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Multiple exportins influence thyroid hormone receptor localization

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ABSTRACT

The thyroid hormone receptor (TR) undergoes nucleocytoplasmic shuttling and regulates target genes involved in metabolism and development. Previously, we showed that TR follows a CRM1/calreticulin-mediated nuclear export pathway. However, two lines of evidence suggest TR also follows another pathway: export is only partially blocked by leptomycin B (LMB), a CRM1-specific inhibitor; and we identified nuclear export signals in TR that are LMB-resistant. To determine whether other exportins are involved in TR shuttling, we used RNA interference and fluorescence recovery after photobleaching shuttling assays in transfected cells. Knockdown of exportins 4, 5, and 7 altered TR shuttling dynamics, and when exportins 5 and 7 were overexpressed, TR distribution shifted toward the cytosol. To further assess the effects of exportin overexpression, we examined transactivation of a TR-responsive reporter gene. Our data indicate that multiple exportins influence TR localization, highlighting a fine balance of nuclear import, retention, and export that modulates TR function.

1. Introduction

Thyroid hormone (triiodothyronine, T_3) is important in regulating genes responsible for metabolism, growth, and development. Encoded by two different genes, thyroid hormone receptors TRα1 and TRβ1 respond to T_3 levels by activating or repressing target gene expression. Although primarily found in the nucleus at steady-state, TRα1 and TRβ1 can rapidly shuttle between the nucleus and cytoplasm (Baumann et al., 2001; Bunn et al., 2001; Grespin et al., 2008). Nuclear import and export of proteins occurs through the nuclear pore complexes, mediated by members of the karyopherin β family called importins and exportins, respectively (Kimura and Imamoto, 2014; Pemberton and Paschal, 2005). By coupling mutagenesis and localization studies, nuclear localization signal (NLS) and nuclear export signal (NES) motifs that interact with the transport machinery have been found in conserved domains of members of the nuclear receptor superfamily (Black et al., 2004; Holaska et al., 2002; Kanno et al., 2005, 2007; Liu and DeFranco, 2000; Lombardi et al., 2008; Mavinakere et al., 2012; Nguyen et al., 2009; Pemberton and Paschal, 2005; Picard and Yamamoto, 1987; Saporita et al., 2003; Sorokin et al., 2007; Umemoto and Fujiki, 2012). This transport process provides a central regulatory point for coordinating cell signaling and gene expression.

From our prior studies, an increasingly complex picture has emerged of the intricate molecular mechanisms that regulate trafficking and function of TRα1 and TRβ1 (collectively referred to as TR hereinafter for simplicity). Previously, we showed that TR can exit the nucleus by a pathway mediated by the export factor CRM1 (chromosome region maintenance 1), also known as exportin 1, in cooperation with calreticulin (Grespin et al., 2008); however, the exact interaction and mechanism remain unclear. Two main lines of evidence suggested that TR might also follow a CRM1/calreticulin-independent nuclear export pathway. First, under conditions in which TR still shuttles, shuttling of p53 and the oncoprotein v-ErbA is completely blocked in the presence of the CRM1-specific inhibitor leptomycin B (LMB) (Bunn et al., 2001; DeLong et al., 2004). Also, during fluorescence recovery after photobleaching (FRAP) experiments, when one nucleus in a multinucleate HeLa cell was photobleached, recovery of fluorescence in the bleached nucleus in the presence of LMB was reduced by only 60%, relative to recovery in the absence of LMB (Grespin et al., 2008). Second, no CRM1-dependent NES in TR has yet been characterized (Mavinakere et al., 2012). Indeed, our studies showed that TR interacts directly with calreticulin but complex formation with CRM1 was not detectable in pull-down assays (Grespin et al., 2008). In an effort to identify and clarify the mode of TR nuclear export, we previously carried

Abbreviations: T_3, thyroid hormone (triiodothyronine); TRα1, thyroid hormone receptor α1; TRβ1, thyroid hormone receptor β1; NLS, nuclear localization signal; NES, nuclear export signal; CRM1, chromosome region maintenance 1; LMB, leptomycin B; FRAP, fluorescence recovery after photobleaching; RNAi, RNA interference; GFP, green fluorescent protein; shRNA, short hairpin RNA; qPCR, quantitative PCR; TRE, thyroid hormone response element; WGA, wheat germ agglutinin; miRNA, microRNA.

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out a comprehensive analysis of TR to screen for NES motifs. We identified a region spanning helices 3 and 6 of the ligand-binding domain, that either houses two monopartite NESs or a single, bipartite NES, and we fully characterized another NES in helix 12 of the ligand-binding domain. These NES motifs were able to export a nucleus-localized fusion protein to the cytosol (Mavinakere et al., 2012). Intriguingly, these NES motifs were shown to be insensitive to LMB. In the presence of LMB, they were still able to direct the fusion protein to the cytosol, suggesting that they follow a CRM1-independent export pathway (Mavinakere et al., 2012).

In the present study, we sought to determine whether other exportins are involved in this alternative nuclear export pathway of TR, and to determine their relative contributions to TR export overall. To this end, we coupled RNA interference (RNAi) with FRAP experiments in live HeLa cells. Shuttling dynamics of TR were assessed upon knockdown of transportins 1 and 2, and exportins, 4, 5, 6, and 7. Additionally, we used overexpression assays and T_3-responsive reporter gene assays to further assess the role of a panel of exportins in modulating TR function. Exportin-t and exportin 2 (CAS/CSE1L) were not included in our study, since they are specific for tRNA export (Arts et al., 1998) and importin α export (Kutay et al., 1997), respectively. We also excluded RanBP17; although a close homolog of exportin 7 (RanBP16), it is primarily expressed in the testis and pancreas, and a direct role for this protein in nuclear export has not been demonstrated (Koch et al., 2000; Kutay et al., 2000). Taken together, results presented here provide evidence that multiple exportins influence cellular localization of TRα1 and TRβ1 and, in this way, may play a role in modulating T_3-mediated gene expression.

2. Methods

2.1. Plasmids

The plasmid pGFP-TRα1 encodes a functional green fluorescent protein (GFP)-tagged rat TRα1 fusion protein (Bunn et al., 2001). pGFP-TRβ1 encodes a functional GFP-tagged human TRβ1 (Mavinakere et al., 2012). Pre-designed SureSilencing™ short hairpin RNA (shRNA) plasmid sets consisting of four different shRNA expression plasmids for each target mRNA were purchased from SABioscience (Frederick, MD) for human transportin 1 (TNPO1), transportin 2 (TNPO2), exportin 4 (XPO4), exportin 5 (XPO5), exportin 6 (XPO6), exportin 7 (XPO7), and a scrambled sequence negative control. pk-Myc-exportin 5, pCMV-Myc, and pCMV-HA were obtained from Addgene (Cambridge, MA), Clontech Laboratories, Inc. (Mountain View, CA), and BD Biosciences (San Jose, CA), respectively. The HA-tagged exportin 7 expression plasmid (pMT2SM-RanBP16) was a gift from C. Smas (University of Toledo College of Medicine, Ohio). The mCherry-tagged exportin 4 expression plasmid (pmCherry-XPO4) was obtained from GenScript (Piscataway, NJ) and pmCherry-C1 was from Clontech. 2xDR4-SV40-Luc was a gift from J. L. Jameson (Northwestern University) and consists of two copies of a positive, direct repeat TRE (DR+4) in the firefly luciferase vector pGL3. pGL4.74 encodes *Renilla* luciferase (Promega, Madison, WI).

2.2. Fluorescence recovery after photobleaching (FRAP)

HeLa cells (ATCC, #CCL-2) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY), at 37 °C under 5% CO_2 and 98% humidity. Cells were seeded at $2.0–2.5 \times 10^5$ cells per 3-cm dish with a cover glass bottom (MatTek Corporation, Ashland, MA). Twenty four hours after seeding, cells were co-transfected with 1 μg GFP-TRα1 expression plasmid, and 1 μg of the appropriate target-specific or control shRNA expression plasmids, using the two shRNAs from each set of four (see Section 2.1) that showed the greatest knockdown efficacy as assessed by quantitative PCR (see Section 2.4). Transfection medium containing Lipofectamine 2000 (Life Technologies) was replaced with complete medium 9 h post-transfection. Twenty seven hours post-transfection, cells were prepared for live-cell imaging: cells were incubated in 2 mL of complete media containing 100 μg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO), 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL wheat germ agglutinin conjugated to Alexa Fluor® 350 (Life Technologies). Cells were washed twice with Dulbecco's phosphate-buffered saline and imaged. During the experiment, cells were incubated in MEM-α without phenol red, containing 50 μg/mL cycloheximide, 50 units/mL penicillin, and 50 μg/mL streptomycin. In preliminary studies, we tested a range of post-transfection incubation times (17 h, 24 h, 27 h, and 30 h), varied the amount of Lipofectamine 2000 and the time cells were exposed to the reagent, selected for knockdown cells with puromycin, and varied the shRNA plasmid amounts and combinations. The conditions described earlier were determined to have high transfection efficiency (70–80% of cells were transfected), effectively reduce the levels of exportins in cells (at least 50% knockdown), while still retaining cell viability. Altered conditions either decreased transfection efficiency, decreased knockdown efficiency, or led to increased cell mortality. Cell mortality was assessed by visual inspection of the number of adherent cells prior to transfection, compared with the number of cells remaining adhered post-transfection, with the standard set at >60% retention.

All FRAP experiments were performed in an OkoLab Incubation System (Warner Instruments, Inc., Hamden, CT) at 37 °C under 5% CO_2. Images were collected from an inverted Nikon A1Rsi confocal microscope Ti-E-PFS using a 40× water objective (Nikon Inc., Melville, NY). The 488-nm line of a krypton–argon laser with a band-pass 525/50 nm emission filter was used for GFP detection; the 405-nm line with a band-pass 450/50 emission filter was used for Alexa Fluor® 350 detection. Images were obtained using the stimulation/bleaching acquisition module of NIS-Elements AR (Nikon). An initial image was recorded from an area containing a GFP-expressing cell with two or more nuclei, using 1–4% laser power from the 488 nm line and 8–20% laser power from the 405 nm line. One nucleus within the multinucleated cell was exposed at 100% laser power for 10–12 s using the 488 nm line. Post-bleach sequential images were then taken every 5 min for 24 cycles at the lower laser intensities noted earlier. For quantitative analysis of digitized images, fluorescent intensity values were generated using NIS-Elements AR (Nikon). Bleached and unbleached nuclei were each considered as independent regions of interest. In addition, these values took into account the background brightness levels during each experiment. Intensity values were subsequently normalized so that the total fluorescence within each multinucleated cell after bleaching was equal to 1.0 (arbitrary units). After normalization, convergence of the representative curves for bleached and unbleached nuclei toward one another represents the degree of fluorescence equilibration between these compartments. When one bleached and one unbleached nucleus are present, complete equilibration occurs at 0.5 fluorescence units (Grespin et al., 2008).

2.3. Fixation, immunofluorescence, and cell scoring

HeLa cells were seeded at $2.5–3.0 \times 10^5$ cells per well of a 6-well plate with glass coverslips (Fisher Scientific, Pittsburgh, PA). Twenty four hours after seeding, cells were transfected with 2 μg plasmid DNA, using Lipofectamine 2000 Reagent. Approximately 18 h post-transfection, cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton-X-100. The following antibodies were used at 1:500: anti-c-Myc (Clontech), Cy3-goat anti-mouse (Life Technologies/Zymed), and anti-HA tag (Abcam, Cambridge, MA). Texas Red anti-rabbit IgG (H + L) (Vector Laboratories, Burlingame, CA) antibody