Mavinakere et al, 2012).

The TR DBD is the most conserved domain among TRα and TRβ isoforms and the nuclear receptor superfamily. This region is responsible for TRs ability to bind target genes independent of ligand (Aastapova et al., 2008). Two zinc fingers in this region allow for binding to the DNA helix through protein interaction with nitrogenous bases and phosphate groups of genomic DNA (Nelson et al, 1995). Within this zinc motif, residues of the upstream P-box bind
the TRE DNA major groove, while the down-stream D-box amino acids are critical for dimerization of the receptor (Aranda and Pascual, 2001).

The hinge region connects the LBD and the DBD and is poorly conserved across TRs. This region is crucial in subcellular localization of TRα1 and TRβ1 as it possesses the strong lysine-rich NLS, NLS-1 (Robbins et al., 1991; Mavinakere et al., 2012). This sequence allows importin proteins to interact with TR and target the receptor into the nucleus to carry out its function on T3-responsive genes. While this hinge NLS accounts for a significant amount of TRα1 nuclear localization, it works in combination with other sequences to ultimately determine TRs subcellular distribution (Mavinakere et al., 2012).

T3 is able to bind TR at the C-terminal LBD. T3 and T4 bind a hydrophobic pocket formed by the LBD which consequently causes a conformational change in the interaction between TR and co-regulators (Wagner et al., 2005). This hormone binding and conformational change occurs in helix 12 of the C-terminal transactivation region (AF-2) (Souza et al., 2011). Interaction between cofactors and TR allows for the subsequent transcriptional regulation of T3 target genes (Fondell, 1996). Though all six isoforms have marked homology among their domains, TRα2 and TRα3 are unable to bind T3 due to truncation of this AF-2 domain (Ortiga-Carvalho, 2014). Despite remaining unliganded, these isoforms are still able to dimerize with other TR isoforms and constitutively downregulate TR transcriptional activity in the presence of T3 (Koenig, 1989). This domain has also been identified for its role in nucleocytoplasmic shuttling of the TRα1 isoform (Mavinakere, 2012), and further characterization of the region’s function in TRα1 nuclear export is the focus of this thesis work.
Functions of Thyroid Hormone Receptor

TRs that bind T₃ can exert positive or negative regulation on gene expression at hormone response elements. TR can bind these regions of gene expression control in its monomer, homodimer, or heterodimer form, and this control of target genes can occur in both the presence and absence of ligand. The mechanism of repression and activation is mediated by both ligand binding and heterodimerization with corepressors. Ligand binding of T₃ in the LBD causes a conformational change in the AF-2 region. This change in position of the domain allows TH to recruit co-activators and co-repressors (Ortiga-Carvalho, 2005; de Araujo, 2010) (Figure 3).

In the absence of activation by T₃, unliganded TR can bind and inhibit expression of genes under positive TH transcriptional regulation. Unliganded TRα1 heterodimerizes with RXR and binds DNA at the TRE. This dimer can then bind a co-repressor which acts to block transcription of the TH-responsive gene (Hu and Lazar, 2000). These co-repressors can act by inhibiting formation of the basal transcription complex and by modifying the chromatin structure using recruitment of histone deacetylases to repress gene transcription (Iwasaki et al, 1996; Astapova et al., 2015). In the presence of T₃, a conformational change of helix 12 in the LBD of TR disrupts binding with the co-repressor, and recruits a co-activator protein (Souza, 2011). This co-activator can then recruit the transcription machinery and up-regulate expression of the positive TRE. Mechanisms for T₃ control of negative TREs are proposed to be the reverse of that for positive TREs where DNA binding of TR activates expression in the absence of ligand (Santos, 2006; Ortiga-Carvalho, 2014).
Figure 3: Mechanism for TR regulation of TREs

A. TR forms a heterodimer with RXR and binds DNA at the TRE. In the absence of T3, this dimer recruits a corepressor which prevents binding of the basal transcription complex to the promoter of the target gene, and no mRNA is synthesized. B. In the presence of T3, binding of T3 to TR disrupts association between corepressors and the RXR-TR heterodimer. The dimer is then able to bind a coactivator which recruits transcription machinery to the TRE and promotes expression of the associated target gene.
Nucleocytoplasmic Shuttling

Subcellular localization is an important level of functional regulation for transcription factors such as nuclear receptors (Weis, 2003). Because DNA binding is imperative for TR to carry out its physiological effects, localization to the nucleus is essential to affect transcriptional change. The membrane functions to create physical separation between mRNA transcription and protein synthesis. However, this compartmentalization also presents a barrier between these cytosolic cellular processes and transcriptional elements within the nucleus that can affect change in their target gene expression.

The nuclear envelope allows for transport across the membrane through the nuclear pore complex (NPC). Small molecules (no bigger than approximately 40 kD) are able to passively diffuse through the NPC while larger molecules require association with karyopherin transport proteins in order to enter the nucleus. These transport proteins are able to bind cargo proteins containing an NLS or NES and target them through the NPC, across the membrane to their respective destinations (Poon, 2005). Karyopherins that recognize NLSs and NESs in carrying out these roles of nuclear import and export are titled importins and exportins, respectively.

The NPC is comprised of approximately 30 nucleoporin proteins that arrange to form a channel with eight-fold radial symmetry (Rout et al., 2000). This ring is split into three ring layers termed the cytoplasmic ring, inner ring, and nuclear ring (Schwartz, 2005). A basket structure is attached to the nuclear ring, and long filaments extend into the cytosol from the cytoplasmic ring. The main permeability barrier of the NPC is established by extensions from nucleoporins within the channel comprised of repeating phenylalanine-glycine (FG) residues. In
addition, FG repeats coordinate nuclear transport as a binding site for translocating transport proteins (Terry and Wente, 2009).

Nuclear import is mediated by importin chaperones that recognize and bind specifically to proteins with basic NLS residues. In the classical model, an importin $\alpha/\beta$ heterodimer binds a protein targeted to the nucleus in the cytosol and facilitates passage through the NPC. A member of the $\beta$-karyopherin family, importin $\beta\_1$, can associate directly with cargo protein or bind an adaptor protein, $\alpha$-karyopherin, specific to the cargo. The $\alpha$-importin C-terminus binds the basic residues in the NLS based on the cargo sequence charge and hydrophobicity (Pemberton and Paschal, 2005). The points of interaction between the NLS and the $\alpha$-importin are characterized by tri-helical structures called armadillo (ARM) repeats. These repeats form a binding pocket that can recognize the basic NLS residues (Kobe, 1999). The $\alpha$-importin N-terminus consists of the importin-$\beta$-binding domain (IBB). In this classical pathway binding of the IBB to the $\beta$-importin completes the assembly of the transport cargo. Nucleoporin FG residues along the inner face of the channel bind transiently with the assembled transport complex as it passes into the nucleus. Inside the nucleus, RanGTP binds the $\beta$-importin causing a conformational change allowing the complex to disassociate and the nuclear protein to carry out its role within the nucleus (Lee et al., 2005). The importin-RanGTP complex is passed through the NPC back into the cytosol. RanGAP in the cytosol assists in hydrolyzing the importin-RanGTP complex to Ran-GDP disassociating the two proteins, and the importin chaperone is able to be recycled. Specific cargo proteins are able to interact with multiple importins, and importins are able to bind promiscuously to different cargos (Mosammaparast, 2004; Cingolani et al, 1999; Cingolant et al, 2002; Fukuhara et al, 2004).
Translocation of proteins through the NPC is bidirectional with the import and export pathways utilizing overlapping factors in their respective roles. Export is also mediated by members of the β-karyopherin family that recognize and bind specific localization signals. Export of tRNA is a main function of the identified export protein, Exportin-t; however, nuclear export of proteins is less understood. The general mechanism of export is analogous to the reverse of import (Figure 4). A transport complex consisting of RanGTP, exportin, and an NES-cargo are translocated from the nucleus to the cytoplasm. Hydrolysis of RanGTP to RanGDP allows for disassembly of the complex releasing the cargo protein (Turner et al, 2012). The best-characterized member of the β-karyopherin family involved in nuclear export is the export receptor, CRM1 (Fuluda, 1997). CRM1 has been identified as the major exportin involved in controlling nucleocytoplasmic distributions. This exportin binds leucine-rich sequences on cargo targeted for export. Consequently, NESs are classically characterized as short leucine-rich hydrophobic regions; however, export cargo proteins without this hydrophobic trait have been identified within the nuclear receptor superfamily (Saporita et al, 2003; Mavinakere et al, 2012). Leptomycin-B (LMB) is a specific inhibitor of CRM1 (Wolff et al, 1997). Therefore, LMB is a powerful tool in characterization of CRM1 independent export pathways (Bunn et al, 2001). At least three CRM1-independent nuclear export pathways have been previously identified in TRα using LMB in export assays (Mavinakere et al, 2012). The complete function of extra-nuclear TR is still under investigation. In relation to TRE transcriptional control, export is a form of regulation of DNA binding. However, TRα1 has been implicated in other non-genomic functions. For instance, a variant of TRα can carry out transcriptional regulation of mtDNA following import into the mitochondria (Wrutniak-Cabello et al., 2001).
Figure 4: Classical model for nuclear export

In the nucleus, cargo protein with an NES associates with an exportin protein and RanGTP to assemble the transport complex. The complex can then pass through the NPC while retaining the native protein structure of the cargo protein. Hydrolysis of RanGTP to RanGDP causes dissociation of the complex and the cargo is free to carry out its extra-nuclear functions.
Other karyopherins have been identified as export receptors; however, their target cargo proteins and mechanistic details of their function are poorly understood. Exportins 4, 5, 6, and 7 have all been characterized for their roles in both broad and specific transport of diverse molecules (Mingot et al., 2004; Stuven et al., 2003; Lipowsky et al., 2000; Bohnsack et al., 2004). Exportin 5 has been shown to influence cytosolic localization of the nuclear transcription factors eEF1A and ILF (Calado et al., 2002; Brownawell and Macara, 2002). It also interacts directly with pre-micro RNA (miRNA) as a key player in the pre-miRNA export pathway (Leisegang et al., 2012). More relevantly, exportin 5 has been shown to mediate nuclear export of the androgen receptor as well (Shank et al. 2008). Exportin 4 is the least conserved member of the importin-β family and is also able to target the nuclear transcription factors eEF1A and Smad3 to the cytosol (Lipowsky, 2000; Chook and Suel, 2011). Export of nuclear profilin-actin complex to the cytosol is mediated by exportin 6, and this function serves as a regulator of actin polymerization in the nucleus (Stuven, 2003). Finally, exportin 7 seems to have a wide range of non-specific interaction with nuclear export cargo including the cytosolic anchor 14-3-3σ, p50RhoGAP, and histones in erythroid cells (Mingot et al., 2004; Hattangadi et al., 2014). TR is known to interact with three of these exportins, 4, 5, and 7, as regulators of its subcellular localization (Subramanian et al., 2015).

**Nuclear Import and Export of Thyroid Hormone Receptor**

There is a wide variety of shuttling characteristics among the nuclear receptors. In the absence of ligand, nuclear receptors can be localized to the cytosol, the whole cell, or the nucleus. Progesterone and estrogen receptors, for instance, are localized to the nucleus regardless
of ligand binding. Meanwhile, glucocorticoids and androgen receptors are primarily cytosolic in the absence of ligand, and in the presence of ligand are targeted to the nucleus. This wide range of distribution patterns indicates the diversity across this class of receptors and further suggests study of an individual pattern of unliganded TR (Kumar et al, 2006).

Unliganded TR is predominantly located in the nucleus where it can carry out its repressive functions. However, the receptor also shuttles quickly between the nucleus and the cytoplasm in an energy-dependent fashion thus requiring use of both nuclear import and export pathways (DeLong et al, 2004; Grespin et al, 2008; Roggero et al, 2016). This additional control of import and export of TR provides another level of regulation of TREs (Kaffman et al. 1999). TR has several known interactions with members of the β-karyopherin superfamily. Recently, it has been shown that import of TRα1 into the nucleus is mediated by at least two transport pathways. TRα1 uses the classical importin α1/β1 heterodimer pathway, in addition to a novel importin-7 mediated pathway. These studies revealed direct protein interaction between these transport proteins and the TR cargo (Roggero et al, 2016). Export pathways, however, are less well-characterized. Knockdown of exportins 4, 5, and 7 has recently been shown to disrupt shuttling dynamics of TRα1, while overexpression of exportins 5 and 7 increase the cytosolic distribution of TRα1 (Subramanian et al, 2015). However, direct interaction between TRα1 and these exportins has yet to be elucidated. Overall, the nuclear shuttling that contributes to our current understanding of TR transcriptional regulation is a meticulous balance among nuclear import, nuclear export, and nuclear retention. Consequently, identification and characterization of the NLSs and NESs of TR is a crucial piece of information in fully understanding shuttling dynamics of this nuclear receptor.
**Thyroid Hormone Receptor Localization Signals**

There have been two NLSs identified in TRα1. A classical bipartite NLS (NLS-1) located in the hinge region is suggested to interact with the importin α1/β1 pathway. An additional NLS (NLS-2) located in the N-terminal A/B domain may act as an alternative importin-7 mediated pathway (Roggero et al, 2016). Export of TR uses multiple transport pathways as well. A CRM1/calreticulin-dependent and a CRM1-independent pathway have both been identified (Grespin et al, 2008; Mavinakere et al, 2012). These findings imply that multiple export pathways could be utilized by TRα1 in regulation of its cellular distribution. Targeted mutagenesis of the LBD residues was used to pinpoint these candidate sequences. This previous work in the lab has identified multiple transferable CRM1-independent sequences that influence nuclear export of TRα1. Two characterized sequences, NES-H12, located in helix 12 within the LBD, and NES-H3/H6, located in the helix 3 to helix 6 region of the LBD, have significant effects on nucleocytoplasmic shuttling of TRα1. Deletion of these residues results in a significant shift in localization of a cytosolic protein to the nucleus; while introduction of these motifs results in a significant shift in localization of a nuclear protein to the cytosol (Mavinakere et al, 2012).

**Thesis Objective: Characterization of Novel NESs in the Thyroid Hormone Receptor**

The previously characterized region of nuclear export activity residing in the helix 3-6 region spans residues 209-265 and is much larger than both a classical NES and the helical NES-H12. This lead to investigation of whether this region contains a single, or multiple NES motifs in addition to identifying a minimal sequence capable of export function. While previous work has shown that each helix (NES-H3 and NES-H6) does contain some export functionality, the
regions of interest examined in these studies possessed overlapping residues, and did not establish minimal independent regions necessary for export activity. Additionally, though TRα1 is established to possess a CRM1-dependent NES, this region has yet to be determined (Grespin et al., 2008; Mavinakere et al, 2012). An additional region of potential export regulation is located between residues 188-206 in the most N-terminal region of the LBD. This sequence has been identified as a candidate for a CRM1-dependent export pathway due to its sensitivity to LMB treatment. Given this information, this thesis sought to accomplish the following:

- Characterize of NES-H3 and NES-H6 as a single bipartite NES or two individually acting monopartite NESs by identifying localization patterns of two non-overlapping sequences.
- Determine if the potential CRM1-dependent NES spanning residues 188-206 has export function by observation of its ability to target a nuclear protein to the cytosol.
- Identify whether interaction is direct between the identified NESs and exportins using pull-down assays and immunoblot detection.

MATERIALS AND METHODS

Plasmids

The NES domain plasmids were designed from rat TRα1 sequences using GeneArt gene synthesis services (Life Technologies), and were digested from the GeneArt vector with endonucleases [HindIII; BamH1 (NEB)]. These inserts were then size-separated in a 1.5% agarose gel and purified using a gel extraction kit (Qiagen). Digested constructs were ligated into
the MBP-mCherry expression vector (constructed in the Allison lab), and transformed into 5-alpha competent *E. coli* cells (NEB). Plasmids were isolated using a plasmid midiprep kit (Zymo Research).

**Cell Culture and Transient Transfection**

HeLa cells were grown at 37°C in Minimal Essential Media (Gibco) supplemented with 10% fetal bovine serum. For fluorescence analysis, HeLa cells were plated at 2.5-3.5 × 10^5 cells/mL on glass coverslips in a six-well plate. Twenty-four hours after plating, cells were transfected with 2 µg of expression construct plasmid DNA and 4 µL Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). Cells were fixed with 3.7% formaldehyde 24 hours post-transfection, and coverslips were mounted on microscope slides with Fluoro-Gel II containing DAPI (Electron Microscopy Sciences).

**Semi-quantitative Scoring**

Cells were scored blindly based on observation of their red fluorescence distribution patterns and assigned a score of: whole-cell, nuclear, or cytoplasmic (n=200 cells). For NES construct semi-quantitative analysis, cytosolic and whole cell scores were combined into one cytosolic category. Percent of cells with a particular distribution pattern per replicate were calculated and the mean of these percentages were used. Nuclear export function of NESs was measured as a significant increase in average percent of cells from a nuclear score (obtained from the Hinge control) to a cytosolic distribution score.
Mean fluorescence scoring

Mean brightness of an ROI in both the nucleus and the cytoplasm of 100 cells per replicate were measured, and a nuclear-to-cytoplasm ratio for fluorescence values was recorded. Consequently, a ratio of 1.0 represents equal fluorescence values for the nucleus cytoplasm or a “whole-cell” localization pattern. Cells transfected with NES constructs often presented fluorescent aggregates throughout the cytoplasm. Though fluorescence values were primarily obtained from ROIs of homogenous expression, some replicates presented a significant number of cells with congregation of fluorescence in the form of cytoplasmic aggregates. Quantitation of fluorescence in these cells was obtained from a mean between the fluorescence of a region containing the aggregate and a region absent of one. This average was then used to calculate the Nuclear-to-Cytoplasm ratio value for that cell. Quantitation of fluorescence values was obtained using Nikon NIS Elements software and transferred to Microsoft Excel. Students t-test was used to determine the statistical significance of any changes in distribution patterns.

Pull-down assays

For co-immunoprecipitation assays, HeLa cells were plated at 10-11 × 10^5 cells/mL in a 100mm vented dish. 24 hours after plating, cells were transfected with 10 µg of plasmid DNA and 20 µL Lipofectamine 2000 (Invitrogen) in Opti-Mem (Invitrogen). Twenty-six hours post-transfection, cells were trypsinized, washed three times in D-PBS, and collected for immunoprecipitation (Chromotek). Cell lysates were tumbled with RFP-Trap Agarose Beads (Chromotek) at 4° C for 2.5 hours. Beads were separated by centrifugation and washed. Beads and unbound supernatant samples were both collected and re-suspended in 2X SDS-PAGE Sample Buffer for subsequent separation with SDS-PAGE.
**SDS PAGE and Western Blot**

Kaleidoscope Protein Standards (Bio-Rad) was used to detect protein size. 20 µl of each immunoprecipitation sample was separated using 8% SDS-PAGE then transferred to a PVDF membrane using the iBlot 7-minute Blotting System (Invitrogen). Membranes were then incubated shaking overnight at 4°C in a 1% BSA Tris-buffered Saline (TBS) plus 0.1% Tween 20 blocking solution. Membranes were then washed six times in TBS with 0.1% Tween 20 and incubated with appropriate primary antibodies for 2 hours in blocking solution at room temperature. A second set of six washes was performed and followed by incubation with respective secondary antibodies for 1.5 hours [anti-RFP, 1:2,000 dilution: horseradish peroxidase-conjugated goat anti-mouse IgG, 1:25,000 dilution (Chromotek); anti-exportin 7, 1:2,000 dilution: horseradish peroxidase-conjugated mouse anti-goat IgG, 1:25,000 dilution; anti-exportin 5, 1:4,000 dilution: horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:25,000 dilution (GE Healthcare)]. After a final set of six washes, blots undergo chemiluminescent detection using ECL Prime detection reagent (GE Healthcare).

**RESULTS**

**MBP-mCherry expression vectors provide a nuclear and cytoplasmic fluorescence control**

Plasmids were designed containing mCherry-maltose binding protein (MBP) (to create a fusion protein too large to diffuse through the nuclear pore complexes), the TRα1 hinge region NLS-1 (to allow nuclear import), and each NES motif of interest. We also designed an MBP-mCherry construct as a control for cytosolic localization and MBP-mCherry-Hinge construct as a control
for nuclear distribution. Upon initial observation, both the nuclear and cytosolic control groups had the expected subcellular distribution. MBP-mCherry has a diffuse whole cell distribution with 98% (±1%) of cells displaying fluorescence localized to the cytosol in the form of diffuse expression and cytoplasmic aggregates. MPB-mCherry-Hinge has an obvious nuclear distribution (89% ±5%) (Figure 5) characteristic of proteins which function mainly in the nucleus. Quantitative analysis of mean fluorescence intensity reflected similar results with the Hinge control having an average N/C value of 1.98 (±0.01) and the MBP-mCherry control with a mean fluorescence of 1.51 (±0.03) (Figure 6).

**Helix 3 and Helix 6 each have individually functioning NESs**

The motifs of each helix were designed to exclude the proline and methionine residues located between helix 3 and helix 6 (Figure 7). This created two, non-overlapping nuclear proteins containing their respective putative NESs. The construct containing helix 3 residues (NES-H3) displayed a range of subcellular distributions (Figures 5 and 8). Cells with homogenous fluorescence signal had noticeably more cytosolic fluorescence than the nuclear Hinge-only construct. When quantified using mean fluorescence intensity these cells had an N/C ratio of 1.63 (±0.05) (Figure 6). The helix 6 construct (NES-H6) has a similar distribution pattern with an N/C value of 1.75 (±0.09). These constructs both had a significantly more cytosolic distribution than the nuclear control (NES-H3, p=0.045; NES-H6, p=0.026) (Figure 6). However, the most commonly observed distribution pattern between these two proteins was a markedly aggregated cytosolic localization with little fluorescence detected in the nucleus (Figure 9). Semi-quantitative analysis was able to represent this characteristic of this construct expression more completely than ROI mean intensity. The semi-quantitative scoring of these constructs reflects a
subcellular localization consistent with the quantitative observations. There is a significant difference in the percent of cells with a cytosolic distribution of both NES-H3 (p= 0.028) and NES-H6 (p= 0.030) when compared with the hinge control (Figure 10). Thus, NES-H3 and NES-H6 are both able to individually target a nuclear protein to the cytosol in a fashion indicative of using export pathways.

**NESH3 and NESH6 may lie on the peripheral sides of each motif**

A third fusion protein, H3/H6-219-254, was designed to include central residues from each helix (residues 219-254) (Figure 7). Subcellular localization of this protein elucidates relative location of the NESs within these helical structures. From initial qualitative observations, this construct had some cytoplasmic aggregates in addition to a nuclear localization pattern (Figure 11).

Semi-quantitative analysis of this construct revealed it does not have significant export function (p=0.056) (Figure 12). Similarly, quantitative scoring indicates an insignificant difference between the N/C ratio of the nuclear control and this overlapping construct (p=0.129) (Figure 6). However, there was also not a significant difference between the localization patterns of NES-H3, NES-H6 and NES-H3/H6 constructs (p=0.07; p=0.06). This result may be an artifact of a truncated NES created by this construct design and its consequent weak interaction with respective transport machinery. Thus, NESs in each of these helices most likely lie in the two regions spanned by NES-H3 and NES-H6 constructs and not this overlapping domain.

**The putative NES, LBD-188-206, does not have significant influence on subcellular localization of nuclear proteins.**
In a similar manner to NES-H3 and NES-H6, constructs for the CRM1-dependent NES located in the LBD residues 188-206 were designed to assay for export functionality using an MBP-mCherry-hinge vector. This construct was not able to localize nuclear fluorescence in a significantly different distribution to the hinge construct (p=0.121) (Figure 13; Figure 14). The additional quantitative fluorescence intensity analysis shows that this construct does not have a significant effect on targeting the nuclear protein to the cytosol (p=0.566) (Figure 6).

**MBP- mCherry NES constructs were unable to interact with identified TRα1 exportins**

Full length TRα1 has been shown to have direct interaction with exportins 4, 5, and 7 as identified by coimmunoprecipitation assays (Zhang, unpublished data). However, “RFP-trap” coimmunoprecipitation assays for these constructs performed here failed to reveal any interaction between these NES fusion proteins and specific exportins. Several replicate Western blots were unable to detect binding between any NES fusion proteins and exportin 4, 5 or 7 (Figure 15). RFP-trap was able to isolate mCherry bound proteins as detected by anti-RFP antibody; however, only unbound lysates showed the presence of exportin 4, 5, and 7. A noticeably lower level of mCherry-TRα1 protein was detected in blots for exportins 4 and 7, and this may account for a lack of detection of any “trapped” exportins. However, relatively high levels of TRα1 in exportin 5 western blots suggest this is not a reason for a lack of trapped protein interaction.
Figure 5: Total percent cellular localization of TRα1 NES fusion proteins obtained from semi-quantitative analysis of cells following transient transfection with plasmid constructs.

Various TRα1 NES candidate sequences expressed in the MBP-mCherry fluorescent vector were assigned a score based on their observed subcellular distribution: Nuclear, nuclear>cytoplasm; Cytoplasmic, cytoplasm>nucleus; Whole Cell, nuclear = cytoplasm. Replicate experiments were quantified (n=4-5 replicates, with >200 cells per replicate). Error bars indicate ±SEM.
Figure 6: Mean fluorescence values of TRα1 NES fusion proteins obtained from nuclear and cytosolic ROIs.

Mean ROI intensity presented as a ratio of Nuclear/Cytosolic (N/C) fluorescence ratio of cells transfected with constructs of interest. ROI area size was controlled between the nuclear and cytosolic measurements for each cell. Each cell’s individual N/C score was calculated, and averages of this ratio were taken across replicate experiments (n=2 replicates, with 100 cells per replicate). Error bars represent ±SEM. *p<0.05; **p < 0.005.
Figure 7: Design of NES-H3 and NES-H6 fluorescent expression vectors

MBP-mCherry contains Maltose Binding Protein (MBP) to make a protein too large to passively diffuse through the NPC and mCherry for use in visualizing red fluorescence. The Trα1 hinge-region NLS1 was fused to target this protein to the nucleus. Non-overlapping regions of NES-H3 and NES-H6 were added to test for their sufficiency for cytoplasmic accumulation. A third fusion protein, H3/H6-219-254, was designed to include central residues from each helix (residues 219-254) to determine if the functional NES lies within the overlapping regions of the helices.
Figure 8: Patterns of fluorescence distribution for transfected NES constructs.

MBP-mCherry constructs displayed diffuse whole cell distribution with cytoplasmic aggregates. MBP-mCherry-Hinge constructs were localized to the nucleus. NES-H3 and NES-H6 sequences were able to increase cytosolic distribution of the fusion protein.

Figure 9: Representative distribution of cytosolic aggregate pattern.

An aggregate pattern was present in all three NES construct groups and was scored as “cytosolic” for semi-quantitative analysis. For quantitative analysis, ROIs were placed on strongly fluorescent aggregates and a region absent of aggregates and the average of these values was used as the cytoplasmic mean for N/C ratios.
Figure 10: Semi-quantitative scoring of fluorescence distribution of NES-H3 and HES-H6 constructs

Data for cytoplasmic distribution were calculated as total percent cells with cytosolic distribution plus total percent cells with whole cell distribution. NESH3 and NESH6 constructs are both sufficient for significant cytosolic accumulation when compared to the nuclear control (NESH3 $p=0.028$; NESH6 $p=0.030$). Error bars represent ±SEM *$p<0.05$. n=4-5 replicates, with >200 cells per replicate.
Figure 11: Patterns of fluorescence distribution for transfected NES overlap constructs.

Cells transfected with overlap construct containing medial residues between helices 3 and 6 inserted into the MBP-mCherry-Hinge expression vector. NESH3/H6-219-254 sequences had a predominantly nuclear localization, with minimal cytoplasmic accumulation.

Figure 12: Semi-quantitative scoring of fluorescence distribution of overlap construct

Cells were transfected with H3/H6 overlap construct (Figure 7), and replicate experiments were scored semi-quantitatively. n=4-5 replicates, with >200 cells per replicate. Overlapping residues 219-254 failed to influence significant cytosolic accumulation with semi quantitative analysis (p=0.056).
Figure 13: Patterns of fluorescence distribution for candidate CRM1-dependent NES construct

Cells were transfected with CRM1/calretiuclin-dependent candidate NES, LBD-188-206, in the MBP-mCherry-Hinge fluorescence vector. Cells had a primarily nuclear distribution similar to expression of the Hinge nuclear control construct. This distribution pattern is indicative of minimal cytosolic accumulation.

Figure 14: Semi-quantitative scoring of fluorescence distribution of LBD-188-206 construct

Data for cytoplasmic distribution was calculated as total percent cells with cytosolic distribution plus total percent cells with whole cell distribution. Residues failed to influence significant cytosolic accumulation as indicated by semi quantitative analysis. Error bars represent ±SEM.
Figure 15: Western blots following RFP-trap of TRα1 fusion proteins, did not coimmunoprecipitate with exportins.

RFP-trap pull down assays were used to identify potential protein interaction between NES constructs and exportins previously identified to influence TRα1 trafficking. Detection of RFP were used for quality control of the assay. RFP-trap was able to pull-down a majority of RFP bound proteins as indicated by detection with anti-RFP. There was no detectable interaction between trapped proteins and exportins 4, 5, or 7. mCherry-TRα1 was also unable to coimmunoprecipitate with any of these exportins.
DISCUSSION

Prior work had identified nuclear export capabilities in the helix 3/helix 6 motifs (Mavinakere et al, 2012); however, whether this region possessed two independently acting NES sequences or a single bipartite sequence was yet to be determined. The results of this thesis, taken with previous studies on nuclear export of TRα1, indicate several factors important in influencing TR’s subcellular trafficking. Primarily, TRα1 possesses three monopartite, CRM1-independent NESs. NES-H12, NES-H3, and NES-H6, are all located in helical motifs of the LBD, and each possess independent, non-overlapping sequences capable of targeting a nuclear protein to the cytosol. These results indicate the possibility for three individually acting export pathways each with its own NES or transport proteins. Differences among localization patterns between the two distinct constructs and the single, 219-254, overlapping construct provide insight to the pinpointed location of these distinct NESs. The H3/H6-219-254 segment was not able to significantly target the nuclear fusion protein to the nucleus; there was not a significant difference in cytosolic accumulation between the overlapping sequence and the separate H3 and H6 constructs. This indicates that at least some export function is retained in this overlapping sequence, perhaps a portion of NES-H3 or NES-H6. However, if both NESs were located in this overlapping region, the export signal is predicted to be stronger as seen when two NES-H12 sequences are combined in expression (Mavinakere et al, 2012). The sequences of these helices possess an isoleucine and leucine-rich region in the N-terminal region of the NES-H6 construct that is also included in the overlapping construct. Although this pathway was previously determined to be CRM1-independent, leucine residues within NES sequences have been implicated in CRM1-independent nuclear export in prior studies (Sato et al., 2006). Based on
These results, it is predicted that at least one of the two NESs in this region is located on the peripheries of the H3-H6 motifs.

This work also eliminated the LBD-188-206 region as a candidate sequence sufficient for determining nuclear export. This sequence does not match the CRM1-dependent consensus sequence rich in leucine residues (Fornerod et al., 1997). However, the current study does not negate the possibility for this sequence mediating TRα1 export in a CRM1/calreticulin-dependent fashion. CRM1 is still able to mediate export in the absence of the leucine-rich NES, and it has been shown that TR coordinates with calreticulin in conjunction with CRM1 for its export (Grespin et al., 2008; Savory et al. 1999). Therefore, the classical CRM1-leucine rich NES association does not necessarily apply to all LMB sensitive pathways. Nevertheless, unlike NES-H3 and NES-H6, the sequence of interest was unable to significantly and independently influence cytosolic accumulation of a nuclear protein on its own.

Previous mutagenesis studies show that disruption of the helical structure in NES-H12 significantly decreases its ability to mediate export function (Mavinakere et al., 2012). Similarly, the highly conserved helix 12 is essential in the conformational change necessary for TR ligand binding (Souza et al., 2011). Helix 3 and helix 6 are also involved in formation of the ligand binding pocket necessary for TR response to T_3 (Wagner et al, 1995). There are several characteristics of these regions indicating their critical role in transcriptional regulation of TR. First, NES-H3 and NES-H6 are conserved across isoforms of TRα1 and TRβ1 in multiple species. Additionally, a cluster of mutations spanning these regions are linked to the genetic disease Resistance to Thyroid Hormone and subsequently were associated with reduced ligand binding affinity and diminished transactivation of TR (Refetoff et al., 1993; Collingwood et al.,
In a similar manner, mislocation of TR could be linked to consequent misregulation of TR associated with thyroid cancer (Cheng et al, 2010).

The predominant presence of cytoplasmic aggregates indicates some kind of interruption in the fusion protein folding. The sequences of interest are small with many hydrophobic residues capable of congregating in these characteristic aggregates. Therefore, the highly cytosolic distribution pattern observed in NES-H3 and NES-H6, may be an artifact of these aggregates disrupting normal protein function. Whether this inhibits these constructs’ interaction with specific export proteins is unknown. However, at least some shuttling pathways remained intact as indicated by the nuclear localization of these constructs. Similarly, diffuse cytosolic accumulation was still observed, and here is classified as evidence for nuclear export. Aggregates in the MBP-mCherry control vector also indicate imperfect expression vector structure, and ideally, a completely cytosolic vector can be used for further characterization experiments. Regardless of this, the MBP-mCherry-hinge construct had characteristic nuclear patterning, and NES-H3 and NES-H6 were sufficient to shift this distribution towards the cytosol.

Immunoblot experiments presented here failed to indicate any direct protein interaction between NES constructs and exportins previously identified in TR shuttling. This may be a result of a large proportion of RFP-tagged fusion proteins being unable to properly bind their respective exportins with high affinity due to improper folding as indicated by aggregates. However, failure to reveal interaction between the mCherry-TRα1 and exportins as well may suggest mCherry-TRα1 is not able to interact with exportins 4, 5, and 7 in the same manner as GFP-TRα1 constructs do (Zhang, unpublished data).
FUTURE DIRECTIONS AND CONCLUSION

There are other factors involved in influencing TR shuttling that are yet to be fully characterized including direct interaction with various export proteins and the effects of post translational modifications on TR localization. Identified modifications on TRs include acetylation, ubiquination, and sumoylation (Sanchez-Pacheco et al, 2009; Liu et al, 2012; Wadosky, 2012). In particular, sumoylation is a modification of interest regarding this thesis work due to the presence of a sumoylation site identified on residue 283 near NES-H6 (Liu et al, 2012). Whether subcellular trafficking is influenced by this post-translational modification is currently under investigation. Further targeted mutagenesis of these individual sequences is a future direction for determining a definitive consensus sequence responsible for export function. Additionally, establishing a primarily cytosolic fluorescence control vector is another area of interest for improving detection of export function. Because the LBD-188-206 sequence examined here failed to show individual export capabilities, the exact NES mediated by the previously characterized CRM1/calreticulin mediated pathway is yet to be identified. Further mutagenesis studies in conjunction with LMB treatment assays will contribute to isolating and defining this sequence of interest. Once a CRM1-dependent sequence has been identified for influencing cytoplasmic accumulation, subsequent pull-down assays similar to those presented here could be used to confirm CRM1/calreticulin interaction. Finally, establishing a protocol for detecting receptor NES-exportin interaction is a crucial step in for fully elucidating the conclusive role that these signals play in export pathways.

This thesis concludes that there are three independent, monopartite sequences in TRα1 sufficient for targeting a nuclear protein to the cytosol: NES-H12, NES-H3, and NES-H6.
Though a fourth CRM1-dependent, signal is indicated by TR treatment with LMB (Grespin et al., 2008), the candidate residues presented here do not have export capabilities. The NESs detailed in the current study, though located in close proximity in the LBD, are not dependent on each other to induce cytosolic accumulation. These findings also have implications for shuttling of other TR variants. Helix 3 and Helix 6 motifs are highly conserved in TRβ1 despite this isoform having a distinct physiological role. Several diseases present accumulations of mutations in regions determining TR localization, and nuclear receptor misregulation is implicated in a wide range of cancer and metabolic dysfunction (Cheng et al, 2010; Bonamy et al, 2005; Bunn et al., 2001; Mavinakere et al., 2012). This, taken with the general robustness of the nuclear receptor family’s role in many critical cellular processes, indicates the significance of export regulation regarding human health. Overall, identification of these export sequences in conjunction with the current body of knowledge concerning TRα1 shuttling contributes to our understanding of how TRα1 regulates T₃-responsive gene expression. Furthermore, the effects of nuclear receptor export on normal and dysfunctional endocrine function is of particular significance regarding a number of pathologies affecting human health.
REFERENCES


