2008

Nucleocytoplasmic Shuttling of the Thyroid Hormone Receptor

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A Thesis presented to the Graduate Faculty

of the College of William and Mary in Candidacy for the Degree of

Master of Science

Department of Biology

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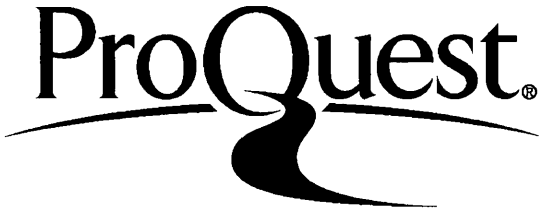
May, 2008



APPROVAL PAGE

This thesis is submitted in partial fulfillment
of
the requirements for the degree of

Master of Science

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Approved by the Committee, April 2008

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ABSTRACT PAGE

Thyroid hormone receptor α (TRα) functions as either an activator or repressor of target genes in response to thyroid hormone. TRα shuttles between the nucleus and the cytoplasm across the nuclear membrane. This thesis research focused on the mechanisms regulating nuclear import and export of TRα. Prior studies have shown that TRα nuclear import in *Xenopus* oocytes proceeds by two coexisting mechanisms, through a passive diffusion pathway, or a signal-mediated process. Here, to determine the general mechanism of nuclear import in mammalian cells, *in vitro* nuclear import assays were performed, using digitonin permeabilized HeLa (human) cells. To demonstrate an energy requirement, import reactions containing recombinant FITC-labeled TRα were performed in the presence of complete cytosol or import buffer alone and visualized by fluorescence microscopy. The import reaction containing cytosol was shown to support strong TRα nuclear import at 30°C, but when the import reaction was carried out at 4°C nuclear import was inhibited. TRα nuclear import could be fully restored by further incubation at 30°C. Reversible inhibition suggests that chilling blocked TRα import by inhibiting specific transport components, rather than by preventing import by way of non-specific cellular damage. Additionally, depletion of ATP (and GTP) using apyrase treatment strongly inhibited nuclear import, suggesting that TRα import occurs by a process requiring metabolic energy. To assess factors required for TRα nuclear import, import reactions were reconstituted by addition of recombinant proteins. Import reaction mixture containing RanGDP, NTF2, importin α1, and importin β1, along with an energy regeneration system was able to mediate TRα import. Taken together, these results show that, in mammalian cells, TRα import follows a signal-mediated pathway. Import is temperature and energy-dependent and classical import factors are able to support TRα nuclear import in this *in vitro* system.

The process of nuclear export is of equal importance to nuclear import in a nucleocytoplasmic shuttling protein. Studies in mammalian cells have shown TRα does not directly follow a pathway mediated by the export factor CRM1. Here, *in vitro* nuclear export assays were performed using digitonin permeabilized HeLa cells to study the role of the Ca^{2+} binding protein calreticulin (CRT) in TRα nuclear export. Permeabilized HeLa cells expressing GFP-TRα were incubated in an export reaction containing CRT alone, complete cytosol which contains endogenous CRM1 but not CRT, or export reactions containing complete cytosol supplemented with CRT. Export reactions containing both CRT and cytosol were able to support >95% loss of nuclear fluorescence of GFP-TRα indicative of nuclear export, while reactions with cytosol alone were only able to support <80% loss of nuclear fluorescence of GFP-TRα. Reactions containing only CRT did not support nuclear export. Data presented in this thesis research thus provide evidence of a cooperative interaction of CRM1 and CRT to mediate the nuclear export of TRα

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Figures & Tables

Acknowledgements:

I would like to thank Dr. Allison for her help and guidance and friendship throughout my time at William and Mary. Without her continued support this research would not have been possible. I would also like to thank Dr. Zwollo and Dr. Kerscher for their guidance, suggestions, and encouragement during the writing of this thesis. In addition, I would like to acknowledge the members of the Allison lab, especially Matt Grespin and Manu Mavinakere.. Finally, I would like to thank my wife for her love and support and also for her help with statistical analysis.

Funding for this research was provided by:

The National Science Foundation

The National Institutes of Health

Chapter 1 – General Introduction

Thyroid hormone (TH) is a tyrosine-based hormone produced by the thyroid gland that requires dietary iodine for its production. Thyroid hormone binds to the thyroid hormone receptor (TR) in the cell nucleus affecting a wide range of tissues (Brook and Marshall, 2001). The regulation of basal metabolism, normal growth, central nervous system development, stimulation of liver function, and maintaining proper cardiac output are just some of the processes affected by TH (Ahmed et al., 2008; Brook and Marshall, 2001; Burris and McCabe, 2001; Yen, 2001). TH has even been shown to be critical in the adaptation from freshwater life to seawater survival in some salmon species (Specker, 1988). TR is thus critical in many systems and a better understanding of the regulation of its intracellular trafficking along with activation or repression of its target genes is of potential importance for understanding TH related diseases and developing treatment strategies.

The thyroid hormone receptor is a transcription factor that can either activate or repress TH-responsive genes bearing thyroid hormone response elements (TREs) depending on its liganded state. The thyroid hormone receptor is a member of the type II nuclear receptor superfamily including the vitamin D receptor and the retinoid X receptor, as well as steroid hormone receptors such as the estrogen, androgen and glucocorticoid receptors (Tsai and O'Malley, 1994; Yen, 2001). Thyroid hormone receptor shuttles between the nucleus and the cytoplasm (Bunn et al., 2001) and, therefore, must cross the nuclear

membrane. This shuttling is a critical aspect of nuclear import and export via nuclear pore complexes (NPC).

Molecules and ions smaller than ~40kD are able to passively diffuse through the NPC while larger molecules are required to follow signal mediated import and export pathways. One of the two main forms of TR, TRα, is a 46kD protein, close to the limits for passive diffusion through the NPC. Studies have shown that TRα nuclear import in *Xenopus* oocytes proceeds by two coexisting mechanisms, through a passive diffusion pathway, or a signal-mediated process (Bunn et al., 2001). Prior to this thesis research, the general mechanism of nuclear import in mammalian cells was unknown and, although TR had been shown to follow an energy and temperature dependent export process in *Xenopus* oocytes, the transport factors had not been characterized (Bunn et al., 2001). Signal-mediated transport requires additional soluble factors to facilitate translocation into the nucleus (Allison, 2007; Bunn et al., 2001; Pemberton and Paschal, 2005; Stewart, 2007). These factors, known as importins and exportins, can bind to a cargo protein at either a nuclear localization signal (NLS) for import or a nuclear export signal (NES) for export and facilitate transit of the cargo through the NPC by interacting with the proteins of the NPC. The use of a signal mediated process adds an additional regulatory step in the modulation of TH-responsive genes.

The overall aim of this thesis was to characterize the general mechanisms for TR transport by using *in vitro* nuclear import and export assays. The following sections will review TR structure and function, nuclear import and export

mechanisms, and the use of *in vitro* transport assays to characterize as well as reconstitute import and export pathways.

Thyroid Hormone

Production & Regulation –

The thyroid hormone system is composed of two hormones secreted from the thyroid gland, 3,3′,5,5′-tetraiodothyronine (T_4) and 3,5,3′-triiodothyronine (T_3) (Brook and Marshall, 2001) (Figure 1.1). These hormones are secreted by a hormone cascade starting at the hypothalamus with thyrotropin-releasing hormone (TRH) stimulating the release of thyroid stimulating hormone (TSH) from the pituitary gland (Berne and Levy, 1990; Conn and Melmed, 1997) (Figure 1.2). TSH then stimulates the thyroid gland to produce T_4 and, in much lower quantities, T_3. Free T_3 in circulation causes autoinhibition by a negative feedback loop in which free T_3 down regulates the production of TRH and therefore TSH (Berne and Levy, 1990). T_4 and T_3 circulate through the blood stream bound predominantly to serum proteins (Burrow, 1993; Seo, 1996). T_4 is converted by tissue specific deiodinases to T_3, its more active form, by deiodination upon entering the target tissue (Kohrle, 1999; Kohrle, 2000). The removal of one iodine greatly enhances the receptor/ligand complex binding affinity and stability of liganded TR. When T_4 binds to TR, the ligand binding pocket must accommodate the large 5′ iodine while T_3, which is lacking the 5′ iodine, fits more tightly in the binding pocket (Sandler et al., 2004).

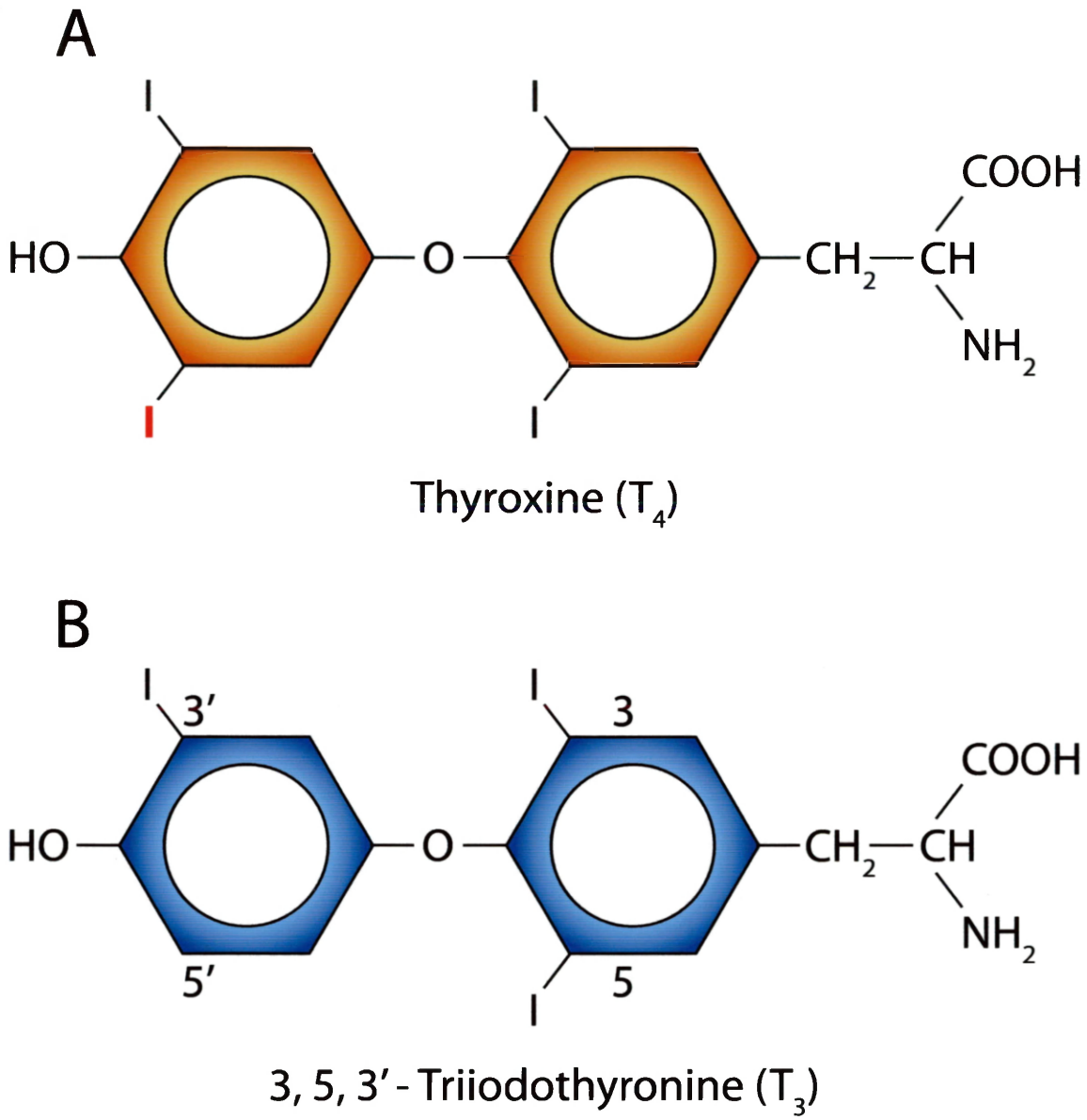

Figure 1.1 Structure of Thyroxine (T_4) and Triiodothyronine (T_3). (A) The predominantly biologically inactive thyroid hormone form Thyroxine (T_4) with the 5′ iodine shown in red. (B) Biologically active thyroid hormone form triiodothyronine (T_3) formed by a deiodination reaction removing the 5′ iodine.

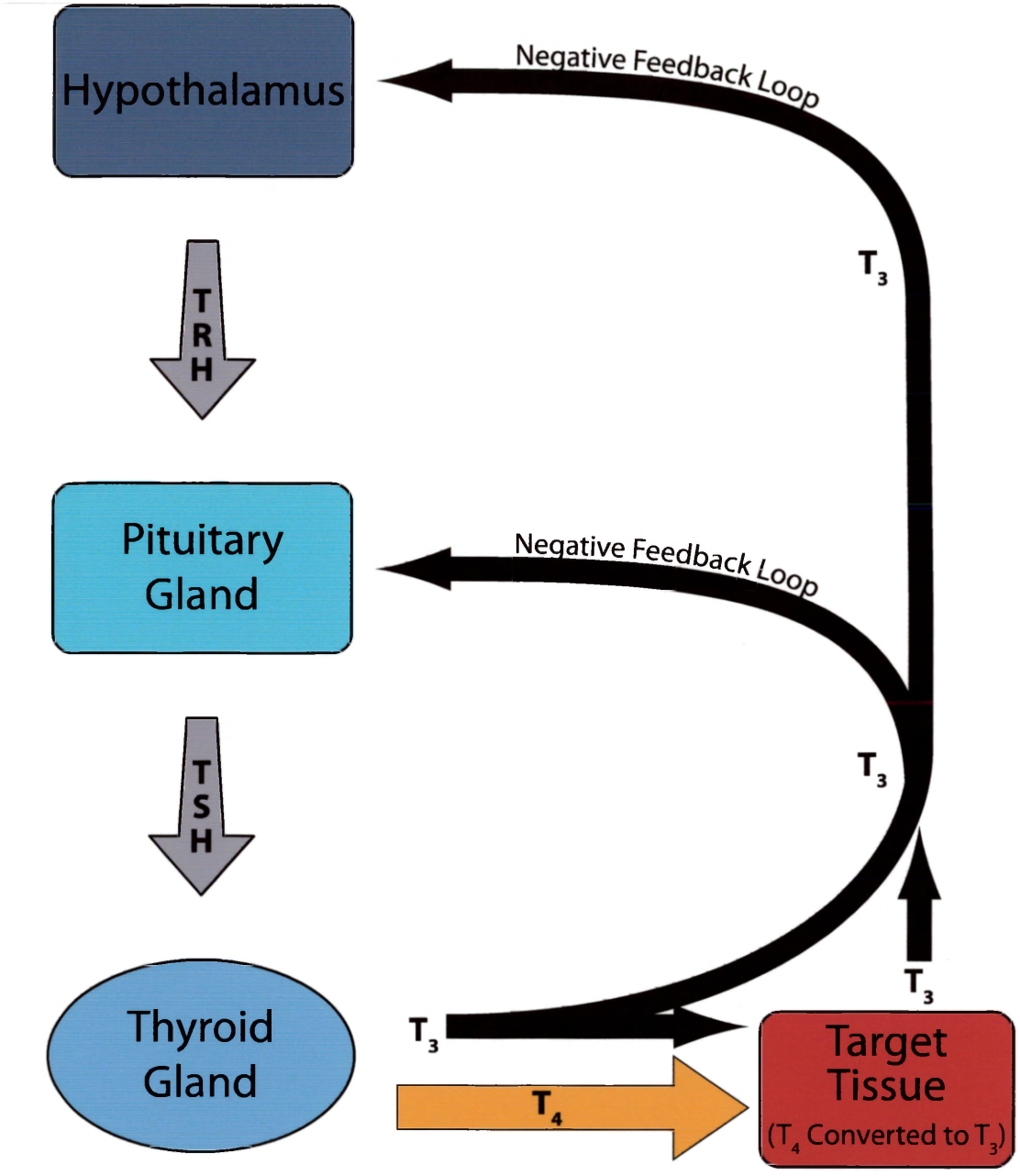

Figure 1.2 Thyroid Hormone Pathway. The production of thyroid hormone begins at the hypothalamus with the production of thyrotropin-releasing hormone (TRH) which stimulates the release of thyroid stimulating hormone (TSH) from the pituitary gland. TSH then stimulates the thyroid gland to produce T_4 and lower quantities of T_3. Free T_3 in circulation causes autoinhibition by a negative feedback loop in which free T_3 down regulates the production of TRH and TSH. T_4 and T_3 circulate through the blood stream bound predominantly to serum proteins, and T_4 is converted by tissue specific deiodinases to active T_3 by deiodination upon entering the target tissue.

Receptor Structure –

Thyroid hormone receptor (TR) has two main forms, thyroid hormone receptor alpha (TRα), and thyroid hormone receptor beta (TRβ) (Laudet and Gronemeyer, 2002; Lazar, 1993), each encoded by two different genes. Both TRα and TRβ have two major isoforms generated by alternative splicing (Lazar, 1993; Yen, 2001) (Figure 1.3). TRα1 is the focus of this thesis research, and hereafter is refered to as TRα for simplicity. The TRα2 isoform does not bind hormone and does not form dimmers, therefore, was not part of this study. The TRα gene is on human chromosome 17 while the TRβ gene is on human chromosome 3. These receptors have the ability to bind DNA and either activate transcription in the presence of T_3 or repress transcription in the absence of T_3, or vice versa in some cases.

TR is comprised of three major domains; an N-terminal activation domain (A/B), a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) (Figure 1.3). The N-terminal A/B region is the least conserved region among TR isoforms and is thought to play a role in transcriptional activation (Laudet and Gronemeyer, 2002; Lazar, 1993; Tsai and O'Malley, 1994; Yen, 2001), though its role is poorly understood.

The DBD is the most highly conserved central region with approximately 88% sequence similarity among TR isoforms (Laudet and Gronemeyer, 2002). This region contains two zinc fingers responsible for protein-DNA binding; each coordinating four cysteine residues around a zinc ion. The P Box of the first finger contacts the major groove of target DNA that makes up the TRE and

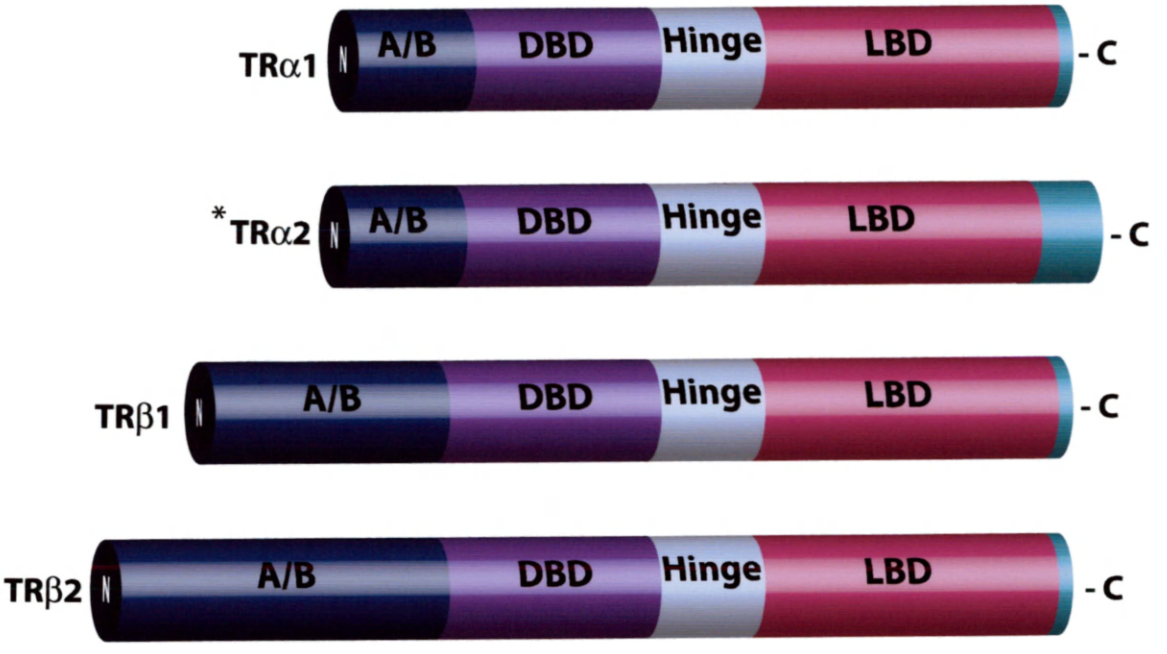

Figure 1.3 Structure of Thyroid Hormone Receptors. Thyroid hormone receptor (TR) has two main forms, thyroid hormone receptor alpha (TRα), and thyroid hormone receptor beta (TRβ). They are encoded by two different genes, each having two major isoforms that are generated by alternative splicing. TRα1 is the focus of this thesis research and is referred to as TRα for simplicity. TRα2 is a variant form that does not bind ligand. Three major TR domains; the N-terminal activation domain (A/B), a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). TR also has a flexable linker or hinge region between the DBD and LBD. The domains indicated above are not drawn to exact proportions, and are for reference only.

* The TRα2 isoform lacks the ability to bind T_3 and does not form heterodimers

imparts a sequence specific binding ability to the receptor (Tsai and O'Malley, 1994; Yen, 2001). The D Box of the second zinc finger binds to the minor grove in the TRE adding additional DNA contact points. The DBD also contains regions, both upstream and downstream of the zinc fingers, which aid in dimer formation and the stabilization of the dimers.

Joining the C-terminal end of the DBD to the LBD is the hinge region. This region contains a short lysine-rich amino acid sequence known as a nuclear localization sequence (NLS). Research on the TRβ NLS has found this short sequence to be required for the recognition and binding of karyopherins involved in nuclear import (Zhu et al., 1998). The hinge region of TRα is thought to contain a classical NLS as well, though currently it has not been as well characterized as the TRβ region. Prior to this thesis, little research had been done on any aspect of TRα NLS mediated import.

The LBD region plays a primary role in the binding of T_3, and also in transactivation and dimerization (Ribeiro et al., 2001). The ligand binding pocket formed by the LBD has been shown to bind T_3 as well as T_4. As noted earlier, the hormone receptor/T_4 complex is less stable than the complex that forms in the presence of T_3, but interestingly the receptor is still able to close around the larger T_4 (Sandler et al., 2004). When hormone is bound in the hydrophobic pocket of the LBD, conformational changes cause a "closing" of the hydrophobic pocket around the hormone (Brook and Marshall, 2001; Laudet and Gronemeyer, 2002). This conformational change allows coactivators to bind the receptor/ligand complex and modulate transcription.

Thyroid Hormone Response Elements & Receptor Dimerization –

TR binds to DNA via its DBD; however, this region is also important for dimerization with the retinoid X receptor (RXR). TR is able to bind DNA at TREs preferentially as a heterodimer with RXR but can also bind as a monomer or a homodimer (Velasco et al., 2007; Yen, 2001). Recently, unliganded TRβ DBD-LBD constructs have been shown to form tetramers in solution via interactions of their LBD. The formation of RXR tetramers in the absence of ligand has been shown to have an auto-silencing function by blocking transcription activation domains (Kersten et al., 1998). The function of unliganded TR tetramers is not yet understood; however, it is possible they may have some similar auto-silencing function (Figueira et al., 2007).

Each dimer state has a different binding preference for a certain type of TRE. Thyroid hormone response elements (TRE) are composed of repeat sequences of DNA (AGGTCA or AGTTCA) that exist in several forms. They can be formed as direct repeats, inverted repeats or as palindromes. Additionally, most TREs are activated by liganded TR (positive TREs) while a few others are repressed by liganded TR (negative TREs) (Glass, 1994). Finally, it has also been shown that all receptor forms can respond to T_3, not solely the TR-RXR heterodimer (Velasco et al., 2007).

Coactivation & Repression –

As summarized in the previous section, thyroid hormone responsive genes can be either activated or repressed by TR binding to a TRE, depending on its ligand state (Figure 1.4). Many of the known thyroid diseases arise from

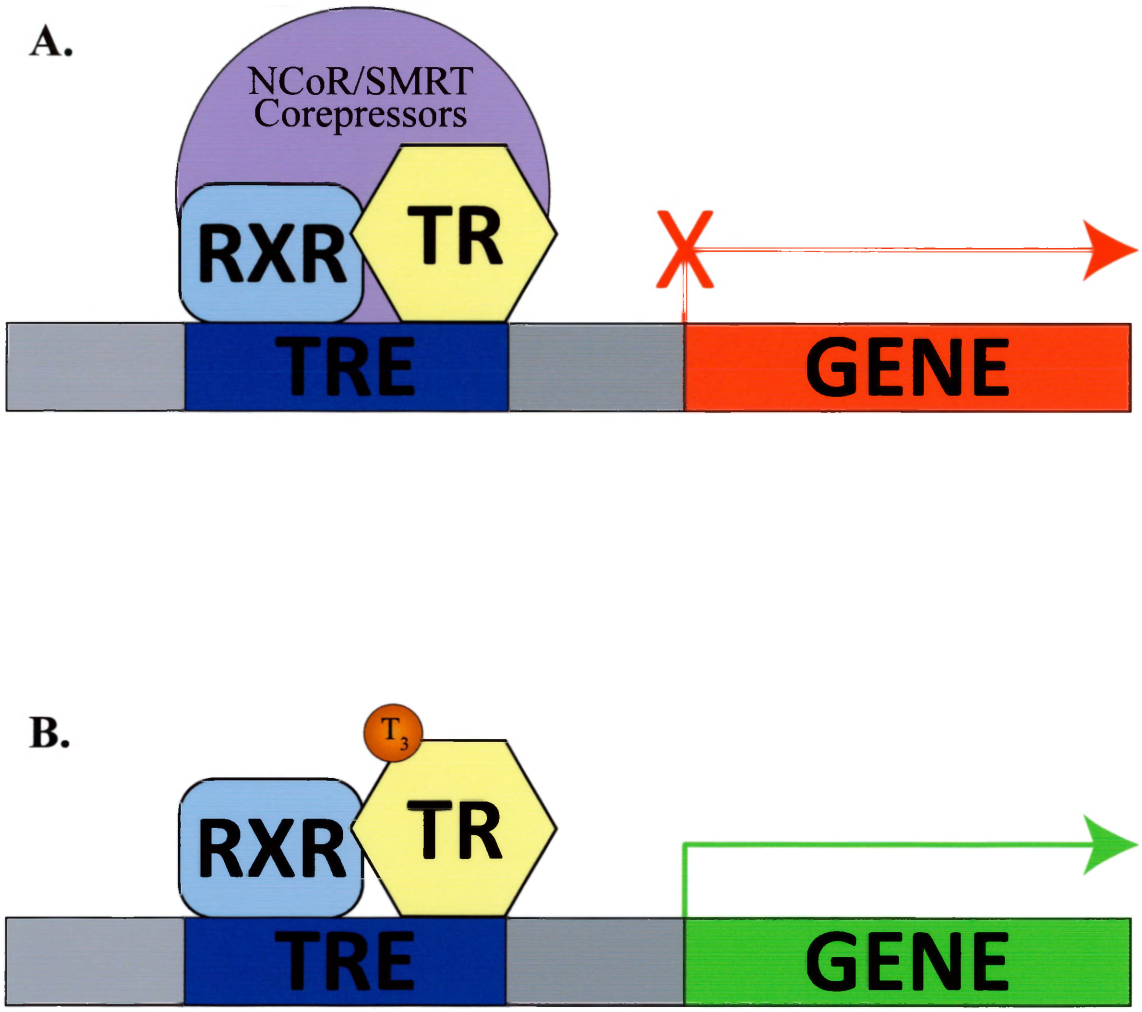

Figure 1.4 Coactivation & Repression of the Thyroid Hormone Receptor. (A) In the absence of T_3, TR has an active corepressor site which, when TR is bound to a positive TRE, represses transcription of TH responsive genes in target tissues. Repressor proteins such as NCoR and SMRT (●) inhibit the formation of the transcriptional preinitiation complex, repressing gene expression. (B) When T_3 binds to TR (●), the receptor undergoes a conformational change disrupting the corepressor binding site and forming a site for coactivator binding. The formation of the preiniation complex can proceed and gene expression occurs.

mutant forms of the receptor with altered gene activation properties or from their localization to an incorrect cellular compartment. As previously mentioned, TR plays several different roles in growth and development and therefore altered gene activity can lead to abnormal cell growth. The study of import and export pathways will provide a better understanding of TH related diseases that arise from mutant forms of TR, as well as general TR gene regulation.

TR is primarily localized to the nucleus at steady state. This is a key difference distinguishing TR from steroid hormone receptors, which are generally sequestered in the cytoplasm in an inactive state when their ligand is not present (Shank and Paschal, 2005). In the absence of T_3, TR has an active corepressor site which, when TR is bound to a positive TRE, represses transcription of TH responsive genes in target tissues. Two of the most well studied repressor proteins are nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). These large proteins are thought to repress transcription by interacting with the hinge region of TR and with components of the basal transcription machinery such as TATA binding protein (TBP) and transcription factor IIB (TFIIB) (Aranda and Pascual, 2001; Choi et al., 2008; Yen, 2001). By binding to these factors, the formation of the preinitiation complex is hindered at an early stage preventing transcription (Allison, 2007; Fondell et al., 1996).

Alternatively, when T_3 binds to TR, the receptor undergoes a conformational change disrupting the corepressor binding site and forming a site for coactivator binding. When the liganded TR binds a positive TRE,

transcription of TH responsive genes in target tissues is activated (Glass & Rosenfeld 2000). It is likely that chromatin remodeling factors play key roles in both repression and activation of TH responsive genes (Aranda and Pascual, 2001; Yen, 2001).

Nuclear Import & Export

Nuclear Pore Structure –

In eukaryotic cells the nuclear membrane forms a barrier, separating DNA into the nuclear compartment, and protein synthesis into the cytoplasmic compartment. Many molecules produced cytoplasmically such as TR, must cross this barrier to carry out their function in the nucleus. This provides an additional level of gene regulation that is controlled by the passage of the protein/receptor or "cargo" through a highly structured channel in the nuclear membrane called a nuclear pore complex (NPC). This process is known as nucleocytoplasmic transport.

The NPC is a proteinaecous structure of eight fold symmetry that is comprised of multiple copies of ~30 different nucleoporins, and approaches 50MDa in mass. These proteins form loose cytoplasmic filaments, a structured nuclear basket, and the membrane spanning proteins which line the central channel. Nucleoporins contain unstructured phenylalanine-glycine (FG) repeats which project into the central channel and from the nuclear and cytoplasmic faces of the NPC (Alber et al., 2007). These FG repeats also interact with each other and may form a diffusion barrier permeable only to small metabolites and to macro-molecules complexed with soluble factors known as importins or exportins

(Alber et al., 2007; Frey and Gorlich, 2007; Lim et al., 2007a; Lim et al., 2006; Lim et al., 2007b).

The NPC creates a regulated channel between the cytoplasm and the nuclear compartment (Figure 1.5). Ions and small molecules up to a size range of ~40kD can passively diffuse through the NPC (Ribbeck and Gorlich, 2002). Larger molecules must use a signal mediated process involving a short lysine and arginine rich amino acid sequence, called the nuclear localization signal (NLS), a Ran gradient, and additional soluble factors to pass through a FG interaction barrier in the central channel of NPC (Lim et al., 2007a; Lim et al., 2006; Lim et al., 2007b).

Importins Mediate Nuclear Import –

Nuclear import and export pathways utilize a group of proteins collectively known as importins to aid macromolecules in reaching their target subcellular compartment. Nuclear import of a protein "cargo" bearing a classical NLS uses soluble factors called importins which are thought to aid the cargo in passing through the FG barrier by a direct importin-FG repeat interaction. In the classical NLS mediated import pathway, importin α binds to a cargo protein and acts as an adaptor molecule allowing a trimeric cargo/α/β complex to form (Pemberton and Paschal, 2005; Stewart, 2007) (Figure 1.6). This cargo/α/β complex is then able to translocate through the NPC. In general, importin α acts as an adaptor molecule between the cargo and importin β. By doing so, proteins which would be unable to bind importin β directly are able to take advantage of its ability to facilitate entry into the nucleus (Cook et al., 2007). Some cargo proteins are able

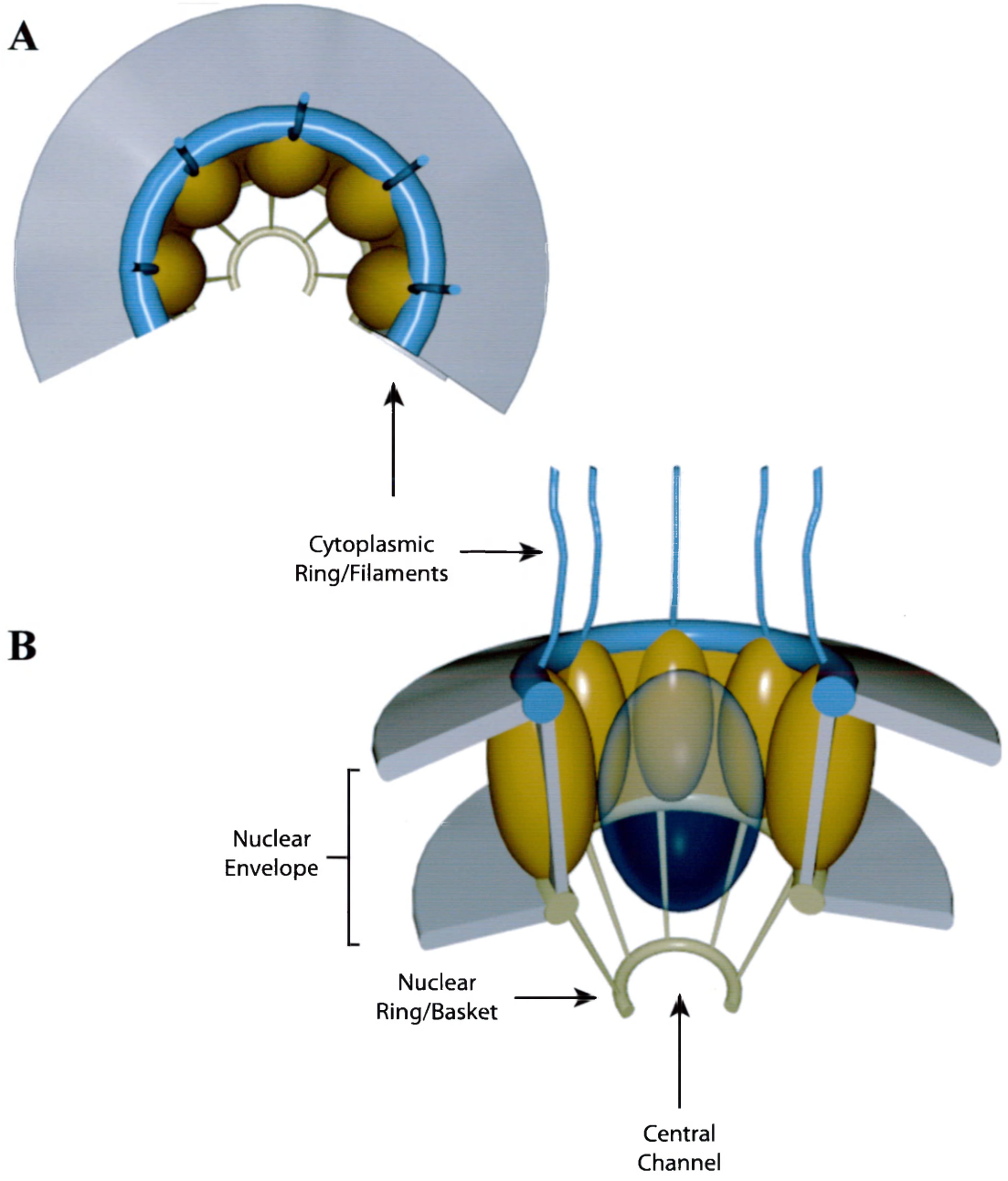

Figure 1.5 Nuclear Pore Complex. Blue components are cytoplasmic filaments and the cytoplasmic ring. Gray components are the inner and outer membranes comprising the nuclear envelope. Dark brown components are nucleoporins that form the barrel or central channel. Light brown components are the nuclear ring and basket. (A) View from the cytoplasm down into the cutaway NPC model (B) Side profile of the NPC with a "central plug" included (transparent blue). Both top and side views show 5 of the 8 subunits that form the complete NPC.

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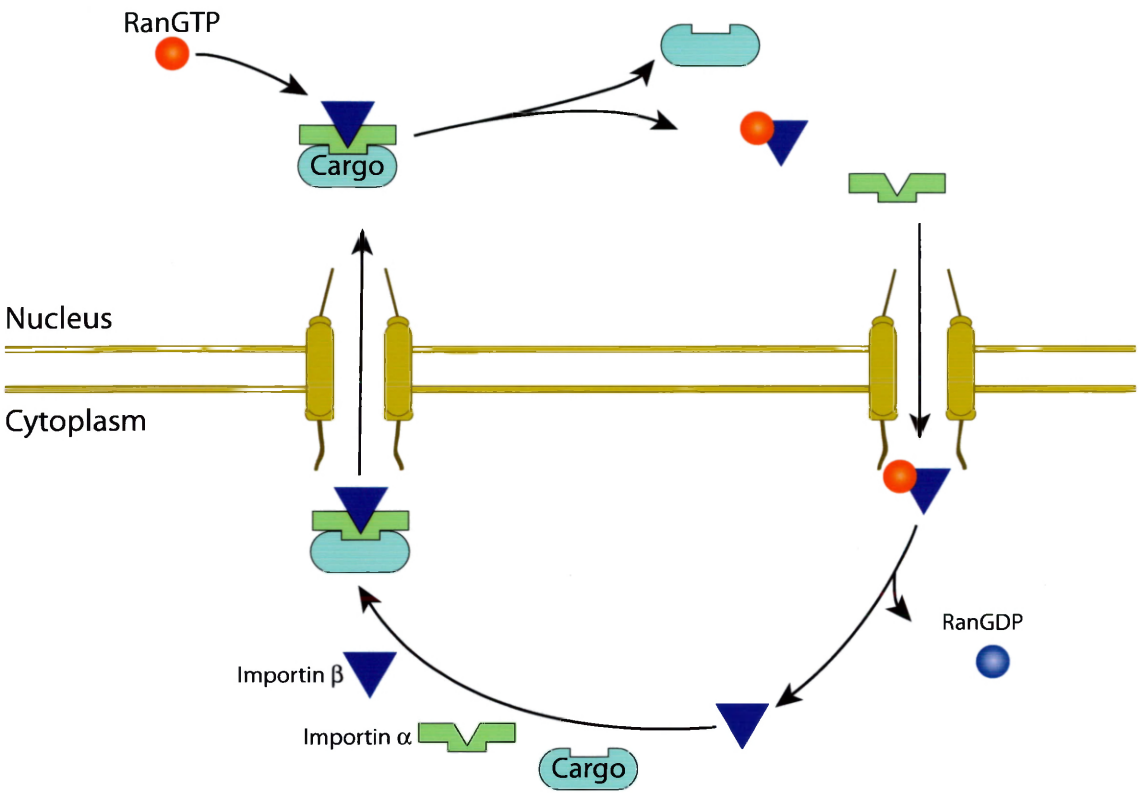

Figure 1.6 Nuclear Import Cycle. The classical nuclear import cycle begins with importin α binding to the NLS of a cargo. Importin a then acts as an adaptor protein for importin b, binding the cargo/α/β in a trimeric import complex. The import complex translocates throught the NPC and upon reaching the nuclear side of the NPC, dissociates upon interaction with RanGTP. The cargo is released freely in the nucleus, and the importins are recycled to the cytoplasm.

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to directly bind importin β to facilitate their movement through the NPC FG barrier.

Importin α is a 58kD protein composed of three major domains, an importin β binding (IBB) domain, a NLS binding domain, and CAS or cellular apoptosis susceptibility protein domain (Kutay et al., 1997).

The IBB domain is composed of a helical region extended away from the main body of the protein, and serves as the binding site for importin β. The NLS binding domain in the central region of the protein provides the binding site for the lysine/arginine rich NLS of the cargo protein, and the C-terminal end contains the CAS binding domain. CAS is an export factor in the importin β-like family that can bind importin α in the presence of RanGTP (Gorlich and Kutay, 1999; Kutay et al., 1997; Riddick and Macara, 2005). When the trimeric complex containing importin α has dissociated in the nucleus, the export receptor CAS binds to importin α and facilitates its recycling to the cytoplasm (Cook et al., 2007; Kutay et al., 1997; Strom and Weis, 2001).

Importin β is a 97kD protein and the most well studied member of the importin β-like family. This family contains both import and export receptors as well as several orphan receptors for which no cargo is currently known. In general, members of the importin β-like family all have the ability to bind RanGTP as well as a cargo or adaptor molecule. Additionally, these proteins contain binding sites for interaction with nucleoporin FG repeats, a feature which is key to their import/export function. Importin β is able to interact directly with the FG repeats to "open" a path through the FG repeat meshwork. It is not yet known if

the interactions between unstructured FG repeats form a solid hydrogel *in vivo* or if the interactions are less rigid, but it is clear that there is great deal of FG repeat interaction in the central channel of the NPC (Alber et al., 2007; Frey and Gorlich, 2007; Lim et al., 2007a; Lim et al., 2006; Lim et al., 2007b). After releasing its cargo, importin β is recycled to the cytoplasm complexed with RanGTP (Riddick and Macara, 2005; Stewart, 2007)

The Role of Ran in Nuclear Import –

A relaxing or opening of the FG barrier for cargoes complexed with import receptors allows them to pass to the nuclear side of the membrane where a high concentration of a small GTPase Ran in its GTP bound form (RanGTP) resides. Ran is a 24kD member of the Ras superfamily of small GTPases which can switch between binding GTP and GDP. Ran has critical functions in the nuclear import pathway, a pathway which is dependent on an asymmetrical distribution of RanGDP/GTP. In the nucleus, a high concentration of Ran in its GTP bound state exists. When the import complex encounters RanGTP on the nuclear side of the NPC, the complex dissociates leaving its cargo in the nucleus. A high RanGTP concentration is required to facilitate the dissociation of any import complex successfully passing through the NPC. The importins then are recycled to the cytoplasm complexed with RanGTP where, upon exiting the cytoplasmic side of the NPC, RanGTP is hydrolyzed to RanGDP by GTPase-activating protein (RanGAP). This causes a release of importins into the cytoplasm, allowing them to participate in additional rounds of import. To maintain the high nuclear concentration of RanGTP, RanGDP in the cytoplasm binds to nuclear

transport factor 2 (NTF2) and is recycled to the nucleus where guanine exchange factor (RanGEF), associated with chromatin, converts it back to RanGTP by nucleotide exchange (Cook et al., 2007; Ribbeck et al., 1998; Stewart, 2007). The action of these cellular compartment-specific exchange factors maintains the asymmetrical distribution of high RanGDP in the cytoplasm and high RanGTP concentrations in the nuclear compartment (Figure 1.7)

Exportins Mediate Nuclear Export –

Export of a protein in the nuclear compartment follows a similar, though less well understood path, compared to nuclear import. A cargo to be exported generally has an exposed leucine rich nuclear export signal (NES). Export factors called exportins can, in the presence of RanGTP, recognize and bind to an NES forming a trimeric complex with the cargo which can then translocate through the NPC (Fried and Kutay, 2003). Currently, more than 75 proteins are known to have a NES that is able to bind exportin1, also known as CRM1 (Pemberton and Paschal, 2005). CRM1 is a member of the importin β-like family of proteins (Mosammaparast and Pemberton, 2004; Strom and Weis, 2001), and is one of the most well studied "classical" exportins. CRM1 is responsible for the export of a broad range of proteins and RNA (Fried and Kutay, 2003; Pemberton and Paschal, 2005) (Figure 1.8).

The Role of Ran in Nuclear Export –

As described above, Ran GTP plays an opposite role in nuclear export compared to its role in nuclear import; import complexes are dissociated in the presence of RanGTP, but export complexes are formed. As RanGTP containing

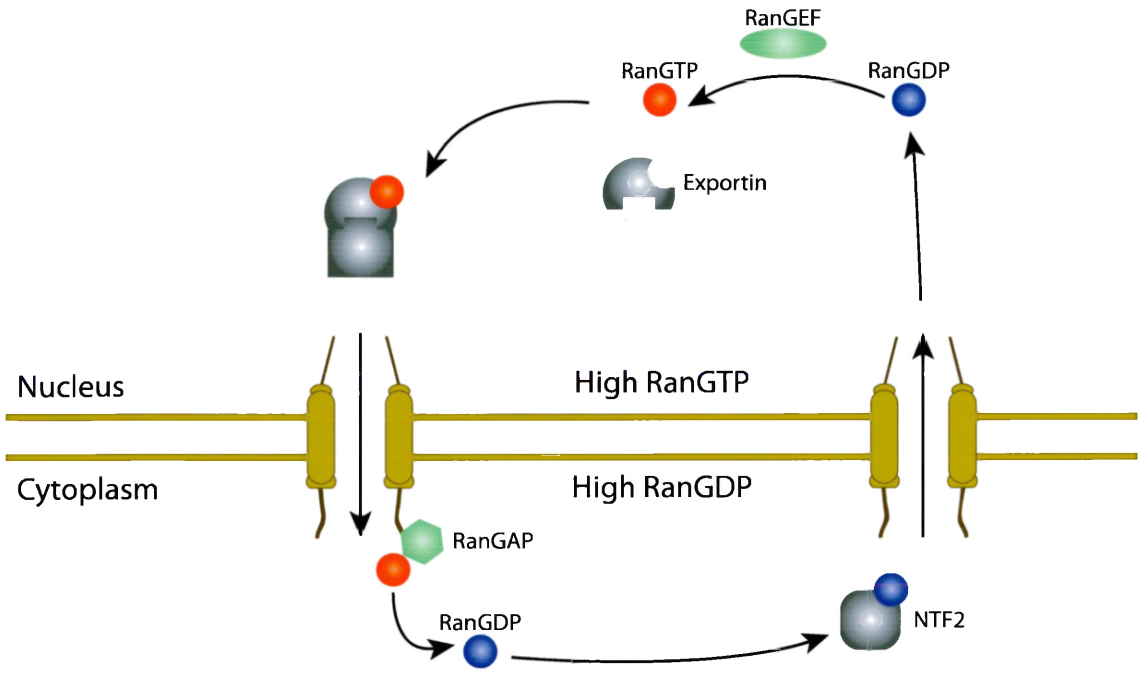

Figure 1.7 Ran Cycle. Signal-mediated nuclear transport depends on a cellular Ran gradient for proper function. A high concentration of RanGTP (red) is needed in the nucleus to dissociate import complexes, while the hydrolysis of RanGTP to RanGDP (blue) by a GTPase-activating protein RanGAP, is responsible for dissociation of export complexes. Free cytoplasmic RanGDP is imported to the nucleus in association with NTF2 where Ran guanosine-nucleotide exchange factor exchanges a GTP for the GDP resulting in nuclear RanGTP.

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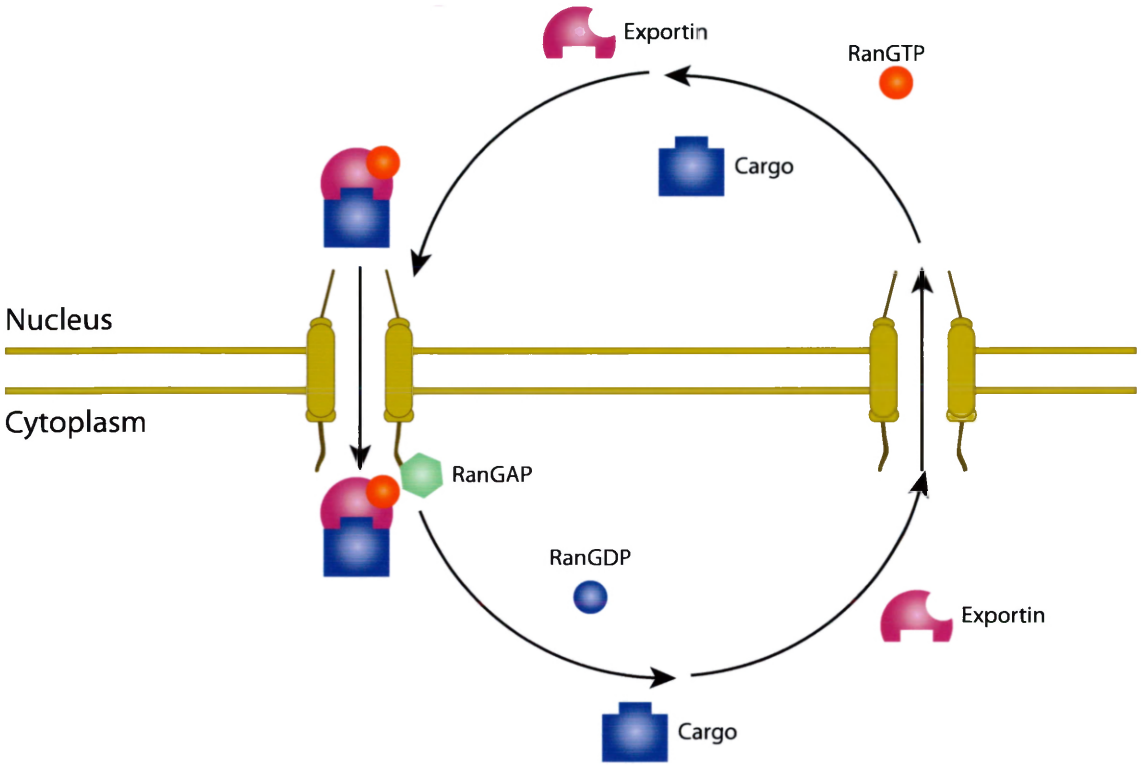

Figure 1.8 Nuclear Export Cycle. The nuclear export cycle begins with exportin, RanGTP, and a NES bearing cargo forming a trimeric export complex in the nucleus. The export complex translocates throught the NPC and upon reaching the cytoplasmic side of the pore, the complex dissociates upon hydrolysis of RanGTP by RanGAP. The cargo is released freely in the cytoplasm. Exportin, and RanGTP are recycled to the nucleus.
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export complexes reach the cytoplasmic side of the NPC, the complex dissociates when GTP is hydrolyzed to GDP by the GTPase activating protein RanGAP. This protein, bound to the cytoplasmic filaments of the NPC, is responsible for maintaining Ran in its predominant cytoplasmic form, RanGDP. Through the actions of RanGEF and RanGAP, each localized to a specific cellular compartment, a gradient of high nuclear RanGTP and high cytoplasmic RanGDP is maintained.

Nuclear Receptors and Importins –

Characterizing the importins that mediate nuclear receptor import is a complex and daunting task. The study of nuclear receptors would be greatly simplified if a receptor were to contain a single NLS, not in any way influenced by ligand induced conformational changes. This is not generally the case. Nuclear receptors typically have several NLSs in different domains, as well as exhibiting variable import characteristics based on receptor ligand state.

For example the glucocorticoid receptor, has two NLS regions one in the DBD region (NL1) and the other in the LBD region (NL2) each with varying properties (Savory et al., 1999). NL1 is more closely related to the "classical" NLS and is able to bind importin α as well as importin 7 and 8 regardless of ligand state. NL2 however can only bind importin 7 and 8, but the binding can occur in the absence of ligand, indicating the hormone binding process is downstream of importin binding (Freedman and Yamamoto, 2004). It has been proposed that importin 7 and 8 can act as cytoplasmic chaperones as well as

import factors, and therefore may be serving a dual role in this system (Jakel et al., 2002).

Another member of the type II nuclear receptor superfamily, RXR has some additional interesting properties. RXR is a common binding partner for TR, VDR and RAR (Laudet and Gronemeyer, 2002). Instead of binding the adaptor importin α, RXR exhibits a strong direct association with importin β, and is localized to the nucleus in the absence of ligand. This binding and import was "moderately" enhanced by the addition of its ligand (Yasmin et al., 2005).

In contrast, VDR associates with importin α weakly in the absence of ligand but strongly when ligand is present. Nuclear import of the RXR-VDR heterodimer is somewhat more complex. Heterodimer formation is inhibited in the presence of the RXR ligand, but it is stabilized when the ligands for both receptors are present. When complexed together, the heterodimer is imported using the VDR association with importin α (Yasmin et al., 2005).

Other nuclear receptors such as the estrogen receptor (ER), mineralocorticoid receptor (MR), androgen receptor (AR), and progesterone receptor (PR) have all been found to contain one or more NLS region. Point mutations in some of these NLS regions have been shown to alter the cellular localization of the receptor. This change in localization is likely due to the receptors loss of ability to bind importins needed to facilitate nuclear import (Sebastian et al., 2004; Shank and Paschal, 2005; Walther et al., 2005; Ylikomi et al., 1992). However, little work has been done to characterize which importins

interact with these receptors, a fact which may be due to the difficulty of expressing and purifying full length nuclear hormone receptors for use in *in vitro* binding assays.

Nuclear Receptors and Exportins –

The nuclear export of receptors is of equal importance to their import, though the process is less well understood. Nuclear export can attenuate the effects of ligand on transcriptional activity of a nuclear receptor by recycling the receptor to the cytoplasm where it may be degraded.

There are several exportins (members of the importin β-like family) which have only single cargoes, such as the previously discussed CAS or the export of tRNA by exportin-t. To date most of the information on export factors focuses on exportin 1 also known as chromosome region maintenance 1 or CRM1 (Hutten and Kehlenbach, 2007). As noted earlier, nuclear export using this factor is generally thought of as the "classical" export pathway, and nuclear receptors such as GR, ER, and PR as well as the oncoprotein v-ErbA , tRNA and mRNA have been shown to follow a CRM1 export pathway (DeLong et al., 2004; Kutay et al., 1998; Shank and Paschal, 2005).

The antifungal agent leptomycin B (LMB) has been used extensively in determining CRM1 dependent export, such as in the above mentioned examples as it was found to be a potent inhibitor of CRM1 mediated export. LMB is an unsaturated, branched-chain fatty acid that can bind covalently to a cysteine residue in the NES binding region of CRM1 thereby preventing the binding of CRM1 to a cargo (Yashiroda and Yoshida, 2003).

Recently, there has been increased research into the Ca^{2+} binding protein calreticulin (CRT) as an export receptor that may facilitate or participate in the nuclear export of nuclear receptors (Black et al., 2001; Burns et al., 1994; Dedhar et al., 1994; Holaska et al., 2001; Holaska and Paschal, 1998). GR has been shown to follow a CRT-dependent nuclear export pathway (Holaska et al., 2001; Holaska and Paschal, 1998), and a cooperative CRM1/CRT-mediated nuclear export pathway has been proposed for TRα (see Chapter 4).

Permeabilized Cell *In Vitro* Nuclear Transport Assays

In Vitro Nuclear Import Assays –

In 1990, Adam et al. developed a novel *in vitro* system for reconstituting nuclear import in digitonin-permeabilized cells. Digitonin is a weak nonionic detergent that selectively permeabilizes the plasma membrane due to its higher cholesterol content, while the nuclear membrane, which has lower cholesterol content, remains intact. Additionally, the NPC retains its structure as well as its transport abilities. Soluble endogenous factors are washed from the cell and purified recombinant import factors/cargo and or "cytosol" can be added back to study import characteristics (Figure 1.9). The ability to add back selected recombinant factors provides a powerful tool for determining specific factors required for import of a target cargo (Table 1). Several exogenous cytosol replacements are commonly used, such as rabbit reticulocyte lysate (RRL), as well as HeLa cell and *Xenopus* oocytes extracts.

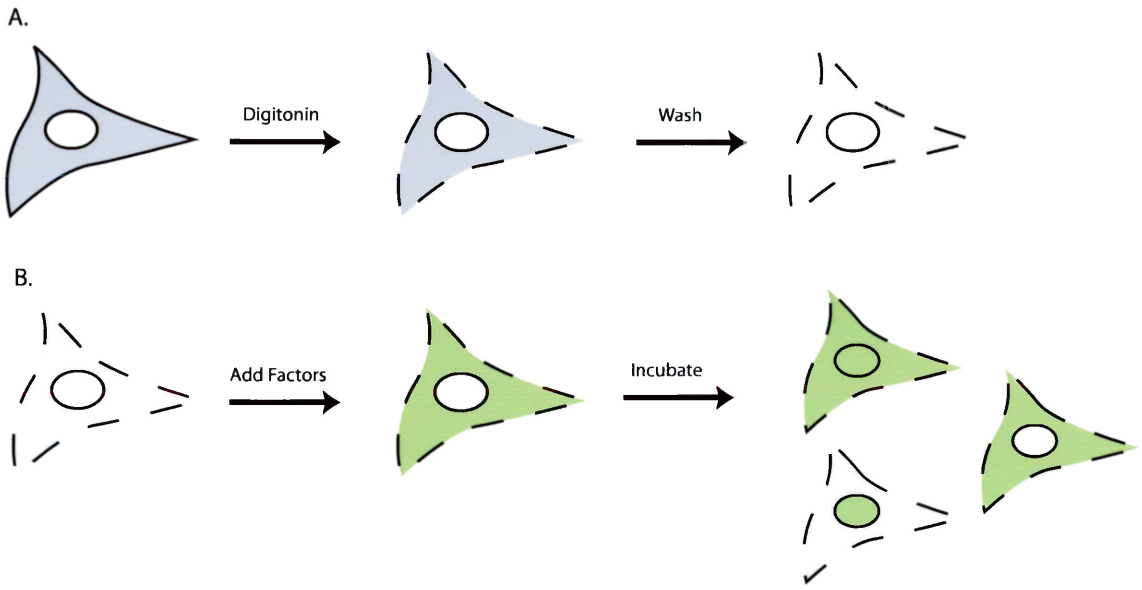


Figure1.9 Permeabilized Cell In Vitro Nuclear Import Assay. (A) HeLa cells grown on coverslips are permeabilized with 50µg/ml digitonin (Dashed line), and washed with ice cold import buffer to remove endogenous factors (gray) from the cytoplasm. (B) Washed and permeabilized cells are then incubated with fluorescently-tagged cargo and recombinant import factors or cytosol. After a 30 minute incubation at 30°C the cells are fixed and the subcellular distribution of the cargo is visualized by fluorescence microscopy.

`Table 1 – Permeabilized Cell *In Vitro* Nuclear Import Assays

Receptor / Cargo	Factors	Treatment *	Source
GR	Importin 7,8 α/β	RC Chilling Apyrase RanQ69L	(Freedman and Yamamoto, 2004) GR-407-525 & full GR import w/cyto+energy Chilling & apyrase stopped import. Not able to import full length with RC factors alone.
Allophycocyanin + SV40 NLS (APC+NLS)		± RRL RRL ± ATP WGA Import buffer ± ATP	(Adam et al., 1990) Demonstrated nuclear membrane remained intact after permeabilization. Cargo + buffer or supplemented with ATP had no import. With RRL and energy import occurred, but failed when RRL was depleted of ATP. Import complete at 15minutes. Compared cytosol replacements. RRL/HeLa/*Xenopus* oocyte. All functional as replacements, *S. cerevisiae* cytosol extract was not.
Imp *α/β*	Importin α/β	RC ± RRL Temp Apyrase	(Miyamoto et al., 2002) Imp *α* migrated into the nucleus in an Imp *β*, Ran independent manner not requiring exogenous ATP/GTP GFP-imp *α* imports w/import buffer alone Sensitive to temperature and WGA 3 mouse isoforms also imported w/o cytosol
REV protein HIV type 1	Importin β,5,7	RC Chilling ± RRL	(Arnold et al., 2006a) Importin α independent import Non "classical" NLS GST-Rev imported at 18°C with cytosol & Wt Ran, but Inhibited at 4°C, -RRL, or with RanQ69L
p35 (GST)	Importin β,5,7	RC Chilling ± RRL Apyrase WGA RanQ69L	(Fu et al., 2006) Imp β,5,7 all actively imported p35 in HeLa cells Chilling, Apyrase, WGA and Import buffer alone blocked import Import occurred +RRL, didn't not occur –RRL Mutation K61-63A blocked RRL import
c-Fos	Transportin	RC Chilling ± Cyto RanQ69L	(Arnold et al., 2006b) At 25°C, c-Fos was imported by transportin in HeLa cells. Chilling, RanQ69L, and Import buffer alone blocked import. HeLa cell cytosol used in place of RRL
UbcM2	Importin 11	RC ± Cyto Apyrase RanQ69L	(Plafker and Macara, 2000) UbcM2 imported with Imp 11 cytosol Cytosol HEK cells transfected with importin 11 or without (mock) was used in place of RRL + importin 11. Recombinant Imp 11 precipitated out when added to import buffer

*RC = reconstituted import Cyto =Cytoplasm replacement RRL = Rabbit Reticulocyte Lysate WGA = wheat germ agglutinin

In Vitro Nuclear Export Assays –

Permeabilized cells have been widely used for the study of import characteristics, but much less frequently used for the study of nuclear export factors and pathways. This is likely due to the added difficulty of properly reconstituting both the cytosolic and nuclear environments. In fact, the export process in general is not as well understood as the import process. Nonetheless, *in vitro* nuclear export assays have been used successfully to study calreticulin (CRT)-mediated export of the glucocorticoid receptor, RXR, and VDR as well as NFAT export (Holaska et al., 2002; Kehlenbach et al., 1998; Prufer and Barsony, 2002; Walther et al., 2003) (Figure 1.10).

Aims of this Thesis

As highlighted in the preceding review, the interaction of import/export cargoes with karyopherins and how these factors facilitate transport is of great importance for a complete understanding of gene regulation and signal transduction pathways in cells. An understanding of transcriptional regulation by nuclear receptor trafficking, whether due to the ability of a nuclear receptor to bind a karyopherin, or the ability of a karyopherin to interact with the NPC, will prove invaluable in treating current TH related diseases as well as developing future drug therapies.

Although the many effects of TR and its ligand TH have been extensively studied, very little research has been done on how TR import/export occurs and what karyopherins are involved. Given the broad and vital role TH plays, a better understanding of how it performs its role is clearly beneficial. This research

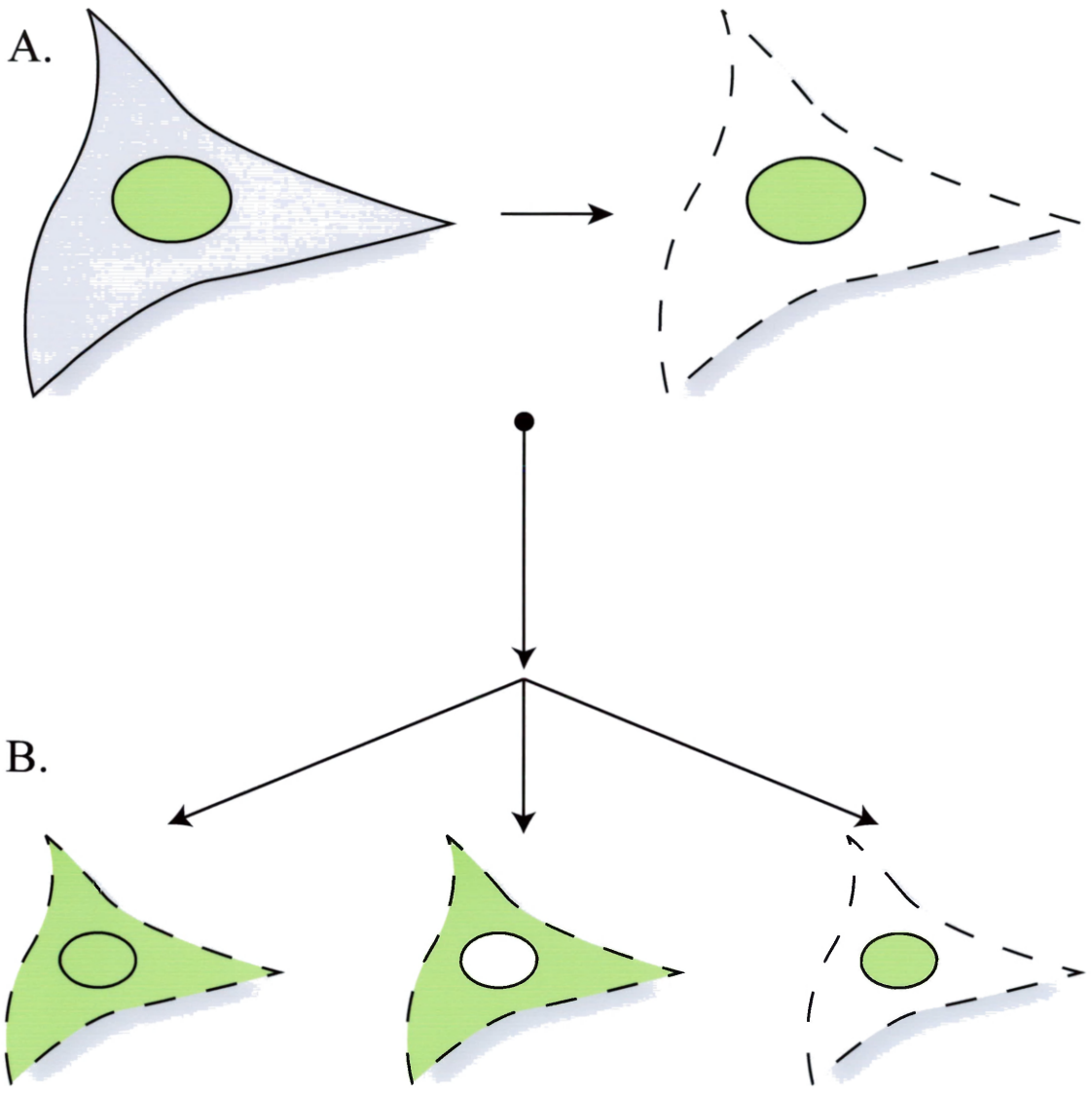


Figure 1.10 Permeabilized Cell In Vitro Nuclear Export Assay. (A) HeLa cells are grown on coverslips, transfected with GFP-TRα, and after 16h, are permeabilized with 50μg/ml digitonin (Dashed line). Permeabilized cells are washed with ice cold export buffer to remove endogenous factors (gray) from the cytoplasm. (B) Washed and permeabilized cells are then incubated with recombinant export factors and/or cytosol. After a 30 minute incubation at 30°C the cells are fixed and the subcellular distribution of the cargo is visualized by fluorescence microscopy.

provides insight into the requirements of TRα for nuclear import and evidence for a cooperative CRT-CRM1 mediated export pathway.

The following three specific questions were addressed:

1) Does TRα follow a temperature and energy dependent import pathway in mammalian cells?
2) What factors are required to reconstitute TRα nuclear import?
3) Does calreticulin (CRT) mediate TRα nuclear export?

Chapter 2 – Methods

Plasmids–

The plasmid pGFP-TRα encodes a functional GFP-TRα fusion protein expressed under human cytomegalovirus (CMV) promoter control. This plasmid was previously constructed in the lab by subcloning the PCR product of rTRα1 (rat) cDNA into the enhanced GFP expression plasmid pEGFP-C1 (CLONTECH Laboratories, Inc. Palo Alto, CA) using *Sac*I and *Bam*HI enzymes (Bunn et al., 2001).

The plasmid pGEX-CRTwt was a gift from B. Paschal (University of Virginia) and encodes full length calreticulin subcloned into the pGEX-KG vector for over expression in bacteria (Holaska et al., 2001).

The plasmid pGEX-2T-T_3Rα was a gift from M. Privalsky (University of California) and encodes chicken thyroid hormone receptor α cDNA cloned into the pGEX-2T GST vector for over expression in bacteria (Tzagarakis-Foster and Privalsky, 1998).

The plasmids encoding wild-type Ran-GST (pGST-RanWt), and the Ran mutant RanQ69L unable to hydrolyze GTP (pGST-RanQ69L) were a gift from R. Truant (Truant and Cullen, 1999; Truant et al., 1998; Truant et al., 1999). Both wildtype and mutant coding regions were inserted into the pGEX (Pharmacia) GST vector for over expression in bacteria.

The plasmids PKW 230 encoding His_6-Importin α (pRSET hSRP1α) and PKW 485 encoding His_6-Importin β (pETImpβ) were a gift from K. Weis (Nachury and Weis, 1999; Weis et al., 1996). The importin α and importin β cDNAs were inserted into N- terminal His tag vectors for over expression in bacteria; pRSET-C (Invitrogen) and pET, respectively.

All plasmids were propagated in *E.coli*-DH5α and purified using a Qiagen Plasmid Midi Kit (Qiagen Inc., Valencia CA). DNA purity and concentration were measured using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer.

Protein Overexpression –

Plasmids coding for the protein of interest were transformed into competent *E.coli* (BL21 DE3-RIL) (Stratagene, La Jolla, CA), per the manufacturer's protocol, and grown to an OD_{600} of 0.6-0.8 at 37°C. Expression was induced with 0.5mM IPTG (Isopropyl-β-D-Thiogalactoside) (Fisher Scientific, Pittsburgh, PA) and grown 3-5h at 30°C. Post-expression cultures were centrifuged at 1700 X g for 15 min at 4°C; bacterial pellets were stored at -80°C prior to protein purification.

GST Protein Purification –

Bacterial pellets were resuspended in 10 ml B-PER® Bacterial Protein Extraction Reagent (Pierce, Rockford, IL), 1 ml 5.0 mg/ml lysozyme (Fisher) 10mM Tris, pH 8.0, and one Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN). Resuspended pellets were incubated on ice for 30 min. The pellets were subsequently sonicated 2X at a setting of "6" for 5 to 10 seconds each time (Sonic Dismembrator Model 100; Fisher) on ice to fully lyse the bacteria. The lysed mixture was then centrifuged at 17,950 X g for 15 min at 4°C, and the supernatant was applied to 200µl of 50% Glutathione Sepharose 4B resin (GE Healthcare, Uppsala, Sweden). Samples were incubated for 60 min at 4°C with gentle rotation, and then centrifuged for 5 min at 500 X g at 4°C to pellet the resin. The resin pellet was washed 3 times with 10 ml ice cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and then transferred to a Microfilter Spin Column (Pierce) and washed an additional 2 times with 600µl ice cold PBS. 100µl Glutathione Elution Buffer (10mM glutathione) was added to the column, incubated at room temperature for 2-4 min with agitation, and then centrifuged at 700 X g for 30 sec at 4°C to collect eluted protein. The elution step was repeated 3X. The eluted fractions were pooled and dialyzed (Slide-A-Lyzer® Mini Dialysis Units, 7000MWCO, Pierce) against D-PBS overnight at 4°C. Protein samples were then concentrated using Micron Ultracel YM-30 Centrifugal Filter Devices (Millipore, Bedford, MA). Concentrated protein samples were analyzed by SDS-PAGE using 8% or 12% gels depending on protein size. Protein concentration

was estimated using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer. Samples were stored at -80°C.

His Tag Protein Extraction –

Bacterial pellets were resuspended in 10 ml B-PER® Bacterial Protein Extraction Reagent (Pierce), 1 ml 5.0 mg/ml lysozyme (Fisher), 10mM Tris, pH 8.0, and one Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN). The suspension was transferred to a glass Corex tube and subsequently sonicated 2X at a setting of "6" for 5 to 10 seconds each time (Sonic Dismembrator Model 100; Fisher) on ice to fully lyse the bacteria. The lysed mixture was then centrifuged at 17,950 X g for 15 min at 4°C. The supernatant containing soluble proteins was transferred to a 15 ml Falcon tube containing 200 μl of pre-equilibrated 50% Talon resin (Clontech/BD Biosciences). Samples and Talon resin were incubated for 60 min at 4°C with gentle rotation, then centrifuged for 5 min at 500 X g at 4°C to pellet the resin. The resin pellet was washed 3 times with 10 ml ice cold 1X Equilibration/Wash buffer (pH 7.0) (50 mM sodium phosphate; 300 mM NaCl) and then transferred to a Microfilter Spin Column (Pierce) and washed an additional two times with 600μl 1X Equilibration/Wash buffer. 100μl 1X Imidazole Elution buffer (50 mM sodium phosphate, pH 7.0; 300 mM NaCl;150 mM imidazole) was added to the column, incubated at room temperature for 2-4 min with agitation, then centrifuged at 700 X g for 30 sec at 4°C to collect eluted protein. The elution step was repeated 3X. The eluted fractions were pooled and dialyzed (Slide-A-Lyzer® Mini Dialysis Units, 7000MWCO, Pierce) against D-PBS overnight at 4°C. Protein samples

were then concentrated using Micron Ultracel YM-30 Centrifugal Filter Devices (Millipore, Bedford, MA). Concentrated protein samples were analyzed by SDS-PAGE. Protein concentration was estimated using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer. Samples were stored at -80°C.

SDS-PAGE –

Protein samples were visually analyzed using SDS-PAGE. A BioRad Mini Protean II gel apparatus (Hercules, CA) was set up according to the manufacturer's specifications and acrylamide (30% Acrylamide/Bis solution, 29:1, 3.3% C) gel percentages of either 8% or 12% were used depending on protein size.

Protein samples of varying concentrations were added to 1X SDS-PAGE Sample Buffer (125mM Tris, pH 6.8; 1% SDS; 5% glycerol; 0.005% bromophenol blue; 20mM DTT), and boiled for 5 min to denature the proteins.

All gels were loaded with one lane of Kaleidoscope Protein Standards (5μl) or one lane of Precision Plus Kaleidoscope Protein Standards (8μl) (BioRad). Samples were electrophoresed in 1X SDS-PAGE Running Buffer (192mM glycine; 25mM Tris; 3mM SDS, pH 8.2) at 150 V until the size standard band closest to the protein sample size was midway through the separating phase of the gel.

After electrophoresis, the gel was washed 3X for five min with sterile deionized water. The gel was then immersed in Simply Blue Safe Stain (Invitrogen) for one hour followed by immersion in 100ml deionized water for

destaining. Images were captured either on Polaroid 667 film, or digitally using a BioRad Gel Doc XR documentation system with Quantity One analysis software (v4.6.1).

FITC Labeling –

Proteins to be visualized in import assays were fluorescently labeled using the FluoReporter® Protein Labeling Kit (Invitrogen/Molecular Probes). Briefly, 200μl of concentrated protein and 20μl of 1M bicarbonate solution were loaded into the reaction tube provided in the kit. Using a stock solution of the manufactures reactive dye (prepared separately), an amount of dye (calculated based on protein sample concentration) was added to the protein sample in the reaction tube. The sample was then incubated in the dark with stirring for 1h at room temperature. The labeled sample was dialyzed (Slide-A-Lyzer® Mini Dialysis Units, 7000MWCO, Pierce) against D-PBS over night at 4°C; protein concentration was estimated based on A280 readings using a NanoDrop® ND-1000 full-spectrum UV/Vis Spectrophotometer. Protein samples were then concentrated using Micron Ultracel YM-30 Centrifugal Filter Devices (Millipore) if needed. Samples were stored at -80°C

Cell Culture –

HeLa cells (ATCC CCL-2) were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) containing penicillin (10,000U/ml) and streptomycin (100 μg/ml), at 37°C under 5% CO_2 and 98% humidity. Cells were grown to 70-90% confluency.

Nucleotide Preloading of Ran –

For the preloading of Ran with either GTP or GDP (Sigma, St. Louis, MO), 40µl of purified recombinant Ran was incubated in the presence of 15mM EDTA, 2mM DTT and 0.6mM of the desired nucleotide for 60 min at room temperature. After the incubation period the reaction was quenched with the addition of 30mM magnesium acetate for an additional 15 min at room temperature. Loaded Ran was then aliquoted and stored at -80°C. This loading protocol was adapted from Schwoebel et al. (2002).

Permeabilized Cell Nuclear Import Assays –

HeLa cells were seeded on 22mm Coverslips for Cell Growth™ (Fisher) in 6 well culture dishes (Nunc, Rochester, NY) at a density of 2-3x10^5 cells per well. 16-24 hours post-seeding the medium in each well was replaced with fresh MEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) for 4h. After 4h, cells were washed 2X with 2ml per well cold import buffer (20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$,), then permeabilized with 50µg/ml digitonin (Calbiochem, San Diego, CA) in import buffer for 4 min at room temperature. Cells were then rinsed 1X with 2ml per well cold import buffer for 10 min. Subsequently, coverslips were inverted over 50µl drops of import reaction mix (energy regeneration system composed of 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 0.5 mM ATP, 0.5 mM GTP, 10X import buffer, 0.67µm fluorescein isothiocyanate (FITC) labeled GST-TRα, and ±25µl rabbit reticulocyte lysate (RRL)) on parafilm in a moist chamber for 30 min at 30°C. Subsequently, cells were fixed in 3.7% formaldehyde (Fisher) for 10

min followed by a 5 min rinse with import buffer. Coverslips were subsequently mounted on slides with 8μl GelMount containing 4′,6-Diamidino-2′phenylindole dihydrochloride (DAPI) (Sigma) (0.5μg/ml). Slides were examined by fluorescence microscopy.

Permeabilized Cell Nuclear Export Assays –

HeLa cells were seeded on 22mm Coverslips for Cell Growth™ (Fisher) in 6 well culture dishes (Nunc) at a concentration of 2-3x10^5 cells per well. 24 hours post-seeding each well was transiently transfected with 2μg plasmid DNA and 10μl Lipofectamine reagent (Invitrogen) in Opti-MEM I Reduced Serum Medium, and incubated 12-16h. After 12-16h Opti-MEM I was replaced with MEM containing 10% fetal bovine serum for 4 hours. After 4 h cells were washed 3X with 2ml per well ice-cold export buffer: 20mM HEPES, pH 7.3, 110mM KOAc, 5mM NaOAc, 2mM $Mg(OAc)_2$, 1mM EGTA, 2mM DTT, 1mM PMSF, Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (1 tablet/10 ml) (Roche), then permeabilized with 50μg/ml digitonin (Calbiochem) in export buffer for 4 ½ min. Cells were then rinsed 1X with 2ml per well ice-cold export buffer for 10 min. Subsequently, coverslips were inverted over 50μl drops of export reaction mix (energy regeneration system composed of 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 0.5mM ATP, 0.5mM GTP, 5x export buffer, ±670nM GST-CRT, and ± 25μl RRL) on parafilm in a moist chamber for 30 min at 30°C. Cells were then fixed in 3.7% formaldehyde (Fisher) for 10 min followed by a 5 min rinse with export buffer. Coverslips were subsequently

mounted on slides with 8μl GelMount with DAPI (0.5μg/ml), and viewed by fluorescence microscopy.

Fluorescence Microscopy –

Images were collected using either of two microscopes. For some analyses, an inverted Nikon ECLIPSE TE 2000-E fluorescence microscope, with Nikon Ultraviolet Excitation: UV-2E/C filter block for DAPI visualization, and a Blue Excitation: B-2E/C filter block for GFP/ FITC visualization were used with a Nikon Plan Apo 40x/0.95objective. A CoolSNAP HQ_2 CCD camera (Photometrics, Tucson, AZ) and NIS-Elements AR software (Nikon) were used for image acquisition and primary image processing. For other analyses, an Olympus BX60 microscope with U-MNU filter cube for DAPI, and Omega Optical XF100-2 for GFP were used with an Olympus 40xUPlanFL 40x/0.75 objective. A Cooke SenisCam^{QE} camera and IPlab software (BD Biosciences Bioimaging Rockville, MD) were use for image acquisition and primary image processing. ImageJ and Adobe PhotoshopCS3/IllustratorCS3 were also used for any secondary image processing.

Cell Scoring & Statistical Analysis –

For permeabilized cell nuclear *in vitro* import assays, the localization of fluorescence in cells was scored as either wholly nuclear, or whole cell\cytoplasmic. All experiments consisted of a minimum of three replicates, each replicate counting 200 healthy nuclei. The integrity and morphology of the nucleus was assessed visually using a DAPI stain. All cell counts were performed blind, without prior knowledge of the treatment.

Once cells were counted, results were tested for statistical significance using SPSS 11.0. Data were examined for homogeneity of variances using a Levene test; variances were all homogeneous therefore no transformations were necessary. A one-way ANOVA was performed for each experiment; a p-value of 0.05 or less was considered significant. Post hoc Student-Newman-Keuls tests were performed to distinguish significant subgroups.

Chapter 3 – Nuclear Import of TR*α*

Results

With a size of 46kD, the TR*α* protein is at or slightly above the upper theoretical limit for passive diffusion through the NPC. Previous research done in *Xenopus* oocytes has shown that TR*α* is capable of nuclear entry in two distinctly separate ways, a passive diffusion pathway and a signal-mediated pathway (Bunn et al., 2001). To test for a passive diffusion pathway, wheat germ agglutinin (WGA) was used. WGA is a lectin that can bind NPC proteins and inhibit signal-mediated nuclear import but not affect passive diffusion. TR*α* was able to enter the nucleus when in the presence of WGA, a result indicative of a passive diffusion pathway. To test the signal-mediated pathway, Bunn et al. tagged TR*α* with glutathione-S-transferase (GST) to increase its mass from 46kD to 73kD. The mass of the fusion protein is well above the upper limit for passive diffusion through the NPC. The nuclear entry of GST-TR*α* in the absence of WGA and the lack of nuclear entry upon WGA treatment indicated that TR*α* can also follow a signal-mediated nuclear import pathway in *Xenopus* oocytes (Bunn et al., 2001).

Given the specialized nature of oocytes, it was of interest to determine whether TR*α* would behave in a similar fashion in mammalian cells. There are three common criteria or questions asked when establishing whether a cargo is following a signal-mediated import pathway vs a passive diffusion pathway. First, are soluble factors required for import or can import occur in the absence of soluble factors? Second, does import require physiological temperature or can

import occur under chilled conditions? Third, does import require an energy source, or is import energy-independent? Thus, to determine whether TR*α* follows a signal-mediated pathway for nuclear entry in mammalian cells, these three criteria were tested using permeabilized cell *in vitro* nuclear import assays.

TRα Import in Mammalian Cells Requires Soluble Factors –

Permeabilized cell *in vitro* nuclear import assays require an abundance of purified cargo. One method for obtaining large quantities of recombinant protein is by overexpression in bacteria, yeast, or by commercially available "cell free" systems. Purification is typically through the use of affinity tags attached to the recombinant protein, and there are a number of different tags available with different properties. Of the available tags, a His tag was selected to purify TR*α* because it would keep the total protein mass nearly identical to that of un-tagged TR*α*. This should allow import via passive diffusion if mammalian cells behave in a similar fashion to *Xenopus* oocytes. Thus, the first objective of this research was to produce His-tagged TR*α* for use in mammalian import assays that would be comparable to the *in vitro* synthesized TR injected into *Xenopus* oocytes. To this end several different plasmids were engineered for bacterial overexpression of His-tagged TR*α* (Table 2). Purification proved extremely difficult. This is not without precedence; purification and overexpression of nuclear receptors is generally quite challenging. Further, it has been reported that "mammalian TRs are notoriously difficult to produce in *Escherichia coli*" (Diallo et al., 2005). Whether the overexpressed recombinant protein proves to be toxic to the bacteria during expression, or there is a codon bias (Kane, 1995), or if the protein

Table 2 – His-Tagged TRα1 Plasmid Constructs

Plasmid Construct/ Vector	Expression System	Company	Purified Protein Yield *
pHAT20-TRα1 pHAT20	Bacterial	Clontech	Low - Very Low Detectable by Western Blot
pQE-30-XA-TRα1 pQE-XA30	Bacterial	Qiagen	Low - Very Low Detectable by Western Blot
pIVEX 2.4D-TRα1 pIVEX 2.4D	Cell-Free (Bacterial)	Roche	Low - Very Low Detectable by Western Blot
TRα1-His_6-TOPO5 pYES2.1/V5	Yeast	Invitrogen	Low - Very Low Detectable by Western Blot
pKLAC1	Yeast	New England Biolabs	Future.....

* Low – Very Low indicate yields of 0.0 to 0.05μg/μl. Approximately 1μg/μl purified protein would be required for use in transport assays.

produced is incorporated into bacterial inclusion bodies, the end result is low or no purified protein with a native conformation (Swietnicki, 2006; Tsumoto et al., 2003; Villaverde and Carrio, 2003). Denaturing extraction may lead to higher yields, but is less useful for functional studies. To date, no amount sufficient for use in permeabilized cell in vitro nuclear import assays was obtained, regardless of numerous attempts at optimizing expression conditions.

Without a suitable His tagged TRα construct, a GST tagged TRα expression plasmid capable of producing $\geq 1 \mu g/\mu l$ of recombinant protein was already available in the lab was used. GST-TRα allowed investigation of the signal-mediated questions posed in this thesis, but the size of the GST tag precluded passive diffusion studies.

To test whether soluble factors are required for nuclear entry, FITC labeled recombinant GST-TRα was used for import assays in the presence or absence of a rabbit reticulocyte lysate (RRL) "cytosol replacement" and an energy regeneration system (Figure 2.1). When combined with RRL, GST-TRα was found to be predominantly nuclear after a 30 minute incubation at 30°C. When RRL was absent, TRα was almost fully cytoplasmic in its cellular distribution. Due to the 73 kD size of GST-TRα, it was not able to passively diffuse into the nucleus. The need for cytosol (RRL) to achieve nuclear entry is an excellent indicator that one or more soluble factors are facilitating signal-mediated nuclear import. These findings are consistent with the *Xenopus* oocytes studies (Bunn et al., 2001) which used a similar protein construct, and suggest that TRα is following a signal-mediated import pathway in mammalian cell.

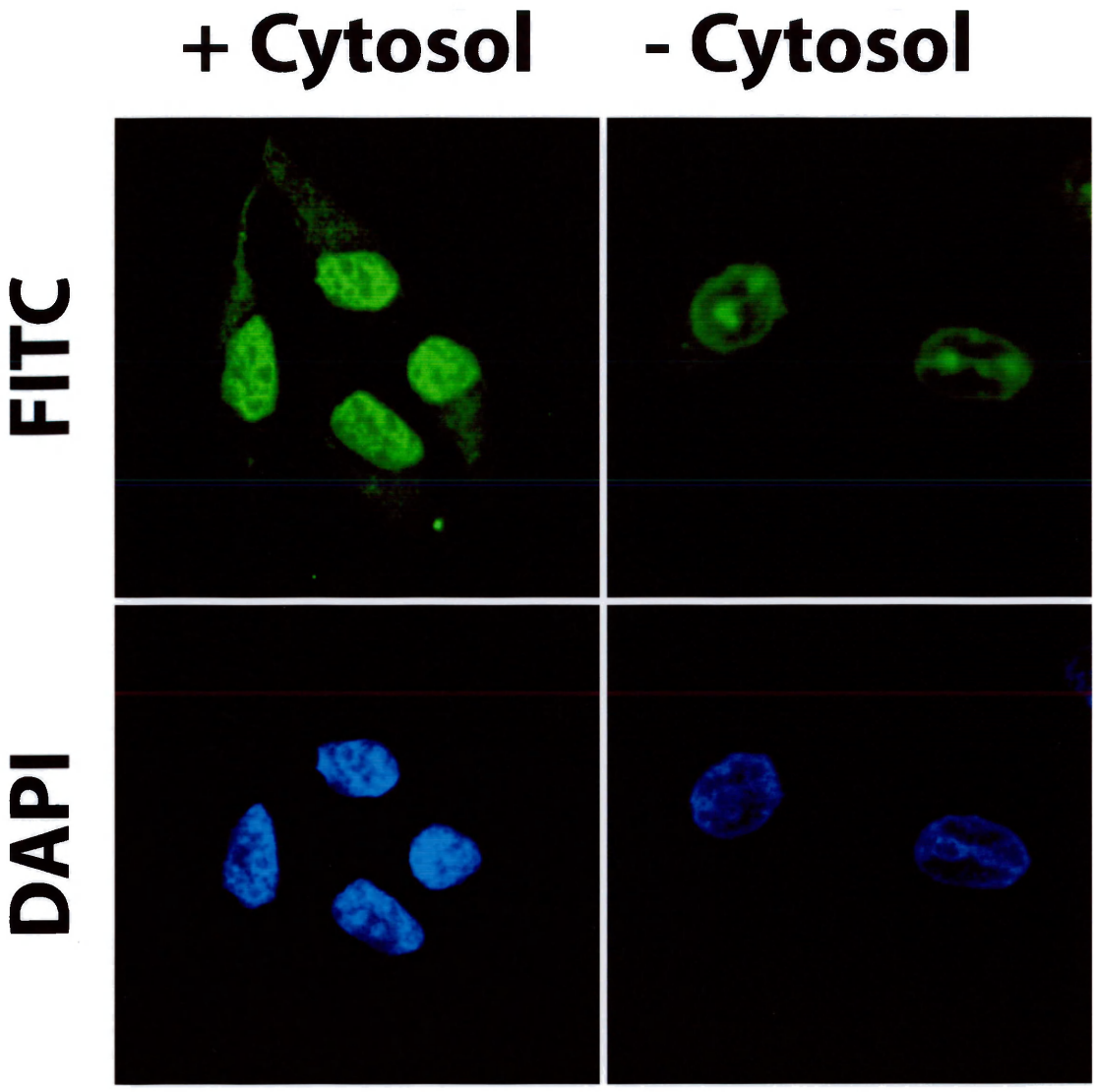


Figure 2.1 **TRα Nuclear Import Requires Soluble Factors in Digitonin-Permeabilized Cells.**
HeLa cells were grown on coverslips in 6-well plates, permeabilized with digitonin, and incubated at 30°C with import reaction mix containing an energy regeneration system, either in the presence or absence of cytosol and FITC-GST-TRα as substrate. After 30 min, samples were fixed, mounted, and viewed by epifluorescence microscopy. In the presence of cytosol TRα is localized to the nucleus. In the absence of cytosol, TRα remains cytoplasmic, or docked at the cytoplasmic face of the nuclear pore complex as indicated by nuclear rim-staining.

TRα Import in Mammalian Cells is Temperature-Dependent –

Given that TR requires soluble factors for nuclear import, it was of interest to determine whether import was temperature-dependent, as this is an additional criterion for signal-mediated transport. Again, using permeabilized cell *in vitro* nuclear import assays, import reactions containing TR*α* with RRL as a "cytosol replacement" were tested for TR*α* nuclear entry in a chilled environment. Chilling has been shown to abolish active transport while only marginally effecting passive transport (Breeuwer and Goldfarb, 1990; Freedman and Yamamoto, 2004). Import reactions were incubated at 4°C while a control import reaction was incubated at 30°C. After 30 min, the import reactions incubated at 30°C and half of the reactions at 4°C were fixed. The remaining reactions were moved from 4°C and incubated for an additional 30 min at 30°C. Prior to fixation the import reactions incubated at 30°C exhibited strong TR*α* nuclear import (Figure 2.2); however, when the import reactions were carried out at 4°C, nuclear import was significantly inhibited (Figure 2.3) (ANOVA, P=0.007). TR*α* nuclear import could be fully restored by further incubation at 30°C. Reversible inhibition suggests that chilling blocked TR*α* import by inhibiting specific transport components, rather than by preventing import by way of non-specific cellular damage (Bunn et al., 2001).

TRα Import in Mammalian Cells is Energy-Dependent –

In an effort to further characterize the requirement of energy for the nuclear import of GST-TR*α* in permeabilized HeLa cells, the effects of energy depletion were studied. Treatment with apyrase, an enzyme that hydrolyzes ATP

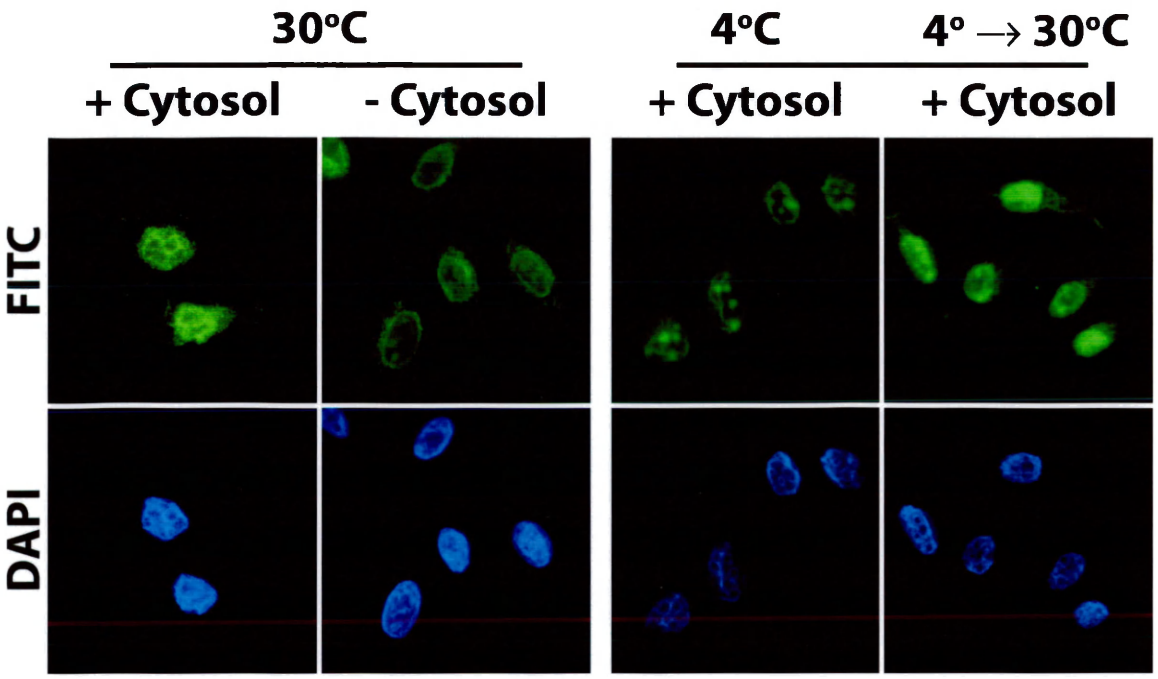


Figure 2.2 TRα Nuclear Import is Temperature Dependent in Digitonin-Permeabilized Mammalian Cells.
HeLa cells were grown on coverslips in 6-well plates, permeabilized with digitonin, incubated with import reaction mix and FITC-GST-TRα as substrate. After 30 min, samples were fixed, mounted, and viewed by epifluorescence microscopy. In the presence of cytosol at 30°C or at 4°C followed by 30°C as a control of cell viability, TRα is localized to the nucleus. In the absence of cytosol, and at low temperature (4°C), TRα remains cytoplasmic, or docked at the cytoplasmic face of the nuclear pore complex as indicated by nuclear rim-staining.

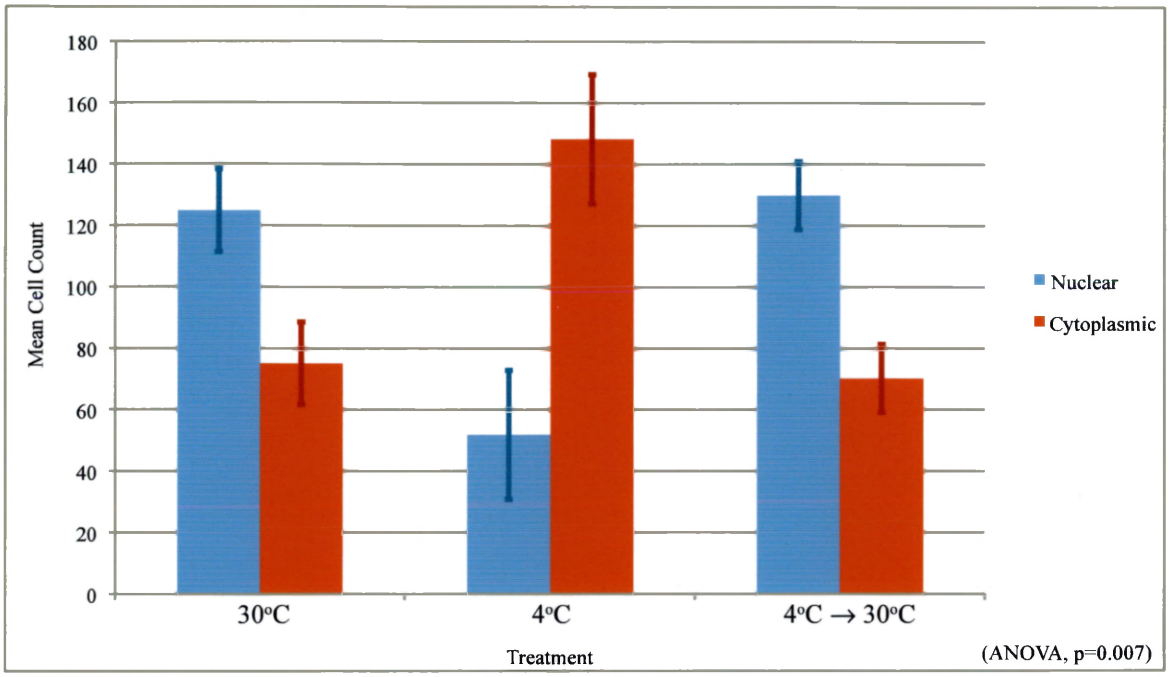


Figure 2.3 TRα Import in Mammalian Cells is Temperature-Dependent. FITC-GST-TRα localization was scored as either Nuclear or Cytoplasmic in each replicate. Bars indicate mean cell counts for the temperature treatments, and error bars indicate SEM for 5 replicates. Total cell count was 1000 cells.

is commonly used to determine whether nuclear import is energy dependent. For example, the nuclear import of TRα in *Xenopus* oocytes occurred in the presence and absence of apyrase indicating the ability to follow a passive diffusion pathway (Bunn et al., 2001). In contrast, apyrase treatment of PR (Haverinen et al., 2001) and GR (Freedman and Yamamoto, 2004) effectively blocked nuclear import in mammalian cells, indicating that cellular energy was a requirement in their import.

Apyrase depletes cellular ATP by acting as the catalyst for phosphate removal from ATP and ADP. Nuclear transport does not use ATP directly as an energy source; however, GTP is required to maintain the Ran gradient across the nuclear membrane (Cook et al., 2007; Fried and Kutay, 2003; Nachury and Weis, 1999). Depletion of cellular GTP by apyrase occurs due to the requirement for ATP in the phosphorylation of GMP to GDP and then to GTP (Zalkin and Dixon, 1992). By depleting ATP, new cellular GTP production is effectively stopped.

Import assays were performed in the presence of RRL, and contained either an energy regeneration system or apyrase. These reactions were compared to a control reaction containing import buffer in place of RRL (Figure 2.4). When incubated at 30°C for 30 minutes with apyrase, nuclear import of FITC-GST-TRα was significantly inhibited (ANOVA, $p=0.001$; Student-Newman-Keuls, $p \leq 0.05$) compared to reactions containing RRL and an energy regeneration system. However, TRα import in the presence of apyrase was not inhibited to the same extent as the reaction containing import buffer alone (Figure

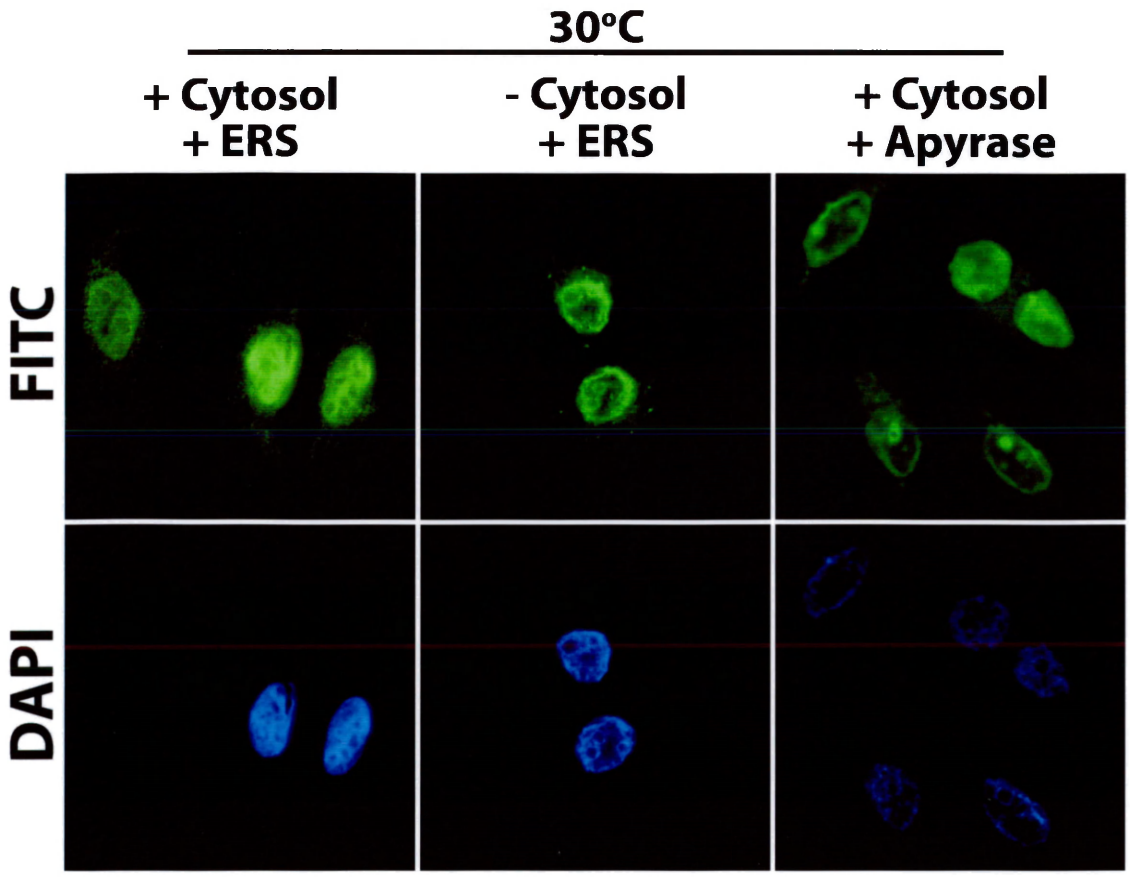


Figure 2.4 TRα Nuclear Import in Mammalian Cells is Inhibited by Apyrase. Import assays were performed in the presence of RRL (+Cytosol) or absence of RRL (-Cytosol), and contained either an energy regeneration system (ERS) or apyrase. When incubated at 30°C for 30 minutes with apyrase, nuclear import of FITC-GST-TRα was significantly inhibited (ANOVA, $p=0.001$; Student-Newman-Keuls, $p \leq 0.05$) compared to reactions containing cytosol and an energy regeneration system (ERS), but not inhibited to the same extent as the reaction containing import buffer alone.

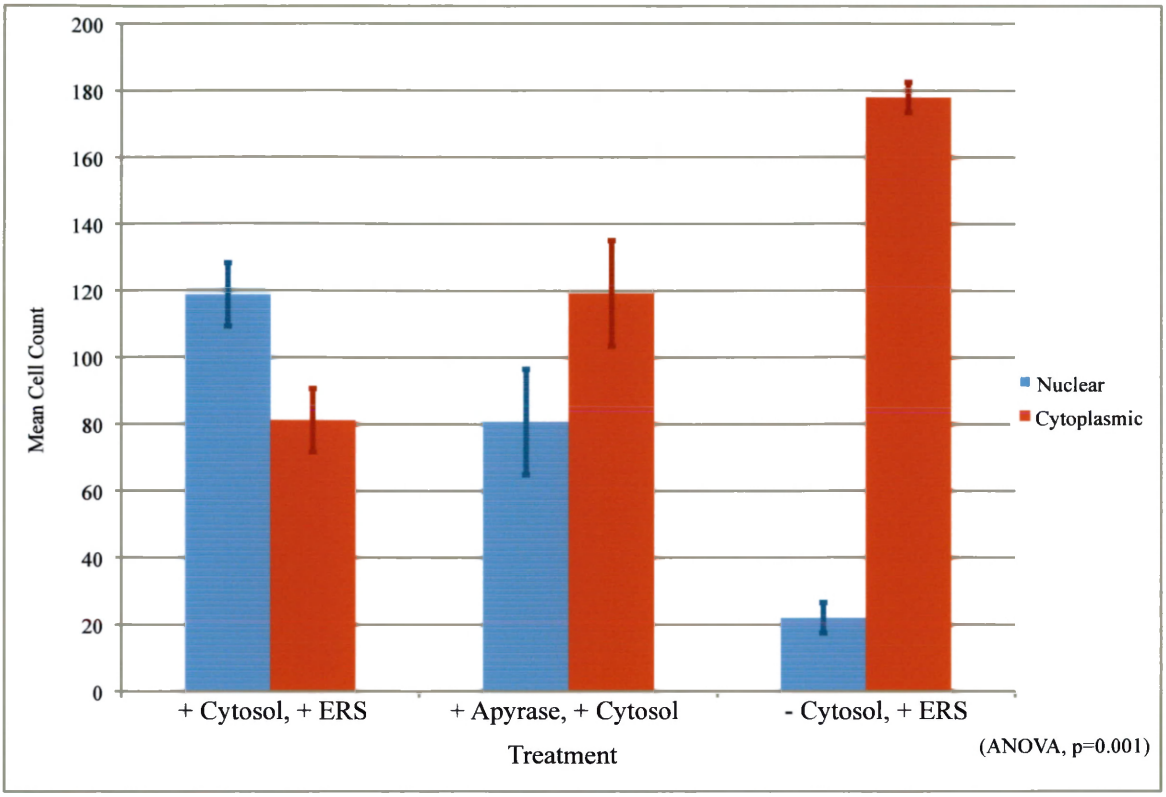


Figure 2.5 TRα Follows an Energy Dependent Pathway in Mammalian Cells. TRα localization was examined in the presence and absence of cytosol (+/- Cytosol) and included either an energy regeneraton system (+ERS) or apyrase. FITC-GST-TRα localization in permeabilized cells was scored as either Nuclear or Cytoplasmic in each replicate. Bars indicate mean cell counts for the energy treatments, and error bars indicate SEM for 4 replicates. Total cell count was 800 cells.

2.5). These results clearly demonstrate an energy requirement for signal-mediated nuclear import of TR*α* in mammalian cells.

TRα Nuclear Import in Mammalian Cells is Mediated by Importin α/β –

To characterize the soluble components required for nuclear import of TR*α* in mammalian cells, a panel of recombinant factors was used to reconstitute (or rebuild) the components of the pathway. Prior studies suggested that the NLS found in the hinge region of TR*α* is a classical NLS (cNLS) (Lee and Mahdavi, 1993). Import from a cNLS would require the use of classical importins; therefore, recombinant importin *α*, importin β, and FITC labeled GST-TR*α* were incubated along with an energy regeneration system in the absence of RRL for 30 minutes at 30°C. Ran preloaded with GDP was also included to maintain the normal cellular Ran gradient providing directionality in transport. Reactions containing import factors were compared to control reactions containing import buffer and an energy regeneration system. In the presence of importin *α/β* and Ran, TR*α* was localized to the nucleus; however, import was not as efficient as import in the presence of RRL (Figure 2.6). In addition, the efficiency of import was highly variable from replicate to replicate. These finding suggest that factors in addition to the importin α/β complex may be required for complete TRα nuclear import.

Discussion

Nuclear import is required for transcription factors, which are translated in the cytoplasm, to have an effect on gene regulation. In the present study, permeabilized cell *in vitro* nuclear import assays showed that TR*α* requires soluble factors for import in mammalian cells. Furthermore, using the same

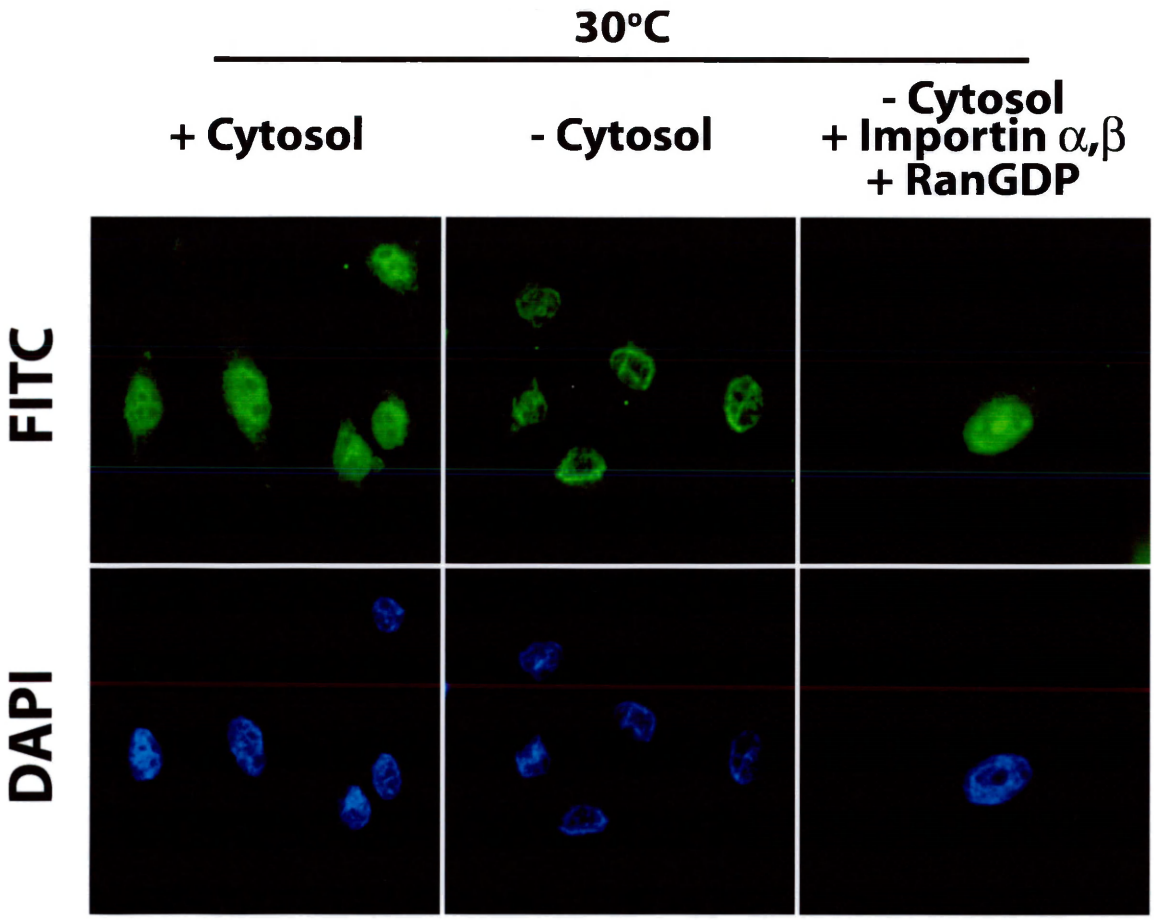


Figure 2.6 TRα Nuclear Import in Mammalian Cells is Mediated by Importin α/β. HeLa cells were grown on coverslips in 6-well plates, permeabilized with digitonin, incubated with import reaction mix and FITC-GST-TRα as substrate. After 30 min, samples were fixed, mounted, and viewed by epifluorescence microscopy. In the presence of cytosol at 30°C, and in the absence of cytosol but suplemented with recombinant importin α, importin β, FITC labeled GST-TRα and an energy regeneration system, TRα is localized to the nucleus. In the absence of cytosol or additional factors TRα remains cytoplasmic, or docked at the cytoplasmic face of the nuclear pore complex as indicated by nuclear rim-staining.

assays, the import pathway was shown to be temperature and energy dependent. Taken together, these data fulfill three main criteria required to demonstate that TR*α* follows one or more signal-mediated nuclear import pathways, and that at least one pathway is partially reliant on importin *α*/*β*.

TRα Import in Mammalian Cells is Temperature & Energy Dependent –

Chilling and apyrase treatments in permeabilized cell *in vitro* nuclear import assays are often used to demonstrate signal-mediated import. In permeabilized HeLa cells, nuclear import of TR*α* was significantly reduced when cells were chilled during *in vitro* nuclear import assays and upon treatment of import reactions with apyrase. Prior results from other receptors and nuclear proteins are consistent with TR data presented here, further confirming the use of a signal-mediated pathway (Table 1).

Although the chilling data clearly demonstrate a signal-mediated pathway is being followed, they do not define the exact importin or process effected during the assay. It is likely that the decreased temperature reduces the binding kinetics of importin/cargo complex interactions. Alternately, if an importin/cargo complex does form, the chilled environment could interfere with its interaction with nuclear pore complex proteins, reducing the cargo's ability to translocate through the pore.

It is not surprising that depletion of GTP from the import system by treatment with apyrase impaired TRα nuclear import. The signal-mediated import process depends on the RanGDP-RanGTP gradient between the cytoplasm and

the ncleus. Without GTP to bind Ran in the nucleus, importin/cargo complexes would not be able to dissociate in the nuclear compartment and import would essentially be stalled at the NPC.

TRα Nuclear Import in Mammalian Cells is Mediated by Importin α/β –

The data in this thesis demonstrate that importin *α/β* can mediate TR*α* import, though not to the same degree as a complete cytosol replacement. This would be somewhat puzzling if import was fully dependent on the conserved, classical NLS located in the hinge region of TR*α*, as previously believed based on data from TRβ studies (Zhu et al., 1998). However, the discovery of a second non-classical NLS (M. Mavinakere, L. Allison unpublished observations) helps to explain this result. Although previously unknown for TR*α*, more than one NLS is not uncommon among the nuclear receptor family. The GR, AR, ER and PR all contain more than one sequence thought to be NLS regions (Freedman and Yamamoto, 2004; Savory et al., 1999; Shank and Paschal, 2005; Ylikomi et al., 1992). Based on the data presented here, a model could be proposed in which one NLS is using importin *α/β* for partial import, while complete, efficient import, would require an additional importin to interact with the second NLS. Only when the requirements of the two NLS regions are met would complete, efficient import, be expected. Alternatively, it is also possible that the delicate balance of cargo and import factors, as well as cellular energy levels was not optimal. This "import equation" must be balanced for import to occur. For example, the addition of too much importin *β* can allow it to import without a cargo and bind RanGTP in the nucleus. This complex can then export naturally and RanGDP is hydrolyzed

to RanGDP in the cytoplasm. This process termed "futile cycling", depletes the nucleus of RanGTP without import of any cargo effectively blocking import (Riddick and Macara, 2005). Given this complication it is tempting to reduce the concentration of importin β, but then there is a risk of negatively impacting transport efficiency by not providing enough importin β to participate in cargo import (Yang and Musser, 2006).

When studying nuclear protein import, isolated protein domains are sometimes used either in place of, or in conjunction with, the full length protein. The reasons for using isolated domains vary, but range from characterizing a specific isolated domain to providing a substitute if the full length protein can't be purified. For example, the GR NLS regions as well as the c-Fos transportin binding sites previously mentioned (Table 1) were characterized using isolated sections of the proteins (Arnold et al., 2006b; Freedman and Yamamoto, 2004). This type of analysis has both positive and negative points. On the positive side, by using isolated domains, it is possible to separate out the effects different regions have on nuclear import. On the negative side, using isolated domains may result in an artifact generated from using that fragment, rather than naturally occurring effects. While studying isolated domains may be useful for identifying properties of that specific domain, it is likely that the nuclear import characteristics of the full length protein reflect the "combined melding" of the individual domains properties. A study of the GR, using a domain fragment as well as full length GR illustrates the complexity of reconstituting nuclear import (Freedman and Yamamoto, 2004). Both the GR fragment and full length GR

were localized to the nucleus in the presence of cytosol in import assays, in a manner similar to results for TR presented in this thesis. However, in the presence of importin 7 as well as importin *α/β* the GR fragment retained the ability to import while the full length GR could not be imported without the addition of cytosol.

As discussed earlier, the results in this thesis provide evidence that TR*α* can follow a signal-mediated import pathway. Several questions remain to be answered. First, is it possible that TRα could be diffusing passively in addition to the active signal-mediated pathway shown in this thesis? Considering the size of TR it is still possible that passive diffusion could occur naturally in HeLa cells as was seen in *Xenopus* oocytes (Bunn et al., 2001). The purification of a full length, His-tagged TRα would help to answer this question. Second, does TRα follow multiple import pathways? There are multiple importin β-like import factors (Strom and Weis, 2001) (Table 1) and, in addition, a suite of different importin α adaptors (importin α1-6) (Goldfarb et al., 2004). Until all of TRα's NLS sites and the importins which bind those sites are known it will be difficult to say for certain whether multiple signal-mediated pathways exist. The discovery of two or more NLS regions in a nuclear receptor, even if their binding partners are unknown, would suggest multiple import pathways contributing to the nuclear receptor overall import characteristics.

In summary, the data in this thesis chapter demonstrate TRα import can occur by a classical signal-mediated nuclear import pathway, and point to the

possibility that more than one import pathway is followed for complete nuclear import.

Chapter 4 – Manuscript from Thesis Research: Nuclear Export of TR*α*

Statement of Candidate's Contribution to Manuscript

The work reported in the following manuscript was performed by various members of the Allison lab. M. Grespin and G. Bonamy shared first-authorship, thus, although third in the list of coauthors, the candidate held the role of "second" author. The candidate was responsible for the permeabilized cell export assay data collected throughout the course of this research and wrote the related manuscript sections. The candidate also helped to supervise the work of undergraduate students included in the project. The following is a summary of the candidate's contribution towards generating each individual figure in the manuscript presented in the following pages.

Figures 1-5 & 7-8 – The candidate's coauthors collected the data presented in figures 1-5, and 7-8.

Figure 6. – The candidate was responsible for collection of the data presented in this figure, as well as for performing all relevant techniques and technical skills required for data acquisition. These include, but are not limited to, purification of recombinant proteins, maintaining cell cultures, preparation of cells for experimentation, performing fluorescence microscopy, and image analysis.

Chapter 5 – General Discussion & Future Directions

The data presented in Chapter 3 of this thesis provide solid evidence that TR*α* follows a signal-mediated nuclear import process in mammalian cells. A more complex task will be to obtain data to clarify exactly which soluble factors are mediating nuclear import and whether there are alternative pathways for nuclear entry. The discovery of an NLS in the A/B domain of TR*α* in addition to the classical NLS (cNLS) in the hinge region complicates this task (M. Mavinakere, L. Allison unpublished observations). It is likely TR*α* can follow a classical importin *α*/*β* signal-mediated import process using its hinge region cNLS, but, it is also possible the second NLS (which does not have a classical sequence) follows an alternative pathway either at a different time, in a different cell type, or in some cooperative fashion to create an overall TRα import "fingerprint". In fact many members of the nuclear receptor family have multiple NLSs or proto-NLSs; thus, it is possible the use of several importins is a common family characteristic (see Introduction: Nuclear Receptors and Importins).

The existence of two NLS regions may explain the less efficient results from *in vitro* import assays attempting to reconstitute the import process using only importin *α*/*β*. It seems quite logical that the requirements of both NLS regions must be met before nuclear import in a reconstituted system could compare to nuclear import supported by a cytosol replacement presumably containing a full complement of soluble factors.

One approach to finding the requirements of the second NLS is to sequentially add additional recombinant importins to the reconstituted import reaction already containing importin α/β, until an import level similar to that of RRL is achieved. This could prove to be a complicated and labor intensive task as it would require the overexpression and purification of many additional importins as well as optimizing their use in the import assay itself. Alternatively, by cloning individual domains of TRα into a GFP-GST-GFP expression plasmid (to increase the molecular weight above that of passive diffusion), the two NLS regions and their importin requirement can be separated. By transfecting the A/B domain construct into mammalian HeLa cells, RNA interferences (RNAi) can be used to knockdown or "block" either single importins or multiple importins simultaneously, and determine exactly which importin is required for that individual NLS. Once the specific factors are determined by RNAi, *in vitro* nuclear import assays could be carried out in a more focused manner using both full length receptor and isolated domains. Only the identified importin or importins would need to be purified to further confirm the RNAi results.

In contrast to the well studied importins discussed above, the endoplasmic reticulum protein calreticulin (CRT) has only recently begun to be considered to have an additional role as an export factor, and little data exist on its localization to or role in the nucleus. The manuscript in Chapter 4 provides strong and compelling evidence for cooperative CRT/CRM1 mediated export of TRα, though it should be noted that NES binding locations for these export factors remain to be determined.

Currently experiments are in progress to demonstrate the formation of such a trimeric protein complex (CRT/CRM1/TRα) using co-immunoprecipitation assays and GST pulldown assays. Demonstrating this complex using these methods would further validate this model for TRα export.

The thyroid hormone receptors are responsible for a wide range of development and metabolic processes. It is vital to understand all aspects of TR's regulation including T_3/receptor and receptor /DNA interactions and, specific to this thesis, transport of TR across the nuclear membrane. Without this critical transport, TR would simply remain a cytosolic protein and be unable to function as a transcription factor.

The study of nucleocytoplasmic shuttling proteins and nuclear import/export pathways in general is a vital topic of research. The existence of a nuclear envelope separating the DNA from protein synthesis adds an additional level of control over gene expression, and upsetting a shuttling proteins cellular localization can lead to disease.

Appendix

Table 3 – Protein Expression

Protein	Expression	Organism
GST-TRα1	≥1 μg/μl	*E.coli* (Bl21-DE3-RIL)
RanWt	≥1 μg/μl	*E.coli* (Bl21-DE3-RIL)
RanQ69L	≥1 μg/μl	*E.coli* (Bl21-DE3-RIL)
Importin β	≥1 μg/μl	*E.coli* (Bl21-DE3-RIL)
Importin α	≤ 0.05 μg/μl	*E.coli* (Bl21-DE3-RIL)
TRα1-His_6	≥ 0.1μg/μl /Extraction Issue	Yeast
GST-CRT	≥ 1 μg/μl	*E.coli* (Bl21-DE3-RIL)
His-CRM1	≤0.05 μg/μl Extraction Issue	*E.coli* (Bl21-DE3-RIL)

Statistical Analysis for Energy and Factor Dependence

Oneway ANOVA

Descriptives

Nuclear counts

	N	Mean	Std. Deviation	Std. Error
+RRL, +ERS	4	118.7500	18.92749	9.46375
Apyrase (+RRL)	4	80.7500	31.65833	15.82917
-RRL, +ERS	4	22.0000	9.09212	4.54606
Total	12	73.8333	46.06090	13.29664

Descriptives

Nuclear counts

	95% Confidence Interval for Mean		Minimum	Maximum
	Lower Bound	Upper Bound		
+RRL, +ERS	88.6321	148.8679	103.00	146.00
Apyrase (+RRL)	30.3745	131.1255	35.00	102.00
-RRL, +ERS	7.5324	36.4676	10.00	32.00
Total	44.5676	103.0990	10.00	146.00

Test of Homogeneity of Variances

Nuclear counts

Levene Statistic	df1	df2	Sig.
1.904	2	9	.204

ANOVA

Nuclear counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19008.167	2	9504.083	19.757	**.001**
Within Groups	4329.500	9	481.056		
Total	23337.667	11			

Post Hoc Tests

Homogeneous Subsets

Nuclear counts

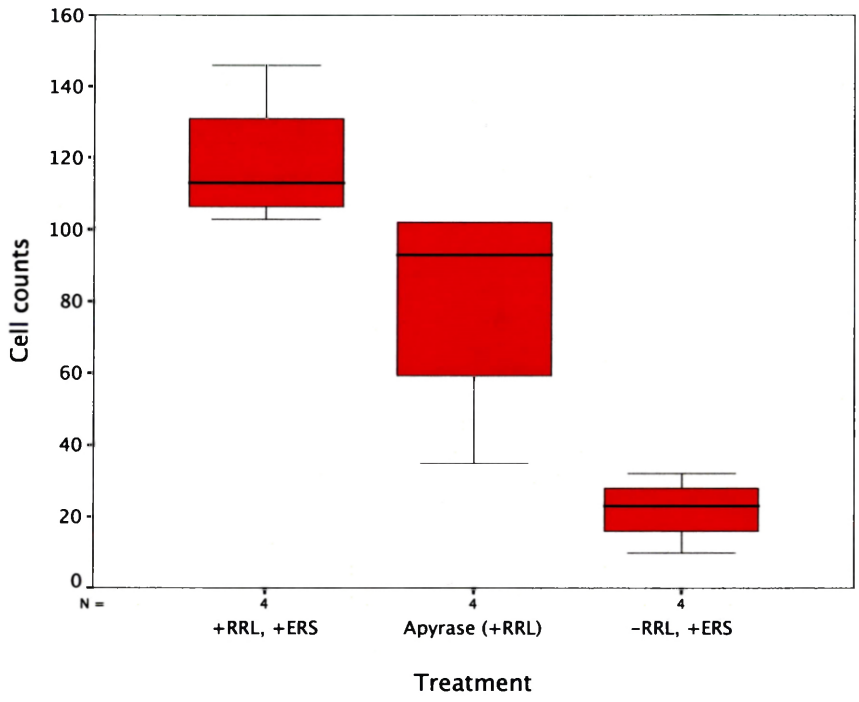
Student-Newman-Keuls[a]

		Subset for alpha = .05		
Treatment	N	1	2	3
-RRL, +ERS	4	22.0000		
Apyrase (+RRL)	4		80.7500	
+RRL, +ERS	4			118.7500
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Cell counts


160
140
120
100
80
60
40
20
0
Cell counts
N =
4
4
4
+RRL, +ERS
Apyrase (+RRL)
-RRL, +ERS
Treatment

Statistical Analysis for Temperature Dependence

Oneway ANOVA

Descriptives

Cell Count

	N	Mean	Std. Deviation	Std. Error
30 Deg C	5	124.8000	30.04497	13.43652
4 Deg C	5	51.8000	47.11369	21.06988
4 & 30 Deg C	5	129.8000	24.73257	11.06074
Total	15	102.1333	49.28034	12.72413

Descriptives

Cell Count

	95% Confidence Interval for Mean		Minimum	Maximum
	Lower Bound	Upper Bound		
30 Deg C	87.4942	162.1058	84.00	159.00
4 Deg C	-6.6994	110.2994	3.00	129.00
4 & 30 Deg C	99.0905	160.5095	95.00	159.00
Total	74.8428	129.4239	3.00	159.00

Test of Homogeneity of Variances

Cell Count

Levene Statistic	df1	df2	Sig.
.383	2	12	.690

ANOVA

Cell Count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19063.333	2	9531.667	7.658	**.007**
Within Groups	14936.400	12	1244.700		
Total	33999.733	14			

Post Hoc Tests

Homogeneous Subsets

Cell Count

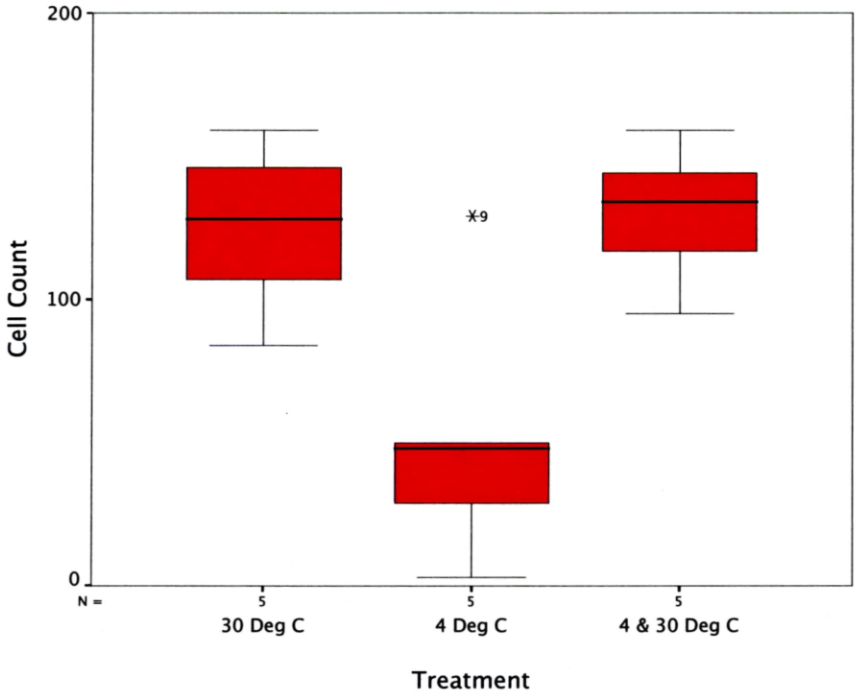
Student-Newman-Keuls[a]

		Subset for alpha = .05	
Treatment	N	1	2
4 Deg C	5	51.8000	
30 Deg C	5		124.8000
4 & 30 Deg C	5		129.8000
Sig.		1.000	.826

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Cell Count



Nuclear

Replicates	30 Deg C	4 DegC	4 & 30 Deg C
1	128	29	159
2	146	3	95
3	107	129	144
4	159	48	117
5	84	50	134
Mean	124.8	51.8	129.8
SEM	13.437	21.070	11.061

Cytoplasmic

Replicates		30 Deg C	4 DegC	4 & 30 Deg °C
	1	72	171	41
	2	54	197	105
	3	93	71	56
	4	41	152	83
	5	116	150	66
Mean		75.2	148.2	70.2
SEM		13.437	21.070	11.061

	30 Deg C	4 Deg C	4 & 30 Deg C
Nuclear	124.8	51.8	129.8
Cytoplasmic	75.2	148.2	70.2
SEM	13.4365174	21.0698837	11.0607414

4 & 30 deg

Date 5/17/06

actual	Blind		Nucle	Cyto		Nuclear	Cyto
1	5		75	25	normal	128	72
2	3		5	95	4deg	29	171
3	6		78	22	4 & 30 deg	159	41
4	4	*	53	47	normal		
5	1		24	76	4deg		
6	2		81	19	4 & 30 deg		

Date 6/2/06

actual	Blind		Nucle	Cyto		Nuclear	Cyto
1	5		89	11	normal	146	54
2	3		2	98	4deg	3	197
3	6		80	20	4 & 30 deg	95	105
4	1	*	57	43	normal		
5	2		1	99	4deg		
6	4	*	15	85	4 & 30 deg		

Date 6/20/06

actual	Blind		Nucle	Cyto		Nuclear	Cyto
1	3	*	22	78	normal	107	93
2	6	*	85	15	4deg	129	71
3	4		56	44	4 & 30 deg	144	56
4	2		85	15	normal		
5	1		44	56	4deg		
6	5		88	12	4 & 30 deg		

Date 9/6/06

actual	Blind		Nuc	Cyto		Nuclear	Cyto
1	4		87	13	normal	159	41
2	5		23	77	4deg	48	152
3	2		43	57	4 & 30 deg	117	83
4	6		72	28	normal		
5	3		25	75	4deg		
6	1		74	26	4 & 30 deg		

Date 9/15/06

actual	Blind		Nucle	Cyto		Nuclear	Cyto
1	2		18	82	normal	84	116
2	3		24	76	4deg	50	150
3	5		57	43	4 & 30 deg	134	66
4	6		66	34	normal		
5	1		26	74	4deg		
6	4		77	23	4 & 30 deg		

Nuclear

Replicate	Control	Apyrase	Import Buffer
1	110	102	24
2	146	35	32
3	116	102	22
4	103	84	10
Mean	118.75	80.75	22
SEM	9.46374662	15.8291661	4.54606057

Cytoplasmic

Replicate	Control	Apyrase	Import Buffer
1	90	98	176
2	54	165	168
3	84	98	178
4	97	116	190
Mean	81.25	119.25	178
SEM	9.46374662	15.8291661	4.54606057

	+RRL, +ERS	Apyrase (+RR	-RRL, +ERS
Nuclear	118.75	80.75	22
Cytoplasmic	81.25	119.25	178

Energy Depletion

Date: 9/22/2006

Actual	Blind		Nuclear	Rim/Cyto	
1	5		73	27	normal
2	3		13	87	apyrase
3	2		6	94	Import buffer
4	6	*	37	63	
5	4	*	89	11	
6	1		18	82	

Date: 9/26/2006

Actual	Blind		Nuclear	Rim/Cyto	
1	6		73	27	normal
2	3		14	86	apyrase
3	4		15	85	Import buffer
4	5		73	27	
5	1		21	79	
6	2		17	83	

Date: 10/6/2010

Actual	Blind		Nuclear	Rim/Cyto	
1	5	*	25	75	normal
2	6		10	90	apyrase
3	4		6	94	Import buffer
4	3		78	22	
5	1	*	74	26	
6	2		4	96	

Date: 10/11/2010

Actual	Blind		Nuclear	Rim/Cyto	
1	2		60	40	normal
2	4		36	64	apyrase
3	5		12	88	Import buffer
4	4		56	44	
5	6	*	66	34	
6	3		10	90	

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