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Distinct roles for classical nuclear import receptors in the growth of multinucleated muscle cells☆

Monica N. Hall [a], Christine A. Griffin [b], Adriana Simionescu [b], Anita H. Corbett [c,*], Grace K. Pavlath [d,**]

[a] Graduate Program in Genetics and Molecular Biology, Emory University, Atlanta, GA 30322, USA
[b] Graduate Program in Biochemistry, Cell and Developmental Biology, Emory University, Atlanta, GA 30322, USA
[c] Department of Biochemistry, Emory University, Atlanta, GA 30322, USA
[d] Department of Pharmacology, Emory University, Atlanta, GA 30322, USA

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ABSTRACT

Proper muscle function is dependent on spatial and temporal control of gene expression in myofibers. Myofibers are multinucleated cells that are formed, repaired and maintained by the process of myogenesis in which progenitor myoblasts proliferate, differentiate and fuse. Gene expression is dependent upon proteins that require facilitated nuclear import, however little is known about the regulation of nucleocytoplasmic transport during the formation of myofibers. We analyzed the role of karyopherin alpha (KPNA), a key classical nuclear import receptor, during myogenesis. We established that five karyopherin alpha paralogs are expressed by primary mouse myoblasts *in vitro* and that their steady-state levels increase in multinucleated myotubes, suggesting a global increase in demand for classical nuclear import during myogenesis. We used siRNA-mediated knockdown to identify paralog-specific roles for KPNA1 and KPNA2 during myogenesis. KPNA1 knockdown increased myoblast proliferation, whereas KPNA2 knockdown decreased proliferation. In contrast, no proliferation defect was observed with KPNA4 knockdown. Only knockdown of KPNA2 decreased myotube growth. These results identify distinct pathways involved in myoblast proliferation and myotube growth that rely on specific nuclear import receptors suggesting that regulation of classical nuclear import pathways likely plays a critical role in controlling gene expression in skeletal muscle.

Introduction

Skeletal muscle myofibers are multinucleated cells that contain many hundreds of nuclei spread across the length of the cell in a common cytoplasm. Each myonucleus is believed to express protein products for a defined volume of surrounding cytoplasm called the myonuclear domain (Allen et al., 1999). How a myofiber with hundreds of nuclei coordinates and regulates the transport of macromolecules into and out of these nuclei is unknown. Spatial and temporal regulation of nucleocytoplasmic transport into individual nuclei must occur within a single myofiber since transcriptional activity of a specific gene locus can differ among nuclei within the same myofiber (Newlands et al., 1998). Furthermore, the transcription factors, NFATc1 and NFAT5, as well as the endonuclease EndoG localize to some myonuclei but not others within the same multinucleated cell (Abbott et al., 1998; Dupont-Versteegden et al., 2006; O'Connor et al., 2007). Skeletal muscle is a very plastic tissue that readily undergoes changes in mass in response to aging, injury or disease. Changes in muscle mass can impact breathing, locomotion and metabolism and affect motility and lifespan. Understanding how nucleocytoplasmic transport is regulated in myofibers will lead to a greater understanding of how external signals are sensed by muscle cells and translated into changes in gene expression necessary for tissue homeostasis.

All macromolecular transport between the nucleus and the cytoplasm is mediated by the nuclear pore complexes (NPC) that perforate the nuclear envelope (Lim and Fahrenkrog, 2006). Passive diffusion of ions and small molecules can occur; however, macromolecules require an appropriate targeting signal for transit through nuclear pores (Freitas and Cunha, 2009). Classical nuclear import, which is best characterized of the nuclear import mechanisms, depends on a classical nuclear localization signal (cNLS) in a cargo protein to be imported into the nucleus (Lange et al., 2007). cNLS motifs are comprised of basic residues present as either a single cluster (monopartite) or two clusters separated by a linker (bipartite)

Abbreviations: cNLS, classical nuclear localization signal; DM, differentiation media; eMyHC, embryonic myosin heavy chain; GM, growth media; KPNA, karyopherin alpha; KPNB1, karyopherin beta1; NFATc2, Nuclear Factor of Activated T cells cytoplasmic 2; NPC, nuclear pore complex.

☆ Note: For journal matters related to reviews and publication contact Grace Pavlath.

* Correspondence to: A.H. Corbett, Department of Biochemistry, Rollins Research Building, Rm 4117, 1510 Clifton Rd, Emory University, Atlanta, GA 30322, USA. Fax: +1 4047273954.

** Correspondence to: G.K. Pavlath, Department of Pharmacology, Rollins Research Building, Rm 5027, 1510 Clifton Rd, Emory University, Atlanta, GA 30322, USA. Fax: +1 4047270365.

E-mail addresses: acorbe2@emory.edu (A.H. Corbett), gpavlat@emory.edu (G.K. Pavlath).

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(Kalderon et al., 1984; Robbins et al., 1991). Proteins that contain a cNLS are imported into the nucleus by a heterodimeric receptor consisting of the classical nuclear import receptor karyopherin alpha (KPNA) and the pore targeting subunit karyopherin beta1 (KPNB1) (Fig. 1A) (Lange et al., 2007). Once in the nucleus, the cNLS-containing protein is released and both KPNA and KPNB1 are recycled separately back to the cytoplasm for another round of import (Hood and Silver, 1998; Kutay et al., 1997).

While *Saccharomyces cerevisiae* contains a single, essential karyopherin alpha, Srp1, the situation is more complex in *Homo sapiens* where a single KPNB1 can function with any of seven KPNA paralogs: KPNA1, KPNA2, KPNA3, KPNA4, KPNA5, KPNA6 and KPNA7 (Kelley et al., 2010; Kohler et al., 1997; Kohler et al., 1999; Tsuji et al., 1997). Six KPNA paralogs exist in mouse with which the corresponding human homologues share 80–90% amino acid identity (Fig. 1B) (Hu et al., 2010; Tsuji et al., 1997). KPNA paralogs in mouse and human are categorized into three subtypes based on their percentage of amino acid identity (Tsuji et al., 1997). Mouse subtypes are Subtype S: KPNA1 and KPNA6; Subtype P: KPNA2 and KPNA7; and Subtype Q: KPNA3 and KPNA4, with placement of recently discovered murine KPNA7 into its subtype being tentative (Hu et al., 2010). Subtype members share 80% to 90% amino acid identity, whereas different subtypes share 40% to 50% amino acid identity. While KPNA paralogs all function in classical nuclear import, their roles can differ in importing specific cNLS-containing proteins that are required for cell differentiation and function (Huenniger et al., 2010; Kohler et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007).

To begin to understand how nucleocytoplasmic import is regulated in multinucleated muscle cells, we utilized an established *in vitro* model of myogenesis using primary mouse muscle cells (Rando and Blau, 1994). In this model, precursor mononucleated myoblasts proliferate in high serum-containing media but upon switching to a low mitogen media the cells exit the cell cycle, differentiate into myocytes that migrate and adhere to other myocytes and undergo membrane fusion to form multinucleated nascent myotubes. Further rounds of myocyte fusion with nascent myotubes yield large mature myotubes with many myonuclei. We used this model to analyze classical nuclear import in muscle cells, specifically the role of different KPNA subtypes represented by KPNA1, KPNA2 and KPNA4. This model offers the advantage that the role of KPNA-mediated nuclear import can be studied both in the context of mono- and multinucleated muscle cells. We determined that five mouse karyopherin alpha paralogs are expressed in primary myoblasts *in vitro* and their steady-state levels increase as myoblasts progress through myogenesis to form multinucleated myotubes. Through the use of RNAi, we demonstrate that KPNA1 and KPNA2 have differential roles in regulating myoblast proliferation as well as myotube size. Furthermore, we detect changes in the steady-state localization of a key cNLS-dependent cargo required for growth of myotubes, Nuclear Factor of Activated T cells, cytoplasmic 2 (NFATc2). In contrast to KPNA1 and KPNA2, knockdown of KPNA4 has no effect on myogenesis. These data provide evidence for distinct classical nuclear import pathways in skeletal muscle that rely on specific KPNA import receptors. We suggest that classical nuclear import may provide a novel regulatory mechanism during the formation and growth of multinucleated cells.

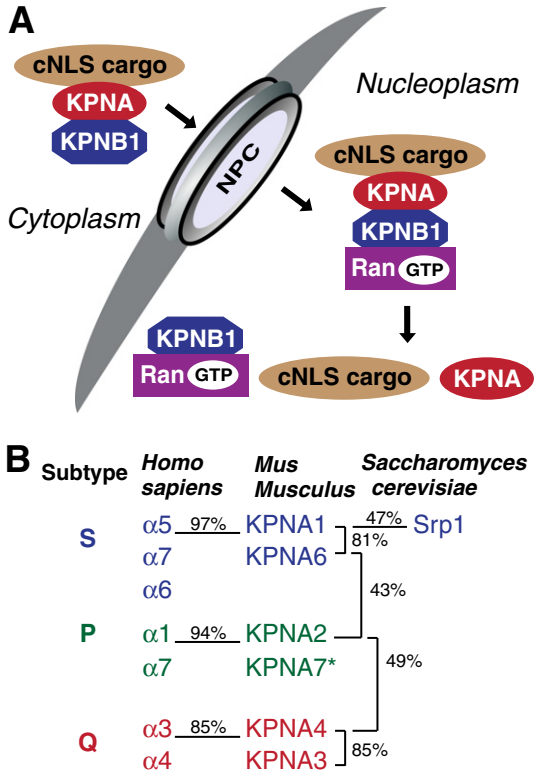

Fig. 1. Classical nuclear import. (A) In the cytoplasm, proteins containing a classical nuclear localization sequence (cNLS) are recognized by the classical nuclear import receptor complex consisting of karyopherin alpha (KPNA) and karyopherin beta1 (KPNB1). KPNA recognizes and binds cNLS-containing proteins, while KPNB1 mediates nuclear import of the complex through interactions with the nuclear pore complex (NPC). In the nucleus, KPNB1 is bound by Ran-GTP which induces a conformational change that dissociates the import complex leading to release of the cNLS protein. (B) KPNA paralogs are categorized into three subtypes, S, P and Q. The percent identity between a few subtypes is shown for human, mouse and budding yeast to illustrate the homology between and within subtypes for KPNA paralogs. * The placement of recently discovered mouse KPNA7 into subtype P is tentative (Hu et al., 2010).

Material and methods

Primary muscle cell culture

Primary myoblasts were isolated from the hind limb muscles of adult Balb/c mice between 8 and 12 wk of age as described previously (Jansen and Pavlath, 2006) and cultured in growth medium (GM: Ham's F-10, 20% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin and 100 μg/ml streptomycin) on collagen coated plates. Primary cultures were enriched for myogenic cells by using the preplating technique as described previously (Rando and Blau, 1994) and determined to be 97% pure by MyoD immunostaining.

To induce differentiation and fusion, myoblasts were seeded in GM onto 6-well plates (2×10^5 cells per well) coated with entactin–collagen–laminin (ECL, MilliPore) and allowed to adhere for ~1 h before switching to differentiation media [DM: DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% insulin–transferrin–selenium-A (Invitrogen)].

RT-PCR analyses

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The reverse transcriptase reaction was performed using 2 μg of total RNA/sample using random hexamers and M-MLV reverse transcriptase (Invitrogen). cDNA was amplified using 2.5 μM of each primer and the Expand High Fidelity PCR system (Roche). All KPNA primers spanned intron–exon boundaries to control for genomic contamination and were specific to individual KPNA paralogs as determined by Blast search. In addition, RNA was tested by PCR for DNA contamination. Primer sequences were: KPNA1 (F: 5′-TCCTGCTTTGCGGGCTGTGG-3′ and R: 5′-GGGGTGCGATGCTGCTGTCC-3′); KPNA2 (F: 5′-CTGCTGGGCCATTTCCTACCTGA-3′ and R: 5′-ACGCGGCCTCCTTCTGTGCTT-3′); KPNA3 (F: 5′-CAAGGGCCGCGATGTG-GAGA-3′ and R: 5′-CTGATGTGGGGAATGGAGGAGTCG-3′); KPNA4 (F: